Clinical and functional heterogeneity associated with the disruption of Retinoic Acid Receptor beta

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ABSTRACT

Purpose: Dominant variants in the retinoic acid receptor beta *(RARB)* gene underlie a syndromic form of microphthalmia, known as MCOPS12, which is associated with other birth anomalies and global developmental delay with spasticity and/or dystonia. Here, we report 25 affected individuals with 17 novel pathogenic or likely pathogenic variants in *RARB*. This study aims to characterize the functional impact of these variants and describe the clinical spectrum of MCOPS12.

Methods: We used in vitro transcriptional assays and in silico structural analysis to assess the functional relevance of *RARB* variants in affecting the normal response to retinoids.

Results: We found that all *RARB* variants tested in our assays exhibited either a gain-of-function or a loss-of-function activity. Loss-of-function variants disrupted RARB function through a dominant-negative effect, possibly by disrupting ligand binding and/or coactivators' recruitment. By reviewing clinical data from 52 affected individuals, we found that disruption of *RARB* is associated with a more variable phenotype than initially suspected, with the absence in some individuals of cardinal features of MCOPS12, such as developmental eye anomaly or motor impairment.

Conclusion: Our study indicates that pathogenic variants in *RARB* are functionally heterogeneous and associated with extensive clinical heterogeneity.

Introduction

Regulation of the retinoic acid (RA) pathway is essential for the development of several organs, including the brain and eye in both humans and animals.[1] In target cells, RA binds to a heterodimer complex formed of retinoic acid receptor (RAR) and retinoid X receptor (RXR). There are 3 subtypes of RAR (RARA, RARB, and RARG), which, upon dimerization with RXR, bind to retinoic acid response elements (RAREs) contained in target genes to modulate their transcription with the help of coregulators. RARs are members of the nuclear receptor superfamily that share highly conserved DNA-binding domain (DBD) and ligand-binding domain (LBD).[2] Structural analysis of the LBD has determined the presence of 12 alpha helices and 3 beta turns, which define the formation of a ligand-binding pocket and a hydrophobic cleft involved in coregulator recruitment. The binding of RA triggers the appropriate repositioning of helix-12 of RARs to adopt a proper docking conformation for coactivator recruitment, resulting in transcription of target genes.

We have reported that de novo missense variants in *RARB* cause a syndromic form of microphthalmia (MCOPS12; MIM 615524) associated with diaphragmatic hernia/eventration, cardiac defects, Chiari malformation type 1, global

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developmental delay, and spasticity and/or dystonia.[3,4] These variants are all located in the LBD of RARB and induce a gain-of-function (GOF) effect characterized by an increase of RA responsiveness in a cell-based transcriptional assay.[3,4] Here, we report 17 novel variants in *RARB*, identified in 25 individuals with MCOPS12 features. Using transcriptional assays, we ascertained that some of the variants induced a GOF, as previously described,[3,4] whereas others caused a dominant-negative effect, suggesting that MCOPS12 is associated with some functional heterogeneity. Finally, compilation of the clinical data of 52 individuals carrying novel and known pathogenic variants indicates that the phenotypic spectrum associated with the disruption of *RARB* is broader and more variable than initially reported.

Materials and Methods

Variant identification and classification

RARB variants were identified using clinical exome or targeted sequencing. Description of the variants is based on the National Center for Biotechnology Information reference sequence NM_000965.5, using complementary DNA numbering with position 1 corresponding to the A of the ATG translation initiation codon (Supplemental Table 1). Variants are also described at the chromosomal level (GRCh38) using the reference sequence NC_000003.12 and at the protein level using the reference sequence NP_000956.2 (Table 1, Supplemental Table 1).

Transfection studies

One-hybrid luciferase reporter transcriptional assay was performed as previously described.[3] Briefly, HEK293 cells were seeded in 24-well plates and transfected with 100 ng per well of expression plasmid encoding either *Gal4* DBD fusion plasmids of human wild-type or mutant RARB in the presence of 500 ng of UAStkLuc reporter-gene construct. To determine the dominant-negative effects of mutants, HEK293 cells were transfected with increasing concentrations of untagged RARB variants in the presence of a RAREtkLuc reporter to assess their impact on endogenously expressed wild-type RARB receptor. Validation of the C98,101A defective mutant was performed in RAR triple knockout mouse embryonic fibroblasts. Cells were treated with various concentrations of all-*trans* RA (atRA) or 9-*cis* RA, or with vehicle (dimethyl sulfoxide; 1/1000, v/v) for 16 hours. Luciferase values were normalized to β-galactosidase activity and expressed as a fold response compared with vehicle-treated cells. Data were derived from at least 4 independent experiments performed in triplicate.

Molecular dynamics simulation of RARB

Molecular dynamics (MD) simulations of the ligand-free form of RARB and the p.(Met290Arg) and p.(Leu402Pro) receptors were carried out in GROMACS software version 2019.2,[5] using AMBER99SB-ILDN force field.[6] Starting models were derived from X-ray crystal structure (PDB ID: 4DM8, resolution 2.3 Å) reported previously.[7] Initial positions for mutant residues were found in Coot by optimizing fit for mutant residue to wild-type electron density and avoiding clashes with surrounding residues.[8] During setup, the ligand-free and coactivator-free monomer of the LBD encompassing residues 171 to 409 was placed in a dodecahedron box (10 Å padding) with TIP3P water and neutralized by adding sodium and chloride ions to a final concentration of 150 mM. Following energy minimization, a modified Berendsen thermostat (2 groups, 0.1 ps time constant, 310 K reference temperature) followed by Berendsen barostat (isotropic, coupling constant 0.5 ps, reference pressure 1 bar) were sequentially coupled to the system over 100 picoseconds. The unconstrained MD simulations ran for 100 nanoseconds. The resulting trajectories corrected for periodic boundary condition artifacts were analyzed in Chimera[9] and R (https://www.R-project.org).

Results

Functional impact of novel *RARB* variants

We identified 17 novel *RARB* heterozygous variants, all absent from gnomAD, in 25 individuals with developmental eye abnormalities and/or other clinical features previously associated with MCOPS12 (Figure 1, Table 1, Supplemental Table 1). All of these variants are missense alleles, except for 3 canonical splice sites and 1 truncating variant. The missense variants affect amino acids that are conserved in all RARB isoforms and all RAR vertebrate proteins. We classified these 17 variants as pathogenic or likely pathogenic variants based on the American College of Medical Genetics and Genomics framework (Supplemental Table 1).[10]

With the exception of p.(Gly103Cys) and the splicing variants, the novel *RARB* variants are located in distinct clusters within the LBD (Figure 1). Interestingly, 5 amino acid residues affected by LBD missense variants (Trp218, Arg269, Phe279, Gly384, and Arg387) are thought to establish contact with atRA,[11] whereas the remaining ones are located in close vicinity of the ligand-binding pocket or in the helix-12 involved in coactivator recruitment. Previously described LBD variants were all shown to exert a GOF effect when tested using a one-hybrid luciferase reporter assay, which provides a readout of the transcriptional activity of complementary DNA products in response to RA (1 μM) without any interference from endogenously expressed RARB.[3,4] We thus sought to determine the transcriptional response of the novel LBD variants using the same approach. We found that 5 variants (p.(Tyr201Cys), p.(Arg269Thr), p.(Asp281Val), p.(Gly282Ser), and p.(Leu402Val)) exhibited a GOF effect with significant increase in their transcriptional response to atRA and 9-*cis* RA when compared with the wild-type

Table 1 Clinical characteristics of individuals with pathogenic/likely pathogenic variants in *RARB*

Variant (NC_000003.12)	Variant (NP_000956.2)	Inheritance[a]	Functional Impact[b]	No. of Individuals (Sex)	Developmental Eye Defect[c]	Diaphragmatic Hernia/ Eventration[c]	Swallowing Difficulties[c]	Heart Defects[c]	Gross Motor Delay[c]	Language Delay[c]	Intellectual Disability[c]	Hypotonia[c]	Spasticity[c]	Dystonia[c]	Chiari Malformation Type 1[c]
g.25501182G>T	p.(Gly103Cys)	de novo	LOF	1 (M)	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	nr
g.25501285G>A	p.(Arg137Gln)	inherited	LOF	2 (1 M:1 F)	2/2	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
g.25569911A>G	p.(Tyr201Cys)	de novo	GOF	1 (M)	0/1	0/1	0/1	0/1	1/1	1/1	na	1/1	0/1	0/1	1/1
g.25580574T>C	p.(Leu213Pro)	de novo	GOF	3 (F)	3/3	0/2	1/1	3/3	2/2	2/2	2/2	2/3	2/2	2/2	1/3
g.25580590G>C	p.(Trp218Cys)	de novo	LOF	1 (F)	1/1	1/1	0/1	0/1	na	na	na	na	na	na	nr
g.25593522G>C	p.(Arg269Thr)	inherited	GOF	4 (1 M:3 F)	4/4	0/4	2/3	1/1	4/4	2/2	1/1	2/3	3/4	1/4	1/1
g.25593551T>G	p.(Phe279Val)	de novo	GOF	1 (M)	1/1	1/1	nr	nr	nr	na	na	nr	nr	nr	nr
g.25593558A>T	p.(Asp281Val)	de novo	GOF	1 (F)	0/1	0/1	0/1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	0/1
g.25593560G>A	p.(Gly282Ser)	de novo	GOF	3 (2 M:1 F)	2/3	2/3	1/3	0/3	3/3	3/3	na	3/3	2/3	0/3	1/3
g.25593570T>G	p.(Leu285Arg)	de novo	LOF	1 (F)	0/1	0/1	0/1	0/1	1/1	0/1	0/1	1/1	1/1	1/1	0/1
g.25593585T>G	p.(Met290Arg)	inherited	LOF	1 (F)	1/1	0/1	0/1	0/1	1/1	nr	1/1	nr	nr	nr	0/1
g.25593588A>T	p.(His291Leu)	de novo	GOF	1 (M)	1/1	1/1	0/1	0/1	1/1	0/1	nr	1/1	1/1	1/1	0/1
g.25593590A>G	p.(Asn292Asp)	de novo	LOF	1 (M)	0/1	0/1	0/1	0/1	1/1	0/1	nr	1/1	1/1	0/1	1/1
g.25593603G>C	p.(Gly296Ala)	de novo	GOF	1 (F)	1/1	0/1	nr	0/1	1/1	1/1	Nr	1/1	1/1	1/1	0/1
g.25593708G>A		inherited	nd	1 (F)	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	nr
g.25594679G>T		inherited	nd	1 (F)	1/1	0/1	0/1	0/1	1/1	1/1	nr	0/1	0/1	0/1	nr
g.25596419G>C		de novo	nd	1 (M)	1/1	0/1	0/1	0/1	1/1	1/1	nr	1/1	1/1	0/1	0/1
g.25596420G>A	p.(Gly384Asp)	de novo	LOF	2 (F)	1/2	0/2	0/2	0/2	2/2	?	0/1	2/2	1/2	1/2	nr
g.25596428C>T	p.(Arg387Cys)	de novo	GOF	15 (8 M:7 F)	15/15	12/15	7/8	8/15	10/10	9/9	1/1	9/9	6/8	3/5	6/8
g.25596428C>A	p.(Arg387Ser)	de novo	GOF	2 (M)	2/2	1/2	1/1	1/2	1/2	1/2	0/1	1/1	1/2	1/2	0/1
g.25596429G>T	p.(Arg387Leu)	unknown	GOF	1 (F)	1/1	0/1	nr	0/1	1/1	1/1	nr	1/1	nr	nr	nr
g.25596457dup	p.(Gly397T rpfs*15)	de novo	LOF	1 (M)	1/1	0/1	0/1	1/1	1/1	0/1	0/1	0/1	1/1	1/1	0/1
g.25596462C>G	p.(Ser398*)	de novo	LOF	1 (M)	1/1	1/1	1/1	0/1	1/1	0/1	0/1	1/1	1/1	1/1	0/1
g.25596473C>G	p.(Leu402Val)	de novo	GOF	1 (F)	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1
g.25596474T>C	p.(Leu402Pro)	de novo	LOF	3 (1 M:2 F)	3/3	1/3	2/2	0/3	2/2	2/2	1/1	1/2	2/2	1/2	2/2
g.25596489T>C	p.(Leu407Pro)	de novo	LOF	1 (M)	1/1	1/1	1/1	1/1	na	na	na	1/1	1/1	0/1	0/1
Total number of individuals with a given feature (%)				52 24 M:28 F	45/52 (87)	21/49 (43)	16/36 (44)	16/46 (35)	36/40 (90)	25/34 (74)	6/15 (40)	30/38 (79)	25/37 (68)	15/34 (44)	14/30 (47)

F, female; *GOF*, gain-of-function; *LOF*, loss-of-function; *M*, male; *na*, not ascertained; *nd*, not determined; *nr*, not reported.

[a]Mode of inheritance reported in at least 1 individual.

[b]Impact of the variant based on the functional studies performed herein.

[c]The denominator corresponds to the number of individuals for whom the presence or absence of a given feature was reported. Variants in bold were not previously reported in the literature.

receptor (Figure 2A). The similar responses using both RAR activating ligands indicate that the GOF effects were not isomer specific. We also found that the variants p.(Phe279Val), p.(His291Leu), and p.(Arg387Leu) recently identified in individuals with MCOPS12,[12-14] also conferred GOF potential of activation when compared with the wild-type receptor (Figure 2A, Supplemental Table 1). Therefore, all 3 reported substitutions at Arg387(Cys/Ser/Leu) resulted in a GOF receptor.[3,4] Finally, we found that the de novo variant p.(Gly294Val) reported in a DECIPHER individual (277774) with MCOPS12 also induces a GOF effect (Figure 2A, Supplemental Table 1).

In contrast, the novel LBD variants p.(Trp218Cys), p.(Leu285Arg), p.(Met290Arg), p.(Asn292Asp), p.(Gly384Asp), p.(Leu402Pro), and p.(Leu407Pro) were significantly less effective than the wild-type receptor in responding to RA ligands (1 μM), indicating that loss-of-function (LOF) variants can also cause MCOPS12 (Figure 2A). We also found that the de novo variant p.(Ile403Thr) reported in a DECIPHER individual (265740) with MCOPS12 exhibited a reduced response to ligands (Figure 2A, Supplemental Table 1). This variant and the closely located p.(Leu402Pro) and p.(Leu407Pro) affect residues within helix-12 that form the coactivators' interacting motif. Finally, we found that the de novo variant p.(Ser398*) recently described in a child with MCOPS12 exhibited LOF activity (Figure 2A).[15] Because this variant is in the last exon of the gene, it is unlikely to induce nonsense-mediated decay of the transcript and may thus result in the production of a truncated protein without helix-12. We have not tested the effect of the adjacent variant p.(Gly397Trpfs*15) reported here, but we predict a response similar to that of p.(Ser398*) because both are missing a functional helix-12.

We next investigated whether the regulation of mutant LOF receptors is dose dependent. Interestingly, significant increases in activity were observed at higher RA concentrations, suggesting a potential for LOF receptors to be activated (Figure 2B). Given the dose-dependent regulation of LOF receptors, we then tested the response of GOF receptors to diminished levels of retinoids. We noticed that though some GOF receptors remained activated at lower concentrations of RA, others became less responsive compared with wild-type RARB (Figure 2C), suggesting that these might exhibit LOF activity in conditions of minimal access to retinoids. Such behavior is not well understood and deserves future investigation. Still, this divergent response to lower levels of retinoids might support the presence of distinct subclasses within the family of variants with a GOF effect at the concentration of retinoids (1 μM) used in our assay.

Dominant-negative effect of LOF variants

We next addressed the possibility that the LOF variants decrease the transcriptional responsiveness to RA through a dominant-negative mechanism. HEK293 cells produce RARB at high levels compared with the other RAR isoforms, and transfection of these cells with a luciferase reporter under the control of a genuine RARE binding site provides a strong transcriptional readout to the endogenous response to RA ligands (1 μM) (Figure 3A). Under such condition, the expression of all tested LOF variants caused a significant and dose-dependent reduction in RA response, consistent with a dominant-negative behavior (Figure 3B-E). Notably, helix-12 LOF variants were among the most effective in inducing a dominant-negative effect (Figure 3E), along with the p.(Met290Arg) LBD variant (Figure 3C). On the other hand, expression of the wild-type RARB or the GOF p.(Arg387Cys) (not shown) and p.(Arg269Thr) receptors did not downregulate ligand responsiveness under the same conditions (Figure 3B). We also analyzed the biallelic truncating variants p.(Arg119*) and p.(Ile403Serfs*15), which were identified in siblings with MCOPS12.[3] Both variants dramatically decreased the transcriptional response of RARB to RA.[3] Here, we found that p.(Ile403Serfs*15), which disrupts helix-12, induced a dominant-negative effect, whereas p.(Arg119*), which lacks the second zinc finger essential for DNA binding, had no dominant-negative activity (Figure 3E and F).

To address whether DNA binding is required for promoting the dominant-negative activity of LOF variants, we replaced 2 Cys residues (Cys98 and Cys101) that are essential for RARB DNA binding and transcriptional activation by Ala residues (Figure 3G). The insertion of the C98A;C101A mutation in the context of the p.(Met290Arg), p.(Ser398*), and p.(Leu407Pro) receptors strongly impaired their dominant-negative effects when compared with intact DBD mutants (Figure 3H). These results suggest the requirement of DNA binding capacity and RARE occupancy as a mechanism by which the LOF variants may exert their dominant-negative potential.

Among the novel variants described here, p.(Gly103Cys) is the only one that is located in the DBD, affecting position +2 from the first of the 2 zinc fingers critical for DNA binding. We found that p.(Gly103Cys) reduced RARB response to RA ligands via a dominant-negative effect (Figures 2A and 3F). Thus, despite a possible lower DNA binding to RARE elements, p.(Gly103Cys) remains effective at disrupting wild-type RARB response.

Structural impact of dominant-negative variants

To gain insight into the effect of the dominant-negative variants at the protein level, we performed all-atom MD simulations on the ligand-free LBD of the p.(Met290Arg) and p.(Leu402Pro) mutants compared with wild-type RARB. We observed that the 2 variants were stable over 100-ns simulations when compared with the wild-type receptor (Figure 4A), suggesting that both variants are not sufficient to disrupt the overall fold of the LBD. However, the p.(Met290Arg) variant placed a charged residue into a

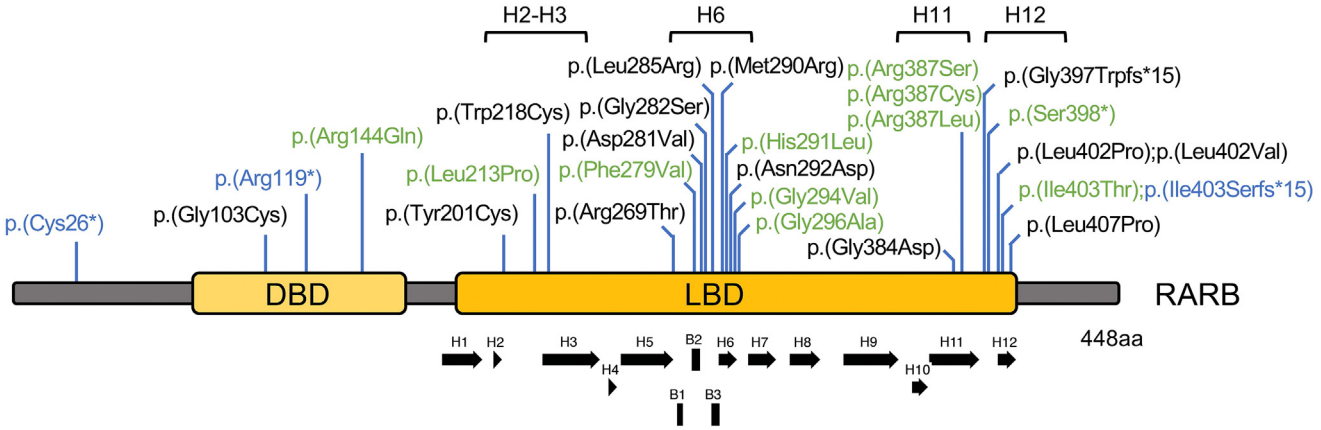

Figure 1 Localization of RARB variants. Schematic representation of the position of biallelic (blue) and dominant (novel in black, previously reported in green) coding variants along the RARB protein. Also shown are the positions of the 12 α-helices (H1-H12) and the 3 β-turns (B1-B3) of the protein. *DBD*, DNA binding domain; *LBD*, ligand binding domain; *RARB*, retinoic acid receptor beta.

hydrophobic part of the ligand-binding pocket. During simulation, Arg290 relaxed into the space normally occupied by the adjacent Phe295, a key ligand-binding residue, displacing it away and reconfiguring the ligand-binding site into a binding-incompetent conformation considering the unfavored burying of a charged residue in a hydrophobic environment (Figure 4B). The p.(Met290Arg) variant is therefore likely to interfere with ligand binding. Leu402 is part of helix-12 required to recruit transcriptional coactivators. The p.(Leu402Pro) variant breaks surface complementarity, removing important hydrophobic contacts, which affects helix-12 integrity and cofactor binding (Figure 4C). These findings are consistent with a scenario where both mutants could normally interact with DNA and RXR, yet fail to engage the ligand and/or the coactivator for transcriptional activation.

Phenotypic spectrum associated with *RARB* variants

In addition to the 25 affected individuals with novel variants, we report here 5 new individuals with known variants, including p.(Arg387Cys) (n = 3), p.(Arg387Ser) (n = 1), and p.(Leu213Pro) (n = 1), and we provide additional clinical information about previously described individuals with the variants p.(His291Leu) and p.(Ser398*) (Supplemental Table 1).[13,15] In total, by combining these 32 cases with 20 previously reported ones,[3,4,12,14,16-19] we obtained a series of 52 individuals carrying likely pathogenic or pathogenic dominant variants in *RARB* (total of 26 variants) for whom we have access to some clinical data (Table 1, Supplemental Table 1). Two cases were fetuses from terminated pregnancies and 6 individuals were deceased, including 4 neonatal deaths caused by respiratory failure related to diaphragmatic hernia/eventration and 2 deaths in older children from infections in the context of severe motor impairment.[4] Inheritance is known in the case of 43 families: variants occurred de novo in 38 of them and were inherited in 5, either from an affected (2 families) or unaffected (3 families) parent. Only the parents with clinical features are included in Table 1 and Supplemental Table 1 and discussed hereafter. The 2 DECIPHER cases that we characterized at the molecular level were not included in Table 1 and in this analysis of RARB-related phenotypes.

Developmental eye anomalies were the most common feature with a prevalence of 45 of 52 (87%) (Table 1, Supplemental Table 1). Affected individuals displayed a variable combination of unilateral or bilateral microphthalmos/anophthalmos (n = 34); anterior segment dysgenesis (including corneal opacification, sclerocornea, and Peter's anomaly and/or iris strands) (n = 22); coloboma of the iris, retina, choroid and/or optic nerve (n = 22); cataracts (n = 5); optic nerve hypoplasia (n = 5); foveal hypoplasia (n = 2); lens subluxation (n = 2); blepharophimosis (n = 2); and retinal dysplasia (n = 2). Other ophthalmological findings were only reported once in our series (Supplemental Table 1). Four individuals did not have any of these developmental eye anomalies clinically recognized.

In addition, 21 of 49 (43%) affected individuals showed diaphragmatic hernias and/or eventrations. Heart defects were found in 15 of 45 participants (33%) who had an echocardiography. Swallowing difficulties (16 of 36, 44%) were frequently observed, necessitating G-tube or nasogastric feeding in at least 13 affected individuals. Four individuals had intestinal malrotation, and 4 others had congenital hip subluxation. Episodes of hypoglycemia of unclear etiology were reported in 5 affected individuals.

Most affected individuals showed severe gross motor delay. Among the 36 individuals who were 24 months of age or older and whose motor developmental history is known, 17 (47%) could not sit unassisted even as late as 18 years of age (including 3 individuals who lost the ability to sit unassisted), 7 (19%) could sit unassisted but could not walk, 8 (22%) started to walk after 24 months of age, and 4 (11%) had no gross motor delay. Motor impairment was initially associated with axial hypotonia (30 of 38, 79%) and subsequently with spasticity (25 of 37, 68%) and/or dystonia

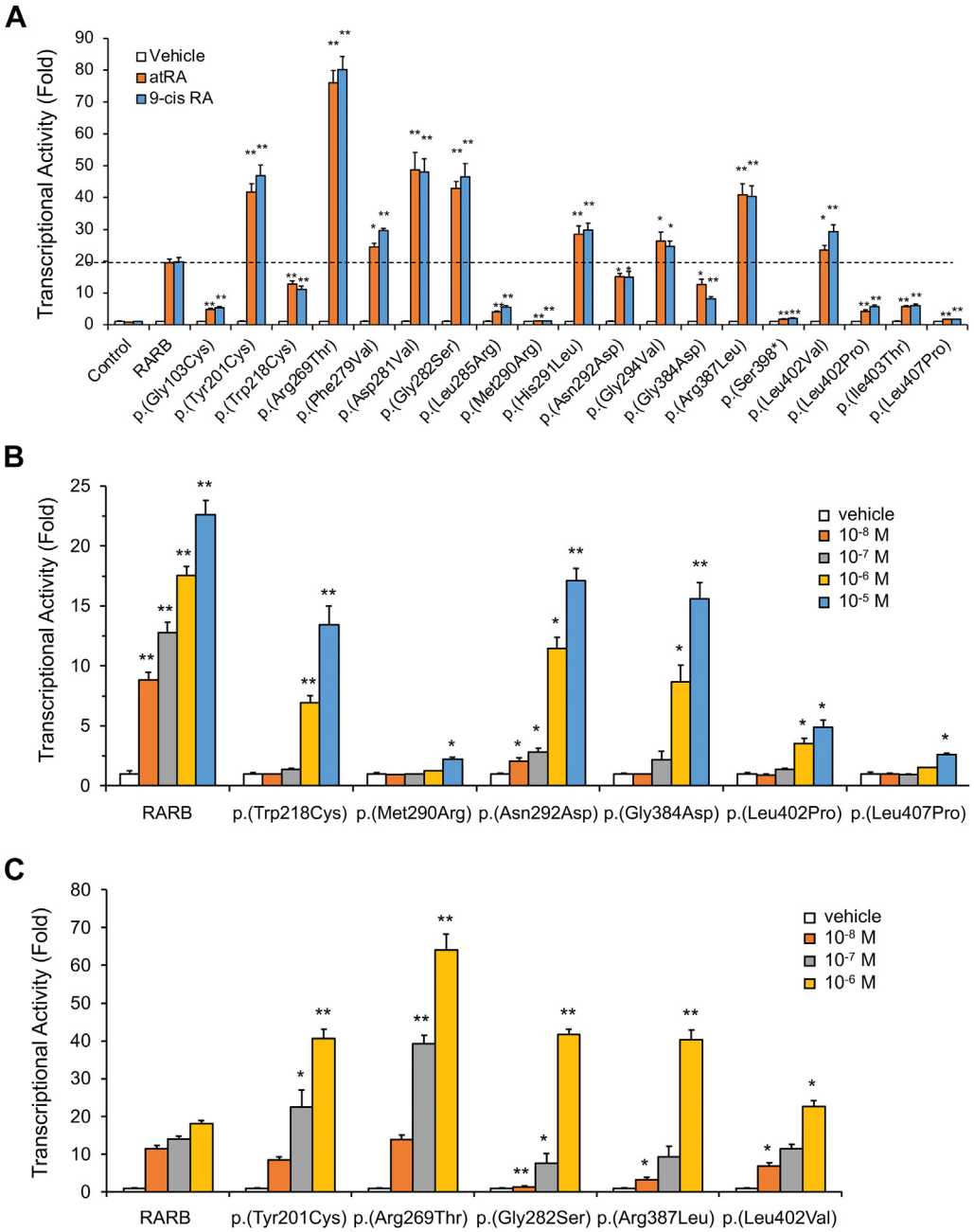

Figure 2 **Transcriptional response of RARB variants to RA ligands.** A. Human embryonic kidney HEK293 cells were transfected with Gal4 fusion plasmids of wild-type human RARB or the indicated genetic variants in the presence of UAStkLuc reporter luciferase gene construct. Cells were then treated with 1 μM atRA, 1 μM 9-*cis* RA, or vehicle (dimethyl sulfoxide; 1/1000, v/v) for 16 hours. Luciferase values were normalized to β-galactosidase activity and expressed as a fold response compared with vehicle-treated cells set at 1.0 for each mutant. Empty Gal4-transfected cells were used as a negative control. Data (mean ± SEM) were derived from at least 4 independent experiments performed in triplicate. *$P < .05$; **$P < .01$ vs wild-type RARB response to each respective RA ligand. B. The same assay as in (A) was used to assess the impact of increasing concentrations of atRA on the transcriptional response of LOF variants. *$P < .05$; **$P < .001$ vs untreated mutant RARB response. C. Similar as in (B) except that GOF variants were tested to decreased concentrations of atRA. *$P < .05$; **$P < .001$ vs wild-type RARB treated in the same condition. *atRA*, all-*trans* retinoic acid; *DMSO*, dimethyl sulfoxide; *GOF*, gain of function; *LOF*, loss of function; *RARB*, retinoic acid receptor beta.

(15 of 34, 44%), which was reported as progressive in 5 individuals.

Among the 36 individuals who were 24 months of age or older and whose language developmental history is known, 24 (67%) individuals had some language delay, including 10 who were nonverbal even as late as 18 years of age and 14 who could say a few isolated words or make short sentences, 10 (28%) seemed to display normal language

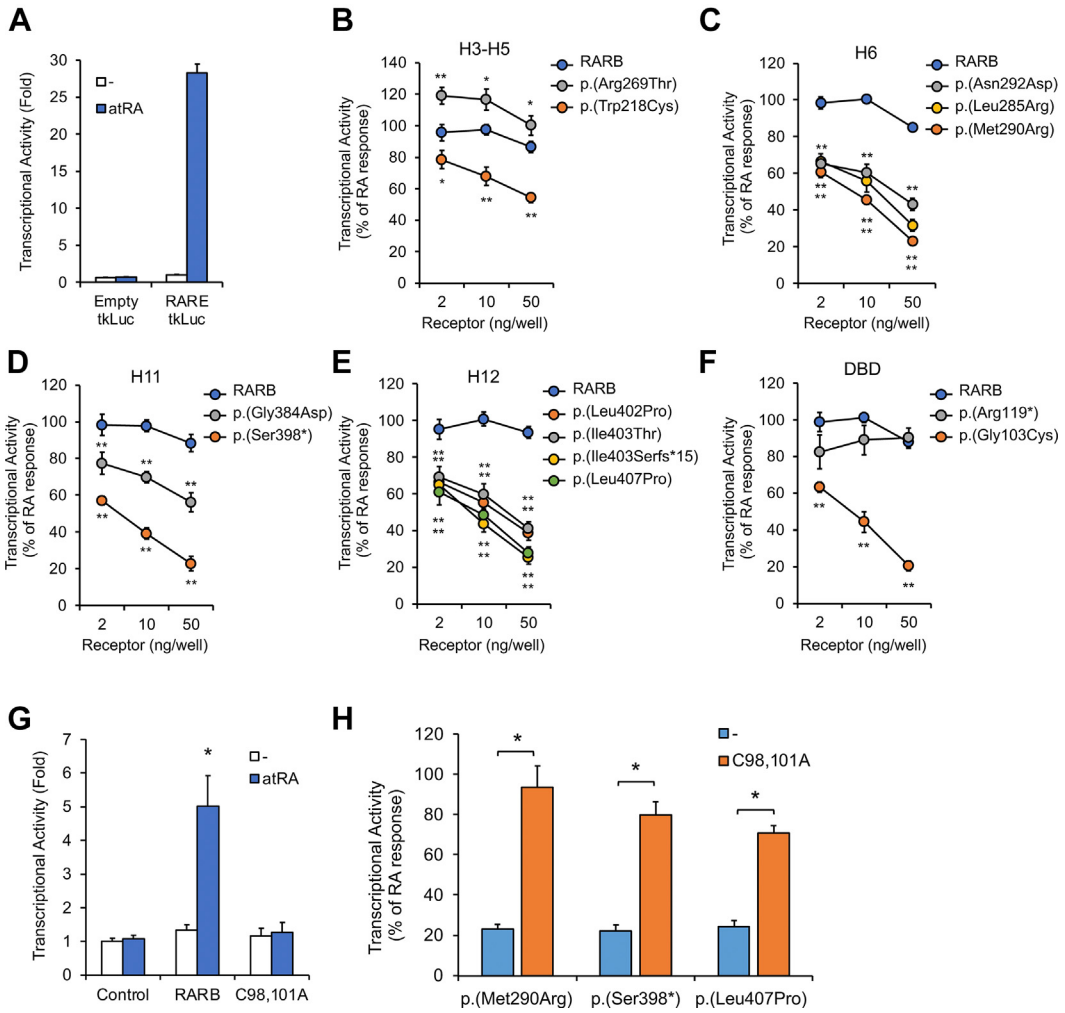

Figure 3 **Dominant-negative effects of RARB variants.** A. Endogenous response of HEK293 cells to atRA. Cells were transfected with a RAREtkLuc reporter luciferase gene construct or an empty tkLuc reporter as a negative control and then treated with 1 μM atRA or vehicle (DMSO; 1/1,000, v/v) for 16 hours. Luciferase values were normalized to β-galactosidase activity and expressed as a fold response compared with vehicle-treated RAREtkLuc-transfected cells set at 1.0. B-F. HEK293 cells were seeded in 24-well plates and transfected as in (A) with an RAREtkLuc reporter in the presence of increasing concentrations of plasmids encoding wild-type RARB or each indicated variant. Luciferase values were normalized to β-galactosidase activity, and dominant-negative activity was determined as the % change of endogenous RA response determined as in (A) and set at 100%. Data (mean ± SEM) were derived from at least 4 independent experiments performed in triplicate. *$P < .05$; **$P < .005$ vs wild-type RARB-transfected cells in the same conditions. G. The C98,101A mutation in the DNA binding domain abolished RARB response to RA. RAR triple knockout mouse embryonic fibroblasts were transfected or not (control) with wild-type or C98,101A mutated RARB in the presence of the RAREtkLuc reporter and treated with RA (1 μM atRA, 16h). Luciferase values were expressed as fold (mean ± SEM) compared with untreated control cells. Data were derived from at least 4 independent experiments performed in triplicate. *$P < .01$. H. DNA binding domain integrity is indispensable for dominant-negative activity of LOF variants. HEK293 cells were transfected with each indicated LOF variant in the context or not of the C98,101A mutation, and treated as in (G) in the presence of the RAREtkLuc reporter. Dominant-negative activity was determined as the % change of endogenous RA response set at 100%. Data (mean ± SEM) were derived from at least 4 independent experiments performed in triplicate. *$P < .05$. *atRA*, all-*trans* retinoic acid; *DBD*, DNA binding domain; *DMSO*, dimethyl sulfoxide; *LOF*, loss of function; *RA*, retinoic acid; *RARB*, retinoic acid receptor beta.

development, and 2 (6%) individuals had language delay of unspecified severity. It is noteworthy that 6 individuals with severe motor impairment were reported as having normal language or mild language impairment. Cognitive impairment was not systematically characterized. Intellectual disability was reported in 5 individuals, whereas normal cognition was described in 4 individuals with severe motor impairment. Brain magnetic resonance imaging showed a variable combination of Chiari malformation type 1 (14 of 30, 47%), enlargement of the lateral and/or third ventricles (12 of 30, including 8 individuals with Chiari malformation), changes in the shape or size of the corpus callosum

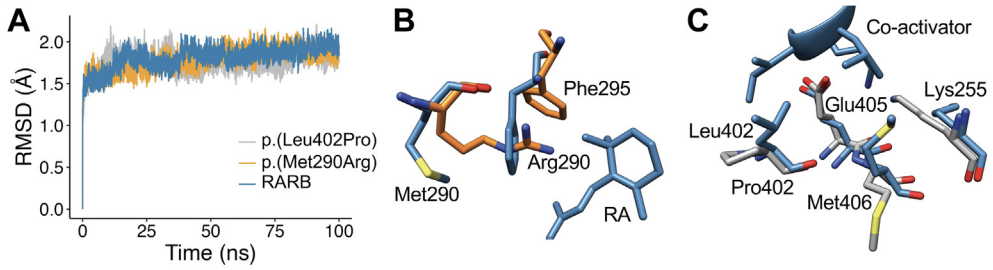

Figure 4 Molecular dynamics simulation of RARB variants structural changes. A. Root mean square deviation traces over 100 ns all-atom MD trajectories have been performed for p.(Met290Arg) and p.(Leu402Pro) variants and compared with wild-type RARB. Results show that all receptor forms were stable under simulation. B. The p.(Met290Arg) variant results in repacking of side chain residues in the binding pocket: wild-type structure used to set up simulation (blue, PDB ID 4DM8) vs a frame from the end of the mutant simulation (orange). Arg290 extends toward the ligand pocket, pushing aside Phe295, a key contact residue. C. The p.(Leu402Pro) variant changes the shape of coactivator binding surfaces, removing important hydrophobic contacts to the coactivator helix (top): wild-type structure used to set up simulation (blue, PDB ID: 4DM8) vs a frame from the end of the mutant simulation (gray). *MD*, molecular dynamics; *RA*, retinoic acid; *RARB*, retinoic acid receptor beta; *RMSD*, root-mean-square deviation.

(5 of 30), and white matter anomalies (5 of 30) (Supplemental Table 1). Interestingly, the size of basal ganglia was decreased in 2 individuals with severe motor impairment.

GOF and LOF variants were found in 34 and 15 participants, respectively (Supplemental Table 2). Statistical analysis (Fisher test) did not show any significant difference between the phenotypic manifestations associated with these 2 classes of variants. It is noteworthy that p.(Arg387Cys), which is present in about a third of affected individuals, seems to be associated with a relatively homogeneous phenotype with a high prevalence of diaphragmatic anomalies, swallowing difficulties, motor and language impairment, and Chiari malformation type 1.

Of interest, p.(Leu402Val) is the only variant in helix-12 with a GOF effect. This variant occurred de novo in an individual with Chiari malformation type 1 and growth hormone deficiency but without any developmental eye anomaly or motor impairment. Although this presentation is atypical, we conclude that it is likely explained by p.(Leu402Val) as discussed in detail in the Supplemental material.

Discussion

We report here the most extensive series of individuals with pathogenic variants in the *RARB* gene. Our study indicates that these individuals display a broader and more variable phenotype than previously described. For instance, although individuals with *RARB* variants reported until now displayed developmental eye anomalies, we report here that such variants can be associated with the absence of a clinically recognized eye phenotype even in individuals with global developmental delay and severe motor impairment. Conversely, we described individuals with developmental eye anomalies but without any other clinical manifestations. The clinical heterogeneity of MCOPS12 is also illustrated by both the intrafamilial and interfamilial heterogeneity as observed by the variable presence of some cardinal features of MCOPS12 in members of the same family or in unrelated individuals carrying the same variant allele. Given this heterogeneity, the use of the term MCOPS12, which refers to a syndromic form of microphthalmia, seems inappropriate to designate the disorders found in individuals who display isolated eye involvement or who show neurodevelopmental involvement without any eye anomaly. As the clinical manifestations associated with RARB become better delineated, a dyadic approach combining the gene name with the appropriate phenotypic descriptors or an alternative disorder naming system could potentially be considered, as recently suggested.[20,21]

Functional heterogeneity of *RARB* variants

The genetic landscape associated with *RARB*-related disorders is characterized by the predominance of missense variants distributed in clusters along the LBD. This pattern raises the possibility that these variants disrupt RARB function by modulating RA ligand effect on the receptor rather than by causing haploinsufficiency. Indeed, we have previously reported that dominant LBD variants associated with MCOPS12 exhibited a GOF effect in promoting their response to RA ligands at the concentration of 1 μM.[3,4] Here, we show that not all of the LBD pathogenic variants exert GOF activity; rather, some of them display LOF activity. Given their distribution in multiple clusters across the LBD, we envision that the LOF variants affect the transcriptional activity of RARB via distinct mechanisms. For example, variants located in the vicinity of the ligand-binding pocket could perturb proper positioning of RA in RARB. This is likely the case for the p.(Met290Arg) variant, which is predicted, based on our molecular simulation analysis, to interfere with the optimal binding of RA by disrupting the hydrophobic ligand-binding pocket

without affecting the overall folding of the protein. A different mechanism could be invoked for the LOF activity of helix-12 variants. It is known that the integrity of helix-12 is an absolute requirement in establishing contacts with transcriptional coactivators to mediate proper ligand activation of nuclear receptors. Accordingly, our simulation analysis of p.(Leu402Pro) MD has indicated a major change to the shape of the surface used to engage the coactivator, resulting in a disorganized helix-12 configuration.

Our observation that LOF variants display cross-regulation with endogenous RARB strongly suggests that they behave as dominant-negative receptors. We found that an intact DBD is required for the dominant-negative activity of LOF variants. Such requirement is consistent with the formation of RXR heterodimers with LOF receptors, thereby occupying RARE elements on DNA and hence preventing normal RXR-RARB binding to target genes. Therefore, we propose that the mechanism underlying the dominant-negative effects of these LOF variants involves a competition between functional and nonfunctional complexes for binding to cognate RARE elements on DNA, thereby decreasing the ligand-binding transcriptional activation of target genes. Interestingly, we also observed that the DBD variant p.(Gly103Cys) decreased ligand responsiveness via a dominant-negative effect. This variant resides in the first zinc finger of the DBD in a region that is required for proper binding of nuclear receptors to DNA.[22] We thus suspect that the p.(Gly103Cys) receptor may form nonfunctional heterodimers through ineffective binding to DNA, thereby impeding the access of DNA sites to wild-type receptor.

We were able to functionally classify mutant receptors based on their increased or decreased responsiveness to retinoids at the concentration of 1 μM, which has been classically used in cellular assays.[23-26] Interestingly, we found that some GOF receptors remained activated at lower concentrations of RA, whereas others displayed decreased responsiveness in conditions of minimal access to retinoids, raising the possibility that they have a LOF activity, possibly involving a dominant-negative effect, at these lower RA levels. Although tissue-specific RA synthesis and degradation levels remain hard to determine, studies have raised the possibility that physiological RA signaling could be triggered in the low to high nanomolar range depending on target regions during early embryonic development.[27,28] Further studies will be necessary to fully characterize the subclasses of mutant receptors and the optimized gene dosage "window" to achieve biological balance.

Impact of *RARB* haploinsufficiency

Biallelic truncating variants in *RARB* have been reported in 2 MCOPS12 families. In 1 of them, 2 affected siblings inherited the compound heterozygous variants p.(Arg119*) and p.(Ile403Serfs*15),[3] whereas in the other family, a single child inherited the homozygous variant c.78C>A:p.(C26*).[29] Here, we show that p.(Ile403Serfs*15), similar to the closely located p.(Gly397Trpfs*15) and p.(Ser398*), induces dominant-negative effects, most likely by producing a protein without a functional helix-12, whereas the upstream p.(Arg119*) variant is likely to disrupt RARB function by inducing haploinsufficiency of the gene. Thus, truncating variants in *RARB* have distinct effects depending on their position along the gene, as previously described for other disorders.[30]

The fact that the parents of these affected individuals carrying the p.(C26*) or p.(Arg119*) variant were not reported to display any MCOPS12 phenotypic anomalies suggests that *RARB* haploinsufficiency may be clinically silent. However, gnomAD indicates that RARB has a low tolerance to haploinsufficiency (pLI: 1), reporting the presence of only 7 different heterozygous truncating variants (12 alleles; gnomAD v2.1.1 and v3.2), including 3 variants located at the very end of the coding region. Interestingly, Kalaskar et al[19] identified the DBD variant p.(Arg137Gln) in individuals with isolated bilateral colobomas and found that it was causing haploinsufficiency. Altogether, these observations raise the possibility that *RARB* haploinsufficiency may have some variable outcomes ranging from being silent in some individuals to causing a non-syndromic form of developmental eye anomalies in others.

The presence of the dominant-negative p.(Ile403Serfs*15) in *trans* with the haploinsufficient p.(Arg119*) in MCOPS12 siblings is puzzling. Even though the parent carrying p.(Ile403Serfs*15) appears asymptomatic, we cannot exclude the possibility that MCOPS12 in this family is caused by this dominant-negative variant, with minimal contribution of the haploinsufficient variant. Additional studies will be required to further investigate the clinical impact of *RARB* haploinsufficiency.

Three *RARB* variants occur at canonical splice sites. The individual with c.991+1G>A shows an isolated developmental eye anomaly, whereas the individual with c.1150+1G>T displays developmental eye anomalies with a history of mild developmental delay. Both of these variants were inherited from unaffected parents. In contrast, c.1151-1G>C occurred de novo in an individual with a much more severe disorder, including eye anomalies, global developmental delay, and motor impairment (Table 1, Supplemental Table 1). In silico splice site analysis using varSEAK (https://varseak.bio/) predicts that c.991+1G>A and c.1150+1G>T induce the skipping of exons 6 and 7, respectively, resulting in the creation of a frameshift and that c.1151-1G>C activates a cryptic splice acceptor site in intron 7, 42 bp from the boundary with exon 8 (the last exon of the gene), also introducing a frameshift. A possible interpretation of these results is that c.991+1G>A and c.1150+1G>T cause haploinsufficiency by inducing nonsense-mediated decay and/or the production of a truncated protein without any ability to dimerize and bind DNA, whereas the more distal splice variant c.1151-1G>C induces

a dominant-negative effect by selectively disrupting helix-12. Unfortunately, validation of this hypothesis was not possible because we could not have access to RNA samples from the participants.

Parallel with other nuclear receptors

To date, defects in almost half of the 48 nuclear receptor genes have been involved in human disorders.[31] Both GOF and dominant-negative variants have been described in these disorders.[32-37] Interestingly, some of these variants act through similar mechanisms to those described here for RARB. For example, most LOF variants in the LBD of the thyroid hormone receptors THRA and THRB, including missense and truncated variants located in helix-12, have been reported to cause thyroid hormone resistance via a dominant-negative effect over the wild-type receptor, either by decreasing ligand affinity and/or by disrupting coactivator recruitment.[36-39] Of note, dominant-negative THRA and THRB receptors could be rescued using higher concentrations of the ligand in vitro, and administration of THR agonists has been shown to decrease the biochemical and clinical anomalies associated with thyroid hormone resistance.[36,37] Our findings suggest that a similar mechanism might prevail to rescue the transcriptional response of dominant-negative RARB receptors with higher RA concentrations, opening up the possibility of using selective RARB agonists to treat MCOPS12.

Data Availability

Data and materials individually are available upon request.

Acknowledgments

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Ethics Declaration

This study was approved by the CHU Sainte-Justine's research ethics board. Informed consent was obtained from all participants, and data were deidentified. The DDD study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC).

Conflict of Interest

J.R.L. owns stock in 23andMe and is a paid consultant for Genome International. All other authors declare no conflicts of interest.

Additional Information

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