

SHORT THESIS 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**

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1. Introduction

Nuclear receptors (NRs) are a structurally related superfamily of proteins involved in responses to hormonal, metabolic, and environmental signals. In humans, the NR superfamily comprises 48 members involved in distinct regulatory pathways. Ligands are small signaling molecule that bind directly to NRs and regulate their activity. Well-established endogenous ligands have been identified for approximately half of the NR members in humans (1, 2). NRs mainly function as ligand-dependent transcription factors (TFs) that regulate gene expression through binding to specific genomic regulatory elements. NR activation by their agonists facilitates their dimerization, binding to specific DNA response elements (REs), and the recruitment of co-regulatory complexes. The recruited Mediator complex and/or other coactivators increase local histone acetylation, chromatin openness, and the activation of transcriptional machinery at the promoter of the regulated genes. REs recognized by NRs typically consist of repeated DNA half-sites arranged as inverted repeats (IRs) or direct repeats (DRs), separated by spacer sequences of any nucleotide with variable lengths (2-4).

Retinoic acid receptors (RARs) and the vitamin D receptor (VDR) are members of NR superfamily and mediate the biological actions of retinoids (naturally occurring forms of vitamin A and synthetic analogs of retinol) and vitamin D, respectively. 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 (5-7). 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 gene regulatory regions are occupied by both receptors support the idea that cross-talk between these pathways may occur at the chromatin level. However, the prevalence, genomic features, and functional relevance of such common binding regions remain poorly defined.

RARs, VDR and many other NRs primarily function by forming heterodimers with the retinoid X receptors (RXRs). Therefore, RXRs serve as a central component of multiple NR signaling pathways. In addition to forming heterodimers with other NRs, RXRs can also form and function through homodimers (1, 8). RXRs can be activated by endogenous ligands such as 9-cis RA, as well as by synthetic agonists (9). “Permissive” RXR heterodimers including LXR-RXR, PPAR-RXR, FXR-RXR, and PXR-RXR can be activated solely by RXR ligand, independent of ligand binding by the partner receptor. 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 (10). 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 in different cellular models (11, 12), a comprehensive systematic genome-wide assessment of RXR genomic binding and transcriptional outcomes under simultaneous pathway activation has not yet been addressed.

Macrophages are immune cells that detect and respond to environmental and metabolic signals via receptors recognizing NR ligands, pathogens, and cytokines. NRs such as ER, GR, LXR, PPARs, RARs, and VDR play important roles in regulating macrophage functions, including lipid metabolism, inflammation, phagocytosis, and differentiation. These receptors also influence cellular processes such as cholesterol handling, cell mobility, energy use, and antimicrobial activity through interconnected signaling pathways, where activation of one NR can affect others (8). The human monocytic cell line THP-1 serves as a useful model for studying monocyte and macrophage biology, as it can differentiate into macrophage-like cells and expresses multiple NRs, allowing researchers to investigate the functional effects of NR ligands and the genomic pathways regulated by NRs in macrophages (13, 14).

2. Aims of the study

The first objective of this thesis was to investigate RAR α and VDR genomic pathway interactions by identifying and characterizing genomic regulatory regions co-occupied by RAR α and VDR in macrophage-like cells. We aimed to examine the convergence of gene sets up-regulated by RAR α and VDR ligands, explore the role of shared binding regions in mediating this convergence, and further characterize these regions in terms of chromatin accessibility and the presence of DNA binding motifs.

The second objective was to investigate how ligands of RXR and its heterodimeric partners reshape the RXR binding landscape, and to determine whether RXR availability becomes limiting when multiple ligands for RXR and its partners are present and how this potential limitation contributes to pathway interference in macrophage-like cells.

3. Materials and Methods

To address our questions, we performed an integrated genome-wide analysis of receptor binding, chromatin accessibility, and transcriptional changes in vehicle- and ligand-treated cells using macrophage-like cells as a model (PMA-stimulated THP-1 cells).

3.1. Cell culture and ligand treatment

The human monocytic THP-1 cells were cultured under standard conditions recommended by the supplier and were passaged every three

days. To induce macrophage-like differentiation, cells were treated with PMA for 16 hours, followed by incubation in hormone-depleted medium to minimize background signaling. The differentiated cells were then exposed to vehicle or specific NR ligands at defined concentrations, either individually or in combination, and harvested at defined time points depending on the downstream experiment. The ligands included agonists for RAR α , VDR, PPAR δ , PPAR γ , LXR, TR, and RXR.

3.2. RNA-seq experiments and data analysis

For RNA-seq experiments, PMA-THP-1 cells were treated for 6 hours with vehicle or NR agonists, and total RNA was extracted, quality was checked, and used for library preparation followed by sequencing on the Illumina platform. Reads were aligned to the human reference genome (hg38) and analyzed for differential gene expression. Protein-coding genes were selected based on HGNC list, and transcription start sites (TSS) were defined using GRCh38.p14 annotations. Cooperatively up-regulated genes (with enhanced expression under ligand co-treatment) were identified by comparing the response to single ligands (AM580 or 1,25-vitD) to combined-ligand treatment, and using fold-change (FC) and p-value cutoffs.

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

mRNA and eRNA expression levels of selected genes and regulatory regions were measured by RT-qPCR in PMA-THP-1 cells treated with vehicle or ligands in biological triplicates. Cells were treated for 1-6

hours for mRNA analysis and 6 hours for eRNA analysis, followed by RNA extraction, cDNA synthesis, and quantitative PCR. Expression levels were calculated using the comparative threshold cycle method, normalized to cyclophilin A (PPIA).

3.4. ChIP-seq experiments and data analysis

ChIP-seq experiments were performed on PMA-THP-1 cells stimulated with vehicle or ligands for 1-6 hours. Cells were cross-linked and lysed, and chromatin was sheared by sonication, followed by immunoprecipitation using antibodies against RAR α , VDR, MED1, RXR α , and H3K27ac and using Protein A and/or G magnetic beads in biological duplicates or triplicates. DNA was purified, quantified, and used for library preparation, then sequenced on the Illumina platform. ChIP-seq data were processed using an in-house pipeline. Reads were aligned to the hg38 genome, and peaks were identified using MACS2 peak calling software.

3.5. Downstream analysis of ChIP-seq data

Summits were extended by ± 100 bp or MACS2 narrow peaks were used directly to define ChIP-seq peaks. Normalized tag densities (reads per kilobase per million mapped reads (RPKM)) were calculated for each peak. Consensus peak sets detected in at least two biological replicates were defined. Analysis of ligand-dependent changes in ChIP-seq data sets identified ligand-induced or reduced peaks and ligand-unresponsive peaks using an R Bioconductor package, DiffBind. ChIP-seq peak-to-

gene association analyses were performed by assessing the enrichment of peaks within a TSS ± 25 kb of upregulated genes, or by assigning peaks to the nearest gene TSS using `annotatePeaks.pl` (HOMER).

DNA motif analysis in ChIP-seq datasets was performed by calculating the prevalence of position weight matrices (PWMs) within peak sets. PWMs were obtained from the HOMER and JASPAR databases. PWM alignment scores, reflecting the similarity of sequences to the PWMs, were calculated using a control set of 5,000 randomly selected, size-matched genomic regions. Score cutoffs were defined based on the threshold corresponding to 5% positive matches in the control set. Motifs exceeding these cutoffs were then scanned genome-wide, and their frequencies were assessed within peak sets. Distances between motifs were calculated using `bedtools`. Additionally, *de novo* motif discovery was performed using `findMotifsGenome.pl` (HOMER) to identify enriched DNA motifs within top-ranked peaks.

3.6. ATAC-seq experiments and data analysis

ATAC-seq experiments were performed on PMA-THP-1 cells treated with vehicle or AM580 for 1 hour in biological duplicates. Nuclei were isolated and the DNA was subjected to tagmentation, indexing, and library preparation, followed by PCR amplification for nine cycles. DNA was then purified and quality was assessed prior to sequencing. Libraries were sequenced on an Illumina platform. Raw reads were processed using a pipeline similar to that used for primary analysis of ChIP-seq data.

3.7. Meta-analysis of NR ChIP-seq datasets

Publicly available NR ChIP-seq datasets were obtained from the Sequence Read Archive (SRA) database. Samples were selected by filtering metadata, retaining only ChIP-seq experiments with antibodies targeting the NR of interest, appropriate control and ligand-treated samples (up to 24 h treatment), and association with a peer-reviewed publication. FASTQ files were downloaded and merged, when necessary, followed by processing through our in-house ChIP-seq pipeline using the hg38 or mm10 reference genomes. Consensus peak sets were generated for each study. Up to ten studies per NR were included based on dataset availability and predefined selection criteria. Finally, RPKM values were calculated for each consensus peak set, and ligand-to-control ratios were determined for comparative analysis across studies and different NRs.

4. Results

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.

4.1. 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 vehicle or the selective agonists AM580 (RAR α) and 1,25-vitD (VDR). Consensus peak sets

contained 5,936 RAR α peaks and 14,505 VDR peaks under ligand-treated conditions. Ligand treatment increased both the number of binding sites and occupancy levels, with a more pronounced effect observed for VDR. Comparison of ligand-induced cistromes revealed substantial overlap between RAR α and VDR binding regions. Genomic regions were categorized as RAR α -only, VDR-only, or common (n = 3,551). The common regions represented approximately 60% of the RAR α cistrome and 24% of the VDR cistrome. These shared regions were further classified based on the ratio of ChIP-seq signals into RAR α -dominant, similarly occupied, and VDR-dominant groups, together forming five clusters used for subsequent analyses.

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

RNA-seq analysis was performed to link receptor binding with transcriptional responses to AM580 and 1,25-vitD. AM580 regulated 1,094 genes, 1,25-vitD regulated 387 genes, and combined treatment regulated 1,507 genes. Upregulated genes were classified into AM580-specific (n = 537), 1,25-vitD-specific (n = 158), and commonly induced (n = 91) groups, and included known target genes for retinoids and vitamin D. Analysis of combinatorial effects of ligands revealed that many common target genes exhibited cooperative induction under combined (AM580 + 1,25-vitD) treatment. We found that among the common target genes, 48 protein-coding genes were cooperatively upregulated by the two ligands. Time-course RT-qPCR confirmed that

these cooperative effects were evident at early time points (2-3 hours) for representative target genes.

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

The association between RAR α and VDR binding and gene regulation was assessed using two complementary approaches. First, binding regions were assigned to the nearest gene, revealing that most regions (79-89%) were associated with genes not differentially expressed. Ligand-specific patterns emerged among the remaining regions: RAR α -only and RAR α -dominant regions were mainly associated with AM580-induced genes, whereas VDR-only and VDR-dominant regions were more often associated with 1,25-vitD-induced genes. A subset of VDR-only and VDR-dominant regions was associated with genes induced exclusively by AM580. Second, enrichment of binding regions within TSS $\pm$ 25 kb of upregulated genes showed that genes induced by both ligands were associated with a higher number of nearby binding regions than ligand-specific genes. Observed-to-expected analyses confirmed preferential enrichment of RAR α -only and RAR α -dominant regions near AM580-responsive genes and VDR-only and VDR-dominant regions near 1,25-vitD-responsive genes, while similarly occupied and VDR-dominant regions were enriched near commonly induced genes. Overall, these results indicate that common binding regions contribute to common gene regulation; however, they are not uniquely associated with common genes.

4.4. MED1 is recruited in a ligand-responsive manner at many RAR α and VDR common binding regions

The activity of RAR α and VDR binding regions was assessed using MED1 ChIP-seq and eRNA expression analysis as indicators of enhancer activity. MED1, a co-activator recruited by NRs, was used to evaluate the transcriptional activity of regulatory regions in PMA-THP-1 cells treated with vehicle, AM580, 1,25-vitD, or their combination. A large fraction of RAR α and VDR binding regions overlapped with MED1 peaks, although only a subset showed ligand-dependent MED1 recruitment. This ligand-responsive MED1 recruitment was most frequent in common RAR α and VDR binding regions. We further analyzed MED1 occupancy at RAR α and VDR binding regions near various gene sets. Common binding regions showing significant MED1 induction after combined (AM580 + 1,25-vitD) treatment were largely absent near non-regulated and downregulated genes and were most abundant near genes upregulated by both ligands, compared with the AM580-only and 1,25-vitD-only groups. MED1 occupancy was examined within TSS $\pm$ 25 kb of protein-coding genes that were cooperatively upregulated by both ligands (48 genes). MED1 binding was generally stronger following combined (AM580 + 1,25-vitD) treatment than after either AM580 or 1,25-vitD alone at many of these loci.

eRNA measurements confirmed that selected regulatory regions exhibited ligand-dependent transcriptional activity patterns consistent

with MED1 occupancy. Common binding regions showed cooperative eRNA induction, whereas RAR α - or VDR-only regions responded primarily to their respective agonists. Overall, these findings suggest that common RAR α and VDR binding regions are particularly associated with cooperative enhancer activity, as reflected by MED1 recruitment and eRNA expression. 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.

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

Chromatin accessibility was analyzed to investigate whether common RAR α and VDR binding regions are more accessible compared to regions exclusively occupied by either receptor. Signal-regulated transcription factors (SRTFs) have limited ability to bind DNA motifs within closed chromatin; therefore, their binding landscape is largely determined by pre-existing accessible regulatory elements, typically established by lineage-determining transcription factors (LDTFs). Comparative analysis of chromatin accessibility in vehicle-treated cells across RAR α and VDR binding clusters showed that common binding regions were more frequently associated with accessible chromatin and exhibited stronger ATAC-seq signals than receptor-specific regions. Overall, these results indicate that commonly occupied RAR α and VDR binding regions represent more readily accessible genomic sites for these NRs.

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

De novo motif discovery and PWM-based motif mapping in RAR α and VDR ChIP-seq peak sets were performed to characterize underlying DNA REs in these regions. De novo analysis of the top peaks identified DR5 and DR2 motifs as the dominant RE in RAR α binding regions and the DR3 motif as the dominant RE in VDR peaks, along with motifs associated with macrophage LDTFs. PWM-based analysis confirmed these motif enrichment patterns, with DR5, DR2, and DR1 representing the most common RAREs in the RAR α cistrome and DR3 the most common VDREs in the VDR cistrome. DR1/2/5 were therefore retained as putative RAREs for subsequent analyses.

Across binding clusters, RAR α -only and RAR α -dominant clusters were enriched for RAREs. Common regions showed enrichment for both motif classes. Unexpectedly, in the VDR-dominant and VDR-only clusters, not only DR3 but also RAREs were enriched. This suggests that motif presence alone does not fully explain receptor binding specificity, and that other factors such as motif score (similarity to the canonical motifs) and chromatin accessibility are also involved in determining the pattern of receptor binding. Finally, analysis of co-occurring RARE (DR1/2/5) and VDRE (DR3) motifs showed that such combinations were relatively rare and most frequent in VDR-dominant common regions. Most of the common RAR α and VDR binding regions contained either a single motif class or none, suggesting that only a small portion of RAR α

and VDR co-binding was mediated by “strong” co-occurring canonical motifs.

4.7. Common RAR α and VDR binding regions display diverse RARE-VDRE configurations

The spatial arrangement of RARE (DR1/2/5) and VDRE (DR3) motifs was analyzed within common RAR α and VDR binding regions by measuring distances between motif centers. Motif pairs were classified as largely overlapping (≥ 7 shared bases), partially overlapping (1-6 shared bases), or non-overlapping. All configurations were observed in common binding regions, with non-overlapping motifs being the most frequent, followed by partially overlapping and largely overlapping configurations.

4.8. Overlap of NR cistromes and co-occurrence of motifs in other studies

We investigated how NR cistromes overlap and how their motifs co-occur, using both our data and previously published studies. Earlier research reported substantial overlap between NR cistromes, but differences in analysis methods made comparisons difficult. To resolve this, we reanalyzed selected ChIP-seq datasets alongside our own using a standardized workflow. For each NR pair, we identified binding regions, measured cistrome overlap, and analyzed motif prevalence and co-occurrence. The size ratios between larger and smaller cistromes varied widely (e.g., 2.1 for VDR vs. RAR α and up to 11.7 for PPAR α vs.

GR). The fraction of overlapping regions in the smaller cistrome ranged from 22% to 85%, confirming that shared binding regions are common among NRs. Consensus motif prevalence differed considerably across datasets, ranging from about 14% to 55%. (For simplicity, only the DR5 motif was analyzed as RARE). Despite frequent overlap in binding regions and moderate-to-high motif prevalence, motif co-occurrence within common regions was consistently low (3.6%-14.3%). Even in cases like ER α vs. GR, where the prevalence of motifs was high in the entire cistromes, motif co-occurrence remained limited. Overall, this suggests that while NR cistromes often overlap, simultaneous presence of strong motifs in the same regions is relatively rare and likely a general feature of NR genomic binding.

Part II: RXR has a non-limiting role in macrophage-like cells stimulated with multiple NR agonists

4.9. ChIP-seq meta-analysis identifies RXR as one of the least ligand-sensitive NRs with respect to the overall genomic occupancy

We analyzed publicly available ChIP-seq datasets to assess how ligand binding affects genome-wide occupancy of RXR and other NRs. Studies were selected based on strict criteria, requiring both control and ligand-treated conditions. The selected datasets were processed using a standardized pipeline, and for each study we calculated the ratio of genomic occupancy in ligand-treated versus control conditions. As

expected, steroid receptors showed strong ligand-dependent increases in genomic binding, although the magnitude varied between studies, likely due to differences in cell types, endogenous ligand levels, and experimental setups. Fewer datasets were available for RXR and its heterodimeric partners. In contrast to steroid receptors, RXR and its heterodimeric partners showed only modest changes in genome-wide occupancy upon ligand treatment, with VDR displaying the strongest response within this group. However, even changes in VDR occupancy were lower than those observed for steroid receptors.

4.10. Overall genomic occupancy of RXR is largely unchanged following ligand stimulation in THP-1 cells

Using PMA-THP-1 cells as a model, we investigated how ligands targeting RXR and its heterodimeric partners affect RXR chromatin binding. RNA-seq data from vehicle-treated PMA-THP-1 cells were used to identify ligand-inducible RXR partners expressed in these cells. RXRA was the most abundant RXR isotype, while RXRB and RXRG were expressed at very low levels. Several RXR partner receptors (including PPARs, LXRs, RARs, VDR, and THRA) were moderately to highly expressed. Analysis of mRNA data from the Human Protein Atlas showed that RXR expression was generally comparable to or higher than its partners in many primary human cell types, including macrophages.

For ChIP-seq experiments, we used the RXR agonist LG268 and ligands targeting the most expressed RXR partners. Genomic regions occupied by RXR were identified. Although most RXR peaks were

shared between control and ligand-treated samples, a smaller fraction (14-36%) appeared only after ligand stimulation. These ligand-specific sites generally showed low signal intensity. The RXR binding changes were typically minimal, with ligand versus control signal ratios close to 1.0. The strongest effects were seen with LG268 and T3, but even these increases were modest. These results suggest that RXR genomic occupancy remains largely stable in PMA-THP-1 cells regardless of ligand stimulation, consistent with previous findings in primary macrophages.

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

Although overall RXR occupancy did not indicate robust changes based on overall signal ratios, this does not rule out redistribution at specific genomic loci. To examine this, ligand-dependent increases and decreases in RXR binding were determined using DiffBind. LG268 produced the strongest effect, generating the highest number of responsive peaks even after stringent filtering by FC and FDR cutoffs. In contrast, ligands targeting RXR partners showed weaker effects, with the RAR α agonist AM580 having the least pronounced effect on RXR redistribution. Because strict FC and FDR filtering yielded few significant peaks for several ligands, an FC-only approach was used to define ligand-induced, ligand-reduced, and unresponsive peaks for downstream analyses, although these sets are considered “exploratory” due to a higher likelihood of false-positive results.

Motif analysis of RXR peaks in vehicle and ligand-treated conditions showed distinct patterns. In control cells, canonical DR0-DR5 motifs were enriched. Ligand-induced peaks mainly reflected expected heterodimer-specific preferences (e.g. 1,25-vitD-induced peaks were enriched for DR3 motifs). Ligand-reduced peaks often contained motifs other than the canonical motifs of the activated receptor. Overall, this supports a model in which activated receptors recruit RXR to their target binding sites while displacing it from other complexes. Comparison of LG268-induced peak set with those induced by other ligands showed patterns consistent with permissive versus non-permissive heterodimers. VDR-induced peaks showed little overlap with LG268-induced peak set, consistent with non-permissive behavior, whereas LXR and PPAR agonists showed strong overlap, consistent with their permissive behavior. Analysis of RXR occupancy within TSS ± 25 kb of selected target genes indicated that ligand-induced RXR peaks were frequently located near known target genes of RXR partners.

4.12. 1,25-vitD-responsive genes are associated with regulatory regions at which RXR binding is either induced or unchanged by treatment with 1,25-vitD

We analyzed the gene regulatory activity of RXR binding regions, focusing on genes induced by the VDR agonist 1,25-vitD as a case study. We performed RNA-seq, integrated previously generated VDR ChIP-seq data, conducted new RXR ChIP-seq experiments using a 1 h ligand

treatment to match the VDR dataset, and analyzed MED1 and newly generated H3K27ac ChIP-seq data to assess enhancer activity.

RNA-seq identified 252 protein-coding genes upregulated by 1,25-vitD, including known VDR target genes. RXR peaks within TSS $\pm$ 25 kb of these genes were mapped, yielding 851 ligand-unresponsive, 99 ligand-induced, and 2 ligand-reduced peaks. Comparative analysis showed that ligand-induced RXR binding regions exhibited stronger VDR recruitment, higher MED1 and H3K27ac signals, and higher basal VDR occupancy. Notably, ligand-unresponsive regions also showed increased VDR binding and enhancer-associated signals upon treatment, indicating regulatory activity independent of induction in RXR binding. Moreover, increases in VDR occupancy were greater than those of RXR at the same regions following 1,25-vitD treatment. Ligand-induced peaks also showed stronger DR3 motif enrichment, consistent with canonical VDR-RXR binding motif. Together, these results suggested that both ligand-induced and unresponsive RXR binding regions contribute to gene regulation.

4.13. 1,25-vitD differentially affects VDR and RXR occupancy at shared genomic regions

We observed that 1,25-vitD-induced increases in VDR binding occurred even at unresponsive RXR binding regions. This raised the question of whether this difference in partner dynamics is a general phenomenon. We analyzed all overlapping RXR-VDR binding regions in PMA-THP-1 cells and found that ligand-induced signal changes

consistently favored VDR, indicating that this imbalance is a general genome-wide phenomenon. To validate this finding, we reanalyzed four independent studies with paired RXR and VDR ChIP-seq data in murine cells. Despite variability between datasets, all showed the same trend with VDR occupancy increased more than RXR upon ligand treatment. Overall, these results demonstrate that 1,25-vitD differentially affects VDR and RXR binding.

4.14. Treatments with ‘cocktails’ containing multiple agonists induce only minor changes in overall RXR occupancy

We examined how RXR binding is affected by simultaneous activation of multiple receptors using two ligand “cocktails” including one with six RXR partner agonists and another combining these with the RXR agonist LG268. Compared to single-ligand treatments, these combinations caused only modest increases in overall RXR occupancy. Analysis of binding changes showed that fewer than 15% of RXR peaks were affected by the combined treatments. The most notable increases and decreases in RXR binding occurred under AM580 and 1,25-vitD conditions, respectively. Overall, co-activation of multiple RXR partners leads to only minor global changes in RXR genomic binding, with effects limited to a small subset of genomic regions.

4.15. Combined agonist treatment minimally impacts RXR occupancy and transcriptional responses compared to 1,25-vitD

We analyzed how changes in RXR binding relate to gene expression following co-activation of multiple RXR partners, using 1,25-vitD treatment as a case study. Comparing RXR occupancy between 1,25-vitD alone and combined six agonists showed that only a small fraction of RXR peaks changed in RXR occupancy, with 7% decreasing and 11% increasing. These patterns could reflect the net effect of multiple RXR dimers on the same regions and the redistribution of RXR between different dimers across genomic regions upon combined ligand treatment.

To link RXR binding with transcription, ChIP-seq and RNA-seq data were integrated. A total of 241 1,25-vitD-upregulated genes with nearby RXR peaks were identified. Genes were grouped based on RXR occupancy changes. No overall decrease in gene expression was observed for genes with reduced RXR binding, while genes with increased RXR occupancy showed moderately higher expression under combined treatment. Finally, only a small overlap was found between genes with reduced expression and those with decreased RXR binding. Overall, co-activation of RXR partners has a modest effect on transcription, and coordinated decreases in both RXR binding and gene expression occur in only a small subset of genes.

5. Summary

NRs are ligand-dependent TFs that regulate gene expression through genomic binding, in many cases as heterodimers with RXR. RARs and VDR mediate retinoids and vitamin D signaling pathways, respectively. Previous studies suggested that RAR and VDR mediated pathways are distinct but partially overlapping, and certain genomic regulatory regions are occupied by both RAR and VDR receptors. This raised questions about how these commonly occupied genomic regions contribute to convergence and cooperation between RAR- and VDR-mediated pathways. RXR is a common heterodimer partner for many NRs; therefore, simultaneous activation of multiple NRs could potentially lead to competition for RXR and altered transcriptional responses. We asked whether and how RXR, as a shared partner, limits or coordinates NRs' activity under co-activation of multiple RXR partners.

To address these questions, genome-wide analyses of receptor binding, chromatin openness, and transcription were performed in ligand-treated PMA-THP-1 cells, complemented by validation experiments and reanalysis of published datasets.

New findings:

Our results with respect to the first aim can be summarized as follows:

1- 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.

2- 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.

3- Genes upregulated by both agonists were often associated with common binding regions.

4- Common RAR α and VDR binding regions were typically localized within highly accessible chromatin.

5- These common binding regions were frequently, though not exclusively, involved in cooperative regulation between retinoid and vitamin D pathways.

6- DNA REs 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.

7- 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 common RAR α 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:

1- 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.

2- 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.

3- 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.

4- 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.

5- Although 1,25-vitD markedly enhanced VDR binding, the corresponding increase in RXR occupancy was comparatively modest.

6- Both ligand-induced and ligand-unresponsive RXR binding regions contributed to gene regulation.

7- The overall RXR binding exhibited only moderate changes under combined ligand treatment compared with the single-ligand stimulations.

8- Only a small fraction of 1,25-vitD-upregulated 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.

6. References

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7. List of publications



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Registry number: DEENK/182/2026.PL
Subject: PhD Publication List

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., Szirák, 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.
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4. Ghalamkari, S., **Mianesaz, H.**, Chitsaz, A., Ghazavi, M., Salehi, M.: Proband-Only Exome Sequencing for Intellectual Disability in Iran: Diagnostic Yield and Genetic Insights.
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11. Ghalamkari, S., Khosravian, F., **Mianesaz, H.**, Kazemi, M., Behjati, M., Hakimian, S. M., Salehi, M.: A Comparison Between Full-COLD PCR/HRM and PCR Sequencing for Detection of Mutations in Exon 9 of PIK3CA in Breast Cancer Patients.
Appl. Biochem. Biotechnol. 187 (3), 975-983, 2018.
DOI: <http://dx.doi.org/10.1007/s12010-018-2859-3>
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