Hypoxia-inducible factor 1a is a \textit{Tsc1}-regulated survival factor in newborn neurons in tuberous sclerosis complex

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Tuberous sclerosis complex (TSC) is a genetic disorder caused by mutations in \textit{TSC1} or \textit{TSC2} resulting in hyperactivity of the mammalian target of rapamycin and disabling brain lesions. These lesions contain misplaced neurons enriched in hypoxia-inducible factor 1a (HIF1a). However, the relationship between \textit{TSC1/2} and HIF1a and the function of HIF1a in TSC neurons remain unexplored. Here, we examine the degree of HIF1a activity and its function in newborn \textit{Tsc1}null neurons in a mouse model of TSC. Using single cell electroporation in the neurogenic subventricular zone (SVZ) of neonatal mice, we deleted \textit{Tsc1} and generated olfactory lesions containing misplaced \textit{Tsc1}null neurons as previously reported. These newborn neurons displayed elevated HIF1a-mediated transcriptional activity when compared with \textit{Tsc1} heterozygote neurons and a marked resistance to cell death induced by a HIF1a antagonist. Electroporation of \textit{Hif1a} targeting short hairpin RNA (shRNA) or dominant negative HIF1a constructs resulted in 80–90% loss of \textit{Tsc1}null newborn neurons although sparing SVZ stem cells. Consistent with this later finding, induction of \textit{Hif1a} shRNA expression during synaptic integration thus bypassing neuron production also resulted in newborn neuron death. Collectively, these results suggest that HIF1a acts as a molecular determinant of newborn neuron survival and that its \textit{Tsc1}-dependent up-regulation gave \textit{Tsc1}null neurons a survival advantage, despite their misplacement in a novel microenvironment.

INTRODUCTION

Tuberous sclerosis complex (TSC) is a developmental disorder caused by mutations in the tumor suppressor genes, \textit{TSC1} or \textit{TSC2}, resulting in benign growths, malformations and neurologic dysfunction (1–5). Lesions within the central nervous system contain misplaced giant cells and neurons that display increased activity of the mammalian target of rapamycin (mTOR) signaling pathway (6,7). These diseased cells also display increases in the expression of the basic helix-loop-helix transcription factor hypoxia-inducible factor 1a (HIF1a) in human TSC tissue (8).

HIF1a is master transcriptional regulator of the adaptive response to hypoxia through activation of the transcription of many genes, including those involved in energy metabolism, angiogenesis and apoptosis (9–11). Through its transcriptional activity, HIF1a has been implicated in many vital cellular functions (11). Recently, it has been shown to regulate the proliferation and differentiation of adult neural stem cells in the hippocampal subgranular zone and the survival of retinal neurons during development (12,13). In TSC neurons, increased HIF1a expression is expected to result from hyperactivity of the mTOR complex 1 (mTORC1) because mTORC1 signaling pathway regulates HIF1a levels and activity in several systems (14–18). mTOR is the catalytic subunit of the two molecular complexes mTORC1 and mTORC2. HIF1a has also been shown to be elevated in \textit{Tsc2}null and \textit{Tsc1}null mouse embryonic fibroblasts (19–21). However, it remains to be examined whether \textit{TSC1} or \textit{TSC2} loss in neuronal cells leads to cell-autonomous increases in HIF1a expression and activity. In addition, the function of HIF1a in TSC neurons remains unknown.

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Here, we examine HIF1α activity and function in newborn Tsc1null neurons in a mouse model of TSC. In this model, we recently reported that Tsc1 deletion in neonatal stem cells of the neurogenic subventricular zone (SVZ) using electroporation leads to olfactory bulb (OB) lesions containing ectopic Tsc1null neurons (22). Using RT-PCR and in vivo gene reporter assays, we found that HIF1α levels and transcriptional activity were elevated in newborn Tsc1null neurons. Loss-of-function experiments using Hif1a short hairpin RNA (shRNA) or dominant negative constructs revealed that Tsc1-dependent HIF1α up-regulation allows newborn Tsc1null neurons to survive within TSC lesions, despite their placement in a heterotopic microenvironment.

RESULTS

Generation of TSC-like lesions containing newborn Tsc1null neurons

To generate TSC-like lesions containing Tsc1null neurons, we used R26R-Stop-tdTomato (noted R26R RFP) mouse pups containing a conditional (floxed, fl) and a wild type (wt) or Tsc1 mutant (mut) allele (noted Tsc1fl/wt and Tsc1fl/mut, Fig. 1A). CRE recombinase (CRE) and enhanced green fluorescent protein (GFP)-encoding DNA plasmids were injected into the lateral ventricle and were electroporated into neural progenitor cells of the SVZ in postnatal day (P) 0–1 pups (Fig. 1B) as previously performed (22–24). Neural progenitor...
cells are known to divide and generate transit amplifying cells that themselves generate neuroblasts. These neuroblasts migrate along the rostral migratory stream (RMS) to reach the OB, where they differentiate into interneurons, including granule cells and periglomerular cells (Fig. 1C). Following CRE electroporation, we detected recombination at the R26R locus by visualizing red fluorescent protein (RFP) fluorescence in SVZ cells and in newborn neurons that entered the OB circuitry by 5 days post-electroporation (dpe, P28 in Fig. 1D and I for RMS and OB images, respectively) (14,25–27). Using this approach, we previously reported that Tsc1 was efficiently knocked out in RFP+ neurons of Tsc1fl/wt/R26R mice resulting in hyperactive mTORC1 assessed with phosphorylated S6 immunostaining in Tsc1null cells when compared with Tsc1mut cells (het for heterozygote) in Tsc1null mice (22). S6 is phosphorylated by p70 S6 Kinase 1, a downstream target of mTORC1. As previously reported, GFP+/RFP+ ectopic cells resembling neurons were found in or outside the RMS (Fig. 1D and H) marked with doublecortin staining (DCX, in Fig. 1H) and in the OB of P28 Tsc1fl/wt/R26R mice (Fig. 1F, G, and I). In contrast, littermate Tsc1fl/mut/R26R mice did not show ectopic Tsc1mut cells (OB image in Fig. 1E). Consistent with Tsc1 loss in Tsc1mut mice, mTORC1 was elevated in ectopic Tsc1null cells as shown by immunostaining for phosphorylated S6 (pS6) in P28 RMS and OB sections (Fig. 1D in RMS and I in OB). Thus, Tsc1 removal from newborn neurons in Tsc1fl/mut mice results in the formation of TSC-like lesions containing ectopic Tsc1null cells with hyperactive mTORC1.

### Hif1a mRNA and HIF1α transcriptional activity are up-regulated in newborn Tsc1null neurons

Considering that mTORC1 regulates HIF1α levels and activity in several systems (14–18), we examined Hif1a mRNA level and transcriptional activity in the OB of Tsc1 mice. First, we performed endpoint RT-PCR from cDNA generated from CRE-electroporated (ipsilateral) and contralateral OB of P21 Tsc1fl/wt/R26R (black) mice (Fig. 2A). Five sets of primers were designed to amplify Hif1a transcripts in the OB containing Tsc1null neurons (Fig. 2B). To better quantify the increases, we performed quantitative RT-PCR (qRT-PCR) using the standard curve method with the most sensitive Hif1a primer set and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer for normalization. There was a 3-fold increase in the Hif1a transcript levels in the OB containing Tsc1null neurons when compared with the contralateral OB (N = 3 mice for each condition, P < 0.05, P21, Fig. 2C). This increase in Hif1a is underestimated because of the low percentage of RFP+ cells in the OB. As a control for CRE, we found that the levels of the stress regulated mRNA transcript Erol1 did not change in Tsc1null mice electroporated with or without CRE (N = 3 out of 3, P21, Fig. 2C). HIF1α forms a heterodimer with ARNT and regulates transcriptional activity of genes containing HIF response elements (HRE) within the promoter. Second, we thus examined whether HIF1α-dependent gene transcription was increased in Tsc1null neurons of the OB. We performed in vivo gene reporter assays by co-electroporating plasmids encoding CRE, a constitutively active renilla luciferase, or Photinus pyralis luciferase under the control of HRE in Tsc1null mice (Fig. 2D). The HRE-Photinus luciferase gene reporter activity was significantly increased in relation to the control Renilla luciferase in P28 OB containing Tsc1null neurons when compared with OB containing Tsc1het.
neurons (Fig. 2E, N = 6, n = 18, P < 0.001, mean = 3.1 versus 0.4). To further confirm increased HIF1α transcriptional activity, we examined the mRNA level of a well-accepted HIF1α target, vascular endothelial growth factor (VEGF) (11,28). There was a significant 3-fold increase in VEGF mRNA levels in OB containing Tsc1<sup>fl/wt</sup> neurons when compared with OB containing Tsc1<sup>fl/mut</sup> neurons (N = 6 out of 6, P < 0.05), whereas the levels of β-actin, a negative control, were unaltered (Fig. 2F). Collectively, these experiments show that Tsc1 deletion in newborn neurons is sufficient to increase HIF1α levels and induce transcriptional transactivation.

**Tsc1<sup>fl/mut</sup> neurons are partially resistant to echinomycin-induced cell death**

To assess the role of HIF1α in newborn TSC neurons, the HIF1α antagonist, echinomycin (29), was injected into CRE/GFP-electroporated Tsc1<sup>fl/wt</sup>/R26R and Tsc1<sup>fl/mut</sup>/R26R mice. Injections of echinomycin [10 μg/kg, (30)] or vehicle were performed once a day for 4 days starting at P6 or P8 (Fig. 3A). To examine whether echinomycin resulted in HIF1α activity in newborn neurons, we performed once a day for 4 days starting at P6 or P8 (Fig. 3). Nine days post-echinomycin treatment, vehicle treatment, suggesting that echinomycin decreased VEGF levels at 24 and 48 h when compared with echinomycin or vehicle. We found that echinomycin significantly decreased VEGF protein levels in the OB of wild-type mice at several time points post-treatment with echinomycin or vehicle. We found that echinomycin significantly decreased VEGF levels at 24 and 48 h when compared with echinomycin or vehicle. We found that echinomycin significantly decreased VEGF levels at 24 and 48 h when compared with vehicle treatment, suggesting that echinomycin decreased HIF1α activity (Fig. 3B). Nine days post-echinomycin treatment (i.e. P18–P21), whole brain imaging revealed a significant decrease in GFP and RFP fluorescence in the OB of Tsc1<sup>fl/wt</sup> R26R mice when compared with Tsc1<sup>fl/mut</sup>/R26R mice (P < 0.01, N = 4 each, Fig. 3C). We thus counted the number of newborn GFP<sup>+</sup> Tsc1<sup>fl</sup> or Tsc1<sup>null</sup> neurons in the granule cell layer of OB coronal sections. Echinomycin treatment resulted in a ~40 and 80% decrease in the number of newborn Tsc1<sup>fl/mut</sup> and Tsc1<sup>fl/wt</sup> granule cells when compared with vehicle-treated Tsc1<sup>fl/mut</sup> and Tsc1<sup>fl/wt</sup> mice, respectively (Fig. 3D, N = 4 versus 5 for Tsc1<sup>fl/mut</sup> and for N = 4 versus 3 for Tsc1<sup>fl/wt</sup> treated with vehicle and echinomycin, respectively, P-values in the figure). Tsc1<sup>null</sup> neurons were thus more resistant to echinomycin treatment than Tsc1<sup>fl</sup> neurons. This finding is in agreement with the increased levels and activity of HIF1α in Tsc1<sup>null</sup> neurons when compared with Tsc1<sup>fl</sup> neurons (see Fig. 2). In addition, the numbers of electroporated SVZ cells surviving echinomycin treatment were similar to vehicle-treated CRE/GFP electroporated littermate Tsc1<sup>fl/mut</sup> mice (N = 4 and 6, Fig. 3E). These results suggest that HIF1α may be a pro-survival factor in newborn neurons.

**HIF1α is essential for the survival of newborn Tsc1<sup>null</sup> and Tsc1<sup>fl/wt</sup> neurons**

To further assess the function of HIF1α on Tsc1<sup>null</sup> newborn neuron survival, we performed HIF1α loss-of-function experiments by electroporating vectors encoding a dominant negative HIF1α (dnHIF1α) or shRNA targeting HIF1α (shHIF1α). These vectors were co-electroporated with CRE- and GFP-encoding plasmids into Tsc1<sup>fl/mut</sup>/R26R mice (Figs 4A and 5A).

We first tested the effect of a dnHIF1α-encoding vector. In control condition, the dnHIF1α vector was replaced by the GFP-encoding vector to inject the same amount of plasmid in the control and the dnHIF1α conditions. HIF1α was detected in Neuro2a 24 h post-transfection with the dnHIF1α vector or the HIF1α overexpressor (Fig. 4B). Electroporation of the dnHIF1α significantly reduced the number of Tsc1<sup>null</sup> neurons in the OB when compared with control (P17, P < 0.001, N = 4 and 5 mice, respectively) without changing GFP<sup>-</sup> cell density in the SVZ (N = 4 control, N = 5 Echi, P < 0.001). These data suggest that HIF1α activity is required for the survival of newborn Tsc1<sup>null</sup> neurons.

We next performed knockdown experiments. The shHIF1α vectors were previously reported to efficiently knockdown HIF1α (12). In agreement with previous results, 48 h post-transfection of Neuro2a cells, shHIF1α eliminated the HIF1α signal induced by co-transfection with a HIF1α expressing plasmid (Fig. 5B, blot for shHIF1α plasmid 1). Experimental conditions included: (i) Two shHIF1α together or only shHIF1α plasmid 1 compared with an empty shRNA vector (shEV, control) and examination at P28 (N = 5 and N = 16 each, respectively), (ii) the shHIF1α plasmid 1 compared with either a scrambled shRNA or a shEV and examination at P19 (N = 4 shHIF1α compared with three scrambled shRNA and N = 7 shHIF1α compared with seven shEV). All the conditions gave similar results; quantification is shown for P28 with shRNA plasmid 1 versus shEV. Following electroporation of either one (plasmid 1) or two shHIF1α-encoding vectors, we found an absence of GFP fluorescence in the OB upon whole brain examination at P19 and P28 (Fig. 5C for P19.
with shRNA plasmid 1 versus shEV). Closer examination of OB coronal sections revealed a drastic reduction in the number of GFP+ shHif1a-expressing Tsc1null neurons when compared with control (P < 0.001, N = 16 each, Fig. 5D and E for shRNA plasmid 1 versus shEV at P28). Cell number was examined per section (2–3 sections per mouse), and statistical analysis was performed per mouse (N). In the SVZ of the same mice, electroporated GFP+ cells persisted along the lateral ventricle and were equal between the control and HIF1a knockdown conditions (N = 4 and 3, respectively, Fig. 5E and F). This latter result suggests that HIF1a had no effect on SVZ cell survival in agreement with the low levels of HIF1a immunostaining in the postnatal SVZ (Data not shown). Of note, electroporation of shHif1a plasmid 1 into the SVZ of Tsc1fl/wt mice led to a complete elimination of newborn neurons (N = 4 and four each, P < 0.01, Supplementary Material, Fig. S1) that is in agreement with the echinomycin data showing Hif1a requirement for Tsc1null cell survival. In considering that shRNAs can have off-target effects, we examined whether co-electroporation of a shHif1a and a HIF1a overexpressor insensitive to HIF1a knockdown would prevent the effect of shHif1a on newborn neurons. We designed a new shRNA directed to the mouse 3′ untranslated region (3′UTR) of Hif1a mRNA that lacked homology with human HIF1a (hHIF1a). The efficiency of the UTR-shHif1a was first tested in vitro using antibodies recognizing either the mouse or the human HIF1a (Fig. 5G). We found that UTR-shHif1a knocked down mouse HIF1a, but did not affect human HIF1a expression as expected. To detect baseline mouse HIF1a, Neuro2a cells were exposed to hypoxic conditions. Next, we co-electroporated the UTR-shHif1a with or without the overexpressor that encodes human HIF1a. In both cases, a GFP-encoding vector was included, but at twice the concentration in the condition without the overexpressor. We found a significant increase in the number of Tsc1null newborn neurons in the P28 OB that contained UTR-shHif1a with the hHIF1a overexpressor when compared with the condition without hHIF1a (N = 5 shHif1a and N = 4 shHif1a + HIF1a, Fig. 5H and I). The number of cells in the SVZ was similar in both conditions (same N, Fig. 5I). These data suggest that shHif1a led to the loss of newborn neurons through knockdown of endogenous HIF1a.

**HIF1a knockdown in newborn neurons, but not in neural progenitor cells, leads to neuronal loss**

To circumvent any effects of HIF1a knockdown on SVZ neural progenitor cells, we used an inducible Cre-Lox-based plasmid vector to express shHif1a (plasmid 1) at a precise developmental stage of neuroblasts. The vector, called pSico [i.e. plasmid for stable RNA interference conditional (31)], contains a U6 promoter followed by LoxP sites around a CMV promoter driving GFP, acting as a stop sequence prior to the HIF1a sequence or a non-coding sequence (Fig. 6A). Transfection of Neuro2a cells with the inducible shHif1a together with a CRE- and a mouse HIF1a-encoding plasmid led to knockdown of HIF1a levels in 48 h (Fig. 6B). Following co-electroporation of pSico and a vector encodingE12CREERT2 (and a GFP-encoding reporter vector) in Tsc1fl/wt/R26R mice, intraperitoneal tamoxifen injections are expected to allow timed shHif1a expression in pSico-containing newborn neurons. We recently reported that this experimental approach worked for another shRNA using the same inducible vector (26,27). To test tamoxifen efficiency in vivo, we examined GFP fluorescence 2 days post-injection of tamoxifen R26R-GFP mice electroporated with the inducible CRE. We found GFP+ cells as early as 2 days post-injection of tamoxifen (injected at 3 or 7 dpe, Data not shown) (26,27). Tamoxifen was injected at 10 dpe when newborn neuroblasts were integrating in the OB circuitry. It was recently reported using neonatal electroporation that newborn neurons are synaptically integrated by 2 weeks post-electroporation.
using morphometry analysis and patch clamp recordings (25–27). By 2 weeks of post-tamoxifen injection (P24), the number of shHif1a/GFP+ Tsc1null neurons was significantly reduced when compared with control (i.e. in mice electroporated with a shRNA empty vector (shEV) or shHif1a plasmid 1. (D) Confocal images of coronal P28 OB sections containing Tsc1null cells expressing shEV or shHif1a plasmid 1. (E) Bar graphs of the relative number of Tsc1null cells containing either shEV (gray) or shHif1a plasmid 1 (black) in the P28 OB and SVZ. (F) Confocal images of P28 SVZ containing Tsc1null cells expressing shEV or shHif1a plasmid 1. (G) Western blots of mouse and human HIF1α using species-specific antibodies and GAPDH under different conditions. Neuro2a cells were exposed to hypoxic conditions to increase baseline mouse HIF1α. shHif1a (UTR) results in decreased mouse HIF1α, but not human HIF1α present due to the transfection with a vector encoding human HIF1α. (H) Confocal images of coronal P28 OB sections containing Tsc1null cells expressing UTR-shHif1a or UTR-shHif1a + hHIF1α. (I) Bar graphs of the relative number of Tsc1null cells containing either UTR-shHif1a (gray) or UTR-shHif1a + hHIF1α (black) in the OB and SVZ. *P < 0.05, ***P < 0.001. NS, not significant. Scale bars: 100 μm (D and F) and 140 μm (H).

**DISCUSSION**

Here, our data show for the first time that HIF1α acts as a molecular determinant of newborn neuron survival in the context of TSC. More precisely, our findings suggest that TSC1-dependent HIF1α up-regulation gave Tsc1null neurons a survival advantage, despite their ectopic location in a novel microenvironment. Tsc1het neurons were also sensitive to decreased HIF1α levels and more sensitive to echinomycin-induced death consistent with their lower levels of HIF1α when compared with Tsc1null neurons.

We used a recently established neonatal model of TSC-like lesions. These lesions are located in the OB and along the route from the SVZ to the OB and recapitulate several aspects of the TSC lesions seen in patients (22). Indeed, TSC patients exhibit OB hamartoma or malformations (32,33). Although several models of TSC pathologies have been reported (22,34–44), our neonatal model recapitulates the discrete nature of the human lesions and allows easy access to tissue containing lesions and Tsc1null cells. The TSC-like lesions consisted of migratory heterotopias that were a collection of misplaced Tsc1null cells (neurons and glia) and ectopic clusters of Tsc1null neurons. Tsc1null neurons that reached the OB displayed hypertrophic dendritic tree and formed micronodules (22). Here, we illustrated olfactory heterotopias and misplaced enlarged neurons in the OB. OB containing Tsc1het neurons did not display such lesions.
and appeared normal. We found that the transcript levels of Hif1a were significantly increased in the OB containing Tsc1 null cells when compared with the contralateral OB. In addition, we measured the transcriptional activity of HIF1a using in vivo luciferase reporter assays and found a significant increase in HIF1a activity in the OB containing Tsc1 null cells when compared with that containing Tsc1 het cells. Finally, the transcript levels of VEGF, a HIF1a target, were also elevated in the OB containing Tsc1 null cells. These data are in agreement with published data showing increased HIF1a expression and increased protein expression of HIF1a-dependent genes (e.g. VEGF) in human TSC tissue (8,14,19,20,45–47).

To assess HIF1a function on Tsc1 null neuron development, we pharmacologically blocked HIF1a and performed loss-of-function experiments. The former experiment revealed that Tsc1 null neurons are partially resistant to inhibition with echinomycin when compared with Tsc1 het neurons. This is in agreement with the finding that Tsc1 null neurons have increased HIF1a activity when compared with Tsc1 het neurons. Knockdown of HIF1a led to complete elimination of Tsc1 het neurons consistent with the echinomycin data. Knockdown of HIF1a or expression of a dnHIF1a in Tsc1 flav mice led to a dramatic reduction in the number of newborn Tsc1 flav neurons. The number of Tsc1 flav SVZ cells was not affected suggesting that changes in SVZ neural progenitor cells did not account for the loss of newborn neurons. The effect of HIF1a on cell survival was not limited to Tsc1 flav neurons, but also applied to Tsc1 het neurons.

To avoid an effect of HIF1a knockdown on neuroblast production from neural progenitor cells, we used inducible vectors to selectively knockdown Hif1a when newborn neurons had migrated into the OB, thus bypassing the majority of SVZ neural progenitor cells. HIF1a knockdown in newborn Tsc1 flav neurons undergoing synaptic integration led to a dramatic reduction in their number in the OB. Thus, these data suggest that HIF1a is important for neuroblast survival at the time of synaptic integration. It remains to be examined whether HIF1a is also important for neuroblast survival during migration. Upon examination of the RMS at 6 days post-electroporation of shHif1a, we observed no obvious loss of neuroblasts, suggesting that HIF1a may not be required for their survival during their migration.

Collectively, we suggest that HIF1a is required for newborn Tsc1 flav neuron survival, despite their ectopic location in Tsc1 mutant brains. We propose that up-regulation of HIF1a transcriptional activity in newborn neurons promotes the growth and persistence of TSC CNS lesions.

**MATERIALS AND METHODS**

**Animals**

Research protocols were approved by the Yale University Institutional Animal Care and Use Committee. Experiments were performed on littermate Tsc1 flav/R26R and Tsc1 flav/mut/R26R mice of either gender obtained by crossing the following two lines of transgenic mice: Tsc1 flav (Jackson Laboratories) and Tsc1 flav/mut (NCI) with that we had crossed with R26R-Stop-RFP mice (R26RFP, Jackson Lab, RFP for tdTomato). These two lines of mice were generated by David J. Kwiatkowski (Brigham and Women’s Hospital, Harvard Medical School,
Cambridge, MA, USA). Mice were prescreened for successful electroporation prior to sacrificing by viewing an epifluorescence microscope or a Kodak 4000 imager.

Genotyping
Tail or toe samples were taken and were subjected to DNA isolation, PCR amplification using previously published primers (22,41,48), and ampiclons separated by standard electrophoresis methods. The floxed allele contains the LoxP sites surrounding the sequence to be excised (exon 17 and 18) upon Cre recombination (49). The mutant allele lacks the sequence flanked by LoxP sites, thus making the hamartin protein non-functional.

Vectors
The vectors pCAG-HIF1α, PBS/pU6-Hif1α RNAi plasmid 1, PBS/pU6-Hif1α RNAi plasmid 2 (Addgene, C. Cepko for all three Hif1α vectors), pCAG-GFP, pCAG-CRE and pCAG-ERT2CREERT2 were purchased from Addgene (donated by Dr C. Cepko). For rescue experiments, an shRNA directed to the mouse 3’UTR Hif1α mRNA, which lacked homology with human HIF1α, was inserted into the pSico plasmid (Addgene, Dr M. Jacks). The hairpin sequence for UTR-Hif1α shRNA is GCACAAACTCAGATAGTTTAG (sense)-GGAC AGCACAC (loop)-CTAAACTATCTGAGTTTGTGC (anti-sense) and was synthesized with 5′-gtagaattc and 3′-tcgag g 3′-flanking ends for cloning using HpaI and XhoI. For conditional shRNA expression, the Cre-Lox conditional vector p5sico was used to place the Hif1α shRNA behind the floxed-GFP cassette. The pSico plasmid was co-injected with the tamoxifen-inducible CRE expression vector pCAG-ERT2CREERT2 (Addgene, Dr C. Cepko) and a pCAG-tdTomato (noted RFP) vector that was constructed using the pCMV-tdTomato vector from Clontech. Upon inducing ERT2CREERT2-recombinase activity with tamoxifen, the enhanced green fluorescent protein cassette would recombine out and place shRNA sequence directly downstream from the U6 RNA promoter. The control vectors for shRNA contained an empty sequence.

Neuro2a cell culture, transfection and western blots
The Neuro-2a mouse neuroblastoma cell line (American type culture collection) was maintained in multi-well plates or flasks (BD Falcon) at 37°C and 5% CO2. Culture medium consisted of DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/l penicillin and 100 μg/l streptomycin. PolyJet (Signagen) was used to transfect the vectors when transfected cells or tissues were treated with 100 mM CoCl2. Transfected cells or tissues were harvested 24 and 48 h post-transfection. In some conditions (mentioned in the text) to increase baseline HIF1α levels, cells were treated with vehicle or 100 mM CoCl2. Transfected cells or tissues were lysed in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor cocktail (Sigma). Proteins were resolved on SDS PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% milk and 0.1% Tween in tris-buffered saline for 1 h and probed overnight at 4°C with primary antibodies to detect VEGF (Abcam, 1:100), mouse HIF1α (Novus, 1:1000), human HIF1α (Novus, 1:1000), tubulin (Abcam, 1:5000) and GAPDH (SCBT, 1:5000). Membranes were washed and then incubated with HRP-conjugated secondary antibodies (1 h, room temperature). Specific protein bands were detected using standard enhanced chemiluminescence and film.

Neonatal electroporation
Electroporations were performed as previously described (22,24). For postnatal electroporations, plasmids (2–3 μg/μl) were diluted in PBS containing 0.1% fast green as a tracer. 0.5–1 μl of plasmid solution were injected into the lateral ventricles of neonatal pups using a pulled glass pipette (diameter < 50 μm). Five square pulses of 50 ms duration with 950 ms intervals at 100 V were applied using a pulse ECM830 BTX generator and tweezer-type electrodes (model 520, BTX) placed on the heads of P0–P1 pups.

Luciferase assays
Mice that had experienced postnatal electroporation were anesthetized with Nembutal and decapitated. OBs were flash frozen in liquid nitrogen and placed in 1.5 ml ice cold lysis buffer and subjected to a dual luciferase assay (Promega) according to the manufacturer’s recommendations. Tissue was passed through a pre-chilled 20 gage needle attached to a 1 ml syringe 10 times. Samples were centrifuged at 10 000 g for 10 min at 4°C. The supernatant was then placed on ice for ~15 min. Twenty microliters of sample were preloaded into 100 μl of luciferase assay substrate II and briefly mixed. The luminescence was measured in a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Hundred microliters of stop and glo reagent were then added, mixed, and the luminescence from Renilla reniformis was determined.

Slice preparation and immunostaining
P28 mice were deeply anesthetized with pentobarbital (50 mg/kg). The brain was then quickly removed and placed in 4% paraformaldehyde overnight at 4°C, then washed in 1x PBS. The region of electroporation was imaged using a Kodak 4000 imager. The next day, 100-μm-thick slices were prepared using a vibratome (Leica VTS 1000). Immunostaining was performed in free-floating 100-μm-thick slices as previously described (50). Free-floating sections were blocked in PBS containing 0.1% Triton X-100, 0.1% Tween-20 and 2% BSA and incubated in primary antibodies (see below) overnight at 4°C. After several washes in PBS containing 0.1% Tween-20, slices were incubated with the appropriate secondary antibody [Alexa Fluor series at 1:1000 (Invitrogen) or cyanine series at 1:500 (Jackson Immunoresearch)] for 1 h at room temperature. Primary antibodies were rabbit anti-pS6 (1:1000; Cell Signaling; 240/244). Each staining was replicated in slices from three different mice. Z-section images were acquired on a confocal microscope (Olympus FluoView 1000) with a 20× dry objective (NA 0.75). Low-magnification images were acquired with a 10× dry objective or a dissecting scope (SZX16 with a SDF PLAP0 1× PF objective). Images were analyzed using Imaris 4.0 (Bitplane AG).
and reconstructed using ImageJ 1.39t software (Wayne Rasband, NIH) or Photoshop CS3.

Cell counts and echinomycin treatment

Cell number was determined by an observer blinded to conditions and genotypes in two to three slices per animal for several mice from multiple litters for each condition (for exact n, see text). Four micrometers of Z-sections were taken with a 10× dry objectives with a confocal microscope (Olympus Fluoview 1000) with a 1024 × 1024 resolution.

In both the OB and the SVZ of electroporated and echinomycin-treated mice, images were opened with image J, and Z-projections were generated from each fluorescent channel. The stacked image was inverted, subjected to thresholding and converted to a binary image. The image was then processed using image J binary watershedding. Blinded automated computational analysis was then performed (pixel area = 0.001–0.02 and circularity = 0.2–1.0).

Echinomycin was solubilized in ethanol prior to adding sunflower oil. Echinomycin was injected daily at 10 μg/kg.

RNA isolation and qRT-PCR

RNA isolation and qRT-PCR were performed as described by Feliciano et al. (22). Briefly, Trizol reagent and 21% chloroform were added to each electroporated OB and passed through a 22 gage 1.5 inch needle and then vortexed. Following centrifugation for 15 min at 4°C and 12 000g, the top aqueous phase was transferred to a fresh reaction tube. After adding 1.0 ml of ethanol, the sample was vortexed for 1 min and centrifuged at 8000× g for 10 min. Pellets were rinsed three times with 75% ETOH. Following centrifugation, the RNA was eluted with RNase-free deionized H2O prior to determining its concentration and purity on a spectrophotometer. The RNA isolation and qRT-PCR were performed as described by Le-Hashemite et al. (2003) Molecular genetic advances in tuberous sclerosis. Hum. Genet., 107, 97–114.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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