A Recurrent *De Novo PACS2* Heterozygous Missense Variant Causes Neonatal-Onset Developmental Epileptic Encephalopathy, Facial Dysmorphism, and Cerebellar Dysgenesis

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Developmental and epileptic encephalopathies (DEEs) represent a large clinical and genetic heterogeneous group of neurodevelopmental diseases. The identification of pathogenic genetic variants in DEEs remains crucial for deciphering this complex group and for accurately caring for affected individuals (clinical diagnosis, genetic counseling, impacting medical, precision therapy, clinical trials, etc.). Whole-exome sequencing and intensive data sharing identified a recurrent *de novo* PACS2 heterozygous missense variant in 14 unrelated individuals. Their phenotype was characterized by epilepsy, global developmental delay with or without autism, common cerebellar dysgenesis, and facial dysmorphism. Mixed focal and generalized epilepsy occurred in the neonatal period, controlled with difficulty in the first year, but many improved in early childhood. PACS2 is an important PACS1 paralog and encodes a multifunctional sorting protein involved in nuclear gene expression and pathway traffic regulation. Both proteins harbor cargo(furin)-binding regions (FBRs) that bind cargo proteins, sorting adaptors, and cellular kinase. Compared to the defined *PACS1* recurrent variant series, individuals with *PACS2* variant have more consistently neonatal/early-infantile-onset epilepsy that can be challenging to control. Cerebellar abnormalities may be similar but *PACS2* individuals exhibit a pattern of clear dysgenesis ranging from mild to severe. Functional studies demonstrated that the PACS2 recurrent variant reduces the ability of the predicted autoregulatory domain to modulate the interaction between the PACS2 FBR and client proteins, which may disturb cellular function. These findings support the causality of this recurrent *de novo PACS2* heterozygous missense in DEEs with facial dysmorphim and cerebellar dysgenesis.

Epilepsy is a common neurologic disorder of childhood, affecting approximately 7 in 10,000 children before 2 years of age and often associated with developmental delay/intellectual disability (ID).[1] The developmental and epileptic encephalopathies (DEEs) are a group of severe infantile- and childhood-onset epilepsies characterized by developmental slowing or regression in the context of recurrent seizures and frequent interictal epileptiform discharges,

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often on a background of developmental delay.[2] The DEEs have a wide range of etiologies, including both acquired and genetic causes. These include specific congenital or acquired structural brain lesions, metabolic disorders, chromosomal anomalies, copy-number variants (CNV), or single gene defects.[3–5] Variants in hundreds of genes have been associated with epilepsy to date, with a variety of inheritance patterns or arising *de novo*.[6] Although genes encoding ion channels correspond to one third of the epilepsy-associated genes, others can affect diverse molecular pathways involved in membrane excitability, synaptic plasticity, presynaptic neurotransmitter release, postsynaptic receptors, transporters, cell metabolism, and many processes important in early brain development.[7,8]

The identification of pathogenic genetic variants related to the epileptic disorders, including the DEEs, remains crucial, providing more precise definition of the clinical diagnosis, allowing accurate genetic counseling, impacting medical management including precision therapy in some cases, linking families to appropriate support/family groups, and opening options for gene-specific clinical trials.[9] Currently available next-generation sequencing strategies exhibit a diagnostic yield of 20%–50% in epilepsy broadly.[10–15] The yield is higher (~60%–83%) in individuals with onset <2 months of age and in certain groups such as Ohtahara syndrome.[16,17] Whole-exome sequencing (WES) studies demonstrated the importance of *de novo* single-nucleotide variations (SNVs) in DEEs.[18–20] WES has also become a powerful approach for identifying new genes that underlie Mendelian disorders when previous approaches, including chromosomal microarray analysis and epilepsy gene panel testing, have failed.[21–29]

Previously, WES identified a *de novo* missense variant, GenBank: NM_018026.2 (c.607C>T), in *PACS1* (MIM: 607492) in two unrelated individuals with unexplained ID and strikingly similar facial dysmorphisms.[30] This variant is highly recurrent and is now reported in 19 unrelated individuals with ID.[30–32] *PACS1* encodes a trans-Golgi-membrane traffic regulator that directs protein cargo and several viral-envelope proteins, with high expression during human embryonic brain development and downregulation after birth.[33–36] The p.Arg203Trp substitution triggers cytoplasmic aggregates from altered PACS1, leads to protein-trafficking defects, and most likely abrogates the ability of the protein to perform its normal function. This was the first report of variants in a phosphofurin acid cluster sorting protein leading to human disease. Mutant *pacs1* zebrafish embryos showed craniofacial defects driven by aberrant specification and migration of cranial neural-crest cells, most likely due to a dominant-negative effect.[30]

We identified a recurrent *de novo* missense variant in PACS2, in individuals with neonatal/early-infantile-onset DEEs, with or without extra-neurological features. Using trio WES, we first identified (Supplemental Data) a heterozygous missense variant (chr14:g.105834449G>A; GenBank: NM_001100913.2; c.625G>A) (ClinVar SUB3731210) in *PACS2* (MIM: 610423), predicted to result in a glutamate-to-lysine substitution (p.Glu209Lys) (Figure 1, Table 1; individuals 1 and 2) in two unrelated individuals with DEE and facial dysmorphism. WES had been performed according to standard procedures using the Agilent CRE Capture kit on an Illumina HiSeq 2000. Raw data had been processed as previously describe.[37] Sanger sequencing (polymerase chain reaction, PCR) in both individuals and their parents confirmed the presence of the variant in the individuals and absence in the parents, consistent with *de novo* occurrence. This variant, absent from the gnomAD and EVS databases (see Web Resources), involves a highly conserved amino acid located in an acid hydrophobic domain of the PACS2 protein that leads to polarity and protein conformation changes and is predicted to be damaging by PolyPhen-2 and SIFT (see Web Resources). By data sharing through GeneMatcher,[38] GeneDx (see Web Resources), and French AnDDI-Rares

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https://doi.org/10.1016/j.ajhg.2018.03.005.

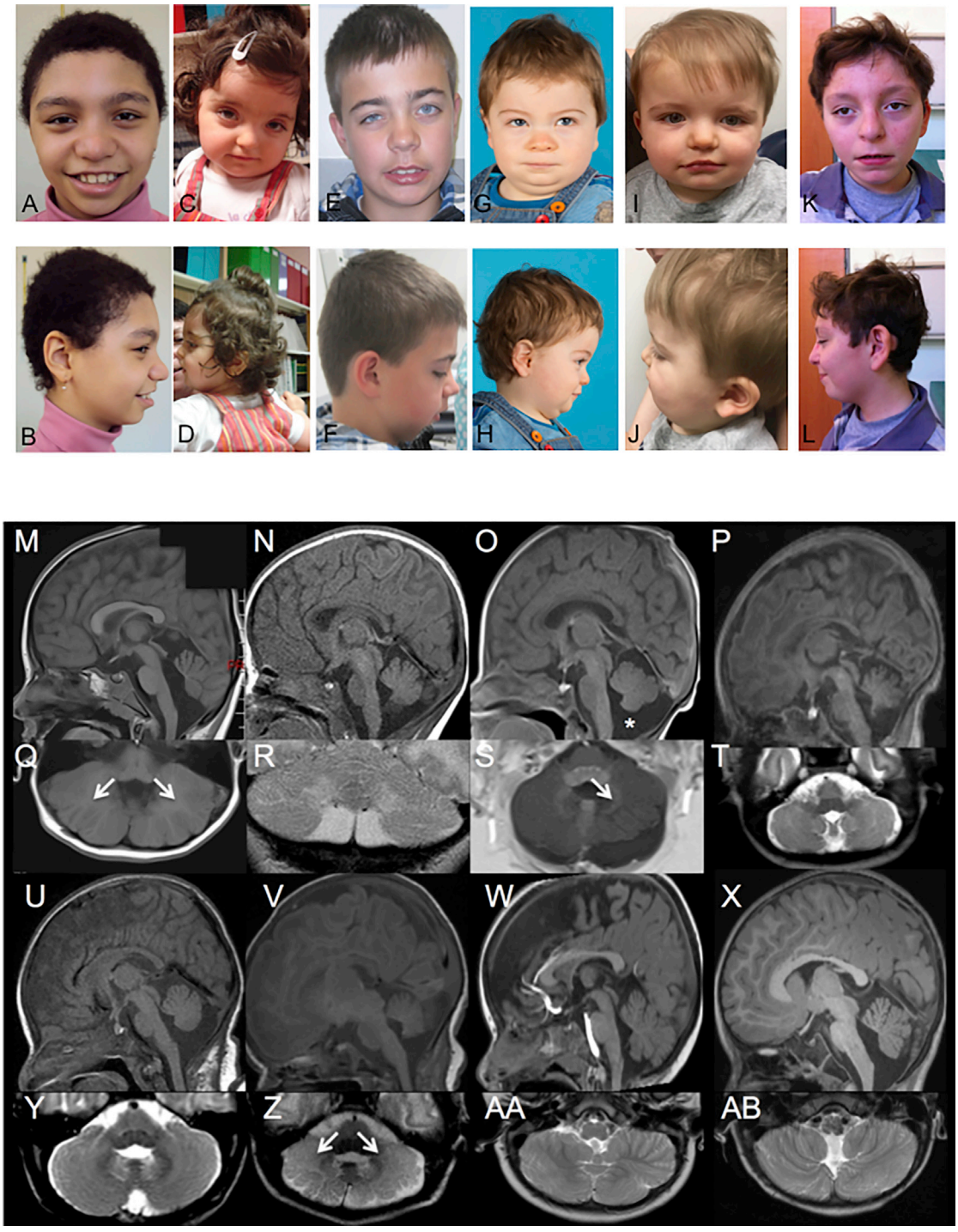

Figure 1. Clinical and Imaging Features

(A) Pictures of individuals 1 (a, b), 2 (c, d), 3 (e, f), 5 (g, h), 7 (i, j), and 11 (k, l): variable facial dysmorphism.

(B) Spectrum of posterior fossa abnormalities in the *PACS2* cohort. Sagittal T1 weighted (m–p, u–x), axial T2 weighted (t, y–ab), axial T1 weighted (q, s), and coronal T2 weighted (r) imaging for subject 2 at 5 years of age (m, q), subject 4 at 3 weeks of age (n, r), subject 5 at 7 days of age (o, s), subject 9 at 1 week of age (p, t), subject 10 at 1 month of age (u, y), subject 12 at 3 months of age (v, z), subject 13 at 23 months of age (w, aa), and subject 14 at 2.5 years of age (x, ab). Of 8 subjects with centrally reviewed imaging, there was prominence of the cisterna magna (asterisk in o) in all but subject 13 (w) and widening of the foramen Magendie in all subjects except subject 12 (v). Mild inferior vermian hypoplasia was also evident in subjects 2 (m), 4 (n), 5 (o), and 14 (x). Cerebellar hemisphere dysplasia was present in subjects 2 (q), 4 (r), 5 (s), 12 (z), and 13 (aa) manifest as unusual centrifugal orientation of the folia bilaterally in subjects 2 (q, arrows) and 12 (z, arrows) and on the left side only in subject 5 (s, arrow). Distortion of the foliar pattern was present without centrifugal orientation in subjects 4 (r) and 13 (aa). Subtler foliar distortion was visible in subjects 9 (t) and 14 (ab).

Table 1. Detailed Extra-neurological Phenotype of Individuals with PACS2 p.Glu209Lys

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6
Gender	F	F	M	F	M	M
Gestational age (WG)	38	37	38	35	37	40
Birth Parameters						
Weight	−0.3 SD	median	+2 SD	+1.8 SD	−1.7 SD	+2.5 SD
Length	+0.6 SD	NA	NA	NA	−0.9 SD	+2 SD
OFC	+0.4 SD	NA	NA	NA	−2.3 SD	>3 SD
Growth Parameters						
Age at last follow-up	16 year	4 year	15 year	8 year	19 mo	8 year
Weight	+1 SD	median	+1.6 SD	+1.5 SD	−1.0 SD	+0.5 SD
Height	+2 SD	median	+0.7 SD	−0.8 SD	−1.8 SD	+2 SD
OFC	median	+1 SD	−0.7 SD	+0.4 SD	−1.9 SD	+2 SD
Facial Dysmorphism						
Synophris	+	+	+	+	−	−
Hypertelorism	+	+	+	+	−	−
Down-slanting palpebral fissures	+	+	+	+	−	+
Broad nasal root	−	+	−	+	+	−
Thin vermillon of upper lip	+	+	+	+	+	+
Wide mouth with downturned corners	+	+	+	+	+/−	−
Prominent incisors	+	−	+	+	NA	−
Widely spaced teeth	+	−	+	−	NA	−
Everted vermillon of lower lip	+	−	+	−	−	+
Distal limb anomalies	slender fingers	−	2/3 syndactyly of toes	−	V finger clinodactyly, variant transverse palmar crease	finger pads
Hematological Anomalies						
Neutropenia	moderate	−	−	−	−	NA
Anemia	−	+	−	+	+	NA
Eye/hearing features	moderate myopia	−	−	strabismus	strabismus	strabismus astigmatism, myopia, anisocoria
Additional clinical features	metatarsus varus	dextrocardia	cryptorchidism	none	none	small ventricular septal defect, testis ectopia

Abbreviations: F, female; M, male; m, median; mo, months; NA, not available; SD, standard deviation; year, years; * no details available.

network (see Web Resources), we ascertained 12 additional individuals harboring the same *de novo* heterozygous missense variant, GenBank: NM_018026.2; c.607C>T (p.Glu209Lys) (Figure 1). Three of the individuals had been detected through a targeted method to identify genes with significant clustering patterns of *de novo* variants in a dataset of 4,061 *de novo* missense mutations from published trio WES studies of 5,302 individuals with ID and developmental anomalies.[29] All individuals' variants were identified by research or clinical diagnostic WES, initially as candidate gene variants, and there were not alternative genetic or non-genetic diagnoses.

This recurrence strongly supported the implication of the PACS2 c.607C>T (p.Glu209Lys) missense variant in

Individual 7	Individual 8	Individual 9	Individual 10	Individual 11	Individual 12	Individual 13	Individual 14
M	F	F	M	M	F	F	F
at term	37	at term	at term	39.5	34	33	39
+1 SD	−3 SD	median	NA	median	+0.5 SD	−0.5 SD	+0.6 SD
+1 SD	−1.5 SD	−2 SD	NA	−1 SD	−1.5 SD	median	+0.9 SD
median	−2.3 SD	median	NA	median	NA	median	−0.1 SD
16 mo	5 year	3 year	7 year	12.5 year	9 mo	3.5 year	5.5 year
+1.5 SD	−1 SD	median	+1.3 SD	+2.5 SD	+1 SD	median	−2 SD
−1 SD	−0.5 SD	−0.7 SD	+1.8 SD	−1 SD	-	median	−0.4 SD
−1 SD	−1 SD	−1.0 SD	+0.4 SD	median	−1.5 SD	+1 SD	−1.4 SD
−	−	+	"mildly dysmorphic"*	−	−	−	−
−	+	−		−	+	+	−
+	+	−		−	−	+	−
+	+	+		+	+	+	+
−	+	+		+	+	+	+
−	+	−		+	+	+	−
NA	−	−		−	NA	NA	−
NA	−	−		+	NA	NA	−
−	−	−		−	−	+	+
−	−	−	bilateral palmar crease	broad and tapering short fingers, V finger brachyclino-dactyly	V finger clinodactyly	-	right transverse palmar crease, V finger clinodactyly
−	−	−	−	−	−	−	−
mild	−	−	−	−	−	−	−
−	hypermetropic astigmatism	myopia, astigmatism, cortical visual impairment	−	hypermetropic astigmatism, frequent otitis	−	−	−
cryptorchidism, 1 cm café au lait birthmark, mild conductive hearing loss, poor feeding, frequent ear and respiratory infections	none	dysphasia, accessory caudally placed nipples	none	micropenis, unilateral cryptorchidism, infantile hypertrophic pyloric stenosis, velopharyngeal hypotonia, central precocious puberty	brachycephaly, inverted nipples	atrial septal defect, sacral pit	features consistent with Chinese heritage

human disease, supported further by its absence in gnomAD and EVS. However, because WES frequently identifies *de novo* variants in individuals with ID and epilepsy and more than 60% of the variants are missense, interpretation represents a great challenge. Many factors are considered when evaluating the significance of candidate genes, including recurrence, strikingly similar phenotypic outcomes in recurrent variants, previous evidence of overlap with pathogenic copy-number variation, localization of the variant in the protein, variant burden among healthy individuals, and membership of the candidate gene in disease-implicated protein networks.[39]

Detailed retrospective phenotyping of the 14 individuals with the recurrent PACS2 p.Glu209Lys variant

Table 2. Detailed Neurological Features of Individuals with *PACS2* p.Glu209Lys

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6
Developmental Features						
Sitting age	8 mo	20 mo	6 mo	NA	16 mo	16 mo
Walking age	22 mo	NA	18 mo	18 mo	18 mo	22 mo
Speech delay	+	+	+	+	+	+
DD/ID	+	+	+	+	+	+
Neurological Features						
Hypotonia	+	NA	NA	NA	–	–
Nystagmus	–	+	–	–	+	–
Stereotypies	–	+ (transient)	–	–	–	+
Others	increased tendon reflexes	wide-based gait	–	–	visual problems	–
Psychiatric/ behavioral features	–	sleeping and behavioral disturbances	–	mild autistic disorder	–	obsessive compulsive disorder
Epilepsy Details						
Age of onset	6 days	4 days	4 days	7 days	2 days	2 days
Seizure types	focal	GTCs	NA	GTCs	clonic and GTC	NA
Longest seizure-free interval or age at last seizure	NA	6 mo	NA	2 years, immediate recurrence after withdrawal of valproate	9 mo	status epilepticus 3 mo, 3.5 years; status epilepticus without fever: 3.5 year:
EEG features	NA	neonatal to 3.5 mo: focal spikes, normal background; 1 year: normal	NA	neonatal: excess discontinuity, excessive multifocal sharp waves → generalized bursts of epileptic activity + MF sharp waves	neonatal: normal; 4 months: generalized slowing with MF sharp waves and frequent focal seizures	neonatal: left temporal spikes; 3.5 year (awake only): left paroxysmal temporal rolandic spikes, generalized slowing.
Brain MRI (age)	mild foliar distortion of the left cerebellar hemisphere, mega cisterna magna* (5 yr)	inferior vermian hypoplasia with prominent foramen Magendie and cisterna magna, severe foliar distortion of cerebellar hemispheres with centrifugal orientation, hypothalamic fusion anomaly (5 yr)	increased subarachnoid spaces* (NA)	mild inferior vermian hypoplasia with a patulous foramen Magendie and mega cisterna magna, mild distortion of the cerebellar folia (3 wk)	retrocerebellar arachnoidal cyst, inferior vermian hypoplasia with prominent foramen Magendie and a mega cisterna magna, severe foliar distortion of the left cerebellar hemisphere with centrifugal orientation, hypothalamic fusion anomaly (7 d)	normal* (10 d); normal* (4 mo)

(*Continued on next page*)

Individual 7	Individual 8	Individual 9	Individual 10	Individual 11	Individual 12	Individual 13	Individual 14
NA	10 mo	18 mo	12 mo	9 mo	7 mo	12 mo	11 mo
not walking	27 mo	not walking	24 mo	24 mo	NA	36 mo	36 mo
+	+	+	+	+	NA	few single words	+
mild	+	+	+	+	+	mild	mild
axial	–	diffuse	diffuse	–	+	axial	diffuse
–	–	–	–	+ (transient)	–	–	–
–	–	+	+	–	+ (transient)	+	–
slightly increased tone in hands	–	–	–	–	–	increased tendon reflexes	wide-based gait
–	atypical social and behavioral features	atypical social and behavioral features	autism spectrum disorder	autism spectrum disorder	–	–	selective mutism
2 days	2 weeks	2 days	1–2 mo	1 day	3 days	2 weeks	3 days
focal with tonic stiffening and autonomic features, later clonic	focal, later tonic	focal tonic,tonic-clonic, and myoclonic, later GTCs and generalized tonic	clonic seizure with eye deviation, later GTC	focal (stopped at 2 months), later GTCs	focal tonic-clonic and tonic; status epilepticus	focal tonic-clonic, later focal or generalized	tonic, later tonic or GTCs
NA	2 years, off AEDs since 3.5 year	2 years	2 years	NA	NA	NA	NA
6–7 wk: MF epileptiform activity; 9 mo: normal	6 wk: subtle aberration R frontocentral and L temporal	neonatal: excess discontinuity, excess MF sharp waves; 2 year: intermittent generalized slowing, intermittent L temporal slowing	4 mo: normal; 17 mo: rare generalized spikes	neonatal: epileptic discharges, Lrolandic region	neonatal: excess MF spikes and sharp waves, especially bilateral temporal regions; 2 mo: background poorly organized high amplitude background, lack of state change, MF spikes	6 d: normal; neonatal/infantile: MF epileptiform activity, high amplitude slow spikes bilateral temporal; 17 mo: diphasic spikes at vertex, field to right frontocentral region, enhanced in sleep 3 year: ESES	neonatal: excessive L and R central and temporal sharp waves; 10 mo: frequent L frontocentral region spikes; 22 mo: diffuse, frontally predominant 2-3 Hz spike or polyspike and wave, up to 500uV. 3 year: mild generalized slowing, frequent Left temporal epileptiform discharges
inferior vermian hypoplasia, left retrocerebellar cyst, causing distortion of the smaller left cerebellar hemisphere and thinning of the overlying bones* (2 mo)	normal* (neonatal)	mega cisterna magna and patulous foramen Magendie, subtle cerebellar foliar distortion, hypothalamic fusion anomaly (1 wk)	mega cisterna magna, patulous foramen Magendie (1 mo)	thick corpus callosum, inferior vermian hypoplasia* (12.5 yr)	mega cisterna magna, severe foliar distortion with centrifugal orientation (3 mo)	moderate cerebellar foliar distortion (23 mo)	mild scattered subarachnoid hemorrhage structurally normal (2 mo); prominent cisterna magna and patulous foramen magendie with subtle foilar distortion (left side predominant) (31 mo)

(*Continued on next page*)

Table 2. *Continued*

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6
Treatment	carbamazepine	phenobarbital, valproate	carbamazepine	phenobarbital, P5P, pyridoxine, valproate	levetiracetam, phenobarbital carbamazepine	topiramate

Abbreviations: d, day; DD/ID, developmental delay or intellectual disability; ESES, electrical status epilepticus of sleep; GTC, generalized tonic clonic seizure; L, left; m, months; MF, multifocal; mo, month; NA, not available; P5P, pyridoxal-5-phosphate; R, right; unsup., unsupported; * brain MRI not reviewed by neurologist E.Y. from Boston Children's hospital.

demonstrated a consistent phenotype characterized by neonatal- to early-infantile-onset epilepsy, global developmental delay with variable autistic features, facial dysmorphism, and cerebellar dysgenesis (Tables 1 and 2, Figure 1; see Supplemental Note). All individuals presented with early epilepsy, the majority with onset in the first 2 weeks of life (13/14 case subjects), with one individual presenting in the 2nd month. The predominant seizure types were focal motor, and some had accompanying autonomic, tonic, and generalized tonic-clonic seizures (GTCs). One individual had myoclonic seizures. Neonatal seizures captured on EEG had either clear focal onset (often multifocal) or at times diffuse attenuation with later focal features. Most often neonatal seizures were focal and over time GTCs and tonic seizures were seen, including at times episodes of status epilepticus. Data from older individuals suggest that the epilepsy may resolve in early childhood for at least a subset. Early EEGs often showed excess sharp waves with or without mild excess discontinuity but more severe encephalopathy patterns such as burst suppression or hypsarrhythmia were not seen in this cohort. Over time, focal, multifocal, or generalized epileptiform activity were reported, and electrical status epilepticus of sleep was seen in a single individual in correlation with developmental regression. While late follow-up EEG data are limited, it has shown (when available) generalized or focal slowing in some and normalization of the background in others, with or without epileptiform activity. EEG and evolution for this cohort of individuals are summarized in Table 2. Overall, the epilepsy appears to start as focal neonatal and evolve to mixed focal and generalized epilepsy with status epilepticus in many individuals. Epilepsy appears most difficult to control in infancy with improvement after the first year of life. While larger cohorts and longer follow-up are needed for further epilepsy phenotypic characterization, there are clear patterns seen even in this initial cohort of 14 individuals. Variable degrees of global developmental delay were observed in all case subjects and behavioral disturbances in a subset (8/14 case subjects). Neurological examination evidenced hypotonia (7/11 case subjects), hand stereotypies (6/14 case subjects), nystagmus (3/14 case subjects), increased reflexes or pyramidal syndrome (2/14 case subjects), and wide-based gait (2/14 case subjects). Aside from wide-based gait, no definitive cerebellar features were noted, but the individuals were not systematically screened prospectively and many individuals have severe motor impairment. Facial dysmorphisms were variable including coarse features, hypertelorism, broad nasal root, and thin superior lip (Figure 1). Additional features included variable minor distal limb features (8/14 case subjects) and hematological disturbances (5/13 case subjects) (Table 1). Brain MRI demonstrated dysgenesis of the cerebellar folia in at least 9/14 case subjects including 7/8 case subjects available for review of original data by a board-certified pediatric neuroradiologist (E.Y.) (Figure 1B, Table 2). It was at times subtle and not mentioned on the clinical reports. Mega cisterna magna and inferior vermian hypoplasia were additionally seen in 8/14 and 6/14 case subjects, respectively (Figure 1B, Table 2). Three individuals had a hypothalamic fusion anomaly (Figure 1B, Table 2).

In the literature, 16 individuals have been described with microdeletion of chromosome 14q32.33, encompassing *PACS2*.[40] The common phenotype of these individuals includes varying degrees of developmental delay or ID and facial dysmorphism, but less consistently epilepsy (4/12 case subjects) or febrile seizures with normal EEG (1/12 case subjects). Indeed, although seizures seem prevalent in individuals with a ring chromosome 14,[41] their relationship with terminal chromosome 14q deletions remains unclear, especially since seizures in the 4-year-old boy with the smallest deletion (0.31 Mb) were possibly related to an independent family susceptibility, since his mother and maternal grandmother also had epilepsy.[40] The relationship between PACS2 haploinsufficiency has been less clearly demonstrated and may be less penetrant than in this series of individuals with a recurrent missense variant in PACS2.

The present findings are consistent with prior reports of developmental delay/ID, facial dysmorphism, and variably epilepsy in individuals with a recurrent missense variant in the related gene *PACS1*. Indeed, 19 unrelated individuals affected with ID have been reported with a recurrent causal *de novo* variant (p.Arg203Trp) in PACS1.[30–32] Despite the important PACS1/PACS2 homology, these hotspot variants do not occur at homologous positions but rather appear to affect specifically each protein function, resulting in disease (Figure S1). Moreover, individuals with PACS1

Individual 7	Individual 8	Individual 9	Individual 10	Individual 11	Individual 12	Individual 13	Individual 14
phenobarbital	phenobarbital sodium valproate	levetiracetam, phenobarbital	levetiracetam	valproate	levetiracetam, phenobarbital, oxcarbazepine	vigabatrin, levetiracetam, pyridoxalphosphate, pyridoxine, lamotrigine, valproate, clobazam	phenobarbital, pyridoxine, levetiracetam, lacosamide

p.Arg203Trp or PACS2 p.Glu209Lys variants present with some clinical similarities such as constant ID, speech delay, dysmorphic facial appearance (arched eyebrows, hypertelorism with downslanting palpebral fissures, bulbous nasal tip, wide mouth with downturned corners, and thin upper lip), as well as frequent hypotonia, behavioral disturbances, cryptorchidism, and cerebellar abnormalities. Cerebellar dysgenesis is now well defined in both cohorts of individuals with PACS1 p.Arg203Trp and PACS2 p.Glu209Lys, making a strong argument to consider this feature as a genetically determined abnormality. While neonatal seizures appear a consistent feature in individuals with the recurrent *PACS2* variant, febrile or afebrile seizures occurred in 12/19 individuals with the *PACS1* recurrent variant, being well controlled with anti-epileptic drugs.[30–32] Details of the epilepsy in this cohort are limited. The epilepsy appears to have earlier onset and be more often refractory in infancy for the individuals with the recurrent *PACS2* variant. While cardiac malformations occur commonly in individuals with the recurrent *PACS1* variant, only one individual with the recurrent *PACS2* variant present with dextrocardia.[31] These differences may reflect differences between the role of PACS1 and PACS2 proteins, or alternately may reflect differential impacts of the variants on protein function.

The *PACS1* and *PACS2* genes are broadly expressed, with selective enrichment in peripheral blood lymphocytes and spinal cord, respectively (GTEx data). Although they are both transcribed in brain tissue, PACS1 and PACS2 protein levels are differentially distributed at the cellular level, the former being enriched in neuronal centers while the latter is enriched in glial cells-enriched white matter. *PACS2* encodes a multifunctional sorting protein involved in nuclear gene expression and pathway traffic regulation, whereas PACS1 is a trans-Golgi-membrane regulator that directs phosphorylated cargo molecules.[36,42] Both proteins harbor cargo(furin)-binding regions (FBRs) that bind cargo proteins, sorting adaptors, and cellular kinase.[36] PACS1 and PACS2 appear highly localized during human prenatal brain development (see Allen Brain Atlas).[33,35] The canonical 963-amino acid PACS1 and 889-amino acid PACS2 proteins are important paralogs, sharing overall 54% sequence identity and nearly 80% sequence identity in the ~150 aa cargo (furin) binding regions (FBRs) which binds client proteins at acidic clusters that can be phosphorylated by casein kinase 2 (CK2), as well as at α helices.[43] The ID-associated PACS1 p.Arg203Trp mutation is located in the PACS1 FBR and, consequently, reduces the interaction of PACS1 with client proteins. However, the PACS2 p.Glu209Lys mutation reported here is located C-terminal to the FBR in the disordered middle region (MR) (Figure 2A). In PACS1, the MR contains an autoregulatory domain, which includes a CK2 phosphorylatable acidic cluster that reversibly binds the FBR to modulate access to client proteins.[44] Because the PACS2 p.Glu209Lys mutation is located in the corresponding acidic cluster in the PACS2 MR, it suggests that mutation of this segment may also alter the ability of the nearby PACS2 FBR to interact with client proteins. We therefore compared the ability of HA-tagged PACS2 or PACS2 p.Glu209Lys to interact with Flag-tagged client proteins, including the histone deacetylases SIRT1 and HDAC1 as well as the ion channel TRPV1. Co-immunoprecipitation analysis showed that each of the client proteins interacted, to a greater extent with PACS2 p.Glu209Lys than with WT PACS2 (Figure 2B), suggesting that the PACS2 mutation reduces the ability of the predicted autoregulatory domain to modulate the interaction between the PACS2 FBR and client proteins, which may disturb cellular function.

PACS2 has roles in both the nucleus and cytoplasm.[43] In the nucleus, PACS2 controls the SIRT1-p53-21 axis to promote cell cycle arrest following DNA damage response by directly inhibiting SIRT1-dependent deacetylation of p53.[45] In the cytoplasm, PACS2 regulates endoplasmic reticulum (ER) homeostasis, ER-mitochondria communication, autophagy, and endosomal trafficking of ion channels, receptors, and enzymes.[42,46] In response to death ligands, PACS2 switches to a pro-apoptotic effector that coordinates trafficking steps leading to Bim- and Bid-dependent lysosomal and mitochrondria outer membrane permeabilization, respectively, to trigger activation of executioner caspases and cell death.[46,47] Phosphorylation of PACS2 Ser437 by mTORC2/Akt promotes binding to 14-3-3 proteins. The phosphorylation state of PACS2 Ser437 acts like a molecular switch that separates PACS2's broad anabolic (survival) and catabolic (apoptotic) roles.[43]

The multi-functional roles for PACS2 in cell and tissue homeostasis suggest that the p.Glu209Lys mutation may

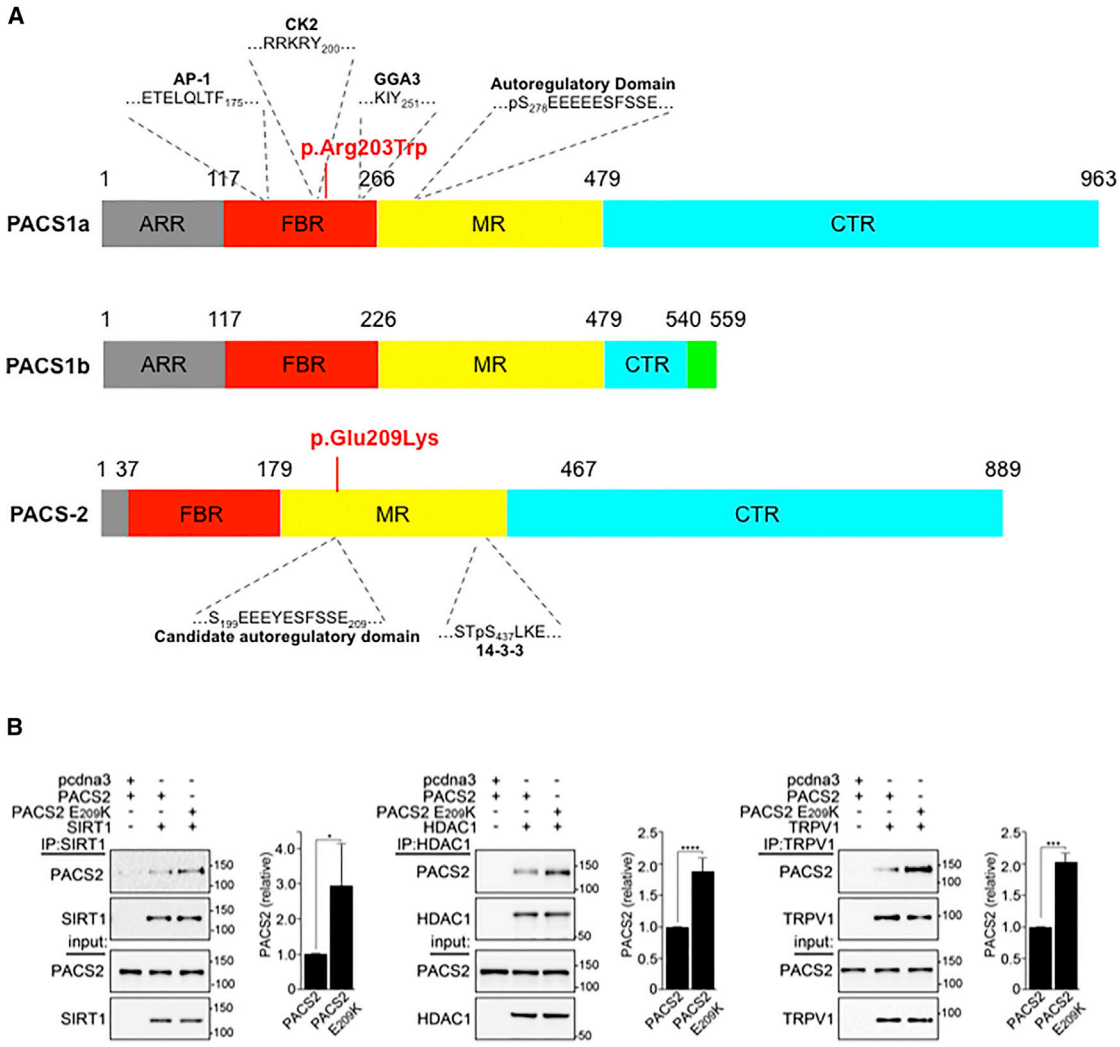

Figure 2. PACS2 Variant Location

(A) Schematic of PACS1 and PACS2 illustrating the proposed domains (ARR, atrophin-1-related region; FBR, furin (cargo)-binding region; MR, middle region; CTR, C-terminal region) and residues important for partner protein binding (AP-1, adaptor protein complex 1; CK2, protein kinase CK2; GGA, Golgi-associated γ-adaptin ear homology domain ARF-interacting protein), with location of PACS1 and PACS2 missense variants responsible for intellectual disability.

(B) HCT116 cells, which can be efficiently transfected with plasmids, expressing the indicated proteins were harvested in mRIPA (50 mM Tris-HCl [pH 8.0] plus 1% NP-40, 1% deoxycholate, and 150 mM NaCl) containing proteinase inhibitors (0.5 mM PMSF, 0.1 μM each of aprotinin, E-64, and leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 20 mM NaF). The FLAG-tagged cargo proteins SIRT1, HDAC1, or TRPV1 were immunoprecipitated with anti-Flag antibody (Sigma #F7425) and co-precipitating HA-tagged PACS2 or PACS2 p.Glu209Lys was detected by western blot using anti-HA antibody (Cell Signaling Technology #3724S) and developed with the Pierce ECL Western Blotting Substrate (ThermoFisher) using a FluorChem E image acquisition system (ProteinSimple). Signals were quantified using the AlphaView image analysis software package (ProteinSimple) and normalized to wild-type PACS2. Data are mean ± standard deviation, n = 4.

alter a putative regulatory domain that alters binding of PACS2 to one or more client proteins critical for neuron communication, neurogenesis, or cerebellar development. The increased interaction between PACS2 p.Glu209Lys and SIRT1 or HDAC1 suggest the mutation may alter deacetylase functions, such as the control of p53, that impact epilepsy or cerebellar development.[48–50] Similarly, the increased interaction between PACS2 p.Glu209Lys and TrpV1 suggest an altered function of one or more ion channels, contributing indirectly to channelopathies associated with excitability disorders.[51] Finally, the p.Glu209Lys mutation may affect important roles for mTORC2/Akt and 14-3-3 in neuronal migration and dendritic arborization.[52,53]

In conclusion, the recurrent *de novo* missense variant resulting in PACS2 p.Glu209Lys in 14 unrelated individuals with a well-defined phenotype supports causality of PACS2 variant in DEE with facial dysmorphism and cerebellar dysgenesis. The pathogenic p.Glu209Lys variant disturbs the interaction between PACS2 and its related proteins, which may alter one or more cellular functions that underlie this neurodevelopmental disease.

Supplemental Data

Supplemental Data include one figure and supplemental notes and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.03.005.

Consortia

The C4RCD Research Group includes the clinical team and laboratory research team involved in individual enrollment, sample processing, exome sequencing, data processing, preparation of variant annotation files, data analysis, validation of data, and return of research data to families. Candidate genes are identified and discussed at data analysis meetings of the entire group. The following members of the group (listed in alphabetical order) have contributed significantly to this work: Chris Balak, Newell Belnap, Ana Claasen, Amanda Courtright, David W. Craig, Matt de Both, Matthew J. Huentelman, Madison LaFleur, Sampathkumar Rangasamy, Ryan Richholt, Isabelle Schrauwen, Ashley L. Siniard, and Szabolcs Szelinger.

Acknowledgments

We thank the affected individuals and their families involved in the study and the University of Burgundy Centre de Calcul (CCuB, see Web Resources) for technical support and management of the informatics platform. The authors also thank the Genome Aggregation Database (gnomAD) and the groups that provided exome and genome variant data to this resource. A full list of contributing groups can be found at http://gnomad.broadinstitute.org/about. We thank the Integragen society and CNG for exome analysis. pTRPV1/f was a gift from D. Julius and pHDAC1/f was a gift from E. Verdin (Addgene #13820). We also thank P. Narvakar and S. Luan for assistance. The authors acknowledge the contributions of all members (current and past) of The C4RCD Research Group. This work was funded by the Regional Council of Burgundy / Dijon University hospital (PARI 2013), the French Ministry of Health (PHRC N° 2013-A00103-42), and NIH (grants R01 CA151564, DK112844, and DK114855).

Received: December 22, 2017
Accepted: February 27, 2018
Published: April 12, 2018

Web Resources

Allen Brain Atlas, http://www.brain-map.org/
AnDDI-Rares network, http://www.anddi-rares.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
GeneDx, https://www.genedx.com
GeneMatcher, https://genematcher.org/
gnomAD Browser, http://gnomad.broadinstitute.org/
GTEx Portal, https://www.gtexportal.org/home/
Human Gene Mutation Database (HGMD), http://www.biobase-international.com/product/hgmd
IGV, http://www.broadinstitute.org/igv/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
Primer3, http://bioinfo.ut.ee/primer3
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SeattleSeq Annotation 131, http://snp.gs.washington.edu/SeattleSeqAnnotation131/
SIFT, http://sift.bii.a-star.edu.sg/

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The American Journal of Human Genetics, Volume *102*

Supplemental Data

A Recurrent *De Novo PACS2* Heterozygous Missense Variant Causes Neonatal-Onset Developmental Epileptic Encephalopathy, Facial Dysmorphism, and Cerebellar Dysgenesis

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SUPPLEMENTAL DATA

Case reports

Clinical, genetic/metabolic data were reviewed, as well as EEGs and MRIs data from the medical records. Each group contributed phenotypic details for affected individuals from their center, in accordance with local Internal Review Board approved protocols. De-identified primary EEG and MRI data were reviewed by neurologist/epileptologist HEO and neuroradiologist EY from Boston Children's Hospital when available. We specifically evaluated age at seizure onset, seizure types, evolution of epilepsy over time, EEG patterns, developmental phenotype, physical and neurologic exam features, and medical signs/symptoms. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and proper informed consent were obtained.

Individual 1: a 16-year-old girl was the first child of healthy unrelated parents, with a healthy maternal half sister. Born at 38 weeks of gestation after normal pregnancy, she presented normal birth measurements (weight 3270g (-0,3 SD), height 51cm (+0,6 SD), and OFC 34 cm (+0,4 SD), as well as hypotonia, metatarsus varus and increased tendon reflexes. At day 6, she presented with focal seizures. Psychomotor development was delayed: she sat without support at 8 months of age and walked at 22 months of age. She spoke her first words at 20 months of age and her first sentence just before the age of 4 years. Her stature and weight gain were normal. Her last examination demonstrated pyramidal syndrome, slender fingers and facial dysmorphism (figure 1) including hypertelorism, long and down-slanting palpebral fissures, thick eyebrows, wide mouth with downturned corners, thin vermillion of upper lip

and everted vermillion of the lower lip, prominent frontal incisors and wide spaced teeth. She also presented with asymptomatic permanent moderate neutropenia (unclear if medication related), epilepsy requiring carbamazepine treatment and a moderate ID. Brain MRI, at 5 years of age, showed mild foliar distortion of the left cerebellar hemisphere and mega cisterna magna. Standard chromosomal analysis and metabolic assessment were normal, as well as *COH1* and *STK9* Sanger sequencing.

Individual 2: a 4-year-old girl was the second child of healthy unrelated parents. Her father had 3 maternal uncles affected with ID. Prenatal ultrasound survey had been marked by dextrocardia. At 37 weeks of gestation, weight was 3295g (median). Neonatal convulsions started at day 4 with generalized tonic-clonic seizures with eye deviation, head back, opisthotonos, and facial erythrosis. Neonatal EEG showed right central and occipital spike waves. At 3 ½ months the EEG showed right and left temporal sharp waves but an otherwise normal background and no seizures. EEG was normal after 1 year of age. Psychomotor and speech delay occurred with sitting acquired at 20 months of age and first words at 26-30 months of age. She also had a poor eye pursuit, horizontal nystagmus, sleeping and behavioral disturbances with stereotypies, and agitation. At last examination at 4 years of age, she presented with facial dysmorphism including long and down-slanting palpebral fissures, synophris, low posterior and anterior hairline, wide mouth with downturned corners and thin vermillion of the upper lip, and sacral dimple. Neurological features included a wide based gait and difficulties in fine motor skills. With valproate treatment, she was seizure free since 6 months of age. She also had a mild chronic anemia with normal white count. Examination of fundus, visual and auditory evoked potentials, and electroretinogram were normal. Brain MRI, at 5 years of age, showed inferior vermian

hypoplasia with prominent foramen Magendie and cisterna magna, severe foliar distortion of cerebellar hemispheres with centrifugal orientation, and hypothalamic fusion anomaly (Figure 1B). Standard chromosomal analysis, array-CGH and metabolic assessments were normal.

Individual 3: a 15-year-old boy was the third child of healthy unrelated parents, with a healthy older brother and sister. Born at 38 weeks of gestation after normal pregnancy, he presented with macrosomia (birth weight = 4535g (+2 SD)). Neonatal convulsions started at day 4. Psychomotor development delay was normal with independent sitting at 6 months of age and walking at 18 months of age. Speech was delayed with the first word at 24 months of age. At last examination, he presented with normal growth measurements, moderate ID needing special educational school and facial dysmorphism with long and down-slanting palpebral fissures, prominent pillars of philtrum, wide mouth with downturned corners, thin vermillion of the upper lip and everted vermillion of the lower lip, prominent frontal incisors and diastemas of the teeth (Figure 1). She also had 2/3 syndactyly of toes and cryptorchidism. Neurological examination was normal and seizures are currently controlled by carbamazepine. Brain MRI showed increased subarachnoid spaces. Standard chromosomal analysis, array-CGH, FRAXA screening and metabolic assessment were normal.

Individual 4 : a 8-year-old girl was the second child of healthy unrelated parents. Born at 35 weeks of gestation, she weighted 3380g (+1,8 SD). Generalized tonic-clonic seizures with eye deviation started at day 7. Awake EEG showed excessive multifocal sharp waves, excess discontinuity, and seizures consisting of generalized attenuation followed by frontal-central spike waves. Seizures treatment was started with phenobarbital, pyridoxal phosphate, and

pyridoxine. From age 6 months she has been treated by valproate. Brain MRI, at 3 weeks of age, showed mild inferior vermian hypoplasia with a patulous foramen Magendie and mega cisterna magna, as well as mild distortion of the cerebellar folia (Figure 1). She walked independently at 18 months of age. Speech was delayed. Seizures were well controlled on medication after 2 years of age, but convulsions recurred immediatly upon withdrawal of valproate. At last examination, she presented with normal growth parameters and facial dysmorphism with long and down-slanting palpebral fissures, short philtrum, wide mouth with downturned corners, thin superior lip and everted inferior lip, broad nasal root, and low-set ears. She also had ID and mild autistic spectrum disorder. Standard chromosomal analysis, array-CGH, and metabolic assessment were normal.

Individual 5: a 19-month-old boy was born at term with mild congenital microcephaly (birth measurements: weight = 2570g (-1.7 SD); length = 48cm (-0.9 SD); OFC = 32.9 cm (-2.3 SD)). Facial dysmorphism included epicanthic folds, short mild up slanting palpebral fissures, a thin upper lip, a wide mouth, and a broad flat nasal bridge. He had variant transverse palmar creases and clinodactyly of the fifth fingers, but the latter feature was also present in his mother and maternal grandmother. Neonatal seizures, started at day 2, presented with saturation dips to 70% and stiffening of the right arm with flexion of the left arm or general hypotonia, initially treated by levetiracetam that was stopped at 5 months of age. At 2 months he showed clonics of both arms while the eyes widened and turned up, at 4 months his seizures started with a scream, the eyes widened, the arms flexed, consequently the arms showed contractions which secondarily generalisation to all limbs, at 10 months he had his last seizure which was generalized tonic-clonic with a nystagmus. Twice he has been re-admitted because of recurrence of ongoing seizures. Both times he reacted well to a loading

dose of phenytoin and was treated by carbamazepine. Brain MRI, at 7 days of life, showed a retrocerebellar arachnoidal cyst, inferior vermian hypoplasia with prominent foramen Magendie and a mega cisterna magna, as well as severe foliar distortion of the left cerebellar hemisphere with centrifugal orientation, and hypothalamic fusion anomaly (Figure 1). Initial two long lasting neonatal EEG 's were unremarkable. EEG at 4 months showed intensive multifocal epileptic discharges at the left hemisphere more than at the right, with once a clinical seizure starting left parieto-occipitally, with secondary generalization. Two days later the EEG showed frequent ictal generalized discharges, with a possible focal start tempero-occipitally at both sides. Four days thereafter, i.e. the last EEG recording, just after his seizures were stopped, showed diffuse slowing without epileptic discharges. At last examination (19 months), growth parameters were: weight -1,0 SD, height -1,8 SD, and OFC -1,9 SD. Psychomotor development was delayed but improved after phenobarbital treatment was discontinued at 14 months of age. He crawled and made a few assisted steps at 18 months of age. He spoke his first words at 19 months of age. Ophthalmologic evaluation revealed strabismus and he had a mild chronic anemia. SNP-array was normal. Whole Exome Sequencing showed, besides the variant in *PACS2*, a de novo variant of unknown significance in *CHD1*.

Individual 6: a 8 year-old boy was the fifth child of healthy unrelated parents. He was born at 40 weeks of gestation after a normal pregnancy, and initial exam demonstrated macrosomia (Birth mensurations : Weight = 4730g (+2.5 SD); Length = 54.5cm (+ 2SD); OFC = 40.5 cm (> 3SD)). Epileptic seizures occurred on day of life 2. EEG showed left temporal spikes. Psychomotor development was delayed: he sat without support at 16 months of age and walked autonomously after 22 months of age. No verbal language (except 3- 4 words).

He presented also obsessive compulsive disorder and stereotypies. Physical examination showed facial dysmorphism including long and down-slanting palpebral fissures, thin vermillion of upper lip, fine eyebrows, large ears and small mouth. Brain MRIs at 10 days and 4 months of age was normal. At 3 years and 6 months of age, he presented seizure without any fever.

Individual 7: a 13-month-old boy was born at term with normal birth mensurations (weight = 3860g (+1 SD); length 53.4cm (+ 1SD); OFC 35cm (median)). Facial dysmorphism included slight down-slanting palpebral fissures, break in the left brow, short, depressed and slightly broad nasal bridge, rounded nasal tip, wide mouth with downturned corners, and bilateral uplifted ear lobes. Initially focal seizures started at day 2 with autonomic features and consisted of isolated stiffening with apnea and bradycardia, without convulsions. Seizures later evolved to include clonic movements. He developed epilepsy controlled by phenobarbital. Brain MRI, at 2 months of age, showed inferior vermian hypoplasia and left retrocerebellar cyst causing distortion of the smaller left cerebellar hemisphere and thinning of the overlying bones. This cyst was in communication with the fourth ventricle through the widened foramen of Magendie. No cyst membrane was identified. The differential considerations included a Blake pouch cyst or arachnoid cyst. EEG recording at 7 weeks of age showed bi-temporal sharp waves with right predominance and right mixed frequencies of predominantly delta and theta waves. During sleep, the background was higher in amplitude and contains more slow activity with brief periods of relative suppression (persistent trace alternans). The last 24 hour EEG recording at 9 months of life was normal. Psychomotor development was delayed with decreased axial tone and slightly increased tone in hands: no independent sitting at 8 months of age and no walking/cruising at 16

months of age. At last examination (16 months), growth parameters were normal. He presented with undescended testicle and decreased hearing thought to be due to ear infections/fluid in ears. Ophthalmologic evaluation was normal and he had a mild chronic anemia with normal white count. Standard chromosomal analysis, array-CGH, FRAXA screening, and metabolic assessment were normal.

Individual 8: a 5-year-old girl, is the first child of healthy unrelated parents. Born at 37 weeks of gestation after pregnancy complicated by preeclampsia, she presented small for gestational age (weight = 2010g (-3 SD); length = 48cm (-1.5 SD); OFC = 32.5cm (-2.3SD)). Two weeks after birth, she presented with focal epilepsy followed by generalized seizures with sudden cramping/stiffness, turning away eyes, and no reaction. Convulsions occurred during several minutes but total recovery took several hours. Her last seizure occurred at 2 years of age. At last examination she did not need any medication. Brain Magnetic Resonance Imaging (MRI) in neonatal period was normal. Psychomotor development was delayed: she sat without support at 10 months of age and walked at 27 months of age. Speech was delayed, she spoke her first words around 2 years of age. Speech development took a giant stride when she was 3,5 years old and antiepileptic drugs were stopped. Physical examination showed facial dysmorphism including hypertelorism and a broad nasal bridge, mild down-slanting of the palpebral fissures, wide mouth with thin lips and a broad forehead. SNP-array analysis showed two paternally inherited duplications (1p13.3 and 11q22.3), as well as one maternally inherited duplication (Xq23). Sequencing of a targeted NGS panel on epilepsy revealed a variant of unknown significance in the *SLC9A6* gene (X-linked intellectual disability type Christianson), which turned out to be paternally derived as well. Whole-exome Sequencing showed, besides the variant in *PACS2*, a *de novo*

heterozygous variant of unknown significance in the *FBXO31* gene [MIM 609102], that has been one time reported in autosomal recessive mental retardation [MIM 615979].

Individual 9: a 3-year-old girl, the third child of healthy unrelated parents, was born full term by repeat c-section (weight = 3470g (median), length = 48cm (- 2SD) and OFC = 34.5cm (median)). She has congenital hypotonia, severe global developmental delay without regression, neonatal-onset epilepsy with tonic and tonic-clonic seizures including episodes of status epilepticus, oropharyngeal dysphasia, and visual dysfunction. She first presented with seizures on day of life 2, described as head thrusting back and eyes rolling up or head and eye deviation with variable progression to a generalized convulsion. The predominant early seizure type was generalized tonic. Initial EEG pattern showed excess discontinuity, multifocal sharp waves (frequent left frontal-central), and focal tonic seizures associated with generalized decrement followed by focal left fronto-central rhythmic sharp waves (age 3-5 days). Subsequent seizures continued to involved bilateral tonic activity, at times with a more complex semiology ending in myoclonic jerks associated with generalized periodic discharges (3 weeks of age), with generalized and bifrontal electrographic features. She had multifocal myoclonus, variably correlating with focal spikes on EEGs in the first month. She developed over time bilateral tonic and tonic-clonic seizures with head deviation, including episodes of status epilepticus at 5 months and 6 months. Seizures spaced in the second half of her first year to every 1-2 months, at times in clusters then as of 11 months she was seizure free for >2 years. She had recurrence of two GTCs, 1 month apart only in the setting of weaning off of phenobarbital to off at age 3.5 years. She continued on levetiracetam monotherapy. Formal developmental testing at 33 months of age evidenced an overall age-equivalent level of 10 months. She obtained the following composite scores on the Bayley-

III: 55 for cognitive, 53 for language and 46 for motor composites. She sat at 18 months, and has been cruising but not walking independently since approximately 2.5 years. She has behavioral challenges, limited understanding and communication skills (nonspecific babbling only, no communicative gestures), and sensory sensitivities. Using BITSEA and M-CHAT-R measure, she demonstrated somewhat more behavior concerns and somewhat less social/behavioral competence than expected for her age. She had twisting type hand stereotypies. On last exam, she had coarse facial features, diffuse hypotonia, limited eye contact but some tracking, unable to follow commands but responsive to mother's voice. She has oropharyngeal dysphasia requiring thickened liquids. Gastrostomy tube was used only for medications at this time. MRI brain, at 1 week of age, showed mega cisterna magna and patulous foramen Magendie, subtle cerebellar foliar distortion, and hypothalamic fusion anomaly (Figure 1B). Extensive blood, urine and CSF metabolic investigations were normal, as well as array-CGH. GeneDx infantile epilepsy panel evidenced a missense variant of unknown significance in the *NRXN1* gene (c.2310G>T, p.Gln770His).

Individual 10: a 7-year-old boy, 4th child of parents who are consanguineous (3rd cousins), was born at term with no perinatal distress. Birth mensurations were not available. He presented initially with seizures at 1 month of age. Seizure types were predominantly tonic clonic seizures especially with illness or fever. Breath-holding spells were another trigger. Initial EEG pattern was normal. Initially he had up to 5 admissions in 1 month for seizures, but gradually they spaced out to approximately 5 in one year then seizure free for 2 years at last follow-up. Last EEG showed rare generalized spikes. Brain MRI, at 1 month of age, showed mega cisterna magnaa with patulous foramen Magendie (Figure 1B). He had a diagnosis of autism spectrum disorder. He sat independently at 12 months, cruised at 18

months and walked independently at 2 years. He had a few words which are repetitive. Last examination showed mild facial dysmorphim and bilateral palmar creases, limited words and echolalia, and hand stereotypies (flapping, hitting own head). Extensive blood, urine and CSF metabolic investigations were normal, as well as array-CGH. GeneDx infantile epilepsy panel evidenced a heterozygous variant of unknown significance in the *RELN* gene (c.8005G>A - p.Val2669Ile, NM_005045.3) which is not thought to explain his symptoms.

Individual 11: a 12.5-year-old boy was the second child of healthy unrelated parents. At 39.5 weeks of gestation, after normal pregnancy, he was born by caesarian section. He presented with normal birth measurements (weight = 3,510g (median); length = 48cm (- 1 SD); OFC = 35cm (median)). At day 1, he presented with feeding difficulties, transient nystagmus, focal seizures evolving in generalized tonic clonic type, treated with valproate and vigabatrin. The last seizures occurred at 2 months of age, and he has been off antiseizure medications since 2 years of age. EEG showed focal epileptiform discharges in the left rolandic region. Growth and weight mensuration's were normal with OFC at - 1, 8 SD. He presented with language and developmental delay (walking at 2 years of age), behavioral disturbances with self-harm behavior and stereotypies. Facial dysmorphism included brachycephaly, long horizontal palpebral fissures, large nasal bridge, bulbous nasal tip, and long smooth philtrum and thin superior lip. Additional features include V finger clinodactyly, micropenis, testicular ectopia, partial hypothyroid and central precocious puberty. High resolution chromosomal, metabolic screening *SNRPN* methylation analyses were normal. *PACS1* causing gene was also clinically evocated. Brain MRI, at 12.5 years of age, revealed thick corpus callosum and inferior vermian hypoplasia.

Individual 12: a 9-month-old female was the second child of healthy, unrelated parents. The pregnancy was complicated by gestational diabetes requiring insulin and pre-term labor, treated with terbutaline. She was delivered at 34 weeks of gestation (weight = 3090 g (+0.5 SD); length = 45.7 cm (-1.5 SD)) and was kept in the neonatal intensive care unit for blood sugar monitoring. On the third day of life she had her first seizures, which started with eyelid fluttering. Intermittent EEG monitoring between 3 and 30 days of life showed excessive multifocal spikes and sharp waves especially in the bilateral temporal regions, interpreted as indicative of underlying cortical irritability with increase susceptibility for seizures. Brain MRI at 3 days showed small scattered subarachnoid and intraventricular hemorrhages. A head ultrasound was normal. Seizures became stereotyped: she would bring her arms to her chest, keep her hands fisted, head and eyes would turn to the right, and she would have shaking of the right leg. Seizures lasted less than a minute, and occurred 6-7 times a day. She was treated with levetiracetam and phenobarbital, but seizures persisted. She was discharged home at 35 days of age. At 2 months of age, she was having 3-4 seizures each day presenting as one-minute episodes of stiffening. An EEG at that time was significantly abnormal with persistent multifocal seizure discharges and an abnormal background. At 3 months of age, she had an episode of status epilepticus and was placed on oxcarbazepine, and has not had any further seizures. Brain MRI, at 3 months of age, showed mega cisterna magna and severe foliar distortion with centrifugal orientation (Figure 1B). Review of prior imaging studies showed that the cerebellar abnormality was indeed present on her initial MRI done at 3 days of age. She made slow progresses in her development. At 7 months, head control was improving, she was able to grab objects with her hands, brought her hands to her mouth, could push up in a prone position, and was attempting to roll. She was babbling and “razzing”, smiling, giggling, and playing social games. At most recent

examination, at 7 months of age, physical findings included hypertelorism, broad nasal root, with depressed nasal bridgen bulbous nasal tip, thin superior lip, wide mouth with downturned corners, brachycephaly, long philtrum, and inverted nipples. Appendicular tone was normal but she had truncal hypotonia. She had minimal head lag. She was attentive, babbled, smiled appropriately, and fixed and follows with her gaze. She performed constant tongue thrusting. Blood screen for CDGs was normal.

Individual 13 : a 3.5-year-old female was the second child of healthy, unrelated parents. Her older sister, one half-brother and one half-sister were healthy and there was no family history of epilepsy or intellectual deficiency. She was delivered at 33 weeks of gestation, after an uncomplicated pregnancy (weight = 2270 g (-0.5 SD); height = 47 cm (median); OFC = 32.5 cm (median)). She had atrial septal defect and sacral pit. Seizures started during the first week of life and were characterized by bilateral, asymmetrical tonic stiffening, leading to opisthotonus, apnea and bradycardia with erythrosis or cyanosis, eye and head deviation, followed by a right or left side hemiclonic or a bilateral clonic seizure, which could end by focal clonic shaking of one limb. Seizures were organized in clusters of 10 – 13 over 10 hours, lasting 1-5 minutes each. Seizure activity remained high during the first year, with nearly weekly seizure clusters. Then seizures frequency slowed down, mainly triggered by fever and infections with 1-5 seizures per cluster. At age of 3 years, she has seizure free intervals of 3 to 6 months. No other seizure type occurred. Initial EEG at 6 days of age was normal. At age of 2 months (43 weeks of gestation) multifocal epileptiform activity appeared with prominent high-amplitude slow spikes in both temporal regions and small rapid spikes on vertex and right, rarely left fronto-central regions. Two seizures were recorded showing prominent diffuse ictal decremental activity, which was diffuse or predominant on anterior

regions, followed rhythmical theta activity emerging from right frontocentral area, evolving then over both central regions, intermixed with spikes, with highest amplitude on the vertex. Paroxysmal activity stopped earlier on right frontocentral region, and was then more prominent over the left side, diffusing to temporo occipital region. One of the seizures were followed by a marked postictal depression. A postictal alternating pattern with amplitude attenuations of 1-2 seconds resolved after a few minutes. At evolution, interictal abnormalities became rare, background rhythm nearly normalized with normal sleep spindle development. At age 17 months, diphasic spikes occurred at vertex spreading to the right frontocentral region, enhanced in sleep. She was treated initially with Vigabatrin, Levetiracetam, Valproate and Lamotrigine, which was stopped and switched to Clobazam at age of 3 years because of significant potentiation of the right fronto-central spike wave focus becoming subcontinuous at wake with diffusion to right temporal - occipital and to left frontal - central region. In sleep there was continuous right hemispheric paroxysmal activity with maximal amplitude over the right frontocentral area, spreading significantly to left side. Some degree of regression was noticed, concomitant to electrical status epilepticus predominating on right hemisphere. After withdrawal of Lamotrigine, she recently started to make a few steps wit support. She can speak a few words. She has good visual and interactive contact but has some stereotypies (rocking and hand friction with emotion). Neurological examination showed increased tendon reflexes, without other features of pyramidal syndrome. She had some dysmorphic features: hypertelorism, broad nasal root, thin vermillon of upper lip, and wide mouth with downturned corners. Brain MRI, at 23 months of age, showed moderate cerebellar foliar distortion (Figure 1B). Metabolic screening and array-CGH were normal. A 90-genes panel for monogenic epilepsies was negative.

Individual 14: a 5.5-year-old Chinese female was the first child born to this set of healthy unrelated parents. Her 18 year-old paternal half-sister was healthy. The family history was negative for learning disabilities, intellectual disabilities, autism spectrum disorders, birth defects, multiple miscarriages, sudden death, seizures, and consanguinity. She was delivered at 39 WG with macrosomia (weight = 4.035 g (+ 0.6 SD); length = 49.5 cm (+ 0.9 SD); OFC = 34.5 cm (+ 0.1 SD). She had several episodes of tonic posturing of her upper extremities, some associated with bradycardia and duskiness around 50 hours and was placed on video EEG monitoring which demonstrated that these episodes did have electrographic correlates, suggestive of tonic seizures. She was treated with phenobarbital with resolution of the seizures. She was initially treated empirically with ampicillin and gentamicin for neonatal sepsis, aciclovir for HSV infection, which was discontinued after a negative infectious work-up. Her newborn state screen was negative. Her neurological exam was normal. Her work-up in the NICU included a MRI brain, showing mild underopercularisation and white matter heterogeneity, which was felt to be within normal limits and a small hemorrhage in the left superior parietal cortex likely due to birth trauma, magnetic resonance spectroscopy which was normal, lactate/pyruvate which was negative for infection, normal cerebrospinal fluid and serum lactate, and elevated serum pyruvate due to feeds. Due to her clinical stability and seizures, which were easily controlled, she was discharged home on day of life 9. phenobarbitol was weaned around 6 weeks of life, but at 2 months of life she presented with seizures consisting of initial eye deviation then whole body stiffening and was restarted on phenobarbitol as well as pyridoxine and levetiracetam, the later was subsequently weaned and lacosamide was added after recurrent breakthrough partial seizures. She had several recurrent partial as well as generalized seizures with viral illnesses. Her last clinical

seizure was at about 4 years old. A trial of weaning of levetiracetam was halted after demonstration of electrographic seizures, and she has been stable maintained on pyridoxine, levetiracetam, and Lacosamide. Her development was delayed as she was noted to be hypotonic with an ataxic gait. A comprehensive developmental assessment at 4 years of age demonstrated cognitive and language skills at an approximate 3 to 3.5 year level, with normal receptive but decrease expressive language skills. She continues to demonstrate low tone, normal deep tendon reflexes, and a wide based steady gait, with selective mutism. Her laboratory evaluations included comprehensive cerebrospinal fluid, urine and blood metabolic studies including cerebrospinal fluid neurotransmitters that were normal. At 31 months of age, brain MRI showed prominent cisterna magna and patulous foramen magendie with subtle foilar distortion, predominant on left side (Figure 1B). Genetic testing included a pyridoxine-dependent seizure panel (*SCN1A, ALDH7A1*), *AASA, PC6, FOXG1, CDLK5, MEF2C* del/dup studies and GeneDx Comprehensive Epilepsy panel (53 genes). All were normal except the latter returned a heterozygous missense variant of unknown clinical significance in the *PNKP* gene. Biallelic mutations in this gene are associated with microcephaly, Seizures and developmental delay. Whole exome sequencing revealed compound heterozygosity of variants of unknown significance in the *AHNAK* and *XRCC1* genes. Collaboration with Keith Caldecott and lab members Richard Hailstone and Nicholas Hoch, failed to demonsttrate a functional significance in *XRCC1* activity in lymphoblasts derived from this individual (data not shown).

Acknowledgments

The following members of the C4RCD group (listed in alphabetical order) have contributed significantly to this work: Chris Balak, Newell Belnap, Ana Claasen, Amanda Courtright, David

W. Craig, Matt de Both, Matthew J. Huentelman, Madison LaFleur, Sampathkumar Rangasamy, Ryan Richholt, Isabelle Schrauwen, Ashley L. Siniard, Szabolcs Szelinger.

Figure S1 : Alignment of the PACS1 and PACS2 proteins with location of the p.Arg203Trp and p.Glu209Lys showing protein homoly.