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**Splice variant in *ARX* leading to loss of C-terminal region in a boy with intellectual disability and infantile onset developmental and epileptic encephalopathy.**

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## **Abstract**

Pathogenic variants in the X-chromosome *Aristaless*-related homeobox (*ARX*) gene contribute to intellectual disability, epilepsy and associated co-morbidities in affected males. Here we report a novel splice variant in *ARX* in a family with three affected individuals. The proband had early onset developmental and epileptic encephalopathy, his brother and mother had severe and mild intellectual disability respectively. Massively parallel sequencing identified a novel c.1449-1G>C in intron 4 of the *ARX* gene, predicted to abolish the splice acceptor site, retaining intron 4 and leading to a premature termination codon immediately after exon 4. As exon 5 is the last exon of the *ARX* gene, the premature termination codon at position p.L484\* would be predicted to escape nonsense mediated mRNA decay, potentially producing at least some C-terminally truncated protein. Analysis of cDNA from patient lymphoblastoid cells confirmed retention of intron 4 and loss of detectable expression of *ARX* mRNA across exon 4 to exon 5. We review published cases of variants that lead to altered or early termination of the *ARX* protein, but not complete loss-of-function, and are associated with phenotypes of intellectual disability and infantile onset developmental and epileptic encephalopathies, including Ohtahara and West syndromes. Taken together, this novel splice variant

retaining intron 4 is likely to be the cause of the early onset developmental and epileptic encephalopathy in the proband.

**Keywords**

ARX, Intellectual disability, splice, Ohtahara syndrome, epilepsy

## **Introduction**

*Aristaless*-related homeobox (*ARX*) gene [NM\_139058.2] is frequently mutated to cause X-linked intellectual disability (XLID) with or without comorbidities including autism spectrum disorder, epilepsy, lissencephaly and ambiguous genitalia. *ARX* belongs to the paired-type homeobox transcription factor family, which is characterised by the presence of a conserved 60 amino acid DNA-binding domain called the homeodomain. Other important domains present in *ARX* include an octapeptide domain, four polyalanine tracts, three nuclear localisation signals and an *Aristaless* domain at the C-terminal end of the protein. There are more than 60 different pathogenic variants reported across *ARX* which give rise to a broad range of phenotypes, with the most common pathogenic variants being expansions of the first and second polyalanine tracts (c.304ins(GCG)<sup>7</sup> and c.429\_452dup) (reviewed in [1]).

Being a homeodomain transcription factor, *ARX* is crucial in the regulation of early embryogenesis and development. Pathogenic variants within the homeodomain region are well characterised and generally abolish binding of *ARX* to target DNA sequences [2]. There are several variants, including insertion variants in exon 5 of *ARX* that are predicted to cause frameshift variants that are pathogenic, resulting in altered or early termination of the *ARX* protein, but not complete-loss-of function [3-14]. These cases invariably lead to infantile onset developmental and epileptic encephalopathies such as Ohtahara Syndrome (also called early infantile epileptic encephalopathy) and West syndrome. Malformations or and progressive structural abnormalities are recognized on MRI scans including neuronal

migration disorders, cerebral atrophy, agenesis of the corpus callosum, and hypoplasia / atrophy of the corpus callosum and cerebral atrophy. Congenital and acquired microcephaly are also observed. The pathogenic mechanisms underlying these phenotypes are not well understood. However, the loss of exon 5 or the C-terminal region of ARX would mean the loss of the *Aristaless* domain of ARX. The exact molecular function of the *Aristaless* domain remains to be established, although a role in transcription repression has been proposed [15, 16]. Here we report a variant in *ARX* abolishing an acceptor splice site leading to retention of intron 4 and exon 5 at least in some transcripts in a boy with early onset developmental and epileptic encephalopathy.

## **Materials and Methods**

### **Phenotype of proband and family**

A 2 year 4 month old boy (II-2 on Figure 1A) presented at age four weeks with focal tonic and tonic-clonic seizures. Seizures lasted 20-40 seconds, and escalated in frequency over two weeks, with multiple seizures occurring per day. Infrequent focal tonic seizures occurred from two to seven months and then settled. Seizures recurred when topiramate was weaned at 2 years 1 month.

Mild hypotonia was present prior to seizure onset, and developmental plateauing accompanied early seizures. Visual regard, fixing and following emerged at eight weeks. Vision and fine motor development were normal. Gross motor development was mildly delayed with rolling at six months, commando-crawling at 12 months, and walking

independently at 19 months. Expressive and receptive language and social skills were delayed. Vocalization and monosyllabic babble began at 12 months. At 2 years 4 months, he still had no words and no protoimperative pointing. He met diagnostic criteria for Autism Spectrum Disorder.

Growth parameters were normal with head circumference following the 25-50<sup>th</sup> centile. There was mild plagiocephaly and genitalia were normal. Neurological examination was normal.

Interictal EEGs at four and five weeks of age were normal. By six and seven weeks, EEGs showed posteriorly predominant sharp-, spike- and polyspike-wave discharges. EEG at ten months of age was normal. MRI brain was only performed at one month and showed a thin hypoplastic corpus callosum, confirmed on cranial ultrasound, without cerebral atrophy or cortical malformation (Figure 1B). Chromosomal microarray, urine metabolic and paired blood/CSF metabolic investigations were normal.

At five weeks, he was treated with Levetiracetam, with Topiramate added after six weeks. Levetiracetam was successfully weaned at 12 months. Topiramate withdrawal was attempted at two years and one month, however breakthrough seizures occurred two weeks after cessation. Reinstitution of topiramate monotherapy has been successful. The electroclinical features are consistent with early onset developmental and epileptic encephalopathy.

His six year-old brother (II-1 on Figure 1A) began ambulation later at 22 months, fulfilled autism spectrum disorder diagnosis aged 4 years, has severe intellectual disability, and remains non-verbal and without epilepsy at 6 years of age. There are no dysmorphic features or abnormal genitalia. Neuroimaging has not been performed. There is one younger brother aged 11 months who is developing normally (II-3 on Figure 1A). The boys' mother has mild intellectual disability, without epilepsy (I-2 Figure 1A). She completed grade 9 schooling and has been employed in service industries. Neuroimaging has not been performed.

#### **Molecular analysis of the ARX variant**

The study was approved by the University of Tasmania Human Research Ethics Committee and written informed consent was obtained from the boys' parents. Genomic DNA was extracted from whole blood using standard techniques. Massively parallel sequencing (MPS) was performed using the TruSight One panel (FC-141-1007) on an Illumina NextSeq550 at the Department of Molecular Genetics at The Children's Hospital at Westmead, NSW, Australia. Target regions of interest were restricted to coding regions and the canonical splice sites. Target rate indicates percentage of bases with a minimum depth of coverage of 20x. Alignments and variant calls were generated using SoftGenetics NextGene (version 2.4.1) to the February 2009 human genome assembly (GRCh37/hg19) and variant calls were limited to the requested panel of genes. Variants identified are classified according to the American College of Medical Genetics (ACMG) criteria [17].

Variants identified are accessed using Alamut Batch (version 1.4.3) and only variants with an allele frequency of <0.1% for dominant disorders, or <1% for recessive disorder are reported. Sanger sequencing (performed at AGRF, Westmead laboratory) was used to confirm the variant.

### **Quantitative real-time polymerase chain reaction**

For cDNA expression analysis, total RNA was extracted from lymphoblastoid cell lines (LCL) using TRIzol<sup>®</sup> (Life Technologies) and RNeasy Mini Kit (Qiagen) and treated with DNase I (Qiagen) following the manufacturer's instruction. Routinely, 2 µg of total RNA was primed with random hexanucleotides and then subjected to reverse transcription with SuperScript III reverse transcriptase (Life Technologies). Validation of the reaction was done by PCR using primers specific to the ubiquitously expressed *EsteraseD*.

## **Results**

### **Molecular analysis of an intronic variant in *ARX* leads to altered splicing and predicted c-terminal truncation of *ARX*.**

Massively parallel sequencing of an Epileptic Encephalopathy panel (V2.2, 80 gene) of proband (II-2 on Figure 1A) DNA demonstrated a novel nucleotide change, ChrX(GRCh37)g:25023028G>C, c.1449-1G>C in intron 4 of the *ARX* gene (NM\_139058.2). Segregation analysis showed that the proband's mother (I-2), and brother (II-1) also have the same variant (Figure 1A). The *ARX* variant is an intronic single



nucleotide substitution that is predicted to abolish the consensus splice acceptor site at c.1449 at the end of intron 4 (Figure 1C). *In silico* analysis indicates this may lead to retention of the 2,201-nucleotide intron 4. This in turn would lead to a translated protein that would contain a premature termination codon (PTC) immediately at the end of exon 4. As exon 5 is the last exon of the *ARX* gene, the PTC at position p.L484\* would be predicted to escape nonsense mediated decay, potentially producing (some) c-terminally truncated protein.

The level of *ARX* mRNA expression from lymphoblastoid cells (LCLs) is very low, but detectable. Amplification of *ARX* mRNA with primers spanning exons 4-5 showed a single product at ~479bp in LCLs from unaffected controls (control 1 and control 2) (Figure 2A). There was no product of this size detected in the patient sample even when an increasing amount of cDNA template was used (patient II-2) or repeat cDNA stocks were generated and analysed (Patient (repeat) in Figure 2A). To demonstrate that the loss of this product was not due to the quality of the patient cDNA, amplification across exons 5-8 of another X-chromosome gene, *IQSEC2*, yielded the expected band at ~ 691bp in all samples tested. Moreover, the level of expression of *IQSEC2* in the proband was similar to the expression in the two control samples (Figure 2B). To establish if intron 4 is retained due to the splice variant in patient II-2, a forward primer binding within intron 4 and a reverse primer binding within exon 5 amplified a weak but detectable band at the predicted size of ~347bp in the patient sample, but as expected no product was detected in the control samples (Figure 2C). We confirmed that intron 4 – exon 5 sequence of *ARX* was present in this PCR amplicon from the patient cDNA via Sanger sequencing (Figure 2D). As the

splice pathogenic variant occurs between exon 4 and exon 5, we predict that some mRNA expression across earlier exons may also be detectable. Primers amplifying across exon 1 and 2 in the *ARX* transcript clearly show that although the level of expression in LCLs is weak, there is a detectable band at the predicted 185bp in both the control samples and the patient samples (Figure 2E). Taken together, we demonstrate that *ARX* is transcribed in the affected patient, but due to retaining intron 4 and introduction of a predicted premature termination codon, that there is no detectable expression of full-length wildtype *ARX mRNA*, at least in LCLs generated from blood. It is unclear to what extent this altered splicing impacts on protein expression in patients, as *ARX* is not expressed adequately in available patient material.

### **Discussion**

A novel variant within intron 4 of the *ARX* gene was identified in a proband with early onset developmental and epileptic encephalopathy, and his brother and mother with severe and mild intellectual disability, respectively. The variant abolishes the splice acceptor site, producing an aberrantly spliced transcript retaining intron 4, leading to a premature termination codon (PTC) introduced into the spliced mRNA product. Generally, unnatural splice sites introducing a PTC into mRNA lead to non-sense mediated decay (NMD) or production of a truncated protein lacking functional domains that are encoded in subsequent exons. The variant in this case occurs within the last intron-exon junction of the *ARX* gene. Hence, even though there is a predicted PTC introduced at p.L484\* of the normally 562 amino acid protein, the resulting mRNA is likely to escape NMD and is

predicted to instead produce a C-terminally truncated protein, lacking the *Aristaless* domain.

There are only two other cases of splice variants in *ARX* reported to contribute to disease [18]. Both lead to skipping of earlier exons, and the subsequent loss of *ARX* function as demonstrated by the severe phenotype of X-linked lissencephaly with abnormal genitalia (XLAG). However, the patient and family reported here do not present with this malformation phenotype associated with the complete loss of *ARX* function. Instead, the phenotype of the proband resembles other cases in which variants lead to altered or early termination of the *ARX* protein, but not complete loss-of-function. The phenotype of patient II-2 falls within the spectrum of infantile developmental and epileptic encephalopathies associated with *ARX*, including Ohtahara syndrome and West syndrome. His presentation is more in keeping with an early onset (defined as less than three months) developmental and epileptic encephalopathy, as he did not have the burst-suppression EEG signature of Ohtahara syndrome. All reported cases of pathogenic variants in *ARX* that lead to phenotypes of seizure onset prior to three months are captured in Table 1 [3-14]. Interestingly, several insertion variants in exon 5 of *ARX* that are predicted to cause altered or early termination of the *ARX* protein lead to severe and early onset epilepsy syndromes rather than the brain malformation / XLAG outcomes associated with complete loss-of-function variants (Table 1). These variants are generally in affected males, but there are two reports in affected females [4, 8], both with Ohtahara syndrome phenotypes.

In keeping with these other reported cases, our experimental work indicates that the splice variant leads to retention of intron 4 with a subsequent frameshift and PTC, predicted to truncate the ARX protein. Our data from cDNA generated from patient lymphoblastoid cells indicates that this variant contributes to the *ARX* mRNA transcript being altered, abolishing the acceptor splice site. As the levels of ARX expression are very low in LCLs, we cannot rule out that in tissues of higher *ARX* expression levels, such as the brain, that some normally spliced *ARX* mRNA may also be transcribed, leading to a full length and fully functional protein. This may account for the overall less severe clinical phenotype observed in our patient who could walk although speech acquisition was clearly delayed.

A common finding across many of the patients listed on Table 1 is the lack of the C-terminal region of the ARX protein. This region contains the *Aristaless* domain, and is a characteristic feature also found in as many as 16 members within the subgroup of paired-class homeoproteins [16]. The exact molecular function of the *Aristaless* domain is still enigmatic, although a role in transcription repression and activation has been proposed [15]. As well as functioning as an activator domain, the *Aristaless* domain likely facilitates interaction with cofactor proteins. Future work is warranted to dissect how this C-terminal *Aristaless* domain of *ARX* regulates protein activity in terms of protein interaction, DNA binding, transcriptional activity and protein stability, and how this dysfunction leads to early onset developmental and epileptic encephalopathy in some and intellectual disability in other individuals.

#### **Acknowledgements**

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### **Data Availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### **Conflict of Interest**

Ingrid Scheffer has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin and Nutricia; editorial boards of the *Annals of Neurology*, *Neurology* and *Epileptic Disorders*; may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has received speaker honoraria from GlaxoSmithKline, Athena Diagnostics, UCB, BioMarin, Eisai and Transgenomics; has received funding for travel from Athena Diagnostics, UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai; and receives/has received research support from the National Health and Medical Research Council of Australia, National Institutes of Health, Australian Research Council, Health Research Council of New Zealand, CURE, and March of Dimes.

The remaining authors declare no conflict of interest.

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## Figure Legends

**Figure 1: Identification of a c.1449-1G>C missense variant in intron 4 of the *ARX* gene (NM\_139058.2) leading to a splice variant and predicted p.(L484\*).** (A) Pedigree of family. Open symbol represents unaffected individual. Filled quadrants represent phenotypes listed in each legend in affected males (squares) and female (circle); Intellectual disability (top left quadrant), Autism Spectrum Disorder (top right quadrant), Non-verbal (bottom right quadrant) and seizures (bottom left quadrant). Individual generations are numbered with Roman numerals on the left-hand side of pedigree. Individuals tested for the nucleotide substitution are indicated either M (mutant allele) or M/N (mutant and normal allele). (B) Neuroimaging of II-2. MRI brain Coronal T2 sequence and Sagittal Ultrasound showing thin hypoplastic corpus callosum. (C) The exon-intron structure of the *ARX* gene [GenBank:NM\_139058.2] has five exons, with the ATG start codon, open reading frame and stop codon positions in black and 5' and 3' untranslated regions in light grey. The corresponding nucleotides are listed above each exon. Below is the predicted protein structure of the 562-residue protein (NP\_620689.1) with known functional domains highlighted; octapeptide (OP) in horizontal stripes, nuclear localisation sequences (NLS) in black, polyalanine tracts (PA) in white, acidic domain in vertical stripes, homeodomain (HD) in diagonal stripes and Aristaless domain (OAR) in crosshatching. The exon 4 to exon 5 boundary highlights the intronic single nucleotide substitution predicted to abolish the consensus splice acceptor site at c.1449 at the end of intron 4 (dotted red vertical line), leading to retention of the 2,201-nucleotide intron 4, with the translated protein containing a premature termination codon immediately at the end of exon 4. As exon 5 is the last exon of the *ARX* gene, the stop codon at position p.L484\* would be predicted to escape NMD, potentially producing (some) c-terminally truncated protein.

**Figure 2: Analysis of *ARX* cDNA in patient LCLs.** In each panel, the first column corresponds to 1 kb plus DNA ladder. The next lane in each panel show the amplification outcomes across exon-exon boundaries from cDNA generated from control LCLs (C1 and C2) followed by proband patient II-2 with the neg lane showing patient sample generated without superscript enzyme (-RT). (A) Amplification spanning exons 4 to 5 yields a single band at the expected size of 479bp across for each of two control samples at all concentrations of cDNA tested (listed above the panel). There was no band detected from the proband at any concentration (2 to 8 µg) of cDNA template tested. (B) The integrity of the cDNA from all samples is demonstrated by a consistently strong single band of expected size of 691bp amplified from exons 5 to 8 of X-linked gene, *IQSEC2*. (C) Retention of intron 4 in the patient cDNA is demonstrated by a band amplified from intron 4 to exon 5 of *ARX* at the expected size of 347bp. As expected, this retained intron band is not present in either control. (D) Sanger sequencing of the 347bp PCR product from cDNA of P(II-2) from figure 2C demonstrates the c.1449-1G>C variant in the amplicon that spans the intron 4- exon 5 boundaries. (E) Transcription of *ARX* mRNA from exons 1 to 2 is demonstrated by a weak but detectable band of expected size at 185 bp in controls and patient cDNA.

**Supplementary Figure 1. RNA extraction and cDNA generation from control and patient lymphoblastoid cells.** A) Total RNA was extracted from LCLs of controls and patient II-2. B) Routinely, 2  $\mu$ g of RNA was used to generate cDNA, and confirmed by expression of Esterase D (a house keeping gene) at expected band size of 452bp present at similar levels in control and patient samples with superscript enzyme added (+RT) but not in samples generated without superscript enzyme (-RT), or in the no template (neg) control lane. DNA ladders are shown in first lane of each panel, 1 kb plus for panel A and puc19/Hap1 digest for panel B.



Table 1: Pathogenic variants in *ARX* (outside the polyalanine tracts) leading to phenotypes with early (< 3 months) onset of seizures

cDNA (nuc) Protein (AA)	Domain	Variant Type	#	sex	Epilepsy Syndrome / Age seizure onset	MRI findings; serial (ages at scans)	Clinical features (age)	Refs
c.81C>G p.Y27X	<b>Oct</b>	nonsense	1	M (x2)	(M1) West / 1mo  (M2) / OS 5d	Cerebral atrophy (5mo); progressive cerebral atrophy + white matter signal abN (21mo)  CC hypoplastic (1mo)	Profound global developmental delay (5y)  No developmental progress, CVI (5 mo)	[3]
c.869C>A p.S290X		nonsense	1	F	OS / 1mo	Not available	Severe developmental delay, microcephaly, dystonia, CVI (6 mo)	[4]
c.1072A>T p.R358W	<b>HD</b>	missense	1	M	West / 2mo	CC agenesis, ventriculomegaly (13y)	Severe global delay, acquired microcephaly, dystonia, spastic quadriplegia (23y)	[5]
c.1074G>T p.R358S	<b>HD</b>	missense	1	M	West / 3mo	CC agenesis, polymicrogyria and pachygyria (not stated)	Severe global developmental delay. AbN genitalia- micropenis + cryptorchidism.	[6]
IVS4-816Ex570del p.R483fs		deletion	1	M	West / 1mo	Delayed myelination (not stated)	Severe developmental delay, microcephaly, central hypotonia, spasticity, unilateral microphthalmia (2y)	[7]
c.1449-1G>C p.L484X		Splice	1	M	EODEE 1mo	CC hypoplastic (1mo)	Severe language delay, autistic features, mild motor delay. No microcephaly (2y4mo)	This report
c.1459delA p.487fsX491		deletion	1	F	OS / 3mo	Normal (3mo; repeated at 2yo)	Severe language delay, autistic features, moderate motor delay (3y)	[8]
c.1471_1472insC p.491fsX531	<b>Arist</b>	insertion	1	M	OS, later West / 1d	Cerebral atrophy, hippocampal sclerosis, CC hypoplastic/atrophic (22mo)	Severe developmental delay, microcephaly, central hypotonia, spasticity, AbN genitalia- micropenis + cryptorchidism (7mo)	[9]
c.1564_1568dupAC GGC p.A524fsX534	<b>Arist</b>	insertion	1	M (x2)	(M1) OS / 1mo  (M2) OS / 46d	Cerebral atrophy, basal ganglia signal change (early infancy; repeated at 33mo)	Severe developmental delay, congenital microcephaly, dystonia, spastic quadriplegia, scoliosis. (28 mo)	[10]
c.1600G>C p.A534P	<b>Arist</b>	Missense	1	M	OS, later West / 53d	Normal	Severe ID, SUDEP (2y6mo)	[11]
c.1604T>A p.L535Q	<b>Arist</b>	missense	1	M (x2)	(M1) OS / 20d  (M2) OS, later West 6d	Normal (6mo); Cerebral atrophy (22mo)  Normal (6d); Cerebral atrophy (2y)	Severe developmental delay, congenital microcephaly, spastic quadriplegia (22 mo). Severe developmental delay, acquired microcephaly, spastic quadriplegia (2y)	[12]
c.1604_1605insT p.E536fsX672	<b>Arist</b>	insertion	1	M	OS / 22d	Static ventriculomegaly (44mo; repeated at 13y)	Severe developmental delay, acquired microcephaly, spastic quadriplegia (13y)	[10]

c.1611T>C p.L537P	<b>Arist</b>	missense	1	M	OS / 1d	Normal (1y); Cerebral atrophy, CC atrophy (13y)	Severe developmental delay, dystonia, spastic quadriplegia (16y)	[8]
c.1616C>A p.A539D	<b>Arist</b>	Missense	1	M (x2)	(M1) OS / 21d (M2) OS / 19d	Normal No MRI, CT normal	No microcephaly. Died 5 yo.	[14]

**AG:** Abnormal genitalia, **CC:** Corpus callosum, **CVI:** cortical visual impairment, **EODEE:** Early onset developmental and epileptic encephalopathy, **OS:** Ohtahara syndrome, **West:** West syndrome. (MIM 308350), **WEST:** West Syndrome - Infantile spasms X-linked (MIM 308350). Age ~ d: days, mo: months, y: years, Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence for the ARX gene [GenBank: NM\_139058.2]

Figure 1

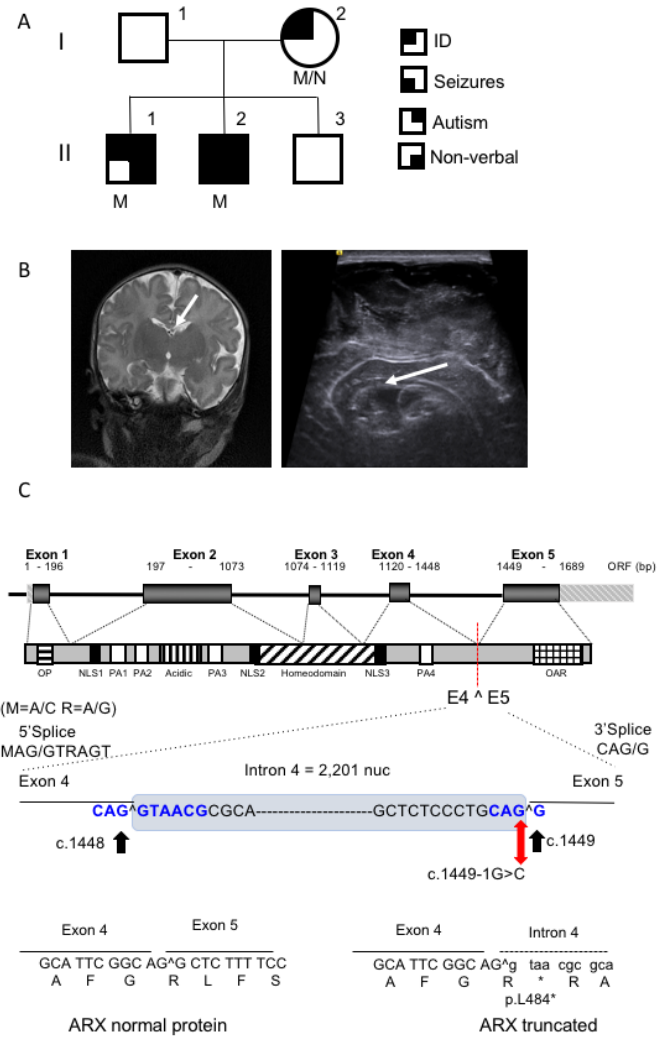






Figure 1

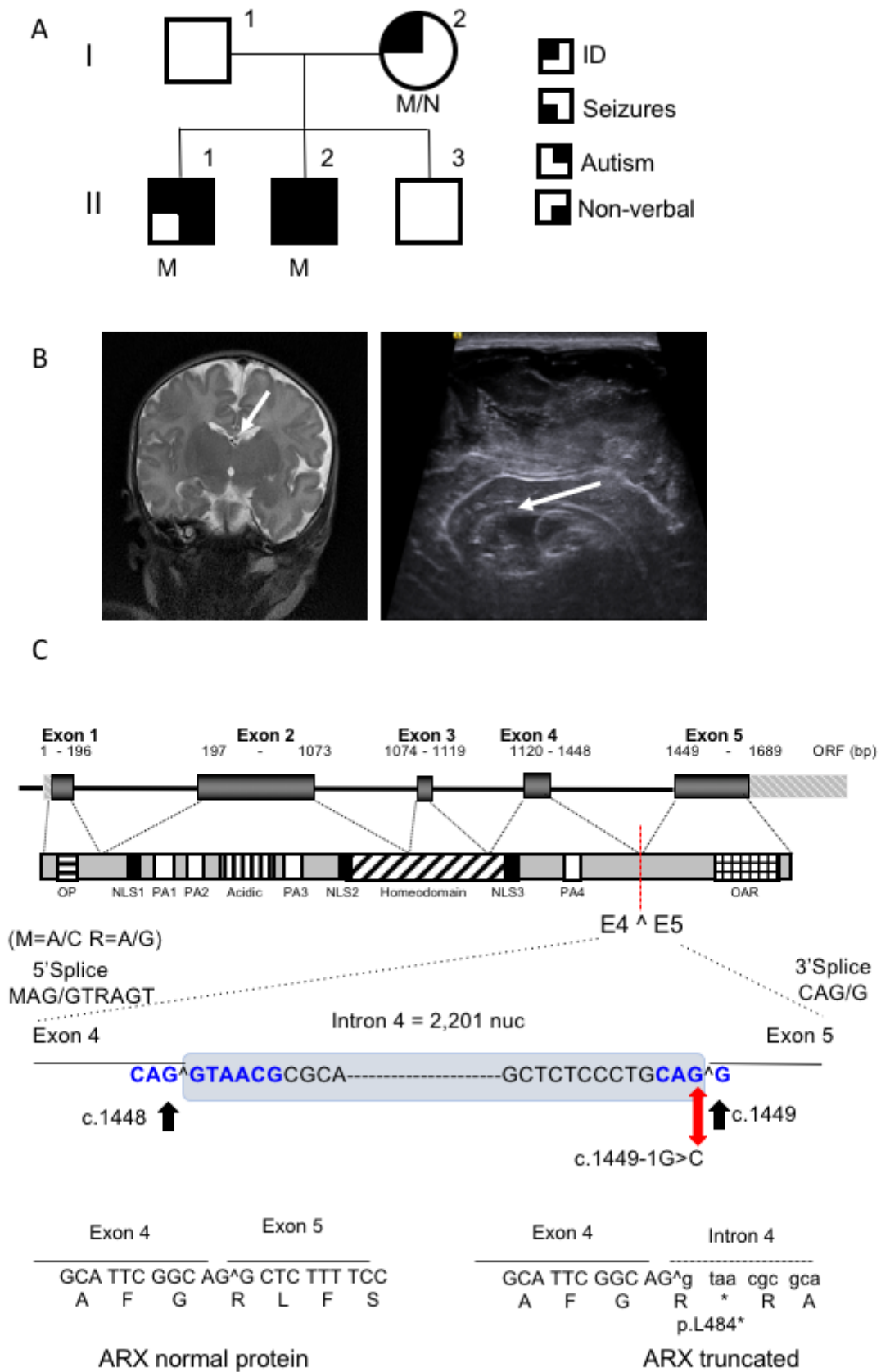
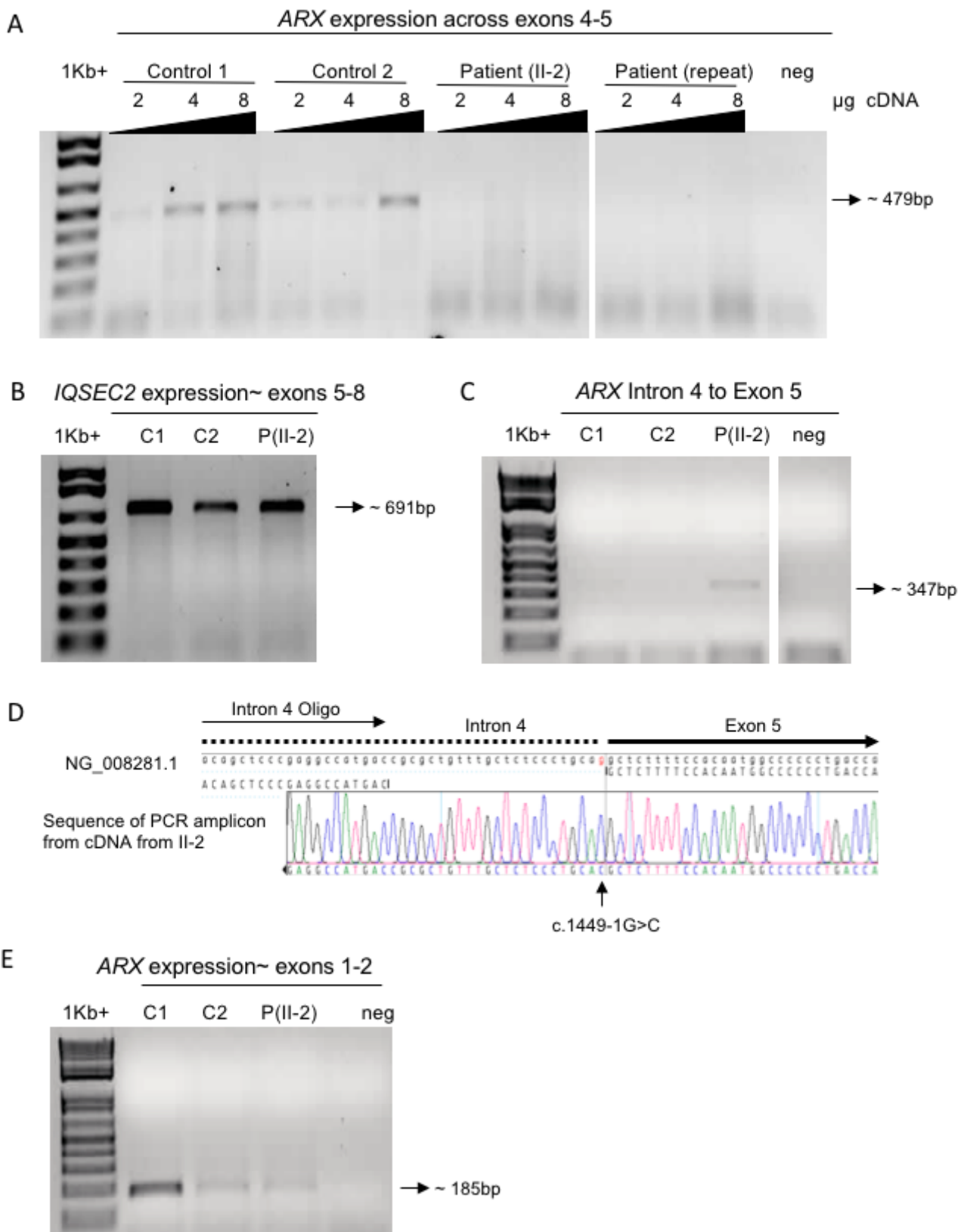


Figure 2





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