The American Journal of Human Genetics, Volume *106*

Supplemental Data

De novo EIF2AK1 and *EIF2AK2* Variants Are Associated with Developmental Delay, Leukoencephalopathy, and Neurologic Decompensation

Dongxue Mao, Chloe M. Reuter, Maura R.Z. Ruzhnikov, Anita E. Beck, Emily G. Farrow, Lisa T. Emrick, Jill A. Rosenfeld, Katherine M. Mackenzie, Laurie Robak, Matthew T. Wheeler, Lindsay C. Burrage, Mahim Jain, Pengfei Liu, Daniel Calame, Sébastien Küry, Martin Sillesen, Klaus Schmitz-Abe, Davide Tonduti, Luigina Spaccini, Maria Iascone, Casie A. Genetti, Mary K. Koenig, Madeline Graf, Alyssa Tran, Mercedes Alejandro, Undiagnosed Diseases Network, Brendan H. Lee, Isabelle Thiffault, Pankaj B. Agrawal, Jonathan A. Bernstein, Hugo J. Bellen, and Hsiao-Tuan Chao

SUPPLEMENTAL TABLES

TABLE S1: Variant prediction for *de novo EIF2AK1* and *EIF2AK2* variants

Variant prediction:	Proband 1	Proband 2	Proband 3	Proband 4	Proband 5	Proband 6	Proband 7	Proband 8	Proband 9
CADD	23.8	13.14	18.03	2.28	19.81	20.8	7.98	6.411	4.672
GERP	4.79	-1.2	-10.5	-10.1	0.308	3.82	-6.52	-4.67	-0.791
M-CAP	Tolerated	Tolerated	Damaging	Tolerated	Damaging	Damaging	Damaging	Damaging	Damaging
PolyPhen2 HumDiv	Possibly Damaging	Benign	Probably Damaging	Benign	Probably Damaging	Probably Damaging	Benign	Benign	Benign
PolyPhen2 HumVar	Possibly Damaging	Benign	Probably Damaging	Benign	Probably Damaging	Probably Damaging	Probably Damaging	Benign	Benign
Phylop Vertebrate	4.56	-0.215	-1.854	-5.231	-0.385	2.94	-1.53	-0.323	-0.791
SIFT	Tolerated	Damaging	Tolerated	Tolerated	Damaging	Damaging	Tolerated	Tolerated	Tolerated

EIF2AK1 and *EIF2AK2* missense variants were assessed using multiple variant prediction algorithms. CADD (Combined Annotation Dependent Depletion)[1], GERP (Genomic Evolutionary Rare Profiling)[2], M-CAP (Mendelian Clinically Applicable Pathogenicity)[3], PolyPhen2 (Polymorphism Phenotyping v2) HumDiv and HumVar[4], phyloP (phylogenetic *P*-values)[2,5], SIFT (Sorting Intolerant From Tolerant)[6]

TABLE S2: Summary of clinical findings in individuals with heterozygous *de novo EIF2AK1* and *EIF2AK2* variants

General:	Proband 1	Proband 2	Proband 3	Proband 4	Proband 5	Proband 6	Proband 7	Proband 8	Proband 9
Age at onset	10 months	9 months	18 months	14 months	1 month	birth	7-8 months	first months of life	first months of life
Presenting features	delayed development	seizure, delayed development	motor and language delay, ataxia	seizure, hypotonia, delayed development	delayed development, nystagmus	congenital nystagmus, delayed development, microcephaly	delayed development, neurologic regression with febrile illness	developmental delay	bilateral horizontal nystagmus, seizures
Family history of neurologic findings	mild toe-walking, no spasticity	negative	mild speech delay, ADHD, autism	negative	older sister with PKU	autism spectrum disorder	muscular dystrophy; hypermobility; autism; dyspraxia; hearing loss; cognitive impairment	Parkinson's Disease	epilepsy; sudden infant death syndrome
Mother's age at conception (years)	22 years	40 years	34 years	30 years	38 years	29 years	20 years	30 years	24 years
Father's age at conception (years)	23 years	36 years	34 years	37 years	39 years	37 years	21 years	34 years	37 years
Gestational age	Full term	36 weeks	Full term	Full term	38 weeks	36 weeks	41 weeks	40 weeks, 6 days	39 weeks
Height at most recent assessment	112.5 cm (Z = -0.46)	123.2 cm (Z = -2.80)	149.2 cm (Z = -1.11)	90.9 cm (Z = -0.99)	77 cm (Z = -2.0)	81.5 cm (Z = -0.95)	92.5 cm (Z = -1.21)	120 cm (at 8.5 years)	95.7 cm (Z = -2.28)
Weight at most recent assessment	19.1 kg (Z = -0.66)	26.2 kg (Z = -1.72)	37.1 kg (Z= -1.29)	12.2kg (Z = -0.80)	7 kg (Z = -3.0)	10.3 kg (Z = -0.82)	13.4 kg (Z = -0.94)	20 kg (at 8.5 years)	12.8 kg (Z = -2.99)
Status	alive	alive	alive	alive	deceased at 22 months	alive	alive	alive	alive
Co-morbidity	n/a	n/a	n/a	n/a	Phenylketonuria	n/a	n/a	n/a	n/a
Consanguinity	negative	negative	negative	negative	negative	parents are first cousins once removed	negative	negative	negative
Dysmorphisms	negative	short philtrum, mild retrognathia	bilateral pre-auricular pits	negative	acquired microcephaly at 9 months	acquired microcephaly	bilateral 5th toe clinodactyly	hypotelorism, mild epicanthus, large superior incisors, 5th finger clinodactyly with hypoplastic nails	none
Other abnormalities	spasticity and toe-walking at 21 months	movement disorder at 6 years old	elevated cholesterol, recurrent mouth blisters, eczema	pancytopenia, dry skin, arthritis	SGA, IUGR	SGA, IUGR, oligohydramnios	dysphagia	n/a	dysphagia
Development:									
Head control	n/a	n/a	n/a	n/a	3-4 months	8 months	n/a	n/a	n/a
Sat independently	6-8 months	n/a	6 months	n/a	5-6 months	n/a	14-15 months	9 months	1 year
Stood with support	n/a	n/a	n/a	n/a	nonambulatory	nonambulatory	7 months	16 months	3 year
Walked independently	21 months, abnormal gait	16 months	12-18 months, abnormal gait	16 months, abnormal gait	nonambulatory	nonambulatory	21-23 months, abnormal gait	19 months	nonambulatory
Speech	first word at 13 months, short phrases at 26 months	first word at 18 months, short phrases at 3 years	first word at 15 months but slow to develop and words are difficult to understand	10 words at 17 months	nonverbal	babbling	speaks 50 words at 3 years, 2-word phrases at 4 years	first word at 20 months	nonverbal
Current speech ability and features	speech is 50% intelligible, dysarthric	mixed receptive-expressive language delay, echolalia, dysarthric	dysarthric	dysarthric	nonverbal	nonverbal	dysarthric, abruptly nonverbal following RSV illness at 4 years	dysarthric, nasal voice	nonverbal but has a few signs and follows simlpe commands
Cognitive impairment	no	yes	yes	yes	yes	yes	yes	yes	yes
School	mainstream with accomodations	special	special	n/a	n/a	not yet school age	special	special	special
Ophthalmology:									
Nystagmus	no	no	no	no	yes	yes	no	no	yes
Saccadic abnormalities	no	yes	no	no	no	no	no	no	no
Jerky eye movements	no	no	no	no	no	no	no	no	no
Additional features	no	astigmatism	no	no	congenital nystagmus, strabismus	left esotropia, congenital nystagmus	left esotropia, hyperopia, and ambylopia	no	no
Musculoskeletal:									
Scoliosis	no	yes	no	no	no	no	no	no	no
Camptocormia	no	yes	no	no	no	no	no	yes	no
Muscle wasting	no	yes	no	no	no	no	no	no	yes
Muscle biopsy	no	yes	no	no	yes	no	no	no	no
Muscle biopsy findings	n/a	nonspecific Type II fiber atrophy	n/a	n/a	post-mortem tissue, changes in complex I	n/a	n/a	n/a	n/a
Nerve conduction study	yes	yes	no	no	no	no	no	yes	no
Nerve conduction study findings	normal	normal	n/a	n/a	n/a	n/a	n/a	normal	n/a
Neuropsychiatry:									
Anxiety	yes	yes	no	n/a	n/a	n/a	no	no	no
Depression	no	no	no	n/a	n/a	n/a	no	no	no
ADHD	yes	no	no	n/a	n/a	n/a	no	no	no
Autism	no	no	yes	n/a	n/a	n/a	no	no	no

Abbreviations: attention deficit and hyperactivity disorder (ADHD), phenylketonuria (PKU), not available (n/a), small for gestational age (SGA), intrauterine growth restriction (IUGR),

TABLE S3: MRI spine and laboratory findings for individuals with *de novo EIF2AK1* and *EIF2AK2* variants

MRI Spine:	Proband 1	Proband 2	Proband 3	Proband 4	Proband 5	Proband 6	Proband 7	Proband 8	Proband 9
Age at most recent assessment	3 years	9 years	13 years	17 months	not performed	not performed	4 years	not performed	not performed
T1W signal	isointense	isointense	isointense	isointense	n/a	n/a	isointense	n/a	n/a
T2 hyperintensity	isointense	hyperintense, dorsal-most upper cervical cord, dorsal medulla, dorsal pons	hyperintense, patchy signal in the brainstem, prominent signal in the central gray of cervical cord	hyperintense, central grey matter of the spinal cord	n/a	n/a	isointense	n/a	n/a
Contrast enhancement	no	dorsal brainstem, upper cervical cord	no	no	n/a	n/a	no	n/a	n/a
Conus medullaris termination	normal	normal	normal	normal	n/a	n/a	normal	n/a	n/a
Cauda equina	normal	normal	normal	normal	n/a	n/a	normal	n/a	n/a
Metabolic:									
Mitochondrial genome	n/a	n/a	normal	normal	normal	normal	normal	n/a	no
Plasma amino acids	normal	n/a	n/a	normal	normal	normal	normal	normal	normal
Plasma acylcarnitine profile	n/a	n/a	n/a	normal	normal	normal	normal	normal	normal
Plasma very long chain fatty acids	n/a	n/a	n/a	n/a	normal	n/a	normal	normal	normal
Urine organic acids	n/a	normal	n/a	normal	normal	normal	normal	normal	normal
Urine amino acids	n/a	n/a	n/a	n/a	n/a	n/a	normal	normal	n/a
Plasma & urine creatine and guanidinoacetate	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Plasma total homocysteine	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Plasma 7-dehydrocholesterol	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Urine MPS	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Urine oligosaccharides	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Urine purines and pyrimidines	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Blood alpha glucosidase (Pompe)	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Blood AFP	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Leukocyte coenzyme Q10	n/a	n/a	n/a	n/a	n/a	n/a	normal	n/a	n/a
Congenital disorders of glycosylation screen	n/a	n/a	n/a	n/a	n/a	n/a	normal	normal	n/a
Lysosomal activity	n/a	n/a	n/a	n/a	normal	n/a	n/a	normal	n/a
Hematologic and Immunologic:									
Immunoglobulin levels	n/a	n/a	normal	normal	normal	normal	n/a	n/a	n/a
Leukopenia	no	no	no	yes	yes	no	no	no	no
Additional features	n/a	n/a	n/a	normocytic anemia, pancytopenia	splenic hyperplasia	n/a	mild normocytic anemia	n/a	n/a

Abbreviations: not available (n/a)

SUPPLEMENTAL METHODS

Human subjects and sequencing studies: Informed consent for all subjects was obtained in accordance with research protocols that were approved by the institutional review board at Baylor College of Medicine, Stanford University, or at local institutions prior to testing. DNA was extracted for all subjects from peripheral blood mononuclear cells for trio exome sequencing (ES) in CLIA certified laboratories and variants were confirmed by Sanger sequencing.

For proband 1 and 7, trio ES was performed at GeneDX with Illumina SureSelect XT kit reagents and a HiSeq2500 platform (Illumina). For proband 2, 6, and 9, trio ES was performed at Baylor Genetics through the Whole Genome Laboratory (https://www.bcm.edu/research/medical-genetics-labs/index.cfm?PMID=21319) using methods described[7]. Produced sequence reads were aligned to the GRCh37 (hg19) human genome reference assembly using the HGSC Mercury analysis pipeline (http://www.tinyurl.com/HGSC-Mercury/). Variants were determined and called using the Atlas2 suite to produce a variant call file[8].For the population comparisons we utilized data from the Exome Aggregation Consortium (ExAC), Cambridge, MA (http://exac.broadinstitute.org), Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/), and Genome Aggregation Database (gnomAD), Cambridge, MA (http://gnomad.broadinstitute.org).

Probands 1 and 2 were enrolled at the BCM Undiagnosed Diseases Network (UDN) site. Baylor Genetics provided research reanalysis of proband 1's trio ES and UDN researchers used Codified Genomics for variant interpretation. Proband 3 was enrolled at the Stanford UDN site. Trio ES was performed at Ambry Genetics with research analysis and variant interpretation by researchers at the Stanford UDN site. For proband 4, trio ES was performed at Children's Mercy Hospital Center for Pediatric Genomic Medicine as previously described[9]. For proband 5, trio ES

was performed by the Center for Mendelian Genomics and the Broad Institute, analyzed the results with SEQR (https://seqr.broadinstitute.org) and VexP[10]. For proband 8, trio ES was conducted using genomic DNA from the proband and parents, the exonic regions and flanking splice junctions of the genome were captured using either the Clinical Research Exome v.2 kit (Agilent Technologies, Santa Clara, CA). Sequencing was done on a NextSeq500 Illumina system with 150bp paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19, and analyzed for sequence variants using a custom-developed analysis tool[11]. Additional sequencing technology and variant interpretation protocol have been previously described[11]. Coverage on target for the index was ≥ 10x for 98.6% with a mean coverage of 200x.

All variant nomenclature uses GRCh37 (hg19) human genome reference assembly with GenBank: NM_014413.4 (*EIF2AK1*) and GenBank: NM_002759.3 (*EIF2AK2*).

cDNA mutagenesis: The cDNAs encoding the EIF2AK1/2 WT protein were subcloned from the respective Gateway donor vectors into the mammalian vector, pcDNA-DEST40 (with a CMV promoter and C-terminal V5 tag), via the Invitrogen Gateway LR Clonase II protocol. The stop codon was removed by mutagenesis to tag the protein with V5 tag. EIF2AK1/2 variants were introduced via site-directed mutagenesis using the QuikChange II site-directed mutagenesis kit (Agilent, 200523) or NEB Q5 Site-Directed Mutagenesis Kit (E0554). Clones were sequenced and confirmed to be correctly subcloned into the pcDNA-DEST40 destination vector. Primers used for the mutagenesis are listed below:

Primer	Sequence (5'-3')
EIF2AK1-A1342G-F	atcagggccatgaagaaaaacatttcttggcttcagatctc
EIF2AK1-A1342G-R	gagatctgaagccaagaaatgtttttcttcatggccctgat
EIF2AK2-A31T-F	ggtatgtattaagttcctccaagaagaaacctgctgaaagatc
EIF2AK2-A31T-R	gatctttcagcaggtttcttcttggaggaacttaatacatacc
EIF2AK2-A227G-F	gccttcttttccttactaagtatctcaacagctaatttggctg
EIF2AK2-A227G-R	cagccaaattagctgttgagatacttagtaaggaaaagaaggc
EIF2AK2-A227G-F	gccttcttttccttactaagtatctcaacagctaatttggctg
EIF2AK2-A227G-R	cagccaaattagctgttgagatacttagtaaggaaaagaaggc
EIF2AK2-C326T-F	ttacagttagtcttttcttctggacaattctattgataaggcctatg
EIF2AK2-C326T-R	cataggccttatcaatagaattgtccagaagaaaagactaactgtaa
EIF2AK2-T341A-F	cacactgttcataatttacagtttgtcttttcttctgggcaattcta
EIF2AK2-T341A-R	tagaattgcccagaagaaaagacaaactgtaaattatgaacagtgtg
EIF2AK2-A398T-F	ctgtcccattttgcatttaaaatgaaatccttctggccc
EIF2AK2-A398T-R	gggccagaaggatttcattttaaatgcaaaatgggacag
EIF2AK2-G973A-F	atcccaacagctattgtagtgaacaatatttacatgatcaagt
EIF2AK2-G973A-R	acttgatcatgtaaatattgttcactacaatagctgttgggatggatt
EIF2AK2-C1382G-F	atagtcttgcgaacaaatctgttctgggctcatgtatc
EIF2AK2-C1382G-R	gatacatgagcccagaacagatttgttcgcaagactat

Mammalian tissue culture: HEK293T or HeLa cells were grown in high glucose Dulbecco's modified Eagle's medium (ThermoFisher Scientific, 11960) supplemented with 10% fetal bovine serum (Sigma, F0926) 1% (v:v) GlutaMAX (ThermoFisher Scientific, 35050061), and 1% (v:v) penicillin-streptomycin (GenDEPOT, CA005-010) and grown in a humidified incubator at 37°C with 5% CO_2.

Poly-IC treatment: Control or proband-derived skin fibroblasts were incubated in regular media with or without poly-IC (Sigma, P1530) (final concentration 10 µg/ml) for 24hrs. Cells were lysed for protein collection and Western blot analysis.

DNA and siRNA Transfection: Generation of mammalian expression vectors with EIF2AK1/2 WT or variants cDNAs are discussed above. GFP-RFP-MAP1LC3A tandem tagged constructs were provided by Marco Sardiello (Baylor College of Medicine). siRNAs used for transient interference of EIF2AK1/2 are listed below:

siRNA	Source	Catalog	Sequence (5'-3')
EIF2AK1-1	Sigma	SASI_Hs01_00086018	CGUUGUAUUUAGUAAGCCU
EIF2AK1-2	Sigma	SASI_Hs01_00086021	CCUUUACAAGACUUGUUAA
EIF2AK1-3	ThermoFisher	s25822	GACGGAAAGACUUACGUUAtt
EIF2AK2-1	Sigma	SASI_Hs01_00019634	GAGGUUUACAUUUCAAGUU
EIF2AK2-2	Sigma	SASI_Hs01_00019640	GUCAGAAGCAGGGAGUAGU
EIF2AK2-3	ThermoFisher	s11185	GAUUAAGGGUGCAACUAAAtt

For DNA and siRNA transfection, HEK293T or HeLa cells were seeded in 6-well plates till 60% confluence. To introduce siRNA, cells are transfected with scramble or EIF2AK1/2 siRNAs using Lipofectamine RNAiMAX for 3 days using standard protocol. To introduce DNA, cells are transfected with mammalian expression vectors using Lipofectamine 3000 for 2 days following standard protocol. The transfection ratio of DNA (μg) to Lipofectamine 3000 (μl) was 1 to 2. Transfected cells were lysed with 300 μl of Lysis Buffer (2% SDS, 50mM Tris, 2mM EDTA).

Protein sample collection and Western blotting: To collect protein samples, cultured cells were washed with PBS once, and then lysed with Lysis Buffer (2% SDS, 50mM Tris, 2mM EDTA). Protein concentration was measured and normalized with Pierce™ BCA Protein Assay Kit (ThermoFisher, Catalog No. 23225). The whole cell lysates were fractioned by SDS-PAGE (Bio-Rad 4–20% precast polyacrylamide gel) and transferred to nitrocellulose membranes using tank electroblotting transfer system according to the manufacturer's protocols (Bio-Rad). Nitrocellulose membranes were then incubated with 5% BSA in TBST (10 mM Tris, pH7.4, 150mM NaCl, 0.1% Tween-20) before incubating with primary antibodies. Primary antibodies used for Western blotting are listed below:

Antibody	Dilution	Source	Catalog
V5	1:5000	Invitrogen	R960-25
EIF2AK2	1:1000	Cell Signaling	12297S
GAPDH	1:5000	Cell Signaling	2118S
Phospho-EIF2S1	1:1000	Cell Signaling	3597S
GFP	1:2000	Invitrogen	A11122
ATF4	1:2000	Cell Signaling	11815S
EIF2S1	1:2000	Abcam	ab26197
ACTIN	1:5000	MP Biomedicals	ICN691001

Western blot image collection and analysis: Western blot images were acquired using a Bio-Rad ChemiDoc™ Imaging Systems and all images were collected by the imaging system within the linear range. Densitometric measurement of the bands were performed with ImageJ. Statistical analysis of the quantification was performed with R. We first confirmed the normality of the data (p = 0.05, Shapiro-Wilk test), and then used Student's t-test to measure the difference between groups. Data shown as mean ± standard error of mean. Number of replicates, n, quantified per test group is stated in the figure.

SUPPLEMENTAL REFERENCES

1 Kircher, M. *et al.* A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* **46**, 310-315, doi:10.1038/ng.2892 (2014).

2 Cooper, G. M. *et al.* Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res* **15**, 901-913, doi:10.1101/gr.3577405 (2005).

3 Jagadeesh, K. A. *et al.* M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. *Nat Genet* **48**, 1581-1586, doi:10.1038/ng.3703 (2016).

4 Adzhubei, I. A. *et al.* A method and server for predicting damaging missense mutations. *Nat Methods* **7**, 248-249, doi:10.1038/nmeth0410-248 (2010).

5 Pollard, K. S., Hubisz, M. J., Rosenbloom, K. R. & Siepel, A. Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res* **20**, 110-121, doi:10.1101/gr.097857.109 (2010).

6 Vaser, R., Adusumalli, S., Leng, S. N., Sikic, M. & Ng, P. C. SIFT missense predictions for genomes. *Nat Protoc* **11**, 1-9, doi:10.1038/nprot.2015.123 (2016).

7 Lupski, J. R. *et al.* Exome sequencing resolves apparent incidental findings and reveals further complexity of SH3TC2 variant alleles causing Charcot-Marie-Tooth neuropathy. *Genome Med* **5**, 57, doi:10.1186/gm461 (2013).

8 Danecek, P. *et al.* The variant call format and VCFtools. *Bioinformatics* **27**, 2156-2158 (2011).

9 Caylor, R. C. *et al.* Incidental diagnosis of tuberous sclerosis complex by exome sequencing in three families with subclinical findings. *Neurogenetics* **19**, 205-213, doi:10.1007/s10048-018-0551-y (2018).

10 Schmitz-Abe, K. *et al.* Unique bioinformatic approach and comprehensive reanalysis improve diagnostic yield of clinical exomes. *Eur J Hum Genet* **27**, 1398-1405, doi:10.1038/s41431-019-0401-x (2019).

11 Pezzani, L. *et al.* Atypical presentation of pediatric BRAF RASopathy with acute encephalopathy. *Am J Med Genet A* **176**, 2867-2871, doi:10.1002/ajmg.a.40635 (2018).