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Gain-of-Function Mutations in *RARB* Cause Intellectual Disability with Progressive Motor Impairment

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ABSTRACT: Retinoic acid (RA) signaling plays a key role in the development and function of several systems in mammals. We previously discovered that the de novo mutations c.1159C>T (p.Arg387Cys) and c.1159C>A (p.Arg387Ser) in the RA Receptor Beta (*RARB*) gene cause microphthalmia and diaphragmatic hernia. However, the natural history of affected subjects beyond the prenatal or neonatal period was unknown. Here, we describe nine additional subjects with microphthalmia who have de novo mutations in *RARB*, including the previously described p.Arg387Cys as well as the novel c.887G>C (p.Gly296Ala) and c.638T>C (p.Leu213Pro). Moreover, we review the information on four previously reported cases. All subjects who survived the neonatal period (n = 10) displayed severe global developmental delay with progressive motor impairment due to spasticity and/or dystonia (with or without chorea). The majority of subjects also showed Chiari type I malformation and severe feeding difficulties. We previously found that p.Arg387Cys and p.Arg387Ser induce a gain-of-function. We show here that the p.Gly296Ala and p.Leu213Pro *RARB* mutations further promote the RA ligand-induced transcriptional activity by twofold to threefold over the wild-type receptor, also indicating a gain-of-function mechanism. These observations suggest that precise regulation of RA signaling is required for brain development and/or function in humans.

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KEY WORDS: retinoic acid; RARB; gain-of-function; movement disorder; developmental delay; intellectual disability

Introduction

Retinoic acid (RA) signaling is required for the development of several organs, including the eye, heart, diaphragm, lungs, and limbs. Both loss and gain of RA signaling cause structural defects of these organs, indicating that precise regulation of this pathway is required for appropriate development [Ross et al., 2000; Cunningham and Duester, 2015). RA is a metabolite of retinol, a derivative of vitamin A. In target cells, RA binds to a heterodimer RA receptor complex formed of retinoic acid receptor (RAR) and retinoid X receptor (RXR). There are three subtypes of RAR (RARA, RARB, and RARG) and three subtypes of RXR (RXRA, RXRB, and RXRG). The RA receptor complex binds to DNA motifs known as RA response elements (RAREs) to modulate transcription of target genes by interacting with transcriptional corepressors and coactivators.

We previously reported the identification of two de novo missense mutations in *RARB* (MIM #180220; NM_000965.3), c.1159C>T (p.Arg387Cys) and c.1159C>A (p.Arg387Ser), in three subjects with microphthalmia, diaphragmatic hernia, and

Additional Supporting Information may be found in the online version of this article.

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variable pulmonary hypoplasia [Srour et al., 2013]. These subjects were mainly assessed during the prenatal or neonatal period and their long-term course had not yet been characterized. Here, we identified, by targeted or exome sequencing, nine additional individuals with de novo mutations in *RARB* harboring either the recurrent c.1159C>T (p.Arg387Cys) mutation or the novel mutations c.887G>C (p.Gly296Ala) or c.638T>C (p.Leu213Pro). Moreover, we obtained follow-up information on a previously described individual with the p.Arg387Cys mutation and review the information on three additional reported individuals [Srour et al., 2013; Slavotinek et al., 2014]. These patients have bilateral microphthalmia and a complex neurologic phenotype characterized by severe developmental delay, progressive spasticity, movement disorder, Chiari type I malformation (Chiari-I; MIM #118420), and feeding difficulties. We show that the two novel de novo variants exhibit a significant increased response to RA ligands compared with wild-type RARB receptor, indicating that they confer a gain-of-function, like the previously described de novo mutations in *RARB*. Increased RA signaling mediated by RARB thus appears to specifically disrupt brain development and/or function.

Materials

Subjects and Mutation Analysis

Previously undescribed individuals with de novo mutations in *RARB* were recruited from Canada (subjects 1, 2, and 6), the USA (subjects 3, 5, 7, 8, and 9), and the UK (subject 4). Informed consent was obtained from each participant or legal guardian and the study was approved by the ethics research board of CHU Sainte-Justine (Montreal, Quebec, Canada).

Exome capture and sequencing was done on a clinical or research basis at four different facilities: Whole Genome Laboratory at Baylor College of Medicine (BCM) (subjects 2, 5, and 7; Roche Nimblegen VCRome v2.1 exome capture and HiSeq2000 sequencing), GeneDx (subject 3 and 9; Agilent SureSelect XT2 v4 capture and HiSeq2000 sequencing), Ambry Genetics (subject 8; Roche Nimblegen VCRome v.2.0 exome capture and HiSeq2500), and McGill University and Genome Quebec Innovation Centre (Montreal, Canada, subject 6; Agilent SureSelect XT v5 exome capture, HiSeq2000). Read processing, mapping to human genome reference hg19, variant calling, annotations, and filtering for rare variants (minor allele frequency $\leq$ 1%–5%) affecting the coding sequence and/or consensus splice sites were performed as previously described [Neveling et al., 2013; Yang et al., 2013; Dhamija et al., 2014; Trakadis et al., 2014). Briefly, variants affecting coding and splice sites that were present at minor allele frequencies $\leq$1%–5% in public databases (e.g., 1000 Genomes, NHLBI Exome Sequencing Project [ESP] Exome Variant Server [EVS]) and in in-house control datasets were selected). Among these variants, only those present in known disease genes (OMIM [http://omim.org], HGMD [http://www.hgmd.cf.ac.uk/ac/index.php]) or in specific sets of disease genes relevant to the phenotype of interest were prioritized for further analyses.

Nucleotide numbering of the mutations herein reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the NCBI reference sequence NM_000965.3, whereas the amino positions are based on the corresponding NCBI reference sequence NP_000956.2. All de novo *RARB* mutations identified herein were submitted to the ClinVar database: http://www.ncbi.nlm.nih.gov/clinvar/?term = RARB (accession numbers: SCV000257491.1; SCV000257490.1; SCV000106029.2; SCV000106028.2). Predictions of the effects of the de novo missense *RARB* mutations on the protein function were done using SIFT (v1.03; http://sift.jcvi.org/) and PolyPhen-2 (v2.2r398; http://genetics.bwh.harvard.edu/pph2/).

Transfection Studies

The transcriptional potential of *RARB* was determined using a cellular one-hybrid luciferase reporter transcriptional assay, as previously described [Srour et al., 2013]. Briefly, human embryonic kidney HEK293 cells were seeded in 24-well plates and transfected with 100 ng per well of expression plasmid encoding either Gal4 fusion of wild-type human RARB, or p.Arg387Cys, p.Arg387Ser, p.Leu213Pro, and p.Gly296Ala RARB variants in the presence of 500 ng of UAStkLuc reporter–gene construct. All variant constructs were generated by site-directed mutagenesis and confirmed by automated sequencing. Cells were treated with 1 μM all-*trans* RA (atRA), 1 μM 9-cis RA, or vehicle (DMSO; 1/1,000, v/v) for 16 hr. Luciferase values were normalized to β-galactosidase activity and expressed as a fold response compared with those of empty Gal4-transfected control cells. Data were derived from three to five independent experiments performed in triplicate.

Results

De Novo Mutations in *RARB*

We identified de novo missense mutations in *RARB* in nine previously undescribed individuals (subjects 1–9), including two monozygotic twins (subjects 1–2) (Table 1). These mutations were identified by exome sequencing in seven unrelated individuals (subjects 2, 3, 5–9) and by targeted sequencing in a newborn (subject 4). Sanger sequencing confirmed that these mutations were present in the blood DNA of the affected individuals (including in subject 1, the twin brother of subject 2) but not their parents, indicating that they are de novo. These mutations included the previously-reported recurrent c.1159C>T (p.Arg387Cys) in seven individuals and the novel c.887G>C (p.Gly296Ala) and c.638T>C (p.Leu213Pro) variants, each in a single individual (Table 1). Both the c. 638T>C (p.Leu213Pro) and c.887G>C (p.Gly296Ala) missense mutations are predicted to be damaging according to SIFT (score = 0.00) and Polyphen-2 (HumVar score = 0.855 and 1.000, respectively), and affect well-conserved residues (Fig. 1A). These mutations are absent from public SNP databases (1000 Genomes, EVS, or ExAC [http://exac.broadinstitute.org/]).

Clinical Phenotype of Individuals with De Novo Missense Mutations in *RARB*

Combining these nine subjects with four previously described cases, we are aware of a total of eight females and five males with de novo mutations in *RARB* (Table 1; Supporting Information). Four individuals are deceased: subject 1 died at 13 years of age from a pneumonia possibly caused by lung aspiration, and his twin brother (subject 2) died at 10 years of age from dehydration secondary to gastroenteritis; subject 4 and subject 12 died in the first few days of life from respiratory failure caused by diaphragmatic hernia. One case (subject 11) was the product of a pregnancy terminated at 34 weeks gestation because of the presence of structural defects.

All individuals had micro/anophthalmia, with blindness or poor vision. The microphthalmia was bilateral in 11/13 subjects. In

Table 1. Clinical Features of Individuals with De Novo Mutations in *RARB*

Subject	1	2	3	4	5	6	7	8	9	10[a]	11[b]	12[c]	13[d]
Mutation (NM_000965.3)	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.887G>C p.Gly296Ala	c.638T>C p.Leu213Pro	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.1159C>A p.Arg387Ser	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys	c.1159C>T p.Arg387Cys
Gender	M	M	F	F	F	F	F	F	M	M	M	F	F
Age (age at death)	(10 y)	(13 y)	4 y	(7 d)	5 y	3 y	5.5 y	8 y	5 y	18 y	(Fetus)	(Few hours)	NA
Microphthalmia	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral	Unilateral[f]	Bilateral	Bilateral	Unilateral	Bilateral	Bilateral
Diaphragmatic hernia (eventration)	(Yes)	No	No	Yes, left	(Yes) left	No	No	No	Yes	Yes	Yes, left	Yes, left	Yes, left
Cardiac abnormalities	PPS	No	VSD, PDA	PDA, PFO	ASD	No	PDA, left SVC	No	PFO, PPS	No	No	No	No
Chiari type 1	Yes	Yes	Yes	NA	Yes	No	No	Borderline	Yes	No	NA	NA	Yes
Developmental delay	Severe	Severe	Severe, mainly motor	NA	Severe	Severe	Severe	Severe	Yes	Severe	NA	NA	NA
Ambulation	Nonambulat.	Nonambulat.	Nonambulat.	NA	Nonambulat.	Nonambulat.	Nonambulat.	Nonambulat.	Nonambulat.	Nonambulat.	NA	NA	NA
Language	Sentences, reads Braile	Words	Short sentences	NA	No words[e]	Words	Words	Sentences		Words?	NA	NA	NA
Motor regression	Yes	Yes	Yes	NA	Yes	Yes	Yes-with illness	Yes	No	Yes	NA	NA	NA
Progressive spasticity	Yes	Yes	No	NA	Yes	Yes	No	Yes	Yes	Yes	NA	NA	NA
Movement disorder	No	No	No	NA	Yes dystonia and choreoatheto-sis	Yes dystonia	Yes dystonia and choreoathetosis	Yes dystonia and choreoathetosis	No	NA	NA	NA	NA
Other	Dysphagia, gastrostomy, sagittal cran-iosynostosis	Dysphagia gastrostomy, Nissen fundoplication	Hypoglycemia	Anterior placement of anus, rectovaginal fis-tula,hepatosplenomegaly	Hydrocephalus, hypoglycemia	Transient neonatal apnea	Intestinal malrotation, gastrostomy, severe GERD, breath-holding spells, hypoglycemia	Severe GERD, vitamin B12 deficiency, premature adrenarche, laryngomalacia	Intestinal malrotation, severe GERD, dysphagia, gastrostomy, Nissen fundoplication, congenital hearing loss, kypho-sis,dysmorphisms, mirror movements		Intestinal malrotation, mild IUGR	Bilateral lung hypoplasia, bicornate uterus	NA
Cause of death	Respiratory illness	Gastroenteritis		Cardiorespiratory failure							Terminated pregnancy	Respiratory failure	

[a]Subject II-3 (Family B)
[b]Subject II-1 (Family C), and
[c]Subject II-1 (Family D) in Srour et al. (2013).
[d]Subject described in Slavotinek et al. (2014).
[e]was putting three words together, but lost ability after an episode of regression.
[f]right microphthalmia and left coloboma and sclerocornea.
ASD, atrial septal defect; d, days; F, female; GERD, gastroesophageal reflux; IUGR, intrauterine growth restriction; M, male; NA, not applicable/not available; nonambulat., nonambulatory; PDA, patent ductus arteriosus; PFO, patent foramen ovale; PPS, peripheral pulmonic stenosis; SVC, superior vena cava; VSD, ventral septal defect; y, year.

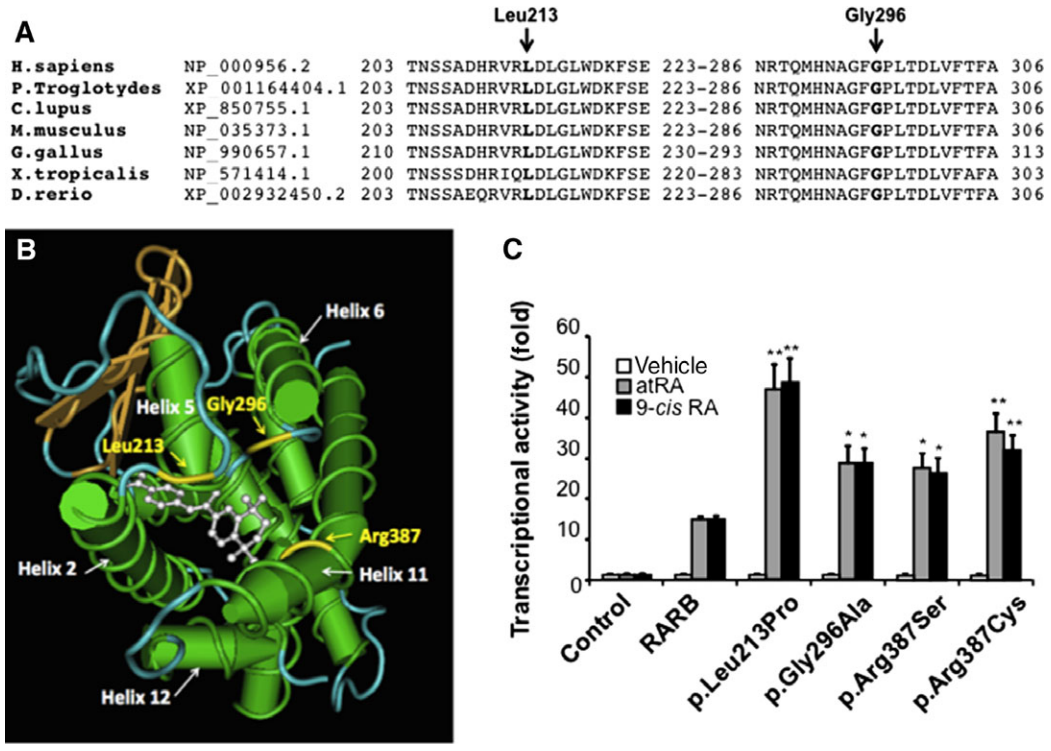

Figure 1. **A**: Conservation of residues Leu213 and Gly296, which are affected by the novel de novo mutations in RARB. **B**: Localization of the residues affected by the de novo mutations c.1159C>T (p.Arg387Cys), c.1159C>A (p.Arg387Ser), c.887G>C (p.Gly296Ala), and c.638T>C (p.Leu213Pro) in a crystallographic model of RARB. Three-dimensional structure of RARB ligand-binding domain (Protein Data Bank ID 4DM8) in the presence of RA ligand (white) shows the proximity of the Arg387, Leu213, and Gly296 residues surrounding the hydrophobic ligand pocket. **C**: Transcriptional activation of human RARB variants to RA ligands. HEK293 cells were transfected with expression plasmid encoding either Gal4 fusion of wild-type human RARB or the indicated genetic variants in the presence of UAStkLuc reporter–gene construct. All variant constructs were generated by site-directed mutagenesis. Cells were treated with 1 μM atRA, 1 μM 9-*cis* RA, or vehicle (DMSO; 1/1,000, v/v) for 16 hr. Luciferase values were normalized to β-galactosidase activity and expressed as a fold response compared with those of empty Gal4-transfected control cells. Data were derived from three to five independent experiments performed in triplicate. $^{*}P < 0.005$, $^{**}P < 0.0001$ versus wild-type RARB response to each respective RA ligand. Values represent means, and error bars represent SEMs. The crystal structure of RARB was visualized using Cn3D (vs 4.3.1) through the NCBI's Website (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid = 97846).

addition to microphthalmia, most subjects had sclerocornea (10/11) and coloboma (7/10) consistent with anterior segment defects. Congenital diaphragmatic hernia or eventration was present in 8/13 and cardiac abnormalities in 6/13 (patent ductus arteriosus, patent foramen ovale, peripheral pulmonic stenosis, left superior vena cava, atria and ventral septal defects). Other structural abnormalities included malrotated bowel (3/13), anterior placement of anus with a rectovaginal fistula (1/13), sagittal craniosynostosis (1/13), and bicornate uterus (1/13).

All the individuals who survived the neonatal period and for whom we have follow-up information ($n = 10$; age range 2–16 years) had global developmental delay, with the motor domain being most severely affected (Table 1; Supporting Information). These individuals were nonambulatory. Language development was more variable, with the majority of individuals saying only few words, but some able to make sentences. One individual was able to read Braille (subject 1). Patients were initially hypotonic, but most (7/10) developed increasing spasticity in their limbs (between ages 2 and 8 years). In addition, a large proportion of individuals (4/9) developed dystonia, with or without choreoathetosis. Dystonia in one of these patients (subject 7) improved with levodopa treatment. Cerebrospinal fluid concentration of the dopamine metabolite homovanillic acid was normal prior to treatment in this subject. In all affected individuals, the progressive spasticity and movement disorder resulted in regression of their motor function, with loss of milestones, such as the ability to sit or stand with support.

Episodes of recurrent severe apneas were noted in 5/10 individuals in the neonatal period and early infancy. These events had a mixed central and obstruction pattern, and were often believed to be either associated with severe gastroesophageal reflux and laryngospasm or laryngomalacia. Feeding issues were prominent. Many patients had dysphagia and severe gastro-oesophageal reflux, resulting in failure to thrive (5/10) and requiring a gastrostomy in 5/10 individuals. Three individuals had recurrent episodes of unexplained hypoglycemia despite extensive endocrinologic testing. Three individuals also developed episodes of developmental regression either after general anesthesia (subjects 5 and 7) or during intercurrent illness (subject 3), followed by gradual improvement but without return to baseline. No individual had epilepsy. Chiari-I was observed in seven of the 10 individuals in whom a brain MRI was performed (Fig. 2). Prominent ventricles or hydrocephalus was noted in six individuals (of whom five have a Chiari-I), including one individual who required a third ventriculostomy and Chiari-I decompression surgery (subject 3) and a second requiring a ventriculoperitoneal shunt (subject 5). No other structural or white matter abnormalities were noted.

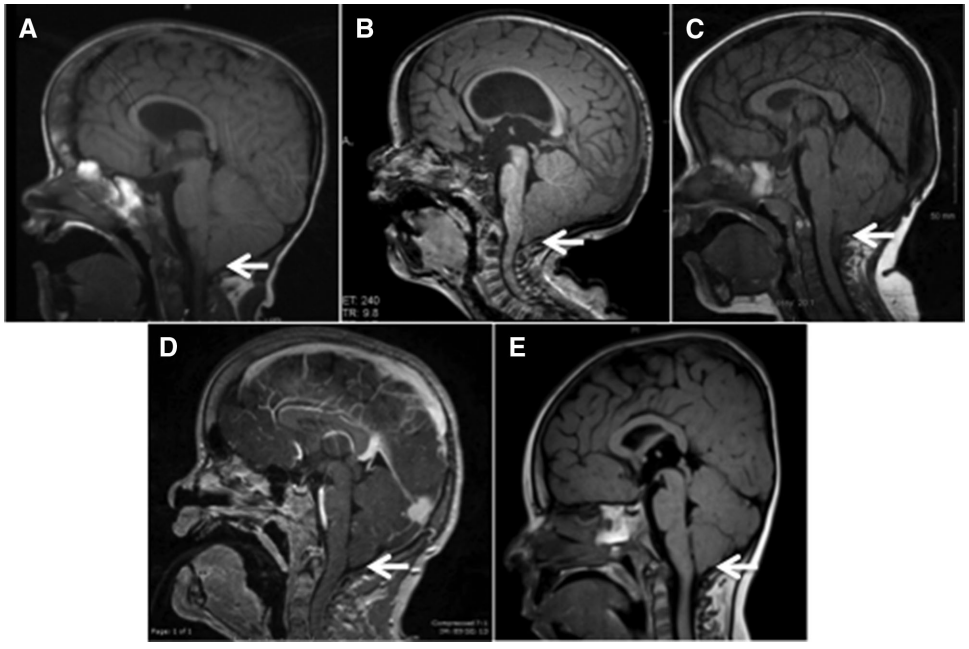

Figure 2. Brain MRI showing signs of Chiari type I malformation. Sagittal T1 MRI image from cases 2(**A**), 3(**B**), 5(**C**), 8(**D**), and 9(**E**) showing Chiari type-I malformation (arrows).

Functional Impact of *RARB* Mutations

RA controls gene expression at the transcriptional level by interacting with RA receptors, promoting their direct binding to RAREs contained in responsive genes. The retinoid receptors have a basic modular design consisting of an N-terminal A/B activation domain, a central DNA-binding domain (region C), a linker/flexible hinge (region D), a ligand-binding domain (region E), and a C-terminal region F of unknown structure and function. The ligand-binding domain, which is formed by 12 conserved alpha helices and a beta turn, contains the ligand-binding pocket, the main dimerization domain, and a hydrophobic cleft involved in coregulator binding. The binding of RA triggers the appropriate repositioning of helix 12 (also known as activating function-2) of RA receptors to hinder corepressor interaction and concomitantly creating a proper docking site to allow recruitment of coactivators, resulting in an increase in transcription of target genes. The crystallographic structure of the ligand-binding domain of RARB has determined the exact position of amino acids forming the hydrophobic core embedding the ligand. Interestingly, the residues Leu213, Gly296, and Arg387, which are affected by the mutations described here (c.638T>C [p.Leu213Pro], c.887G>C [p.Gly296Ala], c.1159C>T [p.Arg387Cys], and c.1159C>A [p.Arg387Ser]), are located within the ligand-binding site of RARB and in close proximity to the retinoid ligand (Fig. 1B). The Leu213 residue is located near helix 2, Gly296 between helix 5 and 6 and Arg387 near helix 11. More strikingly, these residues are facing inward relative to the position of RA (Fig. 1B).

We previously found that the variants p.Arg387Ser and p.Arg387Cys induced a gain-of-function effect in RARB. We thus sought to determine whether the novel de novo variants, p.Leu213Pro and p.Gly296Ala, confer the same properties by using a one-hybrid functional assay, as previously described [Srour et al., 2013]. Both altered RARB exhibited potent twofold to threefold increases in their transcriptional response to atRA and 9*cis*-RA ligands compared with wild type. The p.Leu213Pro RARB variant had a striking 46–48-fold increase in transcriptional response, and the p.Gly296Ala RARB variant a 28–29-fold increase compared with 14–15-fold induction for wild-type RARB (Fig. 1C). For comparison, the p.Arg387Ser and p.Arg387Cys RARB variants exhibited a 25–26-fold and 30–35-fold induction, respectively (Fig. 1C). These results suggest that all the de novo mutations studied here are associated with a gain-of-function mechanism with excessive transcriptional responsiveness to RA ligands.

Discussion

In this study, we report nine previously undescribed subjects bearing de novo mutations in *RARB*. In total, we are aware of 13 subjects with such mutations [Srour et al., 2013; Slavotinek et al., 2014). We previously established that these individuals have microphthalmia with variable diaphragmatic hernia [Srour et al., 2013]. We show here that they also display a complex neurological phenotype, which includes intellectual disability, progressive motor deterioration, and Chiari-I, reflecting the involvement of RA signaling in multiple developmental and physiological processes.

Our transcriptional assay revealed that all of the documented mutations (p.Arg387Cys, p.Arg387Ser, p.Gly296Ala, and the p.Leu213Pro) conferred gain-of-function properties to RARB, increasing its transcriptional activation potential by twofold to threefold over the wild-type receptor in response to both atRA and 9*cis*-RA ligands. Interestingly, Leu231, Gly296, and Arg387 are all located adjacent to residues establishing direct contacts with retinoid ligands, based on the crystal structure of RARB [Germain et al., 2004] and of the highly similar RARG isoform [Renaud et al., 1995]. One possibility is that the corresponding mutations induce a gain-of-function effect by increasing RA-binding affinity. Alternatively,

these substitutions could induce a conformational change in RARB affecting protein stability and coactivator recruitment.

Progressive Motor Impairment

The neurologic phenotype observed in individuals with de novo mutations in *RARB* described herein appears to be progressive in the majority of the individuals who survived the neonatal period. Indeed, almost all individuals (8/9) experienced regression in their motor function, noted between the ages of 1 and 6 years, with loss of the ability to sit or stand. This was mainly associated with the appearance of progressive spasticity (7/11) with or without dystonia and choreoathetosis (4/11). Thus, affected individuals exhibit both corticospinal deficits, as evidenced by the progressive spasticity, as well as extrapyramidal deficits, as evidenced by the movement disorder. Of note, dystonia, especially in patients with the chronic form of the disorder, may be difficult to differentiate from spasticity. Thus, we may be underestimating the presence of dystonia in our patients.

Movement disorders such as dystonia and chorea are typically explained by some dysfunction of the striatum, a subdivision of the basal ganglia that plays a critical role for the control of voluntary movements by integrating incoming information from all regions of the cortex. The striatum is composed principally of GABAergic medium spiny neurons, which project to the substantia nigra and globus pallidus (reviewed in Crittenden and Graybiel, 2011). Dopamine signaling plays a major role in the control of voluntary movements via the D1 dopamine receptor in the striatonigral neurons and the D2 dopamine receptor (D2R) in the striatopallidal neurons. The lateral ganglionic eminence (LGE), which gives rise to the striatum, is the main source of RA in the forebrain during late embryonic development [Molotkova et al., 2007]. Genetic knockout studies in mice have shown that loss of RA production in the LGE impairs GABAergic differentiation in the striatum [Chatzi et al., 2011]. *Rarb* is predominantly expressed in the LGE during mouse forebrain development [Liao et al., 2005; Chatzi et al., 2011). Moreover, *Rarb* mutant mice have a reduction of DR1-producing striatonigral neurons, which is caused by decreased proliferation and premature differentiation of neuronal progenitors through a RA-dependent mechanism [Liao et al., 2008; Rataj-Baniowska et al., 2015]. These mice have impaired motor coordination and balance [Chiang et al., 1998; Krezel et al., 1998]. In addition, administration of apomorphine, a dopamine agonist, induces exaggeration of head bobbing movement and reduction of rearing activity when compared with wild-type controls, suggesting that their movement abnormalities are dopamine-sensitive, as observed in Subject 7 [Liao et al., 2008]. It is possible that the development of these striatal circuits is sensitive to both a decrease and increase of RA signaling via RARB. Gain-of-function mutations in *RARB* would then cause a movement disorder by affecting their development. It is important to note that dystonia due to abnormal development of striatal circuits may not be necessarily obvious at birth but emerge later during life, as is observed in many genetic forms of dystonia or dystonic cerebral palsy.

Another possibility is that the motor impairment observed in our subjects involves a mechanism that is impacting postnatal striatal function. RARB and its partner RXRγ are both expressed in the striatum of adult mice [Krezel et al., 1999]. Interestingly, the D2R promoter contains a functional RARE [Samad et al., 1997]. Although the expression of D2 receptors is not altered in the striatum of *Rarb* adult mutant mice, it is dramatically reduced in the striatum of double *Rarb:Rxr*γ mutant mice, suggesting that RARB has the potential of modulating D2R expression [Samad et al., 1997; Krezel et al., 1998]. These observations raise the possibility that gain-of-function mutations could affect the control of voluntary movement by increasing RARB transcriptional activity and enhancing the expression of dopamine receptors.

Cognitive Impairment

Subjects with gain-of-function mutations in *RARB* display cognitive deficits. Several observations suggest that *RARB* is required for synaptic processes involved in cognition. *Rarb* mutant mice show impaired spatial memory, which does not appear to be related to their motor or visual deficits [Chiang et al., 1998]. In these mice, the hippocampus, which plays a key role in spatial memory and learning, shows impaired long-term potentiation and long-term depression, two forms of synaptic plasticity involved in memory and learning [Chiang et al., 1998]. *RARB* thus appears to be required for synaptic processes involved in cognition, which might be disrupted by the gain-of-function mutations identified in our cases. Striatal dysfunction per se can also affect cognition. For instance, overexpression of D2R specifically in the striatum results in selective cognitive impairments in working memory by directly disrupting the function of the prefrontral cortex [Kellendonk et al., 2006]. As suggested above, it is possible that the gain-of-function mutations in *RARB* increase the expression of D2R, contributing to the cognitive impairment of the affected individuals. Finally, increased RA signaling could affect cognition via a developmental mechanism. For instance, abolition of RA synthesis during development markedly reduces the differentiation of a population of cortical GABAergic interneurons that originate from the LGE [Chatzi et al., 2011]. Disruption of cortical GABAergic circuits has the potential of causing cognitive impairment. It is unclear, however, whether RARB regulates the development of these cells.

Chiari Type I Malformation

Remarkably, seven patients, including our pair of twins and a previously published patient, all with the p. Arg387Cys mutation, showed Chiari-I [Slavotinek et al., 2014]. This abnormality is defined by the observation of a descent of the cerebellar tonsils across the foramen magnum. Hypoplasia of the occipital bone, resulting in a shallow posterior cranial fossa, is believed to cause Chiari-I. Tonsillar herniation then occurs because the posterior cranial fossa is unable to house a normal cerebellum. Previous studies suggest that increased RA signaling affects occipital bone development. Marin-Padilla and Marin-Padilla (1981) reported that vitamin A administration during embryogenesis cause hypoplasia of the basioccipital bone in hamsters, resulting in a small posterior cranial fossa and downward displacement of the developing cerebellum. Interestingly, an association was observed between the occurrence of Chiari-I in humans and variants in *ALDH1A2*, which codes for one of the key enzymes catalyzing RA synthesis [Urbizu et al., 2013].

The occurrence of Chiari-I in our cases suggests that RARB signaling regulates the development of occipital bones. Interestingly, a homozygous null allele of *CYP26B1*, which codes for a RA-degrading enzyme, has been described in siblings with severe hypoplasia of the occipital and parietal bones [Laue et al., 2011]. This mutation is predicted to increase RA signaling pathways, possibly mimicking the effect of the gain-of-function mutations described here. Studies performed in mice and zebrafish indicate that RA promotes the differentiation of osteoblasts into (pre)osteocytes [Maclean et al.,

2009; James et al., 2010; Laue et al., 2011). It has been proposed that increased RA signaling would lead to a depletion of osteoblasts and decreased generation of osteoid, resulting in calvarial hypoplasia [Laue et al., 2011]. A similar mechanism could explain the development of Chiari-I in our subjects.

Phenotypical Convergence of Loss- and Gain-of-Function Mutations in *RARB*

Gain-of-function mutations in *RARB* cause microphthalmia and diaphragmatic hernia [Srour et al., 2013]. Moreover, human and mice studies have shown that exposure to vitamin A or RA during embryonic development results in various congenital malformations, including microphthalmia and diaphragmatic hernia [Padmanabhan et al., 1981; Balkan et al., 1992; Ozeki et al., 1999; Sulik et al., 1995; Ozeki and Shirai, 1998; Lee et al., 2012]. Interestingly, we previously described fetal and newborn siblings with microphthalmia and diaphragmatic hernia, carrying biallelic truncating mutations in *RARB* [Srour et al., 2013]. Therefore, both deficiencies and excess of RA have the potential of inducing the same phenotypes. A recent study showed that RA exposure during embryonic development in mice was followed by decreased levels of *Raldh* transcripts encoding RA-synthesizing enzymes and increased levels of Cyp26a1 and Cyp26b1, mRNAs encoding enzymes that catabolize RA [Lee et al., 2012]. Overall, these changes resulted in a decrease in RA levels. Restoration of RA levels by maternal supplementation with low doses of RA after the teratogenic insult rescued several developmental defects. Paradoxically, increased RARB signaling could thus result in a secondary state of RA deficiency, which could have an impact on this pathway at specific stages of development. Alternatively, it is possible that some developmental processes require a tight regulation of RARB targets, given that too little or too much signaling has the same consequence on these pathways.

Conclusion

We have shown that gain-of-function mutations in *RARB* cause a complex developmental and progressive disorder of the brain, providing novel insight into the role of RA in neural networks in humans. A better understanding of the molecular and cellular pathophysiology of this disorder might result in the development of pharmacological treatments that would aim to modulate RA signaling.

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