

EIF2S3 Mutations Associated with Severe X-Linked Intellectual Disability Syndrome MEHMO

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ABSTRACT: Impairment of translation initiation and its regulation within the integrated stress response (ISR) and related unfolded-protein response has been identified as a cause of several multisystemic syndromes. Here, we link MEHMO syndrome, whose genetic etiology was unknown, to this group of disorders. MEHMO is a rare X-linked syndrome characterized by profound intellectual disability, epilepsy, hypogonadism and hypogenitalism, microcephaly, and obesity. We have identified a C-terminal

frameshift mutation (Ile465Serfs) in the *EIF2S3* gene in three families with MEHMO syndrome and a novel maternally inherited missense *EIF2S3* variant (c.324T>A; p.Ser108Arg) in another male patient with less severe clinical symptoms. The *EIF2S3* gene encodes the γ subunit of eukaryotic translation initiation factor 2 (eIF2), crucial for initiation of protein synthesis and regulation of the ISR. Studies in patient fibroblasts confirm increased ISR activation due to the Ile465Serfs mutation and functional assays in yeast demonstrate that the Ile465Serfs mutation impairs eIF2 γ function to a greater extent than tested missense mutations, consistent with the more severe clinical phenotype of the Ile465Serfs male mutation carriers. Thus, we propose that more severe *EIF2S3* mutations cause the full MEHMO phenotype, while less deleterious mutations cause a milder form of the syndrome with only a subset of the symptoms.

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KEY WORDS: MEHMO syndrome; XLID; *EIF2S3*; translation initiation; integrated stress response; unfolded-protein response

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Introduction

Neurodevelopmental disorders comprise MEHMO syndrome (MIM# 300148), a rare form of severe X-linked intellectual disability (XLID) with additional clinical symptoms including microcephaly, epileptic seizures, hypogonadism, hypogenitalism, diabetes, and obesity. The acronym MEHMO was coined by Steinmüller et al.

[Steinmüller et al., 1998] who described a large three-generation family with five affected boys and assigned the disease locus to the short arm of chromosome X (Xp11.3-22.13). Only three other affected individuals from two families were clinically described and communicated in the literature [Delozier-Blanchet et al., 1989, 1999; Leshinsky-Silver et al., 2002]. Till now, the genetic cause of this condition has not been resolved.

We took advantage of next-generation sequencing in three unreported families with this condition, in addition to the family reported by Steinmüller et al. [Steinmüller et al., 1998], to identify the underlying genetic cause of this severe disorder. We report that in these families MEHMO syndrome is associated with frameshift and missense mutations in the X chromosome gene *EIF2S3*. We previously identified a missense mutation in *EIF2S3* present in a consanguineous family with XLID (MIM# 300987) [Borck et al., 2012], and while this manuscript was in preparation, two novel *EIF2S3* mutations were reported in two unrelated families with variable XLID (MIM# 300987) [Moortgat et al., 2016].

EIF2S3 encodes the γ subunit of the heterotrimeric eukaryotic translation initiation factor 2 (eIF2), which forms a stable ternary complex (TC) with GTP and the initiator methionyl-tRNA ($\text{Met-tRNA}_i^{\text{Met}}$). The eIF2-GTP-Met-tRNA $_i^{\text{Met}}$ TC binds along with the factors eIF1, eIF1A, eIF3, and eIF5 to the small 40S ribosomal subunit to form a 43S preinitiation complex (PIC). Subsequent binding of this complex to an mRNA forms a 48S PIC. According to the scanning model of translation initiation, the 43S PIC first associates with the 5' end of an mRNA and then linearly scans in a 3' direction to select a translation start site [Hinnebusch, 2011]. Base pairing interactions between the anticodon loop of tRNA $_i^{\text{Met}}$ and an AUG codon of the mRNA trigger the ribosome to stop scanning, promote completion of GTP hydrolysis by eIF2, and prompt release of eIF2-GDP from the complex. Conformational changes in the ribosome accompanying these events culminate in joining of the large 60S ribosomal subunit to form the 80S initiation complex that is poised to begin translation elongation, the codon-dependent stepwise addition of amino acids to the growing polypeptide chain [Hinnebusch, 2014].

The active eIF2-GTP is regenerated by the guanine nucleotide exchange factor eIF2B. This is a key control point for eukaryotic protein synthesis. Phosphorylation of the α subunit of eIF2 by members of a family of stress-responsive protein kinases impairs general translation, yet enhances the translation of specific mRNAs in a process known as general amino acid control in yeast [Dever, 2002; Hinnebusch, 2005] or as the integrated stress response (ISR) in other organisms [Harding et al., 2000, 2003; Dever, 2002, 2007; Pavitt and Ron, 2012]. The eIF2 α kinase PERK feeds into the ISR by sensing endoplasmic reticulum (ER) stress and triggering the translational arm of the unfolded-protein response (UPR) to alleviate the burden of misfolded protein accumulation. The phosphorylation of eIF2 α converts eIF2 into an inhibitor of eIF2B and thereby limits the availability of eIF2-GTP for TC formation [Hinnebusch, 2005]. The decreased TC level impairs global translation, providing the cell time to cope with the stressor, and paradoxically increases the translation of selected mRNAs bearing regulatory upstream open reading frames (uORFs). This class of translationally upregulated mRNAs includes those encoding the yeast transcription factor *GCN4* and the mammalian activating transcription factor 4 (*ATF4*), which in turn promote expression of genes necessary for the adaptive response to the stress situation [Harding et al., 2003; Hinnebusch, 2005; Ron and Walter, 2007]. One of the *ATF4* target genes is *DDIT3* encoding the transcription factor C/EBP-homologous protein (CHOP) that plays an essential role in response to a variety of cell stresses by inducing cell cycle arrest or, in the case of unresolved or prolonged stress, apoptosis [Su and Kilberg, 2008; Tabas and Ron, 2011]. The ISR

and translation control of the *GCN4* and *ATF4* mRNAs have proven to be useful in vivo reporters of eIF2 function [Harding et al., 2000; 2003; Lu et al., 2004; Vattem and Wek, 2004; Hinnebusch, 2005; Alone et al., 2008; Borck et al., 2012; Young and Wek, 2016].

Here, we connect *EIF2S3* with MEHMO syndrome, show via functional assays in yeast and human cells that the *eif2s3/EIF2S3* mutations identified in this study have a deleterious effect on eIF2 γ function, and suggest genotype-phenotype relations in affected subjects.

Materials and Methods

Patients

All subjects provided written informed consent for this research study, which was approved by the ethics committees of the participating centers and adhered to the tenets set forth in the Declaration of Helsinki. For subjects under the age of 18 years, written consent was obtained from a parent.

Cell Culture

HEK293-T, HeLa, and fibroblast cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM glutamine at 37°C with 5% CO $_2$. Transient DNA transfections were carried out using PeqFECT DNA or jetPRIME (both PEQLAB, Erlangen, Germany) according to the manufacturer's instructions.

Next-Generation Sequencing and Sanger Sequencing

Genomic DNA of patients and their family members was extracted from peripheral blood using standard procedures. Probands from all families had next-generation sequencing performed (whole exome or X chromosome exome). In case of Slovak patients, whole exome sequencing (WES) including basic bioinformatics up to annotated vcf files was performed by service providers. DNA of the index patient from family 1 was sequenced by Theragen BiO Institute (South Korea) on HiSeq2500 (Illumina, San Diego, CA) using SureSelect Whole Human All Exon V4+UTR (Agilent, Santa Clara, CA, USA) for exome capture. Sequencing reads were mapped to hg19 reference using BWA-MEM algorithm and variants were called using GATK UnifiedGenotyper pipeline. Variants with PHRED score quality >10 were annotated using snpEFF and snpSIFT tools. Variants were prioritized according to their population frequency, predicted effect, and position on the X chromosome. DNA of the index patient from family 2 and his parents were sequenced by BGI (Hong Kong) using the Human 59M Exon kit and Complete Genomics platform. Base calling, alignment to reference b37, and variant calling were performed using Complete Genomics' specific bioinformatic pipeline by the service provider. The index patient from family 3 had X-chromosome exome sequencing performed as previously described [Hu et al., 2016]. Afterward, sequencing reads were mapped to hg18 via RazerS as described in Hu et al. [Hu et al., 2016]. Accordingly, short insertions/deletions were called from those primary alignments as well as by applying a split-read mapping approach (SplazerS, [Emde et al., 2012]). Final variant calls were lifted over to hg19 in order to allow evaluation and prioritization based on more recent gene annotations. WES was performed on DNA of the index patient from family 4 and his parents by GeneDx (Gaithersburg, MD) on HiSeq2500 (Illumina) using SureSelect V4+UTR (Agilent

Technologies Inc., Santa Clara, CA) for exome capture and on DNA of the patient from the family published by DeLozier-Blanchet et al. [DeLozier-Blanchet et al., 1989, 1999] as previously described [Borck et al., 2012].

Conventional Sanger sequencing for mutation search in *EIF2S3* coding exons and flanking intronic sequences, confirmation of variants, segregation studies, and haplotyping was performed on ABI 3500 sequencer (Applied Biosystems, Foster City, CA) using standard procedures. Primers can be obtained upon request. For *EIF2S3* cDNA numbering, the A of the ATG translation initiation codon in the GenBank reference sequence NM_001415.3, with the initiation codon as codon 1 was used. For haplotyping of family 1, regions containing positions of 18 previously unreported variants found on chromosome X of the index patient (Supp. Table S1A), as well as 16 SNPs from 11 other regions (Supp. Table S1B), were sequenced in all available members of his family. Haplotypes within the pedigree were resolved manually.

EIF2S3 variants identified through this study were submitted to the LOVD (databases.lovd.nl/shared/genes/EIF2S3; variants EIF2S3_000008 and EIF2S3_000018) and ClinVar (SCV000321299 and SCV000321546) databases.

Identity by Descent Analysis

For identity by descent (IBD) analysis, genotyping was performed using the Illumina Human Omni 2.5 BeadChip (Illumina). SNP array data for the index patients from families 1 and 3 and exome data for the index patients from families 1 and 2 and the mother from family 2 were run through LINKDATAGEN [Bahlo and Bromhead, 2009] with parameter $\text{bin} = 0.0002$ to produce a collection of markers with high heterozygosity. XIBD [Henden et al., 2016] was used to estimate the number of meiosis separating the pair of individuals, the probabilities of sharing zero or one allele IBD, and genomic regions IBD via posterior probabilities and the Viterbi algorithm. SNPs in near perfect linkage disequilibrium ($R^2 \geq 0.99$) and SNPs with minor allele frequencies $\leq 3\%$ according to HapMap phase II were removed.

In Silico Analysis

The Clustal Omega alignment was performed using the Uniprot web service (<http://www.uniprot.org/align> [McWilliam et al., 2013]). Protein structure analyses were performed using PyMol software (The PyMOL Molecular Graphics System, Version 1.7.6.6 Schrödinger, LLC). Combined annotation-dependent depletion (CADD) tool was used for estimation of variant deleteriousness [Kircher et al., 2014].

Plasmids for Work in Yeast

The single copy number *URA3 GCN4-lacZ* plasmid p180 [Hinnebusch, 1985] as well as the single copy number *URA3 HIS4(AUG)-lacZ* (p367) and *HIS4(UUG)-lacZ* (p391) plasmids [Cigan et al., 1988] were described previously. The single copy number *LEU2* plasmid pC2860 that expresses His₈-tagged *GCD11* (yeast eIF2 γ) under control of the native promoter [Alone et al., 2008] as well as the derivatives of pC2860 expressing the mutants *His₈-GCD11-V281K* (pC4187) and *His₈-GCD11-V281T* (pC4188) were described previously [Borck et al., 2012]. Derivatives of pC2860 were generated by site-directed mutagenesis to express *His₈-GCD11-D167R* (pC5343) as well as the yeast/human eIF2 γ chimera

His₈-GCD11(Sc¹⁻⁵¹⁴/Hs⁴⁵⁷⁻⁴⁷²) (pC5051) and its mutant derivative *His₈-GCD11(Sc¹⁻⁵¹⁴/Hs⁴⁵⁷⁻⁴⁷²)-I465Sfs** 4 (pC5052).

Yeast Strains and Genetic Methods

Yeast strains expressing eIF2 γ mutants were generated by plasmid shuffling [Boeke et al., 1987]. Briefly, the single copy number *LEU2* plasmid pC2860 expressing wild-type yeast eIF2 γ (*GCD11*) or its mutant derivatives were introduced by transformation into the strain J292 (*MAT α leu2-3 leu2-112 ura3-52 his3 gcn2 Δ ::loxP gcd11 Δ ::KanMX p[GCD11, URA3]*) [Alone et al., 2008]. The resulting transformants were plated on medium containing 5-fluoroorotic acid to evict the *URA3* plasmid containing wild-type *GCD11* and to leave only the *LEU2* plasmid expressing eIF2 γ . For growth assays, the resulting strains were colony purified, grown to saturation in liquid synthetic dextrose (SD) medium containing uracil and histidine, and 4 μ l of serial dilutions (of OD₆₀₀ = 1.0, 0.1, 0.01, 0.001, 0.0001) were spotted on SD medium containing the same supplements. Plates were incubated at 30 or 37°C for 3 days.

For measurement of *GCN4-lacZ* expression, derivatives of strain J292 expressing wild-type or mutant forms of eIF2 γ were transformed with the *GCN4-lacZ* plasmid p180. Transformants were grown in SD medium with amino acid supplements to A₆₀₀ ~0.8, and β -galactosidase activities were determined as described previously [Hinnebusch, 1985]. To measure the UUG/AUG ratio, the J292 derivatives were transformed with the plasmids p3989 (*HIS4(AUG)-lacZ*) or p3990 (*HIS4(UUG)-lacZ*). Transformants were grown to A₆₀₀ ~0.8, and β -galactosidase activities were determined. All assays were performed in triplicate at least three times.

RNA Extraction and Reverse Transcription

Total RNA was extracted from 5×10^6 fibroblast cells using the RNeasy[®] Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For qRT-PCR, RNA samples were treated with DNaseI (Qiagen) prior to cDNA synthesis. RNA samples were qualitatively assessed and quantified using an Agilent 2100 Bioanalyser with RNA 6000 Nano kit (Agilent Technologies Inc.). cDNA was prepared from 1 μ g of total RNA with High-Capacity Reverse Transcription Kit (Applied Biosystems) using the random priming method. The mRNA level of *DDIT3* was assessed by RT-qPCR using TaqMan[®] assays (Hs00358796_g1) and TaqMan[®] Gene Expression Master Mix on 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to *YWHAZ* (TaqMan[®] assay Hs03044281_g1) as a reference gene. Results are presented as $2^{-\Delta\Delta Cq}$. The data were assessed with a two-sample *t*-test (***) $P < 0.001$.

Protein Extraction from Fibroblast Cells

A total of 2×10^6 patient-derived or control fibroblasts were seeded in 10 cm dishes. After 24 hr, cells were washed twice with PBS and incubated with 1 ml lysis buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 0.1 % NP40 supplemented with EDTA-free protease inhibitors and phosphatase inhibitors (both Roche, Basel, Switzerland) for 15 min on ice. Lysates were then collected and immediately frozen in liquid nitrogen. After thawing the lysates on ice, they were centrifuged at 15,000 rcf at 4°C for 15 min and the supernatants were concentrated using vivaspin 500 columns (GE Healthcare Life Sciences, Freiburg, Germany) to 100 μ l according to the manufacturer's recommendations.

Plasmids Used for Studies in Mammalian Cells

pCDNA4_EIF2S3 and pCDNA4_EIF2S3_I222T were described previously [Borck et al. 2012]. For cloning the EIF2S3 del TCAA construct, we used the pCDNA4/TO/myc-HisA vector double digested with *EcoRI* and *XhoI* (New England Biolabs, Ipswich, MA) and PCR products generated using pCDNA4_EIF2S3 as template with the following primer pairs: EIF2S3for_ EcoRI 5'-TAGAATTCA TGGCGGGCGGAGAAGCTGG-3' and EIF2S3rev_ XhoI-CAAT_myc 5'-ATCTCGAGCTGTTGGCTTGTCACTCCTCTTCTATCTG ACCCAACCAA-3'. For cloning N-terminal FLAG-tagged EIF2S3 wild-type and mutant constructs, we used the pCMV-Tag 2b vector (STRATAGENE, La Jolla, CA) double digested with *EcoRI* and *XhoI* (New England Biolabs) and PCR products generated using pCDNA4_EIF2S3 or pCDNA4_EIF2S3_delTCAA as template with the following primer pairs: EIF2S3for_ EcoRI_FLAG 5'-TAGAATTCGCGGGCGGAGAAGCTGGAG-3', EIF2S3rev_ XhoI_FLAG 5'-ATCTCGAGCTAGTCATCATCTACTGTTG-3', EIF2S3rev_ XhoI_TAAC_FLAG 5'-ATCTCGAGCTACTGTTGGCTTGTCACT C-3'. For cloning N-terminal GFP-tagged EIF2S3 wild-type and mutant constructs, we used the pEGFP-C2 vector (BD Biosciences Clontech) double digested with *EcoRI* and *SallI* (New England Biolabs) and PCR products generated using pCDNA4_EIF2S3 or pCDNA4_EIF2S3_ΔTAAC as template with the following primer pairs: EIF2S3forw_ EcoRI 5'-TAGAATTCATGGCGGGCGGAGAA GCTGG-3' and EIF2S3rev_ SallI 5'-ATGTCGACCTAGTCATCATC TA-3' or EIF2S3rev_ SallI_TAAC 5'-ATGTCGACCTACTGTTGGC TTG-3'. The p.Ser108Arg derivative was generated by site-directed mutagenesis using the QuikChange II site-directed Mutagenesis Kit (Agilent Technologies Inc.). All constructs were verified by Sanger sequencing.

Transient Expression and Western Blotting

For transient expression experiments, HeLa and HEK293T cells were transfected with 8–12 μg plasmid DNA as described above. Twenty-four hours after transfection, cells were collected and resuspended in lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, and 1% Triton-X100 supplemented with EDTA-free protease inhibitors (Roche). Supernatants were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat milk in phosphate buffered saline Triton X-100 (PBST), and incubated with primary antibody overnight at 4°C. Membranes were then washed three times with PBST and incubated with HRP-conjugated secondary antibody for 30 min at room temperature followed by chemiluminescence detection.

The following antibodies were used: monoclonal anti-Flag-HRP (A8592, 1:3,000; Sigma-Aldrich, St. Louis, MO), polyclonal rabbit anti-EIF2S3 (PA5-31177; 1:1,000; Thermo Fischer Scientific, Rockford, IL), monoclonal mouse anti-CHOP (MA1-250; 1:1,000; Thermo Fischer Scientific), monoclonal mouse anti-Myc antibody (631206, 1:500; Clontech Laboratories, Inc., Mountain View, CA, USA), monoclonal rat anti-tubulin (ab6160 1:20,000; Abcam, Cambridge, UK), anti-mouse-HRP (115-035-003, 1:5,000; Dianova, Hamburg, Germany), and anti-rabbit-HRP (NA9340, 1:3,000; Amersham, Piscataway, NJ). Western blot quantification was performed using ImageJ software.

Immunofluorescence and Confocal Microscopy

HEK293T cells were seeded on laminin/poly-D lysine covered glass cover slips in six-well plates and were transiently transfected

with 2 μg plasmid DNA per well. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde, permeated with 0.2% Triton-X100, and blocked with 3% bovine serum albumin for 1 hr at room temperature. Incubation with primary monoclonal mouse anti-myc antibody (631206, 1:500; Clontech Laboratories, Inc.) was performed at 4°C overnight followed by detection with the secondary Alexa 594 anti-mouse antibody (A21200, 1:1,000; Thermo Fischer Scientific) for 1 hr at room temperature. Nuclei were counterstained with 0.5 μg/ml DAPI and coverslips were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). Images were acquired using a Zeiss LSM 700 confocal microscope (Zeiss, Jena, Germany).

Mitochondrial OXPHOS Activities

Activities of complexes II and IV were measured in saponin permeabilized muscle fibers using oxygen consumption measurements with Clark's electrode (Oxytherm, Hansatech, UK). Approximately 10 mg of muscle was taken into an ice cold relaxing and biopsy preservation solution (BIOPS) prepared as previously described [Larsen et al., 2014]. Briefly, myofibers were gently separated by forceps in ice-cold BIOPS and saponin (50 μg/ml) was added for 30 min. Permeabilized sample was transferred into an ice-cold mitochondrial respiration medium (MiRO5) prepared as described in [Pesta and Gnaiger, 2012]. COX activity was assessed as a difference in respiration rate between the complex IV activation state (subsequent addition of 5 mM ascorbate and 0.25 mM TMPD) and the KCN-inactivated state (3 × 10 mM KCN). All measurements were made in triplicates. Outer mitochondrial membrane integrity was routinely tested by the addition of cytochrome c (19 μM) in the complex IV activated state. Activity of complex II was measured after complex I blocking with rotenone (10 μM) by the addition of complex II substrate succinate (10 mM) and ADP (5 mM) and stopped by the addition of malonate (1 mM), a complex II inhibitor. Complex II activity was calculated as a difference in respiration rate between the complex II activated state and the malonate-inactivated state. Three and two patient 1 tissue biological replicates were used for complexes II and IV measurements, respectively. Three and nine tissues from control individuals were used for complexes II and IV measurements, respectively.

Results

Clinical Phenotypes

Four families with MEHMO syndrome were investigated by massively parallel sequencing to identify the underlying genetic cause. The pedigrees of the families are shown in Figure 1A. Clinical characteristics of all affected males from these families are summarized in Table 1, together with all previously published patients with MEHMO syndrome.

Family 1

The index patient from family 1 was born to nonconsanguineous parents of Slovak origin and has one healthy older sister. His mother has sideroblastic anemia and his maternal grandmother suffered from a heart attack at 54 years, but were otherwise normal. The maternal grandmother had one miscarriage and her brother died in the first months of life of unknown cause. There are no other living male members in the maternal lineage. The proband had prenatally

Table 1. Summary of Phenotypes and Genotypes of All Cases with Resolved and Unresolved MEHMO Syndrome

Reference	This study	This study	This study, [Steinmuller et al., 1998]	This study	[Borck et al., 2012]	[Moortgat et al., 2016]	[Tarpey et al., 2009]	[Delozier-Blanchet et al., 1989]	[Leshinsky-Silver et al., 2002]	
Family EIF2S3 genotype (NML001415.3)	F1 c.1394_1397delTCAA (p.Ile465Serfs*4)	F2 c.1394_1397delTCAA (p.Ile465Serfs*4)	F3 c.1394_1397delTCAA (p.Ile465Serfs*4)	F4 c.324T>A (p.Ser108Arg) VOUS	F5 c.665T>C (p.Ile222Thr)	F6 c.777T>G (p.Ile259Met)	F7 c.1394_1397delTCAA (p.Ile465Serfs*4)	F8 c.451G>C (p.Val151Leu)	F9 EIF2S3 excluded	F10 EIF2S3 excluded, SERAC1 (p.Tyr430X) (MEGDEL syndrome) Ethiopian Jewish
Origin	Slovakia	Slovakia	Germany	United States	Morocco Jewish	Belgium	Spain	n.a.	Southern Italy	Ethiopian Jewish
No. of described patients	1	1	4* (+1)	1	3	2	4	n.a.	2	1
Lifespan	Alive at 5 y	Alive at 1.5 y	2 y/7/2/10 mo	Alive at 5 y	Alive at 14 and 11.5 y	17 y/alive at 18 y	12 mo	n.a.	4.5 y/alive at 14 y	Alive at 12 mo
Low birth weight	+	+	+(1/4)	n.a.	n.a.	+	+	n.a.	n.a.	+
Low birth length	+	+	+(1/2)	n.a.	n.a.	+	+	n.a.	-	n.a.
Full MEHMO syndrome (all clinical aspects of the acronym present)	+	+	+	-	-	-	+	n.a.	+	+
Microcephaly	+	+	+	+	+	+	+	n.a.	+	+
Seizures/abnormal EEG	+	+	+	+	+	+	+	n.a.	+	+
Seizures + abnormal EEG	+	+	+	-	+	+	+	n.a.	+	+
Brain MRI changes	+	+	n.a.	n.a.	+	+	+	n.a.	+	+
Hypotonia	+	+	n.a.	+	n.a.	+	+	n.a.	n.a.	n.a.
Hypertonia	+	+	+	+	+	+	+	n.a.	+	n.a.
Hyperreflexia	-	-	n.a.	-	+	n.a.	n.a.	n.a.	+	-(no reflexes)
Hypogonadism/genitalism	+	+	+	+	+	+	+	n.a.	+	+
Developmental delay	+	+	+	+	+	+	+	n.a.	+	+
Infancy-onset obesity	+	+	+	+	+	+	n.a.	n.a.	+	+
Postnatal growth retardation	+	+	+	-	+	+	+	n.a.	+	n.a.
Hypoglycemia	Neonatal	Neonatal	n.a.	n.a.	n.a.	Episodic	Neonatal	n.a.	n.a.	n.a.
Diabetes	Onset at 10 mo	Onset at 10 mo	Onset at age of 6 mo (1/4)	-	-	-	-	n.a.	Neonatal/episodic hyperglycemia	n.a.
Other Endocrinopathy	Panhypopituitarism	Partial hypopituitarism	Small thyroid (1/4)	-	Low GH levels (2/3)	-	-	n.a.	n.a.	n.a.
Other	atrial septal defect, lactose intolerance, elevated hepatic enzymes, severe combined dyslipidaemia	-	Increased levels of acylcarnithine and fatty acids, single palmar crease (left hand), facial telangiectasia (1/5)	Chronic lung disease, congenital scoliosis, delayed gastric emptying, grade 5 vesico-ureteral reflux	Ataxic gait, Achilles tendon shortening, III:1 -cleft lip and palate, walked independently at 2 y, speaks short sentences, can feed himself; III:2 - speaks 3-4 words, cannot feed himself	Skeletal immaturity	-	n.a.	Chronic constipation	Lactic acidosis, elevated urine 3-methylglutaconic acid, no teeth, large lips

cDNA nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence NM_001415.3, with the initiation codon as codon 1, VOUS, variant of unknown clinical significance; GH, growth hormone; n.a., not available; y, years; mo, months; *patient IV:8 was diagnosed prenatally with microcephaly and the pregnancy was terminated at week 24. MEGDEL, 3-methylglutaconic aciduria type IV with sensorineural deafness, encephalopathy, and Leigh-like syndrome.

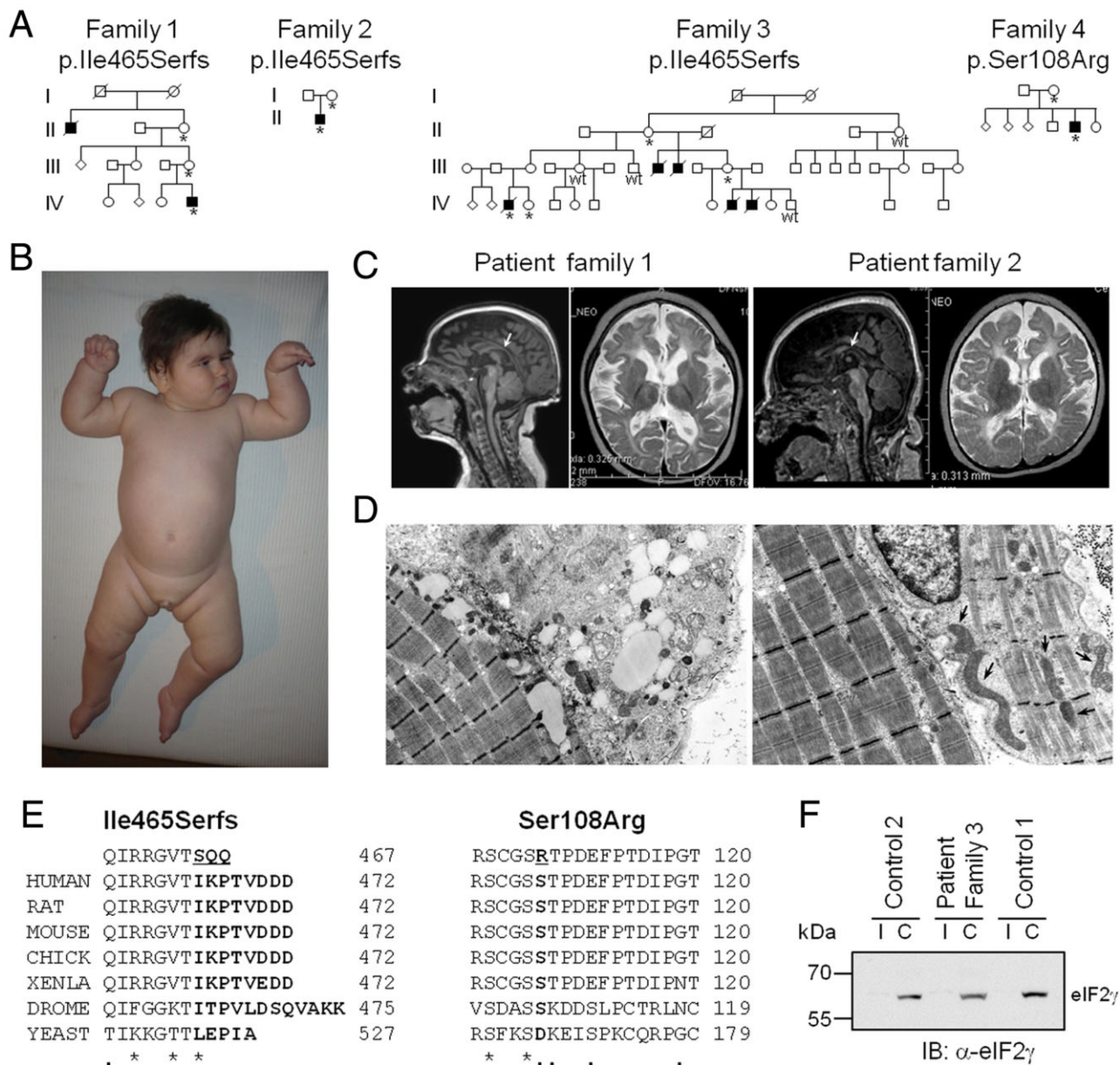


Figure 1. Cosegregating truncating and missense mutations in *EIF2S3* cause MEHMO syndrome. **A:** Pedigrees of families 1–4 with cosegregating truncating and missense mutations in *EIF2S3*. Open circles represent females, open squares represent unaffected males, and closed squares represent affected males; *mutation carrier; wt, wild type. **B:** Physical appearance of index patient from family 1 at 10 months—obese with full cheeks, large ears, downturned corners of mouth, epiblepharon, long eyelashes and thick eyebrows, tapered fingers, and talipes. Microgenitalism is visible. **C:** Cranial MRI—patient 1: (left) Sagittal T1W image at 4 months showing thin and flat corpus callosum (arrow), and (right) axial T2W image at 19 months showing cerebral atrophy, delay of myelination, acute demyelinating changes in the periventricular white matter, enlarged ventricular system, and subarachnoid fronto-temporal space. Patient 2: (left) T1W image at 10 months showing cerebral atrophy and thin corpus callosum (arrow), and (right) axial T2W image showing enlargement of ventricular system, delay of myelination, and acute demyelinating changes in the periventricular white matter. **D:** Electron microscopy of patient 1 muscle biopsy shows myofibril degradation (left) and presence of huge mitochondria with abnormal cristae arrangement (right, arrows). **E:** Clustal Omega analysis of eIF2 γ C-terminus (left) and the mutated serine residue (right) in humans and other species. The C-terminus is conserved in vertebrates, and the Ser108 is conserved down to *Drosophila melanogaster*. **F:** Western blot analysis of protein cell lysate from control and patient fibroblast cell lines immunoblotted with anti-eIF2 γ antibody shows the presence of eIF γ protein. I, input; C, concentrated protein.

diagnosed microcephaly at 28th week of gestation. He was delivered at 39+6 gestational weeks with birth length of 47 cm (–2.0 SD) and weight 2,920 g (–1.4 SD), Apgar score 8/10 at 5 and 10 min, and occipitofrontal circumference (OFC) 32 cm (–2.6 SD). Micropenis, cryptorchidism, and peripheral hypertonia were noticed.

Diagnosis of MEHMO was made at the age of 10 months based on the microcephaly, partial complex epileptic seizures (diagnosed at the age of 4 months) resistant to anticonvulsives, hypogenitalism, severe mental and motor delay (corresponding to third month of life), central obesity (rapid weight gain developed after feeding via

Table 2. Respiratory Chain Complexes II and IV Activity Measurements in Permeabilized Muscle Fibers of Index Patient from Family 1

	Controls mean \pm SD	Patient mean \pm SD	P
Complex II	3.11 \pm 0.4	1.22 \pm 0.15	0.009
Complex IV	1.66 \pm 0.75	2.1 \pm 0.84	0.409

Activities are expressed in nM/min of consumed oxygen per milligram of tissue wet weight.

nasogastric tube inserted due to the dysphagia and hypotrophy at the age of 3 months—BMI SDS increased from -2.1 to $+1.0$ SD at the age of 8 months, and to $+3.7$ SD at the age of 2 years), and typical dysmorphic features (full cheeks, large ears, downturned corners of mouth, epiblepharon, long eyelashes and thick eyebrows, tapered fingers, and talipes; Fig. 1B).

Other findings. Brain magnetic resonance imaging (MRI) at the age of 4 months showed mild myelination delay corresponding to second and third month, and the myelination delay was more pronounced at the age of 19 months with further acute demyelinating changes and cerebral atrophy (Fig. 1C). Nonautoimmune diabetes mellitus was diagnosed at 10 months and insulin treatment was started. During the first 18 months of life, he suffered from repeated episodes (6–8 per year) of acute bronchitis and bronchopneumonia, during which he was lethargic, had worsened diabetes control, and his seizures exacerbated (up to seventy 2-min episodes a day before the antiepileptic treatment was optimized). The seizures were induced also by increased ambient temperature. At age of 17 months, histological examination of muscle biopsy revealed abnormal striated muscle and muscle mitochondria (Fig. 1D). Measurements of activities of respiratory chain complexes II and IV in patient 1 muscle (Table 2) showed decreased complex II and normal complex IV activities. For accessory signs, see Table 1.

Current status. Currently, at 5 years, his height is 88 cm (-5 SD), weight is 15.3 kg (BMI 19.8 kg/m², 2.3 SD), and head circumference is 39 cm (-8.4 SD). Epileptic seizures are well controlled under combination of vigabatrin, phenobarbital, and topiramate treatment. Seizures emerge only during respiratory and urinary infections, which are now less frequent (the patient is under immunoglobulin therapy). He is fed per os with additional nasogastric feeding. He has central hypotonia and peripheral hypertonia, and reacts only to strong tactile stimuli.

Family 2

The index patient from family 2 is a first child from a Slovak non-consanguineous family with no other affected individuals known. His mother is unaffected. He was delivered in 40+0 gestational weeks with birth length of 49 cm (-0.95 SD) and weight 2440 g (-2.65 SD), Apgar score 8/10, and OFC 29 cm (-5.1 SD).

MEHMO features included microcephaly, therapy-resistant seizures (diagnosed at the age of 6 months) without electroencephalogram (EEG) correlation, hypogenitalism, severe mental and motor delay (corresponding to the third month of life at the age of 2 years), central obesity, and dysmorphic features very similar to the proband of family 1. He has nonautoimmune diabetes mellitus treated with insulin from 10 months. Brain MRI showed myelination delay at 10 months of age (Fig. 1C). For accessory signs, see Table 1.

Current status. At the age of 3 years, his height is 78 cm (-4.7 SD) and BMI is 19.1 kg/m² ($+1.9$ SD). He is hypotonic with no voluntary movements and he does not show any social interaction.

Family 3

This family has been previously described by Steinmüller et al. [1998]. The index patient (IV:3) was diagnosed prenatally with microcephaly and was delivered by Caesarean section during the 32nd gestational week with birth weight 1,420 g (-0.8 SD), length 40.5 cm (-0.4 SD), and OFC 26.5 cm (-1.7 SD). All females from this family who carry the *EIF2S3* mutation on one of their X chromosomes were unaffected.

MEHMO features included microcephaly, therapy-resistant epileptic seizures (diagnosed at the age of 7 weeks), hypogenitalism, severe mental and motor delay, central obesity, and dysmorphic features including narrow forehead, full cheeks, facial telangiectasias, downturned corners of the mouth, large ears, edematous hands and feet, tapered fingers, and bilateral talipes. Diabetes was diagnosed at age of 6 months due to a ketoacidotic crisis. For accessory signs, see Table 1.

Current status. At 2 years, his height was 74 cm (-4 SD), BMI was 19.2 kg/m² ($+2$ SD), and head circumference was 37.7 cm (-8.2 SD). Toward the end of his life at 2 years, he had permanent seizures refractory to treatment.

Family 4

The proband is the second child from the fifth pregnancy (three miscarriages early on in the pregnancies) of nonconsanguineous Caucasian parents of Northern European origin living in the United States with no other affected individuals known including his mother, older brother, and younger sister.

MEHMO features included microcephaly, hypospadias and cryptorchidism, developmental delay, and obesity with BMI at the 97th centile (after a longer period of failure to thrive). He does not have epileptic seizures and his EEG was normal.

Current status. At 4 years and 7 months, his height was 93 cm (-3.2 SD), weight was 12.3 kg (-2.2 SD), BMI was 14.2 kg/m² (-0.8 SD), and his head circumference was 46.5 cm (-3.4 SD). He has central hypotonia and peripheral hypertonia and spasticity, chronic lung disease, dysphagia, delayed gastric emptying, and grade 5 vesicoureteral reflux (Table 1).

Exome Sequencing Identified Likely Deleterious Mutations in *EIF2S3*

Index cases from all families (families 1–4) were investigated by massively parallel sequencing. For three patients (families 1, 2, and 4), we performed WES and included the parents from families 2 and 4. The index patient from family 3 was investigated as part of a larger study on X-chromosome exome resequencing on families with XLID as previously described [Hu et al., 2016]. Sequence analysis indicated that all patients carried a hemizygous mutation in *EIF2S3* located on chromosome X as the most likely disease-relevant change. *EIF2S3* lies in the linkage interval determined for family 3 [Steinmüller et al., 1998]. Strikingly, families 1–3 carried the identical 4 bp deletion c.1394_1397delTCAA, which cosegregated with the disease in the families and is predicted to cause a frameshift and a premature

stop codon (p.Ile465Serfs*4) in the *EIF2S3* encoded protein eIF2 γ . The deletion alters the C-terminus with the last eight amino acids being replaced by three different amino acids (Fig. 1E). The patient from family 4 carried a point mutation, c.324T>A, which he inherited from his healthy mother (Fig. 1A) and could not be tested in his healthy brother. The single nucleotide exchange alters a highly conserved serine residue into arginine (p.Ser108Arg) (Fig. 1E). The mutations were not present in public databases, including dbSNP147 (<https://www.ncbi.nlm.nih.gov/SNP/>) and ExAC (<http://exac.broadinstitute.org>). Both mutations were predicted to affect function by in silico software analysis with CADD scores (<http://cadd.gs.washington.edu/>) of 35 for the deletion and 24 for the missense variant. Accordingly, both mutations would be predicted to be within the 1% most deleterious substitutions in the human genome.

The fact that males affected by MEHMO syndrome from three independent families carried the same *EIF2S3* 4 bp deletion prompted us to study their possible relatedness. The families themselves had no knowledge of any common ancestors. IBD analysis (see Supporting Information) using SNP array data from index patients of families 1 and 3 did not reveal any regions on the X chromosome to be identical by descent greater than 0.5 cM, excluding relationships between the affecteds of up to fourth-degree cousins with high probability. Subsequent comparison of WES data from index patients of the Slovak families 1 and 2, the mother from family 2, and X-chromosome sequencing data from the index patient from family 3 suggested a founder effect in these three families but with more distant relatedness (Supp. Tables S1 and S2).

No variants were found in the coding and adjacent intronic regions of *EIF2S3* in the index patients described by Leshinsky-Silver et al. [2002] and Delozier-Blanchet et al. [1989].

MEHMO Syndrome Mutations in Yeast eIF2 γ Impair Translation Initiation and Decrease Fidelity of Start Codon Selection

Recent high-resolution cryo-EM images of the yeast 48S PIC have provided insights into the structure of eIF2 and its interactions with other components of the PIC [Hussain et al., 2014; Llacer et al., 2015]. As shown in Figure 2A and B, the eIF2 TC docks on the intersubunit face of the 40S subunit with the primary contacts between the TC and the ribosome limited to the Met-tRNA_i^{Met} (green in the figure) and the N-terminal portions of the eIF2 α subunit (light yellow). The eIF2 γ subunit consists of three domains: an N-terminal GTP-binding (G) domain and two β -barrel domains DII and DIII (Figs. 2C and 3A). The eIF2 β subunit binds to the backside of the G domain (opposite the face that contacts GTP), while eIF2 α binds to domain DII. The acceptor arm of Met-tRNA_i^{Met} binds into a groove between the G domain and domains DII and DIII (Fig. 2B).

The human eIF2 γ -Ile465Serfs*4 mutation lies at the C-terminus of human eIF2 γ . While the C-terminus of yeast eIF2 γ is not fully resolved in the structure (Figs. 2B and C), the corresponding residues, Leu523 (L523) to the C-terminus, lie on the backside of the protein, remote from binding sites for eIF2 α , eIF2 β , Met-tRNA_i^{Met}, and the ribosome. The eIF2 γ missense mutation identified in this study, p.Ser108Arg, also lies in a region not fully resolved in the yeast eIF2 γ structure. The corresponding residue in yeast eIF2 γ is Asp167 (D167), which is in a flexible loop that is part of a zinc-binding ribbon inserted within the G domain (Fig. 2C). As the function of the Zn²⁺-binding element in eIF2 γ is unknown and it does not appear to interact with any other components in the 48S PIC [Llacer et al.,

2015], it is currently not possible to predict how the p.Ser108Arg mutation within a flexible loop of the element impacts eIF2 function. The p.Ile259Met mutation identified in the patient described by Moortgat et al. [2016] lies on the face of domain II that forms the binding cleft for Met-tRNA_i^{Met} (Figs. 2B and C). The corresponding residue in yeast eIF2 γ is Ile318. Interestingly, mutation of the adjacent conserved Arg319 residue in yeast eIF2 γ to Asp was lethal, indicating a critical function for this region of eIF2 γ [Roll-Mecak et al., 2004].

To test if the newly identified *EIF2S3* mutations impair eIF2 function, yeast models of the mutants were generated. To test the impact of the C-terminal mutation on eIF2 γ function, a yeast/human (*Sc/Hs*) chimeric form of eIF2 γ was generated by replacing the C-terminal 13 residues of yeast eIF2 γ (residues 515–527) with the corresponding C-terminal 16 residues of human eIF2 γ (residues 457–472) (Figs. 3A and B). This fusion point was chosen because, as shown in Figure 3B (and in Supp. Fig. S1), human and yeast eIF2 γ show strong sequence similarity with identical amino acids at 18 of the last 19 residues preceding the fusion point in the chimera (compare yeast residues Glu497-Trp514 with human residues Glu439-Trp456). In addition to the wild-type chimera (*Sc*^{L514}/*Hs*⁴⁵⁷⁻⁴⁷²; *Sc/Hs*), the *Sc/Hs-Ile465Serfs*4* mutant was generated by deleting the same four nucleotides deleted in the patients. As shown in Figure 3B, the mutation replaces the eight C-terminal residues of human eIF2 γ (-IKPTVDDD) with three novel residues (-SQQ).

Yeast expressing the *Sc/Hs* eIF2 γ chimera as the sole form of eIF2 γ grew like yeast expressing wild-type yeast eIF2 γ with no discernable growth defect at either 30 or 37°C (Fig. 3C). In contrast, yeast expressing the *Sc/Hs-Ile465Serfs*4* mutant exhibited a slight slow growth (Slg⁻) phenotype at 30°C that was exacerbated at 37°C (Fig. 3C). The Slg⁻ phenotype was not due to reduced protein levels as Western blot analysis of whole cell extracts revealed that both the *Sc/Hs* chimera and the *Sc/Hs-Ile465Serfs*4* mutant were expressed at levels equivalent to wild-type yeast eIF2 γ (data not shown). As a positive control in these growth assays, we tested the yeast eIF2 γ -Val281Thr and eIF2 γ -Val281Lys mutants, corresponding to the human eIF2 γ -Ile222Thr mutation in the previously described patients with XLID [Borck et al., 2012]. As observed previously, the Val281Lys mutation conferred a striking Slg⁻ phenotype (Fig. 3C).

To further explore the impact of the eIF2 γ C-terminal frameshift mutation on eIF2 function, a *GCN4-lacZ* reporter was introduced into isogenic yeast strains expressing wild-type yeast eIF2 γ , the *Sc/Hs* chimera, the chimera with the Ile465Serfs*4 mutation, or, as a positive control, yeast eIF2 γ -Val281Thr, which was previously shown to impair eIF2 function and cause derepression of *GCN4* expression [Borck et al., 2012]. Mutations that lower eIF2 activity lead to elevated *GCN4* (yeast homolog of *ATF4*) expression by limiting reinitiation [Hinnebusch, 2005]. Thus, ribosomes scanning downstream after translation of the first uORF (uORF1) on the *GCN4* mRNA fail to reinitiate at the inhibitory uORFs 3 and 4 and continue scanning. The added scanning time enables the ribosomes to acquire an eIF2 TC and reinitiate translation at the *GCN4* ORF instead. Although *GCN4-lacZ* reporter expression was similarly repressed in yeast expressing wild-type eIF2 γ or the *Sc/Hs* chimera, the Ile465Serfs*4 mutation derepressed *GCN4* expression nearly threefold, comparably to cells expressing eIF2 γ -Val281Thr (Fig. 4A). These results demonstrate that the C-terminal mutation in eIF2 γ impairs eIF2 function to a similar extent as the previously examined eIF2 γ -Val281Thr mutation.

In addition to derepressing *GCN4* expression, mutations that impair eIF2 function have also been found to lower the fidelity of translation initiation and enable ribosomes to initiate at non-AUG

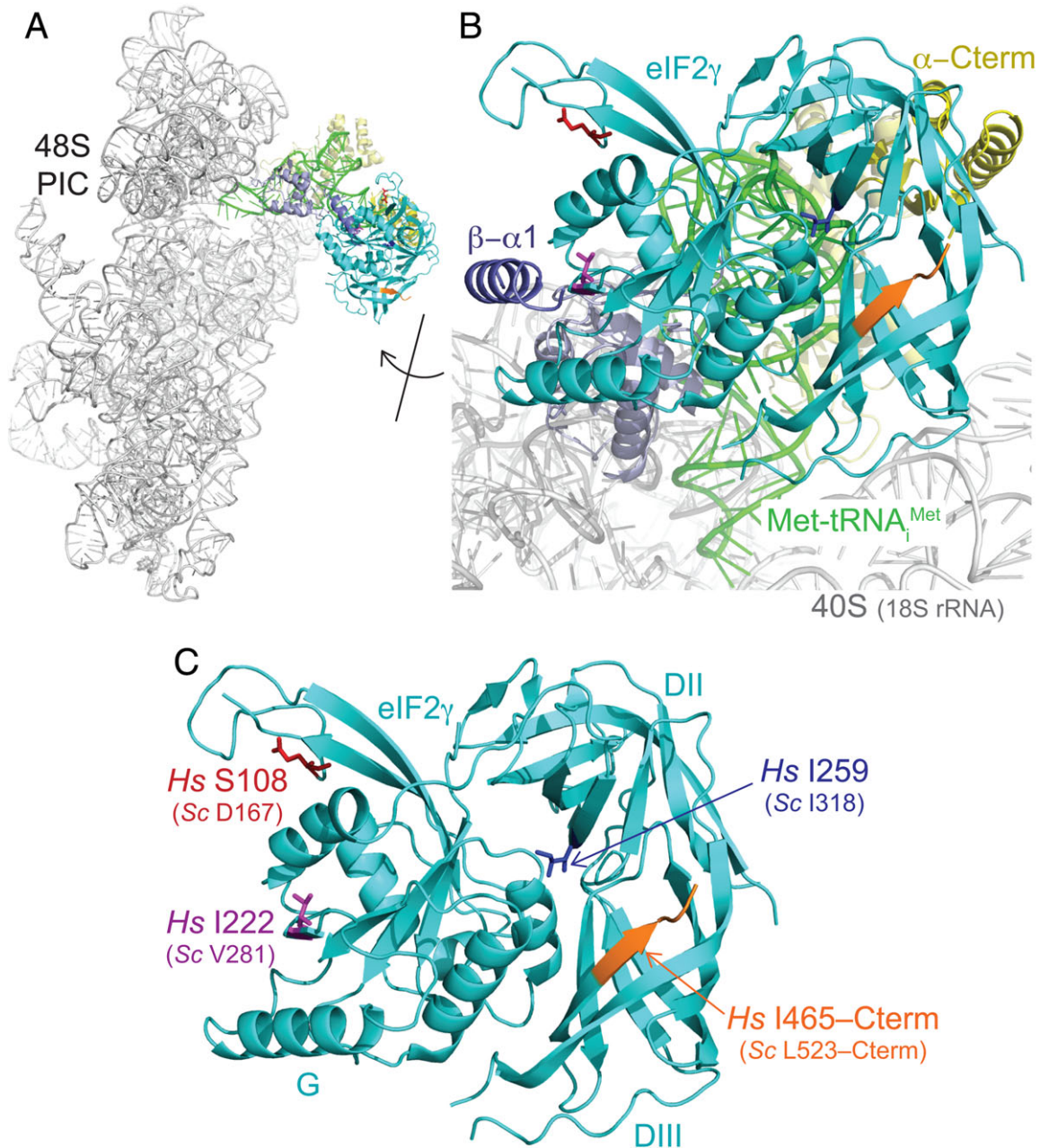


Figure 2. Mutations map to distinct regions of the eIF2 γ structure. **A:** Ribbons representation of the yeast 48S preinitiation complex (PIC; pdb 3JAP). Only the 18S rRNA of the 40S subunit (gray), Met-tRNA $_{Met}$ (green), eIF2 α (N-terminal domains, light yellow; C-terminal domain, yellow), eIF2 β (helix α 1, blue; C-terminal domain, slate), and eIF2 γ (cyan) are depicted. **B:** Image from panel A rotated as shown and zoomed to highlight the eIF2 γ subunit. Sites of the four human mutations are highlighted: S108R (*Sc* D167; red), I222T (*Sc* V281; purple), I259M (*Sc* I318; blue), and I465Sfs*4 (*Sc* L523–C-terminus; orange). **C:** Zoom of eIF2 γ from panel B. The other eIF2 subunits and 48S complex components have been removed for clarity. Sites of eIF2 γ mutations are colored as in panel B and labeled.

codons. Although translation is normally stringently restricted to initiate at an AUG codon, mutations that weaken Met-tRNA $_{Met}$ binding to eIF2, enhance eIF2 GTPase activity, or disrupt eIF2 integrity, enhance initiation at non-AUG codons and confer a suppressor of initiation (*Sui*⁻) phenotype in yeast [Hinnebusch, 2011]. To assess translation initiation fidelity, *HIS4-lacZ* reporter constructs with an AUG or UUG start codon were introduced into the same strains used to examine *GCN4* expression. Consistent with previous reports, expression of the *HIS4(UUG)-LacZ* reporter was substantially poorer than expression of the *HIS4(AUG)-LacZ*

reporter in cells expressing wild-type and *Sc/Hs* chimeric eIF2 γ leading to a low UUG/AUG initiation ratio ([Donahue and Cigan, 1988; Castilho-Valavicius et al., 1990; Dorris et al., 1995]; Fig. 4B, first bar). Consistent with the notion that the eIF2 γ -Ile465Serfs*4 mutation impairs eIF2 function, the UUG/AUG ratio was increased approximately twofold in cells expressing this eIF2 γ mutant compared to cells expressing the *Sc/Hs* chimera (Fig. 4B, second and third bar). This twofold increase in the UUG/AUG initiation ratio is similar to what was observed in cells expressing the eIF2 γ -Val281Thr mutant (Fig. 4B, fourth bar). Taken together, these results

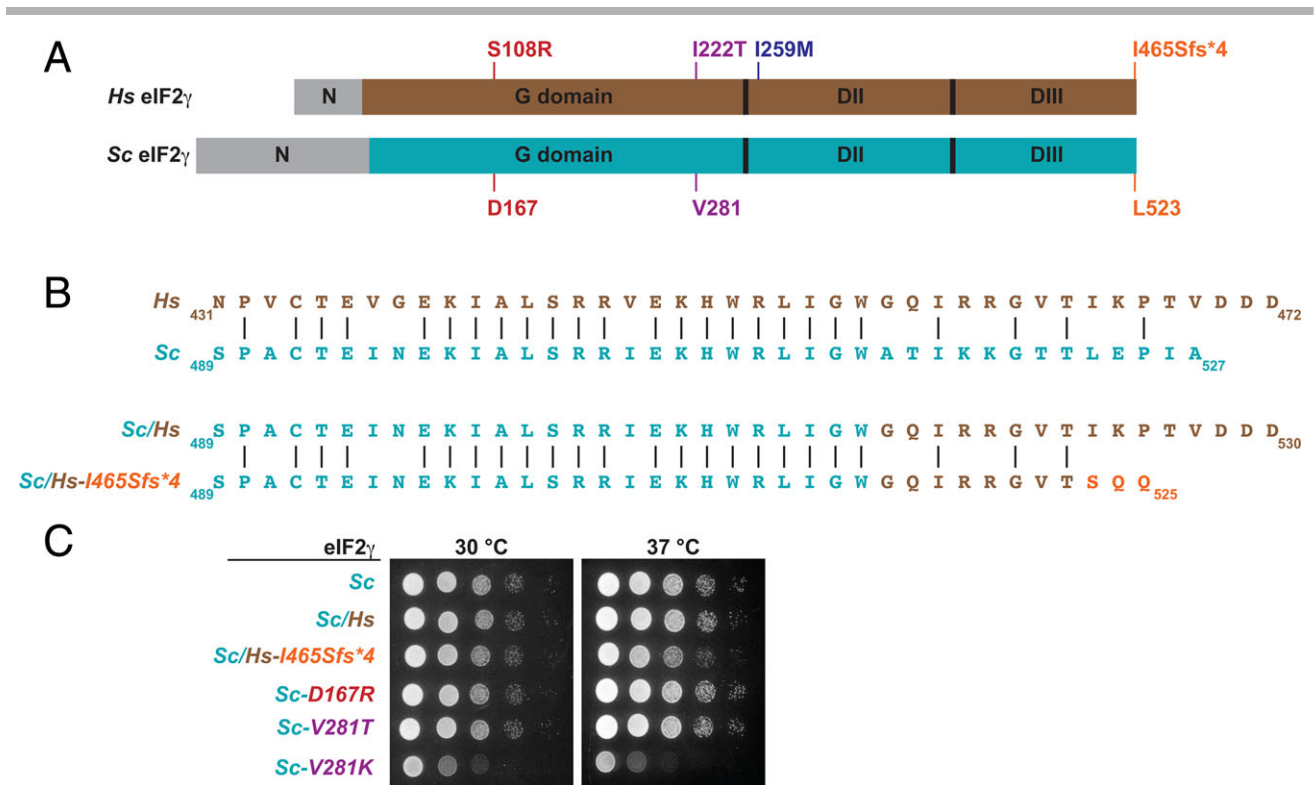


Figure 3. Impact of mutations in yeast eIF2 γ on cell growth. **A:** Schematics of human (top, brown) and yeast (*Saccharomyces cerevisiae*, Sc, bottom, cyan) eIF2 γ highlighting the N-terminal extensions (N), G domains and domains DII and DIII. Sites of mutations in human eIF2 γ and the corresponding residues in yeast eIF2 γ are labeled and colored as in Figure 2. **B:** Alignments (top) of the C-terminal sequences of human (*Hs*, brown) and yeast (*Sc*, cyan) eIF2 γ and (bottom) of a yeast/human eIF2 γ chimera with either the native human C-terminus (*Sc/Hs*) or with the C-terminal frameshift mutation (*Sc/Hs-I465Sfs*4*). **C:** Serial dilutions of yeast cells expressing, as the sole source of eIF2 γ , the indicated eIF2 γ mutants were grown on minimal synthetic dextrose (SD) medium at 30 or 37°C for 3 days.

demonstrate that the C-terminal mutation in eIF2 γ impairs eIF2 function.

As stated above, the p.Ser108Arg mutation in human eIF2 γ lies in a flexible loop within the putative Zn²⁺-binding element. Based on sequence alignment of human and yeast eIF2 γ (Supp. Fig. S1), yeast eIF2 γ residue Asp167 corresponds to human eIF2 γ residue Ser108. As shown in Figure 3C, yeast expressing eIF2 γ -Asp167Arg as the sole source of eIF2 γ exhibited no growth defects at either 30 or 37°C, nor did the mutation affect *GCN4* expression or translation start site selection (UUG initiation; data not shown). Combining the results obtained in yeast with the limited number of family members tested for the variant, we currently interpret the p.Ser108Arg substitution as a variant of unknown significance according to American College of Medical Genetics guidelines [Richards et al., 2015].

Elevated *DDIT3*/CHOP Expression in MEHMO Patient-Derived Fibroblasts

Given that the 4 bp deletion identified in families 1–3 is located in the last exon of *EIF2S3*, we assumed that the corresponding mutant mRNA would likely not undergo degradation by nonsense-mediated decay and instead would be translated into a truncated protein. To test this, we performed Western blot analysis for endogenous eIF2 γ protein using whole cell lysates from patient and control fibroblast cell lines. In all samples with concentrated protein, a specific product could be detected, whereas only very faint signals could be obtained without prior protein concentration (Fig. 1F).

These results demonstrate that eIF2 γ is expressed in human fibroblasts as expected given its essential role in cellular protein synthesis, and that the C-terminal truncated protein is produced in the patients. Furthermore, we examined transiently expressed N-terminal (FLAG) and C-terminal (myc) tagged wild-type and mutant eIF2 γ proteins in HeLa and HEK293T cells and could detect the proteins using anti-FLAG and anti-myc antibodies (Supp. Fig. S2). Next, we investigated the subcellular localization of transiently expressed eIF2 γ in HEK293T cells and the results did not reveal any major differences between the wild-type and mutant proteins (Supp. Fig. S3).

Having found that the C-terminal frameshift mutation impairs eIF2 γ function and derepresses *GCN4* expression in yeast, we investigated whether the ISR is induced in patient-derived fibroblasts. A key control point in the ISR is ATF4. Like its yeast homolog *GCN4*, expression of *ATF4* is controlled by uORFs and both proteins are transcription factors that direct the expression of key stress response proteins. We assessed the transcript level of the ATF4 target gene *DDIT3* by RT-qPCR and the levels of the *DDIT3*-encoded CHOP protein by Western blot in patient and control fibroblast samples. RT-qPCR experiments were performed using RNA extracted from patient IV:1 from family 3 (six biological replicates) and at least four control fibroblasts (three biological replicates) from healthy male individuals. The *DDIT3* transcript level was significantly increased (2.5-fold) in the patient relative to the controls (Fig. 5A), and Western blot analysis of whole cell lysates confirmed significantly elevated CHOP protein levels in the patient compared to control fibroblasts (see representative Western blot in Fig. 5B).

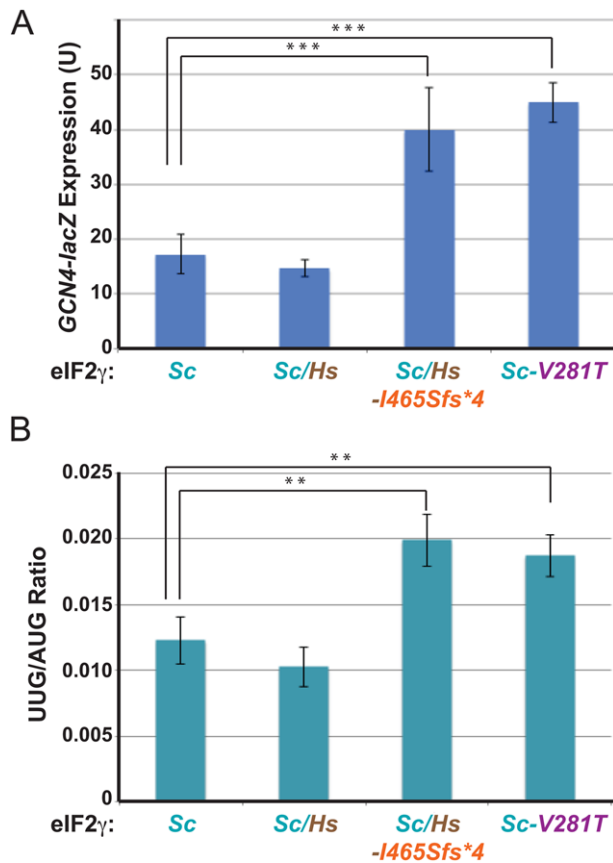


Figure 4. Mutations in yeast eIF2 γ impair translation initiation. **A** and **B**: A *GCN4-lacZ* (**A**) or *HIS4(AUG)*- or *HIS4(UUG)-lacZ* (**B**) reporter construct was introduced into yeast strains expressing the indicated WT or mutant form of yeast (*Sc*) or of yeast/human chimera (*Sc/Hs*) eIF2 γ . β -Galactosidase activities (**A**) or mean ratios of activities (**B**) and standard deviations were determined from at least three independent transformants. The data were assessed by Student's *t*-test and *P*-values relative to wild-type (*Sc*) eIF2 γ (***P* \leq 0.01; ****P* \leq 0.001).

Discussion

This study demonstrates that mutations in *EIF2S3* encoding the translation initiation factor eIF2 γ are associated with the XLID syndrome MEHMO. Since its initial description more than 25 years ago [DeLozier-Blanchet et al., 1989], only five families have been reported with full MEHMO syndrome (including two new families from this study). In three of the families, we have found the C-terminal p.Ile465Serfs mutation. Inspection of sequencing data and haplotype analysis on available family members suggests that the families could be distantly related. An additional family carrying this pathogenic variant has been described very recently [Moortgat et al., 2016]; however, the phenotype was not described as MEHMO syndrome, probably due to early death of the patient at 12 months. In two families [DeLozier-Blanchet et al., 1989; Leshinsky-Silver et al., 2002], no mutation was found in the *EIF2S3* gene. While the patient reported by Leshinsky-Silver et al. [2002] carried a deleterious (p.Tyr430X) mutation in the *SERAC1* (MIM# 614725) gene, the genetic defect in the family reported by DeLozier-Blanchet et al. [1989, 1999] could be located outside the *EIF2S3* region examined by WES or the disease in this family might have been caused by a mutation in another gene mimicking the MEHMO syndrome.

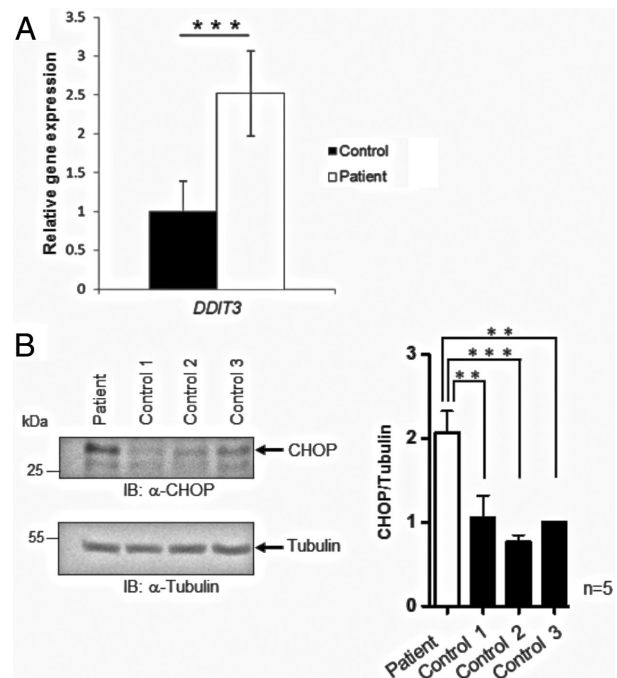


Figure 5. The EIF2S3 C-terminal mutation is associated with elevated *DDIT3*/CHOP transcript and protein levels in patient fibroblasts. **A**: Expression of *DDIT3* mRNA in patient and control fibroblasts measured using RT-qPCR. Levels are normalized to the control and expressed as 2^{-dCq} with SD. ****P* < 0.001, *t*-test. Patient fibroblasts show an increased *DDIT3* transcript level compared to controls. **B**: Representative Western blot analysis of protein lysates from patient fibroblasts (index from family 3) with C-terminal-deleted EIF2S3 and three controls. Whole cell lysate from patient and control fibroblasts was concentrated and separated on SDS-PAGE. The gel was blotted and probed with an anti-CHOP antibody (upper panel). The blot was subsequently probed with an antitubulin antibody as a loading control (bottom panel). Quantification of the specific bands from Western blots was performed using ImageJ software, and CHOP levels were normalized to tubulin levels. Histogram of five independent experiments shows a statistically significant increase in CHOP levels in patient fibroblasts compared to controls. The data were assessed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (***P* \leq 0.01; ****P* \leq 0.001).

Interestingly, to date four different mutations have been found in the *EIF2S3* gene in patients described as having syndromic XLID. Phenotypic overlap with MEHMO syndrome is now evident in all cases. All four index patients carrying the C-terminal frameshift mutation presented the full spectrum of MEHMO syndrome including microcephaly, epileptic seizures, hypogonadism, hypogonitalism, and obesity. Moreover, three of the patients developed insulin-dependent diabetes in the first year of life. All of the patients with the frameshift mutation failed to achieve head control, were not able to make any voluntary movements, and did not have any social interactions. In addition, typical facial dysmorphism were present in these patients. In contrast, the patients with the missense mutations identified to date are less severely affected and present only subsets of symptoms that can differ even between family members carrying the same mutation. For example, the boy reported in this study with the maternally inherited p.Ser108Arg substitution of currently unknown clinical significance does not have diabetes or epilepsy. Moreover, in a family with the p.Ile259Met mutation, epilepsy was present only in one (III:2) of the two affected males. Both patients were able to perform movements and were capable of social interaction, and one of them even spoke several words [Moortgat et al.,

2016]. In our previously published family with the p.Ile222Thr mutation, the patients had only moderate-to-severe intellectual disability (ID) (one of them can speak in short sentences and feed himself). One of these latter patients lived into adulthood, and they were able to walk (ataxic gait). Other clinical features of MEHMO syndrome were present individually in this family—generalized seizures (patient III:2) and postpubertal microgenitalism and obesity (patient II:3) [Borck et al., 2012]. The final reported missense mutation p.Val151Leu was found in one XLID family, but further phenotypic information was unavailable [Tarpey et al., 2009].

Although a larger case series will be required to define genotype–phenotype relations in MEHMO syndrome, the variable clinical severity may depend on the type and localization of the *EIF2S3* mutations with the eIF2 γ C-terminal frameshift mutation being more deleterious than the reported amino acid substitutions. We assessed the impact of the mutations found in our patients in a yeast model. Consistent with the more severe patient symptoms, the eIF2 γ -Ile465Serfs*4 mutation significantly impaired yeast cell growth (Fig. 3), while yeast expressing the eIF2 γ -Asp167Arg and eIF2 γ -Val281Thr mutants (corresponding to the Ser108Arg and Ile222Thr mutations in human eIF2 γ , respectively) grew comparably to the control strain expressing wild-type eIF2 γ (Fig. 3). Interestingly, measurement of *GCN4* expression and the fidelity of translation start site selection demonstrated that the C-terminal truncating mutation in eIF2 γ impairs eIF2 function to a similar extent as the eIF2 γ -Val281Thr mutation, while the eIF2 γ -Asp167Arg missense mutation did not influence these assays. The lack of observable phenotype for this latter mutation in yeast suggests that the impact of the mutation may be more notable in multicellular organisms or that the mutation specifically impairs a function of human eIF2 γ that is not shared with the yeast factor.

Pathogenic variants of eIF2 γ could impact eIF2 function in various ways including altering the binding of eIF2 γ to its counterparts eIF2 α and eIF2 β , altering the binding of eIF2 to Met-tRNA_i^{Met}, GTP, the ribosome or other translation factors, or altering eIF2 γ expression or stability. We have shown that the C-terminal frameshift mutation does not result in loss of the eIF2 γ protein, suggesting instead that it impairs eIF2 function. In this context, it is interesting to note that two mutations causing similar impairments in eIF2 function were previously identified near the C-terminus of yeast eIF2 γ . The *gcd11-503* mutant, which generates eIF2 γ -Arg510His, derepressed *GCN4* expression and increased initiation at non-AUG codons, while the *gcd11-508* mutant, which causes a frameshift that replaces the C-terminal three residues in yeast eIF2 γ (normally –PIA, see Supp. Fig. S1) with the sequence –VGIEINYHLLTSYITSILSYTVLEDDANDEK, derepressed *GCN4* expression, but did not lessen the fidelity of translation start site selection [Dorris et al., 1995]. Thus, an intact C-terminus is critical for proper eIF2 function in vivo, although the precise molecular role remains to be determined. The remaining eIF2 γ mutations linked to XLID are located in distinct regions of the protein. As reported previously [Borck et al., 2012], the Ile222Thr mutation in human eIF2 γ , corresponding to the residue Val281 in *Saccharomyces cerevisiae* eIF2 γ (Fig. 3A and Supp. Fig. S1), lies in the docking site for helix α 1 of eIF2 β (Fig. 2B) and mutation of this residue impaired the binding of eIF2 β to both yeast and human eIF2 γ . The recently identified p.Ile259Met mutation [Moortgat et al., 2016] (Ile318 in yeast) lies near the Met-tRNA_i^{Met} binding site and mutation of the adjacent Arg319 to Asp in yeast eIF2 γ was lethal [Roll-Mecak et al., 2004]. Neither the C-terminal frameshift mutation nor the novel p.Ser108Arg variants are localized in functionally defined regions of eIF2 γ and their exact functional consequences are hard to predict.

In mammals and other metazoans, downregulation of eIF2 function through phosphorylation of eIF2 α activates the ISR [Harding et al., 2003], which corresponds to the translational arm of the UPR. As originally observed for the *GCN4* mRNA in yeast [Hinnebusch, 2005], regulated reinitiation at uORFs governs the translation of the *ATF4* mRNA in a manner controlled by eIF2 function [Harding et al., 2000; Lu et al., 2004; Vatter and Wek, 2004; Young and Wek, 2016]. We confirmed in patient-derived fibroblasts that the C-terminal eIF2 γ frameshift mutation leads to activation of the ISR, consistent with the notion that the mutation lowers eIF2 function. Increased expression of *DDIT3* and its corresponding protein CHOP show that MEHMO syndrome belongs to the group of disorders caused by dysregulation of translation or the UPR (Table 3). Moreover, the clinical picture of MEHMO syndrome shares common features with these disorders. Mutations in eIF2 γ , like mutations in *EIF2B1* (MIM# 606686), *EIF2B2* (MIM# 606454), *EIF2B3* (MIM# 606273), *EIF2B4* (MIM# 606687), *EIF2B5* (MIM# 603945), and *PPP1R15B* (MIM# 613257), impair the activity or availability of TC. This could lead to a general insufficiency of protein synthesis that most significantly impacts processes requiring high rates of protein synthesis and will also cause increased sensitivity in states of elevated metabolic requirements. Moreover, the decreased translation rate and attendant chronically elevated ATF4 and CHOP levels result in apoptosis [Tabas and Ron, 2011; Walter and Ron, 2011; Han et al., 2013; Matsumoto et al., 2013]. In vanishing white matter (VWM) disease (MIM# 603896) caused by mutations in any of the subunits of eIF2B, mutated cells are hypersensitive to stressors that lead to eIF2 α phosphorylation [Kantor et al., 2005; van der Voorn et al., 2005]. Similarly, patients with VWM syndrome can undergo rapid deterioration following stress such as fever, minor head trauma, or even fright [Schiffmann et al., 1994; Vermeulen et al., 2005; Labauge et al., 2009]. Like the VWM patients, the condition of patient 1 in our study was markedly aggravated during periods of illness. Interestingly, the VWM syndrome pathology is mainly localized to the brain and ovaries, while MEHMO syndrome is more generalized (though the white matter changes are reminiscent of VWM). In contrast, patients with mutations in *PPP1R15B* share additional symptoms with the *EIF2S3* male mutation carriers including diabetes, severe microcephaly, growth retardation, developmental delay, ID, and bone dysplasia [Abdulkarim et al., 2015; Kernohan et al., 2015].

In contrast to diseases such as VWM and MEHMO syndrome, which are linked to induction of the ISR or UPR, insufficient activation of the UPR is also linked to disease. In Wolcott–Rallison syndrome (WRS) (MIM# 226980), mutated PERK (encoded by *EIF2AK3*, MIM# 604032) fails to phosphorylate eIF2 α in response to ER stress. This results in impaired downregulation of protein synthesis and further ER overload [Harding et al., 2001; Volchuk and Ron, 2010]. The UPR is important for proliferation and differentiation of pancreatic β -cells [Zhang et al., 2006; Kaufman et al., 2010], and dedifferentiation from a secretory phenotype in chondrocytes may play a role in adaptation to chronic stress [Tsang et al., 2007; Rajpar et al., 2009]. The dysregulation of the UPR may thus explain the main features of WRS—permanent neonatal diabetes and epiphyseal dysplasia. Despite their different impacts on the UPR, another frequent symptom of WRS—episodes of acute liver failure triggered by intercurrent diseases such as upper airway infections [Julier and Nicolino, 2010]—is similar to the progressive deterioration in MEHMO and VWM syndromes. Moreover, MEHMO patient 3 (this report) had congenital scoliosis, a feature commonly associated with WRS.

In addition to the PERK mutations that cause WRS by altering ER homeostasis, ER proteins that likewise play a role in ER

Table 3. Similarities among Syndromes Caused by Mutations in Genes Involved in eIF2 Function or Regulation or in the UPR

Gene Protein	<i>EIF2S3</i> γ Subunit of eIF2	<i>EIF2B1, 2, 3, 4, 5</i> Subunits of eIF2B	<i>PPP1R15B</i> CrpP (p-eIF2α phosphatase)	<i>EIF2AK3</i> PERK (eIF2α kinase)	<i>IER3IP1</i> ER and Golgi located	<i>WFS1</i> ER transmembrane protein	<i>DNAJC3</i> BiP cochaperone	<i>SIL1</i> BiP cochaperone
Role in protein metabolism	Translation initiation	eIF2-GTP recycling for translation initiation	Constitutively restores translation initiation	UPR—downregulation of translation in case of ER stress	Modulation of apoptosis and differentiation	Ca ²⁺ homeostasis important for correct protein folding	Ensures proper folding of proteins	Ensures proper folding of proteins
Inactivating mutations lead to	Attenuation of translation, inappropriate ATF4-CHOP induction	Attenuation of translation, inappropriate ATF4-CHOP induction	Attenuation of translation	Impaired ER stress induced attenuation of translation, lack of ATF4-CHOP branch of UPR induction, ER overload	Increased susceptibility to cellular stress	ER Ca ²⁺ depletion and misfolding of proteins	Increased ER stress	Increased ER stress
Syndrome*	MEHMO (MIM# 300148), syndromic XLID	CACH/VWM (MIM# 603896)	Syndromic ID with diabetes (MIM# 616817)	WRS (MIM# 226980)	MEDS (MIM# 614231)	WFS (MIM#222300)	ACPHD (MIM# 616192)	MSS (MIM# 248800)
Inheritance	X-linked recessive	AR	AR	AR	AR	AR	AR	AR
No. of patients/families (including this study)	18/8	~290	4/2	~60	7/5	>450	5/2	~70
Onset	Congenital	From congenital to adult	Congenital	Neonatal, first year of life	Congenital	Childhood	From child to adult	Congenital to infancy
Life span	Few months, oldest patient alive at 18 y	Stress-induced death, childhood form— months to years from onset; adult form—years from onset	Oldest patient alive at 31 y	Few weeks to few years, exceptionally over 30 y	The oldest patient deceased at 8 y	30–40 y	Oldest patient alive at 41 y	
Diabetes	±	–	+	+	+	+	+	–
Obesity	+	–	–	–	–	–	–	–
Endocrinopathies	Panhypopituitarism, hypogonadism, ± hypogonadism, ±	–	Hypothyroidism ±	Central hypothyroidism	Hypogonadism, hypogonadism, ±	Diabetes insipidus	n.r.	Hypergonadotrophic hypogonadism, ±
ID	Moderate to profound	None to profound	Severe	None to severe	Profound	±	– isolated cognitive deficit	Mild to moderate
Microcephaly	+	±	+	–	+	–	n.r.	±
Neurodegeneration	+	+	+	n.r.	+	+	+	+
Developmental delay	+	+ After disease onset	+	+	+	±	–	+
Epilepsy	+	+	n.r.	n.r.	+	–	–	±
Short stature	+	–	–	+	±	–	+	+
Bone abnormalities	±	–	+	Multiple epiphyseal dysplasia	±	–	n.r.	+
Hearing loss	–	–	–	–	–	+	+	–
Dysmorphic features	+	–	+	±	+	+	+	±
Special features		Ovarian failure, optic nerve atrophy		Liver dysfunction, recurrent infections	Optic atrophy, diabetes insipidus			Congenital cataracts, myopathy
Progressive course	+	+	+	+	+	+	+	+
Episodic aggravation due to stress/illness	+	+	n.r.	+	+	–	n.r.	n.r.
References	This study; Borck et al., 2012; Moortgat et al., 2016; Steinmuller et al., 1998; Tarpey et al., 2009	Fogli et al., 2004; Labarge et al., 2009	Abdel-Salam et al., 2012; Kernohan et al., 2015	Delepine et al., 2000; Julier and Nicolino, 2010; Rubio-Cabezas et al., 2009	Abdel-Salam et al., 2012; Poulton et al., 2011; Shalev et al., 2014	de Hereclia et al., 2013; Fonseca et al., 2005	Synofzik et al., 2014	Ezgu et al., 2014; Hasegawa et al., 2014; Senderek et al., 2005

CACH/VWM, childhood ataxia with central hypomyelination/vanishing white matter disease; WRS, Wolcott-Rallison syndrome; MEDS, microcephaly, epilepsy, and diabetes syndrome; WFS, Wolfram syndrome; ACPHD, ataxia, combined cerebellar and peripheral, with learning loss and diabetes mellitus; MSS, Marinesco-Sjorgen syndrome; BiP, immunoglobulin heavy-chain binding protein; AR, autosomal recessive; n.r., not reported; y, years.

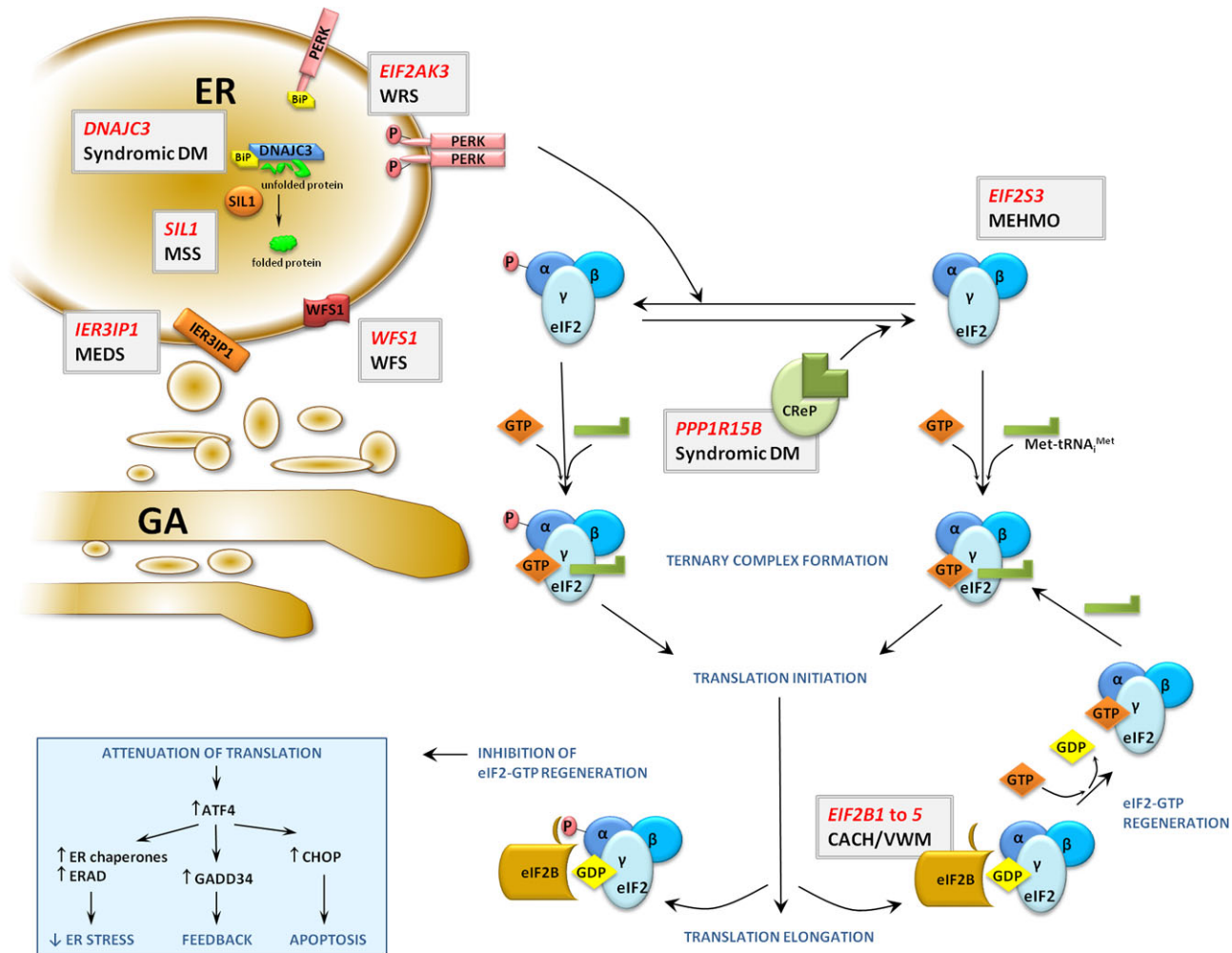


Figure 6. MEHMO syndrome belongs to the disorders associated with alterations in eIF2 function and regulation or involved in the UPR. To initiate translation of the factor eIF2, a heterotrimeric complex of α , β , and γ subunits binds GTP and initiator Met-tRNA^{Met} to form a ternary complex (TC). The TC associates with a ribosome, and during the course of translation initiation the GTP is hydrolyzed to GDP enabling release of eIF2-GDP from the ribosome. In order for eIF2 to participate in subsequent rounds of initiation, the GDP bound to eIF2 is exchanged for GTP in a reaction catalyzed by the guanine nucleotide exchange factor eIF2B. During ER stress, the recognition and refolding of misfolded proteins is assisted by the chaperones DNAJC3 and BiP with the help of the nucleotide exchange factor SIL1. The products of the *IER3IP1* and *WFS1* genes are also important for ER function. Under nonstress conditions, BiP binds to the kinase PERK blocking its activation. The overload of misfolded proteins under stress conditions titrates BiP from PERK leading to activation of the kinase. Activated PERK phosphorylates the α subunit of eIF2, converting eIF2 into a competitive inhibitor of eIF2B. The subsequent decreased availability of eIF2-GTP for TC formation leads to downregulation of overall protein synthesis and increased translation of the *ATF4* mRNA. The ATF4 protein, a transcription factor, upregulates genes necessary for the adaptive response to the stress situation including *DDIT3* encoding CHOP. Prolonged activation of the stress response and elevated CHOP levels eventually leads to apoptosis. Mutations in genes involved in these processes (gray boxes: gene in red, disorder in black) account for syndromes with overlapping clinical pictures (summarized in Table 3).

homeostasis and correct protein folding were found to be mutated in syndromes with clinical features that overlap MEHMO syndrome. For example, mutations in *DNAJC3* (MIM# 601184) [Synofzik et al., 2014], *SIL1* (MIM# 608005) [Senderek et al., 2005], *WFS1* (MIM# 606201) [Fonseca et al., 2005], and *IER3IP1* (MIM# 609382) [Abdel-Salam et al., 2012] (Table 3, Fig. 6) result in accumulation of misfolded proteins in the ER leading to UPR activation. The microcephaly, epilepsy, and diabetes syndrome (MEDS) syndrome (MIM# 614231) caused by *IER3IP1* mutations strikingly resembles MEHMO syndrome with affected patients suffering from severe developmental delay, seizures difficult to control, insulin-dependent diabetes, and hypotonia. Moreover, some MEDS syndrome patients were obese and one had hypogonadism. The facial features of MEDS syndrome patients are also similar to MEHMO

patients with full cheeks and large ears. In addition to these similarities with MEHMO syndrome, some MEDS patients shared features with WRS syndrome including skeletal abnormalities and repeated bone fractures. The function of *IER3IP1* protein is not fully understood, but the protein seems to play a role in maintaining vesicular trafficking between the ER and Golgi, and cells with nonfunctional *IER3IP1* are more susceptible to cellular stress and subsequent apoptosis [Poulton et al., 2011].

Mutations in other components of the translation apparatus as well as regulators of translation can also cause human disease. We and others showed that mutations in *FTSJ1* (MIM# 300499), *TRMT1* (MIM# 611669), and *NSUN2* (MIM# 610916), which are implicated in tRNA modification, are associated with ID (MIM# 309549, MIM# 611091) [Freude et al., 2004; Abbasi-Moheb et al.,

2012; Khan et al., 2012; Davarniya et al., 2015; Guy et al., 2015]. Similarly, mutations in *LASIL* (MIM# 300964), which is important for the biogenesis of the 60S ribosomal subunit, are associated with Wilson–Turner syndrome (MIM# 309585) characterized by obesity, gynecomastia, speech difficulties, emotional lability, tapering fingers, and small feet [Hu et al., 2016]. Finally, mutations in the X-linked *RPL10* gene (MIM# 312173), which codes for a highly conserved multifunctional component of the large 60S ribosomal subunit, are associated with a variable phenotype with XLID or normal cognitive function (MIM# 300847) [Klauck et al., 2006; Chiocchetti et al., 2011; Brooks et al., 2014; Thevenon et al., 2015; Zanni et al., 2015].

In brief, the clinical symptoms associated with mutations in eIF2 γ (MEHMO syndrome), eIF2B (childhood ataxia with central nervous system hypomyelination (CACH)/VWM syndrome), and *PPP1R15B* that encodes CReP indicate that sustained low-level protein synthesis is poorly tolerated. At the same time, the overlapping phenotypes in WRS result from impaired downregulation of translation in response to a variety of stress conditions. Thus, tight regulation of protein synthesis and its adaptation to cellular stress are critical for health and development. Moreover, the lack of appropriate regulation helps to explain the significant influence of environment that can modulate the course and severity of these diseases.

In conclusion, this study establishes the link between mutations in the *EIF2S3* gene and MEHMO syndrome. Our findings contribute to an increasing list of disorders caused by dysregulation of protein synthesis and underscore the importance of considering genes involved in translation and its regulation as candidates for ID-associated syndromes.

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