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 congenital 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 a2-chimaerin. In vitro functional studies of the co-inherited mutation were performed, including a Rac-GTP activation assay, quantification of a2-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 a2-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/iovs.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 HOX A1 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 a2-chimaerin and shorter a1-chimaerin protein,9,11 and previous work supports hyperactivation of the a2-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 a2-
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 supradduction 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.

**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 with the polymorphic markers 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 ancestry, 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 α2-chimaerin, and V5 mutant α2-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 stained with Ponceau S. Rac1 activation was detected using an anti-Rac GTP antibody (ab1790; 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% 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, West Grove, PA). Y143K, A223V, and P252Q mutants were included as positive controls.

**Co-immunoprecipitation Experiments**

Three micrograms V5 wild-type α2-chimaerin vector, V5 mutant α2-chimaerin vector, or empty vector was transiently co-transfected with 5 μg GFP-fused wild-type α2-chimaerin vector into HEK293T cells. After 48 hours’ incubation, the cells were pretreated with 5 μM of PKC inhibitor for 30 minutes and then a subset was stimulated with 10 μM of PMA for 30 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% 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.). Y143K was included as a positive control.

**Results**

Six family members (I:2, II:2, III: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, III: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 superior function, supranuclear function, ductions, saccades, pursuit, and stereopsis and visual field, slit lamp, intraocular pressure, and diluted 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 supradduction bilaterally, with large V-pattern exotropia. Visual acuity, stereopsis, and diluted 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 levosversion with mild supradduction 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. MRI 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 III: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
revealed left hypertropia, larger in dorsumversion, and a right supraduction deficit (Fig. 3A). Corrected visual acuity was /H11002 0.1 and /H11002 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 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**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Abducens Nerve in Subarachnoid Space</th>
<th>CN3 Cross Section in Subarachnoid Space (mm²)</th>
<th>Motor Nerves within the Orbit</th>
<th>ON Cross Section within the Orbit (mm²)</th>
<th>SO Muscle Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
<td>Normal</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>II:2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>II:3</td>
<td></td>
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<tr>
<td>III:1</td>
<td></td>
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<tr>
<td>III:5</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Average (±SD)</td>
<td>1.72 ± 0.36†</td>
<td>2.01 ± 0.36</td>
<td>1.90</td>
<td>2.01</td>
<td>OS: abducens not visualized</td>
</tr>
<tr>
<td>Controls (±SD)</td>
<td>2.01 ± 0.36</td>
<td>7.41 ± 1.34†</td>
<td>14.10</td>
<td>3.16</td>
<td>135 ± 15†</td>
</tr>
</tbody>
</table>

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.
were small and horizontally oval. 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), consistent with findings frequently seen in DRS. 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,
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 \( \beta \)-2-chimaerin (Figs. 7C, 7D). Y148 is encoded as part of the \( \beta \)-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 \( \text{CHN1} \) 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 \( \text{CHN1} \) mutations8,10; the behavior of Y143H was examined 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 DUFS2 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 \( \text{CHN1} \) 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 \( \text{CHN1} \) mutations, in which all affected members had DRS with or without less prominent vertical movement disorders, and a few individuals were...
asymptomatic mutation carriers.\textsuperscript{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\textsuperscript{H}1/1022T missense mutation results in a CHN1\textsubscript{2}-chimaerin Y148F amino acid substitution, which is predicted to alter an CHN1\textsubscript{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 CHN1\textsubscript{2}-chimaerin and its PMA-associated translocation to the cell membrane and lowered Rac-GTP levels in the cell, compared with wild-type CHN1\textsubscript{2}-chimaerin. Thus, this mutation results in hyperactivation of CHN1\textsubscript{2}-chimaerin function in a fashion indistinguishable from four of the previously reported CHN1 mutations that cause DRS.\textsuperscript{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-

\textbf{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 oculomotor nerve (CN3) with the left lateral rectus (LR) muscle, correlating with left DRS.

\textbf{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\textsuperscript{H}1022T 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 CHN1\textsubscript{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) The proteins share identical C1 and Rac-GAP domains. Only CHN1\textsubscript{2}-chimaerin contains an SH2 domain, whereas CHN1\textsubscript{1}-chimaerin has unique N-terminal sequence highlighted in black. The novel c.443 A\textsuperscript{H}1/1022T mutation alters residue Y148 (boxed in red) which is only present in CHN1\textsubscript{2}-chimaerin (red arrowhead). Previously reported mutations are indicated (arrows, arrowheads) below. (D) Intramolecular interactions that stabilize the closed conformation of CHN1\textsubscript{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.
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 \textit{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 supradduction limitations resulting from mutations in \textit{KIF21A} or \textit{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 \textit{a2}-chimaerin protein than previously recognized, and \textit{CHN1} mutations should be considered in the differential diagnosis of patients with supradduction 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 \textit{CHN1} mutations and suggest that this pedigree may reflect one end of a clinical spectrum resulting from genetic modification of the primary \textit{CHN1} mutation. Additional clinical studies of \textit{CHN1} and of its role in the development of the ocular motor axis should provide additional insights into this phenotypic variation.

**Acknowledgments**

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**References**

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**FIGURE 8.** Functional studies of the novel \textit{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 \textit{a2}-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- \textit{a2}-chimaerin (bottom blot) were equal (n = 7). (B) Representative Western blot results from PMA-stimulated \textit{a2}-chimaerin membrane translocation experiment. Although total \textit{a2}-chimaerin is comparable in all conditions, PMA stimulates translocation of \textit{a2}-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 = 5). (C) Representative Western blot showing that increase in the self-assembly of WT \textit{a2}-chimaerin with Y148F- and Y143H-mutated \textit{a2}-chimaerin compared to self-assembly of WT with WT \textit{a2}-chimaerin (n = 4).


