Pathogenic MAST3 variants in the STK domain are associated with epilepsy

Running title: MAST3 pathogenic variants in DEE

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Summary for social media

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*MAST3 de novo* variants are a novel cause of developmental and epileptic encephalopathies, and act through a putative gain-of-function mechanism in excitatory neurons.
Abstract

Objective: The MAST family of microtubule-associated serine-threonine kinases (STK) have distinct expression patterns in the developing and mature human and mouse brain. To date, only MAST1 has been conclusively associated with neurological disease, with de novo variants in individuals with a neurodevelopmental disorder, including a mega corpus callosum.

Methods: Using exome sequencing we identify MAST3 missense variants in individuals with epilepsy. We also assess the effect of these variants on the ability of MAST3 to phosphorylate the target gene product ARPP-16 in HEK293T cells.

Results: We identify de novo missense variants in the STK domain in 11 individuals, including two recurrent variants p.G510S (n=5) and p.G515S (n=3). All 11 individuals had developmental and epileptic encephalopathy, with 8 having normal development prior to seizure onset at < 2 years of age. All patients developed multiple seizure types, while 9/11 had seizures triggered by fever and 9/11 had drug-resistant seizures. In vitro analysis of HEK293T cells transfected with MAST3 cDNA carrying a subset of these patient-specific missense variants demonstrated variable but generally lower expression, with concomitant increased phosphorylation of the MAST3 target, ARPP-16, compared to wildtype. These findings suggest the patient-specific variants may confer MAST3 gain-of-function. Moreover, single-nuclei RNA sequencing and immunohistochemistry shows that MAST3 expression is restricted to excitatory neurons in the cortex late in prenatal development and postnatally.

Interpretation: In summary, we describe MAST3 as a novel epilepsy-associated gene with a potential gain-of-function pathogenic mechanism that may be primarily restricted to excitatory neurons in the cortex.
Introduction

Developmental and Epileptic Encephalopathies (DEEs) encompass a group of disorders featuring frequent epileptic activity and developmental impairment. Development plateaus or regresses when seizures are worse but can also occur independent of seizures and are thus attributable to the underlying cause alone.\(^1,2\) DEE causes can be varied, but a genetic etiology is known or suspected in most cases without metabolic or structural causes. The highest yield for genetic testing is in the early onset DEEs where up to 80% of patients have a known underlying genetic etiology.\(^3\) In patients with DEEs, the goal of genetic investigations is not only to end the diagnostic odyssey for families but ideally, to identify a potentially actionable target that could lead to precision medicine therapies.

Despite the high diagnostic rate of patients with DEEs, many patients still lack a genetic diagnosis, likely at least in part to pathogenic variants in undiscovered genes. Here we describe variants in MAST3, a microtubule-associated serine/threonine kinase.\(^4\) This gene is a member of a family of serine/threonine kinases (MAST1-4 and MAST-like) that is predominantly expressed in the human cortex, hippocampus, and striatum.\(^5\) The role of MAST3 in the brain is not fully understood, but recent studies in adult rodent striatal medium spiny neurons suggest a role in coordination of cAMP/protein kinase A/protein phosphatase 2A signaling pathways that mediate the effects of dopamine.\(^6\)

To date, disease-associations of MAST3 variants are rare; an intronic SNP has been associated with inflammatory bowel disease\(^10\) and a single individual has been reported with a de novo MAST3 variant and clinical features of atypical MECP2-associated disorder.\(^11\) However, pathogenic variants in the MAST3 homolog, MAST1, have been identified in individuals with developmental brain abnormalities that include a particularly striking mega-corpus callosum along with cerebellar hypoplasia and cortical malformations. More recently, three additional patients with additional neurodevelopmental disorders with and without malformations of cortical development and MAST2 de novo variants have been reported.\(^12-14\) Overall, individuals with de novo MAST1 variants present with a spectrum of developmental impairments and a subset have epilepsy.\(^15\) The proposed pathogenic mechanism of MAST1 variants is a dominant negative effect, given the reduced abundance of MAST1 protein to around 20% of wildtype as well as a decrease in MAST homologs (MAST2/3) in a mouse model carrying a heterozygous patient-specific variant.\(^15\)

As part of an international collaboration, we identified 11 individuals from 4 continents with DEEs and de novo variants in the serine/threonine kinase (STK) domain of MAST3. In vitro modeling of a subset of these variants showed a potential gain of function effect on MAST3 kinase activity. We propose MAST3 as a novel cause of DEEs and implicate a second member of the MAST family in neurodevelopmental disorders though with distinct phenotypes, likely related to gene expression timing and/or localization during neurodevelopment.

Materials and Methods

Study Participants

All patients were identified by either clinical or research investigations to determine the underlying etiology of their DEE. Patients were ascertained through personal communication or GeneMatcher\(^14\). Exome sequencing was performed by a commercial clinical laboratory in the USA or Europe (Patients 1, 2, 6, 7, 10, 11)\(^17\), in research laboratories/in-house sequencing facilities (Patients 3, 4) or by exome sequencing of the proband by the Epi25 Collaborative (in press) (Patients 5, 9). The variant of patient 8 was identified by targeted resequencing in a research laboratory using single-molecule molecular inversion probes designed to cover the exons +5bp into the intron.\(^14\) Variant allele frequencies were assessed using GnomAD and TopMed (see list of URLs). All variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines for variant interpretation.\(^19\) This study was approved by the local institutional review board at each site and all patients and parental or legal guardians provided consent for this study and publication of clinical and genetic data. Missense Tolerance Ratios\(^20\) were plotted with ggplot2, combined annotation dependent depletion (CADD), and polyphen scores were generated by variant effect predictor. Clinical histories were analyzed and seizure types and epilepsy syndromes were classified according to the International League Against Epilepsy classification criteria.\(^21\)

MAST3 expression in the developing human and mouse brain

To determine the expression of MAST3 in the mature and developing human brain we analyzed RNA-seq and single nucleus-RNA-seq data from the Allen Cell Types Database and the Allen Developing Human Brain Database. In addition to RNA expression analysis, we performed immunohistochemistry in 11-week-old human cerebral organoids generated with the STEMdiff cerebral organoid kit \(^{22}\) and 10-micron thick sections were cut with a cryostat (Leica). Sections were permeabilized and blocked for 1-hour at room temperature with 0.3% Triton X-100 and 10% normal donkey serum, respectively, and then overnight at 4°C with the following primary antibodies: MAST3 (Novus Biologicals, Cat # NBP1-82993), CUX1 (Santa Cruz Biotechnology, sc-514008), SATB2 (Abcam, ab51502), TBR1 (ProteinTech Group, 66564-1-g) and Ctip2 (Abcam, ab18465). After a 2-hour incubation at room temperature with secondary antibodies (Donkey anti-rabbit AlexaFluor 594, anti-rat AlexaFluor 488, anti-mouse AlexaFluor 647), slides were counterstained with Hoechst 33342 (ThermoFisher Scientific) and mounted with ProLong Gold Antifade (ThermoFisher Scientific). Images were acquired with a Nikon A1R laser scanning confocal microscope.

Analysis of effect of MAST3 variants on phosphorylation of ARPP-16 in HEK293 cells

To assess their kinase activity, MAST3 wild-type and a subset of variants were individually expressed in human embryonic kidney (HEK) 293T cells (ATCC CRL-3216) with the established MAST3 substrate protein ARPP-16.\(^6,7\) All MAST3 constructs were cloned into the pCMV plasmid
compared to other individuals in this cohort, and the variant was outside of the STK domain, we interpret this case as a variant of uncertain significance (VUS).

Six unique missense variants were identified in 11 individuals occurring in the STK domain and two variants were recurrent (Fig 1A): c.1963T>C, p.F655L variant (CADD = 24.3 and polyphen = 0.604) in the proband only. Language development was normal until a regression at 15-18 months leading to both receptive and expressive language delays. Similarly, there was a regression in social development leading to poor eye contact, smile, and joint attention. Given that this variant was associated with an atypical presentation as de novo, 12 individuals with variants in the STK domain (Table 1, Table S1 and S2, Fig 1A) are represented (Table S1 and S2); 3 patients are currently taking sodium channel blockers. Antiepileptic medications that elicited some improvement in seizure control included valproic acid (5), topiramate (1), zonisamide (2), phenobarbital (1), felbamate, oxcarbazepine (1), and cannabidiol. The ketogenic diet was trialed in 3 patients represented (Table S1 and S2); 3 patients are currently taking sodium channel blockers. Antiepileptic medications that elicited some improvement in seizure control included valproic acid (5), topiramate (1), zonisamide (2), phenobarbital (1), felbamate, oxcarbazepine (1), lamotrigine (1), clobazam and cannabinidiol. The ketogenic diet was trialed in 3 patients with improvement in seizure control for patient 7 but not for patient 6 or 10.

Epilepsy phenotype in patients with MAST3 STK domain variants

All 11 patients had DRE with seizure onset at or before 2 years of age (median: 11 months, range: 2 to 24 months), with 7/11 patients having seizure onset before 12 months of age. The first seizure was a febrile convulsion, in 4 patients, an afebrile, tonic-clonic seizure in 4, myoclonic seizure in 1 and a myoclonic-atonic seizure in 1 patient (Table 1, Table S1). Three of 11 patients (1, 2 and 4) were reported to have a Dravet-like phenotype, primarily due to the febrile-induced seizures and status epilepticus associated with fever. However, in contrast with those with Dravet Syndrome, the hemiclonic/myoclonic seizures that are hallmarks of this DRE were not present.

All patients developed multiple seizure types, including atonic (3), myoclonic (2) myoclonic absence with eye flutter, myoclonic-atonic (1), tonic (5), generalized tonic-clonic with or without fever (11) and focal with impaired awareness (6). Seizures evolved to include other types in most patients, and these included atonic, myoclonic, myoclonic absence with eye flutter, myoclonic, atonic, tonic, tonic-clonic, and focal with impaired awareness. All patients experienced generalized tonic-clonic seizures with and without fevers (n = 11). Ten of 11 patients reported provoking factors for seizures (such as illness or fever and sleep deprivation). Eight patients experienced episodes of convulsive status epilepticus of whom 3 also experienced episodes of non-convulsive status epilepticus.

Two patients were seizure free at their most recent follow up: patient 3, now 8 years old, has been seizure free since age 2.5 years and is on no medications while patient 2, now seven years old, has been seizure-free since 2 years nine months on valproate alone. The remaining 9 patients had drug-resistant seizures and were taking 1-3 (mean: 2) antiepileptic medication with a variety of pharmacological targets represented (Table S1 and S2); 3 patients are currently taking sodium channel blockers. Antiepileptic medications that elicited some improvement in seizure control included valproic acid (5), topiramate (1), zonisamide (2), phenobarbital (1), felbamate, oxcarbazepine (1), lamotrigine (1), clobazam and cannabinidiol. The ketogenic diet was trialed in 3 patients with improvement in seizure control for patient 7 but not for patient 6 or 10.
Initial EEGs performed within 12 months of seizure onset were available for 8/11 patients; 6 were reported as normal whilst background slowing was reported in patient 1 and generalized and focal abnormalities were reported in patient 3. Follow up EEGs were available for 9/11 patients; 3 were reported as normal whilst 4 patients had background disorganized or slowing, while patient 9 had bisynchronous frontal spikes and polyspikes and patient 7 had generalized seizures with eyelid flutters captured on EEG. Where this information was available, neither burst suppression nor hypsarrhythmia were features of the EEG (n=8).

**Neurodevelopmental phenotype of patients with MAST3 STK pathogenic variants**

Early development was normal in all 8 individuals with data available (Table S2). All individuals except patient 5, experienced developmental regression or plateau before 2 years of age (mean: 16 months, range: 11-24 months) coinciding with seizure in 6 patients and status epilepticus in 5. Data was unavailable for patient 5. In 4 individuals, regression was persistent, while in 3 individuals (patient 3,4 and 6) there was a period of recovery after initial regression associated with seizure control. Regression was associated with seizure onset in three individuals, and status epilepticus in seven individuals (Table S2). Developmental regression most commonly affected language (7) followed by gross motor (4), social (1), fine motor (1) and toilet training (1). The majority of patients (10/11) are described as non-verbal or with limited speech with expressive speech affected more than receptive speech. Eight patients exhibited delayed gross motor milestones with walking attained at a mean age of 17.5 months (range 14-31 months). Intellectual disability was severe in 6 individuals, moderate in 3, mild-moderate in patient 9 and severe-profound in patient 10 (data not available for patient 5).

Nine patients were noreomphicale whilst patient 3 had macrocephaly and patient 9 had microcephaly. Three of 11 patients had dysmorphic features; patients 10 and 11 had dysmorphic facies, patient 10 also had short stature and patient 7 had 2-3rd toe syndactyly. Six patients had hypotonia with patient 1 developing progressive axial hypotonia and appendicular hypotonia. Eight patients had an abnormal gait; 5 were unsteady, had poor balance or fell frequently, 2 had knee flexion, patient 1 uses a wheelchair and patient 9 had wide-based walking. Sleep difficulties were reported in 7 patients, movement disorder in 2, a high pain threshold in 2, drooling in 2, stereotypies in 2 and hypothyroidism in 2. Three patients reported gastrointestinal difficulties including celiac disease in patient 3, cyclical vomiting in patient 7 and gastroesophageal reflux (resolved) – in patient 11.

**Neuroimaging for patients with MAST3 pathogenic variants**

Brain MRI was reported as normal in all patients with reports available (n=5) except for patient 4 who had a smaller left anterior hippocampus. MR images for 5 patients were reviewed independently by a single pediatric neuroradiologist (S.M.); all 5 were abnormal demonstrating a small pituitary (2), thin corpus callosum (1) or both (1) (Fig 2) Patients showed no evidence of clinical features associated with a hypo-active pituitary.

**Expression of MAST3**

We generated a heat map of MAST1/MAST3 expression, as well as other epilepsy associated genes (SCN1A/SCN3A), neuronal marker genes using bulk RNA-seq data from the Brainspan atlas of the developing human brain (see list of URLs) (Fig 3A). MAST3 expression is low over the period of human fetal neurodevelopment, with expression increasing at 26 weeks post-conception and steadily increasing postnatally (Fig 3A). Single-nuclei RNA-seq of post-mortem motor cortex from the Allen Brain Map demonstrates that MAST3 expression is restricted to excitatory neurons in the human cortex (Fig 3B). At the protein level, MAST3 is present in a fraction of early-born (CTIP2+), neurons, and in the majority of late-born (CUX1+) excitatory neurons in 11-week-old human cerebral organoids (Fig 3C). In mouse cortex, MAST3 is primarily cytosolic (Fig 3D) and present in postmitotic excitatory neurons, mostly colocalizing with CTIP2 and TBR1 at E14.5, and mainly with the upper-layer neuron marker SATB2 at E16.5 (Fig 3E).

**Assessment of kinase activity of MAST3 variants**

Wild-type and four of the MAST3 variants described in this study were expressed in HEK293T cells together with its substrate, ARPP-16 (Fig 4). A kinase-dead control (p.K396H) was also co-expressed with ARPP-16. Compared to wild-type, the expression levels of the MAST3 variants was consistently lower, with the exception of the p.V551L variant which exhibited intermediate expression (Fig 4A). As expected, wild-type MAST3 was active as demonstrated by robust phosphorylation at Ser46 of ARPP-16, while there was minimal phosphorylation by kinase-dead MAST3. Despite lower expression, the levels of Ser46 phosphorylation for each of the variants (p.G510S, p.G515S, p.L516P, p.V551L) was similar to that of wild-type MAST3. When normalized to expression levels of MAST3 protein, the activities of each variant was therefore higher than wild-type, reaching significance for the p.G510S-containing mutant (*p = 0.0159, one-way ANOVA with Dunnett’s posthoc test). The reason for the lower expression of the MAST3 variants is not clear but could involve decreased protein synthesis or increased protein turnover. Nevertheless, these initial studies suggest the possibility of some sort of gain-of-function phenotype for these MAST3 variants through more effective phosphorylation of ARPP-16 and possibly other substrates.

**Discussion**

In this study, we describe de novo missense variants located in the STK domain of MAST3 as a novel cause for DEE in 11 patients. There were two recurrent variants (p.G510S and p.G515S present in 8 individuals collectively), and an additional three variants (p.R406P, p.L516P, p.V551L) located nearby11. A twelfth patient harboured a genetic change outside of the STK domain; he did not have epilepsy but rather a diagnosis of ASD and additional patients and functional studies are required to interpret the pathogenicity of variants outside the STK domain. Moreover, the first variant is located within the third position of the “Asp-Phe-Gly+1” (DFG+1) domain, which determines the specificity of the target residue that will be phosphorylated and is highly conserved among protein kinases11, 21. Modulation of MAST3 by Protein Kinase A (PKA) involves phosphorylation of position 512, again close to the variants’ location in this series6. Alteration of a subset of these specific variants in the STK domain resulted in lower expression in an in vitro cell culture model, but more effective phosphorylation
of the MAST3 target, ARPP-16, consistent with a potential gain-of-function phenotype. Notably, a gain-of-function Gly to Ser mutation (p.G2019S) is found in an analogous position in the LRRK2 kinase and is the most common genetic cause of Parkinson’s disease24-26.

Patients with pathogenic variants in the STK domain of MAST3 typically presented with DEE, including 3 patients with Dravet syndrome-like phenotype. However, while these three individuals all presented with febrile-induced seizures and status epilepticus, age of onset was more variable, and individuals did not present with hemiconic/myoclonic seizures typical of Dravet-syndrome. All patients had seizure onset before 2 years of age, with 7/11 having onset before 12 months of age. Most patients had normal development prior to the onset of seizures but then experienced developmental regression or plateau which coincided with seizure onset or episodes of status epilepticus. All patients developed multiple seizure types: all 11 patients had generalized tonic seizures on 2-3 anti-epileptic drugs and 2 had been seizure free since age 2.5-2.75 years. All patients had intellectual disability, with most (5/11) having severe impairment. Most patients developed comorbidities including 6 patients with ASD or autistic features, 8 with gait disturbances, 7 experienced sleep issues, 6 had hypotonia, 3 had dysmorphic features and 3 had gastrointestinal problems.

MAST3 is the third of the five-member MAST family of serine/threonine kinases, which share a STK and a postsynaptic density protein-95/discs large/zona occludens-1 domain (PDZ). These genes have an overlapping but unique expression in the brain, with MAST3 expressed predominantly in the striatum, hippocampus, and cortex. It has been studied most extensively in the rodent striatum, where the majority of MAST3 expression is localized to medium spiny inhibitory GABAergic projection neurons5. Conversely, our transcriptomic analysis illustrates that MAST3 expression is primarily restricted to late neurodevelopment and may be restricted to cortical excitatory neurons in humans. This pattern contrasts to MAST1, which is expressed early and at high levels in the developing brain and all cell types, including excitatory, inhibitory, and non-neuronal cells. De novo MAST3 variants were previously described as a cause of a mega-corpus callosum syndrome with cerebellar hypoplasia23. Intriguingly, this includes a p.Gly510Ser variant identified in three individuals, the same amino acid position as the MAST3 p.Gly510Ser variant described in five individuals here (Fig 1D). The authors suggest MAST2 variants result in a dominant-negative mechanism for reduced expression of MAST2 and MAST3 expression in a mouse model. However, here we show MAST3 variants may act in a gain-of-function manner as demonstrated by more efficient phosphorylation of its substrate, ARPP-16. Therefore, another possible explanation for the reduced expression of MAST2 and MAST3 in MAST1 knockout models is compensation for a hyperactive MAST1. Finally, the MRI abnormalities in individuals with MAST1 pathogenic variants are extensive, while those with MAST3 pathogenic variants we analyzed revealed very mild abnormalities (Fig 2). These differences/likely lie in the expression patterns of the two genes, where MAST1 is expressed throughout development with disruption resulting in extensive malformations of cortical development while MAST3, expressed later and exclusively in excitatory neurons, is associated with DEE. This expression pattern is similar in sodium channels, where SCN3A, which is expressed early in development, is associated with malformations of cortical development. In contrast, SCN1A is expressed much later and is associated with DEE generally devoid of MRI findings (Fig 3A)27, 28. Moreover, the restriction in MAST3 expression to excitatory neurons in the cortex (but not striatum) is somewhat reminiscent of SCN8A-associated DEE, where SCN8A is expressed in excitatory neurons, with gain-of-function variants leading to excessive excitatory neuron activity29. We speculate that MAST3 gain-of-function variants may lead to the same excessive excitatory neuronal activity, though this requires further investigation in model systems. For instance, the study of patient-specific induced pluripotent stem cells (iPSCs) and isogenic controls to glutamatergic and GABAergic neurons could be used as a model system to address this question, as well as assess the functional impacts of the variants on MAST3 stability, MAST homolog abundance and phosphorylation of putative targets6-9.

In both in vitro and in vivo models of striatum, MAST3 has been shown to phosphorylate ARPP-16 at Ser466-7, and this shifts the balance of its downstream target, protein phosphatase 2A (PP2A), from active to inactive8. The in vitro work from this study suggests that four MAST3 variants result in a potential gain-of-function phenotype and therefore increased phosphorylation of ARPP-16. This would subsequently lead to a greater decrease in PP2A activity and a concomitant overall increase in phosphorylation of substrates for PP2A. Changes in PP2A have been drug-resistant whilst 3 had achieved moderate seizure control on 2-3 antiepileptic drugs and 2 had been seizure free since age 2.5-2.75 years. All patients had intellectual disability, with most (5/11) having severe impairment. Most patients developed comorbidities including 6 patients with ASD or autistic features, 8 with gait disturbances, 7 experienced sleep issues, 6 had hypotonia, 3 had dysmorphic features and 3 had gastrointestinal problems.

In summary, we propose MAST3 as a novel gene for DEE, thereby expanding the genetic landscape of DEEs. Notably, CDKL5, which is one of the most common causes of DEE, also functions like MAST3 as a kinase in the brain. Patients with pathogenic MAST3 variants present with DEE with normal development prior to seizure onset at <2 years. Status epilepticus and fever-sensitive seizures are common, severe intellectual disability was seen in most as were significant comorbidities. We also identified a single individual with a neurodevelopmental disorder, including ASD but without epilepsy, with a de novo variant outside the kinase domain. With the addition of MAST3 to clinical diagnostic sequencing panels, the prevalence of de novo kinase variants in DEE will be determined, along with the spectrum of neurodevelopmental phenotypes associated with this gene. MAST3 is the second member of MAST family of kinases to be implicated in CNS dysfunction. We propose a potential gain-of-function pathogenic mechanism that is restricted to MAST3 dysfunction in cortical excitatory neurons, reminiscent of SCN8A-associated epilepsy.

URLs:
Gene Matcher: https://genematcher.org/
gnomAD: https://gnomad.broadinstitute.org/ for both allele frequencies and LOEUF scores
TOPMed/Bravo: https://bravo.sph.umich.edu/freez3a/ht19/
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Authorship contributions

ES, KRC, AGG, ACN, GLC contributed to the conception and design of the study. ES, KRC, EB, AS, JR, AMM, JG, ROL, BS, WGW, EJM, KI, SK, TP, CB, AV, EHB, RHvJ, NM, CMEA, HG, PC, PIA, SB, LHR, UDN, KM, RG, IES, HCM, SM, LL and JJM contributed to the acquisition and analysis of data. ES, KRC, AGG, JR, SM, ACN, and GLC contributed to drafting the text or preparing the figures.

Potential conflicts of interest

The authors report no competing interests.

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Allen Brain Map single-nucleus transcriptome: https://portal.brain-map.org/atlas-and-data/rnaseq/human-m1-10x
Figure legends

Figure 1. MAST3 patient-specific variants occur at conserved sites that are intolerant to genetic variation. (A) MAST3 patient-specific variants (number of individuals in parenthesis) in relation to ST kinase (amino acids 367-640) and PDZ domains (amino acids 958-1038). (B) Corresponding graph with missense tolerance ratio (MTR, y-axis) and protein position (x-axis). The tolerance ratio measures cDNA intolerance to missense variants with 5% representing the cutoff for more intolerant segments. Regions of the ST kinase domain which are intolerant to variations portions of the ST kinase domain overlap with the distribution of pathogenic variants. (C) Multispecies alignment of the STK domain harboring the MAST3 patient variants (highlighted in green) show all pathogenic variants are highly conserved across 11 species (identical/similar amino acids in red). (D) Alignment of the MAST1 and MAST3 protein sequences. The serine threonine kinase (STK) domain has high homology and the MAST1 p. G517S and MAST3 p. G510S (highlighted in green) occur at the same position in the STK domain. Overall, these protein sequences have 61% identity.

Figure 2. MRI changes in 5 individuals with MAST3 variants. (A-C) - Sagittal FLAIR images (i) Patient 7 (p.G515S) 4 years old, normal corpus callosum and small pituitary. (B) Patient 11, 15 years old, thin corpus callosum and normal pituitary. (C) Patient 3, 2 years 9 months old, thin corpus callosum and small pituitary. D. Table summarizing changes found in corpus callosum and pituitary gland in the 5 individuals in whom we had access to MRIs. All patients had either a thin corpus callosum and/or a small pituitary gland, but not the extensive abnormalities observed in in individuals with MAST1 pathogenic variants.

Figure 3: Expression of MAST3 and MAST1 in whole brain throughout prenatal development and postnatally. (A) Heatmap of bulk RNA-seq data from the Brainscan atlas of the developing human brain (see list of URLs). MAST3 expression begins at about 26 weeks in prenatal development. In contrast, expression of MAST1 is highest early in prenatal development (9 weeks) and then is reduced throughout the lifespan. This pattern is similar in other DEE-associated genes, SCN1A and SCN3A, where only the latter is associated with malformation of cortical development. Key neuronal and developmental markers NESTIN, EOMES, SOX2 and TUBB3 are also shown. FPKM = Fragments Per Kilobase of transcript per Million mapped reads. (B) Heatmap of single-nucleus RNA-seq data from post-mortem adult human motor cortex for MAST3 and MAST1 (see list of URLs). MAST3 is expressed exclusively in excitatory neurons, while MAST1 is expressed in both inhibitory and excitatory neurons in the cortex. (C) Immunofluorescence in 11-week-old cerebral organoids shows the presence of MAST3 in a fraction of excitatory CTIP2-positive neurons (upper panels, arrows) and in the majority of CUX1-positive neurons (lower panels, arrowheads). (D) Immunofluorescence performed in E14.5 (left panels) and E16.5 (right panels) mouse coronal brain sections show MAST3 is mainly cytosolic, surrounding Tbr1+ cell nuclei (arrowheads). And some cells do present some MAST3 nuclear speckles (arrows). Scale bar=20 microns. (E) Immunofluorescence performed in E14.5 (left panels) and E16.5 (right panels) mouse coronal brain sections confirm MAST3 expression in postmitotic upper layer neurons, co-markers evolving as cerebral development progresses. TBR1 and CTIP2 are neuronal markers specific to deep cortical layers. SATB2 is a neuronal marker for upper cortical layers. Scale bars = 50 µm.

Figure 4. Phosphorylation of ARPP-16 at Ser46 by MAST3 kinase. (A) HA-tagged ARPP-16 alone, or together with MAST3, were expressed in HEK293T cells. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting for MAST3 protein, phospho-Ser46-ARPP-16, and HA-ARPP-16. Representative blots show that WT and all MAST3 mutant phosphate ARPP-16. There was minimal, background, phosphorylation of ARPP-16, as shown by the expression of ARPP-16 alone (lane 2) or when co-expressed with the dead-kinase mutant K396H (lane 4). Lanes: 1) GFP Only, 2) ARPP-16 Only, 3) Wild-Type MAST3 + ARPP-16, 4) K396H dead kinase control + ARPP-16, 5) G510S MAST3 + ARPP-16, 6) G515S MAST3 + ARPP-16, 7) L516P MAST3 + ARPP-16, 8) V551L MAST3 + ARPP-16. (B) Quantification of immunoblots revealed an increase in ARPP-16 phosphorylation when co-expressed with the G510S mutant compared to WT MAST3 (*p = 0.0159, one-way ANOVA with Dunnett’s posthoc test). Note that the GFP control, ARPP-16 alone control, and the dead kinase control quantifications were omitted from the graph due to the presence of zero values.
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<table>
<thead>
<tr>
<th></th>
<th>Normal Pituitary</th>
<th>Small Pituitary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Corpus Callosum</td>
<td></td>
<td>n = 2</td>
</tr>
<tr>
<td>Thin Corpus Callosum</td>
<td>n = 1</td>
<td>n = 2</td>
</tr>
</tbody>
</table>
Table 1 Genetic details and seizure-specific clinical features for individuals with MAST3 STK domain de novo variants

<table>
<thead>
<tr>
<th>Patient</th>
<th>Variant (CADD, Polyphen)</th>
<th>Degree of ID</th>
<th>Epilepsy Diagnosis</th>
<th>Age at sz onset in months</th>
<th>Sz type at onset</th>
<th>Subsequent sz types (age of onset if known)</th>
<th>Current sz control (age if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.1217G&gt;C, p.R406P (34.0, 1.00)</td>
<td>Severe</td>
<td>DEE</td>
<td>18</td>
<td>Convulsive status epilepticus with fever</td>
<td>Atonic, tonic, GTCS; often in clusters with fever</td>
<td>Moderate-control</td>
</tr>
<tr>
<td>2</td>
<td>c.1528 G&gt;A, p.G510S (32.0, 0.999)</td>
<td>Severe</td>
<td>DEE</td>
<td>11</td>
<td>Febrile tonic-clonic</td>
<td>Tonic-clonic, Focal (motionless staring, oromotor automatisms, diffuse tonic) Absences Often in clusters with fever</td>
<td>Seizure control since age 2 y 9 mo</td>
</tr>
<tr>
<td>3</td>
<td>c.1528 G&gt;A, p.G510S (32.0, 0.999)</td>
<td>Severe</td>
<td>DEE</td>
<td>6-7</td>
<td>Myoclonic</td>
<td>Absence (14 mo) Atonic (Drop attacks) (14 mo) Tonic-clonic (14 mo)</td>
<td>Well-controlled (2.5 yrs)</td>
</tr>
<tr>
<td>4*</td>
<td>c.1528 G&gt;A, p.G510S (32.0, 0.999)</td>
<td>Severe</td>
<td>DEE</td>
<td>2</td>
<td>Generalized tonic clonic</td>
<td>Atonic Tonic TCS Often in clusters with fever</td>
<td>Drug-resistant</td>
</tr>
<tr>
<td>5</td>
<td>c.1528 G&gt;A, p.G510S (32.0, 0.999)</td>
<td>Severe</td>
<td>DEE</td>
<td>24</td>
<td>Generalized tonic-clonic (&lt;24 hours of vaccination)</td>
<td>TCS Absence, FIAS</td>
<td>Drug-resistant at age of last follow-up (27 yrs)</td>
</tr>
<tr>
<td>6</td>
<td>c.1528 G&gt;A, p.G510S (32.0, 0.999)</td>
<td>Moderate</td>
<td>DEE</td>
<td>10</td>
<td>Generalized tonic-clonic in context of viral illness</td>
<td>GTCS Drop attacks Absence, FIAS Focal status</td>
<td>Drug-resistant</td>
</tr>
<tr>
<td>7</td>
<td>c.1543 G&gt;A, p.G515S (27.3, 0.989)</td>
<td>Moderate</td>
<td>DEE</td>
<td>11-12</td>
<td>Myoclonic-atonic</td>
<td>Myoclonic absence (eye flutter) Myoclonic atonic Myoclonic Atonic (head drops) GTCS (17 mo) Tonic (vibratory)</td>
<td>Drug-resistant</td>
</tr>
<tr>
<td>8</td>
<td>c.1543 G&gt;A, p.G515S (27.3, 0.989)</td>
<td>Moderate</td>
<td>DEE</td>
<td>20</td>
<td>Tonic-clonic</td>
<td>Tonic (20 mo) FIAS with manual automatisms evolving to tonic seizure (30 years) FBTCS</td>
<td>Moderate-control</td>
</tr>
<tr>
<td>9</td>
<td>c.1543 G&gt;A, p.G515S (27.3, 0.989)</td>
<td>Mild-moderate</td>
<td>DEE</td>
<td>7</td>
<td>Febrile convolution</td>
<td>Absence (1 year) Myoclonic Tonic-clonic Convulsive status epilepticus (single event at 7 years)</td>
<td>Moderate control</td>
</tr>
<tr>
<td>10</td>
<td>c.1547 T&gt;C, p.L516P (26.4, 0.999)</td>
<td>Severe-Profound</td>
<td>DEE</td>
<td>24</td>
<td>ND</td>
<td>FIAS (mouth automatism, head and eye deviation to one side and generalized shaking – nocturnal) GTC</td>
<td>Drug-resistant</td>
</tr>
<tr>
<td>11</td>
<td>c.1651G&gt;T, p.V551L (25.3, 0.915)</td>
<td>Severe</td>
<td>DEE</td>
<td>11 months</td>
<td>Febrile convolution</td>
<td>FIAS (11 years), myoclonic, absence, atonic, GTCS, tonic</td>
<td>Moderate control</td>
</tr>
</tbody>
</table>

Abbreviations: Sz – seizure, FBTCS, focal to bilateral tonic clonic seizures, FIAS – focal impaired awareness, GTCS – generalized tonic clonic seizures, TCS -tonic clonic seizures, ND – not determined

*patient previously described in Iwama et al, 2019
Author/s:
Spinelli, E; Christensen, KR; Bryant, E; Schneider, A; Rakotomamonjy, J; Muir, AM; Giannelli, J; Littlejohn, RO; Roeder, ER; Schmidt, B; Wilson, WG; Marco, EJ; Iwama, K; Kumada, S; Pisano, T; Barba, C; Vetro, A; Brilstra, EH; Jaarsveld, RH; Matsumoto, N; Goldberg-Stern, H; Carney, PW; Andrews, P; El Achkar, CM; Berkovic, S; Rodan, LH; McWalter, K; Guerrini, R; Scheffer, IE; Mefford, HC; Mandelstam, S; Laux, L; Millichap, JJ; Guemez-Gamboa, A; Nairn, AC; Carvill, GL

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