

## De Novo Mutations in *SIK1* Cause a Spectrum of Developmental Epilepsies

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Developmental epilepsies are age-dependent seizure disorders for which genetic causes have been increasingly identified. Here we report six unrelated individuals with mutations in salt-inducible kinase 1 (*SIK1*) in a series of 101 persons with early myoclonic encephalopathy, Ohtahara syndrome, and infantile spasms. Individuals with *SIK1* mutations had short survival in cases with neonatal epilepsy onset, and an autism plus developmental syndrome after infantile spasms in others. All six mutations occurred outside the kinase domain of *SIK1* and each of the mutants displayed autophosphorylation and kinase activity toward HDAC5. Three mutations generated truncated forms of *SIK1* that were resistant to degradation and also showed changes in sub-cellular localization compared to wild-type *SIK1*. We also report the human neuropathologic examination of *SIK1*-related developmental epilepsy, with normal neuronal morphology and lamination but abnormal *SIK1* protein cellular localization. Therefore, these results expand the genetic etiologies of developmental epilepsies by demonstrating *SIK1* mutations as a cause of severe developmental epilepsy.

Developmental epilepsies are age-dependent pediatric seizure disorders that include early myoclonic encephalopathy,<sup>1,2</sup> Ohtahara syndrome,<sup>3,4</sup> malignant migrating partial seizures of infancy,<sup>5</sup> infantile spasms,<sup>6</sup> and Lennox-Gastaut syndrome.<sup>7</sup> These disorders are often devastating, with outcomes ranging from catastrophic neonatal epilepsy with short survival to severe developmental disability, autism, and life-long intractable epilepsy.<sup>8,9</sup> Some children present with early myoclonic encephalopathy or Ohtahara syndrome and then progress to infantile spasms and Lennox-Gastaut syndrome as they mature.<sup>10,11</sup> Mutations have been identified in several genes in individuals with developmental epilepsies, clustering in several biological pathways, and it is common for persons with similar mutations in genes such as *ARX*<sup>12,13</sup> (MIM 308350), *CDKL5*<sup>14,15</sup> (MIM 300672), *MEF2C*<sup>16,17</sup> (MIM 613443), and *STXBP1*<sup>18,19</sup> (MIM 612164) to present with a spectrum of developmental epilepsies. However, in many the cause remains unknown.

As part of an ongoing genetic research program on developmental epilepsies, we performed genetic analysis in 101 individuals for whom clinically available molecular genetic tests had failed to detect a mutation. The genetic analysis was performed in accordance with informed consent procedures approved by the Institutional Review Boards at the University of Rochester Medical Center, Washington University, and Seattle Children's Hospital. Clinical records, electroencephalogram (EEG) tracings and reports, and MRI scans were reviewed retrospectively.

In this study, we present six unrelated children with developmental epilepsies in whom mutations in the salt-inducible kinase *SIK1* (RefSeq accession number NM\_173354.3) were identified.

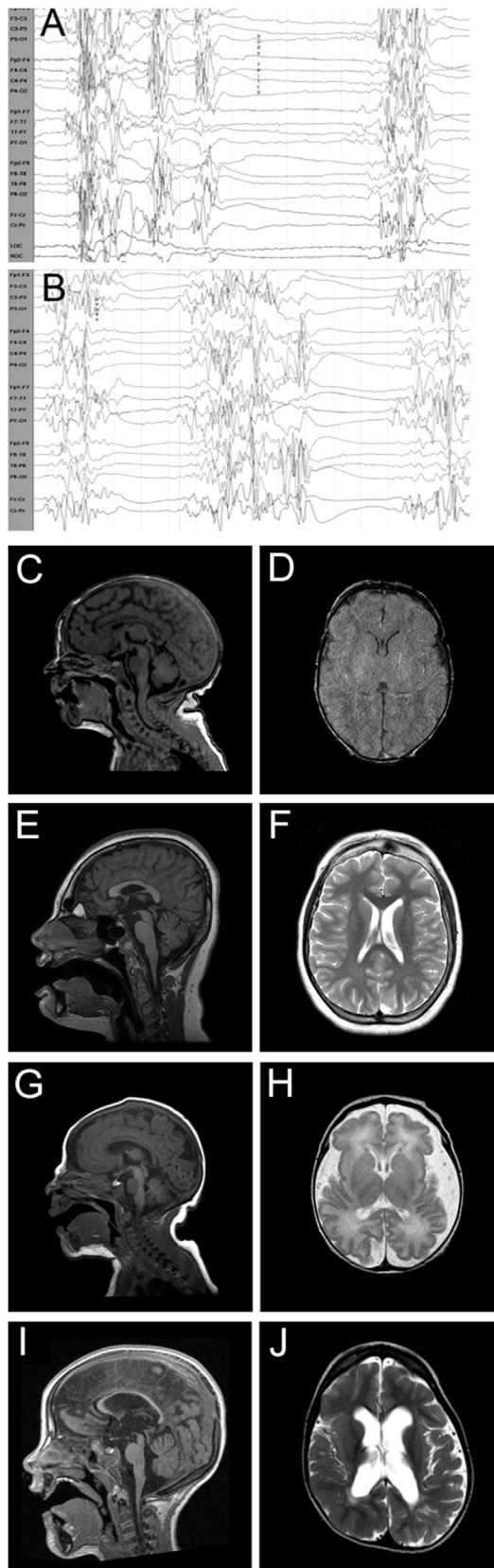
The first mutation in *SIK1* was identified through research-based exome sequencing in subject DB13-001, a boy born at term who presented with seizures consistent with early myoclonic encephalopathy at 20 min of life. An EEG on the second day of life showed a discontinuous background with burst suppression pattern (Figure 1A). He developed intermittent myoclonic jerking movements, and EEG on day 14 of life showed continuous high-amplitude burst activity alternating with periods of voltage suppression. Seizures did not respond to anticonvulsants, and an extensive metabolic and genetic workup was negative. He developed tonic seizures, burst suppression pattern persisted at 8 months of life (Figure 1B), and myoclonic seizures worsened. Brain MRI showed no structural malformations (Figures 1C and 1D). He died after complications of a respiratory illness at 10 months of age. Further clinical details are available in the Supplemental Data.

After clinical chromosomal microarray and epilepsy gene panel sequencing was negative, we performed whole exome sequencing (WES) on saliva-derived DNA from subject DB13-001 and both parents. We used the Agilent SureSelect 50 Mb whole exome capture kit, and 100 bp paired-end reads were generated on an Illumina HiSeq2500 machine at the University of Rochester Genomics Research

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**Figure 1. EEG and Brain Imaging Data for Individuals with *SIK1* Mutations**

(A) Representative EEG tracings from subject DB13-001 at 10 days of age showing burst suppression pattern with myoclonic activity, consistent with a diagnosis of early myoclonic encephalopathy.

Center. Sequence was aligned to hg19 via BWA v.0.6.2 and analyzed with Picard v.1.84, SAMtools v.0.1.18, and GATK v.2.3-9. Mean read depth across the exome was  $72\times$  determined by GATK's DepthOfCoverage walker, with 89% of bases covered at more than  $20\times$ . Annotation of variants was performed with ANNOVAR, and de novo, autosomal-recessive, and X-linked variants were identified and common variants in dbSNP 137 excluded via SOLVE-Brain v.1.0.1. Common population variants were identified with the NHLBI Exome Variant Server. Ensembl's Variant Effect Predictor provided SIFT and PolyPhen predictions of pathogenicity. GERP scores were obtained from the UCSC Genome Browser. Our analyses revealed four de novo heterozygous and two compound heterozygous variants in this individual (Table S1).

Of particular interest was a de novo heterozygous c.895C>A (p.Pro287Thr) variant identified in *SIK1* (RefSeq NM\_173354.3) and validated with standard bidirectional Sanger sequencing (primer sequences provided in Table S2) (Figure 2A). Though the p.Pro287Thr amino acid substitution in *SIK1* was not predicted to be deleterious by SIFT or PolyPhen, and the amino acid residue was not highly conserved (Table 1), this genomic variant was not present in  $>6,500$  individuals in the NHLBI Exome Variant Server or in the  $>61,000$  individuals in the Exome Aggregation Consortium (ExAC) Browser. The other de novo heterozygous and compound heterozygous variants were determined not to be compelling disease-causing candidates, as detailed in Table S1.

*SIK1* is a member of the AMP kinase subfamily with several roles in the central nervous system including regulation of the circadian clock<sup>20</sup> and transcription of corticotropin-releasing hormone in the hypothalamus.<sup>21</sup> *SIK1* abundance and activity are also increased by stimulation with adrenocorticotrophic hormone (ACTH),<sup>22</sup> which is interestingly a first-line treatment for infantile spasms.<sup>23</sup> ACTH triggers phosphorylation of *SIK1* in a Protein Kinase A (PKA)-dependent manner,<sup>24</sup> resulting in movement of activated *SIK1* from the cytoplasm into the nucleus, where it in turn phosphorylates HDAC5.<sup>25</sup> Phosphorylation of HDAC5 allows *MEF2C* transcriptional activity to proceed.<sup>26</sup> *MEF2C* is a transcription factor important in both dorsal and ventral neuronal developmental pathways<sup>17</sup>

(B) At 8 months of age, the EEG had not improved, with continued burst suppression pattern associated with both myoclonic and tonic seizures.

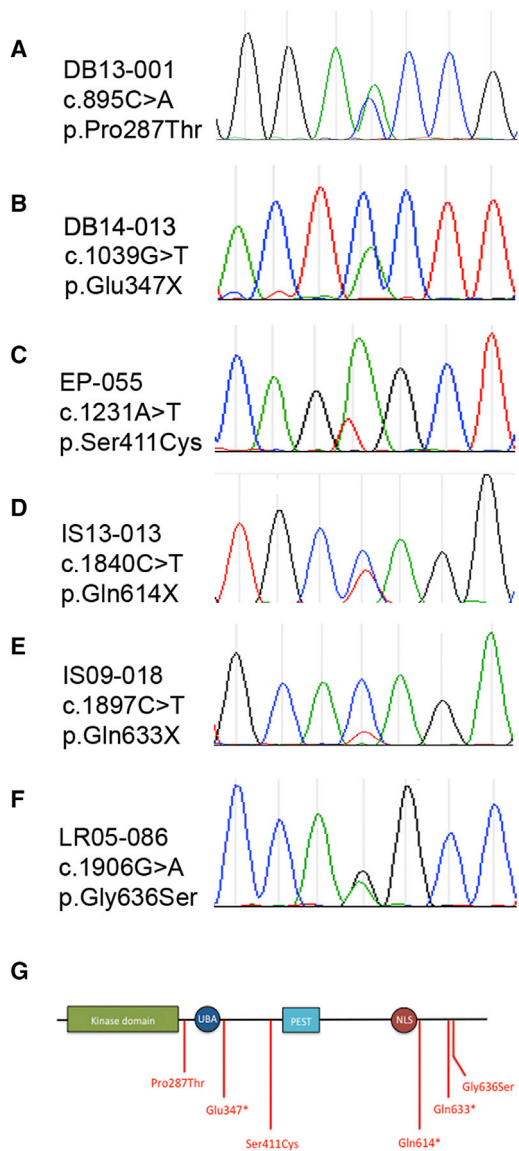
(C–J) MRI scans of subjects with *SIK1* mutations showed subtle abnormalities.

(C and D) Subject DB13-001 (p.Pro287Thr) at age 1 week showed normal morphology.

(E and F) Subject IS13-013 (p.Gln614\*) at age 12 years showed low forehead, mild shortening of the corpus callosum, and mild atrophy of the cerebellar vermis.

(G and H) Subject LR05-086 (p.Gly636Ser) at 3 days of age with increased extra-axial spaces and simplified gyral pattern in the frontal lobes.

(I and J) Subject LR05-086 at age 16 months showed thinning of the corpus callosum and asymmetric thinning of the white matter.



**Figure 2. Heterozygous Mutations in *SIK1***

(A) Whole exome sequencing identified a c.895C>A variation resulting in a p.Pro287Thr amino acid substitution, confirmed de novo by Sanger sequencing of proband and parents.

(B–F) Unidirectional Sanger sequencing of the coding and intron-exon boundaries of *SIK1* in 100 subjects with developmental epilepsy identified heterozygous mutations in 5 additional unrelated subjects, including c.1039G>T (p.Glu347\*) (B), c.1231A>T (p.Ser411Cys) (C), c.1840C>T (p.Gln614\*) (D), c.1897C>T (p.Gln633\*) (E), and c.1906G>A (p.Gly636Ser) (F). *SIK1* sequences were compared to reference NM\_173354.3. Primer sequences used are provided in Table S2. Sequence variations not found in dbSNP 137 were then confirmed with bidirectional sequencing, and all were confirmed de novo except for DB14-013 and IS09-018 where parental DNA was unavailable.

(G) *SIK1* contains a protein kinase domain (green), a ubiquitin binding area (UBA), a proline-glutamate-serine-threonine (PEST) domain, and a nuclear localization signal (NLS). Amino acid substitutions (red) resulting from mutations in *SIK1* occurred in regions outside of known domains and in a cluster between the nuclear localization signal and the C terminus.

and its expression is decreased with deficiency of the early forebrain transcription factor *ARX*.<sup>27</sup> Loss-of-function mutations in both *ARX* and *MEF2C* cause developmental epilepsies including infantile spasms.<sup>12,16</sup>

*SIK1* also regulates CREB1 transcriptional activity via phosphorylation of TORC1 in an LKB1-dependent manner,<sup>28</sup> resulting in downstream effects on dendrite growth during neuronal development.<sup>29</sup> Recent transcriptome studies of epilepsy have identified CREB-target genes as involved in seizure pathogenesis.<sup>30</sup> *SIK1* is therefore positioned to interact with the *MEF2C* pathway known to be important in developmental epilepsies and the *CREB1* pathway implicated in epilepsy pathogenesis.

Because some authors have found mutation tolerance scores helpful in determining a gene's likelihood to cause disease,<sup>31</sup> we performed this evaluation for *SIK1*. With the NHLBI EVS, we confirmed that *SIK1* has 13 common (mean allele frequency > 0.1%) variants out of 105 coding variants, with a residual variance intolerance score (RVIS) of  $-0.2626$ , which falls at the 35<sup>th</sup> percentile.<sup>32</sup> Other authors have arbitrarily chosen RVIS scores below the 25<sup>th</sup> percentile as associated with pathogenicity in epileptic encephalopathies.<sup>31</sup> However, given that several known epileptic encephalopathy genes have RVIS scores between the 35<sup>th</sup> percentile (for *FOXG1* [MIM 613454]) and the 58<sup>th</sup> percentile (for *MEF2C*),<sup>32</sup> we question the usefulness of this statistical approach to identifying causal mutations in genes, particularly in genes associated with rare but very specific phenotypes.

To determine whether mutations in *SIK1* are present in other subjects with developmental epilepsies, we performed Sanger sequencing of the coding regions of *SIK1* in 100 subjects with developmental epilepsies and identified five additional mutations (Figures 2B–2F), including three truncations and two missense mutations. The mutations were de novo in all subjects where parental DNA was available (four out of six children, including our initial proband). None of these mutations were present in >6,500 individuals in the NHLBI EVS or in >61,000 individuals in the ExAC Browser. The three nonsense mutations were all predicted to result in a truncated protein. None of the mutations identified in *SIK1* in our subjects with developmental epilepsies localized to the functional kinase domain of the protein (Figure 2G) or other well-characterized regulatory regions.

A truncating mutation (c.1039G>T [p.Glu347\*]; RefSeq NM\_173354.3) was identified in postmortem brain tissue from subject DB14-013, a male infant who presented with early myoclonic encephalopathy shortly after birth with death at 3 months of age attributed to intractable seizures. Three subjects presenting with infantile spasms (EP-055, IS13-013, and IS09-018) subsequently developed intractable epilepsy and an autism plus developmental disorder with absent speech, impaired socialization, and repetitive behaviors. Brain MRI in our subjects was either normal (Figures 1C and 1D), showed mild hypoplasia of the frontal lobes (Figures 1E and 1F), or in one subject



**Table 1. Summary of *SIK1* Mutations Found in Six Subjects with Developmental Epilepsy**

Subject	Genomic Coordinates (hg19)	cDNA	Amino Acid Substitution	Inheritance	Present in EVS	Present in ExAC	SIFT/PolyPhen	GERP
DB13-001	chr21: g.44840227G>T	c.895C>A	p.Pro287Thr	de novo	no	no	0.22/0.081	-1.55
DB14-013	chr21: g.44839819C>A	c.1039G>T	p.Glu347*	ND	no	no	-	5.16
EP-055	chr21: g.44839247T>A	c.1231A>T	p.Ser411Cys	de novo	no	no	0.03/0.751	-0.88
IS13-013	chr21: g.44837559G>A	c.1840C>T	p.Gln614*	de novo	no	no	-	4.07
IS09-018	chr21: g.44837502G>A	c.1897C>T	p.Gln633*	ND	no	no	-	4.07
LR05-086	chr21: g.44837493C>T	c.1906G>A	p.Gly636Ser	de novo	no	no	0.46/0.093	-0.48

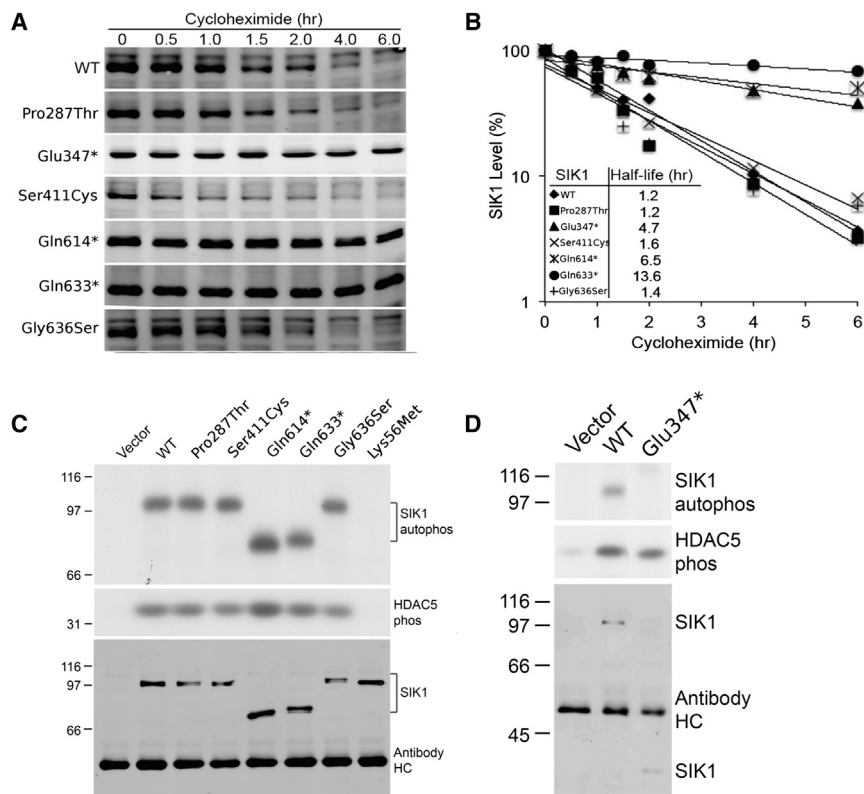
Abbreviations are as follows: ND, no data because parental samples not available; EVS, NHLBI Exome Variant Server; ExAC, Exome Aggregation Consortium; SIFT, Sorting Intolerant From Tolerant score; PolyPhen, Polymorphism Phenotyping score; GERP, Genomic Evolutionary Rate Profiling score.

exhibited simplification of the gyral pattern and asymmetric thinning of the white matter (Figures 1G–1J). These are nonspecific findings commonly identified in individuals with severe developmental disability. Genomic features of the *SIK1* mutations are summarized in Table 1 and further clinical details are in the Supplemental Data.

Because *SIK1* is known to exhibit a high rate of turnover in C2C12 cells and primary myoblasts,<sup>33</sup> we first tested whether the mutations identified affected protein stability. Wild-type and variant forms of *SIK1* were expressed in HEK293T cells by transfection, and the half-life of each protein determined by cycloheximide treatment and quantitative immunoblotting. Our analysis revealed that the c.1039G>T (p.Glu347\*), c.1840C>T (p.Gln614\*), and c.1897C>T (p.Gln633\*) proteins exhibited an increase in stability compared to wild-type *SIK1* (Figures 3A and 3B). Phosphorylation of Thr473 by PKA increases the half-life of *SIK1*, as does deleting an internal segment (residues 451–472) that resembles a PEST sequence.<sup>33</sup> Thus, the absence of these residues in the p.Glu347\* protein probably explains the increase in protein stability. Even more striking was the stability of the p.Gln614\* and p.Gln633\* mutants, which might indicate that interdomain interactions are critical for kinase stability and occur after the Ser575, but might interact with the stability domains. The variant *SIK1* proteins p.Pro287Thr, p.Ser411Cys, and p.Gly363Ser had turnover rates similar to that of wild-type *SIK1*.

We were next interested in whether the observed *SIK1* mutations altered kinase activity. Using immunoprecipitation-kinase assays, we evaluated both autophosphorylation of *SIK1* and phosphorylation of HDAC5, a well-characterized substrate of *SIK1*.<sup>26,34</sup> We used recombinant fragments of HDAC5 expressed as GST fusion proteins that contained *SIK1* phosphorylation sites Ser359 and Ser498. A kinase-dead form of *SIK1* (p.Lys56Met) was used as a negative control in our activity assays.<sup>33</sup> All *SIK1* variant proteins identified in individuals with developmental epilepsy demonstrated both autophosphorylation activity as well as the ability to phosphorylate HDAC5 fragments in vitro (Figures 3C and 3D). These data suggest that both variant and truncated *SIK1* proteins retain kinase activity.

We then sought to determine whether variant and truncated *SIK1* proteins exhibited normal protein localization patterns. In control conditions, wild-type and kinase-dead p.Lys56Met *SIK1* localize to a punctate pattern in the nuclei of HEK293TN cells (Figures 4A and 4C). Whereas the p.Pro287Thr, p.Ser411Cys, and p.Gly363Ser proteins showed a similar pattern of punctate nuclear localization, the p.Glu347\*, p.Gln614\*, and p.Gln633\* truncated proteins exhibited a broader pattern of localization within the nucleus and in the cytoplasm. Because PKA is known to regulate the expression and distribution of *SIK1*,<sup>22</sup> we treated HEK293TN cells with Forskolin (Fsk) and 3-isobutyl-1-methylxanthine (IBMX) to activate PKA signaling and determine its effects on the *SIK1* mutant proteins. As expected, wild-type *SIK1* protein levels increased with prolonged Fsk/IBMX treatment and its localization shifted to both the cytoplasm and the nucleus (Figure 4B). A similar response to Fsk/IBMX was seen with the kinase-dead protein, although additional foci of protein product were observed in the cytoplasm of these cells (Figures 4C and 4D). A similar observation was made in cells expressing the p.Glu347\* variant (Figures 4G and 4H). Additionally, all three truncated proteins (p.Glu347\*, p.Gln614\*, and p.Gln633\*) showed a broader nuclear localization and increased cytoplasmic localization compared to wild-type *SIK1* (Figures 4G–4L). The protein localization pattern for the missense variant proteins after Fsk/IBMX treatment resembled that of wild-type protein (Figures 4M–4R). Furthermore, unlike the wild-type *SIK1* protein, the expression level of the p.Gln614\* and p.Gln633\* proteins did not increase as much under Fsk/IBMX treatment, although their overall expression levels were higher than that of the wild-type protein or the missense mutants (Figure S1). These data suggest that the missense *SIK1* proteins localized to the nucleus and shuttled to the cytoplasm with PKA stimulation, similar to wild-type protein. The truncated proteins appear to have improper protein localization and regulation, perhaps due to lack of a regulatory function normally contained in the C terminus that promotes nuclear localization. Our biochemical and cellular observations on the effects of these mutations are summarized in Table 2.



### Figure 3. SIK1 Biochemical Studies

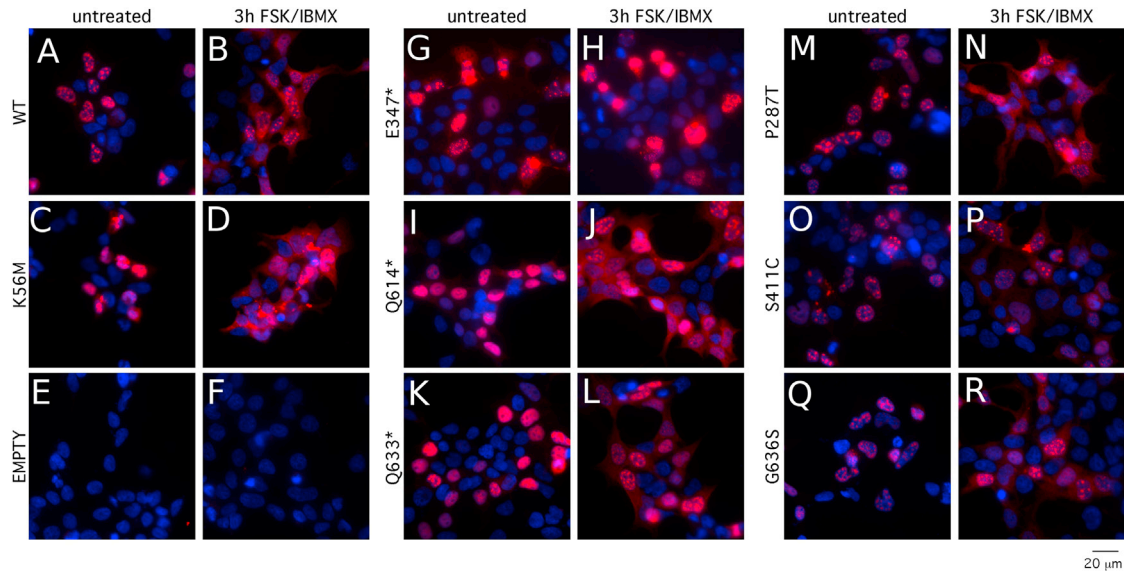
(A and B) To study the effects of *SIK1* mutations in vitro, myc-*SIK1* expression constructs were created by using human *SIK1* amplified by PCR (Phusion Polymerase, NEB) from a cDNA construct (Thermo Scientific, clone ID, 4831049; accession, BC038504). Primer sequences are available in Table S3. Both myc and h*SIK1* fragments were cloned with T4 ligase (NEB) into the XhoI and NotI site of a lentiviral bi-cistronic farnesylated GFP construct derived from a combination of pGIPZ non-silencing (Thermo Scientific), pLVX-IRES-tdTomato (Clontech), pDsRed-Monomer-F (Clontech), and pIRES2-acGFP (Clontech) vectors. Individual mutations were introduced into *SIK1* through PCR amplification with Phusion polymerase (NEB) and mutagenic primers, followed by recombination with an In-Fusion HD cloning system (Clontech). HEK293 cells (Systems Biosciences) were cultured in growth medium with DMEM/F12 ([+] L-glut and [+] sodium bicarbonate from Life Technologies), 5% FBS (Atlanta Biologicals), 1% MEM NEAA (Life Technologies), and 1% sodium pyruvate (Life Technologies) in a 5% CO<sub>2</sub> humidified incubator (Forma Scientific). *SIK1* constructs were transfected into cells with Fugene-6 (Promega) according to the manufacturer's procedure. Six hours after transfection, cells were

split into smaller plates, grown overnight, and given drug treatments (cycloheximide 100 µg/ml [Sigma]) for various durations. Cells were washed once with PBS, lysed in SDS-loading buffer, heated at ~97°C for 5 min, and then sonicated. Lysates were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane for immunoblots. Membranes were blocked with 5% NFDm (non-fat dry milk) in PBS-T solution and probed with an anti-myc primary antibody (9E10, DSHB), followed by Alexa Fluor 680-goat anti-mouse-IgG (H<sup>+</sup>L) (Invitrogen). The signal was visualized and quantified with Odyssey (LICOR). The truncating p.Glu347\*, p.Gln614\*, and p.Gln633\* mutants showed increased protein stability (A) and prolonged half-life (B) compared to wild-type *SIK1*. The missense mutations were comparable to wild-type in their protein stability and half-life. *SIK1* kinase activity (C and D) was measured by IP kinase assay with two GST-tagged fragments of human HDAC5 (243–276, 423–538; expressed in *E. coli*) that contain published sites for *SIK1* (Ser259, Ser498). Myc-tagged WT and *SIK1* mutants were expressed in HEK293 cells, immunoprecipitated with anti-myc antibody preloaded onto protein G beads, and incubated with 1 µg substrate and <sup>32</sup>P-ATP (100 µM ATP total) in kinase buffer (50 mM HEPES [pH 7], containing 5 mM MgCl<sub>2</sub>, 5 mM B-glycerophosphate, 150 µM Na<sub>3</sub>CO<sub>4</sub>, 1 mM DTT, and 1 mg/ml BSA) for 30 min at 30°C. *SIK1* mutants p.Glu347\*, p.Gln614\*, and p.Gln633\* express at higher levels, and therefore bead volume inputs were adjusted accordingly. Kinase assay samples were subjected to SDS-PAGE and phosphorimaging. *SIK1* (WT and mutants) does not phosphorylate GST under these assay conditions (data not shown). Auto-phosphorylation and HDAC5 phosphorylation was observed for all mutants, compared to wild-type *SIK1* and the p.Lys56Met kinase-dead control (C and D).

Because of the availability of a brain specimen from subject DB14-013 with the p.Glu347\* *SIK1* mutation, we were able to compare *SIK1* distribution in the presence of this mutation as compared with control and mutation-negative epileptic infant brain. Brain weight for subject DB14-013 was 584 g (average 516 g). On microscopic examination, no malformations were identified and neuronal morphology and lamination were normal. *SIK1* immunofluorescence (IF) was detected in neurons and proximal dendrites within the hippocampus and cortex (Figures 5A–5C). *SIK1* IF was also seen in GFAP<sup>+</sup> astrocytes in the cortical white matter (Figures 5D–5F). However, the intracellular distribution of *SIK1* IF differed between control infant brain, subject DB14-013 (*SIK1* p.Glu347\*), and that of an infant with epileptic encephalopathy but normal *SIK1* sequencing. In subject DB14-013, neuronal *SIK1* IF was predominant in the cytoplasm only, whereas in the other

specimens it was equivalent in both the nucleus and cytoplasm (Figures 5G–5I). These findings indicated that *SIK1* protein expression levels were not substantially altered by the presence of the heterozygous p.Glu347\* mutation and that this *SIK1* mutation did not result in gross abnormalities of brain formation or neuronal morphology. However, alteration of *SIK1* intracellular distribution was detected, suggesting that nuclear localization of the p.Glu347\* mutant might be impaired, consistent with our in vitro studies.

Our individuals with *SIK1* mutations had universally severe outcomes. Subjects DB13-001 and DB14-013 both presented with early myoclonic encephalopathy and died in infancy from intractable epilepsy with respiratory failure. The other subjects had severe developmental impairment with autistic features and continued refractory seizures, and subject LR05-086 had respiratory failure requiring



**Figure 4. SIK1 Protein Localization Studies**

To study protein localization, HEK293TN cells were plated on coverslips in a plasma cleaner and coated with poly-L-lysine (Sigma). The day after plating, cells were transfected with CMV::myc-SIK1-IRES2-acGFP-F expression constructs via Lipofectamine 2000 according to the manufacturer's procedure. The day after transfection, cells received drug treatments (Forskolin 10  $\mu$ M [Cayman Chemicals], IBMX 18  $\mu$ M [Calbiochem]) for 3 hr. Cells were fixed with a 4% paraformaldehyde PBS solution and blocked with PBS containing 1% serum and 0.1% Triton X-100. Cells were incubated with a mouse anti-myc (9E10, DSHB) primary antibody diluted in blocking solution, followed by a second incubation with goat anti-mouse Alexa594 conjugated secondary antibody (Invitrogen Molecular Probes) and Hoechst 33342 nuclear stain (Sigma) also diluted in blocking solution. Coverslips were mounted on slides with Mowiol and imaged with an Olympus BX61 fluorescent microscope and Metamorph software. Wild-type SIK1 (A and B), the kinase-dead p.Lys56Met (C and D), and empty vector (E and F) were used as controls. The mutant p.Glu347\* (G and H), p.Gln614\* (I and J), and p.Gln633\* (K and L) SIK1 proteins showed abnormal localization in both control (A, C, E, G, I, K, M, O, and Q) and Fsk/IBMX-treated conditions (B, D, F, H, J, L, N, P, R). The missense mutants p.Pro287Thr (M and N), p.Ser411Cys (O and P), and p.Gly636Ser (Q and R) had localization patterns resembling wild-type protein.

tracheostomy placement and long-term ventilation. None of the subjects with infantile spasms responded to ACTH. Nearly all of the subjects required the placement of gastrostomy tubes for nutrition, and two of the four persons who survived past infancy remained nonambulatory. Scoliosis was present in two of the subjects. The clinical features of *SIK1*-related developmental epilepsy are summarized in Table 3 as well as in the Supplemental Data.

In conclusion, we identified de novo heterozygous mutations in *SIK1* in children presenting with developmental epilepsies including early myoclonic encephalopathy, Ohtahara syndrome, and infantile spasms. Overall, our data

indicated that truncating mutations resulted in increased protein stability and abnormal cellular localization and that all mutant proteins were capable of phosphorylating HDAC5. We also present human neuropathology of *SIK1*-related developmental epilepsy and confirm our in vitro observations of abnormal SIK1 protein cellular localization in that specimen. Our study identified 6 *SIK1* mutations in 101 individuals sequenced, suggesting that this might be a relatively common cause of developmental epilepsy and that kinases involved in the nuclear control of gene transcription can contribute to neurodevelopmental disease and severe epilepsy.

**Table 2. Summary of Effects of SIK1 Mutations on Protein Stability, Cellular Localization, and HDAC5 Phosphorylation**

Subject	Amino Acid Substitution	Increased Protein Stability	Abnormal Cellular Localization	HDAC5 Phosphorylation
DB13-001	p.Pro287Thr	-	-	+
DB14-013	p.Glu347*	+	+	+
EP-055	p.Ser411Cys	-	-	+
IS13-013	p.Gln614*	+	+	+
IS09-018	p.Gln633*	+	+	+
LR05-086	p.Gly636Ser	-	-	+

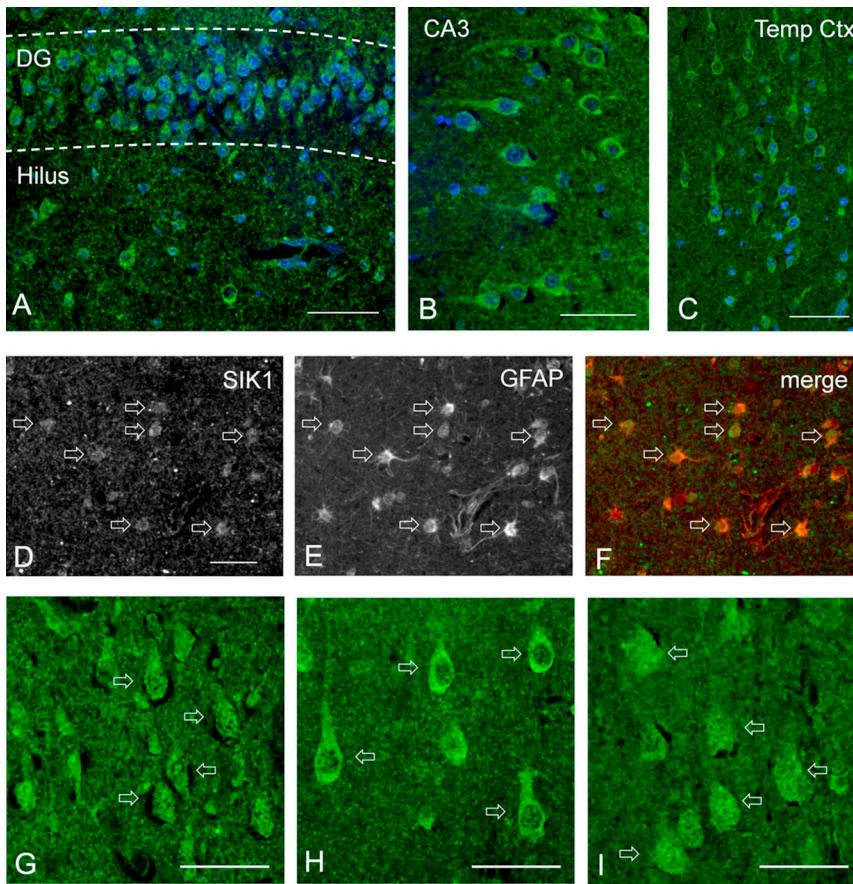
### Supplemental Data

Supplemental Data include case reports, one figure, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2015.02.013>.

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**Figure 5. SIK1 Brain Immunohistochemistry**

Formalin-fixed human brain sections from subject DB14-013 (p.Glu347\* mutation) and two other infants were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, embedded in paraffin, and sectioned at a thickness of 4  $\mu$ m. Specimens were deparaffinized by incubation in xylene, followed by rehydration through graded ethanol/water solutions and equilibration in PBS. Antigen retrieval was performed by heating in 0.01 M sodium citrate (pH 6.0) in a 90°C water bath for 45 min. Specimens were then blocked in PBS with 10% donkey serum, 0.1% Triton X-100, and 2% BSA for 30 min and incubated overnight at 4°C in primary antibodies diluted in blocking solution. The SIK1 primary antibody (M01, clone 2C12, Abnova) is a mouse monoclonal IgG2a directed against an epitope mapping to the N-terminal 100 amino acids of human SIK1. Other primary antibodies included goat polyclonal MAP2 and GFAP (Santa Cruz). Slides were then incubated in fluorescent secondary antibodies at room temperature for 2 hr. After incubation in DAPI nuclear counterstain and Sudan Black to block endogenous fluorescence, sections were examined and photographed with a Nikon Eclipse Ni-U epifluorescence microscope. (A–C) SIK1 immunofluorescence (IF) in subject DB14-013 was detected in neuronal somas and proximal dendrites

within the dentate gyrus and hilus (A) and CA3 of the hippocampus (B), as well as in the temporal cortex (C).

(D–F) SIK1 expression in GFAP<sup>+</sup> astrocytes in white matter of the temporal cortex in subject DB14-013, illustrated by staining for SIK1 (D), GFAP (E), or both (F). Scale bar represents 100  $\mu$ m.

(G–I) SIK1 IF in temporal cortex of a control infant (G), subject DB14-013 (H), and an epileptic infant with normal *SIK1* sequencing (I). Pyramidal neurons exhibited both nuclear and cytoplasmic SIK1 IF in the control and epileptic subjects, but localization predominantly in the cytoplasm in subject DB14-013. Scale bars represent 50  $\mu$ m.

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**Table 3. Genotype-Phenotype Correlations in Six Subjects Presenting with Developmental Epilepsy and *SIK1* Mutations**

Subject	Amino Acid Substitution	Presenting Epilepsy	Death in Infancy	Death in ID	Absent Language	Impaired Socialization	Repetitive Behaviors	Non-ambulatory Tube	Gastrostomy	Respiratory Failure	Other Findings
DB13-001	p.Pro287Thr	EME	+	NA	NA	NA	NA	NA	+	+	–
DB14-013	p.Glu347*	EME	+	NA	NA	NA	NA	NA	+	+	–
EP-055	p.Ser411Cys	ISS	–	+	+	+	+	–	–	–	anomalous pulmonary arterial circulation
IS13-013	p.Gln614*	ISS	–	+	+	+	+	–	+	–	disordered sleep, breath-holding spells, scoliosis
IS09-018	p.Gln633*	ISS	–	+	+	+	+	+	+	–	–
LR05-086	p.Gly636Ser	OS	–	+	+	+	+	+	+	+	diabetes insipidus, scoliosis

Abbreviations are as follows: EME, early myoclonic encephalopathy; OS, Ohtahara syndrome; ISS, infantile spasms; ID, intellectual disability; NA, not applicable.

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## Web Resources

The URLs for data presented herein are as follows:

ANNOVAR, <http://www.openbioinformatics.org/annovar/>  
Burrows-Wheeler Aligner, <http://bio-bwa.sourceforge.net/>  
dbSNP, [http://www.ncbi.nlm.nih.gov/SNP/snp\\_summary.cgi](http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi)  
ExAC Browser, <http://exac.broadinstitute.org/>  
GATK, <http://www.broadinstitute.org/gatk/>  
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
Picard, <http://picard.sourceforge.net/>  
RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>  
SAMtools, <http://samtools.sourceforge.net/>  
SOLVE-Brain, [https://paciorkowski-lab.urmc.rochester.edu/solve\\_brain](https://paciorkowski-lab.urmc.rochester.edu/solve_brain)  
Variant Effect Predictor, <http://useast.ensembl.org/info/docs/tools/vep/index.html>  
UCSC Genome Browser, <http://genome.ucsc.edu>

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Supplemental Data

## **De Novo Mutations in *SIK1* Cause a Spectrum of Developmental Epilepsies**

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## **Supplemental Notes: Case Reports**

### DB13-001

This was an infant boy born at term after an uncomplicated pregnancy. Delivery was via caesarean section after failure to progress. Apgar scores were 8 and 9 at one and five minutes. Birth weight was 3340 g. At 20 minutes of age he was noted to be dusky and grunting, and was transferred to the NICU for observation. At 80 minutes of life he had repeated episodes of periodic breathing and oxygen desaturations. These episodes continued. Metabolic evaluation and infectious workup, including cerebrospinal fluid studies, were normal. An EEG on day of life 2 showed discontinuous background with burst suppression. He developed intermittent myoclonic jerking movements and repeat EEG on day of life 14 showed continuous high amplitude burst activity alternating with periods of voltage suppression. Brain MRI was normal. Pyridoxine challenge was performed without changes in the EEG after administration. He was started on phenobarbital, biotin, and clonazepam, and was discharged home. Clinical sequencing of a 70 gene comprehensive epilepsy panel was normal, as was chromosomal microarray. He continued to have frequent myoclonic seizures, worsening at 6 months of life. He had very limited development, and at 8 months of age had no head control, did not roll over, was unable to sit, did not bring hands to midline, and did not track objects or attend to voices. He required a gastrostomy tube for feeding, and was on supplemental home oxygen. Repeat EEG at 8 months of age showed highly discontinuous, mildly asynchronous background with no state change, reactivity, or normal waking or sleep patterns, consistent with burst suppression pattern. He also had multifocal epileptiform



discharges within the bursts, many of which correlated with short random jerks of arms, body and legs. He died after complications of a respiratory illness at 10 months of age.

#### DB14-013

This was an infant boy born at term after an uncomplicated pregnancy. Myoclonic seizures began minutes after an uncomplicated vaginal delivery. Apgar scores were 8 and 9 at 1 and 5 minutes. Birth weight was 3235 g and head circumference was 37 cm. He remained in a phenobarbital coma in the NICU for three months, and continued to have breakthrough myoclonic seizures despite therapeutic phenobarbital levels. EEGs demonstrated burst suppression pattern consistent with early myoclonic encephalopathy and/or Ohtahara syndrome with multifocal spikes and generalized high amplitude multispikes correlating with myoclonic activity. No recognizable background rhythms were noted on repeat EEGs. Pyridoxine did not change the EEG features. Extensive metabolic and infectious evaluations were negative. Muscle, nerve, and skin biopsies were normal. Cerebrospinal fluid neurotransmitters were normal. Brain MRI was normal. Tracheostomy was performed and gastrostomy tube was placed. He continued to have breakthrough seizures with bradycardia and hypoxia requiring increasing ventilator support. The parents elected to remove ventilator support and the infant died at 3 months of age. On autopsy, the cerebral convolutions and sulci were well developed and without malformation. The brain weight was 584 g (average is 516 g).

#### EP-055

This male subject was born at term via an uneventful spontaneous vaginal delivery after a pregnancy complicated by oligohydramnios. His birth weight was 3.2 Kg, birth length 51 cm, and head circumference was 34 cm. At 4 months of age he began having infantile

spasms and was treated with adrenocorticotrophic hormone (ACTH). He then developed generalized tonic-clonic seizures, and was started on valproic acid, zonisamide, and clorazepate. He was also noted to have an anomalous left circumflex artery arising from his pulmonary artery that was reimplanted at 9 months of age, after which he was asymptomatic. He had global developmental delay, began walking at 5 years of age, is unable to feed himself, and did not develop verbal language. Brain MRI was reportedly normal. An EEG at 5 years of age showed generalized slowing. The proband exhibited behavioral problems, aggressiveness, and self-injurious behaviors for which he received guaifenesin, risperidone, and olanzapine. At 11 years of age, his weight was at the 60th percentile, height was at the 25<sup>th</sup> percentile, and head circumference was at the 25<sup>th</sup> percentile. He exhibited deep set eyes, short nose and philtrum, and thin upper lip. He had repetitive movements including head-banging, biting, and other self-injurious behaviors.

### IS13-013

This female subject was born at term after an uncomplicated pregnancy and delivery. Birth weight was 7 pounds ½ ounce. Head circumference was normal at birth. At 4 months of age she had onset of infantile spasms, was initially treated with phenobarbital, and then ACTH. She did not respond to ACTH, and her longest seizure-free interval was 5 weeks. She continued to have a variety of seizure types, the most common were epileptic spasms identical to previous infantile spasms, and occurring in 1-2 clusters a month. She also had atonic seizures with head drops that were refractory to multiple anti-seizure medications. She had global developmental delay, and rolled at 10 months, sat alone at 1 year, and walked at 3 years of age. At 6 ½ years her head circumference was -2

SD below the mean. She had a normal karyotype, normal chromosomal microarray, and normal sequencing of *MECP2* and *PCDH19*. At 15 years of age she had no clear words, appeared to understand some speech. She has repetitive movements such as bruxism and hand-flapping, especially when excited. She has never slept through the night, and has great difficulty with sleep onset. She began having breath-holding spells at age 7 years, which do not have EEG correlate. She has had self-injurious behaviors such as biting. Vagal nerve stimulator was placed at 5 years of age, and gastrostomy tube was placed at 6 years of age. She has a history of scoliosis with a 43 degree curve.

#### IS09-018

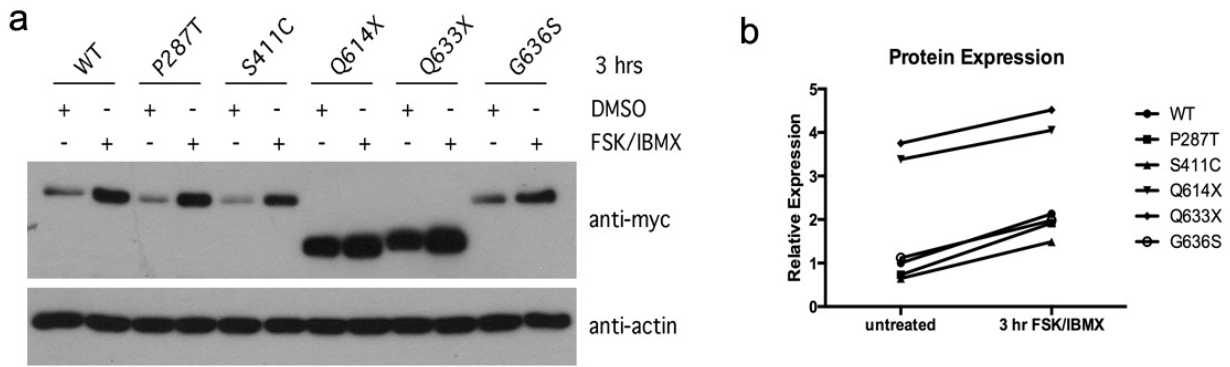
This female patient was born at term after an uncomplicated pregnancy. She developed seizures at 2 months of age and was diagnosed with infantile spasms at 8 months of age. She was treated with levetiracetam and clobazam, but continues to have myoclonic and tonic-clonic generalized seizures. Brain MRI was normal. At 8 years of age, she has no verbal language, is fed through a gastrostomy tube, is nonambulatory, and is dependent on others for care. She occasionally fixes visually on her parents, but does not respond to other visual or auditory stimuli. She has multiple involuntary movements of the extremities.

#### LR05-086

This female patient was born at term after an uncomplicated pregnancy. She had myoclonic jerks at birth and tonic seizures starting at 4 hours of life. An EEG at 8 days showed burst-suppression pattern, and she was diagnosed with Ohtahara syndrome. At 6 months of age she developed infantile spasms with EEGs at 6 months, 1 year, and 2 years showing hypsarrhythmia. Infantile spasms did not respond to ACTH. She has been



treated with phenobarbital, phenytoin, zonisamide, levetiracetam, vigabatrin, rufinamide, and valproic acid. A gastrostomy tube was placed at 6 months of age. A tracheostomy was placed at age 3 years after complications of aspiration pneumonia. Major illnesses have been complicated by diabetes insipidus. Scoliosis surgery was performed at age 4 years. At last follow-up at 10 years of age she continues to have daily myoclonic seizures. She requires mechanical ventilation at night. Developmentally, she makes eye contact and regards when spoken to, and has no head control and few spontaneous movements.



**Figure S1:** The expression of the p.Gln614\* and p.Gln633\* proteins increased under Fsk/IBMX treatment and their overall expression was higher than that of the wild-type protein or the missense variants.

Variant	Status from Exome Analysis	Notes
<i>CYP2A13</i> (NM_000766) c.884C>A p.T295N	<i>De novo</i>	Cytochrome P450 family member. Ubiquitously expressed in many tissues.
<i>GOLGA6B</i> (NM_018652) c.623G>A p.R208Q	<i>De novo</i>	Possible pseudogene. Not brain expressed.
<i>MAGEL2</i> (NM_019066) c.509C>T p.A170V	<i>De novo</i>	Identified as <i>de novo</i> from exome analysis, but found to be paternally inherited by Sanger sequencing.
<i>PSME1</i> (NM_006263) c.460G>A p.E154K	<i>De novo</i>	Implicated in immunoproteasome assembly and required for efficient antigen processing. Not brain expressed.
<i>PRRC2C</i> (NM_015172) c.2161A>G p.M721V c.3875G>A p.R1292Q	AR	Proline-rich coiled coil 2C Evidence for brain expression in Allen Brain Atlas. The c.2161A>G is present in EVS (rs 376665075), predicted benign by PolyPhen2. The c.3875G>A variant is not present in EVS, but SIFT/PolyPhen give “unknown” prediction of effect.
<i>POLRMT</i> (NM_005035) c.2570T>C p.L857P c.526G>A p.E176K	AR	Mitochondrial polymerase. No specific evidence for brain expression in Allen Brain Atlas. The c.2570T>C variant is predicted deleterious (0) by SIFT and probably damaging (0.999) by PolyPhen. However, the c.526G>A variant is present in the EVS (rs367986983), and is predicted to be benign.

**Table S1:** Other *de novo* and autosomal recessive variants identified by whole exome sequencing of subject DB13-001 that were not considered potentially causative after further analysis. No indels or X-linked variants were identified that could be plausibly pathogenic.



Exon	Forward	Reverse
2	GACAGGCGTGGGAGCAG	GACAATCAAGCCTGGAGCAG
3	TGTGTAAATTAGGTTGAGGGTTTC	AAAAGGGAAGCCACCTTCAG
4-5	TTGTAGCGCTGCCTTGTG	AGCTTGATGTCCCTGCTCC
6-7	AGCCCCTTGCCATTTGC	TCCCAGACTCAACAAGGACTC
8	CCTGGCGTGTCAAACCAC	GACAGCTGCCTAGCCCC
9	AAGAATAACCTGGGATCGGG	GGTGCCTGACAGGGGAG
10-11	TGTTTCTTCTCTTGATGGCG	ACGTCGACCGTAAGCCTATC
12	GGGGAGCCTAATCCTTTGC	CGGACAAAGTCACGTACTGC
13	TGGAAACCCCTTAAGCCAAG	AGGAGATAACCTGCCGTGTG
14	CACTCGGAAACCCGAGAAC	AGGCCAGATGTCTTCACCTC

**Table S2:** Primers used for Sanger sequencing of *SIK1* (NM\_173354.3).

Human *SIK1* was amplified by PCR (Phusion Polymerase, NEB) from a cDNA construct (Thermo Scientific, Clone ID: 4831049, Accession: BC038504) using the following primers: 5'-gcggttagcatggttatcatgtcgg-3' and 5'-taaggatccgcgccgctcactgcaccaggac-3'. Myc sequence was introduced by annealing complementary primers 5'-tcgagaccatggaacaaaaactcatctcagaagaagatgcgg-3' and 5'-ctagccgcatcttcttgagatgagttttgtccatggtc-3'.

**Table S3:** Primers used for *SIK1* expression constructs.