Biallelic *ADARB1* variants associated with microcephaly, intellectual disability and seizures

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ABSTRACT

The RNA editing enzyme ADAR2 is essential for recoding of brain transcripts. Impaired ADAR2 editing leads to early-onset epilepsy and premature death in a mouse model. Here, we report biallelic variants in *ADARB1*, the gene encoding ADAR2, in four unrelated individuals with microcephaly, intellectual disability, and epilepsy. In one individual, a homozygous variant in one of the double-stranded RNA-binding domains was identified. In the others, variants were situated in or around the deaminase domain. To evaluate the effects of these variants on ADAR2 enzymatic activity, we performed *in vitro* assays with recombinant proteins in HEK293T cells and *ex vivo* assays with fibroblasts derived from one of the individuals. We demonstrate that these ADAR2 variants lead to reduced editing activity on a known ADAR2 substrate. We also demonstrate that one variant leads to changes in splicing of *ADARB1* transcript isoforms. These findings reinforce the importance of RNA editing in brain development and introduce *ADARB1* as a genetic etiology in individuals with intellectual disability, microcephaly and epilepsy.

INTRODUCTION

The adenosine deaminases acting on RNA (ADARs) are a family of enzymes that catalyze the hydrolytic deamination of adenosine to inosine in double-stranded RNA (dsRNA) (for review see [1]). The editing and RNA-binding activities of ADARs affect different aspects of RNA processing and can also lead to RNA recoding since inosine is usually recognized as guanosine by the translation machinery.[2] Three members of the ADAR family; ADAR1, ADAR2, and ADAR3, (encoded by the *ADAR* (MIM:146920), *ADARB1* (MIM:601218) and *ADARB2* (MIM:602065) genes, respectively) have been identified in humans, but only ADAR1 and ADAR2 are enzymatically active. All ADARs contain the eponymous deaminase domain and several double-stranded RNA-binding domains (dsRBDs). ADARs can assemble into hetero- and homodimers, and the dimerization is important for editing activity.[3-7]

Editing by ADAR1 is essential for innate immunity. Biallelic or rarely, *de novo* heterozygous variants in its encoding gene, *ADAR*, cause Aicardi-Goutières syndrome type 6 [AGS6; MIM: 615010], a genetically heterogeneous interferonopathy characterized by features resembling congenital viral infection, including microcephaly, intellectual disability, brain calcification, dystonia and high interferon levels.[8; 9] Heterozygous variants in *ADAR* have also been reported in association with dyschromatosis symmetrica hereditaria [DSH; MIM: 127400], a macular pigmentary cutaneous condition affecting the face and dorsal extremities.[10] Individuals with features clinically overlapping both AGS and DSH are rare.[11; 12]

ADARB1 (GenBank: NM_001112.4) encodes two major protein isoforms of ADAR2: the shorter ADAR2S (ADAR2a; UniProt ID: P78563-2) and longer ADAR2L (ADAR2b; UniProt: P78563-1) that differ by an alternatively spliced exon within the deaminase domain. This alternatively spliced exon extends the deaminase domain of ADAR2L by 40 amino acids. Both ADAR2 isoforms are expressed in the human brain at different developmental stages.[13; 14] ADAR2L is enzymatically less active than ADAR2S,[15; 16] although *in vitro* editing assays

performed with rat ADAR2 proteins demonstrate that the editing efficiency of the two isoforms at some editing sites is identical.[17]

In general, ADAR2 performs site-specific editing, whereas ADAR1 mediates promiscuous editing of transcripts containing repetitive elements such as *Alu* elements that can form intramolecular duplexes.[18] In humans, adenosine-to-inosine (A-to-I) editing within exons resulting in recoding is rare and predominantly occurs in the brain, within transcripts encoding subunits of neuroreceptors and ion channels. Editing can modulate the properties of encoded neuronal proteins. One of the best-studied transcripts recoded by ADAR2 is *Gria2*, which encodes a subunit of the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. The adenosine at the Q/R site of *Gria2* is edited in up to 100% of transcripts resulting in a change of glutamine to arginine. This change leads to the AMPA receptor being impermeable to calcium[19] and decreases trafficking of the assembled AMPA receptor from the ER to the postsynaptic region.[20; 21] The net effect of a reduction in RNA editing is an increase in the calcium permeability of AMPA receptors and an increase in their number at the postsynaptic region. A recent study reported 28 individuals with intellectual disability and neurodevelopmental abnormalities with *de novo* heterozygous variants in *GRIA2* (MIM:138247). One of the most deleterious variants (p.(Gln607Glu)) occurred at the Q/R site in an individual with a severe epileptic encephalopathy, resulting in a gain-of-function increase in channel current and reinforcing the importance of this editing site.[22] However, other editing substrates of ADAR2 or its editing-independent functions could play a role in brain physiology. Herein we report four unrelated individuals with biallelic *ADARB1* variants associated with microcephaly, intellectual disability and seizures which were intractable in three. We observed reductions in ADAR2 editing activity with four of the five variants and no changes in localization as assayed *in vitro* with recombinant proteins expressed in cultured cells. One of the variants, p.(Arg603Gln), appears to affect the stability of the recombinant ADAR2 protein.

Another variant, c.1492A>G (p.(Thr498Ala)), alters the ratio of two splicing isoforms of *ADARB1* transcript in two tested cell lines and fibroblasts derived from the affected individual. These data suggest that biallelic variants in *ADARB1* cause microcephaly, intellectual disability and intractable epilepsy, reinforcing the importance of RNA editing in human brain development.

SUBJECTS AND METHODS

We use the *ADARB1* exon numbering proposed by Slavov and Gardiner.[23] Based on this numbering, the Alu-J cassette exon of ADAR2L is referred to as exon 5a. The numbering of amino acids in this paper is based on the longer protein isoform ADAR2L.

Participant recruitment and sequencing

Individuals were clinically evaluated in separate centers and contact between researchers was facilitated by the use of web-based tools Matchmaker Exchange[24] and GeneMatcher.[25] Individual 1 underwent trio exome sequencing by the Genomics Platform at the Broad Institute of Harvard and MIT (Broad Institute, Cambridge, MA, USA)[26] as part of the Murdoch Children's Research Institute Undiagnosed Diseases Project (RCH HREC 36291A), Individual 2 underwent exome sequencing through GeneDx (Gaithersburg, MD) on a clinical basis, Individual 3 underwent trio exome sequencing as part of a pilot funded by the Israeli Ministry of Health and Individual 4 underwent trio exome sequencing as described previously.[27]

Cell culture

HEK 293T cells were cultured in MEM w/Earle's Salts (biosera) supplemented with non-essential amino acids (Sigma-Aldrich), 10% FCS, and penicillin/streptomycin (biosera). SH-SY5Y cells were cultured in Ham's F12 medium and MEM w/Earle's Salts (mixed 1:1, biosera) supplemented with non-essential amino acids, 15% FCS, and penicillin/streptomycin. HeLa

cells were cultured in DMEM High Glucose (biosera) supplemented with non-essential amino acids, 10% FCS, and penicillin/streptomycin. Fibroblasts were cultured in DMEM High Glucose (biosera) supplemented with non-essential amino acids, 15% FCS, and penicillin/streptomycin. All the cell lines were grown as monolayers at 37 °C with 5% CO_2.

Plasmid constructs and site-directed mutagenesis

Phusion Hot Start II polymerase (Thermo Fisher) was used for all cloning and mutagenesis PCRs. The full-length *ADARB1* coding sequence was amplified from HeLa cDNA using PCR with gene-specific primers introducing N-terminal FLAG-tag and C-terminal 6xHis-tag (both tags separated by Leu and Val from the coding sequence). A second pair of primers was used to introduce *att*B Gateway Cloning sites. ADAR2S and ADAR2L bands were cut out and extracted from agarose gel and cloned into pc3D destination vector[28] using Gateway Cloning (Thermo Fisher). The vector expressing human mir-376a2 was generated previously.[28] For the splicing assay, primers with *att*B cloning sites were designed to amplify a minigene that contains exons 5, 5a, and 6 of human *ADARB1* gene from HEK 293T gDNA and the PCR product was cloned into pDESTsplice destination vector[29] using Gateway Cloning. For mutagenesis, wild-type plasmids were used as templates for amplification with primers containing the desired variants. The PCR products were treated with *Dpn*I restriction enzyme (Thermo Fisher) at 37 °C for 1 h and transformed into *E. coli* DH5α or TOP10 strains.

Transient transfection

For the editing assay, HEK 293T cells were seeded on 12-well plates 24 h before transfection at ~73,500 cells/cm^2 (255,000 cells per well). 1 µg of *ADARB1* expressing plasmid and 1 µg of the editing reporter plasmid was mixed with 3.5 µl of Lipofectamine 3000 (Thermo Fisher) and used for co-transfection as per manufacturer's instructions. Cells were collected 72 h after transfection by gentle washing with PBS and divided into two tubes. The cell pellet from one

tube was used for RNA extraction with TriPure reagent (Sigma-Aldrich) whereas the other cell pellet was used for immunoblotting.

For the cycloheximide chase assay, the seeding of HEK 293T cells on 12-well plates was performed as for the editing assay. 1 µg of *ADARB1*-expressing plasmid and 2.5 µl of Lipofectamine 3000 were used for transfection as per manufacturer's instructions. Twenty-four hours post-transfection, cells were either treated with 70 µg/ml of cycloheximide (Sigma-Aldrich) for indicated times or left untreated. Cells were collected 48 h after the transfection and the cell pellet was used for immunoblotting. The densitometric analysis of immunoblots was performed with Image Studio Lite program (LI-COR).

For the splicing assay, SH-SY5Y cells and HeLa cells were seeded on 6-well plates 24 h before transfection at ~42,100 cells/cm^2 (400,000 cells per well) or at ~29,500 cells/cm^2 (280,000 cells per well), respectively. 2 µg of *ADARB1* minigene plasmid with 3 µl (HeLa) or 4 µl (SH-SY5Y) of Lipofectamine 3000 were used for transfection as per manufacturer's instructions. Cells were collected 24 h after transfection by adding 750 µl of TriPure reagent directly to plates.

Immunofluorescence

HeLa cells were seeded on coverslips coated with 0.2% gelatin in 24-well plates at ~26,800 cells/cm^2 (50,000 cells per well). The cells were transfected at the time of seeding with 500 ng of *ADARB1*-expressing plasmid and 0.75 µl of Lipofectamine 3000 per well as per manufacturer's instructions. All the following steps were performed at room temperature. After 24 h, the coverslips were briefly rinsed in PBS, fixed in 3.7% formaldehyde (diluted in PBS) for 10 min, and quenched with 50 mM NH_4Cl (diluted in PBS) for 5 min. The cells were then washed with PBS and permeabilised with 0.2% Triton X-100 for 5 min. The coverslips were washed again with PBS and blocked with blocking solution (1% BSA + 0.05% Tween 20) for 60 min. Staining was performed with rabbit anti-FLAG polyclonal antibody (F7425, Sigma-Aldrich) diluted 1:800 in blocking solution for 60 min. After the staining, the coverslips were

washed 3x in PBST and stained with Alexa Fluor 568 goat anti-rabbit IgG (H+L) polyclonal secondary antibody (A-11011, Thermo Fisher) diluted 1:200 in PBS with 0.3 µg/ml DAPI for 60 min. The coverslips were then washed 2x in PBST and 1x in PBS, rinsed with MQ water and mounted in Mowiol 4-88 mounting medium (EMD Millipore). Samples were analysed with inverted microscope Zeiss Axio Observer.Z1 with confocal unit LSM 800 or upright microscope Zeiss AxioImager.Z2.

Immunoblotting

The cell pellet was resuspended in 15 µl of Lysis Buffer (10 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 0.1 M NaCl, 2% SDS) with 1x cOmplete™ Protease Inhibitor (Roche) added and lysed for 30 min at 4°C. Whole cell lysate was pelleted by centrifugation and the clear supernatant used for immunoblotting. Lysates were boiled for 5 min in Laemmli buffer prior to electrophoretic separation on 8% or 10% SDS polyacrylamide gel and blotting on a nitrocellulose membrane. Antibodies used for immunoblots are listed in Supplemental Data.

RNA extraction and reverse transcription

Total RNA was isolated with TriPure reagent according to manufacturer's instructions. RNA samples were treated with 10 U of DNase I (Roche) per 50 µg of total RNA and then purified by phenol-chloroform extraction and ethanol precipitation with sodium acetate. A 2.0-2.5 µg aliquot of DNase-treated total RNA was reverse transcribed into cDNA with RevertAid RT Kit (Thermo Fisher) according to manufacturer's instructions with 2.5 µM of oligo$(dT)_{18}$ (fibroblasts) or with 0.25 µM of both GAPDH Rv and Spl2 (SH-SY5Y and HeLa) used as primers.

Real-time PCR

All quantitative PCR experiments were performed with a LightCycler® 480 Instrument II (Roche). PCR was performed with LightCycler® 480 SYBR Green I Master (Roche) with the

following cycling conditions: 1 cycle of 95 °C for 10 min, then 45 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. Gene-specific primers used for PCR amplification are summarised in Table S1. All samples were tested in technical duplicates and normalised to *GAPDH*. The relative expression of target genes was calculated with the $2^{-\Delta\Delta CT}$ method.[30] Biological quadruplicates were used to calculate the mean and standard deviation. Statistical significance of differences between samples was determined by two-tailed *t*-tests using Microsoft Excel.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplemental data.

RESULTS

Clinical phenotypic characterization

Affected individuals were microcephalic at birth (Individual 1) or developed postnatal microcephaly ranging from -3.6 to -4.0 SD, experienced seizures and severe global developmental delay or intellectual disability. The affected children do not share a specific facial gestalt (Figure 1A-E). All four had severe feeding difficulties and two required percutaneous endoscopic gastrostomy feeds. Neuroimaging of Individuals 1-4 demonstrated various non-specific abnormalities including thinning of the corpus callosum, delayed myelination, and cerebral atrophy, but no calcifications (Figure 1F-K). No cutaneous abnormalities such as chilblains were identified in any of the affected individuals at their most recent evaluations. Salient features are described here while more detailed descriptions are summarized in Table 1 and Supplemental Data.

Individual 1 is one of two children to healthy non-consanguineous Australian parents of European descent. Developmental delay and hypotonia were present from infancy. His head circumference at birth was 30.5 cm (-2.2 SD) but this worsened in infancy such that it now tracks at -3.4 SD. At age 4 ½ years, he needs help to stand and is unable to walk independently. He has limited intelligible speech but vocalizes and understands some sign language. At age 5 ½ years he had his first generalized tonic-clonic seizure and also has frequent staring spells not considered to be absence episodes. His EEG showed a slow and less well-modulated background for age, but no epileptiform activity.

The parents of Individual 2 are healthy non-consanguineous individuals of Hispanic descent. Individual 2 experienced infantile-onset focal clonic and tonic-clonic seizures that were intractable to multiple therapies, including phenobarbital, levetiracetam, oxcarbazepine, topiramate, pyridoxine, pyridoxal-5-phosphate, phenytoin, lacosamide, clonazepam, clobazam, rufinamide, valproic acid, felbamate, the ketogenic diet, quinidine, and vagal nerve stimulation. Individual 2 died at the age of two years due to epilepsy. Prior to his death, he was profoundly delayed. He was unable to visually track objects, roll over or sit, and he never achieved head control or meaningful language. His neurological examination was remarkable for microcephaly with plagiocephaly, severe hypotonia, cortical blindness, and limited voluntary movement. Individual 2's seizure semiology and EEG were consistent with epilepsy of infancy with migrating focal seizures (Figure 1L-M).

Individual 3 is a 2-year-old boy, born to consanguineous parents, with severe developmental delay, and intractable seizures that began at age 4 months at which time antiepileptic treatment was initiated (levetiracetam, phenobarbital, topiramate, pyridoxine and a ketogenic diet). EEG at the age of 8 months was reported with epileptic foci in the left temporal and occipital regions, but a sleep EEG at 18 months recorded no epileptic activity. At age 2 years, Individual 3 had

not achieved any developmental milestones; he was inactive, poorly responsive, made no eye contact, and did not interact with his surroundings.

Individual 4 is an 11-year-old boy born to first cousin Azari parents. Developmental delay was noted in the first months of life and generalized intractable seizures began at age 7 months with developmental stagnation thereafter. EEG at the age of 9 years demonstrated both focal and generalized epileptiform discharges. At age 11 years, he has failure to thrive, feeding difficulties, profound intellectual disability, microcephaly and ongoing generalized tonic-clonic seizures treated with carbamazepine. He is non-verbal, non-ambulatory, does not visually fix, and has central hypotonia with peripheral hypertonia. He has repetitive movements of his left hand and neck.

Exome sequencing results

All four individuals were found to have biallelic missense variants in *ADARB1* (see Table 2 for variant details). All clinically unaffected parents and siblings are either heterozygous carriers or homozygous for the reference allele.

Four of the five variants reported here are observed in heterozygous state at low allele frequencies, and none were found in homozygous state in the gnomAD or epi25 databases (accessed 24 September 2019). In the epi25 database, we did not find any individual compound heterozygous for any combination of the five variants from this cohort. The p.(Lys127Glu) variant is not listed in these databases; only a synonymous variant (rs1210305864) that affects the same codon has been observed previously. No other non-synonymous variants affecting Lys127, Lys367, Thr498, Arg603, or Ala722 were reported.

The location and conservation of *ADARB1* variants

Four of the five *ADARB1* variants identified in the individuals in this cohort are situated in or around the deaminase domain and one is situated in dsRBD1 (Fig. 2A). The variant in dsRBD1, p.(Lys127Glu), affects a lysine residue that contacts the sugar-phosphate backbone of dsRNA (Fig. 2B).[31] The p.(Lys367Asn) variant is located in strand β2 of the deaminase domain, but the affected lysine lies at the surface of the protein and does not appear to make any contact with other residues in the deaminase domain (Fig. 2C). The p.(Thr498Ala) variant is located in the 40-amino-acid-long in-frame insertion in the deaminase domain that is incorporated only in the longer protein isoform ADAR2L. This insertion is encoded by exon 5a and it extends the RNA-binding loop between strands β5 and β6.[32] The p.(Arg603Gln) variant is situated near the C-terminal part of helix α7 of the deaminase domain. Arg603 makes contacts with the Cα carbonyls of Gly518 and Ala599 and with the side chain of Asp558 (Fig. 2D). The p.(Ala722Val) variant lies at the surface of the protein and affects the Ccap residue of a Schellman loop at the C-terminal end of helix α10 (Fig. 2E).[33]

The importance and conservation of the Lys127 residue from dsRBD1 has been previously established.[34] The multiple sequence alignment of ADAR family proteins reveals that Lys367 is conserved in mammalian ADAR2 and in dADAR from *Drosophila melanogaster*. On the other hand, Arg603 is highly conserved across the whole ADAR family (Fig. 2F). Moreover, Arg603 might be important for deaminase-domain-containing proteins in general, as the alignment of various human and mouse deaminases also confirms its conservation, and Asp558 which makes contacts with Arg603 is also conserved (Fig. 2G). However, it should be noted that we have lower confidence in the fidelity of the alignment in Fig. 2G because the sequence around Arg603 is not as conserved as in other highly conserved regions (e.g. Arg562 and Lys669 that interact with IP6), and because of the presence of gaps in the aligned ADAD1 sequence, both of which allow multiple alignment possibilities. Ala722 is highly conserved in ADAR2 and ADAR3 except for *Xenopus tropicalis* and *Takifugu rubripes* (Fig. 2F). Since exon

5a, where the Thr498 residue is located, originates from a primate-specific *Alu* element inserted in *ADARB1* sequence, most of the sequences have a gap in the alignment at this position (Fig. S1).

The ADAR2 variants p.(Lys127Glu), p.(Lys367Asn), p.(Arg603Gln), and p.(Ala722Val) result in decreased editing activity of ADAR2

To test the initial hypothesis that the *ADARB1* variants would cause a decrease in editing efficiency, we employed a cellular editing assay with a plasmid expressing human mir-376a2.[28; 35] A decrease in editing efficiency could indicate that the *GRIA2* Q/R site is not 100% edited in the brains of affected individuals.

Endogenous ADAR2 from HEK 293T cells has a very low editing activity which allows performing editing assays on this cell line with transiently expressed ADAR2 proteins.[28; 36] Plasmids expressing FLAG-tagged ADAR2 mutant or wild-type enzyme were co-transfected into HEK 293T cells with the microRNA expressing plasmid and after 3 days RNA was extracted from the cells. To ensure ADAR2 protein levels were approximately the same in all samples, we used whole cell lysates for immunoblotting and probed them with anti-FLAG antibody (Fig. 3D, S2). RNA was treated with DNase prior to RT-PCR and PCR products were sequenced. The editing efficiency was assessed by calculating the ratio of the guanosine peak height to the sum of adenosine and guanosine peak heights (G/(A+G)) at the editing site +4 of mir-376a2. The peak heights of the chromatograms at this editing site were measured with QSVanalyser program.

As expected, four out of five variants caused a decrease in editing activity of ADAR2 (Fig. 3A, B). The p.(Lys127Glu), p.(Lys367Asn), and p.(Ala722Val) variants caused a mild decrease in editing activity (14.6%, 10.9%, and 4.4% in ADAR2S, respectively), whereas the

p.(Arg603Gln) variant resulted in a severe drop in editing activity (>85% decrease). Note that the severely reduced editing activity of the p.(Arg603Gln) variant is indistinguishable from background editing in untransfected HEK 293T cells. The effect of these variants on editing activity (compared to wild-type protein) was assessed, and the same trend can be observed, in both ADAR2S and ADAR2L isoform, except for the p.(Thr498Ala) variant which is only present in the ADAR2L isoform. The decrease in editing efficiency related to the p.(Lys127Glu) and p.(Lys367Asn) variants was more pronounced in the ADAR2S isoform than in the ADAR2L isoform. These results were further validated with an editing assay employing a plasmid expressing the murine *Gria2* Q/R site as a substrate (Fig. 3C, S2).

The immunoblots demonstrate that the ADAR2 p.(Arg603Gln) recombinant protein attains low protein levels in transiently transfected cells (Fig. 3D); such low protein levels were not observed with other ADAR2 variants (Fig. S2). Furthermore, when protein synthesis was inhibited by cycloheximide, ADAR2 p.(Arg603Gln) protein levels decreased more rapidly compared to ADAR2 wild-type (Fig. 3E). These results indicated that the p.(Arg603Gln) variant affects the stability of ADAR2 protein.

Contrary to our expectations, the editing activity of the p.(Thr498Ala) mutant was not decreased (Fig. 3B). Since ADAR2 forms a homodimer,[6; 7] we investigated the effect of the combination of p.(Lys367Asn) and p.(Thr498Ala) variants as was seen in Individual 1. To accomplish this, plasmids expressing ADAR2L mutants p.(Lys367Asn) and p.(Thr498Ala) were mixed in a 1:1 ratio and used in the same editing assay as above. The results again showed no decrease in editing activity compared to ADAR2L wild-type protein (Fig. 3B). The results of the editing assay suggested that decreased editing activity may contribute to the phenotype, but only for four of the five variants, which led us to further investigate the effects of the p.(Thr498Ala) variant.

The ADAR2 variants properly localise to the nucleoli

In the event that mislocalization of the ADAR2 mutants could contribute to the dysregulation of ADAR2, we analysed the subcellular localisation of all five ADAR2 variants. In this regard, the p.(Lys127Glu) variant is particularly interesting as it affects a critical lysine residue of dsRBD1. The mutation of this lysine to alanine in homologous domains of both human PKR and *Drosophila* Staufen was shown to result in a loss of dsRNA binding.[37; 38] The RNA binding activity of ADAR2 was similarly affected when residues Lys127 from dsRBD1 and Lys281 from dsRBD2 were both mutated to alanines.[39] Binding of dsRNA is required for nucleolar localization of ADAR2,[39] so we were interested to see if the p.(Lys127Glu) variant would cause mislocalization of ADAR2.

HeLa cells were transiently transfected with plasmids expressing FLAG-tagged ADAR2 wild-type or mutant proteins, and indirect immunofluorescence with anti-FLAG antibody and DAPI staining was performed. The results show that all the tested variants in both ADAR2S and ADAR2L are localised in the nucleus (Fig. 3F). Moreover, all the mutants are enriched in the nucleoli as previously reported for endogenous ADAR2.[39; 40]

The c.1492A>G variant alters splicing of exon 5a

Given the location of the c.1492A>G (p.(Thr498Ala)) variant in the alternatively spliced exon 5a (Fig. 4A), we investigated its impact on splicing. A minigene containing *ADARB1* exon 5a, the surrounding exons 5 and 6, and parts of the flanking introns was cloned into pDESTsplice[29] splicing reporter plasmid (Fig. 4A). This construct was then transiently transfected into SH-SY5Y neuroblastoma cells and HeLa cells. These two cell lines were selected because they originate from different tissues, and we wanted to take into account the possible variability in the expression of splicing factors in different cell lines. Twenty-four hours after the transfection,

total RNA was isolated, DNase treated, and RT-PCR was performed to assess splicing of exon 5a with two different primer pairs (Fig. 4A).

No difference in PCR band patterns between the wild-type and mutant minigene reporters was observed, indicating that the recognition of the 5' splice site downstream from the c.1492A>G variant is not affected. However, the relative ratio of long (with exon 5a) and short (without exon 5a) PCR products, as measured by quantification of PCR bands on agarose gel, is shifted towards the short product in the mutant minigene reporter compared to the wild-type minigene reporter (Fig. 4B-D). The same trend was observed in SH-SY5Y and HeLa cells with both primer pairs. The relative decrease of mRNA containing exon 5a compared to mRNA without exon 5a in mutant minigene reporter was also confirmed by qPCR (Fig. 4B, C). Thus, an effect of the c.1492A>G variant on alternative splicing of exon 5a was observed in the transfection experiment.

To check whether the c.1492A>G variant lies in an exonic splicing enhancer (ESE) or silencer region, we performed an *in silico* analysis using online algorithms that predict which sequences have potential to bind splicing activators or repressors. We first tested the wildtype and c.1492A>G mutant sequence of exon 5a with EX-SKIP and HOT-SKIP,[41] tools that integrate several algorithms to determine which exonic variant has the highest chance to result in exon skipping. Both of these tools predict that the wildtype and c.1492A>G mutant exon 5a have comparable chance of exon skipping. Next, we used ESEfinder 3.0[42; 43] and SpliceAid 2[44] to look for differential binding of splicing activators and repressors. ESEfinder 3.0 predicts that the c.1492A>G variant creates a potential binding site for splicing factor SRSF1 (also known as SF2), whereas SpliceAid 2 predicts that the variant abolishes potential binding sites for NOVA1, NOVA2, and TRA2B (also known as Htra2-β1 or SFRS10) (Fig. S3).

RT-qPCR confirms an increase in ADAR2S mRNA and a decrease in the inclusion of exon 5a in fibroblasts derived from Individual 1

Fibroblasts derived from Individual 1 were used to measure *ADARB1* mRNA expression by RT-qPCR. There was a very small increase in both total mRNA level and ADAR2S mRNA level compared to control fibroblasts that were derived from an age- and sex-matched control. No change was observed in ADAR2L mRNA level (Fig. 5A). Furthermore, ADAR2 protein levels from control fibroblasts and Individual 1's fibroblasts were assessed by immunoblot with anti-ADAR2 antibody, which demonstrated the same small increase in ADAR2 protein levels in Individual 1's fibroblasts (Fig. 5B). The effect of the c.1492A>G variant on *ADARB1* mRNA splicing was also investigated by quantification of PCR products on agarose gels which again revealed a decrease in exon 5a inclusion in Individual 1's fibroblasts compared to control fibroblasts (Fig. 5C). This experiment confirms the observation made with the splicing reporter. An attempt was made to directly investigate editing activity of endogenous ADAR2 in these fibroblasts; however, the deaminase activity of ADAR2 in fibroblasts is very low. In addition, the transcript encoding the glutamate receptor subunit *GRIA2* is not expressed in fibroblasts, and transient transfection of plasmid expressing murine *Gria2* Q/R site did not lead to detectable editing in these cells (Fig. S4).

DISCUSSION

Herein we report human neurological disease associated with biallelic variants in *ADARB1*. The clinical phenotype comprises microcephaly, severe to profound developmental delay, hypotonia, epilepsy that is largely intractable and in one individual is consistent clinically and electrographically with epilepsy of infancy with migrating focal seizures (EIMFS). Functional assays of four variants (p.(Lys127Glu), p.(Lys367Asn), p.(Arg603Gln), and p.(Ala722Val)) demonstrate impairments in RNA editing ability of the resultant ADAR2 proteins, which is

hypothesized to lead to increased Ca^{2+} permeability of AMPA receptors. Another variant c.1492A>G (p.(Thr498Ala)) leads to a change in alternative splicing of the *ADARB1* gene to exclude exon 5a. These functional data support the pathogenic role of the *ADARB1* variants in the clinical phenotype observed in this cohort.

Diverse genes are involved in the pathogenesis of epileptic encephalopathies of infancy, with many of them encoding proteins involved in synaptic functions and ion channels. Adding to this complexity, variants in the same gene can manifest as different epileptic syndromes (for review see [45]). The epilepsy observed in Individuals 2-4 is clinically consistent with an intractable epileptic encephalopathy, and the electrographic phenotype of Individual 2 is consistent with epilepsy of infancy with migrating focal seizures (EIMFS). Additional observations are needed to confirm *ADARB1* as a candidate for this intractable epilepsy syndrome alongside other causative genes.[46-58] More broadly, these data implicate *ADARB1* as a genetic etiology for early-onset epileptic encephalopathy, though additional, milder epilepsy phenotypes may be possible.

The effects of ADAR2 on brain function have been comprehensively studied in mouse models.[59-61] Mice with total *Adar2* knockout have seizures and die within 3 weeks, but mice with single-allele *Adar2* knockout are phenotypically normal,[59] suggesting that a single functional allele of *Adar2* is sufficient for *Gria2* Q/R site editing, and supporting the recessive inheritance pattern of *ADARB1* variants observed in this cohort. Furthermore, the importance of *GRIA2* Q/R site editing for proper neuronal development was highlighted by a recent study reporting an individual with a *de novo* heterozygous variant p.(Gln607Glu) affecting the Q/R site of GRIA2.[22] This individual had an intractable epileptic encephalopathy with severe intellectual disability and limited functional capacity, similar to the phenotype we observed in Individuals 2-4 in this cohort. Functional studies of the GRIA2 p.(Gln607Glu) variant demonstrated an increased current amplitude, which was hypothesized to explain the epilepsy in this individual.

Because impaired Q/R site editing by ADAR2 variants in this cohort would be expected to result in increased AMPA receptor current as well, the phenotypic similarities among these individuals strengthen the conclusion that *ADARB1* variants cause the phenotype observed in this cohort.

The editing assay shows a mild decrease of the editing activity of the p.(Lys127Glu) variant located within the dsRBD1. Data from NMR of the dsRBDs of ADAR2 bound to dsRNA reveals that this amino acid makes non-sequence specific contacts with dsRNA.[31] It is part of a highly conserved motif present in dsRBDs, and when this entire motif is mutated, the binding to dsRNA is abolished.[38; 62] Nucleolar localization of ADAR2 depends on binding to dsRNA,[39] but the p.(Lys127Glu) variant alone does not impair the proper localization of ADAR2 (Fig. 3F). The unaffected dsRBD2 of the p.(Lys127Glu) variant probably compensates for its functional effect. However, based on the NMR structure of the dsRBD bound to dsRNA it is highly likely that the ability of the p.(Lys127Glu) variant to bind dsRNA is diminished. It should be noted that other RNA-binding proteins can compete with ADAR2 p.(Lys127Glu) for its natural targets, the full effect of which cannot be assessed in the *in vitro* assay since these proteins are not produced in HEK 293T cells (such as ADAR3). As RNA-binding proteins modulate the editing activity of ADARs,[63-66] we hypothesize that the *in vivo* effects of the p.(Lys127Glu) variant may be broader than what is reflected in the mild decrease of editing activity *in vitro*.

The p.(Lys367Asn) variant has slightly decreased editing activity. Based on the published structure of the ADAR2 deaminase domain (PDB ID: 5ED1),[32] the p.(Lys367Asn) variant lies at the surface of the protein on the side facing away from the edited RNA (Fig. 2C). The affected residue is therefore accessible for potential protein-protein interactions, several of which have been reported.[64; 67-72] ADAR2 forms a homodimer,[3-7] but the residues involved in dimerization remain unknown. Further investigation of the ADAR2 interactome and the mechanism of

ADAR2 dimerization is required to determine whether some of these interactions could be impaired by the p.(Lys367Asn) variant.

The lack of knowledge about the structural role or the function of the 40-amino-acid-long ADAR2L-specific extension complicates the assessment of the effects of the p.(Thr498Ala) variant. We did not observe any decrease of the p.(Thr498Ala) mutant's editing activity; however, we cannot exclude the possibility that this variant might affect the editing of other targets. Since Individual 1 is compound heterozygous for two variants in *ADARB1*, we also tested the editing activity of the p.(Thr498Ala) mutant in a co-transfection experiment with the p.(Lys367Asn) mutant and again observed no decrease in editing activity. Crystallographic studies of ADAR2S revealed that the region where the ADAR2L extension is located is disordered in the RNA-free state[73] but becomes ordered upon RNA binding.[32] The results of the splicing assay demonstrate that the c.1492A>G (p.(Thr498Ala)) variant has a small effect on the ratio of ADAR2S and ADAR2L splicing isoforms. The *in silico* analysis we performed highlights the possibility of differential binding of splicing regulators (SRSF1, NOVA1, NOVA2 and TRA2B) caused by the c.1492A>G variant. Others have shown that the co-transfection of a vector overexpressing SRSF1 with an ADAR2 wild-type minigene has no apparent effect on exon 5a inclusion.[74] TRA2B predominantly promotes exon inclusion by binding to ESE.[75; 76] This is in line with the *in silico* analysis which predicts that binding of TRA2B is impeded by the c.1492A>G variant. However, whether any of the proteins predicted to bind the affected sequence has a role in the alternative splicing of *ADARB1* exon 5a remains to be established.

Interestingly, we observed a change in the alternative splicing of the c.1492A>G mutant in favor of the more active ADAR2S isoform, suggesting that A-to-I editing would increase. However, without assessing alternative splicing of the c.1492A>G variant in developing

neurons directly, we cannot draw a definitive conclusion about the effects of the c.1492A>G variant on alternative splicing in the human brain.

Notably, Individual 1, who is compound heterozygous for the p.(Lys367Asn) and p.(Thr498Ala) variants, has a milder phenotype than the other individuals in this cohort. We hypothesize that this milder phenotype is related to an impact on an ADAR2 function that is editing independent, separate from its role in site-specific mRNA editing. Perhaps the alternative splicing seen in these assays disrupts critical protein-protein or protein-RNA interactions with other binding partners, modifying other downstream targets important for neuronal function. Future studies on the editing-independent roles of ADAR2 will be important in elucidating these potential mechanisms. Ongoing clinical follow-up of Individual 1 will be vital in assessing for evolution of his seizure phenotype or other neurological abnormalities. Reporting of other affected individuals with milder phenotypes associated with biallelic *ADARB1* variants will assist in drawing further genotype-phenotype correlations.

The results of the cycloheximide chase assay indicate that the p.(Arg603Gln) variant leads to faster degradation of ADAR2 protein (Fig. 3E). The severe effects of this variant can be explained by examining the structure of the ADAR2 deaminase domain (PDB ID: 5ED1).[32] Arg603 interacts with Gly518 situated at the C-terminal end of the RNA-binding loop and the Ala599 situated in helix α7 of the deaminase domain (Fig. 2D). However, the most important interaction is with Asp558 situated in helix α5 of the deaminase domain. The zinc ligand residue Cys556 and IP6-interacting residues Lys559 and Arg562 are also located in helix α5. Thus, we expect that the p.(Arg603Gln) variant affects the folding and the stability of ADAR2 by altering the position of the catalytically important helix α5 and possibly by causing additional structural alterations in helix α7 and the RNA-binding loop.

The p.(Ala722Val) variant has a small effect on ADAR2 editing activity. This variant affects the Ccap residue of a Schellman loop that lies at the surface of the protein close to the RNA-

facing side of ADAR2 (Fig. 2E). Alanine is one of the preferred amino acids at the Ccap position of a Schellman loop. On the other hand, valine at the Ccap was previously reported to be detrimental for the stability of the Schellman loop.[77] Besides the observed effect on the editing activity, this variant could additionally disrupt the local folding of ADAR2 protein.

In summary, these findings suggest that the *ADARB1* variants detected in this cohort have multiple deleterious effects at both the RNA and protein level leading to microcephaly, intellectual disability and epilepsy. The established importance of *GRIA2* Q/R site editing and its role in seizures in mouse models and humans leads us to hypothesize that the phenotypes observed in these individuals, particularly the epilepsy phenotype, are at least partially caused by *GRIA2* under-editing. However, we cannot exclude the possibility that other roles of ADAR2, mediated by protein-protein or protein-RNA interactions, play a role in the disease.

Description of Supplemental Data

Supplemental Data include five figures, one table, and one section detailing clinical descriptions.

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Declaration of Interests

JJ is an employee of GeneDx, Inc.

List of acronyms

ADAR	double-stranded RNA-specific adenosine deaminase
AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
DSH	dyschromatosis symmetrica hereditaria
dsRBD	double-stranded RNA-binding domain
dsRNA	double-stranded RNA
ER	endoplasmic reticulum
ESE	exonic splicing enhancer
Gria2	glutamate receptor 2
IP6	inositol hexakisphosphate
PKR	interferon-induced, double-stranded RNA-activated protein kinase
Staufen	Maternal effect protein staufen

Web resources

1000 Genomes Project (https://www.internationalgenome.org/)

CADD Combined Annotation Dependent Depletion (https://cadd.gs.washington.edu/)

Epi25 database (http://epi25.broadinstitute.org/)

EVS Exome Variant Server (https://evs.gs.washington.edu/EVS/)

Functional Analysis through Hidden Markov Models (http://fathmm.biocompute.org.uk/)

GeneMatcher (https://genematcher.org)

GERP Genomic Evolutionary Rate Profiling

(http://mendel.stanford.edu/SidowLab/downloads/gerp/)

GnomAD Genome Aggregation Database (https://gnomad.broadinstitute.org/)

Iranome (http://www.iranome.ir/)

Matchmaker Exchange (https://www.matchmakerexchange.org)

Mutation Assessor (http://mutationassessor.org/)

Mutation Taster (http://www.mutationtaster.org/)

Online Mendelian Inheritance in Man (http://www.omim.org)

PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/)

PROVEAN Protein Variation Effect Analyzer (http://provean.jcvi.org/index.php)

SIFT Sorting Intolerant from Tolerant (https://sift.bii.a-star.edu.sg/)

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Table 1. Clinical phenotype of affected individuals

Patient ID	Individual 1	Individual 2	Individual 3	Individual 4
Gender	Male	Male	Male	Male
Age at last examination	5 years 9 months	24 months (deceased)	2 years	11 years
Phenotype				
Prenatal & neonatal history	Concern about CMV infection in first trimester; but no CMV detected by PCR of amniotic fluid, neonatal blood and urine. Neonatal jaundice treated with 3d of phototherapy	c-section due to pre-eclampsia. No other complications with pregnancy or delivery. Spent 7 wks in NICU, not intubated. Had anemia requiring blood transfusion, and apnea requiring caffeine.	During the pregnancy elevated Nuchal translucency (3.8 mm) microcephaly and polyhydramnios (AFI-27 CM) were detected	No complications during pregnancy or delivery
Prenatal structural anomalies	No	No	Elevated Nuchal translucency (3.8 mm) microcephaly and polyhydramnios (AFI-27 CM)	No
Gestational age at birth	40	31	40	38
Congenital abnormalities	No	No	Yes	No
Growth (age)				
Most recent height in cm (SD)	100 cm (-1.3 SD)	81 cm (-2 SD)	76 cm (-3.6 SD)	114 cm (-4.3 SD)
Most recent weight in kg (SD)	15.5 kg (-0.8 SD)	10.95 kg (-0.9 SD)	Unknown	20 kg (-4.1 SD)
Most recent head circumference in cm (SD)	46 cm (-3.6 SD)	43.5 cm (-4.0 SD)	43 cm (-4.4 SD)	49 cm (-3.3 SD)

Length at birth in cm (SD)	51 cm (+0.5 SD)	Unknown	Unknown	50 cm (-4.3 SD)
Weight at birth in g (SD)	3430 g (+0.17 SD)	1701 g	3500 g	3600 g (+0.51 SD)
Head circumference at birth in cm (SD)	30.5 cm (-2.2 SD)	Unknown	36.5 cm (+0.38 SD)	Unknown- recalled within normal range
Development				
Motor delay	Yes	Yes	Yes	Yes
Age at walking	N/A - can stand with assistance	N/A- unable to roll over or support head	N/A	N/A
Age at first words	12 months	Nonverbal	Nonverbal	Nonverbal
Number of words at most recent evaluation	Two	None	None	None
Intellectual disability	Yes	Yes	Yes	Yes
Degree of intellectual disability	Severe	Profound	Severe	Profound
Neurologic and psychiatric features				
Neurological abnormalities	Epilepsy, global developmental delay, intermittent tremor in leg	Epilepsy, global developmental delay, diffuse hypotonia, symmetric antigravity movements of limbs	Epilepsy, global developmental delay, hypertonia with significant spasticity.	Epilepsy, global developmental delay, axial hypotonia with appendicular hypertonia and distal contractures, muscle atrophy, repetitive movements of right hand and neck
Neuroimaging	MRI (6 months): Thin corpus callosum, incomplete myelination; CT (16 months): no	MRI (22 months): Microcephaly, diffuse supratentorial volume loss, white matter gliosis, and delayed myelination	MRI (23 months): Thin corpus callosum	MRI (3 years): Brain atrophy in temporal lobes

	calcification, no sutural synostosis			
EEG findings	Slow and less well-modulated background for age, but no epileptiform activity	Multifocal epileptiform discharges	Focal epileptiform discharges in the left temporal and occipital regions	Focal and generalized epileptiform discharges
Seizure semiology	Two generalized tonic-clonic seizures	Migrating focal seizures	Intractable generalized seizures	Intractable generalized seizures started at the age of 7 with increased frequency from once a month to once every 15 days
Behavioral problems	No	No	No	No
Sleep disturbance	Frequent waking during night; early morning waking	No	No	No
Facial features	Round face with metopic ridging, brachycephaly, upslanting palpebral fissures, normal corneal reflexes, thin upper lip	Non-dysmorphic with plagiocephaly	Oval face with plagiocephaly and high arched palate	Non-dysmorphic
Miscellaneous				
Hearing	Normal	Normal	Normal	Normal
Vision	Exotropia but normal vision	Cortical blindness	Cortical blindness	Cortical blindness
Abnormality of the heart	No	No	No	No
Abnormality of the respiratory system	Laryngomalacia	Grade 1 subglottic stenosis	No	No

Abnormality of the gastrointestinal system	PEG feeds	PEG feeds	Feeding difficulties	Feeding difficulties
Abnormality of the urogenital system	No	No	Left cryptorchidism	No
Abnormality of the skin / hair / nails	High anterior hairline, sparse scalp hair; no chillblains	No	Single café au lait spot on back	No
Abnormality of the musculoskeletal system	No	No	No	No
Abnormality of the endocrine system	No	No	No	No
Abnormality of the immunological system	No	No	No	No
Family History				
Consanguinity	No	No	Yes	Yes
Miscarriages	No	Unknown	Yes-5 spontaneous early miscarriages	no
Congenital abnormalities	No	No	Mother with congenital heart defect	no
Intellectual disability	No	No	Yes (Supp Data)	no
Other	No	High cholesterol (parents)	Yes (Supp Data)	no
Previous genetic testing				
Karyotype	No	Yes -normal	No	Yes-normal
Fragile X	Yes - normal	No	No	No
Array-CGH	Yes- normal	Yes- normal	Normal	No

Gene panel testing	No	Yes - normal infantile epilepsy panel (genes tested: *ARX, CDKL5, SLC25A22, STXBP1, SPTAN1, SCN1A, GABRG2, KCNQ2, ARHGEF2, PCDH19, PNKP, SCN2A, PLCB1, GABRD, and MEF2C*)	No	No

Table 2. Characteristics of *ADARB1* variants identified by exome sequencing in affected individuals

	Individual 1		Individual 2	Individual 3	Individual 4
	Varian 1	Variant 2			
Chromosome position (hg19)	chr21:46602522 G>C	chr21:46604484 A>G	chr21:46595995 A>G	chr21:46624592 G>A	chr21:46642051 C>T
cDNA change (NM_015833.3)	c.1101G>C	c.1492A>G	c.379A>G	c.1808G>A	c.2165C>T
Amino acid change	p.(Lys367Asn)	p.(Thr498Ala)	p.(Lys127Glu)	p.(Arg603Gln)	p.(Ala722Val)
Inheritance	Paternal	Maternal	Biparental (homozygous)	Biparental (homozygous)	Biparental (homozygous)
NCBI reference SNP number	rs778818769	rs544025652	N/A	rs1364071684	rs1323703791
In silico assessments					
FATHMM	Tolerated (score 1.41)	Tolerated (score 1.63)	Damaging (score -2.33)	Damaging (score -4.34)	Damaging (score -3.35)
MutationAssessor	Low (1.385)	Neutral (-0.55)	High (4.745)	Medium (3.465)	Medium (3.11)
MutationTaster	Disease causing	Polymorphism	Disease causing	Disease causing	Disease causing
PolyPhen2	Benign (0.286)	Benign (0)	Probably damaging (1.00)	Probably damaging (1.00)	Possibly damaging (0.948)
SIFT	Damaging	Tolerated	Damaging	Damaging	Damaging
PROVEAN	Neutral	Neutral	Deleterious	Deleterious	Deleterious
CADD	23.1	0.174	25.9	33	29.3
GERP	1.53	-0.77	5.24	4.77	5.05
Disease database					
epi25	Not observed	Not observed	Not observed	Not observed	Not observed
Population databases					

EVS	Not observed	Not observed	Not observed	Not observed	Not observed
1000G	Not observed	Not observed	Not observed	Not observed	Not observed
ExAC	Not observed	1/1172 (no homozygotes)	Not observed	Not observed	Not observed
gnomAD	2/235014 ($8.5x10^{-6}$); no homozygotes	47/88638 ($5.3x10^{-4}$); no homozygotes	Not observed	1/251444 ($3.98x10^{-6}$); no homozygotes	1/245374 (4.08x10-6); no homozygotes
Iranome	Not observed	Not observed	Not observed	Not observed	Not observed
Other pathogenic or likely pathogenic variants associated with microcephaly, seizures or intellectual disability in humans	Not observed	Not observed	Not observed	Not observed	Not observed

Figure Legends

Figure 1. Clinical and neuroradiographic images of Individuals 1-3.

A: Frontal and **B:** lateral image of Individual 1 at age 10 months; note plagiocephaly

C: Frontal and **D:** lateral image of Individual 1 at 4 years 4 months; note the exotropia but general lack of dysmorphic features

E: Frontal image of Individual 3 at 2 years; note the lack of dysmorphic features

F-K: Axial T2 and T1 weighted MRI images from Individual 1 at six months of age (**F**, **G**), Individual 2 at 22 months of age (**H**, **I**) and Individual 3 at 23 months of age (**J**, **K**), demonstrating cerebral volume loss and thinning of the corpus callosum.

L, **M:** EEG of Individual 2 demonstrating migrating focal seizures. Image L demonstrates a right hemispheric electrographic seizure, while Image M taken 15 seconds later demonstrates the right hemispheric seizure ending while an independent left hemispheric seizure evolves.

Figure 2. The location and conservation of *ADARB1* variants

A: Domain organization of ADAR2 protein with locations of variants indicated. NLS stands for nuclear localization signal. **B:** Cartoon model of ADAR2 dsRBD1 (blue). Lys127 makes contact with dsRNA (wheat) (PDB ID: 2L3C). **C:** Space-filling model of the ADAR2 deaminase domain (blue) with Lys367 highlighted in red (PDB ID: 5ED1). **D:** Close-up view of helices α5 and α7 of the ADAR2 deaminase domain (blue). The contacts made between Arg603 and other residues are represented as black dashed lines. Zinc coordination and interactions with IP6 are represented as green dashed lines. Zinc is illustrated as an orange sphere, IP6 as a stick model (PDB ID: 5ED1). **E:** Stick model of the Schellman loop containing Ala722 (PDB ID: 5ED1). Hydrogen bonds are represented as black dashed lines. Only side

chains of Ala722 (left) or Val722 and Leu724 (right) are shown. PyMOL Mutagenesis Wizard was used to replace Ala722 with valine in the model. Val722 rotamer with the lowest strain is shown (right) with unfavorable van der Waals overlaps represented as colored disks. **F,G:** Multiple sequence alignments of ADAR1, ADAR2, and ADAR3 from diverse organisms (**F**) and of various proteins containing the deaminase domain from human and mouse (**G**) with locations of variants or other important residues indicated above the alignments. Secondary structural elements observed in the ADAR2 deaminase domain are indicated. Human ADAR2 sequence is highlighted.

Figure 3. Editing activity, protein stability, and subcellular localization of ADAR2 mutants

A, B: Graphs showing editing at position +4 of human mir-376a2 by ADAR2S (**A**) or ADAR2L (**B**) proteins in transiently transfected HEK 293T. **C:** Graph showing editing at mouse *Gria2* Q/R site by ADAR2L in transiently transfected HEK 293T. In panels **A**, **B**, and **C**, ratio G/(A+G) is the ratio of the guanosine peak height to the sum of adenosine and guanosine peak heights of the sequencing chromatograms. Editing levels were normalized to the editing by the wild-type protein, which is set as 100% (as indicated by a dashed line). Note that ADAR2L wild-type has lower editing efficiency (~70.6%) compared to ADAR2S wild-type (~74.5%). Data represent means ± s.d. ($n \geq 3$ independent experiments). $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). **D:** Immunoblots probed with indicated antibodies showing protein levels of FLAG-tagged ADAR2 wild-type or p.(Arg603Gln) proteins after co-transfection of HEK 293T with plasmids expressing ADAR2 and mir-376a2. Lanes with the same labels represent replicates. **E:** HEK 293T cells transfected with FLAG-tagged ADAR2 wild-type or p.(Arg603Gln) were treated with cycloheximide (70 μg/ml) for indicated times. Whole cell lysates were used for immunoblotting with indicated antibodies. Representative immunoblots

are shown. Protein levels were quantified by densitometric analysis of immunoblots. Data represent means ± s.d. (n ≥ 4 independent experiments). $p \leq 0.05$ (*), $p \leq 0.01$ (**). **F:** HeLa cells were transiently transfected with plasmids expressing the indicated FLAG-tagged proteins and analyzed by indirect immunofluorescence. Transfected cells were stained with anti-FLAG antibody (red channel). DAPI (blue channel) was used as a DNA stain. Cells with representative staining pattern were selected.

Figure 4. Splicing effects of *ADARB1* c.1492A>G variant

A: Diagram depicting part of the splicing reporter plasmid. An A-to-G variant that was found in Individual 1 is shown in red with parts of flanking sequences in black. Primers used for PCR are represented by arrows below the exons. **B-D:** The wild-type or c.1492A>G splicing reporter plasmids were transfected into SH-SY5Y or HeLa cells, total RNA was isolated, RT-PCR was performed with indicated primers, and splicing products were separated in 1% (Spl1+2) or 1.75% (Spl3+4, GAPDH) agarose gel. Exon 5a inclusion level is calculated as a percentage of the longer PCR product, 100% corresponds to the sum of the longer and shorter PCR product. +S = short product positive control; +L = long product positive control; +P = wild-type plasmid positive control; - = negative PCR control; L = DNA ladder. qPCR was also performed with RNA from transiently transfected cells. qPCR results are normalized to mRNA expression in cells transfected with wild-type plasmid. Data represent means ± s.d. (n = 4 independent experiments). $p \leq 0.05$ (*). **B:** Analysis of splicing in transiently transfected SH-SY5Y cells by quantification of PCR bands on agarose gel (left) and by RT-qPCR quantification (right). **C:** Analysis of splicing in transiently transfected HeLa cells by quantification of PCR bands on agarose gel (left) and by RT-qPCR quantification (right). **D:** Confirmation of splicing analysis results by quantification of PCR bands on agarose gel with a different pair of primers (Spl1+Spl2). Note that the slightly larger band in panels B and C that migrates above the longer

PCR product is a heterodimer of shorter and longer PCR product (verified by Sanger sequencing).

Figure 5. ADAR2 expression and splicing in fibroblasts derived from Individual 1

A: RT-qPCR analysis of short (ADAR2S), long (ADAR2L), and total ADAR2 mRNA isoforms in Individual 1-derived and control fibroblasts. Results are normalized to mRNA expression in control fibroblasts. Data represent means ± s.d. (n = 4 independent experiments). $p \leq 0.05$ (*). **B:** Immunoblots probed with indicated antibodies showing protein levels of ADAR2 in Individual 1-derived and control fibroblasts. Two total protein concentrations were loaded and both α-tubulin and Ponceau staining were used as loading controls. **C:** Total RNA from Individual 1-derived and control fibroblasts was used for RT-PCR and resolved on 1.75% agarose gel. Exon 5a inclusion level is calculated as a percentage of the longer PCR product, 100% corresponds to the sum of the longer and shorter PCR product. +S = short product positive control; +L = long product positive control; +P = wild-type plasmid positive control; - = negative PCR control; L = DNA ladder. $p \leq 0.05$ (*).