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Abstract

The nuclear receptor Peroxisome Proliferator-Activated Receptor alpha (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 SUMO1 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.

MESH Keywords Binding Sites ; Cell Line ; Co-Repressor Proteins ; metabolism ; Gene Expression Regulation ; Humans ; Kinetics ; Lysine ; metabolism ; Nuclear Receptor Co-Repressor 1 ; metabolism ; PPAR alpha ; metabolism ; physiology ; Protein Transport ; SUMO-1 Protein ; metabolism

The nuclear receptor Peroxisome Proliferator-Activated Receptor alpha (PPARα) is a key regulator of energy homeostasis (1 –5) and the anti-inflammatory response (6 –8). PPARα is highest expressed 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 for review (11)).

The structure of PPARα consists of an amino-terminal A/B domain containing a ligand-independent cis-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 carboxy-terminus, a ligand-binding E domain (LBD), which contains the ligand-dependent activation function called AF-2 (figure (12 –14)2A) (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 N-CoR 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 has 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 for review (18)). While this study was in progress, Leuenberger et al. 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 downregulation of this gene (19). Although this study identified a role for SUMO modification in the regulation of 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 for review (21)). Targeted lysine residues are part of the consensus site ΨKxD/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 gentamycine were purchased from Invitrogen Life Technologies (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-SmithKline (Les Ulis, France). JetPEI was purchased from Ozyme (Saint-Quentin en Yvelines, France). Redivue L-(^{35} S)-Methionine was purchased from Amersham Pharmacia Biotech (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-SUMO1-His6 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 K_{138} R, K_{185} R, K_{216} R, K_{310} R, K_{358} R, K_{449} R expression vectors were generated using pSG5-hPPARα WT as template and the QuickChange 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 K_{185} R) 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 hours, cells were incubated in medium containing 0.2 % fetal calf serum; 0.2 % free fatty acid BSA (Sigma, Saint-Quentin Fallavier, France) and Me_2 SO or 600 nM of GW7647 (kindly provided by GlaxoSmithKline). After 24 hours, cells were lysed with 100 µL of reporter lysis buffer (Promega, Charbonnières, France) according to the manufacturer's protocol, and the luciferase activity was analysed with Mithras LB 940 luminometer (Berthold Technologies, Thoiry, France). As transfection control, β-galactosidase activity was analysed 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 ONTARGETplus 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, Courtaboeuf, France). HuH-7 were transfected for 24 hours with 50 nM of siRNA using Dharmafect1 transfection reagent (Dharmacon, Thermo-Fisher Scientific, Saint-Herblain, France) accordingly to the manufacturer's protocol. Knocking-down efficiencies of each siRNA were analysed by RT Q-PCR (figure 1A and 1B).

GST Pull-Down assay

GST pull-down assays have been performed as previously described (22). Briefly, BL21-Star [pGEX4T2-Ubc9] and BL21-star [pGEX4T2] E.coli strains were grown in Terrific Broth medium (Invitrogen, Cergy-Pontoise, France) GST-Proteins expression was induced with 0.1 mM IPTG for 3 hours. Bacteria were mechanically disrupted with FRENCH-Press and GST and GST fusion proteins were isolated using pull-down technique. A total of 15 µg GST, GST-Ubc9 were incubated with 4 µL [^{35}S]-methionine hPPARα WT for 2 hours at 4°C. Finally, bound proteins were boiled at 95°C, separated by SDS-PAGE and analysed 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 HRP-coupled IgG and Immobilon western detection kit (Millipore, St Quentin en Yvelines, France). 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, Saint-Herblain, France). The secondary antibodies against rabbit and mouse IgG were purchased from Amersham Pharmacia Biotech (Orsay, France) and the secondary antibodies against goat IgG was obtained from Thermofisher Scientific (Saint-Herblain, France).

Nickel pull down assay

Transfected cells were lysed in denaturating conditions using 6M-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 analysed 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, Charbonnières, France) according to the manufacturer's instructions. Reaction products were fractionated by SDS-PAGE and analysed by autoradiography.

Coimmunoprecipitation

HuH-7 cells were cross-linked with 1.5 mM Ethylene glycol-bis(SuccinimidylSuccinate) (Thermo Fisher Scientific, Rockford, USA) for 20 minutes at room temperature. After ice-cold PBS 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, St Louis, USA) overnight at 4°C. Beads were washed four times with ice-cold TBS and eluted with Laemmli buffer. Protein amounts were analysed by western blotting.

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 (figure 1). After RNA purification, the expression of hPPARα target genes implicated in different metabolic pathways was analysed 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 upregulated in absence of NCoR but not in absence of

SMRT (figure 1C and 1D , respectively). Inversely, HMGCOAS2 expression is increased in absence of SMRT but not in absence of NCoR (figure 1E). These data suggest that L-CPT1 and PDK4 are NCoR-sensitive hPPARα target genes, whereas HMGCOAS2 is a SMRT-regulated hPPARα target gene.

Since 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 pull-down. GST-Ubc9 WT was incubated with in vitro translated ^{35} S-labeled hPPARα protein. As shown in figure 2B , 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 previously shown that murine PPARα is SUMOylated (26). No data are however available concerning human PPARα. SUMOplot™ prediction algorithm analysis identified six putative SUMOylation sites (K138, K185, K216, K310, K358, K449) in human PPARα, which are conserved between species (figure 2A). In order 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 figure 2C , hPPARα is SUMOylated in the presence of SUMO-1 WT, but not the SUMO-1 mutant.

In order to examine whether hPPARα is SUMOylated in a cellular context, HuH-7 hepatoma cells were transfected with hPPARα WT and SUMO1-His_6 expression vectors (figure 2D). Histidine-tagged SUMOylated proteins were then isolated from whole HuH-7 cell extracts using Ni-NTA beads. When SUMO1-His_6 was co-expressed with hPPARα, an additional high molecular weight band (72 kDa) corresponding to SUMO-1-conjugated hPPARα was observed (figure 2D) 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 SUMO1-His_6 expression vectors, the cells were treated with vehicle (Me_2 SO) or GW7647 and SUMOylated PPAR α was specifically analysed by western blotting using anti-PPARα antibodies. As shown in figure 2E , SUMOylation of hPPARα strongly decreased in presence of GW7647 compared to vehicle. These data suggest that ligand binding either impairs the SUMOylation of hPPAR α 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 presence of GW7647 and Histidine-tagged SUMOylated proteins were analysed by western blotting using anti-His_6 antibodies. As shown in figure 2F , 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 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 (figure 3A). However, when cells were co-transfected with hPPARα WT, SUMO1-His_6 , Ubc9 and FLAG-PIASy expression vectors (figure 3A), SUMO1-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

In order to define the role of the SUMO pathway on hPPARα activity, HuH-7 cells were co-transfected with the J6-TKLuc 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 (figure 3B). 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 absence of Ubc9, the expression of L-CPT1 or PDK4 , which appears to be more sensitive to NCoR expression (figure 1C and 1D), was significantly increased, suggesting that SUMO pathway inhibits their expression (figure 4A–C). Interestingly, HMGCOAS2 gene expression, which was not

altered by NCoR silencing (figure 1E), was not affected by Ubc9 silencing. To evaluate the role of hPPARα on the observed effect of Ubc9 knockdown, we analysed 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 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 (figure 4D–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 figure 1 demonstrate that the SUMO pathway selectively inhibits NCoR-specific hPPARα target genes in a hPPARα-dependent manner.

Human PPARα is SUMOylated on lysine 185

To identify the SUMOylated site in hPPARα, the six potential acceptor lysines (figure 2A) were individually replaced by site-directed mutagenesis by an unSUMOylatable arginine, a residue with a similar steric hindrance. Each hPPARα mutant protein was analysed for their ability to be SUMOylated in vitro . As shown in figure 5A , no band corresponding to SUMOylated protein was visible with the mutant hPPARα K_{185} R, 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 (figure 5B). The cells were transfected with PPARα and SUMO-1, Ubc9 and PIASy expression vectors and SUMOylated PPARα proteins were analysed by western blotting after 48 hours. The significant reduction of the signal corresponding to the PPARα K185R protein compared to 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 K_{185} R expression vectors or pSG5 control (figure 6). HuH-7 cells were then treated with the specific hPPARα ligand GW7647. As expected, basal and ligand-induced activities of the mutant hPPARα K_{185} R were significantly higher compared to the WT protein. While both WT and mutant K_{185} R proteins are equally expressed in cells (figure 6B) 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 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 (figure 7) and SMRT (figure 8) with the hPPARα WT or hPPARα K_{185} R 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 K_{185} R expression vectors, and increasing amounts of VP16-AD (activating domain) or VP16-NCoR vectors (figure 7A). 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 K_{185} R, indicating that the NCoR protein interacts with hPPARα WT with a higher efficiency than hPPARα K_{185} R.

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 K_{185} R, and with increasing amounts of NCoR full-length expression vector (figure 7B). In order to appreciate both hPPARα WT and hPPARα K_{185} R sensitivity to NCoR independently of the difference between their respective transcriptional activities, the transcriptional activity of both hPPARα WT and hPPARα K_{185} R 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 K_{185} R was unaffected by the co-expression of NCoR (figure 7B), showing that the hPPARα K_{185} R mutant is less sensitive to a decrease in activity by NCoR co-transfection compared to hPPARα WT protein. To reinforce these results, HuH-7 cells were cotransfected with a flagged-hPPARα WT or K_{185} R expression vectors and NCoR full-length. Flagged-proteins were immunoprecipitated and associated NCoR proteins were analysed by western blotting. The results presented in figure 7C show that, in contrast to PPARα WT, the SUMOylation-defective hPPARα K_{185} R form did not interact with the NCoR protein, confirming our previous results (figure 7A). 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 (figure 8). For that purpose, HuH-7 cells were transfected with the J6-TK-Luc reporter vector, the hPPARα WT or hPPARα K_{185} R, and increasing amounts of VP16-AD or VP16-SMRT vectors. By contrast to NCoR (figure 7), increasing amounts of SMRT similarly activated WT and K_{185} R hPPARα proteins, indicating that the K_{185} R mutation in hPPARα has no effect on the interaction of hPPARα with the corepressor SMRT (figure 8A).

Additionally, in order 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 K_{185} R, with increasing amounts of either pCI-SMRT or pCI as control (figure 8B). In accordance with the one-hybrid results, the transcriptional activity of both hPPARα WT and hPPARα K_{185} R was decreased to a similar extent by SMRT. Altogether, these data show that the hPPARα K_{185} R 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 weight (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 K_{185} R mutant is still slightly SUMOylated in cells (figure 5B), suggesting that a second minor SUMOylated site could exist. Because the replacement of lysine 185 into an 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 figure 5A that K_{138} R and K_{216} R hPPARα mutants are less SUMOylated in vitro compared to 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 (figure 9) (31).

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 K_{185} R 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 (figure 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 (figure 1). Using the mutant protein hPPARα K_{185} R we showed that SUMOylation of hPPARα promotes NCoR recruitment without influencing the binding to SMRT. These data suggest that hPPARα SUMOylation helps discriminating among the interactions with different corepressors. Accordingly, over-expression of full-length NCoR inhibits transcriptional activity of PPARα WT without changing the transcriptional activity of hPPARα K_{185} R, whereas overexpression of SMRT inhibits transcriptional activity of both hPPARα WT and hPPARα K_{185} R. Such differential recruitment of corepressors has already been observed with the nuclear receptor LXR alpha (LXRα), which preferentially recruits either NCoR and 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 downregulation 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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Figure 1

Selective effect of decreased expression of NCoR and SMRT on PPARα target genes in HuH7 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 ± SD of triplicate determinations. Statistically differences are indicated (t-test; Scramble vs siRNA Me_2 SO: **p<0.01; ***p<0.001; ns: non significant).

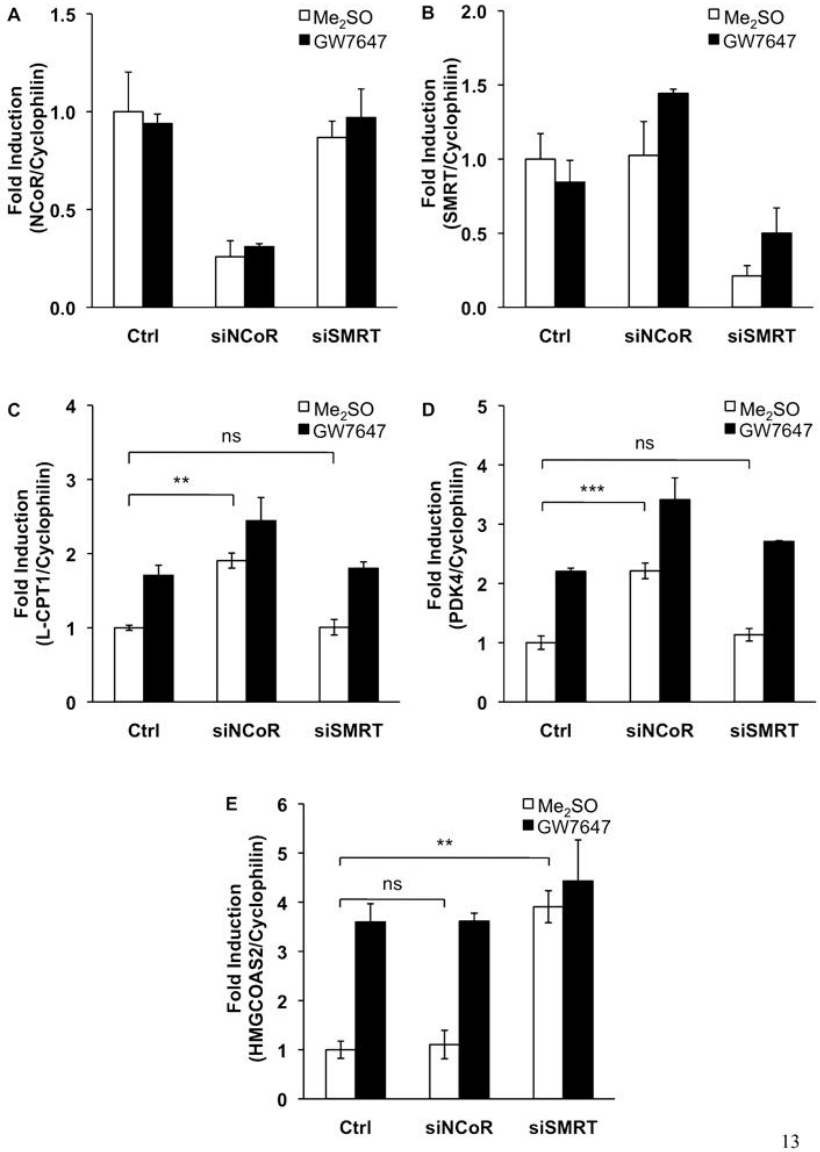

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Figure 2

Human PPARα is a substrate for SUMO-1 modification in vitro and in HuH-7 cells

A . Protein sequence of hPPARα (NM Q07869) was analysed with the SUMOylation prediction site algorithm SUMOplot™ prediction. Numbers correspond to amino acids position. 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 glutathion-sepharose and proteins were analysed 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 analysed 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 hours, cells are 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 analysed 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-SUMO1-His$_6$ expression vectors. Cells were treated with Me_2 SO as control or GW7647 (600 nM). After 24 hours, a SUMOylation test was performed as described above. **F** . HuH-7 cells were transfected with pSG5-SUMO1-His$_6$ expression vector and treated with GW7647 for 24 hours. SUMOylated proteins were analysed by western blotting using an anti-His$_6$ antibodies.

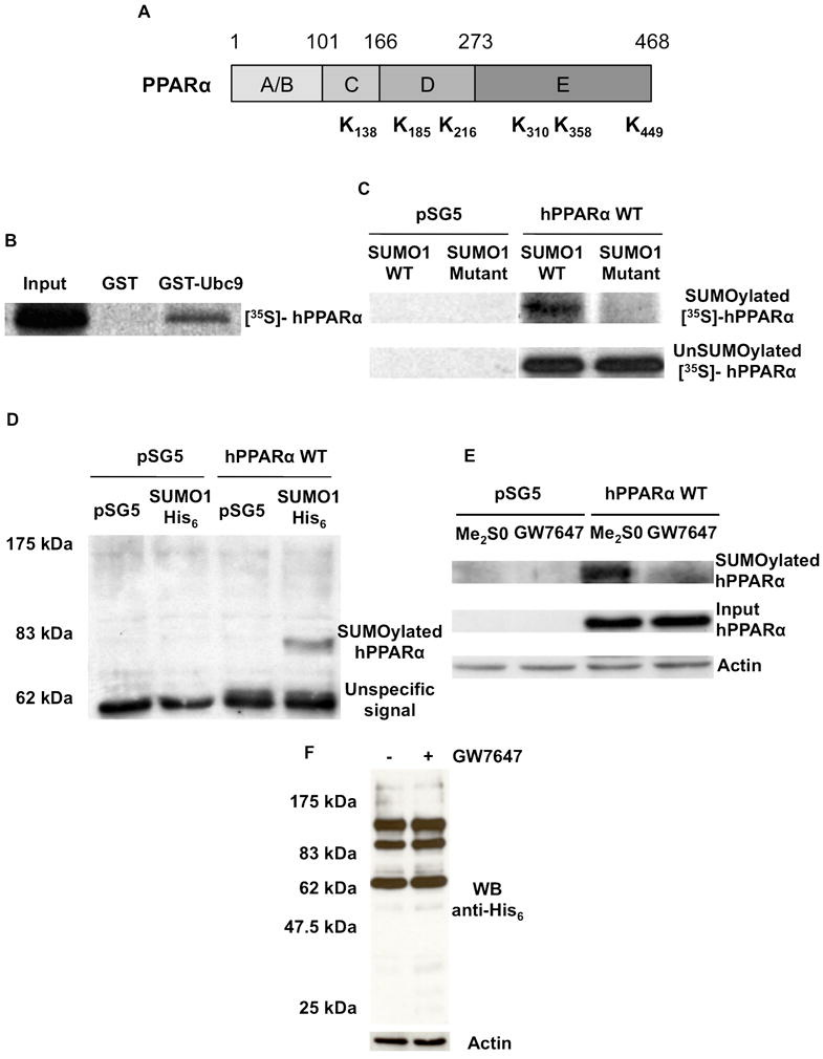


Figure 3

PIASy regulates SUMOylation of human PPARα and inhibits its transcriptional activity

A . Cos-7 cells were cotransfected with pSG5 control vector or pSG5-hPPARα WT expression vector and with pSG5-SUMO1-His_6 , pSG5-Ubc9 and pcDNA3-FLAG-PIASy expression vectors as described. Empty vector pSG5 was used as negative control. After 48 hours, cell extracts were incubated with Ni-NTA beads to isolate histidine-tagged SUMOylated proteins. The SUMOylated hPPARα proteins and hPPARα input proteins were analysed by western blotting using anti-PPARα antibodies. The SUMO1-His_6 input proteins were analysed by western blotting using anti-His_6 antibodies. **B** . HuH-7 cells were cotransfected 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 ± SD of triplicate determinations.

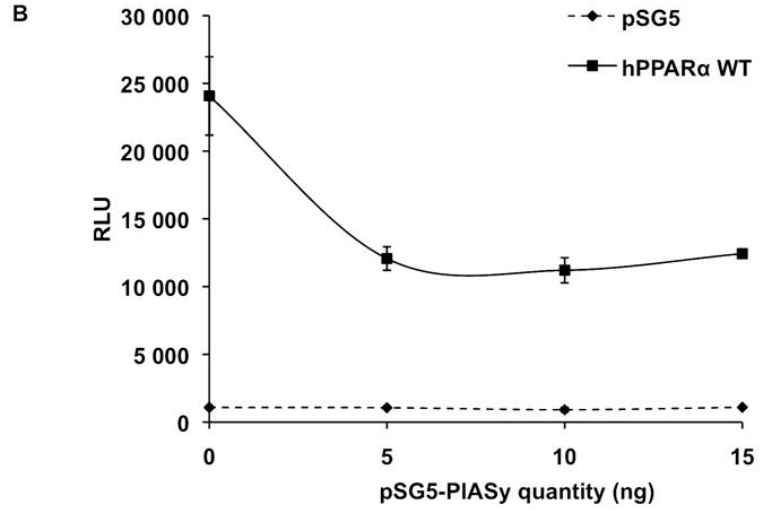


Figure 4

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_2 SO or GW7647 (600 nM) in DMEM medium 0.2% FCS, 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 ± SD of triplicate determinations. Statistically differences are indicated (t-test; Control vs PIASy: *p<0.05; ** p<0.01; ns: non-significant)

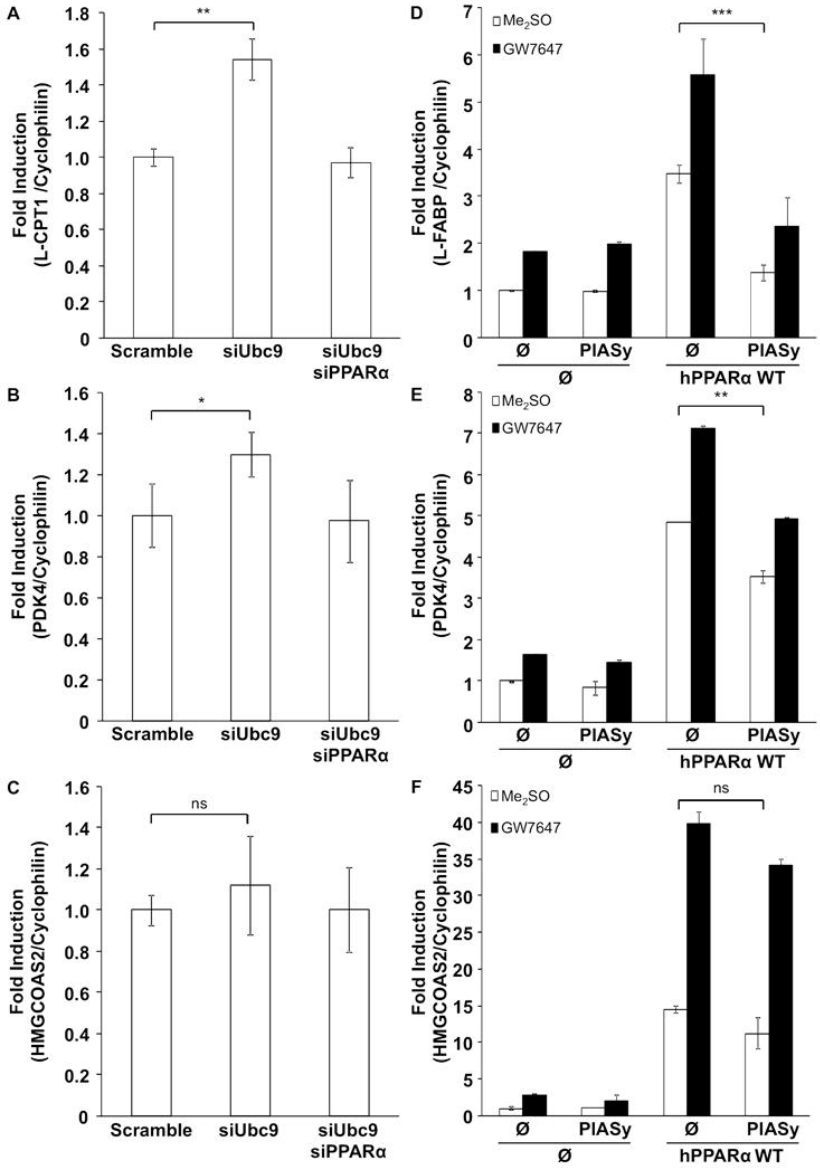


Figure 5

SUMOylation of hPPARα occurs on lysine 185

A. In vitro translated [^{35} S]-methionine hPPARα WT, hPPARα K_{138} R, hPPARα K_{185} R, hPPARα K_{216} R, hPPARα K_{310} R, hPPARα K_{358} R, hPPARα K_{449} R 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 analysed by autoradiography. **B** . Cos-7 cells were cotransfected with pSG5 control vector or pSG5-hPPARα WT or pSG5-hPPARα K_{185} R expression vectors and with pSG5-SUMO1-His_6 , pSG5-Ubc9 and pcDNA3-FLAG-PIASy expression vectors or pSG5 vector and pcDNA3-FLAG as controls. After 48 hours, Cos-7 cells were scraped in ice-cold PBS. One tenth of cells were lysed in RIPA 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 analysed by western blotting using anti-PPARα antibodies. The SUMO1-His_6 input proteins were analysed by western blotting using anti- His_6 antibodies.

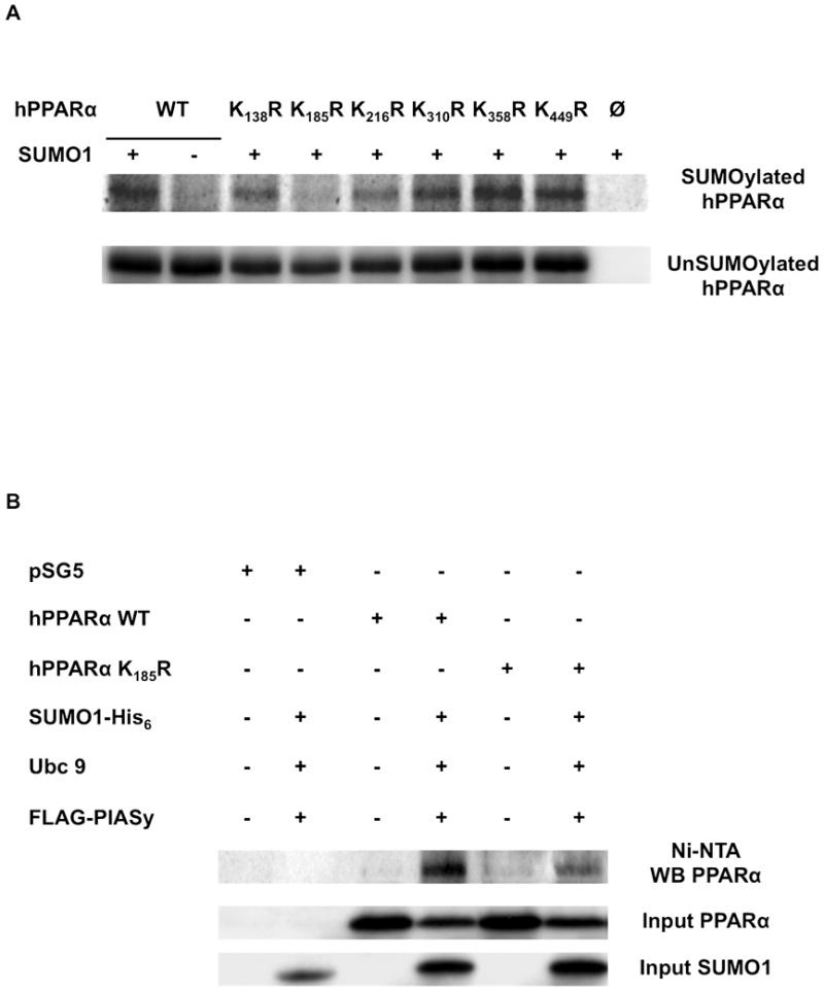


Figure 6

The transcriptional activity of hPPARα K_{185} R is increased compared to hPPARα WT

A . HuH-7 cells were transfected with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K_{185} R expression vectors, with the pSV-β-galactosidase, with the reporter vector J6-TK-Luc. After 24 h of transfection, cells were treated with Me_2 SO 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 ± SD of triplicate determinations. **B** . PPARα protein from transfection assay cell lysates was analysed by western blotting using anti-PPARα antibodies.

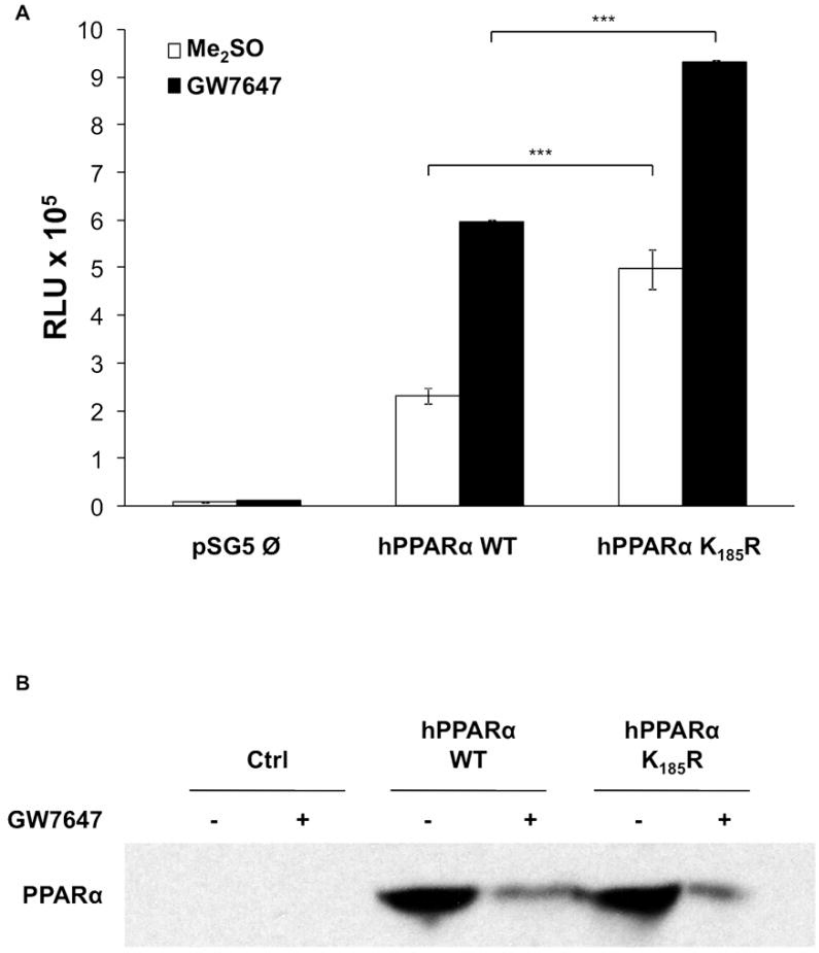
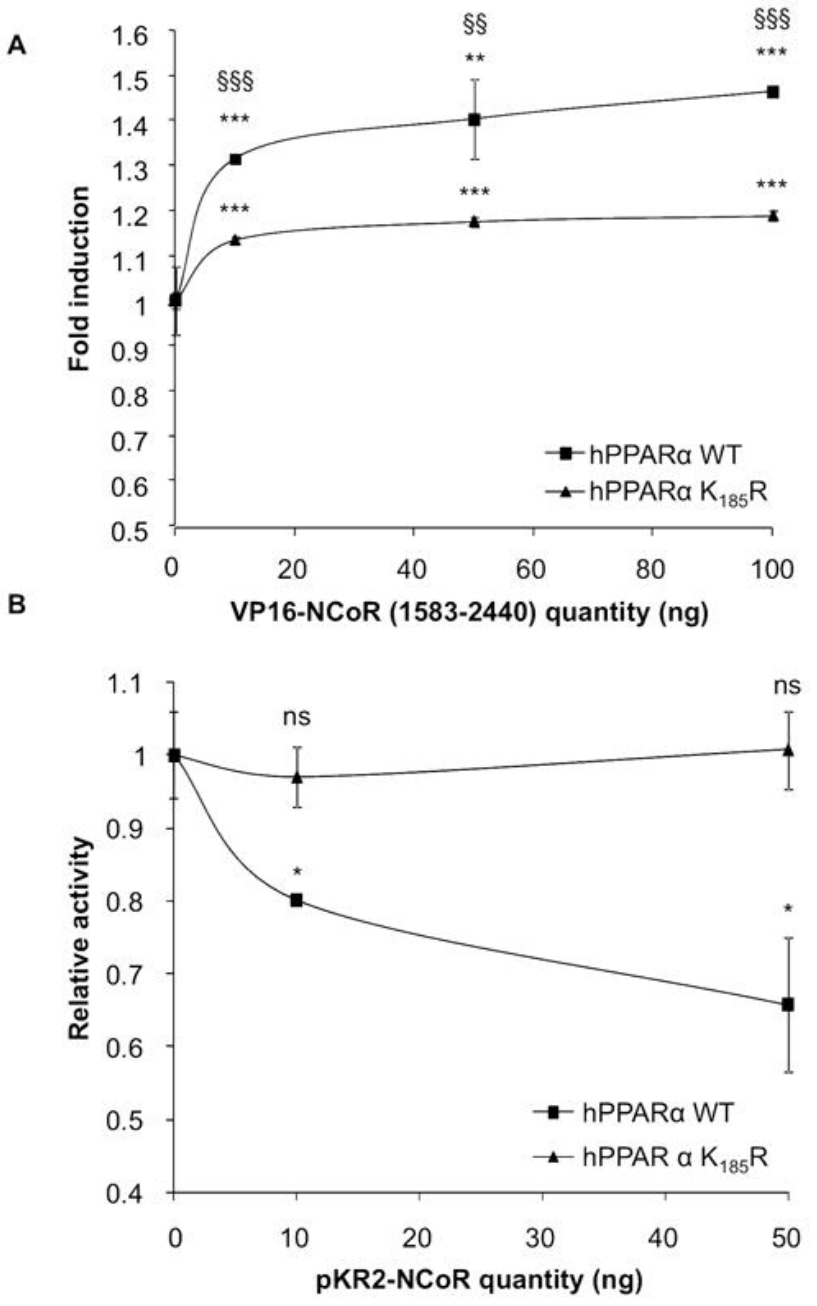


Figure 7

The hPPARα K_{185} R 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 K_{185} R 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 ± SD of triplicate determinations. Statistically differences are indicated (t-test; without VP16-NCoR vs with VP16-NCoR: **p<0.01, ***p<0.001; hPPARα WT vs hPPARα K_{185} R: §§ 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 K_{185} R expression vectors, and with increasing amounts of pKCR2 control vector or pKR2-NCoR full-length 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, pKCR2-NCoR curves were compared to their respective pKCR2 control curves, respectively. Results are expressed as relative inhibition. Each bar is the mean value ± SD of triplicate determinations. Statistically differences are indicated (t-test; without pKR2-NCoR vs with pKR2-NCoR: *p<0.05, ns: non significant). pKCR2 curves are not represented. **C.** HuH-7 cells were cotransfected with pEF-FLAG-hPPARα WT or K185R expression vectors and pKR2-NCoR. Flagged proteins were immunoprecipitated and associated NCoR proteins were analysed by western blotting.


A
Fold induction
VP16-NCoR (1583-2440) quantity (ng)
hPPARα WT
hPPARα K185R
§§§
§§
***
**
B
Relative activity
pKR2-NCoR quantity (ng)
hPPARα WT
hPPAR α K185R
ns
*

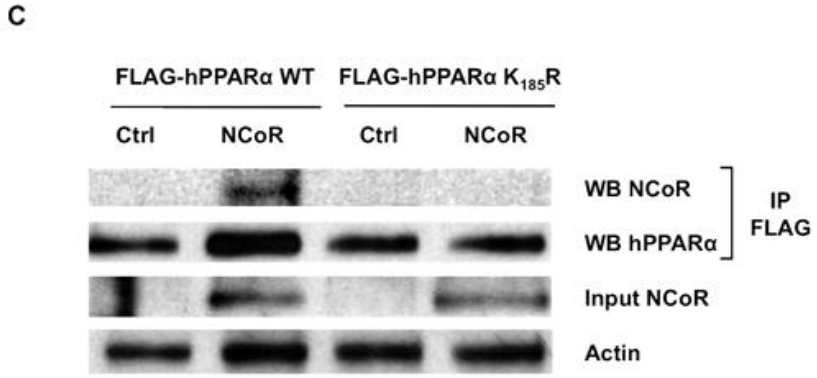

C
FLAG-hPPARα WT
FLAG-hPPARα K185R
Ctrl
NCoR
Ctrl
NCoR
WB NCoR
WB hPPARα
IP FLAG
Input NCoR
Actin

Figure 8

The hPPARα WT and hPPARα K_{185} R 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 K_{185} R 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 ± SD 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 K_{185} R 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 to their respective pCI control curves. Results are expressed as relative inhibition. Each bar is the mean value ± SD of triplicate determinations. pCI curves are not represented.

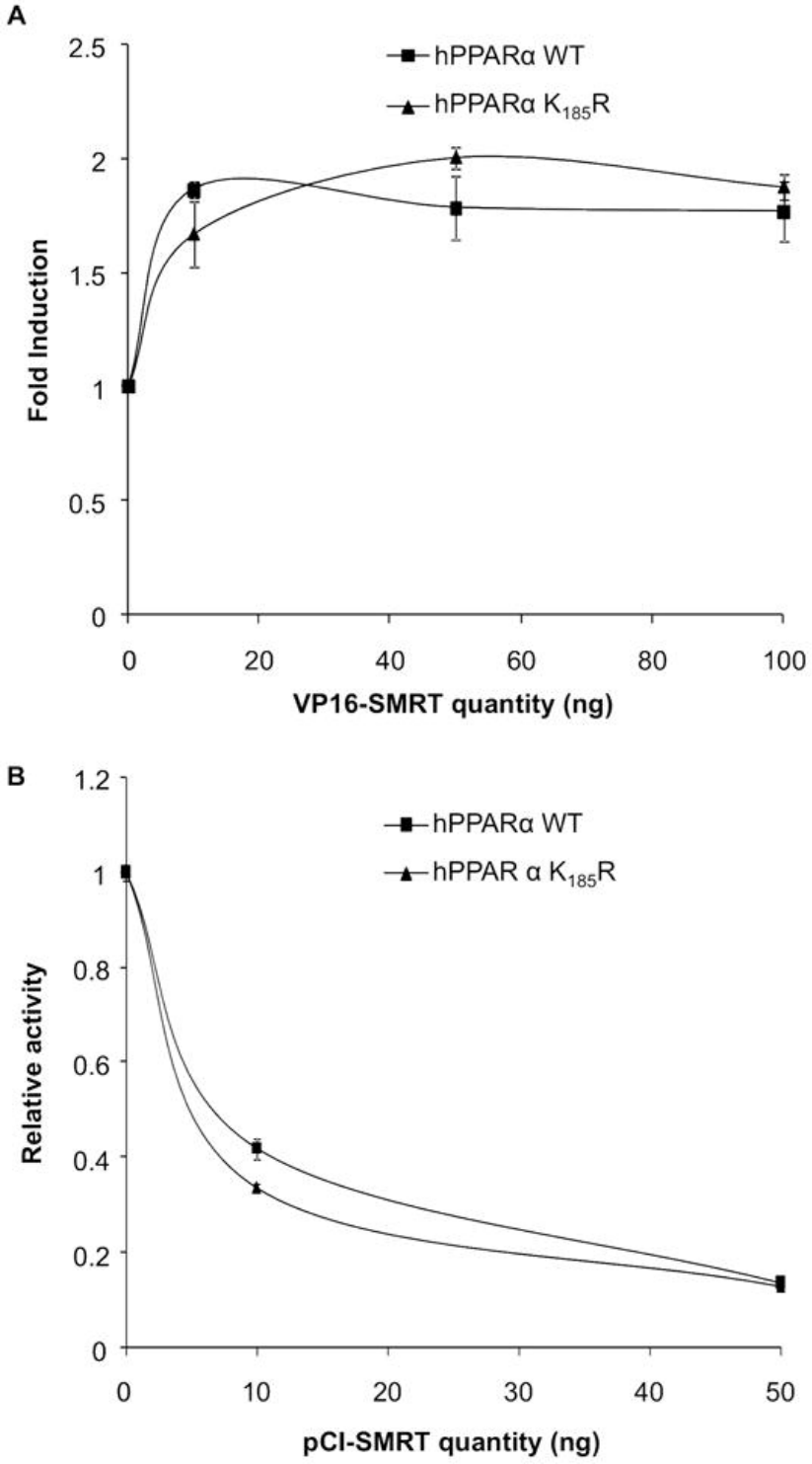


Figure 9

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.

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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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