Impaired eIF5A function causes a Mendelian disorder that is partially rescued in model systems by spermidine

Victor Faundes  
*University of Manchester*  
Jorge L. Granadillo  
*Washington University School of Medicine in St. Louis*  
et al.

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Impaired eIF5A function causes a Mendelian disorder that is partially rescued in model systems by spermidine


The structure of proline prevents it from adopting an optimal position for rapid protein synthesis. Poly-proline-tract (PPT) associated ribosomal stalling is resolved by highly conserved eIF5A, the only protein to contain the amino acid hypusine. We show that de novo heterozygous EIF5A variants cause a disorder characterized by variable combinations of developmental delay, microcephaly, micrognathia and dysmorphism. Yeast growth assays, polysome profiling, total/hypusinated eIF5A levels and PPT-reporters studies reveal that the variants impair eIF5A function, reduce eIF5A-ribosome interactions and impair the synthesis of PPT-containing proteins. Supplementation with 1 mM spermidine partially corrects the yeast growth defects, improves the polysome profiles and restores expression of PPT reporters. In zebrafish, knockdown eif5a partly recapitulates the human phenotype that can be rescued with 1 µM spermidine supplementation. In summary, we uncover the role of eIF5A in human development and disease, demonstrate the mechanistic complexity of EIF5A-related disorder and raise possibilities for its treatment.
Proline is a unique amino acid as its amine nitrogen is bonded to two, instead of one, carbon atoms with a distinctive rigid cyclic structure that prevents it from adopting an optimal position required for rapid protein synthesis. The presence of proline, either as a peptidyl donor or an acceptor, impedes the rate of peptide bond formation by the ribosome, an inhibitory effect that becomes progressively stronger, to the extent that three or more consecutive prolines provoke ribosome stalling. In eukaryotic cells, ribosomal stalling is resolved by the Eukaryotic Translation Initiation Factor 5A (eIF5A), which is critical for the synthesis of peptide bonds between consecutive proline residues. Notably, the frequency of poly-proline tracts (PPTs) is higher in evolutionarily new proteins and of all tandem amino acid repeats, only the proline repeat frequency correlates with functional complexity of eukaryotic organisms. eIF5A1 (hereafter eIF5A) and its normally undetectable paralogue eIF5A2 are the only human proteins that contain the amino acid hypusine, a post-translationally modified lysine at position 50 (K50). Hypusine is synthesised from spermidine, a polyamine, via two sequential enzymatic steps involving highly conserved deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH) enzymes. Hypusinated eIF5A stabilises P-tRNA that facilitates peptide bond formation at stalled ribosomes. Other functions of eIF5A include recognition of the correct start codon, global protein synthesis elongation and termination, promoting the elongation of many non-poly-proline-specific tripeptide sequences, and eliciting nonsense-mediated decay (NMD). It is essential for cell viability and growth in both simple and complex organisms. Somatic overexpression of eIF5A has unfavourable prognostic implications in several cancers, including pancreatic, lung, hepatocellular, bladder, and colorectal carcinomas. However, no human phenotype has been previously attributed to germline EIF5A variants. Here, we demonstrate that de novo heterozygous EIF5A variants cause a previously undescribed syndrome characterised by variable combinations of developmental delay, microcephaly, congenital malformations and dysmorphism. These variants likely result in the loss of eIF5A function through variable combinations of developmental delay, microcephaly, micrognathia and craniofacial-neurodevelopmental disorder.

Results

Germline variants in EIF5A cause a previously undescribed craniofacial-neurodevelopmental disorder. In an individual with intellectual disability, congenital microcephaly, micrognathia and clinical suspicion of a Kabuki syndrome (MIM # 147920)-like intellectual disability, congenital microcephaly, micrognathia and craniofacial-neurodevelopmental disorder, EIF5A variants impair eIF5A function. Peripheral blood mononuclear cells obtained from Individual 3, with the EIF5A frameshift variant c.324dupA, and two healthy controls were transformed by Epstein-Barr virus into lymphoblastoid cell lines (LCLs). Blood samples from other affected individuals were not available. The EIF5A mRNA level in LCLs was significantly reduced in Individual 3 (Fig. 1e) and the transcript with c.324dupA was not detected (Supplementary Fig. 1), suggesting NMD of the mutant transcript. The Saccharomyces cerevisiae (yeast) and human eIF5A share a very high degree of conservation (62% identity/92% similar) (Fig. 1b) and well-established assays to investigate eIF5A function in yeast are available. We synthesised a human EIF5A cDNA (hereafter eIF5A) optimised for yeast codon usage, and added the 5′ and 3′ control regions of the yeast homologue of EIF5A (known as TIF51A or HYP2, yeIF5A hereafter) (Supplementary Note 2 and Supplementary Table 1). Introduction of this construct on a centromeric plasmid using standard techniques in a yeast strain in which both TIF51A and TIF51B (a second yeIF5A gene that is transcribed only in anaerobic conditions) are deleted (Supplementary Table 3 and Supplementary Fig. 2) restored its growth potential similar to the wild-type yeIF5A (Fig. 2a, rows 1 and 3). This confirmed that the synthetic yeIF5A plasmid can replace yeIF5A functions in line with previous reports. Next, we performed site-directed mutagenesis to create heIF5A constructs with the p.T48N, p.G106R, p.R109Tfs*8 and p.E122K variants in centromeric plasmids (Supplementary Tables 1 and 2). The E122K variant impaired growth, but the other variants were functional. Yeast colonies expressing the heIF5A-R109Ts*8 strain were selected for further analysis. Whole genome sequencing (WGS) revealed that the E122K variant, which is absent in the individual’s germline DNA, is somatically acquired, consistent with a clonal origin of the E122K variant in the patient’s LCLs. The E122K variant was located in one of the most constrained coding regions of the human genome (>99th percentile) and affects residues that are highly evolutionarily conserved (Fig. 1b, c). In silico modelling of missense variants (Fig. 1d) onto the structure of yeast eIF5A in complex with the 60S ribosome (PDB entry 5GAk) shows that the missense variants affect surface-exposed residues. T48 is adjacent to the hypusinated lysine 50, G106 and R109 residues are close to the ribosomal protein uL1 and E122 is close to the P-site tRNA. In contrast, P115 has no clear intermolecular interactions. Although the individuals were identified via their genotypes, on reverse phenotyping their clinical features showed remarkable convergence. All patients were affected by variable degrees of developmental delay and/or intellectual disability, microcephaly (either absolute or relative) and overlapping facial dysmorphisms (Table 1, Fig. 1a and Supplementary Note 1). Notably, four individuals in this cohort were clinically suspected to have either a Kabuki syndrome-like or a mandibulofacial dysostosis (MIM #154400)-like condition. In an individual with de novo frameshift variant c.324dupA, and two healthy controls were transformed by Epstein-Barr virus into lymphoblastoid cell lines (LCLs). Blood samples from other affected individuals were not available. The EIF5A mRNA level in LCLs was significantly reduced in Individual 3 (Fig. 1e) and the transcript with c.324dupA was not detected (Supplementary Fig. 1), suggesting NMD of the mutant transcript. The Saccharomyces cerevisiae (yeast) and human eIF5A share a very high degree of conservation (62% identity/92% similar) (Fig. 1b) and well-established assays to investigate eIF5A function in yeast are available. We synthesised a human EIF5A cDNA (hereafter eIF5A) optimised for yeast codon usage, and added the 5′ and 3′ control regions of the yeast homologue of EIF5A (known as TIF51A or HYP2, yeIF5A hereafter) (Supplementary Note 2 and Supplementary Table 1). Introduction of this construct on a centromeric plasmid using standard techniques in a yeast strain in which both TIF51A and TIF51B (a second yeIF5A gene that is transcribed only in anaerobic conditions) are deleted (Supplementary Table 3 and Supplementary Fig. 2) restored its growth potential similar to the wild-type yeIF5A (Fig. 2a, rows 1 and 3). This confirmed that the synthetic yeIF5A plasmid can replace yeIF5A functions in line with previous reports.

Next, we performed site-directed mutagenesis to create heIF5A constructs with the p.T48N, p.G106R, p.R109Tfs*8 and p.E122K variants in centromeric plasmids (Supplementary Tables 1 and 2). The E122K variant impaired growth, but the other variants were functional. Yeast colonies expressing the heIF5A-R109Ts*8 strain were selected for further analysis. Whole genome sequencing (WGS) revealed that the E122K variant, which is absent in the individual’s germline DNA, is somatically acquired, consistent with a clonal origin of the E122K variant in the patient’s LCLs. The E122K variant was located in one of the most constrained coding regions of the human genome (>99th percentile) and affects residues that are highly evolutionarily conserved (Fig. 1b, c). In silico modelling of missense variants (Fig. 1d) onto the structure of yeast eIF5A in complex with the 60S ribosome (PDB entry 5GAk) shows that the missense variants affect surface-exposed residues. T48 is adjacent to the hypusinated lysine 50, G106 and R109 residues are close to the ribosomal protein uL1 and E122 is close to the P-site tRNA. In contrast, P115 has no clear intermolecular interactions. Although the individuals were identified via their genotypes, on reverse phenotyping their clinical features showed remarkable convergence. All patients were affected by variable degrees of developmental delay and/or intellectual disability, microcephaly (either absolute or relative) and overlapping facial dysmorphisms (Table 1, Fig. 1a and Supplementary Note 1). Notably, four individuals in this cohort were clinically suspected to have either a Kabuki syndrome-like or a mandibulofacial dysostosis (MIM #154400)-like condition.
is consistent with our inability to detect this protein in LCLs from individual 3. In contrast, the three missense mutations each supported yeast growth as the sole source of eIF5A showing that they retain sufficient eIF5A function for cell viability. The heIF5A-T48N and heIF5A-G106R yeast cells exhibited slow growth (Fig. 2a, rows 4 and 5), similar to a previously characterised temperature-sensitive mutant heIF5A-S149P mutant (Fig. 2a, row 2)\(^2,5\). No significant difference was observed between growth of heIF5A-E122K and heIF5A-WT (Fig. 2a, rows 6). These results indicate that the p.T48N and p.G106R variants result in partial loss of eIF5A function, and p.R109Tfs*8 variant results in complete loss of viability. However, the impact of the p.E122K variant remained uncertain.

EIF5A variants reduce eIF5A–ribosome interaction through different mechanisms. Next, we asked if the missense variants affected interaction of heIF5A with ribosomes through polysome profiling (Supplementary Fig. 5). All missense variants that were tested, including the p.E122K variant, exhibited aberrant polysome profiles, with elevated polysome-to-monosome (P/M) ratios and an increase in the free 60S peak heights and the 60S/80S ratios (Fig. 2c), consistent with a global translation elongation defect\(^2,5\). Probing for eIF5A and hypusine across polysome fractions revealed that WT eIF5A has ribosome-free (lanes 1 in Fig. 2c) and 80S (lane 6 in Fig. 2c) peaks. In contrast, each missense mutant showed a reduction in the 80S ribosome fraction (Fig. 2c), indicating a reduction in ribosome binding by each

**Fig. 1** Heterozygous variants in EIF5A cause a novel craniofacial-neurodevelopmental disorder. a Individuals with de novo heterozygous missense (individuals 1, 2, 4 and 7) or frameshift (individual 3) EIF5A variants display similar facial dysmorphism, microcephaly and micrognathia. Photographs of individuals 5 and 6 are not available. b The missense EIF5A variants affect highly conserved residues. Evolutionary conservation of residues affected by variants (delimited by yellow rectangles) is shown in five species. BLOSUM62 scores are depicted in purple scale (dark purple = completely conserved, light purple = relatively conserved, white = not conserved). Lysine (K) 50 is hypusinated (Hyp). c The EIF5A variants are novel and located in the functional sites and domains of eIF5A. Top: hypusine site (HS) is the orange bar between residues 48 and 55 (InterPro entry P63241), and the OB fold domain is the cyan bar between residues 83 and 150 (InterPro entry P63241). Bottom: the location and the minor allele frequencies (MAF) of high quality non-flagged missense variants (yellow triangles) and a protein-truncating variant (p.N28Mfs*64, magenta line) seen only in controls of gnomAD v2.1.1 for the transcript ENST00000336458. d In silico modelling of missense variants supports their deleterious nature. Variants are indicated as red spheres at the surface of yeast eIF5A (semi-transparent gold) with hypusinated K51 (human K50, hK50) (black spheres) shown bound to the yeast 60S ribosomal subunit E site (grey secondary structures only, with uL1 in blue), with adjacent P-site (green) and A-site (pink) tRNAs (PDB entry 5gak). Note that T48 (yeast T49, yT49) is in proximity to the hypusinated hK50; hG106 (yG107) and hR109 (yK110) are close to uL1 and hE122 (yS123) is close to the P-site tRNA. e The mutant EIF5A transcript with the truncating variant is not expressed. The mRNA levels in lymphoblastoid cells from individual 3 compared to control 1 (healthy female) and control 2 (healthy male) are shown relative to GAPDH (2−ΔΔCT method). Each data point corresponds to one technical replicates, and the bars show the mean ± SEM. Two-sided P values were determined by unpaired t-test.
Table 1 Phenotypes of patients with EIF5A variants.

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<th>Characteristics</th>
<th>Individual 1</th>
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<td>F (8.4 y)</td>
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**Table Notes:**
- **Sex (age):** F (Female, age in years)
- **Genomic position:** Gene coordinates (chr:pos)
- **cDNA protein consequenced:** Mutation description
- **Inheritance/zygosity:** Presence of the mutation in the DNA (DN) or heterozygous (Het)
- **Perinatal history:** Presence of congenital microcephaly, unknown, or no
- **Congenital microcephaly:** Yes or no
- **Cardiac anomalies:** Yes or no
- **Hypoplasia:** Yes or no
- **Hypotonia:** Yes or no
- **Growth parameters:** Height (SD) and weight (SD) in standard deviation (SD)
- **Facial dysmorphisms:** Presence of specific facial features
- **Other medical issues:** Presence of other medical conditions

**References:**
- For ACCORD, ADHD, ADHD attention deficit hyperactivity disorder, CNS, central nervous system, DD, developmental delay, IUGR, intra-uterine growth retardation, LW, low weight, Mi, microcephaly, N/A, not applicable, OW, overweight, PF, palpebral fissures, SD, standard deviation, SS, short stature.
mutant eIF5A, consistent with the idea that impaired eIF5A–ribosome interactions impact translation elongation of each mutant.

To explore possible mechanisms for the reduction in ribosome binding of missense variants, we performed WB. In cells expressing \( h eIF5A-T48N \), levels of total eIF5A were normal, but levels of hypusination were reduced, suggesting that the p.T48N impairs hypusination of the adjacent K50 residue. As hypusination is necessary for eIF5A function, reduced hypusination may contribute to the observed reduction in ribosome association. In contrast, total levels of eIF5A were modestly reduced in \( heIF5A-G106R \) and \( heIF5A-E122K \) cells, while hypusination was unaffected (Fig. 2b). Overall, these results indicated that eIF5A variants reduce eIF5A–ribosome interaction, likely through different mechanisms.

**EIF5A variants impair synthesis of proteins with PPTs.** Next, we examined if impaired eIF5A function impacted on translation of specific mRNAs. Because eIF5A is especially critical for the optimal synthesis of proteins containing PPTs\(^2,5\), we studied expression of two previously described PPT reporters—a haemagglutinin (HA)-tagged Ldb17 with a single long PPT of nine consecutive prolines and HA-Eap1 with three shorter PPTs (Supplementary Tables 1 and 3 and Fig. 2d)\(^5\). As these reporters require expression with galactose as a carbon source, we first evaluated whether levels of eIF5A and hypusination in these cell growth conditions were consistent with our previous findings. Here in both of the slow-growing mutants (p.T48N and p.G106R) expression of eIF5A was lower than WT. As previously, hypusination was consistently low for p.T48N (Supplementary Figs. 6 and 7). By WB of total cell extracts we observed decreased levels...
Human microcephaly-associated genes (MAGs) are enriched for PPTs. Next, we explored if impaired synthesis of proteins with PPTs could help explain microcephaly, which was the most consistent feature of our patients. To study this, we prepared a catalogue of all known human MAGs according to OMIM and assessed their PPT content (Supplementary Data 1). We observed that 198/685 (28.9%) MAGs and 4366/17981 (24.2%) of all other human protein-coding genes have ≥ 1 PPT (χ² 7.64; OR = 1.27; 95% CI 1.07–1.5; P = 0.0057)). Next we ranked MAGs according to their proline content in PPTs (Supplementary Data 1 and Fig. 2e). KMT2D was ranked as #1 in this list. Loss-of-function KMT2D variants cause Kabuki syndrome 1, which was the clinically suspected diagnosis in three individuals. SF3B4, variants in which cause acrofacial dysostosis 1, Nager type (a subtype of mandibulofacial dysostosis, MIM #154400) and Kabuki syndrome 1 (MIM #147920), respectively, which overlap with the initial clinical suspicions in individual 1, and for individuals 2 and 4, respectively.

Spermidine partially rescues impaired eIF5A function in yeast. Polymamines contribute to the efficiency and fidelity of protein synthesis, and spermidine may overcome absence of eIF5A to some extent to promote peptide synthesis. Furthermore, DHPS mediated transfer of a 4-aminobutylo moiety from a polyamine, spermidine, to K50 is the first step in formation of active hypusinated eIF5A. Previous work has demonstrated that spermidine promotes longevity in yeast, whereas depletion of polyamines has a deleterious effect. We therefore reasoned that spermidine supplementation could potentially overcome the effects of impaired eIF5A function. In addition, because p.T48N hypusination was reduced, it may indicate that polyamine concentrations were limiting for hypusination in our growth conditions. We screened the effect of supplementing growth medium with different concentrations of spermidine on yeast growth, as measured by the rate of colony formation (Fig. 2a and Supplementary Fig. 8). One millimolar spermidine partially corrected the growth defects of p.T48N and p.G106R cells (Fig. 2a). As a growth phenotype was not observed in yeast expressing the p.E122K variant, this assay was uninformative for this allele. Higher spermidine concentrations had progressively deleterious effects impairing the growth of all strains (Fig. 2b and Supplementary Fig. 8).

Next, we performed polysome profiling of WT, p.T48N and p.G106R cells in the presence of 1 mM spermidine. Spermidine treatment improved the global polysome profiles for both mutant strains with no impact on hif5a-WT (compare Fig. 2b with Fig. 2c). We observed full or partial restoration of hif5a interaction with the 80S ribosome in p.T48N and p.G106R cells, respectively (Fig. 2b). Furthermore, 1 mM spermidine restored expression of PPT reporter in both growth-rescued mutants (Fig. 2c, middle and right graph). These results suggest that spermidine can rescue and/or bypass impaired eIF5A functions in protein synthesis independent of its role as a substrate for hypusination of eIF5A K50 (ref. 35).

Spermidine partially rescues phenotypes of impaired eIF5A function in a zebrafish model. We next investigated if spermidine can rescue the impact of loss of eIF5A function in a developing vertebrate model. Zebrafish eif5a shares a high degree of conservation with its human orthologue (74% identity, 86% similarity) (Fig. 1b). Previous studies have demonstrated that morpholino-mediated knockdown of eif5a or transient over-expression of human EIF5A can cause microcephaly in zebrafish larvae. We used a validated and published splice site morpholino (MO) to knockdown eif5a in fertilised nacre zebrafish eggs, which were incubated in a standard, spermidine-free medium. The resulting larvae were fixed and cartilage was stained at 77 h post-fertilisation (hpf). In line with the human disorder phenotypes, we measured the distance between irises (translating to head circumference and therefore serving as a model for microcephaly) and the length of mandible cartilages (translating to mandibular growth and therefore serving as a model for micrognathia) (Fig. 4a, left and right photographs, respectively).

Although we did not recapitulate the previously described microcephaly phenotype (Fig. 4b, c, left graph), the eif5a MO induced micrognathia in zebrafish larvae (Fig. 4b, c, right graph). Next, we explored the effects on zebrafish of supplementation with 10-fold dilutions (from 1 mM to 0.1 µM) of spermidine. One hundred micromolar or higher concentrations killed larvae before...
supplementation with 1 µM spermidine resulted in partial rescue of micrognathia when compared to control MO (Fig. 4b, c, right graph), demonstrating that spermidine supplementation can rescue this developmental defect by bypassing loss of eIF5A. Thus, endogenous concentrations of spermidine within the yolk are not sufficient to overcome loss of eIF5A and spermidine supplementation is likely able to promote peptide synthesis to rescue this developmental defect33.

Discussion

EIF5A is a unique and critical gene for synthesis of proteins, especially those with PPTs. It is highly intolerant to variation, but so far no human condition caused by variants in this gene has been identified. We define a previously undescribed human disorder caused by heterozygous EIF5A variants. This is supported by high constraint for deleterious EIF5A variants in population databases, the de novo nature of all the variants described here, their absence from population databases along with high evolutionary conservation and the phenotypic similarity of patients ascertained via their genotypes (Fig. 1a-c). The disorder can be caused by protein truncating or missense variants. Of note, the codon encoding Arg109 was impacted in three out of seven cases. In humans, this amino acid is encoded by a CpG including codon (CGA) that may be prone to methylation, deamination and CG-TA transition, each of which could explain clustering of the mutations seen in this study38. The phenotype of the condition consists of variable degrees of developmental delay, intellectual disability, microcephaly and craniofacial dysmorphism, including micrognathia (Table 1, Fig. 1a and Supplementary Note 1).

Although both EIF5A and EIF5A2 are hypusinated and widely expressed in adult human tissues, the expression of the former is ~20-fold higher in brain structures than the latter16. While Eif5agt/gt mice are embryonically lethal3, Eif5a2−/− mice are viable and display normal development39. Therefore, EIF5A2 expression may not be sufficient to compensate the loss of EIF5A function16. Pathogenic variants in eIF2B subunits (EIF2B1, EIF2B2, EIF2B3, EIF2B4 and EIF2B5; MIM #603896)40, EIF2S3 (MIM #300148)41, EIF3F (MIM #618295)42, EIF4E (MIM #615091)43–45 and EIF4G1 (MIM #614251)46 have been previously described to cause distinct neurological disorders. Our findings add to this list of translation factors implicated in human developmental disorders.

Yeast growth assays showed the deleterious nature of the truncating and p.T48N and p.G106R missense EIF5A variants (Fig. 2a). Our results with the truncating variant are concordant with a previous study that demonstrated that deletion of either eIF5A amino- or carboxy-termini were lethal in yeast28. For all studied missense variants, including the E122K variant, the polysome profiles were abnormal further indicating their deleterious nature. More specifically, the higher P/M ratios typically
indicate a reduction in translation elongation rates or pausing of ribosomes causing ‘traffic jams’ on individual mRNAs leading to increased ribosome accumulation on mRNAs. The increase in the free 60S peak heights and the 60S/80S ratios (Fig. 2c) suggests that EIF5A missense variants may result in delay of 60S joining during initiation, or reduce the stability of 80S complexes. Both defects are consistent with the known functions of eIF5A in stimulating the first peptide bond formation and later during elongation and demonstrate clearly that each eIF5A missense mutation impacts protein synthesis globally2.

Absence of the mutant transcript with the c.324dupA variant and lower level of EIF5A mRNA levels in the LCLs of individual 3 suggest haploinsufficiency as the underlying mechanism for the protein truncating variant. This is supported by phenotypic overlap of intellectual disability, microcephaly and retrognathia in patients with 17p13.1 microdeletions that encompass EIF5A47. Notably, the minimum critical region of this 17p13.1 microdeletion syndrome includes 17 protein-coding genes and its main phenotypic driver has not been resolved. Our observations suggest that haploinsufficiency of EIF5A could be responsible for the phenotype of 17p13.1 microdeletion syndrome.

The loss of function of the EIF5A missense variants is likely to be due to different mechanisms. The observation of reduced hypusination in cells expressing the p.T48N variant suggests that this variant impairs hypusination of the adjacent K50 residue. Other missense mutations changing residues adjacent to K50 also have reduced hypusination28, consistent with the idea that they impair interactions with DHPS and/or DOHH enzymes responsible for hypusination. This is also supported by our spermidine supplementation experiment (Fig. 3) that showed no improvement of p.T48N hypusination, indicating that spermidine levels are not a limiting factor for hypusination of this mutant. The DHPS molecular structure indicates that the surface surrounding its active site tunnel is highly acidic48, while the eIF5A surface region around K50 has a complementary positive charged. In silico modelling (Fig. 1d) of the variant structure suggests T48N modestly increases the positive charge, but if or how this affects DHPS or DOHH interactions with eIF5A will require further experimentation. Reduction of total eIF5A levels in mutant cells (Figs. 2b and 3c) could be indicative of reduced protein or mRNA stability, but reduced protein levels per se are unlikely sufficient to explain the mutant phenotype. Instead our polysomal profile results are indicative of reduced interaction of eIF5A with ribosomes (Fig. 2c) as the common mechanism in the yeast model. As we did not have access to patient cells we could not test mRNA and protein expression levels or functional changes in individuals with the missense variants. Therefore, we cannot rule out that other mechanisms such as abnormal splicing or mRNA degradation may contribute to the disorder in individuals with missense EIF5A variants. Identification of more individuals in the future with disease-causing variants in this gene will be of great interest to uncover the underlying mechanisms.

![Fig. 4 Knockdown of eif5a in zebrafish embryos induces micrognathia, which can be partially rescued by spermidine.](image)

**Fig. 4** Knockdown of eif5a in zebrafish embryos induces micrognathia, which can be partially rescued by spermidine. a Measurement of iris distance was used for assessing head size (left photograph), and both Meckel’s (M) plus both palatoquadrate (Pq) cartilages for mandible assessment (right photograph). b Spermidine partially rescues micrognathia. Representative images of alcian blue-stained zebrafish larvae injected with 400 pg (1 nL) of either control or eif5a MOs. Larvae were incubated at 28 °C for 3 h in standard E3 embryo medium (SE3EM) and then transferred to either fresh SE3EM or fresh SE3EM plus 1 μM spermidine at 28 °C, and processed after 77 h post-fertilisation. c Quantification of measurements shown in panel a. Each data point corresponds to one fish, the longer horizontal line represents the median, whereas the shorter horizontal lines are the 25th and 75th percentiles. P values were determined by Kruskal-Wallis test with multiple comparisons. P values for comparisons against control MO injected fish are provided when less than 0.05.
We demonstrated that the loss of eIF5A function resulting from the variants has a deleterious impact on synthesis of proteins with PPT (Fig. 2d). The reduction was statistically significant for p.T48N and p.G106R variants. Although we did observe a reduction for the p.E122K variant, it did not reach statistical significance. In all our assays p.E122K was the least affected mutant. This is consistent with the milder individual phenotype of the patient harbouring this variant (Table 1 and Fig. 1a). Human KMT2D and SF3B4 are two MAGs that encode proteins with one of the highest number of PPTs (Fig. 2e). Interestingly, the phenotypes resulting from their loss of function resemble that of the individuals with EIF5A variants. From these data we surmise that impaired synthesis of proteins enriched in PPTs such as KMT2D and SF3B4 may underlie the phenotype(s) caused by defective eIF5A. Genes encoding the highest level of polyprolines display a strong association with biological processes such as actin/cytoskeletal-associated functions, RNA splicing/turnover, DNA binding/transcription and cell signalling. Notably, variants in actin and encoding genes are known to cause human developmental disorders with microcephaly. Similarly mandibular and craniofacial features of spliceosomal disorders overlap with patients described here. Other possible lines of investigation could also be explored. For example, eIF5A regulates pancreatic cancer metastasis by modulating expression of RhoA and ROCK. Germ-line variants in several Rho-GTPases cause developmental disorders with microcephaly. Recently it was shown that hypusinated eIF5A promotes the efficient expression of a subset of mitochondrial proteins involved in the TCA cycle and oxidative phosphorylation. Exploring the effects of these mutations on synthesis of mitochondrial proteins and function could be an interesting avenue. Additionally, eIF5A has functions in translation translation that we did not explore in this study but which may contribute to this developmental disorder. The importance of eIF5A in neurodevelopment is further emphasised by the recent identification of DHPS deficiency in patients with a neurodevelopmental disorder with seizures and speech and walking impairment (MIM # 618480). The role of eIF5A in normal brain and craniofacial development has never been examined before to the best of our knowledge and the mechanism of how impaired eIF5A results in abnormal neurodevelopment will need to be studied in the future. We demonstrated partial rescue of impaired eIF5A function and its resultant phenotypes in yeast and zebrafish models by spermidine (Figs. 3a–c and 4c–d). In yeast, spermidine rescued ribosome association defect and improved the polyproline score and PPT synthesis of the tested mutants. These effects are all consistent with rescuing eIF5A function. However, the molecular mechanism that underlies the rescue remains unclear. As eIF5A expression and hypusination was not increased by spermidine supplement (Fig. 3c), it appears spermidine is not acting via hypusination alone. In agreement with this idea, eIF5A-80S ribosome interaction was enhanced by spermidine (Fig. 3b). How spermidine could boost restoring eIF5A function is not clear. As spermidine rescued transient knockdown of eif5a in zebrafish, it may act similarly to the yeast model, or independently of eIF5A. Of note, our zebrafish model was based on transient knockdown of eif5a, and stable germ-line mutants will need to be studied to resolve these questions in the future. Importantly, spermidine supplementation has been shown to be safe and well tolerated in mice and humans. It promotes longevity in yeast and may extend the lifespan of mice and humans. Higher spermidine intake has been shown to be linked to lower mortality in humans. It has also been shown to protect against α-synuclein neurotoxicity in fruit flies and its levels are important for memory-retrieval and age-related memory-associated brain structures in rodents. There is a growing interest in using spermidine as a therapeutic agent in conditions such as cognitive decline. Although the effects of spermidine may be independent of its role in the synthesis of hypusinated eIF5A, our results raise the interesting possibility of a potential future therapy for individuals with EIF5A variant-associated disease.

In summary, we have defined a potentially treatable previously undescribed human Mendelian disorder caused by EIF5A mutations that result in reduced eIF5A–ribosome interactions via mutation-specific mechanisms. The phenotypes are likely explained by impaired synthesis of specific PPT-rich proteins. These findings uncover the role of eIF5A, and proteins with PPTs, in human brain and craniofacial development. Our findings open the avenue for future studies to identify the specific ‘hard to synthesise’ proteins, and the biological processes, most dependent on eIF5A function.

Methods

Ascertainment and exome sequencing analyses. Seven individuals from seven unrelated families with heterozygous, de novo, variants in EIF5A were included in this study after informed consents were obtained. The Central Manchester, Cambridge South, and the Republic of Ireland RECs approved this study (02/CMI/238, 10/H3035/83 and GEN284/12, respectively). Informed consent for research studies from patients or their legal representatives was obtained in all cases. The authors affirm that human research participants provided informed consent for publication of the images in Fig. 1. The patients were followed up by clinical geneticists from France, the United Kingdom, and the USA. Variants were identified by trio whole-exome sequencing for the detection of an undiagnosed neurodevelopmental disorder associated with multiple congenital anomalies, following published methodology for sample and library preparation, sequencing data production, analysis and interpretation. For the interpretation process we also considered the impact of variants on the preferentially expressed transcript according to the Gtex project and the canonical protein according to UniprotKb, and the tolerance of the gene to variation according to gnomAD, scoring only high-quality non-flagged variants present in the ‘only control’ subset of this database. We considered variants with significant deviations (percentile >90) according to Havilla et al. All the clinical observations were gathered through the Matchmaker Exchange and GeneMatcher initiatives.

DNA samples from affected individual 3 and her parents, as well as informed consent, were obtained for PCR and Sanger sequencing confirmation. PCR was performed using primers 5′-AATGGCGAGGAGGGGTGT-3′ and 5′-TGCA GGTTCAGAGGATCACT-3′. In yeast, spermidine rescued ribosome interactions via modulation of DHPS de

In silico analysis of variants and MAGs. The evolutionary conservation analysis of the residues affected by missense variants was performed using the eIF5A canonical protein sequence from Homo sapiens (UniProtKB entry P63241), Mus musculus (UniProtKB entry P63242), Gallus gallus (UniProtKB entry Q9O121), Danio rerio (UniProtKB entry Q6NS89), Drosophila melanogaster (UniProtKB entry Q6GU68) and Saccharomyces cerevisiae (UniProtKB entry P23301, yeast hereafter) using the ClustalW alignment in Jalview. The patients were followed up by clinical geneticists from France, the United Kingdom, and the USA. Variants were identified by trio whole-exome sequencing for the detection of an undiagnosed neurodevelopmental disorder associated with multiple congenital anomalies, following published methodology for sample and library preparation, sequencing data production, analysis and interpretation. For the interpretation process we also considered the impact of variants on the preferentially expressed transcript according to the GTEX project and the canonical protein according to UniprotKb, and the tolerance of the gene to variation according to gnomAD, scoring only high-quality non-flagged variants present in the ‘only control’ subset of this database. We considered variants with significant deviations (percentile >90) according to Havilla et al. All the clinical observations were gathered through the Matchmaker Exchange and GeneMatcher initiatives.

Gene, variants, morpholinos and plasmid synthesis and expression. A yeast expression plasmid for human eIF5A (hIF5A-WT) was designed to express the human canonical protein sequence, but using the yeast optimised codon usage and placement of the yeast TIS5A (eti5a) 5′ and 3′ regions. This was commercially synthesised (Epoch Life Sciences) and cloned into a pUC19 vector (Supplementary Note 2), resulting in plasmid pAV2578 (Supplementary Table 1). The gene was subsequently excised using Xhol and Sphi and cloned into Sac+ and SpeI digested single-copy-number (sc) LEU2 (Ycplac11), URA3 (YcpLac33) and high-copy-number (hc) LEU2 YEpplac181 vectors, generating plasmids pAV2580, pAV2592 and pAV2593 (Supplementary Table 1). The hIF5A sequence was verified by Sanger sequencing at Eurofins Genomics using the M13 reverse primer.

QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies), following the manufacturer's instructions. An hc version of p.R109T*8 was created in pAV2593. The resulting plasmids pAV2584[hei5A-T48N*8], pAV2585[hei5A-G106R*8], sc pAV2586[hei5A-K109T*8], pAV2587[hei5A-E122K*] and pAV2582[hei5A-T48N*8 LEU2] (Supplementary Table 1) were verified by Sanger sequencing as above.

For genetic knockdown of the elf5A gene in zebrafish, an elf5A splice site morpholino (MO) was synthesized (Gene Tools, Philomath, OR) for inhibition of the elf5A gene. The elf5A morpholino (ACACATCCTGGC-3′) was previously published86. A standard control MO (5′-CCTTATACCTGAGTCATATTATA-3′) by Gene Tools was also used.

Leucocyte transformation and harvesting. Peripheral blood mononuclear cells from one healthy, adult female (Control 1), one healthy, adult male (Control 2), and Individual 3 were transformed by Epstein-Barr virus into LCLs following a published protocol52. While five million LCLs from Individual 3 were harvested from five 150 mm flasks under standard conditions, another five million LCLs from the same individual and number of flasks were treated for 6 h with 200 µg/ml of puromycin before harvesting, and both type of samples were kept at −80 °C before RNA extraction.

Yeast strain construction and growth assays. The J696 haploid yeast strain deleted for both yeast elf5A (elf5A) genes and whose growth is supported by a plasmid bearing YEp351[ACACATCCTGGC-3′] as previously described was used. To study the effect of variants on synthesis of HA-tagged Ldb17 and Eap1 reporters, all GP7469, GP7482, GP7484 and GP7485 were grown in synthetic complete minus eIF5A. Strains were 10-fold serially diluted and spotted on SD-URA media to mid-log phase at 30 °C. To study the effect of variants on humanised eIF5A expression, total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol from one million LCLs of Control 1 and Control 2 each, and from one million, untreated LCLs and one million puromycin-treated LCLs of Individual 3. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA was reverse transcribed with random hexamer primers (Promega) according to the manufacturer’s protocol. qRT-PCR reactions were performed on a Bio-Rad CFX93 Real Time system (Bio-Rad) using Power SYBR Green PCR Master mix (Applied Biosystems) and the forward 5′-CCGTTGAGTAGTAGGATGACG-3′ and reverse 5′-GGAGCAGTGATAGGTACCCA-3′ eIF5A primers (Sigma-Aldrich). The level of eIF5A mRNA was determined using a relative quantification approach (2−ΔΔCT method) with human GAPDH and its primers 5′-ATGGGAAAGGTGAAGGTGC-3′ and 5′-TAAAACGACGCTTAC5′ as the internal reference. To detect if the frameshift-encoding transcript is expressed, we performed bidirectional Sanger sequencing of Individual 3’s elf5A cDNA, obtained from both untreated and puromycin-treated LCLs using the aforementioned forward primer and reverse 5′-TGAAGGATGCTGGACAATC-3′ primer, as previously described.

Zebrafish knockout, staining and imaging. Zebrafish husbandry was approved by The University of Manchester Ethical Review Board and all experiments were performed in accordance with UK Home Office regulations (PPL P1326867). Four hundred picograms (in 1 nL) of either the elf5a or control MOs were injected into 25–75 fertilised naeive embryos per condition, at the single-cell stage. Injected embryos were incubated at 28 °C in standard E3 embryo medium (SE3EM) until 3 hpf and then split into two groups, one of them only in SE3EM and the other one in SE3EM plus Spermidine (85558; Sigma-Aldrich) to a final concentration of 1 µM. Both groups were incubated at 28 °C until 77 hpf, without renewing the media.

At 77 hpf, larvae were terminated using 4% MS222 and fixed for 1 h with 2% paraformaldehyde, and then stained using a two-colour acid-free bone and cartilage staining protocol57. Stained larvae groups were blindfolded to a manipulator, mounted in 4% methylcellulose and imaged using a DFC7000 T Camera (Leica) coupled to a M165 FC Microscope (Leica) using v10 objective and LASS4 image capture software (version 3.4.2; Leica). Head size and mabndles were measured as depicted in Fig. 4a using ImageJ software (version 1.52j).
where Tab is the test antibody and Cab the control antibody. For hypusine quantification, eIF5A was used as its control antibody. Unpaired t-test against control samples was performed for western blots from yeast extracts and for Individual 3 E1F5A RNA expression. These results were depicted using bar plots, which represent the mean (average) plus standard error of the mean with overlap data points representing independent experiments. Kruskal Wallis test with multiple comparisons was performed for zebrafish analyses. These results were depicted using dot plots and the longer horizontal line represents the median, where relevant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability
All relevant data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. Source data are provided with this paper.

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1Division of Evolution & Genomic Sciences, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK. 2Laboratorio de Genética y Enfermedades Metabólicas, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago, Chile. 3Division of Molecular and Cellular Function, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK. 4Manchester Academic Health Science Centre, University of Manchester, Manchester, UK. 5Division of Neuroscience & Experimental Psychology, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK. 6Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK. 7Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, UK. 8Human Development and Health, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton, UK. 9Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, UK. 10Department of Genetics, AP-HP, Hôpital Necker Enfants Malades, Paris, France. 11Laboratory of Embryology and Genetics of Human Malformations, INSERM UMR 1163, Institut Imagine, Paris, France. 12Paris Descartes-Sorbonne Paris Cité University, Institut Imagine, Paris, France. 13Manchester Centre for Genomic Medicine, St Mary’s Hospital, Manchester University NHS Foundation Trust, Health Innovation Manchester, Manchester, UK. 14Division of Genetics and Metabolism, Department of Pediatrics, University of South Florida, Tampa, FL, USA. 15Northern Genetics Service, Institute of Genetic Medicine, Newcastle upon Tyne, UK. 16Division of Clinical Genetics, Children’s Mercy, Kansas City, MO, USA. 17Department of Pediatrics, University of Missouri—Kansas City, Kansas City, MO, USA. 18Center for Pediatric Genomic Medicine Children’s Mercy, Kansas City, MO, USA. 19School of Medicine, University of Missouri-Kansas City, Kansas City, MO, USA. 20Department of Pathology and Laboratory Medicine, Children’s Mercy, Kansas City, MO, USA. 21Division of Genetics and Genomic Medicine, Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, USA. *email: paul.kasher@manchester.ac.uk; graham.pavitt@manchester.ac.uk; siddharth.bank@manchester.ac.uk