



HAL
open science

Design and in vitro characterization of RXR variants as tools to investigate the biological role of endogenous rexinoids

Albane E Le Maire, Martial Rey, Valérie Vivat, Laura Guée, Pauline Blanc, Christian Malosse, Julia Chamot-Rooke, Pierre Germain, William Bourguet

► **To cite this version:**

Albane E Le Maire, Martial Rey, Valérie Vivat, Laura Guée, Pauline Blanc, et al.. Design and in vitro characterization of RXR variants as tools to investigate the biological role of endogenous rexinoids. 2024. <hal-04707267>

HAL Id: hal-04707267

<https://hal.science/hal-04707267v1>

Preprint submitted on 24 Sep 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC 4.0 - Attribution - Non-commercial use - International License



**JOURNAL OF
MOLECULAR
ENDOCRINOLOGY**



Design and in vitro characterization of RXR variants as tools to investigate the biological role of endogenous rexinoids

Journal:	<i>Journal of Molecular Endocrinology</i>
Manuscript ID	JME-22-0021.R1
mstype:	Research paper
Date Submitted by the Author:	24-Jun-2022
Complete List of Authors:	le Maire, Albane; CNRS, Centre de biologie structurale Rey, Martial; Pasteur Institute, Biologie Structurale et Chimie Vivat, Valérie; IGBMC, Génomique fonctionnelle et cancer Guée, Laura; Inserm, Centre de biologie structurale Blanc, Pauline; Inserm, Centre de biologie structurale Malosse, Christian; Institut Pasteur, Biologie Structurale et Chimie Chamot-Rooke, Julia; Institut Pasteur, Biologie Structurale et Chimie Germain, Pierre; Inserm, Centre de biologie structurale Bourguet, William; Inserm, Centre de Biologie Structurale
Keywords:	Nuclear receptors, Retinoids, Ligand, Structure/function, Protein design

SCHOLARONE™
Manuscripts

1 **Design and *in vitro* characterization of RXR variants as tools to investigate the biological**
2 **role of endogenous rexinoids**

3
4 Albane le Maire¹, Martial Rey², Valérie Vivat^{3,*}, Laura Guée¹, Pauline Blanc¹, Christian Malosse², Julia
5 Chamot-Rooke², Pierre Germain¹, and William Bourguet¹

6
7 ¹CBS (Centre de Biologie Structurale), Univ Montpellier, CNRS, Inserm, Montpellier, France

8 ²Institut Pasteur, Université de Paris, CNRS USR2000, Mass Spectrometry for Biology Unit, 75015
9 Paris, France

10 ³IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), Univ Strasbourg, CNRS,
11 Inserm, Illkirch, France

12

13 **Correspondence to P Germain or W Bourguet:** germain@cbs.cnrs.fr or bourguet@cbs.cnrs.fr

14

15

16 ***Current address:** Flare Therapeutics, 215 First Street, Suite 150, Cambridge, MA 02142, USA

17

18

19 **Keywords:** Nuclear receptors, ligand, retinoids, structure-function, protein design

20

21

22

23 **Short Title:** Design and characterization of RXR-based tools

24

25

26

27

28

29

30 Abstract

31 Retinoid X receptors (RXR α , β , and γ) are essential members of the nuclear receptor (NR) superfamily
32 of ligand-dependent transcriptional regulators that bind DNA response elements and control the
33 expression of large gene networks. As obligate heterodimerization partners of many NRs, RXRs are
34 involved in a variety of pathophysiological processes. However, despite this central role in NR signaling,
35 there is still no consensus regarding the precise biological functions of RXRs and the putative role of
36 the endogenous ligands (rexinoids) previously proposed for these receptors. Based on available crystal
37 structures, we introduced a series of amino acid substitutions into the ligand-binding pocket of all three
38 RXR subtypes in order to alter their binding properties. Subsequent characterization using a battery of
39 cell-based and *in vitro* assays led to the identification of a double mutation abolishing the binding of any
40 ligand while keeping the other receptor functions intact, and a triple mutation that selectively impairs
41 interaction with natural rexinoids but not with some synthetic ligands. We also report crystal structures
42 that help understand the specific ligand-binding capabilities of both variants. These RXR variants, either
43 fully disabled for ligand binding or retaining the property of being activated by synthetic compounds,
44 represent unique tools **that could be used in future studies** to probe the presence of active endogenous
45 rexinoids in tissues/organs and to investigate their role *in vivo*. Last, we provide data suggesting a
46 possible involvement of fatty acids in the weak interaction of RXRs with corepressors.

47

48

49

50 **Introduction**

51 The three retinoid X receptor subtypes (RXR α , β , and γ ; NR2B1-3 according to the official
52 nomenclature) are essential members of the nuclear receptor (NR) superfamily acting as obligatory
53 heterodimerization partners of many family members (Evans and Mangelsdorf 2014; Germain, et al.
54 2006; Gilardi and Desvergne 2014). Similar to other NRs, RXRs exhibit a modular structure with several
55 domains, notably a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain
56 (LBD). Numerous 3D structures of NR LBDs, **including in the context of the nearly full-length receptor**
57 **(Chandra, et al. 2017)**, have corroborated the multifunctional nature of this domain, which controls not
58 only ligand binding into the ligand-binding pocket (LBP) and receptor dimerization but also the
59 interaction with transcriptional corepressors or coactivators through a ligand-dependent activation
60 function (Weikum, et al. 2018).

61 RXRs are expressed in virtually every tissue of the body but disparities are observed in their
62 distribution (Germain et al. 2006). RXR α is predominantly expressed in skin, epidermis, intestine, liver,
63 lung, and kidney. RXR β is ubiquitously expressed, whereas RXR γ is mostly restricted to the skeletal
64 muscle and certain part of the brain. By virtue of their ability to form heterodimers with other NRs such
65 as retinoic acid (RARs), thyroid hormone (TRs), peroxisome proliferator-activated (PPARs), or vitamin
66 D3 (VDR) receptors, RXRs control a variety of pathophysiological processes (Evans and Mangelsdorf
67 2014; Mark, et al. 2006; Mark, et al. 2015). The central role of RXRs makes it a potential therapeutic
68 target for treatment of diseases like cancer and metabolic diseases (Dawson and Xia 2012). Accordingly,
69 major research efforts have been directed towards the identification of potent synthetic molecules acting
70 as modulators of RXR transcriptional activity. They led to the development of several classes of
71 compounds with a panel of activities ranging from agonists to antagonists through partial agonists, with
72 some of them even used in clinical trials (Dominguez, et al. 2017; Perez, et al. 2012; Schierle and Merk
73 2019).

74 Although RXRs occupy a central position in NRs signaling, the existence of RXR endogenous
75 ligands and the putative physiological functions of such ligands have remained a matter of debate. Initial
76 studies led to the discovery of 9-*cis*-retinoic acid (9CRA) as a ligand for all three RXR **and RAR**
77 subtypes (Heyman, et al. 1992; Levin, et al. 1992). But, except for the pancreas, it is not detected in cells

78 (Arnold, et al. 2012; Jones, et al. 2015). A number of fatty acids and retinoic acid analogues (**Fig. 1**)
79 have since been proposed to be physiologically relevant endogenous ligands of RXRs, including
80 phytanic acid (Kitareewan, et al. 1996), methoprene acid (Harmon, et al. 1995), and a series of
81 unsaturated fatty acids such as docosahexaenoic acid (DHA) (de Urquiza, et al. 2000; Goldstein, et al.
82 2003; Lengqvist, et al. 2004; Niu, et al. 2017). However, their status as *bona fide* endogenous RXR
83 ligands could be questioned with regard to their low abundance, very low affinity for RXR and/or weak
84 transactivation capacity, or because they were identified in very specific tissues. The saturated analogue
85 of 9CRA, 9-*cis*-13,14-dihydroretinoic acid (9CDHRA) present at high concentrations in mice,
86 particularly in the serum and liver (Ruhl, et al. 2015), was recently proposed as an endogenous
87 physiologically relevant mammalian RXR ligand. **Note that 9CDHRA also activates RARs at high**
88 **concentration** (de Lera, et al. 2016; Krezel, et al. 2021; Krzyzosiak, et al. 2021; Ruhl, et al. 2018; Ruhl
89 et al. 2015). *In vivo* data in mice suggest that the molecule could be involved in the control of stress-
90 adaptation and depressive-like behaviors through RXR-mediated signaling (Krzyzosiak et al. 2021; Ruhl
91 et al. 2015). However, the putative role of 9CDHRA in a broader physiological context has not been
92 explored yet, so the questions regarding the existence, the biological function and the target tissues of a
93 universal RXR physiological ligand still remain.

94 Based on structural information and using biochemical and cell-based assays, we designed two RXR
95 variants that will help answer several crucial questions regarding its endogenous ligand(s). Does RXR
96 principally serve as a heterodimerization partner allowing DNA binding specificity and signaling
97 through the partner receptors or does it require natural activating ligands to achieve its physiological
98 functions? What are the tissues/organs in which an endogenous RXR ligand is functionally required?
99 What are the biological functions of endogenous rexinoids? Prototypical rexinoids (*i.e.* RXR synthetic
100 or natural ligands) proposed so far to act as endogenous ligands contain a carboxylic head group
101 involved in a salt bridge with an arginine residue of the helix H5 in the RXR LBD, and a long aliphatic
102 chain making numerous van der Waals contacts with essentially non-polar residues distributed all over
103 the LBP of RXR (see examples in **Fig. 1**). In contrast, synthetic rexinoids generally harbor aromatic
104 rings, notably one bearing the carboxylic moiety such as in bexarotene, BMS649 or LG268 (**Fig. 1**). By
105 introducing a series of amino acid substitutions into the LBP of RXRs, we identified a variant with fully

106 impaired ligand-binding activity (hereafter termed 2mRXRs), and a second one responding to some
107 synthetic ligands only (hereafter termed 3mRXRs). Extended structural and functional analyses of these
108 RXR variants revealed the structural bases for their novel ligand-binding properties, and showed that
109 the other functions of the receptor, including DNA-binding and dimerization, remain mostly unchanged.
110 We propose that mouse lines expressing these RXR variants that have partially (the transcriptional
111 activity of 3mRXR can be rescued by synthetic compounds) or totally (2mRXR) lost their ligand-
112 binding capacity would represent unique tools to probe the pathophysiological function(s) and the site(s)
113 of action of endogenous RXR ligand(s) in the whole animal. We also provide data suggesting a possible
114 role of fatty acids in the weak interaction between RXRs and corepressors.

115

116 **Materials and Methods**

117 **Ligands and peptides**

118 Ligands (CD3254, BMS649, LG268, Bexarotene and TTNPB) were purchased from Tocris Bioscience.
119 9CRA, DHA, phytanic acid and methoprene acid were purchased from Sigma-Aldrich. 9CDHRA was
120 a kind gift from Angel de Lera (University of Vigo, Spain). All compound stock solutions were prepared
121 at 10 mM in DMSO. The SRC-1 peptide labelled with FITC (FITC-LTERHKILHRLQLQEGSP), the
122 TIF-2 peptide (KHKILHRLQLQDSS) and the corepressor NCoR peptide labelled with FITC (FITC-
123 DPASNLGLEDIIRKALMGSGFD) were purchased from EZbiolab.

124

125 **Plasmids**

126 The DNA encoding mouse wtRXR α , 2mRXR α , 3mRXR α , mouse wtRXR β , 2mRXR β , 3mRXR β , and
127 mouse wtRXR γ , 2mRXR γ , 3mRXR γ were cloned (InFusion cloning kit) into a pSG5 puro vector
128 between the BamHI and BglII restriction sites.

129

130 **Transactivation assays**

131 COS-1 cells were grown in DMEM with Glutamax and 10% (v/v) FCS, and co-transfected with the
132 DR1-tk-Luc reporter gene and the expression vector of one of the different RXRs studied using JetPei
133 transfectant (Ozyme). After 24 h, the medium was changed to a medium containing the indicated ligands

134 or vehicle. Cells were lysed and assayed for reporter expression 48 h after transfection. The luciferase
135 assay system was used according to the manufacturer's instruction (Promega). In each case, results were
136 normalized to coexpressed β -galactosidase. Each transfection was performed in duplicate and repeated
137 three to six times.

138

139 **Preparation of RXR α variants for *in vitro* studies**

140 For the preparation of 2mRXR α and 3mRXR α variants, the DNA encoding mouse RXR α including the
141 two (R321E/C437Q) and the three (I315A/F318A/A332T) mutations, respectively, optimized for
142 bacterial expression, purchased to Integrated DNA Technologies were cloned using InFusion cloning
143 kit into a pDB vector between the NdeI and XhoI restriction sites. pDB-2mRXR α and pDB-3mRXR α
144 constructs code for proteins containing a hexa histidine-tag and a HRV 3C cleavage site (Leu-Glu-Val-
145 Leu-Phe-Gln/Gly-Pro) at the N terminus, where a specific cleavage occurs between Gln and Gly.

146

147 **Expression and purification of proteins**

148 The wtRXR α LBD protein was purified as previously described (Nahoum, et al. 2007). The 2mRXR α
149 and 3mRXR α proteins were overproduced in *E. coli* BL21 (DE3) grown at 37°C until OD_{600nm} reaches
150 0.6 and overnight at 20° (for 2mRXR α) or for additional three hours at 37°C (for 3mRXR α), after
151 expression induction with 0.5 mM IPTG. Cells were harvested and resuspended in 500 mM NaCl, 50
152 mM Tris pH 7.5, plus one tablet of Complete EDTA free protease inhibitor cocktail and lysozyme at 10
153 μ g/ml and sonicated on ice. The lysate was centrifuged at 18,000 rpm at 4 °C for 30 min. The supernatant
154 was clarified and loaded on a 5 ml HisTrap Column (Cytiva) equilibrated with 500 mM NaCl, 50 mM
155 Tris pH 7.5, 10 mM imidazole. The column was washed with the latter buffer during 10 column volumes
156 (CV) and with 500 mM NaCl, 50 mM Tris pH 7.5, 50 mM imidazole during 10 CV and the protein was
157 eluted with 500 mM NaCl, 50 mM Tris pH 7.5, 250 mM imidazole. Fractions containing the protein
158 were pooled and incubated with 3C protease overnight. The cleaved protein was concentrated and loaded
159 on a HiLoad 26/60 Superdex 75 PG (GE Healthcare) equilibrated with 150 mM NaCl, 20 mM Tris pH
160 7.5, 10% glycerol and 2mM DTT. The pure fractions containing proteins were pooled and concentrated

161 by centrifugation using a Vivaspin™ protein concentrator with a 10 kDa cut-off until concentration,
162 determined by UV absorbance at 280 nm, reaches 8 mg/mL.

163

164 **Size-exclusion chromatography-multi angle light scattering (SEC-MALS)**

165 SEC-MALS experiment was performed at 25°C using a Superdex 200 10/300 GL column (GE
166 HealthCare) connected to a miniDAWN-TREOS light scattering detector and an Optilab T-rEX
167 differential refractive index detector (Wyatt Technology, Santa Barbara, CA). The column was
168 equilibrated with 0.1 M filtered 50mM Tris pH7.5, 150mM NaCl buffer. A sample of 25 µL of RXRα
169 protein (wtRXRα, 3mRXRα or 2mRXRα LBDs), alone or pre-equilibrated with an equal molar excess
170 of purified TRα1 LBD, at 1 mg/mL (around 50 µM) was injected at 0.5 mL/min. Data acquisition and
171 analyses were performed using the ASTRA software (Wyatt). Based on measurement on BSA sample
172 under the same conditions, we have estimated an experimental error in molar mass around 5%.

173

174 **Thermal shift assay (TSA)**

175 Solutions of 25 µL containing 5 µM of RXRα protein, in the presence of DMSO (condition without
176 ligand) or in the presence of three molar excess of ligands, and 1X Sypro® Orange in 50 mM Tris pH
177 8.0, 200 mM NaCl, 5% glycerol were added to the wells of a 96-well PCR plate. The plates were sealed
178 with an optical sealing tape (Bio-Rad) and heated in a 7500 Real Time PCR system (Applied Biosystem)
179 from 25 to 95°C at 1°C intervals. Fluorescence changes in the wells were monitored with a
180 photomultiplier tube. The wavelengths for excitation and emission were 545 nm and 568 nm,
181 respectively. The melting temperatures (T_m) were obtained by fitting the fluorescence data with a
182 Boltzmann model using the GraphPad Prism software.

183

184 **Steady-state fluorescence anisotropy**

185 Measurements of the binding affinities of the fluorescent SRC-1 NR2 peptide for RXRα LBDs in the
186 presence or absence of ligands was performed using a Safire2 microplate reader (TECAN). The
187 excitation wavelength was set at 470 nm and emission was measured at 530 nm. The buffer solution for
188 assays was 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and 10% (v/v) glycerol.

189 The measurements were initiated at 10 μ M of protein, and the sample was then diluted successively by
190 a factor of 2 with the buffer until the lowest protein concentration at 2.4 nM. Fluorescent peptides were
191 added to protein samples at 4 nM, allowing establishment of the titration curve. Ligands, when added,
192 were at a final concentration of three molar excess relative to the highest concentration of protein. The
193 reported data are the average of at least three independent experiments, and error bars correspond to
194 standard deviations.

195

196 **Native mass spectrometry**

197 Proteins were mixed with each ligand at a 1:5 molar ratio in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5
198 mM DTT. Protein-ligand samples were then buffer exchanged twice using a biospin 6 (Bio-Rad) against
199 200 mM ammonium acetate buffer. Then protein-ligand complexes were nanoelectrosprayed in native
200 conditions using a Triversa NanoMate (Advion) coupled to a Tribrid Eclipse Orbitrap mass spectrometer
201 (ThermoFisher Scientific). Spectra were recorded at 15K resolution (at m/z 200) with 20 microscans per
202 scan using the intact protein mode in normal pressure mode. The In-Source Collision Induced
203 Dissociation (IS-CID) energy was varied from 0 V to 60 V with 10 V steps to promote dissociation of
204 the ligand from the protein. Ratios between the ligand-bound and free monomeric protein (10+ ion) were
205 measured for each IS-CID energy and compared for each ligand. Spectra were acquired between 600
206 and 3000 m/z .

207

208 **Crystallization and structure resolution**

209 The 2mRXR α and 3mRXR α proteins concentrated at 8 mg/ml were mixed with a five-fold molar excess
210 of LG268 or BMS649, respectively, and a three-fold molar excess of the TIF-2 NR2 peptide. We mixed
211 the complexes in a 1:2 ratio with reservoir solution consisting of 26% (w/v) PEG 3350, 0.2 M NaF or
212 18% (w/v) PEG 3350, 0.2 M Na Nitrate for 2mRXR α and 3mRXR α complexes, respectively, and
213 crystallized the complexes using the sitting-drop vapor diffusion technique. We mounted a single crystal
214 from mother liquor onto a cryoloop, soaked in the reservoir solution containing an additional 25% (v/v)
215 glycerol and flash-frozen it in liquid nitrogen. We collected diffraction data at the PXI beamline of SLS
216 at 1.58 \AA resolution for 2mRXR α complex and at XALOC beamline of ALBA at 3.30 \AA resolution for

217 3mRXR α complex. We processed diffraction data using XDS (Kabsch 2010) and scaled them with
218 SCALA from the CCP4 program suite (Winn, et al. 2011). We obtained the initial phases by molecular
219 replacement with Phenix (Liebschner, et al. 2019) using the structure of the wt RXR α in complex with
220 BMS649 (Egea, et al. 2002) (PDB 1MVC). Initial Fo – Fc difference maps had strong signal for the
221 ligand and for the peptide, which we could fit accurately into the electron density. We built the model
222 with Coot (Emsley and Cowtan 2004) and refined it with Phenix. The RXR α -RAR α LBD heterodimer
223 was purified and crystallized as described previously (Bourguet, et al. 2000a). The structure was solved
224 by molecular replacement using the structure of the mutant RXR α F318A-RAR α LBD heterodimer (PDB
225 1dkf). (Bourguet, et al. 2000b). Data collection and refinement statistics are summarized in
226 **Supplementary Table 1**. Figures were prepared with PyMOL (<http://pymol.org/>).

227

228 **Results**

229 **Design of RXR variants**

230 Guided by the many RXR LBD crystal structures available and our structure of the RXR α -RAR α LBD
231 heterodimer where the RXR α and RAR α protomers are bound to a fortuitous bacterial fatty acid and the
232 antagonist BMS614, respectively (**Supplementary Fig. 1**), we introduced a series of mutations in the
233 mouse RXR α LBP with the aim to disable ligand-binding without altering other receptor functions (**Fig.**
234 **2A**). Note that there is no species or subtype difference in the amino acid composition of RXR LBP so
235 that the results obtained here on mouse RXR α can be directly extended to all mouse and human receptor
236 subtypes. Single and multiple substitutions were introduced in wild-type (wt) RXR α so as to remove
237 essential ligand/receptor interactions or to generate steric hindrances precluding the formation of a stable
238 ligand/RXR α complex. Two RXR α variants for which full functional and structural characterizations
239 are provided below appeared of particular interest to us with regard to their specific ligand binding
240 profiles. One, referred to as 2mRXR α , contains two mutations, namely R321E and C437Q (mouse
241 numbering). Located in helix H5, R321 plays a key role in forming a salt bridge with the carboxylic
242 head group of rexinoids (**Figs 1 and 2A**) so that its replacement by a glutamic acid residue was expected
243 to reduce ligand binding affinity. On the other side of the LBP (helix H11), the small cysteine residue
244 to larger glutamine amino acid substitution was introduced so as to generate steric hindrance and further
245 decrease ligand binding. This second mutation was chosen based on previous work showing that the
246 mutation of human RXR α C432 (equivalent to C437 in mouse) into bulky aromatic residues (*i.e.*
247 tryptophan or tyrosine) resulted in constitutively active RXR α variants, while the mutation into a
248 glutamine residue had no impact on the basal activity of the receptor but was detrimental to 9CRA
249 binding (Zeng, et al. 2015). The second RXR α variant, referred to as 3mRXR α , contains three mutations,
250 namely I315A, F318A, and A332T (**Fig. 2A**). The two small alanine residues were introduced at
251 positions 315 and 318 to remove the essential hydrophobic stabilizing contacts provided by the bulky
252 isoleucine and phenylalanine amino acids, respectively. Last, located at the tip of the S1/S2 β -turn and
253 in very close proximity to the carboxylic head group of rexinoids, alanine residue 332 was substituted
254 for a slightly larger threonine amino acid with the goal to negatively interfere with ligand binding. A

255 battery of cell-based and *in vitro* assays was subsequently used to perform a full characterization of the
256 ligand responsiveness of these RXR α variants.

257

258 **Functional characterization of the 2m- and 3mRXR variants**

259 *The ligand-binding function is fully impaired in 2mRXRs*

260 First, reporter gene assays were conducted in COS-1 cells to assess the transcriptional activity of wt and
261 RXR α variants in the presence of various synthetic and natural compounds previously proposed as
262 RXRs endogenous ligands. As shown in **Fig. 2B**, wtRXR α could be potently activated by LG268 and
263 CD3254, slightly less by bexarotene, and to a lower extent by BMS649, 9CRA and finally 9CDHRA.
264 Note that 9CDHRA was almost as active as 9CRA at 10 μ M, whereas all the other natural compounds
265 displayed no activity at this concentration (**Supplementary Fig. 2A**). A similar experiment using the
266 2mRXR α variant revealed that none of the compounds tested were able to induce a substantial
267 transcriptional response below 1 μ M, although little RXR α activation could be observed with LG268 at
268 1 μ M (**Fig. 2C**). As expected, the mutations produced the same functional alteration when introduced
269 in RXR β and RXR γ (**Supplementary Fig. 2B**).

270 Using fluorescence anisotropy measurements, we then evaluated the ability of the various ligands to
271 induce the recruitment by wtRXR α and 2mRXR α of a fluorescein-labeled peptide derived from the
272 coactivator SRC-1 and encompassing one LxxLL nuclear receptor interacting motif (hereafter referred
273 to as SRC-1). Consistent with transactivation assays, the synthetic ligands and 9CRA were able to
274 strongly increase the affinity of wtRXR α for SRC-1, whereas 9CDHRA or phytanic acid (PA)
275 strengthened the interaction only slightly (**Fig. 2D**). Results obtained with 2mRXR α were also in line
276 with cell-based data as all the compounds used failed to induce a robust recruitment of the coactivator-
277 derived peptide (**Fig. 2E**).

278 Because the aforementioned data suggested that 2mRXR α is unable to bind any kind of ligand, we
279 evaluated the direct interaction of the compounds with wtRXR α and 2mRXR α using thermal shift assay
280 (TSA) and differential scanning fluorimetry (nanoDSF) that monitor the thermal stabilization of the
281 receptor upon ligand binding. In full agreement with cell-based and fluorescence assays, all the synthetic
282 ligands were able to induce an increase of the melting temperature (T_m) of wtRXR α , whereas every

283 natural compound but 9CRA, and to a lesser extent 9CDHRA, failed to do so (**Fig. 2F** and
284 **Supplementary Fig. 2C**). Regarding 2mRXR α stabilization, none of the synthetic or natural compounds
285 induced a stabilization of the protein, even at high ligand/protein ratios (**Fig. 2G** and **Supplementary**
286 **Fig. 2D**). **Although 2mRXR α and wtRXR α have similar secondary structure content (Supplementary**
287 **Fig. 3), the decreased T_m observed for the unliganded 2mRXR α relative to that of wtRXR α could be**
288 **partly due to a slight destabilizing effect of the mutations, but more likely to** the fact that the bacterially
289 expressed wtRXR α LBD co-purifies, and co-crystallizes (**Fig. 2A**), with a fatty-acid of bacterial origin
290 that stabilizes the protein domain. Such binding is precluded in 2mRXR α . Last, the interaction of
291 wtRXR α and 2mRXR α with the synthetic compounds BMS649, LG268 and CD3254 was characterized
292 by using native mass spectrometry. Native mass spectrometry (MS) has the capability to directly detect
293 ligand binding to protein, determine stoichiometry, relative binding affinities and specificity (Heck
294 2008). As expected from the previous experiments, incubation of the rexinoids with wtRXR α resulted
295 in the formation of binary protein-ligand complexes, but not with the 2mRXR α variant (**Fig. 3A, B** and
296 **Supplementary Fig. 4**). Note that nonspecific binding outside the RXR LBP may account for the small
297 amount of binary complex detected with 2mRXR α and CD3254, as a very similar signal was obtained
298 with 3mRXR α (**Fig. 3B, C**).

299 As a whole, these data show that while synthetic ligands act as potent wtRXR α agonists with EC₅₀s
300 ranging from 3 nM to 58 nM, all the natural compounds but 9CRA (EC₅₀ of 130 nM) and 9CDHRA (1
301 $\mu\text{M} < \text{EC}_{50} < 10 \mu\text{M}$) exert weak activation properties, if any (EC₅₀s $> 10 \mu\text{M}$). They also demonstrate
302 that 2mRXRs have lost most of their capabilities to bind natural and synthetic rexinoids.

303

304 *3mRXRs respond exclusively to benzoic acid-containing synthetic compounds*

305 As with 2mRXR α , the cell-based, *in vitro* assays and native MS experiments described above were
306 applied to 3mRXR α in order to reveal its ligand responsiveness. Reporter gene assays in COS-1 cells
307 revealed that LG268, bexarotene and BMS649 retained a strong agonistic activity, whereas all the
308 natural compounds, including 9CRA, and unexpectedly the synthetic compound CD3254 did not induce
309 any measurable transcriptional response, up to a concentration of 1 μM (**Fig. 4A**). As expected, the
310 mutations produced the same effects when introduced in RXR β and RXR γ (**Supplementary Fig. 5A**).

311 Similar observations were made in coactivator recruitment experiments using fluorescence anisotropy
312 where only the three synthetic compounds LG268, bexarotene and BMS649 were able to increase the
313 affinity of 3mRXR α for SRC-1 (**Fig. 4B**). CD3254 and all the natural compounds failed to induce a
314 recruitment of the coactivator-derived peptide above that obtained with unliganded 3mRXR α . Tm
315 measurements in presence of the various compounds confirmed that all the synthetic compounds but
316 CD3254 interact with 3mRXR α , thereby increasing its thermal stability. In contrast, none of the natural
317 compounds tested bind to this RXR α variant (**Fig. 4C** and **Supplementary Fig. 5B**). Last, MS analysis
318 confirmed that in contrast to wtRXR α , 3mRXR α is unable to form a stable binary complex with
319 CD3254, whereas a good interaction with BMS649 and LG268 is preserved (**Fig. 3C**).

320 Interestingly, a close look at the chemical differences between 3mRXRs binders (LG268, bexarotene
321 and BMS649) and non-binders (natural compounds and CD3254), revealed that members of the latter
322 group of compounds contain an aliphatic chain next to the carboxylate moiety, whereas compounds
323 belonging to the other group harbor an aromatic benzoic acid instead (**Fig. 1**). Altogether, these
324 functional data show that we could identify (i) RXR α , β , γ variants whose ligand-binding function is
325 fully impaired (2mRXRs), and (ii) RXR α , β , γ variants that fail to bind natural compounds, whose
326 hallmark is the presence of an aliphatic chain bearing the carboxylate moiety, but can be activated by
327 synthetic compounds harboring a benzoic acid group (3mRXRs).

328

329 *2mRXRs and 3mRXRs retain their DNA-binding and dimerization capabilities*

330 In addition to providing information regarding the ligand responsiveness of wtRXR α and its variants,
331 the transactivation assays, that uses full-length receptors and the direct repeat 1 (DR1) response element
332 of RXR homodimers, also reveals their DNA-binding and homodimerization capabilities. Mutations
333 located in the LBP of RXRs are unlikely to impact these functions, and accordingly, the DNA- and
334 dimerization-dependent signals observed for 3mRXR α in COS-1 cells with some synthetic ligands (**Fig.**
335 **4A**) and to a lesser extent for 2mRXR α with LG268 (**Fig. 2C**) suggested preserved DNA-binding and
336 self-association functions in RXR variants.

337 Having shown the ligand-binding specificities and the DNA-binding and homodimerization
338 capabilities of the two RXR variants, we then evaluated the impact of the mutations on the ability of

339 2mRXR α and 3mRXR α to form heterodimers. To this end, the oligomeric states of wtRXR α , 2mRXR α
340 and 3mRXR α LBDs, alone and in complex with TR α 1 LBD, were determined using electrophoresis
341 under non-denaturing conditions (**Fig. 5A**). For individual RXR α proteins, two predominant bands
342 corresponding to monomers and homotetramers were observed. An additional band indicating the
343 presence of homodimers was apparent with 3mRXR α . In the presence of the heterodimerization partner
344 TR α 1, bands of similar intensities corresponding to heterodimers were clearly visible for wtRXR α ,
345 2mRXR α and 3mRXR α . Independent confirmation of the heterodimerization abilities of wtRXR α and
346 the RXR α variants was brought by size-exclusion chromatography-multi angle light scattering (SEC-
347 MALS) analysis (**Fig. 5B**). All three RXR α /TR α 1 mixtures eluted similarly at the elution volume of
348 heterodimers, in addition to the elution peak corresponding to monomers.

349 As expected, these results show that the mutations do not modify the DNA-binding and dimerization
350 properties of 2mRXR α and 3mRXR α , and confirm that heterodimerization does not require the presence
351 of RXR ligand to occur.

352

353 **Structural characterization of the 2m- and 3mRXR α variants**

354 To gain insights into the structural basis of RXR variants ligand-binding specificities, we solved the
355 crystal structures of the ternary complexes containing the 2mRXR α or 3mRXR α LBDs bound to a
356 coactivator (TIF-2)-derived peptide and the agonists LG268 or BMS649, respectively (**Supplementary**
357 **Table 1**). Both structures show the canonical active conformation with the C-terminal activation helix
358 H12 capping the LBP and the TIF-2 peptide bound to the surface formed by residues from helices H3,
359 H4 and H12 (**Supplementary Fig. 6**). The ligands LG268 and BMS649 could be unambiguously
360 positioned in their respective electron densities (**Supplementary Fig. 6**). Importantly, our ability to
361 obtain these structures is an additional proof that the introduced mutations do not alter proper protein
362 folding.

363

364 *Structure of LG268-bound 2mRXR α*

365 Although our functional analysis demonstrated that the ligand-binding capacity of 2mRXR α is largely
366 impaired, the high protein and ligand concentrations used during the crystallization process allowed

367 formation of the complex. Comparison of the structure with that of human wtRXR β in complex with
368 LG268 (PDB 1H9U) (Love, et al. 2002) shows that the two LG268 ligands superimpose well with no
369 major repositioning (**Fig. 6A**). In contrast to wtRXR β R387 (equivalent to R321 in mouse RXR α) that
370 points towards the LBP to form a salt bridge with the carboxylate moiety of the ligand, E321 in 2mRXR α
371 turns around and points to the solvent. Water molecules occupy the space left vacant by the R321E
372 mutation. In helix H11, Q437 that replaces a cysteine residue in wtRXR resides at a van der Waals
373 distance to LG268 and appears to be well accommodated following a few side chain rearrangements,
374 notably L438 and L441 (**Fig. 6A**). Clearly, the R321E mutation is the major contributor to the binding
375 impairment of LG268 to 2mRXR α . However, when superimposing the 2mRXR α structure with that of
376 human wtRXR α bound to 9CRA (PDB 1FBY) (Egea, et al. 2000), one can see that the latter occupies
377 more space in the helix H11 region than the synthetic compounds so that the presence of a large
378 glutamine residue at the position normally occupied by a small cysteine amino acid generates a severe
379 steric clash with the ligand (**Fig. 6B**). A similar situation can be seen with several ligands, including
380 CD3254 (PDB 3FUG) (Perez Santin, et al. 2009) (**Fig. 6C**) or 9CDHRA (PDB 4ZSH) (Ruhl et al. 2015)
381 (**Fig. 6D**). Overall it appears that both R321E and C437Q mutations play a role in the ligand-binding
382 disability of 2mRXR α , their relative involvements varying according to the chemical structure of the
383 compounds.

384

385 *Structure of BMS649-bound 3mRXR α*

386 Our functional analysis demonstrated that benzoic acid-containing synthetic compounds such as
387 BMS649 can bind to and activate 3mRXR α . Comparison of the structure with that of human wtRXR α
388 in complex with BMS649 (PDB 1MVC) (Egea et al. 2002) shows that the BMS649 molecules in the
389 two structures are not fully superimposable. Both the position and the conformation of the ligands are
390 slightly different in the two structures (**Fig. 7A**). Due to the steric hindrance generated by the A332T
391 mutation, and to the space made available by the I315A and F318A mutations, BMS649 in 3mRXR α is
392 shifted towards helices H5, H7 and H11, and the benzoic acid group is tilted by roughly 50° relative to
393 its orientation in wtRXR α . This rotation appears essential to maintain important stabilizing interactions
394 between the ligand and surrounding residues from helices H3 and H5 (**Fig. 7B**). Such adaptation is not

395 possible for natural compounds and CD3254 where the phenyl ring of the benzoic acid group is replaced
396 by a rigid aliphatic chain. Furthermore, by superimposing the 3mRXR α structure with that of human
397 wtRXR α bound to 9CRA (PDB 1FBY) (Egea et al. 2000), we also noticed that the latter is longer than
398 BMS649 and the other synthetic compounds except CD3254 (**Fig. 7C**). This length is incompatible with
399 the 1.5 Å ligand shift towards helices H7 and H11 measured for BMS649 and imposed by the A332T
400 mutation present in 3mRXR α . Indeed, ligands like 9CRA, 9CDHRA, CD3254 or DHA are rather large
401 and markedly more constrained within the wtRXR α LBP than bexarotene, LG268 or BMS649. In
402 conclusion, our structural analysis reveals that 3mRXR α discriminates between natural and synthetic
403 rexinoids on the basis of their sizes and chemical structures (*i.e.* aliphatic acids *vs* benzoic acids).

404

405 **Discussion**

406 At least one RXR subtype is present in every mammalian cell and participates in diverse essential
407 physiological processes by heterodimerizing with fifteen different nuclear receptors (Evans and
408 Mangelsdorf 2014; Gilardi and Desvergne 2014). Nevertheless, it is still unknown if RXR actually needs
409 a ligand to fulfill its biological roles in all cell types, in a subset of tissues only, or if it primarily serves
410 as a ligand-independent dimerization partner. No conclusive study has been reported to date on the
411 ligand dependency of RXR because none of them has used a mutated form of RXR in which the ligand-
412 binding function of the receptor has been specifically disabled. Many *in vivo* data in mouse, including
413 from RXR knock-outs (Kastner, et al. 1994; Kastner, et al. 1996) or from the less drastic mutation where
414 the RXR α C-terminal helix H12 (activation helix required for ligand-dependent transcription) was
415 deleted (Mascrez, et al. 1998), have provided insight into the physiological functions of these receptors.
416 Owing to the numerous developmental abnormalities observed in the latter study, it was concluded that
417 the RXR ligand-dependent transactivation, and implicitly the involvement of an endogenous ligand, is
418 instrumental during development (Mascrez et al. 1998). However, later studies demonstrated that
419 deletion of the activation helix of RXR induces a strong interaction with corepressors whose binding
420 site is most likely masked by helix H12 in the wild-type receptor (Hu, et al. 2001; Zhang, et al. 1999).
421 Thus, rather than the abolishment of the RXR ligand-dependent activation function, the enhanced
422 recruitment of corepressors by the RXR α -RAR heterodimer (under investigation in the study), and its

423 consecutive transcriptional repression are most likely at the origin of the phenotypes observed in the
424 mutant mouse.

425 In this regard, our structure of the fatty acid-bound wtRXR α -RAR α heterodimer (**Supplementary**
426 **Fig. 1**) reveals a receptor conformation in which the helix H12 of RXR is docked in the groove serving
427 as a primary binding site of corepressors (le Maire and Bourguet 2014; le Maire, et al. 2010). The
428 structure provides a rationale for the poor interaction of wtRXRs with corepressors, and its enhancement
429 upon helix H12 deletion (Hu and Lazar 1999; Hu et al. 2001; Zhang et al. 1999). Accordingly, an
430 increase in corepressor interaction was observed when two mutations (M454A/L455A, human
431 numbering, **Supplementary Fig. 1**), intended to destabilize the interaction between helix H12 and the
432 corepressor binding site, were introduced in the RXR sequence (**Supplementary Fig. 7A**). In addition,
433 2mRXR α and 3mRXR α that are unlikely to copurify with a bacterial fatty acid were shown to interact
434 more avidly than wtRXR α with a fluorescein-labeled peptide containing the LxxIIxxxL interacting motif
435 of the corepressor NCoR (**Supplementary Fig. 7B**). Further investigations will be necessary to validate
436 these data suggesting a possible role of fatty acids in the weak interaction between RXRs and
437 corepressors by inducing a stabilization of helix H12 in the corepressor interacting groove.

438 A fundamental question that remains is whether an endogenous ligand is actually required for RXR
439 knowing that the transactivation function of all RXR heterodimers can be finely tuned by cognate ligands
440 of the partner receptors. In this regard, while some heterodimers can be activated by ligands of the
441 partner receptor, but not by RXR ligands alone (*e.g.* RXR-RAR), other heterodimers are permissive and
442 allow activation by RXR ligands in the absence of a partner agonist (*e.g.* RXR-PPAR) (Germain, et al.
443 2002; Kojetin, et al. 2015; le Maire, et al. 2019; Shulman, et al. 2004). In a physiological context such
444 RXR-mediated activation by endogenous ligands could complicate the precise regulation of permissive
445 heterodimers and generate uncontrolled signaling pathway promiscuity.

446 To our surprise, all the natural compounds used in this study but 9CRA, and to a lesser extent
447 9CDHRA, did not displayed measurable RXR agonistic activity in our cell-based and *in vitro* assays.
448 One possible explanation for this observation is that these natural substances might be relevant in
449 particular cellular environments or in some physiological conditions as reviewed in (Krezel, et al. 2019).
450 Nevertheless, and whatever the nature of the ligand, we believe that the 2m- and 3mRXR variants

451 transposed in mouse models will be critical to evaluate the biological outcome of ligand binding
452 alteration *in vivo* and should provide a wealth of information regarding the requirement of *bona fide*
453 physiological RXR ligand(s), their target organs or tissues, and their pathophysiological importance.
454 Interestingly, treatments of pregnant mothers or adult mice bearing the 3mRXR variant with BMS649
455 or LG268 could be performed with the aim to rescue the pathological outcome of the mutations.

456

457

For Review Only

458 Declaration of interest

459 The authors declare no conflict of interests.

460

461 Funding

462 The Centre de Biologie Structurale is a member of the France-BioImaging (FBI) and the French
463 Infrastructure for Integrated Structural Biology (FRISBI), two national infrastructures supported by the
464 French National Research Agency (ANR-10-INBS-04-01 and ANR-10-INBS-05, respectively). The
465 native MS experiments were performed on an Eclipse Tribrid Orbitrap Mass Spectrometer funded by
466 the Region Ile de France (DIM IHEALTH).

467

468 Author contribution statement

469 AIM, PG and WB conceived the project. AIM and WB performed crystallographic analyses. VV and
470 PG performed cell-based assays. AIM, LG and PB provided the purified proteins and performed
471 biophysical analyses. AIM, PG and WB analyzed data. MR, CM and JCR performed mass spectrometry
472 and data analyses. WB wrote the manuscript. All authors contributed to editing the manuscript.

473

474 Acknowledgements

475 We acknowledge the experimental assistance from the staff of the Swiss Light Source (SLS, Villigen,
476 Switzerland), the Spanish synchrotron facility ALBA (Barcelona, Spain) and the European Synchrotron
477 Radiation Facility (ESRF, Grenoble, France) during data collection.

478

479 **Accession codes:** The atomic coordinates and structure factors have been deposited in the Protein Data
480 Bank under accession codes 7PDT (3mRXR α), 7PDQ (2mRXR α), and 7QAA (RXR α -RAR α).

481

482

483 **References**

- 484 Arnold SLM, Amory JK, Walsh TJ & Isoherranen N 2012 A sensitive and specific method for
485 measurement of multiple retinoids in human serum with UHPLC-MS/MS. *J Lipid Res* **53** 587-598.
- 486 Bourguet W, Andry V, Iltis C, Klaholz B, Potier N, Van Dorsselaer A, Chambon P, Gronemeyer H &
487 Moras D 2000a Heterodimeric Complex of RAR and RXR Nuclear Receptor Ligand-Binding Domains:
488 Purification, Crystallization, and Preliminary X-Ray Diffraction Analysis. *Protein Expr Purif* **19** 284-
489 288.
- 490 Bourguet W, Vivat V, Wurtz JM, Chambon P, Gronemeyer H & Moras D 2000b Crystal structure of a
491 heterodimeric complex of RAR and RXR ligand-binding domains. *Mol Cell* **5** 289-298.
- 492 Chandra V, Wu D, Li S, Potluri N, Kim Y & Rastinejad F 2017 The quaternary architecture of RAR β -
493 RXR α heterodimer facilitates domain-domain signal transmission. *Nat Commun* **8** 868.
- 494 Dawson MI & Xia Z 2012 The retinoid X receptors and their ligands. *Biochim Biophys Acta* **1821** 21-
495 56.
- 496 de Lera AR, Krezel W & Ruhl R 2016 An Endogenous Mammalian Retinoid X Receptor Ligand, At
497 Last! *ChemMedChem* **11** 1027-1037.
- 498 de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjoval J & Perlmann T 2000
499 Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290** 2140-2144.
- 500 Dominguez M, Alvarez S & de Lera AR 2017 Natural and Structure-based RXR Ligand Scaffolds and
501 Their Functions. *Curr Top Med Chem* **17** 631-662.
- 502 Egea PF, Mitschler A & Moras D 2002 Molecular recognition of agonist ligands by RXRs. *Mol*
503 *Endocrinol* **16** 987-997.
- 504 Egea PF, Mitschler A, Rochel N, Ruff M, Chambon P & Moras D 2000 Crystal structure of the human
505 RXR α ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *Embo J* **19** 2592-2601.

- 506 Emsley P & Cowtan K 2004 Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol*
507 *Crystallogr* **60** 2126-2132.
- 508 Evans RM & Mangelsdorf DJ 2014 Nuclear Receptors, RXR, and the Big Bang. *Cell* **157** 255-266.
- 509 Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf
510 DJ & Gronemeyer H 2006 International Union of Pharmacology. LXIII. Retinoid X receptors.
511 *Pharmacol Rev* **58** 760-772.
- 512 Germain P, Iyer J, Zechel C & Gronemeyer H 2002 Coregulator recruitment and the mechanism of
513 retinoic acid receptor synergy. *Nature* **415** 187-192.
- 514 Gilardi F & Desvergne B 2014 RXRs: collegial partners. *Subcell Biochem* **70** 75-102.
- 515 Goldstein JT, Dobrzyn A, Clagett-Dame M, Pike JW & DeLuca HF 2003 Isolation and characterization
516 of unsaturated fatty acids as natural ligands for the retinoid-X receptor. *Arch Biochem Biophys* **420** 185-
517 193.
- 518 Harmon MA, Boehm MF, Heyman RA & Mangelsdorf DJ 1995 Activation of mammalian retinoid X
519 receptors by the insect growth regulator methoprene. *Proc Natl Acad Sci U S A* **92** 6157-6160.
- 520 Heck AJ 2008 Native mass spectrometry: a bridge between interactomics and structural biology. *Nat*
521 *Methods* **5** 927-933.
- 522 Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM & Thaller C 1992 9-cis retinoic
523 acid is a high affinity ligand for the retinoid X receptor. *Cell* **68** 397-406.
- 524 Hu X & Lazar MA 1999 The CoRNR motif controls the recruitment of corepressors by nuclear hormone
525 receptors. *Nature* **402** 93-96.
- 526 Hu X, Li Y & Lazar MA 2001 Determinants of CoRNR-dependent repression complex assembly on
527 nuclear hormone receptors. *Mol Cell Biol* **21** 1747-1758.

- 528 Jones JW, Pierzchalski K, Yu J & Kane MA 2015 Use of fast HPLC multiple reaction monitoring cubed
529 for endogenous retinoic acid quantification in complex matrices. *Anal Chem* **87** 3222-3230.
- 530 Kabsch W 2010 Xds. *Acta Crystallogr D Biol Crystallogr* **66** 125-132.
- 531 Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D, Vonesch JL, Dolle P &
532 Chambon P 1994 Genetic analysis of RXR alpha developmental function: convergence of RXR and
533 RAR signaling pathways in heart and eye morphogenesis. *Cell* **78** 987-1003.
- 534 Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, Decimo D, Krezel W, Dierich A &
535 Chambon P 1996 Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev* **10** 80-92.
- 536 Kitareewan S, Burka LT, Tomer KB, Parker CE, Deterding LJ, Stevens RD, Forman BM, Mais DE,
537 Heyman RA, McMorris T, et al. 1996 Phytol metabolites are circulating dietary factors that activate the
538 nuclear receptor RXR. *Mol Biol Cell* **7** 1153-1166.
- 539 Kojetin DJ, Matta-Camacho E, Hughes TS, Srinivasan S, Nwachukwu JC, Cavett V, Nowak J, Chalmers
540 MJ, Marciano DP, Kamenecka TM, et al. 2015 Structural mechanism for signal transduction in RXR
541 nuclear receptor heterodimers. *Nat Commun* **6** 8013.
- 542 Krezel W, Rivas A, Szklenar M, Ciancia M, Alvarez R, de Lera AR & Ruhl R 2021 Vitamin A5/X, a
543 New Food to Lipid Hormone Concept for a Nutritional Ligand to Control RXR-Mediated Signaling.
544 *Nutrients* **13**.
- 545 Krezel W, Ruhl R & de Lera AR 2019 Alternative retinoid X receptor (RXR) ligands. *Mol Cell*
546 *Endocrinol* **491** 110436.
- 547 Krzyzosiak A, Podlesny-Drabiniok A, Vaz B, Alvarez R, Ruhl R, de Lera AR & Krezel W 2021 Vitamin
548 A5/X controls stress-adaptation and prevents depressive-like behaviors in a mouse model of chronic
549 stress. *Neurobiol Stress* **15** 100375.

- 550 le Maire A & Bourguet W 2014 Retinoic acid receptors: structural basis for coregulator interaction and
551 exchange. *Subcell Biochem* **70** 37-54.
- 552 le Maire A, Teyssier C, Balaguer P, Bourguet W & Germain P 2019 Regulation of RXR-RAR
553 Heterodimers by RXR- and RAR-Specific Ligands and Their Combinations. *Cells* **8**.
- 554 le Maire A, Teyssier C, Erb C, Grimaldi M, Alvarez S, de Lera AR, Balaguer P, Gronemeyer H, Royer
555 CA, Germain P, et al. 2010 A unique secondary-structure switch controls constitutive gene repression
556 by retinoic acid receptor. *Nat Struct Mol Biol* **17** 801-807.
- 557 Lengqvist J, Mata De Urquiza A, Bergman AC, Willson TM, Sjovall J, Perlmann T & Griffiths WJ 2004
558 Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X
559 receptor alpha ligand-binding domain. *Mol Cell Proteomics* **3** 692-703.
- 560 Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C,
561 Rosenberger M, Lovey A, et al. 1992 9-cis retinoic acid stereoisomer binds and activates the nuclear
562 receptor RXR alpha. *Nature* **355** 359-361.
- 563 Liebschner D, Afonine PV, Baker ML, Bunkoczi G, Chen VB, Croll TI, Hintze B, Hung LW, Jain S,
564 McCoy AJ, et al. 2019 Macromolecular structure determination using X-rays, neutrons and electrons:
565 recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75** 861-877.
- 566 Love JD, Gooch JT, Benko S, Li C, Nagy L, Chatterjee VK, Evans RM & Schwabe JW 2002 The
567 structural basis for the specificity of retinoid-X receptor-selective agonists: new insights into the role of
568 helix H12. *J Biol Chem* **277** 11385-11391.
- 569 Mark M, Ghyselinck NB & Chambon P 2006 Function of retinoid nuclear receptors: lessons from
570 genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse
571 embryogenesis. *Annu Rev Pharmacol Toxicol* **46** 451-480.
- 572 Mark M, Teletin M, Vernet N & Ghyselinck NB 2015 Role of retinoic acid receptor (RAR) signaling in
573 post-natal male germ cell differentiation. *Biochim Biophys Acta* **1849** 84-93.

- 574 Mascrez B, Mark M, Dierich A, Ghyselinck NB, Kastner P & Chambon P 1998 The RXR α ligand-
575 dependent activation function 2 (AF-2) is important for mouse development. *Development* **125** 4691-
576 4707.
- 577 Nahoum V, Perez E, Germain P, Rodriguez-Barríos F, Manzo F, Kammerer S, Lemaire G, Hirsch O,
578 Royer CA, Gronemeyer H, et al. 2007 Modulators of the structural dynamics of the retinoid X receptor
579 to reveal receptor function. *Proc Natl Acad Sci U S A* **104** 17323-17328.
- 580 Niu H, Fujiwara H, di Martino O, Hadwiger G, Frederick TE, Menendez-Gutierrez MP, Ricote M,
581 Bowman GR & Welch JS 2017 Endogenous retinoid X receptor ligands in mouse hematopoietic cells.
582 *Sci Signal* **10**.
- 583 Perez E, Bourguet W, Gronemeyer H & de Lera AR 2012 Modulation of RXR function through ligand
584 design. *Biochim Biophys Acta* **1821** 57-69.
- 585 Perez Santin E, Germain P, Quillard F, Khanwalkar H, Rodriguez-Barríos F, Gronemeyer H, de Lera
586 AR & Bourguet W 2009 Modulating retinoid X receptor with a series of (E)-3-[4-hydroxy-3-(3-alkoxy-
587 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)phenyl]acrylic acids and their 4-alkoxy isomers.
588 *J Med Chem* **52** 3150-3158.
- 589 Ruhl R, Krezel W & de Lera AR 2018 9-Cis-13,14-dihydroretinoic acid, a new endogenous mammalian
590 ligand of retinoid X receptor and the active ligand of a potential new vitamin A category: vitamin A5.
591 *Nutr Rev* **76** 929-941.
- 592 Ruhl R, Krzyzosiak A, Niewiadomska-Cimicka A, Rochel N, Szeles L, Vaz B, Wietrzych-Schindler M,
593 Alvarez S, Szklenar M, Nagy L, et al. 2015 9-cis-13,14-Dihydroretinoic Acid Is an Endogenous Retinoid
594 Acting as RXR Ligand in Mice. *PLoS Genet* **11** e1005213.
- 595 Schierle S & Merk D 2019 Therapeutic modulation of retinoid X receptors - SAR and therapeutic
596 potential of RXR ligands and recent patents. *Expert Opin Ther Pat* **29** 605-621.

597 Shulman AI, Larson C, Mangelsdorf DJ & Ranganathan R 2004 Structural determinants of allosteric
598 ligand activation in RXR heterodimers. *Cell* **116** 417-429.

599 Weikum ER, Liu X & Ortlund EA 2018 The nuclear receptor superfamily: A structural perspective.
600 *Protein Sci* **27** 1876-1892.

601 Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie
602 AG, McCoy A, et al. 2011 Overview of the CCP4 suite and current developments. *Acta Crystallogr D*
603 *Biol Crystallogr* **67** 235-242.

604 Zeng Z, Sun Z, Huang M, Zhang W, Liu J, Chen L, Chen F, Zhou Y, Lin J, Huang F, et al. 2015
605 Nitrostyrene Derivatives Act as RXR α Ligands to Inhibit TNF α Activation of NF- κ B.
606 *Cancer Res* **75** 2049-2060.

607 Zhang J, Hu X & Lazar MA 1999 A novel role for helix 12 of retinoid X receptor in regulating
608 repression. *Mol Cell Biol* **19** 6448-6457.

609

610

611

612 **Figure legends**

613

614 **Figure 1:** Chemical structures of natural and synthetic compounds used in this study.

615

616 **Figure 2:** The ligand-binding function is impaired in 2mRXRs. (A) Close-up view of the mouse RXR α
617 LBP in the wtRXR α -RAR α LBD heterodimer structure (PDB 7QAA). The bound fatty acid (FA) is
618 shown as grey sticks together with the light grey 2Fo-Fc map contoured at 1 σ . The mutated residues in
619 2mRXR α and 3mRXR α are shown as grey and black sticks, respectively. (B) Compound activity in
620 transactivation assays in COS-1 cells. Cells were exposed to increasing concentrations of test
621 compounds. Data are expressed as mean (\pm s.e.m). (C) Same experiment as (B) using 2mRXR α . (D)
622 Titration of fluorescein-labeled SRC-1 peptide by wtRXR α LBD in the absence of ligand (DMSO) or
623 in the presence of the indicated compounds. Assays were performed in triplicate and data are expressed
624 as mean (\pm s.e.m). (E) Same experiments as (D) using 2mRXR α . (F) Melting temperatures (T_m)
625 determined for wtRXR α LBD in the absence of ligand (DMSO) or in the presence of the indicated
626 compounds. Assays were performed in triplicate and data are expressed as mean (\pm s.e.m). (G) Same
627 experiments as (F) using 2mRXR α .

628

629 **Figure 3:** Analysis of ligand binding by native mass spectrometry. Characterization of the interaction
630 between wtRXR α LBD (A), 2mRXR α LBD (B) and 3mRXR α LBD (C) and the synthetic compounds
631 CD3254, BMS649 and LG268. Ratios between the ligand-bound and unliganded proteins were
632 measured for each energy and compared for each ligand. Data were normalized by considering the signal
633 obtained for each protein-ligand complex at the lowest dissociation energy (0V) as 100%. Assays were
634 performed in triplicate and data are expressed as mean (\pm S.D.).

635

636 **Figure 4:** 3mRXRs respond exclusively to benzoic acid-containing synthetic compounds. (A)
637 Compound activity in transactivation assays in COS-1 cells. Cells were exposed to increasing
638 concentrations of test compounds. Data are expressed as mean (\pm s.e.m). (B) Titration of fluorescein-
639 labeled SRC-1 peptide by 3mRXR α LBD in the absence of ligand (DMSO) or in the presence of the

640 indicated compounds. Assays were performed in triplicate and data are expressed as mean (\pm s.e.m). (C)
641 Melting temperatures (T_m) determined for 3mRXR α LBD in the absence of ligand (DMSO) or in the
642 presence of the indicated compounds. Assays were performed in triplicate and data are expressed as
643 mean (\pm s.e.m).

644

645 **Figure 5:** 2mRXRs and 3mRXRs retain their DNA-binding and dimerization capabilities. (A)
646 Electrophoresis under non-denaturing conditions revealing the oligomeric states of wtRXR α , 2mRXR α ,
647 and 3mRXR α LBDs individually and in complex with TR α 1 LBD. The white asterisks indicate RXR α -
648 TR α heterodimers. Bovine serum albumin (BSA) was used as a molecular weight marker. (B) Size-
649 exclusion chromatography-multi angle light scattering (SEC-MALS) analysis showing that wtRXR α ,
650 2mRXR α , and 3mRXR α LBDs heterodimerize with TR α 1 LBD in solution.

651

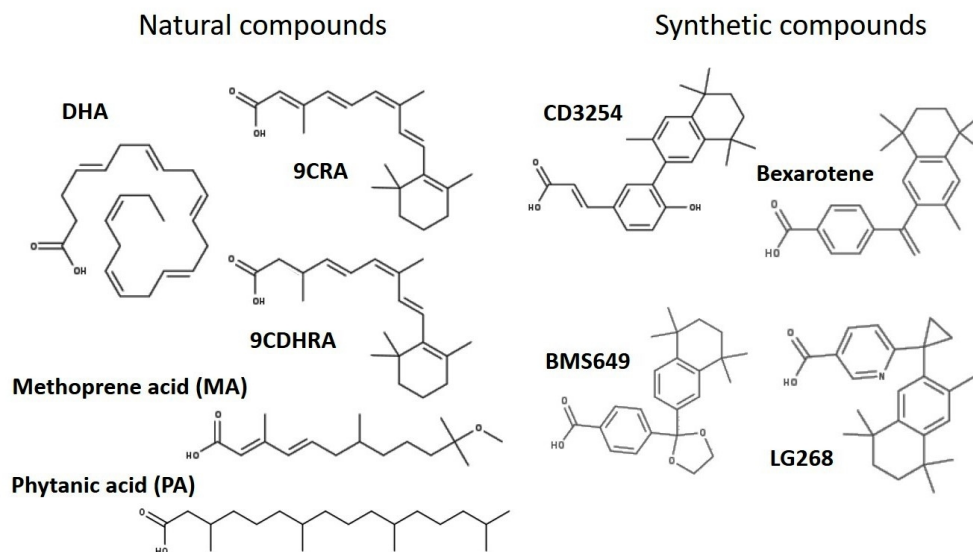
652 **Figure 6:** Structure of LG268-bound 2mRXR α . (A) Superimposition of LG268-bound 2mRXR α with
653 LG268-bound wtRXR β (PDB 1H9U). The ligands and amino acid side chains in 2mRXR α and wtRXR β
654 are displayed as orange and cyan sticks, respectively. (B) Superimposition of LG268-bound 2mRXR α
655 (orange) with 9CRA-bound wtRXR α (cyan, PDB 1FBY). (C) Superimposition of LG268-bound
656 2mRXR α (orange) with CD3254-bound wtRXR α (cyan, PDB 3FUG). (D) Superimposition of LG268-
657 bound 2mRXR α (orange) with 9CDHRA-bound wtRXR α (cyan, PDB 4ZSH). The red asterisk denotes
658 steric incompatibility between Q437 and the ligands displayed in cyan.

659

660 **Figure 7:** Structure of BMS649-bound 3mRXR α . (A) Superimposition of BMS649-bound 3mRXR α
661 with BMS649-bound wtRXR α (PDB 1MVC). The ligands and amino acid side chains in 3mRXR α and
662 wtRXR α are displayed as orange and cyan sticks, respectively. (B) Another view to show the different
663 binding modes of BMS649 in 3mRXR α (orange) and wtRXR α (cyan). Note the shift towards H5, H7
664 (and H11, not shown), and the rotation of the benzoic acid ring to maintain van der Waals contacts with
665 residues in helices H3 and H5 of 3mRXR α (red dotted lines). (C) Superimposition of the indicated
666 ligands as it comes when superimposing the corresponding RXR complex structures. 9CRA and

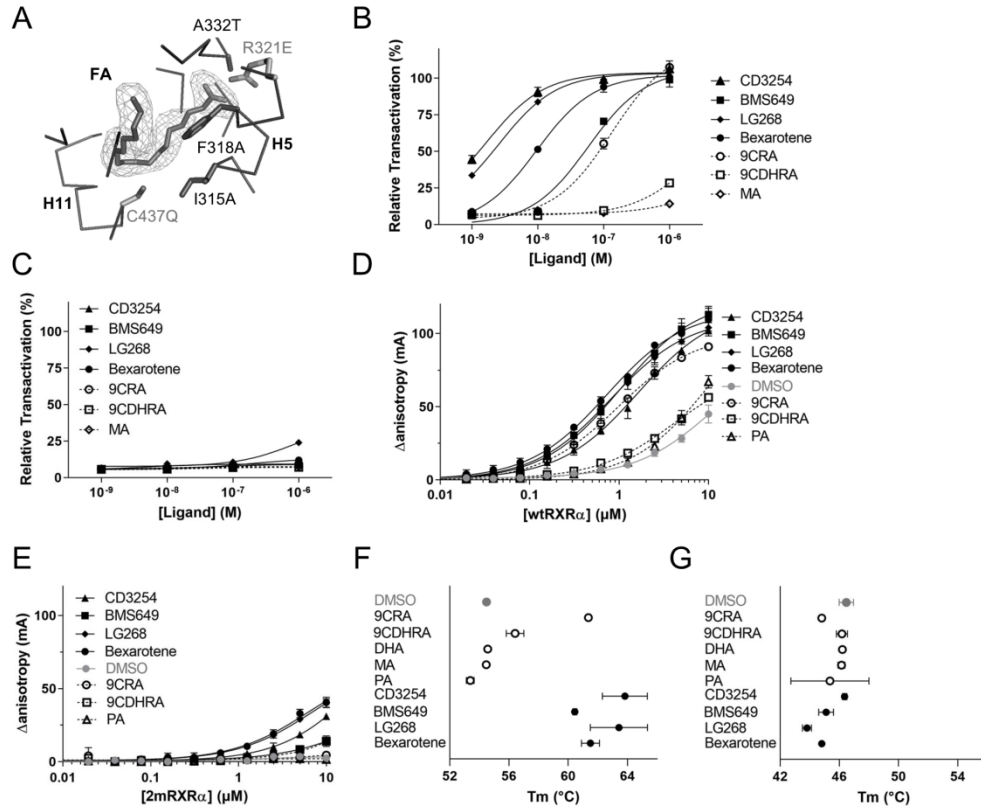
667 CD3254 are markedly longer than LG268, BMS649 and bexarotene. Values in parentheses indicate the
668 longest inter-atomic distance in each ligand.

For Review Only



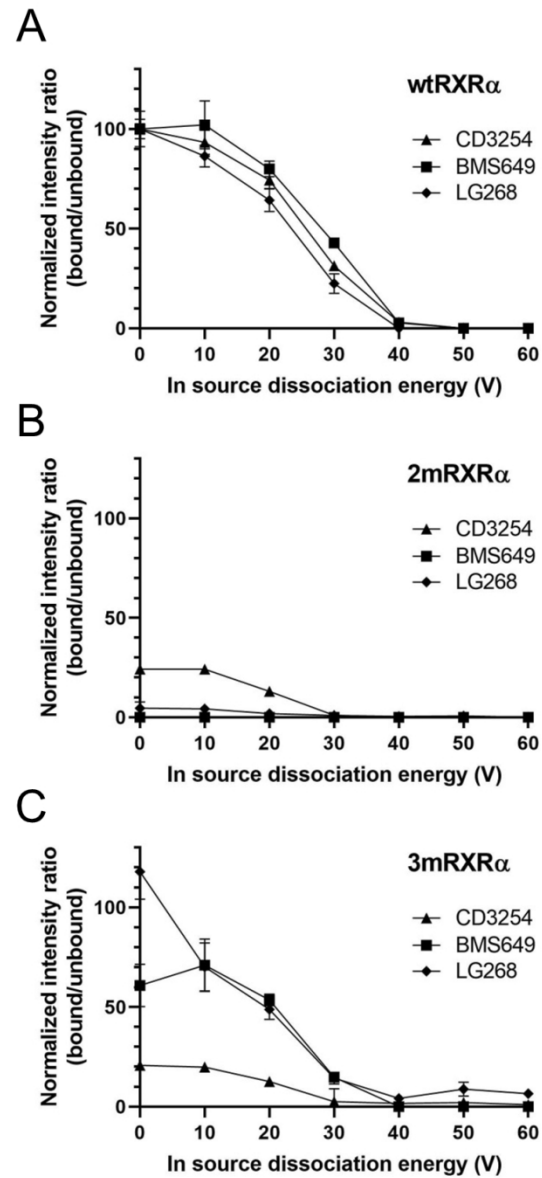
Chemical structures of natural and synthetic compounds used in this study.

226x129mm (150 x 150 DPI)



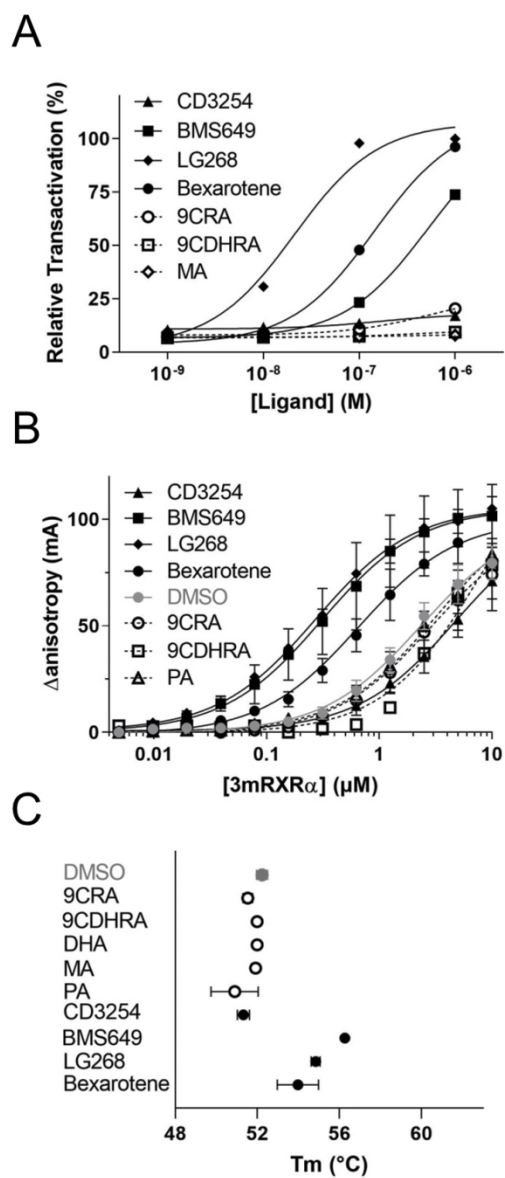
The ligand-binding function is impaired in 2mRXRs.

231x189mm (230 x 230 DPI)



Analysis of ligand binding by native mass spectrometry.

91x188mm (230 x 230 DPI)



3mRXRs respond exclusively to benzoic acid-containing synthetic compounds.

84x190mm (230 x 230 DPI)

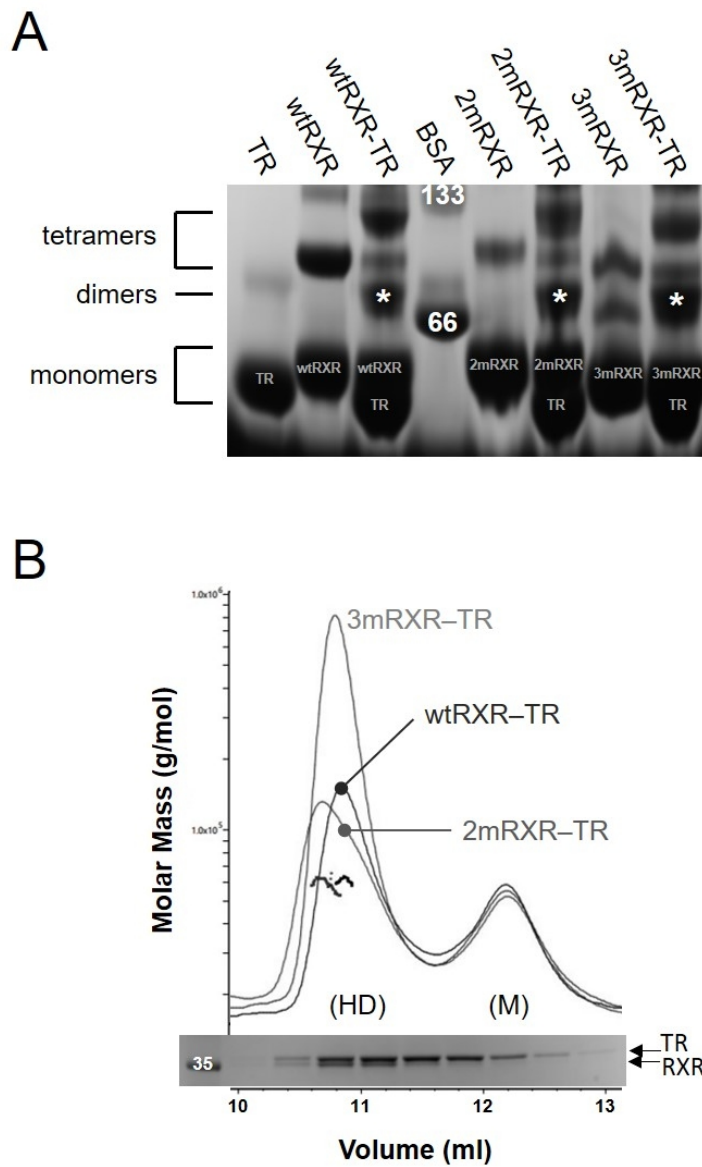
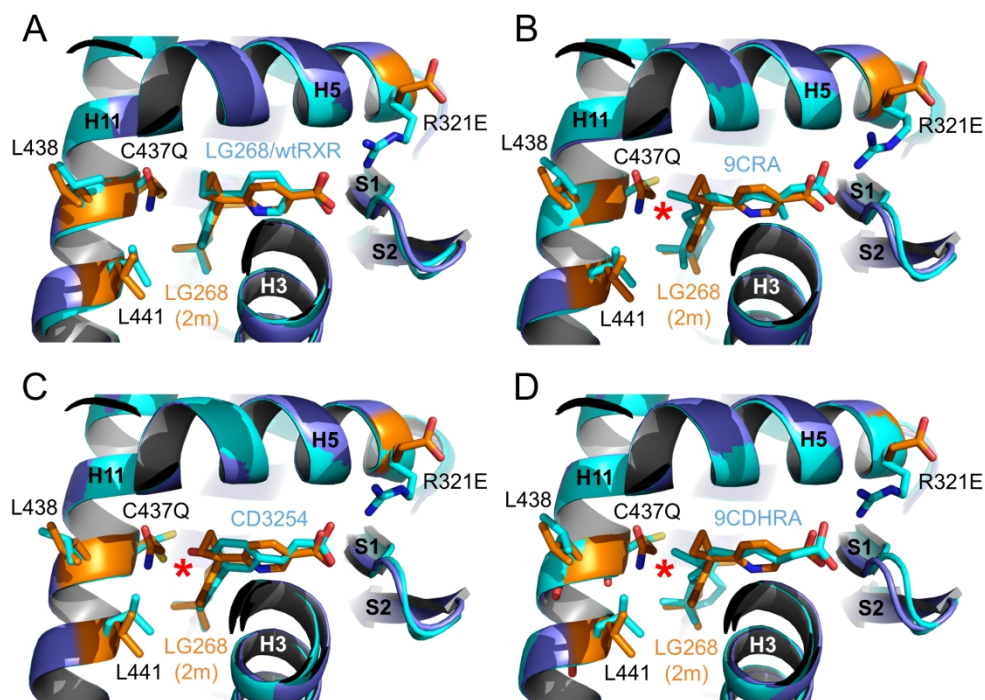


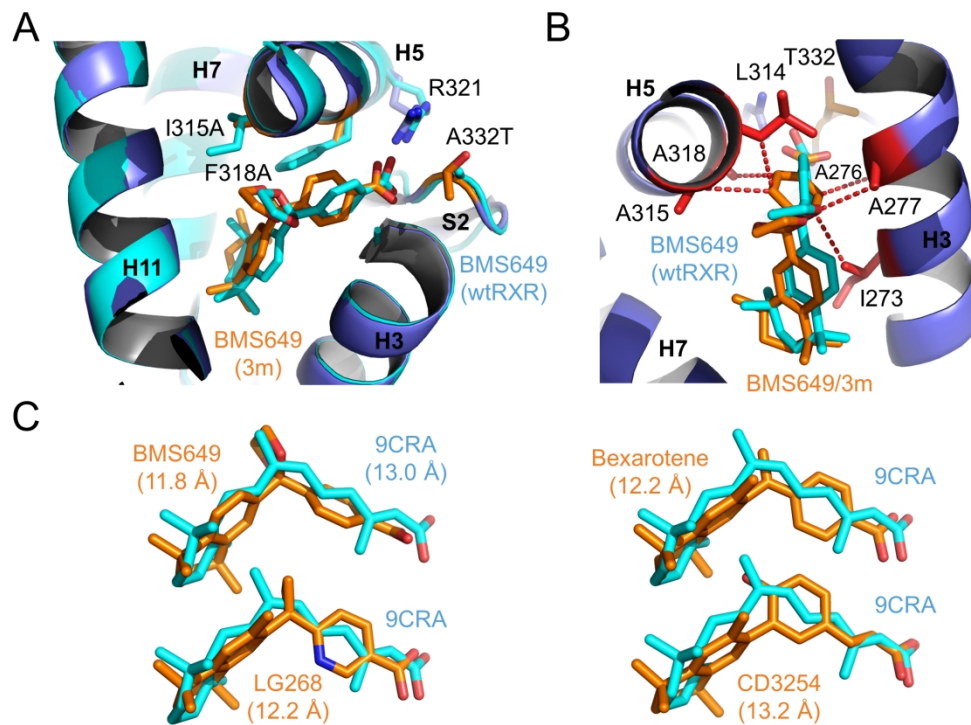
Figure 5

125x186mm (150 x 150 DPI)



Structure of LG268-bound 2mRXRa.

262x186mm (230 x 230 DPI)

Structure of BMS649-bound 3mRXR α .

243x182mm (230 x 230 DPI)

Supplementary material for

Design and in vitro characterization of RXR variants as tools to investigate the biological role of endogenous rexinoids

Albane le Maire¹, Martial Rey², Valérie Vivat^{3,*}, Laura Guée¹, Pauline Blanc¹, Christian Malosse², Julia Chamot-Rooke², Pierre Germain¹, and William Bourguet¹

¹CBS (Centre de Biologie Structurale), Univ Montpellier, CNRS, Inserm, Montpellier, France

²Institut Pasteur, Université de Paris, CNRS USR2000, Mass Spectrometry for Biology Unit, 75015 Paris, France

³IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), Univ Strasbourg, CNRS, Inserm, Illkirch, France

***Current address:** Flare Therapeutics, 215 First Street, Suite 150, Cambridge, MA 02142, USA

Includes:

Supplementary Materials and Methods

Supplementary Figure 1: Structure of wtRXR α -RAR α LBD heterodimer.

Supplementary Figure 2: The ligand-binding function is impaired in 2mRXRs.

Supplementary Figure 3: Far-UV circular dichroism spectra of wt, 2m- and 3mRXR α .

Supplementary Figure 4: Representative mass spectra of wtRXR α , 3mRXR α and 2mRXR α in complex with BMS649.

Supplementary Figure 5: 3mRXRs respond exclusively to benzoic acid-containing synthetic compounds.

Supplementary Figure 6: Structures of 2m- and 3mRXR α in complex with LG268 and BMS649.

Supplementary Figure 7: Interaction of RXR α with the corepressor NCoR.

Supplementary Figure 8: 2mRXRs and 3mRXRs retain their DNA-binding and dimerization capabilities.

Supplementary Table 1: Data collection and refinement statistics.

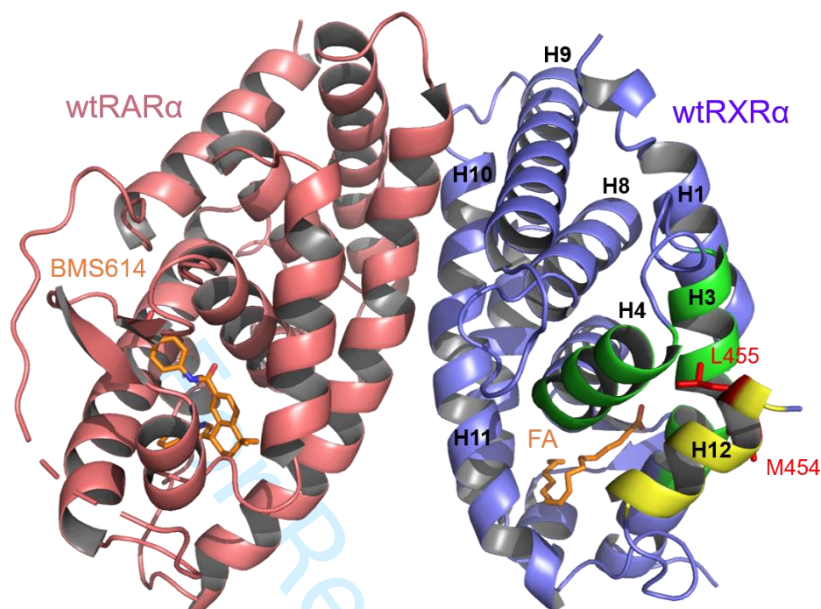
Supplementary Materials and Methods

Label-free thermal shift assay (nanoDSF)

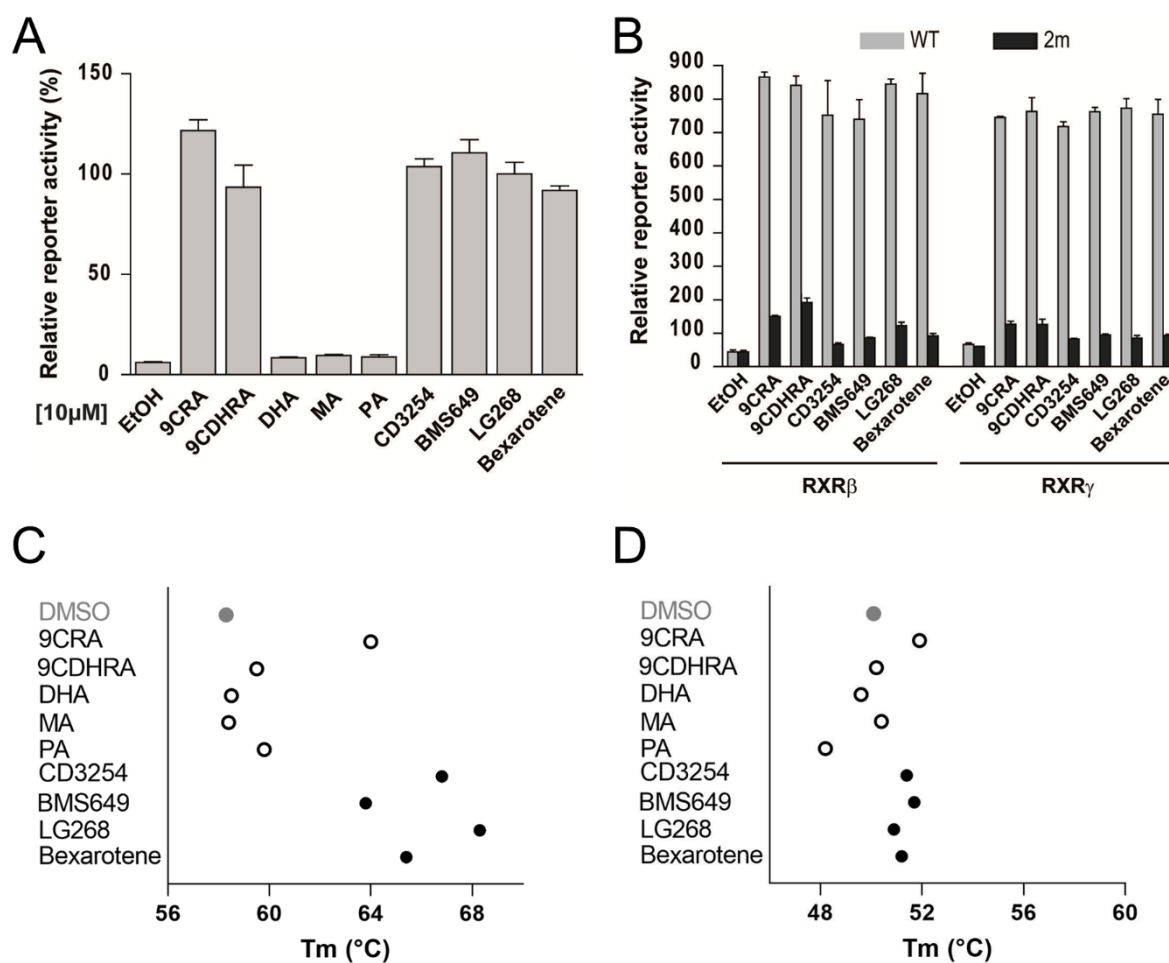
Label-free thermal shift assay experiments were performed with a Tycho NT.6 instrument (NanoTemper Technologies). The samples (20 μ M of RXR α protein), in the presence of DMSO (condition without ligand) or in the presence of three molar excess of ligands, were heated in a glass capillary at a rate of 30 K/min (20°C to 95°C) in 50 mM Tris HCl pH 7.5, 150 mM NaCl, 5% glycerol, and the internal fluorescence at 330 and 350 nm was recorded. Data analysis, data smoothing, and calculation of derivatives was done using the internal evaluation features of the Tycho instrument.

Two-hybrid assays

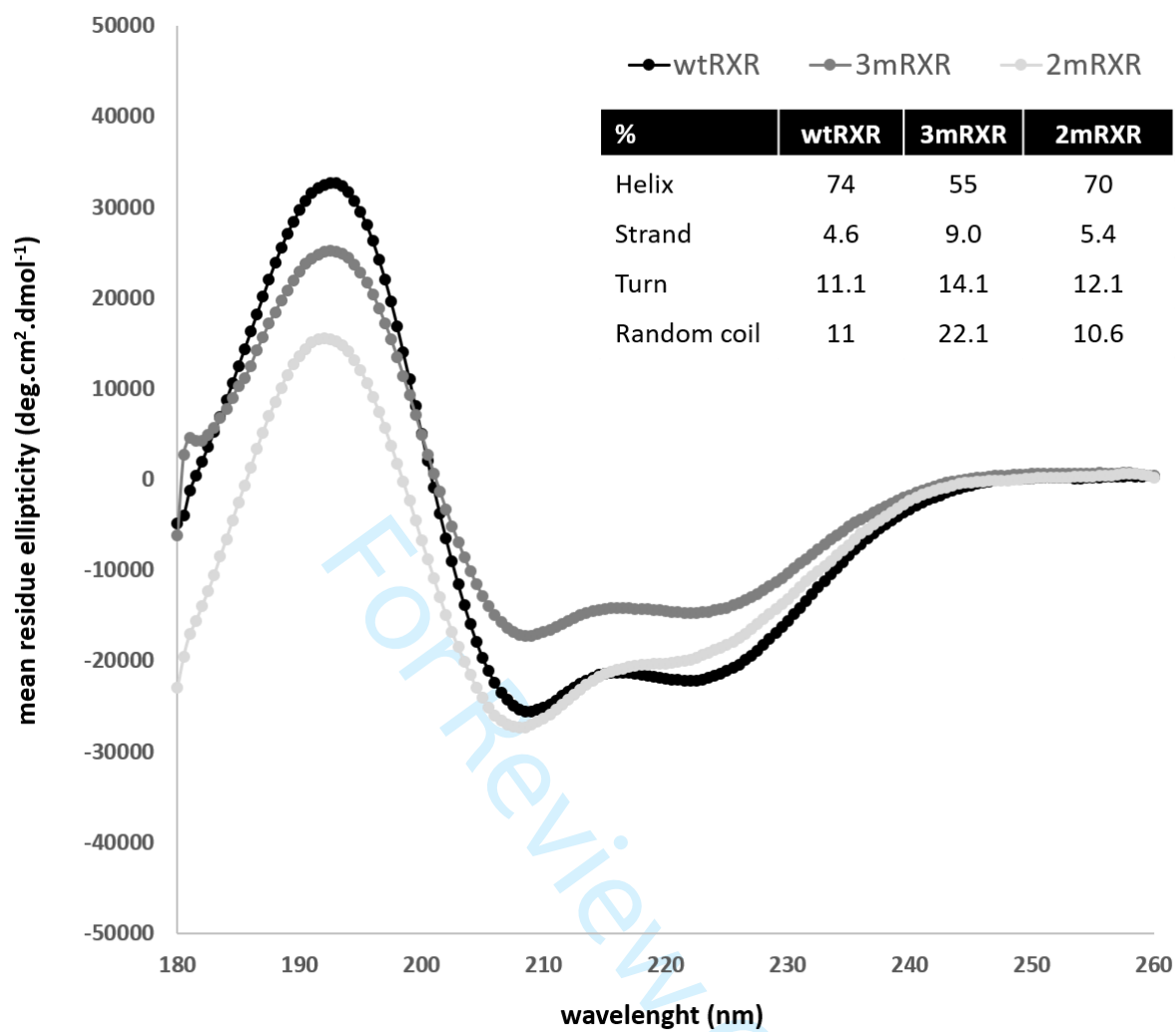
COS cells were cultured in DMEM with Glutamax and 10% (v/v) FCS and transfected using JetPei transfectant (Ozyme). After 24 h, the medium was changed to a medium containing the selective RAR agonist TTNPB or vehicle. Cells were lysed and assayed for reporter gene expression 48 h after transfection. The luciferase assay system was used according to the manufacturer's instruction (Promega). In each case results were normalized to co-expressed β -galactosidase. TTNPB was purchased from Sigma. RXR α mutants were generated into pSG5-RXR α by PCR-assisted site-directed mutagenesis with Deep Vent DNA polymerase (New England Biolabs). The construct was verified by DNA sequencing. The pSG5-based Gal-NCOR and VP16-RAR α LBD (VP16-RAR) expression vectors, and the (17m)5x- β Glob-Luc reporter gene have been described (Germain, et al. 2002).



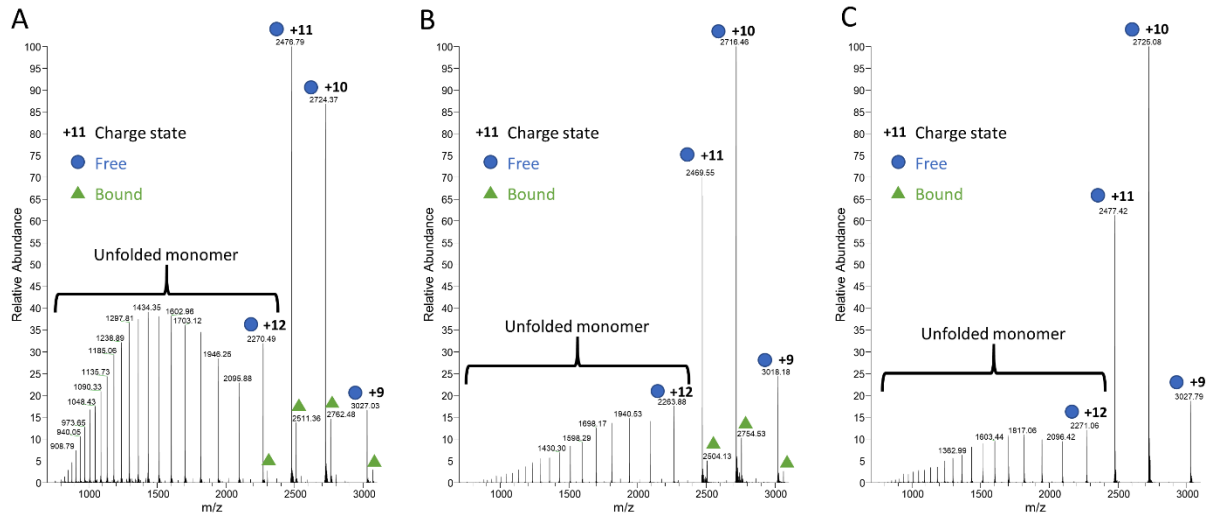
Supplementary Figure 1: Structure of wtRXR α -RAR α LBD heterodimer. The RXR α (blue) and RAR α (pink) subunits are bound to a fatty acid (FA) and the antagonist BMS614, respectively. Ligands are displayed as orange sticks. The corepressor binding groove made of helices H3 and H4 is highlighted in green. The RXR activation helix H12 occupying the corepressor binding site in the fatty acid-bound state is colored in yellow. M454 and L455 that have been mutated to delocalize H12 from the corepressor binding site are shown in red.



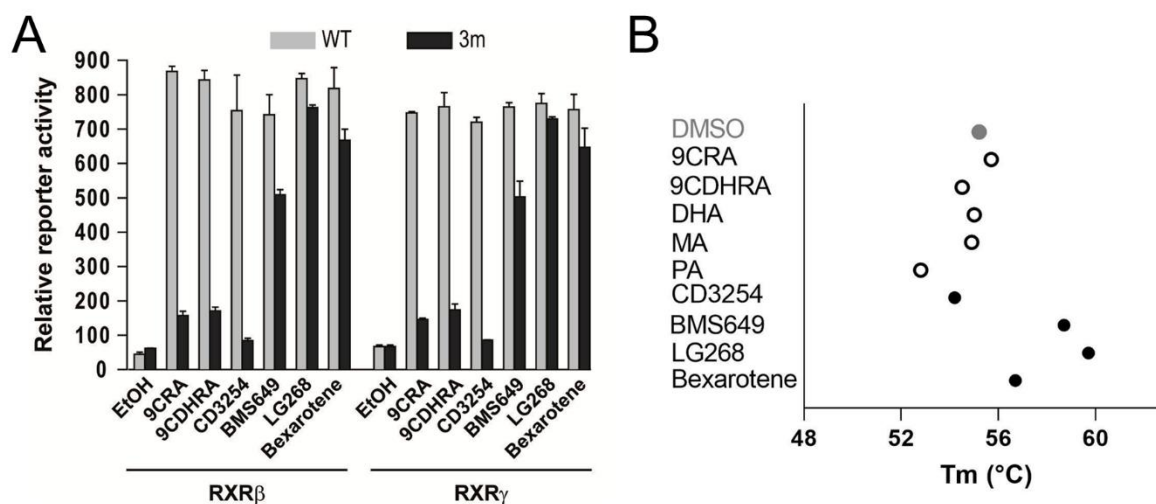
Supplementary Figure 2: The ligand-binding function is impaired in 2mRXRs. (A) COS cells were transiently co-transfected with the reporter gene (DR1)-tk-Luc and RXR α to assess the RXR agonist potential of compounds at 10 μ M. 100% corresponds to reporter gene transcription induced in the presence of the full RXR agonist LG268. All error bars are expressed as s.e.m. (B) COS cells were transiently co-transfected with the reporter gene (DR1)-tk-Luc and either RXR β (light grey) or 2mRXR β (black) (left panel) or RXR γ (light grey) or 2mRXR γ (black) (right panel). All error bars are expressed as s.e.m. (C, D) Label-free thermal shift assay (nanoDSF). Melting temperatures (T_m) determined for wtRXR α (C) and 2mRXR α LBDs (D) in the absence of ligand (DMSO) or in the presence of the indicated compounds.



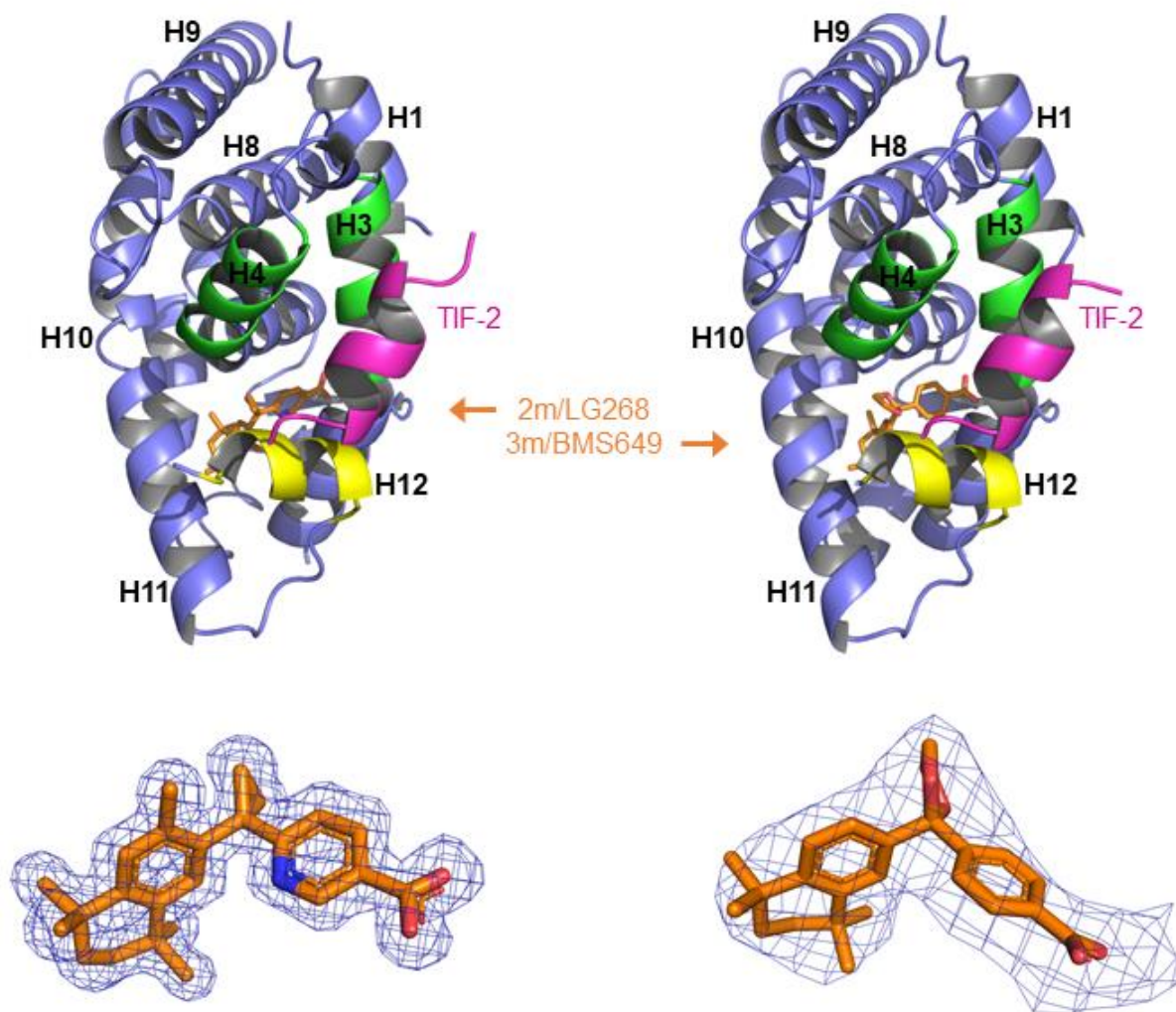
Supplementary Figure 3: Far-UV circular dichroism spectra of wt, 2m- and 3mRXX α at room temperature in purification buffer.



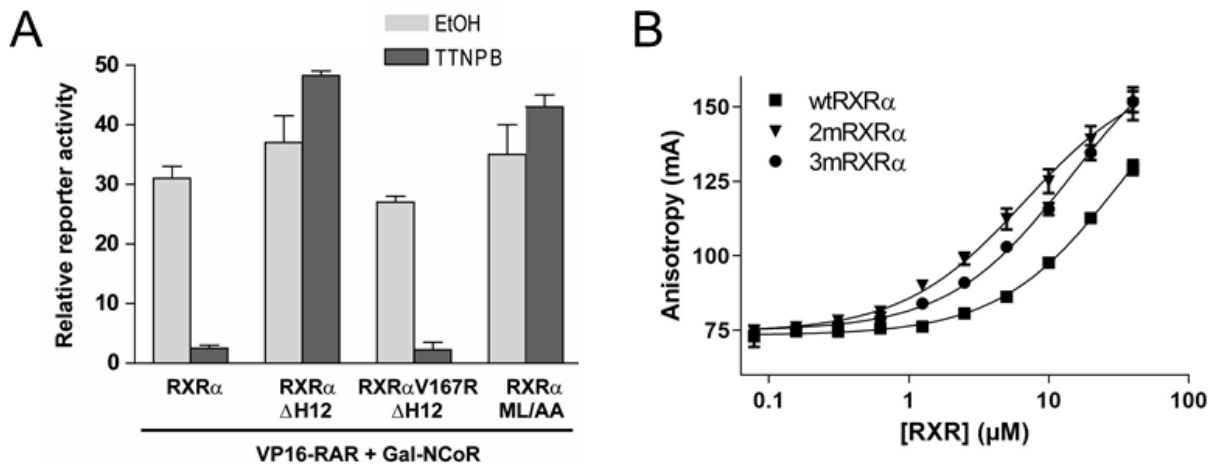
Supplementary Figure 4: Representative mass spectra of the (A) wtRXR α , (B) 3mRXR α and (C) 2mRXR α in complex with BMS649 with no IS-CID energy. Folded monomer signal is spread between 4 different charge states (+12, +11, +10 and +9). The intensities of the bound (green triangles) and free (blue circles) forms are measured in function of the IS-CID energies to generate the graph presented in the Figure 3.



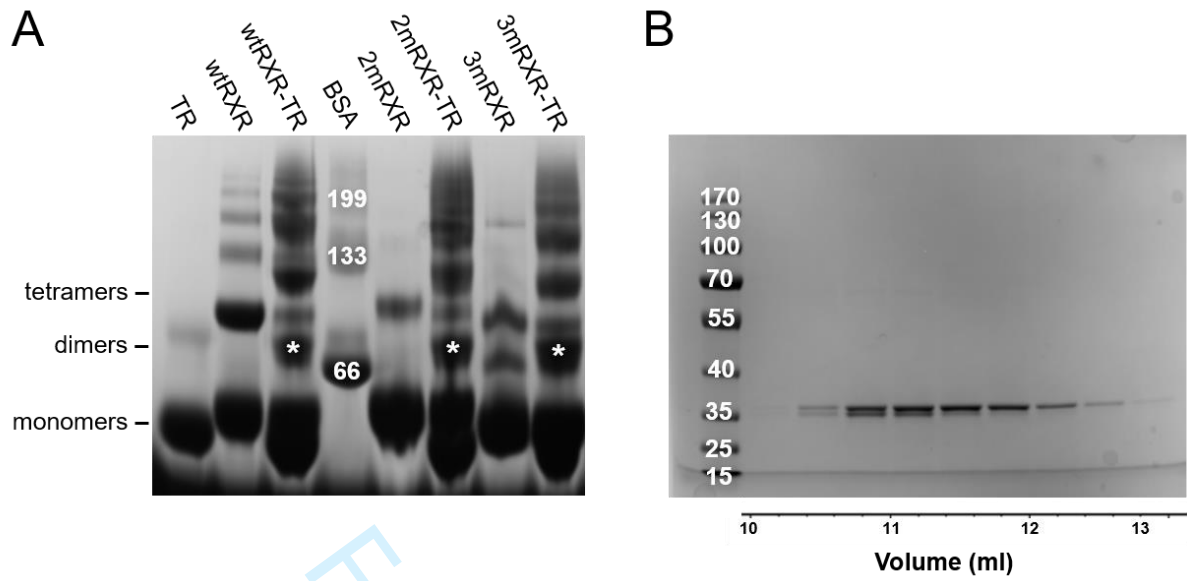
Supplementary Figure 5: 3mRXRs respond exclusively to benzoic acid-containing synthetic compounds. (A) COS cells were transiently co-transfected with the reporter (DR1)-tk-Luc and either RXR β (light grey) or 3mRXR β (black) (left panel) or RXR γ (light grey) or 3mRXR γ (black) (right panel). All error bars are expressed as s.e.m. (B) Label-free thermal shift assay (nanoDSF). Melting temperatures (T_m) determined for 3mRXR α LBD in the absence of ligand (DMSO) or in the presence of the indicated compounds.



Supplementary Figure 6: Structures of 2m- and 3mRXR α in complex with LG268 and BMS649. Ligands are displayed as orange sticks. The coregulator binding groove made of helices H3 and H4 is highlighted in green. The activation helix H12 in the transcriptionally active position is colored in yellow. The TIF-2 coactivator-derived peptide is displayed in magenta. The electron densities shown are unbiased omit Polder maps contoured at 2.0σ , with model bias reduction and exclusion of solvent density.



Supplementary Figure 7: Interaction of RXR α with the corepressor NCoR. (A) Interactions of RXR α -RAR α heterodimers with the corepressor NCoR using mammalian two-hybrid assays with (17m)_{5x}- β Glob-Luc reporter in COS cells and with chimeras containing the GAL4 DNA-binding domain fused to the nuclear receptor interaction region of NCoR (Gal-NCoR) as bait and RAR α LBD fused to the activation domain of VP16 (VP16-RAR) as prey in combination with RXR α , RXR α Δ H12, RXR α V167R Δ H12 or RXR α ML/AA in the presence (light gray) or absence (dark grey) of the RAR selective agonist TTNPB (10nM). All error bars are expressed as s.e.m. In the presence of wtRXR α , addition of TTNPB is sufficient to dissociate NCoR from the heterodimer, indicating that RXR α plays little role in the association. Upon RXR α H12 deletion (RXR α Δ H12), TTNPB fails to dissociate the complex, suggesting that a new interaction surface for NCoR is present in RXR α Δ H12. When preventing the interaction between RXR α Δ H12 and NCoR by introducing an additional mutation in the corepressor-binding site of RXR α (RXR α V167R Δ H12) (Hu and Lazar 1999), the dissociation capacity of TTNPB is restored, confirming that the groove formed by RXR helices H3 and H4 can serve as a corepressor binding surface, provided that helix H12 is not masking it (**Supplementary Fig. 1**). In the same line, the double mutation M454A/L455A (RXR α ML/AA) yields a similar interaction pattern than that observed with RXR α Δ H12, indicating that the introduced mutations in helix H12 (**Supplementary Fig. 1**) cause the release of the RXR α hydrophobic groove and NCoR recruitment. (B) Titration of fluorescein-labeled NCoR peptide by wtRXR α , 2mRXR α or 3mRXR α LBDs. Assays were performed in triplicate and data are expressed as mean (\pm s.e.m). Altogether, these observations suggest that RXR α helix H12 may adopt a fatty acid-dependent conformation that prevents NCoR binding to RXR α , so that in a physiological context, transcriptional activation of the heterodimer is controlled by the partner receptor.



Supplementary Figure 8: 2mRXRs and 3mRXRs retain their DNA-binding and dimerization capabilities. (A) Full-size native gel from Figure 5A. (B) Full-size SDS-PAGE gel from Figure 5B. Molecular weight protein standards are indicated on each gel.

Supplementary Table 1: Data collection and refinement statistics

PDB code	3mRXRα 7PDT	2mRXRα 7PDQ	RXRα-RARα 7QAA**
Wavelength	0.97242	0.99998	0.918
Resolution range	42.83-3.30 (3.41-3.30)*	48.39-1.58 (1.64-1.58)*	19.47-2.76 (2.86-2.76)*
Space group	P 21 21 21	P 43 21 2	P 61 2 2
Unit cell	63.39 68.53 109.72 90 90 90	68.43 68.43 105.61 90 90 90	116.60 116.60 207.80 90 90 120
Total reflections	36709 (3841)	761965 (19995)	
Unique reflections	7562 (749)	34564 (2903)	
Multiplicity	4.9 (5.1)	22.0 (6.9)	
Completeness (%)	98.94 (99.47)	98.25 (83.90)	
Mean I/sigma (I)	10.77 (3.40)	32.76 (2.04)	
Wilson B-factor	83.50	19.68	
R-merge	0.122 (0.548)	0.0604 (0.668)	
R-meas	0.137 (0.610)	0.06176 (0.7216)	
R-pim	0.06108 (0.2639)	0.01247 (0.2585)	
CC1/2	0.994 (0.749)	1 (0.812)	
CC*	0.999 (0.926)	1 (0.947)	
Refl. used in refinement	7530 (750)	34557 (2902)	19728 (2041)
Refl. used for R-free	761 (81)	1708 (130)	1009 (96)
R-work	0.1946 (0.2670)	0.1713 (0.2599)	0.1947 (0.2221)
R-free	0.2098 (0.2786)	0.1967 (0.2954)	0.2450 (0.3012)
Non-hydrogen atoms	3491	2120	3654
macromolecules	3428	1756	3479
ligands	56	27	54
solvent	7	337	121
Protein residues	437	220	447
RMS (bonds)	0.003	0.016	0.005
RMS (angles)	0.66	1.47	0.99
Ramach. favored (%)	97.18	98.60	96.00
Ramach. allowed (%)	2.82	1.40	3.56
Ramach. outliers (%)	0.00	0.00	0.44
Rotamer outliers (%)	0.00	0.00	0.00
Clash score	10.14	6.14	10.71
Average B-factor	70.22	23.70	47.51
macromolecules	70.46	21.81	47.60
ligands	56.95	15.36	38.70
solvent	55.71	34.20	48.00
Number of TLS groups	12	5	0

*Values in parentheses are for highest resolution shell

**Structure deposited at the PDB without data collection statistics (data collected 25 years ago and lost since)