Human CHN1 Mutations Hyperactivate α2-Chimaerin and Cause Duane’s Retraction Syndrome

Noriko Miyake,1,2 John Chilton,3,* Maria Psatha,4,* Long Cheng,1,2 Caroline Andrews,1,2,5 Wai-Man Chan,1 Krystal Law,1† Moira Crosier,6 Susan Lindsay,7 Michelle Cheung,8 James Allen,2 Nick J. Goutowski,7,8 Sian Ellard,8,9 Elizabeth Young,8 Alessandro Iannaccone,10 Binoy Appukuttan,11 J. Timothy Stout,11 Stephen Christiansen,12 Maria Laura Ciccarelli,13 Alfonso Baldi,14 Mara Campioni,14 Juan C. Zenteno,15 Dominic Davenport,4 Laura E. Mariani,5 Mustafa Sahin,5,5 Sarah Guthrie,4 Elizabeth C. Engle1,2,5,16,17†

Duane’s retraction syndrome (DRS) is a complex congenital eye movement disorder caused by aberrant innervation of the extraocular muscles by axons of brainstem motor neurons. Studying families with a variant form of the disorder (DURS2-DRS), we have identified causative heterogeneous missense mutations in CHN1, a gene on chromosome 2q31 that encodes α2-chimaerin, a Rac guanosine triphosphatase—activating protein (RacGAP) signaling protein previously implicated in the pathfinding of corticospinal axons in mice. We found that these are gain-of-function mutations that increase α2-chimaerin RacGAP activity in vitro. Several of the mutations appeared to enhance α2-chimaerin translocation to the cell membrane or enhance its ability to self-associate. Expression of mutant α2-chimaerin constructs in chick embryos resulted in failure of oculomotor axons to innervate their target extraocular muscles. We conclude that α2-chimaerin has a critical developmental function in ocular motor axon pathfinding.

Fig. 1. Duane’s retraction syndrome (DRS) and corresponding CHN1 mutations. (A) Affected member of pedigree JH with limited outward gaze (abduction) and narrowing of the palpebral fissure on attempted inward gaze (adduction) most obvious on leftward gaze. He also has bilateral exotropia on downgaze. (B) The seven DURS2-DRS pedigrees and corresponding heterozygous CHN1 mutations. (C) Schematic representation of α1-chimaerin (top, 334 amino acids) and α2-chimaerin (bottom, 459 amino acids) protein. The isoforms contain identical C1 and RacGAP domains; only α2-chimaerin contains an SH2 domain. Mutations alter residues unique to α2-chimaerin or common to both proteins, as indicated by the arrows. No mutations were found in the α1-chimaerin N-terminal sequence (highlighted in black).
Fig. 2. Human developmental expression profile of α2-chimaerin mRNA by in situ hybridization. (A) Transverse section of CS15 human embryo showing α2-chimaerin mRNA expression (purple deposit) in midbrain, hindbrain (rhombomere 2 indicated), and spinal cord. (B) Higher magnification of (A) showing expression in the ventricular layer of rhombomeres 3 and 4. (C) At CS16, expression is also seen in midbrain, hindbrain, spinal cord, and vestibulocochlear (viii) and vagus (x) nuclei. Higher magnifications of (C) show (D) expression in developing oculomotor neurons and (E) in neurons of rhombomeres 5 (developing abducens neurons) and 6. In CS19 sagittal section (F), expression has declined in basal midbrain and hindbrain and is now found in dorsal root ganglia, cerebellum, diencephalon, and telencephalon. At later stages (G), expression is located in specific regions of the cortical plate and the intermediate and ventricular zones of the forebrain (11 weeks post-ovulation). No signal was detected in corresponding sections hybridized with sense probe (fig. S4). Abbreviations: mb, midbrain; hb, hindbrain; rh, rhombomere; sc, spinal cord; o, oculomotor nucleus; drg, dorsal root ganglia; d, digit; cb, cerebellum; dc, diencephalon; t, telencephalon; c, cortical plate; i and v, intermediate and ventricular zones of the forebrain. Scale bars, 1000 µm [(A) and (C)], 100 µm [(B) and (E)], 200 µm (D), 2000 µm (F), 500 µm (G).

Fig. 3. DURS2-DRS mutations enhance α2-chimaerin function in vitro. (A) Rac-GTP levels were measured in HEK 293T cells transfected with plasmids encoding myc-ephexin, epitope-tagged V5-empty vector, V5–α2-chimaerin wild-type, or V5–α2-chimaerin mutants. Rac-GTP levels are reduced by overexpression of wild-type α2-chimaerin relative to empty vector, and are further reduced in cells expressing each mutant, but are elevated with overexpression of a guanine nucleotide exchange factor, myc-ephexin (27). (B) Densitometric analysis of Rac-GTP levels normalized to total Rac and V5–α2-chimaerin levels. Values are expressed as percent of wild-type α2-chimaerin (mean ± SEM, n = 6 to 10). The difference between the reduction of Rac-GTP levels for each mutant compared to wild-type α2-chimaerin is significant by one-way analysis of variance (ANOVA) with Dunn’s adjustment (F = 9.89, *P < 0.03, **P < 0.005, ***P < 0.0001). (C) α2-Chimaerin translocation examined by immunoblots of total, soluble, and pellet fraction of wild-type and mutant α2-chimaerin with or without 10 µM PMA stimulation. (D) Graphical representation of translocation after PMA treatment, expressed as percent of α2-chimaerin remaining in the soluble fraction (mean ± SEM, n = 3). Enhanced translocation compared to wild-type is significant for L20F, Y143H, A223V, and P252Q by one-way ANOVA with Dunnett’s adjustment (F = 21.00, *P < 0.0001). (E) GFP–α2-chimaerin immunoprecipitates with V5–wild type or V5–L20F α2-chimaerin in the presence of PMA, and minimally in its absence. (F) In the presence of PMA, immunoprecipitation of wild-type α2-chimaerin is enhanced by all mutant α2-chimaerins relative to the wild type except G228S and E313K, which were equivalent to wild-type α2-chimaerin. Results were consistent over at least four independent experiments (see also fig. S6, F and G).
primary development of the abducens and, to a lesser degree, the oculomotor nerve (fig. S1C).

To identify the DURS2 gene, we further analyzed the recombination events that defined the published DURS2 critical region (6, 7), reducing it from 9.9 to 4.6 Mb (fig. S2, A and B), and then sequenced 22 positional candidate genes (fig. S2B) in a proband from each of the four published pedigrees. We identified in each a unique heterozygous missense change in CHN1, which encodes two Rac specific guanosine triphosphatase (GTPase) activating Isoforms of CHN1 activating α2 chimaerin isoforms. We then screened 16 smaller pedigrees that segregated DRS in a dominant fashion, and identified three additional heterozygous CHN1 missense changes in pedigrees RF, IS, and AB (Fig. 1B and figs. S1E and S2C). All seven nucleotide substitutions cosegregated with the affected haplotypes, and none were present in online single nucleotide polymorphism databases or on 788 control chromosomes. Five of the substitutions are predicted to result in nonconservative amino acid substitutions [Leu20(→Phe), Tyr143(→His), Gly228(→Ser), Pro252(→Gln), and Gln313(→Glu)] and two in conservative amino acid substitutions [Ile126(→Val) and Ala223(→Val)] (fig. 1B). All are predicted to alter amino acids that are conserved in eight different species (fig. S2D).

The Rho family member Rac is a GTPase that is active when GTP bound; it serves as a regulator of downstream intracellular signaling cascades controlling cytoskeleton dynamics, including the growth and development of dendrites and axons. Rac is inactivated by 12 Rac GTPase activating proteins (GAPs) in the mammalian genome (11), including α1 and α2 chimaerin (encoded by CHN1) and paralogs β1 and β2 chimaerin (encoded by CHN2). In the rodent brain, α2 chimaerin has been shown to serve as an effector for axon guidance (12, 16), whereas α1 chimaerin appears to play a later role in dendritic pruning (17, 18).

CHN1 is alternatively spliced, and the α1 chimaerin promoter lies in intronic sequence upstream of α2 chimaerin exon 7 (19). Thus, the two isoforms share a RacGAP domain that interacts with and down-regulates Rac activity, as well as a C1 domain that binds to diacylglycerol (DAG), a membrane associated phorbol ester signaling lipid. Only α2 chimaerin contains an N-terminal Src homology 2 (SH2) domain (20, 21).

Three DURS2 mutations alter amino acids unique to α2 chimaerin, whereas four alter residues shared by α1 and α2 chimaerin (Fig. 1C and table S1). Because we cannot distinguish between the two groups clinically, the DURS2 phenotype most likely results from altered α2 chimaerin function.

In situ studies in rats (20, 21) revealed widespread embryonic neuronal expression of α2 chimaerin mRNA. Expression in the caudal brainstem and cephalic flexure peaked at embryonic day (E) 12.5, whereas mouse embryonic expression peaked overall at E10.5 (fig. S3, A and B), both consistent with expression of α2 chimaerin in developing ocular motor neurons. We found similar widespread expression of α2 chimaerin mRNA during human development, strongest at Carnegie stage (CS) 15 and CS16 in the midbrain and hindbrain (Fig. 2, fig. S3, C to E, and fig. S4). Therefore, although expressed in developing ocular motor neurons, the expression pattern alone does not account for the striking restriction of the DURS2 phenotype.

All seven amino acids altered by DURS2 mutations are conserved in α2 chimaerin’s paralog.

Fig. 4. α2-Chimaerin overexpression results in stalling of developing chick oculomotor nerves. (A) Transverse section through E4 whole chick embryo, showing wide neuroepithelial expression of α2-chimaerin mRNA including the hindbrain (hb), forebrain (fb), and trigeminal ganglion (tg). (B) Transverse section through E5–6 chick midbrain, showing α2-chimaerin mRNA expression in the oculomotor nuclei (left nucleus circled in white). (C) Tabulated results of electroporated constructs. (D to I) Confocal image montages (white hatches denote image breaks) at E6 of electroporated oculomotor nerves (green) and extraocular muscles (red) labeled with antibody to myosin [(D), (E), and (G) to (I)] or α-bungarotoxin (f); constructs as labeled. All GFP control (D), 28% of wild type (E), and only 5 to 13% of mutant α2-chimaerin electroporated oculomotor nerves extend normally from the midbrain neuroepithelium, at left, past the dorsal rectus muscle (dr), ciliary ganglion (*), and ventral (vo) and medial (mr) recti to innervate the first target, the ventral oblique (vo) muscle. Nerves expressing mutant α2-chimaerin have a higher incidence of aberrant branching [arrow in (F) with higher-magnification inset] and defasciculation than the wild type (fig. S7). Remarkably, 72% of wild-type α2-chimaerin (G), 87% of L20F α2-chimaerin (H), and 71% of G228S α2-chimaerin (I) electroporated nerves stall in the vicinity of the dr muscle. Scale bars, 200 μm; lr, lateral rectus.
β2 chimaerin (fig. S5, A and B). Both molecules are predicted to exist in inactive, closed conformations in the cytoplasm, and to unfold and translocate to the membrane in response to DAG signaling, exposing their RacGAP domains and inactivating Rac (12, 22). β2 Chimaerin crystallization revealed that its inactive conformation is maintained by intramolecular interactions that impede access to the Rac and DAG binding sites (22). Modeling the DURS2 mutations onto the β2 chimaerin structure (fig. S5, C to E) (22) led to several predictions: (i) α2 Chimaerin Leu143 and Ile230 correspond to two of nine residues predicted by Canagajah et al. to stabilize the β2 chimaerin closed conformation and, when mutated to alanine, were shown to enhance β2 chimaerin translocation to the membrane in vitro. (ii) Tyr143 to alanine, were shown to enhance activate us to hypothesize that DURS2 mutations hyperactivate DAG or Rac binding. (iii) Glu313 is adjacent to the translocation to the membrane in vitro. (ii) Tyr133 (fig. S6G); this result supports a direct or indirect association of α2 chimaerin with itself that may involve its SH2 domain.

On the basis of our findings that DURS2 mutations hyperactivate α2 chimaerin, we hypoth esized that overexpressing α2 chimaerin may result in aberrant axon development in vivo. To test this idea, we used the chick in ovo system to overexpress α2 chimaerin in the embryonic ocu lomotor nucleus. This nucleus is more amenable than the abducens to electroporation, its devel opment in chick has been defined (25), and we previously showed that some DURS2 DRS indi viduals have clinical and MR findings supporting a primary defect in oculomotor nerve development (8 10). Similar to rodents and humans, chick α2 chimaerin mRNA is expressed in neuroepithelium at stages of cranial motor neuron development (E4), and specifically in the developing ocu lo motor nucleus at the stage of axon extension and branching (E6) (Fig. 3, A and B). We electro porated embryonic chick midbrains with green fluorescent protein (GFP) tagged wild type and mutant α2 chimaerin (L20F and G228S) and GFP alone control constructs at E2. These were ana lyzed between E5.5 (fig. S7), when oculomotor axons have extended along an unbranched tract from their distal target, the ventral oblique muscle (vo), and E6, when branching to the other target muscles has ensued (Fig. 4, C to I) (23). All 18 GFP control embryos showed a normal projection pattern in which the oculomotor nerve reached the ventral oblique muscle and branched correctly into other target muscles by E6 (Fig. 4D) (23). In the majority (71 to 87%) of embryos overexpressing wild type or mutant constructs, the oculomotor nerve stalem and its axons terminated prematurely adjacent to the dorsal rectus muscle (Fig. 4, G to I). In addition, 67% of embryos overexpressing mutant constructs displayed aberrant branching and/or defasciculation of the oculomotor nerve, whereas only 24% of embryos overexpressing wild type constructs did so (Fig. 4F and fig. S7, A to H). Regardless of the construct we used, the electroporated ocul omotor nucleus appeared normal in size and neuron cell bodies displayed normal sorting, including normal migration across the midline (fig. S7, I and J) (23), consistent with a pri mary defect in axon rather than cell body de velopment. Taken together, these observations suggest that elevated RacGAP activity as a result of hyperactivated mutant or overexpressed wild type α2 chimaerin results in deregulation of normal oculomotor axon development.

Eph receptors and ephrins (24), as well as neuropilin receptors and semaphorins (25), are expressed in developing cranial motor nuclei in the chick and/or rodent. Several recent papers report that α2 chimaerin interacts with the EphA4 receptor and inactivates Rac in response to ephrin/ EphA4 signaling (13 16). Loss of α2 chimaerin impairs EphA4 forward signaling in vivo and eliminates ephrin induced growth cone collapse in vitro (13 16). α2 Chimaerin has also been implicated in semaphorin 3A induced growth cone collapse (12). EphA4 receptor stimulation can recruit and activate phospholipase C γ1, elev ating DAG levels (26). Therefore, mutant α2 chimaerin may be hyperactivated in response to chemorepellents such as ephrins or semaphorins, resulting in pathological inactivation of Rac and altered transduction of downstream signals (fig. S8, A to C).

Mice with loss of α2 chimaerin have disrupted ephrin/EphA4 signaling and elevated Rac GTP levels, with a phenotype limited to a hopping rabbit like gait resulting from excessive and aber rant midline crossing of corticospinal tract axons and spinal interneuron projections, with no cran i nerval nerve defects reported (13 15). We have now identified human α2 chimaerin mutations that enhance its function, reduce Rac GTP levels, and lead to an ocular motor phenotype as a result of errors in cranial motor neuron development. It is remarkable that the up and down regulation of such a widely expressed signaling molecule results in two restricted and apparently non overlapping phenotypes. It remains to be deter mined in which signaling pathways α2 chimaerin functions in corticospinal and cranial motor axons and why these different motor circuits are uniquely vulnerable to different perturbations in Rho GTPhase activity.

References and Notes
1. See supporting material on Science Online.

842 8 AUGUST 2008 VOL 321 SCIENCE www.sciencemag.org
immunological tolerance to self is essential in the prevention of autoimmune disease. Mechanisms of central tolerance are mediated in part through the expression of a wide array of otherwise tissue specific self antigens (TSAs) such as insulin and thyroglobulin in specialized medullary thymic epithelial cells (mTECs) (1-3). The thymic expression of many of these TSAs is dependent on the autoimmune regulator (Aire) gene. Here we report the identification of extrathympic Aire-expressing cells (eTACs) resident within the secondary lymphoid organs. These stromally derived eTACs express a diverse array of distinct self-antigens and are capable of interacting with and deleting naıve autoreactive T cells. Using two-photon microscopy, we observed stable antigen-specific interactions between eTACs and autoreactive T cells. We propose that such a secondary network of self-antigen–expressing stromal cells may help reinforce immune tolerance by preventing the maturation of autoreactive T cells that escape thymic negative selection.

The prevention of autoimmunity requires the elimination of self-reactive T cells during their development and maturation. The expression of diverse self-antigens by stromal cells in the thymus is essential to this process and depends, in part, on the activity of the autoimmune regulator (Aire) gene. Here we report the identification of extrathympic Aire-expressing cells (eTACs) resident within the secondary lymphoid organs. These stromally derived eTACs express a diverse array of distinct self-antigens and are capable of interacting with and deleting naıve autoreactive T cells. Using two-photon microscopy, we observed stable antigen-specific interactions between eTACs and autoreactive T cells. We propose that such a secondary network of self-antigen–expressing stromal cells may help reinforce immune tolerance by preventing the maturation of autoreactive T cells that escape thymic negative selection.

Deletional Tolerance Mediated by Extrathympic Aire-Expressing Cells

James M. Gardner,¹ Jason J. DeVoss,² Rachel S. Friedman,² David J. Wong,³ Ying X. Tan,¹ Yuyu Zhou,³ Kelsey P. Johannes,¹ Maureen A. Su,¹,4 Howard Y. Chang,³ Matthew F. Krummel,² Mark S. Anderson¹*

I. Immunological tolerance to self is essential in the prevention of autoimmune disease. Mechanisms of central tolerance are mediated in part through the expression of a wide array of otherwise tissue specific self antigens (TSAs) such as insulin and thyroglobulin in specialized medullary thymic epithelial cells (mTECs) (1-3). The thymic expression of many of these TSAs is dependent on the autoimmune regulator (Aire) gene (4, 5), and mutations in Aire lead to severe, multiorgan, tissue specific insulitis. Although these results reveal a role for thymic Aire, self tolerance must continue to be enforced after T cells leave the thymus. Consistent with this fact, Aire expression is also detectable outside the thymus, notably in the secondary lymphoid tissues (4, 9), although the identity and function of such extrathympic Aire expressing cells remain unclear (10, 11). Here we identify a population of extrathympic Aire expressing cells and examine a potential role for Aire in maintaining peripheral tolerance.

To accurately label Aire expressing cells in vivo, we employed a bacterial artificial chro mosome (BAC) transgenic approach (12) using the murine Aire locus modified to drive expres sion of green fluorescent protein (GFP) fused to an autoimmune diabetes related self antigen gene, islet specific glucose 6 phosphatase related pro tein (Igrp) (Fig. 1A) (13). IGRP is a pancreatic β cell specific protein against which autoreactive CD8 T cells are produced in both mouse and human autoimmune diabetes (14-17). We elected to include Igrp in our transgenic construct because it is not detectable in the thymus (fig. S1A) and because an IGRP specific T cell receptor (TCR) transgenic line (8.3) (14) is used to monitor interactions of Igrp Gfp expressing cells. To verify the fidelity of the BAC transgene in recapitulating endogenous Aire expression in the resultant Adig (Aire driven Igrp Gfp) transgenic mice, thymic sec tions were co stained for Aire and GFP, reveal ing thymic GFP expression highly restricted to Aire expressing cells in the medulla (Fig. 1B and fig. S1B). By flow cytometry, GFP+ cells were detectable specifically within the mTEC compartment (Fig. 1C), with approx imately 30 to 40% of mTECs being GFP positive. GFP+ mTECs expressed uniformly high levels of class II major histocompatibil ity complex (MHC) and CD80 (Fig. 1D), as in previous studies of Aire expressing mTECs (3, 11, 18).

To test how the introduction of IGRP into the thymic medullary epithelium might affect T cell selection, we compared 8.3 TCR transgenic and 8.3/Adig double transgenic mice. Tetramer staining confirmed that the 8.3/Adig double transgenic mice showed a significant decrease in the percent and avidity of IGRP specific CD8+ T cells in the thymus (Fig. 1E) and in the periphery (fig. S1C). Further, although IGRP reactive CD8+ T cells were readily detected in the polyclonal wild type NOD background, they were completely absent in Adig NOD mice (fig. S1D). To test the functional impact of this negative selection, 8.3 and 8.3/Adig mice were followed for diabetes incidence, and 8.3/Adig mice were completely protected from disease (Fig. 1F).

In a broad tissue survey using immuno fluorescent anti GFP staining, expression of the transgene was undetectable in most tissues, but distinct populations of extrathympic transgene expressing cells were observed within the lymph nodes and spleen (fig. S2A). These extra thymic Aire expressing cells (eTACs) were generally con fined to the T cell zones of the secondary lymphoid organs and preferentially localized to T cell B cell boundary regions (Fig. 2, A and B). Immuno nofluorescent co stains showed these cells to be negative for B cell (B220), fibroblastic re ticular cell (gp38 and ERTR 7), and dendritic cell (CD11c) markers, but positive for class II MHC (Fig. 2, A and B). Reciprocal bone marrow chimera demonstrated that many of
Supporting Online Material for

Human CHN1 Mutations Hyperactivate α2-Chimaerin and Cause Duane’s Retraction Syndrome

Noriko Miyake, John Chilton, Maria Psatha, Long Cheng, Caroline Andrews, Wai-Man Chan, Krystal Law, Moira Crosier, Susan Lindsay, Michelle Cheung, James Allen, Nick J Gutowski, Sian Ellard, Elizabeth Young, Alessandro Iannaccone, Binoy Appukuttan, J. Timothy Stout, Stephen Christiansen, Maria Laura Ciccarelli, Alfonso Baldi, Mara Campioni, Juan C. Zenteno, Dominic Davenport, Laura E. Mariani, Mustafa Sahin, Sarah Guthrie, Elizabeth C. Engle*

*To whom correspondence should be addressed. E-mail: elizabeth.engle@childrens.harvard.edu

Published 24 July 2008 on Science Express
DOI: 10.1126/science.1156121

This PDF file includes:

Materials and Methods
Figs. S1 to S8
Table S1
References
Materials and Methods

Supporting Figures

Figure S1. Duane’s Retraction Syndrome.

Figure S2. DURS2-DRS results from heterozygous mutations in CHN1.

Figure S3. Expression analysis of α2-chimaerin mRNA by Northern-blot hybridization in mouse and human.

Figure S4. Partial survey of the expression of α2-chimaerin specific CHN1 mRNA in human embryonic stages from CS12 (~26 dpo) to CS22 and 11 weeks post ovulation (wpo).

Figure S5. Alignment of human α2- and β2-chimaerin and schematic representation of the α2-chimaerin DURS2 mutated amino acid residues superimposed on the β2-chimaerin molecule.

Fig S6. Supporting α2-chimaerin hyperactivation and interaction data.

Figure S7. Developing chick oculomotor nerves electroporated with wild-type, G228S, and L20F α2-chimaerin show axon stalling and defasciculation, with normal motor neuron cell sorting.

Fig. S8. Model of α2-chimaerin activation and signaling in the developing ocular cranial motor neurons of normal and DRS individuals.

Supporting Table

Table S1. Summary of DURS2-DRS genetic and functional data for each CHN1 mutation identified.

Supporting Online References
Materials and Methods

Subjects and clinical data. The clinical phenotype and linkage to the DURS2 region for pedigrees IJ (S1, S2), UA (S3), JH and FY (S4, S5) were reported previously, while pedigrees RF, IS, and AB are reported here for the first time. Participating members of pedigrees IJ and UA were enrolled by Dr. Timothy Stout and Dr. Nick Gutowski, respectively, with approval of their institutional review boards / research ethics committees. The remaining five pedigrees were enrolled in the ongoing Institutional-Review-Board approved congenital cranial dysinnervation disorder study in the Engle Laboratory at Children’s Hospital Boston. Each participating individual provided informed consent, was interviewed and examined by one or more of the authors, and provided a peripheral blood or salivary sample from which genomic DNA was extracted using Puregene (Gentra Systems, Inc.) and purifier solution kit (DNA Genotek Inc.). The diagnosis of Duane’s retraction syndrome in each family member was based on ophthalmologic examination using Huber classification (S6). Orbital and MRI data from members of pedigrees JH and FY were previously reported (S5).

Genotyping and reduction of the DURS2 region. Linkage to the DURS2 region was tested in familial Duane’s retraction syndrome pedigrees by the analysis of polymorphic markers D2S2330, D2S335, D2S326, D2S2314 and D2S364 as previously described (S4). The DURS2 region was reduced by refining previously reported recombination events in affected members of pedigrees IJ and UA (S1, S3). Polymorphic di-, tri- and tetranucleotide repeats within the region of potential reduction were identified using the UCSC Genome Browser [http://genome.ucsc.edu/cgi-bin/hgGateway] and primers for amplification of the repeat regions were designed and purchased (Invitrogen). Primer sequences are available on request.

Mutation screening. We screened affected members from pedigrees IJ, FY, UA, and JH (the four published pedigrees that mapped independently to the DURS2 region) for mutations in the coding exons of the 22 genes in the refined DURS2 region. The 16 smaller pedigrees were screened for mutations in CHN1 only. The primers sets for CHN1 and the remaining genes that were screened are available on request. Amplicons were analyzed through a combination of DHPLC performed by Transgenic WAVE DNA Fragment Analysis system and analyzed with WAVEMAKER, and direct sequencing. SNP databases were interrogated for detected changes (NCBI SNP databases [http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp], JSNP database [http://snp.ims.u-tokyo.ac.jp/index.html] and HapMap project [http://www.hapmap.org/index.html.en]). Co-segregation of a putative mutation and affection status was tested for any change of potential significance that was not present in these SNP databases. At least 394 control DNA samples from unrelated individuals of multiple different ethnicities were screened for each putative CHN1 mutation, including 200 Caucasian and 100 Mexican DNA samples from the Coriell Cell Repositories catalog ID HD200CAU, HD100MEX.

Northern Blotting. Alpha2-chimaerin specific regions were amplified by Marathon-ready mouse embryo cDNA (Clontech) and Marathon-ready human fetal brain cDNA (Clontech) with Advantage2 Taq (Clontech). Mouse α2-chimaerin specific probe (400 base pairs (bps) probe corresponding to 61-461 bps in NM_001113246) was amplified using the following primers: 5’- GCTCTGACCCCTGTCTGATACAG ATGA-3’ and 5’-GCAATGTATTCTGCTGCCTTGGTTTCAA-3’. The human α2-chimaerin specific probe (540 bps corresponding to 471-1010 bps in NM_001822) was amplified using the following primers; 5’- TTACAATGGCCCTGACCCTGTGGTTTGGTTTCAA-3’ and 5’-CCCCATCCCTGCGCTGTAGAATCT-3’. After purification with QIAquick Gel Extraction Kit (Qiagen), the PCR products were labeled by α-32P-dCTP by PCR. The control probes (β-actin) were labeled with α-32P-dCTP by Random primed DNA labeling kit (Invitrogen). The blots (Seegene and Clontech) were incubated in prehybridization solution at
68 °C for 30 minutes followed by incubation in hybridization solution containing 1.7-2.0 cpm/ml of α-32P-dCTP labeled probe and 1mg herring sperm DNA (SIGMA) at 68 °C for two hours. Blots were then washed with Wash Solution I (2xSSC/0.05%SDS) twice for 15 minutes at room temperature followed by Wash Solution II (0.1x SSC/0.1%SDS) twice for 15 minutes at 50 °C. Blots were exposed at -80ºC for one day to two weeks and the films developed.

Human embryonic and fetal tissue collection. Human embryonic and fetal samples were collected through the Joint MRC-Wellcome Trust Human Developmental Biology Resource (HDBR) at Newcastle University. The samples were collected following British national guidelines (S7) from terminated pregnancy material, with appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee. Tissues were collected into cold 1x phosphate buffered saline (PBS), separated from surrounding tissue, and fixed overnight in 4% paraformaldehyde at 4°C, before short-term storage at 4°C in 70% ethanol. Placental tissue for karyotype analysis was sampled prior to fixation of the tissue. The stage of development was assessed on the basis of external features according to the Carnegie staging protocol (S8) modified for use with individual embryonic samples rather than in comparisons of several embryos simultaneously (S9, S10).

Human embryonic and fetal tissue in situ hybridization. The human α2-chimaerin specific probe (574-1031bps in NM_001822) for in situ hybridization was amplified from Marathon-ready human fetal brain cDNA (Clontech) with Advantage2 Taq (Clontech) using the following primers; 5'-TACCTGTACTGGCAGGTTGGAAAACAGA-3' and 5'-ATGTCAACCTTTTCTCTGACACCCCATC-3'. PCR product was cloned into pCRII-TOPO vector (Invitrogen) and linearized by EcoRV or BamH1, for SP6 (antisense) or T7 (sense) transcription, respectively. The HDBR gene expression service [http://www.hdbr.org/] performed in situ hybridization to human embryonic samples from Carnegie stages (CS) 12 to 23 (approximately 26 to 56 days post ovulation [dpo]) and from 11 weeks post ovulation [wpo]. In situ hybridization steps included pre-hybridization treatment, probe hybridization, post-hybridization washes, antibody treatment, antibody detection and mounting of slides were carried out as previously described (S11). Stained sections were viewed on a Zeiss Axioplan 2 microscope and images captured with a Zeiss Axiocam and Axiovision system.

Mutation modeling. The α2-chimaerin protein structure was predicted based on the β2-chimaerin crystallization ([1XA6] in Protein Data Bank [http://www.rcsb.org/pdb/home/home.do]), using the protein structure prediction program MOLMOL [http://hugin.ethz.ch/wuthrich/software/molmol].

Expression vector preparation. For mammalian expression constructs, full-length α2-chimaerin was amplified from marathon-ready human fetus brain cDNA (Clontech) using KOD Hifi DNA polymerase (TOYOBO/Novagen) with the following primers; 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTC AATGGCCTGACCCCTGTGATA-3’ and 5’-GGGGACCACTTTGTACAAAAAAGCAGGCTTC AAAATAAAATGTCTTCTTTTG-3’ (with the underlined sequence the linker region for BP recombination in Gateway system). The PCR product was confirmed by appropriate restriction enzyme digestion and direct sequencing, and was cloned into the entry vector (pDONRTM221) of Gateway system (Invitrogen). Each of the seven CHN1 missense mutations detected in DURS2 pedigrees was individually introduced into entry clones using Quick change Site-Directed Mutagenesis Kit (Stratagene) and subsequently confirmed by direct sequence. Each insert was then cloned into pcDNA3.1/nV5-DEST™ (N-terminal V5 tag) (Invitrogen) and pcDNA-DEST™53 (N-terminal GFP-fusion) (Invitrogen) by LR recombination. The sequence of each construct was again confirmed by direct sequencing. The molecular weights of the generated proteins and equal expression levels were confirmed by Western blotting. For the chick expression constructs, wild-type, L20F and G228S mutant full length cDNA derived from the entry clone were isolated from the each entry clone and were cloned into a bicistronic pCAGGS-IRES-GFP vector in which expression is driven by a chick β-actin promoter with a CMV enhancer (S12).
Rac-GTP Activation Assay. V5-empty, V5-wild-type-α2-chimaerin, V5-mutant-α2-chimaerin constructs were transiently transfected into HEK293T cell using Fugene6 (Roche). Myc-ephexin transfection was used as a control, as it is predicted to elevate RacGTP levels (S13). After 48 hours incubation in full media, the Rac1 Activation Assay Kit (Upstate) was used to isolate Rac-GTP by binding to the p21 binding domain of PAK1. Cells were lysed with 500 μl of ice cold 1xMLB buffer, and the lysate was then pre-cleared with glutathione sepharose (Amersham Biosciences) and reacted with 10 μl of a 50% slurry of p21 binding domain (PBD)-beads for 1 hour at 4 ºC. The beads were then washed, boiled in loading buffer and spun down. The supernatant was run in a 12% Bris-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen) for Western blot analysis using a mouse monoclonal anti-Rac1 antibody (BD Transduction Laboratories). Immunoblot images were quantified using Scion Image [http://www.scioncorp.com/], background was subtracted, and each quantity was normalized to total Rac and then to total V5-α2-chimaerin. Next, to mimic the effect of heterozygous DURS2-DRS mutations on RacGAP activity, we compared the RacGTP levels following transfection of 6 g of V5-WT, a mixture of 3 g of V5-WT and 3 g of V5-L20F, or 6 g of V5-L20F-α2-chimaerin into HEK293T cells without and with the addition of PMA. For statistical analysis, V5-wild-type-α2-chimaerin was arbitrarily set to 100% and each mutant value expressed as a percent of V5-wild-type-α2-chimaerin. One-way analysis of variance (one-way ANOVA) with Dunnett’s adjustment was conducted using GraphPad Prism 5 for Mac OS X Software v. 5.0a (www.graphpad.com) and SAS statistical software (v.8.1, SAS Institute, Cary, NC).

Quantification of α2-chimaerin translocation. Subcellular fractions were prepared from HEK293T cells 48 hours after transfection with V5-α2-chimaerin-wild-type or each of the seven V5-α2-chimaerin-mutants using Fugene6 (Roche). Cells were pretreated for 30 min with 5 μM of bisindolylmaleimide I (LC Labs) to inhibit PKC, and then stimulated with 0-10 μM of PMA for 20 min as reported by Canagarajah et al. (S14). Cells were sonicated in hypotonic buffer (S15) and were centrifuged at 100,000 g 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 (S15). Immunoblotting was performed with mouse monoclonal anti-V5 antibody (Invitrogen) and HRP-conjugated Donkey anti-mouse IgG (Jackson Immuno Research Lab). The signal intensities for wild-type and each of the seven mutants were quantified using Scion Image and plotted in a graphical format with the Y-axis representing: [soluble fraction /total fraction following 10 μM PMA treatment] as a percent of [soluble fraction / total fraction without PMA treatment]. The significance of the statistical differences in the behavior of the mutants to wild-type was calculated by one-way ANOVA with Dunnett’s adjustment as described above.

Co-immunoprecipitation experiments. 3 μg V5-α2-chimaerin vectors and 3 μg GFP-fused α2-chimaerin vectors were transiently co-transfected into HEK293T cells. After 48 hours incubation, the cells were pretreated with 5 μM of PKC inhibitor for 30 minutes and then a subset were stimulated with 10 μM of PMA for 30 minutes. Cells were solubilized in lysis buffer [50mM Tris-HCl (pH7.6), 150mM NaCl, 1mM EDTA (pH8.0), 0.5% NP-40, 10% glycerol, 0.5mM DTT, 10mM NaF, 1mM Na₃VO₄ protein inhibitor cocktail-EDTA free (Roche)] on ice for 30 minutes, and the lysate was centrifugated at 14000 rpm at 4°C for 20min. The supernatant was pre-cleared 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) and rec-Protein G-Sepharose 4B Conjugate at 4°C for 16 hours. The rec-Protein G-Sepharose was then washed with cold 1x PBS for 15 minutes x 4, mixed with loading buffer, and boiled. Samples were run in a 12% Bris-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Signal was detected by mouse monoclonal anti-V5 antibody (Invitrogen) and HRP-conjugated Donkey anti-mouse IgG (Jackson Immuno Research Lab). As controls, first V5-wildtype- or L20F-α2-chimaerin vectors at concentrations of 0.6 μg and 1.0 μg were transiently co-transfected with 1.0 μg GFP-α2-chimaerin into HEK293T cells and co-immunoprecipitation performed as
above. Given positive co-IP at these transfection levels in the presence of 10 μM of PMA, 5μg V5-α1-chimaerin vectors and 1 μg GFP-fused wild-type or L20F-α2-chimaerin vectors were transiently co-transfected into HEK293T cells and co-immunoprecipitation and western blotting was performed as above.

**Overexpression of wild-type and mutant α2-chimaerin in primary hippocampal neurons.** Rat primary hippocampal neurons were isolated from E18.5 rat embryonic brain and 1 x10⁶ neurons were plated on 6-well plates coated with 0.5 mg/ml poly D-lysine (Chemicon) and cultured in neurobasal medium (Invitrogen) supplemented with B27 supplement (Invitrogen), 0.5mM L-glutamine, 25 μM glutamate, 100 U/ml penicillin, and 100 mg/ml streptomycin. After 48 hours incubation, the neurons were co-transfected with 3.5 μg V5-α2-chimaerin and 0.5 μg pEGFP-N3 vectors using lipofectamine 2000 (Invitrogen). 48 and 72 hours following transfection, neurons were rinsed once with cold 1x PBS and then lysed on ice for 30 minutes in RIPA lysis buffer [50mM Tris-HCl (pH7.6), 150mM NaCl, 1mM EDTA (pH8.0), 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 0.5mM DDT, 10mM NaF, 1mM Na3VO4, protein inhibitor cocktail-EDTA free (Roche)]. The lysate was then centrifuged at 14000 rpm at 4°C for 10 min and the supernatant mixed with loading buffer and boiled. Western blotting was performed as described above. As a control for α2-chimaerin protein molecular weight, one lane was loaded with wild-type V5-α2-chimaerin overexpressed in HEK293T cells.

**Chick in situ hybridization.** Chick α2-chimaerin clone (NM_001012952) was a kind gift of Dr. Randy Caldwell (S16). The chick α-chimaerin riboprobe (nucleotides 386-819) was amplified using the following primers: 5' AGGCAGCAGAATACATTGCC-3' and 5' TAGTGTGGTAAGGTCACAGC-3' and cloned into pCRII-TOPO (Invitrogen). For in situ hybridization on cryostat sections, slides were washed briefly in 1x PBS and processed as described by Myat et al. (S17).

**Electroporation of chick embryos in ovo.** Hens’ eggs were incubated to Hamburger-Hamilton (HH) stage 10-11 (E2) and processed for electroporation as described by Momose et al. (S18). The midbrain vesicle or hindbrain, as appropriate, was microinjected with the DNA construct. Following electroporation, eggs were sealed and reincubated for 3 - 4 days to HH stage 27-30 (E5-6). Embryos were partially bisected and immunohistochemistry was performed on whole-mounts as described previously (S19), using chicken anti-GFP antibody (Abcam, UK) and mouse monoclonal anti-sarcomeric myosin antibody mf20 (Developmental Studies Hybridoma Bank) or using rabbit polyclonal anti-GFP antibodies (Molecular Probes) and 4 μM Alexa647-conjugated α-bungarotoxin (Molecular Probes). Flattened preparations were viewed from the medial side to visualise the oculomotor nerve. Flatmounted brainstems were stained with anti-Islet1/2 (clone 39.4D5, Developmental Studies Hybridoma Bank, University of Iowa) and anti-GFP (rabbit polyclonal, Molecular Probes) antibodies, and were viewed from the ventral side to visualize the oculomotor nucleus. Both preparations were imagined using a laser-scanning confocal microscope (Nikon EC1 or Zeiss LSM510 Meta).
fig. S1
Fig S1. Duane’s Retraction Syndrome.

(A-C) Schematic representation of extraocular muscle (EOM) innervation in (A) normal individuals, (B) sporadic Duane’s retraction syndrome (DRS), and (C) DURS2-DRS. In the normal state (A), four recti and two oblique EOM move the globe and the levator palpebrae superioris (LPS) muscle raises the eyelid. The oculomotor nucleus (depicted in blue) is composed of five motor subnuclei that send their axons in the oculomotor nerve; the axons in the superior branch of the oculomotor nerve innervate the LPS and superior rectus (SR) muscles, and the axons in the inferior branch innervate the medial rectus (MR), inferior rectus (IR), and inferior oblique (IO) muscles. The trochlear nucleus (depicted in brown) sends its axons in the trochlear nerve to innervate the superior oblique (SO) muscle. The abducens nucleus (depicted in green) is composed of motor neurons and interneurons. The motor neurons send their axons to innervate the lateral rectus (LR) muscle, which has been cut into two pieces in the schematic in order to visualize the oculomotor nerve. In Duane’s retraction syndrome (B), human postmortem studies (S20, S21) have reported that the abducens motor neurons are depleted (depicted as a dotted abducens nucleus), and the abducens cranial nerve is absent or markedly depleted (hatched thin green line). The lateral rectus muscle can be inappropriately innervated by aberrant branches off the oculomotor nerve (thin blue branches off oculomotor to LR muscle). This misinnervation of the lateral rectus by axons destined for the medial and inferior recti is proposed to result in co-contraction and globe retraction, a hallmark of DRS. (C) In our clinical and MRI studies of DURS2-DRS, we found evidence of the changes reported in sporadic DRS (B). In addition, however, we found that a subset of DURS2-DRS patients have a hypoplastic oculomotor nerve and can have small oculomotor-innervated muscles. This is depicted as thin blue oculomotor nucleus in (C).

(D) Schematic pedigree drawings of the four DURS2 families previously reported to map to the DURS2 locus. Squares = males, circles = females, hatches = deceased, filled black squares/circles = affection with Duane’s retraction syndrome, and “+” sign = participation in the study.

(E) Schematic pedigree drawings of the three previously unpublished DURS2 pedigrees harboring CHNI mutations. The DRS phenotype in RF is linked to the DURS2 locus, while in IS and AB it is consistent with linkage. Affection status co-segregates with the DURS2-DRS affected haplotype and CHNI mutation in all participants within each pedigree and, as predicted by haplotype analysis, clinically unaffected individuals FY V1:4 (S4), RF III:7, and AB III:2 harbor disease mutations, supporting 95% penetrance of CHNI mutations underlying DRS.
**A**

- Drosophila melanogaster
- Pan troglodytes
- Homo sapiens
- Macaca mulatta
- Mus musculus
- Rattus norvegicus
- Gallus gallus
- Xenopus tropicalis

**B**

- 2q31 2q32

**C**

- Published DURS2 critical region (9.9 Mb)
- Refined DURS2 region (4.6 Mb)

**D**

- Homo sapiens
- Pan troglodytes
- Macaca mulatta
- Mus musculus
- Rattus norvegicus
- Gallus gallus
- Xenopus tropicalis
- Drosophila melanogaster

---

**fig. S2**
Fig S2. DURS2-DRS results from heterozygous mutations in CHN1.

**(A & B) Reduction of the DURS2 region by linkage analysis.** Critical recombination events are shown for pedigrees UA and IJ in (A), while cytogenetic and physical map of the published and the refined DURS2 critical region is shown in (B). In (B), the top depicts a schematic cytogenetic representation of chromosome 2, indicating the published 2q31-q32 DURS2 critical region, while the bottom is a schematic physical map from ~171 to ~184 Mb along chromosome 2. In both (A) and (B), arrows indicate the relative position of CHN1. Evans *et al.* published the DURS2 centromeric recombination event that occurred in UA III:7 (A) within a 2.7 Mb region between recombinant marker D2S326 (small green font on left in B) and first internal nonrecombinant marker analyzed, D2S188 (small red font in B) (S3). Appukuttan *et al.* published the DURS2 telomeric recombination events in IJ III:3 (A) which fell within the 5 Mb region between recombinant marker D2S364 (small green font on right in B) and first analyzed nonrecombinant marker D2S138 (small red font in B) (S1). By designing primers to short tandem repeats found on of the UCSC Genome Browser Human, we reduced the critical region by maximally reducing the distance between the internal nonrecombinant and first recombinant marker (A and B). This reduced the centromeric recombination in UA III:7 to between recombinant DSCA3 (chr2; 174556614-174556903 bps) and DSCA4 (chr2; 174637754-174638136 bps) and the telomeric recombination in IJ III:3 to between D2SATT3 (chr2; 178561724-178561878 bps) and D2TA4 (chr2; 179218986-179219179 bps). The new refined 4.6 Mb DURS2 region is denoted by brackets between the new recombinant markers, DSCA3 and D2TA4, indicated in (B) by large bold green font with (+) above them. The refined first internal nonrecombinant markers, DSCA4 and D2SATT3, are indicated in (B) by large bold red font with (-) above them. Thirty known genes fell within the newly refined DURS2 region. Among these, 22 were sequenced and mutations were identified only in CHN1. CHN1 is located between polymorphic short tandem repeat markers D2S188 and D2S314 as denoted by a blue arrow. The eight genes not sequenced were GTPBP9, WIPF1, OSBPL6, PRKRA, DFNB59, FKBP7, PLEKHA3, TTN.

**(C) Automated genomic sequence analysis of one affected individual from each DURS2-DRS pedigree showing the seven heterozygous mutations in CHN1.** The nucleotide substitution and predicted corresponding normal and mutated amino acid residue are indicated under each triplet codon. For pedigree FY, note that the second nucleotide of exon 2 is mutated, and the corresponding amino acid is encoded by the last nucleotide in exon 1 (T) and the first two nucleotides in exon 2. Thus, wild-type TTA encodes a leucine while mutant TTT encodes a phenylalanine.

**(D) Human α2-chimaerin protein sequence aligned with homologs surrounding each mutation (boxed in black).** The predicted human α2-chimaerin protein shares 99.8%, 98.5%, 94.4%, 94.2%, 93.1%, 88.8% and 39.1% identity with the chimpanzee, rhesus monkey, mouse, rat, chick, frog, and fruit fly, respectively. The CHN1 mutations associated with DURS2-DRS alter residues that are highly conserved among these homologs.
Fig S3. Expression analysis of α2-chimaerin mRNA by Northern-blot hybridization in mouse and human.

(A-E) For each blot, the upper hybridization is with an α2-chimaerin specific CHN1 probe that recognizes a band of 2.6 kb, and the lower hybridization is with a control β-actin probe. (A) In mouse whole embryos, expression begins at ~E10.5 and is maximal between E10.5-E12.5. (B) In mouse adult brain, high expression is observed in brain and heart with lower expression in testis and kidney. (C) In human fetal tissue, mRNA is abundant in brain, with lower levels in lung and kidney. (D) In human adult tissue, a transcript is expressed primarily in brain and heart, with low expression observed in small intestine, placenta and lung. (E) In human adult brain tissue, α2-chimaerin mRNA is expressed abundantly in the cerebral cortex and at a lower level in putamen, cerebellum, medulla, and spinal cord.
Fig S4. Partial survey of the expression of α2-chimaerin specific \textit{CHN1} mRNA in human embryonic stages from CS12 (~26 dpo) to CS22 and 11 weeks post ovulation (wpo).

(A) to (D) and (K) to (T) are in pairs and in each case the section on the left has been hybridized with the antisense α2-chimaerin specific \textit{CHN1} probe while the section on the right has been hybridized with the sense α2-chimaerin specific \textit{CHN1} probe. (E), (F), (H) and (I) have been hybridized with the antisense probe while (G) and (J) have been hybridized with the sense probe. In each case the sections hybridized to antisense and sense probes have had the same treatment post-hybridization, i.e. they have had the same stringency post-hybridization washes and been incubated with the signal detection reagents for the same periods of time. The sections are not counter-stained but have been treated briefly with a 1% hydrochloric acid/methanol solution to reduce background. This gives the tissue a bluish tinge that is particularly evident in sections hybridized with sense probe where no signal was detected (B, D, G, J, L, N, P, R and T). Between two and fifteen slides were hybridized from two embryos at stages CS12 (A, B), CS16 (H, I, J, K, L) and CS19 (M, N) and between two and five slides from one embryo at stages CS14 (C, D), CS15 (E, F, G) and CS22 (O, P) and at 11 wpo (Q, R, S, T). The corresponding Carnegie Stage (CS) is indicated to the left of each panel.

Panel 1(A-L): (A) At CS12 (~26 days post ovulation), the expression was observed in neural tube around the optic vesicle (o) and in the hindbrain (hb). (C) By CS14, expression is clearly detected throughout the hindbrain and in trigeminal (nV) and facial (nVII) cranial nerve nuclei. Weak expression is also seen in the midbrain (mb) as is the case at CS15 (E) where there is also expression in hindbrain and neural tube. The closest section hybridized with the sense probe is (D), in which the ‘staining’ of the tissue is background and is a different color to that seen with the antisense hybridizations. Interestingly, as in chick (Fig 4) and unlike reported expression in the rat ([22]), expression is in both the ventricular layer and in post-mitotic neurons (Fig. 2, fig. S3C-E, S4). (H) At CS16, expression is in midbrain, hindbrain, spinal cord (sc) and vestibulocochlear (nVIII) and vagus (nX) nuclei. Expression in the midbrain is largely in the basal plate although there is some signal in the alar plate. At higher magnification, expression is seen in the developing oculomotor nucleus (I), and in neurons of rhombomere (rh) 5 where the abducens neurons are located and in neurons in rh 6 (K).

Panel 2 (M-T): Sagittal section of CS19 showing expression in cerebellum (cb), midbrain, diencephalon (dc), telencephalon (t) and dorsal root ganglia (drg). Expression is also detected in the digits (d). The apparent signal in the liver is an artifact (compare M and N). At CS19 expression is largely seen in the alar regions of the midbrain (M), while being very much reduced in basal midbrain and basal hindbrain, and is now detectable in the diencephalon and telencephalon as well. As in rat ([22]), human data show a caudal to rostral progression of expression in the brain. (O) At CS22 (~54 dpo) expression was clearly detected in the developing lateral ventricles (lv) where it continues to be expressed in the final stage examined (11 wpo). (Q, S) At 11 weeks post ovulation (wpo), α2-chimaerin mRNA is also expressed in the intermediate layer (i) and cortical plate (c). As in the chick and unlike reported expression in the rat ([22]), however, expression was seen both in the ventricular layer (F) and in post-mitotic neurons (I, K, S). c – cortical plate; cb- cerebellum; cp- choroid plexus; d- digits; dc- diencephalon; drg- dorsal root ganglia; g- ganglionic eminence; hb- hindbrain; i- intermediate zone; lv- lateral ventricle; mb- midbrain; o- optic vesicle; nIII - oculomotor nucleus; nV - trigeminal nucleus; nVII - facial nucleus; nVIII - vestibulocochlear nucleus; nx - vagus nucleus; rh- rhombomere; sc - spinal cord; t- telencephalon; v- ventricular layer. Scale bars: 100μm in (A), (B), (F), (G), (K) and (L); 200μm in (I) and (J); 500μm in (C), (D), (S) and (T); 1000μm in (E) and (H); 2000μm in (M), (N), (O), (P), (Q) and (R).
**Fig S5. Alignment of human α2- and β2-chimaerin and schematic representation of the α2-chimaerin DURS2 mutated amino acid residues superimposed on the β2-chimaerin molecule.**

(A) Human α2-chimaerin (NP_001813) and β2-chimaerin (1XA6) protein sequence alignment with amino acid numbering indicated above and below the protein sequence, respectively. The SH2, C1 and RacGAP domains are underlined in blue, orange and pink, respectively. Alignment reveals 72.5% identity overall, with 78.2% identify in the SH2 domain, 51.7% in the linker between the SH2 and C1 domains, 93.8% in the C1 domain, and 75.5% in the RacGAP domain (ClustalW alignment). The seven CHN1 mutations associated with DURS2-DRS are boxed in red and all are conserved between these two paralogs.

(B) Table indicating the equivalent β2-chimaerin residues for each of the α2-chimaerin mutations.

(C) Three-dimensional schematic of the predicted closed β2-chimaerin crystal structure with the SH2, C1 and RhoGAP domain regions highlighted in blue, yellow, and pink, respectively. The predicted positions of the α2-chimaerin residues altered by DURS2-DRS mutations are highlighted by dark blue amino acid side chains and generally cluster in and around the C1 domain.

(D & E) Two-dimensional schematics of (D) β2- and (E) α2-chimaerin structures in their closed states showing the β2-chimaerin intramolecular interactions based on the crystallization reported by Canagarajah et al. (S14) and the predicted paralogous α2-chimaerin intramolecular interactions. (D) β2-chimaerin. Open circles represent the positions of amino acids involved in intramolecular interactions, while the two green squares represent two of the residues targeted for mutational analysis by Canagarajah et al. and shown to decrease the EC_{50} for phorbol ester-induced translocation (S14). G235 is the DAG binding site, while residues R311, F315, and E317 interact with Rac. (E) α2-chimaerin. The positions of the seven α2-chimaerin amino acid residues altered by DURS2-DRS mutations are indicated in red. Of these, G228S and Y143H are indicated as red circles as they are predicted to be involved in intramolecular interactions as indicated, and G228S is predicted to be the DAG binding site. L20F and I126M are indicated as red squares as they correspond to the green squares in (D) and would be predicted to alter translocation to the membrane when mutated. E313K, P252Q, and A223V are indicated by red triangles because no direct predictions could be made as to their behavior. Note, however, that E313K is adjacent to the predicted Rac binding region, while A223V is one amino acid removed from a residue involved in an intramolecular interaction.
Fig S6. Supporting α2-chimaerin hyperactivation and interaction data. (A) The levels of over-expressed wild-type, L20F-, and G228S-α2-chimaerin in hippocampal neurons at 48 and 72 hours after transfection are approximately equal when corrected for transfection efficiency based on the level of GFP (n=2). V5-wildtype-α2-chimaerin over-expressed in HEK293T cells provides a size marker. The upper band present in all hippocampal lanes is nonspecific. (B) Rac-GTP levels were measured in HEK293T cells transfected with plasmids encoding V5-α2-chimaerin wild-type, a equal mixture of V5-wild-type- and V5-L20F-α2-chimaerin without (left blot) or with (right blot) PMA. Rac-GTP levels are reduced by co-overexpression of mixed and L20F-α2-chimaerin compared to wild-type alone. (C) Densitometric analysis of Rac-GTP levels in the presence of PMA normalized to total Rac and V5-α2-chimaerin levels. Values are expressed as percent of wild-type α2-chimaerin (mean±SEM, n=3). The difference between the reduction of Rac-GTP levels for either mixed- or L20F- alone is significant when compared to wild-type α2-chimaerin by one-way ANOVA with Dunnett’s adjustment (**p<0.005, ***p<0.0001). There is no significant difference between mixed- and L20F-α2-chimaerin. (D) A greater percentage of mutant-α2-chimaerin than wild-type translocates to the particulate fraction in HEK293T cells in response to PMA, and the degree of translocation is PMA dose dependent. (E) Graph of the densitometric analysis of α2-chimaerin immunoreactivity in the soluble fraction following 0, 1 μM, and 10 μM PMA. The results are expressed as a percentage of α2-chimaerin in the soluble fraction following PMA compared to the pre-treated cells (mean± SEM, n=6-11). At 10 μM PMA, the difference between the percent of wild-type versus L20F that has translocated is statistically significant (P<0.01) using one-way ANOVA followed by Dunnett’s post-test. (F) A representative immunoblot demonstrating that wild-type-α2-chimaerin complexes with itself in the presence of PMA in a dose-dependent manner. (G) α2-chimaerin immunoprecipitates with wild-type or L20F-α2-chimaerin in the presence of PMA when transiently over-expressed in HEK293T cells. In contrast, α1-chimaerin does not immunoprecipitate with wild-type or L20F-α2-chimaerin under the same experimental conditions (n=4).
Fig S7. Developing chick oculomotor nerves electroporated with wild-type, G228S, and L20F α2-chimaerin show axon stalling and defasciculation, with normal motor neuron cell sorting.

Electroporated oculomotor nerves (OMNs) at E5.5 extend from the midbrain neuro-epithelium, left, to the ciliary ganglion (asterisk) and beyond toward the ventral oblique (VO) muscle target. Confocal images of the constructs are as labeled; higher power views of the same preparation are labeled ' or ''. Arrowheads show axon stalling and arrows show axon branching / defasciculation. GFP control embryos showed a normal projection pattern (A, A', and A'') comparable to that seen with anti-neurofilament staining (S23). The nerve expands in the vicinity of the ciliary ganglion, which is the first intermediate target of the OMN, and the nerve then continues past this region towards the VO muscle. Higher power views of the nerve show that these axons have simple unbranched profiles in the proximal part of the nerve (A') and expanded regions with some branching in the vicinity of the ciliary ganglion (A'' & Fig 4D).

Neither wild-type nor mutant overexpression altered the initial projection of the OMN (B, D, and E). Defasciculation and ectopic branching was most striking in G228S-expressing embryos, where the nerve showed a frayed appearance with stalling and branches diverging / defasciculating from the main nerve trunk over some distance (D, D', F and Fig. 4F). This was also seen, however, in L20F-expressing embryos (E, E', G, H) and to a lesser frequency and extent in wild-type (B, C). The somewhat less severe wild-type overexpression phenotype is consistent with the finding that DURS2-DRS mutations enhance α2-chimaerin activity. (I and J) Flatmounted brainstems following electroporation of the right OMN nucleus with wildtype α2-chimaerin at E2 and stained with anti-Islet1 and anti-GFP antibodies at E5.5 (I, HH stage 28) and E6.0 (J, HH stage 30). Compared with the contralateral unelectroporated nucleus, organization of the oculomotor nucleus cell bodies, including the migration across the midline of the ventromedial pool (asterisk in J), appears unaffected by electroporation with wildtype α2-chimaerin (n=11 wild-type and n=7 G228S embryos examined). Scale bars: 200 μm (A, B, D, E, I, J); 100 μm (A', A'', C, E', F, G); 70 μm (D', H).
Fig. S8. Model of α2-chimaerin activation and signaling in the developing ocular cranial motor neurons of normal and DRS individuals.

(A) Tyrosine kinase receptors including EphA4 can both phosphorylate α2-chimaerin and increase DAG by activating phospholipase Cγ1 (PLCγ1), resulting in a conformational change of α2-chimaerin from a closed to open state. Experimentally, the phorbol ester PMA behaves as DAG. The specific receptor(s) upstream of α2-chimaerin in developing ocular motor neurons and axons are not known.

(B) Wild-type open α2-chimaerin can bind EphA4 and DAG at the membrane. Once α2-chimaerin is activated, the RacGAP domain can interact with Rac and hydrolyzes Rac-GTP to Rac-GDP, thus inhibiting Rac signaling.

(C) DURS2-DRS mutant α2-chimaerin has enhanced RacGAP activity either directly, or indirectly through increased translocation to the membrane. The result is enhanced inhibition of downstream Rac signaling.
<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>Isoform altered</th>
<th>MRI data</th>
<th>RacGAP activity</th>
<th>Translocation</th>
<th>α2-α2 Co-IP</th>
<th>In ovo data</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY</td>
<td>60A&gt;T</td>
<td>L20F</td>
<td>α2</td>
<td>Yes</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Yes</td>
<td>Equivalent to L28 in β2-chimaerin&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RF</td>
<td>378T&gt;G</td>
<td>I126M</td>
<td>α2</td>
<td>No</td>
<td>↑</td>
<td>→</td>
<td>↑</td>
<td>No</td>
<td>Equivalent to I134 in β2-chimaerin&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>JH</td>
<td>427T&gt;C</td>
<td>Y143H</td>
<td>α2</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>No</td>
<td>Phosphorylated by EphA4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>UA</td>
<td>668C&gt;T</td>
<td>A223V</td>
<td>α2 + α1</td>
<td>No</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>IS</td>
<td>682G&gt;A</td>
<td>G228S</td>
<td>α2 + α1</td>
<td>No</td>
<td>↑</td>
<td>→</td>
<td>→</td>
<td>Yes</td>
<td>Equivalent to G235 β2-chimaerin Predicted to be DAG binding site&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IJ</td>
<td>755C&gt;A</td>
<td>P252Q</td>
<td>α2 + α1</td>
<td>No</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>937G&gt;A</td>
<td>E313K</td>
<td>α2 + α1</td>
<td>No</td>
<td>↑</td>
<td>→</td>
<td>→</td>
<td>No</td>
<td>Predicted to be adjacent to Rac binding site</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> MR images published (S5)
<sup>b</sup> β2-chimaerin L28A increases β2-chimaerin translocation to the membrane following PMA stimulation (S14).
<sup>c</sup> β2-chimaerin I134A increases β2-chimaerin translocation to the membrane following PMA stimulation (S14).
<sup>d</sup> α2-chimaerin Y143 is phosphorylated by EphA4 stimulation (S24).
<sup>e</sup> β2-chimaerin G235 binds phorbol ester (S14)
**Supporting Online References**


