RESEARCH ARTICLE

Dystonia Linked to *EIF4A2* Haploinsufficiency: A Disorder of Protein Translation Dysfunction

Philip Harrer, MD,[1,2] Matej Škorvánek, MD, PhD,[3,4] Volker Kittke, MSc,[1,2] Ivana Dzinovic, MSc,[1,2] Friederike Borngräber, MD,[5] Mirja Thomsen, MSc,[6] Vanessa Mandel, MSc,[1] Tatiana Svorenova, MD,[3,4] Miriam Ostrozovicova, MD,[3,4] Kristina Kulcsarova, MD,[3,4] Riccardo Berutti, PhD,[1,2] Hauke Busch, PhD,[7] Fabian Ott, MSc,[7] Robert Kopajtich, MSc,[1,2] Holger Prokisch, PhD,[1,2] Kishore R. Kumar, MBBS, PhD, FRACP,[8,9] Niccolo E. Mencacci, MD, PhD,[10] Manju A. Kurian, PhD,[11,12] Alessio Di Fonzo, MD, PhD,[13] Sylvia Boesch, MD,[14] Andrea A. Kühn, MD,[5] Ulrike Blümlein, MD,[15] Katja Lohmann, PhD,[6] Bernhard Haslinger, MD,[16] David Weise, MD,[17,18] Robert Jech, MD, PhD,[19] Juliane Winkelmann, MD,[1,2,20,21] and Michael Zech, MD[1,2*]

[1]*Institute of Neurogenomics, Helmholtz Zentrum München, Munich, Germany*
[2]*Institute of Human Genetics, School of Medicine, Technical University of Munich, Munich, Germany*
[3]*Department of Neurology, P.J. Safarik University, Kosice, Slovak Republic*
[4]*Department of Neurology, University Hospital of L. Pasteur, Kosice, Slovak Republic*
[5]*Movement Disorder and Neuromodulation Unit, Department of Neurology, Charité—Universitätsmedizin Berlin, Berlin, Germany*
[6]*Institute of Neurogenetics, University of Lübeck, Lübeck, Germany*
[7]*Institute of Experimental Dermatology and Institute of Cardiogenetics, University of Lübeck, Lübeck, Germany*
[8]*Translational Neurogenomics Group, Molecular Medicine Laboratory and Neurology Department, Concord Clinical School, Concord Repatriation General Hospital, The University of Sydney, Sydney, New South Wales, Australia*
[9]*Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia*
[10]*Ken and Ruth Davee Department of Neurology, Simpson Querrey Center for Neurogenetics, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, USA*
[11]*Department of Developmental Neurosciences, UCL Great Ormond Street Institute of Child Health, London, UK*
[12]*Department of Neurology, Great Ormond Street Hospital, London, UK*
[13]*Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Neurology Unit, Milan, Italy*
[14]*Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria*
[15]*Department of Pediatrics, Carl-Thiem-Klinikum Cottbus, Cottbus, Germany*
[16]*Department of Neurology, Klinikum rechts der Isar, Technical University of Munich, School of Medicine, Munich, Germany*
[17]*Department of Neurology, Asklepios Fachklinikum Stadtroda, Stadtroda, Germany*
[18]*Department of Neurology, University of Leipzig, Leipzig, Germany*
[19]*Department of Neurology, Charles University in Prague, 1st Faculty of Medicine and General University Hospital in Prague, Prague, Czech Republic*
[20]*Lehrstuhl für Neurogenetik, Technische Universität München, Munich, Germany*
[21]*Munich Cluster for Systems Neurology, SyNergy, Munich, Germany*

ABSTRACT: Background: Protein synthesis is a tightly controlled process, involving a host of translation-initiation factors and microRNA-associated repressors. Variants in the translational regulator *EIF2AK2* were first linked to neurodevelopmental-delay phenotypes, followed by their implication in dystonia. Recently, de novo variants in *EIF4A2*, encoding eukaryotic translation initiation factor 4A isoform 2 (eIF4A2), have been described in pediatric cases with developmental delay and intellectual disability.
Objective: We sought to characterize the role of *EIF4A2* variants in dystonic conditions.

*__Correspondence to:__ Dr. Michael Zech, Institute of Neurogenomics, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany, E-mail: michael.zech@mri.tum.de

Philip Harrer, Matej Škorvánek, and Volker Kittke contributed equally to this work as co-first authors.

Juliane Winkelmann and Michael Zech contributed equally to this work as co-last authors.

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Methods: We undertook an unbiased search for likely deleterious variants in mutation-constrained genes among 1100 families studied with dystonia. Independent cohorts were screened for *EIF4A2* variants. Western blotting and immunocytochemical studies were performed in patient-derived fibroblasts.

Results: We report the discovery of a novel heterozygous *EIF4A2* frameshift deletion (c.896_897del) in seven patients from two unrelated families. The disease was characterized by adolescence- to adulthood-onset dystonia with tremor. In patient-derived fibroblasts, eIF4A2 production amounted to only 50% of the normal quantity. Reduction of eIF4A2 was associated with abnormally increased levels of IMP1, a target of Ccr4-Not, the complex that interacts with eIF4A2 to mediate microRNA-dependent translational repression. By complementing the analyses with fibroblasts bearing *EIF4A2* biallelic mutations, we established a correlation between IMP1 expression alterations and eIF4A2 functional dosage. Moreover, eIF4A2 and Ccr4-Not displayed significantly diminished colocalization in dystonia patient cells. Review of international databases identified *EIF4A2* deletion variants (c.470_472del, c.1144_1145del) in another two dystonia-affected pedigrees.

Conclusions: Our findings demonstrate that *EIF4A2* haploinsufficiency underlies a previously unrecognized dominant dystonia-tremor syndrome. The data imply that translational deregulation is more broadly linked to both early neurodevelopmental phenotypes and later-onset dystonic conditions.

Key Words: *EIF4A2*; loss-of-function variants; translational dysfunction; dystonia; tremor

Introduction

Dystonia defines a phenotypically heterogeneous group of movement disorders that can be underlined by neurodegenerative lesions, neurodevelopmental abnormalities, or combinations of both.[1] Although genetic causes have been established for a growing number of syndromes involving dystonic features, many individuals with overlapping phenotypes remain undiagnosed, and the molecular mechanisms associated with known etiologies are diverse and incompletely understood.[2] A theme shared by several monogenic dystonias involves the maintenance of protein homeostasis,[3] which requires appropriate regulation of protein synthesis and turnover.[4] Defects of the translational machinery represent an important cause of human diseases related to proteome disturbance,[5,6] but only very few components of the translation apparatus have been shown to play a role in the pathogenesis of dystonic conditions. A neurodevelopmental disorder with infantile dystonia has been associated with biallelic variants in *SHQ1*, encoding a factor responsible for ribosome formation.[7] In addition, dystonia has been reported to result from pathologies in eukaryotic initiation factor 2α (eIF2α)-mediated processes,[8] known to be involved in neuronal development and survival.[9,10] In the early steps of protein synthesis, eIF2α functions in concert with the eukaryotic initiation factor 4F (eIF4F) complex consisting of eIF4E, eIF4G, and eIF4A, initiating or inhibiting the scanning of mRNAs.[11] Studies in animal models have demonstrated eIF2α perturbation in relation to mutations of the dystonia-linked genes *TOR1A*[8,12] and *THAP1*.[13] Furthermore, two upstream regulators of eIF2α have been implicated in hereditary dystonia: biallelic variants in *PRKRA* cause dystonia 16 (MIM: 612067),[14] whereas dominant *EIF2AK2* variants underlie dystonia 33 (MIM: 619687)[15]; both diseases are thought to arise as a consequence of translation-inhibition impairments.[15] Interestingly, the original descriptions of *EIF2AK2*-related disease were of individuals with neurodevelopmental phenotypes characterized by milestone delay and cognitive dysfunction,[16] followed by discovery of *EIF2AK2* variants in patients with dystonia.[15,17] Recently, variants in another component of the protein synthetic pathway, eukaryotic translation initiation factor 4A isoform 2 (eIF4A2, encoded by *EIF4A2*), were found to lead to neurodevelopmental disorders with developmental delay, intellectual disability, and epilepsy.[18] Especially de novo missense and deletion mutations (besides biallelic variants in two recessive pedigrees) were described, many of which were demonstrated to induce heterozygous loss of eIF4A2 function.[18] To date, no movement disorders have been reported with variants in *EIF4A2*. In this study, we mined large-scale genomic datasets of patients with dystonia[19,20] to identify two independent families with multiple affected individuals who segregated an identical unique frameshift *EIF4A2* variant. We found that the variant reduced eIF4A2 protein amounts consistent with haploinsufficiency, resulting in deregulation of translation control. We showed that this effect was likely related to an impaired ability of eIF4A2 to associate with Ccr4-Not, a master regulatory complex involved in microRNA-mediated translational inhibition.[21] Two additional rare *EIF4A2* deletion changes were prioritized from dystonia genetics consortia, suggesting a broader role for *EIF4A2* variants in causing dystonic phenotypes.

Subjects and Methods

Subjects and Molecular Methods

The study cohort used for primary analysis consisted of 1100 unrelated index patients with a diverse range of dystonic phenotypes, including isolated dystonia (59%) and dystonia with other neurological features

and/or extraneurological involvement; detailed demographics and clinical characteristics of the patients' conditions have been described elsewhere.[19,20] All subjects in the cohort had been recruited from movement disorders clinics through the practices of the investigators or by referral for dystonia genetics research from various international collaboration partners. The herein described patient II-4 from family A (A-II-4) and patient III-2 from family B (B-III-2) were part of this primary analysis cohort. Written informed consent had been obtained from the participating individuals or, in the case of children or those with intellectual impairment, from parents or legal representatives. Data collection and molecular studies were conducted in accordance with the standards of respective ethics institutional review boards. Each individual had undergone in-depth phenotypic evaluation with clinical examination, magnetic resonance imaging and routine laboratory studies when available, review of medical records, and assessment of affected family members. As part of an ongoing endeavor to uncover the genetic causes of dystonia, the individuals had received research whole-exome sequencing (WES) in different family-based analysis designs (sequencing of at least one additional affected or unaffected family member in 30%, including patients II-2 and II-5 from family A [A-II-2 and A-II-5] and patient II-2 from family B [B-II-2]). Our local WES protocols using Agilent enrichment kits and Illumina machines for generation of 100-bp paired-end reads have been reported previously.[19,20] Data were annotated and filtered according to established procedures with an in-house bioinformatics pipeline, as described previously.[19,20] Variant filtering included consideration of allele frequencies in population databases, expected impact on protein, gene constraint, pathogenicity predictions, and inheritance. Variants surviving the filtering steps were manually evaluated and prioritized.[22,23] In this study, we chose a prioritization strategy different from our previously applied methods designed for discovery of novel candidate genes,[22-24] combining the following lines of evidence for the variant(s) of interest: (1) protein-altering alteration absent from controls; (2) variant located in a mutation-constrained gene as determined by recommended statistical metrics[25]; (3) variant recurrent among unrelated patients; and (4) variant present in WES data of affected family members only. Pathogenic or likely pathogenic variants in established dystonia-associated genes were ignored. To identify additional putative disease-related variants in the selected candidate gene *EIF4A2*, we queried independent dystonia genomic sequencing datasets acquired in the context of multi-institutional consortia or center-specific research projects (Australian dystonia genomes; Lübeck dystonia exome project, Germany; dystonia exomes/genomes at UCL Great Ormond Street Institute, London, UK; Fondazione Ca' Granda IRCCS, Milan, Italy; and Ken and Ruth Davee Department of Neurology, Chicago, IL, USA); respective cohorts and sequencing initiatives have been described before.[22,24,26] Candidate *EIF4A2* variants were confirmed and tested for cosegregation in all available family members by Sanger sequencing.

Human Cell Culture

Fibroblast lines were established from skin biopsies of a patient with heterozygous *EIF4A2* variant (patient A-II-4), healthy control subjects, as well as a previously reported pediatric patient with mixed neurodevelopmental-neurodegenerative disease and biallelic variants in *EIF4A2*.[18] The sample of the latter individual was included in this study to investigate the molecular effect of eIF4A2 protein loss in a dosage-dependent manner and to assess further a recently proposed correlation[18] between residual eIF4A2 amounts and differences in phenotypic outcomes. Cells were cultured according to established procedures.[23]

Western Blotting

Fibroblast protein extracts were prepared for Western blotting by standard methods.[23] Antibodies were used against the following proteins: eIF4A2 (1:20,000, ab31218; Abcam), eIF4A1 (1:20,000, ab31217; Abcam), IMP1 (1:500, 2852S; Cell Signaling), and DDX6 (1:1000, BLD-674402; BioLegend). All primary antibodies were used according to the manufacturer's instructions. Densitometric analyses were carried out with ImageJ, and statistical comparisons were performed with R; significances were calculated by unpaired 2-tailed *t* tests.

Proximity Ligation Assay

Proximity ligation assays (DUO92008; Sigma-Aldrich) were performed in accordance with the manufacturer's recommendations and by using a modified version of the previously published protocol for studying cellular interactions between eIF4A2 and CNOT1.[27] In short, primary antibody incubations were performed at 4°C overnight with antibodies against the following proteins: eIF4A2 (1:2000, sc-137,148; Santa Cruz) and CNOT1 (1:2000, 14,276-1-AP; Proteintech). Negative control reactions were performed using only one primary antibody on cells of control individuals. Nuclei were stained with DAPI. Cells from three biological replicates of each line were imaged on an Axio Imager Z1 (Zeiss) using an EC Plan-Neofluar 20×/0.50 M27 objective, recording 10–15 images per biological replicate. Images were evaluated using the image analysis software Definiens Developer XD 2 (Definiens AG, Munich, Germany). A specific rule set was defined to automatically detect nuclei, as well as fluorescent spots originating from the proximity ligation assay. The quantified parameter was the average number

of spots per nucleus for each image (n = 35–39 per patient/control individual with an average of 10–15 cells per image), which was compared between patient and control cells using Student *t* test with Bonferroni correction for multiple testing.

Results

Genetics Data

Through integrated analysis of rare variants expected to be damaging to protein function and shared by independent dystonia-affected patients from 1100 exome-sequenced families,[19,20] we filtered out as top disease-causal candidate a single heterozygous 2-bp deletion (c.896_897del) in exon 8 of *EIF4A2* (NM_001967.4) (Fig. 1A,C, Table 1). Consistent with the stringent prioritization scheme that we applied, this frameshifting allele was predicted to lead to a loss-of-function effect either by giving rise to generation of a deleteriously truncated polypeptide (p.Thr299Serfs*7) or, more likely, by triggering nonsense-mediated mRNA decay with no protein production. The variant was unobserved in >140,000 control individuals from gnomAD (version 2.1/version 3.1 releases) and 40,000 in-house control chromosomes, and it affected a gene heavily depleted for loss-of-function variation in the general population (gnomAD probability of being loss-of-function intolerant (pLI) score = 1.0, loss-of-function variant observed vs. expected ratio = 0.04, confidence interval = 0.01–0.2).[25] As shown in Fig. 1A, c.896_897del was identically present in WES data of five dystonia-affected individuals from two separate pedigrees, including three siblings from a

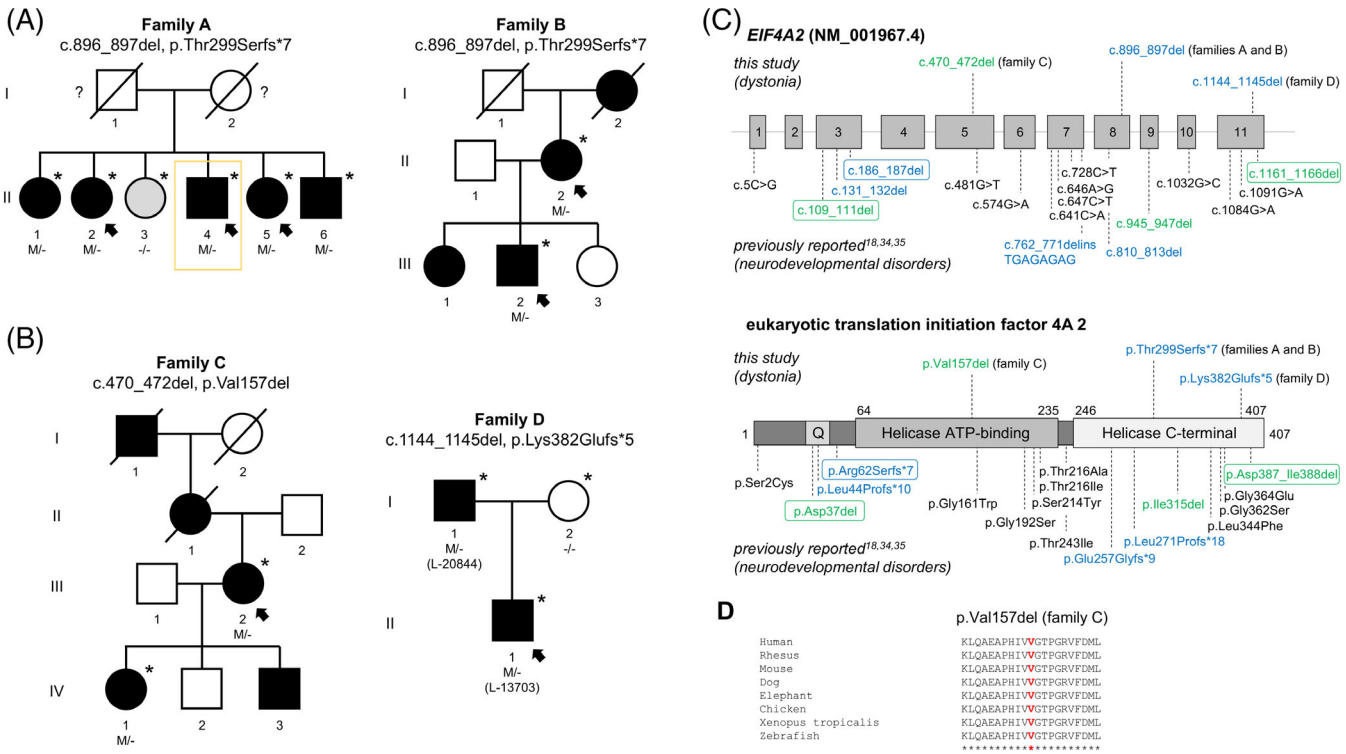

FIG. 1. Family trees and *EIF4A2* variants. (**A** and **B**) Pedigrees of the two unrelated families with the recurrent heterozygous c.896_897del (p.Thr299Serfs*7) frameshifting allele (**A**) and the additionally recruited families with *EIF4A2* deletion alterations (**B**). The dystonia-/tremor-affected individuals are represented by solid black symbols, white symbols indicate healthy family members, and a gray shaded shape in family A indicates a sister of the siblings with dystonia who displayed a clinically different neurological syndrome characterized by progressive dementia and ataxia. Diagonal lines denote deceased subjects. Phenotypic status was unknown for parents in family A (?). The family members marked by asterisks were clinically examined. Arrows point to individuals who were exome sequenced. Subjects with DNA available are labeled according to *EIF4A2* variant status: M/−, heterozygous carrier; −/−, homozygous wild-type. Patient A-II-4 from family A for whom a fibroblast line was obtained and characterized in functional studies is highlighted with an orange box. (**C**) Schematics of the *EIF4A2* gene and the encoded protein's primary structures. Functionally important protein regions are illustrated: the Q motif, the helicase ATP-binding domain, and the helicase C-terminal domain. Locations of herein identified and previously reported[18,28,29] variants are shown at the cDNA (upper panel) and the protein (lower panel) level. The dystonia-associated variants observed in families A–D are plotted above and variants described in patients with neurodevelopmental disease syndromes[18,28,29] below the gene/protein. Variants depicted in blue, green, and black represent loss of function (frameshift), in-frame deletion, and missense changes, respectively. All literature-reported variants were heterozygous de novo mutations, except for three biallelic variants that are marked with boxes; a newly acquired, previously undescribed fibroblast line established from a compound heterozygous carrier of c.186_187del (p.Arg62Serfs*7) and c.1161_1166del (p.Asp387_Ile388del)[18] was analyzed in the functional characterization studies performed as part of this work. (**D**) Multiple alignment of eukaryotic translation initiation factor 4A 2 orthologs showing evolutionary conservation of the amino acid residue deleted by the c.470_472del variant in family C. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 *Clinical characteristics of individuals harboring* EIF4A2 *heterozygous variants identified in this study*

Patient	Gender/ Ethnicity	*EIF4A2* variant	Age at last examination, y	Movement disorders at last examination	Involved areas (distribution)	Age at movement disorder onset, y (site of onset)	Additional neurological features (cognitive dysfunction and/or behavioral problems)	Brain MRI
A-II-4	M/European	c.896_897del (p.Thr299Serfs*7)	67	Dystonia, tremor, jerky movements, dyskinesia	Cranial, cervical, brachial, truncal (generalized)	55 (arms)	Yes	Normal
A-II-2	F/European	c.896_897del (p.Thr299Serfs*7)	70	Dystonia, tremor, jerky movements	Cervical, brachial (segmental)	60 (arms)	Yes (mild)	ND
A-II-1	F/European	c.896_897del (p.Thr299Serfs*7)	72	Dystonia, tremor, jerky movements	Cervical, brachial (segmental)	65 (arms)	Yes	ND
A-II-5	F/European	c.896_897del (p.Thr299Serfs*7)	66	Dystonia, tremor, jerky movements, dyskinesia	Cranial, cervical, brachial (segmental)	45 (arms)	Yes	ND
A-II-6	M/European	c.896_897del (p.Thr299Serfs*7)	59	Dystonia, tremor, jerky movements	Cervical, brachial (segmental)	51 (arms)	Yes (mild)	ND
B-III-2	M/European	c.896_897del (p.Thr299Serfs*7)	35	Dystonia, tremor, jerky movements, dyskinesia	Cranial, cervical, brachial, truncal (generalized)	16 (neck)	Yes (mild)	Normal
B-II-2	F/European	c.896_897del (p.Thr299Serfs*7)	61	Dystonia, tremor	Cervical, brachial (segmental)	13 (neck)	Not reported	ND
C-III-2	F/European	c.470_472del (p.Val157del)	45	Dystonia, tremor, jerky movements	Cranial, cervical, brachial, truncal (generalized)	3 (NK)	Not reported	Normal
C-IV-1	F/European	c.470_472del (p.Val157del)	23	Dystonia, tremor	Cervical, brachial, truncal (generalized)	10 (arms)	No	ND
D-II-1	M/European	c.1144_1145del (p.Lys382Glufs*5)	33	Dystonia, tremor	Cranial, cervical, brachial, truncal (generalized)	6 (arms)	Yes (mild)	Enlarged CSF spaces
D-I-1	M/European	c.1144_1145del (p.Lys382Glufs*5)	59	Tremor	Brachial (focal)	20–25 (arms)	Not reported	ND

Note: Variants are annotated according to genome build GRCh37/hg19 and *EIF4A2* transcript NM_001967.4. None of the variants except for c.1144_1145del (patient D-II-1) are found in gnomAD or in-house control databases (>160,000 control datasets in total); c.1144_1145del is observed in one single gnomAD individual. pLI (probability of being loss-of-function intolerant) for heterozygous *EIF4A2* loss is 1.00 (observed vs. expected ratio = 0.04).
Abbreviations: MRI, magnetic resonance imaging; F, female; M, male; ND, not performed; NK, not known; CSF, cerebrospinal fluid.

Slovak family (family A, patient A-II-4 was part of the primary analysis cohort; see Subjects and Methods) and a mother–son pair of German descent (family B, patient B-III-2 from the primary analysis cohort; see Subjects and Methods). These patients' exomes contained no alternative rare variants considered to be responsible for their dystonic phenotypes. Sanger sequencing in additionally recruited members of family A detected c.896_897del in another two siblings with similar dystonic features, whereas the variant was not found in a sixth sister presenting a clinically distinct condition with progressive dementia and ataxia of suspected neurodegenerative origin (Fig. 1A). Genetic material of two further dystonia-affected relatives in family B was not available for segregation testing. Together, c.896_897del (p.Thr299Serfs*7) fulfilled criteria for classification as a "likely pathogenic" variant according to the American College of Medical Genetics and Genomics standards.[30] Our subsequent search for more *EIF4A2* candidate dystonia-associated variants singled out a heterozygous one-amino acid deletion, c.470_472del (p.Val157del), in a multigenerational pedigree with five affected individuals (family C) (Fig. 1B,C, Table 1); this solo WES-identified variant, absent from all aforementioned control databases, was predicted to disturb a phylogenetically highly conserved residue within the functional helicase ATP binding domain,[18] and cosegregation work demonstrated its presence in an affected offspring of family C's index case (Fig. 1B–D). Moreover, a fourth unrelated patient (family D) was identified who harbored a rare heterozygous frameshift variant, c.1144_1145del (p.Lys382Glufs*5) (Fig. 1B,C, Table 1); c.1144_1145del was located in the last exon of *EIF4A2*, present in a single gnomAD control, and inherited from a tremor-affected father (Fig. 1B,C). The c.470_472del (p.Val157del) and c.1144_1145del (p.Lys382Glufs*5) changes were formally classified as "variants of uncertain significance" according to American College of Medical Genetics and Genomics criteria.[30] Screening of available WES data for families C and D did not identify any other suspicious monogenic variant hits in the context of the observed dystonic presentations.

Clinical Findings

Five siblings in family A had overlapping phenotypes characterized by adult-onset dystonia associated with marked tremor and occasional myoclonic features (Table 1). Subject A-II-4 manifested involuntary tremulous movements of both arms at age 55 years, followed by appearance of constant head deviation, writing difficulties, and jerks with upper-body predominance at around age 60. Examination (age 67) indicated right torticollis, mild dystonic finger posturing, upper-limb postural tremor, irregular jerky movements of the shoulder girdle musculature, and facial dyskinesia. His sister, A-II-2, reported impairments of fine motor skills and abnormal head postures since age 60; at age 70, she displayed jerky action and postural tremor of the hands, involuntary forearm pronation, and tremulous cervical dystonia. All other affected siblings (A-II-1, A-II-5, and A-II-6) developed similar signs of dystonia, tremor, and intermittent myoclonus-like jerks between 45 and 65 years of age; on assessment they had variably expressed combinations of jerky head and/or limb tremor, impaired finger dexterity, dystonia with craniocervical involvement, and perioral dyskinesia. In family B, the son (B-III-2) first noticed involuntary movements of his neck and right shoulder at age 16; over the following years, bibrachial tremor emerged, and symptoms spread to the trunk and face. During follow-up evaluations (age 30–35 years), he showed nonprogressive tremulous cervical dystonia with torti-retrocollis, trunk deviation, postural arm tremor, myoclonic jerks of the left hand, and orofacial abnormal movements. His mother (B-II-2) was diagnosed with adolescence-onset segmental dystonia; she presented with left torticollis and mild dystonic action tremor of both arms. Movement disorder features shared between seven individuals from two families with the c.896_897del (p.Thr299Serfs*7) variant are summarized in Table 1. Further clinical findings for some of these patients included relevant degrees of stable cognitive dysfunction and behavioral comorbidities (depressive-like behavior, anxiety, social withdrawal). The index patient in family C (C-III-2) demonstrated generalized dystonia, with pronounced craniocervical involvement, arm tremor, and intermittent hand jerky movements; her daughter (C-IV-1) experienced mild laterocollis, trunk dystonic movements, and postural tremor of the hands with involuntary finger cramps (Table 1). Finally, family D's patient (D-II-1) had bilateral arm tremor since childhood, followed by manifestation of generalized dystonia in adolescence; his father (D-I-1) presented upper-limb postural and action tremor since the age of 20 to 25 years (Table 1).

Functional Studies

To define the impact of the recurrent c.896_897del variant on eIF4A2, we performed immunoblotting on available fibroblasts from control individuals and family A patient A-II-4. In addition, cells from a published patient with biallelic *EIF4A2* variants,[18] for whom no cellular phenotypes have been described before, were included in the analysis. The abundance of eIF4A2 was reduced to ~50% in patient A-II-4 and to ~10% to 20% in the patient with biallelic variants relative to control individuals (Fig. 2A,B), confirming a variant zygosity-dependent loss of eIF4A2 levels in the mutation carriers. It is well appreciated that the DEAD-box

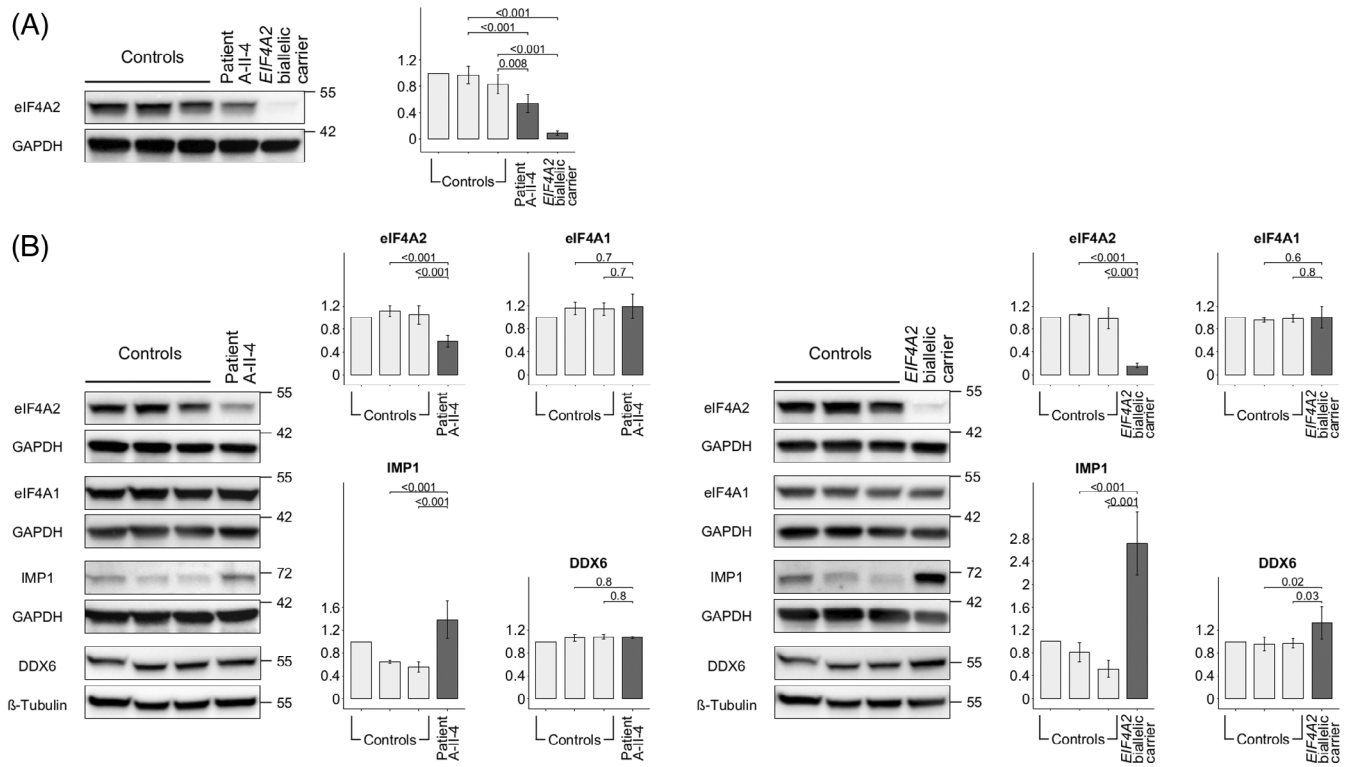

FIG. 2. Western blot analyses of fibroblasts obtained from patients with *EIF4A2* variants and control donors. (**A** and **B**) Primary skin fibroblasts derived from the dystonia-affected individual A-II-4 (family A with heterozygous *EIF4A2* variant c.896_897del), a compound heterozygous carrier of *EIF4A2* c.186_187del/c.1161_1166del[18] (*EIF4A2* biallelic carrier), and three unrelated control subjects were cultured under identical conditions. Total cell lysates were prepared for determination of protein steady-state levels. Western blot assays were performed using antibodies specific for eIF4A2 (**A**) and eIF4A2, eIF4A1, IMP1, as well as DDX6 (**B**). Anti-GAPDH and anti–β-tubulin antibodies were used as a loading reference. Bar charts represent densitometric measurements of eIF4A2, eIF4A1, IMP1, and DDX6 levels (normalized to GAPDH or β-tubulin); bars indicate the means ± standard deviation in lysates of three separate fibroblast cultures. Sizes of detected proteins are shown in kilodaltons, and *P* values are provided for the statistical analyses. An *EIF4A2* variant-specific decrease in eIF4A2 levels (**A** and **B**) and a corresponding, inversely correlated increase in the levels of the downstream translational target IMP1 (**B**) were observed.

RNA helicase eIF4A2, unlike its paralog eIF4A1, exerts dual functions in translational regulation.[21] Besides playing a role in the stimulation of translation initiation via interactions with other eukaryotic initiation factors in the eIF4F complex and eIF2α, eIF4A2 is known as a key effector in microRNA-mediated repression of translation through association with the Ccr4-Not complex.[21] Previous in vitro experiments have demonstrated that artificial knockdown of eIF4A2 critically altered protein levels of Ccr4-Not–related microRNA targets such as IMP1.[27] In light of these findings, we sought to assess whether IMP1 expression was deregulated in the presence of patient *EIF4A2* variants. As shown in Fig. 2B, basal IMP1 concentrations were significantly higher in both mutant fibroblast lines compared with controls, with a clear eIF4A2 protein dosage loss-dependent effect (~40%–50% and ~150%–170% IMP1 expression increase in cells of patient A-II-4 and the patient with biallelic variants, respectively). Remarkably, the abundance of eIF4A1 was not affected by the *EIF4A2* variants, whereas the expression of DDX6, another DEAD-box RNA helicase involved in Ccr4-Not-associated translational inhibition,[27] was upregulated to ~20%–30% only in the cells with biallelic variants (Fig. 2B). This suggested that eIF4A1 and DDX6 were unable to compensate for the heterozygous loss of eIF4A2 in patient A-II-4. Collectively, these studies established that c.896_897del induced *EIF4A2* haploinsufficiency, and that the variant was associated with alterations of translational control suggestive of Ccr4-Not complex dysfunction. To validate a potential effect of the *EIF4A2* variants on eIF4A2-Ccr4-Not interactions, we performed proximity ligation assays[27] in patient and control fibroblasts. Again, we observed a correlation between the extent of eIF4A2 loss and cellular outcomes: compared with control cells, mutants harboring c.896_897del exhibited an ~35%–45% decrease (adjusted $P < 0.001$ for all comparisons) in colocalization of eIF4A2 and the Ccr4-Not component CNOT1, whereas this colocalization was almost completely lost in the patient line with biallelic variants (Fig. 3A,B). These experiments implied that because of the reduced eIF4A2 protein levels, the functionally important association with Ccr4-Not was impaired in patient A-II-4, although less significantly than in the pediatric case with recessive disease.

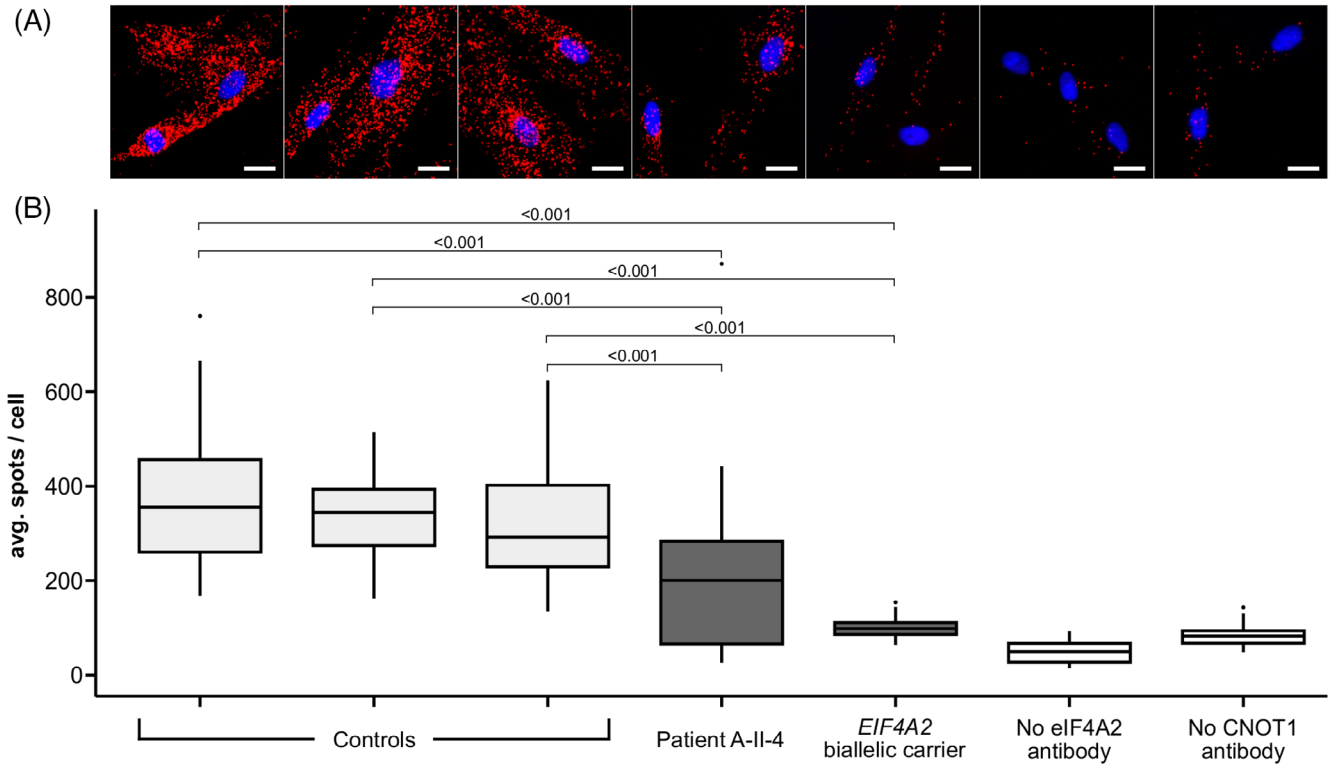

FIG. 3. Proximity ligation assay (PLA) on patient and control fibroblasts. (**A**) Epifluorescence microscopy images showing the association of eIF4A2 with the Ccr4-Not complex factor CNOT1 (from left to right): PLA reaction (red) in skin-derived primary fibroblasts from three unrelated control subjects, the dystonia-affected individual A-II-4 (family A with heterozygous *EIF4A2* variant c.896_897del), a compound heterozygous carrier of *EIF4A2* c.186_187del/c.1161_1166del (*EIF4A2* biallelic carrier),[18] and negative controls for each antibody on control subjects. Nuclei were stained with DAPI (blue). Images are representative of three independent experiments. Scale bars, 25 μm. (**B**) Quantification of PLA reaction dots, as assessed in three biological replicates. Raw data were analyzed using the Definiens Developer XD 2 software (Definiens AG, Munich, Germany); boxplots represent the median, first quartile, and third quartile, and whiskers extend to a maximum of 1.5× interquartile range. Statistically significant differences are indicated (unpaired 2-tailed *t* tests). The number of PLA dots was substantially diminished in cells bearing *EIF4A2* variants, indicating reduced but still functional interactions between eIF4A2 and the Ccr4-Not complex. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

By molecular and clinical characterization of individuals with heterozygous *EIF4A2* variants, we provide evidence for a previously unrecognized monogenic movement disorder. Our findings substantially broaden the clinical spectrum of *EIF4A2*-associated neurodevelopmental disorders to include dystonia-predominant manifestations, similar to observations in *EIF2AK2*-related disease, another condition linked to the protein translation machinery, which is characterized by presentations of both intellectual developmental syndromes[16] and isolated dystonia.[15,17] Our patients' phenotypes comprised dystonic features of variable severity, tremor, and jerky movements resembling myoclonus. The conditions bore some distinct similarities to presentations related to variants in *ANO3* (dystonia 24; MIM: 615034) and *KCTD17* (dystonia 26; MIM: 616398), with onset in adulthood or adolescence and leading involvement of the upper body (craniocervical region, arms).[31,32] The observed distribution of dystonia may help to distinguish patients with *EIF4A2* variants from those with variants in other recurrently mutated genes for dystonia, such as *TOR1A* and *KMT2B*, where prominent leg involvement is often seen.[33,34] The type of spreading of movement disorder features among patients from our four different families was variable, but initial manifestation in the upper extremities with secondary affection of neck and facial muscles was frequently noted (for details, see Table 1). Some patients also displayed nonprogressive cognitive impairments and behavioral/neuropsychiatric disturbances that might be regarded as signs of *EIF4A2*-associated developmental dysfunction[18]; there were, however, no reports of milestone delays or epileptic comorbidities, although we could not precisely assess early neurodevelopment because of advanced age of all subjects. In affected individuals of family A, the reported age at movement disorder onset was considerably later than in patients of family B (late adulthood vs. adolescence), although both families segregated the exact same *EIF4A2* variant. This difference might be explained by phenotypic heterogeneity related to modifying genetic, epigenetic, and/or environmental factors,

as commonly recognized in rare and more prevalent neurogenetic disease conditions.[35] In contrast, most affected members of family A did not actively seek medical attention for many decades in their life, whereas others never visited a neurologist before family-based movement disorder assessment as part of this study; therefore, we cannot exclude that milder undiagnosed dystonic and/or tremulous signs may have pre-existed during adolescence/younger adulthood in some of these individuals. Difficulties with cognition and abnormalities in behavior were more pronounced in some older persons from family A, an observation that could be associated with either incidental clinical variation or family-specific disease progression over a lifetime.

For the frameshift variant c.896_897del (p.Thr299Serfs*7), we offer strong arguments for causal implication in the observed phenotypes, including demonstration of its rarity, segregation with disease in nonrelated pedigrees, and effect on protein and the downstream biological pathway. First, we demonstrated that c.896_897del led to ~50% reduction of eIF4A2 protein amounts, indicative of degradation of the mutant transcript and/or the truncated polypeptide. *EIF4A2* haploinsufficiency has to be considered disease causing given that (1) several recently reported neurodevelopmental disorder–associated *EIF4A2* variants, including missense and frameshift alterations, were shown to represent dominant loss of eIF4A2 function mutations[18]; (2) *EIF4A2* heterozygous predicted loss-of-function variants exhibit significant enrichment in de novo variation catalogs derived from large neurodevelopmental disease cohorts[28,29] (Fig. 1C); and (3) loss of one *EIF4A2* copy is not tolerated among population controls.[25] Second, our studies in patient-derived cells uncovered a specific role for *EIF4A2* variants in producing perturbance of translational regulation, demonstrating an increased expression of the Ccr4-Not complex target IMP1 in association to c.896_897del; this finding strikingly recapitulated published in vitro observations from eIF4A2 knockdown systems.[27] In vivo work has established that *EIF4A2* variants identified in neurodevelopmental disorder cases compromised neuromotor function and morphological development,[18] but the underlying molecular mechanisms have not been examined. Our results thus represent the first evidence that impaired Ccr4-Not–dependent microRNA pathway function, as well as defects of protein-synthesis repression, may be primary contributors to *EIF4A2*-related phenotypes. This is supported by observations from our colocalization assays, indicating diminished direct interactions between eIF4A2 and Ccr4-Not. We further excluded compensatory upregulation of eIF4A2′s paralog eIF4A1 in c.896_897del-bearing fibroblasts, consistent with their nonredundant functions in translational control.[21] Third, we analyzed molecular correlations between heterozygous and biallelic loss-of-function effects in patient cells, generating experimental support for the recent proposition that phenotype severity in *EIF4A2*-associated disease may be determined by residual eIF4A2 functional dosage.[18] Our phenotypic and functional data align with the concept of severe encephalopathic recessive disease in biallelic *EIF4A2* mutation carriers and milder, more variable expressions with a strong neurodevelopment component, now also encompassing movement disorders, in heterozygous carrier individuals.[18]

For the additional herein identified variants, c.470_472del (p.Val157del) and c.1144_1145del (p.Lys382Glufs*5), patient-derived fibroblasts were unobtainable for functional analyses. We highlight, though, that c.470_472del was located in a domain where pathogenic *EIF4A2* variants have previously been documented, and that deletions of single, highly conserved amino acids are part of the genotypic spectrum of *EIF4A2*-related conditions.[18] Further studies are required to firmly establish their pathogenicity, as are studies that help to understand the mechanisms contributing to the wide range of phenotypic expressions in disorders resulting from translational dysfunction, which appears also to include nonmanifestation in heterozygous parents from recessive families.[18]

A growing number of human disease genes, including genes implicated in movement disorders, have now been associated with both dominant and recessive inheritance patterns.[36,37] Our present study adds *EIF4A2* as another movement disorder–related gene to this catalog, which may be important to consider during clinical management and counseling of affected families. Even in heterozygous carriers of *EIF4A2* loss-of-function variants, the penetrance of movement disorder manifestations may be high, as demonstrated by the identification of the herein described pedigrees. However, it is also possible that the apparently complete penetrance in our families reflects an ascertainment bias, and it should be taken into account that genetic alterations linked to highly penetrant disease traits in patient families can have much lower effect sizes in the general population.[38] How heterozygous *EIF4A2* variants lead to predominant movement disorders on the one hand and neurodevelopmental syndromes on the other remains unknown, although this breadth of clinical variability is recognized for many developmentally important genes,[39] including the functionally related *EIF2AK2* locus.[15] It could be that there are specific phenotype-determining molecular effects of the individual variants that have yet to be identified. Another hypothesis might be that there is more generally a phenotypical continuum ranging from early neurodevelopmental features to later-onset dystonia, tremor, and other movement abnormalities, occurring in relation to similar or identical mutational

mechanisms, in which genotype–phenotype correlations are defined by modulation through environment, background (epi)genetic variation, or stochastic factors.[39,40] Identifying these mechanisms underlying variable expressivity for different developmental gene-related neurological diseases should be a priority of future research. ●

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Data Availability Statement

Data available on request from the authors.

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