Expansion of the *CHN1* Strabismus Phenotype

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PURPOSE. Hyperactivating *CHN1* mutations have been described in individuals with Duane retraction syndrome with or without vertical gaze abnormalities. This was a study of five family members with distinctive ocular dysmotility patterns that co-segregated with a novel hyperactivating *CHN1* mutation.

METHODS. Participating members of a family segregating pleomorphic incomitant strabismus underwent ophthalmic examinations, and several underwent high-resolution magnetic resonance imaging (MRI) of the orbits and brain stem. Participant DNA was extracted and amplified for haplotype analysis encompassing the *CHN1* region on chromosome 2q31.1, and mutation analysis of the *CHN1* gene, which encodes the Rac-GAP signaling protein $\alpha 2$ -chimaerin. In vitro functional studies of the co-inherited mutation were performed, including a Rac-GTP activation assay, quantification of $\alpha 2$ -chimaerin translocation, and co-immunoprecipitation.

RESULTS. All five clinically affected family members exhibited monocular or binocular supraduction deficits, three in the absence of Duane retraction syndrome. MRI in four affected individuals demonstrated small or absent abducens nerves in all four, small oculomotor nerve in one, and small optic nerves in three. Superior oblique muscle volume was also decreased in three of the individuals, supporting trochlear nerve hypoplasia. Strabismus segregated with the *CHN1* locus and affected individuals harbored a c.443A>T *CHN1* mutation (p.Y148F). In vitro, this novel mutation behaved similarly to previously

reported *CHN1* mutations underlying familial Duane syndrome, hyperactivating $\alpha 2$ -chimaerin by enhancing its dimerization and membrane association and lowering total intracellular Rac-GTP.

CONCLUSIONS. Analysis of the current pedigree expands the phenotypic spectrum of hyperactivating *CHN1* mutations to include vertical strabismus and supraduction deficits in the absence of Duane retraction syndrome. (*Invest Ophthalmol Vis Sci.* 2011;52:6321–6328) DOI:10.1167/iows.11-7950

The congenital cranial dysinnervation disorders (CCDDs) result from mutations in genes essential to normal development and/or connectivity of cranial motor neurons.^{1,2} To date, seven CCDD genes have been reported. Mutations in three CCDD genes cause both vertical and horizontal gaze deficits and alter development of the oculomotor or oculomotor and trochlear motor neurons more than that of the abducens motor neurons. These include dominant missense mutations in *KIF21A* or *TUBB3* that underlie congenital fibrosis of the extraocular muscles type I (CFEOM1; MIM 135700)³ or congenital fibrosis of extraocular muscles type III (CFEOM3; MIM 600638),⁴ respectively, and recessive mutations in *PHOX2A* that underlie CFEOM2 (MIM 602078).⁵ Mutations in three other genes cause primarily horizontal gaze deficits and alter development or connectivity of abducens motor neurons: dominant mutations in *SALL4* cause Duane radial ray syndrome (DRRS; MIM 607323)⁶; recessive mutations in *HOXA1* cause syndromic horizontal gaze palsy (MIM 601536)⁷; and recessive mutations in *ROBO3* cause horizontal gaze palsy with progressive scoliosis (HGPPS; MIM 607313).⁸

The seventh reported CCDD gene is *CHN1* (MIM 604356)^{9,10}; members of nine unrelated families who segregate Duane retraction syndrome (DRS) as a dominant trait have been reported to harbor heterozygous missense mutations in *CHN1*. Two alternative *CHN1* promoters encode a longer $\alpha 2$ -chimaerin and shorter $\alpha 1$ -chimaerin protein,¹¹ and previous work supports hyperactivation of the $\alpha 2$ -chimaerin protein as underlying autosomal dominant DRS.⁹ All clinically affected members of the nine reported families had bilateral or unilateral DRS, whereas some were also noted to have vertical gaze abnormalities, including A- or V-pattern strabismus, dissociated vertical deviation, manifest hypertropia or hypotropia, limited up or down gaze, and/or unilateral ptosis.^{9,12–14} In addition, penetrance is not complete, and a few unaffected family members have been identified as mutation carriers.

Consistent with DRS, magnetic resonance imaging (MRI) of the orbits and brain stem of affected members of several families harboring *CHN1* mutations revealed small or absent abducens nerve(s) often accompanied by structurally abnormal lateral rectus (LR) muscles, and in some cases evidence of oculomotor nerve (CN3) innervation of the LR muscle.¹⁴ A subset of individuals also had hypoplasia of the superior oblique (SO) muscles, the oculomotor nerves, and/or the optic nerves.¹⁴ These data support a role for hyperactivated $\alpha 2$ -

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chimaerin in development of the abducens and, to a lesser degree, the oculomotor, trochlear, and optic nerves.

Here, we report five related family members who had supraduction deficits, three in the absence of DRS. The ocular dysmotility co-segregates with a novel hyperactivating *CHN1* mutation, establishing that *CHN1* mutations have greater ocular motility pleiotropy than previously recognized.

MATERIAL AND METHODS

Human Subjects

All members of a Caucasian family of Northern European ancestry (pedigree JB) were offered participation in an ongoing clinical and genetic study of strabismus at Children's Hospital Boston. Participants gave written informed consent to a protocol conforming to the Declaration of Helsinki and approved by relevant institutional review boards. Participants underwent ophthalmic examination, and subsets underwent MRI of the brain stem and orbits and/or donated saliva or blood for DNA extraction.

MR Imaging

Imaging was performed at the University of California Los Angeles with a 1.5-T scanner (Signa; General Electric, Milwaukee, WI) supplemented with an array of surface coils embedded in a transparent facemask (Medical Advances, Milwaukee, WI). Digital MR images were analyzed with ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Methodological details of imaging approaches and method of quantitative analysis are described in detail elsewhere.^{14,15}

Genetic Studies

Each genetic study participant provided a peripheral blood or salivary sample from which genomic DNA was extracted using (Puregene kit; Qiagen, Hilden, Germany) or DNA purifier solution (DNA Genotek, Ottawa, ON, Canada). Co-segregation to the DURS2 region was analyzed with the polymorphic markers *D2S2330*, *D2S335*, *D2S2314*, and *D2S364*. DNA from one affected individual (I:2) was then amplified and analyzed for mutations in the coding region of the *CHN1* gene (NM_001822) using a combination of dHPLC and direct sequencing, as previously reported.⁹ Variants identified by dHPLC were confirmed by Sanger sequencing. After identification of a putative mutation, DNA from each family member was sequenced to determine co-segregation with affection status. Public polymorphism databases were queried for the change, and 394 control DNA samples from unaffected individuals (200 Caucasian of Northern European ethnicity, 100 of Mexican ethnicity, and 94 of mixed ethnicity) were screened for the presence or absence of the change. Primer sequences, PCR, and dHPLC conditions are available on request.

Rac-GTP Activation Assay

V5 empty, V5 wild-type $\alpha 2$ -chimaerin, and V5 mutant $\alpha 2$ -chimaerin constructs were transiently transfected into HEK293T cells (Fugene6; Roche, Indianapolis, IN). After 48 hours' incubation in full medium, an Rac1 activation assay kit (Millipore, Billerica, MA) was used to isolate Rac-GTP by binding to the p21-binding domain of PAK1. The supernatant was run in a 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane (Invitrogen) for Western blot analysis with a mouse monoclonal anti-Rac1 antibody (BD Transduction Laboratories, San Jose, CA). E313K and A223V mutants were included as positive controls.⁹

Quantification of $\alpha 2$ -Chimaerin Translocation

Subcellular fractions were prepared from HEK293T cells 48 hours after transfection with V5- $\alpha 2$ -chimaerin constructs (Fugene6; Roche). Cells were pretreated for 30 minutes with 5 μ M of bisindolylmaleimide I (LC Laboratories, Woburn, MA) to inhibit PKC and then stimulated with 0

to 10 μ M of PMA for 20 minutes.¹⁶ Cells were sonicated in hypotonic buffer¹⁷ and were centrifuged at 100,000g at 4°C for 1 hour to separate the soluble and pellet fractions. The pellet fraction, containing both membrane and cytoskeleton, was then resuspended with equal volume of CSK buffer.¹⁷ Immunoblotting was performed with mouse monoclonal anti-V5 antibody (Invitrogen) and HRP-conjugated Donkey anti-mouse IgG (Jackson ImmunoResearch Laboratory Inc., West Grove, PA). Y143H, A223V, and P252Q mutants were included as positive controls.⁹

Co-immunoprecipitation Experiments

Three micrograms V5 wild-type $\alpha 2$ -chimaerin vector, V5 mutant $\alpha 2$ -chimaerin vector, or empty vector was transiently co-transfected with 3 μ g GFP-fused wild-type $\alpha 2$ -chimaerin vector into HEK293T cells. After 48 hours' incubation, the cells were pretreated with 5 μ M of PKC inhibitor for 30 minutes and then a subset was stimulated with 10 μ M of PMA for 30 minutes. The cells were solubilized in lysis buffer on ice for 30 minutes, and the lysate was centrifuged at 18,000g at 4°C for 20 minutes. The supernatant was precleared by incubation with rec-protein G-Sepharose 4B conjugate (Zymed Laboratories, Invitrogen) at 4°C for 1 hour and then incubated with rabbit polyclonal anti-GFP antibody (ab6556; Abcam, Cambridge, UK) and rec-protein G-Sepharose 4B conjugate at 4°C for 16 hours. The rec-protein G-Sepharose was then washed with cold 1 $\times$ PBS for 15 minutes four times, mixed with loading buffer, and boiled. Samples were run in a 12% Bis-Tris gel and transferred to nitrocellulose membrane. Signal was detected by mouse monoclonal anti-V5 antibody and HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Y143H was included as a positive control.⁹

RESULTS

Six family members (I:2, II:2, II:3, III:1, and III:5, who were reportedly affected, and III:2, who was reportedly unaffected) were examined by at least one of the authors. Four affected members (II:2, II:3, III:1, and III:5) participated in the MRI study, and nine family members participated in the genetic study (four reportedly affected, three reportedly unaffected, and two spouses; Fig. 1).

Clinical and Magnetic Resonance Imaging. The results of the ophthalmic examination and MRI are detailed below for each individual, and MRI results are also summarized in Table 1. Unless noted otherwise, levator palpebrae superioris function, pupillary function, ductions, saccades, pursuit, and stereopsis and visual field, slit lamp, intraocular pressure, and dilated fundus examinations were normal.

Family member I:2 was diagnosed with bilateral DRS in childhood. As an adult (Fig. 2), she exhibited bilateral horizontal rectus muscle co-contraction and bilateral abduction limitation. Abduction limitation was mild in the right eye and moderate in the left. She also exhibited mild limitation in supraduction bilaterally, with large V-pattern exotropia. Visual acuity, stereopsis, and dilated fundus examination data were not available, and MRI was not obtained.

Examination of individual II:2 was performed by two authors. The first examination revealed a small incomitant left hypertropia, worse in levoversion with a mild supraduction deficit of the right eye, whereas motility was found to be normal by a second examiner 1 year later. Corrected visual acuity of the right and left eyes was -0.1 logMAR. MR imaging performed at the time of the second examination revealed bilaterally small abducens and left oculomotor nerves within the subarachnoid space, with normal-appearing EOMs and intraorbital nerves (Table 1).

Family member II:3 had a history of a left head tilt since early childhood. He underwent strabismus surgery of unknown type in the right eye at 8 years of age. Examination as an adult

FIGURE 1. Family structure and haplotype analysis of pedigree JB. Schematic of pedigree JB structure, segregating haplotypes surrounding the *CHN1* gene and *CHN1* mutation status for participating individuals. *Squares*, males; *circles*, females; *filled symbols*, strabismus; *asterisk*, imaging by MRI. Alleles for four genetic markers are presented in order along chromosome 2 below genotyped individuals, with the paternal allele on the left and maternal allele on the right. Parentheses around an allele indicate that it is assumed. The segregation of the disease-associated haplotype is indicated by a *black bar*, and segregation of non-disease-associated haplotypes is indicated by *white or hatching*; combinations of patterns denote the presence of genetic recombination events. *CHN1* is located between *D2S335* and *D2S2314*, and the presence or absence of the *CHN1* mutation is indicated by a + or −, respectively.

revealed left hypertropia, larger in deorsumversion, and a right supraduction deficit (Fig. 3A). Corrected visual acuity was −0.1 and −0.2 logMAR in the right eye and left eye, respectively. Stereopsis was absent. Fundus examination was normal except for bilaterally small optic nerve heads without optic cups (Figs. 4A, 4B). MR imaging revealed small abducens and oculomotor nerves bilaterally within the subarachnoid space, with reduced size of all motor nerves, the right optic nerve, and SO muscle volume bilaterally within the orbit (Fig. 3B, Table 1).

Proband III:1 was diagnosed with left double-elevator palsy (monocular elevation deficiency) at age 6 months. Examination at 3 years of age revealed a left hypotropia with limited supraduction that was worse in adduction and mildly impaired stereopsis. Under anesthesia upward rotations were bilaterally unrestricted. She underwent a 10-mm recession of the right superior rectus muscle. Postoperative examination at 8 years of

age revealed left hypertropia that increased in attempted supraduction. Corrected visual acuity was 0.05 logMAR in the right eye and −0.05 in the left eye. MR imaging revealed normal oculomotor and small abducens nerves bilaterally within the subarachnoid space, with reduced size of all motor nerves and the optic nerves, and reduced SO muscle volume bilaterally within the orbits (Fig. 5).

Family member III:2 had normal examination results with physiological hyperopia.

Family member III:5 had childhood diagnoses of Brown syndrome in the right eye and DRS in the left eye. Examination at 22 years revealed limited supraduction of the right eye, greater in adduction than abduction. In the left eye, there was limited infraduction, abduction, and adduction, with retraction on attempted adduction and slow adducting and abducting saccades (Fig. 6A). Convergence was absent. Fundus examination demonstrated dysversion of both optic nerve heads, which

TABLE 1. Summary of MRI Data

Subject	Abducens Nerve in Subarachnoid Space	CN3 Cross Section in Subarachnoid Space (mm ²)		Motor Nerves within the Orbit	ON Cross Section within the Orbit (mm ²)		SO Muscle Volume (mm ³)	
		Right	Left		Right	Left	Right	Left
II:2	Small bilaterally	1.51	1.20*	Normal	8.10	8.69	152.4	154.5
II:3	Small bilaterally	1.45	1.36	Small	7.42*	9.05	134.8*	133.0*
III:1	Small bilaterally	1.62	2.17	Small	6.06*	5.57*	136.1*	112.9*
III:5	OS: not visualized	1.90	2.01	OS: abducens not visualized	5.96*	7.09*	134.0*	154.7
Average (±SD)		1.72 ± 0.36†			7.41 ± 1.34†		135 ± 15†	
Controls (± SD)		2.01 ± 0.36			14.10 ± 3.16		202 ± 33	

CN3, oculomotor nerve; ON, optic nerve; SO, superior oblique; OS, left eye.
* Specific measurement is significant at *P* < 0.05. Normal lower limit of CN3 width is 1.3 mm. Normal minimum ON cross section is 8.1 mm². Normal lower limit of SO volume is 137 mm³.
† Average of all subject measurements is significant at *P* < 0.001. Due to small size and/or variability along the nerve pathway, precise quantitative assessment of subarachnoid abducens nerve and intraorbital motor nerves cross-sectional areas are not feasible.

FIGURE 2. Diagnostic positions of gaze of subject I:2. Variable degrees of bilateral limitation of abduction and adduction, with mild limitation of supra- and infraduction, and large V-pattern exotropia.

were small and horizontally oval.¹⁸ There was a plethora of vascular crossings of both optic nerve head margins, with absence of the optic cup (Figs. 4C, 4D), and a peripapillary area of hyperpigmentation temporal to the left optic nerve, extending to a larger area of flat choroidal nevus (Figs. 4C, 4D). There were no peripheral retinal abnormalities. Corrected visual acuity was 0.1 and -0.2 logMAR in the right and left eyes, respectively. MRI showed the right subarachnoid abducens nerve to be present and not significantly different from normal, whereas the left subarachnoid abducens nerve could not be visualized in any image plane and was presumably absent (Fig. 6B). This finding correlates with the DRS phenotype on the left. The subarachnoid oculomotor nerves appeared normal. Within the left orbit, the abducens nerve was not directly visualized, and a dysplastic inferior division of the left oculomotor nerve abutted the inferior zone of the left lateral rectus muscle (Fig. 6C),

FIGURE 3. Postoperative clinical and MRI features of subject II:3. (A) Ocular versions demonstrating left hypertropia that increases in deorsumversion. (B) Orbital quasi-coronal MR images (*top* image proximal and *bottom* image distal within the orbit) showing bilateral superior oblique (SO) hypoplasia. Right optic nerve hypoplasia is most evident in the *bottom* image.

FIGURE 4. Optic disks of individuals II:3 and III:5. Fundus photos in color (A, C) and red-free (B, D) illumination, of the right eye (*left column*) and left eye (*right column*). Subject II:3 had small optic nerve heads and absent optic cups (A, B). Subject III:5 had small and horizontally oval optic nerve heads, absent optic cups, excessive vascular crossings of optic nerve head margins, and a peripapillary area of hyperpigmentation temporal to the left optic nerve, extending to a larger area of flat choroidal nevus (C, D).

consistent with findings frequently seen in DRS.¹⁴ Intraorbital motor cranial nerves otherwise appeared to be normal, but the intraorbital optic nerve cross-sectional area and SO muscle volume were reduced bilaterally. The left lateral rectus muscle was divided by a longitudinal fissure into superior and inferior zones with a small bright signal between (Table 1).

Linkage Analysis and Mutation Screening

Analysis of polymorphic markers flanking *CHN1* (DURS2 locus, MIM 604356) revealed co-segregation of a haplotype with the CCDD phenotypes, consistent with linkage to the *CHN1* locus (Fig. 1). Sequence analysis of the *CHN1* coding region in I:2 revealed a novel missense mutation c.443 A>T in exon 6 (Fig. 7A). This mutation co-segregated with the affected haplotype in II:2, III:1,

FIGURE 5. MRI features of subject III:1. (A) Quasi-coronal MRI of both orbits, arranged from posterior at *top* to anterior at *bottom*, demonstrates extraocular muscles, including the inferior rectus (IR), levator palpebrae superioris (LPS), medial rectus (MR), superior oblique (SO), and superior rectus (SR). There was reduction in the SO volume and cross-sectional area of the motor nerves and the optic nerve bilaterally. (B) Oblique axial MRI in the plane of the optic chiasm, revealing the oculomotor nerves (CN3). The right nerve was smaller than the left, but both diameters were within normal range statistically. (C) Quasi-sagittal MRI of both orbits demonstrating bilaterally normal extraocular muscles. IO, inferior oblique; ON, optic nerve.

and III:5 and was inferred to do so in II:3 (Fig. 1). The nucleotide substitution is not present in the UCSC SNP database (<http://genome.ucsc.edu/cgi-bin/hgGateway>; University of California Santa Cruz) and was not observed in any of the 394 control individuals. The missense mutation is predicted to result in a substitution of a polar phenylalanine for an evolutionarily conserved nonpolar tyrosine (p.Y148F; Fig. 7B) located in the SH2-C1 linker region of $\alpha 2$ -chimaerin (Figs. 7C, 7D). Y148 is encoded as part of the $\alpha 2$ -chimaerin but not the $\alpha 1$ -chimaerin protein (Fig. 7C), and thus $\alpha 2$ - but not $\alpha 1$ -chimaerin protein function would be altered by the mutation in pedigree JB.

Functional Analysis of the Y148F Amino Acid Substitution

We previously reported the functional analysis of seven missense mutations in *CHN1* that cause DRS.⁹ All seven mutations were demonstrated to increase Rac-GAP activity and lower Rac-GTP levels in the cell. Four of the seven mutations enhanced dimerization of $\alpha 2$ -chimaerin and its PMA-associated translocation to the cell membrane, presumably by destabilizing the inactive, closed conformation of $\alpha 2$ -chimaerin. Y148 is located in the linker between the SH2 and C1 domains, close to residues Y143 and Y141, which have been predicted to form intramolecular bonds with Y211 (Fig. 7D).¹⁶ We previously reported that residues Y143 and P141 are altered by two of the DRS *CHN1* mutations^{9,10}; the behavior of Y143H was exam-

ined in vitro and resulted in enhanced membrane translocation, dimerization, and Rac-GAP activity.⁹ Thus, we examined the behavior of the c.443A>T mutation in vitro, predicting that Y148F may behave similarly.

Using a Rac-GTP activation assay, we found that the mutant Y148F lowered Rac-GTP levels more than wild-type and to a similar extent as the previously reported E313K and A223V DURS2 substitutions (Fig. 8A). Testing the membrane translocation of the Y148F mutant protein after PMA stimulation, we found that the mutant protein was increased in the pellet fraction and decreased in the soluble fraction compared with WT (Fig. 8B). Finally, testing the self-assembly of the mutant protein after PMA stimulation by co-immunoprecipitation, we found enhanced dimerization of mutant protein compared to WT $\alpha 2$ -chimaerin (Fig. 8C).

DISCUSSION

We present a family who segregates a c.443A>T *CHN1* heterozygous missense mutation in a dominant pattern among five members with congenital ocular dysmotility. All five clinically affected family members have monocular or binocular supraduction deficits in the absence of ptosis, whereas only two also have DRS. This is in contrast to the nine pedigrees previously reported to segregate *CHN1* mutations, in which all affected members had DRS with or without less prominent vertical movement disorders, and a few individuals were

FIGURE 6. Clinical and MRI features of subject III:5. (A) Versions demonstrating limited ab- and adduction of the left eye with left palpebral fissure narrowing on adduction and widening on abduction, as well as limited supraduction and adduction of the right eye. There was limited infraduction of the left eye. (B) Oblique axial MRI demonstrating presence of the abducens nerve (CN6) of the right but not on the left side ipsilateral to DRS. CN5, trigeminal nerve. (C) Deep coronal MRI demonstrating anomalous continuity of the inferior division of the oculomotor nerve (CN3) with the left lateral rectus (LR) muscle, correlating with left DRS.

asymptomatic mutation carriers.^{9,10,12,14} Thus, this family expands the clinical spectrum of human phenotypes resulting from *CHN1* mutations to include isolated vertical strabismus with supraduction deficits.

The *CHN1*c.443A>T missense mutation results in a α 2-chimaerin Y148F amino acid substitution, which is predicted to alter an α 2-chimaerin intramolecular interaction and destabilize its closed inactive conformation. This prediction is confirmed by in vitro functional studies, which demonstrate that the Y148F substitution enhanced dimerization of α 2-chimaerin and its PMA-associated translocation to the cell membrane and lowered Rac-GTP levels in the cell, compared with wild-type α 2-chimaerin. Thus, this mutation results in hyperactivation of α 2-chimaerin function in a fashion indistinguishable from four of the previously reported *CHN1* mutations that cause DRS.⁹

Gain-of-function mutations in the single gene *CHN1* play the predominant role in the development of the ocular dysmotility phenotype among all families harboring *CHN1* mutations. The phenotypic variability found between families, among members of the same family, and even between the two eyes of an affected individual, however, is likely to result from differences in the genetic makeup or possibly the in utero environmental exposure of the affected individual. If genetic modification is indeed the cause of phenotypic variability, family JB may seg-

FIGURE 7. *CHN1* mutation analysis. (A) Chromatograms from two members of pedigree JB, unaffected participant II:5 (left) and affected participant I:2 (right), revealing the heterozygous A>T substitution at residue 443 in the affected but not the unaffected participant. The corresponding wild-type and altered amino acid residues are noted below the unaffected and affected participant's sequence, respectively. (B) The α 2-chimaerin protein alignment among eight different species demonstrates the evolutionary conservation of residue Y148 (boxed in red) as well as residues Y143 and Y141 (boxed in green). Y143 and Y141 are five and seven residues upstream from Y148, respectively, and were previously reported to be altered in DRS. (C) Two-dimensional representations of the α 1-chimaerin (top) and α 2-chimaerin (bottom) proteins. The proteins share identical C1 and Rac-GAP domains. Only α 2-chimaerin contains an SH2 domain, whereas α 1-chimaerin has unique N-terminal sequence highlighted in black. The novel c.443 A>T mutation alters residue Y148 (Y148F) which is only present in α 2-chimaerin (red arrowhead). Previously reported mutations are indicated (arrows, arrowheads) below. (D) Intramolecular interactions that stabilize the closed conformation of α 2-chimaerin. Known intramolecular binding sites are denoted by lines connecting the circles; each circle represents an amino acid residue. Green circles: residues altered by previously reported missense mutations; red circle: position of the novel Y148F substitution.

FIGURE 8. Functional studies of the novel *CHN1* mutation resulting in p.Y148F amino acid substitution compared with WT and previously studied mutations. **(A)** Representative Western blot showing results from the Rac-GTP activation assay. *Top blot*: whereas WT α 2-chimaerin reduced Rac-GTP levels compared to empty vector, Y148F lowered the levels below WT, similar to E313K and A223V. Total Rac in the cell (*middle blot*) and amount of transfected V5- α 2-chimaerin (*bottom blot*) were equal ($n = 7$). **(B)** Representative Western blot results from PMA-stimulated α 2-chimaerin membrane translocation experiment. Although total α 2-chimaerin is comparable in all conditions, PMA stimulates translocation of α 2-chimaerin into the pellet (membrane) fraction. This translocation is enhanced compared to WT in all four mutant proteins tested, including Y148F from this report ($n = 3$). **(C)** Representative Western blot showing that increase in the self-assembly of WT α 2-chimaerin with Y148F- and Y143H-mutant α 2-chimaerin compared to self-assembly of WT with WT α 2-chimaerin ($n = 4$).

regate genetic modifier(s) in a unique pattern, resulting in monocular or binocular supraduction deficits in the absence of DRS. It is also possible that the Y148F amino acid substitution results in a variant phenotype by not only altering the hyperactivation of α 2-chimaerin, but also altering how its SH2 or Rac-GAP domain interacts with other proteins.^{19–21} This seems less likely, however, given that the location of Y148 is predicted to be physically distant from either of these two domains (Fig. 7D).

MRI reveals greater endophenotypic overlap among individuals harboring *CHN1* mutations with or without DRS than is suggested by clinical examination alone.¹⁴ Although three of five affected JB family members did not have findings of DRS on clinical examination, those who underwent MR imaging were found to have small-appearing abducens nerves within the subarachnoid space, supporting subclinical maldevelopment of the abducens nerve and nucleus. Consistent with MRI findings in other DRS patients,¹⁴ the participant with unilateral DRS who underwent MRI (III:5) had no visible abducens nerve on the side of his DRS phenotype and had suggestion of misinnervation of the lateral rectus muscle by the oculomotor nerve on the affected side. In addition, subarachnoid and orbital imaging revealed statistically significant reduction in SO muscle volume presumably from trochlear nerve hypoplasia in three, optic nerve hypoplasia in three, and oculomotor nerve hypoplasia in one of the four individuals who underwent MRI (Table 1). The variable hypoplasia of cranial nerves II, III, IV, and VI, detected by MRI, was similarly reported in DRS patients harboring *CHN1* mutations.¹⁴ These data support a continuum of *CHN1* phenotypes, as one might predict from genetic modification of hyperactivating *CHN1* mutations.

The α 2-chimaerin isoform of *CHN1* is expressed in all developing cranial motor neurons, including abducens, trochlear,

and oculomotor, as well as in most developing neurons throughout the central and peripheral nervous system of both human and mouse, including the retina.⁹ Thus, although the expression pattern is consistent with hypoplasia of multiple cranial nerves, it does not account for the restricted ocular phenotypes found in patients harboring gain-of-function *CHN1* mutations. It is interesting that several other genetically defined CCDDs result from mutations in genes also expressed widely during embryonic development, and they too have MRI evidence of variable cranial nerve hypoplasia. Individuals with CFEOM1 or CFEOM3 have ptosis and supraduction limitations resulting from mutations in *KIF21A* or *TUBB3*, respectively.^{3,4} MRI has shown that patients with either syndrome have prominent thinning of the oculomotor nerve; in addition, however, some have abducens and/or optic nerve hypoplasia.^{22,23} Individuals with CFEOM1 may also have trochlear hypoplasia suggested by small SO volumes,²² but this has not been found to date in individuals with CFEOM3.²³

The clinical data from JB family members establishes greater ocular motility pleiotropy resulting from hyperactivated α 2-chimaerin protein than previously recognized, and *CHN1* mutations should be considered in the differential diagnosis of patients with supraduction deficits, even in the absence of DRS. In addition, the MRI data revealed subclinical cranial nerve hypoplasia similar to that previously reported in DRS patients harboring *CHN1* mutations and suggest that this pedigree may reflect one end of a clinical spectrum resulting from genetic modification of the primary *CHN1* mutation. Additional clinical studies of *CHN1* and of its role in the development of the ocular motor axis should provide additional insights into this phenotypic variation.

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