Recessive Truncating Mutations in *ALKBH8* Cause Intellectual Disability and Severe Impairment of Wobble Uridine Modification

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The wobble hypothesis was proposed to explain the presence of fewer tRNAs than possible codons. The wobble nucleoside position in the anticodon stem-loop undergoes a number of modifications that help maintain the efficiency and fidelity of translation. AlkB homolog 8 (ALKBH8) is an atypical member of the highly conserved AlkB family of dioxygenases and is involved in the formation of mcm5s2U, (S)-mchm5U, (R)-mchm5U, mcm5U, and mcm5Um at the anticodon wobble uridines of specific tRNAs. In two multiplex consanguineous families, we identified two homozygous truncating *ALKBH8* mutations causing intellectual disability. Analysis of tRNA derived from affected individuals showed the complete absence of these modifications, consistent with the presumptive loss of function of the variants. Our results highlight the sensitivity of the brain to impaired wobble modification and expand the list of intellectual-disability syndromes caused by mutations in genes related to tRNA modification.

The discovery of the genetic code and the mechanism by which tRNA performs its central role in protein translation presented an apparent conundrum in that there are more possible codon combinations than there are tRNAs with the corresponding cognate anticodons. The "wobble hypothesis" was proposed as a solution wherein non-standard (i.e., non-Crick and Watson) binding is possible between the first (thus referred to as the "wobble") nucleotide of the anticodon stem-loop of tRNA and the third nucleotide of the trinucleotide codon in mRNA.[1] Among the numerous modifications of tRNA, there are specific modifications of the wobble nucleotide, which are thought to be critical for faithful recognition of the cognate and non-cognate codons and, consequently, correct and efficient translation.[2,3] For example, wobble uridines in eukaryotic tRNAs normally harbor a 5-methoxycarbonylmethyl (mcm^5) or a 5-carbamoylmethyl (ncm^5) side chain, sometimes in combination with a 2-thio (s^2) or ribose 2′-O-methyl group. These modifications modulate the tRNAs' decoding properties—ncm^5U is specifically seen in tRNAs that decode "family codon boxes," i.e., groups of four codons that all encode the same amino acid, whereas tRNAs that harbor mcm^5U decode "split codon boxes" that code for different amino acids by only varying the last purine or pyrimidine.[2]

Members of the 2-oxoglutarate (2OG)- and Fe(II)-dependent oxygenase superfamily are dioxygenases that incorporate oxygen into their product in a reaction that converts O_2 and 2OG to CO_2 and succinate. These enzymes catalyze a multitude of cellular processes at the levels of DNA (e.g., DNA repair), RNA (e.g., hypoxia-induced transcriptional regulation), and protein (e.g., posttranslational modification), as well as the epigenome.[4–6] AlkB homolog 8 (encoded by *ALKBH8* [MIM: 613306]) is a member of the highly conserved AlkB family of dioxygenases defined by the presence of a characteristic 2OG-Fe(II) domain, also called an AlkB-like domain after its ortholog in *E. coli*, AlkB. AlkB removes alkylation damage from DNA and RNA as part of the adaptive stress response.[7,8] Unlike its eight mammalian paralogs (including the better-known FTO alpha-ketoglutarate-dependent dioxygenase, noted for its role in obesity pathogenesis[9,10]), ALKBH8 possesses additional methyltransferase (MT) and RNA recognition domains, the latter thought to confer specificity for targeting modified tRNAs.[11,12] Studies of mouse and human cells demonstrated that ALKBH8 is involved in the formation of 5-methoxycarbonylmethyl-2-thiouridine ($mcm5s^2U$), 5-methoxycarbonylhydroxymethyluridine ([S]-$mchm^5U$) and its diastereomer (R)-$mchm^5U$, 5-methoxycarbonylmethyluridine (mcm^5U), and 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm^5Um) at the anticodon wobble uridines of specific tRNAs.[13–15] More specifically, it has been shown that the MT domain of ALKBH8 provides the mcm^5U precursor of mcm^5s^2U in $tRNA^{Lys(UUU)}$, $tRNA^{Gln(UUG)}$, and $tRNA^{Glu\ (UUC)}$, and of $mchm^5U$ in $tRNA^{Gly\ (UCC)}$ and $tRNA^{Arg\ (UCG)}$, whereas its AlkB-like domain catalyzes the hydroxylation of wobble mcm^5U to (S)-$mchm^5U$ in $tRNA^{Gly\ (UCC)}$. A second unknown oxygenase is predicted to be responsible for the hydroxylation of mcm^5U to (R)-$mchm^5U$ in $tRNA^{Arg\ (UCG)}$ [16].

In humans and mice, prior ALKBH8-dependent methylation to generate mcm^5U is strictly required for 2′-thiolation

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to occur. In the absence of ALKBH8, mcm^5s^2U-tRNAs thus largely contain the precursor 5-carboxymethyluridine (cm^5U) rather than the thiolated 5-carboxymethyl-2-thiouridine (cm^5s^2U).[11] Furthermore, ALKBH8 generates mcm^5U at the wobble position of the specialized $tRNA^{Sec\ (UGA)}$, which is further 2′-O-methylated to mcm^5Um in response to intracellular selenium levels (reviewed reviewed by Hatfield and Gladyshev[16]). In mammals, the lack of a specific anti-codon for selenocysteine is circumvented by this 2′-O-methylation of $tRNA^{Sec\ (UGA)}$ wobble mcm^5U such that UGA is recoded from stop to selenocysteine.[11,17–20] Like thiolation, 2′-O-methylation also requires prior methylation by ALKBH8.[21] Thus, the ALKBH8 activity is likely to be critical for the 25 proteins that contain selenocysteine, including glutathione peroxidases (Gpx) and thioredoxin reductases (TrxRs), which are critical for the detoxification of reactive oxygen species (ROS).[22] Indeed, when exposed to high ROS levels, mouse cells respond by increasing the levels of Alkbh8 and mcm^5Um in order to increase the availability of Gpx1, Gpx3, Gpx4, Gpx6, and Txnrd1.[23]

The yeast ortholog of ALKBH8 is Trm9, and its deficiency sensitizes yeast to DNA-damage-induced killing, most likely due to impaired translation of DNA-damage-response (DDR) proteins through impaired wobble uridine modification.[24] Although several abnormalities have been observed in mouse embryonic fibroblasts (MEFs) derived from *Alkbh8*$^{-/-}$ mice (e.g., increased DNA damage at baseline and in response to DNA-damaging agents),[23] *Alkbh8*$^{-/-}$ mice are described as phenotypically normal.[11] However, these mice do recapitulate some of the MEF abnormalities, specifically the lack of mcm^5U, mcm^5s^2U, and mcm^5Um modifications, and the low levels of Gpx1.[11] Similarly, *ALKBH8* knockdown in human cells leads to decreased mcm5U levels, and its expression is induced by DNA damage.[13]

There are no diseases linked to *ALKBH8* mutations, despite the growing list of Mendelian disorders caused by mutations in various components of tRNA modification machinery. One such component includes ADAT3 (encoded by *ADAT3* [MIM: 615302]), which is required for the deamination of adenosine to inosine at the wobble position of tRNA that decodes "family codon boxes."[25] On the basis of two independent biallelic mutational events in two multiplex families, we suggest in this study that the phenotype of *ALKBH8* deficiency in humans is intellectual disability (ID). Furthermore, we show that ALKBH8 deficiency in these individuals is associated with absent (S)-$mchm^5U$, (R)-$mchm^5U$, mcm^5Um, and mcm^5s^2U modifications in total tRNA.

The index individual in family 1 (IV:13, Figure 1) is a 12-year old boy with ID, epilepsy, and history of global developmental delay (GDD). He had normal prenatal and birth history. Seizure onset was at age 1 year, and his seizures have been well controlled by medication. He sat at age 1 year and walked at age 2 years. Currently, he has very limited expressive language and an IQ (intelligence quotient) of 52. He is hyperactive with a very poor attention span. His complete lack of self-care and understanding of the concept of danger necessitates constant supervision. His medical history is largely unremarkable otherwise. His growth parameters are age appropriate, and he only has mild dysmorphism in the form of an overbite, a small penis, and undescended testicles. Echocardiogram was normal. Brain magnetic resonance imaging (MRI) revealed normal findings except for a well-defined rounded lesion seen in the right transverse sinuses; this lesion is isointense on T1 and hyper-intense on T2 and most likely represents an arachnoid granulation. He has two affected siblings, a 15-year-old brother (family 1_IV:12) with ID, epilepsy, and GDD, and a 5-year-old sister with ID and GDD but no epilepsy (family 1_IV:16).

The index individual in family 2 (IV:12) is a 16-year-old boy with ID, epilepsy, GDD, and congenital heart disease (ventricular septal defect [VSD]). VSD was repaired at age 1 year and a follow-up echocardiogram showed a thickened tri-leaflet aortic valve with no stenosis but with mild regurgitation. Neuropsychological assessment (via the standard tool Beery-Buktenica Developmental Test of Visual-Motor Integration) at age 12 years showed an age equivalency of 66 months and a standard score of 62 (mild degree of cognitive delay) and severe linguistic impairment. Social skills were consistent with his mental age. Like the index individual in family 1, he was diagnosed with attention deficit hyperactivity disorder (ADHD) of the combined type. The first seizure episodes were noted at 9–12 months of age, and his seizures are currently under good control with topiramate. He had a myringotomy tube inserted bilaterally and an adeno-tonsillectomy at age 2 years as treatment for recurrent otitis media and persistent middle-ear effusion. Growth parameters showed macrocephaly (occipitofrontal circumference [OFC] 60 cm at 16 years of age) but normal height and weight. He had curly hair and large and deep-set eyes. He appeared active, had a happy demeanor, and had occasional stereotypic movements (lateral neck shaking). He was mildly hypotonic. An MRI of his brain was normal. His initial electroencephalogram (EEG) was mildly abnormal and showed generalized slowing and episodes of generalized delta discharges, which might indicate underlying cortical irritability. His chromosomal analysis and single-nucleotide polymorphism (SNP) array study were negative. His acylcarnitine profile, biotinidase enzymatic activity, urine organic acid analysis, and plasma very-long-chain-fatty-acid analysis were unremarkable.

Family 2 has three other boys who are affected with same phenotype as the index individual and three children (two girls and one boy) who are unaffected (Figure 1). The first affected brother (family 2_IV:7) is now 28 years old and has severe ID, large ears, and a long face. He developed seizures at age 2 years, but they ceased by age 5 years. The second affected brother (family 2_IV:8) is now 26 years old and has a history of prune belly syndrome (severe hypoplasia of abdominal wall muscles as a result of chronically increased intra-abdominal pressure during fetal

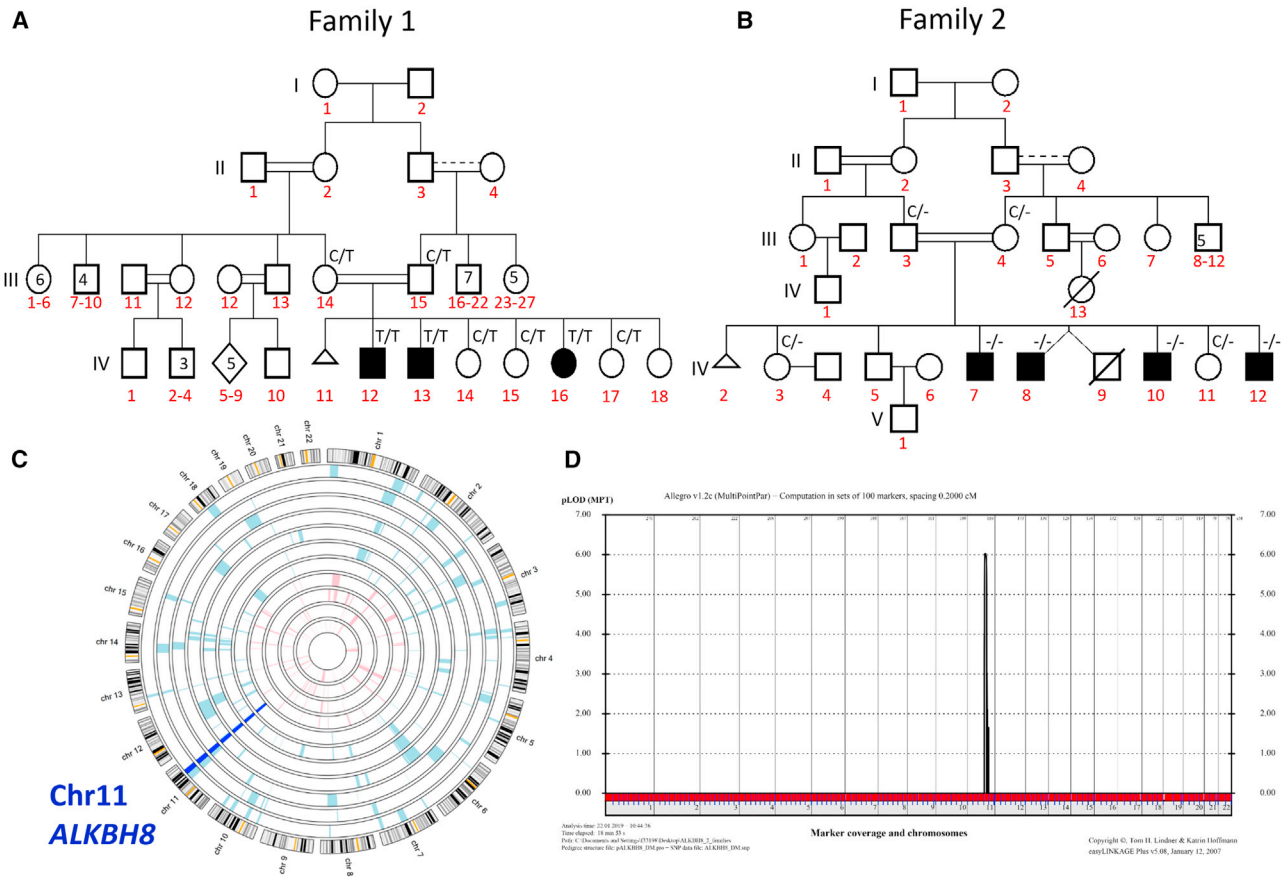

Figure 1. An ID Syndrome Is Linked to ALKBH8
(A) and (B) Pedigrees of the two study families. Squares and circles denote males and females, respectively (solid symbols indicate affected individuals). Genotypes for the two identified mutations in *ALKBH8* are shown below the symbol of each tested family member.
(C) Homozygosity mapping (using AgileMultiIdeogram) showing a single region of homozygosity that is shared among affected members of both families and spans *ALKBH8*.
(D) Linkage analysis confirming the strong linkage (LOD score = 6) of the critical autozygous interval with the disease phenotype in both families.

development, typically in association with urinary tract malformations) and underwent bilateral ureteric reimplantation and bilateral orchiopexy. He has severe ID and a long face and large ears. He developed seizures at around age 2 years, but these ceased at age 15 years. He has normal OFC. The third affected brother (family 2_IV:10) is a 24-year-old male who was also born with prune belly syndrome and was found to have left solitary kidney. His OFC at the age of 16 years was 55.5 cm (95th centile). He has severe ID and epilepsy, which is fairly controlled by multiple antiepileptic medications. He had a normal brain MRI. Table 1 summarizes the clinical features of all affected individuals.

Individuals were initially tested as part of a large clinical exome sequencing effort (manuscript under review). The index individual in each family independently underwent clinical exome sequencing that reported a different homozygous variant of unknown significance in ALKBH8: GenBank: NM_001301010.1, exon12, c.1660C>T (p.Arg554*) in family 1 and GenBank: NM_001301010.1, exon12, c.1794delC (p.Trp599Glyfs*19) in family 2 (Figure 2). Both families were subsequently recruited under an IRB-approved research protocol with informed consent (KFSHRC RAC# 2121053). Blood samples were collected from the affected and unaffected members of each nuclear family in tubes containing ethylenediaminetetraacetic acid (EDTA) and sodium heparin for DNA extraction and establishment of lymphoblastoid cell lines (LCL) for RNA extraction, respectively. Autozygome analysis using AutoSNPa was based on regions of homozygosity $\geq$2 Mb in length as surrogates of autozygosity. By defining the candidate autozygome as the autozygous intervals that are exclusively shared by the affected members, we identified a single candidate locus corresponding to chr11q22.3 (Figure 1). This was further corroborated by linkage analysis (EasyLINKAGE package was used under the default parameters) that confirmed linkage of both families to the same candidate autozygous interval with a LOD (logarithm of the odds) score of 6 (Figure 1). Segregation analysis confirmed strict segregation of the variants with the disease in an autosomal-recessive fashion in both families, as shown on the pedigrees (Figure 1).

Table 1. Summary of Clinical Features in the Affected Individuals

Case ID	Family 1_IV:12	Family 1_IV:13	Family 1_IV:16	Family 2_IV:7	Family 2_IV:8	Family 2_IV:10	Family 2_IV:12
Gender	male	male	female	male	male	male	male
Age	15 years	12 years	5 years	28 years	26 years	24 years	16 years
ID	P	P	P	P	P	P	P
Epilepsy	P	P	A	P	P	P	P
GDD	P	P	P	P	P	P	P
MRI brain	NA	arachnoid granulation	NA	NA	NA	N	N
Other features		mild dysmorphism, ADHD		mild dysmorphism	prune belly syndrome	prune belly syndrome, unilateral renal agenesis, macrocephaly	ADHD, CHD, macrocephaly

Abbreviations are as follows: A, absent; ADHD, attention deficit hyperactivity disorder; CHD, congenital heart disease; N, normal; NA, not available; and P, present.

We analyzed the modification status of nucleosides from affected-individual- and control-derived tRNA by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described before with the mass transitions listed in Table S1.[26] To address whether ALKBH8 deficiency had an effect on the modification status of wobble uridines, we extracted total tRNA from LCL derived from affected and unaffected individuals from Family 1 and Family 2, as well as from independent control subjects, using miRNeasy Mini Kit (QIAGEN). We analyzed the modification status of nucleosides from affected-individual- and control-derived tRNA by LC-MS/MS (as described previously) with the mass transitions listed in Table S1.[26] LC-MS/MS is arguably the modification analysis approach with the highest specificity; it identifies each nucleoside by its molecular mass and molecular fragmentation pattern upon gas-phase collisions of the nucleoside. The liquid chromatography (LC) retention time is decided by the nucleosides' physio-chemical properties, and this offers additional characteristics that are used for identification (see Table S1 for details). These several layers of specificity—molecular mass, molecular fragmentation pattern, and LC retention time—make it possible to identify specific nucleoside modifications with a high degree of certainty even with the extremely complex background of biological samples.

LC-MS/MS revealed that the wobble modifications mcm^{5}s^{2}U, (R)-mchm^{5}U and (S)-mchm^{5}U, and mcm^{5}Um were readily detected in total tRNA from all unaffected family members and independent controls but were completely absent from total tRNA from affected individuals (Figure 3). Similarly, mcm^{5}U was detected in all controls of both families and was completely absent in all affected individuals of family 2 (Figure 3). However, in family 1, a low (2%) LC-MS/MS signal that resembled the characteristics of mcm^{5}U was found in all affected individuals (Figure 4), so although the mcm^{5}U levels were also dramatically decreased in family 2 affected individuals, we cannot exclude the possibility that very low levels of mcm^{5}U may still be present. However, the ribose-methylated form mcm^{5}Um was completely absent in all affected individuals from both families (Figures 3 and 4),indicating aberrant modification of the selenocysteine-specific tRNA^{Sec} that is required for the efficient expression of certain stress-related selenoproteins.[11,17–20]

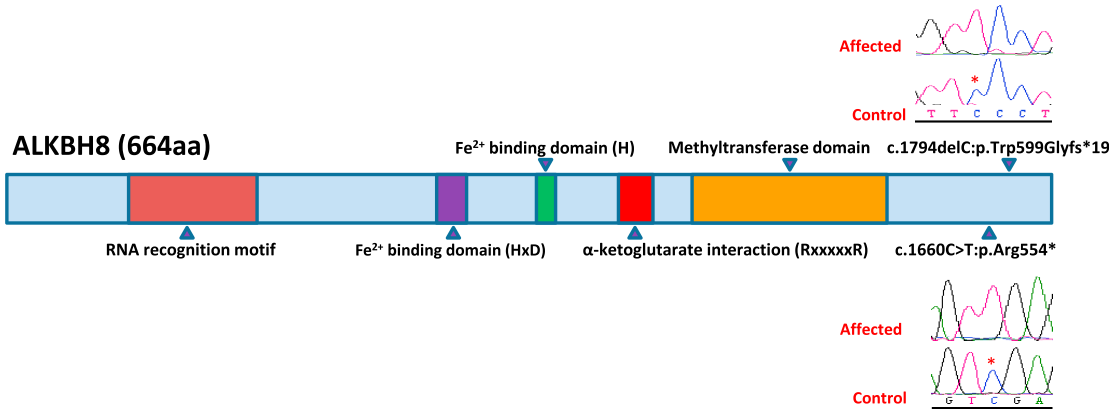

Figure 2. Identification of *ALKBH8* Homozygous Truncating Variants
Cartoon of ALKBH8 indicating the known functional domains and the locations of the two homozygous mutations along with their sequence chromatograms (normal controls are shown for comparison). Note the C-terminal locations of both variants, which were found on RT-PCR to escape NMD (nonsense-mediated decay).

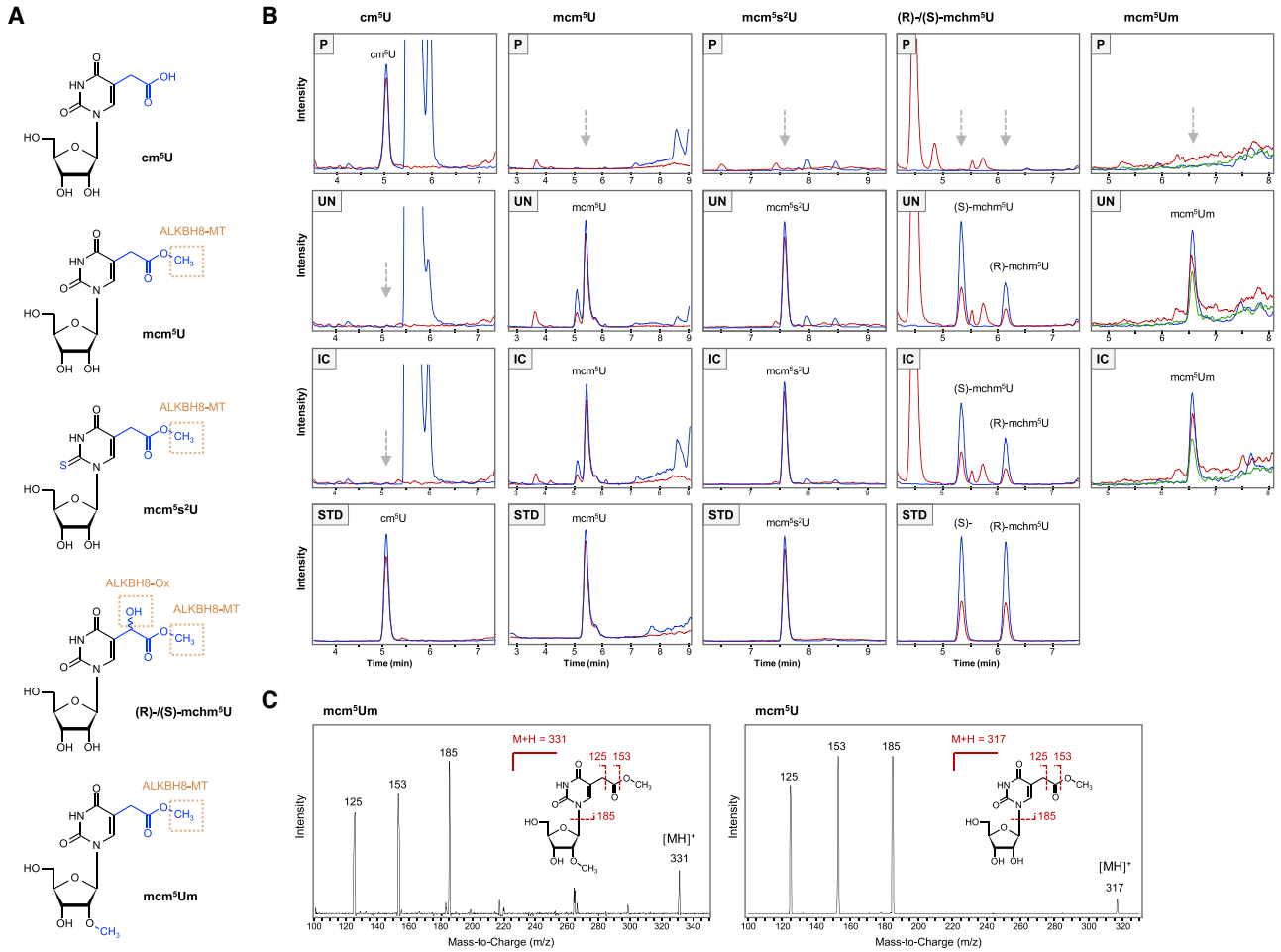

Figure 3. Qualitative Validation of the LC-MS/MS Quantification of Wobble Uridine Modifications
(A) Chemical structures of wobble uridine modifications. Functional groups added by the ALKBH8 MT domain and the 2OG-Fe(II) domain (Ox) are indicated with dashed rectangles.
(B) Representative LC-MS/MS chromatograms comparing wobble uridines in total tRNA from an affected individual (P), a control individual from an unaffected family (UN), and an independent control (IC) to a pure nucleoside standard (STD). For mcm^{5}Um, where no standard was available, the molecular fragmentation pattern shown in (C) was used for identification. Blue chromatogram traces correspond to mass transitions from the characteristic loss of ribose neutrals, and red and green traces correspond to qualifier mass transitions from the loss of additional molecular substructures. An arrow indicates the expected retention time for the nucleoside in cases where it was not detected.
(C) MS/MS product ion spectrum showing the molecular fragmentation pattern of the putative mcm^{5}Um (left) compared to the related and verified mcm^{5}U (right) in total tRNA. The spectrum shows the expected pattern and thus verifies mcm^{5}Um identity.

Thus, and in agreement with previous data on Alkbh8-deficient mice,[11,27] we detected substantial amounts of cm^{5}U, the precursor of mcm^{5}U, in total tRNA from affected individuals, whereas no cm^{5}U was detected in total tRNA from controls (Figures 3 and 4). We could detect negligible amounts of cm^{5}s^{2}U, the thiolated form of cm^{5}U, in all individuals tested, and there were no appreciable differences between affected individuals and controls. The results confirmed that the presence of a methylated mcm^5 side chain is a prerequisite for efficient thiolation by the enzyme responsible for mcm^{5}s^{2}U formation. The amide wobble uridine ncm^{5}U, normally present in tRNA isoacceptors carrying alanine, valine, serine, proline, and threonine, seemed to accumulate slightly in affected individuals as compared to controls ($p = 0.021$, unpaired Student's t test comparing all affected individuals [$n = 7$] to all unaffected family controls [$n = 6$]) (Figure 4). This observation is consistent with previous data on mice[11] and might indicate that the cm^{5}U-containing tRNAs accumulating in affected individuals are partially channeled into the pathway generating ncm^{5}U.

Recent years have witnessed a growing interest in tRNA modification as a cellular process with strong relevance to human health and development, especially in the central nervous system. A point mutation in *ADAT3* originally reported by us in 2013 turned out to be the most common single-gene mutation in Arabian individuals with ID.[25] *ADAT3* encodes an ortholog of yeast, TAD3, which together with ADAT2 forms a complex that is required for I_{34} modification of substrate tRNAs.[28] Subsequently, we identified a *WDR4* (MIM: 605924) founder mutation that impairs a highly conserved and specific

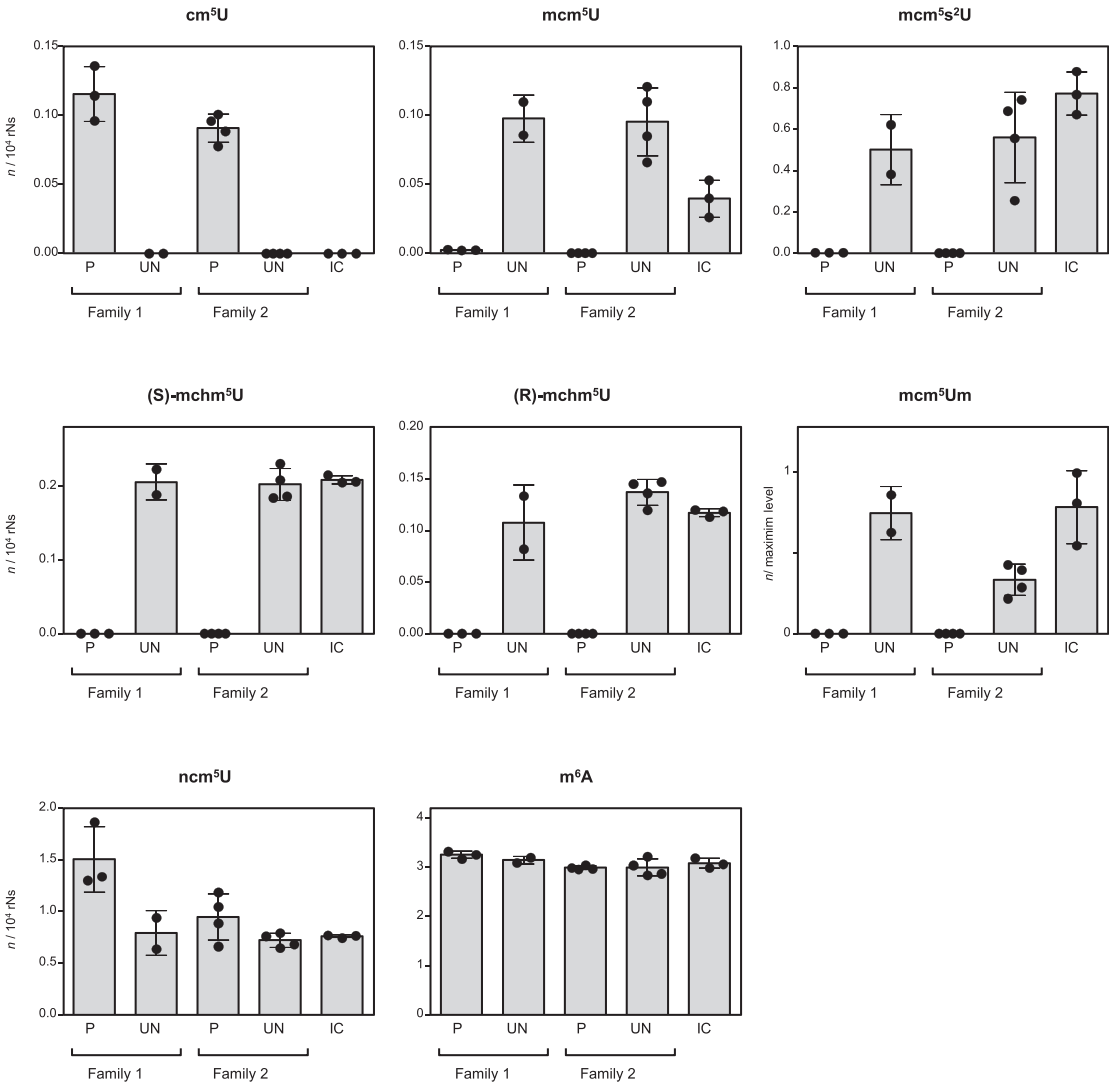

Figure 4. *ALKBH8* Mutations Are Associated with Abnormal Wobble Uridine Modification
LC-MS/MS quantification of wobble uridine modifications and the independent tRNA modification m6A in total tRNA from patients (P, n = 7), unaffected family controls (UN, n = 6), and independent controls (IC, n = 3). Each dot in the bar graphs represents an individual from the indicated group. The levels are expressed as ratios of modified to unmodified nucleosides for all modifications except mcm5Um, where no standard was available. For mcm5Um, the levels are expressed as a ratio to the maximum recorded level in the sample set.

(m7G46) methylation of tRNA in affected individuals with severe encephalopathy and microcephaly.[29] Additional examples include mutations in *TRMT10A* (MIM: 616013) encoding the yeast TRM10 ortholog tRNA methyltransferase 10A required for m^1G_9 modification,[30] and in *NSUN2* (MIM: 610916), encoding NOP2/Sun RNA methyltransferase 2 required for m^5C modification of C_{34} of the anticodon[31,32] in Mendelian syndromes involving ID.[31,33–35] More recently, we have identified mutations in *PUS3* (MIM: 616283) and *PUS7* (MIM: 616261), encoding enzymes required for the modification of uridine in tRNA into pseudouridine, in individuals with ID.[36,37]

Our finding of independent homozygous truncating variants in ALKBH8 in multiplex families with ID seems, therefore, consistent with above-described pattern of brain predilection to disorders of tRNA modification.[38] This is unlikely to be related to brain-specific expression of *ALKBH8* because our analysis of LCL, derived from affected individuals' blood lymphocytes, seems to suggest a systemic deficiency of the wobble uridine modifications in tRNA. This is also in line with previously published work suggesting a wide profile of expression developmentally.[39] However, it should be noted that other studies seem to suggest that the early embryonic broad expression profile becomes progressively more restricted to specific neuronal cells.[12]

The pathogenesis of *ALKBH8*-related ID is unclear. The dramatic reduction of $mchm^5U$ modifications in tRNA is very similar to what has been observed in *Alkbh8* knockout mice, and yet those mice are described as phenotypically normal. However, we note that no specialized cognitive tests were administered, so it remains

possible that cognitive impairment might have been overlooked. In the examples noted above on tRNA modification-related ID syndromes, it was proposed that the presumptive deleterious consequence to protein translation is particularly harmful to brain function. Although this might still be the case here, we note that ALKBH8 has been shown to play an indirect role in the detoxification of ROS (see above). Given the high metabolic demand of the brain, it is tempting to speculate that impaired response to ROS might have played a role in the pathogenesis of this novel syndrome. For example, Endres et al. noted that *Alkbh8*$^{-/-}$ MEFs reprogram stress-response systems to adapt to stress, so it is possible that brain-specific reprogramming could be part of the pathogenesis in the affected individuals.[23]

In conclusion, we propose that *ALKBH8* bi-allelic loss-of-function mutations in humans cause ID, presumably mediated by severe impairment of the wobble uridine modifications in tRNA. The two families were identified in a large clinical exome study involving >2,200 families, so this is clearly a rare disease even in a highly consanguineous population. Nonetheless, it is hoped that this report will facilitate the identification of additional cases to further delineate the phenotype of this disorder. In particular, it will be of interest to observe the phenotypic expression of less severe variants than the ones we report.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.03.026.

Acknowledgments

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Declaration of Interests

The authors declare no competing interests.

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Web Resources

AgileMultiIdeogram, http://dna.leeds.ac.uk/agile/AgileMultiIdeogram/
AutoSNPa, http://dna.leeds.ac.uk/autosnpa/
EasyLINKAGE, http://nephrologie.uniklinikum-leipzig.de/nephrologie.site,postext,easylinkage.html
Online Mendelian Inheritance in Man, http://omim.org/

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