PPARβ/δ controls Mediator recruitment and transcription initiation

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Abstract

Repression of transcription by nuclear receptors involves NCOR and SMRT corepressor complexes, which harbour the deacetylase HDAC3 as a subunit. Both deacetylase-dependent and -independent repression mechanisms have been reported for these complexes. In the absence of ligands, the nuclear receptor PPARβ/δ recruits NCOR and SMRT and represses expression of its canonical targets including the *ANGPTL4* gene. Agonistic ligands cause corepressor dissociation and enable enhanced induction of transcription by coactivators. *Vice versa*, recently developed synthetic inverse agonists lead to augmented corepressor recruitment and repression that dominates over activating stimuli. Both basal repression of *ANGPTL4* and reinforced repression elicited by inverse agonists are partially insensitive to HDAC inhibition. This raises the question of how PPARβ/δ represses transcription mechanistically.

Here, we show that the PPARβ/δ inverse agonist PT-S264 impairs transcription initiation in human cells. Inverse agonist-bound PPARβ/δ interferes with recruitment of Mediator, RNA polymerase II, and TFIIB, but not with recruitment of other basal transcription factors, to the *ANGPTL4* promoter. We identify NCOR as the main ligand-dependent interactor of PPARβ/δ in the presence of PT-S264. In PPARβ/δ knockout cells, reconstitution with PPARβ/δ mutants deficient in basal repression recruit less NCOR, SMRT, and HDAC3 to chromatin, concomitant with increased binding of RNA polymerase II. PT-S264 restores binding of NCOR, SMRT, and HDAC3, resulting in diminished polymerase II binding and transcriptional repression. In the presence of HDAC inhibitors, ligand-mediated repression of PPARβ/δ target genes is only partially relieved. Our findings corroborate deacetylase-dependent and -independent repressive functions of HDAC3-containing complexes. Deacetylase-independent repression mediated by binding of inverse agonists to PPARβ/δ involve NCOR/SMRT recruitment and interference with Mediator, TFIIB, and RNA polymerase II binding.

Introduction

PPARβ/δ (peroxisome proliferator activated receptor β/δ) is a type II nuclear receptor which constitutively binds to DNA as an obligate heterodimer with a retinoid X receptor (RXR). Its target genes function in lipid and glucose metabolism and also in inflammation [1, 2]. An important PPAR target gene is *ANGPTL4* (*angiopoietin-like 4*), a regulator of lipid metabolism, angiogenesis, wound healing, and metastasis [3, 4]. In the absence of ligands, the PPARβ/δ-RXR heterodimer represses its canonical target genes [1] *via* the recruitment of corepressors [5] such as NCOR (nuclear receptor corepressor)- and SMRT (silencing mediator of retinoid and thyroid hormone receptors)-containing complexes [6, 7], which both harbour the catalytic subunit histone deacetylase 3 (HDAC3) [8] whose activity requires binding to NCOR or SMRT [9]. Several fatty acids and their derivatives act as endogenous PPARβ/δ agonists [10, 11, 12, 13]. Agonistic ligands cause dissociation of corepressors from the nuclear receptor at ligand-regulated target genes, while synthetic inverse agonists recently developed in our group lead to enhanced corepressor recruitment [4, 6, 14, 15]. Basal repression of *ANGPTL4* and augmented repression in the presence of inverse agonists are largely insensitive to trichostatin A [4], an inhibitor of class I and II HDACs, suggesting an HDAC-independent repression mechanism. Induction of *ANGPTL4* transcription by activating stimuli is efficiently suppressed by PPARβ/δ inverse agonists, and this coincides with decreased binding of RNA polymerase II (RNAPII) [4]. Agonists alleviate basal repression, and transcription is induced synergistically with other activating stimuli [16, 17].

The preinitiation complex (PIC) is comprised of the general transcription factors (GTFs; TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), the Mediator complex, and RNAPII [18]. Its formation is a prerequisite and a rate-limiting

process for RNAPII-dependent transcription. After clearance by the polymerase, additional rounds of transcription are initiated from the scaffold complex, which contains a subset of general transcription factors which remain bound to the promoter. It was shown that reinitiation of transcription from an immobilized template requires reincorporation of TFIIB, TFIIF, and RNAPII into the scaffold [19], yielding a reinitiation complex (RIC). The re-use of remaining promoter-bound GTFs supersedes the need for recurrent PIC formation and thus enables high-level transcription. Moreover, dephosphorylation of the carboxyterminal domain (CTD) of the large subunit of RNAPII after termination allows for RNAPII recycling, which is enhanced by proximity of the transcription start site (TSS) and the terminator [20]. TFIIB is necessary for the formation of these gene loops [21, 22, 23].

In the present study, we investigated the mechanism of transcriptional repression by PPARβ/δ inverse agonists in human cell lines. Our data show that inverse agonists specifically interfere with stimulus-dependent recruitment of TFIIB, RNAPII, and Mediator subunits to the *ANGPTL4* promoter, while binding of the scaffold subunits TFIIA and TFIIH is unchanged. This suggests an impairment of transcription at the Mediator and RNAPII recruitment step, affecting reinitiation. The binding pattern of RNAPII at the *ANGPTL4* locus in the presence of an inverse agonist is similar to the pattern elicited by the transcription initiation inhibitor triptolide. We identify NCOR as the main ligand-dependent interactor of PPARβ/δ in the presence of the inverse agonist PT-S264. Strikingly, PT-S264–dependent repression is partially insensitive to both trichostatin A, a non-selective HDAC inhibitor, and also to apicidin, an HDAC3-selective inhibitor. Expression of PPARβ/δ mutants in PPARβ/δ knockout cells identified amino acids required for basal repression. These PPARβ/δ mutants revealed diminished NCOR, SMRT, and HDAC3 binding to chromatin in the basal state, concomitant with increased RNAPII binding. Recruitment of NCOR, SMRT, and HDAC3 was restored by the inverse agonist, as was RNAPII loss and repression of transcription. Repression of PPAR target genes by these mutant receptors was largely insensitive to HDAC inhibition. Our data show that chromatin-bound NCOR and SMRT complexes downregulate transcription *via* both deacetylase-dependent and -independent mechanisms.

Materials and methods

Antibodies

Target	supplier	species	clonality	assay	ordering no.	RRID
HDAC3	Santa Cruz	rabbit	polyclonal	ChIP	sc-11417	AB_2118706
LDH	Santa Cruz	rabbit	polyclonal	IB	sc-33781	AB_2134947
MED1	Santa Cruz	rabbit	polyclonal	ChIP	sc-8998	AB_2144021
MED13L	Bethyl	rabbit	polyclonal	ChIP	A302-420A	AB_1907303
NCOR	Abcam	rabbit	polyclonal	ChIP	ab24552	AB_2149005
NCOR	Bethyl	rabbit	polyclonal	ChIP	A301-145A	AB_873085
NCOR	Thermo	rabbit	polyclonal	IB	PA1-844A	AB_2149004
none (IgG fraction)	Sigma	rabbit	polyclonal	ChIP	I5006	AB_1163659
PPARα	Santa Cruz	rabbit	polyclonal	ChIP, IB	sc-9000	AB_2165737
PPARβ/δ	Santa Cruz	rabbit	polyclonal	ChIP	sc-7197	AB_2268420
PPARβ/δ	Santa Cruz	mouse	monoclonal	IB	sc-74517	AB_1128604
PPARγ	Santa Cruz	rabbit	polyclonal	ChIP, IB	sc-7196	AB_654710
RPB1 CTD (RNAPII)	Biolegend	mouse	monoclonal	ChIP	8WG16	AB_2565554
RPB1 NTD (RNAPII)	Santa Cruz	rabbit	polyclonal	ChIP	sc-899	AB_632359
RXR	Santa Cruz	rabbit	polyclonal	ChIP	sc-774	AB_2270041
SMRT	Abcam	rabbit	polyclonal	ChIP	ab24551	AB_2149134
TBLR1	Novus	rabbit	polyclonal	ChIP	NB600-270	AB_10001343
TBP	Santa Cruz	rabbit	polyclonal	ChIP	sc-273	AB_2200059
TFIIA	Santa Cruz	rabbit	polyclonal	ChIP	sc-25365	AB_2116529
TFIIB	Santa Cruz	rabbit	polyclonal	ChIP	sc-225	AB_2114380
TFIIH	Santa Cruz	rabbit	polyclonal	ChIP	sc-293	AB_2262177

Cell culture and treatment

φNX cells [24] were cultivated in DMEM with 10 % FBS. MDA-MB231-*luc2* and Caki-1 cells were cultivated in McCoy's 5A medium with 10 % FBS. The compound or compounds were added for 6 h (expression analyses) or 30 min (ChIP assays) to non-confluent cultures. Control populations were supplied with an equivalent volume of solvent (DMSO for low molecular weight compounds, PBS with 0.1 % (w/v) fatty-acid free BSA for TGFβ).

Compounds

Name	supplier	ordering number or citation
Apicidin	Applichem	A7861
Blasticidin	Fisher Scientific	10648203
DRB	Cayman	10010302
Flavopiridol	Cayman	10009197
L165,041	Tocris	1856
Pioglitazone	Adipogen	AG-CR1-0067
PT-S264	Diederich group	[15]
ST247	Diederich group	[25]
TGFβ from human platelets	Merck	616450
TGFβ from porcine platelets	Sigma	T5300
Trichostatin A	Applichem	A7812
Triptolide	Cayman	11973
Wy-14643	Cayman	70730

ChIP–mass spectrometry (ChIP-MS)

Two technical replicates each were performed by Active Motif Epigenetic Services according to a published protocol [26] from the following MDA-MB231-*luc2* samples: IgG, PT-S264 treatment (0.3 µM); α-PPARβ/δ, PT-S264 treatment (0.3 µM); α-PPARβ/δ, L165,041 treatment (1.0 µM). The unspecific IgG pool from rabbit was obtained from Sigma-Aldrich (no. I5006), the PPARβ/δ antibody from Santa Cruz Biotechnology (sc-7197, rabbit polyclonal). Candidate proteins were filtered with scaffold Viewer 4.8.3 (Proteome Software) according to the following criteria: At least two unique peptides in total and a maximum of two unique peptides each in the negative control replicates (IgG). The protein and the peptide thresholds were each set to 80 %.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

Total RNA was isolated with the NucleoSpin RNA kit (Macherey&Nagel, no. 740955) according to the manufacturer's instructions; the DNase digestion and desalting steps were omitted. Complementary DNA synthesis was carried out with the iScript cDNA Synthesis Kit (Bio-Rad, no. 170-8891SP) according to the manufacturer's instructions with 500 ng of purified RNA per sample. Quantitative PCR analyses were performed in three technical replicates per sample using ABsolute SYBR Green master mix (Thermo Scientific, no. AB-1158B) in Mx3000p and Mx3005

thermocyclers (Stratagene). The *RPL27* transcript was used for normalization. RT-qPCR was carried out using the following primers (250 nM each):

Transcript	forward sequence	reverse sequence
ANGPTL4	TTTTGGTGAACTGCAAGATGA	GAAGTCCACTGAGCCATCGT
PDK4	TTATACATACTCCACTGCACCA	ATAGACTCAGAAGACAAAGCCT
PLIN2	GGAATCTTTAGATGACGTGATGG	CAAGTCTATGGTGGTGAAATCAA
RPL27	AAAGCTGTCATCGTGAAGAAC	GCTGTCACTTTGCGGGGGTAG
TSC22D3	CTTCTCTTCTCTGCTTGGAGGG	CGATCTTGTTGTCTATGGCCAC

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP was essentially performed as described previously [27, 28]. Fixation was performed with 1 % formaldehyde in media for 10 min at room temperature followed by quenching with 125 mM glycin for 5 min. Cells were lysed in buffer L1 (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % (v/v) NP40, protease inhibitor mix (Sigma, no. P8340, 1:1000) for 20–40 min on ice. Nuclei were resuspended in ChIP RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP40 (v/v), 1 mM EDTA) supplemented with 1:1000 protease inhibitor mix (Sigma), incubated on ice for 10–20 min and sheared with a Branson S250D Sonifier (Branson Ultrasonics) using a microtip in 1 ml aliquots in 15 ml conical tubes. 40–52 pulses of 1 s, 4 s pause, 20 % amplitude were applied with cooling of the sample in an ice-ethanol mixture or in a 15 ml tube cooler (Active Motif, no. 53077). A 15 min 17,000×g supernatant was precleared with 10 µg of IgG coupled to 100 µl of blocked sepharose slurry (see below) for 45 min at 4 ℃ with agitation. IP was carried out with 300 µl of precleared chromatin, equivalent to 6–8×10^6 cells. ChIP was performed using 4 µg of antibody per sample. For precipitation, a mixture of protein A and protein G sepharose (GE Healthcare life sciences, no. 1752800 and no. 1706180) or pure protein A sepharose (Zymed) was washed twice with ChIP RIPA buffer and blocked with 1 g/l BSA and 0.4 g/l sonicated salmon sperm DNA (Life Technologies no. 15632011) overnight. 50 µl of blocked bead slurry (1:1 volume ratio with liquid phase) were used per IP. Samples were washed once in buffer I (20 mM Tris pH 8,1; 150 mM NaCl; 1 % (v/v) Triton X-100; 0,1 % (w/v) SDS; 2 mM EDTA), once in buffer II (20 mM Tris pH 8,1; 500 mM NaCl; 1 % (v/v) Triton X-100; 0,1 % (w/v) SDS; 2 mM EDTA), twice in buffer III (10 mM Tris pH 8,1; 250 mM LiCl; 1 % (v/v) NP40; 1 % (w/v) sodium deoxycholate; 1 mM EDTA) on ice and twice in Qiagen buffer EB (no. 19086) at room temperature. Immune complexes were eluted twice with 100 mM $NaHCO_3$ and 1 % SDS (w/v) under agitation. Eluates were incubated overnight at 65 ℃ after adding 10 µg of RNase A and 20 µg of proteinase K in the presence of 180 mM NaCl, 35 mM Tris-HCl pH 6.8 and 9 mM EDTA. An input sample representing 1 % of the chromatin used per IP was reverted in parallel. Samples were purified using the Qiagen PCR purification kit according to the manufacturer's instructions. ChIP-qPCR was performed in three technical replicates per sample with

the ABsolute SYBR Green master mix (Thermo Scientific, no. AB-1158B) in in Mx3000p and Mx3005 thermocyclers (Stratagene). Quantitative PCR was carried out using the following primers (250 nM each):

Genomic region	forward sequence	reverse sequence
ABCA1 TSS	GAGAACCGGCTCTGTTGGT	AATTGCGAGCGAGAGTGAGT
ANGPTL4 –700	AGGCAAGGACTTTTGGTGAG	GGAAGGAGGGAAAGAAAGG
ANGPTL4 –500	CTTTTTCCGTCCTTCCTTCC	GCGACAGAGCCAGAGTACG
ANGPTL4 –275	CGGGCTGGTCTGGAAGTC	GCCCGCCTCTAGTGTGAA
ANGPTL4 TSS	TCCGCACCCAACTTATAAAAAC	GGATCACAGTCGTGTGAGGAT
ANGPTL4 +250	CCTAAGAGGATGAGCGGTGCT	TCGTCCCAGGACGCAAAG
ANGPTL4 +300	CACCGCCGTGCTACTGAG	GCCAGGACATTCATCTCGTC
ANGPTL4 +500	TCCACCGACCTCCCGTTAG	AACCAGCCCTGGGGACAC
ANGPTL4 +750	AGGAATTCAAGACCACCTAAAGC	GTGTGTGTGTGTGTAGAAGAGACG
ANGPTL4 +2,000	CGAATTCAGCATCTGCAAAG	GAGGGACCTTTTCTCCCTTG
ANGPTL4 PPREs (+3,500)	CCCAGAGTGACCAGGAAGAC	CCTTACTGGATGGGAGGAAAG
ANGPTL4 +5,000	TAGGCAGATGGCAGAGAGGT	ACAGTGGATGACCAGGGAAG
ANGPTL4 +7,000	ACGATGGCTCAGTGGACTTC	CTGCCTCTGTCCCCACTAGA
ANGPTL4 +10,500	TGGTGCTGTTGTGTGTAGGTC	GCTTTTATTCCAAGAACTCTGTGAG
PDK4 PPREs (–12,200)	GCAGAGTCAACAAGGGGAAG	ACTAGATGCCTGGGAGCTGA
PDK4 TSS	GTCCCAAACAGGAGGAGTCA	CGGAGCCCATAGTTCTTTCTC
PDK4 –4,500 (control region)	GTATGTGTACTGGGGGGAC	CAGATGGCTCTTTTCGTTCC
PLIN2 PPRE (–34,300)	CCAGTAAGCCTCTTAGCACCAC	TGCAACCTTTCACTTTGTGC
PLIN2 TSS	GACGGACTGCAGCGAAAG	GCGAGGCGGGGTTTATAG
TSC22D3 TSS	GCTGGAGTTGAAGGGAAGTG	AGGAGCCAAAATATCTCCGAGT

Genetic deletion and stable transfection

Vectors for parallel expression of a guide RNA and the Cas9 nuclease were obtained from Santa Cruz Biotechnology. The sequences targeting the *PPARD* coding region were CCCTGTGCAGCTATCCGTTT (1), AACACTCACCGCCGTGTGGC (2), and TCGTACGATCCGCATGAAGC (3). MDA-MB231-*luc2* cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions with the guide/Cas9 expression vectors together with pMSCVbsd [29] for selection of transfected cells. After four hours, the cells were supplemented with fresh medium, and blasticidin was added after 30 hours at a concentration of 10 µg/ml. After ten days, single cells were seeded using limiting

dilution in cell-free medium conditioned by the parental cell line. Wells with more than one colony were terminated. Clones were expanded and screened *via* RT-qPCR (loss of *ANGPTL4* repression by PT-S264) and immunoblotting. The 2B3 clone was transfected with pWZLneo-ecoR [30]. After 24 hours, the cells were selected with 500 µg/ml G418 for 14 days.

PPARD expression vectors

The retroviral vector pMSCVbsd [29] was used to clone the *PPARD* cDNA using the *Bgl*II and *Xho*I sites. Deletions were introduced *via* PCR with specific primers, and the fragment was re-inserted into the empty pMSCVbsd vector. Alterations in the cDNA sequence were introduced with site-directed mutagenesis. Vectors are available upon request.

Retroviral transduction

For production of ecotropic retroviruses, pMSCVbsd-*PPARD* vectors or the empty vector were transfected into subconfluent φNX packaging cells [24]. After four hours, 7 ml of fresh medium were added. Twenty-four hours later, the supernatant was harvested, centrifuged at 800×g, and 3 ml aliquots were snap frozen. The φNX cells received fresh medium, and a second supernatant was harvested 24 hours later. Freshly seeded MDA-MB231-*luc2*-2B3 cells ectopically expressing the ecotropic receptor were incubated with 2 ml of fresh medium, 3 ml of φNX supernatant, and 4 µg/ml polybrene for 24 hours. Selection was performed with 10 µg/ml blasticidin for ten days, and cells were subsequently cultured in the presence of blasticidin.

Results

PPARβ/δ inverse agonists interfere with formation of an initiation complex

PT-S264 is an inverse PPARβ/δ agonist with improved repressive properties and stability [15]. We first tested whether it affects initiation complex formation like the previously used inverse agonist ST247 [25], which reduces RNAPII binding to the *ANGPTL4* promoter [4]. Treatment with PT-S264 diminished RNAPII binding to the *ANGPTL4* TSS (scanning ChIP-qPCR using closely spaced primer pairs, fig. 1A) both in the presence and in the absence of TGFβ, which induces *ANGPTL4* transcription. The binding pattern of RNAPII in PT-S264–treated cells was similar to the binding pattern observed in cells treated with the transcription initiation inhibitor triptolide. In contrast, treatment of the cells with the CDK9 inhibitors DRB and flavopiridol, which interfere with elongation, resulted in RNAPII accumulation

at the TSS. Caki-1 cells highly express *ANGPTL4* [4] and hence allow for ChIP-based detection of GTFs at the endogenous locus. To address which step of initiation complex formation was affected, we measured the occupancy of GTFs and Mediator at the *ANGPTL4* TSS in the presence or in the absence of PPARβ/δ inverse agonists. PT-S264 impedes binding of TFIIB and RNAPII. Yet, GTFs are present, indicating that the TSS is accessible (fig. 1B). Since the occupancy of TFIIA was not altered, we postulate that the repressive mechanism affects the transition from the scaffold complex to the RIC; PIC formation may be impaired too, which cannot be inferred from these data obtained after short-term treatment. *TSC22D3* is not a PPAR target gene; reduced TFIIB and RNAPII occupancy were not observed at its TSS upon treatment with the inverse agonist PT-S264. In the presence of TGFβ, TFIIB binding to the *ANGPTL4* TSS is increased, and this is counteracted by PT-S264 (fig. 1C).

The Mediator complex facilitates the rate-limiting step of TFIIB recruitment, and both Mediator and TFIIB are necessary for RNAPII binding [31]. Previous ChIP-qPCR experiments after treatment with the inverse agonist ST247 showed that both TFIIH, another GTF present in the scaffold, and MED1 (Mediator subunit 1) bind to the *ANGPTL4* TSS after TGFβ treatment. However, in the presence of the inverse agonist ST247, Mediator but not TFIIH recruitment was blocked (fig. 1D, left panel). This result shows that inverse agonists does not generally block recruitment of PIC components. On the other hand, MED1 recruitment to the upstream TGFβ-responsive enhancer [16] was unaltered (fig. 1D, middle panel). We additionally tested whether the Mediator kinase module, which regulates reinitiation by sterically preventing RNAPII binding [32], is recruited in the presence of PT-S264. Scanning ChIP across the *ANGPTL4* TSS with an antibody against MED13L, a Mediator subunit that resides in the kinase module, shows recruitment upon stimulation with TGFβ but not after simultaneous treatment with the inverse agonist PT-S264 (fig. 1E) in the same manner as the core Mediator (fig. 1D, left panel), TFIIB (fig. 1C), and RNAPII (fig. 1A and supp. fig. S1). This argues against an instrumental role of the kinase module in inverse agonist-mediated repression and is consistent with the notion that the kinase module is detected at promoters in metazoans [33]. We conclude that PPARβ/δ inverse agonists perturb transcription initiation at the *ANGPTL4* TSS presumably by interfering with Mediator–TFIIB–RNAPII recruitment to promoter-bound GTFs.

PT-S264 augments NCOR binding to PPARβ/δ

We previously found that depletion of NCOR by RNAi alleviates basal repression to the same extent as PPARβ/δ depletion but does not prevent repression by an inverse agonist [4]. However, RNAi-mediated partial depletion may not suffice for full loss of function. To identify candidate repressors in an unbiased approach, ChIP-mass spectrometry (ChIP-MS) according to the RIME (rapid immunoprecipitation and mass spectrometry of endogenous proteins) protocol [26] was performed with an antibody against PPARβ/δ (see table 1 on the following page). We used

Protein or cluster	Gene(s)	IgG PT-S264	α-β/δ PT-S264	α-β/δ L165, 041	diff. count
Neuropathy target esterase	*PNPLA6*	0	36	41	-5
Envoplakin	*EVPL*	0	32	24	8
Periplakin	*PPL*	0	20.5	10.5	10
Cytospin-A	*SPECC1L*	0.5	16	22.5	-6.5
Nuclear receptor corepressor 2	***NCOR1, NCOR2***	0	14.5	0	14.5
Inorganic pyrophosphatase 2, mitochondrial	*PPA2*	0	14.5	14	0.5
Kinesin-like protein KIF15	*KIF15*	0.5	11	5.5	5.5
Peroxisome proliferator-activated receptor δ	***PPARD***	0	10	16	-6
2-hydroxyacyl-CoA lyase 1	*HACL1*	0	10	12	-2
Inositol 1,4,5-trisphosphate receptor type 2	*ITPR2*	0	9.5	5	4.5
BAG family molecular chaperone regulator 3	*BAG3*	0	8.5	6.5	2
Retinoic acid receptor RXR-β	***RXRB, RXRA***	0	8	14.5	-6.5
Myotubularin-related protein 12	*MTMR12*	0	8	5.5	2.5
Zinc finger protein ZPR1	*ZPR1*	0	7	7	0
TBC1 domain family member 2A	*TBC1D2*	0.5	6	4	2
Melanoma inhibitory activity protein	*MIA3*	0	5.5	2	3.5
Nuclear distribution protein nudE-like 1	*NDEL1, NDE1*	0	5.5	4	1.5
GTPase-activating protein and VPS9 domain-containing 1	*GAPVD1*	0	5.5	2	3.5
Zinc finger and BTB domain-containing 9	*ZBTB9*	0.5	4.5	3.5	1
Membrane-associated progesterone receptor component 2	*PGRMC2*	0	4	2	2
Protein kinase Cδ-binding protein	*PRKCDBP*	1	4	3.5	0.5
Complement C4-A	*C4A*	0	3.5	3.5	0
Non-specific lipid-transfer protein	*SCP2*	1	3	0	3
Igγ-2 chain C region	*IGHG2*	2	3	1	2
5'-3' exoribonuclease 1	*XRN1*	0	2.5	5	-2.5
MIA SH3 domain ER export factor 2	*MIA2/CTAGE5*	0	2.5	0	2.5
m7GpppX diphosphatase	*DCPS*	0	2.5	3.5	-1
EF-hand calcium-binding domain-containing protein 4A	*CRACR2B*	0	2.5	2.5	0
Complement C3	*C3*	0	2	0	2
FGFR1 oncogene partner	*FGFR1OP*	0	2	0.5	1.5
E3 ubiquitin-protein ligase TRIM4	*TRIM4*	1	2	1	1
3-ketoacyl-CoA thiolase, peroxisomal	*ACAA1*	0	1.5	0.5	1
Transducin-β like 1 X-linked receptor 1	***TBL1XR1***	0	1.5	0	1.5
Importin subunit α-1	*KPNA2*	1	1.5	0.5	1
Eukaryotic translation initiation factor 5	*EIF5*	1.5	1	0	1
E3 ubiquitin-protein ligase RFWD2	*RFWD2*	0	0	2	-2

Table 1. PPARβ/δ interactors in the presence of the inverse agonist PT-S264 or the agonist L165,041 (RIME ChIP-MS analysis). The numbers indicate the mean unique peptide count from two technical replicates each. The difference count is peptides(PT-S264)–peptides(L165,041) for the cognate IPs (α-PPARβ/δ). An IgG fraction from non-immunized rabbits served as a negative control. The gene names of the antibody target (*PPARD*) and known interactors are printed in boldface.

chromatin from MDA-MB231-*luc2* cells, in which PPARβ/δ target gene repression by inverse agonists is particularly strong (supp. fig. S2 and [4]). PPARβ/δ and RXR were robustly identified both in the presence of the inverse agonist PT-S264 and the agonist L165,041. Importantly, NCOR (encoded by the *NCOR1* gene) and SMRT (encoded by the *NCOR2* gene) were identified as interactors only in the presence of PT-S264 but not in the presence of L165,041. Other known transcriptional repressors were not detected. The NCOR2 protein cluster revealed 15 and 7 unique peptides for NCOR after treatment with PT-S264 in the two technical replicates, while only 4 and 3 peptides were identified for SMRT. This finding may indicate that NCOR binding to PPARβ/δ is dominant over SMRT binding in MDA-MB231-*luc2* cells in the presence of PT-S264. Of note, TBL1XR1, a subunit of NCOR and SMRT complexes [8], was also only detected in the presence of PT-S264 but not in the presence of L165,041.

Next, we measured NCOR binding to the PPARβ/δ binding sites (PPREs) of the target genes *ANGPTL4* and *PDK4* [1] in the presence and in the absence of PT-S264 by ChIP-qPCR using two different antibodies. In agreement with ChIP-MS data, NCOR was present at the *ANGPTL4* PPRE. Interestingly, we found increased binding of NCOR after treatment with the inverse agonist PT-S264 both in the presence and in the absence of TGFβ (fig. 2A). Further ChIP-qPCR analysis of several NCOR and SMRT complex subunits was in line with the ChIP-MS results, showing increased recruitment to the PPARβ/δ binding site of *ANGPTL4* in the presence of PT-S264 and decreased recruitment in the presence of L165,041 (fig. 2B). Increased NCOR recruitment to the *ANGPTL4* PPRE was also observed in Caki-1 cells upon treatment with PT-S264 (supp. fig. S3). Due to its marked contribution to basal repression [4] and its enhanced recruitment to chromatin-bound PPARβ/δ in the presence of PT-S264, we postulate that NCOR mediates downregulation of *ANGPTL4* by PPARβ/δ in the basal state and in the presence of PPARβ/δ inverse agonists.

A reconstitution screen identifies PPARβ/δ mutants defective in ligand response and basal repression

We were unable to obtain NCOR knockout clones in the MDA-MB231-*luc2* and Caki-1 cell lines with the CRISPR-Cas9 system. In order to investigate the role of NCOR in PT-S264–mediated repression, we therefore aimed to interfere with NCOR binding to PPARβ/δ through targeted mutation of the receptor. To this end, expression of PPARβ/δ was disrupted in MDA-MB231-*luc2* cells using the CRISPR-Cas9 system. MDA-MB231-*luc2* harbour two or three *PPARD* alleles per cell according to FISH analyses (data not shown). Four different PPARβ/δ knockout clones were isolated (fig. 3A). Neither PPARα nor PPARγ expression was disrupted in the four clones (fig. 3B,C). ChIP-qPCR confirmed the absence of PPARβ/δ at the *ANGPTL4* and *PDK4* loci (supp. fig. S4). Interestingly, binding of PPARα and PPARγ to the *ANGPTL4* and *PDK4* loci, which each harbour three adjacent PPRE sequences [1, 12, 16], is

increased in PPARβ/δ knockout cells (supp. fig. S4), suggesting that PPARα, PPARβ/δ, and PPARγ compete for the same binding sites. However, relative to wildtype cells, binding of RXR is reduced in PPARβ/δ KO cells. This finding indicates that PPARβ/δ is the main factor that binds to the PPREs of the *ANGPTL4* and *PDK4* genes in MDA-MB231-*luc2*. Consistently, both induction of the *ANGPTL4* transcript by the synthetic agonist L165,041 and repression by the inverse agonist PT-S264 were disrupted in the knockout clones (supp. fig. S5). In contrast, the PPARγ agonist pioglitazone retained its function and stimulated *ANGPTL4* expression. Surprisingly (for unknown reasons), the PPARα agonist Wy-14643 did not activate expression of the *ANGPTL4* gene. (supp. fig. S5).

For rescue experiments with wildtype PPARβ/δ and corresponding mutants, we used the 2B3 clone, which neither showed growth defects nor stable integration of the Cas9 expression vector. Sequencing of clones from PCR fragments indicated that guide RNAs 1 and 3 resulted in single-base deletions (no. 3) or insertions (no. 1). In line with previous observations [34], ectopic expression of PPARβ/δ driven by the strong cytomegalovirus (CMV) promoter led to formation of intracellular aggregates and failed to restore ligand function (data not shown). We stably transfected this cell clone with a vector encoding the murine ecotropic retroviral receptor [30], allowing for ecotropic retroviral infection. The resulting 2B3-ecoR cells were then transduced with pMSCV vectors to express wildtype or mutant PPARβ/δ, driven by the comparably weak viral LTRs. Expression of the mutants was confirmed on protein level (supp. fig. S7). We assume that PPARβ/δ is weakly expressed in all cellular models we analysed due to low signals on both RNA and protein levels. The level of PPARβ/δ ectopically expressed from pMSCV was above that of wildtype cells (fig. 3D). Nevertheless, both the activation and repression functions of PPARβ/δ were restored (see fig. 4), albeit to lesser extent than that observed in wildtype cells (supp. fig. S6).

Next, we generated a panel of 67 mutants that encode for truncated proteins or proteins with one or more substitutions of amino acid residues. Preferentially, mutations of amino acids at the surface of the LBD (ligand binding domain) were used. The panel also includes a negative control, C91A-E92A ”CE2A”, a double mutant of residues critical for DNA binding [35], and mutants in which the AF2 (activating function 2) domain was disrupted—leucines 429, 432, and 433 in the LXXLL motif of helix 12. These mutants should not respond to agonists. We performed a functional screen, intending to identify residues in PPARβ/δ which are necessary for basal and PT-S264–regulated repression of the *ANGPTL4* gene. *ANGPTL4* expression was monitored after treatment with the solvent, the inverse agonist PT-S264, or the agonist L165,041. Importantly, the ligands did not modulate *ANGPTL4* expression in PPARβ/δ knockout cells transduced with the empty vector or the DNA binding-deficient PPARβ/δ C91A-E92A mutant (fig. 4). Inherent technical and biological variation of the RT-PCR–based readouts hampered direct comparison of cohorts of cells infected at different time points. Therefore, we calculated the *ANGPTL4* mRNA expression levels relative to the mean of twelve biological replicates from wildtype PPARβ/δ-reconstituted 2B3 cells. We plotted the $\log_2$ fold change mutant / fold change wildtype and used a cutoff of $2^{1.5}$, equivalent to 2.8 fold. The results of the

screen are summarised in fig. 4. Raw expression data for all mutants are available in the supplementary material.

The positions of selected mutations referred to below are highlighted in fig. 4E. Our screen identified six types of mutations:

(i) Mutations which disrupt basal repression (fig. 4A). These are K421A-K422A and T427I; in cells expressing these mutants, ligand function is not generally impaired, while *ANGPTL4* expression is not downregulated in the absence of ligand. The R314A and T427F mutants are also deficient in basal repression but scored slightly below the cutoff.

(ii) Mutations which enhance basal repression (fig. 4A). Ligand function is not generally impaired; the I327A, T423A, T427D, and L433F mutants cause diminished *ANGPTL4* expression in the absence of ligand compared to wildtype PPARβ/δ.

(iii) Function of the inverse agonist PT-S264 is defective upon expression of the R321K and T423D-T427D, and LxxLL (L429A, L432A, L433I, L433F) mutants, while basal repression was unaffected (fig. 4B).

(iv) Function of the agonist L165,041 was severely impaired upon expression of LxxLL, AF-2, Y437A, and C-terminal deletion mutants (ΔAF-2 and ΔL-box–AF-2); see fig. 4C.

(v) Ligand binding is presumably affected by the F324A and H413W mutations. These amino acid residues face inside the ligand binding pocket (inferred from structural data, PDB ID 3TKM [36]). The F324A mutation apparently does not interfere with PT-S264 function (fig. 4A–C).

(vi) Mutations which lead to general defects of PPARβ/δ (fig. 4A–C); these affect DNA binding or RXR heterodimerisation (C91A-E92A, G146A and A404K, A404W, L406R, respectively; inferred from structural data, PDB ID 3DZY [37]) and presumably protein folding (V257R) or coregulator interaction in general (V279R).

Deletion of helix 12 (AF2) and its connecting loop (L-box) did not abrogate PT-S264–mediated downregulation of *ANGPTL4* expression (fig. 4B), demonstrating that the AF2 domain is not required for repression by the inverse agonist. Loss of basal repression in cells expressing the K421A-K422A and T427I mutants leads to augmented repression in the presence of the inverse agonist (fig. 4B), resulting from a higher basal transcription. Another striking observation pertains to the role of the synthetic agonist L165,041: The mutants with defective basal repression show only weak upregulation of *ANGPTL4* after agonist exposure (fig. 4C). In the presence of TGFβ, repression in cells expressing these mutants was slightly enhanced (fig. 4D). Induction by the agonist was again weaker upon expression of the mutants. In further analyses we focused on mutants which showed a loss of basal repression because they allow to distinguish between basal and ligand-mediated repression and are thus suitable for investigation of the ligand-inducible repression mechanism.

PPARβ/δ mutants defective in basal repression show diminished NCOR and SMRT recruitment to chromatin and increased RNA polymerase II recruitment in the basal state

ChIP-qPCR analysis revealed that the binding of NCOR and SMRT to PPARβ/δ-responsive elements of the ligand-responsive genes *ANGPTL4*, *PDK4*, and *PLIN2* is markedly reduced in cells that express the T427I, K421A-K422A, and R314A mutants (fig. 5). However, in the presence of PT-S264, NCOR and SMRT binding is also restored by the T427I, K421A-K422A, and R314A mutants. Essentially, the same observation was made for HDAC3 (supp. fig. S8). Moreover, binding of RNAPII to the *ANGPTL4*, *PDK4*, *PLIN2*, and *ABCA1* target gene TSSs is strongly reduced in *PPARD* KO cells reconstituted with wt PPARβ/δ (fig. 6A–D) but not in cells expressing the T427I and K421A-K422A mutants. Strikingly, PT-S264 treatment led to reduced RNAPII binding in cells expressing these mutants. RNAPII binding at the *TSC22D3* TSS, which is not a PPAR target gene, was neither affected by expression of the PPARβ/δ-encoding cDNAs nor by PT-S264 (fig. 6F). This finding shows that the PPARβ/δ mutants deficient in basal NCOR, SMRT, and HDAC3 binding allow for increased RNAPII occupancy in the absence of activating ligands. On the other hand, these mutants recruit NCOR, SMRT and HDAC3 in the presence of inverse agonists, and thus RNAPII binding is strongly reduced.

The role of deacetylase activity in repression by PT-S264

To characterise the function of HDAC3 in the repressive mechanism, we treated cells with PT-S264 together with deacetylase inhibitors. In wildtype MDA-MB231-*luc2*, neither TSA nor the HDAC3-selective compound apicidin abrogates repression of *ANGPTL4* elicited by PT-S264 (fig. 7A, upper left panel) which is however diminished. PT-S264–mediated repression of *PDK4*, which is weaker in comparison, is also impeded by the HDAC inhibitors (fig. 7A, upper right panel), albeit to a lesser extent. Another PPARβ/δ target gene transcript, *PLIN2*, shows the same pattern (fig. 7A, lower left panel).

In KO cells with restored wildtype PPARβ/δ expression, PT-S264–mediated repression of *ANGPTL4* is insensitive to the HDAC inhibitors, whereas in *PPARD* KO cells expressing mutants defective in basal repression, repression of *ANGPTL4* is partially sensitive to HDAC inhibition (fig. 7B). This clearly suggests that the NCOR and SMRT complexes recruited to PPARβ/δ target genes exert both deacetylase-dependent and deacetylase-independent repressive functions; the catalytic activity of HDAC3 contributes to but is not sufficient for full repression. This is in agreement with the observation that *ANGPTL4* expression is only weakly induced by HDAC inhibitors (fig. 7B and [4]).

In general, weaker repression in 2B3 cells reconstituted with the wildtype *PPARD* cDNA relative to the parental cells was observed (supp. fig. S6). We attribute this to sequestration of corepressors by overexpressed wildtype

PPARβ/δ, which recruits NCOR and SMRT considerably stronger than the mutants deficient in basal repression (fig. 5). Sequestration might differentially affect the availability of free NCOR and SMRT.

Discussion

NCOR and SMRT figure in gene regulation by a multitude of transcription factors such as the glucocorticoid receptor [38, 39], other nuclear receptors [7, 40], POZ-containing, basic helix-loop-helix, and basic leucine zipper factors [41], the Notch effector RBP-J [42], and MECP2 (methylated CpG binding protein 2) [43]. Therefore, elucidation of the mechanisms used by NCOR and SMRT complexes is of great interest. We show here that PPARβ/δ inverse agonists interfere with recruitment of Mediator, TFIIB, and RNAPII. PPARβ/δ mutants deficient in NCOR and SMRT recruitment in the basal state allow for enhanced RNAPII binding in the absence of ligands, while the inverse agonist PT-S264 restores both NCOR/SMRT binding to these mutants and loss of RNAPII at target gene promoters. Repression is only partially sensitive to HDAC inhibition, which indicates that NCOR/SMRT complexes exert deacetylase-dependent and deacetylase-independent functions to restrain transcription of PPARβ/δ target genes.

HDAC-independent repression mechanisms exerted by NCOR/SMRT have also been reported in other contexts: (I) NCOR and SMRT complexes carry out essential functions that are independent of HDAC3 activity [44], and (II) HDAC-independent repression of human papillomavirus transcription and replication by NCOR was demonstrated [45]. Moreover, whereas MECP2 represses transcription *via* an HDAC-independent mechanism [46], interaction with NCOR/SMRT is crucial for MECP2 function [43], and it was reported recently that detrimental effects of MECP2 overexpression do not depend on HDAC3 catalytic activity [47] (III).

Our data suggest that deacetylation by HDAC3 contributes to but is not sufficient for basal repression of PPARβ/δ target genes. Thus, an additional enzymatic function of HDAC3-containing complexes or, alternatively, inhibition of an activator-borne enzymatic activity may be required for efficient silencing of gene expression. We speculate that acetyltransferases are inhibited by NCOR and SMRT complexes since (i) acetyltransferase activity is required for transcription in nucleosome-free systems [48], (ii) GTFs that contribute to reinitiation—TFIIB, TFIIE, and TFIIF—are acetylated *in vivo* [49, 50], and (iii) the acetyltransferase activities of CBP and p300 are inhibited by NCOR and SMRT *in vitro* [51, 52]. Moreover, Mediator interacts with the acetyltransferase-bearing SAGA complex, and both Mediator and SAGA are necessary for RNAPII-mediated transcription in yeast [53, 54, 55]. Partial sensitivity of PT-S264–dependent repression to HDAC inhibitors strongly suggests that NCOR and SMRT recruited by PPARβ/δ use an additional mechanism to restrict activator function. Induction of the PPARβ/δ target gene *ANGPTL4* by TGFβ depends on SMAD3 [16], which interacts with CBP and p300 to activate transcription [56]. CBP and p300 are also paramount coactivators of NFκB [57] and AP-1 [58] transcription factors, which are major targets of transrepression

by nuclear receptors [39, 58, 59, 60]. These data underscore a possible role for acetyltransferase inhibition in gene regulation by NCOR and SMRT. Since deacetylation and inhibition of acetyltransferase activity act towards the same outcome, the two functions may conceivably act in parallel for more efficient suppression of transcription. Another possibility would be that ubiquitin ligase and deubiquitinase activities which reside in subunits of HDAC3-containing complexes contribute to deacetylase-independent repression. Ubiquitination by TBLR1 [7] could commit activators for degradation, or H2B deubiquitination by USP44 might figure in repression of *ANGPTL4* by NCOR/SMRT [61].

Mediator supports TFIIB binding to promoters, and both Mediator and TFIIB facilitate recruitment of RNAPII to promoter-bound GTFs [31]. This is in line with recent MS data from *S.cerevisiae* which identifies RNAPII and TFIIB among the strongest interactors of Mediator [53]. Moreover, another recent study revealed that human genes which require *de novo* RNAPII recruitment for induction of transcription depend on TFIIB availability [62]. It is unclear how transcription factors modulate reinitiation, and *in vivo* evidence of reinitiation and scaffold complexes is lacking [63]. Our ChIP data (fig. 1) which demonstrate regulation of Mediator, TFIIB, and RNAPII binding by PPARβ/δ inverse agonists are in agreement with findings obtained from *in vitro* systems which describe recruitment of RNAPII and TFIIB [19, 31] to scaffold factors and thus may represent *in vivo* correlates.

It should be noted that we cannot exclude involvement of other corepressors. Genetic deletion of *NCOR1* was unsuccessful in two cell lines; this is consistent with the notions that NCOR function may be necessary for regulation of the cell cycle [64], genome stability [65], or other critical processes [40]. Cellular models without this limitation or knock-in approaches might be suitable to resolve whether NCOR and SMRT complexes are sufficient for repression by PPARβ/δ inverse agonists. Modification of endogenous coding sequences would also circumvent possible effects of overexpression such as cofactor sequestration. Notably, PT-S264–dependent repression after reconstitution of PPARβ/δ expression was fully insensitive to HDAC inhibition, which is in contrast to partial sensitivity observed in the parental cell line. Moreover, HDAC inhibition led to weaker upregulation of PPARβ/δ target genes in the parental cells compared to cells with reconstituted PPARβ/δ.

Taken together, we propose that PPARβ/δ recruits NCOR and SMRT, either of which or both block Mediator recruitment to promoter-bound GTFs *via* both deacetylase-dependent and -independent functions to interfere with TFIIB and RNAPII binding. A model depicted in fig. 8 summarises our conclusions. Additional insight will require identification of the subunits and domains of NCOR and SMRT complexes which exert deacetylase-independent repression and their target proteins, and it will be interesting to probe possible differential functions of NCOR and SMRT.

Figures and Figure Legends

1. PPARβ/δ inverse agonists suppress transcription initiation of *ANGPTL4* and recruitment of Mediator and TFIIB to a scaffold complex. Caki-1 cells were treated for 30 min as indicated and subjected to ChIP-qPCR analysis. **A**: Scanning ChIP-qPCR of the *ANGPTL4* locus. An antibody against the CTD of the large subunit of RNAPII was used. **B**: Binding of TFIIA, TFIIB, and RPB1 at the *ANGPTL4* and *TSC22D3* TSSs. **C**: TFIIB binding at the *ANGPTL4* TSS in the presence of TGFβ, PT-S264, or both. The right panel shows scanning ChIP around the *ANGPTL4* TSS from the same samples. **D**: Binding of TBP, TFIIH, and MED1 at the *ANGPTL4* TSS and the upstream TGFβ- responsive enhancer (8,500 bp upstream of the TSS) in the presence of TGFβ, ST247, or both. **E**: Scanning ChIP-qPCR analysis of MED13L binding around the *ANGPTL4* TSS after stimulation with TGFβ, PT-S264, or both. Means and standard deviation of three technical replicates from representative ChIP assays are plotted.

2. NCOR and SMRT complex subunits are recruited to PPARβ/δ in the presence of PT-S264. MDA-MB231-*luc2* cells were treated as indicated for 30 min, and ChIP-qPCR was performed with antibodies against subunits of HDAC3-containing complexes using primer pairs encompassing the *ANGPTL4* and *PDK4* PPREs (+3,500 or –12,200 bp relative to the TSS of the gene, respectively). **A**: Binding of NCOR in the presence of 300 nM PT-S264, 1 ng/ml TGFβ, or both. Two different antibodies against NCOR were used. **B**: Binding of PPARβ/δ, RXR, HDAC3, TBLR1, NCOR, and SMRT in the presence of solvent (DMSO), 300 nM PT-S264, or 1 μM L165,041. Both panels: Representative ChIP data are plotted. Values denote means and standard deviation from three technical replicates.

3. Characterisation of *PPARD* knockout clones and reconstitution of PPARβ/δ expression. **A**: Immunoblot analysis of PPARβ/δ expression in the parental MDA-MB231-*luc2* cell line and four knockout clones (2B2, 2B3, 2A3, 2A6). The samples were probed for LDH expression as a loading control. **B,C**: Analysis of PPARα (**B**) and PPARγ (**C**) expression in the KO clones. The arrowhead in **B** indicates the band at the expected molecular weight for PPARα. **D**: Reconstitution of the 2B3 *PPARD* KO clone. 2B3 cells were stably transfected with an expression vector for the murine ecotropic receptor and subsequently transduced with a *PPARD*-encoding retrovirus. Two different exposure times are shown for the PPARβ/δ immunoblot.

4. A retroviral reconstitution screen identifies PPARβ/δ mutants deficient in ligand response and basal repression. 2B3 cells stably expressing the murine ecotropic receptor were transduced with retroviruses derived from the empty vector or carrying *PPARD* cDNAs. The wildtype cDNA and a panel of 67 mutants (N between 1 and 8 per construct) were used. *ANGPTL4* expression was measured by RT-qPCR after treatment for 6 h. **A**: Effects on

basal repression. Mean Ct values relative to the Ct value obtained from cells expressing the WT *PPARD* cDNA (N=12, mean Ct 29.5) were plotted. **B,C**: Effects on repression elicited by 300 nM PT-S264 and activation of transcription by 1 µM L165,041. The $\log_2$ ratio ($\Delta\Delta\Delta$Ct) of the relative expression in the respective mutant to the WT was plotted. The mean relative expression (fold change) to the solvent control in cells expressing the WT cDNA is indicated. **D**: The relative expression of *ANGPTL4* was measured in cells transduced with the empty vector ($N\geq3$), a retrovirus carrying the CE2A DNA binding mutant cDNA ($N\geq4$), the wildtype (≥8), the T427I (≥6), the K421A-K422A ($N\geq5$), or the R314A mutant ($N\geq3$) cDNAs and treated as indicated. The fold change relative to the solvent control sample was plotted. Significance levels were calculated *via* unpaired *t* tests between the fold repression values. **E**: A schematic representation of the PPARβ/δ protein including structural features, sequence motifs, and the locations of selected mutations.

5. Corepressor recruitment by PPARβ/δ mutants defective in basal repression. 2B3 *PPARD* KO cells transduced with the indicated constructs were treated with solvent, 1µM L165,041, or 300 nM PT-S264 for 30 min and subjected to ChIP-qPCR analysis using antibodies against NCOR or SMRT. The PPAR binding sites of *ANGPTL4* (+3,500 bp relative to the TSS, **A**), *PDK4* (–12,200 bp, **B**), *PLIN2* (–34,300 bp, **C**) and a negative control region were amplified as indicated. Non-cognate IPs (IgG) were processed in parallel from the solvent-treated cells (**D**). Means and standard deviations of three technical replicates from a representative ChIP assay are plotted.

6. Regulation of RNA polymerase II binding by PPARβ/δ mutants defective in basal repression. 2B3 *PPARD* KO cells transduced with the indicated constructs were treated with solvent or 300 nM PT-S264 for 30 min and subjected to ChIP-qPCR analysis using an antibody against RNA polymerase II. The TSS regions of *ANGPTL4* (**A**), *PDK4* (**B**), *PLIN2* (**C**), *ABCA1* (**D**), a negative control region (**E**), and the *TSC22D3* TSS (**F**) as an additional negative control were amplified as indicated. Means and standard deviations of three technical replicates from a representative ChIP assay are plotted.

7. HDAC inhibitor sensitivity of repression by PPARβ/δ mutants deficient in basal repression. Parental MDA-MB231-*luc2* and 2B3 *PPARD* KO cells (**A**) or 2B3 *PPARD* KO cells transduced with the indicated constructs (**B**) were treated with solvent, 300 nM PT-S264, 500 nM TSA, and 250 nM apicidin as indicated for 6 h, and RT-qPCR with primers against the *ANGPTL4*, *PDK4*, *PLIN2*, and *TSC22D3* transcripts was performed. Means and standard deviations of (A) three experiments or (B) one representative experiment (three technical replicates) are plotted. Mean normalised Ct values in the control condition are indicated. In A, significance levels were calculated *via* unpaired *t* tests between the fold repression values with and without PT-S264.

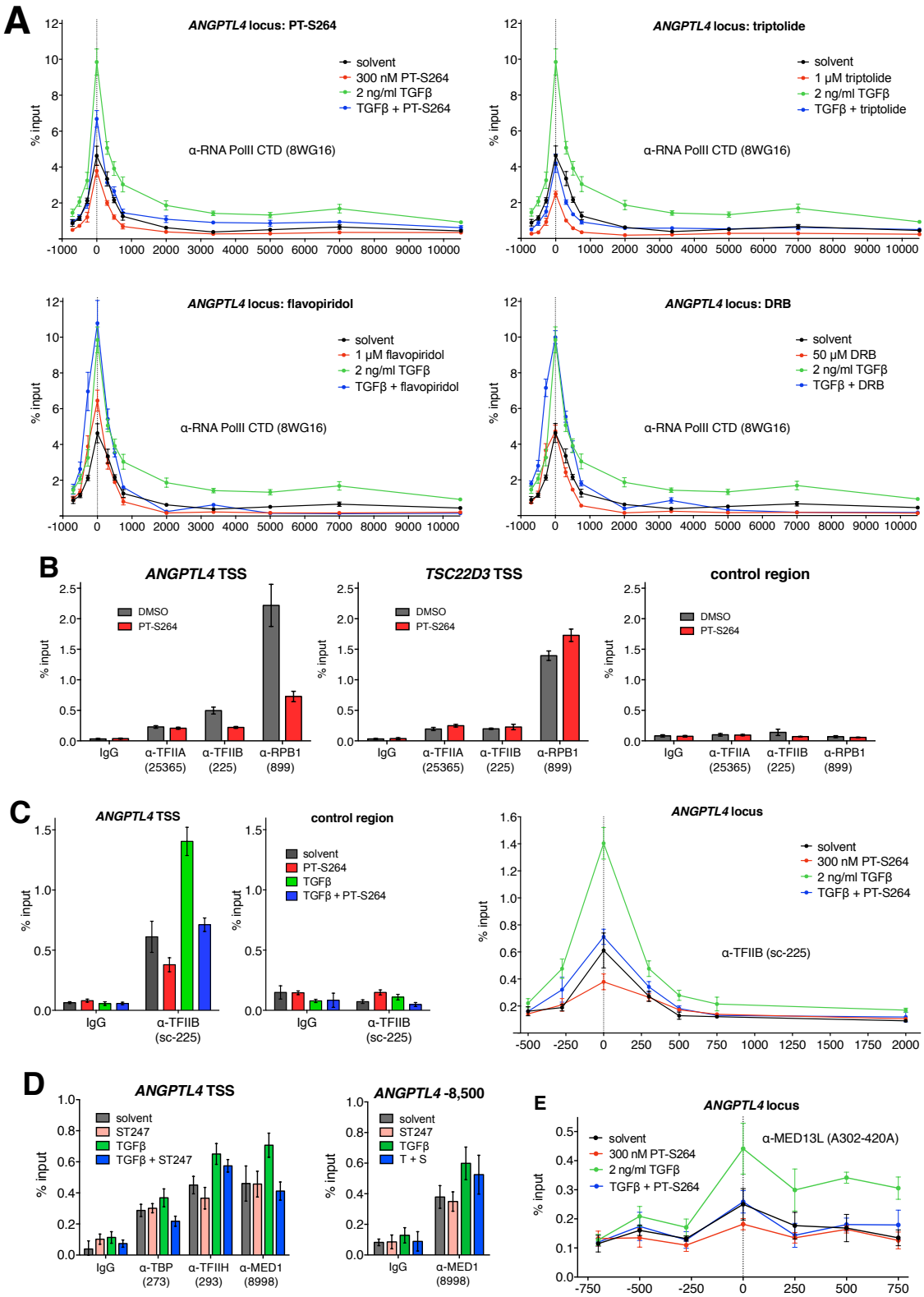


Fig. 1. PPARβ/δ inverse agonists suppress transcription initiation of *ANGPTL4* and recruitment of Mediator and TFIIB to a scaffold complex.

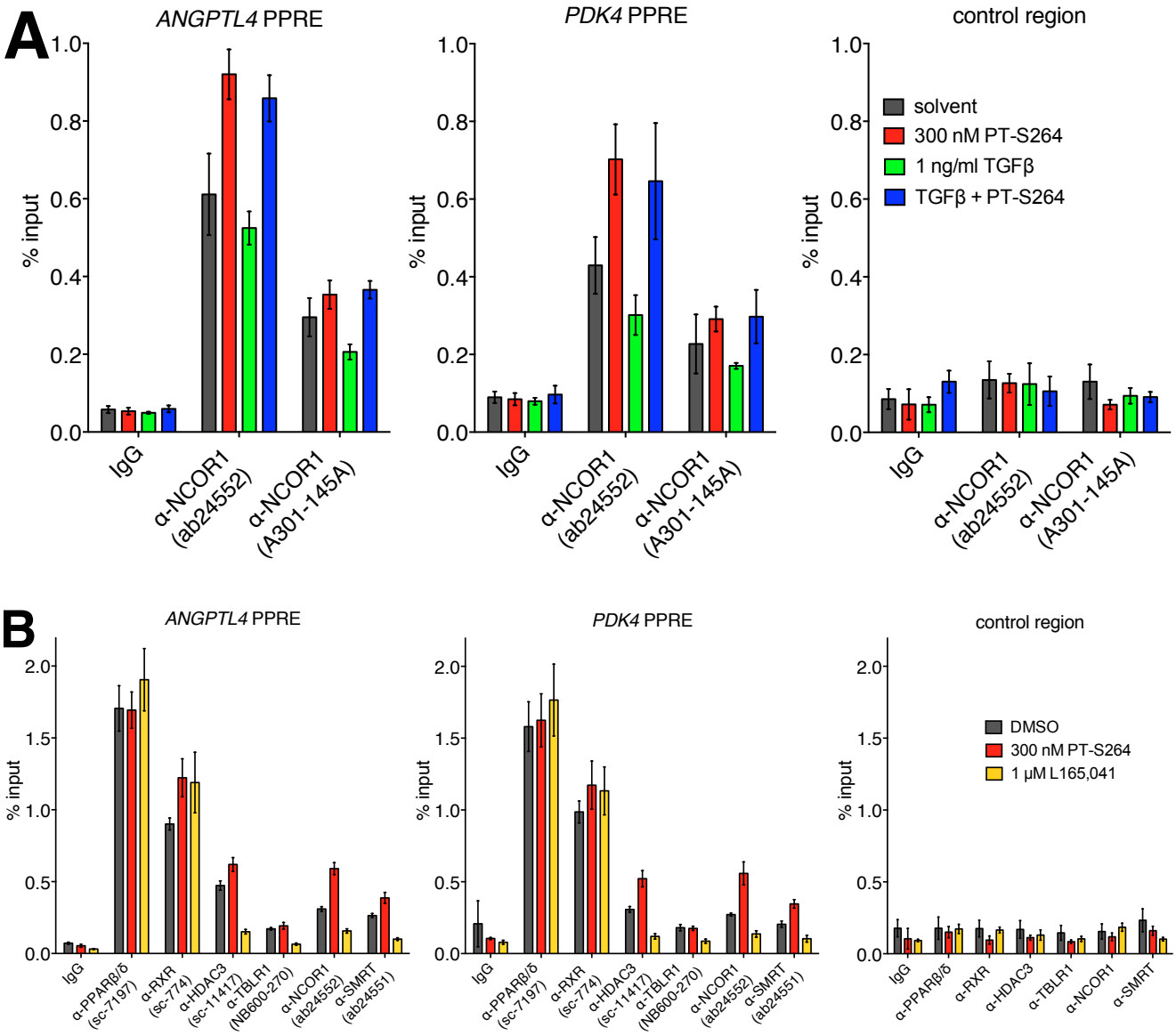


Fig. 2. NCOR and SMRT complex subunits are recruited to PPARβ/δ in the presence of PT-S264.

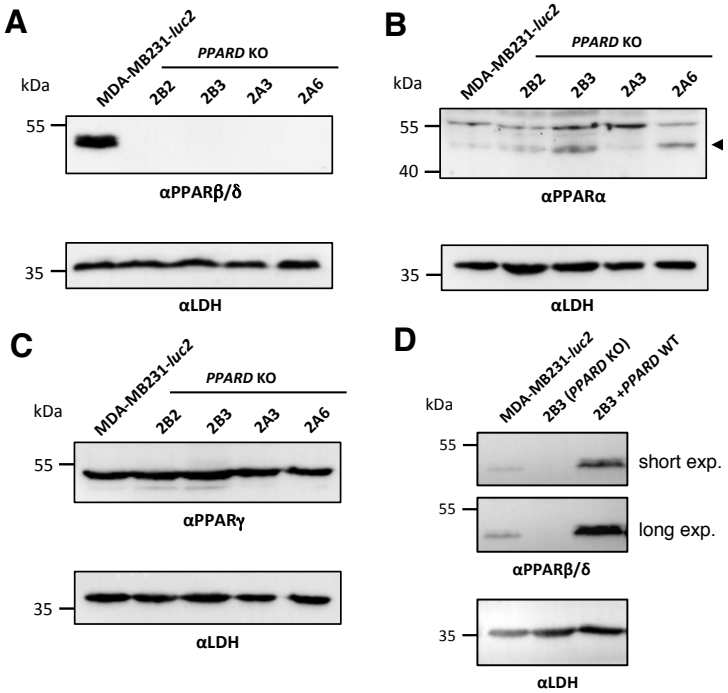


Fig. 3. Characterisation of *PPARD* knockout clones and reconstitution of PPARβ/δ expression.

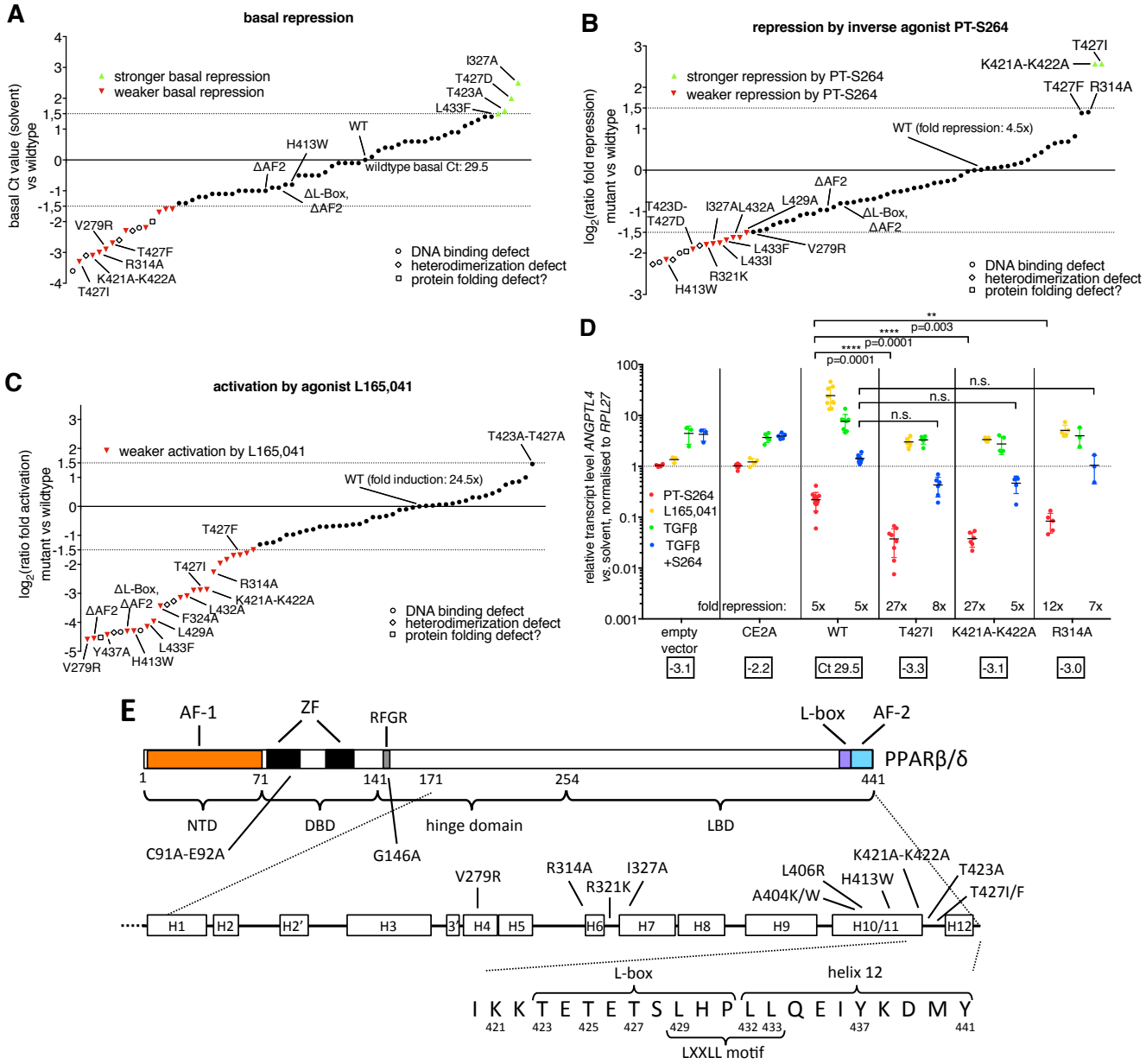


Fig. 4. A retroviral reconstitution screen identifies PPARβ/δ mutants deficient in ligand response and basal repression.

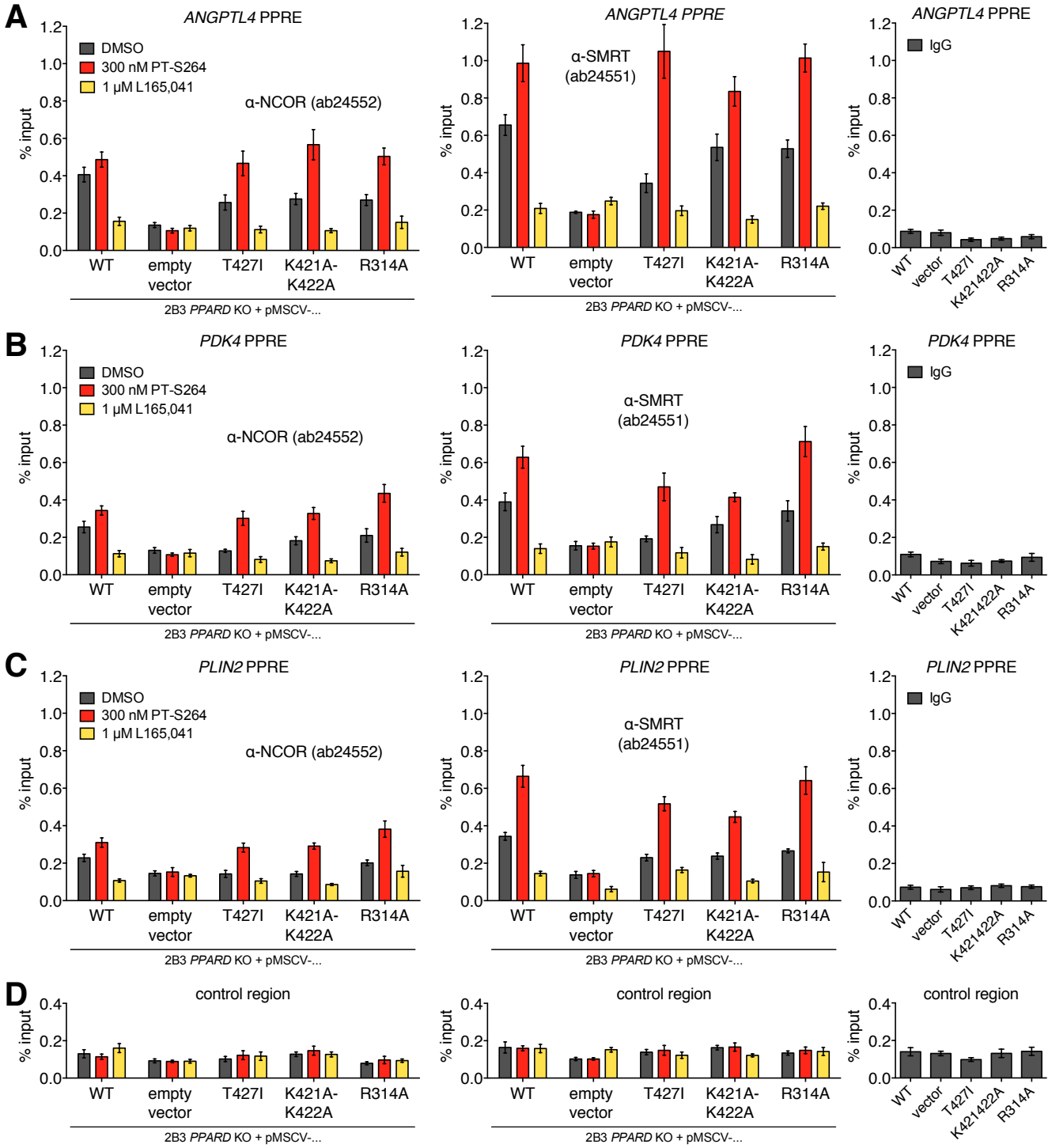


Fig. 5. Corepressor recruitment by PPARβ/δ mutants defective in basal repression.

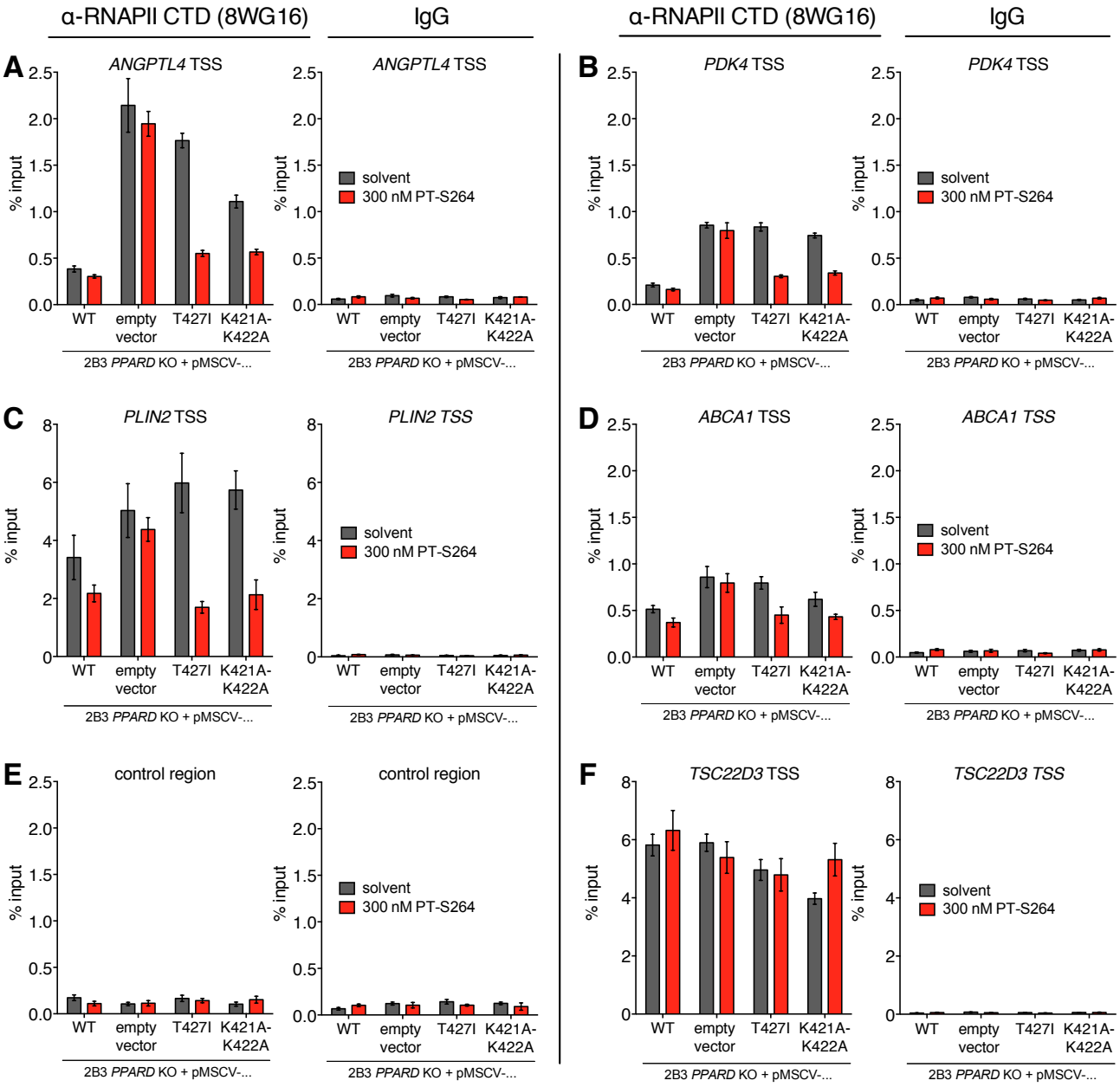


Fig. 6. Regulation of RNA polymerase II binding by PPARβ/δ mutants defective in basal repression.

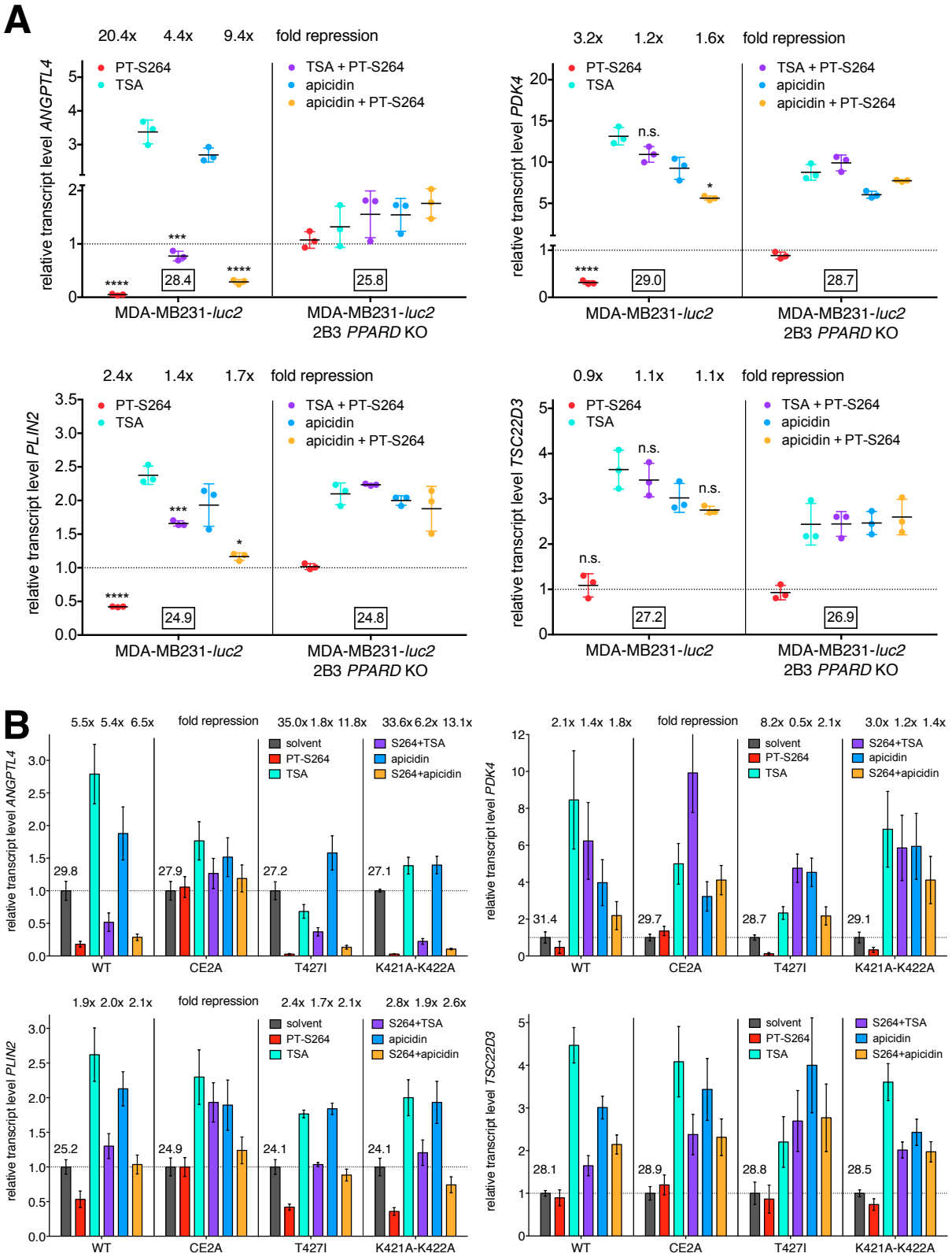


Fig. 7. HDAC inhibitor sensitivity of repression by PPARβ/δ mutants deficient in basal repression.

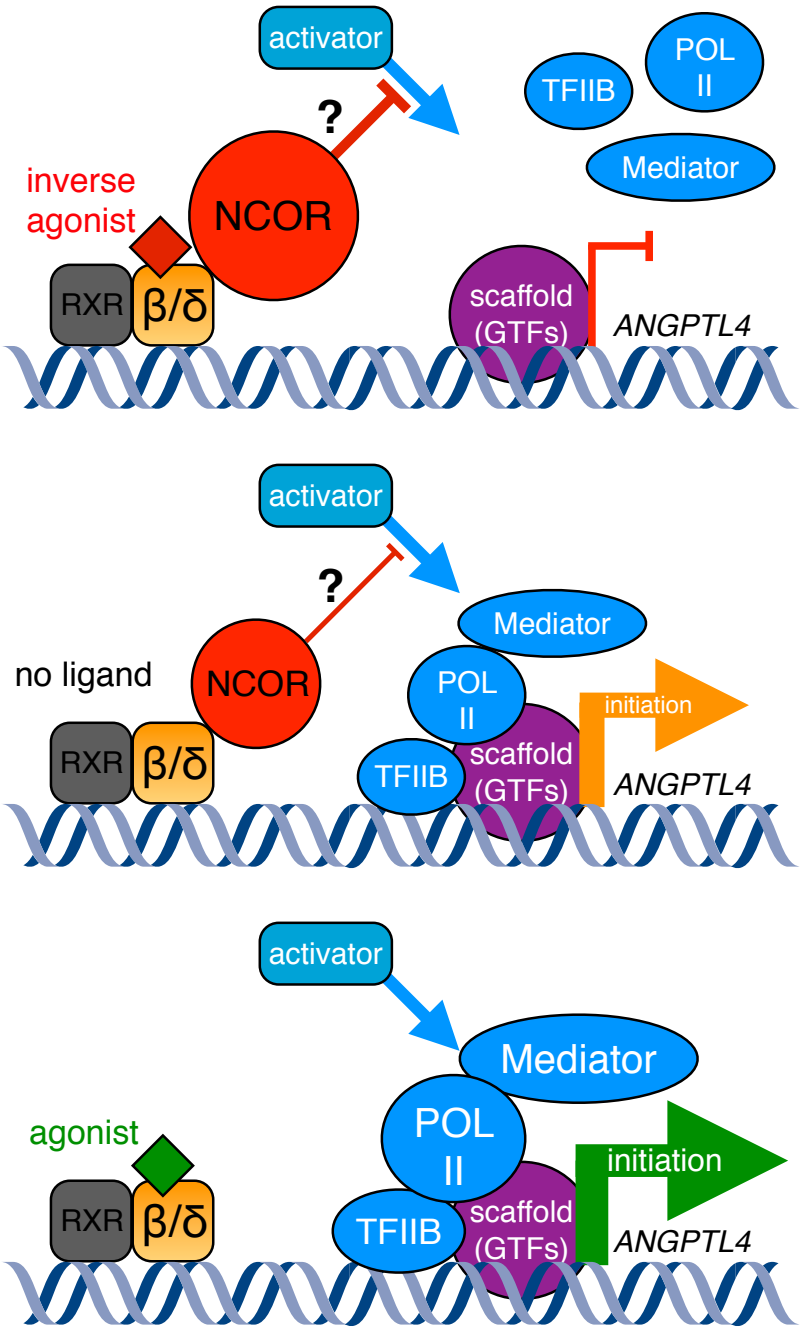
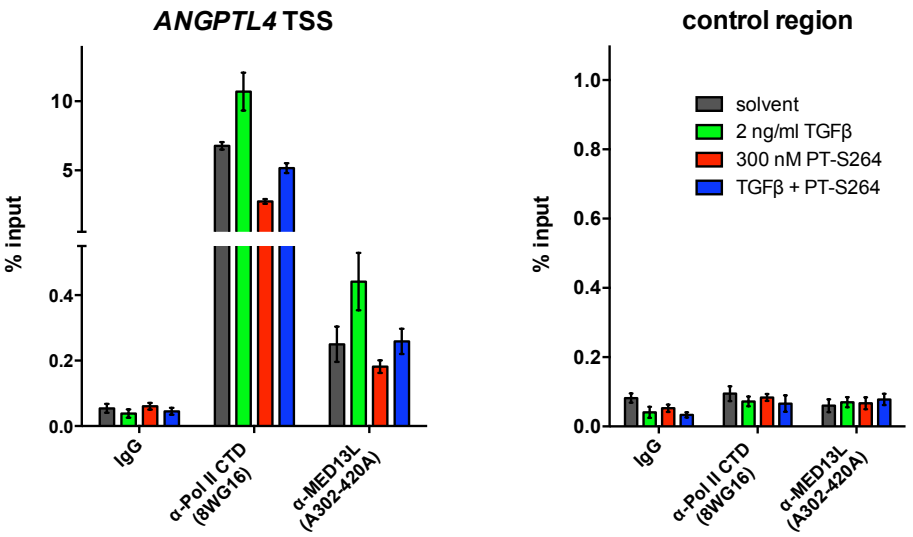
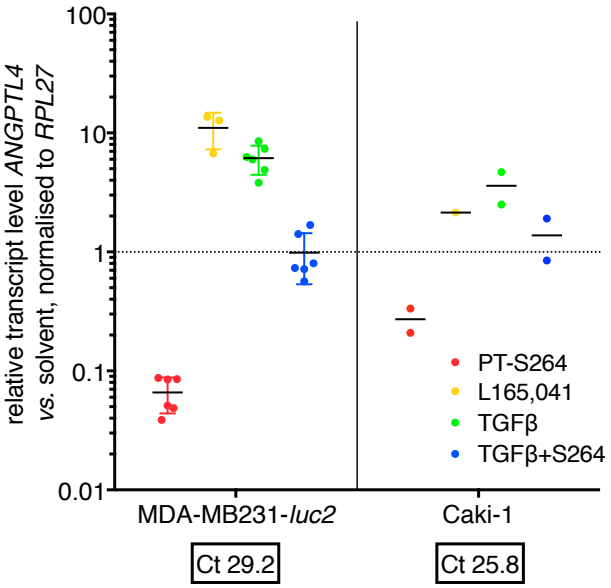


Fig. 8. Ligand-dependent regulation of *ANGPTL4* by PPARβ/δ and NCOR.

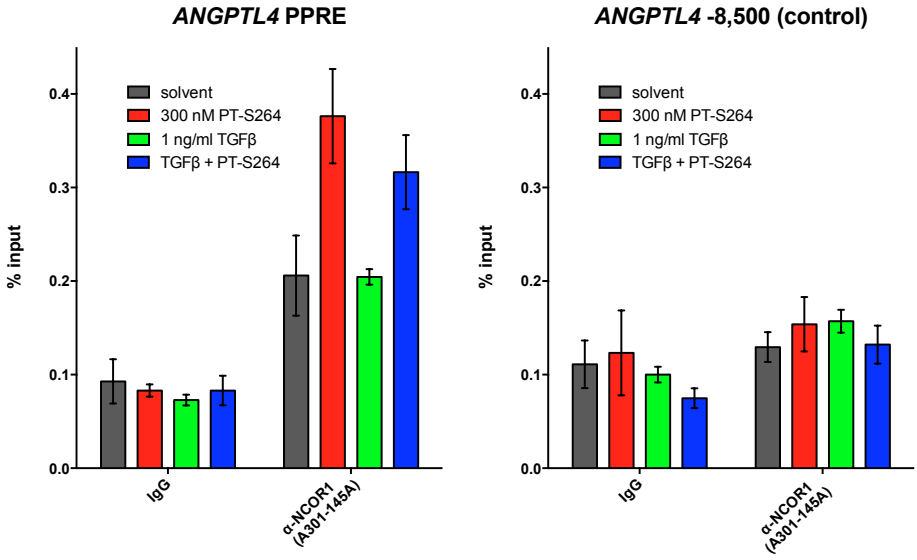
Supporting information



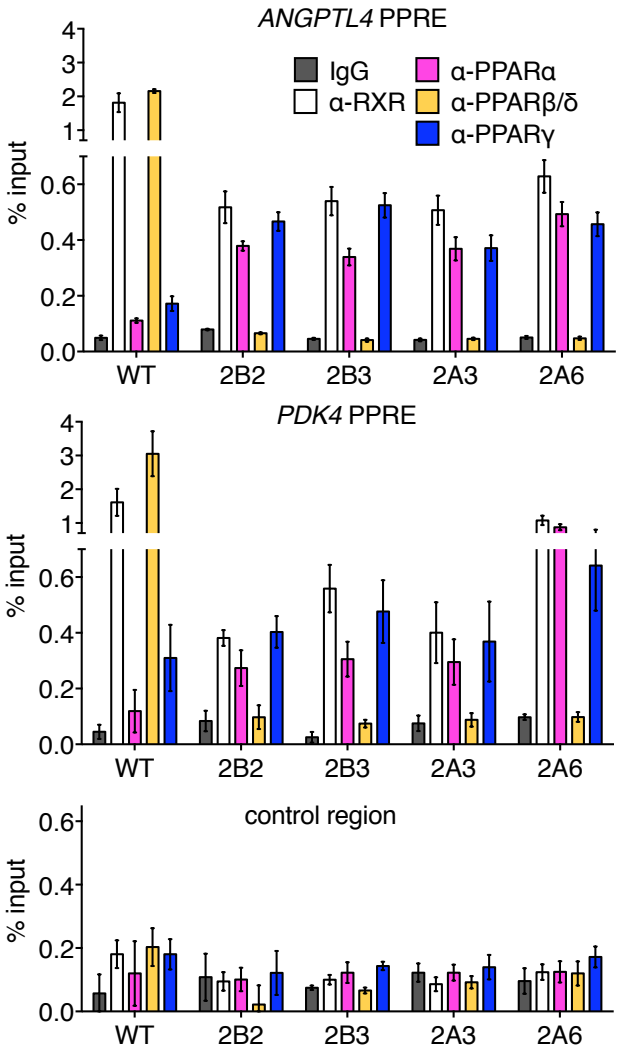
Supp. fig. S1. RNA polymerase II and MED13L binding to the *ANGPTL4* TSS in Caki-1 cells.



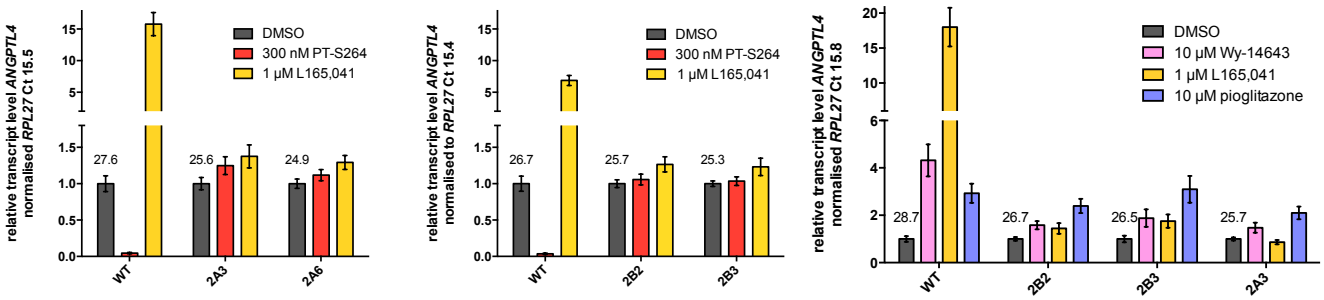
Supp. fig. S2. Analysis of target gene repression by PT-S264 in MDA-MB231-*luc2* and Caki-1 cells.



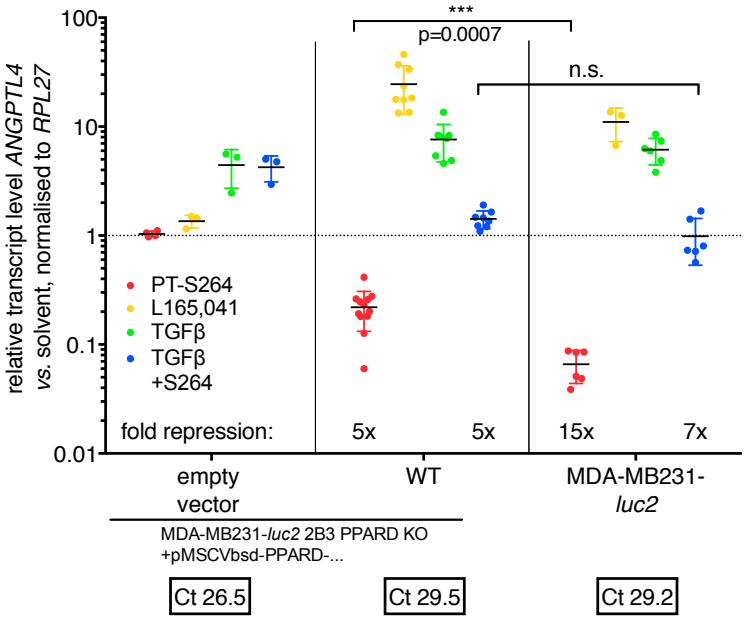
Supp. fig. S3. NCOR recruitment to the *ANGPTL4* PPAR binding site in Caki-1 cells.



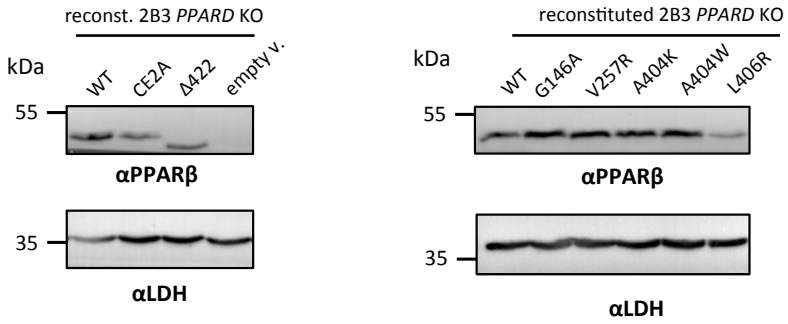
Supp. fig. S4. Analysis of PPAR isoform binding to chromatin in PPARβ/δ knockout clones. ChIP-qPCR analysis of RXR and PPAR binding at the *ANGPTL4* and *PDK4* loci in the KO clones. Parental cells (WT) were processed in parallel.



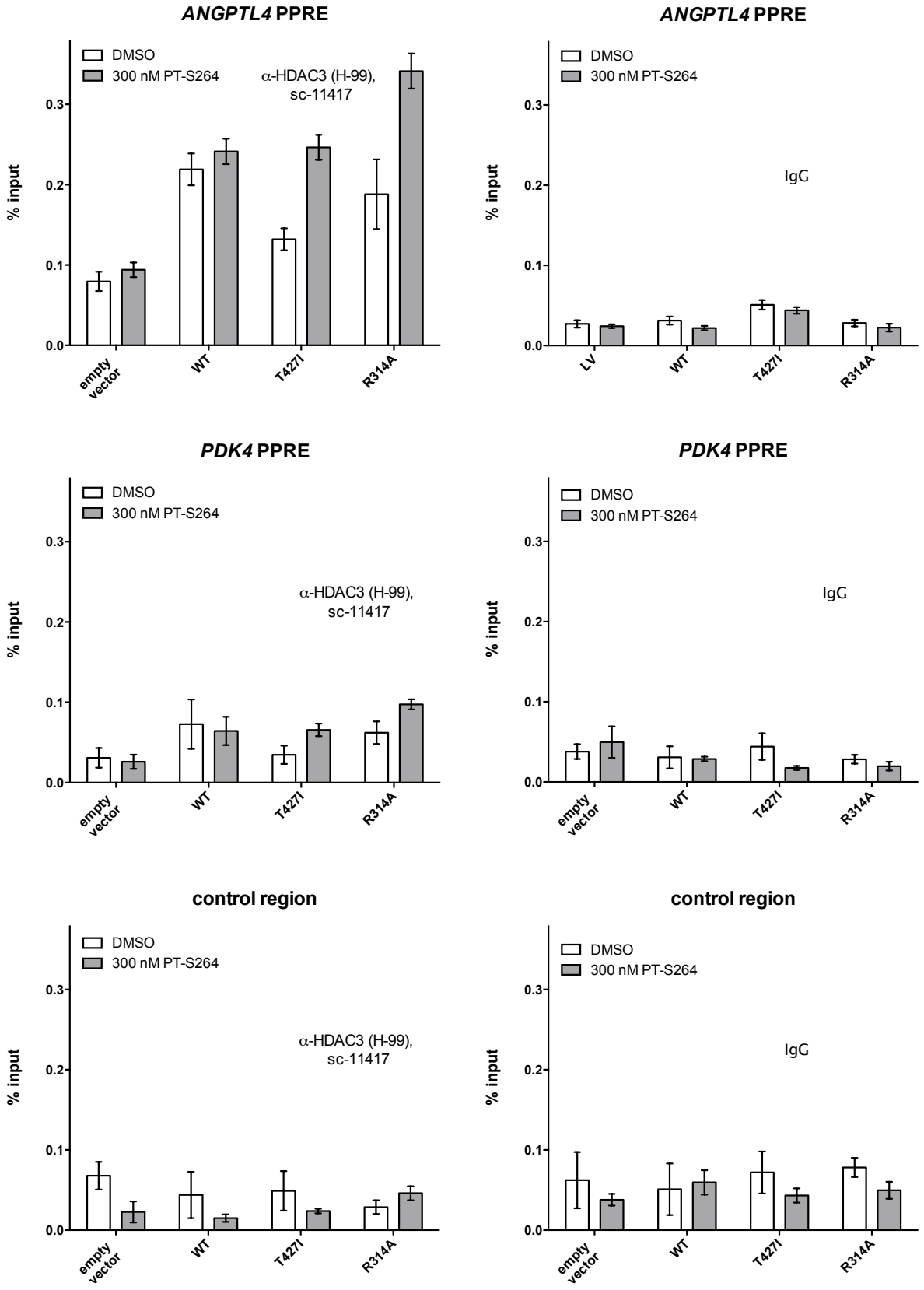
Supp. fig. S5. Regulation of *ANGPTL4* transcription by PPAR ligands in PPARβ/δ knockout clones.



Supp. fig. S6. Repression of *ANGPTL4* transcription by PPARβ/δ ligands in wildtype MDA-MB231-*luc2* cells and in the reconstituted 2B3 PPARβ/δ knockout clone.



Supp. fig. S7. Expression of PPARβ/δ mutants in the 2B3 knockout clone.



Supp. fig. S8. HDAC3 recruitment to PPARβ/δ binding sites in cells expressing mutants deficient in basal repression.

S1 Table. Complete ChIP-MS results. For download

S2 Table. Reconstitution screen data. For download

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

Conceived, designed, and performed the experiments: NL TA. NL performed the vast majority of the experiments. Analysed data: NL BF TA. Generated and contributed reagents and materials: NL CLB SZ BW MD WED TA. Supervised the study and wrote the paper: TA.

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