Gemma Carvill ORCID iD: 0000-0003-4945-3628

Katherine Helbig ORCID iD: 0000-0001-8249-0549

Damaging *de novo* missense variants in *EEF1A2* lead to a developmental and degenerative epileptic-dyskinetic encephalopathy

Gemma L. Carvill*[1], Katherine L. Helbig*[2,3], Candace T. Myers[4], Marcello Scala[5,6], Robert Huether[7], Sara Lewis[8,9], Tyler N. Kruer[8,9], Brandon S. Guida[8,9], Somayeh Bakhtiari[8,9], Joy Sebe[10,11], Sha Tang[7], Heather Stickney[11], Sehribani Ulusoy Oktay[10,11], Ashwin A. Bhandiwad[11], Keri Ramsey[12], Vinodh Narayanan[12], C4RCD Research Group[12], Timothy Feyma[13], Luis O. Rohena[14], Andrea Accogli[6,15,], Mariasavina Severino[5], Georgina Hollingsworth[16], Deepak Gill[17], Christel Depienne[18] Caroline Nava[18], Lynette G Sadleir[19], Paul A. Caruso[20], Angela E. Lin[21], Floor E. Jansen[22], Bobby Koeleman[22], Eva Brilstra[23], Marjolein H. Willemsen[24], Tjitske Kleefstra[24], Joaquim Sa[25], Marie-Laure Mathieu[26], Laurine Perrin[27], Gaetan Lesca[28], Pasquale Striano[5,6], Giorgio Casari[5,6], Ingrid E. Scheffer[16], David Raible[10,11], Evelyn Sattlegger[29], Valeria Capra[#5], Sergio Padilla-Lopez[#8,9], Heather C. Mefford[#4], Michael C. Kruer[#8,9*]

* Denotes shared first author

# Indicates joint senior authors

This is the author manuscript accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.24015.

Correspondence may be addressed to Heather C. Mefford hmefford@uw.edu or

Michael C Kruer kruerm@email.arizona.edu

Affiliations

1 Ken and Ruth Davee Department of Neurology, Northwestern University, Chicago, IL, USA

2 Division of Neurology, Children's Hospital of Philadelphia, Philadelphia, PA, USA

3 The Epilepsy NeuroGenetics Initiative, Children's Hospital of Philadelphia, Philadelphia, PA, USA

4 Division of Genetic Medicine, University of Washington, Seattle, WA, USA

5 IRCCS Istituto Giannina Gaslini, Via Gerolamo Gaslini, Genoa, Italy

6 DINOGMI Università degli Studi di Genova, Genoa, Italy

7 Ambry Genetics, Aliso Viejo, CA, USA

8 Barrow Neurological Institute, Phoenix Children's Hospital, Phoenix, AZ, USA

9 Departments of Child Health, Cellular & Molecular Medicine, and Neurology and Program in Genetics,

University of Arizona College of Medicine Phoenix, Phoenix, AZ, USA

10 Department of Biology, University of Washington, Seattle, WA, USA

11 Department of Biological Structure, University of Washington, Seattle, WA, USA

12 Translational Genomics Research Institute, Phoenix, AZ, USA

13 Gillette Children's Specialty Healthcare, St. Paul, MN, USA

14 Department of Pediatrics, Division of Genetics, San Antonio Military Medical Center, San Antonio, TX, USA and Department of Pediatrics, Long School of Medicine, University of Texas, San Antonio, TX, USA

15 IRCCS Ospedale Policlinico San Martino, Genoa, Italy

16 Departments of Medicine and Paediatrics, University of Melbourne and Austin Health Royal Children's Hospital, Melbourne, Australia

17 Ty Nelson Department of Neurology, The Children's Hospital at Westmead, Sydney, NSW, Australia

18 INSERM UMR 975, Institut du Cerveau et de la Moelle Epinière, Hôpital Pitié-Salpêtrière, Paris, France

19 Department of Paediatrics and Child Health, University of Otago Wellington, Wellington South, New Zealand

20 Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

21 Medical Genetics, Department of Pediatrics, MassGeneral Hospital for Children, Harvard Medical School, Boston, MA, USA

22 Department of Pediatric Neurology, University Medical Center, Utrecht, the Netherlands

23 Department of Genetics, Utrecht University, The Netherlands

24 Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands

25 Serviço de Genética Médica, Centro Hospitalar e Universitário de Coimbra, Portugal

26 Neuropaediatrics Department, Femme Mère Enfant Hospital, Lyon, France and Claude Bernard Lyon 1 University, Lyon, France

27 Department of Paediatric Physical Medicine and Rehabilitation, CHU Saint-Etienne, Hôpital Bellevue, France

28 CRNL Inserm U1028 - CNRS UMR5292 – Claude Bernard University Lyon 1, Lyon, France and Department of Medical Genetics, Lyon University Hospital, Lyon, France

29 School of Natural & Computational Sciences, Massey University, Auckland, New Zealand

Funding information

Portions of this work were supported by a Phoenix Children's Hospital RAC award (SPL), by a Doris Duke Clinical Scientist Development Award (MCK), and by NIH NINDS 1R01NS106298 (MCK). We thank Joseph A. Sisneros for his contributions. GC, MS and VC are members of the Telethon Undiagnosed Diseases Program (TUDP) consortium. GLC is supported by NIH NINDS R00 NS089858. HCM is supported by NIH NINDS R01NS069605. IES is supported by NHMRC of Australia Program grant and Practitioner Fellowship. LGS is supported by the Health Research Council of New Zealand and Cure Kids New Zealand.

ABSTRACT (n=155 words)

Heterozygous *de novo* variants in the eukaryotic elongation factor *EEF1A2* have previously been described in association with intellectual disability and epilepsy but never functionally validated. Here we report 14 new individuals with heterozygous *EEF1A2* variants. We functionally validate multiple variants as protein-damaging using heterologous expression and complementation analysis. Our findings allow us to confirm multiple variants as pathogenic and broaden the phenotypic spectrum to include dystonia/choreoathetosis, and in some cases a degenerative course with

cerebral and cerebellar atrophy. Pathogenic variants appear to act via a haploinsufficiency mechanism, disrupting both the protein synthesis and integrated stress response functions of EEF1A2. Our studies provide evidence that *EEF1A2* is highly intolerant to variation and that *de novo* pathogenic variants lead to an epileptic-dyskinetic encephalopathy with both neurodevelopmental and neurodegenerative features. Developmental features may be driven by impaired synaptic protein synthesis during early brain development while progressive symptoms may be linked to an impaired ability to handle cytotoxic stressors.

Keywords: epilepsy, EEF1A2, de novo, dyskinesia, yeast complementation assay

INTRODUCTION

Heterozygous *de novo* sequence variants in the eukaryotic elongation factor *EEF1A2* have been described in patients with neurodevelopmental disorders, yet such variants have yet to undergo functional genetic validation. Originally, a single patient with intellectual disability (ID) (de Ligt et al., 2012) and then a second with developmental and epileptic encephalopathy (DEE) (Veeramah et al., 2013) carrying *EEF1A2 de novo* variants were identified by cohort-based whole exome sequencing studies. Since then, a series of case reports have described fifteen additional patients with epilepsy and intellectual disability and *de novo* variants in *EEF1A2* (Inui et al., 2016; Lam et al., 2016; Lopes et al., 2016; Nakajima et al., 2015; O'Roak et al., 2014; Ostrander et al., 2018). We followed up these findings with in-depth phenotyping and validation studies. Here we

describe fourteen new patients with *de novo EEF1A2* missense variants, confirm many as pathogenic variants disrupting protein function, and extend the phenotypic spectrum of *EEF1A2*-associated disease in a total of 29 individuals.

The eukaryotic elongation factor 1 alpha family consists of two members, α1 (*EEF1A1*) and α2 (*EEF1A2*). These translation elongation factors deliver amino acyl-tRNAs to the ribosome, growing the nascent polypeptide chain during the elongation phase of protein synthesis. The two EEF1A paralogs are highly homologous (>95% sequence identity; Supp. Figure S1) and have similar protein domains, including a common catalytic guanine nucleotide triphosphate (GTP) hydrolysis domain, structural topologies (domains I, II, III) and two catalytic motifs (switch I/II), which form the catalytic pocket for GTP and cation (Mg^{++}) binding, respectively (Figure 1) (Crepin et al., 2014). The EEF1A proteins are conserved throughout the eukaryotic kingdom. *Saccharomyces cerevisiae* has two genes that code for forms of EEF1A, *TEF1* and *TEF2*, thought to arise as a result of an ancestral duplication event. TEF1 and TEF2 are 80% identical to the human EEF1A2 protein (Supp. Figure S1).

Despite their similarity, *EEF1A1* and *EEF1A2* are differentially regulated and expressed. While *EEF1A1* is ubiquitously expressed, *EEF1A2* expression is predominantly found in brain, muscle and heart in humans, rats and mice (Ann, Lin, Lee, Tu, & Wang, 1992; Knudsen, Frydenberg, Clark, & Leffers, 1993; Newbery et al., 2007; Pan, Ruest, Xu, & Wang, 2004). In zebrafish, expression is limited to the brain (Supp. Figure S2). eEF1A2

knockout mice display early onset neurodegeneration and muscle wasting(Chambers, Peters, & Abbott, 1998), as well as spontaneous seizures and sudden death in another null mouse model(Davies et al., 2017). In contrast heterozygous mice are normal (Chambers et al., 1998; Davies et al., 2017).

Canonically, EEF1A proteins function as integral components of the eukaryotic protein synthesis machinery. EEF1A is part of the EEF1 elongation complex, which is made up of α and β subunits. EEF1A contributes the α portion, complemented by EEF1$\beta\alpha$, EEF1$\beta\beta$ and EEF1$\beta\gamma$ in a 2:1:1:1 ratio (Sasikumar, Perez, & Kinzy, 2012). Some studies have suggested that EEF1A self-dimerization may be important for EEF1 complex function (Bunai, Ando, Ueno, & Numata, 2006). In addition, different faces of the EEF1A protein complex with other proteins, including CDKN2, actin and the guanine exchange factor EEF1G (Gross & Kinzy, 2006; Lee et al., 2013; Vanwetswinkel et al., 2003). Several of these interactions involve differential phosphorylation of Ser/Thr and Tyr residues (Soares, Barlow, Newbery, Porteous, & Abbott, 2009).

In addition to its canonical function, EEF1A has numerous non-canonical functions in normal physiological states including a role in actin bundling (Perez & Kinzy, 2014) and as a facilitator of nuclear-cytoplasmic trafficking of tRNAs during growth states(Mingot, Vega, Cano, Portillo, & Nieto, 2013). Conversely, EEF1A also plays an important role when normal physiology is disrupted by various forms of cytotoxic stressors. For instance, EEF1A co-ordinates the integrated stress response by preventing premature

activation of the GCN2 kinase (Visweswaraiah et al., 2011) and activates *HSP70* transcription in response to heat shock (Vera et al., 2014). Moreover, EEF1A can recognize damaged or misfolded proteins and possesses chaperone activity (Lukash, Turkivska, Negrutskii, & El'skaya, 2004), or, alternatively, can facilitate delivery to the proteasome (Chuang et al., 2004).

All patients with heterozygous *de novo EEF1A2* variants described thus far have exhibited epilepsy and/or intellectual disability (Inui et al., 2016; Lopes et al., 2016; Nakajima et al., 2015; O'Roak et al., 2014). Functional studies evaluating how *EEF1A2* variants might impair protein and ultimately brain function have not been systematically performed. In an effort to bridge this gap in current knowledge we used heterologous expression and functional complementation in yeast, to assess a number of *EEF1A2* variants, and confirmed that these variants likely impair protein function. Our clinical data show that pathogenic variants in *EEF1A2* lead to a wider phenotypic spectrum than previously appreciated, with fully manifesting individuals exhibiting a developmental and epileptic encephalopathy with dystonia-choreoathetosis and neurodegeneration in some cases.

METHODS

Whole exome and targeted resequencing

Individuals with predicted pathogenic *EEF1A2* variants were identified through clinical whole exome sequencing (n=8; Ambry Genetics, Aliso Viejo, CA, USA) (Farwell et al., 2015), through research targeted resequencing using molecular inversion probes (MIPs) (n=3)(Carvill et al., 2013), research whole exome sequencing (n=1), or by panel-based diagnostic next generation sequencing (n=2). Patients were diagnosed with developmental and epileptic encephalopathies (DEEs) as defined by ILAE classification criteria (Scheffer et al., 2017), intellectual disability, and/or dyskinetic movement disorders as described previously (Madeo et al., 2016). We focused on variants that were non-synonymous or altered acceptor/donor splice sites and were not present in the ExAC or gnomAD datasets for further analysis and classified variants according to ACMG criteria (Richards et al., 2015).

Computational modeling

The structural consequences of selected *EEF1A2* pathogenic variants were investigated using computational modeling. The structure of the GDP-bound form of EEF1A2 (PDB:4C0S), GDP-bound form of EEF1A1 (PDB: 1IJF) and the GTP-bound form of EEF1A1 (PDB:3WXM) were used for analysis (Crepin et al., 2014). Since most residues were identical between EEF1A1 and EEF1A2, we did not map EEF1A2 sequences to the

EEF1A1 structures. Alignment between Domain I of GDP-bound EEF1A2 (amino acid 4-236) to EEF1A with GTP and aPelota (amino acid 4-224) and domain I of GDP-bound EEF1A2 (amino acid 4-236) to EEF1A1 with GDP (amino acid 4-236) was performed with Pymol's align feature (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). Instability calculations were performed using the default settings in FoldX with its empirical forcefield (Schymkowitz et al., 2005). Breifly each structure was repaired and then each amino acid change was evaluated for its energy change. Phosphorylation predictions were performed using NetPhos 3.1 Server (Blom, Gammeltoft, & Brunak, 1999).

Complementation assays

The BY4742 wild type (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) yeast strain was purchased from TransOMIC (Huntsville, AL, USA). The *NATMX4* system was used to delete *TEF2* in BY4742 by homologous recombination (Goldstein & McCusker, 1999). The *NATMX4* cassette was obtained by PCR amplification from plasmid p4339 (Goldstein & McCusker, 1999) using primers containing 50-55 bp of the sequence upstream and downstream of the *TEF2* open reading frame. Deletions were tested for resistance to Noursethricin (clonNAT) and confirmed by PCR. Strain MC214 (*MAT* α *ura3-52 leu2-3,112 trp1-*Δ*1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2*Δ *pTEF2 TRP1*) was kindly provided by Dr. Terri Goss Kinzy.

Yeast strains were grown as indicated in YPD medium (1% yeast extract, 2% peptone, 2% D-glucose) or synthetic complete (SC) medium (6.7 g/liter yeast nitrogen base, 2% glucose) supplemented appropriately. When indicated, rapamycin was added (40 ng/ml). For plasmid shuffling experiments, FAA medium containing 5-fluoroanthranilic acid at 0.5 g/l was used as previously described (Toyn, Gunyuzlu, White, Thompson, & Hollis, 2000). *EEF1A2* and V5 N-terminal *TEF2* cDNA sequences and eight V5-tagged variants identified in patients were synthesized (Genscript Inc.), confirmed by direct sequencing and introduced into the p416GPD-URA3(Mumberg, Muller, & Funk, 1995) vector using BamHI and EcoRI restriction sites, allowing us to express them under the strong promoter GPD. The obtained plasmids were then used to transform yeast using the lithium acetate method (Ito, Fukuda, Murata, & Kimura, 1983).

To analyze the expression of the different EEF1A2/TEF2 variants, protein extracts were obtained from 1.0 units of OD_{660}. Briefly, cells were lysed by alkaline extraction (3.5% β-mercaptoethanol in 2M NaoH) and precipitated with trichloroacetic acid as described previously (Camougrand, Kissova, Salin, & Devenish, 2009). Protein extracts were analyzed by SDS-PAGE and western blot using appropriate primary antibodies and a secondary antibody conjugated to HRP. The primary antibodies used in these experiments were the following: anti-V5 (1:5000, Invitrogen cat no R960) and anti-Pgk1 (1:10000, Molecular Probes cat no 459250).

RESULTS

Whole exome and targeted resequencing

We identified sequence variants in *EEF1A2* by either targeted or whole exome sequencing in fourteen patients with epilepsy and/or a dyskinetic movement disorder (dystonia/choreoathetosis) (Table 1 and Figure 1). Overall, we detected nine unique missense variants, three of which were recurrent, including the p.G70S (n=2), p.E122K (n=3) and p.R266W (n=3). These variants arose *de novo* in 13/14 patients, while maternal DNA was unavailable for patient 6 with the recurrent p.E122K variant. All 14 *EEF1A2* missense variants occur at highly conserved residues and are predicted to be deleterious by the *in silico* prediction score, CADD (Table 1), and were absent in population-based databases (gnomAD and TOPMED). In accordance with the ACMG criteria these variants were classified as likely pathogenic or pathogenic (Table 1). We also identified a variant of uncertain significance (VUS) p.M102V in an individual with a milder presentation including ID and epilepsy (Supp. note). This variant was inherited from her unaffected father, was not seen in gnomAD and has a CADD score of 14.

Clinical characteristics

We describe the clinical features of the fourteen newly identified patients and a clinical summary of the fifteen previously reported individuals with *de novo* pathogenic variants in *EEF1A2* (Table 1, Supp. Table S1). All 29 patients presented with significant global

developmental delay identified identified in infancy, ultimately manifesting as moderate to profound intellectual disability in the majority of individuals. Initial fine and gross motor development was only clearly normal in 8/14 (57%) individuals, but these individuals did not differ from those with early delays in terms of cognitive outcomes, as most still had moderate-profound ID and were non-verbal. In an additional previously reported individual with a *de novo* c.370G>A (p.E124K) variant, development was reportedly normal, but then the patient went on to develop significant impairment in receptive language (Lam et al., 2016). This patient's cognitive outcome was milder than the majority of other patients, as she was able to speak in sentences. All patients demonstrate expressive language impairment, and most patients are nonverbal (25/29), although a number of patients could communicate basic needs through signs or pictures. Of the 11/28 (39%) patients who achieved walking, the median age was four years (range 2-7y); though at least half of these individuals required assistance or had an ataxic gait. The remaining patients were non-ambulatory. Developmental regression was seen in a subset of patients (6/19; 32%), coinciding with seizure onset in one patient. Two patients followed a neurodegenerative course and died by age four years. No postmortem tissue was available for analysis.

Among the patients for whom information regarding muscle tone was available, almost all had significant truncal or generalized hypotonia (23/25; 92%). Neonatal hypotonia was noted in multiple patients. Movement disorders were observed in the majority of patients (10/17, 59%) and most commonly consisted of choreoathetosis and/or dystonia

(Supp. Videos S1-S3) although ataxia was described in some patients. Behavioral features were observed in a number of patients (7/19; 37%) and included autistic features, aggression, bruxism, and self-injurious behaviors. In many patients, autistic features were severe, and included not only poor social responsiveness but a generalized hypo-responsiveness to the environment.

Epilepsy was present in all but two patients (26/28; 93%). Median age of seizure onset was four months (range: first day of life to 8 years). Additional data about seizure types were available for 20 patients. Myoclonic seizures were the most common presenting seizure type. Most patients developed additional seizure types, including generalized tonic-clonic, absence, myoclonic, tonic, and epileptic spasms. Seizures were refractory to multiple treatments in a majority of patients and no consistently effective anticonvulsant was identified across patients. However, patients 8 and 10 achieved seizure-freedom on levetiracetam monotherapy, seizures in patients 4 and 12 were reduced or controlled with clobazam, and patients 6, 12 and 14 experienced a reduction or cessation of seizures with valproate. Common EEG features included multifocal discharges and generalized spike- and polyspike-wave, with activation during sleep detected in several patients. A burst-suppression pattern was observed in three individuals.

Neuroimaging data showed variable features, with early life brain MRIs normal for age or exhibiting delayed myelination and/or thin corpus callosum. However, the available

longitudinal neuroimaging showed cortical and/or cerebellar atrophy in six patients that was severe in several cases (Figure 2). Most cases that exhibited developmental regression had evident cortical/cerebellar atrophy if serial MRIs were performed, although in some cases the degree of neurodevelopmental disability present at baseline made it difficult to know if symptoms had progressed.

Although birth parameters were normal for most patients, one patient was noted to have intrauterine growth restriction. Four patients had failure to thrive. Acquired microcephaly was also noted in four cases. Non-specific dysmorphic facial features were noted in just over half the patients (13/23 – 57%), but a consistent facial gestalt was not appreciated. Dysphagia, often necessitating gastrostomy placement and fundoplication, was reported in 7 patients. Notably, three patients had significant respiratory dysfunction, including respiratory failure requiring mechanical ventilation in two patients. Two of these patients were also noted to have episodes of hyperpnea alternating with apnea (Lopes et al., 2016).

Structural modeling of variants

We sought to structurally interpret the impact of the variants through EEF1A2 3D protein and its high homology paralog EEF1A1. Mapping the observed variants on the structure of the GDP-bound EEF1A2 protein showed a visual trend to cluster in the switch I or switch II regions (Figure 1B) allowing us to group variants by protein domain.

The recurrent p.G70S variant is the only residue located in the switch I loop. It is positioned towards the EEF1B/tRNA pocket formed by domains I/II/III (Figure 1B). In the structure of EEF1A1, the catalytically competent form containing bound GTP (pdb: 3WXM), this position is redirected towards the bound GTP and Mg^{++} (Supp. Figure S3). The two structures show a transition of p.G70 after the hydrolysis of GTP to GDP. The Gly is a small apolar amino acid and provides confirmational flexability to the loop. The Ser mutation is a larger polar amino acid that could be phosphoralyted (Supp. results) and decreases structural flexability within the catalytic loop. The variant is anticipated alter the position of the adjacent residue Thr71 which coordinates the binding of Mg^{++} during the hydrolytic cycle. Five of the observed variants are found within or adjacent to the switch II motif. Switch II participates in GTP hydrolysis and GDP dissociation through interactions with switch I and GEF binding (Crepin et al., 2014). The variants p.F98C and p.M102V are buried within the switch II helix and three other observed alterations (p.D91N, p.G384R and p.R423C) are directaly adjacent (Supp. Figure S4). Mutations observed at switch II are anticipated to result in disruption of the helix either through direct instability or through secondary contacts (Supp. results). Together these alterations in the switch II region indicate that disruption of switch II stabilization and dissociation of GDP could be a major mechanism contributing to EEF1A2-associated disease. Residues p.A125E and p.E122K are not directly in the switch I or II region but instead are adjacent to the nucleotide binding pocket (Supp. Figure S5). Both positions are surface exposed and just outside the binding pocket interacting with the nuclotide

through secondary contacts. This implys they might impact another function such as interactions with GEF binding (Crepin et al., 2014) rather than nucleotide binding. The only recurrent variant not in switch I/II or adjacent to the nucleotide binding site is p.R266W. This variant occurs in domain II directed towards the GEF/tRNA binding space in the GDP-bound form (Supp. Figure S4 and Results). Its presence at the interface and change in a physiochemical change in the amino acid, we anticipate p.R266W in EEF1A2 would thus alter heterodimer formation at this interface.

Functional Complementation

We then turned to a series of complementation assays to investigate the functional consequences of potential *EEF1A2* mutations. These included pathogenic and likely pathogenic variants newly-identified in our cohort, the variant of uncertain significance (VUS) (p,M102V) and a previously reported hypomorphic variant (p.P333L)(Cao et al., 2017). We began by introducing *EEF1A2* variants identified in patients into *TEF1*, the yeast counterpart of *EEF1A2*, and determining whether any of the patient-associated variants led to decreased protein stability and/or degradation. Variant steady-state protein abundance was slightly diminished for some variants compared to wild-type (Figure 3A).

Effects on protein synthesis We asked whether *EEF1A2* pathogenic variants impair canonical protein synthesis as measured by growth in nutrient-replete conditions. Double knockout of both the *TEF1* and *TEF2* genes leads to global impairment of protein

synthesis (Cottrelle et al., 1985) and therefore is lethal in yeast. MC214 is a double *tef1Δtef2Δ* strain unable to synthesize tryptophan. The strain is kept alive by exogenous expression of the *TEF1* gene from a plasmid that also carries the selectable auxotrophic marker *TRP1*. *TRP1*, in turn, restores tryptophan synthesis.

To test whether *EEF1A2* variants impair protein synthesis, we used a plasmid shuffling approach in the MC214 strain. We introduced *EEF1A2* variants identified in patients into a second *TEF1-URA3* plasmid, transformed MC214 cells with this plasmid**,** and then selected for variants using fluoroanthracilic acid (FAA). FAA is an analogue of anthracilic acid, a precursor for tryptophan biosynthesis, and cells actively synthesizing tryptophan convert FAA to 5-methyltryptophan, a highly toxic compound that leads to cell death(Toyn et al., 2000). Therefore, FAA medium selects for cells that have lost the original *TRP1-TEF1* plasmid and only express the *EEF1A2* variant of interest. All variants, except the hypomorphic p.P333L variant exhibited impaired growth in FAA-containing medium (Figure 3B), indicating that all of the variants, except p.P333L, have impaired protein synthesis.

Effects on integrated stress-response Subsequently, we sought to determine whether patient variants interfered with a second important function of *EEF1A2*; the cellular responses to cytotoxic stressors. The TOR pathway is a central regulator of growth and proliferation. Diverse cellular stresses, including nutrient depletion, DNA damage, and oxidative stress among others, inhibit TOR and shift cellular programming away from

growth. These stressors activate the integrated stress response, a highly integrated cellular program that activates autophagy(Kroemer, Marino, & Levine, 2010), promotes chaperone activity(Starck et al., 2016), and inhibits growth-associated protein translation in favor of highly selective translation of stress response proteins such as ATF4(Silva, Sattlegger, & Castilho, 2016). EEF1A and TOR signaling intersect at the level of the GCN2 kinase(Silva et al., 2016; Wengrod et al., 2015).

We first generated a yeast strain containing a deletion of the *EEF1A2* ortholog (*tef2Δ*). Loss of *TEF2* causes cells to exhibit growth failure when challenged with the TOR1 inhibitor rapamycin. The yeast *tef2Δ* strain transformed with empty plasmid (p416GPD) was unable to reverse this deficit. However, when introduced into *tef2Δ* cells, wild-type *TEF2* or N-terminally V5-tagged *TEF2* rescue (complement) this phenotype. We next transformed *tef2Δ* cells with *TEF2* modified to incorporate one of the human *EEF1A2* pathogenic variants and the VUS p.M102V identified in this study. None of the variants, except p.P333L was able to complement the growth defect of *tef2Δ* cells (Figure 3C), indicating that all of the variants in this study disrupt cellular stress responses at least when challenged with rapamycin. Steady-state protein abundance of the variants was again similar to wild-type (Figure 3A) under these conditions.

Finally, we sought to determine whether *EEF1A2* variants exert their effects via a dominant-negative mechanism. We expressed eight different variants in wild-type cells,

and scored cells for growth defects in both nutrient-rich and rapamycin-treated conditions. We found that variant expression did not inhibit growth (Figure 3D), indicating that the *EEF1A2* pathogenic variants and VUS p.M102V likely do not exert a dominant-negative effect.

DISCUSSION

In this study, we report the predicted structural and functional consequences of *de novo* *EEF1A2* missense changes and expand the phenotypic spectrum for pathogenic variants. *EEF1A2*-associated developmental and degenerative epileptic-dyskinetic encephalopathy is characterized by epilepsy beginning at a median age of 4 months, typically with refractory myoclonic, generalized tonic-clonic, absence, tonic seizures and epileptic spasms. Early development is impaired, with global developmental delay in infancy, typically evolving to severe to profound intellectual disability. Affected individuals are often non-verbal and non-ambulatory, although some children are higher functioning. Most are hyporesponsive to environmental stimuli, and may receive a diagnosis of autism spectrum disorder or can be described as Rett-like (Lopes et al., 2016). Nearly all patients have severe truncal hypotonia, with some developing dystonia as well. Choreoathetosis is seen in a number of patients. The recognition of acquired microcephaly in some patients suggested a progressive course, with early brain imaging demonstrating abnormal myelination and thin corpus callosum in some patients. Additionally, serial imaging disclosed a progressive cerebral and cerebellar atrophy with

prominent white matter volume loss. Some patients show evident developmental regression, and a subset experienced a frank neurodegenerative course culminating in early death.

EEF1A2 pathogenic variants are characterized by marked clinical heterogeneity. This variability is evident even for patients with the same variant. For instance, within our cohort are the first three patients with a p.R266W variant; a one year-old male with moderate global developmental delay (patient 10), a 3 year-old with severe global developmental delay and neurodegeneration (patient 11), and a 10 year-old female with profound intellectual disability (patient 12). Patient 10 and 12 had early-onset seizures at 4 months and 1 week respectively, with heminclonic and clonic seizures, respectively, and later absence seizures (patient 10) and tonic seizures (patient 12). In contrast, at 10 years of age patient 11 has had no seizures and her EEG shows no epileptiform abnormalities. Moreover, the occurrence of seizures was not predictive of cognitive outcomes, as patient 11 has severe ID despite no apparent seizure activity, while patient 10 had only moderate ID. This variability was also reflected in the movement disorder, as patient 11 had chorea patient 12 had choreiform movements while patient 10 did not have any motor features. Similarly, we were not able to identify genotype-phenotype correlations when considering the location of all 28 pathogenic variants reported to date with either the severity of clinical presentation or with any specific clinical feature.

Our bioinformatic analyses suggested that several *EEF1A2* mutants disrupt EEF1 complex formation/dimerization and/or the protein's ability to cycle GTP. Furthermore, EEF1A2 is a fundamental cellular protein, highly conserved throughout evolution. This permitted us to use heterologous expression in yeast to assess variants identified in this study. Using this approach, we experimentally confirmed that many *EEF1A2* patient-associated variants may disrupt protein function in both protein synthesis-dependent and stress-response paradigms. Our results are intriguing when considered along with our clinical observations that although *EEF1A2* patients typically have a severe neurodevelopmental disorder, neurodegeneration occurs in a subset of affected individuals. We suspect that defects in synaptic protein synthesis during early brain development contribute significantly to the early developmental phenotype as seen in an increasing number of neurodevelopmental disorders, but that suboptimal responses to cellular stressors may predispose to neurodegeneration in parallel. In contrast, the hypomorphic p.P333L variant, previously reported in a homozygous state in an individual with a complex and fatal neurodevelopmental disorder(Cao et al., 2017), failed to disrupt EEF1A2 function and protein synthesis to the same extent as variants described in this study. However, the VUS p.M102V, showed the same effects on EEF1A2 function as pathogenic and likely pathogenic missense variants, despite being paternally inherited and associated with a milder phenotype. One possible explanation is that this variant impacts EEF1A2 function in this assay and thus may be pathogenic with reduced penetrance, though we cannot measure this in our assaym due to it's non-

quantitative nature. Ulitmately more robust genetic studies with a much larger cohort of patients is needed to support this explanation. Of note, *EEF1A2* was recently shown to harbor an excess of ultra-rare, potentially deleterious variants in patients with genetic generalized epilepsy, though this association did not reach genome-wide significance(Collaborative, 2019). Collectively these results may suggest a role for *EEF1A2* more broadly, including in association with the milder, more common genetic epilepsies. Finally, followup studies in higher eukaryotic systems will be valuable for linking disease course with distinct EEF1A2 functions.

A heterozygous null variant of *EEF1A2* has not been identified among the disease-associated variants reported to date. In addition no null variants and only two heterozygous frameshifts are present in over 110,000 individuals in gnomAD (gnomAD v2.1.1 non-neuro). *EEF1A2* is highly intolerant to genomic variation that would lead to loss-of-function (e.g. frameshift, splice-site, premature stop, etc.), with an RVIS = -0.76 and pLI =0.96 scoring *EEF1A2* in the top 15th percentile of genes intolerant to variation by two independent models (Lek et al., 2016; Petrovski, Wang, Heinzen, Allen, & Goldstein, 2013). Moreover, EEF1A2 is intolerant to missense variation, with a z-score of 4.82 (Lek et al., 2016). These observations suggest that heterozygous complete loss-of-function variants may be lethal in humans. In null mouse models, complete loss of eEF1A2 is lethal by 28 days and mice exhibit a muscle wasting phenotype with or without seizures, while heterozygous mice display no overt phenotype (Chambers et al., 1998; Davies et al., 2017). Moreover, in a small study mice carrying a p.Gly70Ser

missense variant either in homozygous state or in combination with a CRISPR-generated indel on the other allele, were phenotypically indistinguishable from complete null mice (Davies et al., 2017). These result suggest that this missense variant acts in a loss-of-function manner in mice. However, the translatability of these findings to human patients with heterozygous, *de novo* mutations is challenging given the lack of phenotype in heterozygous knockout mice. Moreover, the yeast complementation assays we present here are limited in the ability to quanitavely assess the effect of each missense variant on protein function, as evidenced by the same results from both the VUS, p.M102V and the likely pathogenic variants. Our results, however suggest a dominant negative mechanism is unlikely. Ideally, these pathogenic missense variants need to be modelled in patient-derived, or genome edited induced pluripotent stem cells (iPSCs) alongside genome edited heterozygous *EEF1A2* truncation variants to discern between partial and total loss of function as the pathogenic mechanism.

In sum, we have shown that all of the pathogenic/likely pathogenic variants, as well as a VUS we assessed likely impair the normal function of EEF1A2. We have demonstrated that heterozygous *de novo* pathogenic variants in *EEF1A2* lead to a developmental epileptic-dyskinetic encephalopathy (a broader phenotype than previously appreciated), with neurodegeneration in a subset of individuals. Followup studies further delineating the molecular consequences of EEF1A2 haploinsufficiency are likely to bring us closer to effective therapies given recent developments in small molecule therapies targeting the integrated stress response.

Acknowledgements

We wish to thank the patients and their families for participating in this work.

URLs

All variants have been submitted to ClinVar, submission ID: SUB6968208

References:

Ann, D. K., Lin, H. H., Lee, S., Tu, Z. J., & Wang, E. (1992). Characterization of the statin-like S1 and rat elongation factor 1 alpha as two distinctly expressed messages in rat. *J Biol Chem, 267*(2), 699-702.

Blom, N., Gammeltoft, S., & Brunak, S. (1999). Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol, 294*(5), 1351-1362. doi:10.1006/jmbi.1999.3310

Bunai, F., Ando, K., Ueno, H., & Numata, O. (2006). Tetrahymena eukaryotic translation elongation factor 1A (eEF1A) bundles filamentous actin through dimer formation. *J Biochem, 140*(3), 393-399. doi:10.1093/jb/mvj169

Camougrand, N., Kissova, I., Salin, B., & Devenish, R. J. (2009). Monitoring mitophagy in yeast. *Methods Enzymol, 451*, 89-107. doi:10.1016/s0076-6879(08)03208-410.1016/S0076-6879(08)03208-4.

Cao, S., Smith, L. L., Padilla-Lopez, S. R., Guida, B. S., Blume, E., Shi, J.,… Agrawal, P. B. (2017). Homozygous EEF1A2 mutation causes dilated cardiomyopathy, failure to thrive, global developmental delay, epilepsy and early death. *Hum Mol Genet, 26*(18), 3545-3552. doi:10.1093/hmg/ddx239

Carvill, G. L., Heavin, S. B., Yendle, S. C., McMahon, J. M., O'Roak, B. J., Cook, J.,… Mefford, H. C. (2013). Targeted resequencing in epileptic encephalopathies identifies de novo mutations in CHD2 and SYNGAP1. *Nat Genet, 45*(7), 825-830. doi:10.1038/ng.2646

Chambers, D. M., Peters, J., & Abbott, C. M. (1998). The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene. *Proc Natl Acad Sci U S A, 95*(8), 4463-4468.

Chuang, S. M., Chen, L., Lambertson, D., Anand, M., Kinzy, T. G., & Madura, K. (2004). Proteasome-mediated degradation of cotranslationally damaged proteins involves translation elongation factor 1A. *Mol Cell Biol, 25*(1), 403-413. doi:10.1128/mcb.25.1.403-413.2005

Collaborative, E. (2019). Ultra-Rare Genetic Variation in the Epilepsies: A Whole-Exome Sequencing Study of 17,606 Individuals. *Am J Hum Genet, 105*(2), 267-282. doi:10.1016/j.ajhg.2019.05.020

Cottrelle, P., Cool, M., Thuriaux, P., Price, V. L., Thiele, D., Buhler, J. M., & Fromageot, P. (1985). Either one of the two yeast EF-1 alpha genes is required for cell viability. *Curr Genet, 9*(8), 693-697.

Crepin, T., Shalak, V. F., Yaremchuk, A. D., Vlasenko, D. O., McCarthy, A., Negrutskii, B. S.,… El'skaya, A. V. (2014). Mammalian translation elongation factor eEF1A2: X-ray structure and new features of GDP/GTP exchange mechanism in higher eukaryotes. *Nucleic Acids Res, 42*(20), 12939-12948. 10.1093/nar/gku974. Epub 2014 Oct 17.

Davies, F. C., Hope, J. E., McLachlan, F., Nunez, F., Doig, J., Bengani, H.,… Abbott, C. M. (2017). Biallelic mutations in the gene encoding eEF1A2 cause seizures and sudden death in F0 mice. *Sci Rep, 7*, 46019. doi:10.1038/srep4601910.1038/srep46019.

de Ligt, J., Willemsen, M. H., van Bon, B. W., Kleefstra, T., Yntema, H. G., Kroes, T.,… Vissers, L. E. (2012). Diagnostic Exome Sequencing in Persons with Severe Intellectual Disability. *The New England journal of medicine*. doi:10.1056/NEJMoa1206524

Farwell, K. D., Shahmirzadi, L., El-Khechen, D., Powis, Z., Chao, E. C., Tippin Davis, B.,… Tang, S. (2015). Enhanced utility of family-centered diagnostic exome sequencing with inheritance model-based analysis: results from 500

unselected families with undiagnosed genetic conditions. *Genet Med, 17*(7), 578-586 doi:10.1038/gim.2014.154

Goldstein, A. L., & McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. *Yeast, 15*(14), 1541-1553. doi:10.1002/(sici)1097-0061(199910)15:14<1541::aid-yea476>3.0.co;2-k

Gross, S. R., & Kinzy, T. G. (2006). Improper organization of the actin cytoskeleton affects protein synthesis at initiation. *Mol Cell Biol, 27*(5), 1974-1989. doi:10.1128/mcb.00832-06

Inui, T., Kobayashi, S., Ashikari, Y., Sato, R., Endo, W., Uematsu, M.,... Haginoya, K. (2016). Two cases of early-onset myoclonic seizures with continuous parietal delta activity caused by EEF1A2 mutations. *Brain Dev, 38*(5), 520-524. doi:10.1016/j.braindev.2015.11.003

Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J Bacteriol, 153*(1), 163-168.

Knudsen, S. M., Frydenberg, J., Clark, B. F., & Leffers, H. (1993). Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1 alpha. *Eur J Biochem, 215*(3), 549-554.

Kroemer, G., Marino, G., & Levine, B. (2010). Autophagy and the integrated stress response. *Mol Cell, 40*(2), 280-293. doi:10.1016/j.molcel.2010.09.023

Lam, W. W., Millichap, J. J., Soares, D. C., Chin, R., McLellan, A., FitzPatrick, D. R.,… Abbott, C. M. (2016). Novel de novo EEF1A2 missense mutations causing epilepsy and intellectual disability. *Mol Genet Genomic Med, 4*(4), 465-474. doi:10.1002/mgg3.219. eCollection 2016 Jul.

Lee, M. H., Choi, B. Y., Cho, Y. Y., Lee, S. Y., Huang, Z., Kundu, J. K.,… Dong, Z. (2013). Tumor suppressor p16(INK4a) inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction. *J Cell Sci, 126*(Pt 8), 1744-1752. doi:10.1242/jcs.113613. Epub 2013 Feb 26.

Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T.,… MacArthur, D. G. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature, 536*(7616), 285-291. doi:10.1038/nature19057.

Lopes, F., Barbosa, M., Ameur, A., Soares, G., de Sa, J., Dias, A. I.,… Maciel, P. (2016). Identification of novel genetic causes of Rett syndrome-like phenotypes. *J Med Genet, 53*(3), 190-199. doi:10.1136/jmedgenet-2015-103568

Lukash, T. O., Turkivska, H. V., Negrutskii, B. S., & El'skaya, A. V. (2004). Chaperone-like activity of mammalian elongation factor eEF1A: renaturation of aminoacyl-tRNA synthetases. *Int J Biochem Cell Biol, 36*(7), 1341-1347. doi:10.1016/j.biocel.2003.11.009

Madeo, M., Stewart, M., Sun, Y., Sahir, N., Wiethoff, S., Chandrasekar, I.,… Kruer, M. C. (2016). Loss-of-Function Mutations in FRRS1L Lead to an Epileptic-Dyskinetic Encephalopathy. *Am J Hum Genet, 98*(6), 1249-1255. doi:10.1016/j.ajhg.2016.04.008

Mingot, J. M., Vega, S., Cano, A., Portillo, F., & Nieto, M. A. (2013). eEF1A mediates the nuclear export of SNAG-containing proteins via the Exportin5-aminoacyl-tRNA complex. *Cell Rep, 5*(3), 727-737. doi:10.1016/j.celrep.2013.09.030

Mumberg, D., Muller, R., & Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene, 156*(1), 119-122.

Nakajima, J., Okamoto, N., Tohyama, J., Kato, M., Arai, H., Funahashi, O.,… Miyake, N. (2015). De novo EEF1A2 mutations in patients with characteristic facial features, intellectual disability, autistic behaviors and epilepsy. *Clin Genet, 87*(4), 356-361. doi:10.1111/cge.12394

Newbery, H. J., Loh, D. H., O'Donoghue, J. E., Tomlinson, V. A., Chau, Y. Y., Boyd, J. A.,… Abbott, C. M. (2007). Translation elongation factor eEF1A2 is essential for post-weaning survival in mice. *J Biol Chem, 282*(39), 28951-28959. doi:10.1074/jbc.M703962200

O'Roak, B. J., Stessman, H. A., Boyle, E. A., Witherspoon, K. T., Martin, B., Lee, C.,… Eichler, E. E. (2014). Recurrent de novo mutations implicate novel genes

underlying simplex autism risk. *Nat Commun, 5*, 5595. doi:10.1038/ncomms6595

Ostrander, B. E. P., Butterfield, R. J., Pedersen, B. S., Farrell, A. J., Layer, R. M., Ward, A.,… Quinlan, A. R. (2018). Whole-genome analysis for effective clinical diagnosis and gene discovery in early infantile epileptic encephalopathy. *NPJ Genom Med, 3*, 22. doi:10.1038/s41525-018-0061-8

Pan, J., Ruest, L. B., Xu, S., & Wang, E. (2004). Immuno-characterization of the switch of peptide elongation factors eEF1A-1/EF-1alpha and eEF1A-2/S1 in the central nervous system during mouse development. *Brain Res Dev Brain Res, 149*(1), 1-8. doi:10.1016/j.devbrainres.2003.10.011

Perez, W. B., & Kinzy, T. G. (2014). Translation elongation factor 1A mutants with altered actin bundling activity show reduced aminoacyl-tRNA binding and alter initiation via eIF2alpha phosphorylation. *J Biol Chem, 289*(30), 20928-20938. doi:10.1074/jbc.M114.570077

Petrovski, S., Wang, Q., Heinzen, E. L., Allen, A. S., & Goldstein, D. B. (2013). Genic intolerance to functional variation and the interpretation of personal genomes. *PLoS Genet, 9*(8), e1003709. doi:10.1371/journal.pgen.1003709

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J.,… Committee, A. L. Q. A. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of

Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med, 17*(5), 405-424. doi:10.1038/gim.2015.30

Sasikumar, A. N., Perez, W. B., & Kinzy, T. G. (2012). The many roles of the eukaryotic elongation factor 1 complex. *Wiley Interdiscip Rev RNA, 3*(4), 543-555. doi:10.1002/wrna.1118

Scheffer, I. E., Berkovic, S., Capovilla, G., Connolly, M. B., French, J., Guilhoto, L.,… Zuberi, S. M. (2017). ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. *Epilepsia, 58*(4), 512-521. doi:10.1111/epi.13709

Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F., & Serrano, L. (2005). The FoldX web server: an online force field. *Nucleic Acids Res, 33*(Web Server issue), W382-388. doi:10.1093/nar/gki387

Silva, R. C., Sattlegger, E., & Castilho, B. A. (2016). Perturbations in actin dynamics reconfigure protein complexes that modulate GCN2 activity and promote an eIF2 response. *J Cell Sci, 129*(24), 4521-4533. doi:10.1242/jcs.194738. Epub 2016 Nov 16.

Soares, D. C., Barlow, P. N., Newbery, H. J., Porteous, D. J., & Abbott, C. M. (2009). Structural models of human eEF1A1 and eEF1A2 reveal two distinct surface clusters of sequence variation and potential differences in phosphorylation. *PLoS ONE, 4*(7), e6315. doi:10.1371/journal.pone.0006315.

Starck, S. R., Tsai, J. C., Chen, K., Shodiya, M., Wang, L., Yahiro, K.,… Walter, P. (2016). Translation from the 5' untranslated region shapes the integrated stress response. *Science, 351*(6272), aad3867. doi:10.1126/science.aad3867

Toyn, J. H., Gunyuzlu, P. L., White, W. H., Thompson, L. A., & Hollis, G. F. (2000). A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast, 16*(6), 553-560. doi:10.1002/(sici)1097-0061(200004)16:6<553::aid-yea554>3.0.co;2-7

Vanwetswinkel, S., Kriek, J., Andersen, G. R., Guntert, P., Dijk, J., Canters, G. W., & Siegal, G. (2003). Solution structure of the 162 residue C-terminal domain of human elongation factor 1Bgamma. *J Biol Chem, 278*(44), 43443-43451. doi:10.1074/jbc.M306031200

Veeramah, K. R., Johnstone, L., Karafet, T. M., Wolf, D., Sprissler, R., Salogiannis, J.,… Hammer, M. F. (2013). Exome sequencing reveals new causal mutations in children with epileptic encephalopathies. *Epilepsia, 54*(7), 1270-1281. doi:10.1111/epi.12201

Vera, M., Pani, B., Griffiths, L. A., Muchardt, C., Abbott, C. M., Singer, R. H., & Nudler, E. (2014). The translation elongation factor eEF1A1 couples transcription to translation during heat shock response. *Elife, 3*, e03164. doi:10.7554/eLife.0316410.7554/eLife.03164.

Visweswaraiah, J., Lageix, S., Castilho, B. A., Izotova, L., Kinzy, T. G., Hinnebusch, A. G., & Sattlegger, E. (2011). Evidence that eukaryotic translation elongation factor 1A (eEF1A) binds the Gcn2 protein C terminus and inhibits Gcn2 activity. *J Biol Chem, 286*(42), 36568-36579. doi:10.1074/jbc.M111.24889810.1074/jbc.M111.248898. Epub 2011 Aug 17.

Wengrod, J., Wang, D., Weiss, S., Zhong, H., Osman, I., & Gardner, L. B. (2015). Phosphorylation of eIF2alpha triggered by mTORC1 inhibition and PP6C activation is required for autophagy and is aberrant in PP6C-mutated melanoma. *Sci Signal, 8*(367), ra27. doi:10.1126/scisignal.aaa0899

Figure 1. A) Distribution of *EEF1A2 de novo* pathogenic variants described previously or in this study (bold) in patients with neurodevelopmental disorders. The number of patients observed with recurrent variants are shown in parentheses (**bold, this study**), and vertical lines in the bottom panel denote missense variants from the gnomAD dataset seen more than once. The majority of pathogenic variants tend to cluster around the Switch I and II domains and these functional domains tend to be devoid of missense variants in the general population. **B) Structure of eEF1A2 complexed with GDP showing locations of modeled variants**. The locations and orientation of Domain I (green cartoon), domain II (teal cartoon), domain III (peach cartoon), switch I (red), switch II (yellow) and bound GDP (teal stick) and Mg^{++} are shown. Locations of observed variants are shown as sticks with magenta spheres. Positions of ExAc variants are shown in orange.

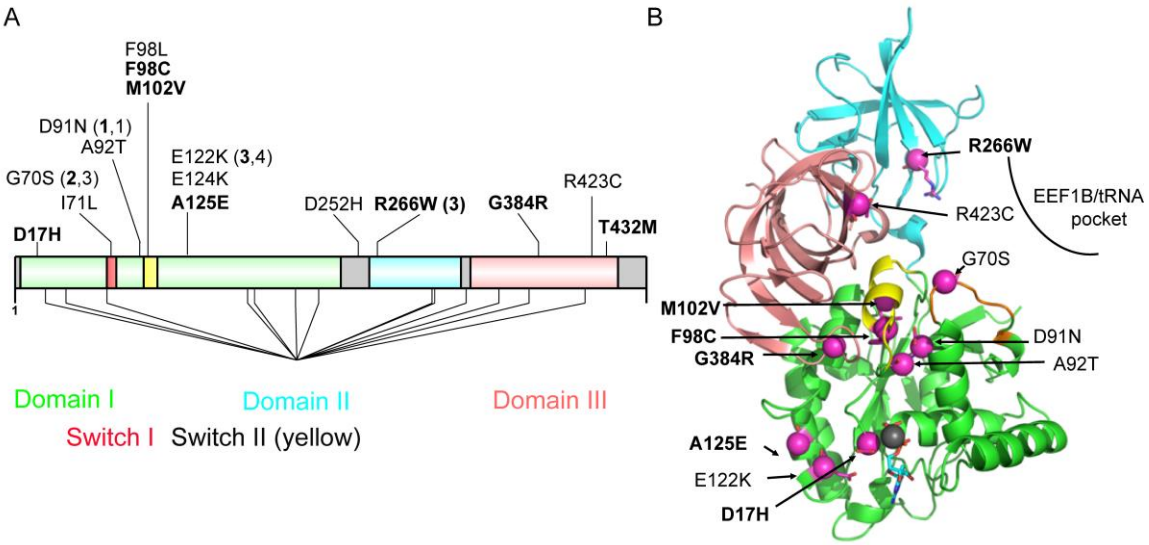

Figure 2: Structural brain images from patients with *EEF1A2* mutations showing cerebral atrophy and abnormal myelination. Sagittal T1-weighted (A) and axial T2-weighted (B,C) of Patient #11 performed at 1.5 months of age show mild diffuse prominence of the supratentorial CSF spaces and absent myelination in the posterior limbs of internal capsules (arrows, B). (D-F) Corresponding MR images performed in the same patient at 5.5 years of age reveal cerebral and cerebellar atrophy with predominant white matter volume loss, *ex vacuo* ventricular dilatation (asterisks, E, F), thin corpus callosum (arrowhead, D) and diffuse enlargement of the sulci. The myelination has slightly progressed (arrows, E), but there is persistent diffuse hazy elevated T2 signal in the deep and subcortical cerebral white matter. Note the severe vermian atrophy (empty arrow, D). Sagittal T1-weighted (G), axial T2-weighted (H) and coronal FLAIR (I) images of Patient #12 performed at 2 years of life reveal diminished white matter volume with thin corpus callosum (arrow, G), mild *ex vacuo* ventriculomegaly and enlarged CSF spaces, associated with markedly reduced myelination. Mild enlargement of vermian and cerebellar hemispheric sulci (empty arrows, G, I) is also present. (J-L) Corresponding images in the same patient at 7 years of age demonstrate progression of the cerebral and cerebellar atrophy (empty arrows, J, L) and minimal interval deposition of myelin in the posterior limbs of internal capsules and deep white matter in the temporo-occipital regions (arrows, K).

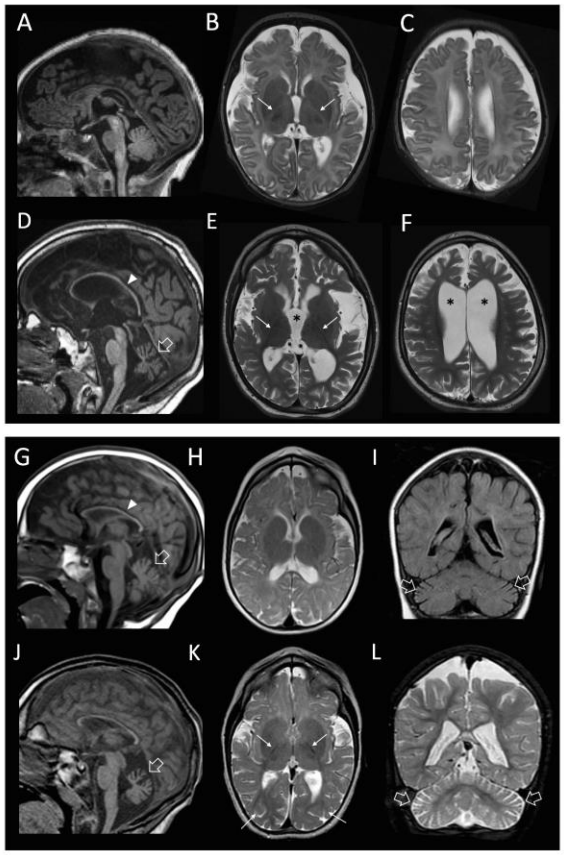

Figure 3: Complementation studies of *EEF1A2* variants using yeast. (A) Representative image from protein extracts obtained from $2x10^7$ cells and analyzed by western blot using antibodies recognizing the V5 epitope or cytosolic protein Pgk1 and graph summarizing normalized protein levels. Some variant steady-state protein levels were significantly diminished ($p<0.05$) compared to wild-type. Similar trends were found when *TEF2* variants were expressed in all yeast strains used un this study. Experiments were performed at least three times. **(B)** Plasmid shuffling findings using the MC214 strain transformed with *TEF2* variants in p416GPD. Fresh cultures in SC medium lacking uracil were grown overnight and equal numbers of cells were deposited on SC-uracil plates with or without 0.5 g/l fluoroanthracilic acid (FAA). FAA medium select for colonies that have lost pTEF2-TRP1 and only have p416GPD derived plasmids. All *TEF2* variants assayed except P333L showed failed growth on FAA plates, indicating a deficit in protein synthesis. All experiments were performed in triplicate; representative image shown. Note that amino acids 159-160 are not found in the yeast TEF2 sequence. For consistency, all variants are listed as human residue equivalents. **(C)** Yeast *tef2-Δ* strain shows sensitivity to the drug rapamycin that is reversed after transformation with the plasmid p416GPD carrying a wild-type copy of the *TEF2* gene. A N-terminal V5-tagged version of TEF2 also rescued growth in presence of rapamycin. Only P333L *TEF2* variant was unable to complement the rapamycin sensitivity phenotype. Yeast strains were cultured overnight in liquid Synthetic Complete (SC) medium lacking uracil and equal numbers of cells were deposited on YPD plates with or without Rapamycin (40 ng/ml) and incubated at 30ºC for 48 hours. Representative image taken from three independent biological replicates. **(D)** In order to test for a possible dominant-negative effect, the same experiment described in (C) was performed using a wild type strain (BY4742).

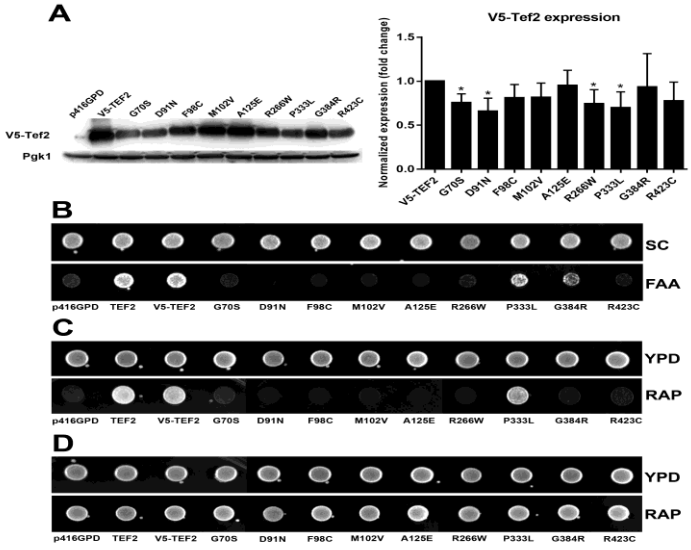

Table 1 Genetic and clinical details for patients with *EEF1A2* pathogenic or likely pathogenic variants

Patient	**Summary new cases (n=14)**	**Previously reported cases (n=15)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Variant NM_001958 (CADD)			c.49G>C p.D17H (27)	c.208G>A p.G70S (28)	c.208G>A p.G70S (28)	c.271G>A p.D91N (27)	c.293T>G p.F98C (27)	c.364G>A p.E122K (28)	c.364G>A p.E122K (28)	c.364G>A p.E122K (28)	c.374C>A p.A125E (27)	c.796C>T p.R266W (24)	c.796C>T p.R266W (24)	c.796C>T p.R266W (24)	c.1150G>C p.G384R (28)	c.1295C>T p.T432M (23)
Inheritance			*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	Not paternal	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	Mosaic mother (<25%)
Classification per ACMG Guidelines			Likely pathogenic	Pathogenic	Pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic	Pathogenic	Likely pathogenic	Likely pathogenic

Variant disrupts protein function by complementation			NR	Yes	Yes	Yes	Yes	NR	NR	NR	Yes	Yes	Yes	Yes	Yes	NR
Sex			M	M	F	F	F	M	M	F	M	M	F	F	M	F
Age at study			13y	Deceased 4y	11y	15y	5y	32y	3y	2y	Deceased 3.75y	1.3y	8y	10y	5y	15y
Failure to thrive	4/14	3/7	No	Yes	No	No	Yes	No	No	No	No	Yes	No	Yes	No	No
Acquired microcephaly	4/14	5/10	No	No	No	No	No	Yes	No	No	No	Yes	Yes	Yes	No	No
Was development ever clearly normal?	8/14		Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	No	No	No	Yes	No
Language impairment	14/14	15/15	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes single words	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes nonverbal	Yes three expressive

																words
Ambulatory	4/14	7/14	No	No	Yes with assistance	No	Yes with assistance	Yes ataxic	No	No	No	No	No	No	No	Yes
Regression age			6 yrs (cognitive deterioration, poor visual attention)	21 mos (lost ability to sit, crawl, and interact with environment)	None	4m (deterioration of head control, interaction; stopped trying to roll)	None	NR	NR	NR	10 m (protracted loss of skills leading to vegetative state)	None	None	None	5 mos (lost ability to pull to sit and social smile)	None
Degree of ID			Severe	Severe	Severe	Profound	Mod era To-Severe	Severe	Moderate to severe	NR	Profound	Moderate	Severe	Profound	Profound	Moderate-severe
Epilepsy	12/14	15/15	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Age of seizure onset			6m	21m	1m	4m	N/A	3m	4m	2m	1d	4m	N/A	1w	1d	35 m

Presenting seizure type			GTCS	Myoclonic	Myoclonic	Myoclonic	N/A	Focal	myoclonic	Myoclonic	Myoclonic	Hemiclonic	N/A	Clonic	Focal	Myoclonic
Additional seizure types			Epileptic spasms, myoclonic, focal	Tonic, GTCS, tonic-clonic	Atypical absence, GTCS	Tonic, GTCS, focal tonic-clonic	N/A	Reflex myoclonic (startle), infantile spasms	Tonic, myoclono-atonic, tonic-clonic, absence	Tonic, atypical absence	Reflex myoclonic - 5m, tonic - 2.5y, TCS <8m, focal - 8m	Absence	N/A	5y: tonic	5m: focal status epilepticus, 7m: infantile spasms; 4y: tonic	N/A
Seizure control			Refractory	Refractory	Reduced freq	Seizure-free from 14yr on CLB	N/A	At age 25 under good control with VPA	Refractory	Seizure-free	Refractory	Seizure-free	N/A	Partial control with CLB and VPA	Infrequent focal seizures	Good but rare myoclonic crises with fever
EEG			Hypsarrhythmia	GSSW, burst-suppression, multifocal	Multifocal; GPSW; PPR	Multifocal with runs of central discharges, ; GPSW with my	Frontoparietal sharp waves, occipital slow waves; focal thet	9m: frequent generalized paroxysms; 14y: sym	Slow background rhythm (theta/delta) with multifoca	Rare generalized polyspikes and spike waves with clinical manifest	Irregular GSW, GPSW with myoclonic seizures; multifo	Right centrotemporal discharges	Normal	Bitemporal epileptiform discharges ; disorganized background	Multifocal discharges, GSW	Multifocal spikes

						oclonic seizures	and delta	metric slowing without epileptiform discharges	l spikes	ation	cal discharges, central ictal rhythm					
MRI			Delayed myelination; thin corpus callosum	Mild cortical atrophy	Normal	Normal apart from small arachnoid cyst	Normal	Normal	Normal at 2y	T2 hyperintensity in left pariето-occipital region, putatively related to recent seizures	Mild cortical atrophy	Mega cisterna magna; thin corpus callosum	Cerebral and brainstem atrophy; delayed myelination; persistent T2 hyperintensity of frontal lobes; thin corpus callosum	Hypomyelination; diffuse cerebral and cerebellar atrophy; thin corpus callosum	Normal	Mild cortical atrophy
AEDs trialed			CBZ, PB, ACTH, VGB, TPM, FBM, CBZ, LEV, CBD oil, ketodiet, VNS;	VPA, PB, CLB, TPM, LTG, LEV, OXC, ketodiet, FBM, RFM	VPA, CLB, LEV, pyridoxine, ETX, ZNS	VPA, CNZ PHT, LEV, LTG, PB, CLB (controlled seizures)	VPA (for chorea; did not impact chorea)	VPA (controlled seizures)	VPA, LEV, TPM, HC, CLB, CLZ, ZNS, B6, LTG, ketogenic die	LEV (controlled seizures)	VPA, TPM, PHT, CLB	LEV (controlled seizures)	N/A	PB, CLB (reduced seizures), VPA (reduced seizures)	PB, prednisolone, LEV, CLB, LTG, VPA, TPM, CBD	VPA (reducted seizures)

			ZNS improved tonic seizures and LTG improved tonic-clonic seizures; CBZ reduced nocturnal seizures						t, RFN, ESM							
Hypotonia	14/14	9/11	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pyramidal tract signs	8/13	2/4	No	Ankle clonus, Hyperreflexia	No	Hyperreflexia	No	No	No	Hyperreflexia	Hyperreflexia	Ankle clonus	Extensor hypertonia; scissoring	Hyperreflexia	Hyperreflexia	NR
Movement disorder	7/13	3/4	No	Yes choreoathetosis, generalized dystonia, stereotypies	No	Yes dystonia	Yes mild dystonia, generalized chorea, stereotypies	Yes ataxia	No	Yes choreoathetosis	No	No	Yes chorea	Yes choreiform movements	No	Generalized dystonia vs. spasticity

Behavioral features	2/14	5/5	No	No	No	No	No	Yes self-injurious in the past	Yes autistic features	No	No	No	No	No	No	No
Dysmorphic features	5/14	8/9	No	No	Yes Synophrys, epicanthic pleat, small chin, small feet	Yes Asymmetric facies, hypertelorism, small feet	No	Yes Deep-set eyes, full nasal alae and tips, full lips	No	No	No	No	No	Yes Low forehead, enlarged nostrils, wide mouth, low set ears with hypoplastic earlobes, hypertrophy of tragus, antitragus, and antihelix	No	Yes Epicanthus, everted inferior lip
Dysphagia	5/13	2/4	Yes	Yes	No	Yes	No	No	No	No	Yes	Yes	No	No	No	NR
Other features			Exotropia; hyperm etropia	Recurrent pneumonia; intermittent hyperpn	Swelling of dorsal surface of ha	Recurrent pneumonia; thoracic scoliosi	Optic nerve atrophy	Recurrent infections as a child;	Obesity	Cortical visual impairment	Cortical visual impairment; high	Recurrent apneic episodes; respiratory	Developmental hip dysplasia; laryngotracheomalacia with	Kyphoscoliosis; amyotrophy	Cortical visual impairment; coarctation	NR

				ea and hypopnea; strabismus, esotropia, hyperopia	nds and feet	s; esotropia		strabismus			frequency sensorineural hearing loss	failure requiring mechanical ventilation	tracheostomy; mildly dilated aortic root		of aorta and VSD; laryngomalacia	

N/A = not applicable; NR= not reported/not assessed; VPA = valproate; LEV = levetiracetam; CLB = clobazam; CLZ = clonazepam; TPM = topiramate; ZNS = zonisamide; LTG = lamotrigine; CBZ = carbamazepine; PB = phenobarbital; CBD = cannabadiol; VGB = vigabatrin; ESM = eslicarbamazepine; FBM = felbamate; PHT = phenytoin; ACTH = adrenocorticotrophic hormone; OXC = oxcabarbazepine; FFM = rufinamide; ETX = ethosuximide

Author/s:
Carvill, GL; Helbig, KL; Myers, CT; Scala, M; Huether, R; Lewis, S; Kruer, TN; Guida, BS; Bakhtiari, S; Sebe, J; Tang, S; Stickney, H; Oktay, SU; Bhandiwad, AA; Ramsey, K; Narayanan, V; Feyma, T; Rohena, LO; Accogli, A; Severino, M; Hollingsworth, G; Gill, D; Depienne, C; Nava, C; Sadleir, LG; Caruso, PA; Lin, AE; Jansen, FE; Koeleman, B; Brilstra, E; Willemsen, MH; Kleefstra, T; Sa, J; Mathieu, M-L; Perrin, L; Lesca, G; Striano, P; Casari, G; Scheffer, IE; Raible, D; Sattlegger, E; Capra, V; Padilla-Lopez, S; Mefford, HC; Kruer, MC

Title:
Damaging de novo missense variants in EEF1A2 lead to a developmental and degenerative epileptic-dyskinetic encephalopathy

Date:
2020-07-01

Citation:
Carvill, G. L., Helbig, K. L., Myers, C. T., Scala, M., Huether, R., Lewis, S., Kruer, T. N., Guida, B. S., Bakhtiari, S., Sebe, J., Tang, S., Stickney, H., Oktay, S. U., Bhandiwad, A. A., Ramsey, K., Narayanan, V., Feyma, T., Rohena, L. O., Accogli, A. ,... Kruer, M. C. (2020). Damaging de novo missense variants in EEF1A2 lead to a developmental and degenerative epileptic-dyskinetic encephalopathy. HUMAN MUTATION, 41 (7), pp.1263-1279. https://doi.org/10.1002/humu.24015.