Hamartin Variants That Are Frequent in Focal Dysplasias and Cortical Tubers Have Reduced Tuberin Binding and Aberrant Subcellular Distribution In Vitro

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Abstract

Focal cortical dysplasia type IIb is characterized by epilepsy-associated malformations that are often composed of balloon cells and dysplastic neurons. There are many histopathologic similarities between focal cortical dysplasia type IIb and cortical tubers in tuberous sclerosis complex (TSC), an autosomal-dominant phakomatosis caused by mutations in the TSC1 or TSC2 genes that encode hamartin and tuberin. We previously found that an allelic variant of TSC1 (hamartinH732Y) is increased in focal cortical dysplasia type IIb. Here, we investigated the subcellular localization of hamartinH732Y and the 2 stop mutants hamartinR692X and hamartinR786X in vitro. Future studies will be needed to characterize the roles of these TSC1 sequence variants in the genesis of dysplastic epileptogenic developmental brain lesions.

Key Words: Amino acid exchange, Hamartin, Insulin receptor signaling pathway, mTOR, Subcellular distribution, Tuberin.

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal-dominant multiorgan disorder characterized by benign tumors and dysplastic lesions in many organs, including skin, kidney, lungs, heart, and brain (1, 2). Cerebral cortical tubers are frequently composed of large dysmorphic neurons and giant cells. Cortical dysplasias associated with focal epilepsies consist of dysplastic cytomegalic neurons and balloon cells (focal cortical dysplasia type IIb [FCDIIb] according to the so-called Palmini classification [3]; Fig. 1). Thus, they resemble cortical tubers. Patients with FCDIIb generally lack additional features of a neurocutaneous phakomatosis (4).

Tuberous sclerosis complex is caused by mutations in TSC1 on chromosome 9q34 (5) or TSC2 on chromosome 16p13.3 (6, 7). Remarkably, TSC1/TSC2 mutations are only detected in approximately 85% of TSC patients (8). Hamartin and tuberin, the corresponding gene products, form a heterodimer operating as a tumor suppressor in the phosphatidylinositol 3-kinase pathway (5, 9, 10). Consistent with the Knudson 2-hit hypothesis, extracerebral neoplasms in TSC patients frequently harbor loss of heterozygosity (11). In contrast, loss of heterozygosity has been rarely found in TSC brain lesions (12).

Recent data suggest that the TSC pathway in neurons is highly sensitive to gene dosage effects, including the observation that TSC1 haploinsufficiency impairs neuronal morphology (13). Furthermore, only little is known about a potentially impaired subcellular distribution of TSC1 sequence variants, that is, aberrant nuclear localization of hamartin in a tuber was demonstrated in a case study (14). It has also been shown that oligomerization of the hamartin carboxyl-terminal coiled coil domain was inhibited in the presence of tuberin (15). The presence of hamartin aggregates in FCDIIb components (Fig. 1) may suggest that aberrant...
interactions between hamartin and tuberin also occur in these epilepsy-associated lesions.

Here, we used in vitro approaches to analyze whether TSC1 coding allele variants in the predicted tuberin-binding domain of hamartin (5) impair this interaction and lead to aberrant subcellular distribution of the resulting hamartin protein variants. Therefore, we focused on the allelic variant known to be increased in FCDIIb patients, that is, a base exchange from C to G in exon 17 of TSC1 at nucleotide 2415 that results in an amino acid exchange from the basic histidine to the neutral tyrosine at codon 732 (hamartin$^{H732Y}$ [16]). This variant is also present at lower frequencies in TSC patients and unaffected individuals (17–19). We also analyzed hamartin$^{R692X}$ and hamartin$^{R786X}$, that is, truncations in the hamartin-tuberin interaction domain that are frequently observed in TSC-associated cortical tubers (18–20). Our data suggest strikingly impaired tuberin binding as well as divergent subcellular distribution patterns of these hamartin variants and provide novel insights into their pathogenetic role in FCD and TSC.
MATERIALS AND METHODS

Surgical Specimens and Immunohistochemistry

Tissue specimens were obtained from FCDIIb (n = 12 patients), cortical tubers (n = 2 patients), and subependymal giant cell astrocytomas (SEGAs, n = 3 patients) (Table). Biopsy samples were collected from patients with chronic pharmacoresistant focal epilepsies in the Epilepsy Surgery Program at the University of Bonn. One patient with SEGA did not have epilepsy. In all epileptic patients (n = 16), surgical removal of the lesion was necessary to achieve seizure control before presurgical evaluation, as previously described (21). Only patients with cortical tubers and SEGAs had additional features characteristic of TSC (5, 8). In a patient with a SEGA (ID 17), neurosurgical intervention was carried out under tumor-surgical considerations. The clinical characteristics of the patients are summarized in the Table. Written informed consent was obtained from all patients concerning the use of brain tissue. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Bonn Medical Center. Surgical specimens were fixed in formaldehyde overnight and embedded in paraffin. The FCDIIb specimens were reviewed by experienced neuropathologists and classified according to international classification schemes (3). Hematoxylin and eosin (H&E) and Nissl stains (22) were performed to assure specific binding affinity (data not shown).

Generation of Plasmid Constructs

To generate pmCherry-TSC1, we first amplified the human TSC1 cDNA coding sequence from pcDNA3.1mycA-TSC1 and subcloned the amplicon into the pCDNA3.1 V5 HIS TOPO vector (Invitrogen, Munich, Germany) using the primer pair TSC1 forward (5’-GCG GCT AGC ATG GCC CAA CAA GCA AAT G-3’) and TSC1 reverse (5’-GCG GTT ACC TTA GCT GTG TTC ATG AGT CTC-3’). Subsequently, an mCherry tag was inserted N-terminally in pcDNA3.1-TSC1 to generate pmCherry-TSC1. Therefore, mCherry was amplified from the pBKS-mCherry plasmid (a generous gift from Dr T. Südhof, Stanford, CA) using the primer pair mCherry forward (5’-GCG TCT AGA ACC ATG GTG AGC AAG GCA GA-3’) and mCherry reverse (5’-GCG GCC GTT ATG CTA CAT GTC GTC CAT GCC-3’). The resulting polymerase chain reaction product was digested with NheI and XbaI (Fermentas, Burlington, Canada) and pcDNA3.1-TSC1 with NheI. DNA sequencing was performed to verify that all sequences were correct (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA). Truncated and mutated hamartin proteins were generated by site-directed mutagenesis of the pcDNA-mcherry-TSC1 plasmids (QuickChange II Site-directed mutagenesis kit, Stratagene, Cedar Creek, TX) following the manufacturer’s manual with the following primers: TSC1R786X forward: 5’-CAT GGC AGC ATT ATA TTC CTC GAG AGC TGC GTG AGC AAG GGC GA-3’; TSC1R786X reverse: 5’-GTA GAA TTC CTC TCA GTC ATG CTG TAG CTG TCT GAT CTG CTG-3’. By base exchange from C to T in codon 786 of TSC1, a stop codon is created, resulting in a truncated TSC1 protein. For rapid screening of clones, a silent base exchange from G to A was generated, which resulted in the deletion of a PstI restriction site (CTGCA/G). For another truncated TSC1 variant, we used the primers: TSC1R692X forward: 5’-GAT GAG ATC CGC ACC CTC TGA GAC CAG CTA CCT TTA CTG CAC AAC-3’; TSC1R692X reverse: 5’-GTT GTG CAG TAA AAG GTT TAG CTC TCA GAG GGT GCC GAG CTC ATC-3’. The base exchange from C to T creates a stop codon at position 692 of TSC1, whereas the silent mutation from T to C resulted in the creation of a new PvuII restriction site (CAT/GG). For another truncated TSC1 variant, we used the primers: TSC1H732Y forward: 5’-GAT GAG ATC CGC ACC CTC TGA GAC CAG CTA CCT TTA CTG CAC AAC-3’; TSC1H732Y reverse: 5’-GTT GTG CAG TAA AAG CAG CTG GTC TCT GAG GGT GGT GCC GAG CTC ATC-3’. The base exchange from C to T creates a stop codon at position 732 of TSC1, whereas the silent mutation from T to C resulted in the creation of a new PvuII restriction site (CAT/GG). For another truncated TSC1 variant, we used the primers: TSC1H732Y forward: 5’-GAT GAG ATC CGC ACC CTC TGA GAC CAG CTA CCT TTA CTG CAC AAC-3’; TSC1H732Y reverse: 5’-GTT GTG CAG TAA AAG CAG CTG GTC TCT GAG GGT GGT GCC GAG CTC ATC-3’. The base exchange from C to T creates a stop codon at position 732 of TSC1, whereas the silent mutation from T to C resulted in the creation of a new PvuII restriction site (CAT/GG). For another truncated TSC1 variant, we used the primers: TSC1H732Y forward: 5’-GAT GAG ATC CGC ACC CTC TGA GAC CAG CTA CCT TTA CTG CAC AAC-3’; TSC1H732Y reverse: 5’-GTT GTG CAG TAA AAG CAG CTG GTC TCT GAG GGT GGT GCC GAG CTC ATC-3’. The base exchange from C to T creates a stop codon at position 732 of TSC1, whereas the silent mutation from T to C resulted in the creation of a new PvuII restriction site (CAT/GG).
TGC TTT GAT CAC-3'. This base exchange from C to T results in an amino acid exchange from histidine to tyrosine, screened by the detection of an additional restriction site of XhoI (C/TCGAG) generated by a silent base exchange from G to C. Mutagenesis was verified by restriction digest and sequencing.

Cell Culture and Transfection Procedures

HEK293T cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C in 5% carbon dioxide. Transfection of HEK293T cells was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol.

Colocalization Assay

HEK293T cells were cultured on coverslips and transfected with TSC2-enhanced green fluorescence protein and the different mCherry-TSC1 expression plasmids, at a confluence of approximately 25%, as previously described. After 48 hours, cells were fixed in 4% paraformaldehyde and analyzed by confocal fluorescence microscopy (Olympus, Munich, Germany). Micrographs were examined using a colocalization assay plug-in on ImageJ.

Western Blot Analysis

For Western blot experiments, HEKT293 cells were transfected with the mCherry-TSC1 plasmid at about 80% confluence and cultured for 2 more days. Cell pellets were resuspended in PBS and 6× loading dye (4× Tris-Cl/sodium dodecyl sulfate [SDS] pH 6.8, 30% glycerol, 10% SDS, 5% mercaptoethanol, 0.012% bromphenol blue) and then lysed by sonification. Equal amounts of cell lysate were separated by electrophoresis on an 8% SDS polyacrylamide gel and transferred to nitrocellulose membranes (Protran Whatman, Kent, UK) overnight at 4°C. After blocking the membrane for 1 hour with PBS/1% cold water fish gelatin (Sigma-Aldrich, St Louis, MO), it was incubated overnight with a monoclonal hamartin antibody (dilution 1:1000) (Hamartin 1B2 Mouse mAB, Cell Signaling, Boston, MA) in PBS, 0.1% TWEEN. IrDye fluorescent secondary antibodies (LI-COR, Lincoln, NE) were diluted 1:20,000 in PBS, 0.1% TWEEN, 0.01% SDS, and incubated for 1 hour. Immunoblots were visualized using the LI-COR Odyssey system (LI-COR).

Immunoprecipitation Assays

For immunoprecipitation analyses, HEK293T cells were harvested 2 days after cotransfection with the various cherry-tagged TSC1 expression plasmids and the green fluorescence protein-tagged TSC2 expression plasmid. Cell pellets were resuspended in lysis buffer (150 mmol/L NaCl, 40 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 0.5% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, protease inhibitor cocktail; Roche, Indianapolis, IN) and incubated for 1 hour at 4°C. Meanwhile, 30 μL of protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) slurry were conjugated with 1 μg of an antibody against hamartin (sc-12082) or tuberin (sc-893) (both from Santa}

### TABLE. Clinical Features and Diagnoses of Patients

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Neuropathologic Diagnosis</th>
<th>Main Clinical Features</th>
<th>Age at Surgery, years, Sex</th>
<th>Age at Epilepsy Onset, years</th>
<th>Seizure Frequency†</th>
<th>Medication at the Time of Surgery</th>
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<td>CPS, sGTCS</td>
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<td>14</td>
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<td>LTG, PGB</td>
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<td>LTG, PB</td>
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<tr>
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<td>2</td>
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<td>SPS, CPS, sGTCS</td>
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<td>3</td>
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<td>OXC, LEV</td>
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<td>LTG</td>
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<td>3</td>
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<td>LEV, LTG, CBZ</td>
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<td>CBZ</td>
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<td>Intraventricular tumor</td>
<td>21, F</td>
<td>—</td>
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*Cases with tuberous sclerosis complex.
†Seizure frequency, events per month or day.

CBZ, carbamazepine; CPS, complex partial seizure; F, female; FCD, focal cortical dysplasia of indicated types; LEV, levetiracetam; LTG, lamotrigine; M, male; ND, not determined (the age at onset of epileptic seizures of Patient 16 and the exact seizure frequency of Patients 5, 8, and 16 are not known. Patient 17 did not have clinically apparent seizures); OXC, oxcarbazepine; PB, phenobarbital; PGB, pregabalin; PHT, phenytoin; SEGA, subependymal giant cell astrocytoma; sGTCS, secondary generalized tonic clonic seizures; SPS, simple partial seizure; TPM, topiramate; VPA, valproate; ZON, zonisamide.
Cruz Biotechnology) in 500 µL lysis buffer at 4°C. After centrifugation of the cell lysate for 10 minutes at 14,000 rpm at 4°C, the supernatant was precleared with 50 µL of protein A/G plus agarose slurry and 1 µg of normal mouse IgG (Santa Cruz Biotechnology) for 1 hour at 4°C. Subsequently, the lysate was added to the conjugated primary antibodies and incubated for 2 hours, rotating at 4°C. The immunoprecipitated complexes were washed 5 times with lysis buffer and analyzed by electrophoresis on a 6% SDS polyacrylamide gel followed by immunoblotting, as previously described.

Immunocytochemistry and Phase-Contrast Microscopy

For immunofluorescence analysis, HEK293T cells were cultured on coverslips for 2 days after transfection and then fixed in 4% paraformaldehyde for 15 minutes at room temperature. After 3 rounds of washing with ice-cold PBS, cells were permeabilized with 0.1% Triton X-100/PBS for 15 minutes and then blocked for 1 hour at room temperature (blocking buffer: 10% normal goat serum, 1% bovine serum albumin in PBS). Cells were incubated overnight with primary antibodies anti-LAMP1 and anti-ERGIC-53/p58 (Sigma-Aldrich) at 4°C and diluted in blocking buffer to a concentration of 1:200. After washing with PBS, secondary antibody was added in a dilution of 1:400 in blocking buffer for 1 hour. The labeling was analyzed by confocal fluorescence microscopy as previously described. Cultured cells were photographed by phase-contrast microscopy (Observer A1; Zeiss, Jena, Germany) in the culture plate.

Statistical Analysis

Two-sided Student t-test was used for statistical analysis of the data.

RESULTS

We have observed that the lesions of FCD$_{1B}$, that is, common epilepsy-associated malformations that resemble cortical tubers in TSC (Figs. 1A–D, I–K), show strikingly aberrant cytoplasmic granular aggregation of hamartin in balloon cells and in dysmorphic neurons (Figs. 1E, F). By contrast, regularly shaped neurons in normal cerebral cortex have a homogeneous cytoplasmic distribution of hamartin (Figs. 1G, H). Moreover, aberrant hamartin aggregation in FCD$_{1B}$ is also observed in dysmorphic neuronal and giant cell elements of cortical tubers (Figs. 1L) and in highly differentiated subependymal SEGAs (Figs. 1M–P). Both cortical tubers and SEGAs are frequently associated with TSC. These observations prompted us to determine whether sequence variants of TSC1 that are frequent in FCD$_{1B}$ and TSC-associated brain lesions are associated with aberrant subcellular distribution or impaired tuberin binding of the resulting hamartin variants. Because numerous components of the phosphatidylinositol 3-kinase cascade operate abnormally in FCD$_{1B}$ and cortical tubers (23), we used a highly controlled in vitro approach for this analysis.

We first generated various plasmids that express TSC1 fused to the fluorescent reporter mCherry. In addition to wild-type (WT) TSC1, we derived TSC1 constructs carrying the amino acid exchanges H732Y, R786X, and R692X (Fig. 2). Mutations in codons 786 and 692 result in truncated hamartin variants that are frequently detected in TSC patients (18–20) and are localized at different sites within the tuberin interaction domain of hamartin (Fig. 2). Next, we tested for protein expression and stability of the TSC1 fusion proteins by transfection of HEK293T cells and immunoblotting. HEK293T cells are a widely used cell line for protein interaction experiments. All proteins were expressed at high levels and migrated at the expected calculated molecular weights (Fig. 3).

![FIGURE 2. Generation of mutant hamartin proteins. A Cherry tag was inserted N-terminally to the tuberous sclerosis complex (TSC1) cDNA (numbered boxes indicating exons); 3 different mutations were introduced into the coding sequence of TSC1 by site-directed mutagenesis (highlighted on gray background); 2 of the sequence alterations resulted in truncating mutations, the generation of stop codons at positions R786X and R692X. The resulting proteins are referred to as hamartin$^{R786X}$ and hamartin$^{R692X}$. Hamartin$^{R692X}$ terminates in the beginning of exon 17; hamartin$^{R786X}$ comprises the amino acids encoded by the entire exon 17 and terminates in the middle of exon 18. The protein parts that are missing as result of introducing the truncating mutations are indicated. A third sequence alteration in the TSC1 cDNA (i.e., a C to G base exchange in exon 17 of TSC1) results in an amino acid exchange at codon 732 of hamartin, from the basic histidine to neutral tyrosine (hamartin$^{H732Y}$). Importantly, this latter alteration does not result in a truncated hamartin protein variant.](https://example.com/figure2.png)
To assess the binding efficiency of WT and mutant TSC1 proteins to tuberin in vitro, we performed immunoprecipitation experiments from extracts of transiently transfected HEK293T cells using an antibody directed against an epitope present in all mutant hamartin protein variants and then probed the precipitates with an antibody against tuberin. As shown in Figures 4A and B, tuberin was efficiently precipitated when coexpressed with hamartin\textsuperscript{WT}, but there was reduction in tuberin-binding affinity for hamartin\textsuperscript{R692X} because this protein variant contains an aberrant stop codon positioned most N-terminal in the tuberin interaction domain, the resulting hamartin product lacks large portions of the coiled-coil domain responsible for tuberin binding (Figs. 4A, B); 5.1-fold binding reduction, for hamartin\textsuperscript{R692X} n = 3 replicates, t-test, p < 0.05. Correspondingly, the hamartin\textsuperscript{R768X} variant also showed a significantly reduced binding affinity to tuberin (Figs. 4A, B); 3.3-fold binding reduction, for hamartin\textsuperscript{R768X} n = 4 replicates, t-test, p < 0.05. In the hamartin\textsuperscript{H732Y} protein, however, a larger part of the tuberin binding coiled-coil domain is present than in hamartin\textsuperscript{R692X}. Strikingly, also a hamartin protein variant (hamartin\textsuperscript{H732Y}), which is found in normal individuals, TSC patients with cortical tubers, as well as in FCD\textsubscript{IIb}, and in which the tuberin binding domain only was precipitated when coexpressed with hamartin\textsuperscript{WT}, but there was no significant reduction in tuberin binding (Figs. 4A, B); 2.3-fold binding reduction, for hamartin\textsuperscript{H732Y} n = 9 replicates, t-test, p < 0.05. Identical results were obtained if the immunoprecipitations were performed vice versa using an antibody against tuberin (data not shown). Together, these experiments show that mutations found in the tuberin-binding domain of hamartin in cortical tubers and in FCD\textsubscript{IIb} directly affect the interaction of these 2 proteins.

We next characterized the subcellular distribution of the different hamartin protein variants. Hamartin\textsuperscript{WT} showed a cytoplasmic localization mostly concentrated in close vicinity to the cellular membrane (Fig. 5). The expression pattern of hamartin\textsuperscript{H732Y} seemed homogeneous. Hamartin\textsuperscript{R692X} and hamartin\textsuperscript{R768X} were more dispersed in the cytoplasm and accumulated in granular-like patterns that are distinct from that of hamartin\textsuperscript{WT} (white arrows, Fig. 5). The granular masses of hamartin\textsuperscript{R692X} and hamartin\textsuperscript{R768X} suggest self-aggregation, a phenomenon that has been previously described (15). Intriguingly, hamartin\textsuperscript{H732Y} revealed a completely different subcellular distribution as it was almost completely localized in the nucleus without any significant cytoplasmic expression. This observation was confirmed by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining (Fig. 5).

We next examined the consequences of the H732Y allelic variant on the interaction of hamartin and tuberin in live cells. We overexpressed both proteins in HEK293T cells

![Figure 3](image1.png)

**FIGURE 3.** Hamartin cherry fusion proteins are expressed in HEK293T cells. To analyze the subcellular distribution of the various hamartin variants, plasmids coding for fusion proteins of hamartin with the fluorescent marker mCherry were generated. The sizes of the fusion proteins were determined by immunoblotting of transfected HEK293T whole cell lysates. As expected, wild-type (WT) hamartin fused to cherry is detected at approximately 158 kd (endogenous tuberous sclerosis complex (TSC1) 130 kd, cherry 28 kd). Hamartin\textsuperscript{H732Y} fused to cherry is the same size as hamartin\textsuperscript{WT} fused to cherry. Hamartin\textsuperscript{R768X} fused to cherry represents the smallest protein (100 kd); hamartin\textsuperscript{R786X} fused to cherry is of intermediate size (110 kd). The arrow marks the HEK293T-endogenous hamartin at 130 kd.

![Figure 4](image2.png)

**FIGURE 4.** Immunoprecipitation of hamartin and tuberin. (A) Hamartin-cherry variants were immunoprecipitated using a hamartin antibody. Subsequently, immunoblots were probed with an antibody against tuberin. The probes were normalized to the whole lysates. Only the tuberin band of the wild-type (WT) hamartin probe can be detected with certainty. This assay was also performed in the reverse order with identical results (data not shown). (B) To examine the binding efficiency between hamartin, WT, and mutant variants and tuberin in vitro, the amount of tuberin that was immunoprecipitated by the different hamartin variants was quantitated after immunoblotting using AIDA software. All 3 hamartin variants show a substantially decreased binding to tuberin compared with that of hamartin\textsuperscript{WT}. Hamartin\textsuperscript{WT}, n = 9; hamartin\textsuperscript{H732Y}, n = 9; hamartin\textsuperscript{R786X}, n = 4; hamartin\textsuperscript{R692X}, n = 3; t-test, *p ≤ 0.05.
FIGURE 5. Subcellular distribution of hamartin variants in HEK293T cells. HEK293T cells were transfected with tuberous sclerosis complex (TSC1)–mCherry expression plasmids (red – view figure in color online-only); nuclei were labeled with DAPI (blue – view figure in color online-only). Cells only stained with DAPI are nontransfected cells. The different hamartin mutants and the wild-type (WT) were analyzed by confocal fluorescence microscopy. HamartinWT showed cytoplasmic localization mostly concentrated near the cellular membrane. In contrast, the hamartinR786X and hamartinR692X variants were more disseminated throughout the cytoplasm and accumulated in granular aggregates (white arrows). HamartinH732Y exhibited a completely different subcellular distribution compared with that of the other proteins as it was almost completely transferred to the nucleus. The exclusively nuclear localization of hamartinH732Y was confirmed by colocalization with DAPI staining. Scale bars = 10 μm.

FIGURE 6. Colocalization assay of tuberin and hamartin in HEK293T cells. (A) HEK293T cells were cotransfected with plasmids coding for mCherry-hamartin (red – view figure in color online-only) and enhanced green fluorescence protein–tuberin (green – view figure in color online-only) fusion proteins. Cells were analyzed by confocal fluorescence microscopy. Wild-type (WT) hamartin shows nearly 100% colocalization with tuberin. Cotransfection of hamartinR786X and tuberin and of hamartinR692X and tuberin, respectively, resulted in a redistribution of the granular pattern observed for hamartinWT into a diffuse pattern, which colocalized with tuberin. HamartinH732Y showed no redistribution when cotransfected with tuberin but stayed strictly in the nucleus indicating reduced colocalization with tuberin (red, cherry fluorescent protein; blue, DAPI; Scale bars = 10 μm. View figure in color online-only). (B) Semiquantitative analysis of the colocalized area of hamartin and tuberin in relation to the entire area of single cells by an ImageJ plug-in developed for the examination of colocalization assays (t-test, p < 0.001; for hamartinH732Y, n = 48 cells counted; for hamartinR786X, n = 46 cells counted; for hamartinR692X, n = 50 cells counted; for hamartinR692X, n = 45 cells counted).
and analyzed their colocalization pattern by confocal fluorescence microscopy. The data corroborated the observation that hamartin\(^{H732Y}\) was translocated to the nucleus even in the presence of increased levels of tuberin. Interestingly, tuberin showed a diffuse cytoplasmic distribution pattern (Fig. 6A). Using the ImagJ plug-in, we analyzed the colocalization of hamartin and tuberin semiquantitatively and detected significantly reduced colocalization of hamartin\(^{H732Y}\) with tuberin compared with that of WT (\(t\)-test, \(p < 0.001\); for hamartin\(^{H732Y}\), \(n = 48\) cells counted; for hamartin\(^{WT}\), \(n = 46\) cells counted; for hamartin\(^{R786Y}\), \(n = 50\) cells counted; for hamartin\(^{R692X}\), \(n = 45\) cells counted; Fig. 6B). Hamartin\(^{WT}\) showed colocalization with tuberin in a largely cytoplasmic distribution (Figs. 6A, B). Interestingly, in these experiments, both truncation mutants exhibited a strong cytoplasmic colocalization with tuberin highly similar

![Image](https://example.com/image.png)

**FIGURE 7.** Analysis of potential colocalization of hamartin in different subcellular compartments. (A) Cells were transfected with various mCherry-hamartin fusion proteins. Immunocytochemistry was performed with anti-LAMP1, which marks lysosomes, and anti-ERGIC-53/p58, which marks endoplasmic reticulum and Golgi apparatus components. These primary antibodies were detected with a green fluorescent secondary antibody. For colocalization of hamartin with mitochondria, HEK293T cells were cotransfected with the hamartin variants fused to mCherry and a plasmid exclusively expressed in mitochondria, pmito–green fluorescence protein (a generous gift from W. Altrock; IFN, Magdeburg, Germany). Results were analyzed by confocal fluorescence microscopy. Hamartin\(^{R692X}\) was present in nuclei; the other variants were located in the cytoplasm in different distribution patterns. There was no reproducible or substantial colocalization of hamartin with any of these compartment-specific markers. (B) Different growth patterns of untransfected HEK293T cells were visualized by phase-contrast microscopy. The panels demonstrate the high degree of density-dependent diversity of sizes and shapes of the HEK293T cells grown in culture. Cell contacts were associated with more rounded shapes (black arrows [B1]), whereas isolated cells exhibit a more spindle-like morphology (white arrows [B2]). At higher magnification, clustered HEK293T cells (black arrow [B3]) reveal round to ovoid shapes, whereas isolated cellular components seem more elongated with the formation of small processes (white arrow [B3]). Scale bars = (A) 10 μm; (B) 100 μm. WT, wild-type.
to the distribution pattern of hamartin\textsuperscript{WT} (Figs. 6A, B). None of the cotransfected hamartin protein variants resulted in an altered distribution of tuberin in the cells.

We performed further immunocytochemistry of transfected HEK293T cells with probes of proteins localized specifically at the Golgi apparatus, the endoplasmic reticulum and lysosomes, and mitochondria to determine whether the individual hamartin variants had different colocalization patterns within these subcellular compartments (Fig. 7A). We did not observe distinct or specific colocalization patterns of the individual hamartin protein variants with these compartments, however. Importantly, the distribution patterns of the distinct hamartin variants were independent of the variable morphology of the HEK293T cells (Fig. 7B). Generally, clustered cells in close contact exhibit a round to ovoid appearance (Figs. 7B1, B3). In contrast, individual cells in more isolated localization reveal a rather spindle-like shape (Figs. 7B2, B3). The variability of HEK293T cellular shape distribution was independent of transfection of cells with the different TSC1 variants.

DISCUSSION

We observed strikingly aberrant subcellular distribution patterns of hamartin in various epilepsy- and TSC-associated lesions. In contrast to neurons in normal cortex that have diffuse cytoplasmic expression of hamartin FCD\textsubscript{IIb}, cortical tubers and SEGAs exhibit cytoplasmic granular aggregates of hamartin (Fig. 1); these are similar to previously reported findings (24). Our present in vitro results also reveal striking differences in tuberin binding and subcellular distribution among hamartin variants that are frequently observed in epilepsy-associated cortical dysplasias and cortical tubers (17–20). Both hamartin variants with a truncated tuberin-binding domain exhibited significantly reduced binding affinity to tuberin. This observation is surprising for hamartin\textsuperscript{R786X}, which still encompasses a substantial part of this domain. Interestingly, hamartin\textsuperscript{H732Y}, an allelic variant of TSC1 present in healthy individuals (17), is highly abundant in FCD\textsubscript{IIb} (16) and is present in TSC patients who lack pathogenic TSC1, and TSC2 mutations (18) also showed a substantially reduced ability to interact with tuberin. Indeed, the affinities of hamartin\textsuperscript{H732Y} and hamartin\textsuperscript{R786X} to tuberin were similar (Fig. 3). This result was rather surprising because 1) in contrast to hamartin\textsuperscript{H732Y}, the hamartin\textsuperscript{R786X} variant has been reported to cause manifest TSC and 2) the hamartin\textsuperscript{H732Y} variant differs from hamartin\textsuperscript{WT} by only a single amino acid exchange from the basic amino acid histidine to the neutral tyrosine within the interaction domain to tuberin. This sequence alteration at codon 732 may alter the 3-dimensional structure of hamartin at the predicted binding domain with tuberin (5). Ablant interaction of hamartin\textsuperscript{H732Y} with tuberin is compatible with downstream activation of the phosphatidylinositol 3-kinase cascade recently described in FCD\textsubscript{IIb} (9, 25, 26).

An important aspect for understanding the pathophysiological relevance of TSC1 allelic variants relates to potentially aberrant subcellular localization of the corresponding hamartin variants compared with tuberin. Factors such as cell type-specific differences and low abundance or antibody limitations may account for previous findings that hamartin and tuberin are localized to different cellular compartments (10, 15, 27–29). It has been demonstrated, however, that tuberin and hamartin associate physically in vivo (10, 30); all of these studies provide evidence for a cytosolic tuberin-hamartin complex. Hamartin has been localized to the membrane/particulate (P100) fraction of cultured cells (30, 31). Recently, nuclear localization of hamartin in pancreatic acinar tissues and individual cell components in the cerebellar granular layer of the cerebellum were also reported (32).

Because of its subcellular localization, hamartin may transmit information about changes in cell adhesion to the nucleus; its involvement in normal cell death may accompany cell detachment or provide an additional potential indirect mechanism through which the cell cycle could be affected. Indeed, it has been suggested that hamartin is a nuclear retinol binding protein that accumulates in the nucleus upon inhibition of nuclear export with leptomycin B, which itself inhibits Exportin 1, a protein required for nuclear export of proteins containing a nuclear export sequence (1). Interestingly, our finding of hamartin\textsuperscript{H732Y} accumulation in the nucleus (in contrast to hamartin\textsuperscript{WT}, hamartin\textsuperscript{R786X}, and hamartin\textsuperscript{R692X}, which are cytoplasmic) implies the following scenarios for allelic variants in contrast to hamartin\textsuperscript{WT}, which is tightly bound to tuberin in a protein complex. Because of the conserved nuclear localization sequence ("RQQHALRNRR" sequence at 708 of hamartin), hamartin\textsuperscript{H732Y} has a reduced binding affinity to tuberin and is shuttled into the nucleus. In view of the relevance of hamartin for cell cycle regulation, this aberrant cytoplasmic–nuclear shuttling of hamartin disrupts the cycling of affected cells. In contrast, hamartin\textsuperscript{R786X} and hamartin\textsuperscript{R692X} are not transported to the nucleus. Accordingly, hamartin\textsuperscript{R692X} lacks the nuclear localization sequence at 708, whereas for hamartin\textsuperscript{R786X}, another mechanism, such as protein misfolding of the truncated protein, may account for its localization. Aberrant nuclear accumulation of hamartin\textsuperscript{H732Y} might impair cell cycling, although the specific effects of this phenomenon were not addressed by the experimental approaches of the present study. It has been shown, however, that hamartin and tuberin influence the activity of molecules critically involved in cell cycle regulation such as CDK1 (33). On the other hand, this aberrant protein variant might also perturb regular nuclear functions in a manner similar to what is known for aberrant protein aggregates in neurodegenerative conditions such as spinocerebellar ataxia type 3 (34). Recent data demonstrated that appropriate phosphorylation, sequestration, and turnover of the TSC protein complex represent critical issues for proper cellular function (35–37). Particularly, the aberrant cytoplasmic aggregates of hamartin we demonstrated in FCD\textsubscript{IIb}, cortical tuber and SEGAs, and nuclear hamartin may impair systemic functions provided by cytoplasmic hamartin. Here, we report the first example of a nuclear hamartin stop variant. Aberration in the nuclear localization of hamartin\textsuperscript{H732Y} may be a factor in the phenotypic variability of hamartin-related diseases.
The hamartin\(^{H732Y}\) allele is also detected in the normal population, with a frequency of 0.5%. Therefore, this sequence alteration cannot be sufficient to cause TSC or FCD. Intriguingly, it has been recently shown that the TSC pathway is sensitive to gene dosage effects, such that loss of a single copy of TSC1, as it is manifest in all neurons of TSC patients, is sufficient to disturb dendritic spine structure (13). Furthermore, loss of heterozygosity has been shown in FCD\(_{IIb}\) components of individuals carrying the hamartin\(^{H732Y}\) allele (16). Our present data suggest that hamartin\(^{H732Y}\) may constitute a predisposing germline variant with low penetrance and a strongly restricted manifestation pattern, which only in individual cells (or e.g. restricted neural precursor clones) overcomes a virtual pathological threshold. This concept is compatible with the remarkable notion that surgical resection of FCD\(_{IIb}\) is curative with respect to seizure events in most patients (38), whereas TSC patients with cortical tubers frequently develop secondary seizure foci after removal of epileptogenic tubers/foci.

It was suggested recently that hamartin could have discrete and specialized functions besides its role in the TSC1/TSC2 complex because of its nuclear localization (39). In line with this idea, hamartin is capable of attenuating the proliferation of mammalian cells (40). Aberrant cellular accumulation of hamartin allelic variants may therefore account for reduced proliferation and cellular densities in cortical dysplasias (41) and the characteristic enlarged and dysplastic cellular phenotype of FCD\(_{IIb}\) elements. Based on these considerations, our data suggest that hamartin, which has mainly been regarded as an interaction partner in the tumor suppressor complex with tuberin, has a pronounced independent functional relevance in the pathogenetic context of TSC and cortical dysplasias.

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