Supplementary Data and Methods for Agonists of the nuclear receptor PPARγ can produce biased signaling.

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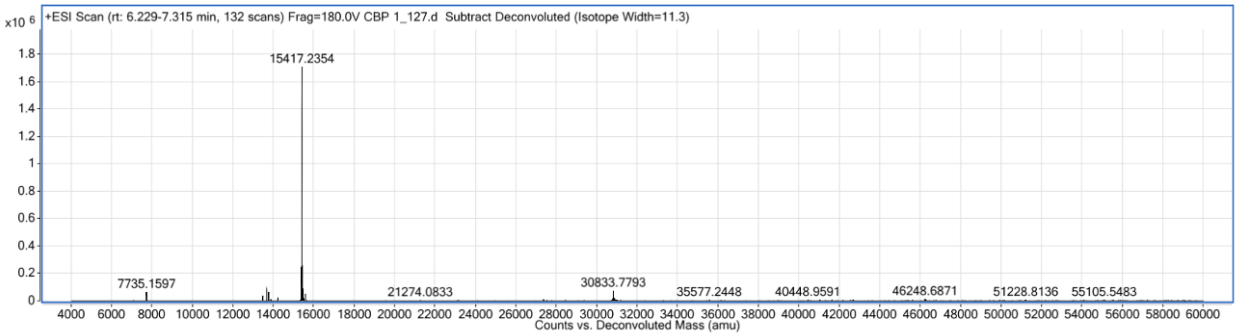
a



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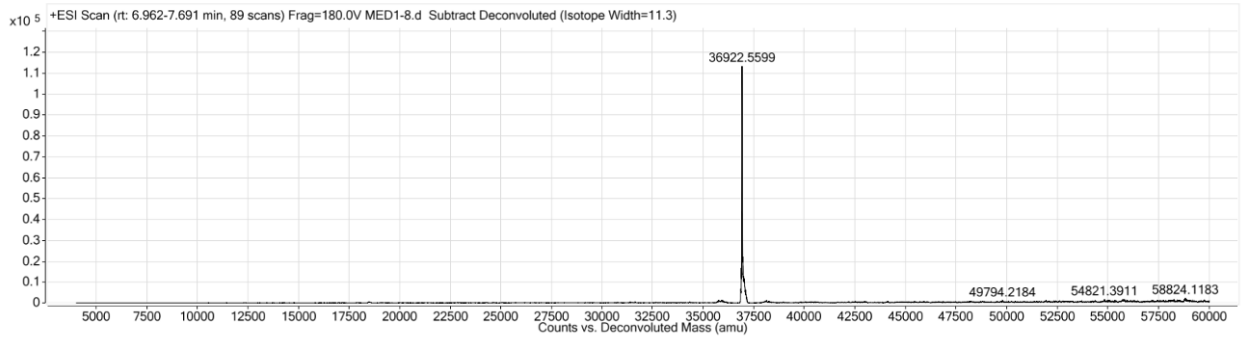

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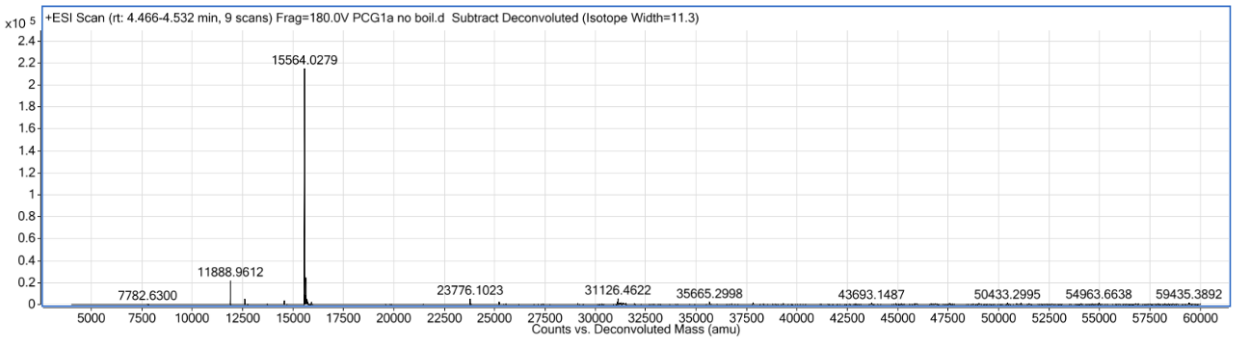
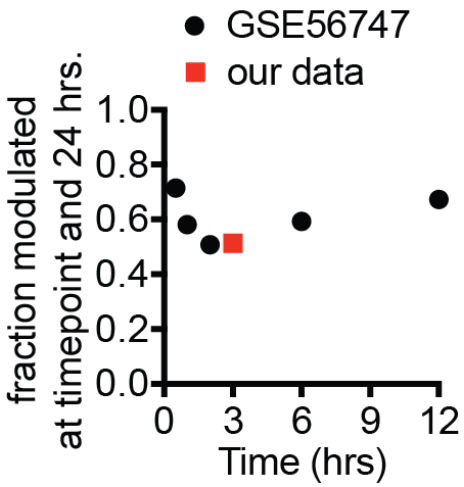
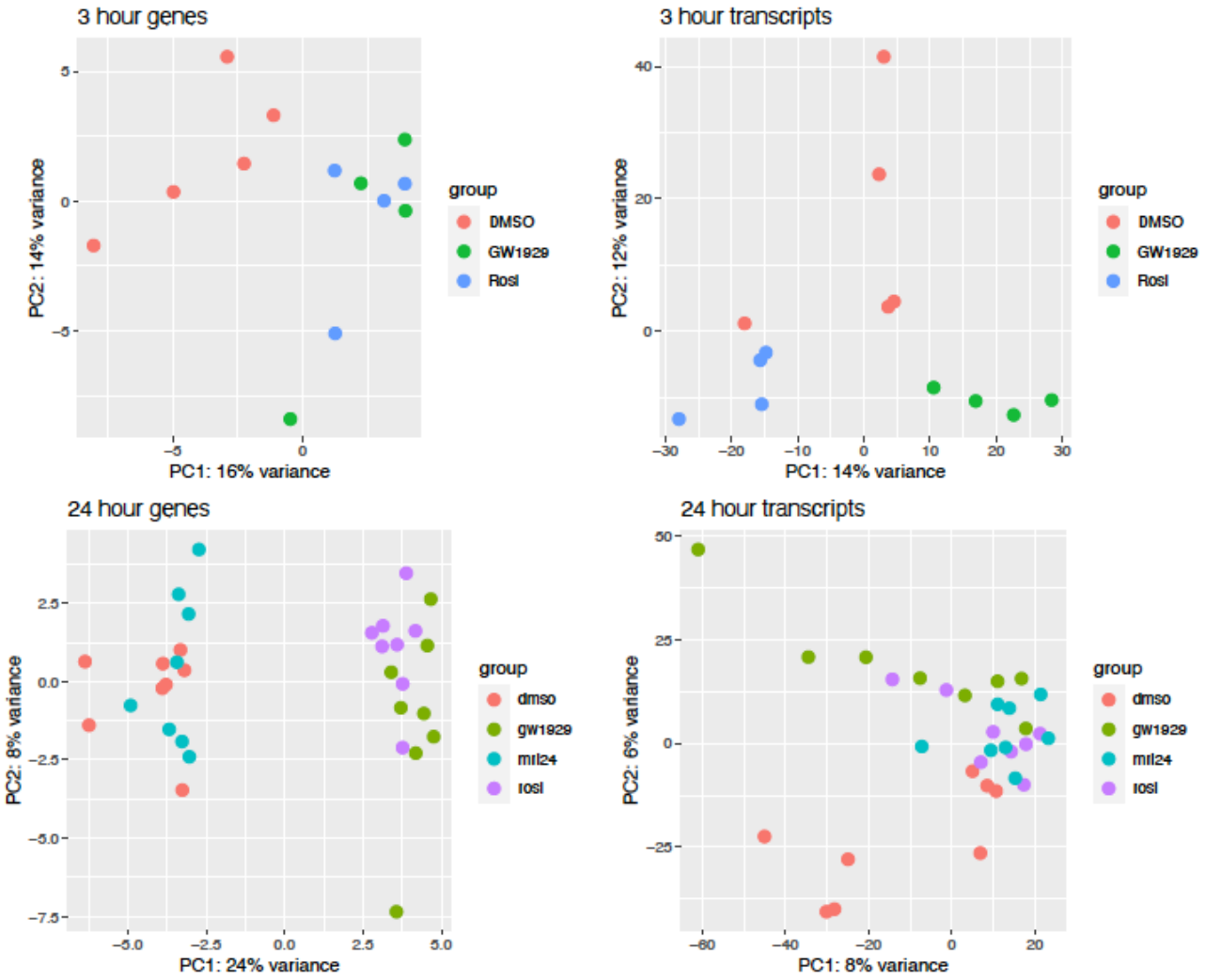


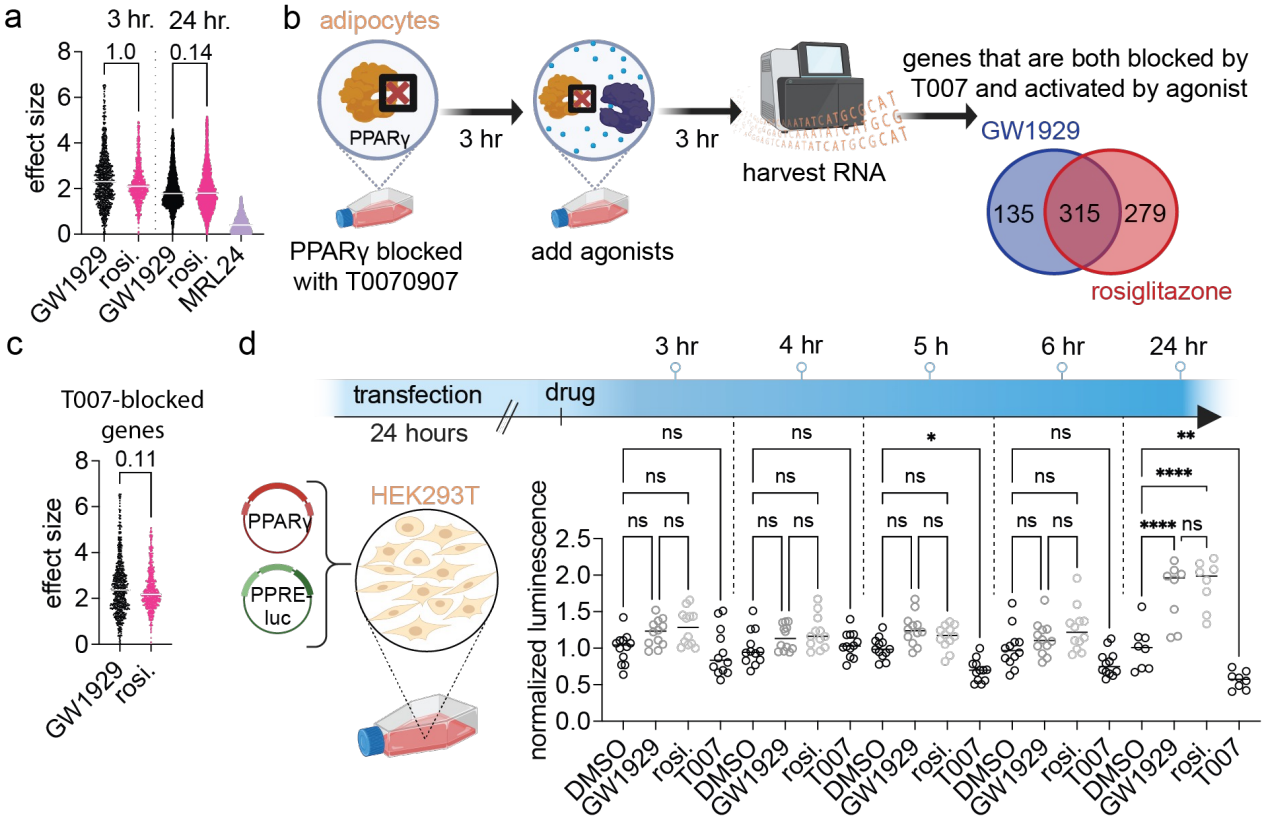
Supplementary Figure 1: Mass spectrometry of coregulator RID proteins purified and used in completive anisotropy. Sequences and expected masses are listed in **Table S2**. Proteins were buffer exchanged into 20% acetonitrile in water with 0.1% TFA prior to mass validation by Q-TOF. A) $CBP_{1\text{-}127}$, B) $MED1_{557\text{-}870}$, and C) $PGC1\alpha_{100\text{-}220}$.



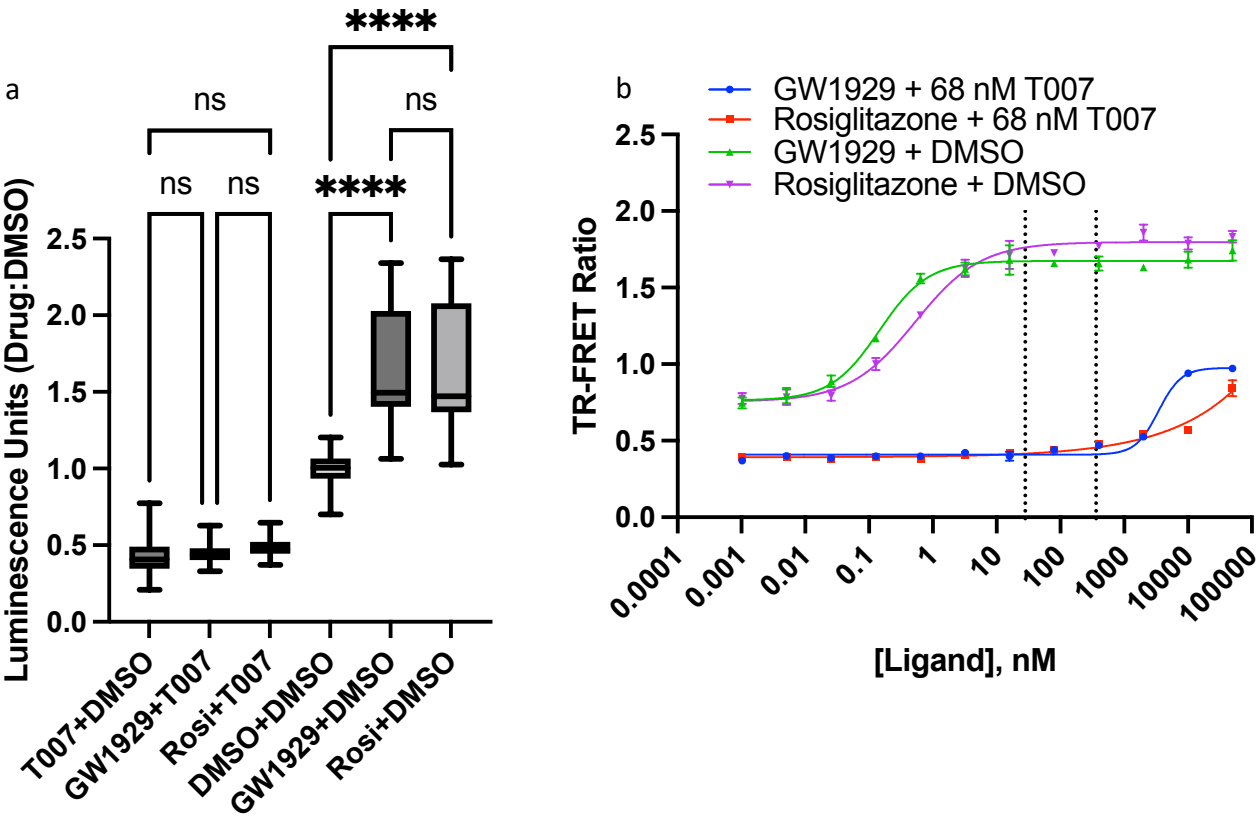
Supplementary Figure 2. Proportion of genes that are significantly differentially expressed at both the indicated early timepoint (0.5, 1, 2, 6, and 12 hours) and at 24 hours in mouse 3T3L1 cells by rosiglitazone (deposited data GSE56747; Step et al. *Genes Dev* **28**, 1018–1028, 2014) are shown as black circles, while the proportion of genes differentially expressed at both 3 and 24 hours in human adipocytes (data in panel a) is shown as a red square.



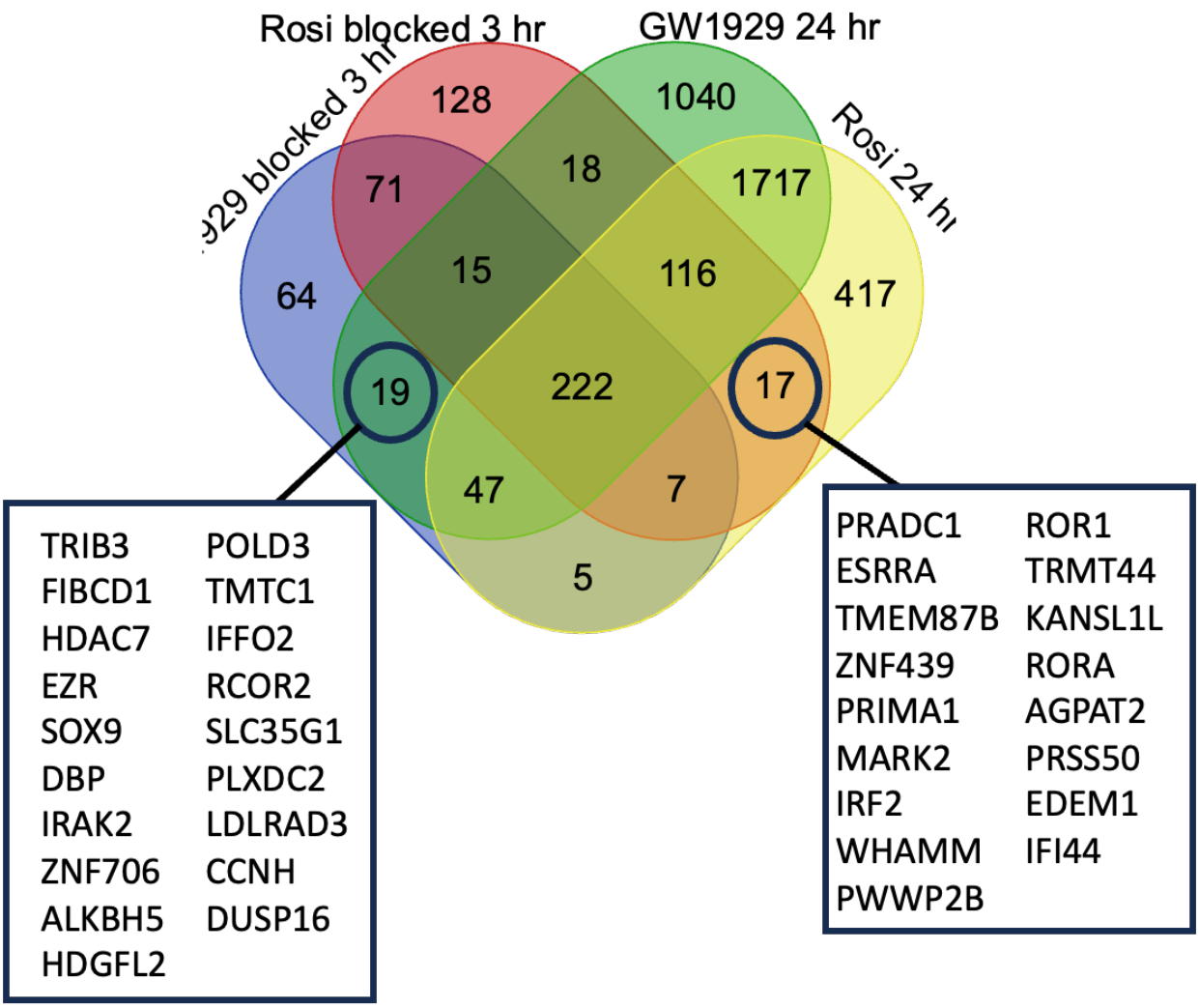
Supplementary Figure 3: Principle component analysis of all differentially expressed genes and transcripts at 3 and 24 hours post ligand exposure in human adipocytes (similar to **Figure 2 panel d-e**). This analysis used variance stabilizing transformation.



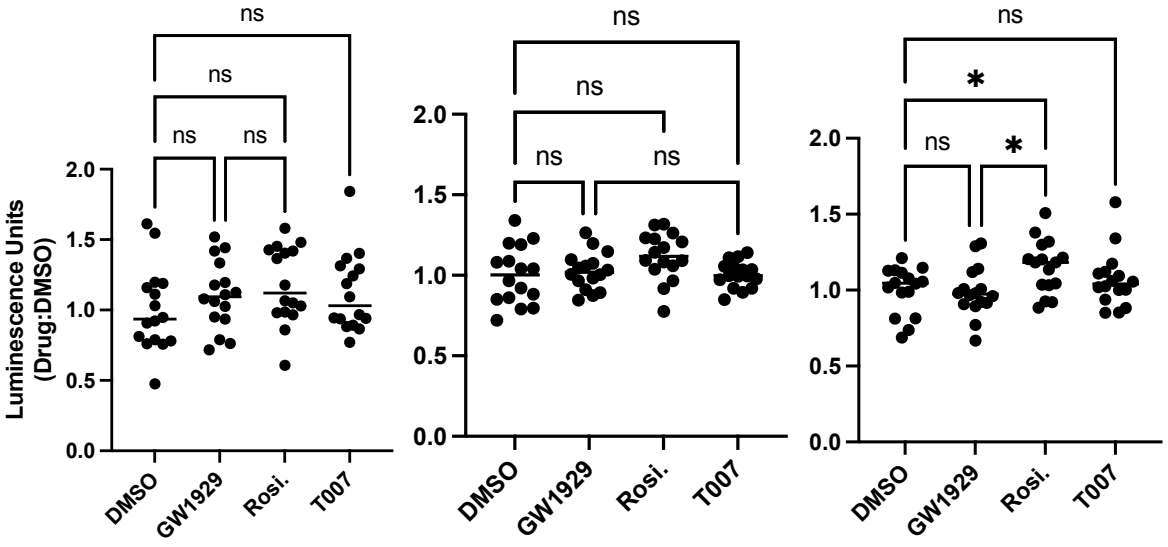
Supplementary Figure 4: Non-ligand bias mechanisms do not account for observed biased signaling. a) Neither ligand displays a stronger effect on the transcriptome, as seen by comparison of effect size at 3 and 24 hours. Outliers were removed with ROUT method Q=1% using Prism 9. The distributions failed normality when tested with the Shapiro-Wilk test in Prism 9. Significance between the comparisons was determined with a 1-way ANOVA using the Kruskal-Wallis test for non-parametric distributions (p-values are displayed). b) We pretreated adipocytes with T0070907 for three hours and then treated with either GW1929 or rosiglitazone for three hours, isolated RNA and performed RNA-seq. Differentially expressed genes (adjusted p-value < 0.05) that were blocked by T0070907 pretreatment show the same trend as in **Figure 2**. This experiment used the same lot of adult stem cells and was performed at the same time as the 3-hour experiment shown in **Figure 2**. c) There is no statistical difference between the effect sizes of the ligands on genes blocked by T0070907 (p-value = 0.1082. Outliers were removed with ROUT method Q=1%, Mann-Whitney test for non-parametric distributions). d) PPRE-reporter-transfected HEK293T cells were treated with drugs at the doses used in RNA-seq for 3, 4, 5, 6, and 24 hours. Neither GW1929 nor rosiglitazone produced significant effects before 24 hours in this assay. One-way ANOVA followed by Holm-Sidak multiple comparisons. See cohen effect size excel files located at https://osf.io/3g2ks/ for data underlying panel a and c and **Datafile2_3h.xlsx** and **Datafile4.xlsx** for data underlying panels b and d respectively. Parts of panels b and d were made using biorender.com.



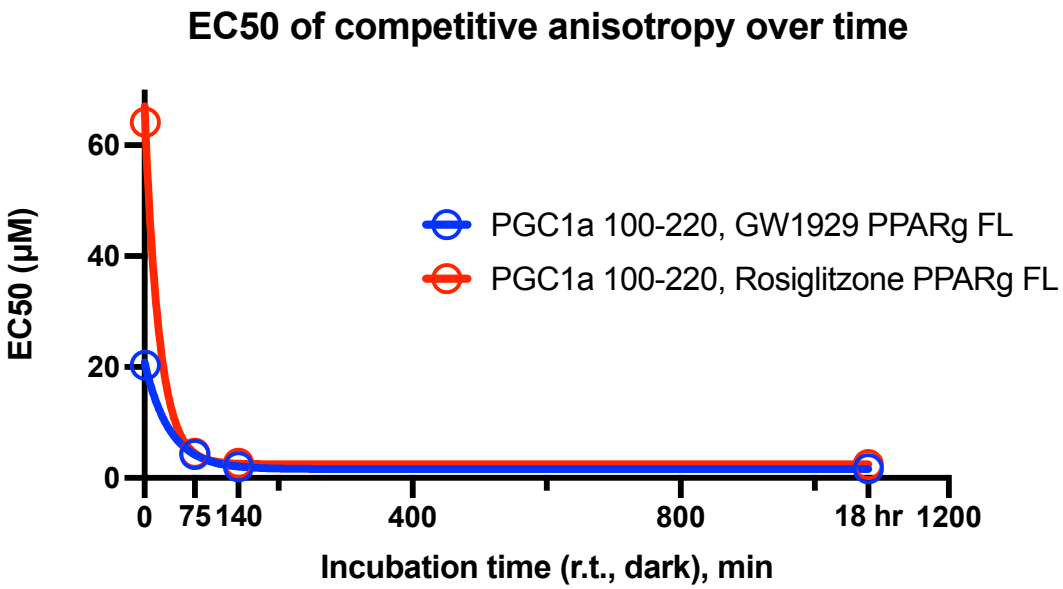
Supplementary Figure 5: PPARγ can be blocked with the covalent inverse agonist T0070907. **a**) HEK293T cells were transfected with a PPARγ plasmid and a 3X-PPRE-Luciferase plasmid. Cells were treated with DMSO or T0070907 (5 µM) for 3 hours and then DMSO or ligand were added. GW1929 was added at 28 nM and rosiglitazone was added at 361 nM. There was no statistical difference between the transactivation for the cells pretreated with T007 (adjusted p-value > 0.9998 for each comparison). GW1929+DMSO and rosiglitazone + DMSO were different than DMSO + DMSO (adjusted p-value < 0.001 for both). GW1929 and rosiglitazone induce the same level of transactivation (adjusted p-value = 0.9656). Significance was determined with a one way ANOVA in Prism9. b) TR-FRET analysis of CBP peptide recruitment to PPARγ using PPARγ (8nM), terbium (0.9 nM), and CBP (400 nM). T0070907 or DMSO was added at 68 nM for 3 hours. After three hours GW1929 or rosiglitazone was titrated in. 3 hours of T0070907 treatment blocked the ability of rosiglitazone and GW1929 to bind PPARγ at the doses used in the adipocyte cell RNA-seq experiments (28 nM for GW1929 and 361 nM for rosiglitazone; vertical dashed lines). See **Datafile4.xlsx** for underlying data.



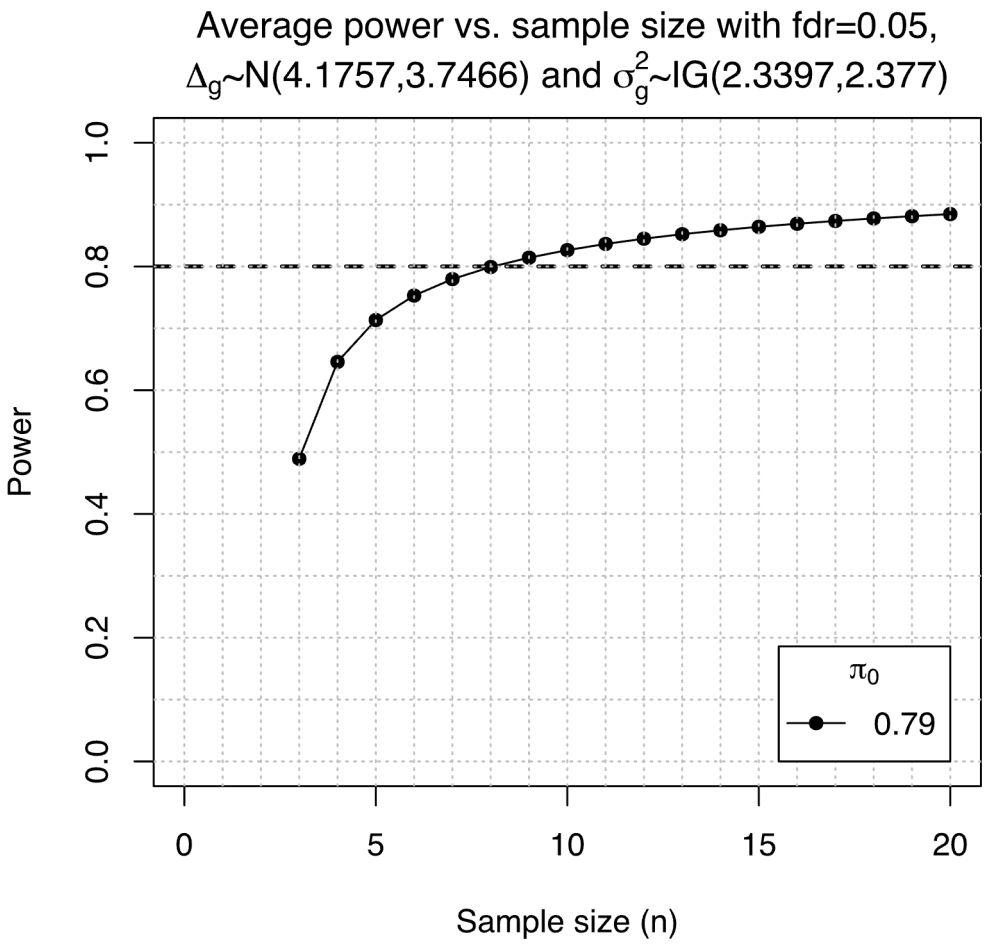
Supplementary Figure 6. Analysis of T0070907-blocked genes. A) Analysis of genes that are both blocked by T0070907 at 3 hours and modulated by GW1929 or rosiglitazone is shown. Similar to Figure 2, at 3 and 24 hours each full agonist differentially expresses unique sets of genes (adjusted p-value < 0.05). Human genes that GW1929 or rosiglitazone uniquely affect at both 3 and 24 hours and are blocked by T0070907 pre-treatment are listed. See **Datafile2_3h.xlsx** for underlying data.



Supplementary Figure 7: Rosiglitazone and GW1929 do not consistently induce changes from DMSO in HEK293T cells at 3 hours. Three independent experiments were carried out. In one othse three rosiglitazone induced changes from DMSO different than GW1929 (adjusted p-value = 0.0325). Significance was determined by three separate one-way ANOVAs in Prism 9. See **Datafile4.xlsx** for underlying data.



Supplementary Figure 8: Competitive anisotropy reaches equilibrium at 2 hours at room temperature. PPARγ FL, 5FAM-CBP peptide, and ligand are mixed at 1:1:1 molar ratio at 800nM with varying concentrations of $PGC1\alpha_{100\text{-}220}$ RID. The effective concentration of PGC1α RID needed to compete out 50% of 5FAM-CBP (EC50) reaches equilibrium at 140 minutes. A one-phase decay equation was fit to each dataset and showed a half-life ($t_{1/2}$) of 26 min and 15 min for GW1929 and Rosiglitazone-bound PPARγ FL, respectively. A 120 min incubation time was chosen for the competitive anisotropy displayed in **Figure 1** of this work.



Supplementary Figure 9: Power analysis indicates that 8 replicates will provide a power of 0.8 at an FDR of 0.05.

Supplementary Table 1. Previous reports of putative PPARγ biased agonists in animal models.

*Ligand**	Apparent favored LxxLL type	Form of coactivator used	Adverse effects in animals compared to ref.[$]	Insulin sensitization in animals compared to ref.[$]	Ref. agonist
F12016[1]	S motif ($PGC1\alpha_{144}$)	peptide	Less weight gain (less inhibition of osteoblast diff. *in vitro*)	similar	Rosi.
[#]***Amorfrutin B***[2]	unclear	peptide	Less weight gain	similar	Rosi.
SR1664[3]	NA	NA	Less weight gain and hemodilution	similar	Rosi.
MBX-102[4]	S motif ($PGC1\alpha_{144}$ and $NCOA1_{1435}$)	peptide	Less weight gain and hemodilution	similar	Rosi.
INT-131[5]	Disfavors N-anchored	peptide	Less hemodilution	similar	Rosi.
S 26948[6]	Unclear; $NCOA1_{459-888}$, $PGC1\alpha_{190-403}$, and $NCOA2_{548-878}$ favored over CBP_{8-93} and $MED1_{459-803}$	Yeast two hybrid of RIDs	Less weight gain	Similar + antiatherogenic effects	Rosi.
KR-6290[7]	Disfavors N-anchored (MED1)	Mammialian two-hybrid FL coreg.	Less weight gain and hemodilution	similar	Rosi.
telmisartan/ irbasartan[8]	unclear	peptide	Less weight gain	Not as good but better than vehicle	Rosi.
FMOC-L-Leucine[9]	Unclear; favors NCOA1	Mammilian two-hybrid FL	Less weight gain	similar	Rosi.
NTZDpa[10,11]	S motif ($PGC1\alpha_{144}$)	peptide	Less weight gain and hemodilution	similar	TZDfa/Rosi.

Ligand is a partial agonist unless otherwise specified

[#]***Significant activity at other receptors***

[$]***Some of these results are inferred. For example, less heart weight gain infers less hemodilution. Some of the data in the referenced articles is suggestive of biased coactivator recruitment, but such implications went unnoticed or unmentioned in the text.***

Supplementary Table 2: Q-TOF mass spectrometry masses of proteins expressed and purified from *E. coli* (BL21-De3) for this work.

Coregulator RID	Protein Sequence	Vector	Predicted mass* (Da)	Experimental mass (Da)
CBP$^{1-127}$	MAHHHHHHVGTENLYFQGVGT MAENLLDGPPNPKRAKLSSPGFS ANDSTDFGSLFDLENDLPDELIPN GGELGLLNSGNLVPDAASKHKQL SELLRGGSGSSINPGIGNVSASSP VQQGLGGQAQGQPNSANMASL SAMGKSPLSQGDWW	pET45b	15416.79	15417.2354
MED1$^{557-870}$	MAHHHHHHVGTGSNDDDDKSP DPENLYFQGMSGTTTPTNTFPGG PITTLFNMSMSIKDRHESVGHGE DFSKVSQNPILTSLLQITGNGGSTI GSSPTPPHHTPPPVSSMAGNTK NHPMLMNLLKDNPAQDFSTLYG SSPLERQNSSSGSPRMEICSGSNK TKKKKSSRLPPEKPKHQTEDDFQ RELFSMDVDSQNPIFDVNMTAD TLDTPHITPAPSQCSTPPTTYPQP VPHPQPSIQRMVRLSSSDSIGPD VTDILSDIAEEASKLPSTSDDCPAI GTPLRDSSSSGHSQSTLFDSDVFQ TNNNENPYTDPADLIADAAGSPS SDSPTNHFFHDGVDFNPDLL	pET45b	36921.92	36922.5599
PGC1α$^{100-220}$	MAHHHHHHVGTGSNDDDDKSP DPVDEDGLPSFDALTDGDVTTDN EASPSSMPDGTPPPQEAEEPSLLK KLLLAPANTQLSYNECSGLSTQNH ANHNHRIRTNPAIVKTENSWSNK AKSICQQQKPQRRPCSELLKYLTT NDDP	pET45b	15565.69	15564.0279

Supplementary Table 3: Characteristics of RNA-seq Experiments.

Experiment 1			Experiment 2		
Ligand	Replicates	Avg RIN	Ligand	Replicates	Avg RIN
DMSO	8	9.7	DMSO	5	9.9
GW1929	8	9.7	GW1929	4	9.9
MRL24	8	9.6	Rosiglitazone	4	9.9
Rosiglitazone	8	9.6	T007	5	9.9
			T007 + GW1929	4	9.8
			T007 + Rosiglitazone	4	9.9
Demographics					
Lot number	ASC0061		Lot number	ASC0065	
Number of donors	5		Number of donors	7	
Mean Age	41		Mean Age	44.3	
Sex	F		Sex	F	
Mean BMI	28.1		Mean BMI	27.6	
Tissue Origin	Abdomen/Thigh/ Flanks		Tissue Origin	Thigh/Flanks/Hips/ Arm/Abdomen	
Ethnicity	Caucasian/African American		Ethnicity	Caucasian	

Supplementary Table 4: Selectivity index genes converted to human gene names

Antidiabetic genes	Adverse genes
AK2	NDUFB11
ANGPTL1	TMEM14C
C9orf72	ACOT8
CAVIN1	SLC25A20
CD36	COX7A2
CFL2	NUDT7
DIXDC1	SLC25A11
EMC4	RAMP1
FABP4	PTPRN
FABP9	WBP1
LRRC27	ABHD12
MSS51	CPT2
MXRA8	ACADVL
NAP1L1	
NKTR	
PIGK	
PPP2R5A	
QKI	
RANBP9	
RGS7	
RORA	
RTF2	
S100A11	
SDSL	
TMEM126B	
YPEL5	
ZWINT	

Supplementary Table 5: Selectivity Index

	GW1929	Rosiglitazone	MRL24
3 hours	5.057	2.331	not performed
24 hours	-4.111	-4.032	0.361

Supplementary Table 6: PPARγ ligands – doses used experimentally

Ligand	Type	Experimental Dose (nM)	PPARγ Ki (nM)	PPARδ Ki (nM)	PPARα Ki (nM)
GW1929	Agonist	28	<1	2344	2626
Rosiglitazone	Agonist	361	11	none detected	35589
MRL24	Partial agonist	166	<1	none detected	15758

Supplementary Methods 1. Derivation of occupancy equation and assumptions used in the derivation.

We define the equilibrium dissociation constant for ligand A (K_A) as:

$$K_A = \frac{[R][A]}{[RA]}$$

Similarly for ligand B:

$$K_B = \frac{[R][B]}{[RB]}$$

Where [R], [A], and [B] are the concentrations of free receptor, ligand A, and Ligand B and [RA] and [RB] are the concentrations of the receptor-ligand A and receptor-ligand B complexes.

The total receptor concentration, $[R_{total}]$, is the sum of free and bound receptors:

$$[R_{total}] = [R] + [RA] + [RB]$$

Rearrange the equilibrium equations to solve for [RA] and [RB]:

$$[RA] = \frac{[R][A]}{K_A}$$

$$[RB] = \frac{[R][B]}{K_B}$$

Substitute expressions for [RA] and [RB] into the equation above for total receptor concentration:

$$[R_{total}] = [R] + \frac{[R][A]}{K_A} + \frac{[R][B]}{K_B}$$

Factor out [R]:

$$[R_{total}] = [R](1 + \frac{[A]}{K_A} + \frac{[B]}{K_B})$$

Solve for [R]:

$$[R] = \frac{[R_{total}]}{(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B})}$$

Calculate bound fractions. Fraction of receptors bound by ligand A, θ_A:

$$\theta_A = \frac{[RA]}{[R_{total}]}$$

Substitute $[R] = \frac{[R_{total}]}{(1+\frac{[A]}{K_A}+\frac{[B]}{K_B})}$ into $[RA] = \frac{[R][A]}{K_A}$

$$[RA] = \frac{\frac{[R_{total}]}{(1+\frac{[A]}{K_A}+\frac{[B]}{K_B})}[A]}{K_A}$$

Simplify and rearrange:

$$\theta_A = \frac{[RA]}{[R_{total}]} = \frac{[A]}{K_A + [A] + \frac{K_A[B]}{K_B}} = \frac{[A]}{[A] + K_A + \frac{K_A[B]}{K_B}} = \frac{[A]}{[A] + K_A(1 + \frac{[B]}{K_B})}$$

Under the assumption that ligand A and ligand B are in large excess compared to the receptor [A]≅ [A_{total}] and [B] ≅ [B_{total}] and $\theta_A = \frac{[A_{total}]}{[A_{total}]+K_A(1+\frac{[B_{total}]}{K_B})}$, which is the equation we used to calculation the bound fraction of ligand A when in competitive binding with ligand B for the same receptor. An analogous equation exists for θ_B,

$$\theta_B = \frac{[B_{total}]}{[B_{total}] + K_B(1 + \frac{[A_{total}]}{K_A})}$$

Data file	Caption for Supplemental Datafiles
Datafile1.xlsx	Data and calculations relevant to Figure 1 panel d and e.
Datafile2_3h.xlsx	RNAseq data for 3-hour ligand treatment.
Datafile2_24h.xlsx	RNAseq data for 24-hour ligand treatment.
Datafile3.xlsx	KEGG pathway analysis, including biased signaling.
Datafile4.xlsx	Data relevant to Supp. Figures 4, 5, and 7.