

Supporting Information

for

Heterodimer formation with retinoic acid receptor RXR α modulates coactivator recruitment by peroxisome proliferator-activated receptor PPAR γ

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Running title: PPAR γ activation in the context of the heterodimer with RXR α

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SI method 1: LBD pulldown

In order to verify proper dimer formation capability of the majority of the protein preparation a pulldown experiment was performed. Therefore, small centrifugal filter columns (nylon membrane with 0.2 μM pore size; VWR) were loaded with 20 μl of Pierce® high capacity streptavidin agarose beads (Thermo Scientific) equilibrated in HTRF buffer [25 mM HEPES pH 7.5, 150 mM KF, 10% (w/v) glycerol, 5 mM DTT (fresh)], then 20 μl of a mixture of biotin coupled RXR α LBD (4 μg) and the respective PPAR LBD (1.4 μg) was applied. After incubation for 10 min at room temperature followed by centrifugation for 2 min and 900 x g the input mixture as well as the flowthrough was analyzed via SDS PAGE. Gels were scanned using an Li-Cor Odyssey imager (LI-COR® Biosciences GmbH, Germany). Missing bands in comparison to the input control were interpreted in two different ways. For RXR α it showed that the streptavidin beads were now coated with the biotin-labeled RXR α LBD. In the case of the PPAR proteins a missing band was evidence of intact LBD:LBD dimer formation between RXR α and the particular PPAR LBD.

SI method 2: Isothermal titration calorimetry

The capability of the RXR α -LBDs to bind the agonist SR11237 was verified via isothermal titration calorimetry (ITC) conducted on an TA Instruments Affinity ITC (TA Instruments, New Castle, Delaware, USA). Recombinant (tag-free) RXR α LBD wt or mutant (120 μM) and SR11237 (10 μM) were dissolved in HTRF buffer [25 mM HEPES pH 7.5, 150 mM KF, 10% (w/v) glycerol, 5 mM DTT (fresh)] supplemented with 1% DMSO (v/v). The ITC instrument was adjusted to 25°C and the stirring rate was set to 75 rpm. Compound solution was filled into the reaction cell (178 μl cell volume) and a solution with one of the RXR α LBDs was titrated (inverse titration). The first injection had a reduced volume of 0.5 μL and was followed by 25 injections of 2.0 μL . An interval of 300 s was maintained between individual injections. The heats of dilution resulting from titrating one of the RXR α LBD proteins into the cell containing only buffer were recorded separately and subtracted from the raw ITC data obtained with compound. Data were analyzed using the NanoAnalyze software package (version 3.7.5). In order to fit reaction enthalpy (ΔH), binding affinity constant (K_d), and stoichiometry (n) an independent binding model was used. Free energy change (ΔG) was calculated using the equation

$$\Delta\text{G} = -RT \cdot \ln K$$

and from there the entropy (ΔS) was calculated from

$$\Delta\text{G} = \Delta\text{H} - T\Delta\text{S}$$

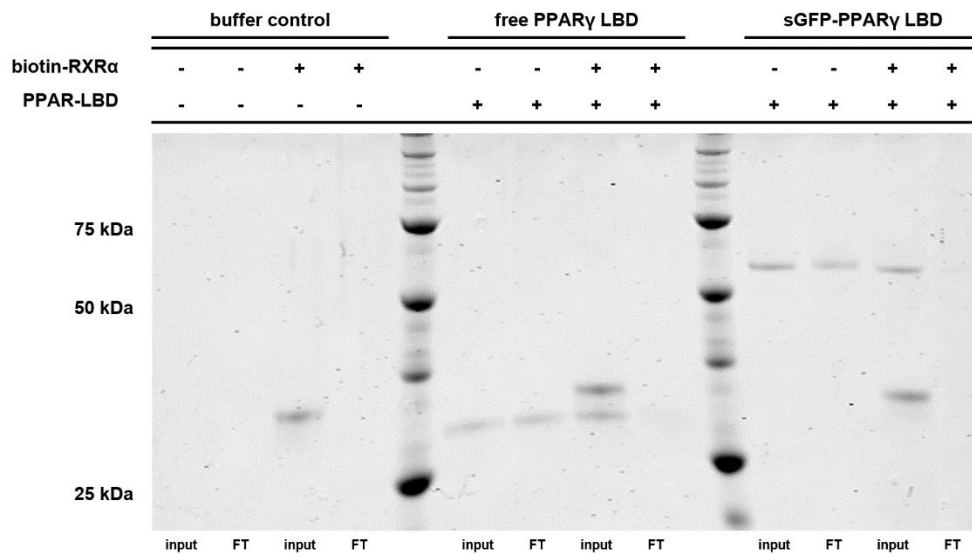


Figure S1. Validation of intact dimer formation capability of PPAR γ protein preparations via pull-down assays. Preparations of both free PPAR γ LBD and sGFP-PPAR γ LBD were tested in a pull-down experiment using RXR α coated streptavidin-agarose beads. Comparison of the input and the flowthrough (FT) demonstrates the retention of the respective PPAR γ LBD through formation of the heterodimer complex with RXR α . Controls prove both proper coating of the streptavidin-agarose beads with the biotin coupled RXR α LBD, and no unspecific binding of the PPAR γ LBDs to the column material.

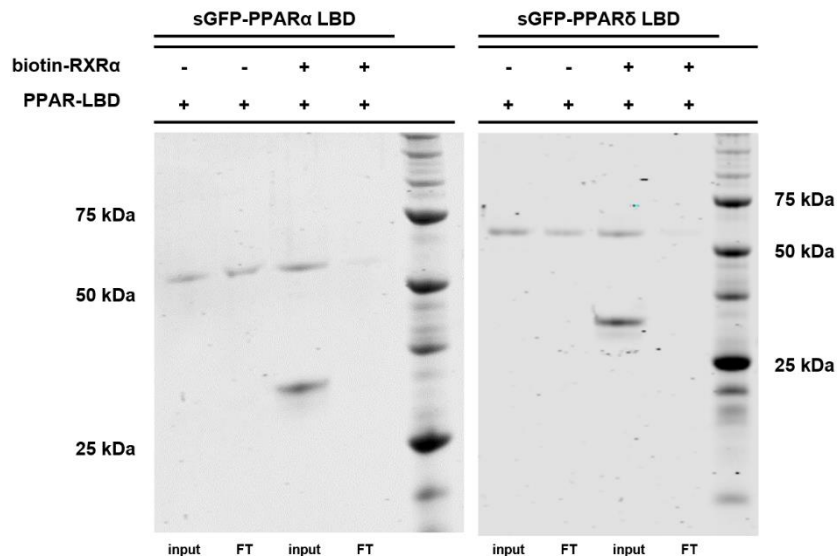
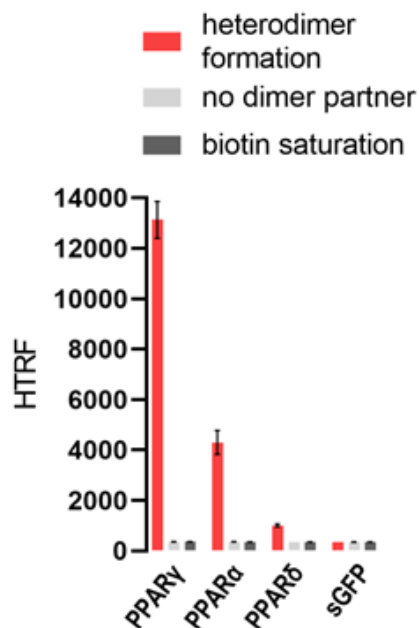
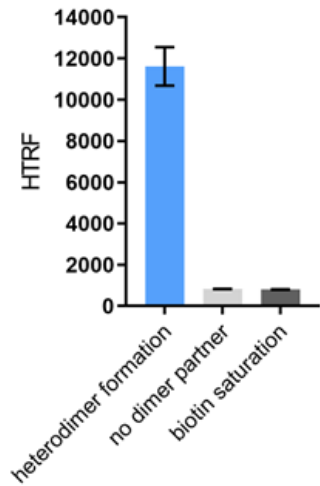


Figure S2. Validation of intact dimer formation capability of PPAR α and PPAR δ protein preparations via pull-down assays. Preparations of both sGFP-PPAR α LBD, and sGFP-PPAR δ LBD were tested in a pull-down experiment using RXR α coated streptavidin-agarose beads. Comparison of the input and the flowthrough (FT) demonstrates the retention of the respective PPAR LBD through formation of the heterodimer complex with RXR α . Controls prove both proper coating of the streptavidin-agarose beads with the biotin coupled RXR α LBD and no unspecific binding of the PPAR LBDs to the column material.



	hetero dimer formation	no dimer partner	biotin saturation
sGFP-PPAR LBD	+	+	+
biotin labeled RXR α LBD	+	-	+
Tb-SA	+	+	+
1.2 μ M biotin	-	-	+

Figure S3. Control experiments for heterodimer formation assays with titrated sGFP-PPAR LBD. LBD of RXR α was labeled with biotin via an N-terminal Avi-tag and coupled to Tb-SA (12 nM RXR α and 12 nM Streptavidin monomers, respectively). 12 nM of the respective sGFP-PPAR LBD was present in all experiments. Binding of sGFP-PPAR to RXR α results in close proximity to Tb-SA and an increase in HTRF in comparison to controls. In samples with biotin saturation the coupling of RXR α to Tb-SA was blocked with an 100-fold molar excess of biotin. This uncoupled heterodimer formation from the recruitment of sGFP into a complex with Tb-SA. HTRF signals of these controls were comparable to controls with no RXR α being present (no dimer partner). Data are the mean \pm SD; N = 4.



	hetero dimer formation	no dimer partner	biotin saturation
sGFP-RXR α LBD	+	+	+
biotin labeled PPAR γ LBD	+	-	+
Tb-SA	+	+	+
1.2 μ M biotin	-	-	+

Figure S4. Control experiments for heterodimer formation assays with titrated sGFP-RXR α LBD. LBD of PPAR γ was labeled with biotin via an N-terminal Avi-tag and coupled to Tb-SA (12 nM PPAR γ and 12 nM Streptavidin monomers, respectively). 12 nM of sGFP-RXR α LBD was present in all experiments. Binding of sGFP-RXR α to PPAR γ results in close proximity to Tb-SA and an increase in HTRF in comparison to controls. When the coupling of PPAR γ to Tb-SA was blocked with an 100-fold molar excess of biotin this uncoupled heterodimer formation from the recruitment of sGFP into a complex with Tb-SA. The HTRF signal of this control was comparable to a second control with no PPAR γ being present (no dimer partner). Data are the mean \pm SD; N = 3.

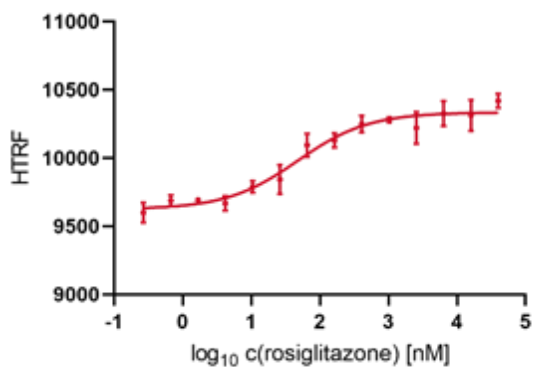


Figure S5. Ligand dependent recruitment of coactivator CBP-1 by biotin labeled PPAR γ LBD coupled to Tb-SA. PPAR γ LBD was labeled with biotin via an N-terminal Avi-tag and subsequently coupled to streptavidin labeled with Terbium cryptate (Tb-SA). The latter was utilized as the FRET donor fluorophore. Reference agonist rosiglitazone was titrated against 100 nM fluorescein (FITC) coupled CBP-1 cofactor peptide (CREB-binding protein; coactivator motif 1; FITC-AASKHKQLSELLRGGSGSS; ThermoFisher; PV4596), 12 nM biotin coupled PPAR γ LBD and 12 nM Tb-SA. HTRF measurements were performed after 1 h incubation at room temperature. Reader settings were the same as described for experiments with sGFP instead of FITC serving as the FRET acceptor. Data are the mean \pm SD; N = 3. R² equals 93 %. The EC₅₀ for rosiglitazone was determined at 48 ± 11 nM.

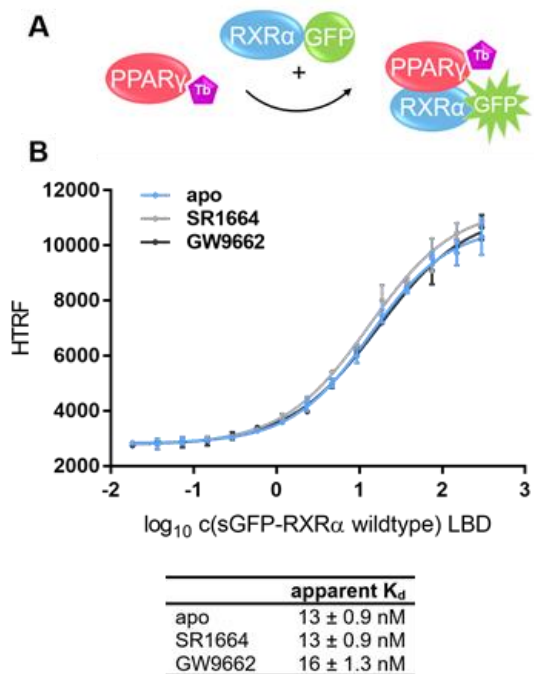


Figure S6. Effect of PPAR γ antagonists on PPAR γ :RXR α heterodimer formation. A, schematic representation of the heterodimer formation assay with PPAR γ LBD being coupled to the FRET donor. B, sGFP-RXR α LBD was titrated against 0.375 nM biotinylated PPAR γ LBD and 0.75 nM Tb-SA with either 1 μ M PPAR γ irreversible antagonist GW9662 (black), non-agonist SR1664 (grey) or no ligand at all (light blue). HTRF measurements were performed after 1 h incubation at room temperature. Adding up free sGFP the total concentration of sGFP was kept constant at 0.3 μ M throughout the entire experiment. Data are the mean \pm SD; N = 3. R² for each curve equals >99 %.

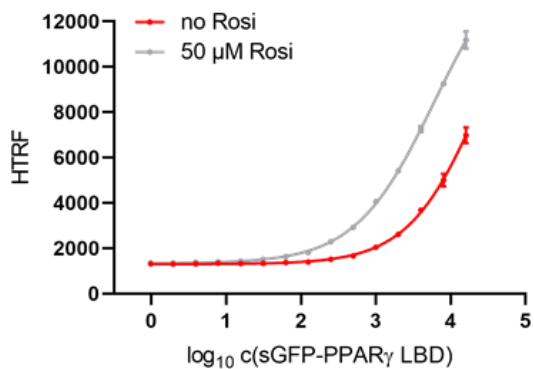


Figure S7. Evaluation of protein concentration as a factor for investigation of rosiglitazone dependent CBP-1 coactivator recruitment to the PPAR γ LBD. Refer to Figure 4 A in main manuscript for schematic presentation of the PPAR γ monomer cofactor recruitment assay. PPAR γ LBD as a fusion protein with N-terminal sGFP was titrated against 12 nM Tb-SA, 12 nM biotinylated CBP-1 cofactor peptide. Recruitment of CBP-1 to the PPAR γ LBD resulted in an increase in HTRF. PPAR γ was activated with 50 μ M of its reference agonist rosiglitazone and compared to apo (DMSO control). HTRF measurements were performed after 1 h incubation at room temperature. Data are the mean \pm SD; N = 4.

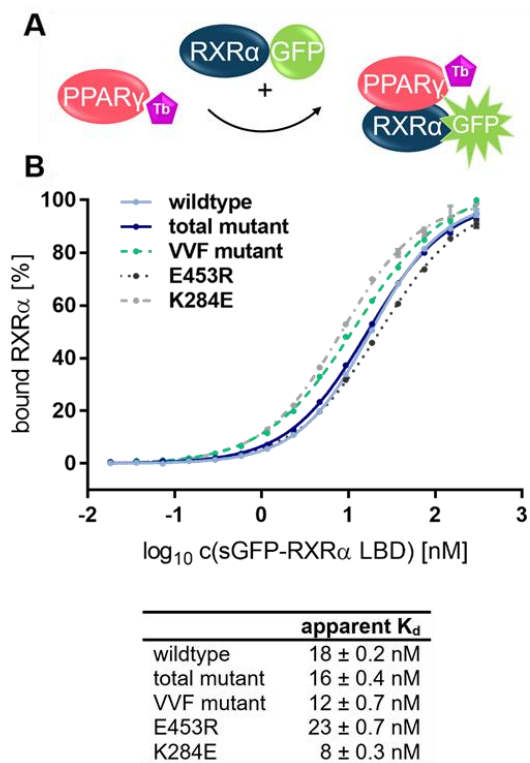


Figure S8. Analysis of LBD:LBD heterodimer formation between PPAR γ and all RXR α mutants generated in comparison to wildtype. A, schematic representation of the heterodimer formation assay with PPAR γ LBD being coupled to the FRET donor. B, sGFP-RXR α LBD wildtype (light blue), mutant E453R (black dotted), mutant K284E (grey dotted), VVF mutant (V280T, V298T, and F450Y) (green dotted), or total mutant (dark blue) harboring all five mutations (V280T, K284E, V298T, F450Y, and E453R) was titrated against 0.375 nM biotinylated PPAR γ LBD and 0.75 nM Tb-SA. Adding up free sGFP the total concentration of sGFP was kept constant at 0.3 μ M throughout the entire experiment. Data are the mean \pm SD; N = 3. R² for each curve equals >99 %.

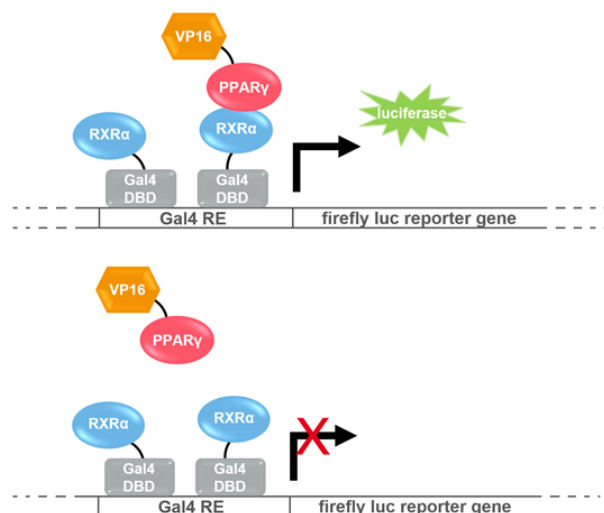


Figure S9. Schematic depiction of the cell-based Gal4-RXR α VP16-PPAR γ heterodimer formation assay. RXR α is expressed as a fusion protein with Gal4 DBD. The latter guides RXR α to the Gal4 DNA response elements in the promoter region upstream of the firefly gene in plasmid pFR-Luc. PPAR γ is expressed as a fusion protein with VP16, a strong trans-inducer of transcription. Formation of the RXR α :PPAR γ heterodimer results in recruitment of VP16 to the Gal4 response elements, and subsequently activates expression of firefly luciferase (Luc). Renilla Luc is constitutively expressed from pRL-SV40 and is utilized as an internal control for transfection and toxicity. Luminescence of firefly Luc divided by the luminescence of renilla Luc is a measure for strength of transactivation resulting from co-expression of the respective NR fusion proteins. When compared to appropriate controls, this strategy can be used to detect dimer formation between VP16-PPAR γ and Gal4-RXR α .

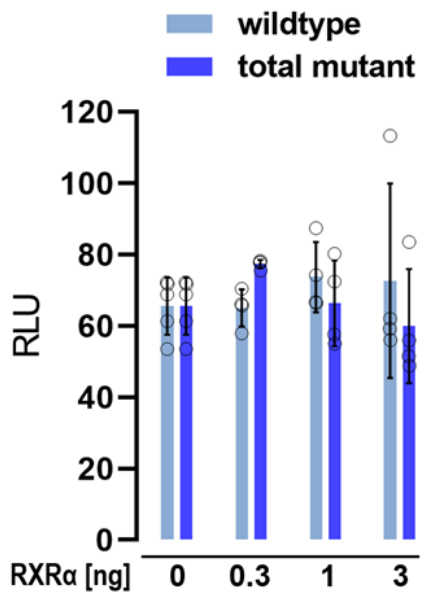


Figure S10. Assessment of transactivation activity of VP16-SRC1 alone and in combination with Gal4-RXRα with no agonist being present. HEK293T cells were co-transfected with 1 ng of the VP16-SRC1-2 plasmid, and various amounts of either Gal4-RXRα wildtype or Gal4-RXRα total mutant plasmid, always in combination with the plasmids for firefly reporter and renilla luciferase. Luciferase fluorescence was detected using the Dual-Glo™ Luciferase Assay System (Promega). Data are the mean ± SD; n = 5.

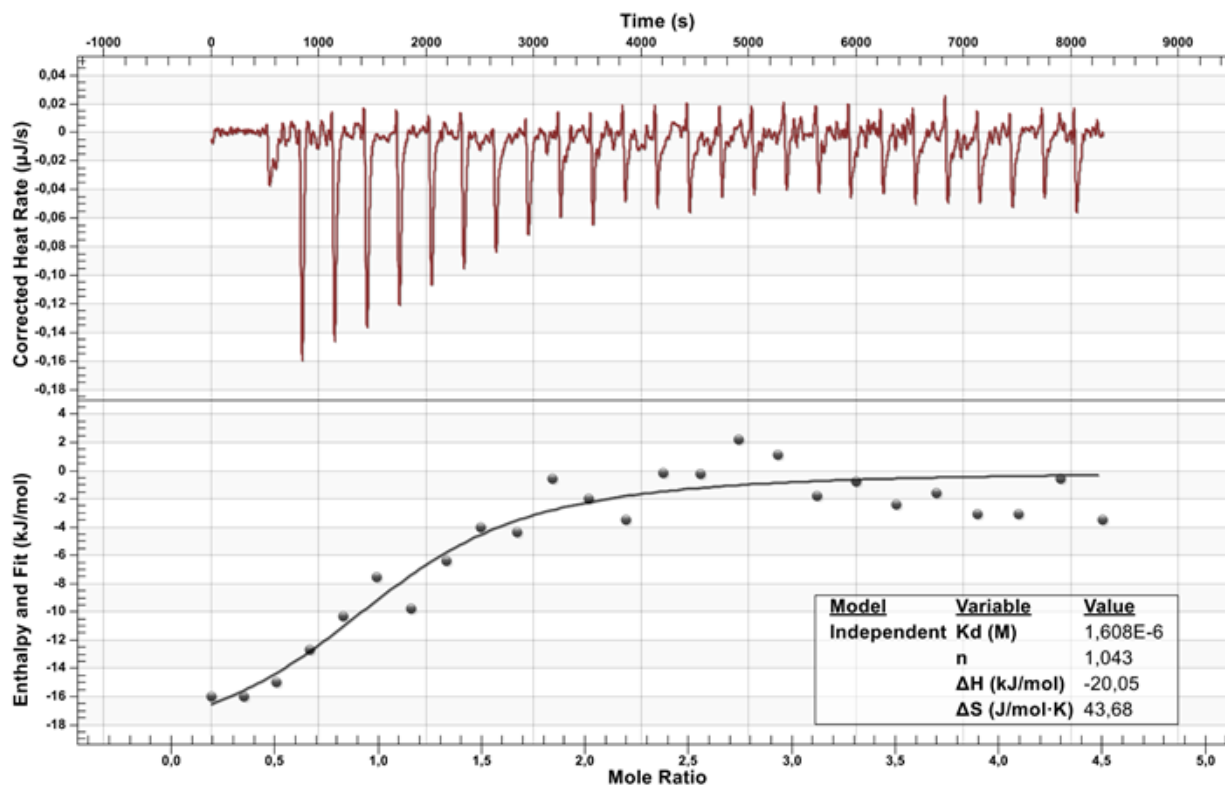


Figure S11. ITC experiment for verification of SR11237 binding to the RXR α wildtype LBD. Recombinant RXR α wildtype LBD (120 μ M) was placed into the syringe and titrated to SR11237 (10 μ M). 25 injections of 2 μ l were performed after an initial injection of 0.5 μ l. An interval of 300 s was maintained between individual injections and the experiments were performed at 25°C and a stirring rate of 75 rpm. The heats of dilution resulting from titrating of protein into buffer were recorded separately and subtracted. Thereafter, data were fitted to an independent binding model.

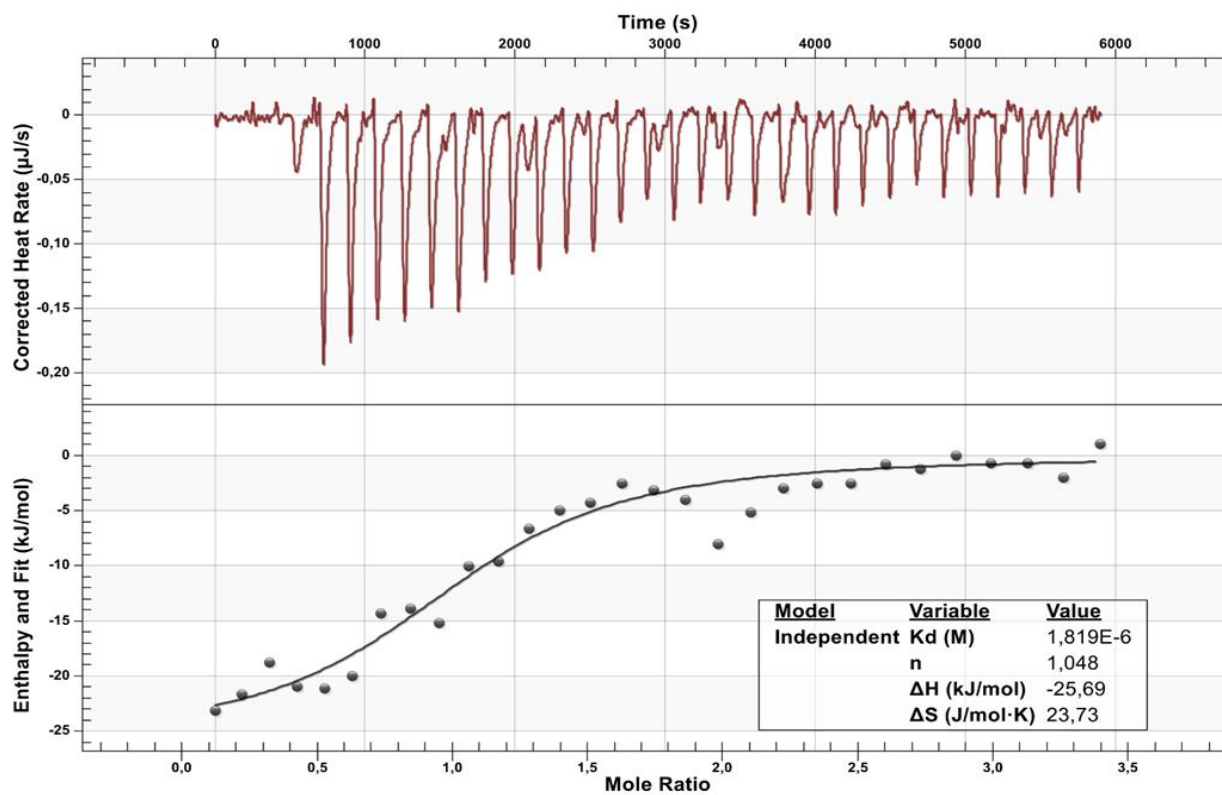


Figure S12. ITC experiment for verification of SR11237 binding to the RXR α total mutant LBD. Recombinant RXR α LBD (120 μ M) harboring the five mutations that prevent recruitment (V280T, K284E, V298T, F450Y, and E453R) was titrated to 10 μ M SR11237. 25 injections of 2 μ l were performed after an initial injection of 0.5 μ l. An interval of 300 s was maintained between individual injections and the experiments were performed at 25°C and a stirring rate of 75 rpm. The heats of dilution resulting from titrating of protein into buffer were recorded separately and subtracted. Thereafter, data were fitted to an independent binding model.

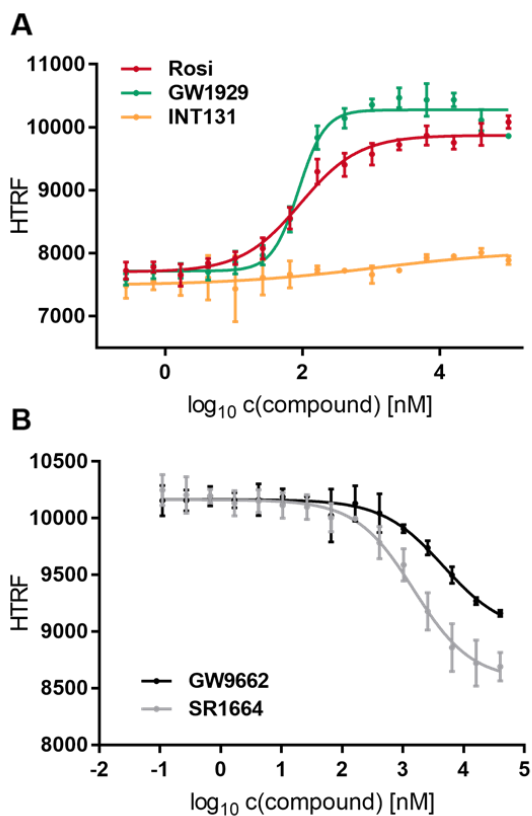


Figure S13. Recruitment of coactivator CBP-1 by PPAR γ LBD in the context of the heterodimer with the RXR α LBD. 100 nM PPAR γ LBD was incubated with 2 μ M sGFP-RXR α LBD harboring mutations (V280T, K284E, V298T, F450Y, and E453R) that block coactivator recruitment by RXR α . The excess of RXR α ensures that throughout the experiments the main share of PPAR γ is being incorporated into the PPAR γ -RXR α heterodimer. Recruitment of biotin-labeled CBP-1 coactivator peptide (12 nM) coupled to Tb-SA (12 nM) is detected as an increase in HTRF. A, CBP-1 recruitment by PPAR γ is modulated by titration of PPAR γ full agonist GW1929 (green), partial agonist INT131 (yellow), or reference agonist rosiglitazone (red). B, CBP-1 recruitment is stimulated with constant 1 μ M rosiglitazone and challenged by titration of GW9662 (black), or SR1664 (grey). Data are the mean \pm SD; N = 3. R² for each curve equals >97 %.

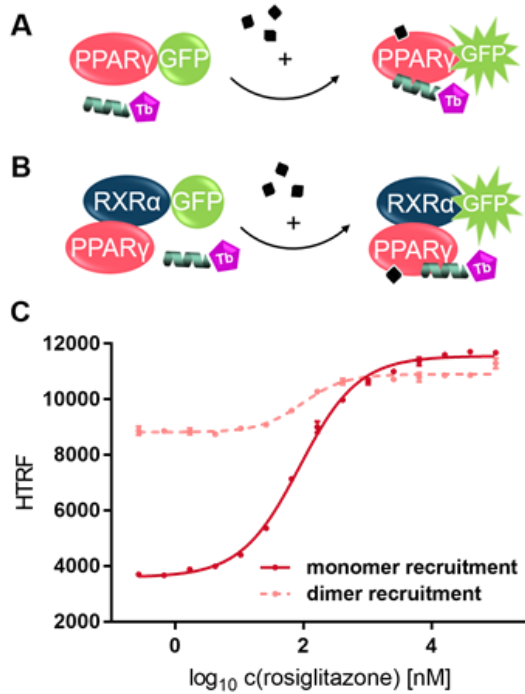
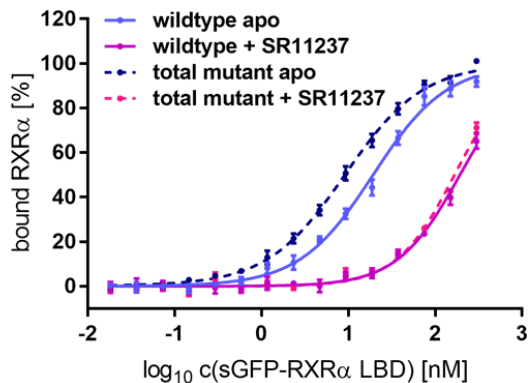


Figure S14. Comparison of recruitment of coactivator CBP-1 by PPAR γ LBD either being present as monomeric LBD or in the context of the LBD:LBD heterodimer with RXR α LBD. A, schematic presentation of monomer recruitment assay. B, schematic presentation recruitment of coactivator by PPAR γ in the context of the heterodimer with mutant RXR α . C, for investigation of coactivator recruitment by isolated monomeric PPAR γ LBD 100 nM sGFP-PPAR was presented. Recruitment by PPAR γ in the context of the heterodimer with RXR α was investigated on 100 nM free PPAR γ LBD of which the main share is incorporated into the LBD:LBD heterodimer with sGFP-RXR α LBD (2 μ M; 20-fold molar excess). The RXR α LBD harbors mutations (V280T, K284E, V298T, F450Y, and E453R) that block coactivator recruitment by RXR α . In either case recruitment of biotin-labeled CBP-1 coactivator peptide (12 nM) coupled to Tb-SA (12 nM) was detected as an increase in HTRF. The recruitment was stimulated by titration of rosiglitazone. Data are the mean \pm SD; N = 4. R² equals 97.4% for dimer recruitment, and 99.7% for monomer recruitment.



	apparent K_d	
	apo	+SR11237
wildtype	19 ± 1 nM	190 ± 8 nM
total mutant	9 ± 0.4 nM	167 ± 8 nM

Figure S15. Effect of RXR α agonists SR11237 on RXR α homodimer formation and comparison between RXR α wildtype and recruitment incapable mutant. sGFP-RXR α LBD was titrated onto 0.375 nM biotinylated RXR α LBD coupled to Tb-SA (0.75 nM Streptavidin subunits). For both wildtype RXR α LBD as well as RXR α mutant (V280T, K284E, V298T, F450Y, and E453R) the experiment was conducted on apo protein, or with constant 10 μ M SR11237 being present. Adding up free sGFP the total concentration of sGFP was kept constant at 0.3 μ M throughout the entire experiment.

Due to the substantial increase in the K_d resulting from treatment with SR11237 the respective curves did no longer reach the upper plateau corresponding to saturation binding. Therefore, the upper plateau (HTRF signal) from the respective experiment with only DMSO (apo) was set as 100% bound for curve fitting and calculation of apparent K_d of dimer formation. Data are the mean \pm SD; N = 3. R^2 for each curve equals >98%.

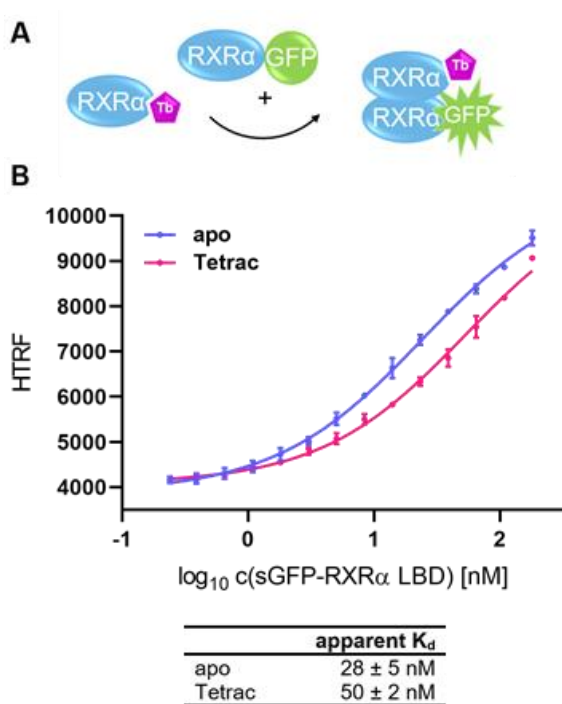


Figure S16. Modulation of RXR α LBD homodimer formation by Tetrac. A, schematic representation of the homodimer formation assay with RXR α LBD being coupled to the FRET donor and titrated with sGFP-RXR α . B, sGFP-RXR α LBD was titrated against 0.375 nM biotinylated RXR α LBD and 0.75 nM Tb-SA. Adding up free sGFP the total concentration of sGFP was kept constant at 0.3 μ M throughout the entire experiment. The effect of a constant concentration of 10 μ M Tetrac was investigated in comparison to no ligand. Data are the mean \pm SD; N = 3. R² for each curve equals >99%. The graph shows one representative experiment out of three independent experiments (n = 3) each conducted with three technical replicates (N = 3). The table reports the mean of apparent $K_d \pm$ SD calculated based on the values derived from the entire set of three independent experiments, respectively.

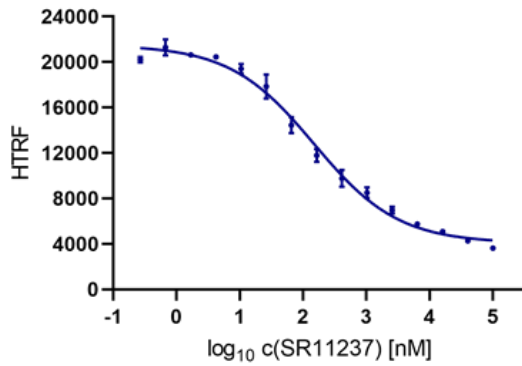


Figure S17. Agonist dependent displacement of NCOR1 corepressor from apo RXR α LBD mutant. SR11237 was titrated against 12 nM Tb-SA, 12 nM biotinylated NCOR1 ID2 (aa 2251-2276) and 100 nM sGFP-RXR α mutant LBD harboring mutations (V280T, K284E, V298T, F450Y, and E453R) that block coactivator recruitment. A reduction in HTRF corresponds to a reduction in complex formed between NCOR1 and RXR α . Data are the mean \pm SD; N = 3. R² for each curve equals >98%..