

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**Interaction between RAR and VDR genomic signaling and the role of RXR
in the interference of nuclear receptor pathways in macrophages**

by

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UNIVERSITY OF DEBRECEN
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1. Abbreviations

1,25-vitD	1 α ,25-dihydroxyvitamin D3
ADH	alcohol dehydrogenase
AF-1	activation function-1
AF-2	activation function-2
AR	androgen receptor
ATAC-seq	assay for transposase-accessible chromatin using sequencing
ATRA	all-trans retinoic acid
BMDM	bone marrow-derived macrophage
BWA	Burrows-Wheeler Aligner
ChIP-seq	chromatin immunoprecipitation coupled with high-throughput sequencing
CUT&RUN	cleavage under targets and release using nuclease
CUT&Tag	cleavage under targets and tagmentation
DamID	DNA adenine methyltransferase identification
DAMP	damage associated molecular pattern
DBD	DNA-binding domain
DBP	vitamin D binding protein
DR	direct repeat
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ER6	everted repeats with a six-nucleotide spacer
ERE	estrogen response element
eRNA	enhancer RNA
FC	fold change
FCS	fluorescence correlation spectroscopy
FDR	false discovery rate
FRAP	fluorescence recovery after photobleaching
FXR	farnesoid X receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTF	gene transfer format
H12	C-terminal helix 12
H3K27ac	histone H3 acetylated at lysine 27
HAT	histone acetyltransferase
HDAC	histone deacetylase
HGNC	HUGO gene nomenclature committee
HMT	histone methyltransferase
IGV	integrative genomics viewer
IP9	inverted palindromes with a nine-nucleotide spacer
IR	inverted repeat
LBD	ligand-binding domain
LDTF	lineage-determining transcription factor
LXR	liver X receptor
LXRE	liver X receptor response element
MACS2	model-based analysis of ChIP-seq version 2
MED1	Mediator complex subunit 1
MR	mineralocorticoid receptor
mRNA	messenger RNA

NR	nuclear receptor
NTD	N-terminal domain
PAMP	pathogen associated molecular pattern
PMA	phorbol-12-myristate-13-acetate
PPAR	peroxisome proliferator-activated receptor
PPARE	peroxisome proliferator-activated receptor response element
PR	progesterone receptor
PWM	position weight matrix
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol-binding protein
RD	read distribution
RDH	retinol dehydrogenase
RE	response element
RNA-seq	RNA sequencing
RPKM	reads per kilobase per million mapped reads
RSG	rosiglitazone
RXR	retinoid X receptor
SERM	selective estrogen receptor modulator
SRA	sequence read archive
SRTF	signal-regulated transcription factor
SRX	SRA experiment
T3	3,3',5-Triiodo-L-thyronine
TDF	tile data format
TF	transcription factor
TR	thyroid hormone receptor
TSS	transcription start site
VDR	vitamin D receptor
VDRE	vitamin D response element

2. Introduction

Nuclear receptors (NRs) are ligand-dependent transcription factors (TFs) that regulate gene expression through binding to specific genomic regulatory elements. Retinoic acid receptors (RARs) and the vitamin D receptor (VDR) are members of this family and mediate the biological actions of retinoids (naturally occurring forms of vitamin A and synthetic analogs of retinol) and vitamin D, respectively. RAR, VDR, and many other NRs primarily function by forming heterodimers with the retinoid X receptor (RXR). RXR is therefore a central component of multiple NR signaling pathways. RAR- and VDR-mediated transcriptional programs regulate a wide range of physiological processes, including immunity, development, reproduction, and metabolic homeostasis. Retinoid and vitamin D deficiencies are associated with several diseases. These vitamins and related compounds are widely used in therapeutic applications ¹⁻³.

The first objective of this thesis was to identify and characterize genomic regulatory regions commonly occupied by RAR α and VDR. Previous studies have demonstrated that a subset of genes can be regulated by both retinoids and vitamin D, often in a cooperative manner, suggesting convergence between RAR- and VDR-mediated signaling pathways. Previous observations that certain regulatory regions are occupied by both RAR and VDR supported the idea that cross-talk between these pathways occurs at the chromatin level ^{4, 5}. However, the prevalence, genomic features, and functional relevance of such common binding regions remain poorly defined. To address this, we performed an integrated genome-wide analysis of receptor binding, chromatin accessibility, and transcriptional changes in ligand-treated macrophage-like cells derived from the THP-1 cell line. Given the importance of retinoids and vitamin D in regulating key biological processes and their potential therapeutic co-application, understanding the mechanisms underlying pathway convergence is of significant interest. Moreover, our study advances our understanding of how NRs with distinct properties can regulate distinct yet largely overlapping gene networks.

For our second objective, we sought to investigate the extent and patterns by which ligands of RXR or its heterodimeric partners reshape the RXR binding landscape. From a systems-level perspective, sharing a common module among multiple pathways introduces the potential for interference, as module availability may become limiting when several pathways are activated simultaneously. Given that multiple RXR heterodimers can be activated either by RXR agonists or by partner-specific ligands, concurrent activation could theoretically lead to competition for RXR and reduced transcriptional responses. Although this concept has been explored using microscopy-based techniques ^{6, 7}, a comprehensive systematic genome-wide assessment of

RXR binding and transcriptional outcomes under simultaneous pathway activation has not yet been addressed. To address this, we performed and integrated genome-wide analyses of RXR binding and transcriptional changes in macrophage-like cells derived from the THP-1 cell line. These cells were treated with agonists of RXR or its six partner receptors, as well as ligand “cocktails” activating multiple NR pathways simultaneously. Macrophages provide an ideal system for this study due to their high RXR expression and the presence of multiple RXR partner receptors. This investigation can contribute to a deeper understanding of NR cross-talk and demonstrate how shared modules involved in transcriptional regulation can support robust gene expression across multiple signaling pathways.

3. Literature review

3.1. Overview of NRs structure and function

NRs are a structurally related superfamily of proteins found in metazoans, which contribute to a complex system that coordinates an organism's responses to hormonal, metabolic, and environmental signals. These receptors regulate diverse physiological processes, including development, homeostasis, metabolism, reproduction, inflammation, and circadian rhythm. NRs primarily function as ligand-activated TFs, as most members of the superfamily bind lipophilic molecules and modulate gene expression in response. In humans, the NR superfamily comprises 48 members encoded by distinct genes and involved in distinct regulatory pathways (Table 1). NR isotypes are distinct proteins within the same receptor group encoded by separate paralogous genes (e.g., $RAR\alpha$, β , and γ encoded by *RARA*, *RARB*, and *RARG*), often exhibiting different tissue distributions. Each NR isotype is considered an individual member of the 48 human NRs. Dysregulation of NR signaling is associated with various cancers, metabolic disorders, and inflammatory diseases. NRs are grouped based on various criteria, including their structural features, modes of dimerization, mechanisms of gene regulation and subcellular localization, the presence or absence of endogenous ligands, and the chemical nature of their ligands (e.g., steroid versus non-steroid). Despite this diversity, all NRs share a conserved structural organization, which forms the basis for their classification as members of this superfamily⁸⁻¹¹.

3.1.1. Modular structure of NRs

NRs typically share a conserved modular structure composed of five regions, commonly referred to as the A-E regions (Figure 1). The A/B region corresponds to the N-terminal domain (NTD), which is structurally flexible, highly variable in length and sequence, and therefore poorly characterized. It contains activation function-1 (AF-1), which mediates ligand-independent transcriptional activity by recruiting co-regulatory proteins in a cell- and promoter-specific manner and is often regulated through post-translational modifications. The C region comprises the DNA-binding domain (DBD), the most conserved domain among NRs, containing two zinc finger motifs formed by cysteine residues, which mediate sequence-specific DNA recognition and receptor dimerization. The D region, or hinge region, is a short, flexible linker connecting the DBD and the ligand-binding domain (LBD). The hinge region shows little sequence conservation, may contain nuclear localization signals, and often includes residues that interact with the DNA minor groove through C-terminal extensions. The E region corresponds to the LBD; a structurally conserved domain of approximately 200-300 amino

acids located at the C-terminus. The LBD is a multifunctional allosteric domain that binds ligands within a predominantly hydrophobic pocket. The size and shape of the pocket, along with the arrangement of hydrogen-bonding residues, vary among NRs. Such differences contribute to the ligand specificity of each receptor. Interestingly, studies have shown that pocket shapes and sizes are highly inducible, which may allow them to accommodate molecules unrelated to endogenous ligands. In addition to ligand binding, the LBD mediates homo- or heterodimerization and provides a platform for the recruitment of transcriptional coactivators or corepressors. Embedded within the LBD is activation function-2 (AF-2), a ligand-dependent regulatory surface formed primarily by helices 3, 4, and the C-terminal helix 12 (H12). Ligand binding induces conformational rearrangements within the LBD, particularly repositioning of H12, which stabilizes or disrupts co-regulator binding and links NRs to chromatin-modifying complexes that ultimately promote or repress gene transcription^{8, 12}.

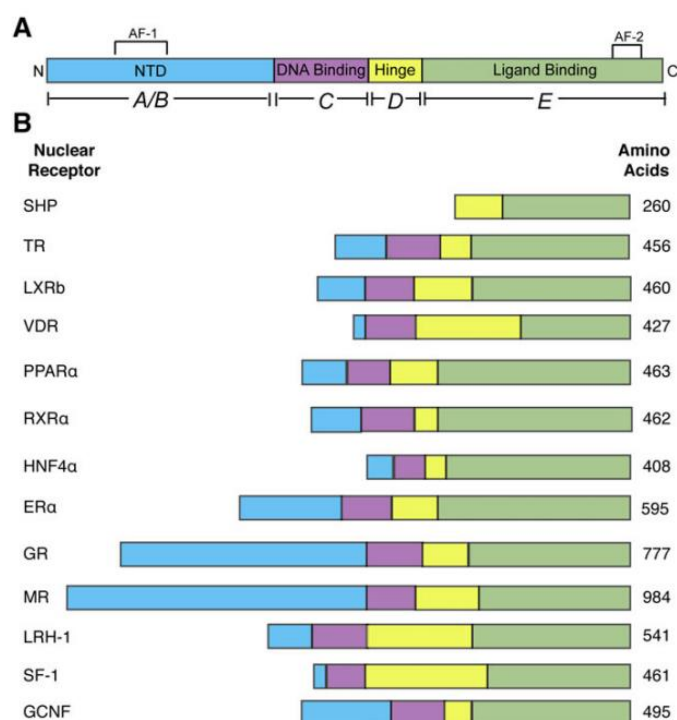


Figure 1. Conserved modular structure of nuclear receptors (NRs). (A) Basic domain architecture of NRs. (B) Domain size and amino acid length of representative NRs. Figure was derived from⁸.

3.1.2. Definition, discovery and modes of action of NR ligands

Ligands are small signaling molecules that bind directly to the NRs and regulate their activity. Physiological NR ligands function as molecular messengers that convey the metabolic, hormonal, or nutritional state of the organism and are present at physiological concentrations sufficient to regulate receptor activity *in vivo*. These ligands are typically small and invariably lipophilic in nature, allowing them to diffuse across cellular membranes and bind within a protected hydrophobic cavity embedded in the highly α -helical LBD. Of the 48 human NRs,

approximately half have well-established endogenous ligands, whereas the existence and identity of ligands for the remaining receptors have not been established or remain controversial (Table 1). Members of the NR superfamily lacking a validated physiological ligand have traditionally been defined as orphan NRs. In several cases, candidate ligands have been proposed based on biochemical or pharmacological evidence but have not been conclusively shown to regulate receptor activity under physiological conditions ^{9, 10, 12}.

Some NR ligands were initially discovered based on their physiological activity, prior to the characterization of their corresponding receptors, a strategy referred to as “classical endocrinology” ¹⁰. The isolation of thyroid hormone from massive amounts of bovine thyroid gland ¹³, followed decades later by the molecular identification of the thyroid hormone receptor ^{14, 15}. Similarly, for some other NRs, their cognate hormones, including sex steroids, glucocorticoids, and vitamin A and D metabolites, were studied by independent fields decades before the molecular mechanisms underlying their activity were elucidated through the cloning of their receptors ^{10, 16, 17}. NR ligand discovery was profoundly accelerated by the advent of “reverse endocrinology,” in which cloned cDNAs of orphan receptors were expressed in cell-based reporter assays to perform high-throughput screening of natural, synthetic, and xenobiotic ligands. This approach greatly increased the sensitivity of ligand discovery and revealed previously unrecognized receptor-ligand pairs ¹⁰.

Receptor-ligand selectivity is primarily determined by the chemical structure of ligands. Differences in the structural and functional groups of various ligands can influence transcriptional outcomes upon binding to their corresponding NR. These structural features dictate whether ligand-receptor binding results in full transcriptional activation, submaximal activation, transcriptional repression, or suppression of constitutive receptor activity below basal levels, corresponding to agonism, partial agonism, antagonism, and inverse agonism, respectively ^{12, 18}. Importantly, a given ligand can regulate distinct gene regulatory programs in different cell types. These divergent responses are dictated by the local gene regulatory environment, including chromatin accessibility and three-dimensional organization, the composition and architecture of promoters and enhancers, and the cell-type-specific availability of transcriptional cofactors ^{19, 20}. While some agonists exhibit selectivity for specific receptor isoforms, others function as pan-agonists that activate multiple receptor isoforms. In addition, cell-specific factors can determine whether a ligand functions as a transcriptional on-switch or off-switch. For example, selective estrogen receptor modulators (SERMs) can mimic estrogen action in certain tissues by activating estrogen target genes while antagonizing estrogen action in other tissues by repressing estrogen target genes. This tissue-selective activity arises from

differences in the abundance of coactivators and corepressors, estrogen receptor isotype expression, and local chromatin context between various cells, ultimately leading to cell-specific transcriptional outcomes ^{12, 18}.

Table 1. List of human NRs. The table provides information on the gene symbols/names according to the HUGO Gene Nomenclature Committee (HGNC), dimerization with RXR and the identified endogenous ligand(s) according to the literature ^{10, 21-25}.

#	approved symbol	alias symbol	approved name	RXR and partners	primary endogenous ligand(s)
1	<i>AR</i>	<i>NR3C4</i>	androgen receptor	no	dihydrotestosterone, testosterone
2	<i>ESR1</i>	<i>NR3A1</i>	estrogen receptor 1	no	estrogens
3	<i>ESR2</i>	<i>NR3A2</i>	estrogen receptor 2	no	estrogens
4	<i>ESRRA</i>	<i>NR3B1</i>	estrogen related receptor α	no	not identified / controversial
5	<i>ESRRB</i>	<i>NR3B2</i>	estrogen related receptor beta	no	not identified / controversial
6	<i>ESRRG</i>	<i>NR3B3</i>	estrogen related receptor gamma	no	not identified / controversial
7	<i>HNF4A</i>	<i>NR2A1</i>	hepatocyte nuclear factor 4 alpha	no	linoleic acid
8	<i>HNF4G</i>	<i>NR2A2</i>	hepatocyte nuclear factor 4 gamma	no	not identified / controversial
9	<i>NR0B1</i>	<i>DAX-1</i>	nuclear receptor subfamily 0 group B member 1	no	not identified / controversial
10	<i>NR1D1</i>	<i>REVERBalpha</i>	nuclear receptor subfamily 1 group D member 1	no	heme
11	<i>NR1D2</i>	<i>REVERBbeta</i>	nuclear receptor subfamily 1 group D member 2	no	heme
12	<i>NR2C1</i>	<i>TR2</i>	nuclear receptor subfamily 2 group C member 1	no	not identified / controversial
13	<i>NR2C2</i>	<i>TR4</i>	nuclear receptor subfamily 2 group C member 2	no	retinol, all-trans retinoic acid
14	<i>NR2E1</i>	<i>TLX</i>	nuclear receptor subfamily 2 group E member 1	no	not identified / controversial
15	<i>NR2F1</i>	<i>COUP-TFI</i>	nuclear receptor subfamily 2 group F member 1	no	not identified / controversial
16	<i>NR2F2</i>	<i>COUP-TFII</i>	nuclear receptor subfamily 2 group F member 2	no	not identified / controversial
17	<i>NR3C1</i>	<i>GR</i>	nuclear receptor subfamily 3 group C member 1	no	cortisol, corticosterone
18	<i>NR3C2</i>	<i>MR</i>	nuclear receptor subfamily 3 group C member 2	no	deoxycorticosterone, aldosterone, cortisol, corticosterone
19	<i>NR4A3</i>	<i>NOR1</i>	nuclear receptor subfamily 4 group A member 3	no	not identified / controversial
20	<i>NR5A1</i>	<i>SF1</i>	nuclear receptor subfamily 5 group A member 1	no	not identified / controversial
21	<i>NR5A2</i>	<i>LRH1</i>	nuclear receptor subfamily 5 group A member 2	no	not identified / controversial
22	<i>NR6A1</i>	<i>GCNF1</i>	nuclear receptor subfamily 6 group A member 1	no	not identified / controversial
23	<i>PGR</i>	<i>NR3C3</i>	progesterone receptor	no	progesterone
24	<i>RORA</i>	<i>NR1F1</i>	RAR related orphan receptor A	no	cholesterol
25	<i>RORB</i>	<i>NR1F2</i>	RAR related orphan receptor B	no	not identified / controversial
26	<i>RORC</i>	<i>NR1F3</i>	RAR related orphan receptor C	no	not identified / controversial
27	<i>NR0B2</i>	<i>SHP</i>	nuclear receptor subfamily 0 group B member 2	yes	not identified / controversial

#	approved symbol	alias symbol	approved name	RXR and partners	primary endogenous ligand(s)
28	<i>NR1H2</i>	<i>LXRb</i>	nuclear receptor subfamily 1 group H member 2	yes	oxysterols
29	<i>NR1H3</i>	<i>LXRa</i>	nuclear receptor subfamily 1 group H member 3	yes	oxysterols
30	<i>NR1H4</i>	<i>FXR</i>	nuclear receptor subfamily 1 group H member 4	yes	chenodeoxycholic acid, lithocholic acid, deoxycholic acid
31	<i>NR1I2</i>	<i>PXR</i>	nuclear receptor subfamily 1 group I member 2	yes	lithocholic acid
32	<i>NR1I3</i>	<i>CAR</i>	nuclear receptor subfamily 1 group I member 3	yes	not identified / controversial
33	<i>NR2E3</i>	<i>PNR</i>	nuclear receptor subfamily 2 group E member 3	yes	not identified / controversial
34	<i>NR2F6</i>	<i>EAR2</i>	nuclear receptor subfamily 2 group F member 6	yes	not identified / controversial
35	<i>NR4A1</i>	<i>NUR77</i>	nuclear receptor subfamily 4 group A member 1	yes	not identified / controversial
36	<i>NR4A2</i>	<i>NURR1</i>	nuclear receptor subfamily 4 group A member 2	yes	not identified / controversial
37	<i>PPARA</i>	<i>NR1C1</i>	peroxisome proliferator activated receptor alpha	yes	polyunsaturated and oxidized fatty acids
38	<i>PPARD</i>	<i>NR1C2</i>	peroxisome proliferator activated receptor delta	yes	polyunsaturated and oxidized fatty acids
39	<i>PPARG</i>	<i>NR1C3</i>	peroxisome proliferator activated receptor gamma	yes	polyunsaturated and oxidized fatty acids
40	<i>RARA</i>	<i>NR1B1</i>	retinoic acid receptor alpha	yes	9-cis retinoic acid, all-trans retinoic acid
41	<i>RARB</i>	<i>NR1B2</i>	retinoic acid receptor beta	yes	9-cis retinoic acid, all-trans retinoic acid
42	<i>RARG</i>	<i>NR1B3</i>	retinoic acid receptor gamma	yes	9-cis retinoic acid, all-trans retinoic acid
43	<i>RXRA</i>	<i>NR2B1</i>	retinoid X receptor alpha	yes	9-cis retinoic acid, docosahexonoic acid, 9-cis-13,14-dihydroretinoic acid
44	<i>RXRB</i>	<i>NR2B2</i>	retinoid X receptor beta	yes	9-cis retinoic acid, docosahexonoic acid, 9-cis-13,14-dihydroretinoic acid
45	<i>RXRG</i>	<i>NR2B3</i>	retinoid X receptor gamma	yes	9-cis retinoic acid, docosahexonoic acid, 9-cis-13,14-dihydroretinoic acid
46	<i>THRA</i>	<i>NR1A1</i>	thyroid hormone receptor alpha	yes	3,3',5-Triiodo-L-thyronine (T ₃) and 3,5,3',5'-tetraiodo-L-thyronine (T ₄)
47	<i>THRB</i>	<i>NR1A2</i>	thyroid hormone receptor beta	yes	3,3',5-Triiodo-L-thyronine (T ₃) and 3,5,3',5'-tetraiodo-L-thyronine (T ₄)
48	<i>VDR</i>	<i>NR1H1</i>	vitamin D receptor	yes	1 α ,25-dihydroxyvitamin D ₃

3.1.3. Molecular mechanism of gene regulation by NRs

NR-mediated transcriptional regulation is an allosteric process in which ligand binding and recognition of specific DNA response elements (REs) are mechanistically coupled to the recruitment of transcriptional co-regulators to chromatin. Binding of a cognate ligand to the LBD and simultaneous engagement of receptor-specific DNA REs induce conformational rearrangements across the receptor, which are transmitted between its domains. These coordinated structural changes determine the composition of co-regulatory complexes

assembled at NR-occupied loci and ultimately drive either transcriptional activation or repression of target genes ^{8, 26, 27}.

Advances in live-cell imaging and single-molecule tracking techniques have revealed that functional NR-chromatin interactions are highly dynamic. This was initially observed for the glucocorticoid receptor (GR) and later for other NRs, including RXR and its partners RAR, VDR, and peroxisome proliferator activated receptor gamma (PPAR γ) ^{6, 7, 28-32}. Moreover, besides direct chromatin binding through REs, NRs can indirectly associate with DNA through protein-protein interactions with other DNA-bound TFs. This mechanism, which is known as tethering, has been documented for several NRs, including ER, GR, PPARs, and LXRs, where ligand-bound receptors modulate transcription by interacting with factors such as AP-1 and NF- κ B rather than by direct binding to DNA ³³⁻³⁵.

Transcriptional activation by NRs is typically initiated when ligand binding stabilizes an active receptor state, in which repositioning of helix 12 within the LBD generates a permissive AF-2 surface that favors recruitment of co-activator proteins. This enables the recruitment of multiprotein co-activator complexes, including the Mediator complex. Binding of co-activators provides a scaffold for histone-modifying enzymes, including histone acetyltransferases (HATs) and histone methyltransferases (HMTs), whose activities increase DNA accessibility. This chromatin remodeling facilitates the recruitment of additional regulatory factors and the basal transcriptional machinery, thereby enabling efficient transcription initiation.

NRs can repress transcription through multiple mechanisms. In the absence of ligand, several NRs, particularly RXR heterodimers, associate with co-repressor complexes that recruit histone deacetylases (HDACs), restricting chromatin and preventing access of the transcriptional machinery to DNA. Additionally, binding of antagonists to NRs can induce conformational changes that favor co-repressor recruitment, leading to transcriptional repression. NRs can also bind to negative DNA REs, which favors co-repressor recruitment even in the presence of agonist ^{8, 26, 27}.

The N-terminal AF-1 domain can drive ligand-independent genomic activity, contributing to the basal transcriptional activity of NRs through the recruitment of co-regulators. The AF-1 domain contains multiple sites for post-translational modifications, such as phosphorylation and SUMOylation, which can modulate NR genomic activity (gene activation or repression) both in the presence of ligand and in the basal, ligand-independent state. The contribution of the AF-1 domain to ligand-independent genomic activity and its modulation by post-translational modifications have been documented for NRs such as estrogen receptors (ER), GR, and PPARs ³⁶⁻³⁹.

3.1.4. Dimerization and subcellular localization of NRs

NRs are generally monomeric in solution but can form higher-order complexes upon ligand and/or DNA binding. Although some NRs remain monomeric on DNA, they are more commonly found as homodimers or in heterodimeric complexes with RXR. This dimerization increases the overall size and structural complexity of the NR, creating new surfaces accessible for co-regulator interactions and post-translational modifications. Steroid receptors, including ER, androgen receptor (AR), progesterone receptor (PR or PGR), mineralocorticoid receptor (MR), and GR, are typically maintained in an inactive conformation by heat shock proteins in the cytoplasm in the absence of ligand; ligand binding induces conformational changes that promote dissociation from heat shock proteins, homodimerization, followed by nuclear translocation and DNA binding. In contrast, many non-steroid NRs are often retained in the nucleus regardless of ligand presence. These NRs often form heterodimers with RXR independently of ligand and can bind DNA constitutively, with ligand primarily modulating co-regulator recruitment (Table 1) ^{8,9,12}.

3.1.5. Co-activators and co-repressors

After binding DNA, NRs recruit a diverse set of co-regulatory proteins. These include over 200 distinct co-activators and co-repressors with cell- and tissue-specific distributions. Co-activators (e.g., SRC-1 and CBP/p300) typically interact with NRs via conserved sequences of amino acids called NR boxes (LXXLL motifs, where L is leucine and X is any amino acid) that bind the AF-2 surface of the ligand-binding domain. Upon binding, the co-activator serves as a scaffold for other proteins involved in chromatin accessibility and facilitating transcription. Co-repressors (e.g., NCoR and SMRT), in contrast, contain CoNR box motifs (LXXXI/HIXXXI/L, where L is leucine, I is isoleucine, H is any hydrophobic residue, and X is any amino acid) that bind the same AF-2 surface but stabilize the receptor in a transcriptionally inactive conformation, recruit HDACs, and prevent the assembly of the basal transcription machinery, thereby repressing transcription. Notably, these interactions are typically modulated post-translational modifications (such as phosphorylation, acetylation, or SUMOylation) on the AF-1 and AF-2 domains ^{8,27}.

3.1.6. DNA response elements and target gene specificity

NRs recognize and bind specific DNA sequences known as REs. The presence and accessibility of these REs within the genome largely determine the repertoire of target genes

regulated by a given NR in a cell-type-specific manner. Canonical REs for various NRs were initially identified through site-specific and promoter-focused functional studies, where individual DNA binding regions were functionally validated for their ability to drive ligand-dependent transcriptional activation or repression. These studies typically employed functional assays such as reporter gene assays, mutational analyses, electrophoretic mobility shift assay (EMSA), and DNase I footprinting to link DNA binding with transcriptional activity^{3, 9, 40}.

Specificity of NR binding to distinct REs is primarily dictated by the DBD, particularly through conserved DNA-recognition surfaces that mediate sequence-specific DNA binding, as well as receptor homo- and heterodimerization. This diversity in DNA sequence recognition enables NRs to generate distinct transcriptional responses. NRs recognize and bind REs, which typically consist of repeated DNA half-sites arranged as inverted (palindromic) or direct repeats (DRs), separated by spacer sequences of any nucleotide with variable lengths, as well as extended monomeric sites (Figure 2)^{8, 12}.

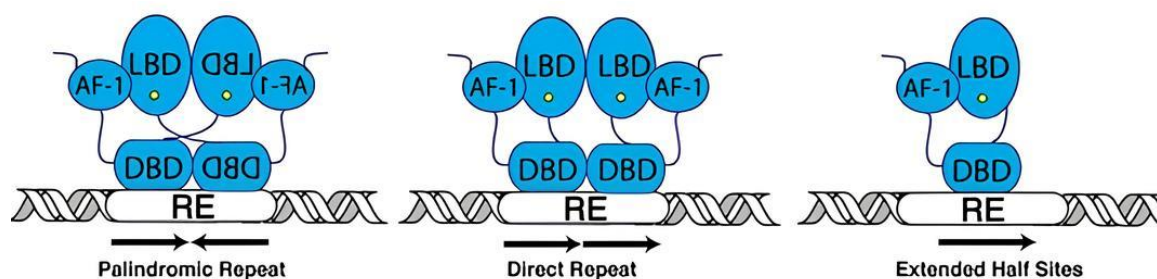


Figure 2. NRs bind DNA response elements (REs) in a variety of configurations. Members of the steroid receptor subfamily typically recognize palindromic repeats, while many other NRs bind direct repeats. In both cases, the repeated half-sites are separated by spacer sequences of variable lengths. Although less common, some NRs (e.g., LRH-1 and SF-1) bind DNA as monomers to extended half-site sequences. Figure was derived from⁸.

Steroid receptors preferentially bind inverted repeat (IR) composed of two AGGACA motifs separated by a spacer, the length of which can allosterically influence receptor activity. For instance, an inverted repeat with a 3 bp spacer (IR3) represents the canonical glucocorticoid response element (GRE) and the estrogen response element (ERE). RXR heterodimers, however, typically bind DRs consisting of two AGGTCA or its variants motifs separated by spacer lengths ranging from 0 to 5 base pairs (DR0 to DR5). For example, DR1 and DR4 represent the canonical peroxisome proliferator-activated receptor response element (PPARE) and liver X receptor response element (LXRE), respectively.^{8, 12}.

3.1.7. Non-genomic functions of NRs

Although NRs are classically studied as ligand-activated TFs, several studies have shown that they can also contribute to non-genomic signaling pathways. These activities typically occur in the cytoplasm or at the plasma membrane, where NRs directly interact with intracellular proteins to rapidly regulate cellular stress responses and participate in signal transduction pathways. A small subset of NRs including steroid receptors (ERs, PR, AR, GR, and MR) as well as TR (thyroid hormone receptor) and VDR, has been reported to localize to the plasma membrane, where they promote membrane-initiated responses. Glucocorticoids can elicit rapid non-genomic effects through interactions with cytosolic or membrane-bound GRs. A truncated variant of TR at the plasma membrane participates in biological processes such as apoptosis, autophagy, and mitochondrial fatty acid oxidation. In response to apoptotic stimuli, RXR α -Nur77 heterodimers translocate to mitochondria, where they regulate apoptosis. Other NRs, including PPAR α , LXR, and farnesoid X receptor (FXR), have also been reported to perform non-genomic functions^{34, 41}.

3.2. Mapping and characterization of NR genomic binding regions

3.2.1. Genome-wide mapping of NR binding by ChIP-seq

The advent of genome-wide approaches has enabled a broader understanding of TFs (including NRs) binding to DNA *in vivo*. Using these approaches, studies have revealed that a single TF can occupy thousands of genomic binding regions, collectively referred to as “cistrome” spanning a wide range of TF-DNA affinities. Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) has emerged as the most widely used approach for genome-wide mapping of protein-DNA interactions. This method is employed to map genomic regions directly occupied by TFs, as well as regions where proteins, such as co-regulators and components of the Mediator complex, associate indirectly with chromatin through protein-protein interactions. ChIP-seq is also frequently applied for genome-wide identification of post-translational histone modifications, which serve as indicators of chromatin state and regulatory activity. Other methods for mapping genome-wide TF binding sites include DNA adenine methyltransferase identification (DamID), cleavage under targets and release using nuclease (CUT&RUN), and its derivative method, cleavage under targets and tagmentation (CUT&Tag) (Figure 3)^{42, 43}.

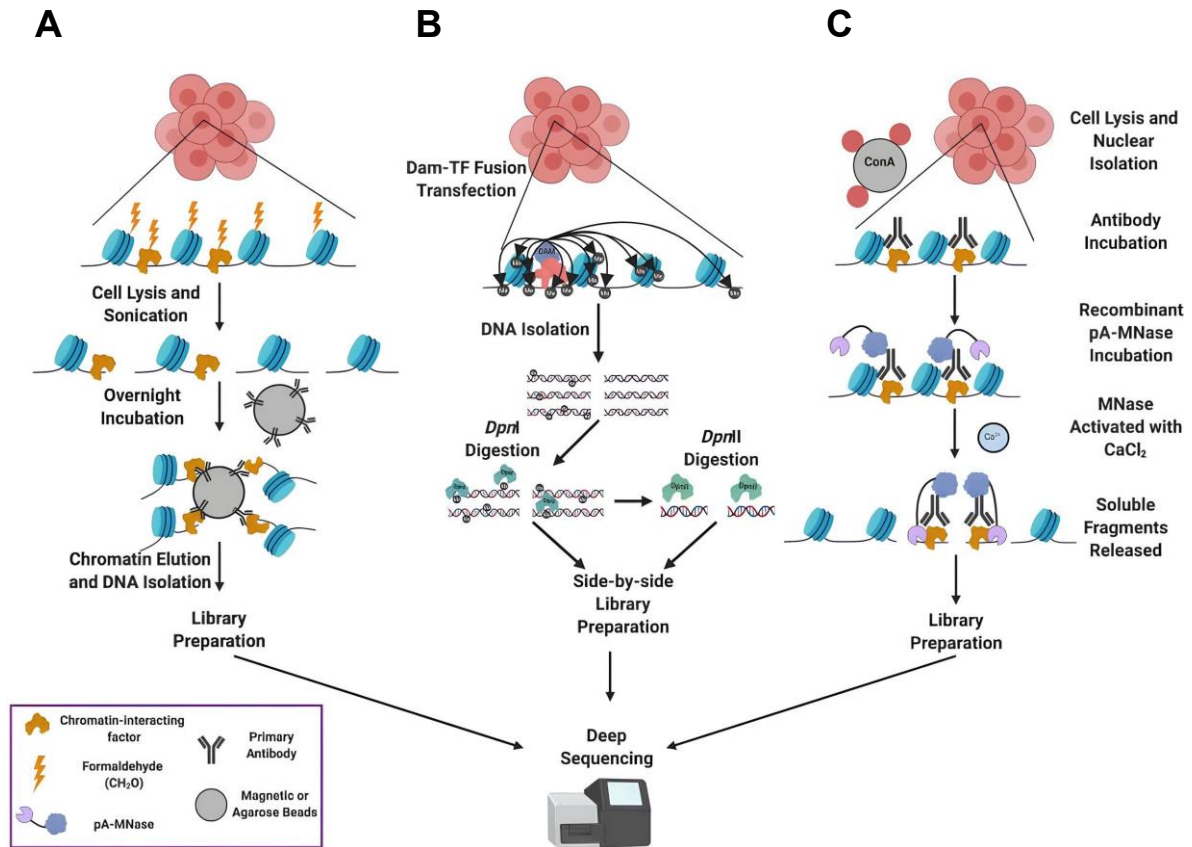


Figure 3. Methods for profiling transcription factor (TF) localization on chromatin. Schematic illustrations of (A) chromatin immunoprecipitation (ChIP), (B) DNA adenine methyltransferase identification (DamID), and (C) cleavage under targets and release using nuclease (CUT&RUN) methods, all of which followed by high-throughput sequencing. Figure was derived from ⁴³.

ChIP-seq captures TF-DNA interactions within the native chromatin context, enabling key determinants of transcriptional regulation, such as cofactors and Pol II, to be involved in the same experiment. The ChIP-seq method has limitations, as it represents a static, population-averaged snapshot of protein-DNA interactions rather than capturing dynamic binding events in individual cells. Additionally, ChIP-seq peaks may encompass multiple binding sites for a given TF, preventing precise quantification of site-specific binding affinity and limiting direct comparisons between different TFs in binding to a given genomic region. Differences in antibody specificity can introduce variability in ChIP-seq results, making direct comparisons between TFs or experiments less reliable when based solely on ChIP-seq data ⁴⁴. Despite these limitations, ChIP-seq allows for the identification of TF binding regions and also provides information on the relative binding intensity of the investigated factors ⁴⁵. Variation in occupancy signal within a sample is biologically meaningful rather than random. This is evidenced by a series of observations, including consistent rankings of binding intensity across

binding regions in biological replicates and correlations with independent biological features such as DNA motif strength, cofactor recruitment, and histone modification patterns. Moreover, transcriptional output correlates with ChIP-seq signal intensity, with highly expressed genes often associated with strong binding signals^{46, 47}. Therefore, ChIP-seq offers a robust and reproducible measure of relative TF occupancy across the genome and can be considered semi-quantitative.

3.2.2. DNA motif enrichment in NR binding regions

DNA motifs summarize TF binding preferences, by capturing recurring sequence patterns enriched within TF-occupied regions. As a result of motif enrichment, each TF recognizes a family of related sequences rather than a single consensus sequence; hence, NRs do not necessarily and exclusively bind canonical RE sequences determined by site-specific and promoter-focused functional studies. DNA motif preferences for TFs are typically presented using position weight matrices (PWMs), which describe the probability of each nucleotide at each position within the motif and can be visualized as sequence logos (Figure 4). Such DNA motifs can be used to scan genomic sequences and predict potential binding regions. However, actual genomic occupancy is influenced by several determinants, including chromatin accessibility, cooperative interactions with other TFs, DNA methylation, and the local regulatory context⁴².

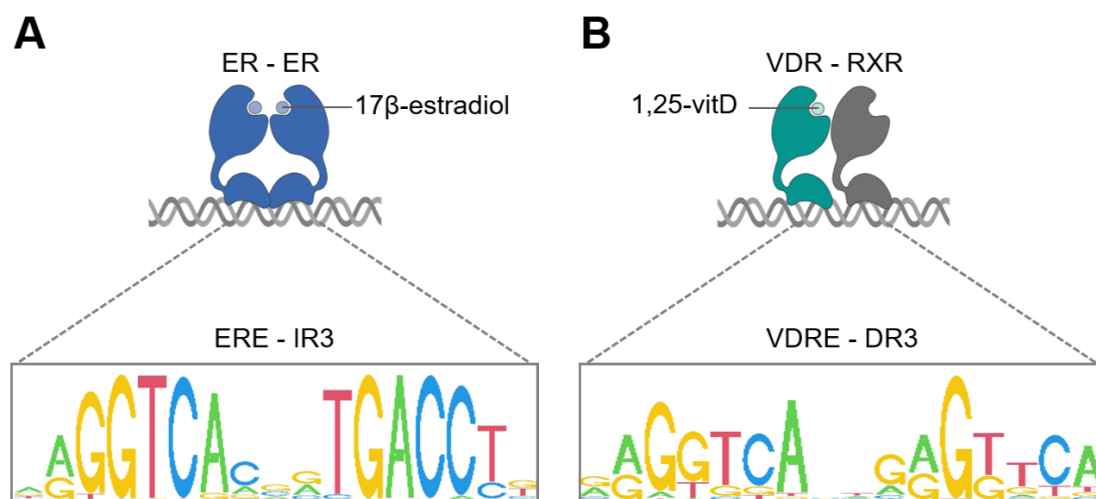


Figure 4. Schematic illustration of DNA motifs recognized by NRs. (A, B) Genomic regions occupied by the ER homodimer (A) and the VDR-RXR heterodimer (B) in the presence of their corresponding agonists, 17β-estradiol (E2) and 1,25-vitD. Corresponding DNA motif logos are shown below each NR complex. Position weight matrices (PWMs) were obtained from JASPAR (ERE-IR3: MA0112.3) and HOMER (VDRE-DR3: GSE22484, motif 394) databases, and motif logos were generated using the R package seqLogo. Figure was created with bioBender.com.

3.2.3. Convergence of NR cistromes

Pairwise analyses of NR cistromes in previous studies showed that the genomic binding regions of different NRs often overlap. In human MCF7 cell line, comparison of the RAR α and ER cistromes in untreated cells identified 2,365 shared sites, representing 49% of the RAR α cistrome⁴⁸. In another study on human MCF7 cell line treated with AM580 (RAR α agonist) and estradiol (ER agonist), comparison of the RAR α and ER cistromes revealed 1,745 shared sites, representing ~38% of the ER cistrome⁴⁹. In murine primary hepatocytes, comparison of the PPAR α and GR cistromes in cells treated with GW7647 (PPAR α agonist) and dexamethasone (GR agonist) showed 1,989 shared sites, representing ~56% of the PPAR α cistrome³⁵. In another study on murine primary liver cells, comparison of the liver X receptor (LXR) and PPAR α cistromes in vehicle-treated cells revealed 3,092 shared sites, representing ~82% of the LXR cistrome⁵⁰. The significant overlap between NR cistromes suggest that NRs may co-regulate subsets of target genes via shared regulatory regions, indicating convergence in their gene regulatory programs.

3.3. RXR-mediated genomic signaling

3.3.1. RXR dimerization and ligand activation

RXRs are essential dimerization partners to derive the genomic activity of numerous NRs, including RARs, VDR, TRs, LXRs, and PPARs. RXRs comprise three isotypes, (α , β , and γ), with distinct patterns of tissue expression^{3, 25, 51}. In addition to forming heterodimers, RXRs can also form homodimers, although the functional significance of RXR homodimers remains incompletely understood⁵¹⁻⁵⁴. RXR activity can be modulated by endogenous ligands such as 9-cis-13,14-dihydroretinoic acid, 9-cis RA and docosahexaenoic acid, as well as by synthetic agonists^{24, 53, 55}. To date, high-affinity endogenous ligands have been identified for roughly two-thirds of the RXR heterodimerization partners in humans (Table 1). Certain RXR heterodimers, termed “permissive” heterodimers, can be activated through RXR ligand binding, independent of ligand binding by the partner receptor; examples include LXR-RXR, PPAR-RXR, FXR-RXR, and PXR-RXR^{3, 51, 53}. In contrast, non-permissive heterodimers, including RAR-RXR, VDR-RXR, and TR-RXR, cannot be activated by RXR ligands alone and require the ligand of the partner receptor for activation. Notably, RAR-RXR is considered a conditional (permissive) heterodimer; in the presence of RAR ligand, combined treatment with RAR and RXR agonists produces a greater effect than the RAR agonist alone^{3, 51, 54}.

3.3.2. Mechanism of gene regulation by RXR dimers

RXR homodimers preferentially recognize DR1 elements, while RXR heterodimers exhibit distinct RE preferences, as discussed earlier. These variations in DNA motif specificity among RXR dimers result in the regulation of distinct but overlapping target gene sets^{9, 51}. In contrast to steroid receptors like GR, which are mainly cytoplasmic and inactive in the absence of ligand, RXR heterodimers primarily act as ligand-dependent molecular switches; in the absence of agonists, they bind DNA and recruit corepressors, while ligand binding facilitates coactivator recruitment and triggers transcriptional activation as described in the mechanisms outlined earlier^{10, 27, 56, 57}.

3.3.3. Determinants of RXR genomic binding landscape

Previous ChIP-seq analyses have demonstrated that the binding landscape of RXR is shaped by cellular differentiation, as well as ligand exposure. Notably, these studies indicated that the cellular differentiation state exerts a more profound influence on RXR chromatin occupancy than the presence of ligands for RXR or its heterodimerization partners. Significant changes in RXR binding upon shifts in cellular state suggest a dominant role for differential cofactor availability, chromatin architecture, and epigenetic context in determining the RXR cistrome⁵⁸⁻⁶⁰. In the following sections, we specifically elaborate on RAR-RXR and VDR-RXR, as these were the receptors analyzed for their genomic signaling interactions in our study.

3.4. Retinoids and the genomic signaling mediated by RAR-RXR

3.4.1. Nomenclature of retinoids

“Retinoids” refer to naturally occurring forms of vitamin A as well as its many synthetic analogs of retinol. This term is also practically used for several compounds that are not closely related to retinol but elicit biological activity of retinol. “Vitamin A” refers to compounds that exhibit the biological effects of retinol and include retinol, retinal (also known as retinaldehyde), and retinoic acid (RA). Certain carotenoids are considered provitamin A because they can be converted into retinal or apocarotenoids that can be metabolized to vitamin A in humans^{61, 62}.

3.4.2. Synthesis, transport, storage, and metabolism of retinoids

No animal species can synthesize vitamin A *de novo*, relying instead on dietary sources. Plants, bacteria, algae, and fungi produce provitamin A carotenoids, some of which animals can convert into vitamin A. Animals can also obtain vitamin A by consuming tissues from other

animals. Upon absorption in the intestine, vitamin A is transported to the liver via the lymphatic and circulation system as retinyl esters in chylomicrons. In hepatocytes, the retinyl esters are hydrolyzed, and the unesterified retinol may then associate with retinol-binding protein (RBP). This is essential for the mobilization of hepatic retinol into plasma and for cellular uptake by extrahepatic target cells. Retinol can also be stored in hepatic stellate cells as retinyl esters. In mammals, 50-80% of the body's total retinol is present in the hepatic stellate cells. This represents an adequate supply of vitamin A for several weeks or months and also ensures a steady blood plasma retinol concentration of about 1-2 μM in spite of normal fluctuations in daily intake of vitamin A. Retinol is oxidized in target cells by retinol dehydrogenases (RDHs), including alcohol dehydrogenases (ADHs), to retinaldehyde, which is further oxidized to RA (the biologically active metabolite) by retinaldehyde dehydrogenases (RALDHs). RA is subsequently catabolized by CYP26 enzymes through mechanisms that control intracellular and tissue RA levels (Figure 5). A distinct branch of retinoid metabolism is essential for vision. In the vertebrate visual cycle, photosensitivity relies on the light-induced isomerization of retinal, involving cis-to-trans or trans-to-cis conversion following photon absorption ⁶².

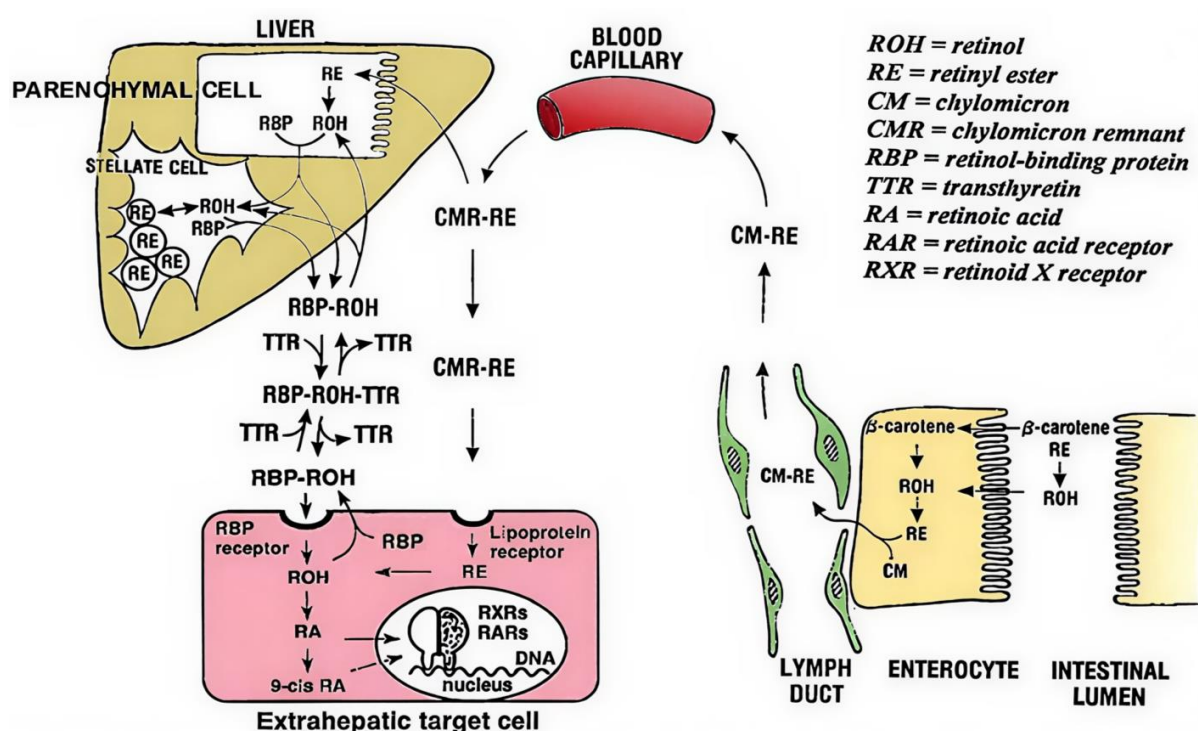


Figure 5. Retinoids distribution and metabolism in target cells. Figure was derived from ⁶².

3.4.3. Retinoids: physiological roles, deficiency and therapeutic applications

Retinoids play essential roles in numerous mammalian physiological processes, including spermatogenesis, fertilization, pregnancy maintenance, morphogenesis, organogenesis, and fetal and perinatal growth. In adults, they are vital for regulating reproduction, immunity, and

vision, and for the proper functioning of the skin, lungs, bone marrow, liver, and nervous system. Vitamin A deficiency remains a major public health concern, increasing susceptibility to severe infections, causing preventable blindness, contributing to skin disorders, and leading to abnormal embryonic development^{1, 62}. Beyond treating deficiencies, retinoids are used therapeutically for dermatological conditions such as acne and psoriasis due to their ability to regulate keratinocyte proliferation and differentiation. Retinoids are also used in the management of several diseases, including age-related macular degeneration, measles, and various malignancies⁶³⁻⁶⁵.

3.4.4. RAR-RXR mediating the ligand-dependent genomic signaling of retinoids

The three RAR isotypes (α , β , and γ) mediate the biological effects of retinoids primarily through heterodimerization with RXR. In addition to signaling via RAR isotypes, the biological effects of certain retinoids can also be mediated through activation of RXR isotypes (as discussed earlier). RARs are activated by all-trans retinoic acid (ATRA) and other retinoic acids, such as 9-cis-RA and 9-cis-13,14-dihydro-RA, as well as by synthetic agonists, including AM580 (RAR α agonist) and TTNPB (pan-RAR agonist)⁶⁶⁻⁶⁸. DR5 was originally identified as the canonical binding site for RAR-RXR through site-specific, promoter-focused studies^{3, 40, 69}. Besides DR5, other retinoic acid response elements (RAREs), including DR0, DR1, DR2, and inverted repeats without a spacer (IR0), have been described using genome-wide approaches^{60, 70, 71}.

3.5. Vitamin D and the genomic signaling mediated by VDR-RXR

3.5.1. Nomenclature of vitamin D

“Vitamin D” is a general term to describe all steroids that exhibit the biological activity of cholecalciferol (vitamin D₃). Two major forms of vitamin D include vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol). Vitamins D₂ and D₃ differ in their side chain structure. This difference does not affect their metabolism or their function as prohormone^{72, 73}.

3.5.2. Synthesis, transport, storage, and metabolism of vitamin D

Humans obtain vitamin D from two principal sources: skin synthesis following exposure to sunlight and dietary intake from foods and supplements. In the skin, solar UV-B radiation converts 7-dehydrocholesterol to previtamin D₃, which is then rapidly isomerized to vitamin D₃ (cholecalciferol). In the diet, vitamin D₃ is primarily present in animal-derived foods such as fatty fish and egg yolks. Vitamin D supplements exist in two main forms: vitamin D₃ and

vitamin D₂, manufactured by UV irradiation of 7-dehydrocholesterol from lanolin (wool fat) and ergosterol from yeast, respectively. Once absorbed in the intestine, vitamin D is transported to the liver via the lymphatic system and circulation. Vitamin D and its metabolites are transported in the blood primarily by vitamin D binding protein (DBP) and, to a lesser extent, by albumin. Vitamin D is primarily stored in adipose tissue and, to a lesser extent, in muscle. The absorbed or skin-derived vitamin D is hydroxylated in the liver by hepatic 25-hydroxylases (CYP2R1 and CYP27A1) to form 25-hydroxyvitamin D (calcidiol, including 25-hydroxyvitamin D₃ and 25-hydroxyvitamin D₂). Subsequently, 25-hydroxyvitamin D bound to DBP is transported to the kidneys, where it is hydroxylated by 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) to generate the biologically active hormone 1 α ,25-dihydroxyvitamin D. This includes both 1 α ,25-dihydroxyvitamin D₃ (calcitriol, hereinafter: 1,25-vitD) and 1 α ,25-dihydroxyvitamin D₂ (ercalcitriol). The renal production of 1 α ,25-dihydroxyvitamin D is tightly regulated by plasma parathyroid hormone levels and serum calcium and phosphate concentrations (Figure 6)^{2, 73-75}.

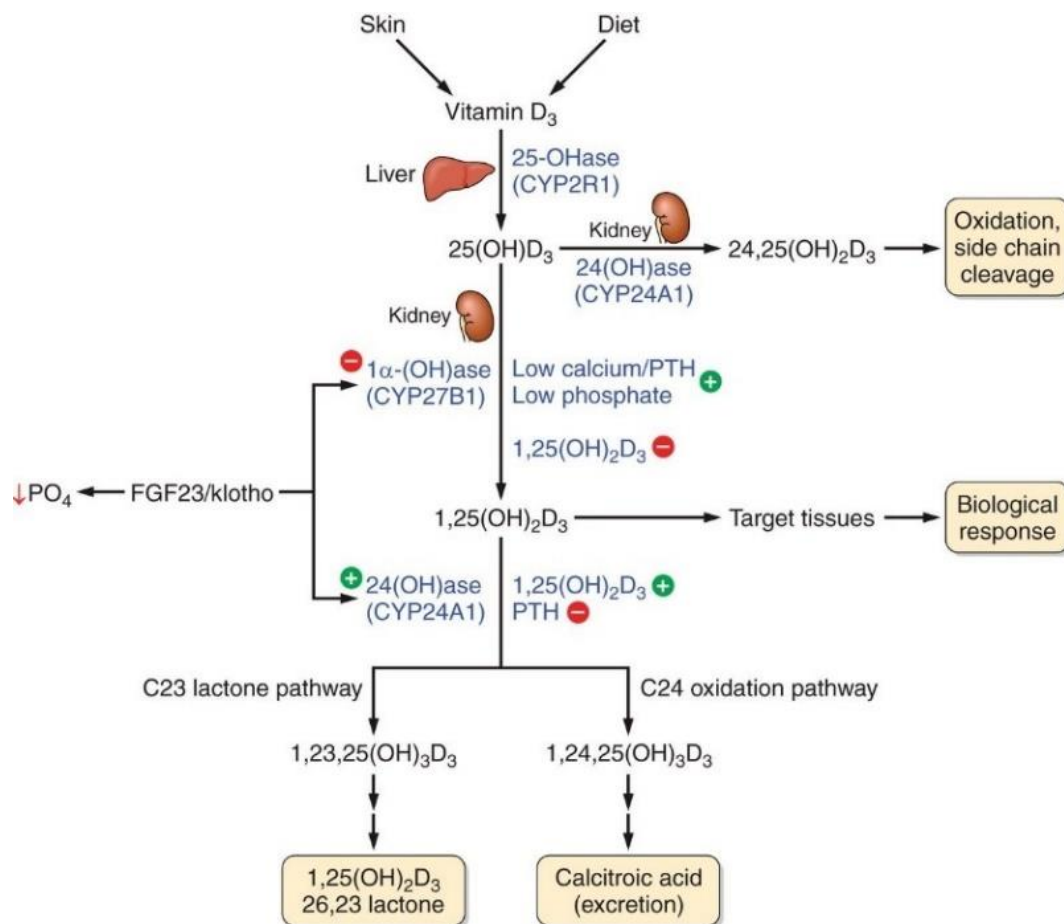


Figure 6. Vitamin D metabolic pathway in the liver and kidney cells. Figure was derived from⁷⁵.

3.5.3. Vitamin D: physiological roles, deficiency and therapeutic applications

Vitamin D plays a critical role in calcium and phosphate homeostasis and is therefore essential for skeletal development and bone health. It acts directly on three principal target tissues, including intestine, kidneys, and bone. Vitamin D maintains optimal serum calcium levels by enhancing the intestinal absorption of dietary calcium and phosphorus, promoting renal calcium reabsorption, and regulating bone remodeling, partly in concert with parathyroid hormone. Vitamin D also regulates additional biological processes, including cell proliferation and differentiation, modulates immune and nervous system functions, and contributes to cardiovascular homeostasis.

Vitamin D deficiency remains highly prevalent and is associated with secondary hyperparathyroidism and reduced bone mineral density, which may progress to osteopenia, osteoporosis, and an increased risk of fractures upon prolonged deficiency. In addition, inadequate vitamin D status has been linked to immune dysregulation (reduced immunological tolerance and increased susceptibility to inflammatory and infectious diseases), as well as elevated risk of certain cancers and cardiovascular diseases^{2, 73}. Vitamin D is primarily used to prevent or treat the manifestations of deficiency. It is predominantly used as a treatment for disorders related to calcium and phosphate metabolism and bone health, as well as for inflammatory and immune-mediated conditions, particularly dermatological and skeletal disorders^{2, 64, 65}.

3.5.4. VDR-RXR mediating the ligand-dependent genomic signaling of vitamin D

VDR mediates the biological effects of vitamin D primarily through heterodimerization with RXR. VDR is activated by high-affinity natural ligands, including calcitriol (1,25-vitD), ercalcitriol, and other synthetic analogs^{76, 77}. DR3 was originally identified as the canonical binding site for VDR-RXR through site-specific, promoter-focused studies^{3, 40, 77}. Genome-wide studies have revealed that vitamin D response elements (VDREs) are less variable than RAREs, with DR3 representing the major VDRE. However, VDR-RXR heterodimers can also bind to DR2 and DR4 motifs, though at considerably lower frequencies. Additional motifs for VDR-RXR, including everted repeats with a six-nucleotide spacer (ER6), DR6, and inverted palindromes with a nine-nucleotide spacer (IP9), may also serve as binding sites, as suggested by analyses of individual regulatory regions of vitamin D-responsive genes^{71, 78-84}.

3.6. Interaction between retinoid and vitamin D signaling

Previous studies have shown that retinoid and vitamin D signaling pathways can converge and interact, either additively or antagonistically, in response to simultaneous stimulation by both ligands. Additive effects on cellular functions and gene expression were reported during the induction of differentiation in leukemia and breast cancer cells, as well as in the inhibition of endothelin-stimulated hypertrophy in rat cardiac myocytes^{85, 86}. By contrast, antagonistic effects were observed during keratinocyte differentiation and in bone resorption^{87, 88}. Notably, previous studies have shown that RARs and VDR can interact at shared DNA binding sites of regulated genes^{3, 5, 31, 69, 89}.

These molecular interactions are mirrored in their overlapping therapeutic applications. Vitamins A and D share overlapping therapeutic applications, particularly in dermatology and oncology. Both retinoids and vitamin D are used in the management of skin diseases such as acne, psoriasis, and atopic dermatitis, where they exert complementary effects on epidermal differentiation and inflammation. Their combined use has been shown to enhance therapeutic outcomes in skin disorders such as acne. Importantly, combined treatment with retinoids and vitamin D has demonstrated beneficial effects in various cellular and animal cancer models, including osteosarcoma, prostate cancer, head and neck squamous cell carcinoma, high-grade glial tumors, and lung cancer^{65, 90, 91}.

3.7. Macrophages and NR signaling

3.7.1. NRs are major regulators of macrophage activity

Macrophages are key immune cells that sense and integrate diverse metabolic and environmental cues, being equipped with a wide array of receptors that recognize NR ligands, pathogen- and damage-associated molecular patterns (PAMPs, DAMPs), and cytokines²⁵. Previous studies have shown that NRs such as ER, GR, LXR, PPARs, RARs, and VDR play important roles in regulating macrophage biology, influencing processes such as lipid metabolism, inflammatory responses, phagocytosis, and cellular differentiation. Notably, there is substantial evidence for crosstalk between NR pathways in macrophages, in which activation of one receptor can alter the expression or activity of others. For example, RAR can induce PPAR γ expression via C/EBPs, and activation of RAR, RXR, or PPAR γ can enhance LXR activity indirectly through increased production of oxysterol ligands via CYP enzymes. Together, these highlight the significance of the NR pathways in orchestrating macrophage development and functions^{25, 92}. In the following sections, we specifically elaborate on the roles of RAR and VDR agonists in macrophage differentiation and function.

3.7.2. Retinoid signaling in macrophage differentiation and function

Retinoid signaling regulates both differentiation and functional properties of macrophages. While the role of retinoids is well established in early hematopoiesis, where they preferentially promotes granulocytic lineage differentiation ⁹³, accumulating evidence shows that retinoids also modulate monocyte-to-macrophage differentiation and polarization. RA promotes M2-like macrophage polarization through activation of the p38 MAPK/STAT6 pathway and synergizes with IL-4-mediated signaling ^{94, 95}. RA promotes a reparative macrophage phenotype characterized by increased oxidative phosphorylation, enhanced tissue repair programs, and elevated phagocytic and efferocytic capacity, all of which are characteristic features of M2 macrophages ^{96, 97}. ATRA has been shown to suppress NF- κ B signaling by downregulating CD14/TLR4 expression in alveolar macrophages, thereby enhancing macrophage phagocytic function ⁹⁸. Retinoids also regulate macrophage lipid metabolism and contribute to atheroprotection by inducing ABCA1 and ABCG1 via RAR/RXR-dependent mechanisms and cooperative interactions with LXRs, promoting cholesterol efflux and limiting foam cell formation ⁹⁹.

3.7.3. Vitamin D signaling in macrophage differentiation and function

1,25-vitD influences macrophage processes including differentiation, polarization, inflammatory responses, antimicrobial activity, and lipid metabolism. 1,25-vitD contributes to monocyte-to-macrophage differentiation ¹⁰⁰, and notably, combined exposure to retinoids and 1,25-vitD more efficiently induces this differentiation, suggesting synergistic crosstalk between these pathways ¹⁰¹. Emerging evidence further indicates that 1,25-vitD promotes polarization toward an M2-like, anti-inflammatory, and tissue-reparative macrophage phenotype ^{102, 103}. 1,25-vitD suppresses inflammatory responses by inhibiting NF- κ B-mediated transcriptional programs and reducing pro-inflammatory cytokines such as TNF, IL-6, and IL-12 ^{103, 104}. It also preserves and supports macrophage antimicrobial function during Toll-like receptor activation by inducing antimicrobial peptides, including cathelicidin and β -defensin 2, which are critical for intracellular pathogen killing ¹⁰⁵. Moreover, 1,25-vitD signaling contributes to macrophage lipid metabolism by suppressing foam cell formation by limiting intracellular lipid accumulation ¹⁰⁶.

3.7.4. THP-1 cell line as a model to study NRs including RARs and VDR in macrophages

THP-1 cells are a human monocytic leukemia cell line widely used as a model to study monocyte and macrophage biology. These cells can be differentiated into macrophage-like cells in response to stimulation with phorbol-12-myristate-13-acetate (PMA), as well as other stimuli, including LPS, IL-4, and TGF- β ^{107, 108}. Importantly, PMA-stimulated THP-1 cells (PMA-THP-1) express RARs and VDR, and transcriptional, phenotypic, and functional changes have been documented upon stimulation with their respective ligands in this cell line^{109, 110}. Of note, genome-wide studies in THP-1 cells have mapped the genomic binding sites of several NRs, including VDR, LXR, PPAR γ , GR, and AR, and have examined ligand-dependent changes in their genomic occupancy and the associated transcriptional pathways^{78, 79, 111}.

4. Aims of the study

In this study, we aimed to investigate the following aspects of NRs' biology and crosstalk:

4.1. Investigation of RAR α and VDR genomic pathway interactions

We aimed to identify and characterize shared RAR α and VDR binding regions and their role in convergence and cooperation between RAR α - and VDR-mediated pathways in macrophage-like cells. This included the following subjects:

- The extent to which RAR α and VDR cistromes overlap.
- The extent to which gene regulatory pathways driven by RAR α and VDR ligands converge. “Convergence” occurs when two or more signaling pathways produce the same or similar cellular responses (e.g., transcriptional changes) by engaging common downstream components.
- Whether RAR α - and VDR-regulated common gene sets undergo cooperative regulation when both pathways are activated. “Cooperation”, in the transcriptional regulation context, refers to the combined action of two or more entities (e.g., ligands or proteins), producing amplified outcomes, such as increased mRNA levels, histone modifications, or enhanced cofactor recruitment, beyond what each entity can achieve individually.

- The extent to which the common RAR α and VDR binding regions are involved in the convergence and cooperative interactions between gene regulatory pathways driven by RAR α and VDR ligands.
- The characteristics of the common binding regions with respect to chromatin accessibility and the presence of consensus motifs for RAR α and VDR.

4.2. Investigation of the role of RXR in the interference of NR pathways

We aimed to explore and characterize the ligand-dependent changes in RXR genomic binding landscape in macrophage-like cells. This included the following subjects:

- The extent and the patterns with which agonists of RXR or its heterodimeric partners reshape the RXR binding landscape.
- Whether RXR serves as a limiting module when multiple ligands for RXR and heterodimerization partners are present simultaneously.
- Whether there is a link between limiting RXR and the interference between pathways, when multiple NRs are simultaneously activated. “Interference” refers to the phenomenon in which activation of one pathway limits or inhibits the activation of another. The interference among NR pathways, including RXR heterodimerization partners, has been documented before suggesting that limited RXR availability may contribute to this interference. However, it has not yet been investigated in the *in vivo* chromatin context.

5. Materials and Methods

5.1. Cell culture and ligand treatment

The human monocytic THP-1 cell line was purchased from the American Type Culture Collection and was cultured in RPMI-1640 medium (Gibco) under humidified conditions at 37°C with 5% CO₂. The culture medium was supplemented with 10% fetal bovine serum (Biosera), 0.05 mM 2-mercaptoethanol (Gibco), 1% penicillin-streptomycin (Sigma-Aldrich), and 1 mM sodium pyruvate (Gibco). Cells were passaged every three days, maintaining a cell density of approximately 2.5×10^5 cells/mL after each passage. To induce macrophage-like differentiation, THP-1 cells were treated with 20 nM PMA (Sigma-Aldrich) for 16 hours. Following differentiation, PMA-treated THP-1 (PMA-THP-1) cells were incubated for 30 minutes in phenol red free RPMI medium (Gibco) containing 10% charcoal-stripped fetal

bovine serum (Biowest, South American origin) and 1% penicillin-streptomycin. Cells were subsequently treated with vehicle control or ligands, including single or combined ligands (see details in Table 2). For combined ligand treatments, ligands were applied simultaneously rather than sequentially; the agonists were first diluted in medium to the required final concentrations, and the resulting ligand cocktail was then added to the cells. Cells were harvested at various time points depending on the experiment.

5.2. RNA-seq experiments and data analysis

RNA-seq experiments

PMA-THP-1 cells were exposed to vehicle or agonist treatments for 6 h. Total RNA was extracted with the Quick-RNA Miniprep Kit (Zymo Research), and RNA quality was evaluated on an Agilent BioAnalyzer using the Eukaryotic Total RNA Nano Kit. Total RNA was used to generate RNA-seq libraries using the Ultra II RNA Sample Prep Kit (New England BioLabs). Libraries were sequenced on an Illumina platform, either a NextSeq 500 or NextSeq 2000, using 75-cycle single-end reads. Altogether, 21 RNA samples were analyzed, representing 7 treatment conditions with three biological replicates per condition (Table 2).

RNA-seq data analysis

The human reference genome (hg38) and HISAT2 were used for sequencing read alignment. Aligned reads were processed with Strand NGS software (<https://www.strand-ngs.com/>) for downstream analysis. Analysis of differential gene expression was conducted as follows. Genes with low expression levels were excluded. ANOVA with Benjamini-Hochberg correction was applied using an adjusted p-value threshold of < 0.05 . Finally, differentially expressed genes were identified by Tukey's post hoc test ($p < 0.05$), and fold-change cut-offs of 1.5 and 0.66 were used to define up- and down-regulated genes, respectively. Protein-coding genes were filtered using a protein-coding gene list from HUGO Gene Nomenclature Committee (HGNC) (<https://www.genenames.org/download/statistics-and-files/>). Transcription start sites (TSSs) of genes were determined using a Gene Transfer Format (GTF) annotation file (GRCh38.p14, NCBI Assembly: GCF_000001405.40).

Table 2. RNA-seq, ChIP-seq and ATAC-seq experiments in PMA-THP1 cells treated with agonist(s).

Aim	Experiment	ChIP factor	Antibody	Treatment	Concentration	Time (h)	# Cells (million)	# Reps
RAR & VDR genomic pathway interactions	RNA-seq	-	-	DMSO-ethanol	1:1	6	0.5	3
		-	-	AM580	100 nM	6	0.5	3
		-	-	1,25-vitD	100 nM	6	0.5	3
		-	-	AM580 + 1,25-vitD	100 nM each	6	0.5	3
	ChIP-seq	RAR α	ab41934, Abcam	DMSO-ethanol	1:1	1	10	2
		RAR α	ab41934, Abcam	AM580	100 nM	1	10	2
		VDR	ab109234, Abcam	DMSO-ethanol	1:1	1	10	2
		VDR	ab109234, Abcam	1,25-vitD	100 nM	1	10	2
		MED1	A300-793A, Bethyl	DMSO-ethanol	1:1	2	7	3
		MED1	A300-793A, Bethyl	AM580	100 nM	2	7	3
		MED1	A300-793A, Bethyl	1,25-vitD	100 nM	2	7	3
		MED1	A300-793A, Bethyl	AM580 + 1,25-vitD	100 nM each	2	7	3
	ATAC-seq	-	-	DMSO-ethanol	1:1	1	0.05	2
		-	-	AM580	100 nM	1	0.05	2
role of RXR in the interference of NR pathways	RNA-seq	-	-	DMSO-ethanol	1:1	6	0.5	3
		-	-	1,25-vitD	100 nM	6	0.5	3
		-	-	AM580, Calcitriol, GW1516, GW3965, RSG, T3	100 nM or 1 μ M	6	0.5	3
	ChIP-seq	RXR α	21218-1-AP, Proteintech	DMSO-ethanol	1:1	2	10	2
		RXR α	21218-1-AP, Proteintech	AM580	100 nM	2	10	2
		RXR α	21218-1-AP, Proteintech	Calcitriol	100 nM	2	10	2
		RXR α	21218-1-AP, Proteintech	GW1516	100 nM	2	10	2
		RXR α	21218-1-AP, Proteintech	GW3965	1 μ M	2	10	2
		RXR α	21218-1-AP, Proteintech	RSG	1 μ M	2	10	2
		RXR α	21218-1-AP, Proteintech	T3	100 nM	2	10	2
		RXR α	21218-1-AP, Proteintech	LG268	1 μ M	2	10	2
		RXR α	21218-1-AP, Proteintech	AM580, Calcitriol, GW1516, GW3965, RSG, T3	100 nM or 1 μ M	2	10	2
		RXR α	21218-1-AP, Proteintech	AM580, Calcitriol, GW1516, GW3965, RSG, T3, LG268	100 nM or 1 μ M	2	10	2
		RXR α	21218-1-AP, Proteintech	DMSO-ethanol	1:1	1	10	2
		RXR α	21218-1-AP, Proteintech	Calcitriol	100 nM	1	10	2
		H3K27ac	ab4729, Abcam	DMSO-ethanol	1:1	6	3	2
		H3K27ac	ab4729, Abcam	Calcitriol	100 nM	6	3	2

Determination of cooperatively up-regulated genes

Cooperatively up-regulated protein-coding genes were identified as follows. First, for each gene up-regulated by either AM580 or 1,25-vitD, the more effective single ligand was determined by comparing the average fold change (FC) induced by AM580 with that induced by 1,25-vitD (both relative to vehicle). Second, using triplicate RNA-seq data, genes showing significant differences between the more effective single ligand and the combined (AM580 + 1,25-vitD) treatment were identified by a t test ($p \leq 0.05$). Third, the ratios of average normalized mRNA counts in the combined (AM580 + 1,25-vitD) treatment versus the more effective single ligand were calculated, and cooperatively up-regulated genes were defined using a cut-off of 1.5.

5.3. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was used to quantify messenger RNA (mRNA) and enhancer RNA (eRNA) expression for selected target genes and their neighboring regulatory regions. PMA-THP-1 cells were treated with vehicle or ligands for 1-6 h for mRNA measurements and for 6 h for eRNA analysis. RNA isolation and cDNA synthesis were carried out with the Quick-RNA Miniprep Kit (ZYMO Research) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), respectively. RT-qPCR was performed using the LightCycler 480 SYBR Green I Master mix (Roche). Relative RNA abundance was determined using the comparative threshold cycle method and normalized to cyclophilin A (*PPIA*). All experiments were performed in biological triplicates. Primer sequences used for mRNA and eRNA measurements are provided in Table 3.

Table 3. List of primer sequences used for mRNA and eRNA measurement by RT-qPCR

Name	Forward sequence	Reverse sequence
<i>CAMP</i> mRNA	GGATGCTAACCTCTACCGCC	CCTCTGGTGACTGCTGTGTC
<i>FBP1</i> mRNA	ACCCCGCTAACAAGAAGAGC	GTCTAACACGGCCTCCTTCC
<i>HBEGF</i> mRNA	TCCCACTGTATCCACGGACC	GTGCTTGTGGCTTGGAGGA
<i>PPIA</i> mRNA	ATGCTGGACCCAACACAAA	TCTTTCACCTTGCCAAACACC
<i>PTGES</i> mRNA	GCCATCATCACGGGCCAA	GGGGTCGCTCCTGCAATAC
<i>RAB20</i> mRNA	TGCAGCGGTATATGGAGCG	GGCAGTACATGGAGCCCAG
<i>TGM2</i> mRNA	GGCTGACCCTGCACTTTGAG	TCTTAGTGGAAAACGGGCCT
<i>CAMP</i> eRNA (+0.6 kb)	TAGCCACCCCCAACCTAGAG	GAGAGCATGTGGTATGGCCT
<i>FBP1</i> eRNA (+0.3 kb)	TCTGAAGATCCAAGCAGGCG	CCCCCGGGAACACTCTTG
<i>HBEGF</i> eRNA (-24 kb)	GAAGAGGCTGCAGAGACAGG	TTCAAGCTCCTTGGCACAGT
<i>PTGES</i> eRNA (+9.8 kb)	AAAATAAGATGCACACATTTCTCC	AATCATCCAAATATGCCAGATGCT

5.4. ChIP-seq experiments and data analysis

ChIP-seq experiments

ChIP-seq experiments were carried out using previously established protocols^{112, 113}. Briefly, three to ten million PMA-THP-1 cells were treated with vehicle or the indicated ligand(s) for 1-6 h, depending on the experimental condition (Table 2). Cells were first cross-linked with 2 mM disuccinimidyl glutarate (Sigma-Aldrich) for 40 min, followed by cross-linking with 1% methanol-free formaldehyde (Thermo Fisher Scientific) for 10 min. The reaction was stopped by adding 0.125 M glycine (Sigma-Aldrich) for 10 min. Cells were lysed in ChIP lysis buffer containing 150 mM NaCl, 1 mM EDTA (pH 8), 20 mM Tris-HCl (pH 8), 1% Triton X-100, and 0.1% SDS, supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail (Roche). Chromatin was fragmented by sonication using a Diagenode Bioruptor. The sheared chromatin was then subjected to overnight immunoprecipitation with antibodies recognizing RAR α , VDR, MED1, RXR α , or H3K27ac (Table 2). Antibody-bound chromatin complexes were collected using Protein A and/or Protein G Dynabeads (Thermo Fisher Scientific), followed by sequential washing and elution. The eluted material was de-crosslinked overnight, and DNA was purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). ChIP DNA concentration was measured using a Qubit fluorimeter. Indexed sequencing libraries were prepared from 1-10 ng of ChIP DNA with the TruSeq ChIP Sample Preparation Kit (Illumina), following the manufacturer's protocol. Libraries were sequenced on an Illumina NextSeq 500 or NextSeq 2000 platform using 75-cycle single-end reads. Altogether, 48 ChIP-seq samples were generated for this study (Table 2).

Primary ChIP-seq data analysis

ChIP-seq data were processed using our in-house ChIP-seq analysis pipeline as described previously^{113, 114}. Reads were aligned to the hg38 reference genome using Burrows-Wheeler Aligner (BWA) with default parameters¹¹⁵. Peak calling and predicting peak summit were performed with the Model-based Analysis of ChIP-seq version 2 (MACS2), applying a q-value threshold of 0.001 and enabling subpeak deconvolution within each peak (call-summits). Regions listed in the ENCODE blacklist were excluded to remove potential artifacts.^{116, 117}

ChIP-seq peak determination and coverage calculation

For our first research aim, identified peak summits ± 100 bp were used to define ChIP-seq “peaks” or “binding regions”. When two summits within the same dataset were separated by less than 200 bp, they were merged, and a new summit was defined at the midpoint between

them. For our second research aim, the MACS2-identified “narrow peaks” were used directly as peaks for downstream analyses. Normalized tag densities, expressed as reads per kilobase per million mapped reads (RPKM), were calculated using bamtools, bedtools, and awk.

Determination of consensus peak sets

In this work, a peak was defined as a "consensus peak" if it was detected in at least two biological replicates. For our first research aim, summits ± 100 bp regions were used to determine consensus peak sets. MACS2-identified “narrow peaks” were also included, as in some cases summits were not assigned by the algorithm in one replicate despite the presence of a corresponding “narrow peak” in that replicate. Accordingly, regions with a summit in one replicate and a “narrow peak” in another were also classified as consensus peaks. For MED1 ChIP-seq, three replicates were generated, enabling more robust statistical identification of ligand-induced binding events. DiffBind package (version 3.16, R Bioconductor) was used to determine consensus peak sets¹¹⁸. MACS2-defined summits ± 100 bp regions, together with the corresponding BAM files, were used as input for DiffBind analysis with default parameters. For our second research aim, MACS2-identified “narrow peaks” were used directly, and consensus RXR peak sets were retained using intersectBed and merged with mergeBed (bedtools).

Analysis of ligand-dependent changes in ChIP-seq datasets

Using DiffBind, a merged peak set was defined from 12 MED1 ChIP-seq samples (minOverlap = 3), and MED1 occupancy was calculated for each MED1 peak across all samples. MED1 signals from combined (AM580 + 1,25-vitD) and single treatments were compared by calculating FCs, identifying the more effective single treatment, and performing statistical analyses using an unpaired t test. This approach enabled the identification of MED1 peaks with significantly increased signals in the combined (AM580 + 1,25-vitD) treatment relative to the more effective single treatment ($FC > 1$, $p \leq 0.05$). For our second research aim, differentially occupied RXR peaks were identified using DiffBind with edgeR. Peaks with FCs > 1.5 or < 0.66 were grouped as ligand-induced or ligand-reduced, respectively. The rest of the peaks were considered ligand-unresponsive.

ChIP-seq peak to gene association analyses

In this work, the TSS ± 25 kb windows of upregulated genes were enriched for different classes of binding regions. Alternatively, identified ChIP-seq peaks were annotated to the TSS

of the nearest gene, and distances between peaks and TSSs were computed using annotatePeaks.pl (HOMER) and a GTF file (GRCh38.p14) ¹¹⁹.

DNA motif analyses in ChIP-seq datasets

The prevalence of PWMs within peak sets was assessed through a four-step analytical workflow. First, PWMs, including those listed in Table 4, were obtained from the HOMER and JASPAR databases (<http://homer.ucsd.edu/homer/motif/HomerMotifDB/homerResults.html> and <https://jaspar2018.genereg.net/collection/core/>). Second, PWM alignment scores were calculated using a control set of 5,000 randomly selected, size-matched genomic regions with NC accession prefixes (complete genome assembly). Motif similarity scores were computed with annotatePeaks.pl (HOMER), and score thresholds were defined as the score corresponding to 5% positive matches in the control set. Third, genome-wide motif scanning was performed across the human genome using the defined PWMs and score cutoffs with scanMotifGenomeWide.pl (HOMER). Fourth, motif frequencies within distinct ChIP-seq peak sets were quantified using intersectBed (bedtools). Genomic coordinates of DR1, DR2, and DR5 motifs were combined to generate a unified set of putative RAREs. Where indicated, PWMs for DR0, DR1, DR2, DR4, and DR6-DR9 were derived by modifying the spacer nucleotides of the DR5 PWM. PWMs corresponding to IR0-IR9 motifs were generated by altering the spacer sequences of the DR5 PWM and reversing the orientation of one half-site. Distances between motifs were calculated using closestBed (bedtools; the -d argument).

De novo motif discovery was carried out using the HOMER tool findMotifsGenome.pl to identify the most significantly enriched DNA motifs within the top-ranked ChIP-seq peaks, based on occupancy levels. Motif lengths of 17 and 18 bp were used for RAR α peaks, 15 and 16 bp for VDR peaks, with a genomic window size of 200 bp was applied.

Table 4. Position weight matrices (PWMs) used for motif enrichment analyses.

#	Motif name	database	Motif accession
1	ERE-IR3	JASPAR	MA0112.3
2	GRE-IR3	HOMER	motif 145 (GSE32465)
3	LXRE-DR4	HOMER	motif 204 (GSE21512)
4	PPARE-DR1	HOMER	motif 290 (GSE47954)
5	RARE-DR0	HOMER	motif 61 (GSE56893)
6	RARE-DR2	JASPAR	MA0859.2
7	RARE-DR5	HOMER	motif 62 (GSE56893)
8	VDRE-DR3	HOMER	motif 394 (GSE22484)

5.5. ATAC-seq experiments and data analysis

Assay for transposase-accessible chromatin using sequencing (ATAC-seq) was performed based on a previously published protocol, with minor adjustments¹²⁰. PMA-THP-1 cells were treated with vehicle or AM580 for 1 h in biological duplicates, after which 50,000 cells were collected and washed with ice-cold PBS (Table 2). Nuclei were prepared using ATAC lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.1% IGEPAL. The isolated nuclei were then processed for tagmentation using the Tagment DNA TDE1 Enzyme and Buffer Kit (Illumina), followed by indexing with the Nextera DNA library preparation kit (Illumina). Indexed DNA fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and amplified for nine PCR cycles with the Kapa HiFi HotStart Kit (Kapa Biosystems). A second purification step was then performed using the same NucleoSpin Gel and PCR Clean-up Kit. Library fragment size profiles were evaluated on an Agilent Bioanalyzer, and sequencing was carried out on an Illumina NextSeq 500 platform.

Raw ATAC-seq data were processed with a command-line workflow adapted from the ChIP-seq analysis pipeline. Sequencing reads were mapped to the human hg38 genome assembly using BWA with default settings¹¹⁵. Open chromatin regions were then detected with MACS2 at a significance cutoff of $q \leq 0.001$. Before downstream analysis, peaks overlapping ENCODE blacklist regions were removed. The dataset was further restricted to regions located on complete genomic assemblies, identified by NC accession prefixes. For comparison purposes, a background set of 5,000 randomly selected genomic regions was generated. These control regions were matched in size to the ATAC-seq regions, were limited to NC accession-prefix sequences, and were subsequently used in downstream analyses of ATAC-seq signal distribution^{116, 117}.

5.6. Analysis of overlap between genomic regions

To assess shared and unique ChIP-seq peaks between vehicle- and ligand-treated samples, across different ChIP-seq factors (e.g., RAR α versus MED1 or RXR versus VDR), and between ChIP-seq and other omics datasets (e.g., RAR α ChIP-seq versus ATAC-seq), overlap analyses were performed using intersectBed (bedtools) as follows. First, overlapping binding regions between two datasets were identified. Subsequently, regions unique to each dataset were determined.

For comparisons between consensus RAR α (AM580) and VDR (1,25-vitD) peak sets, an alternative strategy was employed to ensure that both datasets were treated equally and that no preference was given to either set. Regions from the two consensus peak sets were merged

using mergeBed (bedtools), and merged regions resulting from the overlap of RAR α (AM580) and VDR (1,25-vitD) peaks were defined as shared binding regions.

5.7. Meta-analysis of NR ChIP-seq datasets

Publicly available ChIP-seq datasets were first searched in the Sequence Read Archive (SRA) using keyword combinations containing the name of the NR of interest, “ChIP,” and the species identifiers “human” or “mouse.” For each NR, metadata tables were exported using the “Send to File” and “RunInfo” options. Next, relevant ChIP-seq samples were filtered for inclusion. For datasets linked to GEO accessions, additional sample information was obtained from the GEO database. Entries unrelated to ChIP-seq, such as RNA-seq datasets, were removed. Samples were retained only when the NR of interest was listed in the “antibody” or “chip_antibody” metadata fields. BioProjects were then selected based on predefined criteria: (i) the presence of both control and ligand-treated samples with treatment times of 24 h or less; (ii) linkage to a peer-reviewed publication; and (iii) use of one time point, preferably closest to 2 h, one ligand, preferably the most commonly applied, and one cell type per study. In the next step, FASTQ files were obtained. When multiple SRA run files corresponded to the same SRA experiment (SRX), they were combined into a single FASTQ file. The downloaded ChIP-seq data were then subjected to primary processing using our previously described in-house pipeline, resulting in BAM file generation. Reads from human and mouse samples were aligned to the hg38 and mm10 reference genomes, respectively. Peak calling was subsequently performed with MACS2 as described previously, followed by construction of consensus peak sets. For each study, narrowPeak files from individual samples were combined using mergeBed. If only one replicate was available for each condition, the merged peak file was used as the consensus peak set. For studies containing two or more replicates per condition, only peaks present in at least two samples were included in the consensus peak set. Studies producing fewer than 2,000 consensus peaks were excluded from downstream analyses. When more than ten studies fulfilled the inclusion criteria for a given NR, preference was given to the most recent studies containing at least two replicates per condition. If fewer than ten studies with replicates were available, additional recent single-replicate studies were included until a maximum of ten studies per NR was reached. Finally, RPKM values were calculated for every region in the consensus peak set of each study and assembled into a summary table. For datasets with replicates, average RPKM values were calculated separately for control and ligand-treated samples. Median RPKM values were then determined for control and ligand-stimulated conditions, followed by calculation of ligand-to-control ratios.

5.8. Data visualization

ChIP-seq and ATAC-seq peaks were visualized using the Integrative Genomics Viewer (IGV; Broad Institute). Genome coverage files (BedGraph format) were generated using `makeUCSCfile.pl` and were normalized and converted to Tile Data Format (TDF) files using `igvtools` with the `toTDF` option. Read distribution (RD) histograms were generated using `annotatePeaks.pl` from the HOMER suite with tag directories and BED files, and were visualized using Java TreeView.^{121, 122} Heatmaps were generated using the web-based DisplayR tool or the `pheatmap` package in R (v1.0.12 or v1.0.13) where indicated. For some heatmaps, values were median-normalized or row-normalized (enabling independent scaling within each row), as specified for each heatmap. Motif enrichment heatmaps were generated using frequencies (percentages). Volcano plots displaying RXR signal differences between LG268- and vehicle-treated samples (FC and FDR) were generated using `DiffBind`. PWM logos were generated using `motif2Logo.pl` (HOMER). The overlaps between cistromes and the proportion of regions harboring two motif types were visualized using Circos plots¹²³. Histograms depicting motif-to-motif distances were created using `ggplot2`. Statistical analyses and graph plotting were performed using GraphPad Prism.

6. Results

6.1. Part I: Genomic regions occupied by both RAR α and VDR are involved in the convergence and cooperation of retinoid and vitamin D signaling pathways

6.1.1. RAR α and VDR bind to overlapping regions in PMA-THP-1 cells

RAR α and VDR genomic binding regions were mapped by ChIP-seq in PMA-THP-1 cells treated for 1 hour with either a vehicle or the selective agonists AM580 and 1,25-vitD, using two biological replicates. RAR comprises three distinct isotypes. In our experiments, we focused specifically on RAR α due to its higher expression in THP1 cells and the availability of ChIP-grade antibodies. To selectively activate RAR α while minimizing stimulation of other isotypes, we used the RAR α -specific agonist AM580 (instead of ATRA, a pan-RAR agonist). The potential contribution of other RAR isotypes to the interaction between RA and vitamin D signaling in this cell line remains possible.

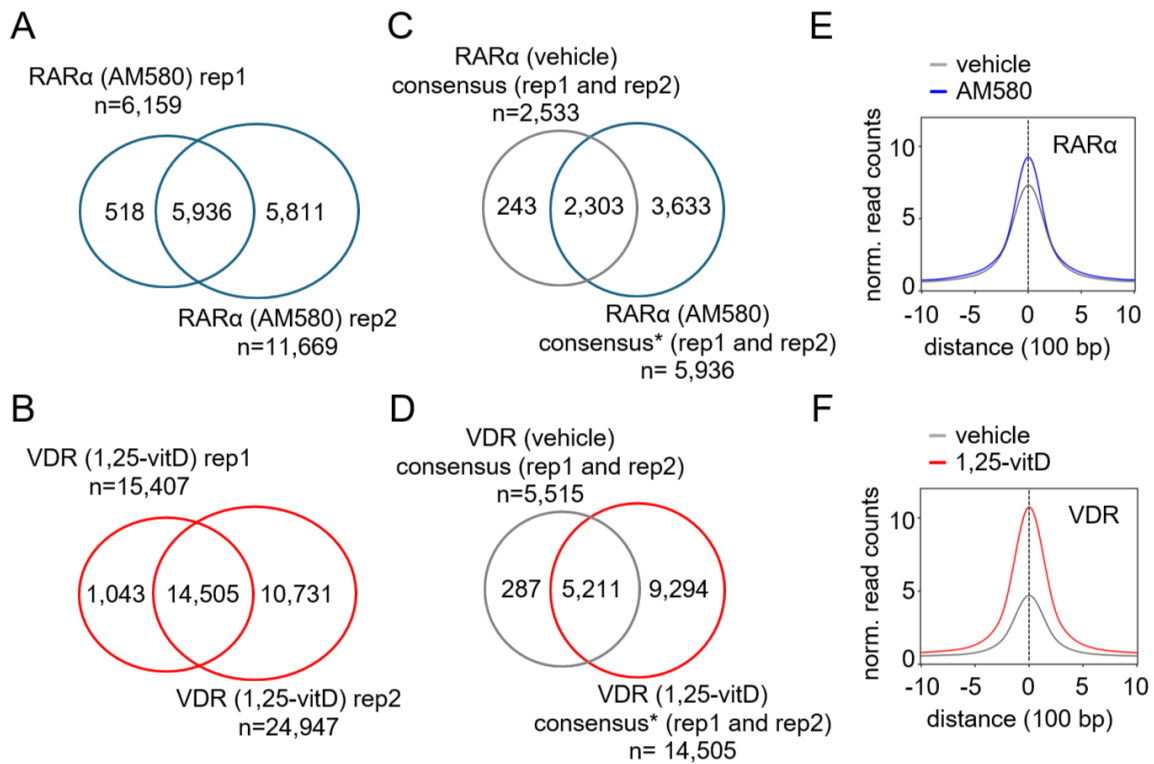


Figure 7. Comparison of RAR α and VDR ChIP-seq peak sets. (A-B) The overlap between two sets of RAR α (A) and VDR (B) peaks (replicates 1 and 2) in PMA-THP-1 cells stimulated with their respective ligands for 1 hour. (C-D) Comparison of consensus RAR α (C) and VDR (D) peak sets obtained from vehicle- and ligand-treated samples. The asterisk indicates the first input file used for intersectBed. (E-F) Normalized read densities within $\pm 1,000$ bp of peak summits for the RAR α (E) and VDR (F) cistromes in vehicle- and ligand-treated cells.

ChIP-seq peak sets from the two biological replicates were compared for RAR α in AM580-treated cells and for VDR in 1,25-vitD-treated cells. The consensus peak sets were defined as peaks shared between the two replicates; under ligand-treated conditions, these sets contained 5,936 RAR α peaks and 14,505 VDR peaks (Figures 7A-B). Ligand stimulation increased both the number of binding regions (Figures 7C-D) and the occupancy levels (Figures 7E-F), with a more pronounced effect on VDR than RAR.

Analysis of the ligand-treated cistromes revealed substantial overlap between RAR α and VDR binding regions. All occupied genomic regions were classified into three groups: RAR α -only (~14%), common (~21%), and VDR-only (~65%). The 3,551 common regions accounted for approximately 60% of the RAR α cistrome and 24% of the VDR cistrome (Figures 8A-B). common binding regions were further classified based on relative binding strength. After normalizing the RAR α and VDR ChIP-seq signals, the ratio of their occupancies was calculated. Using ratio cutoffs of 1.5 and 0.66, common regions were subdivided into RAR α -dominant (n = 1,343), similarly occupied (n = 1,571), and VDR-dominant (n = 637) categories (Figures 8C-D). Together with the RAR α -only and VDR-only groups, these categories formed five clusters that were used in subsequent analyses.

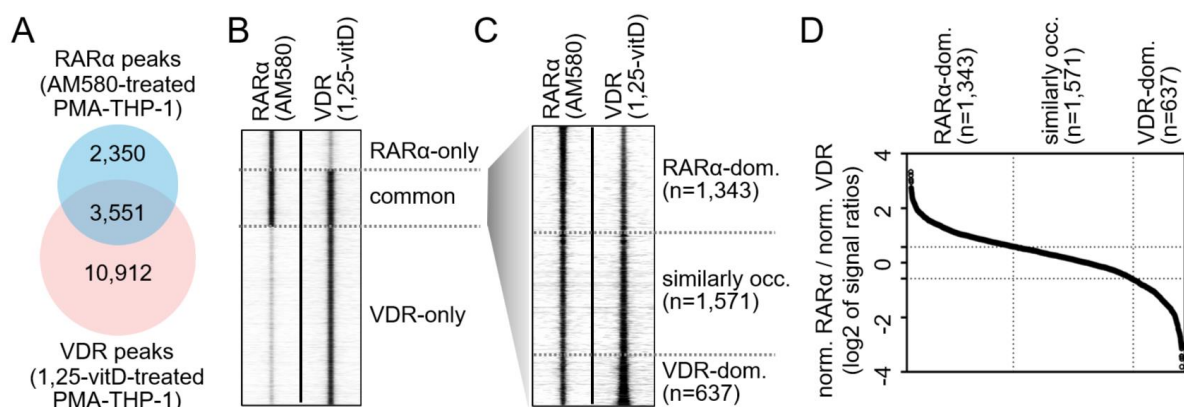


Figure 8. Identification of common RAR α and VDR ChIP-seq peaks in PMA-THP-1 cells. (A) Overlap of genomic regions occupied by RAR α and VDR in cells treated with AM580 and 1,25-vitD, respectively, as identified by ChIP-seq. (B) Read distribution (RD) plot depicting RAR α and VDR ChIP-seq signals in 2 kb windows. (C) RD plot showing RAR α and VDR ChIP-seq signals within three categories of common binding regions in 2 kb windows. Cluster definitions correspond to those shown in panel D. (D) Ratios of normalized RAR α to VDR ChIP-seq signals at regions commonly occupied by both receptors. Binding regions were ranked according to the RAR α /VDR occupancy ratio, and three groups were defined using cutoff values of 1.5 and 0.66 including RAR α -dominant, similarly occupied, and VDR-dominant.

6.1.2. RAR α and VDR ligands up-regulate overlapping gene sets

To examine the link between receptor binding and transcriptional regulation, we analyzed gene expression changes induced by AM580 and 1,25-vitD using RNA-seq. The numbers of differentially expressed genes were 1,094 following AM580 treatment, 387 after 1,25-vitD treatment, and 1,507 in response to combined (AM580 + 1,25-vitD) treatment. Comparison of genes upregulated by each ligand revealed three groups: AM580-only (n = 537), commonly induced by both ligands (n = 91), and 1,25-vitD-only (n = 158) (Figure 9A and B).

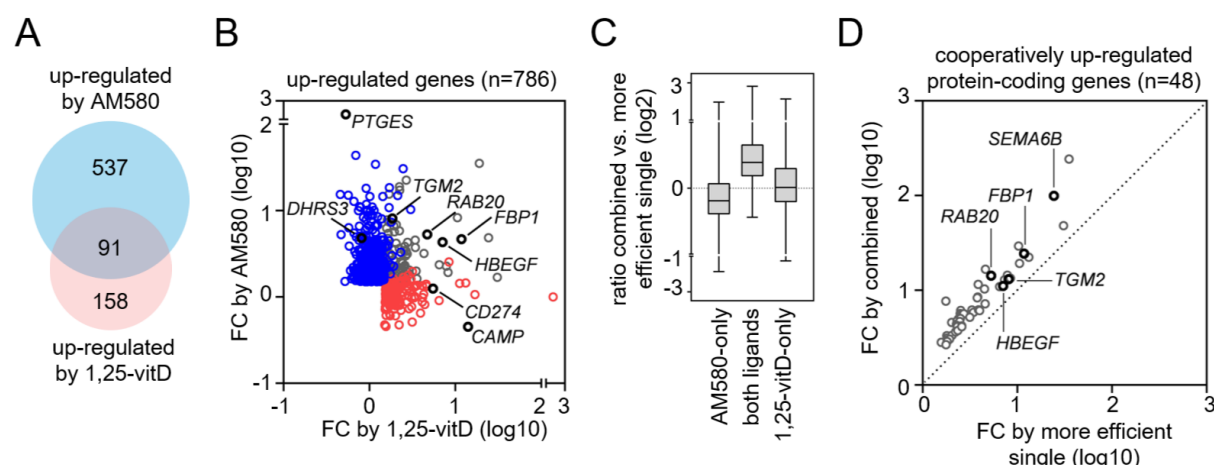


Figure 9. Transcriptional responses to RAR α and/or VDR ligand treatment in PMA-THP-1 cells. (A) Venn diagram showing the overlap among upregulated gene sets in PMA-THP-1 cells, as determined by RNA-seq following 6-hour treatment with the RAR and VDR agonists or vehicle. (B) Scatter plot displaying gene expression fold changes (FC) in response to AM580 and 1,25-vitD. Genes are grouped as follows: induced by AM580 alone (blue), induced by both ligands (grey), or induced by 1,25-vitD alone (red). Representative genes are shown. (C) Box plot illustrating the effect of combined (AM580 + 1,25-vitD) treatment relative to the more effective single-ligand treatment in different gene sets. (D) Scatter plot comparing the FC induced upon combined (AM580 + 1,25-vitD) treatment versus the more effective single agonist in protein-coding genes that showed cooperative up-regulation. Selected cooperatively regulated genes are indicated.

The upregulated gene sets included well-characterized targets of retinoid and vitamin D signaling, several of which linked to known REs¹²⁴⁻¹²⁷. Examples include *PTGES* and *DHRS3*, which were selectively induced by AM580; *CAMP* and *CD274*, which responded specifically to 1,25-vitD; and *FBP1*, *HBEGF*, *TGM2*, and *RAB20*, which were upregulated by both ligands (Figure 9B). To assess combinatorial effects, we calculated fold-change ratios comparing combined (AM580 + 1,25-vitD) treatment with the most effective single-ligand treatment. This analysis revealed that a substantial proportion of shared target genes (57 genes, including 48 protein-coding genes) exhibited cooperative induction upon combined (AM580 + 1,25-vitD) treatment (Figure 9C-D). Notably, 22 of these genes were induced by only one ligand when applied individually but showed enhanced expression under combined (AM580 + 1,25-vitD)

treatment. Time-course analysis of mRNA expression using RT-qPCR for selected genes demonstrated that cooperative effects were already evident at early time points (2-3 h) for representative common target genes, including *FBP1*, *HBEGF*, *RAB20*, and *TGM2* (Figure 10).

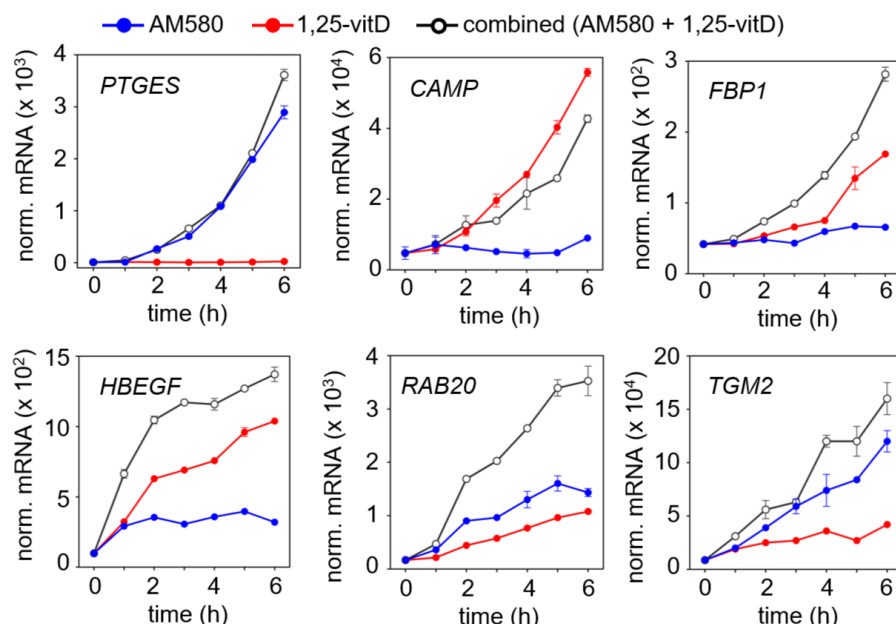


Figure 10. Time-course analysis of mRNA expression for selected genes. PMA-THP-1 cells were treated with AM580, 1,25-vitD, or a combination of both agonists for 1-6 hours. mRNA levels of six representative genes were quantified by RT-qPCR and normalized to *PPIA* expression. Data shown represent one of three independent experiments and are presented as the mean $\pm$ SD of technical triplicates.

6.1.3. Common binding regions are often, but not exclusively, associated with genes induced by both AM580 and 1,25-vitD

The association between gene regulation and the binding profiles of RAR α and VDR was examined using two strategies. In the first approach, each binding region was assigned to its nearest gene, and the expression status of these annotated genes was analyzed (Figure 11A). Approximately 56% of regions bound by RAR α and/or VDR were located within 20 kb of the nearest gene, consistent with previous observations⁶⁰ (Figure 11B). Across all five binding clusters, the majority of regions (79-89%) were linked to genes that did not show differential expression (Figure 11C). The remaining regions were associated with both upregulated and downregulated genes. Among regions annotated with upregulated genes, distinct patterns emerged that reflected ligand-specific gene induction. Regions classified as RAR α -only or RAR α -dominant were more frequently linked to genes induced exclusively by AM580. Similarly occupied regions were associated with all three gene categories. In contrast, VDR-only and VDR-dominant regions were more commonly annotated with genes selectively

induced by 1,25-vitD (Figure 11C). Notably, a subset of VDR-only (3.7%) and VDR-dominant (4.3%) regions was linked to genes induced exclusively by AM580 (Figure 11C). Genes associated with RAR α -only and RAR α -dominant regions exhibited the strongest induction in response to AM580, whereas genes linked to VDR-only and VDR-dominant regions showed the highest fold changes following 1,25-vitD treatment (Figure 11D).

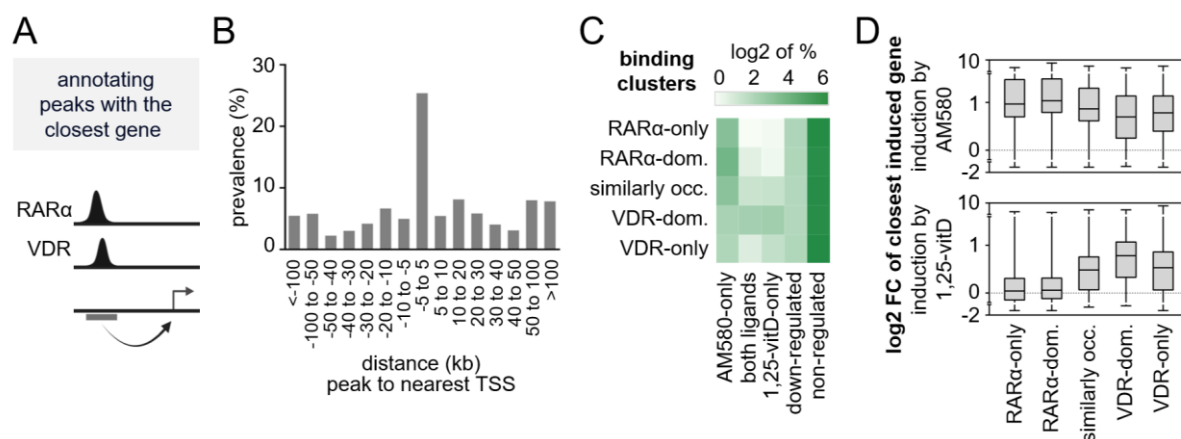


Figure 11. Association between receptor occupancy and transcriptional activation. (A) Schematic illustrating the strategy used to annotate ChIP-seq peaks with nearby genes. (B) Distribution of distances between RAR α - and VDR-bound regions and the nearest annotated TSSs. (C) Heatmap depicting the relative proportions of different gene categories assigned as the nearest genes to each RAR α and VDR binding cluster. Nearest genes were identified for all peaks, and the proportions of gene categories were calculated independently for each binding cluster. (D) Box plots showing gene expression FCs induced by AM580 (top) and 1,25-vitD (bottom) across the different binding clusters. For each binding region, the FC value of the nearest upregulated gene was used.

In a second strategy, we examined the enrichment of distinct binding region categories within ± 25 kb of the TSS of upregulated genes (Figure 12A). Genes induced by both ligands were associated with a greater number of nearby binding regions than genes regulated by either ligand alone (Figure 12B). To quantify these associations, observed-to-expected ratios were calculated to assess the relative representation of each binding cluster within the TSS ± 25 kb of different gene sets (Figure 12C). These ratios were obtained by dividing the actual number of binding regions observed by the number expected based on the total number of binding regions in each cluster. Enrichment results, along with examples of representative target genes, are shown in Figure 12C-D. RAR α -only and RAR α -dominant regions were preferentially enriched near genes induced exclusively by AM580, whereas VDR-only and VDR-dominant regions were over-represented near genes selectively upregulated by 1,25-vitD (Figure 12C).

Binding regions similarly occupied by both receptors, as well as VDR-dominant regions, were enriched within the TSS ± 25 kb of genes induced by both ligands (Figure 12C). Overall, these findings suggest that common RAR α and VDR binding regions contribute significantly to the regulation of genes responsive to both agonists; however, such regions are not uniquely associated with co-induced genes. This suggests that common binding did not necessarily correlate with the regulatory capacity of both receptors.

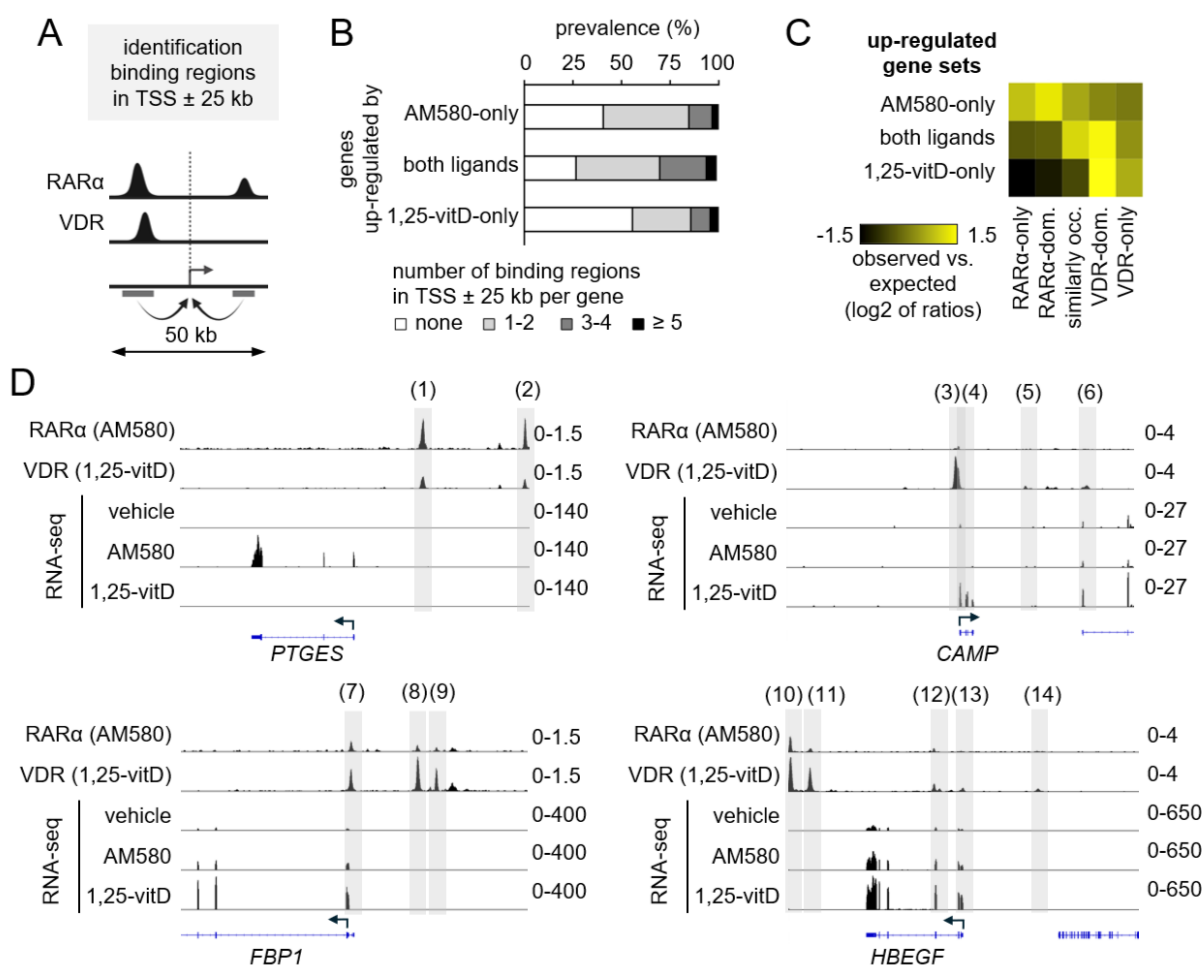


Figure 12. Enrichment of binding region categories within TSS ± 25 kb of up-regulated genes. (A) Schematic illustrating the strategy used for the analysis. (B) Plot showing the number of binding regions per gene located within the TSS ± 25 kb of the three upregulated gene groups including genes up-regulated by AM580 alone, by both ligands, or by 1,25-vitD alone. (C) Enrichment analysis of binding clusters within the TSS ± 25 kb of different gene groups. Enrichment was calculated as the ratio of observed versus expected binding events for each cluster. (D) Integrative Genomics Viewer (IGV) snapshots of representative genes selectively induced by AM580 (PTGES), by 1,25-vitD (CAMP), or by both ligands (FBP1 and HBEGF) in PMA-THP-1 cells. Tracks display normalized ChIP-seq and RNA-seq data. Binding regions correspond to the following clusters: RAR α -dominant [1, 2], similarly occupied [12], VDR-dominant [4, 7, 8, 10, 11], and VDR-only [3, 5, 6, 9, 13, 14].

6.1.4. MED1 recruitment at numerous RAR α and VDR common binding regions is ligand-responsive

The functional activity of regulatory regions can be evaluated using several readouts, such as histone acetylation, coactivator binding, and eRNA production. Here, we assessed genome-wide occupancy of Mediator complex subunit 1 (MED1) by ChIP-seq. We also examined eRNA expression at selected binding regions using RT-qPCR. MED1, also referred to as TRAP220 or DRAP205, is a component of the Mediator complex and is recruited by several NRs, including RARs and VDR^{128, 129}. The Mediator complex contributes to preinitiation complex formation and RNA polymerase II phosphorylation¹³⁰. Elevated MED1 occupancy is commonly linked to super-enhancers and genomic regions bound by multiple TFs¹³¹. Therefore, we used MED1 as a marker to assess the regulatory activity of binding regions.

ChIP-seq was performed with an anti-MED1 antibody in PMA-THP-1 cells exposed for 2 h to vehicle, AM580, 1,25-vitD, or the combination of both ligands (Table 2). Genomic regions with significant changes in MED1 signal between treatment conditions were identified and subsequently analyzed in five ways. First, we examined the overlap between RAR α /VDR peak sets and MED1 peaks. This analysis showed that the majority of RAR α and VDR peaks overlapped MED1-occupied regions, with overlap rates of 84% and 63%, respectively (Figure 13A). However, ligand-dependent MED1 recruitment occurred at only a limited subset of receptor binding sites: 13% of RAR α peaks in AM580-treated cells and 6% of VDR peaks in 1,25-vitD-treated cells (Figure 13B-C).

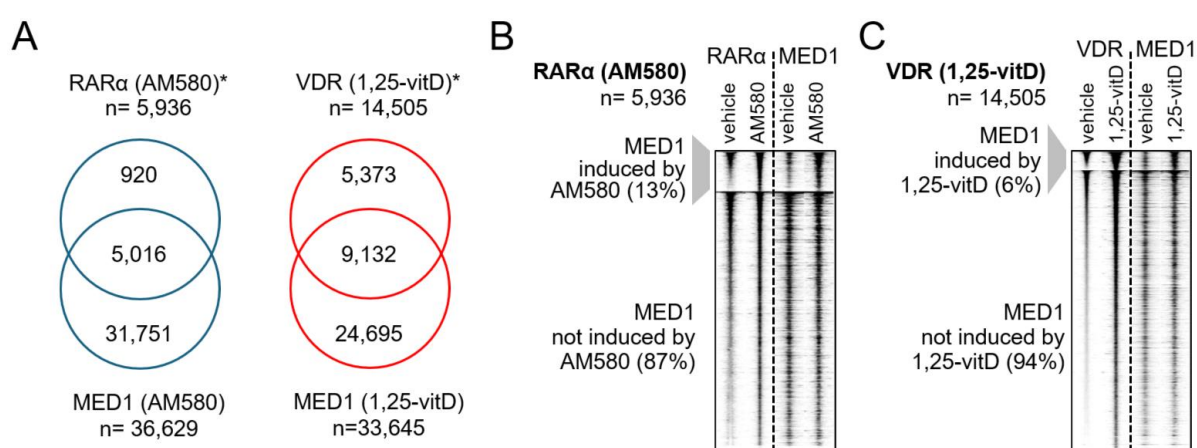


Figure 13. MED1 recruitment to RAR α and VDR binding regions. (A-B) Overlap between RAR α and MED1 consensus peak sets in cells treated with AM580 (A), and between VDR and MED1 consensus peak sets in cells treated with 1,25-vitamin D (B). Asterisks indicate the first input file used for intersectBed. (C-D) RD plots illustrate the colocalization of MED1 with RAR α (C) and VDR (D) peaks in vehicle- or ligand-treated cells within a 2 kb window. RAR α and VDR binding regions were categorized according to ligand-induced changes in MED1 occupancy. Significant increases in MED1 signal (DiffBind FDR ≤ 0.05) were observed at 13% of RAR α (AM580) peaks and 6% of VDR (1,25-vitamin D) peaks.

Second, ligand-responsive MED1 recruitment was evaluated across different receptor binding clusters. Binding regions exhibiting increased MED1 occupancy following AM580, 1,25-vitD, or combined (AM580 + 1,25-vitD) treatment were more prevalent in regions co-occupied by RAR α and VDR than in receptor-specific clusters. In particular, 26% of peaks within the common binding cluster showed induced MED1 signals after combined (AM580 + 1,25-vitD) treatment, compared with 12% and 10% in RAR α -only and VDR-only clusters, respectively (Figure 14A).

Third, we evaluated MED1 occupancy at shared RAR α /VDR binding regions across different gene categories (Figure 14B-C). These categories included three groups of ligand-induced genes, AM580-only, both ligands, and 1,25-vitD-only (Figure 9A), as well as highly expressed non-regulated genes ($n = 250$) and downregulated genes ($n = 559$). Peak numbers were normalized to the total number of binding regions located within ± 25 kb of the TSS for genes in each category. When all shared binding regions were included regardless of MED1 inducibility, their enrichment was comparable across groups or only slightly higher near genes activated by both ligands (Figure 14B). By contrast, shared regions with significant MED1 induction after combined AM580 and 1,25-vitD treatment were rarely found near non-regulated or downregulated genes and were most strongly enriched near genes upregulated by both ligands, compared with the AM580-only and 1,25-vitD-only gene groups (Figure 14C).

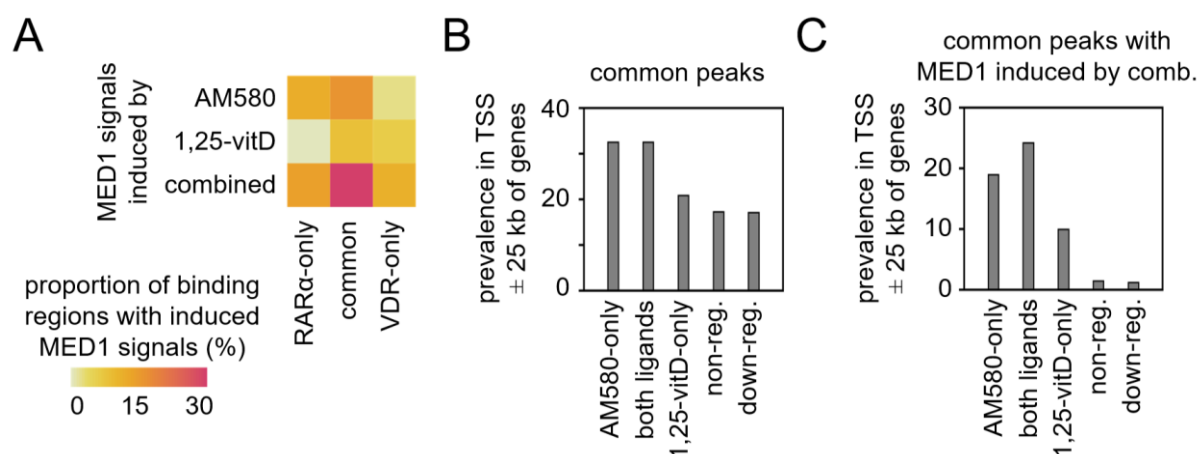


Figure 14. Association of ligand-responsive MED1 recruitment with various binding and gene clusters. (A) The prevalence of RAR α -only, VDR-only, and common binding regions (including RAR α -dominant, similarly occupied, and VDR-dominant) that exhibit a significant increase in MED1 signal (DiffBind, $FDR \leq 0.05$) following treatment with AM580, 1,25-vitD, or their combination. (B-C) Prevalence of RAR α and VDR common binding regions within the TSS ± 25 kb window of genes belonging to different gene categories. The prevalence of all common regions (B) and common regions with MED1 enrichment significantly enhanced by combined (AM580 + 1,25-vitD) treatment (DiffBind, $FDR \leq 0.05$) (C) is shown. Peak numbers were normalized to the total number of peaks in each region. Non-reg., non-regulated; down-reg., downregulated.

Fourth, MED1 occupancy was examined within ± 25 kb of TSS of protein-coding genes that were cooperatively upregulated by both ligands (48 genes; Figure 9D). MED1 binding was generally stronger following combined (AM580 + 1,25-vitD) treatment than after either AM580 or 1,25-vitamin D alone at many of these loci (Figure 15A-B). Fifth, we examined RAR α - and VDR-bound regions in which MED1 was cooperatively recruited following combined (AM580 + 1,25-vitD) treatment. These cooperatively recruited MED1 sites were found across all three binding classes: RAR α -only, common RAR α /VDR, and VDR-only regions. We quantified the distribution of these binding categories within the subset of regions showing cooperative MED1 recruitment that overlapped with either RAR α or VDR peaks (Figure 15C). Compared with the merged RAR α and VDR cistrome (Figure 8A), this subset differed: common binding regions were enriched (43% versus 21%), whereas RAR α -only (8% versus 14%) and VDR-only regions (49% versus 65%) were relatively depleted.

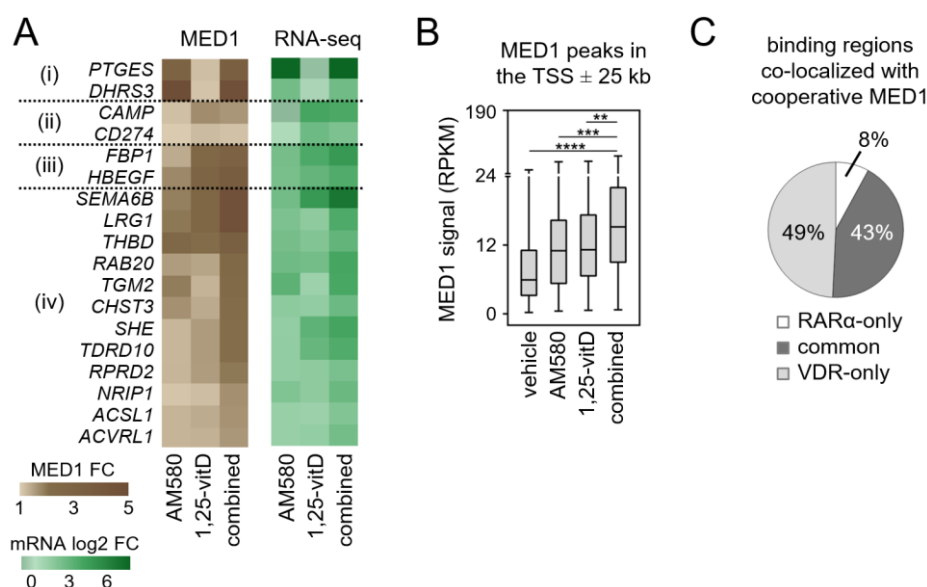


Figure 15. Cooperative MED1 recruitment at RAR α - and VDR-bound regions. (A) Heatmaps illustrating fold changes (FC) in MED1 occupancy (left) and log2 FC in mRNA expression (right) for selected representative genes. Genes include those induced by (i) AM580 alone, (ii) 1,25-vitamin D alone, or (iii) both ligands. In addition, several cooperatively upregulated genes are shown (iv). MED1 occupancy was quantified within the TSS ± 25 kb using ChIP-seq data from cells treated with vehicle, AM580, 1,25-vitamin D, or the combined AM580 + 1,25-vitD. (B) Box plot showing MED1 signal intensity at binding regions ($n = 104$) located within the TSS ± 25 kb of cooperatively upregulated genes. Mean MED1 RPKM values were calculated from three biological replicates per condition. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparison test. ****, $p < 0.0001$; ***, $p = 0.0003$; **, $p = 0.0040$. (C) Pie chart showing the distribution of binding clusters co-localized with cooperative MED1 recruitment, defined as regions where MED1 signals were significantly higher upon combined (AM580 + 1,25-vitD) treatment compared with the more effective single-ligand ($p \leq 0.05$).

Next, we investigated how MED1 recruitment was affected by single versus combined (AM580 + 1,25-vitD) treatments at binding regions located within ± 25 kb of TSS of cooperatively upregulated genes. In total, 104 RAR α - and VDR-bound regions were identified within this window (Figure 16A). Of these, 11 regions, predominantly common binding regions, showed statistically significant cooperative MED1 recruitment upon combined (AM580 + 1,25-vitD) treatment, as determined by comparing MED1 ChIP-seq signals between combined (AM580 + 1,25-vitD) and single ligand treatments using a t test ($P \leq 0.05$). In many additional cases, MED1 occupancy was higher following combined (AM580 + 1,25-vitD) treatment than after the more effective single ligand treatment, although these differences did not reach statistical significance (Figure 16A). Representative ChIP-seq signals for regions associated with cooperatively regulated genes, as well as AM580-only and 1,25-vitD-only genes, are presented in Figure 16B.

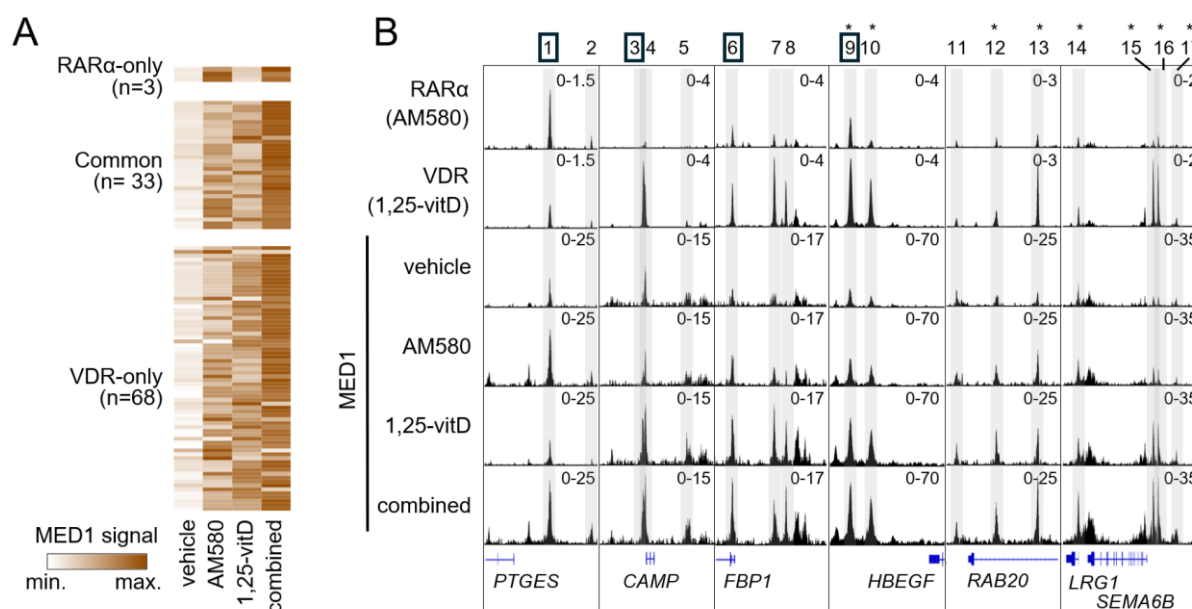


Figure 16. MED1 recruitment at binding regions located within TSS ± 25 kb of cooperatively upregulated genes. (A) Heatmaps showing MED1 occupancy at binding regions ($n = 104$) located within TSS ± 25 kb of cooperatively upregulated genes. Mean MED1 RPKM values were calculated from three biological replicates for each treatment condition. Heatmaps were generated using DisplayR, with row-normalized values (adjusting values in each row independently). (B) IGV snapshots of representative genes induced exclusively by AM580 (PTGES), 1,25-vitD (CAMP), or cooperatively by both ligands (FBP1, HBEGF, RAB20, LRG1, and SEMA6B). Tracks display ChIP-seq signals for RAR α , VDR, and MED1. Highlighted binding regions are classified as RAR α -dominant [1, 2], similarly occupied [11, 14], VDR-dominant [4, 6, 7, 9, 10, 12, 13, 15, 16], or VDR-only [3, 5, 8, 17]. Regions selected for eRNA analysis (Figure 17) are marked with squares. Binding regions co-localized with MED1 peaks showing significantly higher signal following combined (AM580 + 1,25-vitD) treatment (comb.) compared with either single-ligand treatment (t test, $p \leq 0.05$) are indicated by asterisks.

Taken together, these findings indicate that common RAR α /VDR binding regions contribute to the cooperative interaction between retinoid and vitamin D signaling pathways. However, this does not imply that all common regions participate in this cooperation, nor does it exclude the involvement of non-common regions or non-genomic mechanisms.

Following the global analysis of MED1 occupancy, we assessed eRNA expression at selected binding regions using RT-qPCR after 6 hours of stimulation (Figure 17A). Transcription of non-coding eRNAs is a marker of active enhancers, and their expression levels generally correlate with enhancer activity¹³². Four binding regions associated with *PTGES*, *CAMP*, *FBP1*, and *HBEGF*, previously analyzed in Figure 12D, were examined in detail. At two common binding regions, both dominated by VDR and associated with *FBP1* (TSS -0.3 kb) and *HBEGF* (TSS +24 kb), eRNA expression was induced by either AM580 or 1,25-vitD, with clear cooperative effects observed upon combined (AM580 + 1,25-vitD) treatment. In contrast, a third common region, which was RAR α -dominant and linked to *PTGES* (TSS -9.8 kb), showed eRNA induction in response to AM580 alone, while 1,25-vitD was ineffective and appeared to attenuate the AM580 response when both ligands were applied together. The fourth region, a VDR-only region associated with *CAMP* (TSS -0.6 kb), exhibited eRNA induction following 1,25-vitD treatment only, with no response to AM580 and no cooperation. Notably, the eRNA expression patterns closely mirrored the MED1 occupancy profiles observed at these regions (Figure 17).

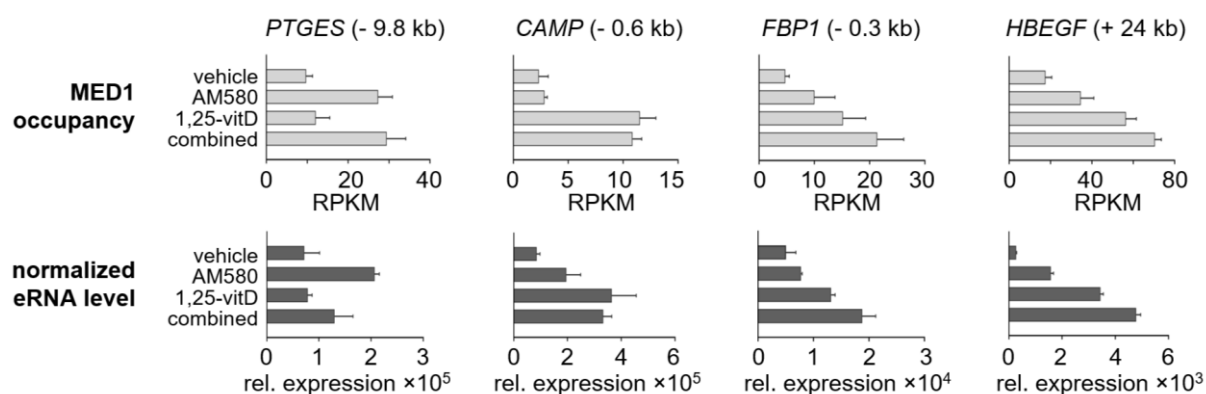


Figure 17. MED1 occupancy and normalized eRNA levels at representative binding regions. MED1 occupancy at each region is shown as the mean RPKM $\pm$ SD from three biological replicates following 2-hour treatment with vehicle or agonist(s). eRNA levels were measured by RT-qPCR after 6-hour treatment with vehicle or agonist(s) and normalized to PPIA mRNA. One representative experiment of three is shown, with values representing the mean of technical triplicates $\pm$ SD.

6.1.5. Common RAR α and VDR binding regions preferentially localize to accessible chromatin

Signal-regulated transcription factors (SRTFs) have limited capacity to bind DNA motifs within closed chromatin. Consequently, their binding profiles are largely determined by pre-existing accessible regulatory elements, which are typically established by lineage-determining transcription factors (LDTFs)¹⁹. Nevertheless, a subset of SRTFs can also bind previously inaccessible genomic regions, referred to as latent or de novo enhancers. Binding at these sites promotes chromatin opening, recruitment of additional TFs, and acquisition of enhancer-associated histone modifications^{19, 133}. We previously showed that genomic regions co-occupied by two or three IRF family members were more accessible prior to stimulation than regions bound by only one IRF¹¹³. Based on this observation, we investigated whether regions co-bound by RAR α and VDR display higher chromatin accessibility compared with regions exclusively bound by either receptor. While the relationship between VDR binding and chromatin accessibility has been extensively characterized across both short and long stimulation periods, including in human myeloid cells^{134, 135}, studies of RARs have largely focused on longer ligand treatments (9-72 h)¹³⁶⁻¹³⁸. To match the timing of our RAR α and VDR ChIP-seq experiments, we therefore performed ATAC-seq in vehicle- and AM580-treated cells after 1 hour of ligand exposure.

The majority (80.1%) of RAR α binding regions were accessible under both vehicle and AM580 conditions (Figure 18A-B). Smaller fractions were accessible only after AM580 treatment (7.5%) or only in vehicle controls (1.5%). Regions inaccessible in both conditions (10.9%) generally showed weak RAR α occupancy. For genes upregulated by AM580, neither RAR α occupancy nor chromatin accessibility changed substantially, even though MED1 recruitment increased following stimulation (e.g., *PTGES*, *DHRS3*, and *TGM2*; Figure 18C). Earlier reports have suggested that RAR ligands can markedly influence chromatin accessibility¹³⁶⁻¹³⁸, at least partly through indirect mechanisms. The discrepancy with our findings is likely due to the shorter treatment duration used here (1 h versus 9-72 h in previous studies).

In a second analysis, we compared chromatin accessibility across five binding clusters in vehicle-treated cells. Common RAR α /VDR binding regions showed a higher proportion of overlap with ATAC-seq-positive regions than regions bound exclusively by either receptor (Figure 18D). Furthermore, these common regions exhibited stronger ATAC-seq signals, both across entire clusters and within ATAC-positive subsets (Figure 18E-F). Together, these results indicate that commonly occupied regions constitute more readily accessible genomic regions for these NRs.

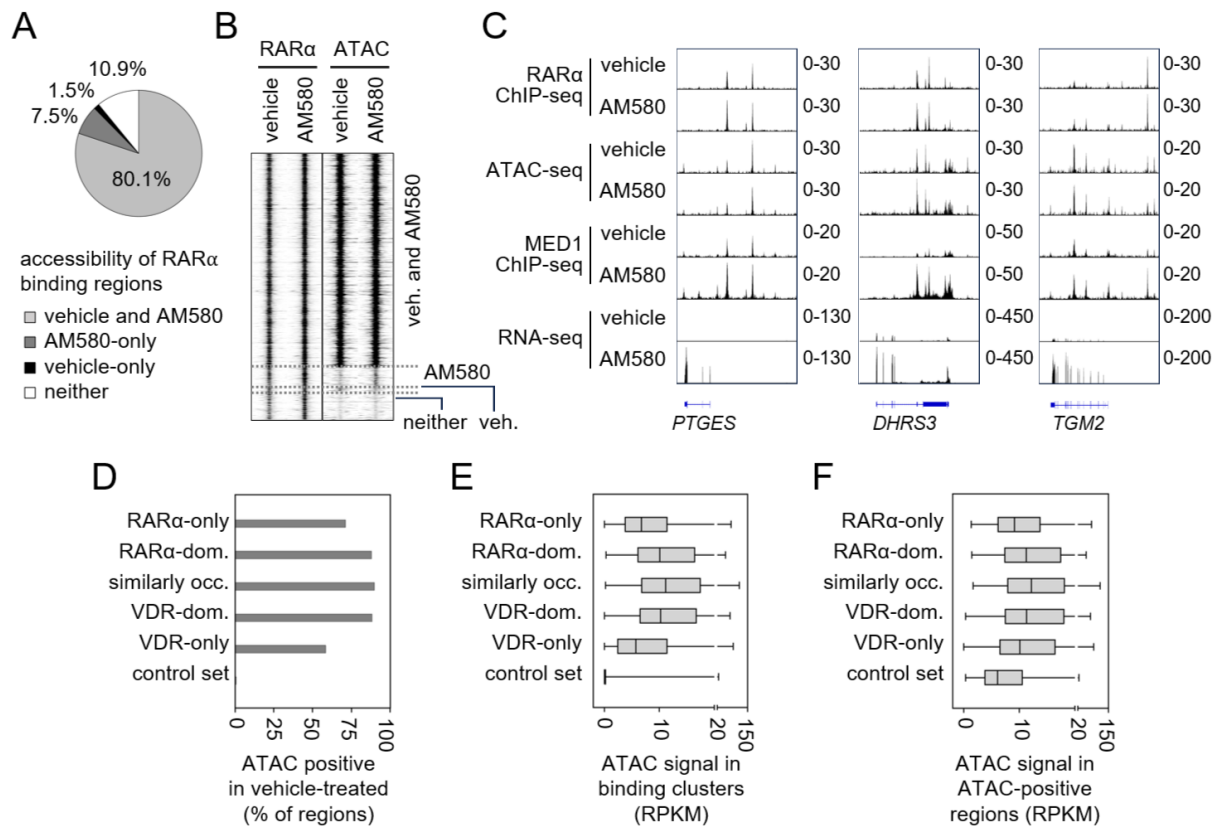


Figure 18. Chromatin accessibility at RAR α and VDR binding regions. (A) Chromatin accessibility of RAR α binding regions assessed by ATAC-seq in PMA-THP-1 cells treated with vehicle or AM580 for 1 h. The proportions of RAR α binding regions accessible in vehicle-treated cells, AM580-treated cells, both conditions, or neither condition are shown. (B) An RD plot displaying RAR α ChIP-seq and ATAC-seq signals across 2 kb windows. (C) IGV snapshots of representative genes upregulated following AM580 treatment. Tracks show RAR α ChIP-seq, ATAC-seq, MED1 ChIP-seq, and RNA-seq data. (D) Percentage of binding regions that overlap with ATAC-positive regions. (E-F) Box plots showing ATAC-seq signal intensity across all regions (E) and specifically within ATAC-positive regions for each RAR α /VDR binding cluster (F) in vehicle-treated cells.

6.1.6. Putative REs in the RAR α and VDR cistromes and binding clusters

We conducted de novo motif discovery and PWM-based motif mapping across the different peak sets. When the top 1,000 RAR α and VDR peaks were analyzed, de novo motif discovery detected DR5 and DR2 motifs in 14.3% and 14.0% of RAR α ChIP-seq peaks, respectively, whereas DR3 motifs were found in 54.0% of VDR peaks (Figure 19). In addition, motifs recognized by macrophage LDTFs were detected, consistent with their established roles in enhancer formation and regulation of SRTF binding^{19, 122}.

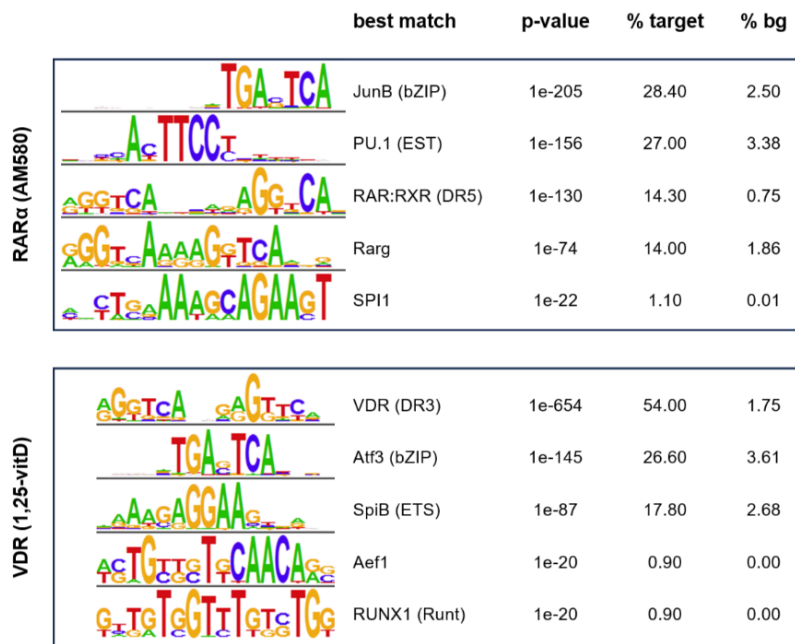


Figure 19. HOMER de novo motif analysis on the top 1,000 peaks from RARα ChIP-seq in AM580-treated cells (top) and VDR ChIP-seq in 1,25-vitD-treated cells (bottom). Motif discovery was conducted using motif lengths of 17-18 bp for RARα and 15-16 bp for VDR, with a region size of 200 bp. bg, background.

Motif mapping using PWMs enabled direct comparison of motif frequencies across datasets. DR3 and DR5 PWMs were retrieved from motif databases and further refined based on de novo motif discovery results. Additional PWMs were created by altering DR5 spacer sequences and half-site orientations. Thresholds for DR3 and DR5 were determined using randomly selected, size-matched control sequences, with the cutoff defined as the score corresponding to 5% positive matches in the control set (Figure 20A-B). These criteria were then applied to evaluate putative RAREs and VDREs within the RARα cistrome after AM580 treatment, the VDR cistrome after 1,25-vitD treatment, and the five predefined binding clusters.

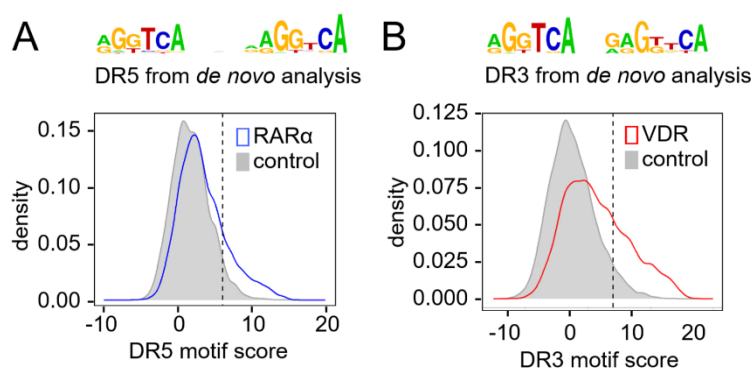


Figure 20. PWM-based motif mapping and selection of score thresholds. (A-B) Density plots showing the distribution of DR5 and DR3 motif scores in RARα (A) and VDR (B) binding regions from AM580- and 1,25-vitD-stimulated cells, respectively. Each plot also includes motif score distributions from randomly selected, size-matched genomic control sequences ($n = 5,000$ regions). Score thresholds (dotted lines) were set at the value corresponding to 5% positive matches in the control set.

First, we analyzed the full RAR α and VDR cistromes. Within the RAR α cistrome, DR5 was the most frequently detected RARE (19.7%), followed by DR2 (18.2%) and DR1 (14.2%). As expected, DR3 was the predominant motif in the VDR cistrome (29.7%), whereas other motifs occurred less frequently (Figure 21A-B). Notably, DR5 was only 1.5-fold more frequent in the RAR α cistrome than in the VDR cistrome, where it was present in 13.3% of regions. When considering the combined presence of the three most common RAREs (DR1, DR2, and DR5; termed DR1/2/5), 42.6% of RAR α regions and 15.8% of control regions contained these motifs. Therefore, only DR1/2/5 were retained as putative RAREs for subsequent analyses to avoid increasing background noise.

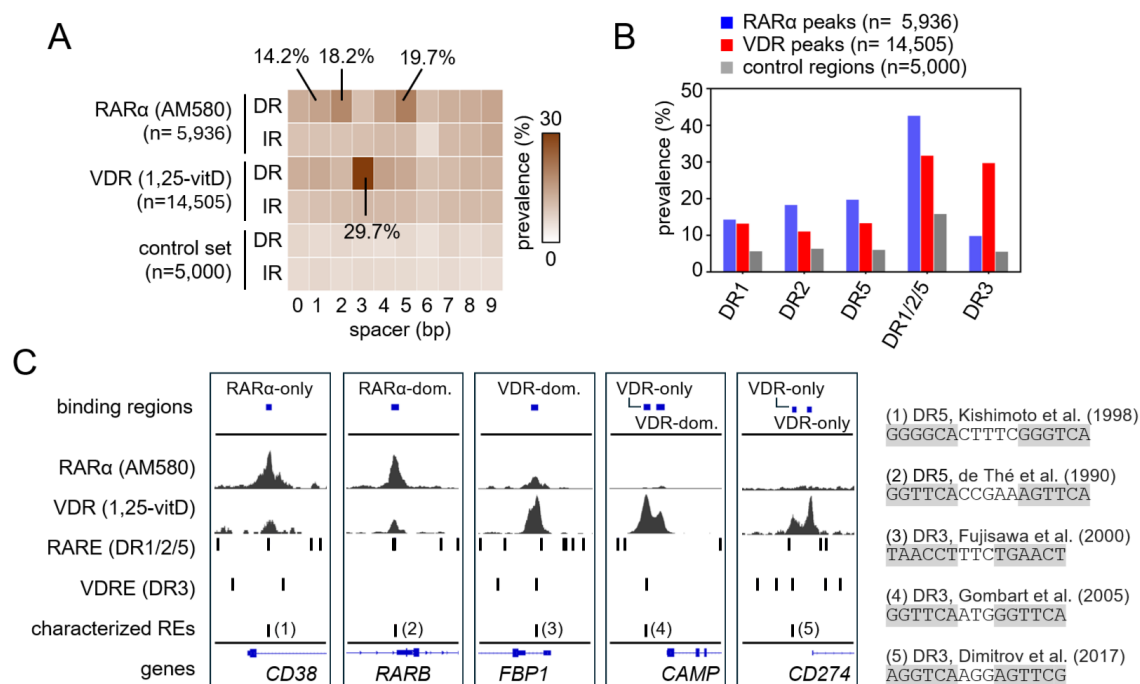


Figure 21. Prevalence and validation of REs within RAR α and VDR cistromes. (A) Heatmap showing the frequency of direct repeats (DRs) and inverted repeats (IRs) with spacer lengths ranging from 0 to 9 nucleotides in RAR α and VDR cistromes, and in a control set. (B) Frequencies of DR1, DR2, DR5, the combined DR1/2/5 group (regions containing any of these motifs), and DR3 within the RAR α and VDR cistromes and the control set. (C) IGV snapshots of representative genomic loci displaying ChIP-seq peaks and predicted motifs. Tracks indicate the classification and positions of binding regions, RAR α and VDR ChIP-seq signals, the locations of RAREs and VDREs identified by our motif enrichment analysis, as well as REs characterized in previous studies. Scales were set independently for each of the five regions. The same scales were used for both RAR α and VDR data within each region.

Motif mapping also recovered several previously characterized REs, including those associated with CD38, RARB, FBP1, CAMP, and CD274, typically located near peak summits (Figure 21C). Interestingly, although the well-established regulatory region of RARB^{5, 139} was classified as RAR α -dominant and contained a DR5 motif, gene expression remained unchanged

following ligand treatment. Together with the observation that many RAR α - and VDR-occupied regions corresponded to non-regulated genes (Figure 11C), this finding indicates that receptor binding alone is insufficient to drive transcriptional changes.

In the second analysis, we compared the distribution of RAREs (DR1, DR2, DR5, and the combined DR1/2/5 category) and DR3 motifs among the five binding clusters (Figure 22A). As anticipated, the RAR α -only and RAR α -dominant clusters were characterized by high RARE frequency and low DR3 occurrence. The similarly occupied cluster was enriched for both motif groups, although DR1/2/5 motifs were substantially more frequent than DR3 motifs (40.6% versus 13.1%; 3.1-fold difference). Unexpectedly, enrichment of both RAREs and DR3 motifs was also observed in the VDR-dominant and VDR-only clusters. The relatively high occurrence of DR1/2/5 motifs in the VDR-only cluster (26.2%) prompted us to ask why RAR α was not recruited to these sites. By integrating ATAC-seq data with motif score analysis, we found that these regions may provide a less permissive environment for RAR α binding, as DR2 and DR5 motif scores were generally lower than in other clusters (Figure 22B), and chromatin accessibility was also reduced (Figure 18D-F).

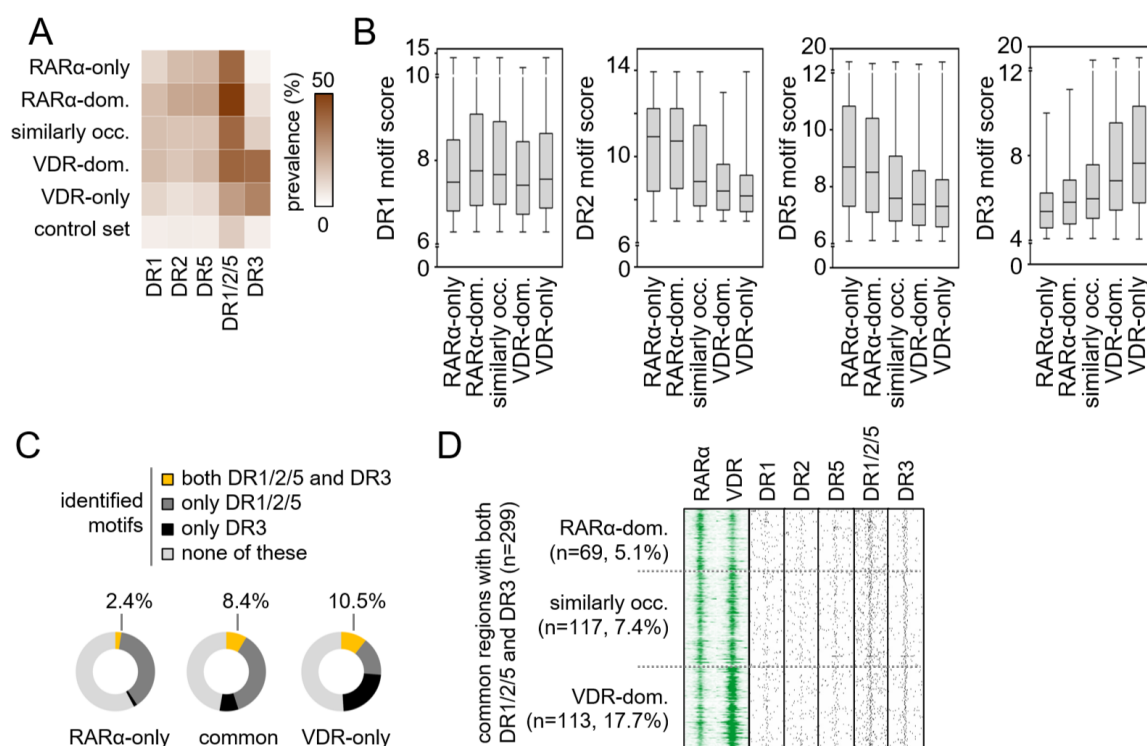


Figure 22. Distribution and strength of REs within binding clusters. (A) Heatmap illustrating the proportion of binding regions within each cluster that contain DR1, DR2, DR5, DR1/2/5, or DR3 motifs. (B) Box-and-whisker plots displaying the highest motif score exceeding the defined threshold for each binding cluster. (C) Proportions of RAR α -only, VDR-only, and common binding regions that contain both DR1/2/5 and DR3 motifs, only DR1/2/5, only DR3, or none of these motifs. (D) ChIP-seq peaks and DNA motifs within common RAR α and VDR binding regions that contain both DR1/2/5 and DR3 elements.

In the third analysis, we examined regions containing both RARE (DR1/2/5) and VDRE (DR3) motifs (Figure 22C-D). Co-occurrence of the two motif types was rare in the RAR α -only cluster (2.4%) but more frequent in common clusters (average 8.4%) and in the VDR-only cluster (10.5%). Among the common clusters, the VDR-dominant group exhibited the highest proportion of regions containing the two motif types (17.7%; 113 of 637 sites). Nevertheless, most common regions contained either only one motif class (44.4%) or none (47.2%). Overall, these findings suggest that only a minority of RAR α -VDR co-binding events are driven by strong, co-occurring canonical REs.

6.1.7. Common RAR α /VDR binding regions display diverse RARE-VDRE configurations

We examined how DR1/2/5 and DR3 motifs are positioned relative to one another within regions commonly occupied by RAR α and VDR. Distances were measured between the central base of a DR3 motif and that of the nearest DR1, DR2, or DR5 motif (Figure 23A).

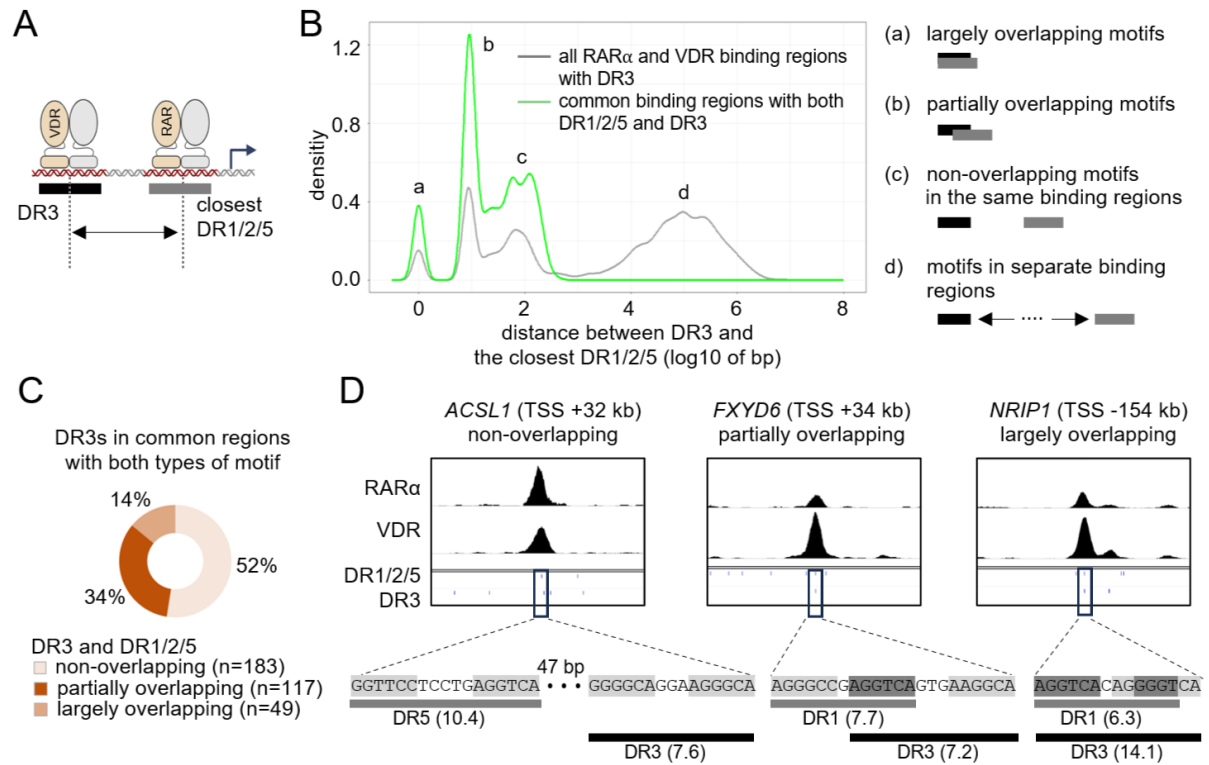


Figure 23. Organization of RARE and VDRE motifs in regions commonly occupied by RAR α and VDR. (A) Schematic illustrating the reference base positions used to calculate distances between DNA motifs. (B) Density plots showing distances between DR3 motifs and their nearest DR1/2/5 motifs. Distances were calculated for DR3 motifs present in all RAR α and VDR binding regions (grey) or specifically in common RAR α -VDR binding regions containing both DR1/2/5 and DR3 motifs (green). The four labeled groups (a-d) correspond to distinct motif arrangements. (C) Distribution of DR3 motif configurations within common RAR α -VDR binding regions that contain both DR1/2/5 and DR3 motifs. (D) IGV snapshots of representative genomic regions (peak summits ± 3 kb) with different configurations of DR3 and DR1/2/5 motifs. Values in parentheses denote motif scores.

The extent of shared bases was computed, and motif pairs were categorized as largely overlapping (≥ 7 shared bases), partially overlapping (1-6 shared bases), or non-overlapping. All three configurations were observed in common binding regions containing both motif types (Figure 23B, green line). A similar pattern emerged when considering all RAR α and VDR binding regions that included DR3 motifs (Figure 23B, grey line), although an additional group was identified in which the nearest DR1/2/5 motif was located at a separate peak from the DR3 peak. Further quantitative analysis of common binding regions revealed that non-overlapping motifs were the most frequent arrangement (52%), followed by partially overlapping (34%) and largely overlapping (14%) configurations (Figure 23C). Representative examples of each motif arrangement are shown in Figure 23D.

6.1.8. Overlap of NR cistromes and motif co-occurrence in previous studies

We aimed to investigate the extent to which NR cistromes overlap and how motifs co-occur, both in our data and in prior studies. Several earlier studies mapped the binding regions of NR pairs with distinct motif preferences in various cell types^{35, 48-50, 140}, many of which reported substantial overlap of cistromes^{35, 48-50}. However, differences in peak-calling algorithms and motif discovery approaches across these studies make direct comparisons difficult. To address this, we reanalyzed ChIP-seq datasets from selected studies^{35, 50, 140} alongside our own using a standardized workflow. (Due to the absence of replicates in some datasets, our original workflow could not be applied.) For each NR pair, we identified binding regions, calculated cistrome overlaps, assessed the prevalence of the most enriched motifs, and examined motif co-occurrence (Figure 24). The size ratios of larger to smaller cistromes were 2.1 for VDR versus RAR α , 1.7 for ER α versus GR, 8.9 for LXR versus PPAR α , and 11.7 for PPAR α versus GR (Figure 24A-D). Overlap fractions within the smaller cistromes varied from 22% (ER α in the ER α versus GR comparison, Figure 24C) to 85% (PPAR α in the PPAR α versus GR comparison, Figure 24D). The RAR α and VDR cistrome sizes and overlaps differ slightly from previous results in this study (Figure 8A) due to the alternative workflow.

For each NR, we determined the PWM of the most enriched motif and motif thresholds were defined using control sets. Motif prevalences were then calculated (Figure 24A-D). To simplify the analysis, only DR5 was evaluated in the RAR α cistrome among all putative RAREs. The standardized workflow produced a wide range of motif prevalences, from 14.3% (GR peaks containing GRE-IR3) to 54.9% (ER peaks containing ERE-IR3).

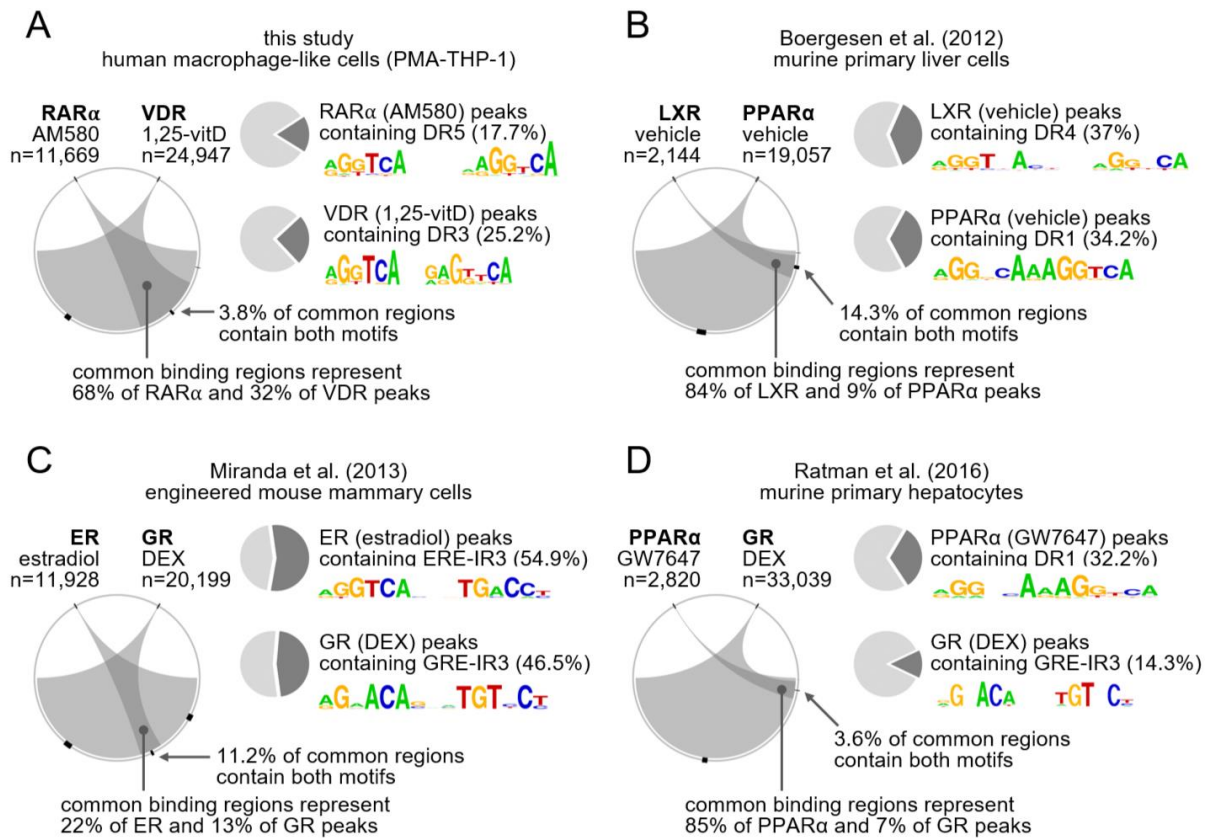


Figure 24. Overlap of NR cistromes and motif co-occurrence. (A) Our datasets and (B-D) publicly available ChIP-seq datasets were reanalyzed. Circos plots illustrate the overlap between NR cistromes. For each NR, the most enriched motifs were identified, with motif logos and their prevalences across the entire cistromes shown in pie charts. Co-occurrence of motif pairs was calculated for three sets of genomic regions: two receptor-specific sets and one set of common regions. Proportions are indicated by block arcs, while exact percentages are provided only for common regions.

Analysis of motif co-occurrence within common binding regions revealed generally low frequencies across all studies, ranging from 3.6% to 14.3% of common peaks (Figure 24A-D). Even in the ER versus GR comparison, where motifs were highly prevalent in total cistromes, co-occurrence was observed in only 11.2% of common binding regions (Figure 24C). Overall, these re-analyses indicate that extensive sharing of binding regions among NRs does not necessarily coincide with a high frequency of motif co-occurrence, suggesting this is a general feature of NR cistromes.

6.2. Part II: RXR availability is not a limiting factor when multiple NR pathways are activated in macrophage-like cells

6.2.1. Meta-analysis of ChIP-seq datasets designates RXR as one of the least ligand-sensitive NRs based on overall genomic binding.

Publicly available ChIP-seq datasets were analyzed to evaluate ligand-dependent changes in the genomic occupancy of RXR and other NRs. Relevant NR ChIP-seq studies were selected based on predefined criteria, including that each study must contain control (vehicle-treated or untreated) and ligand-treated conditions. Although all NRs with identified and non-controversial ligands were surveyed (Table 1), for many of them, there were no ChIP-seq datasets passing the inclusion criteria.

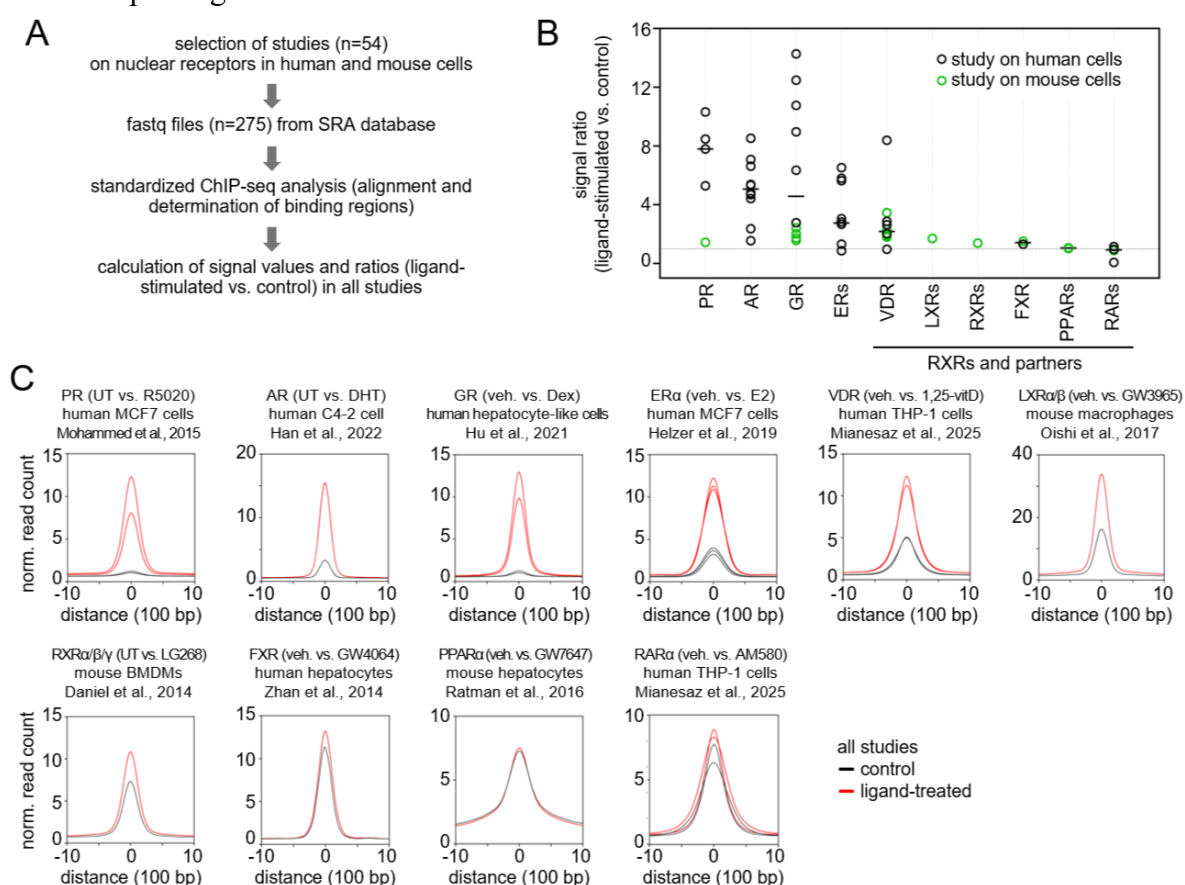


Figure 25. Meta-analysis of ChIP-seq datasets for ligand-dependent effects on genomic binding of NRs. (A) Overview of the ChIP-seq meta-analysis workflow for publicly available datasets. (B) Signal ratios derived from the ChIP-seq meta-analysis are plotted, with each point representing an individual dataset. The ratio of signals from ligand-stimulated to control samples (vehicle-treated or unstimulated cells) was computed by calculating signal intensities at binding sites found within each dataset. When more than one dataset was available for a given NR, the median ratio value is indicated. (C) Histograms displaying normalized read counts in control and ligand-treated cells from the same representative studies within ± 1000 bp of ChIP-seq peak summits. For each NR, one representative study was selected from the available datasets based on having a ligand-to-control ratio close to the median (panel B).

The datasets that met these requirements were downloaded and processed using a uniform analysis pipeline (Figure 25A). The ratio of overall genomic binding in ligand-treated samples versus controls was calculated for each study, yielding a single value per dataset (Figure 25B). In agreement with previous studies, steroid receptors displayed pronounced induction in genome-wide occupancy in response to their ligands. Among studies examining the same receptor, notable variability in occupancy ratios was observed, likely reflecting differences in endogenous ligand levels across cell types, experimental design, and technical factors. Compared with steroid receptors, fewer ChIP-seq datasets were available for RXR and its heterodimeric partners. Within this group, ligand treatment resulted in only modest changes in overall genomic occupancy, and VDR showed the greatest degree of ligand-induced changes. Nevertheless, compared with steroid receptors, these changes were still less pronounced (Figure 25B and representative studies in Figure 25C).

6.2.2. Overall RXR genomic binding is largely unchanged upon ligand treatment in PMA-THP-1 cells

We investigated the impact of ligands that target RXR and its heterodimeric partners on RXR chromatin binding, using PMA-THP-1 cells as a model. Highly expressed ligand-inducible RXR partners in PMA-THP-1 cells were determined (Figure 26A) using RNA-seq data from vehicle-treated cells generated for our first research aim (Table 2). Notably, the order of these receptors could be different if assessed at the protein level. Among RXR isotypes, *RXRA* was the most highly expressed, and *RXRB* and *RXRG* were expressed at very low levels. Highly or moderately expressed RXR partners included *PPARD*, *PPARG*, *LXRB*, *RARG*, *RARA*, *VDR*, *THRA*, *PPARA*, and *LXRA*, and *RXRA* mRNA expression was higher than that of its partner receptors in PMA-THP-1 cells. According to our analysis of mRNA data from the Human Protein Atlas ¹⁴¹, in many primary human cell types, including macrophages, the most abundant RXR isotype was expressed at a level comparable to or exceeding that of its partners (Figure 26B). However, the mRNA expression of the most abundant RXR isotype was significantly lower than that of RXR partner genes in several other cell types (Figure 26B-C).

An RXR agonist and six agonists targeting highly/moderately expressed RXR partners were selected for the ChIP-seq experiments (Figure 26A). Ligand concentrations producing maximal receptor activation were determined from pilot experiments and prior studies ⁵⁴. PMA-THP-1 cells were treated with ligands at the determined concentrations, and ChIP-seq was performed to identify genomic regions occupied by RXR.

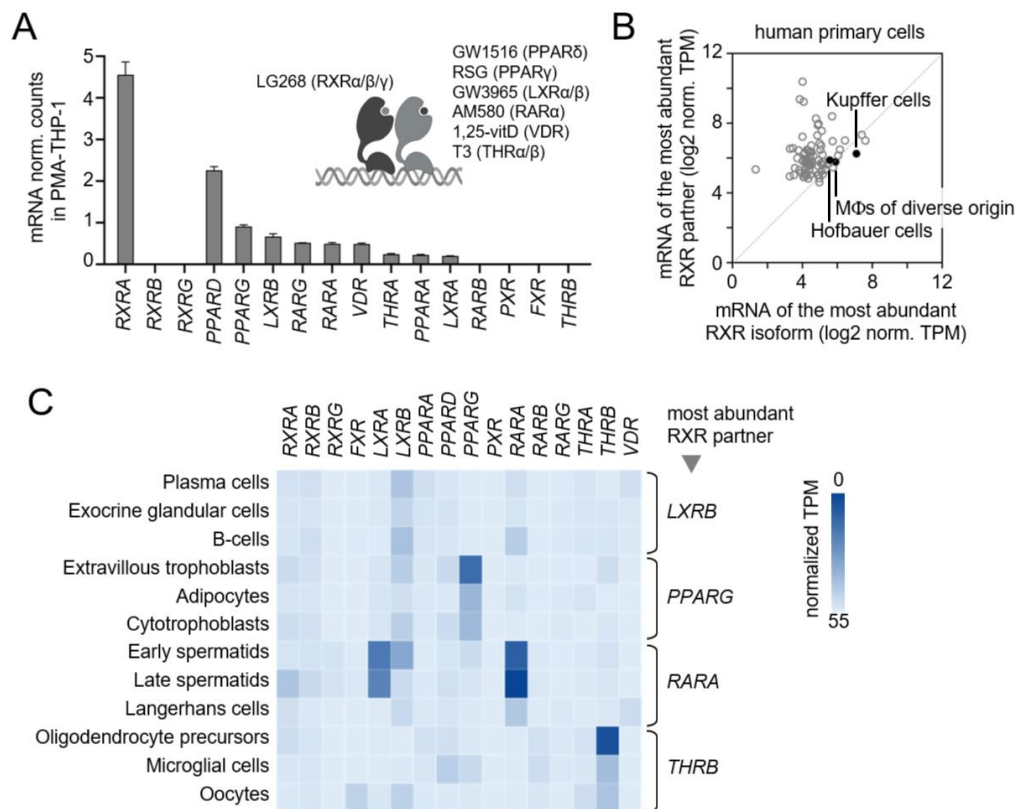


Figure 26. RXR isotype and partner mRNA profiles in different cell types. (A) RNA-seq analysis of RXRs and their heterodimeric partners in PMA-THP-1 cells. The data represent the mean $\pm$ SD of three replicates of vehicle-treated cells. Ligands applied in subsequent ChIP-seq experiments are listed in the inset. (B) mRNA levels of RXR and its most highly expressed partner across multiple primary cell types. Primary human macrophages are shown with black dots. (C) Heatmap displaying the expression of RXR isotypes and their ligand-inducible partners across representative cell types. Single-cell RNA-seq data from the Human Protein Atlas were analyzed for panels B and C.

Reproducible RXR peaks, defined as those detected in both replicates for each condition, were identified for the vehicle control and for each ligand treatment. Most RXR binding regions in ligand-treated samples overlapped with those in control samples, while a notable fraction (14-36%) was unique to ligand-stimulated conditions (Figure 27A). Signal intensity at these ligand-specific regions was generally low (Figures 27B). RXR binding ratios (ligand-stimulated versus control) were typically near 1.0, with the highest increases observed for LG268 and T3 (1.24 and 1.18, respectively) (Figure 27C). In agreement with previous reports in mouse bone marrow-derived macrophages (BMDMs), our results indicate that, regardless of the ligand, overall RXR genomic occupancy remains largely unchanged in macrophage-like cells⁵⁹. Earlier ChIP-seq studies in HeLa cells showed that LG268 enhanced RXR DNA binding to a comparable extent to that observed in our study, which was further confirmed by fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP)³⁰.

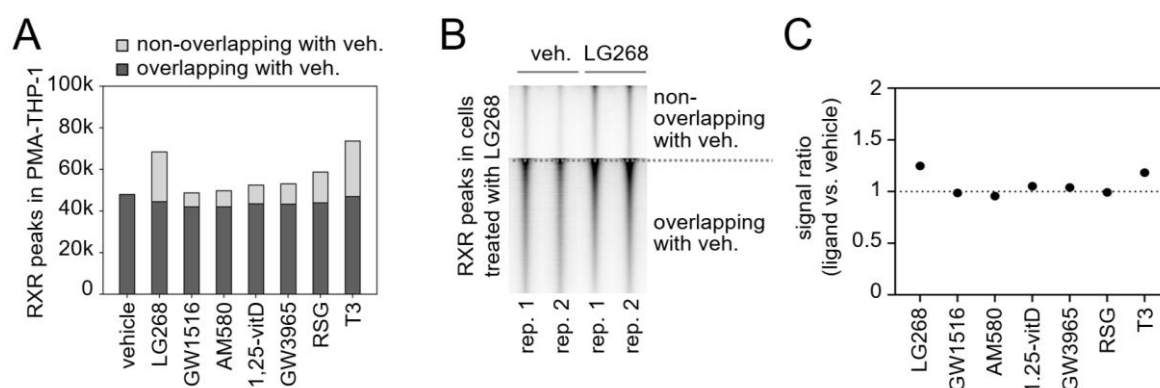


Figure 27. Comparison of RXR binding in vehicle- or ligand-treated PMA-THP-1 cells. (A) The total number of reproducible RXR peaks determined in vehicle- and ligand-treated PMA-THP-1 cells. Regions detected in both vehicle- (veh) and ligand-stimulated samples or uniquely in ligand-stimulated samples are shown in different colors. Cells were treated with vehicle or agonists for 2 h, and RXR ChIP-seq was performed in two biological replicates. *intersectBed* and *mergeBed* (bedtools) were used to retain the reproducible RXR peaks (peaks present in both replicates of each condition). (B) RXR ChIP-seq signals in 2 kb windows are shown in a read distribution plot. Peaks identified in LG268-treated cells are shown. (C) Plot showing RXR ChIP-seq signal ratios. Binding regions identified for each condition were used to quantify the signals, and the ratio of the signals in ligand-stimulated to control was calculated.

6.2.3. Ligand-dependent redistribution of RXR binding is restricted to a subset of genomic regions

Overall RXR occupancy did not indicate robust changes based on signal ratios (Figure 27C); however, RXR may still be redistributed at specific genomic loci, whereby its binding increases or decreases in response to ligand treatment. To investigate this, RXR peaks showing ligand-dependent increases or decreases in occupancy were identified using the DiffBind package (Table 5).

Table 5. The number of RXR binding regions where RXR occupancy changes upon ligand treatment. Percentages in parentheses represent the proportion of total RXR peaks identified in each treatment condition that met the specified filtering criteria.

ligand	RXR peaks FC > 1.5	RXR peaks FC < 0.66	RXR peaks FC > 1.5 and FDR < 0.05	RXR peaks FC < 0.66 and FDR < 0.05
LG268	13,399 (18.0%)	14,929 (20.1%)	8,820 (11.8%)	10,430 (14.0%)
GW1516	1,737 (2.8%)	1,645 (2.6%)	0 (0.0%)	0 (0.0%)
AM580	2,127 (3.3%)	1,227 (1.9%)	325 (0.5%)	0 (0.0%)
1,25-vitD	2,390 (3.8%)	1,987 (3.2%)	802 (1.3%)	55 (0.1%)
GW3965	2,934 (4.5%)	1,671 (2.5%)	9 (0.0%)	0 (0.0%)
RSG	6,789 (10.0%)	2,789 (4.1%)	633 (0.9%)	56 (0.1%)
T3	8,606 (11.2%)	6,566 (8.5%)	1,490 (1.9%)	726 (0.9%)

The RXR ligand, LG268, yielded the highest number of ligand-responsive RXR peaks, thus producing the strongest effect among the tested ligands. Even after applying both FC and FDR thresholds, 8,820 peaks ($FC > 1.5$ and adjusted $p < 0.05$) and 10,430 peaks ($FC < 0.66$ and adjusted $p < 0.05$) were identified. In contrast, ligands targeting RXR partners had more moderate effects, with the RAR α agonist AM580 showing the weakest impact on RXR occupancy. Applying both FC and FDR filtering resulted in few or no significant peaks in many comparisons (Table 5). For subsequent analyses, RXR peak sets were defined based solely on FC thresholds (hereafter termed ligand-induced and ligand-reduced), while other peaks that did not meet either criterion were classified as unresponsive. This FC-only approach was chosen to provide a more complete picture of RXR redistribution and to facilitate the identification of global patterns, motifs, and correlations. Additionally, it enabled classification of peaks into increased, decreased, and unresponsive categories in a more balanced manner. However, because FC-only filtering increases the likelihood of false positives, these peak sets should be considered “exploratory.”

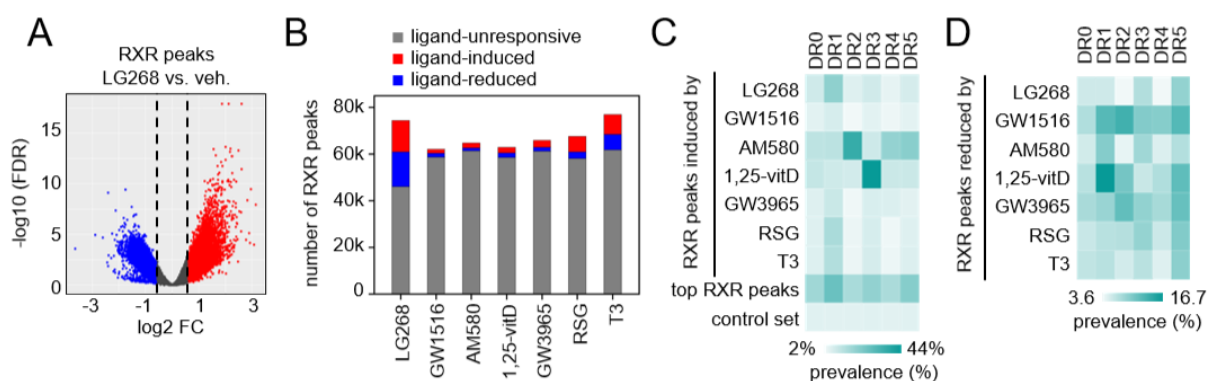


Figure 28. Ligand-dependent changes in RXR genomic binding. (A) RXR binding in PMA-THP-1 cells treated with LG268 and vehicle is compared. Log₂ fold changes in read counts are presented on the x-axis, and statistical significance shown as $-\log_{10} p$ -values is presented on the y-axis. Peaks exhibiting fold changes greater than 1.5 or less than 0.66 relative to vehicle are highlighted in red and blue, respectively. (B) Bar chart indicating the number of ligand-unresponsive, ligand-induced ($FC > 1.5$), or ligand-reduced ($FC < 0.66$) exploratory RXR peaks. (C) Enrichment of DNA motifs within ligand-induced RXR peak sets compared with a background set of randomly selected, size-matched sequences ($n = 5,000$). “Top RXR peaks” correspond to the top 5,000 RXR peaks in vehicle-treated cells after ranking based on occupancy. (D) Enrichment of DNA motifs within ligand-reduced RXR peak sets.

The top RXR peaks in vehicle-treated cells, as well as in ligand-induced and ligand-reduced sets, were analyzed for DNA motif enrichment (Figure 28C-D). In vehicle-treated cells, canonical DR0-DR5 motifs were enriched in the top RXR peaks. Ligand-induced RXR peaks generally reflected heterodimer-specific binding preferences. For instance, 1,25-vitD-induced RXR peaks were enriched for DR3 motifs, consistent with VDR-RXR binding. Peaks induced

by AM580 were enriched for DR1, DR2, and DR5 motifs, consistent with known RAR-RXR preferred motifs. However, unlike previous reports on RAR-RXR motif specificity, DR4 motifs were also enriched in the AM580-induced dataset. Moreover, although DR4 elements are known as the canonical motifs for LXR-RXR and TR-RXR heterodimers, DR4 motifs were only lowly enriched in GW3965- and T3-induced peaks. The reason for this discrepancy remains unclear (Figure 28C). Of note, ligand-reduced RXR peaks were frequently enriched for motifs distinct from the canonical binding motifs of the activated receptor (Figure 28D). This observation suggests that RXR is preferentially recruited to genomic regions bound by activated heterodimers, while being displaced from sites associated with other RXR-containing dimers. This redistribution pattern aligns with previous findings in HEK293 cells, where agonist treatment demonstrated that the ligand-bound NR consistently dominates RXR heterodimer formation ⁶.

We next analyzed ligand-induced RXR peaks in greater detail by comparing those induced by LG268 with peaks induced by ligands for RXR partners. The resulting overlap patterns broadly mirrored the permissive versus non-permissive behavior of RXR heterodimers. For instance, 1,25-vitD-induced RXR peaks showed minimal overlap with those induced by LG268, consistent with the non-permissive behavior of VDR-RXR. In contrast, peaks induced by agonists for permissive heterodimers, such as PPAR γ -RXR, PPAR δ -RXR, and LXR-RXR, showed a high overlap with LG268-induced peaks. Unexpectedly, RXR peaks induced by T3 also overlapped strongly with LG268-induced peaks (Figure 29A).

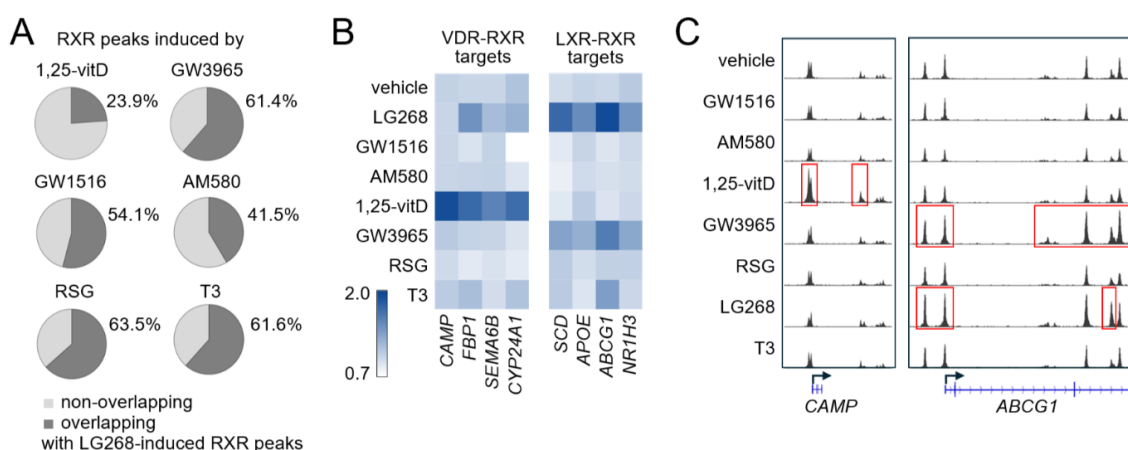


Figure 29. Evaluating the relevance of ligand-induced RXR peaks. (A) Pie charts showing the overlap of RXR peaks induced by ligands for RXR partners with those induced by LG268. (B) Heatmap indicating RXR occupancy (median normalized) within TSS $\pm$ 25 kb of selected target genes for VDR and LXR. (C) IGV snapshots illustrating RXR ChIP-seq signals at representative VDR-RXR (CAMP) and LXR-RXR (ABCG1) target genes under different treatment conditions. Track scales were set to 0-60 for CAMP and 0-50 for ABCG1. Regions highlighted by red boxes correspond to ligand-induced regions (FC > 1.5).

Our results indicated that RXR peaks induced by ligands were frequently located near established target genes of the activated RXR partners. For example, VDR and LXR target genes were often associated with 1,25-vitD- and GW3965-induced RXR peaks, respectively (Figure 29B-C). Notably, consistent with the permissive behavior of LXR-RXR heterodimers, RXR binding near LXR target genes was also enhanced by LG268.

6.2.4. 1,25-vitD-responsive genes are associated with both ligand-induced and ligand-unresponsive RXR peaks

RXR binding regions were characterized in terms of their gene regulatory activity and other genomic characteristics. This analysis focused on genes responsive to stimulation with the VDR ligand 1,25-vitD, and aimed to identify nearby RXR-enriched loci that could participate in their control. 1,25-vitD was selected because the VDR cistrome in PMA-THP-1 cells had already been comprehensively mapped in our first research aim. To carry out this analysis, RNA-seq profiling was conducted in PMA-THP-1 cells following a 6-hour treatment with vehicle or 1,25-vitD, each in three biological replicates (Table 2). In addition, RXR ChIP-seq experiments were repeated using a 1-hour ligand treatment with either vehicle or 1,25-vitD to align with the VDR dataset. To evaluate the transcriptional activity associated with RXR-occupied regions, we examined MED1 ChIP-seq data generated after 2 hours of treatment, as well as H3K27ac ChIP-seq data obtained after 6 hours of exposure to vehicle or 1,25-vitamin D (Table 2).

RNA-seq analysis identified genes significantly upregulated by 1,25-vitD treatment ($FDR < 0.05$, $FC > 1.5$). This yielded 252 protein-coding genes, including several well-established VDR targets (Figure 30A). Earlier studies have demonstrated that validated enhancer-promoter interactions occur most frequently over relatively short genomic distances, with interaction frequency progressively declining beyond this range, particularly above 100 kb ¹¹⁹. To enrich for regulatory regions likely involved in transcriptional control, we assigned RXR binding sites located within ± 25 kb of TSSs. This approach identified 851 ligand-unresponsive, 99 ligand-induced, and 2 ligand-reduced RXR peaks near the 1,25-vitD-upregulated genes (Figure 30B-C).

The identified 1,25-vitD-induced and 1,25-vitD-unresponsive RXR peaks were subsequently compared for their regulatory activity, basal and ligand-responsive VDR binding, and DR3 motif enrichment (the two ligand-reduced RXR peaks were excluded from these analyses). Ligand-induced regions showed stronger increases in VDR, MED1, and H3K27ac signals, and also exhibited higher basal VDR occupancy (Figure 30D-E).

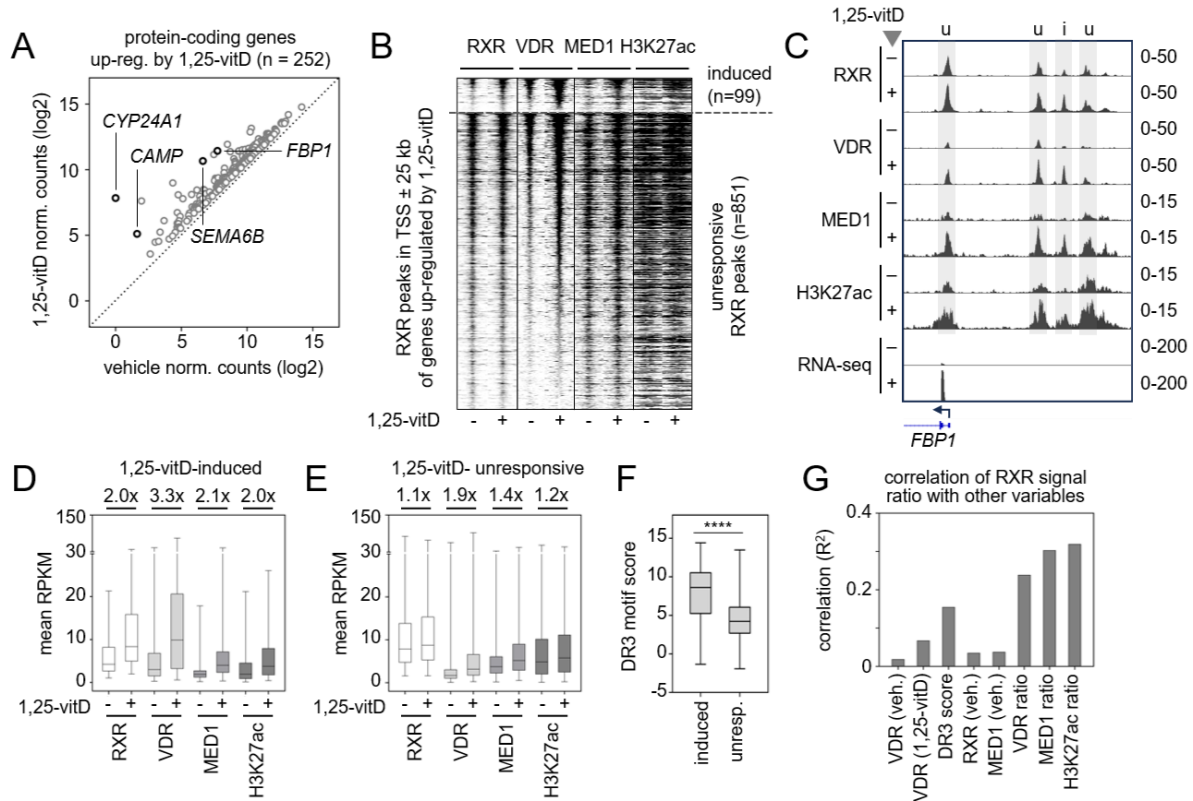


Figure 30. Binding patterns of RXR, VDR, MED1, and H3K27ac within TSS $\pm$ 25 kb of 1,25-vitD-upregulated genes. (A) Normalized mRNA expression in PMA-THP-1 cells following 6 h of 1,25-vitD treatment compared with vehicle control is illustrated in the scatter plot. Selected representative target genes are marked. (B) RD plot showing ChIP-seq signals within a $\pm$ 2 kb region centered on RXR peak summits. RXR peaks located within TSS $\pm$ 25 kb of 1,25-vitD-upregulated genes were categorized as ligand-induced (FC > 1.5, n = 99) or ligand-unresponsive (n = 851). Two ligand-reduced peaks (FC < 0.66) were detected but are not included in the RD plot. (C) IGV snapshot of a representative genomic region displaying normalized ChIP-seq and RNA-seq signal tracks. Ligand-unresponsive and ligand-induced RXR peaks are indicated by “u” and “i,” respectively. (D-E) Box plots comparing ChIP-seq signal intensities across the indicated categories of RXR binding regions located within $\pm$ 25 kb of transcription start sites of 1,25-vitD-upregulated genes. Identical y-axis scales were applied to both ligand-induced and ligand-unresponsive sets to facilitate comparison. Values above the plots represent the ratio of median ChIP-seq signal in 1,25-vitD-treated versus vehicle-treated cells. (F) Box plot comparing DR3 motif scores between ligand-induced and ligand-unresponsive RXR peaks (****, $p < 0.0001$ using an unpaired two-tailed t test; unresp., unresponsive). (G) Bar graph summarizing correlations between ligand responsiveness of RXR peaks and associated genomic features. For each region, the RXR signal ratio between 1,25-vitD and vehicle conditions was determined and correlated with corresponding features.

Notably, ligand-unresponsive RXR regions displayed enhanced VDR binding following ligand treatment, and MED1 and H3K27ac enrichment suggested frequent ligand-dependent enhancer activation at these regions (Figure 30E). In addition, ligand-induced peaks had higher DR3 motif scores, reflecting a higher match to the canonical VDR-RXR binding motif (Figure 30F). Correlation analysis revealed that RXR ligand responsiveness (signal ratio in 1,25-vitD

vs. vehicle) showed a weak correlation with DR3 motif strength as well as VDR, MED1, and H3K27ac signal ratios, but no relationship with other features such as basal VDR or RXR occupancy (Figure 30G). Overall, these findings indicate that both ligand-induced and ligand-unresponsive RXR binding regions contribute to transcriptional regulation, and that changes in VDR and RXR occupancy following ligand stimulation are not necessarily coordinated.

6.2.5. 1,25-vitD differentially affects VDR and RXR occupancy at shared genomic regions

Our previous analysis demonstrated that, following 1,25-vitD treatment, the increase in VDR occupancy exceeded that of RXR at regions located within ± 25 kb of the TSSs of genes upregulated by 1,25-vitD (Figure 30D-E). To assess the magnitude of ligand-induced changes in RXR and VDR binding on a genome-wide scale, we expanded the analysis to include all RXR-VDR co-bound regions in PMA-THP-1 cells. For this purpose, all RXR peaks overlapping with VDR peaks were determined (Figure 31A; $n = 12,329$), and signal ratios comparing 1,25-vitD-treated and vehicle-treated conditions were calculated for both RXR and VDR at these sites. The results showed a consistent shift toward increased VDR occupancy, suggesting that the differential ligand-induced changes between RXR and VDR binding represent a genome-wide effect (Figure 31B).

To determine whether this pattern is also observed in independent datasets, we surveyed published studies that included paired RXR and VDR ChIP-seq data under vehicle and 1,25-vitD treatment conditions. Using the same selection criteria applied for the meta-analysis in Figure 25, four studies conducted in murine cell types were selected¹⁴²⁻¹⁴⁵. Raw sequencing data were downloaded, and signal ratios comparing 1,25-vitD treatment to vehicle control were calculated for RXR and VDR at regions co-occupied by both factors. Although signal ratios varied across the four cell types for both receptors, potentially reflecting species differences (mouse vs. human), receptor expression levels, endogenous ligand availability, or technical variability, a consistent trend was observed. Similar to our finding in PMA-THP-1 cells, the average VDR signal ratio exceeded that of RXR in all reanalyzed datasets (Figure 31C-E). Collectively, these results demonstrate that 1,25-vitD differentially influences VDR and RXR occupancy, suggesting that ligand-induced changes in binding dynamics can vary between heterodimer partners even at the same genomic loci.

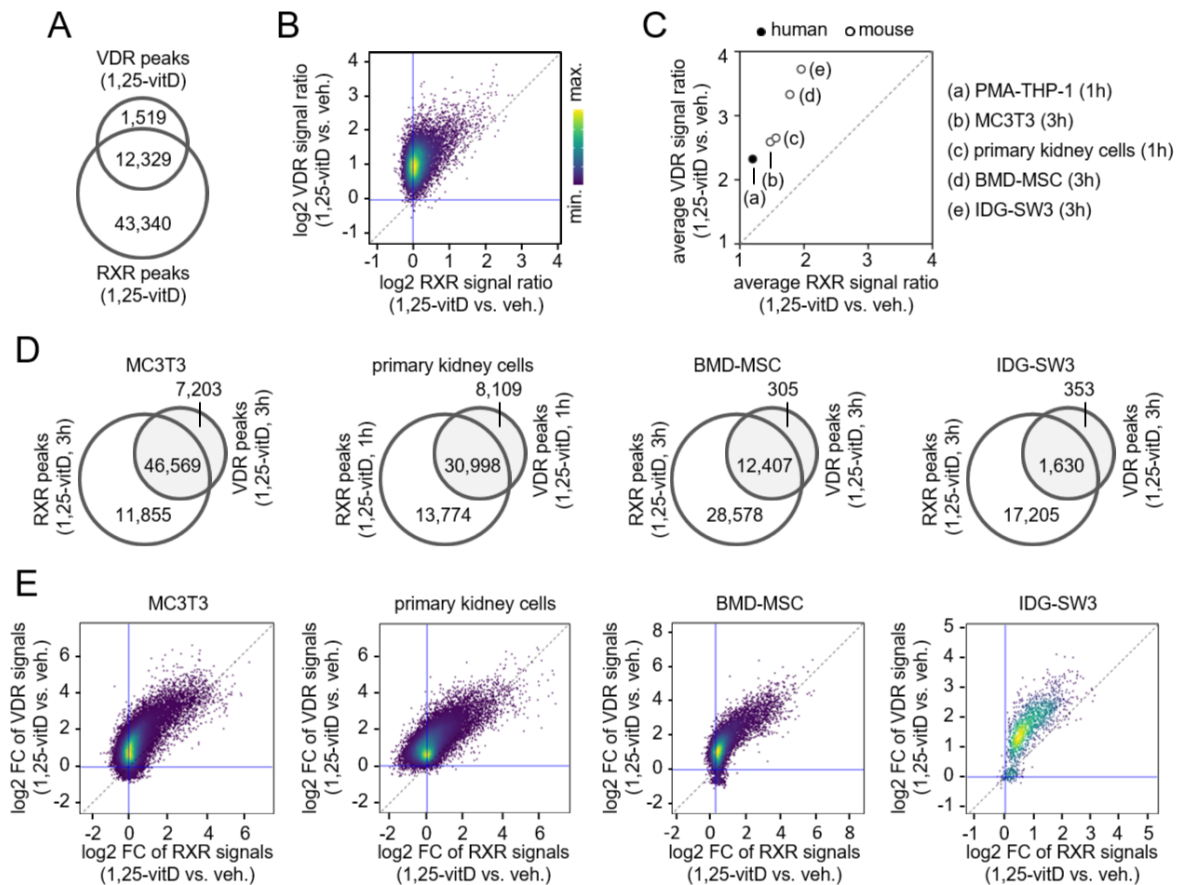


Figure 31. Comparison of ligand-dependent changes in the occupancy of RXR and VDR following 1,25-vitD treatment. (A) Overlap between RXR and VDR ChIP-seq peaks in PMA-THP-1 cells treated with 1,25-vitD for 1 hour. (B) Pseudocolor scatter plot illustrating ligand-induced alterations in RXR and VDR occupancy. For 12,329 regions commonly occupied by both receptors, signal ratios comparing 1,25-vitD-treated and vehicle-treated conditions were determined for RXR and VDR and plotted against one another. (C) Comparison of changes in RXR and VDR binding upon 1,25-vitD treatment across five studies, including the present study (a). For regions commonly occupied by RXR and VDR, signals were measured, and mean signal ratios (1,25-vitD versus vehicle) for each receptor were plotted. Paired RXR and VDR ChIP-seq datasets were obtained from studies on murine cells, including bone marrow-derived mesenchymal stem cells (BMD-MSC), osteoblast precursor cells (MC3T3), primary kidney cells, and the osteocytogenic cell line (IDG-SW3). (D) Venn diagrams showing the overlap of RXR and VDR ChIP-seq peaks in four murine cell types treated with 1,25-vitD. (E) Pseudocolor scatter plots presenting ligand-dependent changes in RXR and VDR occupancy at commonly occupied regions in the same four murine cell types.

6.2.6. Treatments with agonist ‘cocktails’ result in only modest changes in global RXR occupancy

We investigated RXR binding following co-treatment with multiple ligands. Two types of “cocktails” were tested (Table 2): one containing six agonists of RXR partner receptors (“combined six agonists”) and another containing the same six agonists together with LG268 (“combined plus LG268”). In comparison with individual ligand treatments, only modest

increases in overall RXR occupancy were observed when combined cocktails were applied (Figure 32A-B). Changes in RXR distribution were further assessed by identifying peaks with increased or decreased occupancy under combined six agonists or combined plus LG268 treatments relative to single-ligand treatment conditions, using thresholds of >1.5 for increases and <0.667 for decreases (Figure 32C-D). Across all conditions, fewer than 15% of RXR peaks showed altered binding upon treatment with the combined six agonists (Figure 32C). The largest increase and decrease in RXR occupancy were observed at peaks detected under AM580 and 1,25-vitD treatment conditions, respectively. Overall, these findings suggest that simultaneous activation of multiple RXR partner receptors induces only limited global changes in RXR chromatin occupancy, with redistribution occurring at only a restricted subset of binding sites in PMA-THP-1 cells.

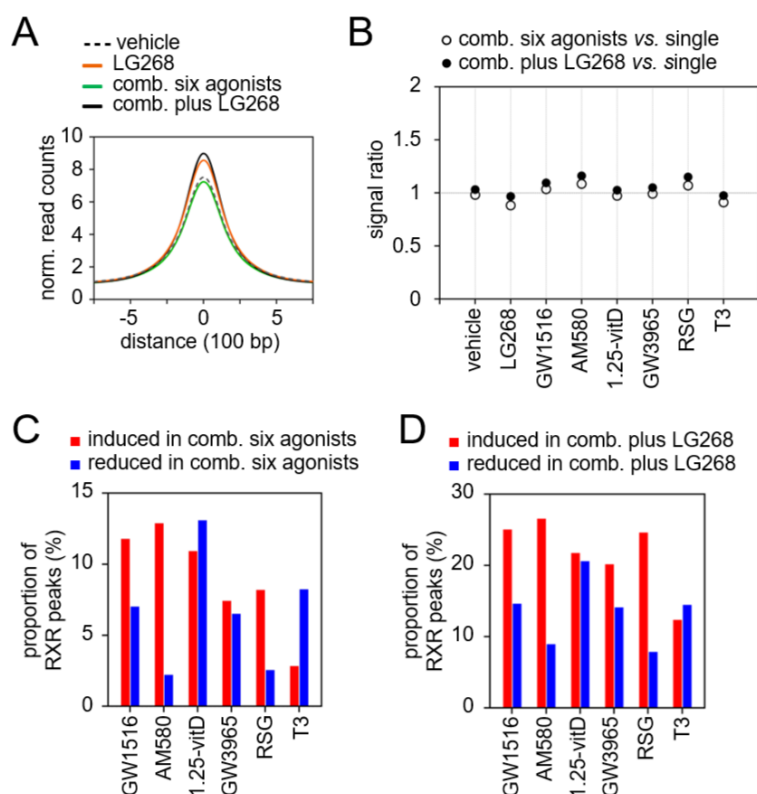


Figure 32. Impact of agonist co-treatment on RXR binding. (A) Normalized read counts within summits ± 750 bp of RXR peaks in PMA-THP-1 cells treated with vehicle or ligand(s). RXR binding was compared between cells treated with all six RXR partner agonists (combined six agonists) or the same six agonists plus LG268 (combined plus LG268) versus vehicle or LG268 alone. (B) RXR signal ratios for the combined six agonists or combined plus LG268 treatments relative to the corresponding single-ligand treatments. For each RXR binding region identified under the different single-agonist treatment conditions, the ratio of signal in the combined six agonists or combined plus LG268 condition to that in the single-ligand condition was calculated. (C-D) Fraction of RXR peak sets showing increased occupancy (RXR signal ratio > 1.5) or decreased occupancy (RXR signal ratio < 0.667) in cells treated with combined six agonists (C) or combined plus LG268 (D) relative to the indicated single-ligand treatments.

6.2.7. Combined agonist treatment minimally impacts RXR binding and gene expression changes in comparison with 1,25-vitD

We investigated the relationship between changes in RXR binding and alterations in gene expression following simultaneous activation of multiple RXR partner receptors. For this analysis, 1,25-vitD treatment was used as a reference to evaluate RXR occupancy and gene regulatory responses under combined ligand conditions.

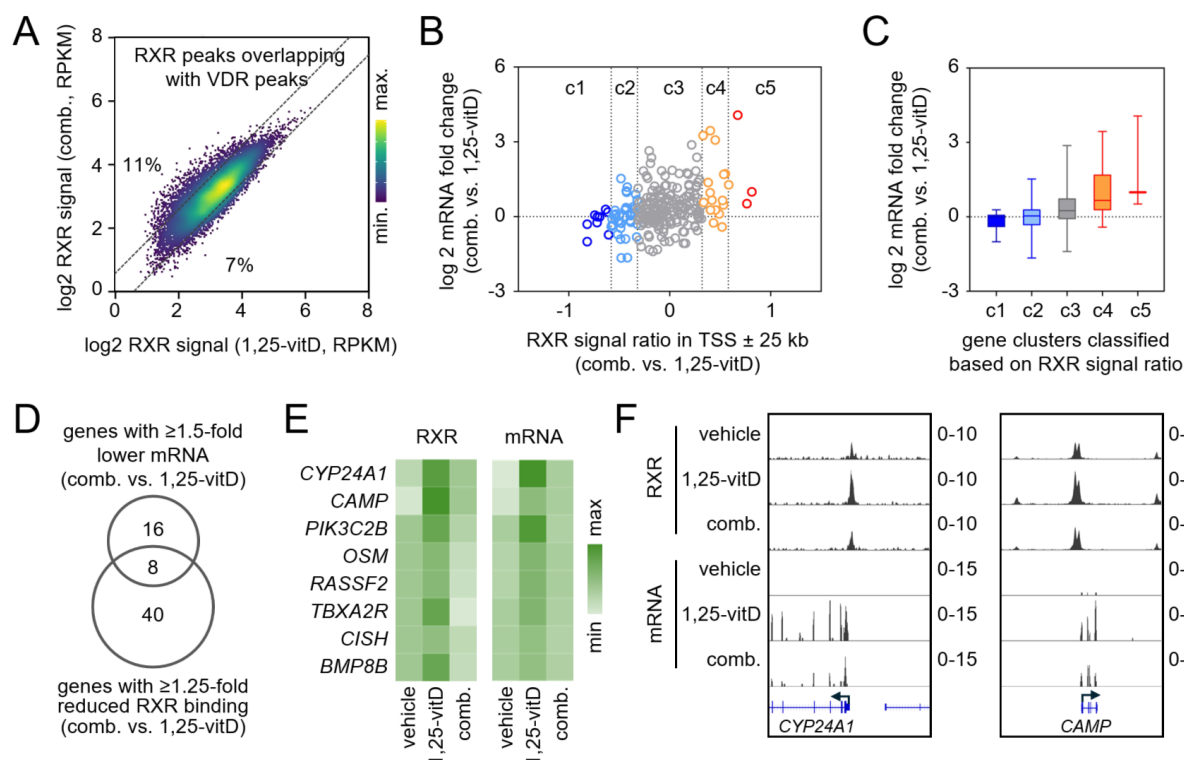


Figure 33. Comparison of the effects of combined six agonist treatment and 1,25-vitD on RXR occupancy and gene expression. (A) Comparison of RXR occupancy in PMA-THP-1 cells treated with the combined six agonists for RXR partner receptors (comb.) versus 1,25-vitD. Only co-occupied RXR-VDR peaks are included. Dotted lines mark RXR signal ratios of 1.5 and 0.667 (combined six agonists vs. 1,25-vitD). (B) Scatter plot showing the relationship between mRNA fold changes (combined six agonists (comb.) vs. 1,25-vitD) and RXR signal ratios (combined six agonists (comb.) vs. 1,25-vitD) within ±25 kb of the TSSs of the associated genes. The analysis includes genes upregulated by 1,25-vitD that were associated with at least one RXR peak within this region (n = 241). Genes were categorized into five different clusters and color-coded based on their RXR signal ratio (R): c1, $R \leq 0.66$; c2, $0.66 < R \leq 0.8$; c3, $0.8 < R \leq 1.25$; c4, $1.25 < R \leq 1.5$; c5, $R > 1.5$. (C) Box plot displaying mRNA fold changes (combined six agonists (comb.) vs. 1,25-vitD) across the five gene clusters (c1-c5). (D) Comparison of two groups of genes upregulated by 1,25-vitD: one group containing genes with at least a 1.5-fold reduction in mRNA expression, and another containing genes with at least a 1.25-fold decrease in RXR occupancy within TSS ±25 kb under combined six agonists (comb.) treatment relative to 1,25-vitD. (E) Heatmaps showing RXR occupancy (TSS ± 25 kb) and mRNA expression for genes common to both subsets identified in panel D (n = 8). (F) IGV snapshots of two representative 1,25-vitD-upregulated genes in which combined treatment resulted in reduced mRNA expression and decreased RXR binding compared with 1,25-vitD alone. Tracks display normalized RXR ChIP-seq and RNA-seq signals within TSS ± 10 kb. Comb., combined six agonists for RXR partners.

RXR occupancy was compared between 1,25-vitD-treated cells and cells treated with six RXR partner agonists (“combined six agonists”) using the RXR-VDR co-occupied peaks. Only a small fraction of RXR peaks showed altered occupancy under combined six agonists treatment relative to 1,25-vitD alone, with 7% showing reduced signals and 11% showing increased signals (Figure 33A). Notably, RXR-VDR binding regions may also be occupied by other RXR heterodimers or RXR homodimers. Therefore, changes in RXR occupancy at these loci likely reflect the combined effects of ligand-induced modulation of multiple RXR-containing dimers, together with possible redistribution of RXR across genomic binding sites.

ChIP-seq and RNA-seq data were integrated for 1,25-vitD-upregulated genes containing at least one RXR peak within TSS $\pm$ 25 kb ($n = 241$) to explore the relationship between RXR binding and transcriptional output. Eleven 1,25-vitD-responsive genes lacking RXR peaks within this interval were excluded from the analysis. Genes were subsequently divided into five clusters based on RXR signal ratio (combined six agonists vs. 1,25-vitD only) (Figure 33B-C). This ratio was obtained by summing RXR occupancy across peaks located within TSS $\pm$ 25 kb under combined treatment or 1,25-vitD treatment conditions. Comparison of mRNA fold changes among the clusters revealed no general reduction in gene expression in clusters with decreased RXR occupancy. In contrast, genes displaying elevated RXR occupancy showed moderately increased mRNA expression following combined six agonists treatment relative to 1,25-vitD alone (Figure 33C).

Finally, the overlap between genes with decreased mRNA expression (≥ 1.5 -fold) and genes with reduced RXR binding in TSS $\pm$ 25 kb (≥ 1.25 -fold) under combined six agonists treatment was examined. Only eight genes were common to both groups (Figure 33D-F). Collectively, these findings indicate that the suppressive effect of combined six agonists treatment on transcription is relatively limited ($\sim 9.9\%$; 24 of 241 genes upregulated by 1,25-vitD), while only a very small subset of genes ($\sim 3.3\%$; 8 genes) exhibited simultaneous reductions in both RXR occupancy and mRNA expression.

7. Discussion

7.1. Convergence and cooperation of retinoid and vitamin D signaling pathways

7.1.1. Common binding regions are involved in convergence between retinoid and vitamin D pathways

Transcriptional networks regulated by NRs often include shared target genes. Multiple molecular mechanisms have been suggested to account for the regulation of an individual gene by agonists acting through two or more NRs (reviewed in ^{25, 146}). One proposed mechanism is: (i) joint regulation by two NRs through their interaction with the same regulatory regions; (ii) regulation through binding to distinct regulatory elements within the same locus; (iii) direct regulation by one NR with indirect regulation by another through tethering mechanisms ¹⁴⁶; and (iv) regulation of the expression of another NR or enzymes responsible for producing endogenous ligands that activate another NR.

Our data showed that shared binding regions were more often associated with commonly upregulated genes than regions exclusively occupied by RAR α or VDR (Figure 11C). Correlation analysis further revealed an enrichment of these common peaks within TSS $\pm$ 25 kb of genes induced by both ligands (Figure 12C). In addition, common regions were located in accessible chromatin regions (Figure 18D), and MED1 recruitment to these regions was frequently induced by combined ligands (Figure 14A). Together, these findings support the first mechanism as a key contributor to the integration of signaling pathways led by retinoid and vitamin D.

7.1.2. RAR α and VDR binding to the DNA is not necessarily coupled with ligand-induced cofactor recruitment and gene regulation

Although our data suggest that shared RAR α and VDR binding regions contribute to the integration of retinoid and vitamin D signaling pathways, a large proportion of these regions, as well as binding regions specific to RAR α or VDR, are unlikely to participate in transcriptional regulation via cofactor recruitment. We observed that the majority of RAR α and VDR binding regions were connected to genes that did not show ligand-dependent transcriptional activation (Figure 11C). Moreover, ligand treatment failed to induce MED1 recruitment at many of these sites (Figure 13B-C and 14A). At shared binding regions, three distinct patterns were identified: MED1 binding was stimulated by both ligands, by only one ligand, or by neither ligand. For instance, both ligands promoted MED1 recruitment and enhanced mRNA expression at common regions located near *FBP1* and *HBEGF*. By contrast, although both receptors occupied the shared binding region near *PTGES* following ligand

treatment, MED1 recruitment was induced only by AM580 (Figures 10, 12D, and 16B). Together with previous findings reported for NRs and other TFs, these results suggest that only a fraction of receptor binding events are associated with ligand-dependent cofactor recruitment and transcriptional regulation.

Multiple factors have been proposed to enable DNA-bound TFs to recruit cofactors and regulate transcription. (i) Physical interaction with the promoter is needed for DNA-bound TFs to act as transcriptional regulators. This interaction is typically mediated by chromatin loops and higher-order chromosomal architecture ¹⁴⁷. (ii) TF binding affinity also contributes to gene-activating capacity, as stronger DNA binding has been suggested to be associated with higher gene-activating potential ⁴⁶. (iii) The DNA sequence of motif, surrounding nucleotides, and overall motif organization can induce allosteric changes in TF conformation, thereby contributing to different regulatory outcomes ¹⁴⁸. (iv) TFs function in concert with other TFs and cofactors; therefore, the gene regulatory capacity of a given TF is also affected by the availability of its co-binding partners ¹⁴⁹. (v) TF activity is further influenced by factors such as strand orientation, the positional arrangement of binding sites, the DNA helical face, and the surrounding chromatin environment ¹⁵⁰. These features act together in a combinatorial and interdependent manner to determine whether a TF, including RAR α or VDR, is capable of recruiting cofactors and regulating gene expression at a particular genomic locus.

7.1.3. A great fraction of RAR α and VDR binding regions lack “strong” REs, resulting in limited co-occurrence of motif within common binding regions

Our analysis showed that fewer than 10% of shared binding regions harbored both categories of “strong” motifs (DR1/2/5 for RAR α and DR3 for VDR) (Figure 22C-D). This observation is in line with earlier reports on LXR and PPAR α ⁵⁰ and with our re-analysis of publicly available ChIP-seq datasets (Figure 24). These findings imply that the relatively low occurrence of overlapping motifs mainly arises because a substantial fraction of RAR α and VDR binding regions lack “strong” REs.

At present, no standard method is available for motif mapping or for clearly discriminating between “strong” and “weak” (degenerate) motifs. The enrichment of a particular motif within a TF cistrome is commonly evaluated using three different strategies: PWM-based motif scanning, de novo motif identification, and direct searches for defined DNA sequences. As outlined in our previous study ¹¹³, each of these approaches has specific benefits and drawbacks. In the current study, we employed a comparatively stringent form of the first strategy, selecting

a threshold corresponding to 5% positive matches in the control dataset. Applying stringent criteria is important for lowering background noise and minimizing false-positive results.

Several findings support the reliability of our motif mapping strategy for the balanced detection of putative RAREs and VDREs. First, the approach successfully identified previously described REs linked to *RARB*, *FBP1*, *CD274*, *CAMP*, and *CD38* (Figure 21C). Second, the distribution of motifs within the RAR α and VDR cistromes (Figure 21A-B) was consistent with the binding preferences obtained from high-throughput SELEX analyses⁷¹. Third, the ERE-IR3 motif was found in approximately 55% of ER cistromes in engineered mouse mammary cells (Figure 24)¹⁴⁰, indicating that our method is capable of detecting the majority of motifs present in a ChIP-seq dataset. Lastly, earlier investigations of RAR and VDR cistromes have also shown that more than half of the binding regions lack cognate motifs. For instance, only ~6% and ~40% of RAR cistromes in mouse embryonic stem cells and embryonal carcinoma cells were enriched with DR5 motif, respectively, and it was not reported as the most enriched RARE in those studies^{60, 70}. In our study, DR5 was the most enriched RARE, detected in 19.7% of RAR α binding regions in PMA-THP-1 cells (Figure 21A). Because RAR-RXR can bind to DR5 and other REs^{60, 70, 71, 151}, we also included DR1 and DR2 as putative RAREs. Similarly, the frequency of DR1/2/5 in mouse F9 embryonal carcinoma cells was reported in a previous study⁶⁰; the lower frequency observed in our study (40.2% vs. 74.3%, Figure 21B) is likely attributable to the use of a more stringent motif mapping approach and the application of an anti-panRAR antibody in the earlier work. For VDR, the DR3 motif, the canonical VDRE, was present in 29.7% of binding regions in our study (Figure 21A-B), compared with ~42% and ~48% in LPS-treated and undifferentiated THP-1 cells, respectively^{78, 79}, and ~37% in monocyte-derived dendritic cells⁸⁰. Together, these data highlight that a substantial portion of RAR α and VDR cistromes lack the canonical DR5 or DR3 motifs.

Several mechanisms may account for the absence of “strong” motifs within NR cistromes (reviewed in^{146, 152}). First, NRs are capable of binding “weak” or degenerate motifs, and chromatin accessibility appears to play a major role in determining binding efficiency; the same motif may act as a high- or low-affinity site depending on whether it is located in open or closed chromatin. Second, many TFs can recognize and act through a broad spectrum of REs^{71, 152}. While we considered DR1/2/5 as RAREs, RAR α -RXR may also bind other motifs, as observed in our study (Figure 21A) and in previous studies. Likewise, VDR-RXR can interact with additional REs such as DR2, DR4, and even DR5 in transcriptionally inactive forms^{5, 71}. Third, protein-binding microarray studies have shown that NRs may bind half-site motifs¹⁵¹. Fourth, DNA binding of NRs may occur indirectly through protein-protein interactions, via tethering

to other NRs or TFs ^{146, 152}. Collectively, these mechanisms likely account for many RAR α and VDR binding events at regions lacking “strong” motifs, although their contributions cannot be quantified without TF mapping with high resolution.

7.1.4. Both shared and non-overlapping DNA motifs were found in the regions commonly occupied by RAR α and VDR

Without TF mapping with high resolution, it is not possible to accurately determine the precise regions where TFs interact with DNA. To our knowledge, ChIP-exo has not yet been applied to the analysis of shared NR binding regions, and implementing this approach was beyond our scope. Nevertheless, detection of cognate DNA motifs can help determine whether receptors mainly bind separate DNA sequences or occupy shared binding sites. To address this, we calculated the distances between DR3 motifs and the closest DR1/2/5 motifs and used these values to estimate the proportions of three motif arrangements within common binding regions. This analysis revealed that about half of DR3 motifs (52%) did not overlap with DR1/2/5 motifs, while the remaining DR3 motifs showed either substantial overlap (14%) or partial overlap (34%) (Figure 23C). The primary distinction between the two overlapping configurations is that, in partially overlapping motifs, only one half-site is shared between the two heterodimers.

Shared DNA motifs in murine primary liver cells were also investigated by Boergesen et al. using a different bioinformatic approach that relied on calculating distances between the centers of peaks ⁵⁰. They found that the mean distances between the centers of LXR and PPAR α peaks were very similar to those measured between the centers of LXR-RXR and PPAR α -RXR peaks, with highly comparable distribution profiles. Based on these results, they proposed that LXR-RXR and PPAR α -RXR mainly occupy the same or overlapping degenerate REs at these genomic regions. In addition, analysis of binding regions for other NRs, such as HNF4 α , FXR, and NR1D1, identified NR “hot spots”, where multiple receptors bind and frequently share degenerate response elements ⁵⁰. Our study and aforementioned study highlight the relevance of shared motifs in promoting convergence between NR signaling pathways, although the frequency of overlapping motifs detected in our analysis is different.

7.1.5. Cooperation and competition of NRs at common REs containing both types of motifs

The observation that different NRs can bind common regions with shared motifs following treatment with combined ligand brings up the question of whether these receptors compete for

occupancy at the same sites or instead co-occupy these sites without competition. Multiple live-cell imaging studies have revealed that TF binding is highly dynamic, with TFs generally interacting transiently with their REs in DNA ¹⁵³.

Voss et al. used GFP-tagged GR and mCherry-tagged ER pBox, a modified ER engineered to recognize the same RE as GR, together with fluorescence microscopy and additional complementary approaches ³². They showed that GR and ER pBox do not strongly compete for the same binding sites. Instead, GR enhanced or “assisted” ER pBox binding at many genomic loci, even when both receptors targeted the same elements. Taken together, these findings suggest that two activated NRs can occupy shared REs without substantial competition.

Our findings suggest that shared binding regions contribute to the interaction and cooperation between retinoid and vitamin D signaling. However, exposure to combined ligands did not uniformly lead to cooperative responses, even at these common sites. Thus, the outcome of simultaneous NR activation at shared genomic regions appears to depend on the local regulatory context and may be shaped by factors such as chromatin accessibility, recruitment of additional TFs, motif strength, and the spatial organization of co-existing DNA motifs. At common binding regions, cooperative activity between NRs is likely driven by increased cofactor recruitment, remodeling of chromatin structure, changes in histone modifications, and more efficient stimulation of the basal transcriptional machinery. Overall, accumulating evidence from genome-wide studies demonstrates that overlapping NR occupancy and shared transcriptional programs are widespread. Our work adds to this understanding by showing how NRs with different RE preferences can control highly overlapping sets of regulatory regions and target genes.

7.2. RXR has a non-limiting role in macrophage-like cells stimulated with multiple NR agonists

7.2.1. ChIP-seq-based analysis of ligand-induced changes in RXR chromatin occupancy

A number of ChIP-seq studies have examined genome-wide RXR occupancy in diverse cellular contexts after activation of RXR itself or its heterodimeric partners. We systematically reviewed all available datasets; however, only one RXR ChIP-seq dataset satisfied the predefined criteria for inclusion in the meta-analysis ⁵⁹. The other datasets were excluded because they lacked appropriate control conditions, involved ligand exposure for more than 24 hours, or failed to meet other selection requirements. In the present work, RXR chromatin binding was analyzed under four defined conditions. Other possible treatments, such as RXR inverse agonist exposure, 9-cis-RA stimulation, or additional ligand combinations, were not

assessed. The analyzed conditions included: (i) vehicle-treated cells without added ligands, (ii) cells treated with the RXR agonist LG268, (iii) cells exposed to agonists of RXR partner receptors, and (iv) cells treated with multi-agonist “cocktails.” The RXR occupancy patterns obtained under each of these conditions are summarized in the following sections.

7.2.2. RXR occupancy in the absence of stimulation with exogenous agonists

In vehicle-treated cells, 48,260 reproducible RXR peaks were identified in both replicates (Figure 27A), which, to our knowledge, represents the highest number of RXR peaks reported so far for any cell type. Motif analysis showed enrichment for DR0-DR5-type motifs, suggesting DNA occupancy by multiple RXR-containing heterodimers (Figure 28C). Although the vehicle condition did not include any added agonists, endogenous ligands may have been present at low concentrations. Earlier *in vitro* studies demonstrated that RXR can recognize specific DNA sequences through its DNA-binding domain without ligand supplementation^{151, 154}, supporting the idea that DNA binding is an intrinsic feature of the receptor. However, in the cellular environment, TFs do not bind all potential DNA motifs. Their occupancy is highly dependent on local chromatin accessibility and protein-protein interactions with dimerization partners, cofactors, and other TFs. This may explain why RXR was found at only a fraction of canonical DR0-DR5 motifs.

7.2.3. Impact of the RXR agonist on RXR occupancy

Among all ligands tested in PMA-THP-1 cells, LG268 produced the most pronounced changes in RXR chromatin occupancy (Figure 27A and C). This result agrees with previous findings in mouse BMDMs, where LG268 caused stronger alterations in RXR binding than RSG or GW3965⁵⁹. Consistently, live-cell FCS experiments in HeLa cells expressing EGFP-RXR showed that RXR ligands, including LG268 and 9-cis-RA, increased the proportion of RXR molecules associated with chromatin, whereas ligands for partner NRs, such as AM580 or RSG, did not³⁰. The effects of LG268 on RXR occupancy across genomic regions may be explained by several mechanisms that are not mutually exclusive. These include: (i) increased intrinsic affinity for DNA due to ligand-induced conformational changes; (ii) dissociation of RXR tetramers upon ligand binding, thereby increasing the pool of dimers capable of DNA binding¹⁵⁵; (iii) enhanced RXR dimer formation and longer residence time on chromatin; and (iv) recruitment of cofactors that stabilize RXR-DNA interactions.

Motif analysis of RXR peaks induced by LG268 showed an approximately twofold enrichment of DR1-type direct repeats relative to other motif categories, although this

preference for DR1 was less apparent when only the strongest RXR peaks were considered (Figure 28C). These observations indicate that LG268 remodels the RXR binding landscape by increasing its association with DR1-containing regions. This redistribution is likely related to enhanced formation of RXR homodimers and/or heterodimers that preferentially bind DR1 elements. The non-uniform redistribution of RXR across the genome after LG268 treatment further supports the idea that increased dimerization and cofactor recruitment contribute substantially to the observed changes in RXR occupancy.

7.2.4. Impact of partner agonists on RXR occupancy

Changes in RXR chromatin occupancy after treatment with partner receptor agonists should be interpreted as indirect effects, mediated through ligand-dependent activation of the corresponding partner receptor and its subsequent impact on RXR. Therefore, evaluating how partner agonists influence RXR binding requires consideration of two key aspects: first, how each ligand alters DNA binding of its cognate receptor; and second, how ligand-induced changes in partner receptor occupancy are connected to changes in RXR binding.

Regarding the first point, our meta-analysis indicated that ligand treatment usually produces only minor changes in the global occupancy of RXR. VDR was the main exception, showing the most pronounced genome-wide increase in binding (Figures 25B and 30B). For other RXR partner receptors, only a small number of datasets fulfilled the selection criteria. Among the eligible RAR ChIP-seq studies ($n = 4$), only the dataset from our previous work showed a slight overall increase in RAR α occupancy, with an occupancy ratio of 1.15 (Figure 25B-C and Figure 7E). Previous reports describing broad RAR cistrome remodeling in stem cells may therefore reflect not only direct ligand-dependent effects on receptor binding, but also wider transcriptional and chromatin alterations linked to RA-induced differentiation⁶⁰.

The influence of ligands on DNA binding remains debated. For example, EMSA-based studies have produced conflicting results, reporting either ligand-stimulated RAR binding¹⁵⁶ or RAR binding that occurs independently of ligand exposure^{139, 157}. By contrast, live-cell FRET and FCS analyses showed ligand-dependent enhancement of both DNA binding and RAR-RXR dimer formation^{7, 29, 31}. However, single-molecule tracking did not reveal ligand-induced changes in RAR-RXR DNA binding¹⁵⁸, and our RAR α ChIP-seq analyses similarly revealed only modest increases in global RAR α binding (Figure 7E). Several mechanisms have been proposed to explain ligand-dependent increases in receptor binding to DNA, including stronger intrinsic DNA-binding affinity, ligand-promoted dimerization, and/or cofactor recruitment that stabilizes receptor-DNA interactions^{6, 7, 27, 30, 159}. In the case of VDR, ligand-

induced nuclear translocation has also been described, similar to the mechanism reported for GR, and this may contribute to increased DNA binding ^{6, 7}. Whether this mechanism fully explains the pronounced ligand-dependent increase in VDR occupancy observed in ChIP-seq datasets, including our own (Figures 25B, 30B and 7F), remains unclear.

Regarding the second point, the influence of a partner ligand on RXR would not be expected to be greater than its effect on the ligand's cognate receptor. However, the relationship between ligand-dependent changes in RXR DNA binding and those of its dimerization partner has been examined only to a limited extent. Even when ChIP-seq datasets are available for both RXR and its partner receptor after stimulation with the same ligand, direct side-by-side comparisons have generally not been performed. To address this issue, we compared the impact of 1,25-vitD on RXR and VDR DNA binding. This analysis showed a marked ligand-induced increase in VDR occupancy, whereas the increase in RXR binding at the corresponding regions was substantially weaker (Figure 31B). This pattern was reproducibly detected across several independent mouse cell datasets (Figure 31C-E). These results suggest that ligand-dependent enhancement of VDR binding is not matched by comparable increases in RXR occupancy. Rather, our findings indicate that the DNA residence times of VDR and RXR may not be closely linked, allowing ligand-induced alterations in binding dynamics to differ between the two dimerization partners. Nevertheless, RXR occupancy remained responsive to 1,25-vitD treatment (Figure 30B), possibly through mechanisms such as increased heterodimer formation and enhanced coactivator recruitment.

7.2.5. Impact of agonist “cocktails” on RXR occupancy

In the present study, co-activation of multiple RXR partner pathways produced only limited alterations in RXR chromatin occupancy (Figure 32B-C). This suggests that, in macrophage-like cells, the available RXR pool is sufficient to support binding with several partner receptors simultaneously. Macrophages are specialized immune cells capable of detecting and integrating a wide range of environmental and metabolic cues, partly through the expression of diverse receptors, including NRs ²⁵. Although THP-1 cells express multiple NRs, RXR mRNA levels are higher than those of its partner receptors, closely resembling the pattern observed in primary macrophages (Figure 26A-B). This suggests that RXR is present in stoichiometric excess relative to its partners in these cells, consistent with earlier observations in U2OS cells ¹⁵⁸. Whether RXR becomes limiting in other cellular contexts characterized by lower RXR expression or higher levels of partner receptors remains an open question.

Our results show that only ~3.3% of protein-coding genes induced by 1,25-vitD exhibited both reduced RXR occupancy and lower transcript levels under combined agonist treatment compared with 1,25-vitD alone (Figure 33D). Moreover, these genes generally showed modest fold changes. Notable exceptions were *CAMP* and *CYP24A1*, two well-characterized 1,25-vitD-responsive genes that display minimal expression in vehicle-treated cells (Figure 30A). Overall, these observations do not support RXR sequestration as the main explanation for changes in 1,25-vitD-responsive gene expression during simultaneous activation of multiple NR pathways. Rather, other forms of NR cross-talk, including competition for DNA REs, limited availability of cofactors, or indirect repression mediated through signaling pathways, are more likely to account for these effects ¹⁴⁶.

From a systems perspective, sharing components among signaling pathways can render networks susceptible to interference when multiple inputs are activated simultaneously, as shared modules may become limiting. Importantly, this principle extends beyond RXR to other transcriptional components, including cofactors and RNA polymerase II. Our data indicate that, in macrophage-like cells, RXR acts as a shared but non-limiting factor across multiple pathways. Even under conditions where numerous RXR-dependent pathways are simultaneously activated, interference observed at the transcriptional level is largely independent of RXR availability.

7.3. Future research directions

Several aspects of the molecular mechanisms underlying the convergence and cooperation of RAR and VDR signaling pathways remain to be further investigated. Exploring higher-order chromatin organization using approaches such as Hi-C or Capture-C would be insightful to identify specific promoter-enhancer contacts that underlie the cooperation and convergence of the two pathways, and to identify functional chromatin loops within which co-regulated gene clusters reside. In addition, functional validation of candidate regulatory elements will clarify their contribution to the convergence and cooperation of the two pathways. This could be achieved by perturbing specific regulatory elements or motifs using CRISPR-based approaches.

Given that only a small fraction of commonly occupied RAR α and VDR binding regions contain canonical REs for both receptors, the mechanisms of co-binding at these regions remain to be further investigated. Whether both receptors bind simultaneously to promiscuous REs or bind through tethering (via protein-protein interactions) could be further explored using approaches such as co-immunoprecipitation and high-resolution chromatin mapping techniques. Furthermore, the convergence of the two pathways in the context of transcriptional

repression remains largely unexplored. It will be important to determine whether downregulated genes are subject to similar cooperative or synergistic regulatory mechanisms as those observed for induced genes. Finally, time-resolved analyses will be helpful in capturing the dynamics of cooperative enhancer activation and gene regulation beyond the early time points examined in this study.

Our findings suggesting a non-limiting role for RXR in macrophages raise several questions to be addressed in future studies. The possibility that other shared components, such as cofactors or elements of the transcriptional machinery, represent limiting factors driving competition could be investigated using the same experimental framework applied here for RXR. Moreover, future studies integrating ChIP-seq analyses of RXR partner receptors will be important to determine whether RXR redistributes among different dimerization partners at the same regulatory elements upon combined ligand treatment. Whether RXR availability becomes limiting in other cellular contexts with lower RXR expression or higher levels of partner receptors also remains to be elucidated. Experimental approaches to artificially reduce the RXR level in the cells (RXR knockdown) may help to clarify whether RXR is present in stoichiometric excess and how this influences NR cross-talk in macrophages.

8. Summary

NRs are ligand-dependent TFs that regulate gene expression by binding to genomic regulatory elements and coordinating chromatin and transcriptional responses. RARs and VDR are members of this superfamily and mediate the biological effects of retinoids and vitamin D, respectively. These receptors, together with many other NRs, primarily function as heterodimers with RXR, giving RXR a central role in NR signaling. The general aim of current research was to investigate mechanisms of NR crosstalk at the genomic level.

Previous studies have indicated that retinoid and vitamin D signaling pathways are distinct yet overlapping, and that certain regulatory regions are occupied by both RARs and VDR receptors. This motivated us to characterize genomic regions occupied by both RAR α and VDR and to assess their role in convergence and cooperation between RAR α - and VDR-mediated pathways in macrophage-like cells as our first aim. Given the role of RXR as a core heterodimerization partner for RAR, VDR, and many other NRs, concurrent activation of RXR and its partner NRs could theoretically lead to competition for RXR and attenuated transcriptional responses. Although this concept has been explored previously with different methods, a comprehensive genome-wide assessment of RXR binding in a native chromatin context has been lacking and constituted our second aim. To address these questions, genome-

wide analyses of receptor binding, chromatin accessibility, and transcriptional changes, as well as validation experiments, were performed in macrophage-like cells derived from the THP-1 cell line treated with specific ligands, and complemented by meta-analyses of published datasets.

Our results with respect to the first aim can be summarized as follows. (i) The cistromes of ligand-activated RAR α and VDR overlapped extensively, with common binding regions representing a large proportion of both the RAR α and VDR cistromes. (ii) Treatment with RAR α and VDR agonists led to the co-regulation of several genes, frequently in a cooperative manner when both RAR α and VDR ligands were applied. (iii) Genes upregulated by both agonists were often associated with common binding regions. (iv) Common RAR α and VDR binding regions were typically localized within highly accessible chromatin. (v) These common binding regions were frequently, though not exclusively, involved in cooperative regulation between retinoid and vitamin D pathways. (vi) DNA response elements for RAR α and VDR were enriched in common binding regions; however, co-occurrence of both motif types was rare, occurring in less than 10% of these regions. (vii) Re-analysis of publicly available ChIP-seq datasets indicated that extensive sharing of binding regions among NRs is a general phenomenon. Collectively, these results support the conclusion that shared RAR and VDR binding regions have a significant regulatory role in convergence and cooperation between retinoid and vitamin D signaling pathways.

Our results with respect to the second aim can be summarized as follows. (i) In the absence of ligands, RXR occupied thousands of genomic regions in the form of various RXR heterodimers, indicating that DNA binding is an intrinsic property of RXR-containing complexes. (ii) Ligand treatment altered RXR occupancy at a subset of regions, with the RXR agonist LG268 inducing a stronger increase in RXR binding than agonists of partner receptors or their combination. (iii) Ligand-induced RXR redistribution occurred at a limited but functionally relevant subset of genomic regions, with motif enrichment analyses suggesting recruitment of RXR to regions occupied by activated RXR heterodimers and withdrawal from regions occupied by other RXR dimers. (iv) Meta-analyses of public datasets and our own results revealed that ligand treatment induced only minor changes in the global occupancy of RXR partner receptors, with VDR being an exception, as it exhibited the strongest overall increase in binding upon calcitriol treatment. (v) Although calcitriol markedly enhanced VDR binding, the corresponding increase in RXR occupancy was comparatively modest. (vi) Both ligand-induced and ligand-unresponsive RXR binding regions contributed to gene regulation. (vii) The overall RXR binding exhibited only moderate changes under combined ligand

treatment compared with the single-ligand stimulations. (viii) Only a small fraction of calcitriol-regulated genes showed a concurrent reduction in RXR binding and decreased mRNA levels under combined agonist treatment. Together, these results support a model in which RXR functions as a non-limiting module in macrophage-like cells, and interference between NR pathways is minimally attributable to RXR sequestration. Instead, alternative mechanisms of NR crosstalk, such as competition for DNA REs, limiting cofactors, or indirect repression via signaling pathways, are likely responsible.

9. References

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10. Keywords

vitamin D receptor (VDR), retinoic acid receptor (RAR), retinoid X receptor (RXR), nuclear receptor, transcription factor, ChIP-seq, binding region mapping, transcriptomics, bioinformatics, macrophage-like cells, nuclear receptor crosstalk, DNA binding motif, genomic signaling pathway, interference, convergence, cooperation

11. List of publications



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Candidate: Hamidreza Mianesaz

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Mianesaz, H.**, Göczi, L., Bojcsuk, D., Ghalamkari, S., Fadel, L., Póliska, S., Penyige, A., Nagy, L., Nagy, G., Vámosi, G., Széles, L.: Genome-wide chromatin profiling reveals a non-limiting role for RXR in macrophage-like cells stimulated with multiple nuclear receptor agonists. *J. Biol Chem.* "Accepted by Publisher", 2026.
IF: 3.9 (2024)
2. **Mianesaz, H.**, Göczi, L., Nagy, G., Póliska, S., Fadel, L., Bojcsuk, D., Penyige, A., Szirá, K., AlHaman, F., Nagy, L., Vámosi, G., Széles, L.: Genomic regions occupied by both RAR α and VDR are involved in the convergence and cooperation of retinoid and vitamin D signaling pathways. *Nucleic Acids Res.* 53 (6), 1-20, 2025.
DOI: <http://dx.doi.org/10.1093/nar/gkaf230>
IF: 13.1 (2024)

List of other publications

3. **Mianesaz, H.**, Ghalamkari, S., Abbasi, F., Razzaghy-Azar, M., Sayarifard, F., Vakili, R., Sedghi, M., Noroozi Asl, S., Hosseini, S., Amoli, M. M., Yaghootkar, H.: Genetic variant profiling of neonatal diabetes mellitus in Iranian patients: unveiling 58 distinct variants in 14 genes. *J of Diabetes Invest.* 15 (10), 1390-1402, 2024.
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