Impaired EIF2S3 function associated with a novel phenotype of X-linked hypopituitarism with glucose dysregulation

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Abstract

Background: The heterotrimeric GTP-binding protein eIF2 forms a ternary complex with initiator methionyl-tRNA and recruits it to the 40S ribosomal subunit for start codon selection and thereby initiates protein synthesis. Mutations in EIF2S3, encoding the eIF2γ subunit, are associated with severe intellectual disability and microcephaly, usually as part of MEHMO syndrome.

Methods: Exome sequencing of the X chromosome was performed on three related males with normal head circumferences and mild learning difficulties, hypopituitarism (GH and TSH deficiencies), and an unusual form of glucose dysregulation. In situ hybridisation on human embryonic tissue, EIF2S3-knockdown studies in a human pancreatic cell line, and yeast assays on the mutated corresponding eIF2γ protein, were performed in this study.

Findings: We report a novel hemizygous EIF2S3 variant, p.Pro432Ser, in the three boys (heterozygous in their mothers). EIF2S3 expression was detectable in the developing pituitary gland and pancreatic islets of Langerhans. Cells lacking EIF2S3 had increased caspase activity/cell death. Impaired protein synthesis and relaxed start codon selection stringency was observed in mutated yeast.

Interpretation: Our data suggest that the p.Pro432Ser mutation impairs eIF2γ function leading to a relatively mild novel phenotype compared with previous EIF2S3 mutations. Our studies support a critical role for EIF2S3 in human hypothalamo-pituitary development and function, and glucose regulation, expanding the range of phenotypes associated with EIF2S3 mutations beyond classical MEHMO syndrome. Untreated hypoglycaemia in previous cases may have contributed to their more severe neurological impairment and seizures in association with impaired EIF2S3.

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1. Introduction

The eukaryotic translation initiation factor (eIF) 2 subunit 3 (EIF2S3) (NM_001415; Xp22.11) gene encodes the gamma (γ) subunit of the translation initiation factor eIF2; the largest of the three eIF2 subunits.

In translation initiation, eIF2 binds GTP via eIF2γ, and the initiator methionyl-tRNA (Met-tRNAiMet), to form a ternary complex that scans mRNA for the AUG start codon [1]. Mutations in several translation factors, including eIF2 subunits, enhance translation initiation at near cognate codons such as AUU and UUG [1–3]. EIF2S3 is located within Xp21.1-p22.13, a region linked to a rare intellectual disability (ID) disorder designated as MEHMO syndrome (OMIM 300148) [4]. MEHMO syndrome exhibits phenotypic heterogeneity and is variably characterized by mental retardation, epileptic seizures, hypogonadism with...
A non-consanguineous white European pedigree with three affected male patients, presented with severe recurrent hypoglycaemia, short stature with GH and TSH deficiencies, an unusual form of glucose dysregulation, and mild learning difficulties. This combination of phenotypes has, to date, not been reported in the literature. There were no publications linking these phenotypes in any online journal listed on Pubmed or elsewhere.

**Added value of this study**

Exome sequencing of the X-chromosome revealed a novel missense variant c.1294C > T in *EIF2S3* (p.Pro432Ser), encoding a subunit of the eukaryotic translation initiation factor 2, eIF2γ, in all three male patients and in the heterozygous mothers. Mutations in *EIF2S3* have been described in patients with MEHMO syndrome, characterized by severe intellectual disability, microcephaly, short stature, epilepsy and accompanying midline and facial abnormalities. The functional and expression data in our study show a moderate but significant eIF2γ impairment, which relates to the milder phenotype in our three male patients.

**Implications of all the available evidence**

This milder loss of function compared with previous *EIF2S3* mutations gives rise to a phenotype that is distinct from the classical spectrum of MEHMO syndrome. Untreated hypoglycaemia in the previously published cases may have contributed to their more severe impairment of neurodevelopment and seizures. We highlight that pancreatic and pituitary phenotypes appear to be associated with *EIF2S3* mutations. Early identification of such patients with a rapid molecular diagnosis may lead to prevention of significant morbidity, and may be critical for the prevention of significant neurodevelopmental delay in these patients.

**2. Materials and methods**

**2.1. DNA sequencing**

The coding regions of the X-chromosome were sequenced in Pedi-gree 1 in the Department of Genetics, University Medical Center Utrecht, Netherlands, in collaboration with GOGene, London UK. Next-generation sequencing of all protein coding sequences on the X chromosome (X-exome) was performed as previously described. Barcoded fragment libraries were pooled in equimolar ratios and enriched using multiplexed targeted genomic enrichment [8] with the Demo X-exome enrichment kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced according to the SOLiD 3 Plus manual (Life Technologies, Carlsbad, CA, USA). Raw sequencing data were mapped against the GRCh37/hg18 reference genome using a custom bioinformatic pipeline based on the BWA algorithm. The percentage cut-off for the non-reference allele (NRA) of candidate single nucleotide variants and small indels was set to 15%. The cut-off for minimum sequence coverage was set to 10 reads. Filtering of variants that fit an X-linked dominant inheritance model was performed using non-stringent criteria for affected males being hemizygous for the candidate causal variant (>50% NRA). Subsequently, all common and rare polymorphisms present in Ensembl 65, ExAC, gnomAD or our in-house X-exome database of ~100 samples, were marked as known and not further considered for this study. The remaining variants were considered to be novel and thus fulfill the criteria for an ultra-rare disease. Protein prediction models were consulted, that use straightforward physical and comparative considerations reflecting pathogenicity of a variant. SIFT scores range from 0 (deleterious) to 1 (tolerated), whilst Polyphen-2 scores range from 0 (benign) to 1 (damaging) respectively.

**2.2. PCR and direct sequencing analysis**

PCR primers were designed using the Ensembl Genome Browser (http://www.ensembl.org/index.html), the UCSC genome browser (https://genome.ucsc.edu/) and the Primer3 input (http://bioinfo.ut.ee/primer3-0.4.0/). DNA was extracted from 103 patient blood samples from our cohort and the coding regions of *EIF2S3* were amplified by PCR using exon flanking primers and the BIOTAQ™ DNA Polymerase kit (Bioline) on an Eppendorf Thermocycler. PCR products were treated with MicroClean reagent (Web Scientific). The precipitate was sequenced with forward and reverse respective primers using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies Ltd) on an Eppendorf Thermocycler. The samples were run on a 3730XL DNA Analyzer (Applied Biosystems/Hitachi, Japan). Detailed PCR and sequencing conditions and primer sequences are available upon request. Control databases ExAC and gnomAD were consulted.

**2.3. Cell culture**

A hybrid cell line (1,184 cells) formed by the electrofusion of a primary culture of human pancreatic islets with PANC-1, a human pancreatic ductal carcinoma cell line, was obtained from Public Health England (PHE). The cells were maintained in a humidified CO₂ incubator at 37 °C in Roswell Park Memorial Institute (RPMI) medium 1640 containing l-glutamine (Life Technologies), supplemented with 10% fetal calf serum (FCS) and 5% penicillin/streptomycin (penstreps). The cells were washed in diluted Hanks Balanced Saline Solution (Gibco) (x1 HBSS), and trypsinised with trypsin (Gibco) diluted in x1 HBSS in line with manufacturer’s instructions. Cells were passaged at >80% confluence.
2.4. Constructs containing shRNA cassettes

GFP-IRES-Puromycin-Zeomycin plasmids (pGIPZ) (11,744 bp), referred to in this study as Clone 1–4, contained small hairpin RNA (shRNA) cassettes targeting the EIF2S3 human gene, validated by the UCL Cancer Institute, and 100% matched with the expected hairpin sequence according to the Open Bionformics Library database. An additional non-silencing (NS) pGIPZ plasmid containing a scrambled shRNA sequence was used as a control in transduction experiments. All five plasmids have a lentiviral (LV) backbone; long terminal repeats (LTR’s) with modified U3 and a packaging signal. The pGIPZ plasmids also contained GFP and a puromycin-resistance cassette. This enabled the cells to be monitored for GFP expression and be puromycin-selected following transduction (the standard map of the pGIPZ plasmid is available upon request). A maxiprep of each plasmid was prepared and used in LV packaging and transduction assays.

2.5. Lentiviral packaging

LV packaging was carried out under appropriate containment by transient co-transfection of HEK293T cells (4.0 × 10⁵ cells/well of a 6 well plate seeded 24 h prior to transfection) for each pGIPZ plasmid in duplicate, with 500 ng of the pGIPZ vector constructs, 333 ng of packaging plasmid (pCMV-dR8.91), 333 ng of VSV-G envelope expressing plasmid (pMD2.G), Fugene HD (3.5 μl/well) transfecting agent (Promega) and opti-mem media (Thermo Fisher Scientific). Media was replaced after 24 h incubation at 37 °C, with subsequent harvesting and filtering of the vector 24 h later. Infectious titres were determined by limiting dilutions of LVs on HEK293T cells. Titres were comprised between 10⁶ and 10⁷ transducing units/ml.

2.6. Transduction of 1.1B4 cells using packaged LV vectors for stable gene knockdown

1.1B4 cells were seeded into 6-well plates and transduced at a multiplicity of infection (MOI) of 5, using the previously generated lentivirus under strict sterile and contained conditions. Cells were incubated at 37 °C for 72 h before media was removed. Fresh RPMI medium containing 10% FCS and 5% penstrep, also used on non-transduced cells, was supplemented with puromycin and added to the cells to positively select the transduced cells expressing shRNA. The cells were monitored and kept under puromycin selection for a further 10 days, trypsinised when confluent and expanded into T25cm² flasks respectively. The cells were washed in x1 HBSS and visualised under an Olympus IX71 inverted fluorescence microscope for the presence of GFP until ready for lysing.

2.7. qPCR analysis

Cells were lysed using RNasey lysis buffer (RLT) and RNA was extracted using the RNasey MiniKit (Qiagen) including a DNase digestion step using the RNase-Free DNase Set (Qiagen). The High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used to yield cDNA from each RNA population. Fast SYBR Green Master Mix (Life Technologies) was used in the qPCR reactions in this study. Intron flanking primers for qPCR were designed using the Universal Probe Library database (Roche) for the target gene (EIF2S3) and the three housekeeping genes (GAPDH, β-ACTIN and HPRT). The cDNA derived from the reverse transcription was diluted 1:5 in all qPCR reactions and nuclease-free water was used as the blank.

2.8. Apoptosis assay

1.1B4 cell populations were seeded into white 96-well sterile tissue culture treated microplates with clear bottoms (PerkinElmer, Cat: 6005181), at 10,000 cells/well in 200 μl of RPMI media, and incubated overnight at 37 °C. Cytokine mix (100 μl/well) containing 1 L-15 (50 U/ml), TNF-α (1000 U/ml), INF-γ (1000 U/ml) was added, diluted in media and incubated for 16 h at 37 °C, to induce caspase activity and thus increase apoptosis in cells. Caspase-Glo 3/7 reagent (Promega, Cat: G8900) was added at a 1:1 ratio of reagent:sample (100 μl) to each well and incubated for 1 h at room temperature before reading the luminescent signal generated on the luminometer. There were triplicate wells of each of the following for Clone 4 (the EIF2S3 knockdown cell line), non-transduced (NT) and NS cell populations: blank wells with no cells and media containing cytokine mix, wells containing cells with media without cytokine mix, and wells containing cells with cytokine mix.

2.9. In situ hybridisation on human embryonic sections

Digoxigenin (DIG) RNA probes were made using the purified pCMV-SPORT6 vector containing full-length human wild-type (WT) EIF2S3 cDNA (IMAGE ID: 4419438) (Source Bioscience). Human embryonic tissue sections were selected at Carnegie stage (CS) 16, 19, 20, 23 (equivalent to gestational age 5.5, 6, 7 and 8 weeks) respectively obtained from the Human Developmental Biology Resource (HDBR) tissue resource. Gene expression studies were performed by in situ hybridisation as previously described [9], to generate a human embryonic expression profile in the hypothalamo-pituitary (HP) region and the pancreas.

2.10. Yeast strains and plasmids

Yeast strain J515 (identical to J212 [10]; MATα leu2-3,-112 ura3-52 his3 gcl11Δ:KanMX p(GCD11, URA3) was used for elf2γ mutant analysis. Mutant and WT GCD11 constructs were introduced into J515 as the sole source of elf2γ by plasmid shuffling [11]. Plasmids used in this study are listed and include p180 [GCN4-lacZ, URA3 12], p367 [His4 (AUG)-lacZ, URA3 13], p391 [His4(UUC)-lacZ, URA3 13], pc2872 [His4-GCD11 (elF2γ), LEU2 10], pCS856 [His4-gcd11-I318M in pc2872 [14]], pc5861 [His4-gcd11-S489 N-A491V in pc2872; this study] and pCS862 [His4-gcd11-S489 N-P490S-A491V in pc2872; this study].

2.11. β-galactosidase assays

For measurement of GCN4-lacZ, HIS4(UUC)-lacZ or HIS4(AUG)-lacZ expression, overnight yeast cultures were grown in synthetic complete (SC) medium and used to inoculate 25 ml of SC medium at OD₆₀₀ = 0.25. For measurement of GCN4-lacZ, cultures were grown for 2 h (untreated or treated with 1 μg/ml sulfometuron methyl (SM), an inhibitor of leucine, isoleucine, and valine biosynthesis). Cultures continued growing for 6 h to OD₆₀₀ ≤ 1 and β-galactosidase activities were determined as previously described [12,15]. For measurement of HIS4 (UUC)-lacZ and HIS4(AUG)-lacZ expression, cultures were left untreated and grown for 6 h to OD₆₀₀ ≤ 1, followed by measurement of β-galactosidase activities. Averages and standard deviations of β-galactosidase activities were calculated for three independent transformants. Statistical significance was calculated using an ANOVA test followed by a post hoc Tukey’s test (P < 0.05).

2.12. Patient phenotypes

Three affected males, monozygotic (identical) twin brothers (III₄, III₅) and their maternal cousin (III₇), born to a non-consanguineous white European pedigree, presented with severe recurrent hypoglycaemia, short stature with GH and TSH deficiencies, and an unique pancreatic phenotype (Table 1). They have an unusual form of glucose dysregulation which fluctuates between hyperinsulinaemic hypoglycaemia and post-prandial hyperglycaemia (Table 1). They were treated with rhGH (Fig. 1A-C), thyroxine and diazoxide together with chlorothiazide. The latter two medications were stopped as the hyperinsulinism resolved at 7 years of age; however, the twins manifested
Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Birth weight kg (SDS)</th>
<th>Age at presentation (years)</th>
<th>Head circumference (cm) (SDS)</th>
<th>Height (cm) (SDS)</th>
<th>Weight kg (SDS)</th>
<th>HbA1c (%)</th>
<th>GH peak (mU/L)</th>
<th>LH peak (IU/L)</th>
<th>FSH peak (mU/L)</th>
<th>Testosterone to HCG ratio (nmol/L) (age in years)</th>
<th>PRL (mU/L) (NR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II3</td>
<td>2.14 (−2.3, 1.3)</td>
<td>3.3 (209.1, 555.1)</td>
<td>11.4 (−0.3)</td>
<td>11.4 (−0.3)</td>
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<tr>
<td>II4</td>
<td>2.13 (−2.2, −0.6)</td>
<td>2.7 (129.3, 449.0)</td>
<td>11.4 (−0.3)</td>
<td>11.4 (−0.3)</td>
<td>11.4 (−0.3)</td>
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<tr>
<td>II5</td>
<td>1.93 (−5.2)</td>
<td>1.5 (12, 22)</td>
<td>11.4 (−0.3)</td>
<td>11.4 (−0.3)</td>
<td>11.4 (−0.3)</td>
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</table>

2.13. Patient II3

Patient II3 was the first born of non-identical twins via emergency caesarean at 38 weeks gestation [birth weight 2.1 kg (−2.8 SDS)]. He presented with poor feeding and hypoglycaemia 18 h after birth. He had congenital heart disease in the form of total anomalous pulmonary venous return at presentation, as well as severe gastro-oesophageal reflux requiring Nissen fundoplication. He was diagnosed with GHD and rhGH was commenced at 1.8 years of age. His thyroid function is currently normal. His gonadotrophin status is unknown as he is prepubertal. He had a blunted testosterone response to a 3-day HCG test; however, he was only 3 years of age when this test was performed (Table 1). He has global developmental delay needing 1:1 support in mainstream school, behavioural problems and mild hepatomegaly with normal liver function. His mother presented with secondary amenorrhoea. He is currently 8.8 years of age, with a weight of −0.2 SDS and height of −0.3 SDS, and continues to respond well to GH therapy. An oral glucose tolerance test (OGTT) performed at 8 years of age was normal with a blood glucose of 4.3 mmol/l at 2 h; however, he had symptomatic hypoglycaemia at 3 h with a blood glucose of 2.9 mmol/L and insulin of 8.6 mU/L, suggesting glucose dysregulation with hyperinsulinaemic hypoglycaemia. Continuous glucose monitoring showed blood glucose concentrations ranging from 3.1–10.3 mmol/L. Ophthalmological examination was normal.

2.14. Patients II4 and II5

Identical twin brothers were born via caesarean section at 34 weeks gestation; patient II4 had a birth weight of 2.15 kg (−0.3 SDS), and patient II5 had a birth weight of 1.93 kg (−1.5 SDS). Initial presentation with gastrointestinal symptoms and failure to thrive led to a diagnosis of intestinal lymphonodular hyperplasia and eosinophilic infiltration. Both patients presented with hypoglycaemic seizures at 2 years of age, and a diagnosis of GHD was made. Both boys had a microphallus increasing in size following commencement of GH treatment at 3.2 years of age. The boys also developed central hypothyroidism at 2 years of age, and were treated with thyroxine. Patient II4 had small undescended testes, however they descended spontaneously by 2 years of age. His twin brother, II5, had normal descended testes on initial examination. The twins had feeding difficulties and poor weight gain, possibly partly due to their dairy-free diet. By 4 years of age, the brothers had delayed speech development with mild conductive hearing loss, and behavioural and mild learning difficulties were present by the age of 6 years. A standard clinical microarray performed in both boys was normal. Behavioural difficulties were more severe in II3; however, patient II5 often had episodes of twitching, possibly related to hypoglycaemia. Patient II5 also had hepatomegaly, myopia and a squint. Both brothers underwent a tonsillectomy and adenoidectomy at 4 years of age due to recurrent tonsillitis and upper airway obstruction. At 10 years of age, the brothers had impaired glucose tolerance upon testing; the glucose concentrations fluctuated between hypoglycaemia and hyperglycaemia (Table 2). Subsequently, the HbA1c was elevated in
both boys [HbA1C 47 mmol/mol (N20-42), 2 h peak glucose on OGTT 13 mmol/L with insulin 33.2 mU/L in III4; HbA1C 43 mmol/mol (N20-42), 2 h peak glucose on OGTT 13.5 mmol/L with insulin 30.5 mU/L in III5]. Both boys had evidence of persisting hyperinsulinaemic hypoglycaemia (fasting glucose of 2.9 mmol/L with insulin of 2.5 mU/L in III5, blood glucose 5 h post-oral glucose load 2.7 mmol/L with insulin 10.9 mU/L in III4) at the age of 13.6 years.

Gonadotrophin secretion tested at 12 years of age was normal (Table 1). Patient III4 had a blunted testosterone response to a 3-day HCG test, while his brother III5 had a borderline testosterone response (Table 1). Nevertheless, both twin brothers have progressed through puberty spontaneously (the most recent pubertal examination at 14.6 years showed Genitalia (G) stage 4 with testicular volumes of 12 and 25 mls in III4 and G4 with testicular volumes of 25 and 20 mls in III5). Both boys had evidence of persisting hyperinsulinaemic hypoglycaemia (fasting glucose of 2.9 mmol/L with insulin of 2.5 mU/L in III4, blood glucose 5 h post-oral glucose load 2.7 mmol/L with insulin 10.9 mU/L in III4) at the age of 13.6 years.

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Their mother had osteoporosis due to secondary amenorrhoea, with menarche at 13 years and cessation of periods at 16 years of age, after which she received oestrogen supplementation (CycloProgynova). In later life she required a hysterectomy. An MRI of the brain was normal in the mother. Maternal height was 162.9 cm and their father had a height of 169 cm (mid-parental height: −0.34 SDS).

3. Results

3.1. Mutational analysis

The segregation of the disease phenotype in affected male individuals only, was suggestive of an X-linked mode of inheritance. Consequently, sequencing of the X chromosome in Pedigree 1 revealed a novel hemizygous variant in EIF2S3 (ENST00000253039.8): ChrX_24091319 C/T, c.1294C > T, p.Pro432Ser (GRCh37) in all 3 affected males, inherited from their heterozygous mothers (sisters). This variant is located at a highly conserved residue in the C-terminal domain of eIF2γ across multiple species (Fig. 2B). Protein prediction models predict this variant to be deleterious (Polyphen2 = 0.971, SIFT = 0). The EIF2S3 c.1294C > T, p.Pro432Ser variant was absent from control databases including the Exome Aggregation Consortium (ExAC) (www.
exac.broadinstitute.org) and the Genome Aggregation Database 2018 (gnomAD) [www.gnomad.broadinstitute.org]. This was the only potential pathogenic variant identified and was considered to be the most likely genetic cause of the disease in the patients.

3.2. EIF2S3 expression analysis using in situ hybridisation in human embryos reveals expression in the developing endocrine organs

EIF2S3 mRNA transcripts were visualised in the hypothalamus and Rathke’s pouch, both the AP and PP, the progenitor cells of the nasal epithelium, and the retina of the eye at both CS20 and 23 of human development respectively (Fig. 3A(i-iv)). The EIF2S3 transcripts were also strongly detected in the pancreas of a 13-week old human fetus (Fig. 3A v, vi). The EIF2S3 DIG-labelled sense control probe had negative staining.

3.3. Cohort screening

Upon identification of the EIF2S3 (p.Pro432Ser) variant in Pedigree 1, 103 patients with variable congenital hypopituitarism phenotypes, +/− structural midline brain defects on MRI, were screened for EIF2S3 variants; isolated GHD (n = 16), SOD (n = 37), hypogonadotropic hypogonadism/Kallmann syndrome (n = 4), and multiple pituitary hormone deficiencies with variable endocrine defects and no other midline defects (n = 46). No further variants were identified in these patients. However, there were no other patients in this cohort that had glucose dysregulation similar to that observed in Pedigree 1.

4. Functional analysis

4.1. Generation of an EIF2S3 knockdown human pancreatic β cell line

A stable EIF2S3 knockdown (KD) β cell line was established in a human hybrid pancreatic β cell line, 1.1B4 cells, using (LV) expressing target-specific short hairpin RNA (shRNA) and green fluorescent protein (GFP). Seventy-two hours after transduction the cells fluoresced green, indicating successful transduction with shRNA-encoding LV. The qPCR expression analysis showed that the most efficient shRNA construct (Clone 4) knocked down EIF2S3 gene expression by 82%, with a relative quantification (RQ) of 0.186 and a 95% confidence interval (CI) ranging between 0.127 and 0.274 when compared to non-transduced cells (RQ: 1, CI: 0.89–1.124) (Fig. 3B).

4.2. Apoptosis assays in the EIF2S3 knockdown cell line demonstrate higher caspase activity and cell death

Following transduction of 1.1B4 cells using shRNA Clone 4, cell viability appeared to be reduced on visual observation. Significantly higher caspase activity was observed in both the KD (Clone 4) and NT control cell populations after the addition of cytokines, compared to basal [Clone 4 (2288 ± 358.31 versus (vs) 1203 ± 63.52 p = 0.0067) and NT (758 ± 84.06 vs 230 ± 59.76 p = 0.0009)] (Fig. 4). Though the NS shRNA transduced control cell population did not reach a statistically significant difference in caspase activity after the addition of cytokines, it followed a similar trend to the other cell populations. Consistent with the essential role of the translational apparatus in cell viability, there was significantly higher caspase activity in the clone 4 EIF2S3 KD cells compared to the two control populations, both basally [NS 1203 ± 63.52 vs 258 ± 100.49, p = 0.00016 and NT 1203 ± 63.52 vs 230 ± 59.76, p = 0.00004] and after the addition of cytokines [NS 2288 ± 358.31 vs 468 ± 261.10, p = 0.002 and NT 2288 ± 358.31 vs 758 ± 84.06, p = 0.002] respectively (Fig. 4).

4.3. Altered translational control and start codon selection stringency in EIF2S3 mutant yeast

As there are no protein structure data available for the human elf2 complex or the isolated human elf2γ protein, the yeast (Saccharomyces cerevisiae) structure presents the best current model of the elf2 complex [16]. The elf2γ docks on domain II of elf2γ and the acceptor arm of Met-tRNAfmet binds into a groove between the G domain and domains II and III of elf2γ (Fig. 5A-C). The yeast P490 residue (homologous to human Pro432) lies on a β-strand within domain III of elf2γ. To test if the Pro432Ser mutation impairs elf2 function, the mutation was introduced into the yeast GCD11 gene encoding elf2γ [17]. As the residues flanking the mutated Pro residue differ between yeast and human, a locally humanized version of yeast elf2γ was generated by replacing Ser489 and Ala491 with Asn and Val, respectively, to

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Fig. 2. (A) Pedigree 1 harbouring the EIF2S3 (p.Pro432Ser) variant. This pedigree consists of three affected individuals that are hemizygous for the EIF2S3 (p.Pro432Ser) variant, represented by the black shaded squares labelled with EIF2S3 p.Pro432Ser. Patients I1, and II3, are monzygotic twins. The circles containing a dot highlight the females that carry the variant in heterozygous form. Unshaded squares/circles represent males and females that were negative for the variant respectively. The roman numerals on the left of the image depict the generation within the pedigree. The numbers ‘1’ – ‘5’ distinguish between each individual within that generation, which are referred to in the text. (B) Conservation of the substituted elf2γ residue. Conservation of the P432 amino acid (highlighted in green) that is substituted in Pedigree 1. Amino acids that differ from the human WT protein sequence are highlighted in red (only in the yeast sequence). The surrounding protein sequence in elf2γ is very highly conserved across multiple species.
mimic human eIF2γ (Fig. 2B). When expressed in yeast cells, the humanized eIF2γ-S489 N-A491V mutant and the patient eIF2γ-S489 N-P490S-A491V mutant derivative had no significant effect on yeast cell growth.

A more sensitive assay to examine eIF2 function in yeast relies on translational control of the GCN4 gene [18]. Conditions that lower eIF2 ternary complex levels, like phosphorylation of eIF2α or mutations that impair eIF2 function [1-3,10,19], elevate GCN4 expression. A GCN4-lacZ reporter was introduced into isogenic strains expressing WT eIF2γ, humanized eIF2γ-S489 N-A491V, the humanized mutant eIF2γ-S489 N.P490S.A491V or eIF2γ-I318M corresponding to the published eIF2γ-Iso259Met mutation [6]. As shown in Fig. 5D, GCN4-lacZ expression was low in cells expressing WT eIF2γ or humanized eIF2γ-S489 N.A491V. The p.P490S mutation resulted in a modest, but statistically significant, ~1.7-fold increase in GCN-lacZ expression, while the I318M mutation [7] conferred nearly an 11-fold increase in GCN-lacZ expression. Treatment with SM to activate eIF2 phosphorylation and lower eIF2 ternary complex levels [20], resulted in a ~6-fold increase in GCN-lacZ expression in cells expressing WT eIF2γ, while GCN-lacZ expression was de-repressed nearly 9-fold in cells expressing the eIF2γ-S489 N.P490S.A491V mutant and over 20-fold in cells expressing the eIF2γ-I318M mutant (Fig. 5D).

Mutations in yeast eIF2 subunits that impair Met-tRNA<sub>Met</sub> binding, enhance GTP hydrolysis, or disrupt complex integrity have been found to relax the stringency of translation start site selection and enhance initiation at differing codons [7,8]. To characterise mutations in this context, a set of HIS4-lacZ reporter constructs with an AUG or UUG start codon were introduced into strains expressing eIF2γ WT or mutant. In eIF2γ WT, the HIS4(UUG)-lacZ reporter was expressed at ~1.6% the level observed with the canonical HIS4(AUG)-lacZ reporter (Fig. 5E) consistent with previous reports [3,13]. A similar low level of UUG initiation was observed in cells expressing the humanized eIF2γ-S489 N.A491V mutant (Fig. 5E, middle bar). Introduction of the P490S mutation, however, increased HIS4(UUG)-lacZ expression to 3% of the value obtained with the AUG-initiated reporter (Fig. 5E, third bar).

5. Discussion

We have identified a novel EIF2S3 mutation in children manifesting a phenotype characterized by mild learning difficulties, hypopituitarism with GH and TSH deficiencies, and an unusual form of glucose dysregulation with early hypoglycaemia and later hyperglycaemia [9]. The 'probability of loss-of-function (LoF) intolerance' (pLI) value for EIF2S3 is 0.92, (calculated from controls in ExAC. A pLI value ≥ 0.9 value is an extremely LoF intolerant gene). The observed number of missense variants from population genetic data is significantly lower than expected with a z score of 3.81 (ExAC). Positive Z scores indicate intolerance to variation, thus indicating that EIF2S3 is highly intolerant to protein coding variation. The p.Pro432Ser variant alters a highly conserved residue in the eIF2γ-C-terminal domain (Fig. 2B) and segregates in an X-linked recessive manner in three generations of Pedigree 1 (Fig. 2A), having been inherited from the heterozygous maternal grandmother.
Murine Eif2cs3 expression in the pancreas, hypothalamus and pituitary (Mouse Genome Informatics), the abundant human EIF2CS3 expression in the postnatal brain and endocrine tissues, and high Elf2y protein expression in the postnatal brain and pancreas (Human Protein Atlas) have previously been established. Prominent EIF2CS3 expression in the hypothalamus, Rathke’s pouch, AP, PP, progenitor cells of the nasal epithelium, and retina of the eye (Fig. 3A[i-iv]) at CS20 and 23 has been established in this study. At CS23, the retina is in the process of differentiating into different cell types. The expression appears to be most prominent in the developing inner nuclear layer, in a region consistent with developing ganglion cells (Fig. 3A(iv)). However, ophthalmological examination has revealed no retinal changes in the three boys. We further examined expression in the human pancreas, due to the previously documented expression of murine Eif2cs3 in the pancreas (MGI), the presence of an unusual pancreatic phenotype in the patients in Pedigree 1, and the early onset diabetes in previously published cases. Expression was most prominent in β-cell progenitors within the Islets of Langerhans in the pancreatic tissue of a 13-week old fetus (Fig. 3A(v-vi)). These data provide a human embryonic EIF2CS3 gene expression profile in a developmental context.

The 1.1B4 human hybrid pancreatic cells [21–23] were used to perform a stable knockdown of the EIF2CS3 gene, using an integrative LV vector expressing Eif2cs3-targeting shRNA to generate an EIF2CS3 KD cell line. The cells transduced with LV vector encoding Eif2cs3-targeting shRNA failed to survive for as long as control cell populations (Fig. 4). Furthermore, the EIF2CS3 KD cell line had significantly higher basal and cytokine-stimulated caspase activities compared to control cells (Fig. 4), suggesting increased apoptosis. The impaired cell survival and increased caspase activities in our EIF2CS3 KD human cell line in this study is consistent with the essential role of Elf2y in initiating protein synthesis within the cell [24].

Elf2y phosphorylation inhibits global protein synthesis under conditions of endoplasmic reticulum (ER) stress [25]. In vivo, Elf2y phosphorylation is essential for the pancreas and liver to provide glucose homeostasis. Defects in Elf2y-mediated translational control in the pancreas can cause a β-cell deficiency and contribute to diabetes. Mice with a homozgyous mutation p.Ser51Ala that abolishes phosphorylation of Elf2y died within 18 h of birth due to prolonged hypoglycaemia associated with reduced glycogen stores and defective gluconeogenesis [26]. Homozygous mutant embryos and neonates displayed a reduction in insulin content 50% and 35% that of WT mice respectively; neonates had 20% of WT serum insulin concentrations, and both had pancreatic β-cell deficiency [26]. A subtle reduction in elf2y phosphorylation in heterozygous p.Ser51Ala mice leads to an increase in β-cell death in some treated cells [27] and type 2 diabetes when the animals are stressed by a physiologically relevant high fat diet (HFD). Delayed folding and/or misfolding of proinsulin in the HFD-fed mutant islets was associated with reduced production of mature insulin [28]. These studies are consistent with the glucose dysregulation observed in Pedigree 1, and suggest that elf2y may also contribute to glucose homeostasis in humans.

The yeast assays conducted in this study show a modest increase in GCN4-lacZ expression in the p.Pro432Ser yeast homologue (p.Pro490Ser), compared to the robust increase in the yeast model of the human p.Ser259Met mutation (p.Ser318Met) [14](Fig. 5D) associated with a more severe phenotype [6], indicating a defect in ternary complex formation or delivery of Met-tRNAiMet to the ribosome. These modest impacts on GCN4-lacZ expression, and the ~2-fold increase in non-AUG initiation seen with p.Pro490Ser (Fig. 5E) suggesting a relaxed start site selection stringency, are comparable to previously reported yeast assays on the human MEHMO syndrome mutations p.Iso222Thr and Iso465Serfs*4 [5,7]. Cumulatively, our results demonstrate that the human p.Pro432Ser elf2y frameshift p.Iso465Serfs*4 [7] were
re-evaluated for the presence of a pancreatic phenotype. Neonatal hypoglycaemia, early onset insulin-dependent diabetes, and variable hypopituitarism have since been noted in some of these patients [29]. However, the cause of the neonatal hypoglycaemia has not, to date, been established. Our studies clearly show impaired glucose dysregulation with initial hypoglycaemia due to hyperinsulinism, followed by an unusual form of diabetes that occurs in the second decade of life and is not insulin-dependent, unlike the early onset diabetes reported in two previously published cases [6,29]. The affected males in Pedigree 1 in our study differ from all previously described cases in having a much milder neurodevelopmental phenotype, with the twin boys attending a mainstream school. Our patients do not have microcephaly, epilepsy, or obesity which are prominent phenotypes in previous patients diagnosed with MEHMO syndrome [5] (Table 3). Patients III5, II11, and III11, from Pedigree 1 had variable generalised white matter loss in the brain, findings which are consistent with previously published reports, where patients with EIF2S3 mutations manifested a global reduction in white matter on MRI [6]. However, this may be attributable to hypoglycaemia at birth. Intriguingly, white matter loss is a prominent feature of Vanishing White Matter (VWM) disease, in which patients have mutations in eIF2B [30,31], the guanine-nucleotide exchange factor for eIF2. Thus, mutations impairing eIF2 and eIF2B may display some phenotypic overlap [9].

We have shown, for the first time to our knowledge, that the hypoglycaemia observed in our pedigree, and likely those previously published, is associated with hyperinsulinism (Table 3), and may then be followed by the evolution of non-autoimmune diabetes, as shown in a previously published murine model [32]. The significantly higher apoptosis in the EIF2S3 KD human cell line, the human HP and pancreatic expression profile, the yeast assays, and the segregation of the EIF2S3 (p.Pro432Ser) variant with the hypopituitarism and glucose dysregulation phenotype in Pedigree 1, all suggest that the p.Pro432Ser mutation moderately, but significantly, impairs eIF2γ function and leads to a relatively mild phenotype affecting human HP and pancreatic function [9]. This milder loss of function compared with previous EIF2S3 mutations, gives rise to a phenotype that is distinct from the classical spectrum of MEHMO syndrome.

Variable effects on brain development are clearly associated with mutations in EIF2S3. It is worth considering that untreated hypoketotic hypoglycaemia in the previously published cases may have contributed to their more severe impairment of neurodevelopment and seizures. The insights provided by our study reporting both pancreatic and pituitary phenotypes associated with EIF2S3 mutations may be critical for the prevention of significant neurodevelopmental delay in these patients. Early identification of such patients with a rapid molecular diagnosis may lead to prevention of significant morbidity in these patients. The identification of further patients with EIF2S3 mutations will allow more geno-type-phenotype correlation studies that will shed further light on the role of eIF2γ in brain, HP, and pancreatic development and function in humans.

**Corresponding author statement**

I, Professor Mehul T Dattani, confirm that I had full access to all the data in the study and had final responsibility for the decision to submit for publication as the corresponding author.
Table 3
Clinical phenotypes of male patients with EIF2S3 mutations from four separate studies. Clinical phenotypes from previous reports by Borck et al5, Moortgat et al6, Skopkova et al7, Stanik J et al8, and from this current study, respectively. Not all unique additional features listed under each study were present in every patient described; each male within the study had various combinations of these features.

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<td>• Hypotonia (axial)</td>
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<td>• Facial dysmorphic features</td>
<td>• Epilepsy</td>
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<td>• Epilepsy</td>
<td>• Thin corpus callosum on MRI</td>
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<td>• Thin corpus callosum on MRI</td>
<td>• Normal pituitary and stalk on MRI</td>
<td>• Developmental delay</td>
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<td>• Enlarged lateral ventricles on MRI</td>
<td>• Global white matter loss on MRI</td>
<td>• Obesity (infancy onset)</td>
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<td>Unique additional features:</td>
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<td>The three unrelated males with severe MEHMO syndrome only:</td>
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<td>Cleft lip/palate</td>
<td>Spastic quadriplegia</td>
<td>• Seizures</td>
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<td><strong>Unique additional features:</strong></td>
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<td>• Growth retardation</td>
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<td><strong>Clinical phenotypes from previous reports by Borck et al5, Moortgat et al6, Skopkova et al7, Stanik J et al8, and from this current study, respectively.</strong></td>
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<td><strong>Unique additional features:</strong></td>
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<td>• Neonatal hypoglycaemia (2 patients only)</td>
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<td><strong>Behavioural problems</strong></td>
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<td>• Early onset insulin-dependent diabetes</td>
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<td><strong>Postpubertal microgenitalism</strong></td>
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<td>• Variable hypopituitarism</td>
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<td><strong>Obesity</strong></td>
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<td>• Convergent strabismus</td>
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<td><strong>Unique additional features:</strong></td>
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<td>• Delayed puberty</td>
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<td>• Genital abnormalities</td>
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<td>• Micronodosa (undersized jaw)</td>
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<td>• Global reduction of white matter on MRI</td>
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<td><strong>Severe combined dyslipidaemia</strong></td>
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<td><strong>Chronic lung disease</strong></td>
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