

# Biallelic Mutations in *ADPRHL2*, Encoding ADP-Ribosylhydrolase 3, Lead to a Degenerative Pediatric Stress-Induced Epileptic Ataxia Syndrome

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ADP-ribosylation, the addition of poly-ADP ribose (PAR) onto proteins, is a response signal to cellular challenges, such as excitotoxicity or oxidative stress. This process is catalyzed by a group of enzymes referred to as poly(ADP-ribose) polymerases (PARPs). Because the accumulation of proteins with this modification results in cell death, its negative regulation restores cellular homeostasis: a process mediated by poly-ADP ribose glycohydrolases (PARGs) and ADP-ribosylhydrolase proteins (ARHs). Using linkage analysis and exome or genome sequencing, we identified recessive inactivating mutations in *ADPRHL2* in six families. Affected individuals exhibited a pediatric-onset neurodegenerative disorder with progressive brain atrophy, developmental regression, and seizures in association with periods of stress, such as infections. Loss of the *Drosophila* paralogue *Parg* showed lethality in response to oxidative challenge that was rescued by human *ADPRHL2*, suggesting functional conservation. Pharmacological inhibition of PARP also rescued the phenotype, suggesting the possibility of postnatal treatment for this genetic condition.

ADP-ribosylation is a tightly regulated posttranslational modification of proteins involved in various essential physiological and pathological processes, including DNA repair, transcription, telomere function, and apoptosis.<sup>1–3</sup> The addition of poly-ADP-ribose (PAR) is mediated by a group of enzymes, referred to as poly(ADP-ribose) polymerases (PARPs), in response to cellular stressors, such as excitotoxicity or reactive oxygen species. PARylated proteins can subsequently initiate cellular stress response pathways. After resolution of the original insult, ADP-ribose polymers are rapidly removed.<sup>4,5</sup> Although PAR modification can protect the cell from death in the setting of cellular stress, excessive PAR accumulation or failure to reverse PAR modification can trigger a cell-death response cascade.<sup>6,7</sup>

Humans have two genes encoding specific PAR-degrading enzymes: *ADPRHL2* (MIM: 610624; Gene ID: 54936) and *PARG* (MIM: 603501). Both are capable of hydrolyzing the glycosidic bond between ADP-ribose moieties and are ubiquitously expressed.<sup>8,9</sup> *ADPRH* (MIM: 603081) and putatively *ADPRHL1* (MIM: 610620) encode proteins that can cleave mono-ADP-ribosylated residues and thus are not functionally redundant with *ADPRHL2* and *PARG*.<sup>8</sup> Studies of *in situ* hybridization have shown high *Adprhl2* expression in the developing mouse forebrain and that its expression remains high in the cerebellum, cortex, hippocampus, and olfactory bulb in early postnatal ages and persists into adulthood.<sup>10</sup> *Parg*<sup>−/−</sup> mice die embryonically as a result of PAR accumulation and cellular apoptosis.<sup>11</sup> There are no reports of *Adprhl2*<sup>−/−</sup> animals,

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introduces a premature stop codon (p.Gln334Ter) predicted to truncate the highly conserved last 30 amino acids of the protein, including part of the ADP-ribosylhydrolase domain. Family 2 harbored the homozygous exon 3 mutation c.316C>T (GenBank: NM\_017825), which also introduces a premature stop codon (p.Gln106Ter) in the ADP-ribosylhydrolase domain. Family 3 revealed the homozygous exon 2 missense mutation c.235A>C (GenBank: NM\_017825), which leads to an amino acid change (p.Thr97Pro) in a residue that is highly conserved among vertebrates (Figure S2A). Using a previously published crystal structure of ARH3, we localized this residue to an  $\alpha$ -helical loop within the ADP-ribosylhydrolase domain and the substrate binding site, which is defined by the position of two Mg<sup>2+</sup> ions located in adjacent binding sites; thus, the residue is predicted to affect protein structure and enzymatic activity (Figure S2B).<sup>19</sup> Family 4 carried the homozygous 5 bp, exon 3 deletion c.414\_418TGCCC (GenBank: NM\_017825), which results in a frameshift (p.Ala139GlyfsTer5) in the ADP-ribosylhydrolase domain. Family 5 carried the homozygous exon 4 missense mutation c.530C>T (GenBank: NM\_017825), which leads to an amino acid change (p.Ser177Leu) that is also highly conserved among vertebrates. It is localized in a critical  $\alpha$ -helical loop within the ADP-ribosylhydrolase domain, also suggesting an effect on protein structure and activity. Family 6 carried the homozygous exon 1 missense mutation c.100G>A (GenBank: NM\_017825), which leads to an amino acid change (p.Asp34Asn) that is highly conserved among vertebrates. This change is also localized in a critical  $\alpha$ -helical loop within the ADP-ribosylhydrolase domain, suggesting a potential impact on protein structure and activity.

The emerging phenotype of recessive *ADPRHL2* mutations is a degenerative pediatric-onset stress-induced epileptic-ataxia syndrome. Individuals with mutations in this gene are asymptomatic early after birth but gradually develop a cyclic pattern of illness-related spontaneous epileptic seizures or present with a neurodegenerative course including weakness, ataxia, and loss of milestones followed by clinical deterioration that ultimately leads to premature death. Most of the subjects succumbed to sudden unexpected death in epilepsy (SUDEP) or an apnoic-attack-like clinical presentation, suggesting a hyperacute presentation prior to the family's recognition of a predisposition. We could not establish an obvious genotype-phenotype correlation given that we show below that the missense mutation also leads to a severe loss of function. Thus, the clinical variability in the age of onset might occur because the genetic background or environmental challenges lead to variable susceptibility to illness-related cellular stress.

The differential diagnosis for this condition was based upon the presentation of a recessive condition with recurrent exacerbations and predominant features of global developmental delay, intellectual disability, seizures, neurogenic changes on electromyography, hearing impair-

ment, regression, and mild cerebellar atrophy but not microcephaly or cataracts. The differential diagnosis in our families included mitochondrial disorders, spastic ataxia, and peripheral neuropathy.

To determine the impact of these mutations on protein folding and binding activity, we generated recombinant proteins in *E.coli* and purified them by His-tag affinity chromatography. Our results showed that the p.Gln334Ter protein was not evident in the soluble fraction, whereas the wild-type (WT) was recovered with good purity (Figure S3A). The p.Thr79Pro protein was expressed and soluble, although possibly recovered with slightly less purity than WT ARH3. We studied the deleterious impact of p.Thr79Pro by using circular dichroism spectroscopy (Figures S3B and S3C). Compared with the WT, this mutant exhibited reduced  $\alpha$ -helical content and an altered secondary structure, in agreement with the fact that p.Thr79Pro occurred within an  $\alpha$ -helical domain. Further, the melting temperature ( $T_m$ ) of p.Thr79Pro was reduced by more than 10°C, confirming destabilization of the mutant (Figures S3D–S3F). We also found that in contrast to WT ARH3, the p.Thr79Pro protein was not stabilized by ligands such as adenosine diphosphate ribose (ADPr) (Figures S3G–S3I). We confirmed the specificity of this assay by using adenosine triphosphate (ATP) and ribose-5-phosphate as negative controls, which were not predicted to bind or stabilize ARH3. Together, these data suggest that both disease-causing, truncating mutants and amino acid substitutions should be destabilized when expressed in cells.

Because the c.1000C>T (p.Gln334ter) mutation of family 1 was in the last exon, we first excluded nonsense-mediated decay (NMD) of the mutant mRNA. We collected skin biopsies from the father (III-II) and two affected individuals (II-IV-6 and II-IV-7), generated primary fibroblasts, and then performed RT-PCR by using primers designed to amplify the last three exons of *ADPRHL2* (Figure S1B). The father's and affected individuals' cells revealed a band of the expected size and of similar intensity to that of a healthy control individual, arguing against NMD. However, when we used an antibody recognizing amino acids 231–245, lysates derived from the affected individuals showed no detectable ARH3 (Figure 2C; Supplemental Data), consistent with a null effect of the truncating mutation. Further, western blot analysis of individual II-2 from family 2 showed an absence of the protein, as predicted by the early stop codon. Fibroblasts from individual II-1 from family 3 showed a severely reduced amount of ARH3 (Figure 2C), consistent with the thermal instability of this mutant protein (Figures S3D–S3F) and the severe alteration of its secondary structure (Figures S3B and S3C).

Whereas humans have two known genes with specific PARG activity (*PARG* and *ADPRHL2*; Figure 3A), *Drosophila* have a single gene that regulates this process: *Parg*. Using the Gal4-UAS system to drive RNAi expression, we found that *Parg* knockdown led to a 60% decrease in total *Parg* mRNA for flies with the ubiquitous *da* promoter and a

**Table 1. Mutations in ADPRHL2 Cause Various Phenotypes, Including Developmental Delay, Cerebellar Atrophy, Ataxia, and Epilepsy**

<b>Family 1</b>								
	<b>I-IV-1</b>	<b>I-IV-2</b>	<b>I-IV-3</b>	<b>I-IV-5</b>	<b>I-IV-11</b>	<b>II-IV-2</b>	<b>II-IV-5</b>	<b>II-IV-6 (A1)</b>
Gender	M	F	M	M	M	F	F	M
Country of origin	UAE	UAE	UAE	UAE	UAE	UAE	UAE	UAE
Parental consanguinity	+	+	+	+	+	+	+	+
Current age or age of death	died at 4 years	died at 2 years	died at 7 years	died at 15 years	4 years	died at 2 years	died at 2 years	died at 9 years
Circumstances of death	in sleep	in sleep	seizure	respiratory failure	–	in sleep 1 week after flu-like illness	seizure after playing	respiratory failure after long airplane trip
<b>Mutation</b>								
Genomic (hg19 <sup>a</sup> )	g.36558895C>T	g.36558895C>T	g.36558895C>T	g.36558895C>T	g.36558895C>T	g.36558895C>T	g.36558895C>T	g.36558895C>T
cDNA	c.1000C>T	c.1000C>T	c.1000C>T	c.1000C>T	c.1000C>T	c.1000C>T	c.1000C>T	c.1000C>T
Protein	p.Gln334*	p.Gln334*	p.Gln334*	p.Gln334*	p.Gln334*	p.Gln334*	p.Gln334*	p.Gln334*
Homozygosity	+	+	+	+	+	+	+	+
<b>Perinatal History</b>								
Normal birth	+	+	+	+	+	+	+	+
Normal early development	+	+	+	+	+	+	+	+
<b>Psychomotor Development</b>								
Speech development	spoke in sentences but then deteriorated	few words at 2 years	normal until 2.5 years but then no further development	normal until 3.5 years but then deteriorated	speaks only a few words	normal speech until death	normal speech until death	normal until 25 years but then deteriorated
Motor development	normal but then deteriorated	normal until death	normal but then deteriorated	normal but then deteriorated	normal but then deteriorated	normal but then deteriorated	normal until death	normal but then deteriorated by 2 years
<b>Seizures</b>								
Seizure onset	18 months	19 months	19 months	24 months	15 months	24 months	15 months	18 months
Seizure types	GTCS	GTCS	GTCS	GTCS	absence, GTCS	GTCS	GTCS	absence, GTCS
<b>Neurological Examination</b>								
Intellect	normal but then delayed	normal until death	normal but then delayed	normal but then delayed	normal but then delayed	normal until death	normal until death	normal but then delayed
EEG	–	–	–	–	–	–	–	generalized epileptiform activity, slow background
MRI (age performed)	–	–	–	normal (5 years)	–	–	–	mild cerebellar atrophy (7 years)

*(Continued on next page)*

	Family 2	Family 3	Family 4		Family 5		Family 6
II-IV-7 (A2)	II-2	II-1	II-1	II-3	IV-1	IV-2	II-3
F	M	F	F	F	M	F	F
UAE	Italy	Turkey	Pakistan	Pakistan	Iran	Iran	Turkey
+	same village	+	+	+	+	+	+
3 years	16 years	15 years	13 years	2 years	died at 6 years	3 years	10 years
-	-	-	-	-	in sleep	-	-
g.36558895C>T	g.36557226C>T	g.36556868A>C	g.36557324_36557328delTGCCC	g.36557324_36557328delTGCCC	g.36557524C>T	g.36557524C>T	g.36554605G>A
c.1000C>T	c.316C>T	c.235A>C	c.414_418delTGCCC	c.414_418delTGCCC	c.530C>T	c.530C>T	c.100G>A
p.Gln334*	p.Gln106*	p.Thr79Pro	p.Ala139Glyfs*4	p.Ala139Glyfs*4	p.Ser177Leu	p.Ser177Leu	p.Asp34Asn
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
+	+	+	+	mild developmental delay	+	+	+
normal speech but then deteriorated	slow speech	normal	normal	delayed	normal until 1.5 years but then deteriorated with difficulty speaking	speaks only a few words	delayed
walked normally at 14 months but then had ataxia and poor balance at 19 months	normal but then deteriorated by 2 years	normal	normal but then deteriorated by 2 years	mildly delayed	normal until 1 year but then deteriorated	normal but then deteriorated by 1.5 years	normal
16 months	-	-	-	9 months	24 months	36 months	-
absence, GTCS	-	-	GTCS with illness	GTCS with illness	multifocal, GTCS	multifocal, GTCS	-
normal but then delayed	normal but then started deteriorating at age 11 years	normal	mild ID (IQ 60)	mild global developmental delay	normal but then stagnated	normal but then stagnated	mild ID
generalized epileptiform activity, slow background	-	-	mild slowing background activity (3 years)	normal	generalized epileptiform activity, slow background	normal	normal
mild cerebellar atrophy (7 years)	cerebellar vermis atrophy (11 years)	mild cerebellar atrophy, spinal cord atrophy (12 years)	mild cerebellar atrophy (4 years)	normal (11 months)	-	normal (3 years)	mild cerebellar vermis atrophy, spinal cord atrophy (15 years)

(Continued on next page)

**Table 1. Continued**

	Family 1							
	I-IV-1	I-IV-2	I-IV-3	I-IV-5	I-IV-11	II-IV-2	II-IV-5	II-IV-6 (A1)
EMG or biopsy	-	-	-	-	-	-	-	nerve biopsy with severe axonal loss
Onset of unsteady gait	-	-	2.5 years	3 years	2.5 years	-	-	2.5 years
<b>Other Clinical Features</b>								
Exacerbated by illness and/or stress	+	+	+	+	+	+	+	+
Other clinical features	-	-	-	hypotonia with repeated pneumonia, ventilator dependent at time of death	can walk but is very unsteady	progressive weakness	progressive weakness	repeated pneumonia, repeated cardiac arrest, profound type II muscle fiber atrophy

Clinical presentation for affected subjects from families 1–6. Abbreviations are as follows: +, yes; -, not available; DTR, deep-tendon reflex; EEG, electroencephalography; EMG, electromyography; F, female; GTCS, generalized tonic-clonic seizure; ID, intellectual disability; M, male; MRI, magnetic resonance imaging; and SNHL, sensorineural hearing loss.

<sup>a</sup>UCSC Genome Browser.

50% decrease with the neuron-specific promoter, *elav* (*embryonic lethal abnormal visual system*) (Figure S4A). Whereas the *da-Gal4* and *Parg*<sup>RNAi</sup> lines showed normal survival, crossing the two together led to *daughterless* (*da*)-mediated expression of *Parg*<sup>RNAi</sup>, which reduced survival substantially (Figure S4B). Ubiquitous knockdown of *Parg* also led to decreased survival when animals were exposed to stress with either hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in their water or environmental hypoxia (2% O<sub>2</sub>) (Figures S4C and S4D). Furthermore, knockdown of *Parg* specifically in neurons largely recapitulated this phenotype by using the same two environmental stressors (Figures S4E and S4F). These data provide evidence that stress leads to premature death in the absence of *Parg* and that neurons play an important role in this phenotype.

However, lethality of these flies was not as severe as in the *Parg*<sup>27.1</sup> line, which carries a p-element insertion that deletes two-thirds of the open reading frame (nucleotides 34,622–36,079 of GenBank: Z98254),<sup>14</sup> suggesting that *Parg*<sup>RNAi</sup> is partially inactivating. These mutant flies with *Parg* loss of function lack the protein Parg and show elevated amounts of PAR, especially in nervous tissue.<sup>14</sup> Mutant flies die in larval stages, but 25% of the animals survive when grown at the permissive 29°C temperature. These adult flies display progressive neurodegeneration, reduced locomotion, and reduced lifespan,<sup>14</sup> consistent with the individuals' phenotypes in our families. We confirmed lethality of the *Parg*<sup>27.1</sup> line and found that forced expression of *Drosophila Parg* under the ubiquitous *da* promoter in the mutant background increased both survival and motor activity as measured by an established "climbing index" (Figures 3B and 3C).<sup>20</sup> Likewise, expression of the human *ADPRHL2* under the same *da* promoter

showed a nearly identical degree of rescue of both survival and locomotor activity (Figures 3B and 3C). These results suggest that human *ADPRHL2* is a functional paralog of *Drosophila Parg*.

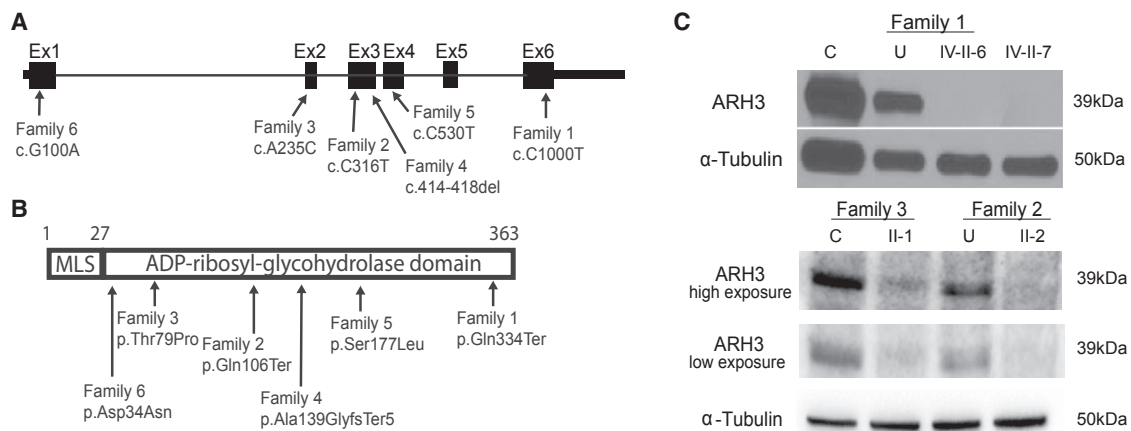
We next tested whether this phenotype might be ameliorated by inhibition of protein PARylation. We reasoned that the requirement for dePARylation should be reduced by the blockage of stress-induced PARylation. Minocycline displays PARP inhibitory activity with an IC<sub>50</sub> of 42 nM in humans<sup>21</sup> and is well tolerated in flies.<sup>22</sup> We fed flies with a range of concentrations from 0 to 1 mg/mL minocycline for 24 hr before stress and measured survival rates 96 hr after stress induction. Drug treatment of flies with ubiquitous knockdown of *Parg* revealed a dose-dependent partial rescue of the lethality (Figure S4G). This rescue was also seen when the drug was given to flies with neuron-specific knockdown of *Parg* (Figure S4H), providing evidence that PARP inhibition can rescue lethality *in vivo*. Although we expect that the effect of minocycline on survival in this assay was due to its effect on PARP, we cannot exclude off-target or non-specific effects.<sup>22</sup>

Given that PARP inhibitors are currently in trials for various types of cancer, it is possible that these drugs could be tested for clinical effectiveness in this orphan disease, where they could have a positive effect. Potentially clinically relevant PARP inhibitors include (1) minocycline, an FDA-approved tetracycline derivative that displays PARP inhibitory activity; (2) dihydroisoquinoline (DPQ), a non-FDA-approved potent PARP-1 inhibitor used in experimental research; and (3) veliparib (ABT: 888), a potent PARP-1 and PARP-2 inhibitor currently in clinical trials for the treatment of various type of cancers (IC<sub>50</sub> = 42, 37, and 4.4 nM, respectively).<sup>21,23</sup>

	Family 2	Family 3	Family 4		Family 5	Family 6	
II-IV-7 (A2)	II-2	II-1	II-1	II-3	IV-1	IV-2	II-3
-	-	axonal polyneuropathy (4 years)	normal muscle biopsy (4 years)	-	normal (4 years)	axonal polyneuropathy (4 years)	axonal polyneuropathy
20 months	11 years	4 years	2.5 years	not yet	1.5 years	1.5 years	10 years
+	+	+	+	+	+	+	+
normal hearing but then developed severe SNHL, severe kyphoscoliosis, one episode of cardiac arrest	myopathic changes on muscle biopsy (11 years)	claw hand and pes cavus deformities, scoliosis, SNHL at 10 years, tracheotomy, ventilation	asthma	-	progressive weakness, tremors, frequent falling	progressive weakness, progressive external ophthalmoplegia	distal muscle atrophy, pes cavus deformity, toe abnormality, scoliosis, brisk DTRs, positive Babinski reflex, intentional tremor, ataxia

The extent to which *ADPRHL2* and *PARG* functionally diverge or converge is not well understood, partly because of a lack of detailed comparative expression analysis and biochemical function. *PARG* demonstrates greater specific activity than *ARH3* for removing PAR from proteins,<sup>8</sup> and loss of *Parg* in mice is embryonically lethal.<sup>13</sup> Together, these data suggest that *PARG* is likely to be the major contributor to PAR removal in cells that express both genes under basal conditions. One possibility is that *ADPRHL2*

acts as a backup for *PARG* to remove excessive PAR moieties under stress conditions. This would be consistent with the clinical presentation of individuals with loss of *ADPRHL2*, where phenotypes appear to be induced by environmental stress. Recent studies have shown that *ARH3* acts on a recently discovered form of Ser-ADP ribosylation.<sup>24</sup> For example, studies have illustrated an excessive accumulation of Ser-poly-ADP-ribosylated enzymes in *ADPRHL2*<sup>-/-</sup> cell lines and that *ARH3* acts mainly on



**Figure 2. Truncating and Missense *ADPRHL2* Mutations in Six Independent Families Are Predicted to Be Inactivating**

(A) Schematic of *ADPRHL2* depicts the coding sequence spanning six exons and the 5' and 3' UTRs. Black arrows indicate the positions of the six identified mutations and their coordinates within the cDNA (Gene ID: 54936).

(B) Schematic of *ARH3* depicts the mitochondrial localization sequence (MLS) and the ADP-ribosyl-glycohydrolase domain. Black arrows indicate the position and coordinates of the impact of the described mutations.

(C) Western blot of fibroblasts from an unrelated control individual (C), the unaffected carrier father (U), and affected individuals IV-II-6 and IV-II-7 from family 1 shows the absence of *ARH3* in affected fibroblasts.  $\alpha$ -tubulin was used as the loading control. Western blot of fibroblasts from an unrelated control individual (C) and affected individual II-1 from family 3 and the unaffected carrier mother (U) and affected individual II-3 from family 2 shows significantly reduced amounts of *ARH3*.  $\alpha$ -tubulin was used as the loading control.



GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>  
 Gene, <https://www.ncbi.nlm.nih.gov/gene>  
 GeneMatcher, <https://genematcher.org>  
 HaplotypeCaller and GATK, <https://www.broadinstitute.org/gatk/>  
 Iranome, <http://www.iranome.ir/>  
 Mutation Assessor, <http://mutationassessor.org/>  
 MutationTaster, <http://mutationtaster.org/>  
 NHLBI Exome Sequencing Project Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
 OMIM, <http://omim.org/>  
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