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

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ABSTRACT

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 mutations in *ALKBH8* with intellectual disability. Analysis of affected individual-derived tRNA showed 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.

REPORT

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 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. all four codons encode the same amino acid, whereas tRNA that harbor mcm^5U decode "split codon boxes" that code for different amino acids by only varying the last purine/pyrimidine [2].

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 2-oxoglutarate 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), 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 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 (mcm^5s^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 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 in [17]). 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; 18-21]. Like thiolation, 2'-O-methylation also requires prior methylation by ALKBH8 [22]. 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) [23]. Indeed, when exposed to high ROS levels, cells respond by increasing the levels of Alkbh8 and mcm^{5}Um in order to increase the availability of Gpx1, Gpx3, Gpx4, Gpx6 and Txnrd1 [24].

The yeast ortholog of ALKBH8 is Trm9 and its deficiency sensitizes yeast to DNA damage-induced killing, likely due to impaired translation of DNA damage response (DDR) proteins through impaired wobble uridine modification [25]. 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) [24], *Alkbh8*$^{-/-}$ mice are described as phenotypically normal [11]. However, these mice do recapitulate some of the abnormalities observed in MEFs, specifically the lack of mcm^{5}U, mcm^{5}s^{2}U and mcm^{5}Um 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 including ADAT3 (encoded by *ADAT3* [MIM 615302]) required for the deamination of adenosine to inosine at the wobble position of tRNA that decode "family codon boxes" [26]. In this study, we suggest that the phenotype of *ALKBH8* deficiency in humans is intellectual disability (ID) based on two independent biallelic mutational events in two multiplex families. Furthermore, we show that ALKBH8 deficiency in these individuals is associated with absent (S)-mchm^{5}U, (R)-mchm^{5}U, mcm^{5}Um and mcm^{5}s^{2}U modifications in total tRNA.

The index 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 has been well controlled by medication. He sat at age 1 year and walked at age 2 years. Currently, he has very limited expressive language with an IQ (intelligence quotient) of 52. He is hyperactive with 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 overbite, 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 which is iso-intense 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 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 follow-up echocardiogram showed thickened tri-leaflet aortic valve with no stenosis but with mild regurgitation. Neuropsychological assessment at age 12-year showed (using the standard tool of Beery-Buktenica Developmental Test of Visual-Motor Integration) age equivalency of 66 months with 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 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 is currently under good control with topiramate. He had a myringotomy tube insertion bilaterally and adeno-tonsillectomy at age 2-year for recurrent otitis media and persistent middle ear effusion. Growth parameters showed macrocephaly (occipitofrontal circumference (OFC) 60 cm at 16 years of age) with normal height and weight. He had curly hair and large and deep-set eyes. He appeared active with happy demeanor and had occasional stereotypic movements (lateral neck shaking). He was mildly hypotonic. MRI brain was normal. Initial electroencephalogram (EEG) was mildly abnormal with generalized slowing and episodes of generalized delta discharges, which may indicate underlying cortical irritability. His chromosomal analysis and single nucleotide polymorphism (SNP) array study were negative. 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 (2 girls and 1 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 long face. He developed seizures at age 2 years but they ceased by age 5 years. The 2nd (Family 2_IV:8) is now 26-year old and has history of Prune Belly syndrome (severe hypoplasia of abdominal wall muscles due to chronically increased intra-abdominal pressure during fetal development typically in association with urinary tract malformations) and underwent bilateral ureteric reimplantation and bilateral orchiopexy. He has severe ID and long face and large ears. He developed seizures at around age 2 years, which

ceased at age 15 years. He has normal OFC. The 3rd (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 on multiple antiepileptic medications. He had a normal brain MRI. Table 1 summarizes the clinical features of all affected individuals.

Cases were initially tested as part of a large clinical exome sequencing effort (manuscript under review). The index case in each family independently underwent clinical exome sequencing that reported a different homozygous variant of unknown significance in *ALKBH8*: NM_001301010.1:exon12:c.1660C>T: p.Arg554* in Family 1 and 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 EDTA and sodium heparin tubes for DNA extraction and establishment of lymphoblastoid cell lines (LCL) for RNA extraction, respectively. Autozygome analysis was based on regions of homozygosity $\geq$2Mb in length as surrogates of autozygosity using AutoSNPa. 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 following 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).

We analyzed the modification status of nucleosides from cases- and controls-derived tRNA by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) as described before with the mass transitions listed in Table S1 [27]. To address whether ALKBH8 deficiency had an effect on the modification status of wobble uridines, total tRNA was extracted from LCL derived from affected and unaffected individuals of Family 1 and 2, as well as independent control subjects using miRNeasy Mini Kit (Qiagen). We analyzed the modification status of nucleosides from cases- and controls-derived tRNA by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) as described before with the mass transitions listed in Table S1 [27]. LC-MS/MS is

arguably the modification analysis approach with the highest specificity, identifying each nucleoside by their molecular mass and molecular fragmentation pattern upon gas-phase collisions of the nucleoside. The LC retention time is decided by the nucleosides physio-chemical properties and 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 dramatically decreased also 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 (Figure 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; 18-21].

Thus, and in agreement with previous data on Alkbh8 deficient mice [11; 16], we detected substantial amounts of cm^{5}U, the precursor of mcm^{5}U, in total tRNA from affected individuals, while no cm^{5}U was detected in total tRNA from controls (Figure 3 and 4). We could detect negligible amounts of cm^{5}s^{2}U, the thiolated form of cm^{5}U, 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 may 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 in intellectual disability cases from Arabia [28]. *ADAT3* encodes an ortholog of yeast TAD3, which forms a complex with ADAT2 that is required for I_{34} modification of substrate tRNAs [29]. Subsequently, we identified a founder mutation of *WDR4* [MIM 605924] that impairs a highly conserved and specific (m7G46) methylation of tRNA in affected individuals with severe encephalopathy and microcephaly [30]. Additional examples include mutations in *TRMT10A* [MIM 616013], encoding the yeast TRM10 ortholog tRNA methyltransferase 10A required for m^1G_9 modification [31], and in *NSUN2* [MIM 610916], encoding NOP2/Sun RNA methyltransferase 2 required for m^5C modification of C_{34} of the anticodon [32; 33], in Mendelian syndromes involving ID [32; 34-36]. More recently, we have shown that 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 [37; 38].

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 [39]. This is unlikely related to brain-specific expression of *ALKBH8* since our analysis of lymphoblastoid cell lines 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 [40]. 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 may have been overlooked. In the examples noted above on tRNA modification-related ID syndromes, it was proposed that the presumptive deleterious consequence on protein translation is particularly harmful to brain function. While this may 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 may 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 [24].

In conclusion, we propose that *ALKBH8* biallelic loss of function mutations cause ID in humans, 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.

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DECLARATION OF INTEREST

Authors declare no conflicts of interest.

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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FIGURE LEGENDS

Figure 1. An intellectual disability syndrome is linked to ALKBH8.

A) and B) Pedigrees of the two study families. Squares and circles denote males and females, respectively (solid symbols indicated affected individuals). Genotypes for the two identified mutations in *ALKBH8* are shown below the symbol of each tested member. C) Homozygosity mapping (using AgileMultiIdeogram) showing a single shared region of homozygosity among affected members of both families spanning *ALKBH8*. D) Linkage analysis confirming the strong linkage of the critical autozygous interval with the disease phenotype in both families with a LOD sore of 6.

Figure 2. Identification of *ALKBH8* homozygous truncating variants.

Cartoon of ALKBH8 indicating the known functional domains and the location of the two homozygous mutations along with their sequence chromatograms (normal controls are shown for comparison). Note the C-terminal location 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 methyltransferase domain (MT) and the 2OG-Fe(II) domain (Ox) are indicated with dashed rectangles. B) Representative LC-MS/MS chromatograms showing wobble uridines in total tRNA from an affected individual (P), unaffected family control (UN), and independent control (IC), compared to a pure nucleoside standard (STD). For mcm^5Um 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 loss of additional molecular substructures. An arrow indicates the expected retention time for the nucleoside in cases it was not detected. C) MS/MS product ion spectrum showing the molecular fragmentation pattern of the putative mcm^5Um in total tRNA (left), compared to the related and verified mcm^5U (right). The spectrum shows the expected pattern and thus verifies mcm^5Um identity.

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 of 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.

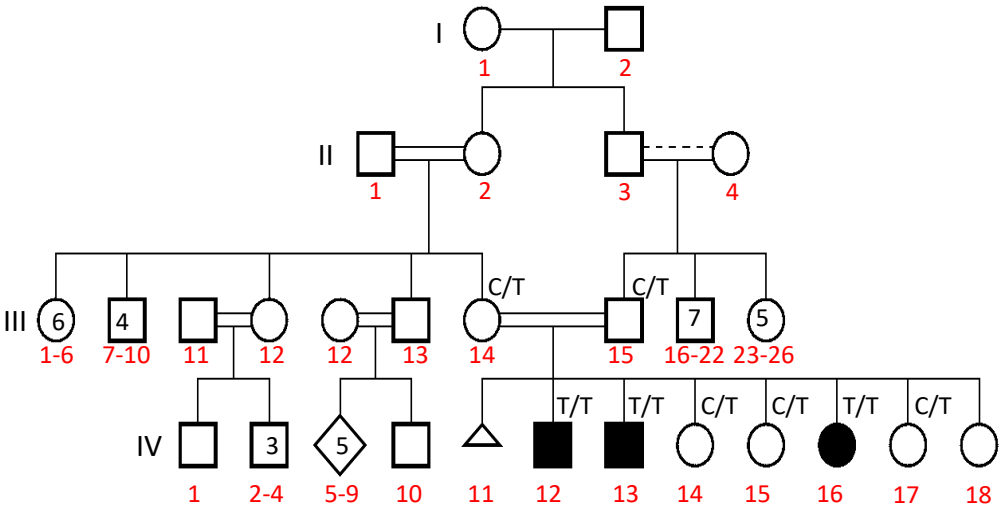
Table 1. Summary of clinical features in the affected individuals

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	15yr	12yr	5yr	28yr	26yr	24yr	16yr
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

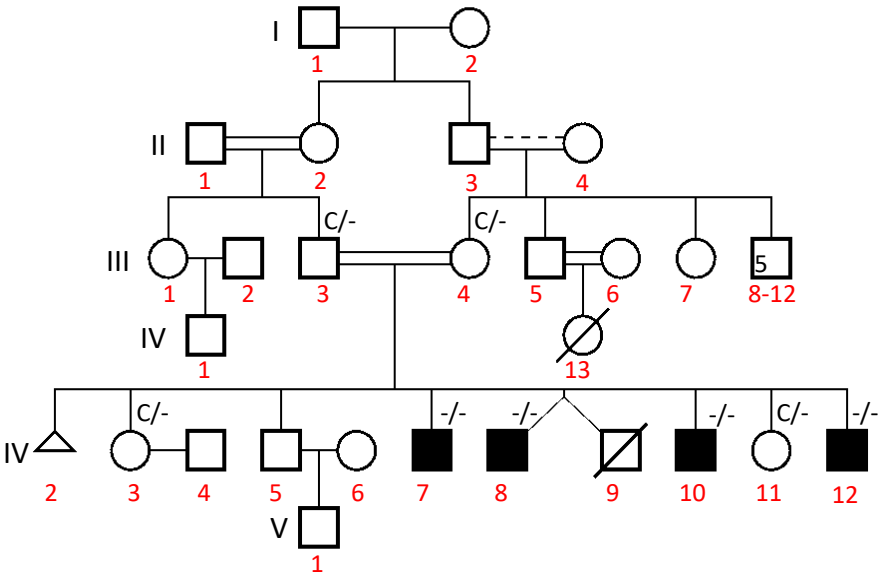
A: absent, ADHD: attention deficit hyperactivity disorder, CHD: congenital heart disease, N: normal, NA: not available, P: present.

Figure 1

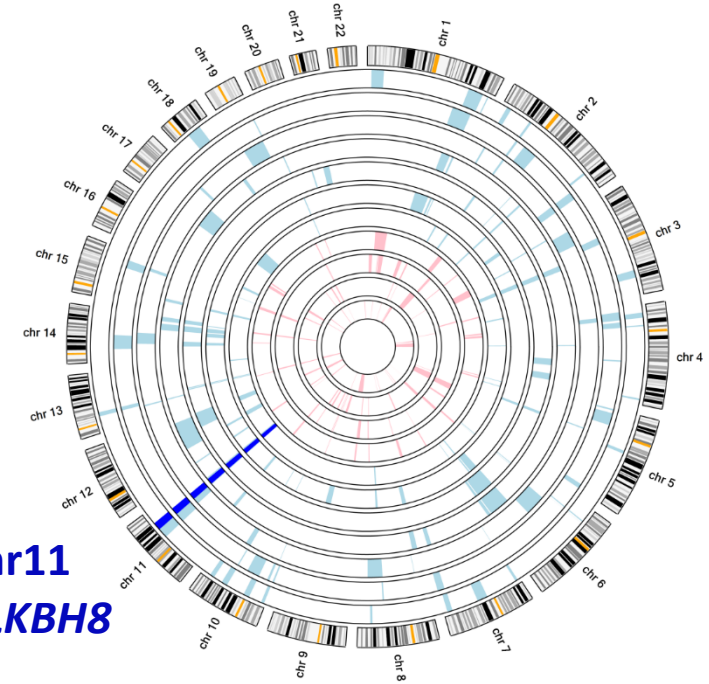
A)



B)



C)



D)

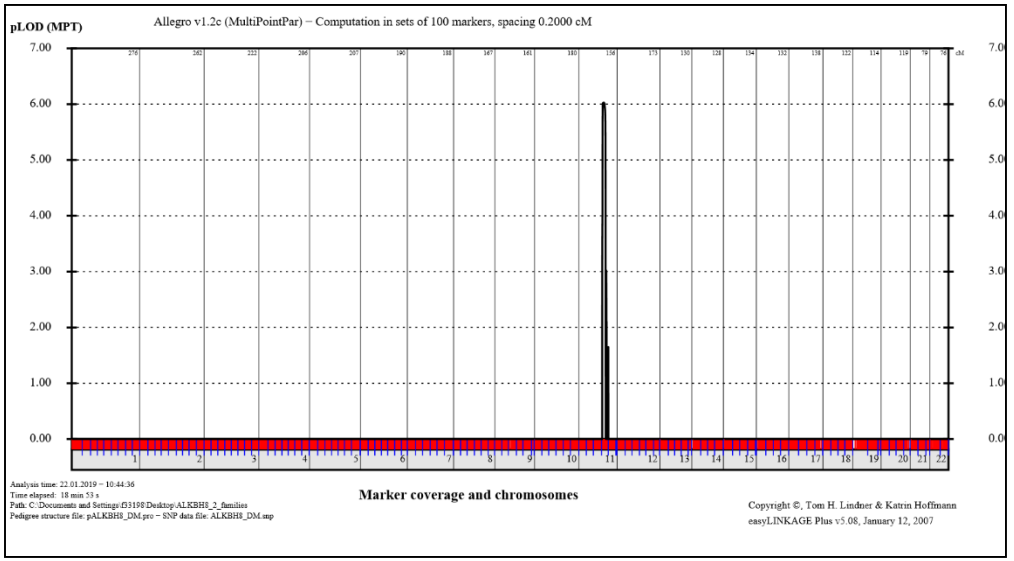


Figure 2

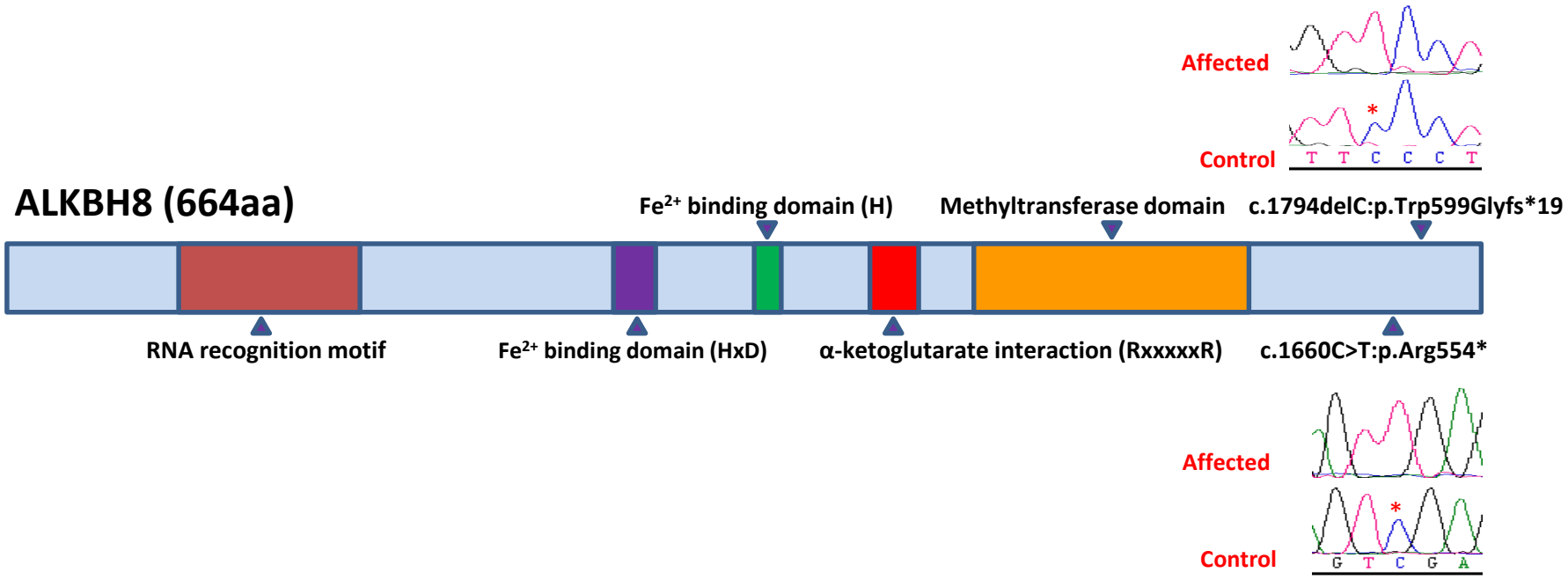

Affected
Control
T T C C C T
ALKBH8 (664aa)
Fe2+ binding domain (H)
Methyltransferase domain
c.1794delC:p.Trp599Glyfs*19
RNA recognition motif
Fe2+ binding domain (HxD)
α-ketoglutarate interaction (RxxxxxR)
c.1660C>T:p.Arg554*
Affected
Control
G T C G A

Figure 3

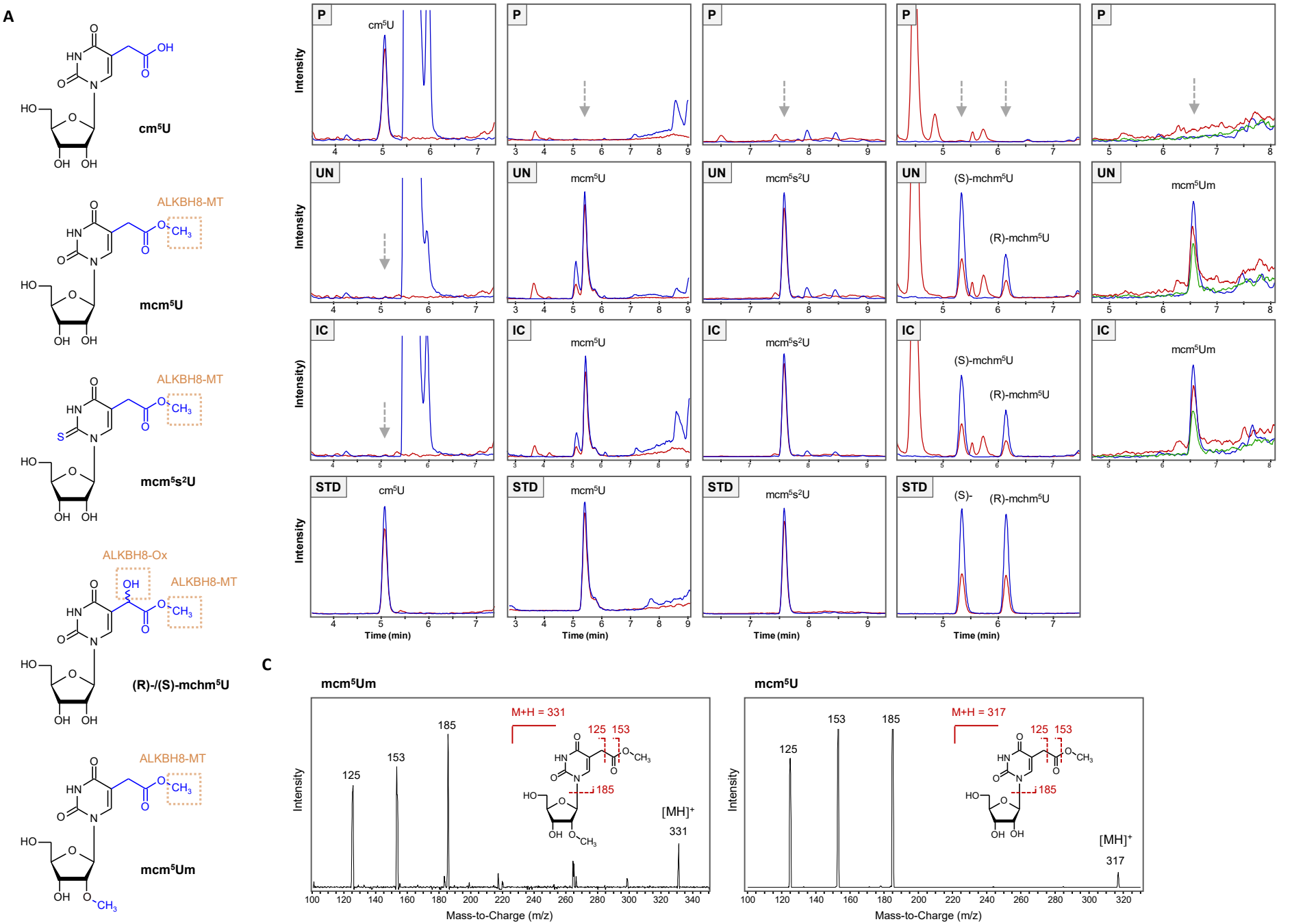
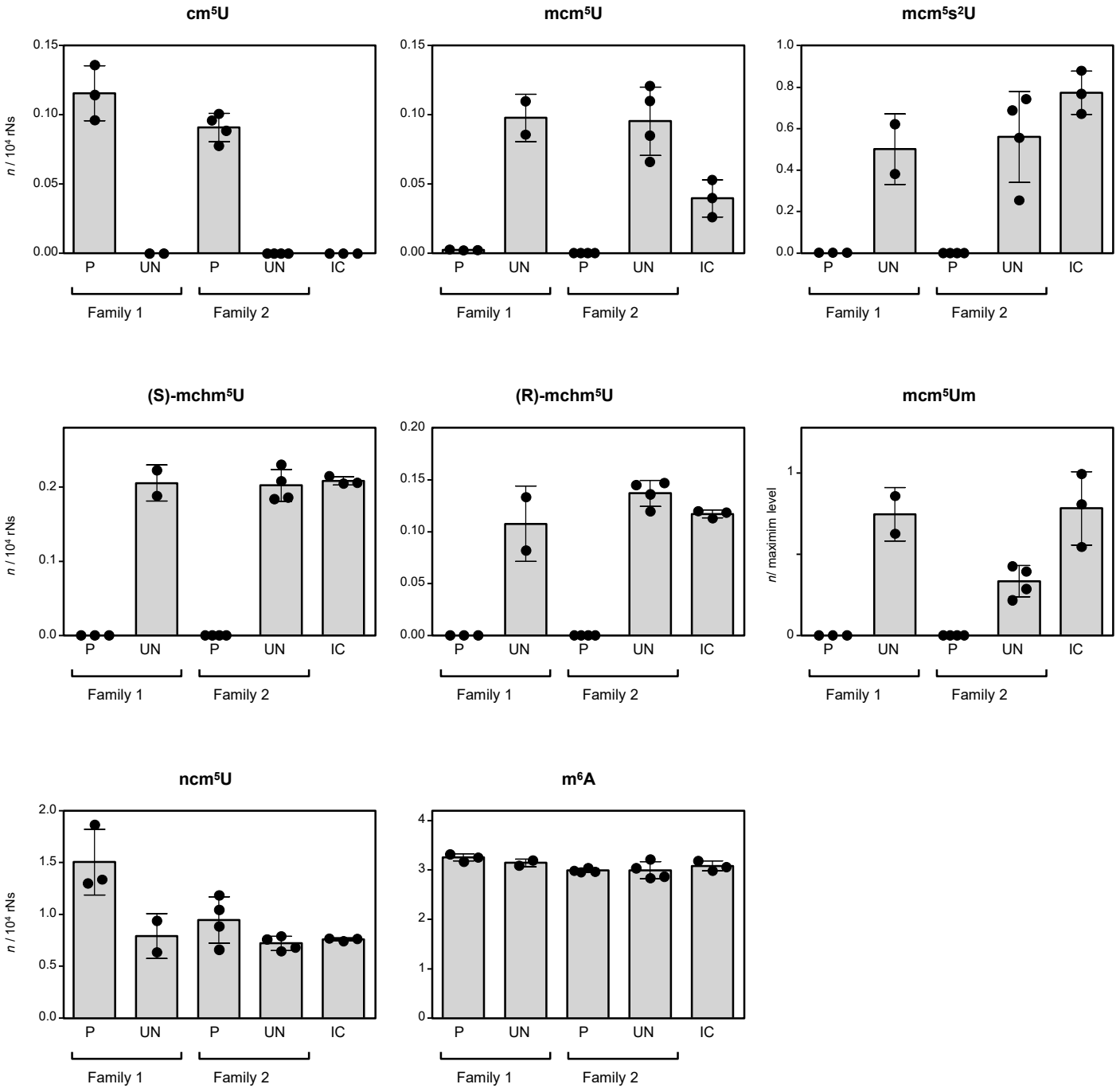

A
cm5U
ALKBH8-MT
mcm5U
ALKBH8-MT
mcm5s2U
ALKBH8-Ox
ALKBH8-MT
(R)-/(S)-mchm5U
ALKBH8-MT
mcm5Um
B
cm5U
mcm5U
mcm5s2U
(R)-/(S)-mchm5U
mcm5Um
P
UN
IC
STD
Intensity
Time (min)
(S)-mchm5U
(R)-mchm5U
C
mcm5Um
mcm5U
M+H = 331
M+H = 317
125
153
185
[MH]+
331
317
Intensity
Mass-to-Charge (m/z)

Figure 4


cm⁵U
mcm⁵U
mcm⁵s²U
(S)-mchm⁵U
(R)-mchm⁵U
mcm⁵Um
ncm⁵U
m⁶A
n / 10⁴ rNs
n/ maximim level
P
UN
IC
Family 1
Family 2