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A homozygous truncating mutation in *PUS3* expands the role of tRNA modification in normal cognition

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Abstract

Intellectual disability is a common and highly heterogeneous disorder etiologically. In a multiplex consanguineous family, we applied autozygosity mapping and exome sequencing and identified a novel homozygous truncating mutation in *PUS3* that fully segregates with the intellectual disability phenotype. Consistent with the known role of Pus3 in isomerizing uracil to pseudouridine at positions 38 and 39 in tRNA, we found a significant reduction in this post-transcriptional modification of tRNA in patient cells. Our finding adds to a growing list of intellectual disability disorders that are caused by perturbation of various tRNA modifications, which highlights the sensitivity of the brain to these highly conserved processes.

Keywords

pseudouridine; post-transcriptional modification; intellectual disability; exome

INTRODUCTION

Intellectual disability (ID) is defined as significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social, and practical adaptive skills, originating before the age 18 (Association 2013). ID is a highly prevalent condition with estimates ranging 1–2% across populations (Maulik et al. 2011). While chromosomal abnormalities are seen in at least 10% of ID patients (Miller et al. 2010), large

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Conflict of Interest

Authors declare no conflict of interest

genomic sequencing studies suggest that single gene mutations are far more frequent and account for another 50%, almost always *de novo* dominant in severe cases (de Ligt et al. 2012; Gilissen et al. 2014; Rauch et al. 2012; Vissers et al. 2010). The contribution of autosomal recessive mutations to ID is unknown but likely to be significant as inferred by the large number of candidate genes identified by recent large scale studies (Alazami et al. 2015; Karaca et al. 2015).

The genes that are mutated in ID patients encode highly diverse groups of proteins, which suggests critical and non-redundant roles of numerous biological processes in normal cognition. One such process that emerged from the discovery of Mendelian forms of ID is tRNA modification. For example, the most common form of autosomal recessive ID in Arabia was found to be due to mutation of *ADAT3*, which edits adenosine to inosine at the wobble position 34 of mature tRNA (Alazami et al. 2013; El-Hattab et al. 2016; Gerber and Keller 1999). More recently, we have identified a form of severe microcephalic primordial dwarfism with global developmental delay that is caused by mutation of *WDR4*, a subunit of the methyltransferase that catalyzes 7-methylguanosine modification of residue G_{46} of tRNA (Shaheen et al. 2015). These findings suggest that normal brain development and function is highly sensitive to perturbation of tRNA modification, presumably by virtue of impaired protein synthesis.

In this study, we show that a homozygous truncation mutation in *PUS3* segregating with ID results in impaired isomerization of uridine to pseudouridine (Ψ) in patient tRNA. PUS3 is a member of the highly conserved TruA/Pus3 family of pseudouridylases, which are important for healthy growth in bacteria and yeast (Bekaert and Rousset 2005; Carbone et al. 1991; Chang et al. 1971; Lecointe et al. 2002; Tsui et al. 1991), and catalyze pseudouridine formation at specific uridine residues in the anticodon-stem loop of tRNAs in all kingdoms of life (Blaby et al. 2011; Chen and Patton 2000; Han et al. 2015; Hur and Stroud 2007; Lecointe et al. 1998; Singer et al. 1972). These findings expand the list of tRNA modification defects linked to ID.

MATERIALS AND METHODS

Human Subjects

Evaluation of affected members by a board-certified clinical geneticist included obtaining medical and family histories, clinical examination, neuroimaging and clinical laboratory investigations. After obtaining a written informed consent for enrollment in an IRB-approved project (KFSHRC RAC#2121053), venous blood was collected in EDTA and sodium heparin tubes for DNA extraction and establishment of lymphoblastoid cell lines (IV:3 15DG1746, IV:4 15DG1747, IV:5 15DG1750, control 15DG0421), respectively. A separate consent to publish photographs was also obtained.

Autozygosity Mapping and Exome Sequencing

Determination of the autozygome of each available family member and the exclusively shared autozygome among the affected members was essentially as described before (Alkuraya 2012). Briefly, runs of homozygosity ≥2Mb were used as surrogates of

autozygosity and determined using AutoSNPa based on a genomewide SNP genotyping file generated using Axiom SNP Array following the manufacturer's instructions (Affymetrix). Exome sequencing was done with an Illumina HiSeq2000TM following exome enrichment using the Agilent SureSelectXT Human All Exon 50Mb Kit. Read alignment was carried out with BWA and variant calling with GATK. Annotation was according to an in-house pipeline that was previously described in combination with autozygome analysis (Alkuraya 2013; Group 2015). Briefly, we only considered homozygous coding/splicing variants, novel/very rare (MAF<0.001) within the shared autozygome of the three affected individuals and such variants were subsequently confirmed by Sanger sequencing and segregation was tested in all available family members.

Extraction of total RNA and purification of $tRNA^{Phe}$ from human cells

LCLs were grown at 37°C in 5% CO_2 in RPMI 1640 medium containing FBS (15%), penicillin (1 U/mL), streptomycin (1 μg/mL), and amphotericin b (0.5 μg/mL) to a density of ~ 1.0×10^6 cells/mL, and bulk RNA from ~1.2×10^8 cells was extracted with TRIzol (Life Technologies) according to manufacturer's instructions. Then $tRNA^{Phe}$ was purified from (1~1.25 mg) bulk RNA using a 5′ biotinylated oligonucleotide (Integrated DNA Technologies) complementary to nucleotides 53–76 of the tRNA, as previously described (Jackman et al. 2003).

HPLC analysis of tRNA

Purified tRNA (1.25 μg) was digested with P1 nuclease and phosphatase as previously described (Jackman et al. 2003), and nucleosides were subjected to HPLC analysis at pH 7.0 as previously described (Guy et al. 2012).

RESULTS

Identification of A Recessive Form of Syndromic Intellectual Disability

Index (IV:3) is a 15 ½ year-old girl with a history of global developmental delay since infancy and failure to thrive (birth growth parameters were normal). She currently ambulates with assistance and has severe ID. There is a history of seizures at 10 months of age but she has been seizure-free on multiple antiepileptic medications. Family history is notable for first cousin parents and two similarly affected sisters (figure 1A) (see below). Examination revealed severe growth deficiency and microcephaly: weight 21 kg (−7.5SD), OFC 50.5 cm (−3.3SD), and height 124 cm (−5.9SD). She has coarse facial features, bilateral strabismus, grey sclera and extensive Mongolian spots (figure 1B). Negative results include urine organic acids and GAGs, Tandem MS, plasma lactate, uric acid, lipid profile, CDG screening, and molecular karyotyping. Brain MRI at 7 years of age showed mild ventriculomegaly with multiple arachnoid cysts in the middle cranial fossae bilaterally and the right cerebellopontine angle with the largest in the right middle cranial fossa, which measures 5.0 × 3.0 cm and causes mild mass effect on the right temporal lobe. In addition, there was volume loss in the frontal lobes anteriorly, and multiple T2/FLAIR white matter signal abnormalities in the subcortical white matter. Current evaluation shows that she is in the severe range of ID (IQ 30).

IV:4 is the 12-year-old sister of IV:3 who similarly presented with a history of global developmental delay, failure to thrive (birth growth parameters were normal) and severe ID. She had no seizures. Examination revealed coarse facies but less striking compared to the sister, severe growth deficiency with borderline microcephaly (weight 16.2 kg (−4.1SD), height 119 cm (−4.3SD), OFC 50 cm (−2.3SD), strabismus, grey sclera and widespread Mongolian spots (figure 1C). Brain MRI showed signal abnormalities within the globus pallidus and dentate nucleus bilaterally with nonspecific symmetrical hyperintensities in the frontal lobes in subcortical location. In addition, there was evidence of dysgenesis of corpus callosum, mild diffuse brain atrophy and a prominent cisterna magna. Similar to her older sister currently she is in the severe range of ID.

IV:5 is the 3.3-year-old sister of the index who also presented with global developmental delay and failure to thrive (birth growth parameters were normal) but no seizures. Assessment at 2 years showed severe axial and appendicular hypotonia with poor interactions. Body weight was 12 kg (48^{th} centile), height was 88 cm (74^{th} centile) and head circumference was 47 cm (37^{th} centile). Brain MRI at 2 years was unremarkable apart from small arachnoid cyst seen along the anterolateral surface of the left cerebellar hemisphere in the posterior fossa. Recent evaluation at the age of 3.3 years showed that her weight is 13.02 kg (16^{th} centile), length 89.5cm (4^{th} centile) and OFC 45.5cm (−2.1SD). She had strabismus, grey sclerae and Mongolian spots (figure 1D). She had global developmental delay and her IQ was in the profound range (<20).

A Homozygous Truncating Mutation in *PUS3* Segregates with a Syndromic Form of Intellectual Disability

Autozygosity mapping revealed that two autozygous intervals on chromosome 11 are exclusively shared by the three affected sisters in the family (Figure S1). Exome sequencing revealed a single coding/splicing novel variant within these critical intervals (figure 1E). Specifically, we identified a homozygous nonsense variant in *PUS3*; Chr11(GRCh37):g. 125763823G>A; NM_031307.3: c.1303C>T: p.(Arg435*) that fully segregated with the phenotype in the family as confirmed by Sanger sequencing (figure 1F). This variant is absent in 1,500 Saudi exomes as well as in the ExAC Browser.

PUS3 protein is a member of the phylogenetically conserved TruA/Pus3 family of proteins that catalyzes isomerization of specific uridines to Ψ in the anticodon-stem loop of tRNAs, at U_{38}, U_{39}, and U_{40} in bacteria (Hur and Stroud 2007; Singer et al. 1972); at U_{38} and U_{39} in the yeast *Saccharomyces cerevisiae* (Lecointe et al. 1998); at U_{39} (and likely U_{38}) in the archaeon *Haloferax volcanii* (Blaby et al. 2011); and based on in vitro studies, at U_{39} and to some extent U_{38} in murine PUS3 (Chen and Patton 2000).

The Arg435 residue at which the stop codon occurs in this family is located in the C-terminal portion of the 481 amino acid PUS3 protein, at the end of a conserved region found widely in teleost vertebrates, but not in the elephant shark, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Dictyostelium discoideum*, *S. cerevisiae* and *E. coli* (figure. 2A), while the subsequent 46 residues of PUS3 comprise a region that is widely conserved in mammals.

Cells Derived from Patients with a Homozygous Nonsense Mutation in *PUS3* Have Decreased Levels of Pseudouridine in tRNA

To further define the defect of the Arg435* allele of *PUS3* and its relationship to ID in this family, we generated lymphoblastoid cell lines (LCLs) derived from the three affected individuals (IV:3, IV:4, IV:5) as well as control LCLs (WT) from a healthy unrelated individual (cell lines from healthy related individuals were not obtained), and analyzed the pseudouridine (Ψ) content of a representative tRNA in which U_{39} is normally modified to $Ψ_{39}$. This modification is found in all 6 of the 18 characterized human tRNAs with an encoded U_{39}, including $tRNA^{Phe}$ (Machnicka et al. 2013), which we chose for analysis because of the six gene copies that specify identical tRNA products. As anticipated if the *PUS3* mutation was responsible for Ψ39 formation, we found that $tRNA^{Phe}$ purified from the WT LCL had almost exactly one additional mole of Ψ/mole tRNA, compared to $tRNA^{Phe}$ from LCLs of each of the three affected individuals (4.04 moles/mole, compared to 3.09, 3.20, and 3.05 moles/mole). By contrast, the levels of the other modifications of $tRNA^{Phe}$ (Cm, Gm, m^2G, m^1A, m^7G, and m^5C) were all very similar in all of the LCLs including WT (figure. 2B, Table 1). Thus, our results strongly suggest that PUS3 with the Arg435* mutation is inactive in the isomerization of U_{39} to Ψ, linking this defect to the ID, consistent with the known biological effects of Pus3 (see below).

DISCUSSION

Pseudouridine is the most abundant post-transcriptional modification of non-coding RNA observed to date across all domains of life (Cantara et al. 2011; Charette and Gray 2000). Pseudouridine possesses an additional free NH group at position 3 allowing for additional hydrogen bonding with other molecules, and it is thought that the presence of this class of modified nucleotides plays a role in increasing the stability of RNA (Auffinger and Westhof 1998). In characterized human tRNAs, pseudouridine is found at 13 different locations (residues 13, 20a, 20b, 31, 32, 27, 28, 35, 38, 39, 54, 55, and e2), and different pseudouridylases are required for modification at each of the different sites or sets of sites. PUS3 was originally identified as the *S. cerevisiae* ortholog of *E.coli* TruA, which synthesizes Ψ at U_{38}, U_{39} and U_{40} (Kammen et al. 1988; Singer et al. 1972). However, the yeast ortholog was found to possess activity only at positions 38 and 39 (Lecointe et al. 1998), and similar results have been obtained in the mammalian ortholog protein, which prefers U_{39} (Chen and Patton 2000).

Lack of PUS3 activity is clearly physiologically consequential. Bacterial *truA* mutants have significant growth defects (Chang et al. 1971; Tsui et al. 1991), while *S. cerevisiae pus3Δ* mutants are slow growing and temperature-sensitive (Carbone et al. 1991; Lecointe et al. 2002), primarily due to reduced function of $tRNA^{Gln(UUG)}$ (Han et al. 2015), and have reduced −1 frameshifting (Bekaert and Rousset 2005). Since $tRNA^{Phe}$ from LCLs with the Arg435* allele of *PUS3* had almost exactly 1 mole/mole less Ψ than control LCLs, we infer that the Arg435* allele nearly completely knocks out PUS3 function due to truncation of the conserved domain and the last 45 amino acids, although the mechanism is not clear.

Our understanding of the physiological context of pseudouridine synthesis, like many other biological processes, was informed by Mendelian disorders that are caused by mutations in

genes involved in this process. In 1998, a severe multisystem disorder known as dyskeratosis congenita (DKC) was found to be caused by mutations in *DKC1*, which encodes a protein that both pseudouridylates rRNA and maintains telomere length (Heiss et al. 1998; Mochizuki et al. 2004). More relevant to the family we present is the finding that a stop mutation in *PUS1*, which is another tRNA pseudouridylase, was associated with mitochondrial myopathy and sideroblastic anemia because PUS1 pseudouridylates tRNA, although the activity of PUS1 on mitochondrial tRNA may have influenced the mitochondrial-specific phenotype (Bykhovskaya et al. 2004; Fernandez-Vizarra et al. 2007).

The phenotype we observe in the context of PUS3 deficiency is largely brain-specific. The expression profile of PUS3 in mammals is unknown but available datasets suggest a wide tissue distribution (Expression Atlas, EBI). Therefore, it is possible that brain-specific phenotype reflects a higher sensitivity to reduced translational efficiency by the brain compared to other organs (Torres et al. 2014). This would be consistent with the finding by us and others that mutations in other genes involved in tRNA modification result in phenotypes with predilection to the brain e.g. *ADAT3*, *WDR4*, *TRM10*, *NSUN2*, and *FTSJ1* (Abbasi-Moheb et al. 2012; Alazami et al. 2013; El-Hattab et al. 2016; Fahiminiya et al. 2014; Freude et al. 2004; Gillis et al. 2014; Guy et al. 2015; Igoillo-Esteve et al. 2013; Khan et al. 2012; Martinez et al. 2012; Ramser et al. 2004; Shaheen et al. 2015). Similarly, many disease-causing mutations in tRNA synthetases and in the ribosome manifest their effects in functional disturbance of the nervous system (Antonellis et al. 2003; Antonellis and Green 2008; Brooks et al. 2014; Yao and Fox 2013).

In conclusion, we show that a nonsense mutation in *PUS3* segregates with an apparently novel autosomal recessive form of ID. Our finding that this mutation results in functional deficiency of PUS3 and a reduction in the availability of tRNA species that are pseudouridylated suggests a disease mechanism that is consistent with the recent identification of ID phenotypes caused by defects in other forms of tRNA modification.

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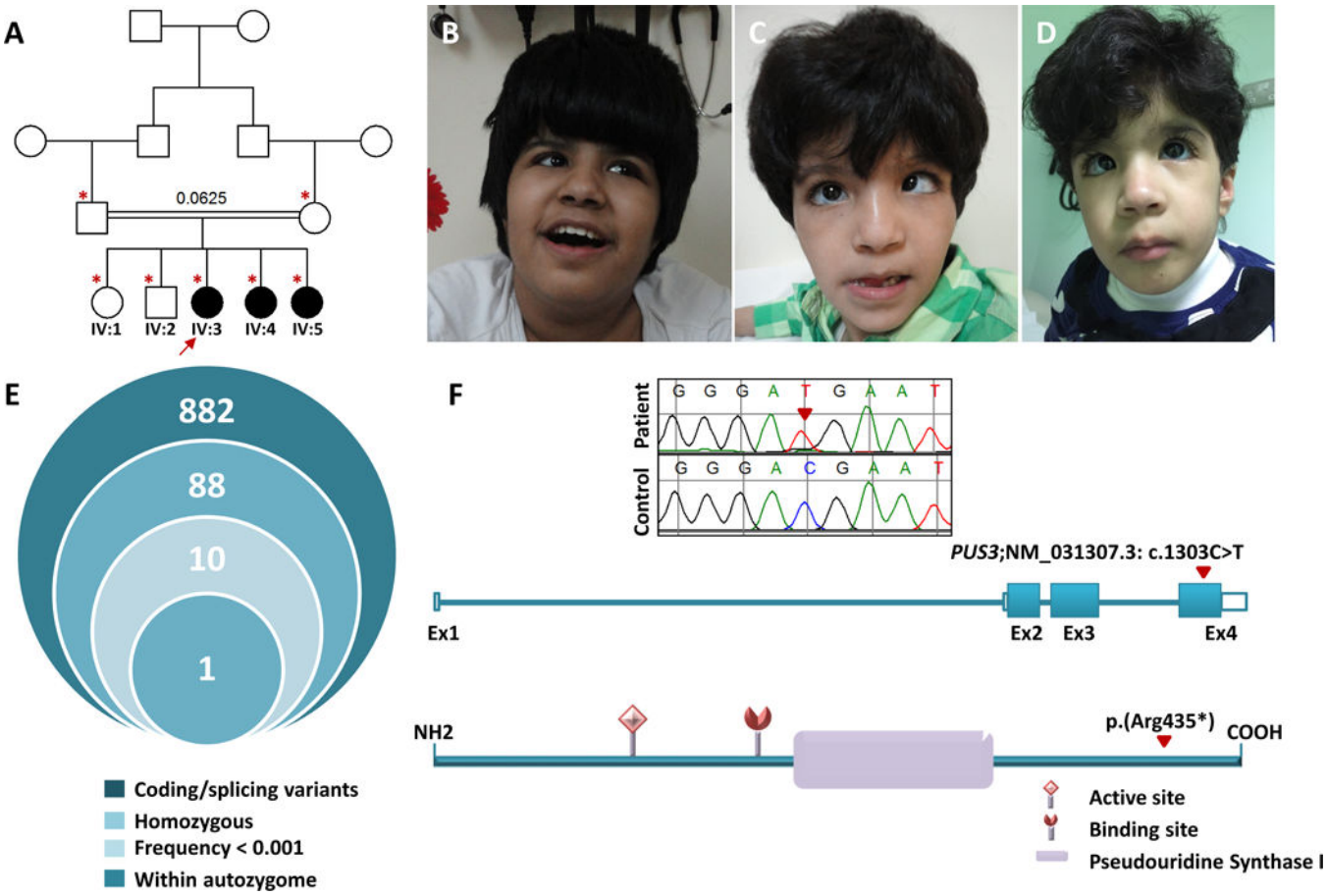

Figure 1.

Identification of an autosomal recessive Syndromic Intellectual Disability and the causative variant. A) Pedigree of the family showing the consanguineous nature of the parents. The index is indicated by an arrow, and asterisks denote individuals whose DNA was available for analysis. B, C and D) facial images of the three sister in the family (IV:3, IV:4 and IV:5 respectively) showing coarse facial features, bilateral strabismus, grey sclera and extensive Mongolian spots. E) Stacked Venn illustrating the exome filtration scheme and the number of surviving variants in each filtration steps. F) Upper panel: Sequence chromatograms of *PUS3* nonsense variant identified in the family (control tracing is shown for comparison and the location of the mutation is denoted by red triangle). Middle panel Schematic representation of *PUS3* transcript, (red triangle indicate the site of the mutations (NM_031307.3: c.1303C>T)). Lower panel: Schematic of PUS3 showing the position of domain" Pseudouridine Synthase I" and the active and binding sites as well as the location of the mutation (p.Arg435*).

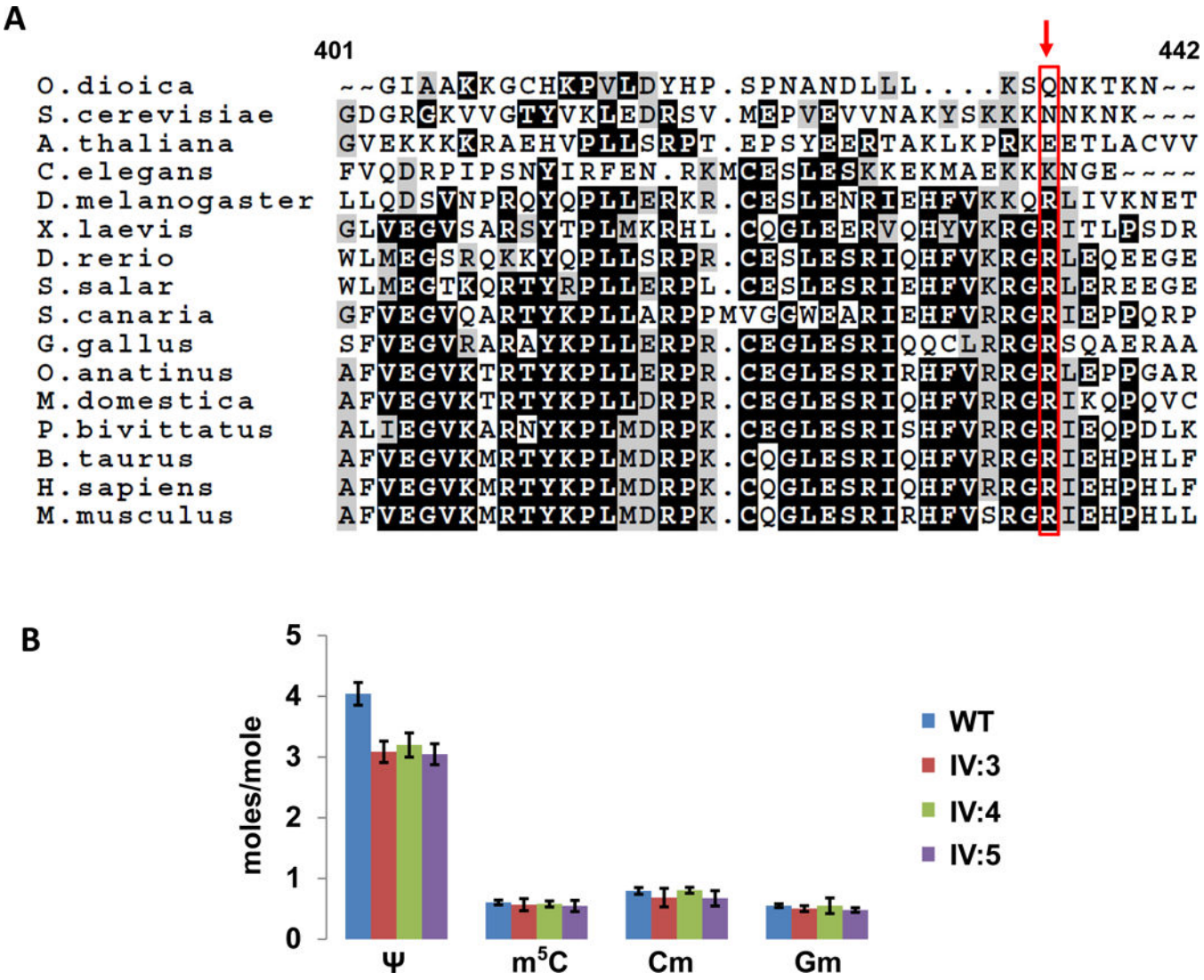

Figure 2.

A) Alignment of the C-terminal region of human PUS3. The figure shows the alignment of human PUS3 (residues 401–442) with the corresponding region from other eukaryotes as indicated. The arrow indicates site of the truncation mutation at Arg435*. B) $tRNA^{Phe}$ purified from LCLs of patients with the Arg435* mutation have reduced Ψ relative to that from control LCLs. Representative data are from Table 1 in the text, to show that Ψ levels are reduced in patient LCLs (by 1 mole/mole), whereas levels of each of three other modifications are not.

Table 1

Analysis of modified nucleotides in $tRNA^{Phe}$ from control (WT) and patient LCLs

Modification[a]	Moles Expected	WT	IV:3	IV:4	IV:5
Ψ	4	4.04 ± 0.19	3.09 ± 0.18	3.20 ± 0.20	3.05 ± 0.17
m^5C	1	0.60 ± 0.04	0.57 ± 0.10	0.58 ± 0.05	0.55 ± 0.09
m^1A	2	0.92 ± 0.07	0.96 ± 0.17	1.02 ± 0.08	0.93 ± 0.17
Cm	1	0.80 ± 0.05	0.69 ± 0.15	0.80 ± 0.05	0.67 ± 0.13
m^7G	1	0.70 ± 0.23	0.49 ± 0.11	0.53 ± 0.07	0.47 ± 0.08
Gm	1	0.55 ± 0.03	0.50 ± 0.05	0.55 ± 0.13	0.48 ± 0.04
M^2G	1	0.68 ± 0.01	0.59 ± 0.10	0.65 ± 0.07	0.54 ± 0.08

[a]Mean and standard deviation based on three individual growths and RNA preparations