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Benoit Pourcet, Inés Pineda-Torra, Bruno Derudas, Bart Staels, Corine Glineur. SUMOylation of human peroxisome proliferator-activated receptor alpha inhibits its trans-activity through the recruitment of the nuclear corepressor NCoR.. *Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology*, 2010, 285 (9), pp.5983-92. <10.1074/jbc.M109.078311>. <inserm-00439808>

HAL Id: inserm-00439808

<https://www.hal.inserm.fr/inserm-00439808>

Submitted on 15 Dec 2010

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SUMOylation of human peroxisome proliferator-activated receptor alpha inhibits its trans-activity through the recruitment of the nuclear corepressor NCoR

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Abstract

The nuclear receptor Peroxisome Proliferator-Activated Receptor alpha (PPAR α) is a key regulator of genes implicated in lipid homeostasis and inflammation. PPAR α 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, PPAR α activity is regulated by post-translational modifications such as phosphorylation and ubiquitination. In this report, we demonstrate that hPPAR α 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 hPPAR α . Finally, our results demonstrate that SUMO1 modification of hPPAR α down-regulates its trans-activity through the specific recruitment of corepressor NCoR but not SMRT leading to the differential expression of a subset of PPAR α target genes. In conclusion, hPPAR α 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 (PPAR α) is a key regulator of energy homeostasis (1 –5) and the anti-inflammatory response (6 –8). PPAR α 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). PPAR α modulates metabolism, especially lipid homeostasis, through its so-called trans-activation activity (10). The use of synthetic PPAR α ligands, such as fibrates, improves lipid profiles in dyslipidemic patients (see for review (11)).

The structure of PPAR α 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).

PPAR α 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, PPAR α 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 PPAR α modifies the conformation of the PPAR α 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 PPAR α /RXR α 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.

PPAR α 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 PPAR α 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 mPPAR α trans-repressive activity, it is unknown whether human PPAR α 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 Ψ KxD/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 hPPAR α is conjugated with SUMO-1 in vitro, in Cos-7 cells and in the human hepatoma cell line HuH-7. In addition, hPPAR α directly interacts in vitro with the E2-conjugating enzyme Ubc9. Furthermore, we demonstrate that the E3-ligating enzyme PIASy regulates hPPAR α SUMO-1 conjugation. The SUMOylation site of human PPAR α 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 hPPAR α suggesting that SUMOylation of this lysine reduces hPPAR α trans-activity, which is explained by a facilitated recruitment of the corepressor NCoR, but not SMRT, upon hPPAR α SUMOylation. We also demonstrate that the SUMO pathway specifically decreases NCoR-specific hPPAR α target gene expression. Finally, we demonstrate that the hPPAR α ligand GW7647 reduces hPPAR α SUMOylation.

EXPERIMENTAL PROCEDURES

Materials

DMEM and fetal calf serum (FCS), glutamine and gentamycin 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-(³⁵S)-Methionine was purchased from Amersham Pharmacia Biotech (Saclay, France). The pSG5-hPPAR α and J6-TK-Luc were described previously (8). The pSG5-hRXR α , 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-hPPAR α K₁₃₈R, K₁₈₅R, K₂₁₆R, K₃₁₀R, K₃₅₈R, K₄₄₉R expression vectors were generated using pSG5-hPPAR α 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 \times 10⁴ cells per well), were transfected with 10 ng of J6-TK-Luc, 100 ng of pSV- β -galactosidase, 5 ng of pSG5-hPPAR α (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 hours, cells were incubated in medium containing 0.2 % fetal calf serum; 0.2 % free fatty acid BSA (Sigma, Saint-Quentin Fallavier, France) and Me₂SO or 600 nM of GW7647 (kindly provided by GlaxoSmithKline). After 24 hours, cells were lysed with 100 μ L 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 PPAR α (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'-GAAGUUUGCGCCUCAUAA-3', 5'-GGCCAGCCAUCACAAUCA-3' and 5'-GAACCACCAUUUUUACCC-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 μ g GST, GST-Ubc9 were incubated with 4 μ L [³⁵S]-methionine hPPAR α 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-PPAR α and anti-actin antibodies were obtained from Tebu-Bio (Le Perray-en-Yvelines, France). The anti-His₆ 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 denaturing conditions using 6M-guanidine hydrochloride. His-SUMO-conjugated proteins were recovered with Ni²⁺-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 ³⁵S-methionine-labeled PPAR α 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 μ g 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 PPAR α 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 hPPAR α . To investigate this hypothesis, HuH-7 cells were transfected either with siRNA for NCoR or SMRT (figure 1). After RNA purification, the expression of hPPAR α 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 hPPAR α target genes, whereas HMGCOAS2 is a SMRT-regulated hPPAR α target gene.

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

Human PPAR α interacts with the SUMO E2-conjugating enzyme Ubc9

We first assessed direct association of hPPAR α with the SUMO E2-conjugating enzyme Ubc9 by GST pull-down. GST-Ubc9 WT was incubated with in vitro translated ³⁵S-labeled hPPAR α protein. As shown in figure 2B , PPAR α interacts with GST-Ubc9 but not with GST alone indicating that hPPAR α interacts directly with Ubc9 in vitro .

Human PPAR α is a substrate for SUMO-1 modification in vitro and in vivo

While this study was ongoing, it has been previously shown that murine PPAR α is SUMOylated (26). No data are however available concerning human PPAR α . SUMOplot™ prediction algorithm analysis identified six putative SUMOylation sites (K138, K185, K216, K310, K358, K449) in human PPAR α , which are conserved between species (figure 2A). In order to examine whether hPPAR α can be SUMOylated in vitro , ³⁵S-labeled hPPAR α 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 , hPPAR α is SUMOylated in the presence of SUMO-1 WT, but not the SUMO-1 mutant.

In order to examine whether hPPAR α is SUMOylated in a cellular context, HuH-7 hepatoma cells were transfected with hPPAR α WT and SUMO1-His₆ expression vectors (figure 2D). Histidine-tagged SUMOylated proteins were then isolated from whole HuH-7 cell extracts using Ni-NTA beads. When SUMO1-His₆ was co-expressed with hPPAR α , an additional high molecular weight band (72 kDa) corresponding to SUMO-1-conjugated hPPAR α was observed (figure 2D) suggesting that hPPAR α is mono-SUMOylated in hepatic cells.

SUMOylation of hPPAR α is ligand-regulated

The presence of ligand has been shown to regulate the SUMOylation of several nuclear receptors such as PPAR γ (26). Hence, we investigated the effect of the PPAR α -specific ligand GW7647 on the SUMO-1 modification of hPPAR α . After transfection of HuH-7 cells with hPPAR α WT and SUMO1-His₆ expression vectors, the cells were treated with vehicle (Me₂SO) or GW7647 and SUMOylated PPAR α was specifically analysed by western blotting using anti-PPAR α antibodies. As shown in figure 2E , SUMOylation of hPPAR α 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-His₆ antibodies. As shown in figure 2F , GW7647 does not modify the amount of other SUMOylated proteins, suggesting that PPAR α ligand GW7647 does not modulate the SUMOylation machinery in a general manner.

PIASy acts as E3 ligating enzyme for hPPAR α 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 ROR α (28). Hence, we investigated the potential role of PIASy in the SUMOylation of hPPAR α . In contrast to HuH-7, transfection of SUMO-1 or both SUMO-1/Ubc9 in Cos-7 cells does not result in SUMOylation of hPPAR α protein (figure 3A). However, when cells were co-transfected with hPPAR α WT, SUMO1-His₆ , Ubc9 and FLAG-PIASy expression vectors (figure 3A), SUMO1-modified hPPAR α was found demonstrating that PIASy can function as an E3 ligating enzyme leading to the SUMOylation of hPPAR α .

SUMOylation of hPPAR α decreases its trans-activation activity

In order to define the role of the SUMO pathway on hPPAR α 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 hPPAR α WT expression vector and increasing amounts of PIASy (figure 3B). The activity of hPPAR α decreased when PIASy was co-transfected, demonstrating that the SUMO pathway regulates human PPAR α trans-activation.

The SUMO pathway inhibits NCoR-specific hPPAR α target gene expression

To address the role of hPPAR α SUMOylation on the trans-activation activity of its target genes, HuH-7 cells were transfected with siRNA Ubc9 and/or siRNA hPPAR α . The expression of different PPAR α 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 hPPAR α on the observed effect of Ubc9 knockdown, we analysed the expression of these genes in cells cotransfected with both siRNA for PPAR α and Ubc9. Our results show that the impact of siRNA Ubc9 on L-CPT1 and PDK4 expression is abolished in presence of siRNA PPAR α , suggesting that the regulation of L-CPT1 and PDK4 gene expression by SUMOylation is mediated by hPPAR α . Conversely, overexpression of PIASy significantly decreased the expression of target genes such as L-FABP and PDK4 induced by hPPAR α (figure 4D–F), suggesting that activation of the SUMO machinery, and subsequent hPPAR α SUMOylation, inhibits hPPAR α 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 hPPAR α target genes in a hPPAR α -dependent manner.

Human PPAR α is SUMOylated on lysine 185

To identify the SUMOylated site in hPPAR α , 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 hPPAR α 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 hPPAR α K₁₈₅R, indicating that hPPAR α 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 hPPAR α WT and K185R in Cos-7 cells (figure 5B). The cells were transfected with PPAR α and SUMO-1, Ubc9 and PIASy expression vectors and SUMOylated PPAR α proteins were analysed by western blotting after 48 hours. The significant reduction of the signal corresponding to the PPAR α K185R protein compared to the WT protein confirms that the lysine 185 in hPPAR α is a SUMO-1 acceptor site.

The lysine 185 is a relevant functional site in the regulation of hPPAR α transcriptional activity

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

Human PPAR α 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 PPAR γ and LXR (24,25). In addition, the hinge region of hPPAR α 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 hPPAR α WT or hPPAR α K₁₈₅R proteins were investigated.

A mammalian one-hybrid assay was performed by transfecting HuH-7 cells with the J6-TK-Luc reporter vector, the hPPAR α WT or hPPAR α K₁₈₅R 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 hPPAR α WT transcription activity more pronouncedly than hPPAR α K₁₈₅R, indicating that the NCoR protein interacts with hPPAR α WT with a higher efficiency than hPPAR α K₁₈₅R.

We also assessed the role of SUMOylation in the functional interaction between hPPAR α and NCoR by co-transfecting HuH-7 cells with the J6-TK-Luc reporter vector, hPPAR α WT or hPPAR α K₁₈₅R, and with increasing amounts of NCoR full-length expression vector (figure 7B). In order to appreciate both hPPAR α WT and hPPAR α K₁₈₅R sensitivity to NCoR independently of the difference between their respective transcriptional activities, the transcriptional activity of both hPPAR α WT and hPPAR α 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 hPPAR α WT was decreased in a dose-dependent manner by NCoR co-transfection whereas the transcriptional activity of hPPAR α K₁₈₅R was unaffected by the co-expression of NCoR (figure 7B), showing that the hPPAR α K₁₈₅R mutant is less sensitive to a decrease in activity by NCoR co-transfection compared to hPPAR α WT protein. To reinforce these results, HuH-7 cells were cotransfected with a flagged-hPPAR α WT or K₁₈₅R 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 PPAR α WT, the SUMOylation-defective hPPAR α K₁₈₅R 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 hPPAR α SUMOylation was assessed on the interaction between the corepressor SMRT and hPPAR α (figure 8). For that purpose, HuH-7 cells were transfected with the J6-TK-Luc reporter vector, the hPPAR α WT or hPPAR α K₁₈₅R, and increasing amounts of VP16-AD or VP16-SMRT vectors. By contrast to NCoR (figure 7), increasing amounts of SMRT similarly activated WT and K₁₈₅R hPPAR α proteins, indicating that the K₁₈₅R mutation in hPPAR α has no effect on the interaction of hPPAR α with the corepressor SMRT (figure 8A).

Additionally, in order to examine the impact of hPPAR α SUMO-conjugation on its functional interaction with SMRT, HuH-7 cells were transfected with reporter vector, hPPAR α WT or K₁₈₅R, 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 hPPAR α WT and hPPAR α K₁₈₅R was decreased to a similar extent by SMRT. Altogether, these data show that the hPPAR α K₁₈₅R protein is still sensitive to repression by SMRT but not to NCoR.

DISCUSSION

In this report, we show that hPPAR α 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 hPPAR α binds to the E2-conjugating enzyme Ubc9 providing evidence that PPAR α is able to interact directly with SUMO pathway components. Therefore, we investigated whether hPPAR α is SUMOylated and whether this SUMOylation could be involved in the regulation of hPPAR α target gene expression by NCoR.

Our results show that inhibition of the SUMO pathway, by knocking-down the E2 conjugating enzyme Ubc9, increases the hPPAR α target genes L-FABP and PDK4. Interestingly, expression of the hPPAR α target gene HMGCOAS2 was not changed under similar conditions suggesting that the SUMOylation pathway regulates some, but not all, PPAR α target genes. Altogether, these results suggest that the selective recruitment of NCoR by SUMO-modified hPPAR α leads to the inhibition of a subset of hPPAR α 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 PPAR α is SUMOylated. In the SUMOylation assays, only one band with a higher molecular weight (72 kDa), corresponding to the size of mono SUMOylated hPPAR α , 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 hPPAR α K₁₈₅R 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 hPPAR α *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 PPAR γ (26), Androgen Receptor (29) or Aryl hydrocarbon Receptor (30). Interestingly, we show in figure 5A that K₁₃₈R and K₂₁₆R hPPAR α 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 PPAR α 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 PPAR γ (32, 33). We have previously shown that Protein Kinase C can phosphorylate hPPAR α 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 hPPAR α protein.

The mutant hPPAR α K₁₈₅R is transcriptionally more active. Conversely, SUMO E3 ligase PIASy-overexpression in HuH-7 cells decreases transcriptional activity of hPPAR α 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 hPPAR α K₁₈₅R we showed that SUMOylation of hPPAR α promotes NCoR recruitment without influencing the binding to SMRT. These data suggest that hPPAR α SUMOylation helps discriminating among the interactions with different corepressors. Accordingly, over-expression of full-length NCoR inhibits transcriptional activity of PPAR α WT without changing the transcriptional activity of hPPAR α K₁₈₅R, whereas overexpression of SMRT inhibits transcriptional activity of both hPPAR α WT and hPPAR α K₁₈₅R. Such differential recruitment of corepressors has already been observed with the nuclear receptor LXR alpha (LXR α), which preferentially recruits either NCoR and SMRT, depending on the target genes (16).

Human PPAR α 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 PPAR β/δ requires SENP1 and various co-regulators to activate gene promoters in response to ligand (34). In contrast to the human protein, mPPAR α SUMOylation is not significantly affected by the presence of the PPAR α ligand Wy-14,643 in NIH3T3 cells (19).

However, mice treated with this ligand show increased SUMOylated PPAR α , suggesting that the ligand could act on PPAR α SUMOylation in a cell type-selective manner.

In conclusion, this study provides the first evidence of SUMOylation of hPPAR α 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 hPPAR α 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 hPPAR α target genes. Finally, our work provides further evidence of the relevance of the hPPAR α hinge region in the regulation of corepressor recruitment.

Acknowledgements:

We express our thanks to A. Dejean, M. Schulz, P. Lefebvre, K. Tabech for their kind gifts of plasmids. We are also grateful to P. Lefebvre, B. Lefebvre, K. Tabech and R. Robillard for their helpful discussions and experimental advices.

This work was supported by grants from Region Nord-Pas de Calais/FEDER, Fondation Cœur et Artères and European Vascular Genomics Network

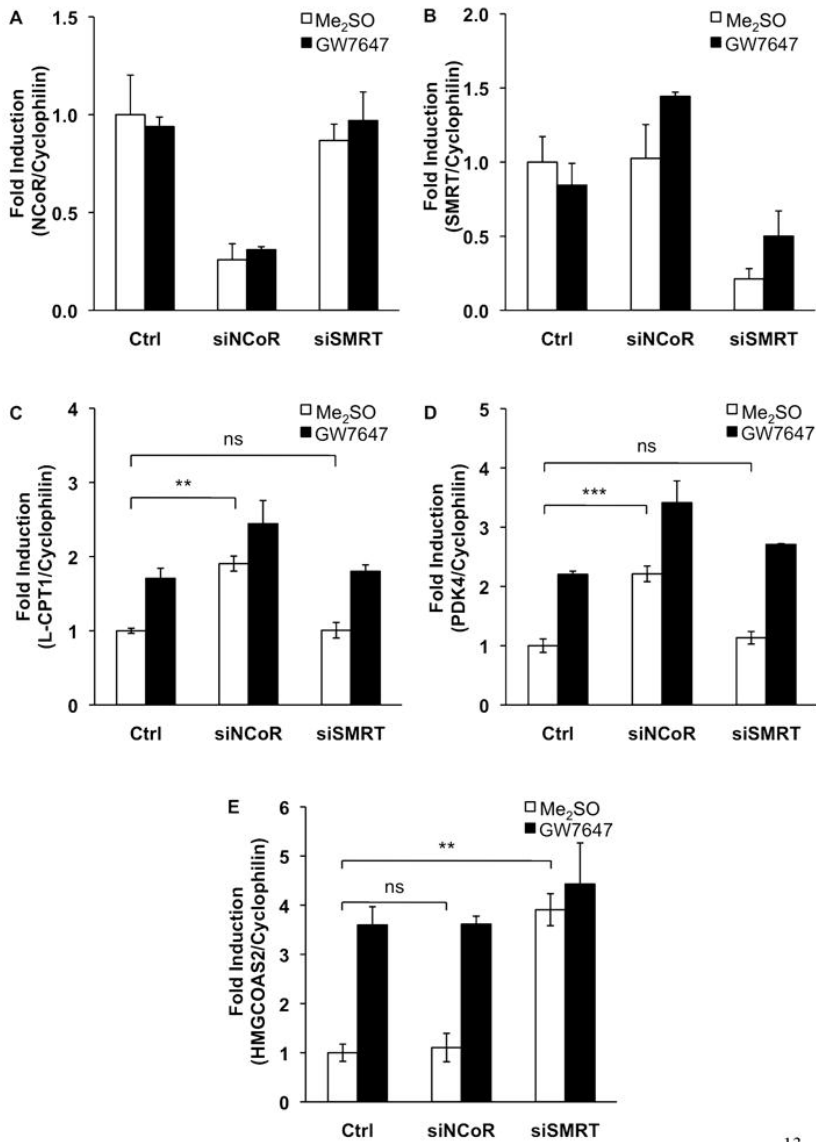
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Figure 1

Selective effect of decreased expression of NCoR and SMRT on PPAR α target genes in HuH7 cells

HuH-7 cells were transfected with control, NCoR or SMRT siRNA and treated with GW7647 (600 nM) or vehicle (Me₂SO). 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 \pm SD of triplicate determinations. Statistically differences are indicated (t-test; Scramble vs siRNA Me₂SO: **p<0.01; ***p<0.001; ns: non significant).



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Figure 2

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

A . Protein sequence of hPPAR α (NM Q07869) was analysed with the SUMOylation prediction site algorithm SUMOplotTM 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** . [³⁵S]-methionine hPPAR α protein was incubated with GST or GST-Ubc9 proteins. Complexes were precipitated with glutathion-sepharose and proteins were analysed by autoradiography **C** . [³⁵S]-methionine hPPAR α WT protein or [³⁵S]-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-hPPAR α WT expression vector and/or pSG5-SUMO-1-His₆ expression vectors. After 24 hours, cells are lysed in denaturing 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-PPAR α antibodies. **E** . HuH-7 cells were transfected with pSG5 control vector or pSG5-hPPAR α WT expression vector, and with pSG5-SUMO1-His₆ expression vectors. Cells were treated with Me₂SO 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-His₆ expression vector and treated with GW7647 for 24 hours. SUMOylated proteins were analysed by western blotting using an anti-His₆ antibodies.

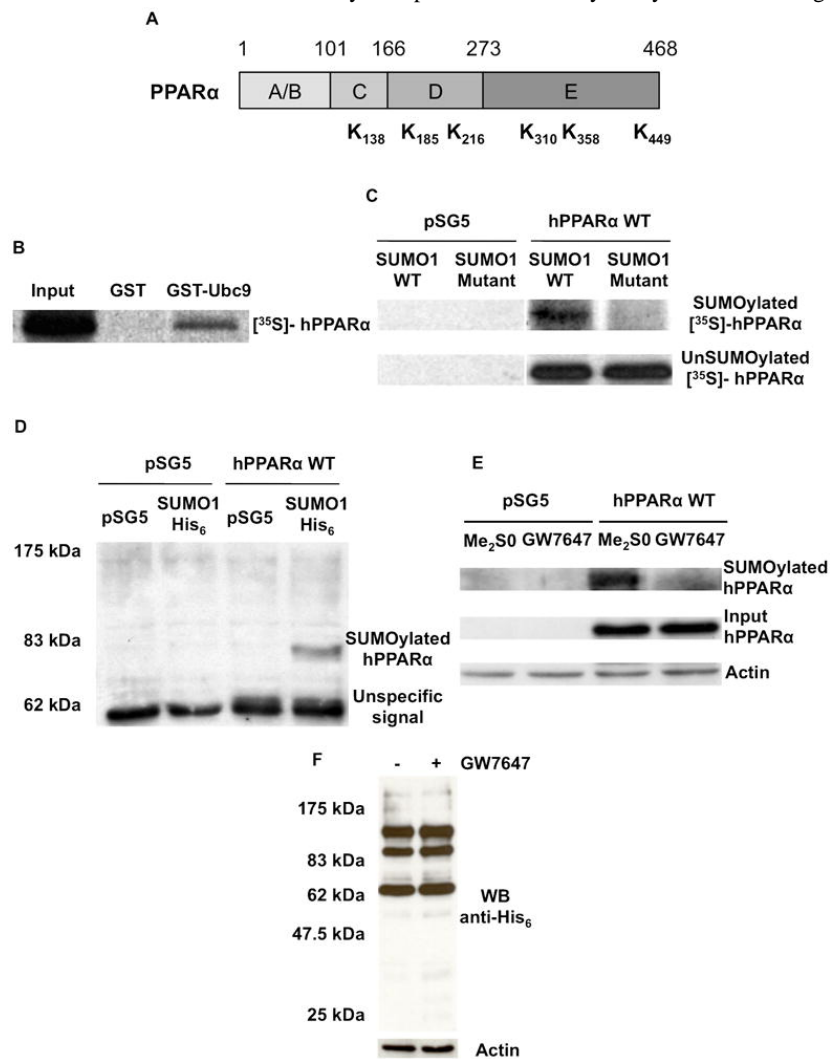


Figure 3

PIASy regulates SUMOylation of human PPAR α and inhibits its transcriptional activity

A . Cos-7 cells were cotransfected with pSG5 control vector or pSG5-hPPAR α WT expression vector and with pSG5-SUMO1-His₆ , 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 hPPAR α proteins and hPPAR α input proteins were analysed by western blotting using anti-PPAR α antibodies. The SUMO1-His₆ input proteins were analysed by western blotting using anti-His₆ antibodies. **B** . HuH-7 cells were cotransfected with pSG5 control vector, pSG5-hPPAR α 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 \pm SD of triplicate determinations.

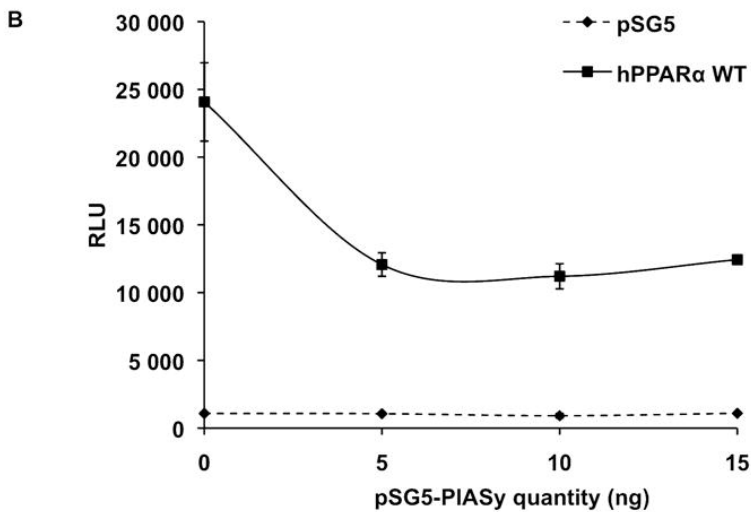
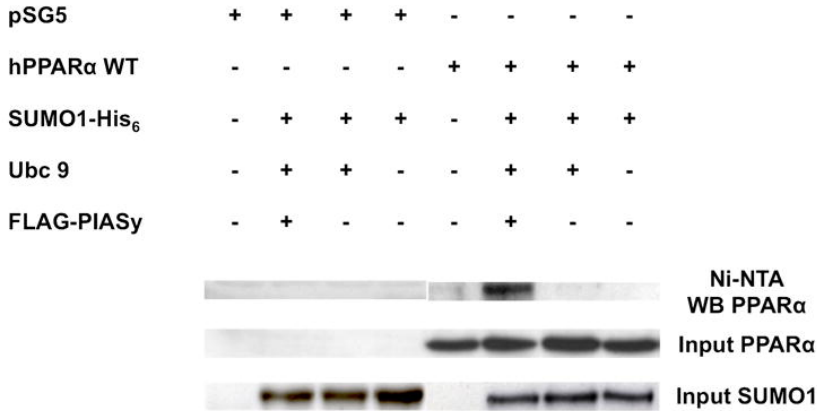
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Figure 4Effect of SUMO pathway modulation on PPAR α target genes in HuH-7 cells

HuH-7 cells were transfected with siRNA Ubc9 and/or siRNA hPPAR α . 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-hPPAR α 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 Me₂SO 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 \pm SD of triplicate determinations. Statistically differences are indicated (t-test; Control vs PIASy: *p<0.05; **p<0.01; ns: non-significant)

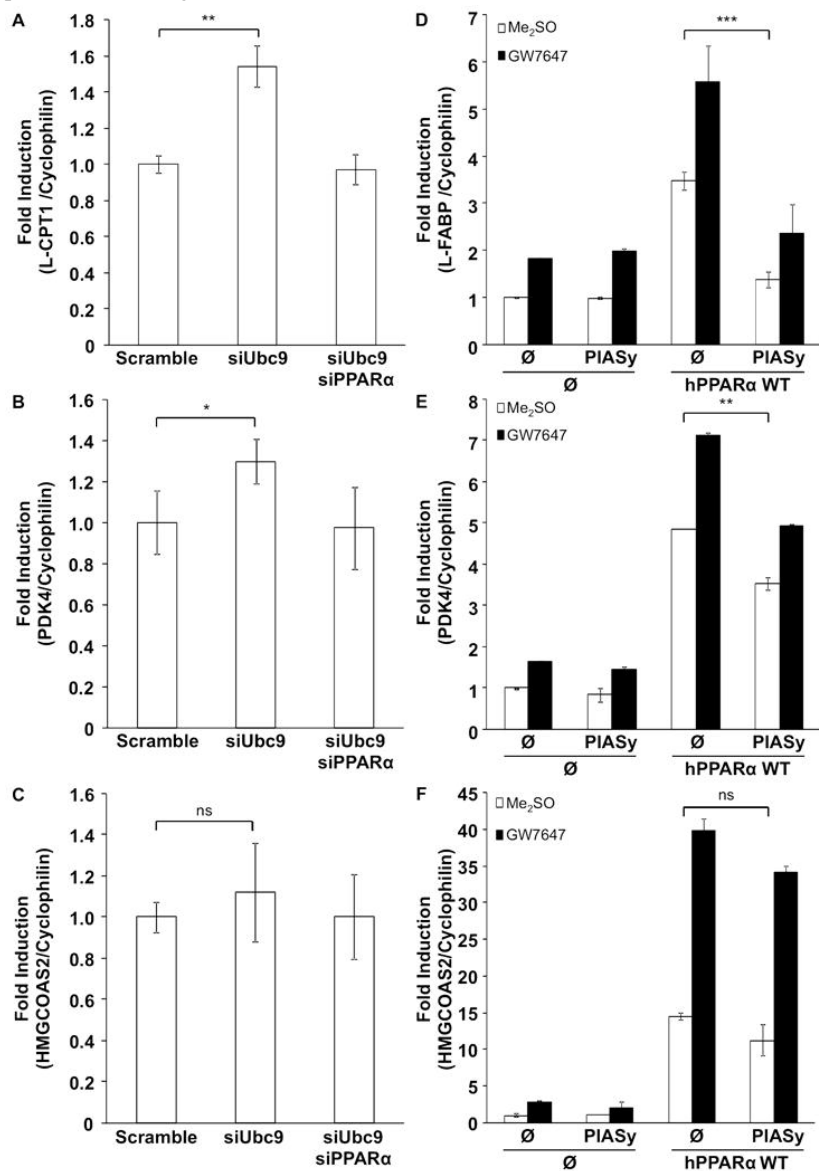
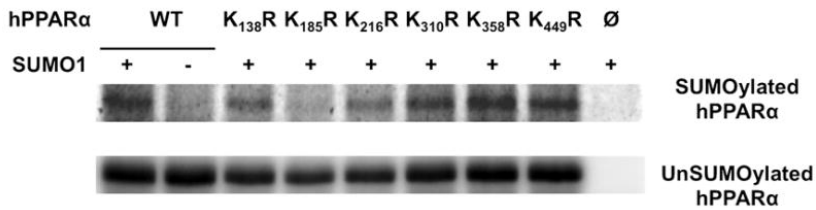


Figure 5

SUMOylation of hPPAR α occurs on lysine 185

A. In vitro translated [35 S]-methionine hPPAR α WT, hPPAR α K $_{138}$ R, hPPAR α K $_{185}$ R, hPPAR α K $_{216}$ R, hPPAR α K $_{310}$ R, hPPAR α K $_{358}$ R, hPPAR α 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-hPPAR α WT or pSG5-hPPAR α 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 hPPAR α proteins and hPPAR α input proteins were analysed by western blotting using anti-PPAR α antibodies. The SUMO1-His $_6$ input proteins were analysed by western blotting using anti- His $_6$ antibodies.

A



B

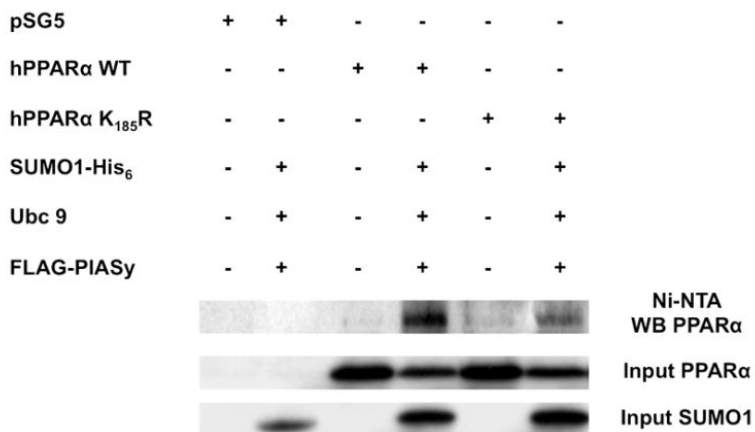
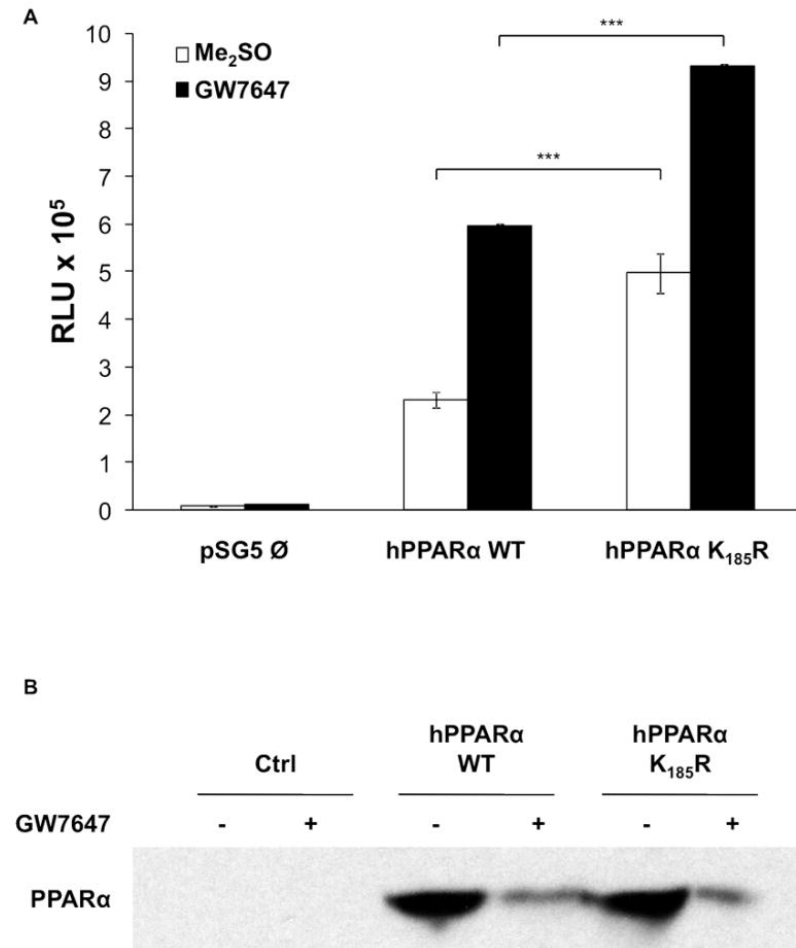


Figure 6

The transcriptional activity of hPPAR α K₁₈₅R is increased compared to hPPAR α WT

A . HuH-7 cells were transfected with the pSG5 control vector, or the pSG5-hPPAR α WT or pSG5-hPPAR α K₁₈₅R expression vectors, with the pSV- β -galactosidase, with the reporter vector J6-TK-Luc. After 24 h of transfection, cells were treated with Me₂SO 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. PPAR α protein amounts were evaluated by western blotting. Each bar is the mean value \pm SD of triplicate determinations. **B** . PPAR α protein from transfection assay cell lysates was analysed by western blotting using anti-PPAR α antibodies.

**Figure 7**

The hPPAR α K₁₈₅R mutant displays a lower physical and functional interaction with NCoR compared with hPPAR α WT

A . HuH-7 cells were transfected with the J6-TK-Luc, with pSV- β -galactosidase, with the pSG5 control vector, or the pSG5-hPPAR α WT or pSG5-hPPAR α 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 \pm SD of triplicate determinations. Statistically differences are indicated (t-test; without VP16-NCoR vs with VP16-NCoR: **p<0.01, ***p<0.001; hPPAR α WT vs hPPAR α 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-hPPAR α WT or pSG5-hPPAR α K₁₈₅R 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 \pm 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-hPPAR α WT or K185R expression vectors and pKR2-NCoR. Flagged proteins were immunoprecipitated and associated NCoR proteins were analysed by western blotting.

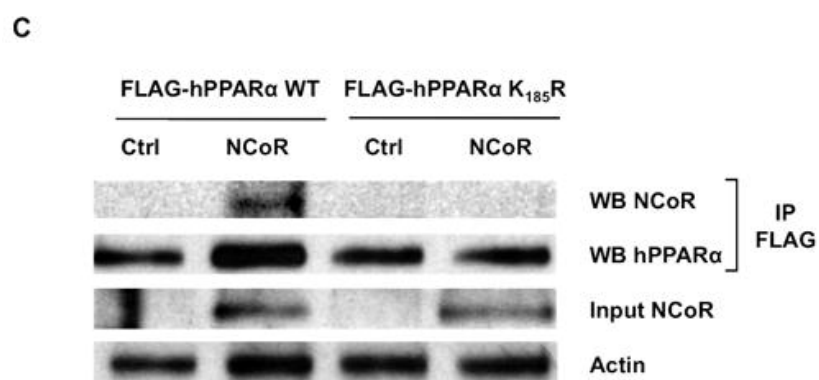
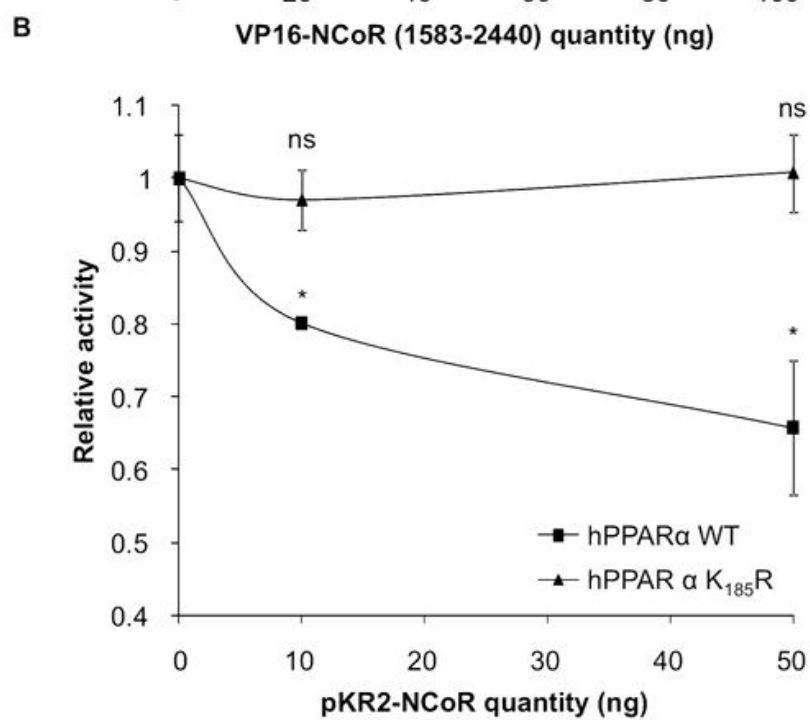
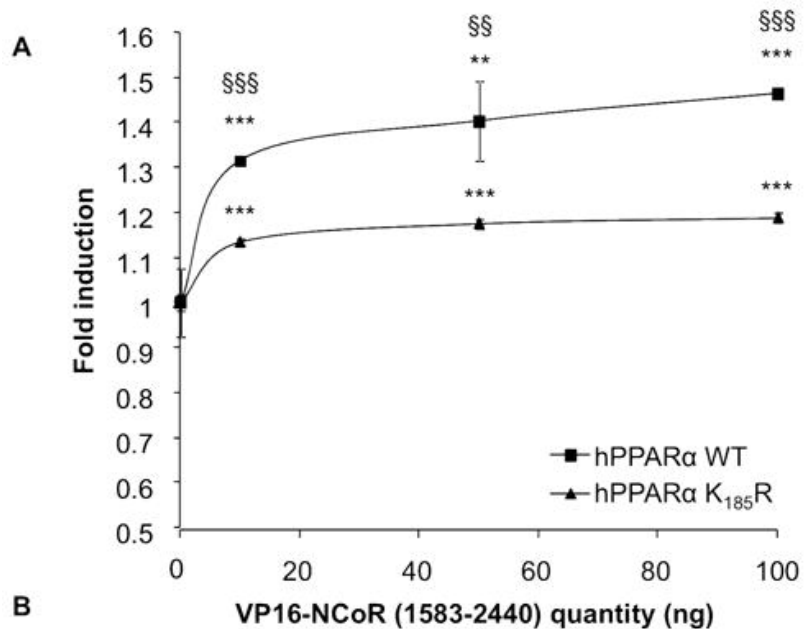


Figure 8

The hPPAR α WT and hPPAR α 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-hPPAR α WT or pSG5-hPPAR α 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 \pm 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-hPPAR α WT or pSG5-hPPAR α 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 to their respective pCI control curves. Results are expressed as relative inhibition. Each bar is the mean value \pm SD of triplicate determinations. pCI curves are not represented.

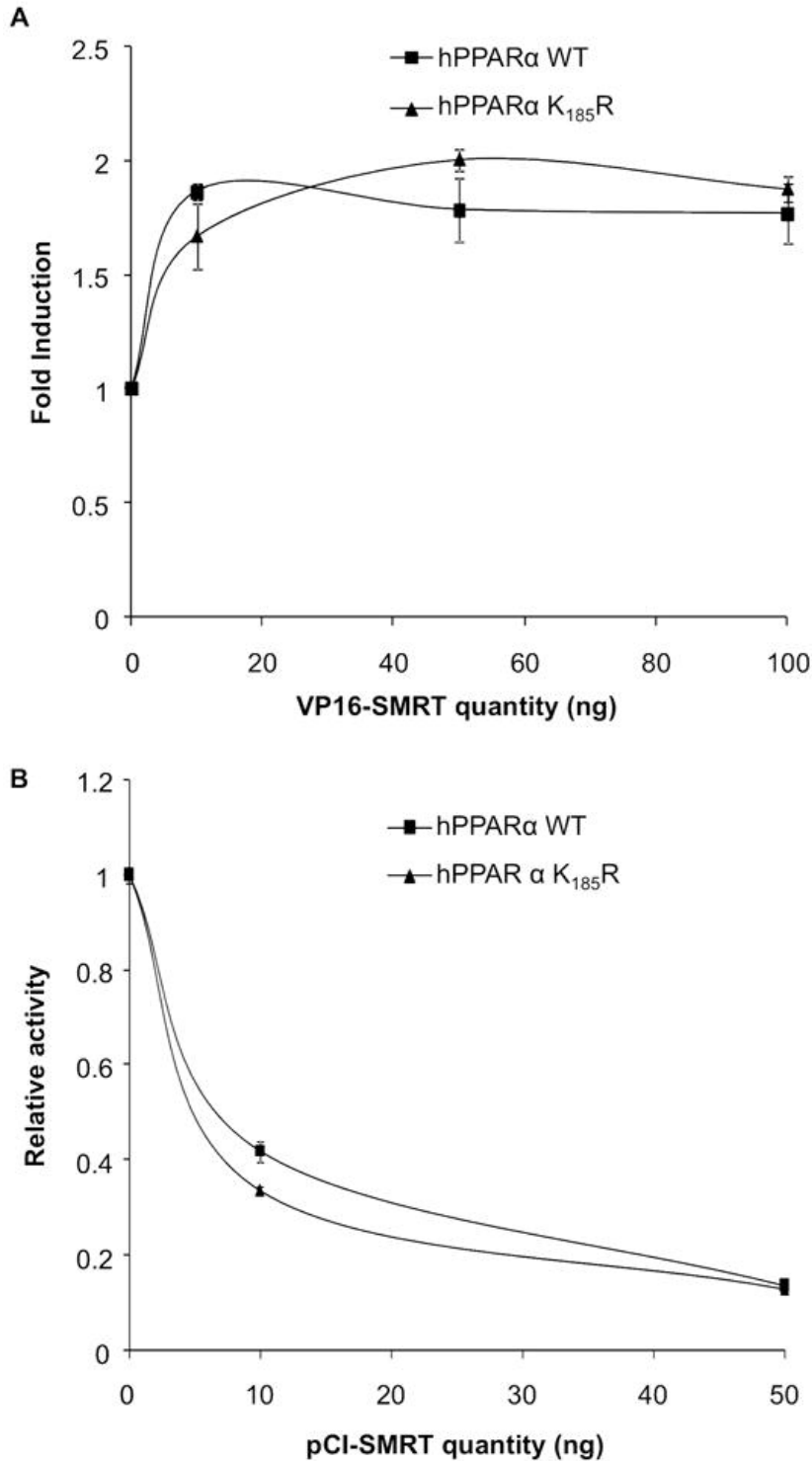


Figure 9

Comparison of the SUMOylation consensus site in mouse, rat and human PPAR α

PPAR α 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|PPAR $\alpha$  MOUSE      170 AIRFGMRPSEKAKLKAEILTCEHDLKDSET 200
sp|P37230|PPAR $\alpha$  RAT       170 AIRFGMRPSEKAKLKAEILTCEHDLKDSET 200
sp|Q07869|PPAR $\alpha$  HUMAN    170 AIRFGMRPSEKAKLKAEILTCEHDIEDSET 200
*****:*****
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