

TWO NOVEL *EIF2S3* MUTATIONS ASSOCIATED WITH SYNDROMIC INTELLECTUAL DISABILITY WITH SEVERE MICROCEPHALY, GROWTH RETARDATION, AND EPILEPSY

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Abstract

X-chromosome exome sequencing was performed to identify the genetic cause of syndromic intellectual disability in two unrelated families with suspected X-linked inheritance. In both families, affected males presented with severe intellectual disability, microcephaly, growth retardation, and epilepsy. A missense mutation (c.777T>G p.(Ile259Met)) and a frameshift mutation (c.1394_1397del p.(Ile465Serfs*4)) were identified in the *EIF2S3* gene in the hemizygous state in affected patients, and in the heterozygous states female obligate carriers. A missense mutation in *EIF2S3*, coding for the gamma-subunit of the translation initiation factor eIF2, was reported once in a family presenting with similar clinical features. Morpholino-based knockdown of the zebrafish *EIF2S3* ortholog (*eif2s3*) recapitulates the human microcephaly and short stature phenotype, supporting the pathogenicity of

the identified variants. Our data confirm that *EIF2S3* mutation is implicated in a rare, but recognizable, form of syndromic intellectual disability.

Key words: X-exome sequencing; microcephaly; epilepsy; X-linked intellectual disability; zebrafish; morpholino

INTRODUCTION

X-linked intellectual disability (XLID) is a group of genetically heterogeneous disorders, accounting for 10–15% of all males with intellectual disability (ID) [Ropers and Hamel, 2005]. Over 150 syndromes have been described [Tarpey et al., 2009; Lubs et al., 2012], among which the most common Fragile X syndrome is responsible for 1–2% of all patients with ID. To date, mutations of a few 100 genes have been reported on the X-chromosome thanks to new approaches of next-generation sequencing [Lubs et al., 2012; Piton et al., 2013; Tzschach et al., 2015]. Hu et al. [2016] investigated by X-exome sequencing 405 families with XLID and resolved up to 58% of patients negative for fragile X mutations. This suggested that new genes involved in XLID syndromes are still to be identified.

Here, we report two unrelated families with a syndromic XLID characterized by severe microcephaly, growth retardation, seizures, and intellectual disability in affected males.

CLINICAL REPORT

FAMILY 1

Patient III.2 (Fig. 1A) was the second male child of non-consanguineous and healthy Belgian parents. Pregnancy was complicated by chronic fetal distress. Cesarean delivery was performed at 37 weeks of pregnancy. Birth weight was 1,910 kg (–2.5 SD), for a length of 42 cm (–2.5 SD), and an OFC of 28.5 cm (3.5 SD). From birth, he showed global developmental delay and spastic quadriplegia. He sat alone at the age of 3 years and never walked independently. Microcephaly became more marked with age and noted at –6 SD at the age of 10, and –8 SD at 15 years. He had severe intellectual disability (FSIQ $\leq$ 40, WISC-IV test), with autistic features characterized by stereotypic movements, restricted and repetitive behaviors, and difficulties in socialization. Language skills were limited to less than five words; he had difficulties in performing daily tasks. Epilepsy with tonic-clonic seizures started at the age of 9 months, and was controlled by lamotrigine and levetiracetam. At the age of 10, several episodes of morning hypoglycemia were noted for which extensive analyses were negative. Low growth hormone levels were observed and delayed puberty was treated by testosterone injection. At 14 years, chronic pancreatitis was diagnosed after a spell of unexplained crying, and the proband was treated by Wirsung sphincterotomy. On his most recent physical examination at 15 years of age, height was 108 cm (–8.7 SD), weight was 22 kg (–7 SD), and his OFC was 43.5 cm (–8 SD). He had large ears (Fig. 1B). He was myopic and had left convergent strabismus. He had axial hypotonia with spasticity of the four limbs. He had brisk reflexes with Babinski sign. He had a micropenis. Brain MRI showed a global reduction of white matter volume and a thin corpus callosum (Fig. 1D–E), without structural anomaly. The pituitary gland and stalk were normal. Metabolic screening (blood, urine, and CSF) was

performed, and was negative. The child died at 17 years from severe respiratory distress and multi-organic failure.

Patient III.3 (Fig. 1A) is the male sibling of patient III.2, born from the same mother but from another father. Pregnancy was unremarkable. Cesarean delivery was performed at 34 weeks of pregnancy. Birth weight was 1,400 kg (−2.5 SD), length was 41 cm (−2.5 SD), and an OFC of 25.5 cm (−3.5 SD). He had progressive developmental delay and spastic quadriparesis. He could stand up a few seconds and walk with aid. Microcephaly was severe and noted at 42 cm at the age of 10 years (−6 SD), and unchanged until 18 years (−8.5 SD). He had severe intellectual disability (FSIQ ≤ 40, WISC-IV test) without autistic features. He had a friendly behavior. He pronounced only a few sounds. He has had no recognized seizures. He had skeletal immaturity (3 years delayed bone age) with partial growth hormone deficiency, and delayed puberty. Hypoglycemia with impaired consciousness was noticed during a functional insulin test. Brain MRI was performed at the age of 7 and showed globally reduced white matter volume with a thin corpus callosum (Fig. 1F–G). At his last examination at the age of 18 years, his weight was 23 kg (−7 SD), height 115 cm (−9 SD), and his OFC was 42 cm (−8.5 SD). Left convergent strabismus was reported (Fig. 1C). He had prepubertal external genitalia. He had axial hypotonia with spasticity of the four limbs and pyramidal syndrome.

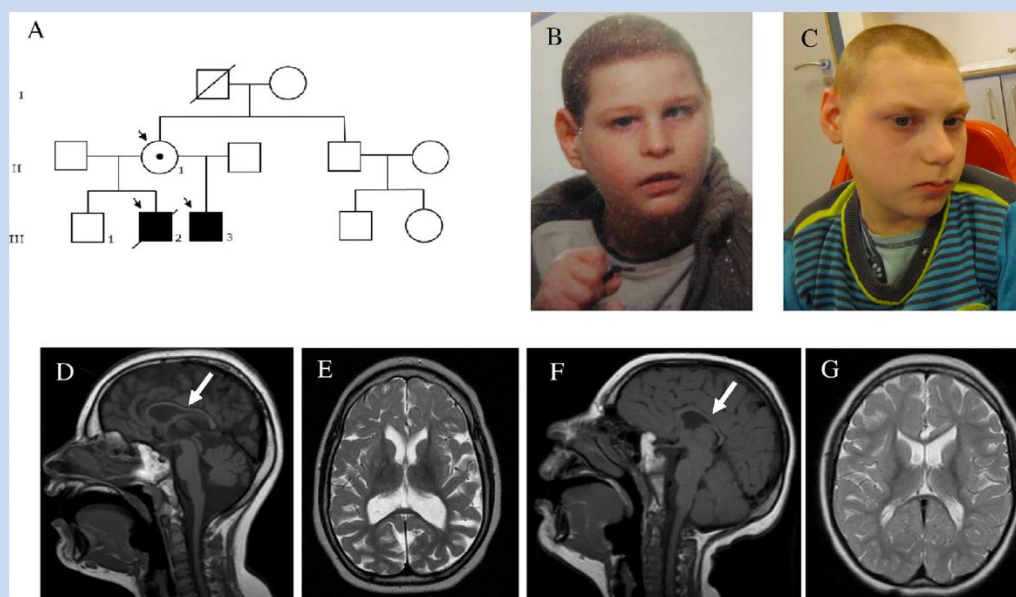
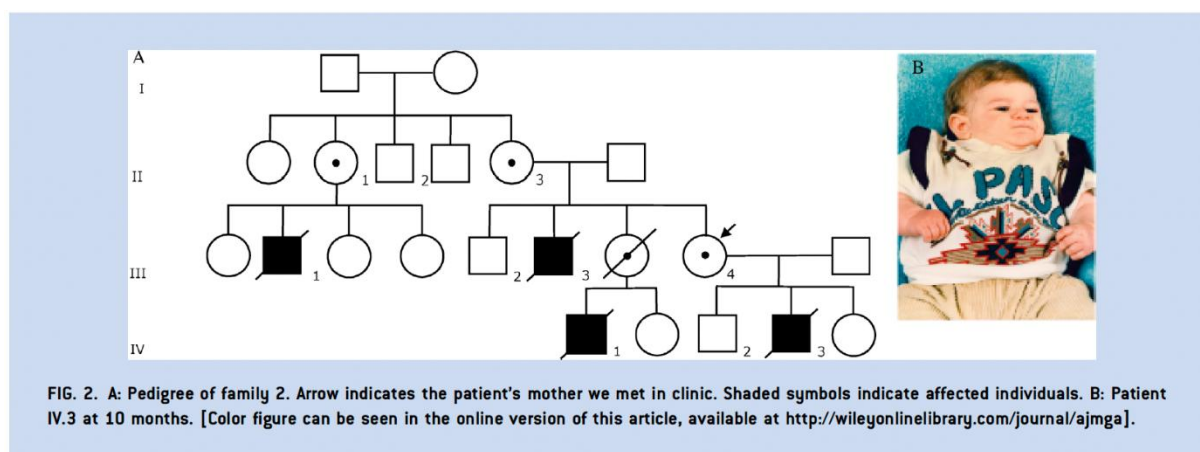


FIG. 1. A: Pedigree of family 1. Probands and their mother (arrows) were seen in clinic. Shaded symbols indicate affected individuals. B–C: Proband III.2 at 15 years (B) and proband III.3 at 18 years (C). Note the severe microcephaly, left strabismus, and large ears. D–G: MRI findings of proband III.2 (D–E) and proband III.3 (F–G). Sagittal T-1 weighted brain MRI images (D, F) and axial T-2 weighted brain MRI images (E, G) show a global low white matter volume and a thin corpus callosum (white arrow). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/ajmga>].

FAMILY 2

This family is originated from Southern Spain. Patient IV.3 (Fig. 2A) was born at 41⁺⁶ gestational weeks after an uneventful pregnancy. His parameters at birth showed a weight of 2,910g (−2SD), a length of 46cm (−3 SD), and an OFC of 31.5cm (−3 SD). He had micrognathia (Fig. 2B), micropenis, and generalized hypertonia with an absence of visual contact. Hypoglycemia was noted after a few hours of life. He developed a severe and global developmental delay. He never achieved head control and had no ocular pursuit at the age of 11 months. He had onset of tonic-clonic seizures at 10 months and died at 12 months of multisystemic failure. The height was 60cm (−4.5 SD) and the OFC was 35.5cm (−7 SD). Brain MRI showed a thin corpus callosum without structural anomalies. Autopsy did not identify any other macroscopic or microscopic anomaly, and confirmed a thin corpus callosum.

Three other male family members (patients III.1, III.3, IV.1) (Fig. 2A) were reported with a similar phenotype including neonatal hypoglycemia, severe microcephaly, developmental delay, micropenis, short stature, epileptic seizures, and early death, but were unavailable for molecular studies as they died long ago.



METHODS AND RESULTS

X-EXOME SEQUENCING

Written consent was provided for genetic studies, performed in routine clinical care and for publication of pictures from both families. This work was not considered research in our Institute. Blood samples were collected from the probands (III.2 and III.3, Fig. 1A), unaffected brother (III.1, Fig. 1A) and their mother (II.1, Fig. 1A) in family 1, and from three obligate female carriers (II.1, II.3, III.4, Fig. 2A) and three unaffected males (II.2, III.2, IV.2, Fig. 2A) in family 2. Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols. First genetic investigations including Fragile X analysis, molecular karyotype and a gene panel sequencing of 150 genes associated with epilepsy (including *WDR62*, *ASPM*, *MECP2*, *CEP135*, *CEP152*, *ZNF335*, *STIL*, *CDK5RAP2*, *CASC5*, *CENPJ*, and *ARX*), were negative in both families.

The two affected male siblings in family 1 and three obligate female carriers in family 2 were selected for X-exome sequencing. For enrichment of genomic DNA, we used Agilent SureSelect Human X-chromosome Exome kit (Agilent, Santa Clara, CA). Targeted regions were sequenced with Ion Torrent (PGM, Thermo Fisher Scientific, Waltham, MA). The coverage was at least 20x for >80% of targeted regions. Reads were aligned against the Hg19 reference genome, using Torrent Suite Software (Thermo Fisher Scientific, Waltham, MA), resulting in a binary alignment map (BAM) file. Variant calling was performed by NextGENe software (SoftGenetics, State College, PA). Generated VCF files were analyzed with Cartagenia tool and in-house informatics. Sanger sequencing using standard protocols was performed for validation of the variants and segregation analysis.

ZEBRAFISH EXPERIMENTS

To study the functional consequences of altered *EIF2S3* expression, we used a morpholino knockdown experience in a zebrafish model. The zebrafish *eif2s3* gene (RefSeqNM_212711) has 12 exons, such as the *EIF2S3* human gene, and shares 80.2% protein identity with the human *EIF2S3*. Zebrafish were housed in the GIGA Zebrafish facility in Liege, according to animal research guidelines, and with approbation of their ethical commission for animal care and use.

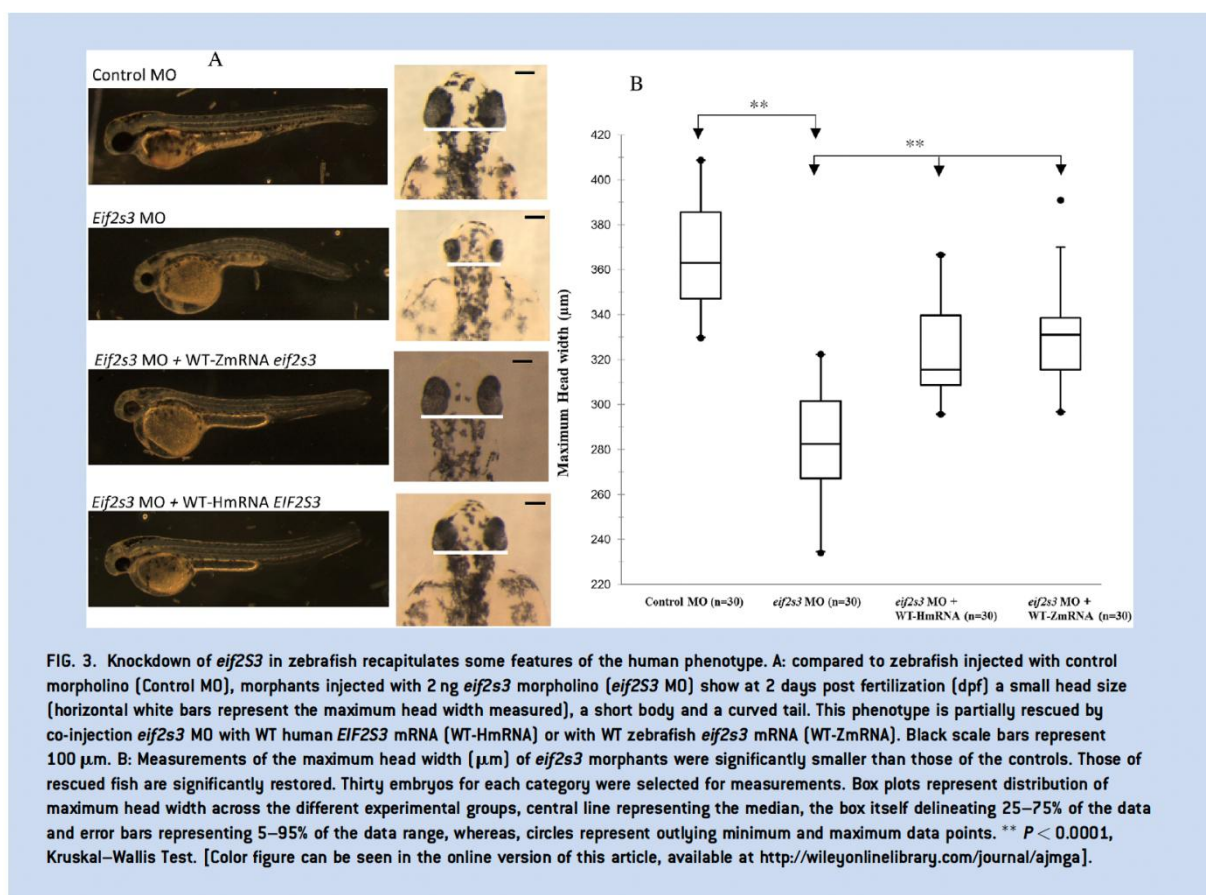
We designed morpholino (MO) oligonucleotides (GeneTools, Philomath, OR) to target gene translation. The MO were resuspended in sterile water to a concentration of 1mM. They were further diluted to 2, 4, and 6µg/µl. One nanoliter of each MO solution was injected into one- to two-cell-stage zebrafish embryos. All studies were performed with a standard control MO obtained from Gene Tools. Injected embryos and uninjected controls were analyzed at 0-, 1-, 2-, and 3 days post fertilization (dpf) for survival, motility, and morphology. For the head width measurements, 30 morphants and 30 controls were selected at 2 dpf. The experiment was done in triplicate. After establishing the knockdown phenotype, rescue experiments were performed by co-injection of *eif2s3* MO with 500pg/egg of either wild-type (WT) zebrafish *eif2s3* RNA (WT-ZmRNA) or human *EIF2S3* RNA (WT-HmRNA). *eif2s3* RNA was transcribed in vitro from *NotI*-linearized pCS2 þ *EIF2S3* constructs using SP6 mMESSAGE Machine Kit (Ambion).

RESULTS

X-exome sequencing showed a c.777T>G p.(Ile259Met) variant (ChrX(GRCh37): g.24084119 T>G) in *EIF2S3* (RefSeq NM_001415.3) in family 1 and a c.1394_1397del p.(Ile465Serfs*4) variant (ChrX(GRCh37): g.24094877_24094880del) in the same gene in family 2. Both variants were confirmed by Sanger sequencing and cosegregated with the disease in both families (Supplementary Fig.S1). In family 1, the missense variant was present in both affected boys (III.2, III.3) and in non-affected obligate carrier mother (II.2), whereas, it was absent in the healthy brother (III.1). In family 2, the frameshift variant was carried by the three mothers (II.1, II.3, III.4) and absent in the healthy males (II.2, III.2, IV.2). The variants were not found in the 1000 Genome database, in dbSNP135, in the Exome Variant Server (EVS), or in ExAC, nor in the literature.

For zebrafish experiments, we tested various concentrations of *elf2s3* MO, and established that the most penetrant and least toxic dose for zebrafish *elf2s3* knockdown experiments was at 2 ng/embryo. *Elf2s3* morphants exhibited hypomotility and morphological deficits at 2 dpf. They were shorter with a curved tail (Fig. 3A). They were affected by a statistically significant reduction of head size (Kruskal–Wallis Test, $P < 0.0001$), with small eyes compared with control MO injected embryos (Fig. 3B). We assessed the specificity of these deficits by performing rescue experiments that partially restored the morphant phenotype, with statistically significant results ($P < 0.0001$) (Fig. 3A–B). Although, no significant differences were observed between the standard control MO and the uninjected embryos, the absence of total rescue is likely due to non-specific, or off-target effects of antisense technologies.

As both variants found by X-exome were not found in databases of population variants and cosegregated with the disease in the families, we considered them as likely deleterious. This hypothesis was strengthened by *elf2s3* knockdown experiments on zebrafish, which recapitulated some features of the human phenotype.



DISCUSSION

We describe two unrelated families in which affected boys presented with similar clinical features consisting of severe microcephaly, growth retardation, epilepsy, and micropenis, with various degrees of severity. By X-exome sequencing, we identified a missense variant c.777T>G p.(Ile259Met) in *EIF2S3* in the first family and a c.1394_1397del p.(Ile465Serfs*4) in the same gene in the second family.

EIF2S3 encodes eIF2 γ . eIF2 γ forms with two distinct subunits (α and β), the eukaryotic translation initiation factor 2, eIF2, essential for protein synthesis. A ternary complex comprised of eIF2, GTP, and an initiator methionyl-tRNA (Met-tRNA_i), binds to the 40 S ribosomal subunit and forms a preinitiation complex. This complex binds to a mRNA and starts protein translation.

Only one mutation c.665T>C, p.(Ile222Thr) in *EIF2S3* has been reported previously. It was found in a family, with three males were affected by apparent ID, microcephaly, and short stature[Borck et al., 2012]. One of them also had generalized seizures, whereas, his brother had postpubertal microgenitalism. Since this first description in 2012, no additional families with *EIF2S3* mutations have been published. We describe in this report, six additional male patients from two unrelated families with similar clinical features, suggesting a rare but specific phenotype related to this gene (Table I).

TABLE I. Clinical Features of Patients With *EIF2S3* Mutations

	Present report		Borck et al. [2012]
	Family 1	Family 2	
Number of affected males	2	4	3
Mutation ^a	c.777T>G p.(Ile259Met)	c.1394_1397del p.(Ile465Serfs*4)	c.665T>C p.(Ile222Thr)
Origin	Belgium	Spain	Morocco
Developmental delay	+	+	+
Speech delay	+	+	+
Intellectual disability	Severe	+	Apparent ID
Growth retardation	+	+	+
Microcephaly	+	+	+
Lower limb spasticity	+	+	+
Seizures	+(1/2)	+	+(1/3)
Thin corpus callosum at brain MRI	+	+	+
Hypoglycemia	+	+	NA
Hypogenitalism	+	+	+(1/3)
Other	Strabismus	NA	Cleft lip and palate (1/3)
	Autistic features (1/2)		Behavioral impairment (1/3)

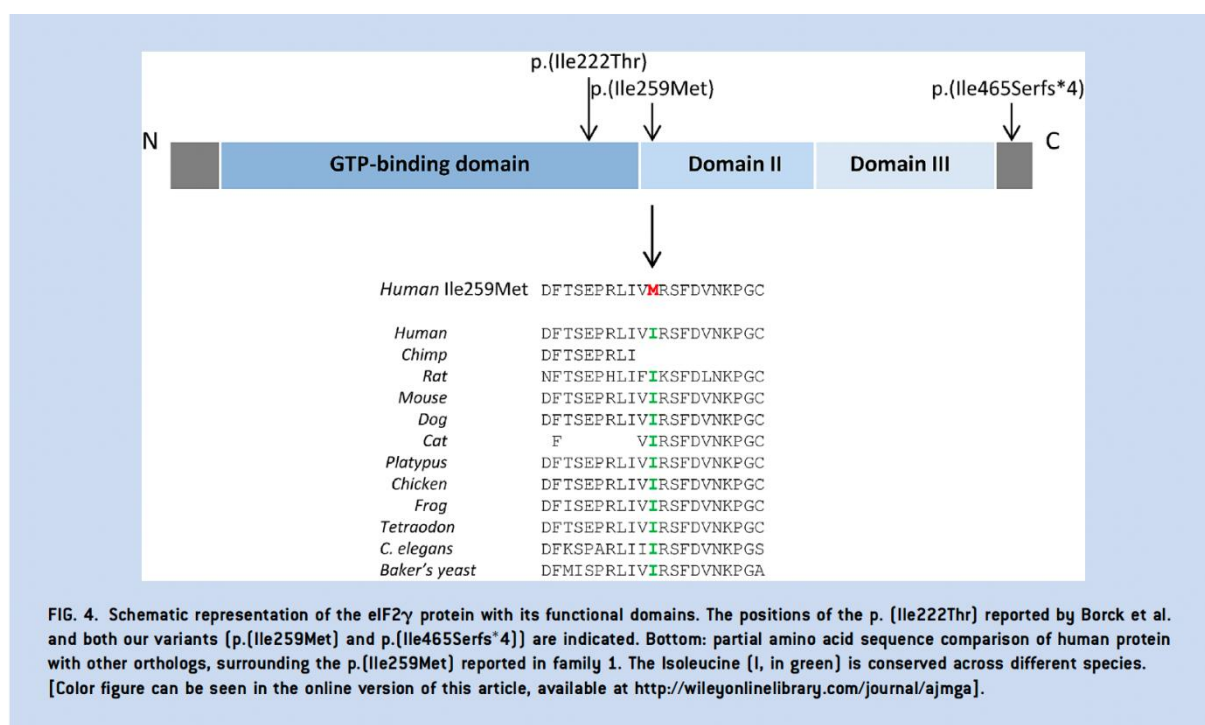
^a*EIF2S3* mutation was annotated according to RefSeq NM_001415.3.

NA: not available.

In family 2, in which affected males presented with a more severe phenotype leading to early death, we found a c.1394_1397del p.(Ile465Serfs*4) mutation that predicts a premature stop codon. Of note, from ExAC the number of loss of function variants in *EIF2S3* is significantly smaller (1) than expected (12), suggesting such variants are deleterious. In family 1, in which one proband is still alive at 18 years, while the brother died at 17 years from severe respiratory distress and multi-organic failure, we found a c.777T>G p.(Ile259Met) in *EIF2S3*. These results could be consistent with some previous reports on XLID pathogenic mutations located in other genes, suggesting that missense variants could be less

deleterious than truncating variants in males with XLID [Tarpey et al., 2009; Moog et al., 2015]. A hypothetical explanation is that transcripts with premature stop codons are targeted for degradation by the nonsense-mediated mRNA (NMD), resulting in absent or very low expression of the truncated protein products; whereas, missense mutations can produce aberrant but stable transcripts, leading to less efficient but still expressed proteins.

We did not find the predicted missense p.(Ile259Met) variant in *EIF2S3* either in our X variants database nor in dbSNP135, the 1000 genome project, EVS, or ExAC. The variant was predicted to be « probably damaging » by SIFT and Polyphen-2. Moreover, the missense variant alters an amino acid residue that is conserved during evolution (Fig. 4). It is located in a functional domain (β -barrel domain, domain II) of the eIF2 γ protein, that binds eIF2 α subunit, and Met-tRNA_i [Yatime et al., 2007; Andaya et al., 2011]. The c.665T>C, p.(Ile222Thr) variant reported by Borck et al. [2012] was located in the GTP-binding domain and impaired eIF2 β subunit binding to eIF2 γ , leading to defects in translation initiation.



The *EIF2S3* conservation from yeast to humans indicates evolutionary importance. To assess the biological impact of mutations in *EIF2S3*, we used the zebrafish vertebrate model, which is a well established approach for the study of genetic microcephaly [Oksenberg et al., 2013; Deciphering Developmental Disorders S, 2015; Nakayama et al., 2015]. By using MO oligonucleotides targeting translation of *eif2s3* gene, we showed that knockdown of the zebrafish *EIF2S3* ortholog reproduces some features of the human phenotype, such as a small head size with small eyes and decreased body length. The observed features seem to be specific as the rescue experiments with human or zebrafish mRNA partially restored the phenotype.

It has long been suggested that translation dysfunction should always be lethal. However, more genes involved in translation pathways are now associated with human diseases: *EIF2B1* (leukoencephalopathy with vanishing white matter) coding for an important subunit of eIF2B (a GTP exchange factor for eIF2) [van der Knaap et al., 2002], *RPL10* (known candidate for XLID and autism) coding for a component of the large ribosomal subunit 60 S [Klauck et al., 2006], or *LAS1L* (novel candidate XLID gene) essential for the biogenesis of the ribosomal subunit 60 S [Castle et al., 2010]. In Wolcott–Rallison syndrome characterized by neonatal diabetes with epiphyseal dysplasia, growth retardation, and microcephaly, homozygous mutations in *EIF2AK3* (PERK, pancreatic eIF2 α kinase) result in diminution of eIF2 α phosphorylation. High expression levels of PERK in pancreatic cells were responsible for pancreatic phenotype in patients with Wolcott–Rallison syndrome [Zhan et al., 2002].

Kernohan et al. [2015] and Abdulkarim et al. [2015] described a novel recessive syndrome characterized by severe microcephaly, short stature, delayed myelination, and intellectual disability. The latter family also had pediatric diabetes. Both teams found a homozygous c.1972 G>A; p.(Arg658Cys) variant in *PPP1R15B*, which encodes a protein that promotes dephosphorylation of the transcription initiation factor eIF2 α . eIF2 α is the third subunit of the heterotrimeric complex eIF2. When eIF2 α is dephosphorylated, it enhances protein translation. While *PPP1R15B* and *EIF2S3* genes are involved in the same translation pathways, the patients with *PPP1R15B* and *EIF2S3* mutations share similar phenotypic characteristics, such as severe microcephaly, intellectual disability, and short stature. Abdulkarim et al. [2015] showed that the p.(Arg658Cys) variant increases eIF2 α phosphorylation and results in β -cell apoptosis. All affected males reported here exhibited episodes of hypoglycemia, without diabetes. Interestingly, proband III.2 in family 1 had chronic pancreatitis treated by pancreatic sphincterotomy, possibly due to β -cell dysfunction or dysregulation. Patients described by Abdulkarim et al. [2015] developed diabetes at the age of 15 and 28 years. Careful attention should therefore, be paid to patient III.3 in family 1, with regular glucose metabolism controls.

In conclusion, we identified two novel variants in *EIF2S3*. Several observations suggest that these variants are pathogenic. First, they were the only candidate variants found by X-exome sequencing cosegregating with the phenotype in both families. Second, the frameshift mutation found in family 2 is predicted to truncate the protein and to likely cause a loss of EIF2S3 function, while the missense mutation in family 1 is located in a conserved region and in a functional domain of the protein. Finally, knocking down *eif2s3* expression in the zebrafish using MO experience recapitulates some features of the human phenotype. We conclude that eukaryotic initiation factor eIF2 plays an essential role in brain development, and that these variants are pathogenic.

It is now important to identify additional individuals with *EIF2S3* mutations to delineate the specific phenotype related to this gene. Further functional studies with human and animal models will be necessary to understand the role of the eIF2 γ protein in the developing brain and in glucose metabolism.

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SUPPORTING INFORMATION

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