Single-cell Tsc1 knockout during corticogenesis generates tuber-like lesions and reduces seizure threshold in mice

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Introduction
Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by mutations in Tsc1 or Tsc2 that lead to mammalian target of rapamycin (mTOR) hyperactivity. Patients with TSC suffer from intractable seizures resulting from cortical malformations known as tubers, but research into how these tubers form has been limited because of the lack of an animal model. To address this limitation, we used in utero electroporation to knock out Tsc1 in selected neuronal populations in mice heterozygous for a mutant Tsc1 allele that eliminates the Tsc1 gene product at a precise developmental time point. Knockout of Tsc1 in single cells led to increased mTOR activity and soma size in the affected neurons. The mice exhibited white matter heterotopic nodules and discrete cortical tuber-like lesions containing cytomegalic and multinucleated neurons with abnormal dendritic trees resembling giant cells. Cortical tubers in the mutant mice did not exhibit signs of gliosis. Furthermore, phospho-S6 immunoreactivity was not upregulated in Tsc1-null astrocytes despite a lower seizure threshold. Collectively, these data suggest that a double-hit strategy to eliminate Tsc1 in discrete neuronal populations generates TSC-associated cortical lesions, providing a model to uncover the mechanisms of lesion formation and cortical hyperexcitability. In addition, the absence of glial reactivity argues against a contribution of astrocytes to lesion-associated hyperexcitability.

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ering that individuals with TSC are born with a systemic loss of one Tsc1 (or Tsc2) allele, we compared data obtained from Tsc1<sup>fl/mut</sup> mice with those obtained from littermate Tsc1<sup>fl/WT</sup> mice. The Tsc1<sup>fl</sup> allele contains LoxP sites surrounding the sequence to be excised upon Cre recombination (Figure 1A). The Tsc1<sup>fl</sup> allele is similar to a recombined allele and contributes to a frame shift and truncated transcript, rendering TSC1 (i.e., hamartin) nonfunctional. Figure 1B illustrates the PCR gels for the genotyping of the different mouse lines. A combination of primers was used to detect the WT (at 295 bp), floxed (at 480 bp), and mutant Tsc1 alleles (at 370 bp).

A combination of pCAG-cre:GFP (Cre fused to GFP) and pCAG-mRFP were electroporated at E15–E16. The fluorescence of the monomeric red fluorescent protein (mRFP) plasmid was visible by eye on dissected brains. Photographs of the fixed brains using

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**Figure 1**

In utero single-cell knockout of Tsc1 in cortical cells. (A) Exons/introns of mutant and floxed alleles before and after Cre-induced excisions of exons 17–18 in the floxed allele. (B) PCR gels of genomic DNA from the mouse lines. (C) P28-fixed brains containing a cortical area electroporated with mRFP. Fluorescent projections from the ipsilateral side containing mRFP<sup>+</sup> cells are visible. (D) Coronal section containing electroporated mRFP<sup>+</sup> cells in the ipsilateral side sent projections to the contralateral cortex from a P15 Tsc1<sup>flWT</sup> mouse electroporated at E15. Shown is a composite of several images. (E) mRFP<sup>+</sup> (red) and Cre:GFP<sup>+</sup> (green) cells in cortex of a P15 Tsc1<sup>flWT</sup> mouse electroporated at E15. (F) PCR gels of DNA plasmid for mRFP and Cre extracted from microdissected mRFP<sup>+</sup> cell–containing cortex and contralateral cortex in P7 Tsc1<sup>flmut</sup> mice. (G) PCR gels from genomic DNA obtained from ipsilateral (mRFP-containing cells) and contralateral cortical tissue microdissected from slices obtained from a P28 Tsc1<sup>flfl</sup> mouse electroporated at E16. (H) PCR gels of Tsc1 and Gapdh cDNA obtained from ipsilateral and contralateral P7 Tsc1<sup>flmut</sup> cortex. (I) Relative abundance of Tsc1 mRNA measured by qRT-PCR and obtained from ipsilateral (mRFP-containing cells) and contralateral cortical tissue microdissected from slices obtained from a P28 Tsc1<sup>flmut</sup> mouse electroporated at E16. *P < 0.005. (J) Hamartin immunostaining (green), mRFP fluorescence (red), and DAPI nuclear counterstain (blue) in ipsilateral cortex from a P28 Tsc1<sup>flmut</sup> mouse electroporated at E15. Arrows indicate the mRFP<sup>+</sup> cell that stained negative for hamartin (green). Scale bars: 3 mm (C), 350 μm (D), 140 μm (E), 30 μm (J). Lanes in B and H were run on the same gel but were noncontiguous (white lines).
A sensitive fluorescent illumination and detection system illustrate both the area of the cortex containing mRFP+ cells and the contralateral area containing mRFP+ projections (Figure 1C). Such ipsilateral and contralateral areas were clearly visible on confocal photographs obtained on coronal brain sections (Figure 1D). In addition, a dual fluorescence from GFP and mRFP was visible (Figure 1E). The vector encoding cre:GFP had a nuclear localization sequence, which resulted in nuclear GFP expression, while mRFP was distributed throughout the cell cytoplasm. Both Cre and mRFP DNA were also detected using PCR from the ipsilateral (i.e., electroporated) but not the contralateral cortex microdissected from P7 brains electroporated at E15 (Figure 1F). Next, to determine whether recombination occurred at the Tsc1fl alleles, we prepared genomic DNA from microdissected ipsilateral and contralateral cortical layer II/III obtained from P28 Tsc1fl/WT mice electroporated at E15. We found a Tsc1 mutant allele band from the ipsilateral tissue but not from the contralateral tissue, suggesting that recombination had occurred, leading to Tsc1 removal in electroporated cells (Figure 1G).

Finally, we examined Tsc1 mRNA and protein expression. RT-PCR for Tsc1 illustrated that there was a significant decrease in Tsc1 but not Gapdh mRNA in the ipsilateral compared with the contralateral P7 cortex (Figure 1G). To better quantify the loss of Tsc1, we performed quantitative RT-PCR (qRT-PCR). Tsc1 mRNA levels were decreased by 70% in the ipsilateral compared with the contralateral cortex microdissected from P28 Tsc1fl/WT mice electroporated at E16 (n = 5 or 6 samples each from 2 mice; Figure 1I). There was not a complete loss, due to contamination of non-electroporated cells in the microdissected cortex. Immunostaining for hamartin in E15-electroporated P28 Tsc1fl/mut mice revealed that all mRFP+ cells exam-
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**Figure 3**

Heterotopic nodule formation with cytomegalic neurons after electroporation at E16. (A) Diagram of a coronal section illustrating the cortical ROI. (B) Diagram illustrating the approximate birthdate of cortical layer II/III neurons. The red arrow indicates the time of electroporation at E16. (C and D) Photographs of mRFP+ cells in the somato-sensory cortex in coronal sections from P15 Tsc1fl/WT (C) and Tsc1fl/mut mice (D) electroporated at E16. (E) Bar graphs illustrating the distribution (as a percentage) of mRFP+ cells across cortical layers in Tsc1fl/WT and Tsc1fl/mut mice. WM, white matter. *P < 0.05. (F–I) Confocal images of mRFP+ cells in one optical section (red, F and H) and a Z-stack (19.5 μm, black, G and I) from the boxed regions in D. Scale bars: 140 μm (C and D) and 70 μm (F–I).

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ind displayed no hamartin staining, while surrounding mRFP+ cells displayed cytoplasmic hamartin staining (n = 3 mice; Figure 1J). In addition, mRFP+ cells in E15-electroporated P28 Tsc1fl/WT mice also displayed hamartin staining (data not shown).

Collectively, these data suggest that in utero electroporation is a reliable method to excise floxed Tsc1 allele(s) upon Cre expression that results in knockout and haploinsufficiency of Tsc1 in mRFP+ cells from Tsc1fl/mut and Tsc1fl/WT mice (hereafter referred to as Tsc1null and Tsc1haplo, respectively).

Single-cell Tsc1 deletion results in increased mTOR activity and cell size. The TSCI gene product, hamartin, negatively regulates mTOR activity, which controls S6 phosphorylation. Phospho-S6 (pS6) is thus commonly used as a marker of mTOR activity. Immunostaining for pS6 (Ser240/244) in E16-electroporated Tsc1null mice revealed that mRFP+ neurons displayed a 3.4-fold elevation in intensity of pS6 staining compared with mRFP+ neurons in the contralateral side (n = 9 mice; P < 0.01; Figure 2, A–E, E15 and E16 electroporation combined). In Figure 2B, mRFP+ neurons in the ipsilateral side also displayed enhanced pS6 compared with neurons in the contralateral side (Figure 2D). This resulted from dilution of the mRFP-encoding plasmid compared with genomic Tsc1 removal (see below). In contrast, loss of a single Tsc1 allele in mRFP+ cells of Tsc1fl/WT mice did not increase pS6 (n = 6 mice; Figure 2, E–G). As a result, the ratio of pS6 staining in Tsc1null cells from Tsc1fl/WT mice was significantly higher than that in Tsc1haplo cells from Tsc1fl/WT mice (Figure 2E).

mTOR hyperactivity leads to increased cell size in other cell types. We thus quantified the soma size of layer II/III mRFP+ and contralateral mRFP+ neurons using NeuN immunostaining. In Tsc1fl/WT mice, the mean soma size of NeuN+/mRFP+ neurons was significantly larger than that of mRFP+ neurons in the contralateral cortex (P < 0.01, n = 8 mice; Figure 2, H–K). As shown for pS6, loss of a single allele in mRFP+ cells of Tsc1fl/WT mice did not result in increased cell size (n = 5 mice; Figure 2K). In addition, measuring the soma of mRFP+ cells in slices from Tsc1fl/WT and Tsc1fl/WT mice revealed that Tsc1null neurons had a significantly larger soma than Tsc1haplo neurons (n = 6 and 5 mice, respectively; 93% increase; Figure 2L).

These data suggest that the mTOR pathway was hyperactivated in mRFP+ cells in Tsc1fl/WT mice (i.e., Tsc1null cells) upon knockout of Tsc1. In addition, loss of heterozygosity using a double-hit strategy resulted in larger mTOR hyperactivity and cell size increase than loss of only a single allele.

Formation of heterotopic nodules with cytomegalic neurons in Tsc1fl/WT mice. Considering the increased mTOR activity in Tsc1null cells, we examined sections for the presence of malformations in the neocortex following electroporation at E16 (Figure 3, A–D). E16 is close to the end of layer II/III neurogenesis in the neocortex of WT mice (ref. 30 and Figure 3B). While 98% ± 1% of Tsc1haplo cells reached layer II/III, 17% ± 3% of Tsc1null cells were misplaced (n = 3 mice; P < 0.05; Figure 3, C–E). In particular, electroporated Tsc1fl/WT cortex displayed misplaced groups of cells, called heterotopic nodules, in layer VI/subplate and in the white matter (Figure 3D). mRFP+ cells in the nodules and in layer II/III were all NeuN+ and were thus identified as neurons (data not shown). Heterotopic nodules contained 5–25 neurons. These Tsc1null neurons in the nodules exhibited enlarged soma size (93% increase...
compared with Tsc1null neurons in layer II/III) and a dysplastic morphology (Figure 3, F–I).

Collectively, these data suggest that single-cell Tsc1 deletion at the end of layer II/III neurogenesis leads to the formation of heterotopic nodules with cytomegalic neurons in and above the white matter that resemble white matter heterotopia seen in TSC individuals. Nevertheless, approximately 80% of the mRFP+ cells reached the proper layer. We hypothesized that only 20% of the cells were affected because of the time necessary to produce Cre following electroporation and for hamartin to be degraded (half-life of ~24 hours; ref. 31). We thus examined whether electroporation at E15, corresponding to the onset of layer II/III formation, would generate larger lesions.

**Single-cell Tsc1 deletion at E15 generates tuber-like lesions in Tsc1fl/mut mice.** Electroporation at E15 is expected to affect a greater proportion of layer II/III-predetermined neurons, the generation of which starts at approximately E14.5 (30, 32). Following E15 electroporation, a simple visual inspection of P28 slices was sufficient
to observe striking misplacement of Tsc1null neurons compared with Tsc1haplo neurons (n = 7 Tsc1fl/WT mice). By contrast, 68% of Tsc1null neurons were misplaced throughout layers IV–VI, leading to a loss of lamination (n = 4 Tsc1fl/mut mice; Figure 4, B–D). The lesion displayed in Figure 4B spanned different cortices in the dorso-ventral axis, from a 6-layer, to a 5-layer, to a 3-layer cortex (piriform cortex). The most dramatically affected cortex was the 6-layer cortex.

Serial sectioning revealed that the lesions spanned about 3 mm, and this size was controlled by the electroporation span (Figure 1C and Figure 4E). Despite a rostro-caudal neurogenic gradient, the lesion was prominent at every level. Nevertheless, the limbic cortex was significantly more affected than the piriform cortex in terms of cell misplacement, giving an appearance of 2 distinct lesions. Finally, cell scattering through the white matter (black arrows in Figure 4E) was visible in all of the sections. The rostral tail of the lesions displayed misplaced cells in a columnar organization reminiscent of a radial migratory track (Figure 4F).

Pathologically in humans, cortical tubers are characterized by a focal loss of normal cortical architecture, with prominent giant cells that have enlarged nuclei and soma, high pS6 levels, and a dysmorphic arborization (24–28). Examination of the cortical lesions in Tsc1fl/mut mice revealed the presence of cytomegalic cells scattered throughout layers IV to VI (Figure 4G and Figure 5, A–C). Immunostaining for pS6 was more intense in enlarged and misplaced cells than in surrounding cells (Figure 4H), giving a mosaic appearance of the cortex in terms of pS6 staining and cell size, as seen in individuals with TSC (27). Such a cortical appearance with pS6 staining is routinely used to characterize cortical tubers at the pathological level. In addition, these enlarged cells had a dysmorphic morphology (Figure 4, G and I, and Figure 5).

Another key hallmark of cortical tubers is increased pS6 in giant cells, which are often multinucleated (25, 33). All ectopic cells displayed high pS6 intensity, suggesting mTOR hyperactivity.

Figure 5
pS6+ enlarged cells are neuronal, dysmorphic, and multinucleated. (A and B) Confocal photographs of mRFP+ cells (red) and pS6 immunostaining (green) in cortical layer V–VI. (C) Black and white image of the red fluorescence in A, illustrating the mosaic nature of cell sizes. (D and E) Confocal photographs of mRFP+ cells (red) and NeuN (green) immunostaining overlay in cortical layer V (E does not show the red channel). Arrows in E denote electroporated cells, which colocalized with NeuN staining. (G) Histogram of cell area in upper and deeper layers in cortical sections from Tsc1fl/mut mice. (H) Bar graph of the ratio of the soma area of mRFP+ cells in layers IV/VI versus II/III in Tsc1fl/mut mice. *P < 0.005. (I and J) Confocal Z-stack photographs and projections of an mRFP+ cell (red) and DAPI (pseudo-colored green) in layer VI. All the images are from P28 Tsc1fl/mut mice electroporated at E15. Scale bars: 70 μm (A–C), 40 μm (D–F), 50 μm (I and J).
Figure 6
Lack of astrogliosis in the tuber-like lesions. (A–F) Photograph of mRFP+ cells (red) and GFAP immunostaining (green) in the ipsilateral (A, B, D, and E) and contralateral (C and F) in P28 and P15 Tsc1flmut mice electroporated at E15 (A–C) and E16 (D–F), respectively. (G–I) Photograph of mRFP+ cells (red) and GS (green) immunostaining in the ipsilateral (G and H) and contralateral (I) in P28 Tsc1flmut mice electroporated at E15. (J and K) Photograph of mRFP fluorescence (red), GS (blue, K), and GFP fluorescence (green) in a P15 CAG-GFP × Tsc1flmut mouse electroporated with pCAG-mRFP and pCAG-Cre.GFP at E16. Inset in J shows PCR gels from genomic DNA obtained from E16-electroporated cortical tissue containing electroporated astrocytes but no electroporated neurons, microdissected from slices obtained from a P28 Tsc1fl/fl mouse in which no mRFP+ neurons were visible. (L and M) Higher-magnification photographs of GS staining and GFP fluorescence from the boxed region in K. Arrows indicate GFP+ astrocytes (i.e., GS+), and arrowheads indicate GFP- astrocytes that exhibit the same soma size. (N) Photograph of GFP fluorescence (green) and pS6 immunostaining (blue) in a CAG-GFP × Tsc1flmut mouse electroporated at E16. (O and P) Higher-magnification photographs of pS6 staining in GFP- cells from the boxed region in N. Arrows point to GFP- astrocytes that are pS6 negative. (Q) Quantification of Tsc1flmut and Tsc1flwt astrocytic soma size, as outlined by GS staining. Scale bars: 300 μm (A–F), 140 μm (G–I), 70 μm (J, K, and N), 20 μm (L, M, O, and P).
(Figure 5, A–C). In addition, ectopic cells were all NeuN+, identifying them as neurons (Figure 5, D–F). They did not express markers of proliferation (Ki67), immature cells (nestin), or immature neurons (doublecortin) (data not shown). Consistent with mTOR hyperactivity, the histogram of soma area and the ratio of soma areas in layer IV/VI versus II/III illustrate that ectopic neurons have a significantly larger soma size than layer II/III neurons (n = 4 mice; Figure 5, G and H). In addition, these also illustrate the large distribution of soma sizes for misplaced cells. Finally, about 10% of the enlarged cells were multinucleated with enlarged nuclei compared with surrounding cells in all mice examined (Figure 5, I and J, and Supplemental Figure 1; available online with this article; doi:10.1172/JCI44909DS1). No mRFP+ cells were multinucleated in any of the Tsc1WT mice examined (n = 3; data not shown).

Collectively, these data suggest that single-cell Tsc1 deletion leads to the formation of focal cortical lesions with hallmarks of cortical tubers, as seen in individuals with TSC. These lesions in Tsc1WT mice are thus referred to as tuber-like lesions.

Lack of astroglial reactivity in tuber-like lesions despite lower seizure threshold. Gliosis or astrogliosis is routinely observed in cortical tubers from individuals with TSC (34). We determined the presence of gliosis using immunostaining for glial fibrillary acidic protein (GFAP). We found no detectable increase in GFAP immunoreactivity in cortical tissue from Tsc1mut mice with cortical tuber-like lesions or heterotopic nodules (Figure 6, A–F). We also immunostained for glutamine synthetase (GS), the expression of which is downregulated in TSC astrocytes and reactive astrocytes associated with other epilepsy disorders (34, 35). We found no detectable decrease in GS immunoreactivity in tuber-like lesions (Figure 6, G–I). These data suggest that cortical astrocytes do not react to the presence of tubers or heterotopic nodules.

Astrocytes with enhanced GFAP and loss of GS in individuals with TSC have been reported to express enhanced pS6 staining and enlarged soma size, possibly as a result of Tsc1 loss (34). In our sections, all mRFP+ cells were NeuN+ and thus neurons. One may presume that astrocytes surrounding the ectopic Tsc1null neurons do not change because they have not lost Tsc1. However, considering that radial glia transform into astrocytes by the end of neurogenesis, genetic removal of Tsc1 at the genomic level using the floxed strategy should also lead to Tsc1 deletion in cortical astrocytes. In addition, the mRFP reporter plasmid is expected to be diluted due to successive cell divisions and therefore is not expressed in the last generated cells. We thus crossed Tsc1mut mice with CAG-GFP fluorescent reporter mice to induce genomic GFP expression in every Cre-containing cell and compare the expression of GFP fluorescence to that of mRFP fluorescence. mRFP was not observed in GFP+ astrocytes exhibiting an astrogial morphology (Figure 6J), suggesting that mRFP was indeed diluted, while GFP was permanently expressed. These stellar GFP+ cells were identified as astrocytes by GS immunostaining (Figure 6, K–M).

Surprisingly, stellate GFP+ cells, and hence Tsc1null astrocytes, did not exhibit increased pS6 immunoreactivity compared with surrounding Tsc1null neurons (Figure 6, N–P). Similarly, Tsc1null astrocytes were the same size as control astrocytes as determined by GS staining (n = 3 mice; Figure 6Q). To determine whether recombination occurred in astrocytes, we microdissected the electroporated cortex from P28 Tsc1fl/fl (E16 electroporation) in a region where electroporated neurons were absent (layer IV). We found a Tsc1 mutant allele band from the ipsilateral tissue (inset of Figure 6J), suggesting that recombination had occurred, leading to Tsc1 removal in GFP+ astrocytes. These data show that cortical control and Tsc1mut astrocytes do not react to the presence of heterotopic nodules and tuber-like lesions.

Because gliosis has been proposed to be responsible for cortical hyperexcitability in TSC (34, 36), we examined whether Tsc1mut mouse containing lesions exhibited a lower seizure threshold. Mice were injected with increasing doses of PTZ (a GABA receptor antagonist), and the latency for generalized tonic-clonic seizures was measured. There was a 25% reduction in seizure latency in Tsc1mut mice containing heterotopic nodules compared with control littermate Tsc1WT mice (Figure 7, A and B). These data suggest that lowered seizure threshold in Tsc1mut mice results from the presence of the cortical malformation.

Discussion

Our findings argue that biallelic inactivation of Tsc1 in cortical neural progenitor cells is necessary for generating malformations resembling cortical tubers and white matter nodules in mice, as seen in TSC patients. In addition, one unexpected finding was the absence of astroglial reactivity and mTOR hyperactivity in Tsc1null astrocytes surrounding the lesions despite a reduced seizure threshold.

The cortical malformations described here recapitulate several key features of the TSC-associated brain lesions in humans. These features include loss of lamination, mosaicism of pS6 expression and cell size, and the presence of ectopic cytomegalic neurons (24–28). In the lesion, the presence of pS6+ and multinucleated cytomegalic neurons with a dysmorphic morphology distinguishes TSC-associated lesions from other cortical malformations (37).

However, our model does not replicate all features of human tubers (i.e., spontaneous seizure activity or neurological phenotype, giant cells and astrogliosis). Regarding the lack of seizure activity, it is important to emphasize that although almost all
patients have seizures, not all tubers are epileptogenic (38–40). In fact, as mentioned below the absence of seizures will allow us to increase cell excitability to examine the outcome on gliosis and inflammation observed in human tubers (41). In addition, it remains speculative whether the tubers contribute to neuroglial phenotypes such as autistic traits (42–44), and we did not examine neuroglial phenotype past 1 month.

At the cellular level, our lesion lacks cytomegalic cells with a mixed neuron-glial or immature phenotype (e.g., immunostaining for nestin or proliferative markers) called giant cells (25, 26, 28, 33, 45). The absence of such cells could result from the innate differences between human and murine neural progenitor cells. Remarkably, none of the rodent models have reported giant cells (12, 21, 23, 46, 47), although these models have recombination in most of the forebrain instead of a section of the cortex, as shown here. The lack of giant cells here and in other models raises the issue of human and rodent difference. Indeed, the developing human neocortex displays an additional population of proliferative radial glia-like cells in the outer subventricular zone that may contribute to the generation of giant cells with mixed phenotypes in humans (48, 49).

Finally, the lesions were not associated with astrogliosis, as shown by the lack of GFAP upregulation despite Tsc1 removal in cortical astrocytes. In addition, Tsc1 removal in astrocytes did not result in a detectable decrease of GS (a functional hallmark of astrogliosis), an elevation of mTOR activity, or a change in cell size. This was unexpected, considering that astrocytes in human tubers show increased GFAP immunoreactivity and cell size and loss of GS (28, 34). We did not explore other features such as K+ channel and glutamate transporter expression that are altered in epileptic astrocytes or Tsc1null astrocytes (36, 50–52). However, human tubers, which have variable degrees of gliosis, are removed from patients who undergo surgical treatment for focal epilepsy mapped to the resected tubers (53). It is unknown whether tubers not associated with seizures have gliosis (38). Regarding another mouse model of TSC, in which Tsc2 was removed in the majority of radial glia and cortical mislamination was reported, Way et al. reported that GFAP upregulation was observed in the neocortex (23). However, these mice had spontaneous clinical seizures, which makes comparison difficult with our data because GFAP upregulation and gliosis might result from the seizure activity (see, for example, ref. 54). In a model by Meikle et al., in which Tsc1 was removed from developing neurons, there was prominent mislamination and increased neuronal size and pS6, but no gliosis (lack of GFAP upregulation) (22). In this model, astrocytes retain Tsc1 but do not react to the lesion, at least prior to the onset of seizures. Similarly in our model, astrocytes lost Tsc1 but did not react to the lesion, as shown by the lack of GFAP upregulation. These findings, including ours, raise an important question of whether GFAP upregulation is downstream of the seizure activity. Our model may help to address this question. Indeed, it would be possible to induce either global seizure activity or focal hyperexcitability of tuber neurons through channelrhodopsin-2 (ChR2) (55) or genetically engineered Gq-coupled receptors (56) in vivo and then examine glial reactivity at different time points. ChR2 or genetically engineered Gq-coupled receptors would not be expressed in astrocytes as a result of plasmid dilution that would result in selective neuronal expression and thus neuronal stimulation.

Collectively, our model replicates most of the hallmarks of tubers, including mislamination and ectopic and cytomegalic neurons with high pS6 intermingled with normal-looking cells that have low pS6 but lack the giant cells with mixed phenotype. For this reason, we refer to our lesions as tuber-like. The issue of astrogliosis raises an important question that our model may help address, as mentioned above.

Our approach has several advantages over previous models. One major advantage of our approach is to allow for a precise spatial-temporal inactivation of Tsc1 and thus the formation of discrete lesions that have not been generated with other TSC models (14, 21–23, 26). In addition, our approach allowed for Tsc1 removal in both neurons and astrocytes and allowed us to precisely choose the timing of recombination by electroporating at different time points from E12 to E18. Compared with other studies, our approach includes the following applications: first, plasmids encoding interference RNA, overexpression system, firefly luciferase for in vivo imaging (ChR2) (55), or engineered Gq-coupled receptors (56) for stimulating tuber-like neurons in slice or in vivo can be co-expressed via electroporation. Second, the tissue surrounding the tuber-like lesion can be studied. This is important because the normal-appearing perituberal tissue has been shown in one study to be the initiation site for epileptiform activity (38). Third, cell migration of Tsc1null neurons in a Tsc1fl/fl cortex can be examined when using fluorescent reporter mice. Using fluorescent rather than LacZ reporter mice is helpful for detecting fluorescence without staining and for future experiments aimed at performing live studies, such as migration assays and patch clamp and calcium experiments. Finally, axonal projections of abnormal neurons can be tracked and their impact on the contralateral neuronal activity can be assessed.

Our data address and raise questions related to the pathogenesis of TSC. First, our data show that biallelic inactivation of Tsc1 (loss of heterozygosity), which has recently been found in tuber cells (18), is necessary for generating tuber-like lesions. Our findings also support the notion that a double-hit strategy to eliminate functional TSC1 leads to tuber formation. In addition, unpublished observations obtained by comparing data in Tsc1fl/fl versus Tsc1fl/mut mice suggest that a double-hit strategy is required for lesion formation. Second, our approach is based on the assumption that tubers are clonal. In a recent article by Crino and colleagues (18), only a single mutation was found from a pool of tuber cells, suggesting that tubers are clonal. This finding also implies that a neural progenitor cell was affected, leading to the generation of multiple cells with similar mutations, as discussed by others (27, 57). However, there are limited data on the clonality of tubers and no data in humans related to the original cell type affected. Third, temporal targeting of the Cre-containing vector reveals that the earlier Tsc1 deletion occurs, the more dramatic is the malformation in terms of size and mislamination. The spatial targeting to different cortices also led to differently sized or mislayered malformations when comparing a 6- versus a 3-layer cortex. These data provide one explanation for the great variability in tuber size and excitability seen in individuals with TSC.

In conclusion, we provide what we believe to be a novel strategy to generate cortical tuber-like lesions with features resembling TSC-associated lesions but without giant cells. Generating discrete cortical tuber-like lesions at a given location will allow us and others to uncover the mechanisms of lesion formation and identify new mTOR-associated targets to limit the formation of lesions and examine cortical excitability. Our strategy can also be applied to study other mTOR-associated pathologies or diseases such as polyhydramnios, megalencephaly, symptomatic epilepsy syndrome, fragile X syndrome, schizophrenia, and autism spectrum disorders.
(58–62). The strategy described here can be used to increase mTOR activity in selective neuronal populations at a given time during embryonic development of Tic10/β mice. Such a strategy may help uncover alterations in the biochemistry and circuitry of TSC and other mTOR-associated neurodevelopmental disorders.

**Methods**

**Animals.** Research protocols were approved by the Yale University Institutional Animal Care and Use Committee. Experiments were performed on littermate animals obtained by crossing the following 2 lines of transgenic mice: Tic10/β (Jackson Laboratories) and Tic10/β (NCI). Tic10/β mice were from a mixed background (C57BL/6J, BALB/cJ, or 129/SvJae mice) and the genetic background of Tic10/β mice was B6.129S. These 2 lines of mice were generated by David J. Kwiatkowski (Brigham and Women’s Hospital, Harvard Medical School, Cambridge, Massachusetts, USA). The breeding lines were also crossed with the GFP fluorescent reporter line CAG-GFP (Jackson Laboratories; B6.Cg-Tg[CAG-Bgeo/GFP]21Lbe/J), in which, upon secondary antibody (Alexa Fluor series at 1:1,000 [Invitrogen]; or Cyanine series at 1:500 [Jackson ImmunoResearch]) for 1 hour at room temperature, lines were also crossed with the GFP fluorescent reporter line CAG-GFP applied using a pulse generator (ECM830; BTX). A combination of immunostaining was performed in free-floating 100-μm-thick sections in sterile 1× PBS. Microdissection of tissues of interest was performed under an epifluorescence dissecting microscope. To lyse tissue and facilitate RNA isolation, the fixed tissue was incubated with proteinase K and digestion buffer for 30 minutes on the shaker (5,200 g and 55°C, as indicated by SABioscience FFPE RNA extraction kit). Trizol reagent (750 μl) plus 200 μl of chloroform were added to 250 μl of digested sample and vortexed for 1 minute. Following centrifugation for 15 minutes at 4°C and 12,000 g, the top aqueous phase was transferred to a fresh reaction tube. After adding 1,000 μl of ethanol, the sample was vortexed for 1 minute, transferred to SABioscience spin columns, and centrifuged at 8,000 rpm for 15 seconds. The columns were rinsed with wash buffer and 75% ETOH. Following centrifugation, the RNA was eluted with RNase-free deionized H2O prior to determining its concentration and purity on a spectrophotometer. The samples with contamination were subjected to an additional ethanol/sodium acetate precipitation.

For RT-PCR, 2.12 μg of RNA was mixed with dNTPs, random primers (Invitrogen), and RNase/DNase-free deionized H2O, heated for 5 minutes at 65°C, and then rapidly chilled on wet ice for 5 minutes, followed by brief centrifugation. RT, RNase out, and SuperScript III were then added to each sample and reverse transcribed in a Bio-Rad MyCycler. cDNA was then subjected to PCR using primers to Gapdh and Tic1. Tic1 primers were 5′-TCA-AGCACCTCTTCTGCCC-3′ and 5′-GTACAGGCGCGGTGTTT-3′.

**Genomic and plasmid DNA isolation.** Fresh, unfixed tissue was subjected to a proteinase K dilution and genomic DNA isolated using a DNeasy kit (DNA extraction). Quantification of DNA purity and concentration was performed, and approximately 0.19 μg of DNA was added to PCR reactions. Invitrogen 10× PCR buffer was added to 10 μl dNTP mix, 50 mM MgSO4, autoclaved DNase-free deionized H2O, and Platinum Taq. The reaction volume was 25 μl after adding 1 μl of each forward and reverse primer with 3 μl of the appropriate sample. PCR was performed in a Bio-Rad MyCycler (32–36 cycles). For plasmid and recombination detection, 5 μl of product was subjected to a second round of PCR. Amplicons were visualized by running samples diluted in 10× blue juice DNA loading buffer loaded onto a 2% agarose gel run at 100 V for 30 minutes alongside a 100-bp DNA ladder (Invitrogen). DNA primers for Cre and mRFP were as follows: 5′-GCAAAGCTGTAGGCTGCGAAG-3′, 5′-TCGGCGCATAACCGAGTGAACACAG-3′ (307 bp), and 5′-TGCGGCCACTGACCATGAGCAG-3′ (352 bp).

**qRT-PCR.** mRNA transcripts were quantified by the standard curve method of qRT-PCR. cDNA from P28 cortical tissue was amplified with the specified primers and detected with SYBR Green (Bio-Rad) by a Chromogen-modified iCycler.
zone to find layer II/III based on cell density). Each layer was identified as a region of interest (ROI) with the polygon tool. Cell counts were automated using the particle analyzer and to exclude debris and cell clumping we used the following settings (area, 0.005 cm² < 0.01 cm²). Three sections were analyzed per mouse.

To measure cell size, Z-stack images of mRFP cells, NeuN+, or G3' cells in coronal sections were acquired with a x20 dry objective (Numerical Aperture, 0.75) using high-low settings to minimize saturation. In ImageJ, the freehand tracing tool was used to outline cell somas. Cell size was represented relative to the indicated controls.

To measure mTOR activity, immunostaining was performed using an antibody to phosphorylated serine 240/244 of the 6s ribosomal protein (pS6). Serial Z-stacks were acquired under the same settings for the ipsilateral and contralateral hemispheres of coronal sections. Because layer II/III cortical neurons project to the contralateral hemisphere, mRFP axons were followed to measure pS6 levels in cells of the non-electroporated region. ROIs were generated using an elliptical selection tool, and average intensity values for each ROI were determined. The ROI did not include the nucleus that had low pS6 staining. For both cell size and pS6 staining measurement, 3 sections per mouse and 38–111 cells per section were analyzed.

Seizure test. Pentylenetetrazole (31.25 μg/g; Invitrogen) was administered via intraperitoneal injection at 10-minute intervals in mice electroporated at E15. The injections were given until the mice exhibited a generalized tonic-clonic seizure (3–4 injections). The time to onset of the generalized seizure was video-recorded. Observers were blinded to the genotypes of the mice and as to whether they were electroporated.

Statistics. Analysis of cell size and pS6 was performed on an average of 43 and 45 cells per slice, respectively, from 3–4 animals in each condition. Laminar distribution of cells was performed on 1–2 slices for 3–4 animals of each genotype. t tests were performed for each comparison made. PTZ-induced seizure assays were performed on 3–4 animals for each condition.

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