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Mutations in *CIZ1* cause adult-onset primary cervical dystonia

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Electronic Database Information

1000 Genomes Project (<http://www.1000genomes.org/>)

Allen Brain Atlas (<http://www.brain-map.org/>)

ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>)

dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>)

ESEfinder (<http://rulai.cshl.edu/tools/ESE2>)

FAS-ESS (<http://genes.mit.edu/fas-ess/>)

HSF (<http://www.umd.be/HSF/>)

ImageJ (<http://rsbweb.nih.gov/ij/>)

MERLIN (<http://www.sph.umich.edu/csg/abecasis/Merlin/>)

MES: MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)

MutationTaster (<http://www.mutationtaster.org>)

NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2/>)

NetPhos2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>)

NHLBI Exome Sequencing Project Variant Server (<http://evs.gs.washington.edu/EVS/>)

NLStradamus (<http://www.moseslab.csb.utoronto.ca/NLStradamus/>)

NNSplice (http://www.fruitfly.org/seq_tools/splice.html)

PESX (<http://cubweb.biology.columbia.edu/pesx/>)

Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>)

PredictProtein (<http://www.predictprotein.org/>)

Primer3 (<http://frodo.wi.mit.edu/primer3/>)

RESCUE-ESE (<http://genes.mit.edu/burgelab/rescue-ese/>)

SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html)

SSF (Splice-Site Finder, <http://www.umd.be/searchSpliceSite.html>)

Potential Conflicts of Interest

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Abstract

Objective—Primary dystonia is usually of adult onset, can be familial, and frequently involves the cervical musculature. Our goal was to identify the causal mutation in a family with adult-onset, primary cervical dystonia.

Methods—Linkage and haplotype analyses were combined with solution-based whole-exome capture and massively parallel sequencing in a large Caucasian pedigree with adult-onset, primary cervical dystonia to identify a cosegregating mutation. High-throughput screening and Sanger sequencing were completed in 308 Caucasians with familial or sporadic adult-onset cervical dystonia and matching controls for sequence variants in this mutant gene.

Results—Exome sequencing led to the identification of an exonic splicing enhancer mutation in Exon 7 of *CIZ1* (c.790A>G, p.S264G) which encodes CIZ1, Cip1-interacting zinc finger protein 1. CIZ1 is a p21^{Cip1/Waf1}-interacting zinc finger protein expressed in brain and involved in DNA synthesis and cell-cycle control. Using a minigene assay, we showed that c.790A>G altered *CIZ1* splicing patterns. The p.S264G mutation also altered the nuclear localization of CIZ1. Screening in subjects with adult-onset cervical dystonia identified two additional CIZ1 missense mutations (p.P47S and p.R672M).

Interpretation—Mutations in *CIZ1* may cause adult-onset, primary cervical dystonia, possibly by precipitating neurodevelopmental abnormalities that manifest in adults and/or G1/S cell-cycle dysregulation in the mature central nervous system.

Primary generalized dystonias usually begin in childhood, whereas focal dystonias typically present during adulthood. Adult-onset primary dystonias are at least 10-fold more prevalent than early-onset cases.¹ Cervical dystonia (CD) or spasmodic torticollis, the most common form of focal dystonia, is characterized by involuntary contractions of the neck muscles, which produce abnormal posturing of the head upon the trunk. Genetic factors are believed to play a major role in the pathogenesis of adult-onset primary dystonia because 10–20% of patients have one or more affected family members.^{2–5}

Although late-onset primary dystonia has a considerable heritable component, large pedigrees adequately powered for linkage analysis are uncommon and only a few have been described in the literature.^{6–9} Moreover, traditional genetic approaches such as linkage analysis have proven to be problematic in focal dystonia given that the characteristic reduced penetrance of mainly early-onset primary dystonias such as DYT1 (*TOR1A*) and DYT6 (*THAP1*) tends to be more pronounced in families with adult-onset disease. To date, a single locus (DYT7) for mainly adult-onset CD has been mapped to Chromosome 18p in a German kindred.¹⁰

In this study, linkage analysis was combined with whole-exome sequencing to identify the cause of CD in a previously described multigenerational Caucasian pedigree from the United States (Family A, Fig. 1).^{6,11} In-solution targeted exome capture and massively parallel sequencing was completed for five definitely affected and three unaffected subjects from the kindred. Using an autosomal dominant model, we employed a stepwise filtering process to identify sequence variants (SVs) common to the five definitely affected subjects but absent from at least two of the three unaffected subjects. SVs were filtered under the assumption that the mutation causing this uncommon familial disease is not present in the general population.

Materials and Methods

Cases and samples

Human studies were conducted in accordance with the Declaration of Helsinki, with formal approval from the institutional review boards at each participating study site. All subjects gave written informed consent. Enrollment of patients with primary dystonia and neurologically normal controls was described in previous publications.^{5,12} All normal controls were examined to exclude dystonia and other neurological disorders. Genomic DNA was extracted from whole blood. All affected subjects had isolated CD with otherwise normal neurological examinations. More specifically, no subject showed clinical evidence of ataxia, spasticity, oculomotor abnormalities, Parkinsonism, or neuropathy.

Linkage analysis

Linkage analysis was performed by genotyping microsatellites at the Genethon facility, Evry, France.¹³ DNA from members of Family A (6 affected and 10 unaffected) was PCR amplified at short tandem repeat loci (microsatellites) in a multiplexing approach. The amplification products were separated by polyacrylamide gel electrophoresis, transferred to nylon membranes, and visualized by electrochemiluminescence with exposure to radiographic film. Allele sizes were scored visually. Data from 238 microsatellite markers, spaced at approximately equal distances across the 22 autosomes, was analyzed using MERLIN (Multipoint Engine for Rapid Likelihood Inference), version 1.1.2.¹⁴ For parametric analysis, heterogeneity logarithm of odds (HLOD) scores were calculated for each marker using an autosomal dominant model with the disease allele frequency set to 0.0001. A nonparametric linkage statistic and associated *P* values were calculated using the Kong and Cox linear model implemented by MERLIN.

Targeted capture and exome sequencing

DNA samples from five members of Family A with definite CD and three unaffected family members were captured with the SureSelect^{XT} All Exome Kit 50 Mb (Agilent, Santa Clara, CA). In brief, 3 µg of genomic DNA was sheared to yield 100- to 150-bp fragments. Shearing was followed by end repairs and adapter ligation. The DNA-adaptor-ligated fragments were then hybridized to the human exome libraries for 24 h at 65°C. After hybridization, washing, and barcoding, DNA was amplified with emulsion PCR. Enriched DNA fragments were sequenced on a SOLiD 4 system (Applied Biosystems, Carlsbad, CA).

Prior to use of the 50-Mb All Exome Kits, four samples (three definite CD, one unaffected) had been captured with the Agilent SureSelect^{XT} Human All Exon Kit 38 Mb and sequenced independently. Reads from samples that were captured and sequenced twice were combined for downstream analyses. Percentage of exome coverage was based on exons targeted by the 50-Mb All Exome Kit, which incorporates Consensus Coding Sequence (CCDS), NCBI Reference Sequence (RefSeq), and GENCODE annotations.

Read mapping and variant analysis

Color space reads were mapped to the human reference genome (NCBI build 37.1) with NextGENe (SoftGenetics, State College, PA). Using the consolidation and elongation functions of NextGENe, instrument sequencing errors were reduced and sequence reads were lengthened prior to variant analysis. Given that short reads generated by the SOLiD system may not be unique within the genome being analyzed, the condensation tool polished the data for adequate coverage by clustering similar reads with a unique anchor sequence. Using this process, short reads were lengthened and reads with errors were filtered or corrected. The average post-processing read length for all eight samples was 74 nt.

To maximize the probability of detecting the causal SV, all base changes occurring in 2 reads in any individual sample were classified as variants for downstream analyses (Supplementary Fig. 1). Variant comparison was done between the five affected and three unaffected subjects to filter the number of SVs to identify the putative causal variant. Variant calls were combined from three independent comparisons of all five definitely affected subjects and two of the three unaffected subjects. The goal of these comparisons was to identify those SVs common to all five affected subjects and absent from at least two of the three unaffected subjects. In this fashion, we reduced the probability of eliminating a true causal variant present in an unaffected carrier. With NextGENe software, all filtering parameters were implemented simultaneously: homozygous SVs, intergenic SVs, deep intronic SVs (> 12 nt from splice sites), SVs reported in dbSNP or 1000 Genomes, synonymous SVs, and nonpathogenic nonsynonymous SVs. Additional details of variant analysis are provided in the Supplementary Information.

To validate the results of exome sequencing, primers were designed with Primer3 to amplify regions harboring SVs. PCR products were examined on agarose gels and cleaned with ExoSAP-IT (United States Biochemical, Cleveland, OH) prior to Sanger sequencing in the forward and reverse directions using BigDye Terminator v3.1 chemistry (Life Technologies, Carlsbad, CA) on an Applied Biosystems 3130XL Genetic Analyzer.

CIZ1 and SETX mutation screening

Sanger sequencing and/or high-resolution melting (HRM) were used for mutation screening in 308 subjects with CD and 724 controls. HRM was performed with the LightCycler 480 Real-Time PCR system and High Resolution Master Mix (Roche, Indianapolis, IN) in accordance with our laboratory protocol.⁵ HRM reactions were performed in either 96- or 384-well plates, using 20 ng of template DNA, 1× HRM Master Mix, 2.5 mM MgCl₂, and 200 nM of each primer in a 10-μl reaction volume. Melting curves and difference plots were analyzed by at least two investigators blinded to phenotype. For the 140 samples with shifted melting curves, PCR products were cleaned using ExoSAP-IT and Sanger sequenced in the forward and reverse directions.

In silico analysis of amino acid substitutions and splicing

Missense variants were analyzed with PolyPhen-2,¹⁵ SIFT,¹⁶ and MutationTaster¹⁷ to predict the pathological character of single amino acid mutations. Protein sequence alignment was performed with ClustalW2.¹⁸ The possible effects of c.790A>G on *CIZ1* splicing were evaluated using a broad array of tools for splice site, exonic enhancer, and silencer site analysis: Relative Enhancer and Silencer Classification by Unanimous Enrichment (RESCUE-ESE¹⁹), Putative Exonic Splicing Enhancers/Silencers (PESX²⁰), Human Splicing Finder (HSF²¹), NetGene2,²² Splice Site Prediction by Neural Network (NNSplice²³), MaxEntScan (MES²⁴), ESEfinder,²⁵ and FAS-ESS.²⁶

CIZ1 expression in human brain and lymphoblastoid cell lines

Adult human whole-brain total RNA (FirstChoice Human Brain Reference RNA, 1 mg/ml) was obtained from Ambion (Austin, TX). Fetal human whole brain and adult human cerebral cortex, cerebellum, substantia nigra, and putamen total RNA were purchased from Clontech (Mountain View, CA). Ambion's TRI Reagent was used to isolate RNA from lymphoblastoid cell lines generated from two affected subjects (Family A, III-2 and III-3) and six normal controls, as previously described.¹² Reverse transcription was performed with Ambion's RETROscript kit using 500 ng total RNA as template along with both random oligonucleotide and oligo(dT) primers. The reaction mixtures were incubated at 44°C for 1 h and then at 92°C for 10 min. Relative quantitative real-time RT-PCR was performed using the LightCycler 480 with gene-specific primers (Supplementary Table 1)

and Universal Taqman probes (Roche) for *CIZ1* and the endogenous control (*PPID*), which encodes 40-kDa peptidyl-prolyl cis-trans isomerase D (cyclophilin D). Quantitative RT-PCR was performed to examine the relative expression of the two major *CIZ1* isoforms. cDNA was amplified with primers *CIZ1_RE6F* and *CIZ1_RE9R*. The agarose gel was imaged (G:BOX gel imaging system, Syngene, Frederick, MA) and bands were analyzed with ImageJ.

Minigene assay of *CIZ1* splicing

To validate the predicted effect of the *CIZ1* c.790A>G variant at the donor splice site of Exon 7, we performed a minigene analysis. The wild-type minigene construct was produced by cloning into the exontrap vector pET01 (MoBiTec, Göttingen, Germany). Genomic DNA encompassing Introns 5–9, including Exons 6–9, was amplified with the SequalPrep Long PCR Kit (Invitrogen, Carlsbad, CA). The PCR product and pET01 were digested with *XhoI* and *BamHI* at 37°C for 2 h and then 65°C for 20 min. This was followed by ligation of cohesive-ended gel-purified digested PCR products into exontrap vector pET01 with the LigaFast Rapid DNA Ligation System (Promega, Madison, WI). After sequence confirmation, the variant c.790A>G was introduced into the WT-minigene with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) to generate a mutant minigene.

Next, HEK 293T (293T) cells were transfected in quadruplicate. Total RNA was extracted with TRI Reagent (Ambion) 72 h after transient transfection. DNA was removed with DNase I (Ambion). Total RNA quality was examined with agarose gel electrophoresis and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed with Ambion's RETROscript kit using 500 ng total RNA as template. PCR products were examined in a 1% agarose gel, bands were quantified with ImageJ, and the effects of genotype were evaluated with *t*-tests.

Cellular localization of wild-type and mutant *CIZ1*

To investigate the effects of p.S264G on the cellular localization of *CIZ1*, we made constructs that expressed full-length human wild-type and mutant (p.S264G) *CIZ1* under CMV promoters. Transient transfections with wild-type and mutant vectors (in quadruplicate, for both) were performed in 293T cells. ImageJ was used to perform particle analysis of GFP-*CIZ1* transfected 293T cells. Detailed methods related to the cellular localization of *CIZ1* are available in the Supplementary Information.

Results

Linkage analysis and exome sequencing

In the five definitely affected and three unaffected subjects from Family A, sequencing generated 2× or greater coverage for 84.7–92.3% of the exome (Supplementary Table 2). After filtering and elimination of read errors with Sanger sequencing, 23 validated SVs remained (Supplementary Fig. 1). Three of these 23 SVs cosegregated with CD: *CIZ1* (c.790A>G and c.1730C>T) and *SETX* (c.2385_2388delAAAG).

In Family A (Fig. 1), CD showed strongest linkage to microsatellite markers D9S159 and D9S1818 on Chr 9q34 (Fig. 1, Table 1). D9S159 is located 1.34 Mb from *CIZ1* on Chr 9q34.11. The marker showing the second highest HLOD score, D9S1818, is located nearby on Chr 9q34.13, 6.11 Mb from *CIZ1* and 1.90 Mb from *SETX*. Haplotype analysis of microsatellite markers on Chr 9 showed that D9S159 cosegregated with mutant *CIZ1* (Supplementary Fig. 2).

The c.790A>G *CIZ1* variant is located within a highly conserved donor splice site in Exon 7 (Fig. 2). In contrast, the c.1730C>T (p.S577F) SV is only partially conserved at the amino acid level and predicted to be damaging by only one of three prediction programs (Table 2). The *SETX* variant (c.2385_2388delAAAG, p.I795Ifs*16) is predicted to cause a frameshift and probably induces nonsense-mediated decay.

Mutation screening and in silico analyses

Using 18 pairs of PCR primers placed on flanking intronic and untranslated regions to encompass the coding regions of the 17 *CIZ1* exons and at least 50 bp of 5'- and 3'-intronic sequence surrounding each exon (Supplementary Table 1), mutation screening in patients with familial and sporadic CD and neurologically normal controls (Table 3) identified five additional missense SVs (p.P47S, p.P50L, p.Q394E, p.S577F, p.R672M) (Table 2, Supplementary Table 3). *In silico* analyses with ClustalW2,¹⁸ Polyphen-2,¹⁵ SIFT,¹⁶ and MutationTaster¹⁷ indicated that p.P47S (Subject C), p.S264G (Family A), and p.R672M (Subject B) were highly conserved and probably damaging to *CIZ1*. Moreover, the p.S264G mutation is located within a putative nuclear localization signal (NLStradamus²⁷) and alters a potential phosphorylation site (PredictProtein²⁸ and NetPhos 2.0²⁹). Two of the novel variants (p.P50L [Subject D] and p.Q394E [Subjects E and F]) detected during screening are predicted to be benign. Moreover, the p.Q394E variant was also found in 1 of 5378 subjects in the Exome Variant Server database. Similarly, none of the validated nonsynonymous *CIZ1* SVs identified in the dbSNP database were highly conserved or predicted to be deleterious by all three prediction programs (Supplementary Table 4).

Although the *SETX* variant (c.2385_2388delAAAG) cosegregated with CD in Family A, it was not required for manifestation of CD in Subjects B and C (Supplementary Table 3). With HRM, we did not detect this variant in 724 neurologically normal control subjects.

Effects of c.790A>G on *CIZ1* splicing

The Exon 7 mutation in *CIZ1* (c.790A>G) may also exert deleterious effects on transcription. RESCUE-ESE,¹⁹ PESX,²⁰ and FAS-ESS²⁶ identified an exonic splice enhancer site in wild-type *CIZ1* that was disrupted by the c.790A>G mutation. ESEfinder predicted that the mutation abolishes the SF2/ASF and SRp55 binding sites present in the wild-type sequence,³⁰ whereas HSF indicated that the splice and enhancer motif sites were broken in the mutant sequence.²¹ Similarly, NetGene2 predicted that the c.790A>G variant abolished the Exon 7 donor splice site.²² NNSPLICE's donor site prediction score was reduced from 0.9 in the wild-type sequence to 0.6 in the mutant sequence, whereas MaxEntScan's donor splice site score fell from 8.77 to 4.69 with introduction of the c.790A>G variant.^{23,24}

To examine the predicted effects of the c.790A>G variant at the donor splice site of Exon 7, we performed a minigene assay (Fig. 3). Wild-type and mutant constructs were produced by cloning gDNA into the exontrap vector pET01 (Fig. 3A). Total RNA was extracted 72 h after transient transfection and analyzed with quantitative RT-PCR (Supplementary Fig. 3). Overall, wild-type and c.790A>G constructs produced bands of identical nucleotide sequences but in differing amounts (Fig. 3B). In particular, a splice variant only containing Exons 7 and 8 had significantly more wild-type 293T cells, and c.790A>G cells were barely detectable in an Exon 7-only variant. Alternate splicing of variable Exon 8 was not identified with the pET01 minigene system. While our findings show that c.790A>G exerts notable effects on splicing patterns, the exact *in vivo* effects of this variant on *CIZ1* splicing in humans may show cell type specificity and developmental regulation.

Expression of *CIZ1* in brain and lymphoblastoid cell lines

With RT-PCR, we identified two major *CIZ1* isoforms in adult and fetal brain (Fig. 2, Supplementary Fig. 4). In adult human brain, both isoforms were present in cerebellum, cerebral cortex, substantia nigra, and putamen. With relative quantitative real-time RT-PCR, *CIZ1* expression was greatest in fetal brain and in cerebellum among adult brain regions (Supplementary Table 5). Relative to *CIZ1* Isoform 1, Isoform 2 was most strongly expressed in substantia nigra and cerebellum. In lymphoblastoid cell lines, the c.790A>G mutation had no effects on overall expression of *CIZ1* and minimal effects on the ratio of Isoform1 to Isoform 2. However, our experiment may have lacked the power to detect an effect of the exonic splicing enhancer mutation on relative expression of Isoforms 1 and 2.

Subnuclear localization of *CIZ1*

To examine the effects of p.S264G on the subnuclear localization of *CIZ1*, wild-type and mutant proteins were expressed in 293T cells (Fig. 4). The p.S264G protein did not fail to enter nuclei or form subnuclear foci. However, particle analysis of GFP-*CIZ1* transfected cells revealed that p.S264G *CIZ1* formed fewer particles per nucleus than the wild-type protein. In comparison to wild-type particles, mutant particles were also larger and encompassed a larger area of the nucleus (Table 4).

Discussion

In a large Caucasian kindred with familial CD, we used linkage analysis and whole-exome sequencing to identify an exonic splicing enhancer mutation in Exon 7 of *CIZ1* (c.790A>G, p.S264G). The microsatellite marker D9S159 on Chr 9q34.11 cosegregated with c.790A>G in all affected subjects. *CIZ1* encodes Cip1-interacting zinc finger protein 1, a DNA replication factor. The c.790A>G variant was shown to alter splicing of *CIZ1* and subnuclear localization of *CIZ1*. We also showed that both major isoforms of *CIZ1* are expressed in motor regions of human brain. Using Sanger sequencing and HRM, 308 subjects with CD and 724 controls were screened for mutations in *CIZ1*. Two additional pathogenic variants (p.P47S and p.R672M) were identified in subjects with CD, but none were found in controls.

Although supported by several lines of evidence, the association between *CIZ1* and CD should be interpreted cautiously. First, we did not identify *CIZ1* mutations in a second multiplex kindred with CD. Second, we cannot exclude the possibility that other coding or noncoding variants on Chr 9q34.11 contribute to the pathogenesis of CD. Finally, with the exception of p.S264G, the predicted pathogenicity of all missense variants was limited to *in silico* analyses. MutationTaster and PolyPhen-2 are associated with accuracy rates of no better than 85.7% and 80.7%, respectively, and the false positive and negative rates for PolyPhen-2 are 21.4% and 16.8%, respectively.¹⁷

Although recessive mutations in *SETX* may be associated with ataxia-ocular apraxia-2 (AOA2; MIM 606002) and occasional subjects with AOA2 may show signs of dystonia, albeit in the lower extremities, all of our manifest CD subjects had otherwise normal neurological examinations without evidence of oculomotor apraxia, ataxia, spasticity, Parkinsonism, neuropathy, or muscle weakness.³¹ Autosomal dominant missense mutations in *SETX* have been associated with amyotrophic lateral sclerosis 4 (ALS4; MIM 602433). Because frameshift mutations have not been described in pedigrees with ALS4, it is possible that missense mutations in subjects with ALS4 function via a gain-of-function mechanism. Therefore, the *SETX* variant identified in Family A is probably benign and not requisite for the expression of CD. However, we cannot exclude the possibility that heterozygous loss-of-function SVs in *SETX* contribute to the expressivity of *CIZ1* mutations.

Primary dystonia may be a neurodevelopmental network disorder of the central nervous system due to dysfunction at one or more nodes of the highly interconnected motor subsystem that includes the cerebellum and basal ganglia.³² The relatively high expression of *CIZ1* in fetal brain and cerebellum are compatible with modern theories of dystonia pathobiology. *CIZ1* is also expressed in a wide range of non-neural tissues such as spleen, thymus, heart, lung, liver, kidney, stomach, and testis.³³ *CIZ1* harbors at least four alternatively spliced exons.^{34,35} Exon skipping may be more common in certain cancers, and a *CIZ1* isoform due to alternative splicing of Exon 8 may be associated with Alzheimer disease.^{34–36} In the context of CD, splicing defects in *CIZ1* may also be critical to the pathogenesis of the c.790A>G variant.

CIZ1 was first identified through its interaction with p21^{Cip1/Waf1}, a cyclin-dependent kinase inhibitor involved in G1/S cell-cycle regulation and cellular differentiation.³³ In cell-free systems, *CIZ1* is able to promote DNA replication after replication complex formation.³⁷ The C-terminal domain anchors *CIZ1* to the nonchromatin nuclear matrix, whereas DNA replication activity resides in the N-terminal half of the protein. Studies of GFP-tagged *CIZ1* have shown that formation of subnuclear particles or foci requires both the N- and C-terminal domains.³⁸

We showed that the p.S264G *CIZ1* did enter nuclei and form subnuclear foci in cultured cells. However, particle analysis revealed that p.S264G *CIZ1* formed larger particles than the wild-type protein. Therefore, p.S264G could exert deleterious effects via a gain-of-function mechanism associated with aberrant DNA synthesis and/or transcription.

Although possibly unique in its association with adult-onset and monosymptomatic focal dystonia, the cellular role and neural localization of *CIZ1* are compatible with current themes in dystonia research. Clinically and biologically, *CIZ1* shows the greatest similarity to the transcription factors THAP1 and TAF1. THAP1 regulates cell proliferation and G1/S cell-cycle progression³⁹ and THAP1 loss-of-function mutations (*DYT6*) cause diverse anatomical patterns of childhood and adult-onset dystonia (including CD) with significant intrafamilial phenotypic variability.^{5,40} While often considered a neurodegenerative disorder, some patients with Lubag (*DYT3*) manifest isolated focal (including CD) or segmental dystonia for years before the development of Parkinsonism. *DYT3* is associated with deficiency of a neuronal isoform of TAF1.⁴¹ TAF1 forms part of the TFIID transcriptional complex. Moreover, TAF1 induces G1/S progression by phosphorylating p53 at threonine-55.⁴² The best characterized primary dystonia, *DYT1*, is due to mutations in *TOR1A* and, like *DYT6*, shows significant intrafamilial variability and is predominantly of childhood onset.⁴³ TorsinA, the protein encoded by *TOR1A*, is concentrated at the nuclear envelope in its mutant form; it appears to play a role in interactions between the nucleus and cytoskeleton⁴⁴ and may participate in transcriptional regulation via the TGF β pathway.⁴⁵

In addition to significant functional overlap at the cellular level, *CIZ1* shows similarities to *THAP1* and *TOR1A* at the systems level. All three genes are robustly expressed in cerebellum, particularly Purkinje cells (Allen Brain Atlas), and recent anatomical studies in genetically engineered *DYT1* mouse models suggest that primary dystonia may be a neurodevelopmental abnormality of cerebellar Purkinje cells.⁴⁶ *CIZ1* gain-of-function mutations could illicit aberrant transcription during development or activation of the cell cycle in terminally differentiated neurons such as Purkinje cells.⁴⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

Family A pedigree and linkage analysis. (A) Filled symbols, definitely affected. Half-filled symbols, possibly affected. Symbols with central dots, unaffected carriers. Arrow, proband. *CIZ1* genotypes: wild-type (+/+) and heterozygous c.790A>G (+/-). (B) Multipoint HLOD scores for Family A generated under an autosomal dominant model with disease allele frequency set to 0.0001 and penetrance of 0.5. Marker positions are plotted on the *x*-axis and vertical dashed lines delimit the 22 autosomes.

FIGURE 2.

Organization of *CIZ1* gene, transcripts, and full-length protein. (A) Structure of *CIZ1* on the reverse strand of Chr. 9 presented in the 5' to 3' direction showing the location of three identified mutations (c.139C>T, c.790A>G, and c.2015G>T) in probands with CD. (B) In brain, *CIZ1* is transcribed into two major isoforms that differ at variable Exon 8. (C) Missense mutations in highly conserved regions of *CIZ1* are shown in relationship to functional domains: QD1, glutamine-rich domain 1; NLS, putative nuclear localization sequence (259–264 aa); QD2, glutamine-rich domain 2; ZF, zinc finger domain; AD, acidic domain; MH3, matrin (MATR3)-homologous domain 3. Other putative NLSs (NLStradamus prediction cutoff of 0.05) are not shown (240–246 aa, 695–723 aa, 832–839 aa, 875–898 aa). (D) Protein sequence analysis with ClustalW2 shows that P47, S264, and R672 are located within highly conserved domains of *CIZ1*. *Mutation in Family A

FIGURE 3.

Minigene assay of *CIZ1* splicing. (A) Segment of *CIZ1* containing Exons 6, 7, 8, and 9 were cloned into the multiple cloning site (MCS) of the pET01 exontrap vector, which contains a short stretch of a eukaryotic phosphatase gene (E) 3' to the eukaryotic strong long terminal repeater promoter of the Rous Sarcoma Virus (RSV). A second segment of E is followed by a polyadenylation site (pA). The c.790A>G mutation at the exonic splicing enhancer is located 2 nucleotides from the Exon 7–Intron 7 splice site. (B) The results of four wild-type and four c.790A>G transfections showed that the A>G mutation exerted significant effects on splicing patterns in HEK 293T cells (* $P < 0.05$).

FIGURE 4.

Cellular localization of wild-type and mutant CIZ1 (S264G) in transiently transfected HEK 293T cells. CIZ1 was visualized with a GFP tag or rabbit anti-CIZ1 antibody. In comparison to wild-type CIZ1, average particle size was larger and the number of particles tended to be smaller in cells expressing S264G. β -tubulin, red; CIZ1, green; and DAPI, violet. Scale bar, 10 μ m.

Table 1

Microsatellite Markers Associated with the Highest HLOD Scores

Marker	Location	HLOD ^a	HLOD ^b	NPL (<i>P</i> value) ^c
D1S235	1q42.3	1.87	1.52	1.025 (0.014)
D1S304	1q43	1.74	1.41	0.990 (0.016)
D3S1300	3p14.2	1.66	1.24	0.725 (0.033)
D3S1595	3p12.1	1.11	1.10	0.916 (0.019)
D9S159	9q34.11	2.71	2.29	1.707 (0.002)
D9S1818	9q34.2	2.27	2.11	1.679 (0.002)
D21S265	21q21.3	1.92	1.50	0.939 (0.018)

^aAutosomal dominant model with penetrance of 1.^bAutosomal dominant model with penetrance of 0.5.^cNPL (nonparametric linkage statistic) and associated *P* values were calculated using the Kong and Cox linear model.

Table 2

In Silico Analysis of Missense Sequence Variants in CIZ1

Family/Subject	Phenotype	Variant	ClustalW2 Conservation	Polyphen-2		SIFT		MutationTaster	
				Prediction ^d	Probability ^e	Prediction	Score ^f	Prediction ^h	Probability ⁱ
A	CD	S264G	Highly	Probably damaging	0.973	Tolerated	0.18	Disease causing	0.81
A	CD	S577F	Partial ^a	Benign	0.000	Damaging	0.05	Polymorphism	0.54
B	CD	R672M	Highly	Probably damaging	1.00	Damaging	0.00	Disease causing	0.87
C	CD	P47S	Highly	Probably damaging	0.997	Damaging	0.02	Polymorphism	0.77
D	Control	P50L	Partial ^b	Benign	0.005	Damaging	0.04	Polymorphism	0.98
E ^j	CD	Q394E	Partial ^c	Benign	0.164	Tolerated	1	Polymorphism	1.00
F ^j	Control	Q394E	Partial ^c	Benign	0.164	Tolerated	1	Polymorphism	1.00

^aS changed to F in monkey.

^bP changed to S in mouse and rat.

^cQ changed to E in pig.

^dQualitative ternary classification (benign, possibly damaging, or probably damaging).

^eProbability of the variation being damaging.

^fSIFT scores range from 0 to 1; an amino acid substitution is predicted to be damaging if the score is 0.05 and tolerated if the score is > 0.05.

^hMutationTaster classifies the results as disease causing or polymorphism.

ⁱProbability ranges from 0 to 1.

^jAlso found in 1 of 5378 subjects in the Exome Variant Server database.

TABLE 3

CIZ1 Mutation Screening

Exon	Cervical Dystonia ^b			Controls ^d		
	HRM	Sanger ^c	Total Subjects	HRM	Sanger	Total Subjects
1	0	140	140	0	100	100
2 ^a	308	236	308	724	300	724
3	308	140	308	724	100	724
4	308	140	308	724	100	724
5	308	140	308	0	100	100
6	308	140	308	0	100	100
7 ^a	308	236	308	724	300	724
8 ^a	308	236	308	724	300	724
9	308	140	308	724	100	724
10 ^a	0	236	236	0	300	300
11	308	140	308	0	100	100
12 ^a	308	236	308	724	300	724
13	308	140	308	724	100	724
14	0	140	140	0	100	100
15	308	140	308	724	100	724
16	308	140	308	724	100	724
17	0	140	140	0	100	100

^aExons harboring novel variants.

^bM:F = 71:237; age: mean = 59.8 yrs, range = 21–92 yrs, SEM = 12.3 yrs. Subjects with CD were divided into two groups for analysis. Group A included 44 probands with a positive family history of dystonia (M:F = 12:32; age: mean = 59.0 yrs, range = 29–85 yrs, SEM = 12.7 yrs; age of onset: mean = 41.1 yrs, range = 7–65 yrs, SEM = 14.6 yrs). Group B included 264 sporadic cases of CD (M:F = 59:205, age: mean = 60.1 yrs, range = 21–92 yrs, SEM = 12.1 yrs; age of onset: mean = 47.6 yrs, range = 14–85 yrs, SEM = 13.7 yrs).

^cSanger sequencing included all samples from group A with the remainder derived from group B. These numbers do not include samples with shifted melting curves, which were subjected to Sanger sequencing after HRM.

^dM:F = 297:427; age: mean = 58.8 yrs, range, 21–95 yrs, SEM = 14.6 yrs.

Table 4

Particle Analysis of GFP-CIZ1 Transfected HEK 293T Cells

Parameter	Wild-type CIZ1	p.S264G CIZ1	<i>t</i> -test <i>P</i> value
Count (number of particles/nucleus)	48.22 ± 1.95	39.07 ± 1.94	1.22E-03
Total area of particles/nucleus (μm ²)	18.15 ± 0.93	26.82 ± 1.57	7.47E-06
Average particle size (μm ²)	0.395 ± 0.020	0.701 ± 0.032	1.96E-12
Average particle perimeter (μm)	2.10 ± 0.06	2.76 ± 0.07	6.38E-10
Circularity ^a	0.902 ± 0.006	0.909 ± 0.005	4.04E-01

^aCircularity = $4\pi \times ([\text{area}]/[\text{perimeter}]^2)$, with a value of 1 indicating a perfect circle. A circularity value approaching 0 indicates an increasingly elongated shape.