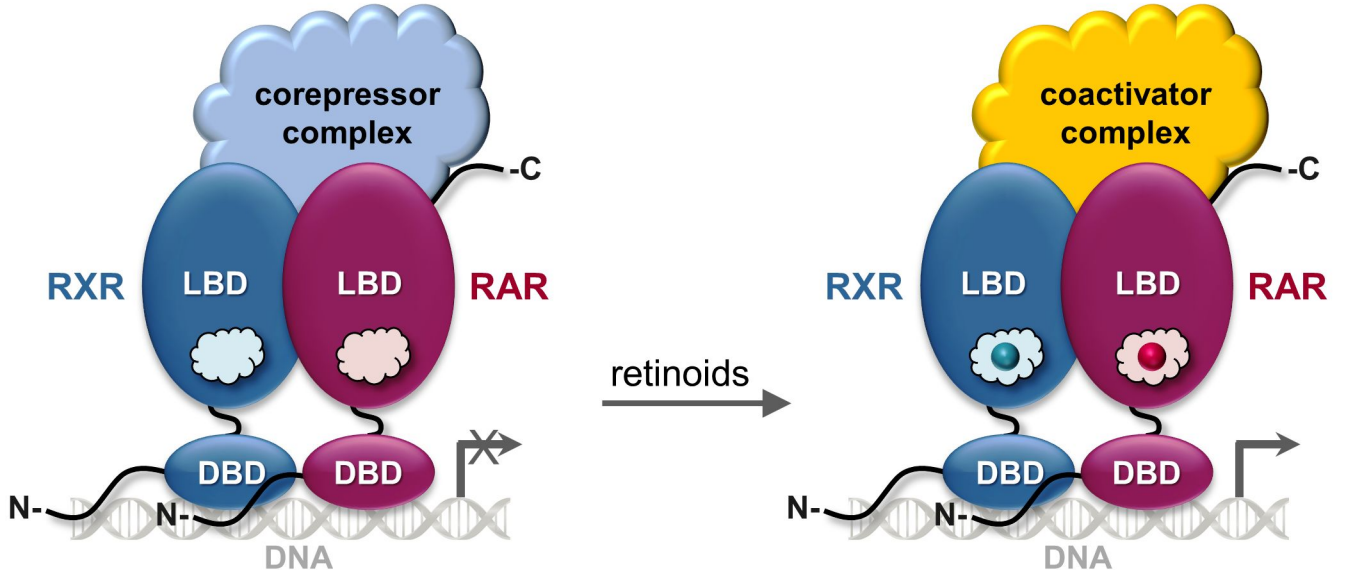
Ligands and DNA in the allosteric control of retinoid receptors function

Pierre Germain, Natacha Rochel, William Bourguet

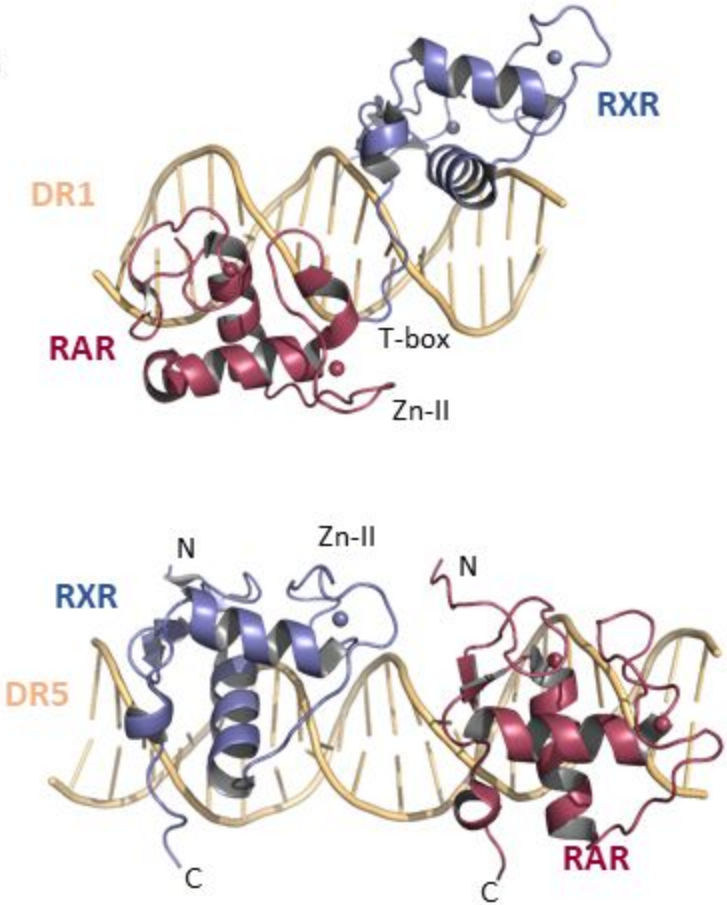

corepressor complex
-C
RXR
LBD
LBD
RAR
DBD
DBD
N-
N-
DNA
retinoids
coactivator complex
-C
RXR
LBD
LBD
RAR
DBD
DBD
N-
N-
DNA

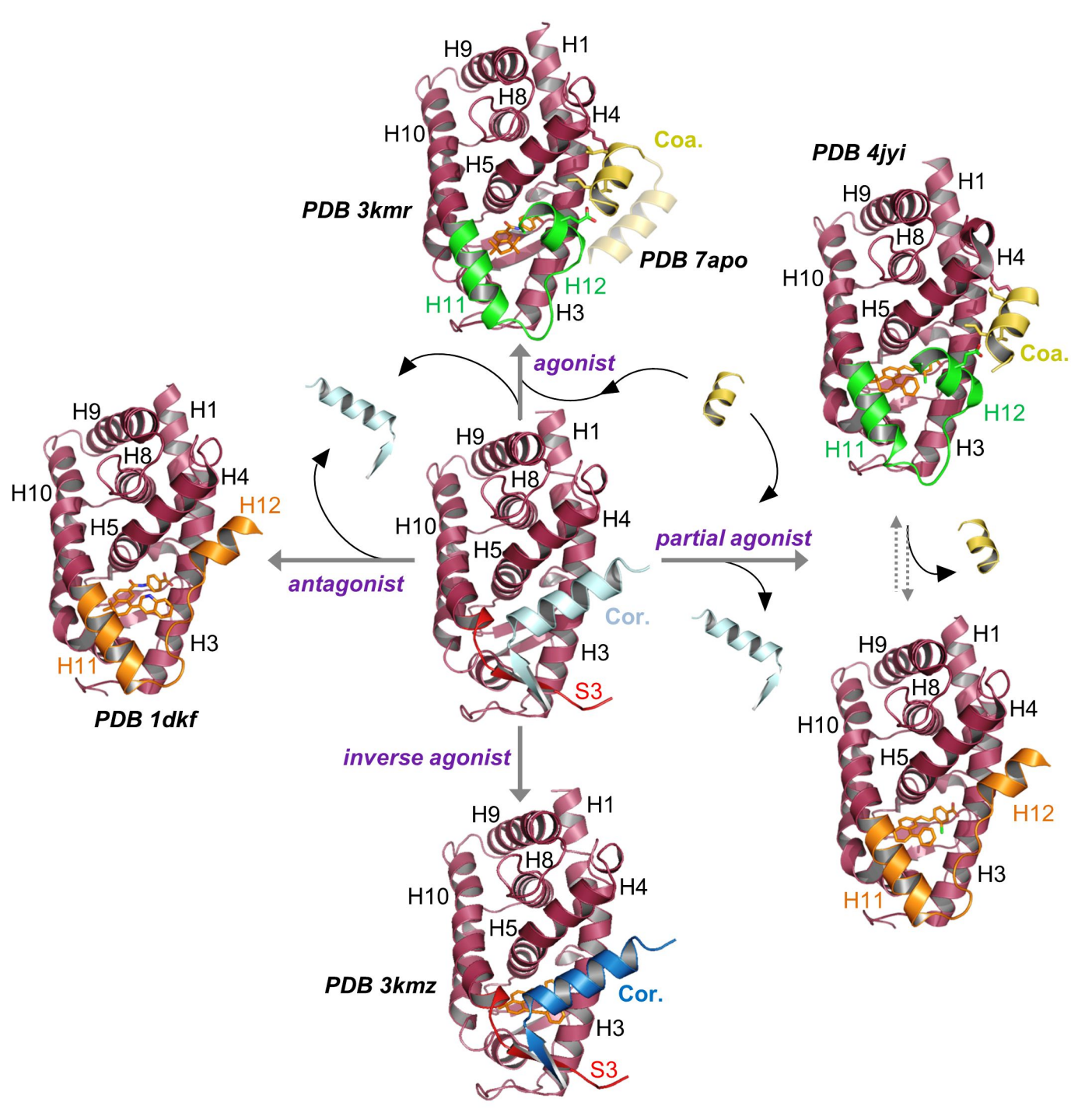
a

RAREs

DR5	*RARb2*	GGTTCAccgaaAGTTCA
DR2	*Hoxa10*	AGTTCAgaAGGTCA
DR1	*Ramp2*	AGTTCAaGGGTCA
DR0	*Hoxb13*	AGGTCAAGGCCA
IR0	*Trim16*	GGGTCATGACCC
DR8	*Mafa*	AGGTCAgaAGTTCAAGGTCA
	Dedd	AGGTCAcgatctggAGTTCA

b




H9
H1
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H5
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PDB 3kmr
PDB 7apo
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antagonist
H9
H1
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H4
H10
H5
Cor.
H3
S3
partial agonist
H9
H1
H8
H4
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H5
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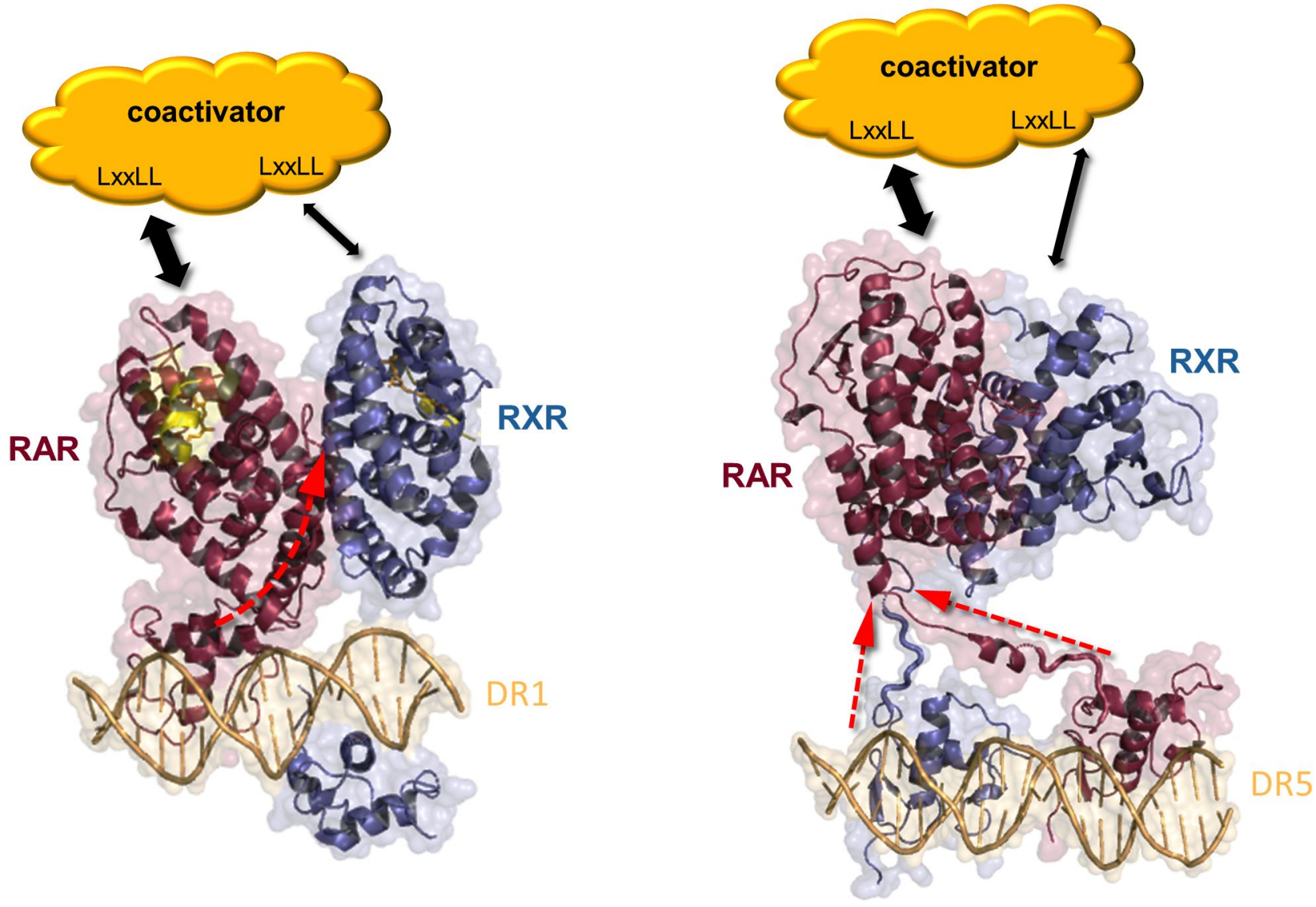
PDB 3kmz
Cor.
H3
S3


coactivator
LxxLL
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RAR
RXR
DR1
coactivator
LxxLL
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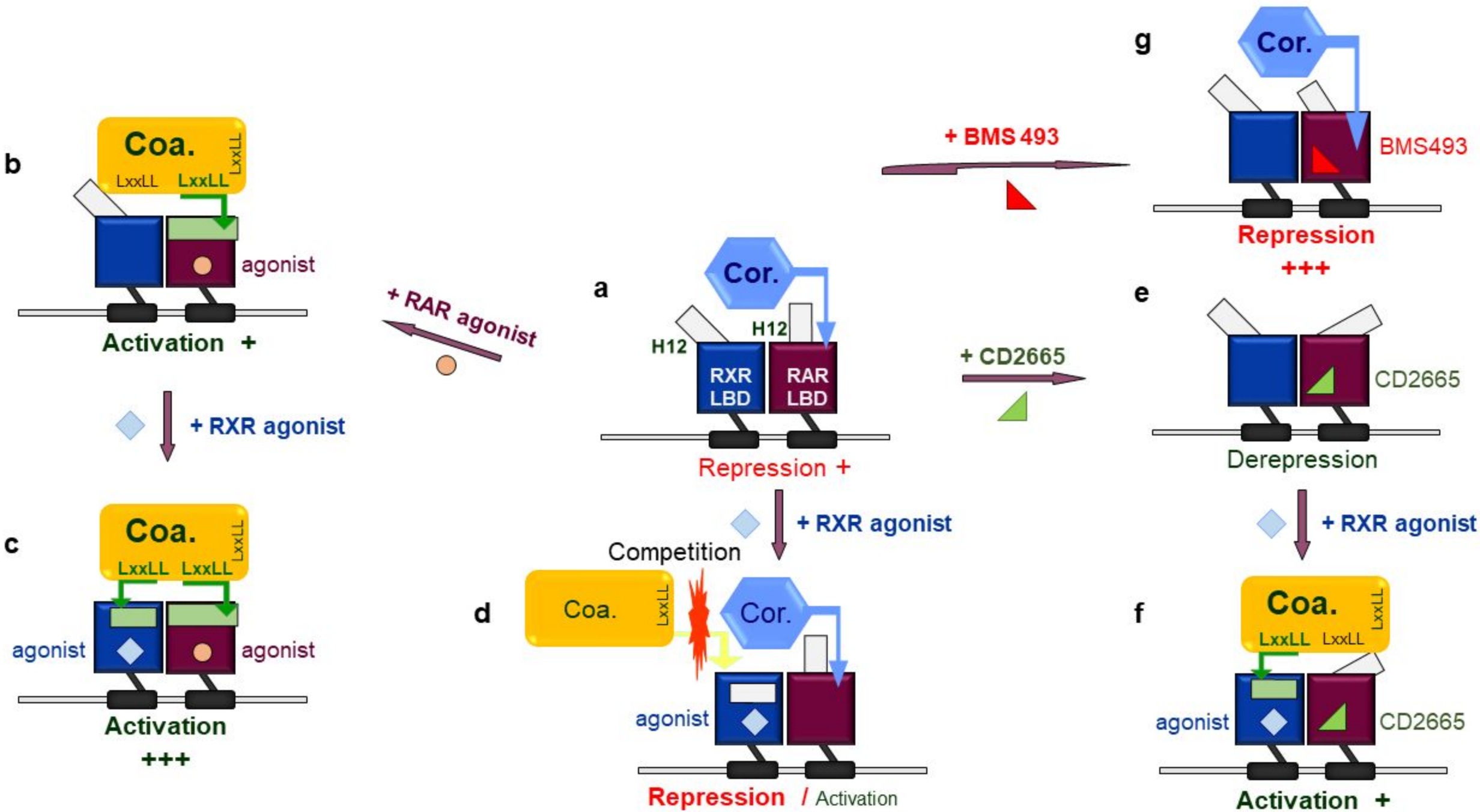
RXR
RAR
DR5


b
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agonist
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+ BMS 493
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g
Cor.
BMS493
Repression
+++
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CD2665
Derepression
+ RXR agonist
f
Coa.
LxxLL
LxxLL
LxxLL
agonist
CD2665
Activation +

Ligands and DNA in the allosteric control of retinoid receptors function

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Summary points

Retinoid receptors control a wide variety of physiological processes and as such represent important drug targets to combat and prevent several cancers, skin and blood diseases or metabolic disorders.

Structural and functional analyses have provided deep insight into the molecular mechanism of their regulation *via* ligand- and DNA-induced allosteric conformational changes which are the basis of their communication with the cellular environment.

The complexity of incoming signals and their integration is still not fully understood and additional studies associating integrative structural biology, genomics and single-cell analysis at various time and size scales are needed to better apprehend retinoid functions.

Abstract

Retinoids are a family of compounds that include both vitamin A (all-*trans* retinol) and its naturally occurring metabolites such as retinoic acids (*e.g.* all-*trans* retinoic acid) as well as synthetic analogs. They are critically involved in the regulation of a wide variety of essential biological processes, such as embryogenesis and organogenesis, apoptosis, reproduction, vision, and the growth and differentiation of normal and neoplastic cells in vertebrates. The ability of these small molecules to control the expression of several hundred genes through binding to nuclear ligand-dependent transcription factors accounts for most of their functions. Three retinoic acid receptor (RARα,β,γ) and three retinoid X receptor (RXRα,β,γ) subtypes form a variety of RXR-RAR heterodimers that have been shown to mediate the pleiotropic effects of retinoids through the recruitment of high-molecular weight coregulatory complexes to response element DNA sequences found in the promoter region of their target genes. Hence, heterodimeric retinoid receptors are multi-domain entities that respond to various incoming signals, such as ligand and DNA binding, by allosteric structural alterations which are the basis of further signal propagation. Here, we provide an overview of the current state of knowledge with regards to the structural mechanisms by which retinoids and DNA response elements act as allosteric effectors that may combine to finely tune RXR-RAR heterodimers activity.

Introduction

The three retinoic acid receptor subtypes α, β and γ (RARα,β,γ; NR1B1-3 according to the official nomenclature) transduce retinoid signaling, in embryo and adult life, through heterodimeric complexes with the three retinoid X receptor paralogs α, β and γ (RXRα,β,γ; NR2B1-3) as partners [1, 2]. By controlling gene transcription, the retinoid receptors play multiple roles in vertebrate development, organogenesis, homeostasis, immune functions, and reproduction [3-5] and represent important drug targets [6-8]. Their activation function is finely tuned by small and mostly hydrophobic ligands (see Table 1 for examples of natural and synthetic retinoids), multiple transcriptional co-regulators (corepressors and coactivators) and post-translational modifications (PTMs), resulting from crosstalk between different signaling routes [9, 10]. Similar to other nuclear receptors (NRs), they display a modular organization with two conserved domains, namely the ligand-binding domain (LBD, moderately conserved) and the DNA-binding domain (DBD, highly conserved), that contain the ligand-dependent activation function 2 (AF-2), and the recognition motif of their binding sites on DNA response elements, respectively (Fig. 1).

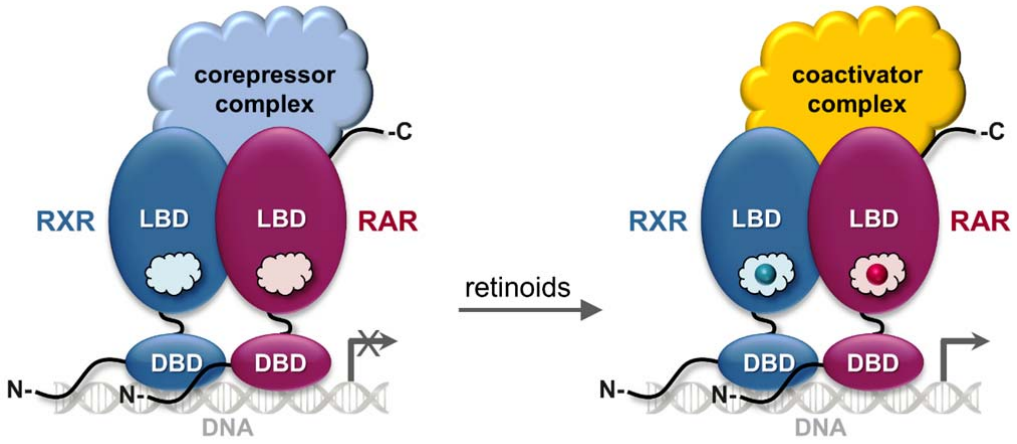

Figure 1: The RXR-RAR heterodimers act as repressors and activators of gene transcription. RXR-RAR heterodimers bind *via* their DNA-binding domains (DBD) to specific retinoic acid response element DNA sequences found in the promoter region of retinoid target genes. In the absence of ligand, the RXR-RAR heterodimer recruits the corepressor proteins NCoR or SMRT and associated enzymatic factors inducing an inactive condensed chromatin structure, preventing transcription. Upon retinoid binding to the RAR ligand-binding domain (LBD), corepressors are released, and coactivators of the SRC family are recruited. The subsequent recruitment of epigenetically active and/or chromatin-modifying complexes such as histone acetyltransferases or histone arginine methyltransferases leads to chromatin alterations that facilitate activation of target gene expression by the basal transcriptional machinery. RXR ligands may act synergistically with RAR ligands to enhance transcription.

The other functional domains are unstructured and less conserved. These regions are the N-terminal domain containing the ligand independent activation function 1 (AF-1), a short linker sequence allowing the proper orientation of DBDs and LBDs within DNA-bound dimers, and a poorly understood C-terminal region which is present in RARs but not in RXRs [11, 12]. RXR-RAR heterodimers control transcription *via* several distinct mechanisms, including both repression and activation. In the absence of ligand, the heterodimer recruits the corepressor proteins NCOR1 (formerly NCoR) or NCOR2 (formerly SMRT) serving as docking platforms to tether the repression machinery to RXR-RAR. Retinoid binding induces conformational changes in the heterodimer, allowing corepressor dissociation and the recruitment of members of the NR coactivator 1-3 (NCOA1-3) families of proteins such as SRC1, TIF2 or RAC3 (Fig. 1). Both corepressor and coactivator complexes act by altering the chromatin structure surrounding the promoter of target genes to allow their repression or activation, respectively [13-16].

Structural studies of NRs at the atomic level began in the early 1990s with X-ray crystallography and nuclear magnetic resonance (NMR) analyses providing the first snapshots of DBDs alone or bound to their DNA targets [17]. In the mid-1990s, the crystal structures of the unliganded (apo) RXRα and RARγ bound to all-*trans*-retinoic acid (ATRA) were the first reported NR LBD structures at atomic resolution. They revealed a new protein fold which was later on demonstrated to be prototypical of all the family members. Comparison of the two structures suggested a molecular mechanism for the ligand-induced activation involving conformational changes upon ligand binding [18]. Later, crystallographic studies using RARs and RXRs in complex with a variety of synthetic ligands ranging from agonists to antagonists through inverse or partial agonists (Table 1) provided extensive insights into the molecular basis of ligand action, revealing how different pharmacological classes of ligands allosterically remodel the receptor surface to enhance or reduce interaction with a given type of co-regulator [11, 19]. Structural studies allowed the precise delineation of the different binding surfaces of LBDs, including interaction sites for co-regulators and partner receptors [19, 20]. Based on these data, a structure-based sequence analysis led to the recognition of two classes within the superfamily with NRs of class I forming homodimers (*e.g.* steroid hormone receptors) while class II members form stable heterodimers with RXR [21]. More recently, crystallographic, cryo-electron microscopy (cryo-

EM) and solution studies have allowed the determination of structures of nearly full-length NRs, including that of the RXR-RAR heterodimer, bound to their cognate response elements, revealing their overall topologies and the role of DNA in domain organization and communication [11, 22, 23]. Solution studies using hydrogen/deuterium-exchange coupled mass-spectrometry (HDX-MS), small angle X-ray scattering (SAXS), fluorescence or NMR approaches further highlighted the importance of structural dynamics, transient interactions and intrinsic disorder in the fine tuning of retinoid receptors function [24]. In this chapter, we provide a current comprehensive view as to how retinoidal ligands and DNA response elements allosterically control the precise regulation of RXR-RAR heterodimers.

Basic principles of DNA recognition by RXR-RAR heterodimers

RXR-RAR heterodimers bind to specific DNA sequences, called retinoic acid response elements (RAREs), that are typically composed of two direct repeats (DR) of a core hexameric motif, (A/G)G(G/T)TCA and are located in the regulatory sequences of target genes. The number of spacer nucleotides between the half-sites was initially defined for various RXR heterodimers in a simplified manner by the '1–5 rule': RXR-RXR and RAR-RXR (DR1), RXR-RAR (DR2), RXR-VDR (DR3), RXR-TR (DR4) and RXR-RAR (DR5) [25]. Genome-wide chromatin immunoprecipitation followed by sequencing data allowed the characterization of RAR cistromes in various cell types [26-32] revealing that in addition to sites comprising the consensus direct repeat DR1, DR2 or DR5 elements, numerous sites comprising non-canonical sequences were detected (Fig. 2a), including DR1, 2 and 5 with non-canonical half-site sequences, inverted repeat 0 (IR0) elements and novel spacing topology elements, the DR0 and DR8 elements [30]. Biochemical and biophysical studies have revealed that RXR-RAR binds with comparable affinities in the nanomolar range to the various RAREs [30, 33]. DNA sequences and topology have been shown to act as allosteric effectors to modulate RAR activity, acting on the recruitment of coactivator or corepressor proteins. Positive or negative regulations have been shown to be strongly influenced by the spacing of the DNA elements to which they bind [34].

Current knowledge of the structural basis of specific DNA recognition by RXR-RAR heterodimer at atomic level is limited to complexes bound to DR1 or DR5 elements, comprising the crystal structures

of the RXRα-RARα DBDs [35] and RXRβ-RARα deleted of RAR and RXR N-terminal disordered regions [36] bound to an idealized or consensus DR1 sequence with identical half-sites and the crystal structure of RXRα-RARα DBDs bound to RARβ2 DR5, the only one with natural RARE [34]. The DBD core is highly conserved among NR family. It is composed of approximately 66 amino acid residues which form a tertiary structure composed of an N-terminal β-hairpin and two α-helices followed by a short C-terminal helix and an extension (Fig. 2b). The N-terminal α-helix fits into the major groove of the DNA and makes direct and water mediated hydrogen bonds with the nucleotide sequence. The C-terminal α-helix is perpendicular to the N-terminal helix and stabilizes the core of the DBD.

On both DR1 and DR5 elements, RAR and RXR bind asymmetrically in a head to tail manner but with reversed polarity (Fig. 2b). The spacing between the two motifs controls the position of the two bound receptors since each base pair leads to a rotation of about 35° and 3.4 Å length increase, thus leading to different dimerization surface in the two complexes. Interestingly, binding of the heterodimer to the DR1 and DR5 induce a DNA curvature not observed in the corresponding RXR homodimer complex on the same DNA element ([35] and unpublished data), to foster heterodimer interactions and resulting in higher affinity of the heterodimer over homodimer. In DR1 complex, the interface is formed by the C-terminal T-box of RXR DBD with the Zn-II region of RAR DBD [35, 36]. While in DR5 complex, the dimerization interface involves residues from the second Zn module of the RXR DBD and the N-terminal region of RAR [34]. In addition to spacing and topology of the response elements, the nature of the half-site sequences in contact with the DBD also modulates DBD geometry and the interactions between the two DBDs, as shown for GR [37] and RXR homodimers [38]. In the RXR-RAR heterodimer DNA complexes [34-36], each DBD forms sequence-specific direct and water mediated base contacts that involve highly conserved residues: Glu153, Lys156 and Arg161 for RXR, and Glu106 and Lys109 for RAR, as well as sequence-specific additional interactions. Different DNA sequences of the RAREs as observed in the genome will exert an allosteric regulation of the DBD heterodimer through changes in contacts at the dimerization interface. This allosteric regulation of DBD positioning may propagate to other domains of the receptor and hence contribute to the fine-tuning of transcription (as discussed further below).

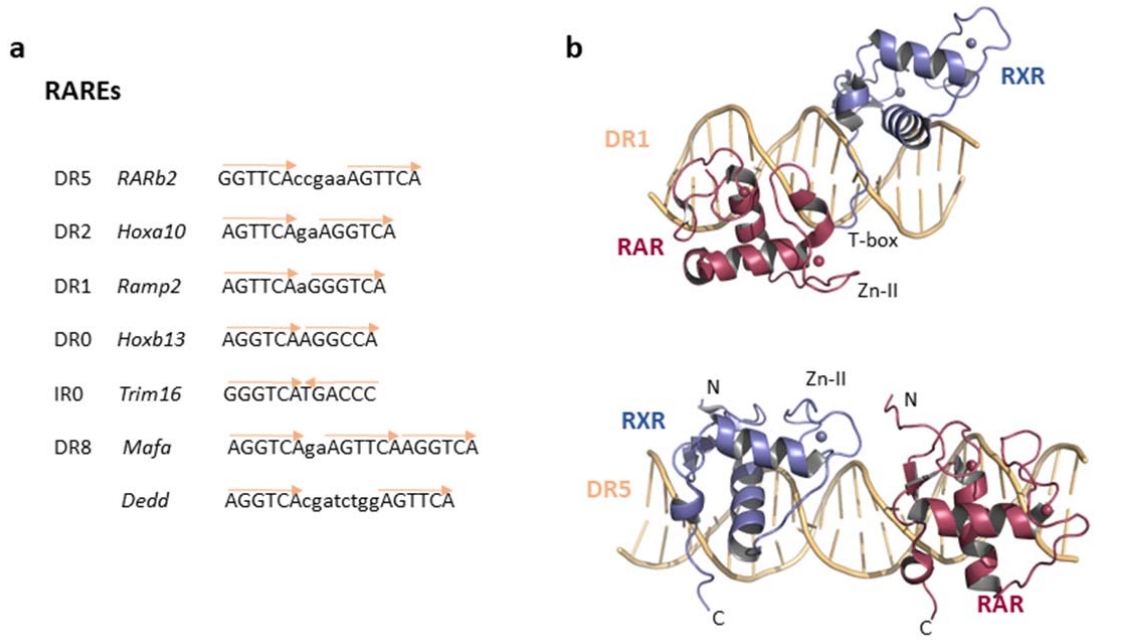

Figure 2: DNA binding of RXR-RAR heterodimers to DNA. (a) RAREs are composed of direct repeats (DR) of the hexanucleotide sequence (5′-(A/G)G(G/T)TCA-3′) separated by separated by 0 (DR0), 1 (DR1), 2 (DR2), 5 (DR5) or 8 (DR8) nucleotides and inverted repeats separated by 0 (IR0). RAR-RXR binds to these elements with a specific polarity. (b) Crystal structure of the heterodimer formed by RAR (in purple) and RXR (in blue) DNA-binding domains in complex with idealized DR1 (PDB ID: 1DSZ) and RARβ2 DR5 (PDB ID: 6XWG).

Structural basis of ligand-regulated co-regulators interaction and exchange

Crystallographic studies of RAR and RXR LBDs in complex with various corepressor and coactivator fragments have provided deep insights into the structural basis of the interactions between these receptors and transcriptional co-regulators, and their modulation by ligands [19]. The LBD of both RARs and RXRs is organized as an antiparallel α-helical sandwich with 12 conserved helices arranged in three layers with a β-turn (S1 and S2) located between helices H5 and H6. Helices H4, H5, H8, H9 and H11 are sandwiched between H1, H2 and H3 on one side and H6, H7 and H10 on the other side (Fig. 3). In contrast, the C-terminal helix H12 (also termed activation or AF-2 helix) is flexible and can adopt various conformations depending on the type of bound ligand. This LBD architecture generates a ligand-binding pocket (LBP) primarily made of hydrophobic residues from helices H3, H5, H7, H11 and the β-sheet segment. Structural studies have revealed that ligands are stabilized in the LBPs through extensive van der Waals contacts and a network of ionic and hydrogen bonds between their carboxylate moieties, a conserved arginine in H5, and water molecules.

In the absence of ligand or in the presence of so-called inverse agonists, RAR exhibits strong repressive activity that is brought about by the recruitment of corepressors. A combination of structural and functional studies revealed that RARs specifically interact with a bipartite and short structural motif found in the NR interaction domain (NRID) of corepressors [39]. This motif comprises a four-turn helix with the conserved LxxxIxx(I/V)Ixxx(Y/F) sequence that docks to a hydrophobic surface of the LBD that is formed by residues from helices H3 and H4, and an N-terminal extended β-strand forming an antiparallel β-sheet with the RAR residues residing after helix H10 (Fig. 3, center panel). Binding of inverse agonist (*e.g.* BMS493) stabilizes this specific interface and therefore enhances transcriptional silencing (Fig. 3, lower panel). In contrast, binding of any other kind of ligands to RARs induces a β-strand to α-helix transition that allows helix H11 formation which in turn provokes corepressor release. In the case of agonists (*e.g.* AM580, Fig. 3, upper panel) and to a lesser extent partial agonists (*e.g.* BMS641, Fig. 3, right panel), this secondary-structure switch is followed by the rearrangement of the C-terminus of RAR LBD into helix H12 that is stabilized in the so-called active position [39, 40]. This particular location of the activation helix defines a hydrophobic surface comprising the C-terminal part of helix H3, helix H4 and H12, and specifically recognized by short LxxLL motifs, reminiscent of the longer LxxxIxx(I/V)Ixxx(Y/F) sequence of corepressors. The short coactivator helix is held in place by interactions of the leucine residues with the hydrophobic groove and by hydrogen bonds with a lysine at the C-terminus of H3 and a glutamate in H12 which together form a "charge clamp". A more recent structure with a longer coactivator-derived peptide revealed how LxxLL flanking regions may drive specificity in coactivator recognition (Fig. 3, upper panel). More specifically, the structure shows that the C-terminal extension of the LxxLL motif folds as a long α-helix making hydrophobic contacts with residues from both the LxxLL helix and RAR helices H3 and H12, thereby inducing a three-fold increase of the affinity of the LxxLL motif for RAR but not for RXR [41]. In contrast to full agonists, partial agonists incompletely stabilize the active form of the receptor (Fig. 3, right panel). The partial activity of these compounds is a consequence of the lower interaction strength between H12 in its active position and the H3/H11 surface that renders this helix more dynamic [42, 43].

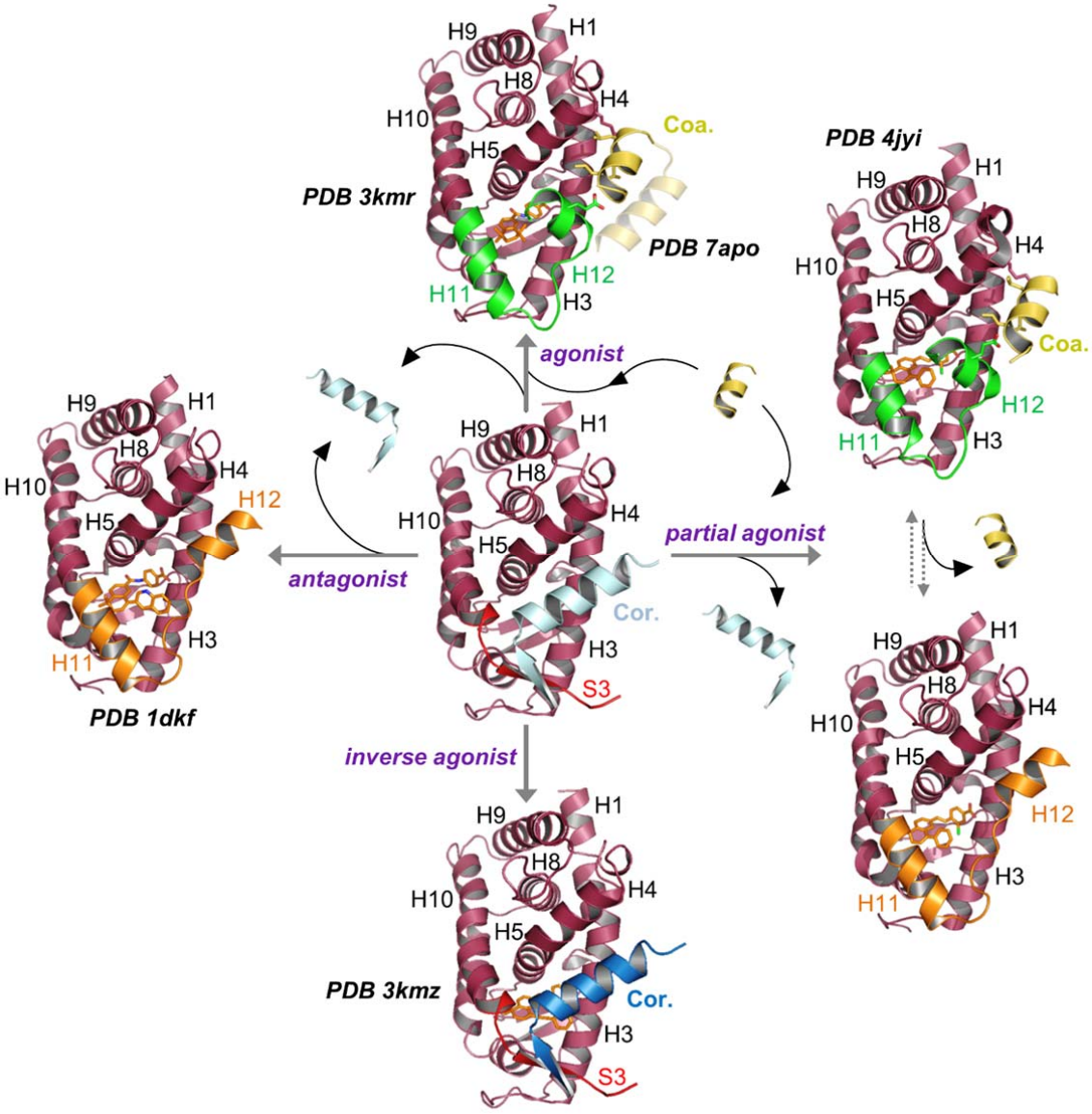

Figure 3: Structural basis of retinoid receptor modulation. Structures of the ligand-binding domain (LBD) of RAR are shown in the absence (center panel) and in the presence of different pharmacological classes of ligands and in complex with various coactivators (Coa.) and corepressor (Cor.) fragments. The core LBD is shown in raspberry color, whereas the color of the varying C-terminal secondary structural elements varies according to the receptor activity state. The corepressor bound conformation (repressive state) is shown in red, the coactivator-bound conformation (active state) is highlighted in green and the antagonist-bound conformation (silent state) is represented in orange. Corepressor and coactivator fragments are shown in blue and yellow, respectively. The dark blue color of the corepressor fragment in the inverse agonist-bound structure denotes higher interaction with RAR LBD relative to the unliganded state (light blue color). Ligands are displayed in their ligand-binding pockets as orange sticks. Important secondary structural elements discussed in the text are labeled (helix: H, strand: S). PDB codes indicate existing structures. PDBs 3kmr and 7apo denote Am580-bound RARα LBD in complex with a short LxxLL-containing peptide (yellow), plus its C-terminal flanking region (transparent yellow), respectively. Images of the unliganded corepressor-bound RAR LBD (center panel) and of the partial agonist-bound LBD in the antagonist conformation (lower right panel) correspond to molecular models deduced from additional structural and functional data.

Note however that the presence of coactivators helps stabilize the active conformation so the transcriptional outcome of partial agonist binding greatly depends on the intracellular concentration of co-regulators. Last, the crystal structure of RAR LBD in complex with the antagonist BMS614 (Fig. 3, left panel) shows that this compound encompasses a bulky extension protruding between helices H3 and H11 which is too long to be contained within the LBP with the AF-2 helix in the active conformation. Thus, in the presence of such compound, H12 adopts an alternative conformation by docking to the binding groove shared by coactivators and corepressors, between helices H3 and H4. This relocation prevents any interaction with both co-regulator types and renders the receptor transcriptionally silent [44]. Note that structural and functional studies performed on RXRs have revealed similar mechanisms of regulation, with the notable exception that this receptor interacts poorly with corepressors.

Signaling through the heterodimer

Lessons from structural studies

In addition to the allosteric effects of RXR and RAR ligands, the DNA response elements not only serve as docking sites for the receptors but act as allosteric modulator of RXR-RAR activity. Structural analyses revealed that the RAREs induce conformational changes in the DBDs [34, 35], that propagate in more distal regions influencing a variety of receptor functions such as dimerization, ligand and coactivator binding [34, 36, 45]. The structures of the multi-domain RXR-RAR complex truncated of their disordered N-terminal domains were determined by X-ray crystallography in complex with an idealized DR1 element [36] or by a combination of solution structural methods for DR1, DR5 and DR0 response elements from regulated genes [34, 45]. In the crystal and solution structures, the hinges remains flexible and only partially ordered, a flexibility necessary to allow the alternative positioning and engagement of the DBDs onto the various RAREs. The crystal structure of RAR-RXR-DR1 complex revealed that the RAR DBD interacts with its LBD, while RXR domains remain flexibly positioned (Fig. 4, left panel) [36]. In the solution structures, the conformations are elongated and the RXR-RAR LBD dimer (Fig. 4, right panel) appears asymmetrically positioned with respect to the DNA response elements [34]. As a consequence of different DNA binding mode on

DR5 and DR0 elements, differences in overall conformations and dynamics of the complexes were observed [34]. These differences are expected to lead to large differences in co-regulator binding and to control transcriptional output, as supported by HDX-MS analyses of RXR-RAR-DNA complexes [34, 36] and as observed for other NR heterodimers [46, 47]. The communications pathway between the DNA, the ligand and the coactivator interacting surface have been proposed to involve either DBD-LBD interactions or long range communication through the hinges domains (Fig. 4).

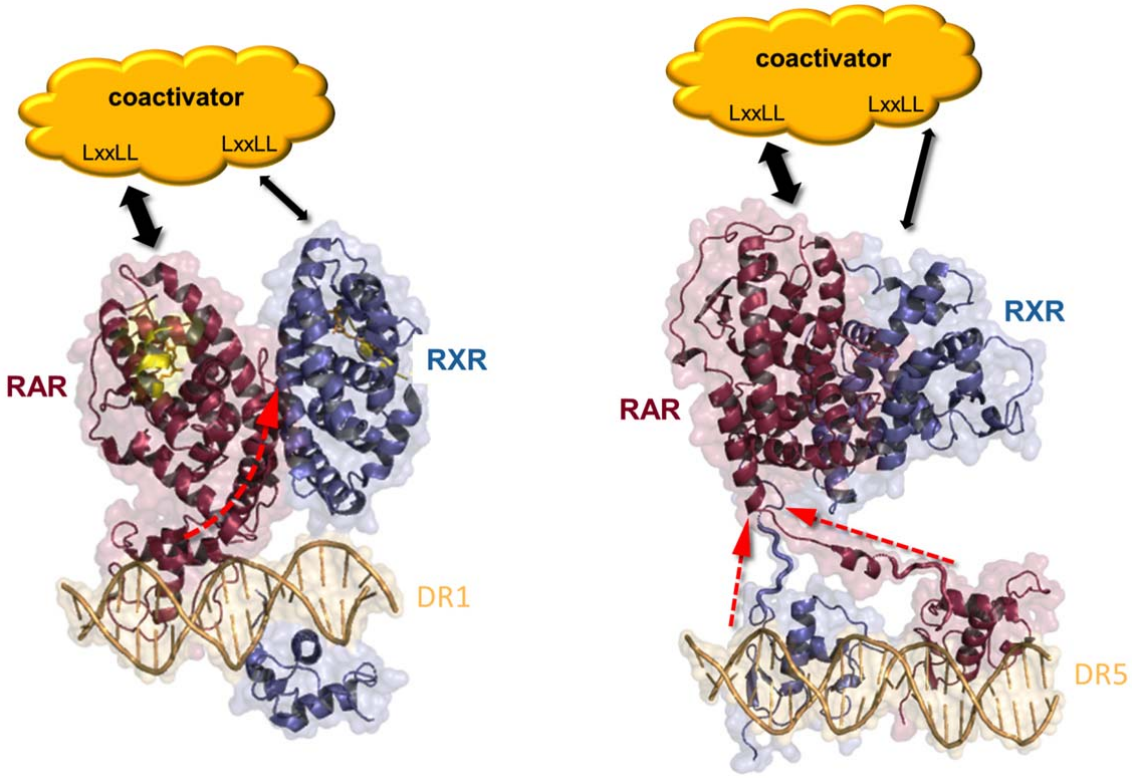

Figure 4: Structures of multi-domain RXR-RAR complexes. Left: Crystal structure of RXR-RAR bound to DR1. Right: Solution structure of RXR-RAR bound to DR5. Modulation of the conformation by DNA binding shown by red arrows leads to ligand-mediated coactivator interactions.

The NRID of corepressors and coactivators are mainly intrinsically disordered regions characterized by the presence of multiple conserved and transiently structured LxxxIxx(I/V)Ixxx(Y/F) and LxxLL motifs, respectively, so that the mechanism by which RAR in the context of the heterodimer with RXR interacts with co-regulators has been a matter of debate. Some studies argued in favor of the deck model where each subunit interacts with one coregulatory binding motif [36, 46, 48], while other studies reported an asymmetric model where RAR is the unique contributor to the interaction with co-

regulators [45]. Two recent studies combining of a large set of biophysical and computational methods allowed to somehow reconcile these seemingly conflicting views by demonstrating that NRIDs form highly dynamic complexes with RAR-RXR, with singly and doubly bound species, and that the equilibrium can be modulated by ligands and mutations [41, 49]. Moreover, they revealed that while the NRID of corepressors and coactivators is mainly disordered, it presents transient but robust intramolecular contacts upon interaction with the heterodimer, indicating that disorder-to-order transitions are key events in the regulation of NR heterodimers.

Lessons from functional studies

The above considerations imply that RXR-RAR heterodimers are the functional entities which regulate expression of target genes raising the question of the relative contributions of each subunit in the transcriptional state of heterodimers. Importantly, both heterodimer subunits retain their own properties in terms of ligand and co-regulator binding [43]. Consequently, an important point is based on the relative affinities of transcriptional co-regulators for RXR and RAR which depend on the ligand binding status of each subunit, making that heterodimer is in equilibrium between repressing and activating states [39, 43, 48]. A recent work indicates that in the absence of ligand for both RXR and RAR, the corepressor may form a transient multisite complex with the heterodimer *via* two binding motifs and, although RAR represents the main surface for corepressor interaction with the heterodimer, RXR would also play a minor role in its recruitment [49]. In contrast to RAR agonists that can activate heterodimers by inducing corepressor dissociation and coactivator interaction (Fig. 5a, b), RXR-selective agonists are unable to trigger RXR-RAR transcriptional response on their own [50, 51]. This phenomenon is referred to as RXR “subordination” or “silencing”. Though the molecular mechanism responsible for this subordination has been highly debated, it can be best explained by the fact that binding of rexinoid agonists to RXR cannot induce corepressor dissociation from heterodimers and thus permits the recruitment of coactivators because of the inaccessibility of the mutually exclusive binding site of the two co-regulator types (Fig. 5d). But when agonists for each subunit are used in combination, RXR ligands enhance the transcriptional activity of the heterodimer in a synergistic manner (Fig. 5c). Previous and recent works suggested that synergy likely originates

from the cooperative binding of two LxxLL motifs from one coactivator molecule to RXR-RAR heterodimer [41, 51]. Accordingly the activating capacity of rexinoids relies only on their ability to induce the recruitment of coactivators and the only way by which rexinoids can significantly contribute to heterodimer activity is in combination with an appropriate RAR ligand. Hence, by releasing corepressor from RARα, the RAR antagonist CD2665 permits agonist-bound RXR to induce the recruitment of coactivators to the RXR-RARα heterodimer which translates into transcription activation (Fig. 5e, f) [43]. In contrast, binding of an inverse agonist to RAR does prevent the coactivator association onto agonist-bound RXR (Fig. 5g) [39, 43, 51, 52]. The degree of corepressor interaction is therefore a crucial determinant on which pharmacological molecules can act for modulating heterodimer activity.

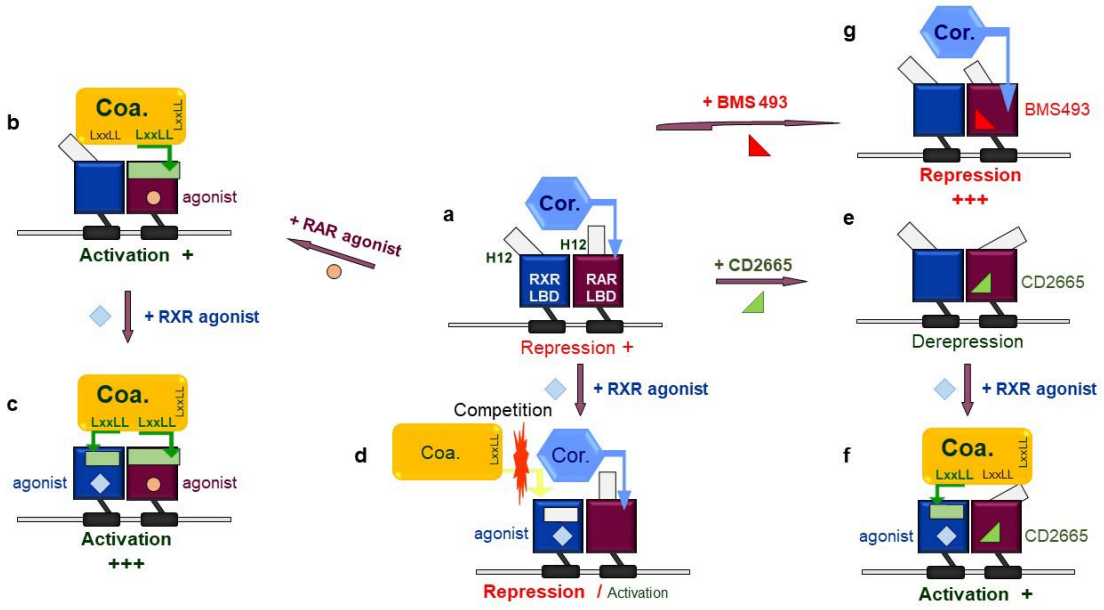

Figure 5: Model for RXR-RAR heterodimer function in the presence of various ligands. The cartoon depicts RXR-RAR bound to a DNA response element and interactions of both corepressor and coactivator as a function of the ligands bound to RAR or RXR or both. (a) In the absence of ligands RXR-RAR heterodimer is associated with a corepressor (Cor.) mainly bound to the RAR subunit. (b) Binding of an RAR agonist provokes a conformational change of RAR LBD such that the corepressor binding interface is disrupted and a novel surface is induced to which the coactivator (Coa.) can bind through a LxxLL motif. As a result RAR agonists dissociate corepressors and recruit coactivators, resulting in transactivation. (c) Further addition of an RXR-selective agonist creates a coactivator binding surface at the RXR LBD allowing a cooperative binding of two LxxLL motifs of one coactivator molecule. (d) In the absence of any RAR ligand an RXR agonist generates a potential coactivator binding interface at RXR LBD but does not enable the dissociation of the corepressor from the RAR subunit. Because of steric interference coactivator is unable to bind to the heterodimer, thus accounting for the so-called RXR subordination. (e) RAR antagonists such as CD2665 destabilize the corepressor interface without generating the surface for coactivator binding. As a result CD2665 is

transcriptionally inactive by its own. (f) By releasing corepressor from RAR CD2665 allows the recruitment of coactivator by the RXR subunit, resulting in transcriptional synergy. (g) Inverse agonists such as BMS493 can reinforce the interaction between the heterodimer and the corepressor that does enhance silencing.

Perspectives

Structurally, RXR-RAR heterodimers interact with coactivators or corepressors that are themselves part of large protein complexes, with enzymatic activities affecting the chromatin structure and the transcriptional output. Current structural studies on RXR-RAR coregulatory complexes are limited to domain of coactivator bound to the heterodimer. Challenging future studies should focus on the structural characterization of intact co-regulator multiprotein complexes with RXR-RAR to decipher the interconnected links between ligand, DNA, PTMs and co-regulator complexes. Beyond structural aspects, the complex mechanistic underpinning of the RXR-RAR heterodimer action needs further exploration. Notably, in addition to the canonical nuclear function of RARs to regulate transcription through the RXR-RAR heterodimer, nonconventional non-genomic effects of these proteins have been reported enlarging the spectrum of their biological activities. In response to ATRA, RARs integrate a variety of signaling pathways, notably through PTMs such as phosphorylation [10, 53-55]. In several cell types, a few percent of RAR proteins are present in the cytosol or in membrane lipid rafts in association with PI3K or Src kinases [56, 57]. ATRA binding to this membrane fraction of RARs rapidly provokes cascades of transient interaction and activation of various kinases, thereby propagating signals to cytosolic or nuclear machineries. These ATRA-activated kinases can phosphorylate nuclear proteins involved in the transcription of RXR-RAR target genes such as histones and co-regulators. Importantly, through both LBDs and AF-1 domains, RARs are substrates for various kinases activated by a variety of signals, but are also themselves phosphorylated by ATRA-activated kinases, affecting RAR binding to DNA, co-regulators recruitment, and degradation [58]. Accordingly, all these phosphorylation mechanisms likely cooperate to coordinate and adjust the dynamic exchanges between RARs, co-regulators and the promoters of target genes [59]. A current challenge is to decipher more precisely how RARs integrate membrane and cytoplasm events, and the crosstalk with the well-established genomic effects.

Such a quest can be achieved through global genomic technologies based on next generation deep sequencing methodology [60]. These approaches now make it possible to integrate global binding of transcription factors, epigenetic chromatin histone and DNA modification patterns with transcriptomes and three-dimensional chromatin structures. In this way genome-wide high throughput sequencing and chromatin immunoprecipitation coupled with deep sequencing contributed insights into how RXR-RAR heterodimers mediate transcriptional regulation [26, 27, 29-32, 61]. Noticeably several studies in various cell types have identified genome-wide RXR-RAR binding sites and gave us a more comprehensive RXR-RAR binding picture [31, 32]. Interestingly, the interaction of heterodimers with regions containing the DR0 motif is predominant in undifferentiated pluripotent embryonic cells [32], while in differentiated cells such as MCF-7, the RXR-RAR bound regions are characterized by a higher frequency of the canonical DR5 sequence [27], highlighting the reorganization of the RXR-RAR binding repertoire during the differentiation process. Further studies on these newly identified RAREs should elucidate their relevance to RXR-RAR function and whether they may be associated with particular transcription factor binding patterns, epigenetic markings and chromatin structures.

Another intriguing fact we learned from the global genomic data analyses is that RAR binding is dynamically regulated by ligand treatment or cell differentiation status [30, 32]. These data revealed the dynamic occupancy of chromatin by pre-existing and de novo recruited RXR-RAR heterodimers, as well as heterodimer replacement or even partner swaps [32]. The observed ATRA-dependent RAR subunit recruitment may therefore likely reflect the increased ability of RXR-phospho-RAR heterodimers to interact with their target DNA sequences [62]. Furthermore, although RXR-RAR heterodimers can bind constitutively to target sites, the massive increase in the number of RXR-RAR bound genomic loci after ATRA stimulation calls into question the classical model for the heterodimer action postulating that a major effect of ligand-dependent activation is to convert preexisting DNA-bound heterodimers from an inhibitory to an activating complex (Fig. 1), as well the widely accepted mechanism of repression by unliganded RXR-RAR. The molecular determinants of such various mechanisms remain to be explored.

This last observation highlights that, while the above genome-wide experiments, although useful, determine average properties of the RXR-RAR signaling in the context of cell population at single

time points, our understanding of RXR-RAR transcriptional action at the level of individual cells or individual target gene alleles remains broadly incomplete. The development of novel single-cell and single-molecule imaging technologies has repositioned biological questions on gene expression [63] and recent works dealing with other NRs have started addressing this issue [64, 65]. These experiments revealed the stochastic nature of transcription which occurs predominantly as burst, but the mechanisms regulating this process are unclear [66]. Together, with recent advances in CRISPR-Cas technology that have greatly facilitated genome editing and RNA labeling for live cell imaging [67], these imaging methods should be applied to RXR-RAR to explore the relationship between ligand binding, heterodimer dynamics, co-regulator association/ disassociation, and RNA bursting. This approach may isolate and question the behavior and influence of each of the regulators of the RXR-RAR signaling on the transcriptional output. Overall, future challenges are to connect data from new, highly sensitive technologies, in real time or on a large scale, to obtain critical new information on the influence of DNA- and ligand-binding, and phosphorylation processes on the regulation of RXR-RAR heterodimer activities.

Author contributions

All authors contributed to writing the manuscript and preparing the Figures.

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Table 1. Chemical structures of the retinoids cited in this review.

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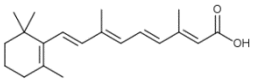
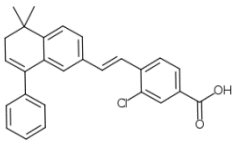
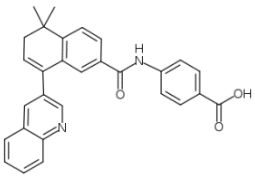
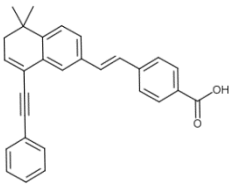
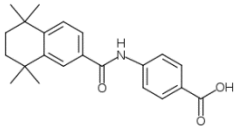
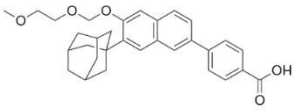
 all-*trans* retinoic acid (ATRA)	 BMS641	 BMS614	 BMS493
 AM580		 CD2665	