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Ectopic HCN4 Provides a Target Biomarker for the Genetic Spectrum of mTORopathies

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Abstract

Background and Objectives

Pathogenic variants in PI3K-AKT-mTOR pathway and GATOR1 complex genes resulting in hyperactivation of mechanistic target of rapamycin (mTOR) complex 1 are a major cause of drug-resistant epilepsy and focal cortical malformations (FCM). Resective neurosurgery is often required to achieve seizure control in patients with mTORopathies due to lack of effectiveness of nonsurgical therapies, including antiseizure medication and mTOR inhibitors. Elevated hyperpolarization-activated cyclic nucleotide–gated potassium channel isoform 4 (HCN4) has been proposed as a key marker in some mTOR-related brain malformations. This study aimed to investigate HCN4 as a biomarker in the brain across the genetic spectrum of mTORopathies in humans.

Methods

Our study investigated the relative steady-state levels and cellular localization of HCN4 in resected human brain tissue from 18 individuals with mTORopathies (3 individuals with tuberous sclerosis complex (TSC) due to *TSC2* variants, 5 individuals with focal cortical dysplasia type IIA (FCD IIA) due to genetic variants in *MTOR*, *AKT3*, and *PIK3CA*, and 10 individuals with FCD IIB due to variants in *TSC1*, *MTOR*, *RHEB*, *DEPDC5*, or *NPRL3*).

Results

Elevated HCN4 was observed to be highly restricted to abnormal cell types (dysmorphic neurons and balloon cells) in brain tissue from all mTORopathy tissues ($p < 0.0001$) compared with those in controls, regardless of genetic cause or variant allele frequency. Elevated HCN4 was not observed in controls or individuals with non-mTOR–related focal epilepsy due to pathogenic variants in *ATP1A3*, *SLC35A2*, or *FGFR1*.

Discussion

HCN4 provides a biomarker for the genetic spectrum of mTORopathies and may present a potential therapeutic target for seizure control in mTOR-related epilepsy.

Introduction

Pathogenic variants in the PI3K-AKT-mTOR pathway and GATOR1 complex genes resulting in hyperactivation of mechanistic target of rapamycin (mTOR) complex 1 are a well-established cause of focal cortical malformations (FCM) causing drug-resistant epilepsy. Collectively, these disorders are known as mTORopathies and include tuberous sclerosis complex (TSC), hemimegalencephaly (HME), and focal cortical dysplasia type II (FCD II).[1]

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Glossary

BC = balloon cell; **BOSD** = bottom-of-sulcus dysplasia; **cAMP** = cyclic adenosine monophosphate; **ddPCR** = droplet digital polymerase chain reaction; **DEE** = developmental and epileptic encephalopathy; **DN** = dysmorphic neuron; **FCD** = focal cortical dysplasia; **FCD II** = focal cortical dysplasia type II; **FCM** = focal cortical malformations; **FFPE** = formalin-fixed paraffin-embedded; **HME** = hemimegalencephaly; **IHC** = immunohistochemistry; **HCN4** = hyperpolarization-activated cyclic nucleotide–gated potassium channel isoform 4; **MOGHE** = mild malformation of cortical development with oligodendroglial hyperplasia in epilepsy; **mTOR** = mechanistic target or rapamycin; **TSC** = tuberous sclerosis complex; **VAF** = variant allele frequency.

FCD is the most common form of FCM. Individuals with FCD typically present with seizures and may have developmental impairments and intellectual disability if drug-resistant epilepsy presents in early childhood. FCD II are FCM characterized by cortical dyslamination and the presence of dysmorphic neurons (DNs), either without (FCD IIA) or with (FCD IIB) balloon cells (BCs).[2-4] TSC is a multisystem disorder, associated with epileptogenic brain lesions known as cortical tubers, which are glioneuronal hamartomas with similar histopathologic features to FCD IIB.[5] The incidence of TSC is estimated to be 1:10,000 live births.[6] Together, TSC and FCD II are the most common disorders requiring surgical intervention to control seizures and represent a substantial medical burden.[7]

Germline or somatic variants in mTOR pathway genes, including in *MTOR, TSC1, TSC2, RHEB, DEPDC5, AKT3, PIK3CA*, and *NPRL3*, are well-established causes of FCD IIB.[8,9] Somatic variants in *TSC1* and *TSC2* can cause TSC but are less common than germline *TSC1* and *TSC2* variants. In FCM with brain somatic mosaicism, a gradient of higher variant allele frequency (VAF) has been observed in abnormal cells such as DNs and BCs in FCD IIB and in the central core of tubers in TSC, compared with adjacent nondysplastic tissue.[10,11]

Despite the genetic basis of mTORopathies being relatively well established, the pathophysiologic and epileptogenic mechanisms are still not fully understood. Epilepsy neurosurgery, which is invasive and expensive, is often the only effective treatment to control seizures and prevent serious developmental consequences in these individuals.[12,13] As such, there is a need to identify novel therapeutic targets for seizure control.

Hyperpolarization-activated cyclic nucleotide–gated potassium channel isoform 4 (HCN4) is a member of the hyperpolarization-activated, cyclic nucleotide–gated cation (HCN) channel family and regulates cardiac and neuronal pacemaker activities.[14] It was recently shown that upregulated HCN4 is a key driver of mTOR-dependent epilepsy in a mouse model with dysregulated RHEB activity.[15] HCN4 was shown to be increased in FCM neurons, and it was found that increasing cyclic adenosine monophosphate (cAMP) in these neurons resulted in repetitive firing, while disruption of HCN4 activity resulted in a rescue of the seizure phenotype in mice. The authors demonstrated abnormal HCN4 steady-state levels in DNs in surgically resected human brain tissue from individuals with TSC and FCD IIB, without reporting the genetic basis in these individuals, and proposed HCN4 as a candidate therapeutic target. A subsequent study provided evidence of shared consequences on cortical pyramidal neuron development and function caused by variants in different mTOR pathway genes.[16] Therefore, in this study we examined the distribution and relative steady-state levels of HCN4 in brain tissue derived from individuals with distinct genetic mTORopathies to determine whether aberrant *HCN4* expression is a consistent feature across the genetic spectrum of FCM-related mTORopathies, mediated by either germline or brain-specific somatic variants.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

This study was approved by the Human Research Ethics Committee at the Royal Children's Hospital (ID 29077). Written informed consent was obtained from participants or legal guardians. Brain tissue samples used for immunohistochemical (IHC) and immunofluorescence analyses were retrieved from the Royal Children's Hospital Pathology Service, the tissue bank associated with the Neurogenetics Group at the Murdoch Children's Research Institute, and the Victorian Brain Bank, all in Melbourne, Australia.

Study Design and Cohort

Brain tissue samples used for IHC and immunofluorescence analyses were retrieved from the Royal Children's Hospital Pathology Service, the tissue bank associated with the Neurogenetics Group at the Murdoch Children's Research Institute, and the Victorian Brain Bank, all in Melbourne, Australia. Brain tissue had been collected during epilepsy surgery or postmortem and stored as formalin-fixed paraffin-embedded (FFPE) tissue. The mTORopathy cohort consisted of 3 individuals with TSC with variants in the *TSC2* gene (cases 1–3), 10 individuals with FCD IIB (cases 4–13, including individuals with bottom-of-sulcus dysplasia [BOSD]) with germline variants in *DEPDC5* (n = 1) and *NPRL3* (n = 1) or brain somatic variants in *MTOR* (n = 6, VAF = 2.1–5.7%), *RHEB* (n = 1, VAF = 13.0%), or *TSC1* (n = 1, VAF = 2.8%), and 5 individuals with FCD IIA and HME (cases 14–18) with brain

somatic variants in *MTOR* (n = 1, VAF = 7.40%), *AKT3* (n = 2, VAF = 4.9–5.1%), or *PIK3CA* (n = 2, VAF = 20–29%). Genetic variants were previously identified using deep targeted sequencing on brain-derived DNA, and variant allele frequencies were validated by droplet digital PCR (ddPCR) as previously described.[11] Non-mTORopathy epilepsy and FCM control specimens consisted of postmortem brain tissue from 1 individual with epilepsy due to a heterozygous variant in *ATP1A3* (control 1) and surgical brain tissue from 4 individuals with mild malformation of cortical development with oligodendroglial hyperplasia in epilepsy (MOGHE) due to a somatic *SLC35A2* variant (control 2–5) and 2 individuals with dysembryoplastic neuroepithelial tumors (DNETs) due to variants in *FGFR1* (controls 6–7) (Table). Four additional healthy control brain tissue samples were collected from adults obtained postmortem from the Victorian Brain Bank (controls 8–11).

Immunohistochemical Analysis

Staining was completed according to established protocols. FFPE tissue slides were incubated overnight with anti-HCN4 (Alomone, #APC-052, dilution 1:500), anti–phospho-S6 ribosomal protein (Ser235/236) (pS6) (Cell Signaling, #4858P, dilution 1:100), and antineurofilament marker (SMI-311) (BioLegend, #837801, dilution 1:1000) antibodies. Hematoxylin staining was completed to identify nuclear components in the tissues.

HCN4 Quantification and Dysmorphic Neuron Identification

Quantification of HCN4 staining was performed using ImageJ analysis software. Regions of interest (ROI) were manually drawn, capturing 15 cells from each slide, and normalized against 5 background regions of equal size to account for background signal intensity. Neuronal cells that met the criteria of >25 μm cell diameter with 15–28 μm nucleus diameter were identified as cytomegalic and labeled as DNs or BCs.[17] Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software La Jolla California, USA).

Immunofluorescence Staining

Staining was completed according to established protocols. FFPE tissue slides were incubated overnight with anti-HCN4 (Alomone, #APC-052, dilution 1:500), anti–phospho-S6 ribosomal protein (Ser235/236) (pS6) (Cell Signaling, #E2R10, dilution 1:500), and antineurofilament H (SMI-32) (Invitrogen, #PA110002, dilution 1:1000) antibodies. DAPI (Abacus, #VEH1500) staining was completed to identify nuclear components in the tissues.

Data Availability

All data are available in the main text or the supplementary materials. All original images are included in the supplementary material, and all clinical data are described in the main text.

Results

Histopathology and Genetic Findings

To assess the steady-state level and distribution of HCN4 in dysplastic tissue derived from the genetic spectrum of mTORopathies, IHC analysis of human surgically resected brain tissue was performed. The cohort consisted of specimens from 18 individuals with causal genetic variants in mTOR pathway genes (Table). Seven age-matched individuals with non-mTOR–related epilepsy and brain malformation due to variants in *ATP1A3* (control 1), *SLC35A2* (controls 2–5), or *FGFR1* (controls 6–7) were used as unrelated epilepsy controls. Brain tissue specimens from 4 deceased adult individuals without FCM were used as healthy controls (controls 8–11).

Strong HCN4 Immunoreactivity Was Observed in Dysplastic Tissues From Individuals With mTOR-Related FCM

Immunostaining of TSC, FCD IIA, and FCD IIB tissues with anti-HCN4 antibody (Figure 1, representative results from 2 cases and 3 controls and eFigure 1, all cases and controls) demonstrated strong immunoreactivity, indicating aberrant HCN4 levels, in individuals with TSC and FCD II (Figure 1, J and M). In all cases with TSC and FCD II, immunoreactivity was restricted to enlarged neurons with irregular shape and increased soma size, features consistent with DNs and BCs. By contrast, adjacent neuronal cells with normal shape and soma size showed weak or absent HCN4 immunoreactivity. Lesional tissue from individuals with FCD IIA/B caused by somatic mTOR pathway variants showed the same pattern of strong HCN4 immunoreactivity in abnormal neurons as seen in lesional tissue from individuals with germline variants. Consistent with previous studies,[14] HCN4 immunoreactivity was very weak or absent in brain tissue from individuals with epilepsy due to non-mTOR–related causes including *ATP1A3* (control 1) (Figure 1A), *SLC35A2* (controls 2–5) (Figure 1D, eFigure 1), and *FGFR1* (controls 6–7) (eFigure 1). Similarly, HCN4 and pS6 immunoreactivity was very weak in control postmortem specimens (Figure 1G, eFigure 1). Quantitative analysis showed that cases with FCD II and TSC had significantly elevated HCN4 in dysmorphic neurons and balloon cells, compared with epilepsies unrelated to the mTOR pathway (Figure 2A) ($p < 0.0001$). Furthermore, HCN4 levels as measured by staining intensity did not exhibit any correlation with VAF in this cohort ($R^2 = 0.63$) (Figure 2B).

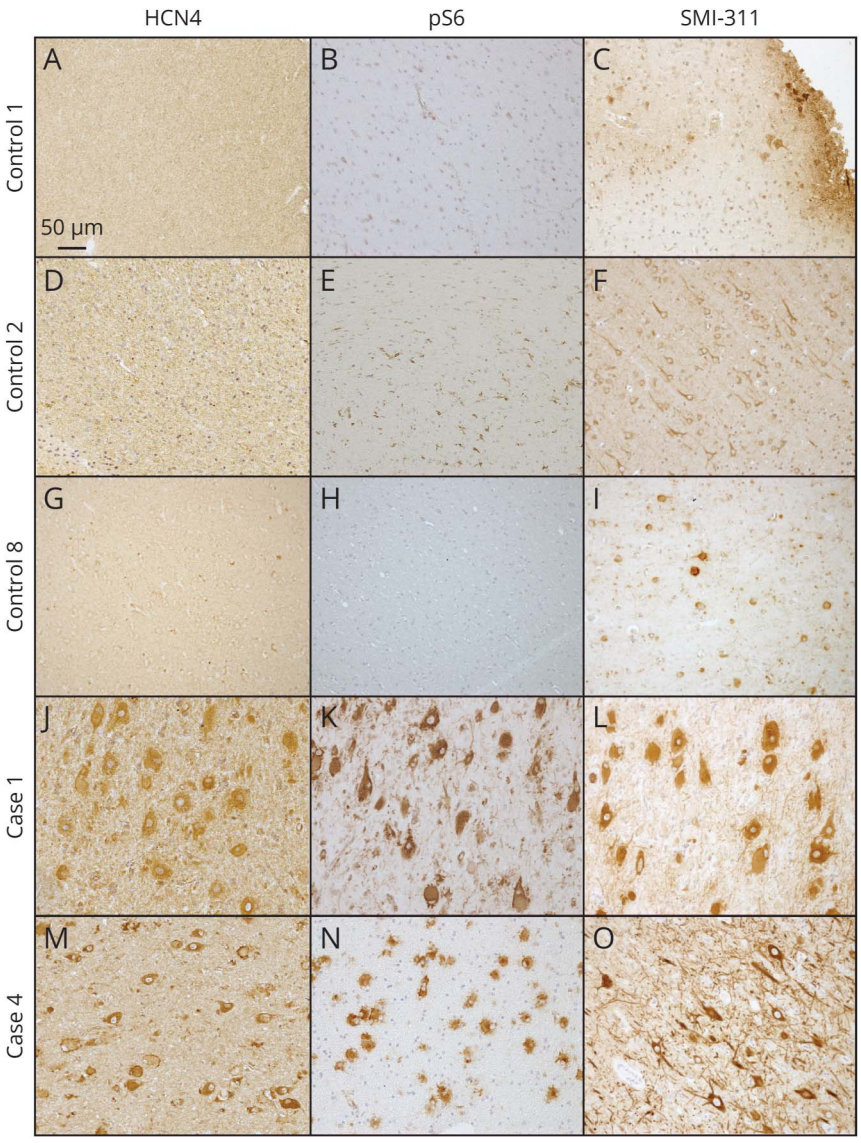
To determine the identity of HCN4-positive cells, IHC analysis was performed using a pS6 antibody. This protein is a key marker of mTOR kinase activity and can be used to identify the abnormal cell types associated with TSC and FCD II (DNs and BCs). Immunostaining of brain sections from affected individuals showed strong immunoreactivity for pS6 in abnormal and enlarged cells with irregular shape, indicating an abundance of DNs and BCs in these tissues (Figure 1, K and N). The density of pS6-positive cells varied between individuals, but all TSC and FCD II tissues exhibited DNs, and BCs were observed in most individuals. Limited and weak immunoreactivity against pS6 was observed in the non-mTOR epilepsy control individuals with PMG, MOGHE, or DNETs (Figure 1, B and E, eFigure 1) and control postmortem specimens (Figure 1H). In addition, IHC analysis was performed using an antibody directed against pan-

Table Cohort of Brain Tissue Specimens

Individual	Age at collection (y)	Sex	Age at seizure onset (y)	Seizure types	Pathology	Gene	Amino acid change	VAF	Tissue region	Specimen type
Control 1	0.3	M	0	Fo	PMG	*ATP1A3*	L924P	Heterozygous	L frontal cortex	Postmortem
Control 2	4	M	1.5	Sp	MOGHE	*SLC35A2*	L120Hfs*7	41.0%	L perisylvian cortex	Surgical
Control 3	2.7	M	0.2	Sp, Fo	MOGHE	*SLC35A2*	Q46*	20%	L superior temporal gyrus	Surgical
Control 4	1.2	F	0.5	Sp	MOGHE	*SLC35A2*	Q183*	1.8%	L superior temporal gyrus	Surgical
Control 5	2	M	0.3	Sp	MOGHE	*SLC35A2*	Q185*	16.5%	R frontal cortex	Surgical
Control 6	3	F	0.3	Sp, Fo	DNET	*FGFR1*	CNV	Heterozygous	L inferior frontal cortex	Surgical
Control 7	17	F	10.5	Fo	DNET	*FGFR1*	N546K	30%	L temporal cortex	Surgical
Control 8	43	M	n/a	n/a	n/a	n/a	n/a	n/a	L temporal cortex	Postmortem
Control 9	49	M	n/a	n/a	n/a	n/a	n/a	n/a	frontal cortex	Postmortem
Control 10	59	F	n/a	n/a	n/a	n/a	n/a	n/a	temporal cortex	Postmortem
Control 11	60	F	n/a	n/a	n/a	n/a	n/a	n/a	frontal cortex	Postmortem
Case 1	2	M	0.9	Sp, Fo	TSC	*TSC2*	Q1192Rfs*18	Heterozygous	L temporal tuber	Surgical
Case 2	2	F	0.3	Sp, Fo	TSC	*TSC2*	Q1281*	Heterozygous	R cuneus tuber	Surgical
Case 3	2	F	1.3	Fo	TSC	*TSC2*	Q204*	Heterozygous	R posterior temporal occipital tuber	Surgical
Case 4	0.5	M	0.5	Sp, Fo	FCD IIB	*DEPDC5*	R843*	Heterozygous	L insular cortex	Surgical
Case 5	6	M	0	Sp, Fo	FCD IIB	*NPRL3*	T459Nfs*21	Heterozygous	R anterior insula long gyrus	Surgical
Case 6	1.9	M	1.8	Fo	FCD IIB	*RHEB*	Y35L	13.0%	L frontal cortex	Surgical
Case 7	2	F	0.8	Fo	FCD IIB	*TSC1*	F581Hfs*6	2.8%	R temporal cortex	Surgical
Case 8	12	M	2	Fo	FCD IIB	*MTOR*	T1977K	5.7%	R frontal cortex	Surgical
Case 9	3	F	1.4	Sp, Fo	FCD IIB	*MTOR*	P2425dup	4.1%	R frontal medial cortex	Surgical
Case 10	9	M	3	Fo	FCD IIB	*MTOR*	S2215Y	3.2%	R subcentral cortex	Surgical
Case 11	18	M	13	Fo	FCD IIB	*MTOR*	C1483R	3.0%	L insular cortex	Surgical
Case 12	17	M	6	Fo	FCD IIB	*MTOR*	S2215Y	2.1%	R precentral cortex	Surgical
Case 13	6	F	0	Fo	FCD IIB/ BOSD	*MTOR*	T1977K	2.9%	R frontal medial cortex	Surgical
Case 14	0.1	M	0	Sp, Fo	FCD IIA/HME	*MTOR*	Y1450_ L1453del	7.4%	R supramarginal gyrus	Surgical
Case 15	1	M	0	Sp, Fo	FCD IIA/HME	*AKT3*	E17K	5.1%	R superior temporal gyrus	Surgical
Case 16	3	M	0	Fo	FCD IIA/HME	*AKT3*	E17K	4.9%	R frontal cortex	Surgical
Case 17	0.6	M	0	Sp, Fo	FCD IIA/HME	*PIK3CA*	E542K	20.0%	R anterior frontal cortex	Surgical
Case 18	5	M	0	Sp, Fo	FCD IIA/HME	*PIK3CA*	H1047R	29.0%	L inferior parietal cortex	Surgical

Abbreviations: DNET = dysembryoplastic neuroepithelial tumor; F = female; FCD IIA = focal cortical dysplasia type IIA; FCD IIB = focal cortical dysplasia type IIB; Fo = focal seizures; HME = hemimegalencephaly; L = left; M = male; MOGHE = mild malformation of cortical development with oligodendroglial hyperplasia in epilepsy; n/a = not applicable; PMG = polymicrogyria; R = right; Sp = epileptic spasms; TSC = tuberous sclerosis complex; VAF = variant allele frequency.

Figure 1 Immunohistochemical Analysis of HCN4 in Patient and Control Brain Tissues



HCN4, SMI311, and pS6 IHC staining for control 1 (PMG) (A–C), control 2 (MOGHE) (D-F), control 8 (healthy adult) (G–I), case 1 (TSC) (J–L), and case 4 (FCD IIB) (M–O) at 20× magnification.

neuronal neurofilaments (SMI-311) (BioLegend, #837801). SMI-311 enables visualization of neurofilaments that accumulate in the soma and dendrites of dysmorphic nerve cells and is used as a specific marker of DNs in FCM tissue. Similar to the results observed for pS6 immunoreactivity, strong specific SMI-311 signal was only observed in tissue from individuals with FCD II and TSC and large abnormal neuronal cells (Figure 1, L and O). Strong SMI-311 immunoreactivity was absent from control tissues (Figure 1, C, F, and I). Collectively, the distribution and localization of HCN4 immunoreactivity was consistent with the abnormal cell types present in FCD II and TSC.

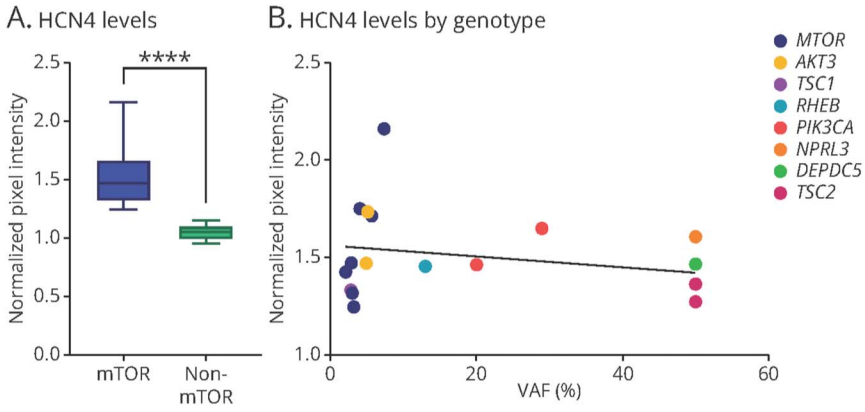
Immunofluorescence Staining Shows Strong HCN4 Signal in FCM Neurons

To validate that HCN4 was elevated primarily in abnormal cell types, colocalization of HCN4 and pS6 was determined by immunofluorescence analysis of specimens from cases 1 (TSC) and 4 (FCD IIB) and control 1 (Figure 3). Immunofluorescence costaining of HCN4 in red, pS6 in purple, and SMI-32 showed clear overlap in the cellular localization of HCN4 and pS6 in DNs in cases 1 and 4 (Figure 3). Presence of HCN4 and pS6 with absence of SMI-32 identified BCs. Strong coexpression of HCN4 and pS6 was not observed in control non-mTOR–related epilepsy tissues or adjacent normal cortical neurons in the FCM specimens (Figure 3A, eFigure 1).

Discussion

We report histopathologic data and elevated HCN4 steady-state levels in FCM tissue from a cohort of individuals with a spectrum of genetically distinct mTORopathies with varying variant allele

Figure 2 HCN4 Quantification in Patient and Control Brain Tissues



(A) Box plot of HCN4 quantification in dysmorphic neurons and balloon cells in all cases vs neurons in all controls and (B) association of HCN4 staining intensity in DNs and BCs with variant allele frequency (VAF) in case cohort. Increased HCN4 is observed in FCD IIB and TSC tissues compared with focal epilepsies caused by non-mTOR genes ($p < 0.0001$). No correlation was observed between VAF and HCN4 levels ($R^2 = 0.63$). Box represents median and interquartile range. Error bars indicate minimum and maximum values.

frequencies in brain tissue. The strong coexpression of pS6 and HCN4 observed using immunofluorescence staining in brain tissue specimens from individuals with TSC and FCD IIB confirms that aberrant HCN4 expression is highly localized to cells demonstrating abnormal mTOR pathway activation.

These findings are consistent with data published recently in *RHEB* and *TSC1*-related FCM.[15] We build on that work to show that increased abundance of HCN4 in DNs and BCs is a common feature across the genetic spectrum of mTOR pathway–related FCM, including *MTOR, TSC1, TSC2, RHEB, DEPDC5, AKT3, PIK3CA,* and *NPRL3.* Furthermore, elevated HCN4 is not observed in brain tissue from individuals with non-MTOR–related epilepsy and FCM due to *ATP1A3, SLC35A2,* or *FGFR1* variants or healthy controls. *ATP1A3* encodes the α3 subunit of Na+/K+-ATPase and has been linked to alternating hemiplegia, cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss syndrome, rapid-onset dystonia-parkinsonism, and developmental and epileptic encephalopathy (DEE).[18,19] *ATP1A3* is not associated with the PI3K-AKT3-mTOR pathway and is not reported to affect pS6 levels.[19] Similarly, individuals with MOGHE typically present with severe drug-resistant epilepsy within the first few months of life and FCM amenable to surgery. However, they are understood to be clinically and genetically distinct from mTORopathies, with somatic *SLC35A2* variants being identified in most cases (20). FGFR1 plays a role in the MAPK-ERK pathway.[20] Notably, DNs and BCs are not a feature of MOGHE, PMG, or DNETs (21). Absence of dysregulated HCN4 in FCM tissue from these controls suggests that HCN4 upregulation is a defining feature of mTORopathies.

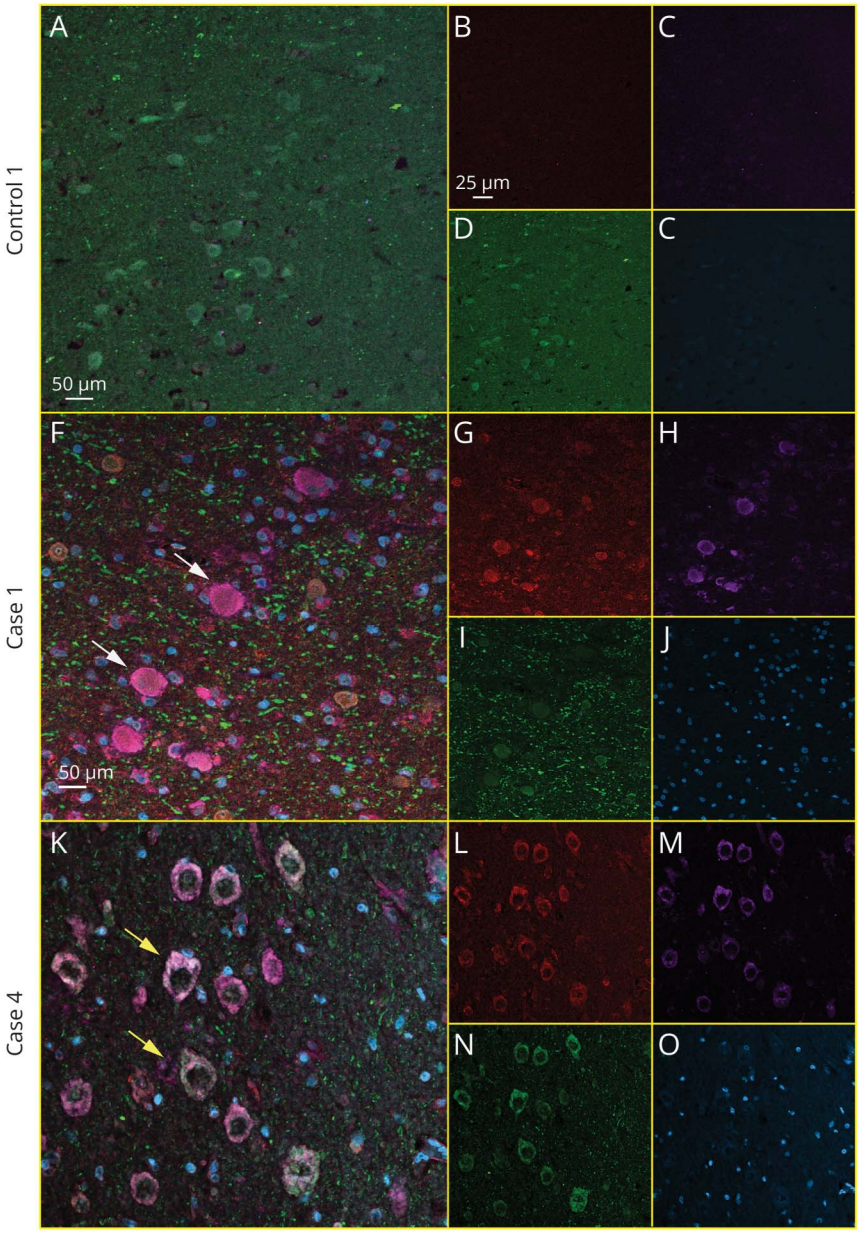
The strong colocalization of pS6 and HCN4 observed via immunofluorescence staining confirms that elevated HCN4 is highly restricted to DNs and BCs, further supporting HCN4 as a potential target for seizure control in mTORopathies more broadly.

Brain somatic mosaicism is common in mTORopathies, particularly in FCD IIB. We observed similar levels of aberrant HCN4 in FCM neurons in specimens from individuals with germline or somatic variants with a variant allele frequency as low as 2.1%, establishing that elevated HCN4 steady-state level is not exclusive to germline mTORopathies. Previously, elevated HCN4 immunoreactivity and channel activity were observed in FCM neurons in a cellular mosaic $Rheb^{CA}$ mouse model of FCD II, achieved by in utero electroporation.[15,21] In this study, we assessed human tissue with variant allele frequencies ranging from 2.1% to 13.0% to demonstrate the consistent distribution and expression of HCN4 in DNs and BCs in human mosaic cases. This is relevant due to the high incidence of low-level brain-specific somatic variants in FCD IIB and TSC and suggests that this mechanism is not exclusive to germline mTORopathies.

Limitations of this work are as follows: (1) this study uses a limited set of controls; 7 pediatric epilepsy specimens and 4 unaffected adult specimens. Unaffected age-matched controls would eliminate any effect of age on HCN4 steady-state levels but are difficult to source. However, the absence of HCN4 staining in adjacent "nondysplastic" cells in individuals with mTORopathies provides confidence that HCN4 in the dysplastic tissue differs from age-expected levels, with the nondysplastic cells acting as internal controls; and (2) this study has only demonstrated dysregulated protein abundance and has not measured HCN-mediated channel activity in human tissue. However, given results observed in the mouse model of TSC-associated and FCD II-associated FCM, we suggest that HCN4 channel activity in dysplastic tissue may be a major contributor to seizure activity in FCD II. This study used an HCN4 antibody that has previously been validated in a conditional mouse knockout model[14] but has not examined other HCN channel proteins. Future studies should explore HCN4 channel activity in a broader set of disorders.

Our results are concordant with an initial study that examined brain tissue from individuals with a clinical, but not molecular, diagnosis of TSC or FCD IIB[15] and collectively suggest that

Figure 3 Colocalization of HCN4 and pS6 in Abnormal Cell Types in Patient Brain Tissue



Immunofluorescence costaining for control 1 (PMG), case 1 (TSC), and case 4 (FCD IIB) at 20× magnification. (A, F, K) Combined HCN4, pS6, and SMI-32 immunofluorescence. (B, G, L) HCN4 staining, indicated by red channel. (C, H, M) pS6 staining, indicated by purple channel. (D, I, N) SMI-32 staining, indicated by green channel. (E, J, O) DAPI stain indicated by blue channel. Immunofluorescence staining shows strong overlap between HCN4 and pS6 immunofluorescence in cases 1 and 4, suggesting that HCN4 is in higher abundance in dysmorphic neurons (SMI-32 positive, marked by yellow arrows) and balloon cells (SMI-32 negative, marked by white arrows).

dysregulated HCN4 is a biomarker of germline and somatic mTORopathies. Recent studies have identified that abnormal cell types appear to be most abundant in the center of tubers and bottom-of-sulcus dysplasias, corresponding to the sites of maximal MRI and electrocorticogram abnormalities.[22,23] Moreover, DNs are reported to be the major epileptogenic cell type in FCD and TSC,[24] and somatic variants causing FCD may be restricted to DNs and BCs.[11] It has previously been shown that surgical resections limited to the tuber center, targeting a nidus of dysmorphic and intrinsically epileptogenic neurons, are sufficient for seizure freedom in TSC.[22] As such, establishment of an effective, targeted nonsurgical therapy for epilepsy in mTORopathies for example by therapeutic targeting of aberrant HCN4 activity could bridge a major treatment gap. Such a strategy could be particularly effective for individuals with multifocal seizures or dysplasia in eloquent cortex, who are poor candidates for resective surgery.

Ectopic HCN4 in DNs is thought to underlie hyperexcitability through a cAMP-mediated activation of the channel causing depolarization and aberrant firing.[15] The expression of nonfunctioning HCN4 channels in DNs is sufficient to stop seizures in the $Rheb^{CA}$ mouse model of FCD II.[15] This suggests that HCN4 channel activation is central to excitability leading to seizures and that "blocking" these channels may be an effective therapeutic strategy. The use of gene technologies, including antisense oligonucleotides, targeted to

reducing *HCN4* expression may prove useful. Brain-specific HCN4 channel knockout is well tolerated in mice arguing that this approach may be feasible in humans.[25] Alternatively, a small molecule approach aimed at developing brain-penetrant HCN4 selective blockers could be another targeting approach. In summary, an elevated abundance of HCN4 in FCM neurons in this genetically distinct cohort of mTORopathies suggests that HCN4 provides a biomarker for mTORopathies and should be investigated as a potential therapeutic target for seizure control in mTORopathies.

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Disclosure

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Appendix Authors

Name	Location	Contribution
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