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A sumoylation-dependent pathway mediating transrepression of inflammatory response genes by PPARγ

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Abstract

The peroxisome proliferator-activated receptor γ (PPARγ) plays essential roles in adipogenesis and glucose homeostasis and is a molecular target of insulin-sensitizing drugs[1–3]. Although the ability of PPARγ agonists to antagonize inflammatory responses by transrepression of nuclear factor kappaB (NF-κB) target genes is linked to anti-diabetic[4] and antiatherogenic actions[5], the mechanisms remain poorly understood. Here we report the identification of a molecular pathway by which PPARγ represses transcriptional activation of inflammatory response genes in macrophages. The initial step of this pathway involves ligand-dependent sumoylation of the PPARγ ligand-binding domain, which targets PPARγ to nuclear receptor co-repressor (NCoR)/histone deacetylase-3 (HDAC3) complexes on inflammatory gene promoters. This in turn prevents recruitment of the ubiquitylation/19S proteosome machinery that normally mediates the signal-dependent removal of corepressor complexes required for gene activation. As a result, NCoR complexes are not cleared from the promoter and target genes are maintained in a repressed state. This mechanism provides an explanation for how an agonist-bound nuclear receptor can be converted from an activator of transcription to a promoter-specific repressor of NF-κB target genes that regulate immunity and homeostasis.

NCoR, and the related factor, silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), are components of corepressor complexes containing HDAC3, transducin beta-like protein-1 (TBL1) and TBLR1 that interact with a subset of unliganded nuclear receptors, mediating active transcriptional repression[6–12]. Ligand-dependent dismissal of these complexes requires Ubc5-dependent ubiquitylation and proteosomal degradation, with Tblr1 functioning as an essential E3 ligase[13]. Recent studies indicate that NCoR/SMRT complexes are also required for basal repression of a subset of NF-κB and AP-1 target genes[13–15], with loss of NCoR resulting in a partially activated phenotype in macrophages[14]. We noted that a number of inflammatory response genes that are de-repressed in NCoR-deficient macrophages are also subject to transrepression by PPARγ agonists, suggesting a possible role of NCoR in this process.

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We focused on the inducible nitric oxide synthase (*iNOS*) gene as a model because it is one of several inflammatory response genes expressed by macrophages that is strongly induced by lipopolysaccharide (LPS)[16] and negatively regulated by PPARγ agonists[17]. Inhibition of NCoR expression using an NCoR-specific siRNA validated for efficacy[13] (FigS1) resulted in a complete reversal of *iNOS* transrepression by synthetic ligands, rosiglitazone, and additionally, GW0072[18] (Fig. 1a). Consistent with these findings, knockdown of NCoR expression, but not SMRT expression, resulted in a reversal of repression of an *iNOS* promoter reporter by PPARγ in RAW264.7 macrophages (Fig. S2a). Potential roles of NCoR-associated HDACs were supported by the finding that treatment with 10 nM of the histone deacetylase inhibitor, Trichostatin A, reversed rosiglitazone-dependent transrepression of *iNOS* (Fig. S2b). To specifically evaluate the role of HDAC3, the effect of a validated HDAC3-specific pool of siRNAs (Fig. S1) was tested in RAW264.7 cells. The HDAC3-specific siRNAs, but not control siRNAs directed against HDAC7, reversed the transrepression observed on the *iNOS* promoter in this system (Fig. S2c).

These observations predicted that NCoR/HDAC3/TBL complexes should associate with the *iNOS* promoter. Chromatin immunoprecipitation (ChIP) experiments confirmed that NCoR, HDAC3, TBL1 and TBLR1 were present on the *iNOS* promoter under basal conditions and that the NCoR and HDAC3 components cleared following LPS stimulation (Fig. 1b). However, in cells treated with rosiglitazone or GW0072, both NCoR and HDAC3 remained on the *iNOS* promoter after LPS stimulation (Fig. 1b). Because signal-dependent induction of NF-κB target genes has been suggested to require removal of NCoR complexes through Ubc5-dependent ubiquitylation[13], we utilized validated siRNAs directed against TBL1, TBLR1 and the ubiquitin conjugating enzyme Ubc5c[35], all of which inhibited *iNOS* induction in response to LPS (Fig.1c). These data are consistent with the hypothesis that the TBL1/TBLR1-recruited ubiquitylation complex is required for LPS-dependent clearance of NCoR and HDAC3 from the *iNOS* promoter, and that PPARγ represses *iNOS* activation by preventing TBL1/TBLR1-dependent corepressor clearance.

We next evaluated whether an ordered sequence of events was required for NCoR clearance in response to LPS stimulation in the presence or absence of rosiglitazone by ChIP assay. While NCoR was cleared from the *iNOS* promoter within 10 minutes of LPS induction, pretreatment of cells with rosiglitazone inhibited clearance at all time-points tested (Fig. 1d). Significantly, Ubc5 was rapidly recruited to the *iNOS* promoter following LPS stimulation in the absence of rosiglitazone, but was not recruited to the promoter in its presence (Fig. 1d). These results suggest that PPARγ acts to repress LPS induction of the *iNOS* gene by preventing recruitment of the Ubc5/19S proteosome machinery required for the clearance of NCoR and HDAC3.

Evaluation of both the *iNOS* promoter and the positively-regulated *CD36* promoter[19] in macrophages revealed that PPARγ was recruited to both promoters in a ligand-dependent manner (Fig. 1e). As expected, the p65 component of NF-κB was recruited exclusively to the *iNOS* promoter in response to LPS, which was not affected by rosiglitazone treatment (Fig. 1e). The recruitment of PPARγ to the *iNOS* promoter did not involve sequence-specific DNA binding because a PPARγ mutant (PPARγ$^{C126A/E127A}$) containing amino acid substitutions in the DNA binding domain that abolish binding to PPARγ response elements and its recruitment to the *CD36* promoter, was efficiently recruited to the *iNOS* promoter (Fig.S3a). This is consistent with previous studies of PPARγ$^{C126A/E127A}$ indicating that it does not activate positive PPARγ target genes but retains transrepression activity[20]. Ligand-dependent interaction of PPARγ with the *iNOS* promoter was abolished by siRNA-mediated knockdown of NCoR, indicating that NCoR is required for PPARγ recruitment (Fig. 1f). Similar results were obtained for four additional LPS-inducible, PPARγ-sensitive promoters; *Ccl3, Ccl7, Cxcl10* and *Tgtp,* (Fig. S3b,c)[14].

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The observation that ligand-dependent recruitment of PPARγ to LPS-responsive promoters required NCoR raised a paradox, because the binding of ligand disrupts direct interactions between NCoR and PPARγ[18]. To identify PPARγ-interacting proteins that might potentially resolve this paradox, a yeast two-hybrid screen was performed using a library constructed from mRNA derived from primary macrophages. One of the clones isolated in this screen encoded the initial 208 amino acids of PIAS1 (protein inhibitor of activated STAT1), initially identified as a suppressor of interferon-dependent transcription[21] and now known to belong to a family of sumo E3 ligases[22]. The region of PIAS1 isolated in this screen (referred to as PIAS1-N, Fig. S4a) contains motifs previously shown to interact with various nuclear receptors[23–25]. The interaction between PPARγ and the PIAS1 clone was confirmed both by yeast survival and α-Galactosidase liquid assays (Fig. S4b). Furthermore, co-immunoprecipitation experiments using antibodies directed against either endogenous or epitope-tagged PPARγ and PIAS1, demonstrated a basal interaction in RAW264.7 cells or in primary macrophages that was modestly enhanced by treatment with rosiglitazone (Fig. 2a).

Sumoylation of transcription factors has previously been correlated with impaired transcriptional activation and/or transcriptional repression[26–28]. The PIAS1-N fragment dominantly inhibited PPARγ-dependent transrepression of the *iNOS* promoter in RAW264.7 cells (Fig. S4c), suggesting a role of PIAS1 or other PIAS proteins in this process. To specifically evaluate the consequences of loss of PIAS1 expression, transfection of validated PIAS1 siRNAs (Fig.S1) resulted in significant inhibition of PPARγ-dependent repression of the *iNOS* promoter, similar to the effects observed for the NCoR siRNA (Fig. 2b), but did not impair transcriptional activation of a positively regulated PPARγ target gene (data not shown). Moreover, siRNA-mediated knockdown of PIAS1 in primary macrophages abolished PPARγ transrepression of the endogenous *iNOS* gene (Fig. 2c). Finally, knockdown of Ubc9, the sumoylation pathway rate-limiting E2 ligase, significantly impaired PPARγ-dependent transrepression of *iNOS* in both RAW264.7 cells and primary macrophages (Fig. S5a and Fig. 2c). These results suggest that PIAS1/Ubc9-mediated sumoylation is required for PPARγ-dependent transrepression.

ChIP assays were next performed in macrophages to determine the roles of PIAS1 and Ubc9 in ligand-dependent recruitment of PPARγ to the *iNOS* promoter and prevention of NCoR clearance. Knockdown of PIAS1 expression abolished recruitment of PPARγ to the *iNOS* promoter, but did not affect recruitment to the positively-regulated *CD36* promoter (Fig. 2d). In addition, knockdown of PIAS1 or Ubc9 prevented the ability of rosiglitazone to retain NCoR on the *iNOS* promoter in the presence of LPS (Fig. 2e).

Sumoylation of the PPARγ2 AF1 domain at K107 (equivalent to K77 of PPARγ1) inhibits ligand-dependent activation of positively-regulated target genes[29]. Examination of the primary amino acid sequence of murine PPARγ revealed an additional sumoylation consensus sequence ψKXE/D[30] at K365 (corresponding to K367 in the human PPARγ sequence, Fig. 3a). Intriguingly, crystal structures of the apo and rosiglitazone-bound forms of PPARγ indicated that the primary amine group of K365 was oriented towards the interior of the ligand-binding domain (LBD) in the apo form, but solvent exposed in the rosiglitazone-bound form (Fig. 3a). Because this amino group is the point of covalent attachment of sumo, the PPARγ crystal structures suggested that K365 could be sumoylated in a ligand-dependent manner. To test this hypothesis, K365 of PPARγ was mutated to arginine and the wild type and mutant proteins tested for sumoylation *in vivo* and *in vitro.* Wild type PPARγ, but not PPARγ^{K365R} exhibited a significant enhancement in sumoylation following treatment with rosiglitazone (Fig. 3b and data not shown)

To determine the functional consequences of K77- and K365-dependent sumoylation, the ability of each mutant to inhibit the *iNOS* promoter and/or transactivate a positively regulated

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PPARγ-dependent promoter were tested in RAW264.7 cells. PPARγ^{K365R} was defective for inhibition of the *iNOS* promoter, while PPARγ^{K77R} retained full transrepression activity (Fig. 3c). PPARγ^{K77R} exhibited enhanced transactivation function, consistent with previous findings[29], while PPARγ^{K365R} exhibited approximately the same activity as wild-type PPARγ on the positively regulated Aox-TK luciferase promoter (Fig. 3d). ChIP assays indicated that wild-type PPARγ and PPARγ^{K77R} were efficiently recruited to the *iNOS* promoter in response to rosiglitazone, while PPARγ^{K365R} was not (Fig. 3e). In contrast, wild-type PPARγ and each of the PPARγ mutants were recruited to the positively regulated *CD36* promoter (Fig. 3f).

Mammalian two-hybrid assays were used to explore effects of sumoylation on interactions of PPARγ with NCoR and HDAC3. Previous studies demonstrated that unliganded PPARγ binds to one of two nuclear receptor interaction domains in the extreme C-terminus of NCoR termed IDC and that this interaction is reversed by ligand[18]. Mammalian two-hybrid assays confirmed this interaction and demonstrated that knocking down expression of PIAS1 or Ubc9 did not influence ligand-dependent dissociation of PPARγ from IDC (Fig 4a). In contrast, PPARγ exhibited a ligand-dependent increase in interaction with an NCoR deletion mutant lacking IDC that was abolished by knocking down Ubc9 or PIAS1 (Fig. 4a, Fig. S5b). Mammalian two-hybrid assays also demonstrated an interaction of PPARγ with HDAC3, but this interaction was only modestly affected by ligand or by knockdown of PIAS1 or Ubc9 (Fig.S5c). Consistent with these results, knockdown of HDAC3 reduced, but did not prevent, recruitment of PPARγ to the *iNOS* promoter in response to ligand as determined by ChIP assays (Fig. 4b). These results suggest that NCoR is required for ligand-dependent recruitment of sumoylated PPARγ to the *iNOS* promoter and that HDAC3 plays a quantitative role in stabilizing this interaction.

The present studies define sequential steps of a pathway mediating ligand-dependent transrepression of inflammatory response genes by PPARγ in macrophages (Fig. 4c). Genes subject to transrepression by this pathway are marked in the basal state by the presence of NCoR/HDAC3/TBL corepressor complexes. LPS signaling results in the clearance of the NCoR and HDAC3 components of this complex in a TBL1-, TBLR1- and Ubc5-dependent manner, allowing a switch from active repression to transcriptional activation. The PPARγ-dependent transrepression pathway is initiated by ligand-induced sumoylation of the ligand-binding domain. This modification targets PPARγ to NCoR complexes associated with the promoter, preventing Ubc5 recruitment in response to LPS signals. As a result, NCoR complexes are not cleared from the promoter and target genes are maintained in a repressed state. Intriguingly, allosteric changes in the PPARγ LBD required for entry into the sumoylation-dependent transrepression pathway are distinct from changes that regulate interactions with conventional coregulators. It will be of interest to define the extent to which this pathway is utilized by PPARγ and other nuclear receptors and to explore how this mechanism can be exploited to develop new drugs for treatment of inflammatory and metabolic diseases.

Methods

Plasmids and cell culture

Primary macrophages were elicited by intra-peritoneal injection with 2ml of thioglycollate. HA-tagged PPARγ WT and mutants at K77R and K365R were cloned into the pcDNA3 backbone (Invitrogen). WT-PIAS1 and PIAS1-N were cloned into a 2xFLAG-pcDNA3 expression vector. PPARγ bait used for yeast two-hybrid including the DNA-binding domain, hinge region and ligand binding domain (PPARγ DHL) was inserted into the pGBK7 vector (Clontech). For RNAi experiments, smart-pool siRNAs (Dharmacon) against PIAS1, Ubc9, HDAC3, HDAC7 or control non-specific and previously validated NCoR were transected

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using lipofectamine 2000 (Invitrogen) into primary macrophages and incubated for 48h. Effects of these siRNAs on cellular protein levels are illustrated in Figure S1.

Yeast two hybrid screen

Yeast two-hybrid library was generated in the pGAD vector (Clontech) with RNA derived from primary peritoneal macrophages elicited from normal and hypercholesterolemic mice. The library was transformed into the AH109 yeast strain and was mated to Y187 strain transformed with PPARγ DHL bait. Colonies were picked 4–6 d post mating. PCR inserts were amplified and sequenced. Yeast plasmids were purified from individual clones. Finally, interactions were verified by α-galactosidase activity in yeast liquid culture assays.

Transient transfection

The RAW264.7 mouse macrophage cell line was transiently transfected with *iNOS* or Aox-TK promoters directing luciferase expression as previously described [17]. For transrepression experiments, wild-type PPARγ or PPARγ mutants were transfected at a 3:1 ratio to reporter plasmids using Superfect reagent (Qiagen). For siRNA experiments, RAW264.7 cells were transfected with siRNAs (40 nM) using Superfect reagent for 48 h prior to activation with PPARγ ligands and LPS induction (6h). In all transfections, cells were treated with 0.1μM rosiglitazone and stimulated with 1 μg/ml LPS and luciferase activity assayed 6 h later. For mammalian two-hybrid based assays, 200ng of UAS-TK luciferase reporter and 100ng each of VP-16 PPARγ WT and GalDBD NCoR constructs were transfected in RAW264.7 cells. Cells were cultured in 0.01μM TSA prior to ligand treatment for 16 h. Transfection experiments evaluated each experimental condition in triplicate and results were expressed as mean +/− standard deviation. Each transfection experiment was independently repeated at least three times. Statistical analysis was preformed using Student's t-test with $P<0.01$ considered statistically significant.

Chromatin immunoprecipitation assays

ChIP assays were performed as previously described[13, 14]. 2–4x10^6 primary macrophages or RAW 264.7 cells were used per experimental point. Cells were pre-treated with 0.1μM Ro (1 h) and stimulated with 1 μg/ml LPS (1h) prior to crosslinking for 10 min with 1% formaldehyde. For PPARγ, H-100 (Santa Cruz Inc.) plus PPARγ antibody #39338 (Active Motif) were used in combination. Antibodies against Tbl1 and TblR1 were described previously[13, 14]. Anti-HA protein A sepharose beads (Covance) were used for wild-type and mutant HA-tagged PPARγ proteins. HDAC3 and p65 antibodies were from SantaCruz. NCoR antibody was from Affinity Bioreagents, Inc. A 150bp region of the *iNOS* promoter was amplified spanning the most proximal NF-κB site to the start of transcription[16]. A 150bp region of the mouse CD36 promoter was amplified spanning the PPRE sequence[19].

RNA isolation, semi-quantitative PCR and Nothern blot analysis

Total RNA (Trizol method) was prepared from primary macrophages pre-treated with 1 μM Ro (2h) prior to 1 μg/ml LPS stimulation (6h). 1μg of total RNA was used for cDNA synthesis and 2μl of cDNA was used for PCR using *iNOS* or inflammatory gene specific primers. For Northern blot analysis, ten μg of total RNA and an *iNOS*-specific probe were utilized.

Co-immunoprecipitations and Western blotting

For co-immunoprecipitations, RAW264.7 cells or 293 cells were transfected using Superfect reagent with HA-PPARγ WT and 2xFLAG-PIAS1 WT in 10 cm dishes. Whole cell extracts (WCEs) were prepared using WCE lysis buffer: 10 mM Tris-HCl pH 8, 420 mM NaCl, 1 mM EDTA and 0.5% NP-40 with protease inhibitor cocktail (Roche Bochem.). Immunoprecipitates were washed 4 times with Wash Buffer containing 10 mM Tris-HCl pH8, 100 mM NaCl, 1

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mM EDTA, 0.5% NP-40 and 0.5% Triton X-100 followed by boiling in 1x sample loading buffer and 10% SDS-PAGE. M2 anti-flag antibody was used at 1:1000 dilution (Sigma). HA immunoblotting was carried out at 1:1000 dilution (Covance). Antibodies utilized for endogenous immunoprecipitation and western blot for PPARγ/PIAS1 interaction experiment were obtained from Active Motifs and Santa Cruz, Inc respectively.

Sumoylation Assays

For *in vivo* sumoylation experiments, 250 μg of total protein extracts were prepared form HeLa cells transfected with HA-tagged PAPRγ WT or sumo point mutants: K77R and K365R, and Myc-tagged SUMO-1. Cell lysates were immunoprecipitated and washed 4 times in lysis buffer containing 0.1% SDS, 0.5% deoxycholate, 0.5% TritonX-100, 1 mM EDTA, 20 mM Tris-HCl pH 7.8 and 150 mM NaCl. Immunoprecipitates were resolved by SDS PAGE and immuno-blotted for anti-HA or anti-Myc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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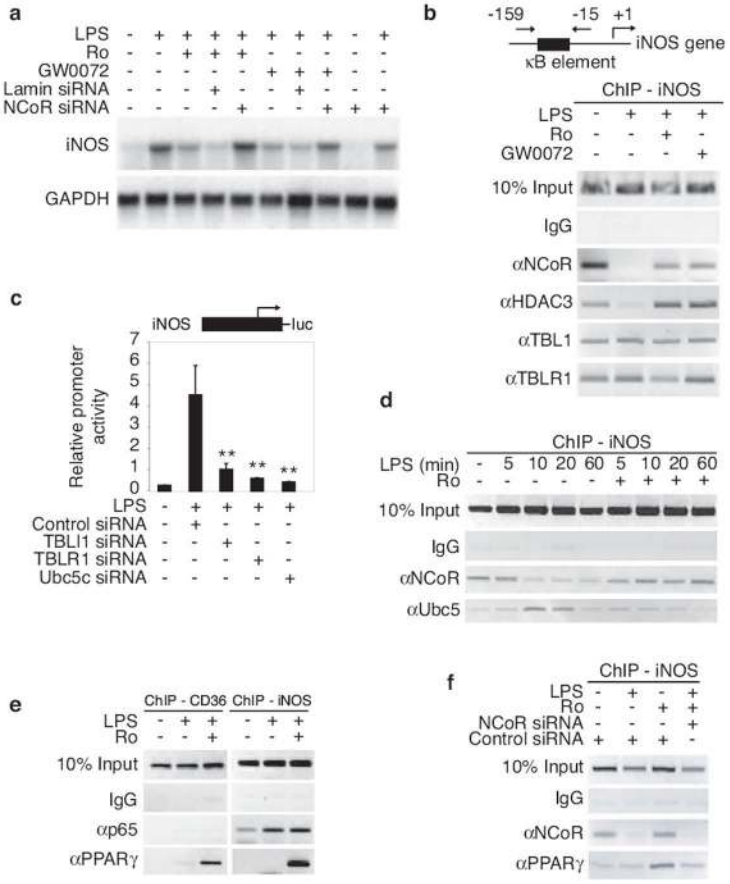

Figure 1.

PPARγ prevents LPS-induced dissociation of the NCoR/HDAC3 complex from the *iNOS* promoter. **a,** Northern blot analysis indicating that siRNAs directed against NCoR abolish rosiglitazone (Ro) and GW0072-dependent repression of LPS-induced *iNOS* expression. **b**, Ro and GW0073 inhibit release of NCoR from the *iNOS* promoter as demonstrated by ChIP assay. **c**, siRNAs directed against TBL1, TBLR1 or Ubc5c prevent LPS induction of the *iNOS* promoter in transiently transfected RAW264.7 macrophages. Error bars in this and succeeding figures represent standard deviations of triplicate, independent determinations. *indicates $p<0.001$ compared to Ro repression; ** refers to $p<0.01$ compared to LPS induction. **d,** Rosiglitazone prevents LPS-dependent recruitment of Ubc5 to the *iNOS* promoter as detected by ChIP assay. **e,** PPARγ binds to the *CD36* and *iNOS* promoters in a ligand-dependent manner as detected by ChIP assay. **f,** Knockdown of NCoR expression prevents PPARγ recruitment to the *iNOS* promoter as detected by ChIP assay.

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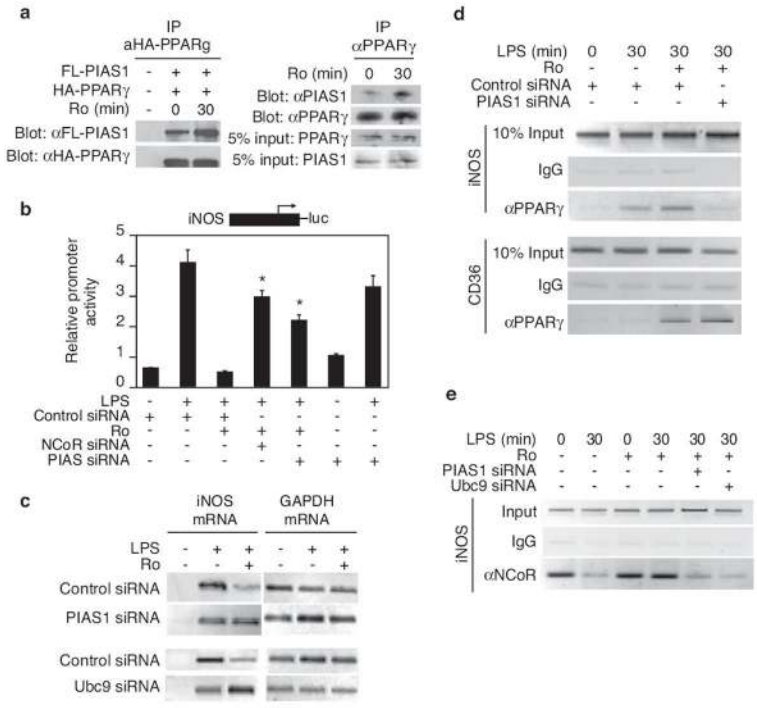

Figure 2.

PIAS1 interacts with PPARγ and is required for transrepression of *iNOS*. **a**, Immunoblots for FLAG-tagged PIAS1 and HA-tagged PPARγ in transfected RAW264.7 macrophages (left panel) or endogenous PIAS1 and PPARγ in primary macrophages (right panel) indicate PIAS1 interaction with immunoprecipitated PPARγ. **b**, siRNAs directed against NCoR and PIAS1 in RAW264.7 macrophages reverse PPARγ transrepression of the *iNOS* promoter. **c,** Primary macrophages transfected with control siRNA, PIAS1 or Ubc9-specific siRNA show reversal of PPARγ transrepression by semi-quantitative PCR for endogenous *GAPDH* and *iNOS* expression. **d,** PPARγ recruitment to the *iNOS* but not the *CD36* promoter in primary macrophages requires PIAS1 as detected by ChIP assays. **e**, PPARγ recruitment to the *iNOS* promoter in primary macrophages requires PIAS1 and Ubc9 as detected by ChIP assays.

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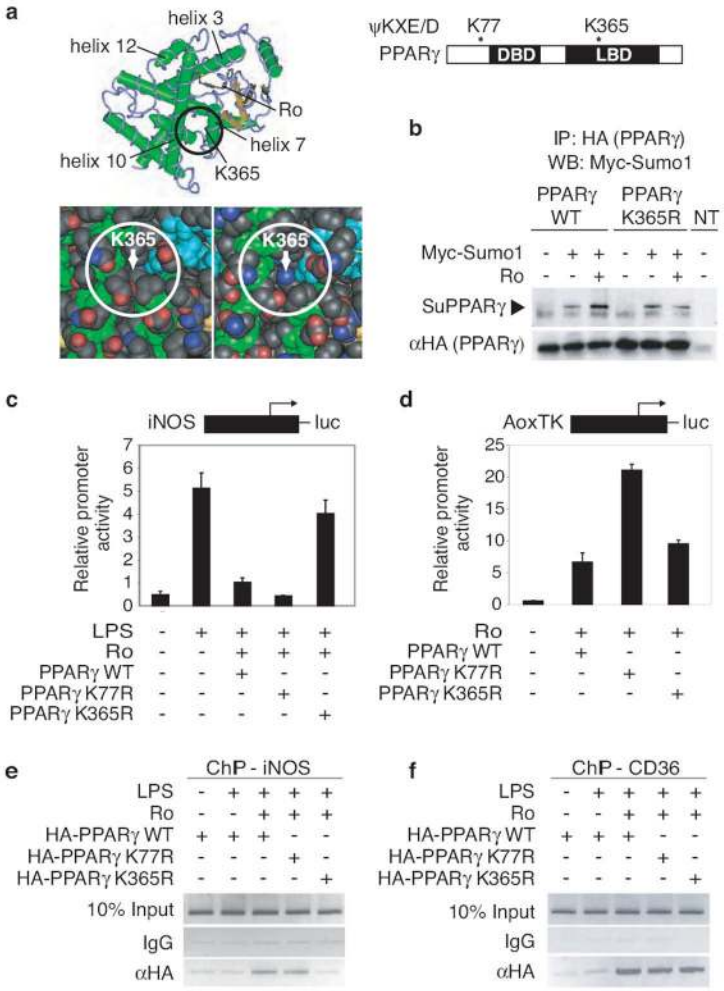

Figure 3.

Ligand-dependent sumoylation of PPARγ is required for transrepression. **a**, Upper right panel shows a schematic representation of PPARγ with two consensus sumoylation sites at K77 and K365. Upper left panel illustrates the PPARγ ligand binding domain, indicating the location of K365 in helix 7. The lower panels illustrate a close-up of this region of the apo PPARγ ligand-binding domain (LBD) surface (left), with the arrow pointing to a hydrogen atom in K365 side chain and liganded PPARγ LBD (right) with the arrow pointing to solvent-exposure of the nitrogen atom of the primary amine of K365 (shown in blue). **b**, Sumoylation of PPARγ at K365 occurs in a ligand-dependent manner. NT refers to non-transfected cells. **c**, **d**, Sumoylation of PPARγ at K365 but not K77 is required for transrepression of the *iNOS* promoter but not transactivation of the AoxTK promoter in transfected RAW264.7 macrophages. **e**, **f**, Sumoylation of PPARγ at K365 is required for recruitment to the *iNOS* promoter but not the *CD36* promoter in RAW264.7 cells as demonstrated by ChIP assays.

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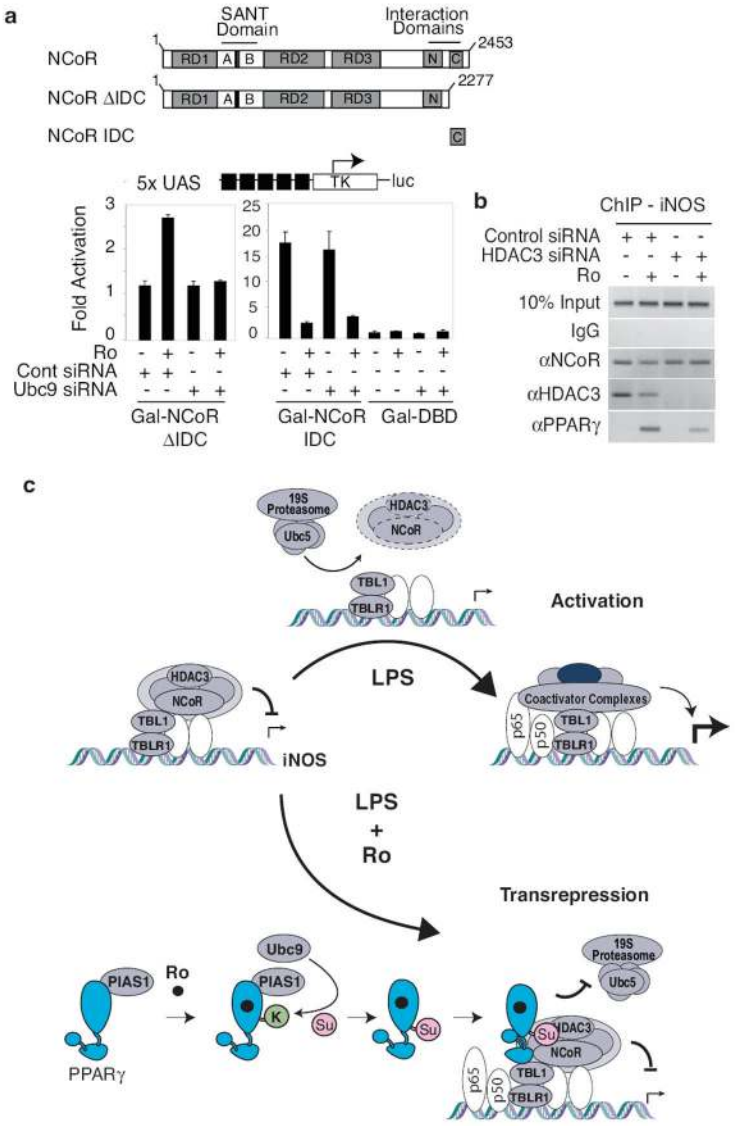

Figure 4.

Sumoylation of PPARγ promotes interaction with the NCoR/HDAC3 complex. **a**, Mammalian two hybrid assay in RAW264.7 cells indicating that VP-16 PPARγWT interacts with GalDBD-NCoR ΔIDC (NCoR a.a. 1-2277 without IDC) but not GalDBD-NCoR IDC (C-terminal nuclear receptor interaction motif) in a ligand-dependent and Ubc9-dependent manner. **b**, siRNAs directed against HDAC3 reduce but do not abolish recruitment of PPARγ to the *iNOS* promoter in primary macrophages as demonstrated by ChIP assays. **c**, Model for mechanisms of LPS activation and PPARγ-dependent repression of the *iNOS* gene. See text for details.