

Biallelic *TSC* gene inactivation in tuberous sclerosis complex

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ABSTRACT

Background: A pivotal developmental question is whether tubers in tuberous sclerosis complex (TSC) form by germline and somatic *TSC1* or *TSC2* gene mutations. Loss of *TSC1* or *TSC2* in vitro and in vivo leads to mTORC1 cascade activation and ribosomal protein S6 phosphorylation (P-S6). Giant cells (GCs) in tubers exhibit S6 phosphorylation, suggesting cell-specific loss of TSC gene function.

Methods: *TSC1* and *TSC2* gene mutations were investigated in DNA extracted from tuber sections (n = 6) and microdissected P-S6-labeled GCs by sequencing and loss of heterozygosity (LOH) analysis to define germline and somatic mutations.

Results: A germline *TSC1* mutation was defined in 1 case and *TSC2* mutations were defined in 5 cases. LOH was not detected in whole tuber sections or microdissected P-S6-labeled GCs. *TSC1* and *TSC2* were sequenced in microdissected P-S6-immunolabeled GCs. In 5 specimens, a somatic mutation was identified in single GCs that was not detected in whole tuber sections or leukocyte DNA. Four somatic mutations were novel variants (1 nonsense and 3 missense mutations) and 1 additional nonsense somatic mutation was previously reported as a germline mutation. In 1 case, no somatic mutation was identified. There was reduced expression of *TSC1* or *TSC2* transcripts in the *TSC1* or *TSC2* associated specimens. In the cases containing a nonsense mutation, no transcript mRNA was detected, suggesting nonsense-mediated degradation.

Conclusions: We provide evidence to support the hypothesis that tubers form by biallelic *TSC1* or *TSC2* gene inactivation reflecting a “2-hit” mechanism of germline and somatic mutational events.

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GLOSSARY

AML = angiomyolipoma; **DN** = dysplastic neuron; **FFPE** = formalin fixed, paraffin embedded; **GC** = giant cell; **H-E** = hematoxylin and eosin; **LOH** = loss of heterozygosity; **TSC** = tuberous sclerosis complex.

Tuberous sclerosis complex (TSC) results from mutations in the *TSC1* or *TSC2* (*TSC2*) genes.^{1,2} Tubers are developmental malformations of the cerebral cortex in TSC that are characterized by disorganized lamination and the presence of a unique cell type, giant cells (GCs), which exhibit cytomegaly.³ Adjacent to GCs are dysplastic neurons (DNs) characterized by a dysmorphic cell body and aberrant dendritic arborizations.

An intensive debate surrounds the molecular mechanisms governing tuber formation during brain development. Loss of heterozygosity (LOH) at either *TSC* gene locus demonstrated in hamartomas in other organ systems from patients with TSC reflects a biallelic or “2-hit” mutational mechanism in which lesion formation depends on the combined effects of germline and somatic mutations.⁴⁻⁶ Biallelic *TSC* gene inactivation leads to activation of the mammalian target of rapamycin complex 1 (mTORC1) cascade as evidenced by phosphorylation of p70 S6 kinase1 and ribosomal S6 proteins.⁷⁻¹⁰ LOH has been demonstrated in TSC subependymal giant cell astrocytomas^{6,11} but not confirmed in tubers.^{4,11-13} Variably reduced *TSC1* or *TSC2* protein expres-

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sion^{14,15} and enhanced protein S6 phosphorylation^{16,17} in tubers suggests that loss of *TSC1* or *TSC2* function and enhanced mTORC1 signaling likely underlies tuber formation. Thus, while a 2-hit mechanism may be responsible for formation of other TSC lesions, it is not known whether tubers form as a result of biallelic gene inactivation. We hypothesized that tubers form by biallelic gene inactivation and we analyzed the *TSC1* and *TSC2* genes by LOH assay and sequencing in 6 tubers as a strategy to define germline and somatic mutations.

METHODS Tubers were obtained from 6 patients (n = 4 male, 2 female; average age at surgery or death 16.7 years) with a clinical diagnosis of TSC.¹⁸ In 5 patients, the tubers were resected as part of epilepsy surgery for refractory seizures and the sixth patient died of cardiopulmonary arrest. In all of the patients, germline *TSC1* or *TSC2* mutations were defined in leukocyte DNA as part of clinical evaluation. In 1 additional postmortem specimen (male, age 35 years), only frozen tuber and angiomyolipoma (AML) tissue were available.

Human tissue specimens. Formalin fixed, paraffin embedded (FFPE) TSC brain specimens were sectioned at 7 μ m and mounted on poly-L-lysine-coated coverslips. Histologic examination of the tubers by hematoxylin and eosin (H-E) staining revealed abnormal cortical lamination, numerous GCs, and DN. Control frontal lobe neocortex was obtained postmortem from 2 patients who died of nonneurologic causes without a history of TSC or epilepsy (1 male, age 22; 1 female, age 26; average postmortem interval to autopsy, 14 hours).

Immunohistochemistry. All FFPE tissue sections were hydrated through graded ethanols, probed with phosphoribosomal S6 antibodies (Ser235/236; 1:500 dilution; Cell Signaling, New England Biolabs, Beverly, MA) and visualized using avidin-biotin conjugation (Vectastain ABC Elite; Vector Labs, Burlingame, CA) with 3,3'-diaminobenzidine. Sections used for histologic analysis were dehydrated through graded ethanols and xylenes and coverslip mounted (Permount). Those used for DNA or mRNA extraction were maintained in diethylpyrocarbonate (DEPC)-treated water at 4°C.

Laser microdissection and DNA extraction. Single P-S6-immunoreactive GCs (n = 100 per case) as well as pyramidal neurons devoid of P-S6 labeling in control frontal cortex (n = 100 per case) were visualized under light microscopy and microdissected with a CellPixIITM laser capture system (Arcturus) or VeritasTM (Westburg) laser capture microdissection and laser cutting systems (see e-Methods on the *Neurology*[®] Web site at www.neurology.org).

LOH analysis, PCR, TSC gene sequencing, and TSC mRNA expression. DNA extracted from cortical tuber sections and single P-S6-labeled GCs was analyzed for LOH and TSC gene sequence (see e-Methods). We carried out fluorescent DNA sequencing using the Big-DyeTM Terminator system (Applied Biosystems) and ABI Prism sequencing. Individual gene sequence for *TSC1* and *TSC2* was compared with either the Leiden Open Variation TSC database (<http://chromium.liacs.nl/LOVD2/TSC/home.php>) or NCBI

SNP database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp>). *TSC1* and *TSC2* mRNA levels were determined as described previously (see e-Methods).¹⁶

Standard protocol approvals, registrations, and patient consents. All human tissue was obtained in accordance with protocols approved by Academic Medical Center, University of Amsterdam, and the University of Pennsylvania Institutional Review Board and Committee on Human Research. Tissue procurement was deemed exempt from patient consent requirement because all patient data were de-identified prior to receipt of tissue in the laboratory.

RESULTS Detection of germline mutations in tuber sections and LOH analysis. We first demonstrated intact *TSC1* and *TSC2* sequence in genomic DNA extracted from whole sections of control cortex and then from microdissected pyramidal cells (n = 100) to confirm the validity and integrity of our PCR primers and amplification conditions from FFPE brain sections. Analysis of *TSC1* and *TSC2* in normal cortex revealed intact gene sequence compared with NCBI GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and the Leiden Open Variation TSC databases. Additionally, the sequencing results from FFPE tissue were reliable for several reasons. First, in both control and TSC associated cases, we identified *TSC1* and *TSC2* SNPs that were previously entered into the NCBI SNP database demonstrating that well-characterized SNPs could be detected in our samples. Second, *TSC1* or *TSC2* mutations were not detected in control samples. Third, germline mutations identified in tubers were also found in leukocyte DNA from the same patients, thus confirming the identity of the germline mutation.

TSC1 and *TSC2* gene sequences were analyzed in DNA extracted from whole tuber sections (n = 6 TSC cases). Germline mutations were identified in all 6 specimens (*TSC1*, 1 case, *TSC2*, 5 cases; table) and among these, 5 mutations had been previously entered into the Leiden Open Variation TSC database as known disease-causing mutations.¹⁹⁻²² The remaining case mutation likely represented a novel mutation variant since this sequence variation was not identified as a SNP in either NCBI or Leiden databases, and it was not identified in any of our control specimens. LOH analysis performed in genomic DNA extracted from the 6 tuber specimens did not reveal loss of heterozygosity at either gene locus. Indeed, as evidence that our LOH assay was valid, in 1 additional case we identified LOH in *TSC2* in a renal AML but not a tuber from the same patient (figure 1). These findings supported previous results¹³ that unlike AMLs, large somatic *TSC1* or *TSC2* gene deletions do not commonly occur in tubers.

S6 protein phosphorylation in tubers. GCs in tubers express P-S6 protein.^{16,17} In keeping with these findings,

Table Summary of germline (G) and somatic (S) mutations detected by sequencing of DNA extracted from a pooled sample of microdissected P-S6-immunoreactive giant cells in tubers^a

Gene	Exon	Nucleotide	Amino acid	DBID	Reference	Type
TSC1	15	2023 delG	D675TfsX49	TSC1_00225	20	G
TSC1	22	3096 G>T	959E>X	Novel	NA	S
TSC2	29	3498_3499insT	Glu1167X	TSC2_00849	20	G
TSC2	29	3412 C>T	Arg1138X	TSC2_00267	19	S
TSC2	33	4397 C>A	S1466X	TSC_00714	20-22	G
TSC2	33	4079 C>T	P1358L	Novel	NA	S
TSC2	16/17	1839+1 G>T	spl	TSC2_00424	20	G
TSC2	4	337 G>A	E114K	Novel	NA	S
TSC2	1-15	del 1-15	NA	Novel	NA	G
TSC2	14	1564 C>T	H522Y	Novel	NA	S
TSC2	3	268 C>T	90Q>X	TSC2_00036	19, 20, 22	G

Abbreviations: del = deletion; fs = frameshift; ins = insertion; NA = not applicable; spl = splice.
^a Sequence pairs depict germline and somatic mutations identified in a single tuber specimen. Bottom row represents germline mutation in a case where no somatic mutation was defined.

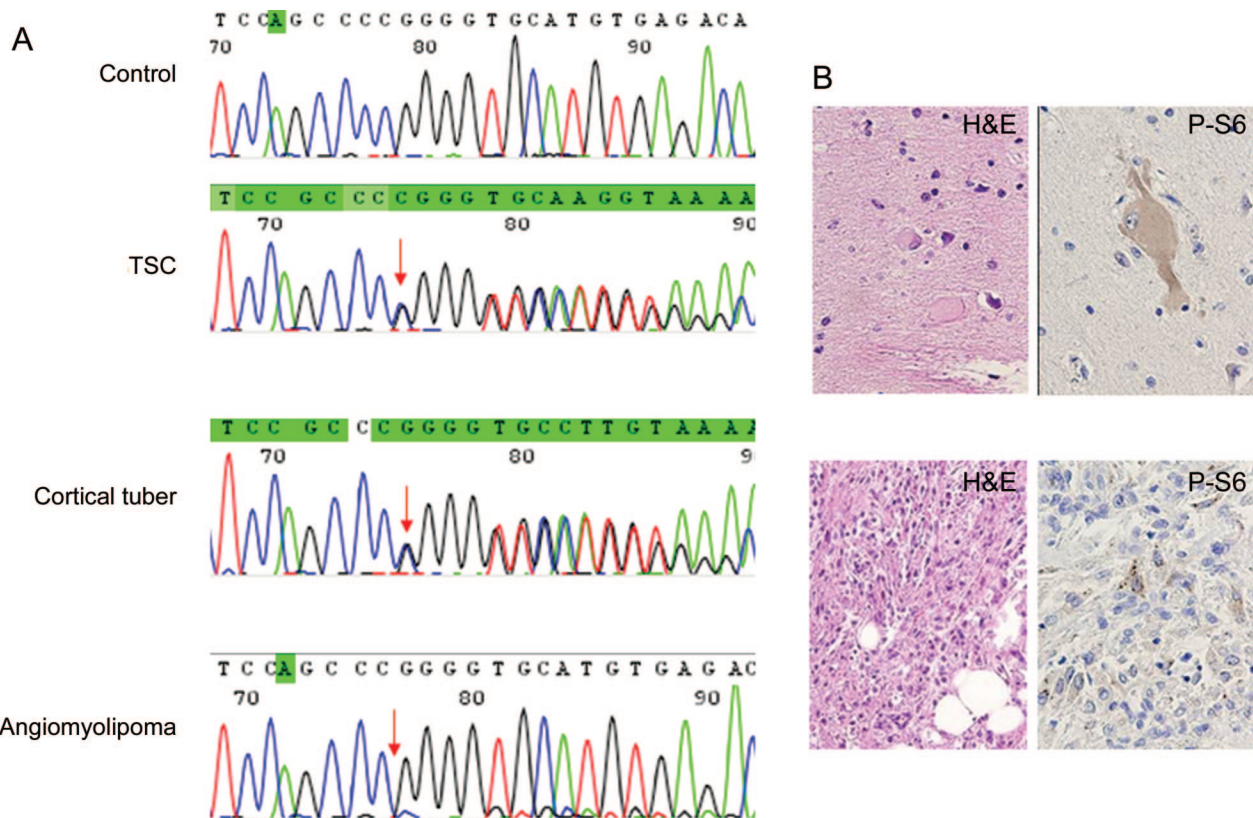
there was robust expression of P-S6 in GCs and large DN in all tuber specimens (figure 2). Morphologically normal neurons near the edge of the resection margin did not exhibit P-S6 labeling. These cells did express

NeuN. There was minimal P-S6 immunoreactivity in control cortex specimens.^{16,17} In the postmortem TSC case, we analyzed an area without obvious cortical lamination defects (normal perituberal cortex), and there were no P-S6-labeled cells observed.

Detection of somatic mutations in P-S6-labeled giant cells. To further investigate whether large deletions did not affect individual cell populations, single P-S6-labeled GCs (n = 100 cells per case) were microdissected from all tuber sections and genomic DNA was extracted. LOH was not detected at either locus in single microdissected cells (data not shown). These findings supported, at the single cell level, the hypothesis that large somatic *TSC1* or *TSC2* gene deletions do not occur in P-S6-immunoreactive GCs.

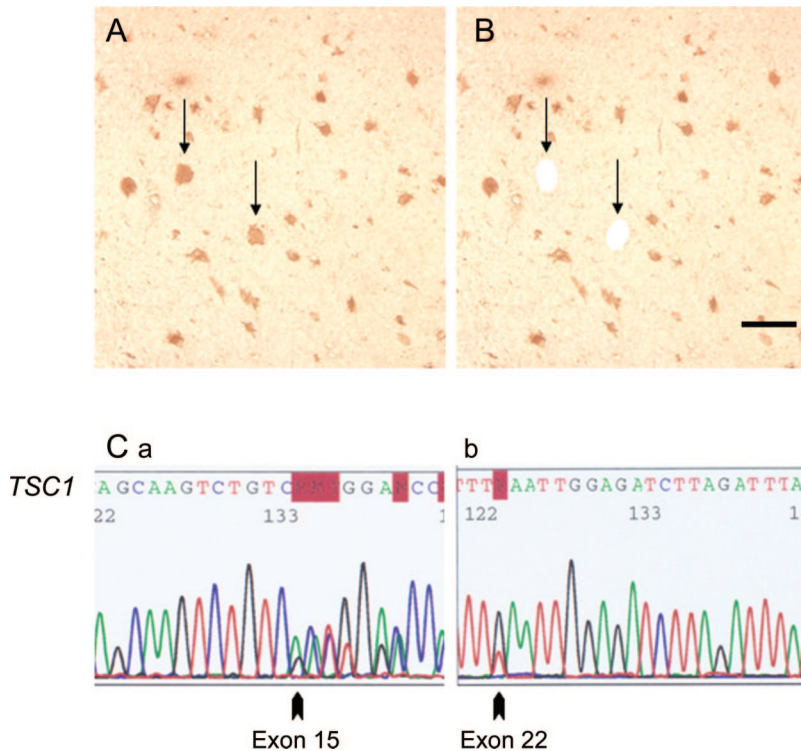
We focused on the TSC specimens with confirmed disease-causing germline mutations (n = 5 cases; 4 surgical, 1 postmortem). All exons of *TSC1* and *TSC2* were sequenced in DNA extracted from the pooled sample of 100 microdissected GCs in each specimen from each case. In one specimen (figure 2), the germline exon 15 *TSC1* mutation (exon 15 delG2023) that led to a frameshift and premature stop codon, detected in

Figure 1 Loss of heterozygosity is not a feature of cortical tubers



(A) Note mutation detected in leukocyte DNA from patient with tuberous sclerosis complex (TSC) (arrow, *TSC2* exon 28 delC) compared with control leukocyte DNA sample. Both wild-type and mutated alleles are detected in cortical tuber homogenates but there is loss of the wild-type allele in angiomyolipoma DNA. (B) Hematoxylin and eosin labeled (H&E) and P-S6-labeled giant cells within a tuber and cells in the angiomyolipoma.

Figure 2 Cell-selective activation of the mTORC1 pathway in tubers and biallelic *TSC1* mutations



(A) P-S6-labeled giant cells (GCs) (arrows) in human tuber specimen. (B) Schematic depicting single P-S6-labeled GCs (arrows, holes) removed by laser capture microdissection. Scale bar 250 μm . (C) Germline and somatic *TSC1* mutations in microdissected P-S6-labeled GCs. (a) Germline mutation, exon 15 delG2023 (arrow) in P-S6-labeled GCs. The same mutation was identified in leukocyte DNA from this patient. (b) Exon 22 nonsense mutation (3096G>T, 959E>X) in single P-S6-labeled GCs was not detected in leukocyte DNA from this patient.

blood and whole tuber DNA from this patient, was found in all P-S6-labeled GCs (figure 2). This mutation had been previously reported²⁰ in the Leiden Open Variation TSC database (database ID: TSC1_00225; <http://chromium.liacs.nl/LOVD2/TSC/home.php>). A previously unreported somatic nonsense *TSC1* mutation was detected in exon 22 (3096G>T, 959E>X) in DNA extracted from microdissected P-S6-labeled GCs (figure 2) that was not detected in leukocyte DNA nor in DNA extracted from the whole tuber in this patient. Sequencing of *TSC2* in P-S6-labeled GCs from this specimen did not reveal mutations.

In another surgical specimen, a previously reported germline *TSC2* mutation (exon 29, 3498_3499insT, Glu1167X, Leiden database ID: TSC2_00849)²⁰ was identified in DNA extracted from a whole tuber section. A nonsense *TSC2* mutation (exon 29, 3412C>T, Arg1138X, Leiden database ID: TSC2_00267)¹⁸ was detected in single microdissected GCs (figure e-1). This mutation was not detected in leukocyte DNA or total DNA extracted from the whole tubers (figure e-1) and thus likely reflected a somatic mutation. Sequencing of *TSC1* in

P-S6-labeled GCs from this case did not reveal mutations.

In a third surgical specimen, a previously reported germline *TSC2* mutation (exon 33, 4397C>A, 1466Ser>X; Leiden database ID: TSC2_00714)^{20,22} was identified in DNA extracted from a whole tuber section and in all P-S6-labeled GCs. A new missense *TSC2* mutation (exon 33, C>T, 1358P>L), not found as a SNP in either the Leiden or NCBI databases, was detected in single microdissected GCs. This missense mutation was not detected in leukocyte DNA or total DNA extracted from the whole tubers and thus likely reflected a novel somatic mutation. Sequencing of *TSC1* in P-S6-labeled GCs from this case did not reveal mutations.

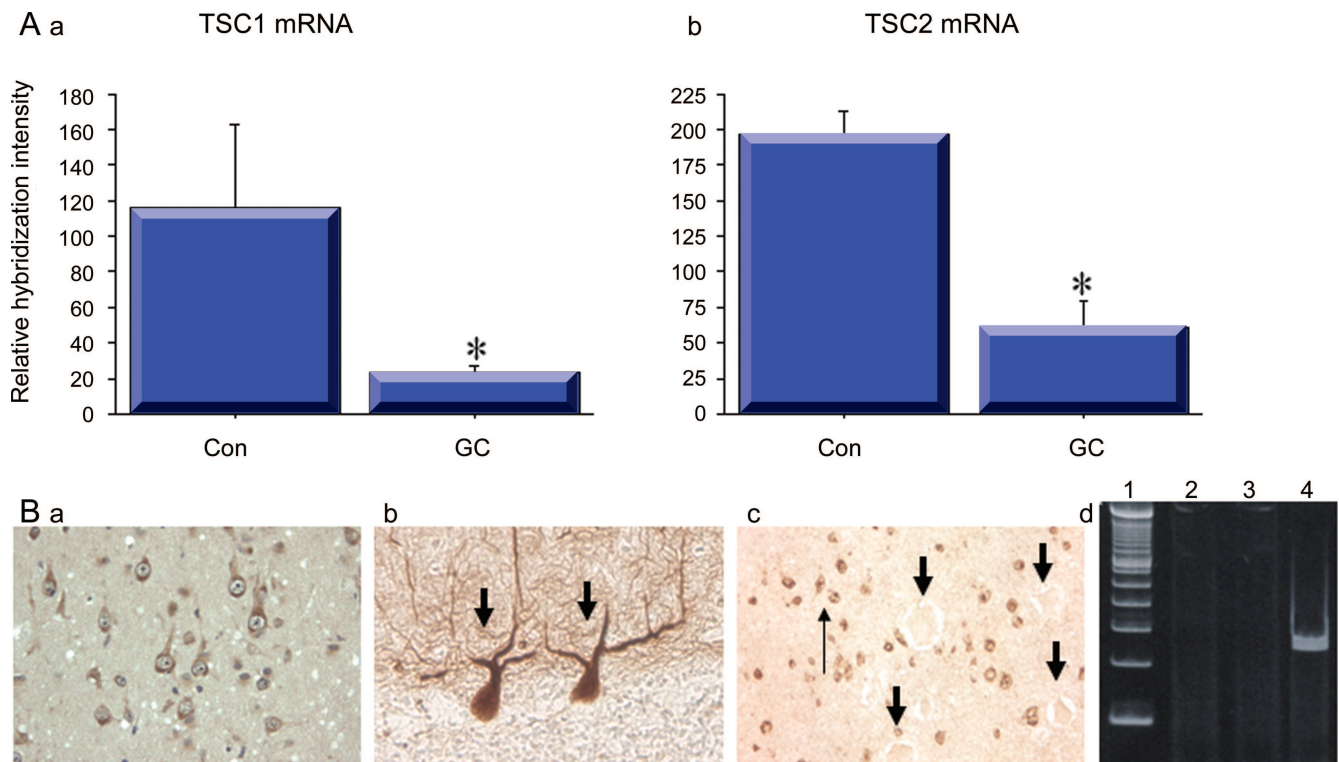
In the postmortem TSC specimen, a previously reported germline *TSC2* mutation was identified in the splice site between exons 16 and 17 (c.1839 + 1G>T; Leiden database ID: TSC2_00424)²⁰ in whole tuber and perituberal tissue homogenates (table). The germline splice site mutation was found in GCs microdissected from the tuber in this specimen. A novel somatic *TSC2* missense mutation (exon 4, 337 G>A, E114K) was detected in single microdissected GCs. Sequencing of *TSC1* in P-S6-labeled GCs from this case did not reveal mutations.

In the fourth surgical specimen, DNA sequencing from the whole lesion revealed a previously reported nonsense mutation in *TSC2* exon 3 (268C>T; 90Q>X; Leiden database ID: TSC2_00036).^{18,20,22} There was no somatic second hit mutation identified and only 1 known synonymous SNP (exon 22, 2686C>T, 860F>F; rs13337626) was found.

In 1 additional surgical specimen, a previously unreported germline deletion of *TSC2* exons 1–15 was detected. A previously unreported missense mutation in *TSC2* exon 14 (1564C>T, 522H>Y) was detected in single microdissected GCs. Analysis of existing NCBI and Leiden databases did not identify this sequence change as a known SNP (of note, 3 known SNPs were detected in this case). Sequencing of *TSC1* in P-S6-labeled GCs from this case did not reveal mutations.

Reduced expression of *TSC1* and *TSC2* mRNA in P-S6-labeled GCs. mRNA was amplified from single microdissected P-S6-labeled GCs as well as pyramidal cells from control cortex and was used to probe arrays containing *TSC1* and *TSC2* cDNAs. *TSC1* mRNA levels were reduced in GCs dissected from the *TSC1*-associated cases and reduced *TSC2* mRNA was detected in GCs from *TSC2*-associated cases when compared with control pyramidal neurons (figure 3). These results are consistent with prior reports, for example, demonstrating reduced *TSC1* gene transcript in individuals with germline *TSC1* mutations²³ and in

Figure 3 Altered TSC1 and TSC2 mRNA expression in giant cells (GCs)



(A) Reduced levels of TSC1 and TSC2 transcripts in GCs microdissected from tubers with either TSC1 (a) or TSC2 (b) germline mutations compared with control pyramidal neurons (Con; * $p < 0.05$). (B) TSC2 protein expression in control pyramidal neurons (a) and Purkinje cells in the cerebellum (b). In (c), there is selective loss of TSC2 protein in GCs (large arrows) compared with surrounding dysmorphic neurons (thin arrow). (d) Failure to detect the exon 15 (lane 2) and exon 22 (lane 3) TSC1 transcripts containing a nonsense mutation but detection of exon 22 in control pyramidal cells (lane 4).

mouse *Tsc1* knockout strains.²⁴ In one TSC2-associated specimen, TSC2 protein levels were reduced in GCs by immunohistochemistry (figure 3).

We further asked whether transcripts containing nonsense mutations could be detected since previous studies have demonstrated that nonsense mutation-containing transcripts are subject to nonsense-mediated mRNA degradation in TSC.²³ Thus, we postulated that, for example, the exon 15 and exon 22 transcripts in the first surgical case might not be detected in single GCs since they could be removed by nonsense-mediated mRNA degradation. Indeed, neither mRNA species (figure 3) was detected by RT-PCR in single microdissected, phospho-S6 immunoreactive GCs ($n = 20$), whereas the germline-encoded transcript was easily detected by RT-PCR in control neurons. These results suggest a putative mechanism to account for reduced TSC transcript levels in GCs.

DISCUSSION We demonstrate evidence for somatic second hit *TSC1* or *TSC2* gene mutations in P-S6-immunoreactive GCs in cortical tubers and suggest that biallelic gene inactivation is a plausible genetic mechanism underlying the pathogenesis of tuber formation in TSC. Since TSC gene inactivation leads to mTORC1 activation, selective expression of P-S6 in GCs in a hu-

man tuber specimen provides a compelling anatomic image of a select cell type in tubers that lacks normal TSC1–TSC2 function. Sequencing of genomic DNA from single GCs exhibiting mTORC1 activation identified somatic *TSC* mutations not detected in whole tuber or leukocyte DNA suggesting that somatic mutations occur within a select cell population within tubers. We focused on cases in which the germline mutations were known disease-causing mutations, and show that in 5 of 6 specimens, a somatic mutation can be defined. While a 2-hit or somatic mutational mechanism has been proposed for a variety of hamartomas in TSC, this is the first report to use single cell gene sequencing to investigate this genetic mechanism in a developmental cortical malformation.

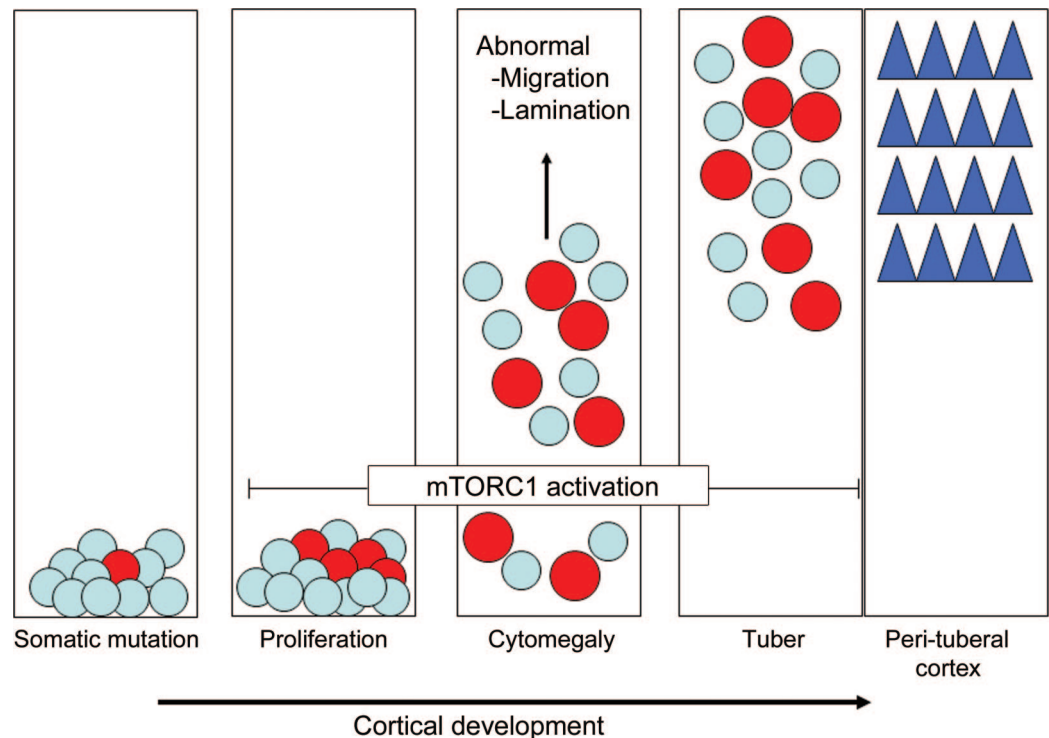
There are several caveats to our results. First, our results leave open the intriguing possibility that haploinsufficiency alone,¹³ e.g., the effects of germline mutation by itself, may be adequate for tuber formation, since in one specimen, a somatic mutation could not be identified. Our findings suggest that the developmental pathogenesis of tubers may be more genomically complex than previously believed. These results support the hypothesis that tubers are formed as a consequence of the combinatorial effects of germline and somatic mutational events detected in a

single cell type⁴ but that the effects of haploinsufficiency alone in a particular cell type, i.e., a cell autonomous effect, may be adequate to induce altered cortical cytoarchitecture. Our results are both similar to and distinct from previous studies that have investigated somatic mutations in tubers.^{4-6,13} For example, in 2 prior reports, somatic mutations could not be detected in tubers; however, these investigators did not use single cell microdissection or gene sequencing.^{5,6,13} In contrast, in 1 previous report, LOH was detected in a single tuber specimen,⁴ whereas in our study, LOH was not identified in any of our specimens, either in homogenates or single microdissected cells. We trust the integrity of our technical approaches since germline mutations detected in tubers were confirmed in blood from the same patients, and in no case were somatic mutations found in GCs identified in blood. LOH was not identified in any of the tubers but was easily identified in a single AML specimen. Further evidence to support the possibility of biallelic gene inactivation is that there was reduced transcript expression of either TSC1 or TSC2 in sin-

gle GCs. Furthermore, the failure to detect a transcript containing a nonsense mutation also suggests that there are reduced TSC1 or TSC2 mRNA levels in cells that likely lead to reduced or even absent protein levels. A similar finding has been demonstrated in existing conditional *Tsc1* mouse knockout strains.²⁴ A final limitation to our results is that we could not screen all possible cell types in tubers, e.g., DNAs, astrocytes, oligodendrocytes, for somatic mutations and cannot conclusively determine how many different cell populations may contain somatic mutations. These findings have important implications for understanding autism and epilepsy in TSC and future studies to examine somatic mutations at the single cell level in distinct cell populations in tubers are clearly warranted.

There are both affected and unaffected brain regions in TSC. Thus, our results allow us to postulate a theoretical view of how tubers may form during brain development in which a somatic TSC gene mutation occurs in a single neuroglial progenitor cell in the embryonic brain, and in combination with the

Figure 4 Developmental model for tuber formation



Far left, a somatic TSC gene mutation occurs in a neuroglial progenitor cell (red circle) superimposed on an existing germline mutation present in all progenitor cells in each patient with tuberous sclerosis complex (blue). Next, cell proliferation results in generation of more cells containing 2 mutational hits. Both haploinsufficient cells (blue) and cells with biallelic mutations (red) migrate into the embryonic cortical plate. Inactivation of the TSC gene leads to mTORC1 pathway activation and progressive cytomegaly. A tuber forms when both haploinsufficient cells and cells sustaining 2 mutations arrive in the cortical plate resulting in a focal, genetic mosaic lesion. There is persistent activation of the mTORC1 cascade as evidenced by enhanced phosphorylation of ribosomal S6 protein and cytomegaly. In contrast, haploinsufficient cells representing structurally normal neurons in perituberal cortex adjacent to tubers (blue triangles) form intact laminae and exhibit preserved cytoarchitecture.

effects of the germline mutation, results in functional TSC protein inactivation (figure 4). Subsequent activation of mTORC1 signaling leads to cytomegaly (giant cells) in the progeny of this progenitor cell as has been demonstrated in several experimental systems.^{25,26} While the cell of origin for GCs remains unknown, GCs likely derive from progenitors in the telencephalic ventricular zone²⁷ and thus could migrate, albeit abnormally, to cortex. Cells lacking functional TSC1 or TSC2 do not migrate appropriately into the cortical plate leading to a focal malformation or tuber.

When examined in toto, tubers likely reflect a mosaic of cell-autonomous and cell-non-autonomous events, that is, direct cellular consequences of altered TSC1/TSC2 protein function and indirect effects of these cells on neighboring cells. We postulate that a small (or large) expansion of a single cell lineage by cell division could give rise to a subset of cells with altered migratory or laminar destinations. Since correct cortical lamination is a sequential process in which each wave of migration may be dependent on the success of the previous wave, altered migration or differentiation in a small population of cells could disrupt a focal group of cells. If neighboring cells, for example, depend on structural support or migratory cues from these abnormal cells, it is reasonable to believe that lamination in this region could be altered for both mutation-containing and non-mutation-containing cells. Future studies will be needed to determine how cells with reduced TSC1 or TSC2 levels disrupt the migratory pathways of adjacent cells lacking a somatic mutation.

In morphologically normal cerebral cortex surrounding tubers, the mTOR pathway is not activated in cells that contain only germline *TSC* gene mutations, e.g., haploinsufficient cells, and these progeny migrate appropriately into the cortical plate (figure 4). Thus, single somatic inactivating mutations may provide one mechanism by which tubers can form directly adjacent to morphologically normal cortex. Future studies will be needed to determine if a distinct somatic mutation occurs in each tuber in any single patient or if multiple second hit mutations can be detected in 1 tuber specimen.

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DISCLOSURE

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