Bi-allelic variants in *DOHH*, catalyzing the last step of hypusine biosynthesis, are associated with a neurodevelopmental disorder

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This article describes mutations in *DOHH* as a cause of intellectual disability. These mutations decrease the activity of the eIF5A protein, whose dysfunction is known to cause intellectual disability.

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Bi-allelic variants in *DOHH*, catalyzing the last step of hypusine biosynthesis, are associated with a neurodevelopmental disorder

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Summary

Deoxyhypusine hydroxylase (DOHH) is the enzyme catalyzing the second step in the post-translational synthesis of hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] in the eukaryotic initiation factor 5A (eIF5A). Hypusine is formed exclusively in eIF5A by two sequential enzymatic steps catalyzed by deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH). Hypusinated eIF5A is essential for translation and cell proliferation in eukaryotes, and all three genes encoding eIF5A, DHPS, and DOHH are highly conserved throughout eukaryotes. Pathogenic variants affecting either *DHPS* or *EIF5A* have been previously associated with neurodevelopmental disorders. Using trio exome sequencing, we identified rare bi-allelic pathogenic missense and truncating *DOHH* variants segregating with disease in five affected individuals from four unrelated families. The *DOHH* variants are associated with a neurodevelopmental phenotype that is similar to phenotypes caused by *DHPS* or *EIF5A* variants and includes global developmental delay, intellectual disability, facial dysmorphism, and microcephaly. A two-dimensional gel analyses revealed the accumulation of deoxyhypusine-containing eIF5A [eIF5A(Dhp)] and a reduction in the hypusinated eIF5A in fibroblasts derived from affected individuals, providing biochemical evidence for deficiency of DOHH activity in cells carrying the bi-allelic *DOHH* variants. Our data suggest that rare bi-allelic variants in *DOHH* result in reduced enzyme activity, limit the hypusination of eIF5A, and thereby lead to a neurodevelopmental disorder.

The ubiquitously expressed eukaryotic translation factor 5A (eIF5A1) and its isoforms are the only cellular proteins that contain hypusine [N^{ε}-(4-amino-2-hydroxybutyl) lysine], an unusual amino acid. Hypusine is a derivative of lysine, formed post-translationally in the eIF5A precursor through two-step enzymatic reactions involving deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH)[1,2] (Figure S1). The first enzyme, DHPS, catalyzes the transfer of the 4-aminobutyl moiety from the polyamine spermidine to a specific lysine residue (Lys50 in human eIF5A) to form the intermediate deoxyhypusine residue.[3] Subsequently, DOHH hydroxylates this intermediate to complete the synthesis of hypusine,[4] which is necessary for activation of elF5A. Both DHPS and DOHH are specific for eIF5A, and no other protein is known to be modified by them.[1,2,5] (Note that in mammals there are two *eIF5A* genes, encoding highly conserved isoforms, *eIF5A1* and *eIF5A2*, both of which undergo hypusine modification. eIF5A1, commonly called eIF5A, is the predominantly expressed isoform. eIF5A can also be used to represent both forms collectively. Because eIF5A mostly exists as the hypusinated form in cells or tissues, eIF5A usually denotes natural eIF5A containing hypusine. However, eIF5A may also designate the unmodified protein. When a clear distinction of different forms is needed, the amino acid residue at or near the hypusination site specified in parentheses.)

All three proteins, eIF5A, DHPS, and DOHH, are highly conserved in eukaryotes.[5] The homozygous whole-body knockout of any of the three genes, *Eif5a*,[6] *Dhps*[6], or

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Dohh[7], is embryonic lethal in mice, suggesting critical roles for eIF5A and the hypusine modification in animal development. Deoxyhypusine containing protein was shown to be partially active in the stimulation of methionyl-puromycin synthesis, an *in vitro* assay for eIF5A activity.[8,9] Whereas eIF5A and DHPS are essential for all eukaryotes, DOHH is not essential in *Sacharomyces cerevisiae*,[4] suggesting that deoxyhypusine containing eIF5A can support growth in the yeast. However, in higher eukaryotes such as *Caenorhabditis elegans*[10] and *Drosophila*,[11] as well as in mammals,[7] DOHH is essential for development.

The structure of eIF5A, a 154-amino-acid-long acidic protein, with two domains (N and C), has been extensively studied. Hypusine is located in a flexible loop in the N domain (Figure S1). The conservation of eight residues flanking hypusine could indicate that these residues are important for eIF5A function or for the hypusine modification.[5] eIF5A is an essential translation factor that facilitates elongation at hard-to-make-bond sites (ribosome stalling sites) such as polyprolines, and it also stimulates translation termination.[9,12] As a key factor in translation, eIF5A is vital for cell proliferation and has also been reported to play a role in cell differentiation, apoptosis, and autophagy.[11] eIF5A has been implicated in several pathological human conditions, including cancer,[13] diabetes,[14] and retroviral infection.[15] The vital role of the deoxyhypusine modification of eIF5A in neurodevelopment in humans has been demonstrated by the identification of bi-allelic variants in *DHPS* (MIM: 600944), leading to loss of function in five individuals with developmental delay, intellectual disability (ID), microcephaly, and epilepsy (MIM: 618480).[16] Furthermore, *de novo* heterozygous variants in *EIF5A* (MIM: 600187) were associated with a neurodevelopmental disorder, the Faundes-Banka syndrome[17] (MIM: 619376) in seven individuals. However, it is not clear whether DOHH and the fully modified, hypusinated eIF5A are required for normal neurodevelopment in humans. Here we report on five individuals with neurodevelopmental disorder from four independent families who harbor bi-allelic variants in *DOHH* (MIM: 611262) and whose affected members have similar neurodevelopmental features.

Individual 1 was recruited through the French HUGODIMS program aimed at deciphering the molecular bases of intellectual disability (ID), and the study was approved by the ethics committee of the University Hospital of Nantes, France (Comité Consultatif sur les Traitements de l'Information en matière de Recherche dans le domaine de la Santé [CCTIRS] number: 14.556). Individuals 2 and 3 were recruited through a study approved by the "Cantonal Research Ethic Commission" of Zurich, Switzerland. Individuals 4 and 5 were recruited through the Discovery project at the University Hospital of Dijon, France (number 2016-A01347-44). All affected individuals were evaluated by a clinical geneticist and/or a pediatric neurologist, and molecular karyotypes performed prior to exome analyses were all normal.

Genetic analyses were performed by exome sequencing at different centers. Family 1 was sequenced at the University Hospital of Angers (France), family 2 at the University of Zurich (Switzerland), and families 3 and 4 at INSERM-Université de Bourgogne (France). Evaluated variant types included SNVs and small indels within exons and exon-intron boundaries. The *in silico* tools used to evaluate the potential impact of the variants included MutationAssessor, PROVEAN, Combined Annotation Dependent Depletion (CADD), and DANN. Specifically, *DOHH* variants were prioritized on the basis of their inheritance pattern, low allele frequencies (minor-allele frequency $< 1/1000$) in publicly available databases, and the predictions of impact of variants as assessed with the *in silico* tools previously cited. All variants were annotated with GenBank: NM_031304.5. Additional details on these methods are provided in Table S1. Exome sequencing identified bi-allelic variants in *DOHH* in individual 1. Subsequent individuals were identified through Genematcher.[18] The parents of each affected individual carry one *DOHH* variant allele and did not exhibit neurodevelopmental features, suggesting that bi-allelic variants are necessary for causing the phenotypes (Figure 1A).

The main clinical findings with regard to the five affected individuals carrying bi-allelic *DOHH* variants are summarized in Table 1 and are compared with features of individuals with *DHPS* or *EIF5A* variants in Table 2. The most prominent clinical features of individuals with bi-allelic *DOHH* variants were developmental delay and/or intellectual disability (5/5), microcephaly (5/5), visual impairment (nystagmus (3/5), strabismus (3/5), and cortical visual impairment (1/5)) and congenital heart malformations (3/5 individuals). Dysmorphic features, especially down-slanted palpebral fissures, were mostly secondary to the microcephaly (Figures 1B–1D). All individuals had an abnormal brain MRI (diffuse atrophy in individuals 1, 4, and 5 and hypomyelination in individuals 2 and 3) (Figures 1E and 1F). In family 2, individual 3 developed severe scoliosis (Figure 1G) requiring surgery at the age of 15. In this family, a heart defect was found only in individual 2, a girl, who also had a less severe epilepsy than her brother, reflecting intrafamilial phenotypic variability. A detailed clinical history of each affected individual is provided in the supplemental note. The affected individuals share a common phenotype involving developmental delay, intellectual disability, microcephaly, and abnormal MRI findings including cortical atrophy or hypomyelination. These phenotypic features partially overlap with those associated with *DHPS* bi-allelic loss-of-function variants or with *EIF5A de novo* variants (Table 2). Most notably, developmental delay and intellectual disability are consistent, and progressive microcephaly is found in 81% (13/16) of these individuals (Table 2). The high prevalence of microcephaly in these disorders is consistent with the involvement of hypusinated eIF5A in neuronal cell growth and survival *in vitro*[19] and is thought to be the consequence of an increased rate of neuronal cell death.

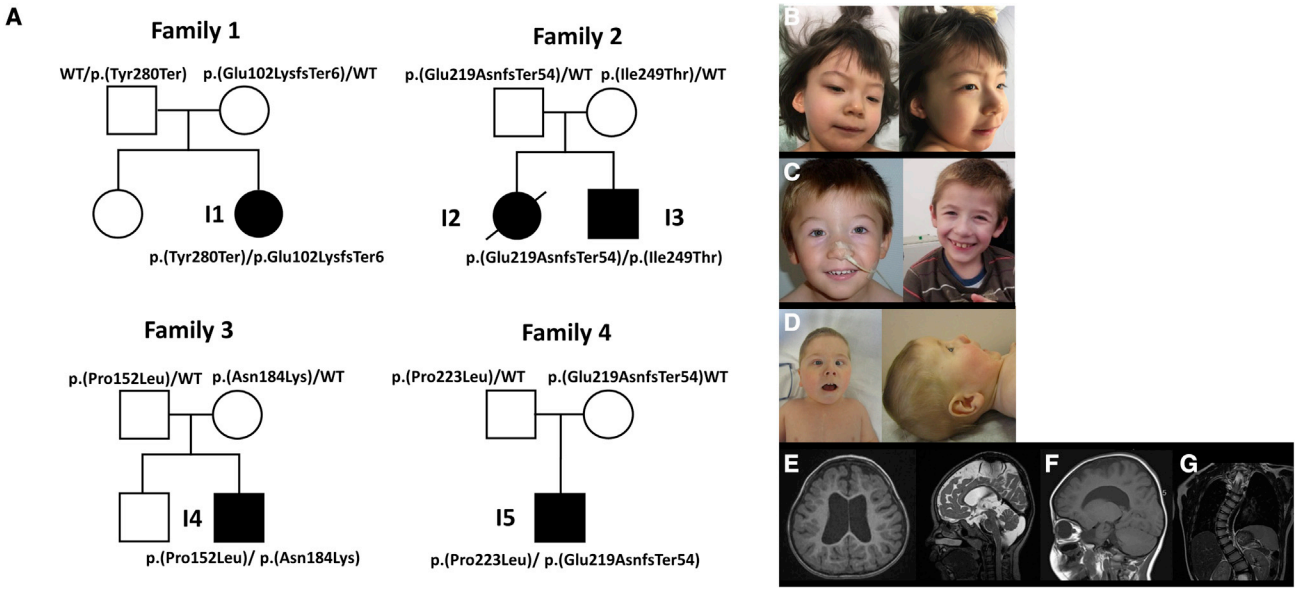


Figure 1. Clinical features of affected individuals
(A) Pedigrees of the four families.
(B) Individual 1 at age 5 years.
(C) Individual 4 at age 4 years (left) and 9 years (right). Note G tube feeding and down-slanted palpebral fissures.
(D) Individual 5 at age at age 22 months.
(E) Brain MRI of individual 1 (T1-weighted axial view on the left and T2-weighted sagital view on the right) at age 3 years. Note the diffuse cortical atrophy with moderate ventricular dilatation, cerebellar atrophy, and thin corpus callosum.
(F) A brain MRI (T1-weighted sagittal view) of individual 4 at age 5 years highlights diffuse cortical atrophy with mild ventricular dilatation and thin corpus callosum.
(G) An MRI of individual 3 at age 10 years shows the severe scoliosis.

The *EIF5A*-associated disorder seems slightly less severe than *DOHH*- or *DHPS*-associated disorders in that most individuals are able to walk and speak and do not have seizures. This might be the consequence of variable residual activity of eIF5A across the three disorders; individuals with *de novo EIF5A* variants have one remaining allele coding a normal protein, whereas individuals with *DHPS* or *DOHH* bi-allelic variants have a marked decrease of eIF5A hypusination. Further descriptions of individuals carrying pathogenic variants in each of these genes are needed to confirm this correlation. Ophthalmologic (strabismus, nystagmus, and cortical visual impairment) and cardiac (congenital malformations, arythmia, cardiomyopathy) abnormalities are found in both *EIF5A*- and *DOHH*-associated disorders. The hypusination of eIF5A in mice was shown to stimulate the expression of MyoD,[20] a known myogenic transcription factor involved in heart differentiation and its reduction might be involved in heart malformations found in individuals 2, 4, and 5 as well as in three of the individuals with *EIF5A de novo* variants. Ophthalmologic impairment was not reported in *DHPS*-related disorders (Table 2). This difference might be due to the limited number of reported individuals, ascertainment bias, and the incomplete penetrance of this feature. Brain MRI abnormalities (5/5) in the DOHH cohort contrast with normal brain MRIs of the seven individuals with *DHPS* or *EIF5A* variants. This difference could be the result of a bias due to the age at which the MRI was performed. Indeed, in DOHH-associated disorders, microcephaly is progressive, and MRIs could have been performed before the onset of lesions.

Spermidine supplementation was reported to partially rescue two different models (i.e., yeast and zebrafish) of eIF5A impairment,[17] hereby suggesting an interesting possibility for treating hypusination-related disorders. However, an accurate description of the natural history of these disorders will be crucial for evaluating the efficacy of this potential therapy. According to our observations, the assessment of head circumference and of the cerebral volume could be good markers for evaluating the efficacy of a therapy with spermidine.

DOHH is a unique nonheme diiron monooxygenase with a superhelical structure (Figure 2A).[21] It is distinct from other protein hydroxylases, such as proline 4-hydroxylases and lysyl hydroxylases, in the structure and the reaction mechanism. It consists of eight tandem helical hairpins, termed HEAT repeats (Figures 2B and 2C). Four strictly conserved His-Glu (HE) pairs critical for anchoring the diiron or the substrate protein and for catalysis are indicated in Figures 2B and 2C. The missense variants reported here are located in the highly conserved regions of the enzyme (Figure 2D) and are predicted to alter the enzyme structure and/or activity. In the case of individual 1 (c.304delG [p.Glu102LysfsTer6]/c.840T>A [p.Tyr280Ter]), the first variant is in exon 3, and its transcript would therefore be predicted to undergo nonsense-mediated decay (NMD); hence,

Table 1. Main findings in individuals with bi-allelic variants in *DOHH*

	Family 1	Family 2		Family 3	Family 4
	Individual 1	**Individual 2**	**Individual 3**	**Individual 4**	**Individual 5**
Gender	female	female	male	male	male
Age at last evaluation	7 years 10 months	15 years (age of death)	17 years	14 years 2 months	25 months (age of death)
Ethnicity	South Korean (mat)/French (pat)	Bosnia (mat)/Croatian (pat)	Bosnia (mat)/Croatia (pat)	French	Belgian
Variants (recurrent variants marked with asterisk)	p.Glu102LysfsTer6 (mat); p.Tyr280Ter (pat)	p.(Ile249Thr) (mat); *p.Glu219AsnfsTer54 (pat)	p.(Ile249Thr) (mat); *p.Glu219AsnfsTer54 (pat)	p.(Pro152Leu) (pat); p.(Asn184Lys) (mat)	p.(Pro223Leu) (pat); *p.(Glu219AsnfsTer54) (mat)
Prenatal issues/ Gestational age	none/39 WG	none/40 WG	none/40 WG	increased nuchal translucency; chylothorax/ 38 WG	cardiopathy/39 WG
Neonatal	hypotonia; temperature instability; poor feeding	none	none	hypotonia; poor feeding	hypotonia
Birth weight	2,670 g (−1.36 SD)	3,000 g (−1.12 SD)	4,210 g (+1.05 SD)	3,290 g +(0.87 SD)	3,040 g (−0.8 SD)
Birth length	46 cm (−1.76 SD)	51 cm (−0.32 SD)	55 cm (+2 SD)	49 cm (−0.44 SD)	49.5 cm (−0.2 SD)
Birth HC	34 cm (0 SD)	NA	NA	34 cm (−0.8 SD)	34.1 cm (−0.8 SD)
Age at last evaluation	5 years 6 months	7 years 9 months	15 years 9 months	14 years, 2 months	22 months
Height or length at last evaluation	95 cm (−3 SD)	114.8 cm (−1.8 SD)	146.5 cm (−3.28 SD)	128.5 (−2.5 SD)	79.2 cm (−2.5 SD)
Weight at last evaluation	11.4 kg (−3 SD)	16.8 kg (−2.56 SD)	29.2 kg (−4.42 SD)	23 kg (−2,5 SD)	10 kg (−2 SD)
HC at last evaluation	44.5 cm (−3.8 SD)	46.5 cm (−4.86 SD)	47.4 cm (−5.98 SD)	50 (−3SD)	43.6 cm (−3.5SD)
Sitting/ walking / speaking	not acquired	13 months	29 months/ /	20 months	not acquired
Walking	not acquired	3 years 6 months	4 years 9 months	7 years	not acquired
Speaking	not acquired	not acquired	not acquired	first words at age 7 years	not acquired
Behavioral	none	shy, stubborn, hyperactive	happy demeanor	happy demeanor	happy demeanor
Clinical seizures	none	2 occurences of tonic-clonic fever-associated seizures at age 5 years and 8 years	generalized epilepsy; drop attacks at the age of 11 years; myoclonus at the age of 15 years; fever-associated focal seizures then generalized seizures at the age of 16 years	none, normal EEG at age 2 years	generalized epilepsy with hyperextension of upper limbs
Tonus	severe hypotonia	hypotonia	Hypotonia; truncal ataxia	hypotonia	severe hypotonia
Brain MRI	3 years: diffuse cerebral atrophy with overly visible furrows and ventricular dilation due to atrophy; cerebellar atrophy; thin corpus callosum	4 years: hypomyelination; cortical atrophy	2 years: hypomyelination; corpus callosum hypoplasia	5 years: cortical atrophy; ventricular dilatation; thin corpus callosum	1 year: cortical atrophy; enlarged ventricles; thin corpus callosum

(*Continued on next page*)

Table 1. *Continued*

	Family 1	Family 2		Family 3	Family 4
	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5
Cardiac features	none	incomplete AV canal; ASD type 2; VSD; global progressive cardiac insufficiency with severe mitral-valve and aortic-valve insufficiency and severe cardiomegaly, which led to exitus letalis; mitral- and aortic-valve replacement and reconstruction was performed; ECG: AV block (age 8 years)	ECG: AV block type 1; reduced ventricular function with EF 82%; Holter ECG: monomorphic ventricular extasystolia; shortened PQ period under effort	ASD, OS	Shone syndrome with aortic coarctation; bicuspid aortic valve; tricuspid-valve insufficiency; ASD; VSD
Visual impairment	horizontal nystagmus; cortical visual impairment	strabismus convergens alternans	strabismus convergens; ablatio retinae left with unilateral amaurosis at age 16 years	horizontal nystagmus	nystagmus; strabismus; severe myopia
Recurrent infections	no	yes	yes (pneumonia)	no	yes (died at 25 months from a pneumonia)

Abbreviations are as follows: ASD, atrial septal defect; AV, atrioventricular; HC, head circumference; NA, not available; OS, ostium secondum; SD, standard deviation; VSD, ventricular septal defect; and WG, weeks of gestation.
Short stature is defined as a length below −2 SD, underweight as a weight below −2 SD, and microcephaly as an HC below −2 SD.

no protein would be produced. Even if a small fraction of the truncated transcript that escaped NMD is translated, the truncated protein, containing only two of the four critical HE sites,[22] would be inactive. The second variant contains the four HE motifs, but because it is missing 22 amino acids from the C terminus, it might have a low partial activity. The truncating variant, c.654_655insAACC (p.Glu219AsnfsTer54], found in individuals 2, 3, and 5 (Figure 1A), is expected to be devoid of activity because it lacks the fourth critical HE (H240-E241) motif (Figures 2B and 2C).[22] The four missense variants, namely, c.455C>T (p.Pro152Leu), c.552C>A (p.Asn184Lys), c.668C>T (p.Pro223Leu), and c.746T>C (p.Ile249Thr), affect amino acid residues highly conserved in most species, including yeast and plants (Figure 2D). After data including functional assessment, frequency in population databases, mode of inheritance, segregation, and phenotypic overlap were taken into consideration, all seven variants were classified as likely pathogenic or pathogenic according to ACMG classification criteria[23] (Table S2), and no other relevant candidate variants were found (Table S3). In line with the lethality of homozygous DOHH knockout in mice,[7] all reported individuals are thought to carry at least one hypomorphic variant with residual DOHH activity.

We examined the amounts of DOHH in fibroblasts derived from three controls and three affected individuals by immunoblotting (Figure 3). No samples from individuals 2 or 5 (both deceased) were available for this eIF5A protein analysis. The amount of DOHH protein (intact 33 kDa, 302 amino acids) was shown to be drastically reduced in individuals 1,3, and 4. Given that the DOHH antibody (ab197587) used for the immunoblot was derived from a synthetic peptide sequence in the C-terminal domain of the protein, we could not determine the presence or stability of the three C-terminal truncated variants, p.Glu102LysfsTer6, p.Tyr280Ter, and p.Glu219AsnfsTer54. In individual 1, who carries two of these C-terminal truncated variants, no DOHH signals were detected (Figure 3). Individual 3, harboring one truncated variant, p.Glu219AsnfsTer54, and a missense variant, p.Ile249Thr, showed a weak DOHH signal (Figure 3A). In the case of individual 4, carrying two missense variants, p.Pro152Leu and p.Asn184Lys, the 33kDa DOHH band was also drastically reduced. The severe reduction of the DOHH missense variants in individuals 3 and 4 (both to ∼20% of controls, average of three immunoblots, Figure 3B) could be due to protein instability, given that SpliceAI and ADA scores do not support an impact on splicing for the three missense variants.

We also assessed the effects of these variants on expression and activity by using GST-DOHH recombinant proteins produced in *E. coli* (Figure S2). The expression of the GST-fusion peptide of the first truncated variant (V1 p.Glu102LysfsTer6, ∼38 kDa) was extremely low, whereas the expression of two other truncated variants (V2 p.Tyr280Ter and V3 p.Glu219AsnfsTer54) and three missense variants appeared normal (Figure S2A). V1 and V3 not containing all four critical HE motifs were inactive as predicted, whereas V2 containing all four HE motifs, but with a truncation of 20 C-terminal residues, showed low partial activity (Figure S2B). All three missense variants (V4–V6) displayed activities at a similar level to that of the WT control, suggesting that the limitation of cellular DOHH activity in individuals carrying these missense variants is most likely due to reduced stability rather than the loss of activities of these missense variants.

Critical *in vivo* biochemical evidence for the impairment of DOHH activities in the affected individuals was obtained by two-dimensional gel electrophoresis of proteins of fibroblasts derived from the control and three affected individuals, individuals 1, 3, and 4 (Figure 4A). Separation of the unhypusinated eIF5A forms from the hypusinated form on 2D gels was previously reported.[24] All three eIF5A forms at

Table 2. Comparison of features evidenced in individuals with variants in *DOHH*, *DHPS*, and *EIF5A*

	Total for *DOHH* individuals	Individuals with bi-allelic *DHPS* variants reported by Ganapathi et al.[16]	Individuals with *de novo* heterozygous *EIF5A* variants reported by Faundes et al.[17]
Gender	3 males, 2 females	1 male, 4 females	3 males, 4 females
Variants	7 variants: one stop gain, two frameshift, 4 missense; compound heterozygous	4 variants: 1 missense (recurrent), one start loss, one splice (recurrent), one inframe deletion; compound heterozygous	7 variants: 5 missense, 1 stop gain, 1 frameshift; *de novo* heterozygous
Prenatal	2/5 (cardiac malformation)	4/5 (preeclampsia)	5/6 (4 IUGR, 1 fetal ascites)
Neonatal	poor feeding 2/5; temperature instability 1/4; hypotonia 3/5	prematurity 2/5; temperature instability 1/5	poor feeding 3/7
Birth: short stature	0/5	0/4	3/7
Birth: underweight	0/5	0/5	3/7
Birth: congenital microencephaly	0/3	0/3	3/7
Last evaluation: short stature	4/5	2/5	2/7
Last evaluation: underweight	4/4	0/5	2/7
Last evaluation: microcelphaly	4/4	3/4	5/7
Sitting	3/5 able to sit	5/5 able to sit	6/7 able to sit
Speaking	2/5 able to walk	5/5 able to walk	6/6 able to walk (delayed in 3)
Walking	1/5 able to speak	2/5 able to speak	6/6 able to speak
Intellectual disability	5/5	5/5	7/7 (1 mild, 1 mild/moderate, 4 moderate, 1 moderate/severe)
Behavioral	happy demeanor 3/5	autism 1/5; hand flapping 3/5	autism 2/7; ADHD 1/7
Clinical seizures	3/5	4/5	0/7
Tonus	hypotonia 5/5	Hypotonia 4/5; spasticity 1/5	hypotonia 1/5
Brain MRI	abnormal 5/5; cortical atrophy 4/5	Normal 4/4	Normal 2/2
Cardiac malformation	3/5	0/5	3/4
Visual impairment	nystagmus 3/5; cortical visual Impairment 1/4	0/5	strabismus 3/7; glaucoma 1/7
Recurrent infections	3/5	1/5	0/7

Abbreviations are as follows: IUGR, intrauterine growth restriction; ADHD, attention deficit hyperactivity disorder

isoelectric points (pIs) of 5.1, 5.2, and 5.3 of Figure 4 were confirmed as eIF5A by mass spectrometry (Table S4). In control fibroblasts, the majority of eIF5A is the hypusinated form [eIF5A(Hpu)] (pI 5.3) (Figure 4A) because eIF5A efficiently undergoes hypusine modification under normal conditions. However, in three affected individuals, an increased accumulation of the unhydroxylated, deoxyhypusine-containing form [eIF5A(Dhp)] (pI ~5.2) was observed (Figure 4A). The identity of this protein (pI 5.2) as eIF5A(Dhp) is supported by a previous study[25] in which a clear separation of eIF5A(Dhp) protein from eIF5A(Hpu) had been shown on a 2D gel of lysates from HeLa cells treated with a DOHH inhibitor, mimosine.[25] A new protein with the same molecular weight but with a lower pI accumulated in the presence of mimosine and was validated as eIF5A(Dhp) by detection of deoxyhypusine in the acid hydrolysate of the protein spot, whereas hypusine was detected in the hydrolysate of eIF5A(Hpu) protein of untreated cells. The identity of eIF5A at pI 5.2 (Figure 4, blue arrow) as eIF5A(Dhp) was further supported by a marked increase in this protein spot on a 2D gel (run under the same conditions as in Figure 4) of lysates from 293T cells treated with another DOHH inhibitor, ciclopirox (CPX) (Figure S3). Importantly, in fibroblasts derived from affected individuals 1, 3, and 4, there were

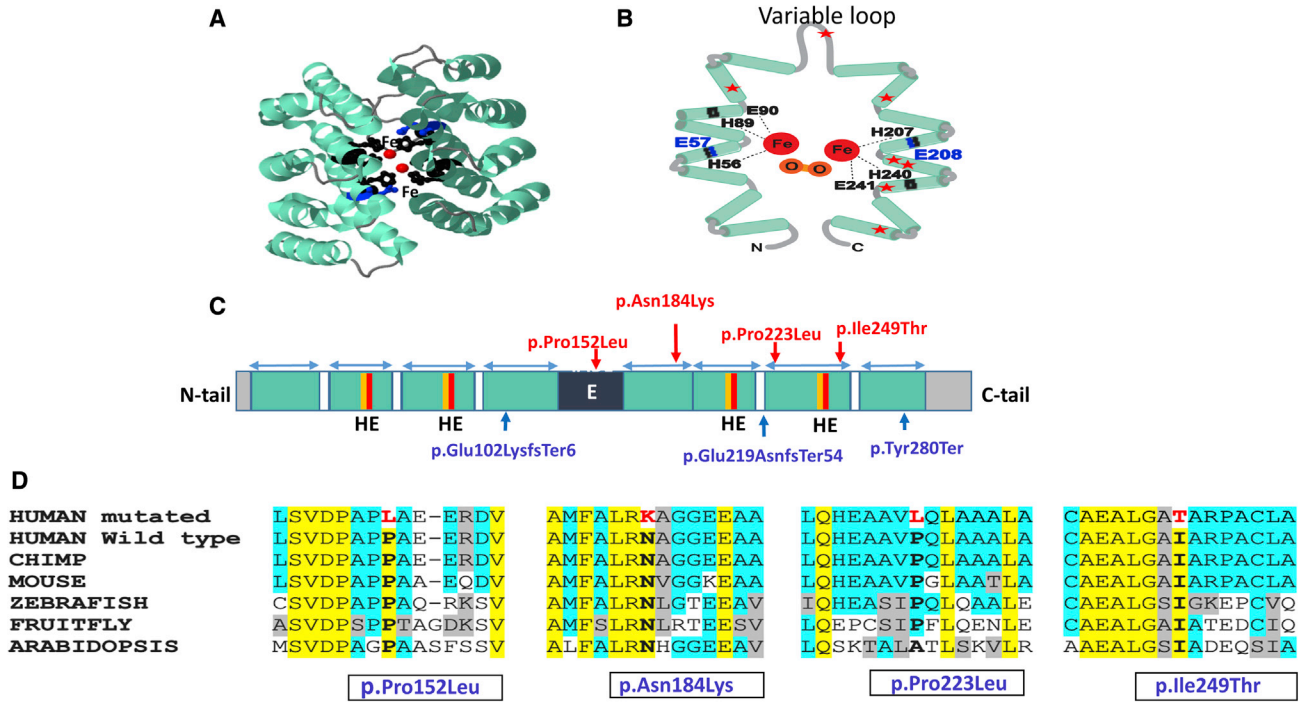


Figure 2. Crystal structure of DOHH, model of its active site, its primary sequence, and the amino acid sequences at the sites of *DOHH* missense variants

(A) Crystal structure of human DOHH peroxo-diiron (III) intermediate (PBD: 4D4Z) (21) consisting of eight helical hairpins (HEAT repeats).

(B) Active-site diagram of DOHH peroxo-diiron intermediate with diiron center (red) and critical amino acid residues involved in binding diiron (black) and the protein substrate (blue). The sites of variants are indicated by red stars.

(C) A bar diagram of the primary sequence of DOHH. The eight HEAT repeats are indicated with green boxes, the sites of DOHH truncation are indicated with blue arrows on the bottom, and the sites of missense variants are indicated with red arrows on top of the bar. The four HE sites critical for DOHH activity are indicated by yellow and red bars.

(D) Amino acid sequences at the missense-variant sites reveal high conservations in six eukaryotic species. Yellow, total conservation; aqua blue, conservation in more than three species; grey, conservative replacement.

remarkable increases (~2.7- to 4.5-fold) in eIF5A(Dhp) compared to the control, whereas the relative levels of hypusinated eIF5A [eIF5A(Hpu)] were reduced (Figure 4B). These results provide strong biochemical evidence that the variant DOHH activities were indeed reduced in fibroblasts of the affected individuals, leading to accumulation of eIF5A(Dhp). Thus, bi-allelic *DOHH* variants might result in a significant reduction in the function of hypusinated eIF5A, thereby impairing translation activity and leading to a neurodevelopmental disorder. Although eIF5A(Dhp) was shown to be partially active in a methionyl-puromycin synthesis assay *in vitro* and can support the growth of a DOHH-null *S. cerevisiae* strain, albeit at a slightly reduced rate (4), it does not support animal development.[7,10,11] From the cryo-electron microscopy structure of hypusinated eIF5A bound to 80S ribosome,[26] the hydroxyl group of the hypusine side chain is predicted to form a hydrogen bond with the phosphate backbone of 25S ribosomal RNA. This hydrogen bond could be critical for the translational activity of eIF5A in animal ribosomes.

Translational fidelity and efficiency are vital for the survival of living organisms. Translational errors can lead to an increase in deleterious proteins, while reducing the functional proteins.[27] The identification of variants of *EIF5A, DHPS*, or *DOHH* as a genetic basis of human neurodevelopmental disorders is not surprising in that variants in other translation factors, including alanyl tRNA synthetase (AARS) and eukaryotic translation elongation factors 2 (EF2) and 1a2 (EF1a2), and eukaryotic translation initiation factor 2S3 (EIF2S3)[28] have been similarly associated with neurodevelopmental disorders.

The brain appears to be the organ most sensitive to a deficiency in active eIF5A; such a deficiency can be toxic to neural cells. However, it is not known whether the neurodevelopmental features of individuals with variants in EIF5A, DHPS, or DOHH are due to a reduced translation efficiency in general or a reduction in specific, eIF5A-dependent factors essential for brain development.

Of note, mutant mice with a temporal or region-specific knockout of Eif5a or Dhps in the forebrain exhibit impairment in growth, lifespan, brain development, and cognitive functions,[29] close to the phenotypes observed in human. These models will be very useful in developing therapies against neurodevelopmental disorders caused by variants of EIF5A, DHPS, or DOHH.

In summary, pathogenic variants in *DHPS* or *DOHH*, both involved in hypusination of eIF5A, as well as pathogenic variants in *EIF5A* itself, result in very similar

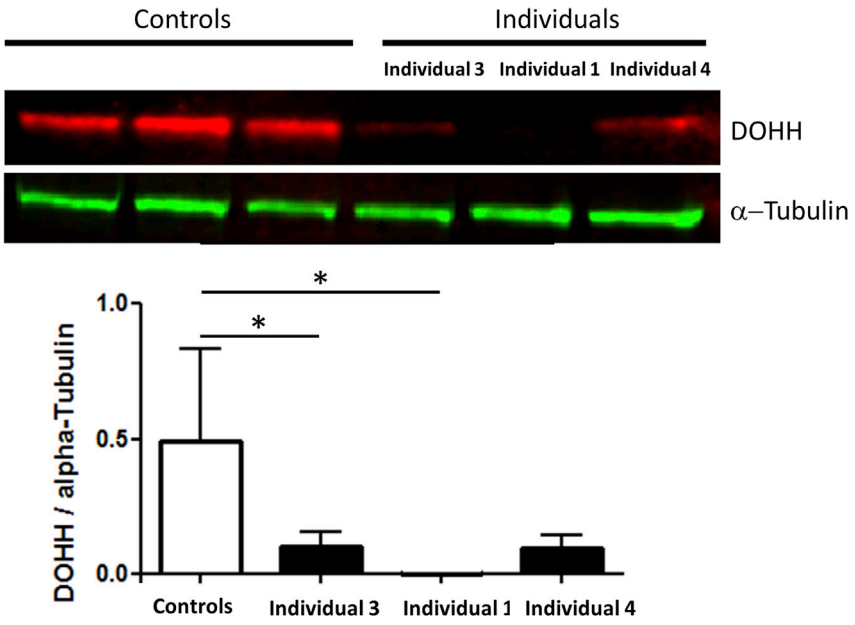
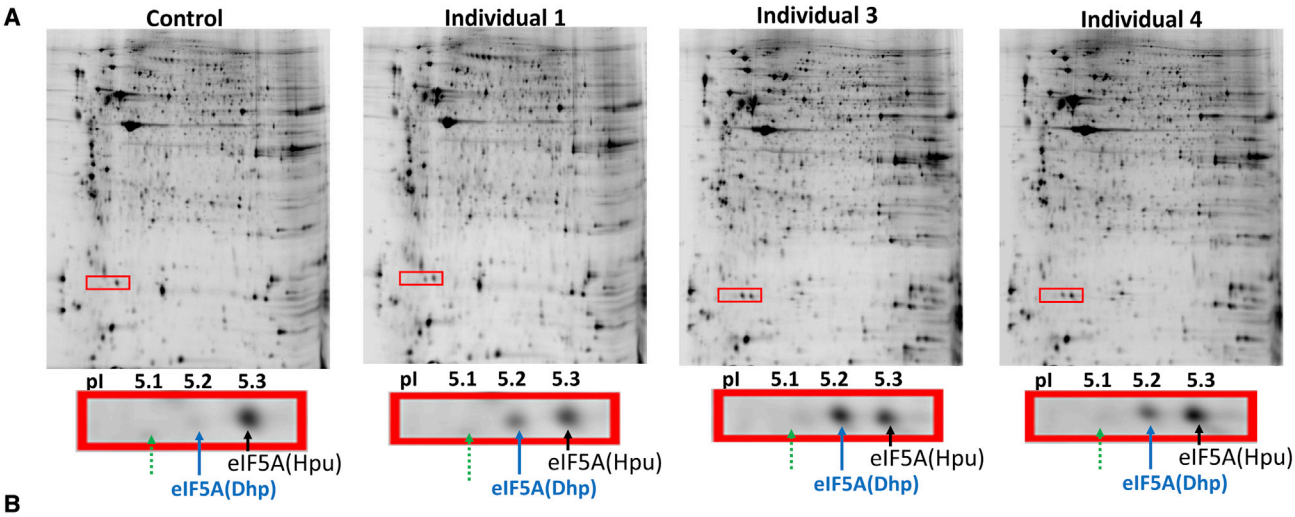


Figure 3. Immunoblot analysis of DOHH proteins in control and affected individuals
Cellular proteins (25 μg) of fibroblasts from three control individuals and affected individuals 1, 3, and 4 were analyzed by immunoblotting with antibodies against DOHH (ab197587) and α-tubulin (ab7291).
Upper panel: Representative blots for three controls and three patients are shown.
Lower panel: Results are expressed as mean values obtained for three controls and three patients ± standard deviations of the mean obtained in four independent experiments. Statistical comparisons were made with the Mann-Whitney U test; differences were considered to be significant at $p < 0.05$.

neurodevelopmental disorders, including global developmental delay, intellectual disability, facial dysmorphism, and microcephaly. We propose to name this group of disorders eIF5A and hypusination-related disorders.

Data and code availability

The *DOHH* variants were submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) (GenBank: NM_031304.5) under accession numbers ClinVar: VCV001285602, VCV001285600, VCV00128



B

pI	Protein	Individual 1/control	Individual 3/control	Individual 4/control
5.1	eIF5A(AcK47, K50)	-1.13	1.38	1.04
5.2	eIF5A(Dhp)	3.08	4.55	2.72
5.3	eIF5A(Hpu)	-1.15	-1.85	-1.16

Figure 4. Accumulation of unhydroxylated eIF5A, eIF5A(Dhp) in fibroblasts from affected individuals harboring bi-allelic *DOHH* variants
2-D DIGE (two-dimensional difference gel electrophoresis) was performed by Applied Biomics as described in the supplemental information. Equal amounts (30 μg) of proteins were first separated by isoelectric focusing, followed by SDS-PAGE.
(A) The control fibroblast contains the main form, hypusinated eIF5A, eIF5A(Hpu) (pI 5.3, black arrow). The fibroblasts from three affected individuals show a reduction in eIF5A(Hpu) and an accumulation of unhydroxylated eIF5A, eIF5A(Dhp), (blue arrow) pI 5.2, indicating a limitation of DOHH activity in these cells. The green arrow indicates the position of the eIF5A precursor, eIF5A(AcK47, K50), pI. 5.1, which accumulates upon inhibition or knockdown of DHPS.
(B) The n-fold changes in the levels of the three eIF5A forms in the affected individuals relative to the control are shown.

5601, VCV001285603, VCV001285606, VCV001285605, and VCV001285604.

The exome-sequencing datasets supporting this study have not been deposited in a public repository because of ethical restriction but are available from the corresponding author (A.Z.) on request.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2022.06.010.

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Declaration of interests

The authors declare no competing interests.

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Web resources

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/submitters/26957.
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
gnomAD browser, http://gnomad.broadinstitute.org/
OMIM, https://www.omim.org/

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