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The tuberous sclerosis complex

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder that results from mutations in the *TSC1* or *TSC2* genes and is associated with hamartoma formation in multiple organ systems. The neurological manifestations of TSC are particularly challenging and include infantile spasms, intractable epilepsy, cognitive disabilities, and autism. Progress over the past 15 years has demonstrated that the *TSC1* or *TSC2* encoded proteins modulate cell function via the mTOR signaling cascade and serve as keystones in regulating cell growth and proliferation. The mTOR pathway provides an intersection for an intricate network of protein cascades that respond to cellular nutrition, energy levels, and growth-factor stimulation. In the brain, *TSC1* and *TSC2* have been implicated in cell body size, dendritic arborization, axonal outgrowth and targeting, neuronal migration, cortical lamination, and spine formation. Antagonism of the mTOR pathway with rapamycin and related compounds may provide new therapeutic options for TSC patients.

Keywords

tuberous sclerosis complex (TSC); focal cortical malformations (FCMs); *TSC1* genes; *TSC2* genes; rapamycin

Focal malformations of cortical development

Focal cortical malformations (FCMs) are the most common cause of medically intractable epilepsy (resistant to antiepileptic drug polytherapy) in the pediatric patient population.¹ FCMs, including tubers in the tuberous sclerosis complex (TSC), focal cortical dysplasia (FCD) with balloon cells (BCs), and hemimegalencephaly (HMEG), are of particular interest because these developmental malformations affect restricted regions of the cerebral cortex, share certain histopathological features^{2–4} and are linked to abnormalities in the mammalian target of rapamycin (mTOR) cell signaling. Tubers, FCD, and HMEG often require neurosurgical resection to achieve adequate seizure control. Unfortunately, Class I outcomes following resection of FCMs are less often attained than for standard temporal lobectomy surgery. FCDs have been classified into subtypes I and II based on distinguishing histopathological features, such as the presence of dysmorphic neurons (DNs) and BCs in type II FCDs but not in type I.⁵ FCDs are often visualized by pre-operative brain MRI although some milder type I FCDs are not detected by routine neuroimaging. HMEG is highly associated with severe intractable neonatal seizures and infantile spasms, a devastating epilepsy syndrome in infants; a classification scheme for HMEG has not yet been formulated. Perhaps most compelling is a recent study demonstrating that of 89 neocortical resections (mean patient age ~25 years), 58

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Conflicts of Interest

The authors declare no conflicts of interest.

exhibited some type of FCM by neuropathological examination despite a normal preoperative brain MRI.⁶ Thus, FCMs may be responsible for the development and manifestation of seizures in patients with presumed nonlesional neocortical epilepsy. Clearly, FCMs are associated with significant healthcare impact for patients including cost, morbidity, and even mortality.

TSC is an autosomal dominant disorder resulting from mutations in one or two genes (*TSC1* and *TSC2*), whereas FCD and HMEG are sporadic disorders that form by unknown mechanisms (for review, see Ref. ⁴). TSC serves as an important disease model for FCMs because of the similarities in histopathology and cell signaling abnormalities. As we advance our understanding of the neurobiology of TSC, it is likely that new insights into other FCMs associated with epilepsy, cognitive, disability, and autism will follow.

Tuberous sclerosis complex: clinical presentation and pathophysiology

TSC is characterized by formation of hamartomas in multiple organ systems.⁷ The birth incidence of TSC is estimated to be approximately 1 in 6000.⁸ Although the majority of organs are susceptible, most patients exhibit dermatological, renal, and/or neurological manifestations.⁹ Dermatological abnormalities are evident in the pediatric population and include hypomelanotic macules, which are found in over 90% of TSC patients, and facial angiofibromas, present in 75% of patients.¹⁰ Renal lesions, collectively occurring in 50–80% of patients with TSC,^{11–13} include angiomyolipomas (AMLs), renal cysts, renal cell carcinoma, and oncocytomas.¹⁴ Multiple bilateral AMLs, comprising abnormally organized blood vessels, smooth muscle cells, and adipose tissue, occur in approximately 80% of individuals with TSC¹² and represent the leading cause of mortality in the TSC patient population secondary to spontaneous hemorrhage.^{7,15} Although the overall incidence of renal cell carcinoma approximates that of the general population, it occurs, on average, 25 years earlier in TSC patients.¹⁴

Neurological abnormalities including epilepsy,¹⁶ neurocognitive dysfunction,¹⁷ and pervasive developmental disorders such as autism¹⁸ are perhaps the most devastating and therapeutically challenging manifestations of TSC. The neurological features of TSC are believed to reflect structural brain abnormalities. Histopathological examination of TSC brain specimens reveals cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas (SEGAs). Cortical tubers are focal developmental malformations of the cerebral cortex exhibiting loss of normal hexalaminar structure and containing several abnormal cellular elements including DNAs, excessive numbers of astrocytes, and giant cells (GCs).^{7,19} Radiological studies demonstrated the presence of tubers *in utero* by 20 weeks gestation, suggesting that tubers form during embryonic cortical development.²⁰ In contrast, subependymal nodules are benign proliferative lesions protruding from the ventricular surface into the ventricular lumen and are believed to be asymptomatic. Subependymal nodules may undergo transformation into SEGAs, which are found in 10% of patients, and may lead to progressive hydrocephalus and death.²¹

Analysis of surgically resected and postmortem TSC-associated lesions provides pivotal clues into the neuropathogenesis of TSC. GCs found in cortical tubers are highly immunoreactive for immature neuroglial markers^{19,22} and show aberrant hyper-activation of the mTOR signaling cascade.^{23,24} For example, GCs and DNAs within tubers are highly immunoreactive for the phosphorylated isoforms of S6K, S6, 4E-BP1, and express abundant vascular endothelial growth factor (VEGF) (Fig. 1). This phosphorylation profile indicates abnormal signaling through the mTOR cascade (see below), which may be responsible for many of the neurological abnormalities found in TSC and implicates rapamycin (an inhibitor of mTOR) as a potential therapeutic agent. Single-cell microdissection of GCs and DNAs from tubers coupled with *in situ* reverse transcription to generate cDNA revealed expression of immature marker

proteins such as nestin and vimentin as well as proliferation markers PCNA and Ki-67,²⁵ suggesting that these cells may be capable of cell division. Recent expression studies of glutamate receptor subtypes revealed an abnormal distribution of GluR subunits in GCs and DN, implicating aberrant glutamate signaling in TSC-associated epilepsy.^{26,27}

Epilepsy, which usually manifests during the first year of life in TSC patients, is the most common neurological disorder in TSC, occurring in 60–90% of individuals.²⁸ A variety of seizure types have been documented, including infantile spasms, simple partial, complex partial, and generalized tonic-clonic seizures.²⁸ Although it is widely believed that tubers are the epileptogenic foci, recent data have raised some controversy.²⁸ While seizures clearly originate from radiographically identified tubers and surgical resection of tubers can alleviate seizures in patients with medically intractable epilepsy,^{29,30} some patients continue to seize following tuberc-tomy.³⁰ Furthermore, epilepsy has been shown to occur in TSC patients in the absence of cortical tubers. A recent study of three TSC patients who underwent detailed intracranial electrocorticography revealed the tubers were electrically silent while the surrounding perituberal cortex demonstrated epileptiform activity.³¹

Neurocognitive and behavioral disturbances are also prevalent in the TSC patient population, but exhibit highly variable expression and severity. Patients with TSC exhibit a bimodally distributed intelligence quotient (IQ), with 55% of patients falling within the normal range, 14% exhibiting mild to severe impairment, and 30.5% exhibiting profound mental retardation.³²

Other clinical manifestations of TSC include lymphangioleiomyomatosis (LAM), which exclusively affects the female patient population and is distinguished by abnormal proliferation of smooth muscle cells and cystic changes within the lung parenchyma.³³ Cardiac rhabdomyomas are common, affecting 50–70% of infants but are rarely symptomatic.³⁴ Retinal astrocytic hamartomas are present in approximately 50% of TSC patients³⁵ and may arise from undifferentiated glial precursors during retinal embryogenesis.³⁶ Despite the recent advent of neurogenetic testing for TSC gene mutations, diagnosis remains based on clinical criteria (Table 1). For example, definitive diagnosis of TSC can be made in a patient who exhibits two major or one major and two minor features of the diagnostic criteria.

Tuberous sclerosis complex: genetics

TSC results from inactivating mutations in either *TSC1*, located on chromosome 9q34,³⁷ or *TSC2*, located on chromosome 16p13.3.³⁸ *TSC1* encodes a 130-kDa protein TSC1/hamartin³⁷ and *TSC2* encodes a 200-kDa protein TSC2/tuberin, which contains a C-terminal GTPase activating protein domain (GAP).³⁹ The *TSC1*-encoded protein hamartin and *TSC2*-encoded protein tuberin bind to each other via their respective coiled-coil domains to form a functional heterodimer (TSC2:TSC1).⁴⁰ TSC exhibits an autosomal dominant pattern of inheritance, but the majority of cases result from sporadic germline mutations. Although *TSC1* and *TSC2* mutations are equally represented in familial TSC, mutations in the *TSC2* gene are more common in sporadic cases.⁴¹ While large deletions and missense mutations have been identified in *TSC2*, the majority of mutations in *TSC1* are small and result in expression of a truncated protein.⁴¹

The TSC2:TSC1 complex functions in a common signaling pathway (Fig. 1), thus inactivating mutations in either gene give rise to the same clinical disorder. Binding of TSC1 to TSC2 appears to stabilize intracellular TSC2 levels since uncomplexed TSC2 is subject to ubiquitin-mediated degradation.^{42,43} Missense mutations in the *TSC2* gene that disrupt its ability to bind to TSC1 have been documented in TSC patients.^{44,45} Furthermore, studies in *Drosophila* revealed that inactivating mutations in the *Drosophila* orthologs of *TSC1* and *TSC2* (*dTSC1* and *dTSC2*) give rise to indistinguishable phenotypes and suggested that the protein products

of these genes regulate a myriad of cellular processes, including cell size, cell cycle, and cellular proliferation.^{46–49} dTSC2 and dTSC1 form a protein complex, and concomitant overexpression of both TSC2 and TSC1—but not individually—results in attenuation of cell size and number.⁴⁸ Cells lacking dTSC1 or dTSC2 are cytomegalic, retain normal ploidy, and continue to inappropriately enter S phase in normally quiescent cell types, suggesting that these proteins play an essential role in facilitating exit from the cell cycle.⁴⁷ TSC1 and TSC2 are known to bind to at least 40 additional proteins,⁵⁰ and thus there are numerous potential and yet undefined effects of *TSC* gene mutations.

Upstream regulation of TSC2:TSC1

The TSC2:TSC1 heterodimer serves as a nexus for integrating the energy status of the cell with nutritional availability and extracellular growth factor signaling (Fig. 2). Both TSC2 and TSC1 are regulated by phosphorylation. TSC1 is phosphorylated at multiple sites (Thr⁴¹⁷, Ser⁵⁸⁴, and Thr¹⁰⁴⁷) by cyclin-dependent kinase 1 (CDK1)/cyclin B during the G₂/M phase of the cell cycle.⁵¹ TSC1 has been shown to localize to the centrosome and interact with the mitotic polo-like kinase (Plk1), the levels of which are negatively regulated by TSC1.⁵² TSC1 has also been shown to interact with actin-binding proteins belonging to the ezrin-radixin-moesin (ERM) family and regulate focal adhesion formation through a Rho-mediated mechanism.⁵³ Additionally, TSC1 is negatively regulated through IKK β -mediated phosphorylation on Ser487 and Ser511 and is possibly linked to cellular inflammatory responses.⁵⁴ Many upstream kinases regulate the activity of TSC2 by phosphorylation, including extracellular signaling-regulated kinase (Erk),^{55,56} Akt,^{57–60} AMP-activated kinase (AMPK),^{61–63} and glycogen synthase kinase 3 (GSK3).⁶⁴ Growth factors generally serve to inhibit TSC2 by activating the MAP kinase (Ras-Raf-MEK1/2-Erk1/2) and PI3K (PI3K-PDK1-Akt) signaling pathways.

Phosphorylation of TSC2 by Erk on Ser664 results in dissociation of the TSC2:TSC1 complex, leading to diminished inhibition of mTOR and hindering the ability of TSC2 to inhibit cell proliferation and oncogenic transformation.^{55,56} This Erk-mediated phosphorylation and inactivation of TSC2 offers an intriguing alternative to loss-of-heterozygosity (LOH) as a pathogenic mechanism underlying tumorigenesis of TSC2^{+/-} CNS lesions. Specifically, the protein product of the wild-type allele may be posttranslationally inactivated by Erk-mediated phosphorylation to produce a functionally null phenotype.⁵⁵ Interestingly, tubers and SEGAs have been shown to be highly immunoreactive for the phosphorylated isoforms of Mek1/2 and Erk1/2, suggesting abnormally hyperactive MAP kinase signaling.^{65,66} Thus, hyperactive MAP signaling in TSC may result in Erk-mediated inactivation of the wild-type allele resulting in functional LOH, producing TSC-associated lesion formation. The exact biological mechanism by which TSC2:TSC1 regulates MAP kinase activation has not been fully elucidated.

Insulin or insulin-like growth factors (IGFs) inhibit the TSC2:TSC1 complex primarily through Akt-mediated phosphorylation and inactivation of TSC2. In brief, binding of IGFs to their receptors results in recruitment and phosphorylation of the insulin receptor substrate (IRS) and subsequent activation of phosphoinositide 3-kinase (PI3K).^{67,68} Activated PI3K converts phosphatidylinositol (4,5) bi-phosphate (PIP2) into phosphatidylinositol (3,4,5) tri-phosphate (PIP3), and leads to recruitment of Akt to the plasma membrane where it is phosphorylated and activated by PDK1.⁶⁷ The PI3K-mediated conversion of PIP2 into PIP3 is inhibited by the lipid phosphatase phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which removes the D3 phosphate from PIP3 to regenerate PIP2.^{69,70} The role of the TSC2:TSC1 heterodimer in the insulin-signaling pathway was first revealed by genetic epistasis experiments in *Drosophila*. Overexpression of dTSC1 and dTSC2 suppressed the lethal phenotype of overexpressed *Drosophila* insulin receptor (*dinr*) and overexpression of dS6K, which lies downstream from dTSC2:dTSC1 (see below) on a *dTSC1*-mutant background

reversed the *dTSC1*-null phenotype.⁴⁸ Additionally, although loss of Akt, which is downstream from the insulin receptor, led to a decrease in cell size, cells lacking both Akt and dTSC1 were similar in size to those lacking dTSC1 alone, confirming that dTSC2:dTSC1 lies downstream of and antagonizes insulin-mediated Akt signaling.⁴⁹ This signaling pathway is well conserved across species.

Addition of growth factors or serum supplementation of mammalian cell lines results in phosphorylation of TSC2 in a PI3K-dependent manner. Addition of PI3K inhibitors LY294002 and wortmannin yields accelerated gel mobility of TSC2, suggesting decreased phosphorylation of TSC2 in response to PI3K inhibition.⁶⁰ This is directly mediated by Akt, which binds to and phosphorylates TSC2.^{58,60} *In vitro* kinase assays revealed that Akt is capable of phosphorylating TSC2 on seven distinct residues.⁶⁰ Mutations of two of these residues, Ser939 and Thr1462, to alanine (TSC2^{S939A/T1462A}), which renders them nonphosphorylatable, as well as expression of a dominant negative form of Akt, inhibits insulin-induced phosphorylation of S6K.⁵⁸ Exogenous expression of Akt enhances phosphorylation of TSC2 while expression of kinase-inactive Akt (Akt-KM) or treatment with a PI3K inhibitor LY294002 decreases phosphorylation of TSC2.⁵⁹ TSC1 or TSC2-depleted cells fail to attenuate downstream mTOR signaling in response to amino acid depletion, serum starvation or growth factor withdrawal.^{71–73} Additionally, exogenous expression of PTEN, an inhibitor of the PI3K-Akt signaling results in decreased phosphorylation of TSC2 and cell lines derived from PTEN-null (PTEN^{−/−}) mouse embryos exhibit constitutively phosphorylated TSC2 on Thr1462.^{58,59} Phosphorylation of TSC2 by Akt may inhibit the function of the TSC2:TSC1 complex either by decreasing the interaction between TSC2 and TSC1 or enhancing the degradation of the heterodimeric complex by the ubiquitin-proteasome pathway.^{59,60}

PTEN inhibits insulin signaling by removing the 3' phosphate of phosphatidylinositol (3,4,5) tri-phosphate (PIP3) to generate phosphatidylinositol (4,5) bi-phosphate (PIP2), thus reversing the PI3K-mediated phosphorylation of PIP2.⁷⁴ *Drosophila* mutants harboring inactivating mutations in the *Drosophila* ortholog of PTEN (dPTEN) exhibit cellular and organ hypertrophy.^{75,76} Overexpression of dPTEN results in attenuated cell size but does not rescue the cytomegalic phenotype of dTSC1-null cell,⁴⁸ while overexpression of dPTEN along with dTSC2 and dTSC1 is additive in the reduction of cell size,⁴⁷ indicating that dPTEN acts upstream of dTSC2:dTSC1. Mice harboring homozygous deletions in *PTEN* (PTEN^{−/−}) die during embryogenesis, while heterozygous mutants (PTEN^{+/-}) contain dysplastic changes in the gastrointestinal tract, prostate gland, and skin and develop cancer in multiple organ systems.⁷⁷ Human mutations in *PTEN* cause several distinct disorders, including Cowden syndrome (CS), an autosomal dominant disorder characterized by hamartomatous lesions in the skin, thyroid, and breast tissue, macrocephaly, and increased incidence of cancer.⁷⁸ Germline mutations in *PTEN* have also been reported in autistic individuals with concomitant macrocephaly.⁷⁹ Mice harboring a conditional deletion of *PTEN* in differentiated cortical and hippocampal neurons (Nse-cre; Pten^{loxP/loxP}) display abnormal social interactions as evidenced by impairments in learning to recognize a previously encountered mouse, diminished nesting, attenuated interaction with a social target, and deficits in maternal behavior.⁸⁰ Macrocephaly in Nse-cre; Pten^{loxP/loxP} mice was found to be secondary to neuronal soma hypertrophy, and cells lacking PTEN exhibited enhanced immunoreactivity for the phosphorylated isoforms of Akt, S6 (Ser235/Ser236) and TSC2 (Ser939), suggesting aberrant hyperactivation of the PI3K-Akt-mTOR pathway.⁸⁰ PTEN-deficient neurons displayed markedly abnormal characteristics, including hypertrophy of the dendritic arborizations, increased dendritic spine density, and abnormal axonal projections in the dentate gyrus of the hippocampus.⁸⁰

In addition to Akt-mediated inhibition, the TSC2:TSC1 complex is negatively regulated by other signaling pathways. For example, TSC2 has recently been shown to bind to the death-associated protein kinase (DAPK), which subsequently phosphorylates TSC2 and leads to dissociation of the TSC2:TSC1 complex.⁸¹ DAPK is a serine-threonine kinase with a diverse repertoire of functions, including regulation of apoptosis in response to a variety of death signals and promotion of autophagy.⁸² Exogenous expression of DAPK in HEK293 cells results in enhanced phosphorylation of S6K and S6 following serum starvation while siRNA-mediated knockout of DAPK attenuates phosphorylation of S6K and S6 primarily secondary to epidermal growth factor (EGF)-mediated activation of the Erk signaling.⁸² Interestingly, DAPK has also been shown to directly phosphorylate S6 on Ser235.⁸³ Additionally, the TSC2:TSC1 complex is inhibited by binding of the forkhead transcription factor FoxO1 to TSC2, leading to subsequent activation of the mTOR-signaling cascade.⁸⁴ FoxO1 belongs to a family of FOXO transcription factors that are degraded in the presence of insulin or growth factors by Akt- and GSK-mediated phosphorylation and translocate into the nucleus upon growth factor withdrawal to regulate cell cycle arrest and DNA repair.⁸⁵

The TSC2:TSC1 complex is activated by AMPK and GSK3. Fluxes in cellular energy stores signal to TSC2:TSC1 through AMPK, which activates TSC2 by phosphorylation on Thr1227 and Ser1345.⁶¹ Depletion of ATP by the glucose analog 2-deoxyglucose (2DG) results in enhanced phosphorylation of TSC2 and decreased phosphorylation of mTOR effectors, including S6K and 4E-BP1.⁶¹ TSC2 is required for inhibition of S6K in response to energy stress since energy depletion fails to decrease S6K phosphorylation in TSC2-null fibroblasts or following siRNA-mediated TSC2 knockdown.⁶¹ AMPK is activated by two conditions: elevation of AMP/ATP and subsequent phosphorylation on its activation loop (Thr173) by upstream kinases. Elevated AMP/ATP levels result in a conformational change of AMPK, rendering it a suitable substrate for upstream kinases, such as LKB1 and CaMKK.^{63,86} Interestingly, in addition to inactivating TSC2, Akt has been shown to inhibit AMPK by decreasing the cellular AMP/ATP ratio.⁶² LKB1-deficient mouse embryonic fibroblasts (LKB1^{-/-} MEFs) fail to phosphorylate and activate AMPK, resulting in aberrant downstream signaling.⁶³ Moreover, LKB1^{-/-} MEFs are similar to TSC2-deficient MEFs in their inability to attenuate downstream mTOR signaling and inhibit apoptosis during periods of energy depletion.⁸⁷ Exogenous expression of LKB1 attenuates signaling downstream from mTOR in an AMPK-dependent manner, since the addition of compound C (an inhibitor of AMPK) or a dominant negative AMPK construct ameliorates the ability of LKB1 to decrease phosphorylation of S6K (Thr389).⁸⁸ Thus, energy depletion induces LKB1-dependent activation of AMPK, which in turn serves to activate the TSC2:TSC1 complex.

Mutations in the LKB1 gene cause the autosomal dominant disorder Peutz-Jeghers syndrome (PJS), characterized by intestinal polyposis and increased incidence of cancer.⁸⁹ Like TSC-associated lesions, PJS polyps exhibit enhanced activation of the mTOR signaling pathway, as evidenced by hyperphosphorylated S6K and S6.⁸⁷ The activity and subcellular localization of LKB1 is regulated by the pseudokinase STRAD α , which relocalizes LKB1 from the nuclear to the cytoplasmic compartment and significantly augments the catalytic activity of LKB1.^{90,91} Homozygous deletions in the *STRAD* α gene have recently been shown to cause Pretzel syndrome (PS), an autosomal recessive disorder characterized by macrocephaly, intractable epilepsy, and severe psychomotor retardation.⁹² PS brain tissue, like TSC- and PJS-associated lesions, is highly immunoreactive for the phosphorylated isoform of S6.⁹² Thus, hyperactive mTOR signaling, secondary to attenuated TSC2:TSC1-mediated inhibition, may serve as the foundation for the pathogenesis of TSC, PJS, and PS.

Like energy and serum deprivation, hypoxia also inhibits mTOR signaling by a TSC2:TSC1-dependent mechanism. In response to hypoxia, TSC2^{-/-} MEFs and TSC2-depleted HEK293 and HeLa cells fail to decrease phosphorylation of S6K and S6.⁹³ Hypoxia modulates mTOR

signaling by upregulating regulated in development and DNA damage responses (Redd1) mRNA, which appears to signal to mTOR through the TSC2:TSC1 complex. Exogenous or inducible expression of Redd1 is sufficient to attenuate phosphorylation of S6K and S6 in wild-type but not TSC2-depleted HEK293 cells.⁹³ Genetic experiments in *Drosophila* indicate that Redd1-homologs inhibit dTOR upstream of the dTSC2:dTSC1 complex.⁹⁴ In fact, overexpression of the mTOR-activator Rheb, which is inactivated by TSC2:TSC1 complex, prevents Redd1 from inhibiting mTOR signaling.⁹⁵ Interestingly, Redd1-deficient cells also fail to attenuate mTOR activity secondary to energy (2DG) or glucose depletion, suggesting a broader role for Redd1 in transferring information about the overall energy status of the cell to mTOR through the TSC2:TSC1 complex.⁹⁵

Phosphorylation of TSC2 by AMPK primes the former kinase for subsequent phosphorylation and activation by GSK3, thus demonstrating a link between Wnt signaling and TSC2:TSC1.⁶⁴ Wnt signaling regulates multiple aspects of cellular physiology including proliferation, differentiation, cell growth, and development.⁹⁶ Wnts are secreted glycoproteins that bind to members of the Frizzled (FZ) family of receptors, leading to activation of disheveled (DSH) and subsequent phosphorylation and inactivation of GSK3.⁹⁶ Exogenous expression of Wnt-1 or application of purified recombinant Wnt-3a to several cell lines yields enhanced phosphorylation of S6K and 4E-BP1 in a rapamycin-dependent manner.⁶⁴ Wnt-mediated activation of mTOR was demonstrated to be dependent on GSK3-mediated phosphorylation of TSC2 on Ser1337 and Ser1341 after a priming phosphorylation of TSC2 on Ser1345 by AMPK.⁶⁴ Moreover, the effects of TSC2 activation by AMPK and GSK3 appear to be additive, since 5-aminoimidazole-4-carboxamide-1- β -ribose (AICAR)-mediated activation of AMPK results in enhanced Wnt-3 mediated phosphorylation of S6K while inhibition of AMPK results in attenuation of GSK3-mediated inhibition of mTOR signaling.⁶⁴ Thus, the TSC2:TSC1 complex integrates information provided by AMPK and GSK3 signaling.

Genomic stress also acts through the AMPK signaling pathway to enhance activation of the TSC2:TSC1 complex, thus leading to attenuation of mTOR signaling. Several factors, such as DNA damage, hypoxia, and ribonucleoside triphosphate depletion, lead to stabilization and activation of p53.⁹⁷ p53 activation results in phosphorylation of AMPK (Thr172) and leads to mTOR inhibition in a TSC2:TSC1-dependent manner.⁹⁸ The p53-mediated phosphorylation and activation of AMPK is orchestrated by two transcriptional targets of p53, Sestrin1 and Sestrin2.⁹⁹ Overexpression of Sestrin1 and Sestrin2 along with S6K1 results in decreased phosphorylation of S6K1 on Thr389 and enhances the phosphorylation of AMPK, while inhibition of AMPK using compound C or shRNA-mediated knockdown of AMPK eliminates Sestrin1- and Sestrin2-dependent attenuation of S6K1 phosphorylation.⁹⁹ In fact, Sestrins form a complex with both AMPK and TSC2:TSC1, thus enhancing the ability of AMPK to phosphorylate and activate the TSC2:TSC1 complex.⁹⁹ Interestingly, p53 regulates expression of several inhibitors of the mTOR-signaling pathway, including AMPK, PTEN, and TSC2.¹⁰⁰ However, the ability of p53 to inhibit downstream mTOR signaling appears to be largely dependent on Sestrins since Sestrin2-null cells and Sestrin1 and Sestrin2 depleted cells fail to down-regulate phosphorylation of mTOR effectors in response to genotoxic stress.¹⁰⁰

Downstream from mTOR

The TSC2:TSC1 complex is the principal cellular inhibitor of the mammalian target of rapamycin. The TSC2 protein acts as a GTPase activating protein toward Ras homolog enriched in brain (Rheb), a Ras family GTPase.^{101,102} GTP-bound Rheb activates mTOR¹⁰³ by preventing the association of mTOR with its endogenous inhibitor FKBP38.¹⁰⁴ TSC2:TSC1 inhibits mTOR activity by stimulating the conversion of active Rheb-GTP to the inactive form, Rheb-GDP.¹⁰¹ Exogenous expression of Rheb enhanced phosphorylation of mTOR effectors

S6K and 4E-BP1 in a rapamycin-dependent manner, while co-expression of Rheb with TSC2:TSC1 attenuates Rheb-mediated mTOR activation.^{101,105}

mTOR is an evolutionarily conserved 280-kDa Ser/Thr protein kinase that regulates a myriad of energy-expansive biological processes including cell growth, translation, transcription, ribosome biogenesis, autophagy, and metabolism.⁶⁷ mTOR is a central component of two complexes: TORC1, which also contains raptor and is inhibited by rapamycin¹⁰⁶ and TORC2, which contains rictor and is not sensitive to rapamycin.¹⁰⁷ TORC2 is implicated in the regulation of actin organization in yeast¹⁰⁸ and mammalian systems¹⁰⁹ and has recently been shown to activate Akt.¹¹⁰ Much more is known about the function of mTOR within the context of TORC1, which is simply referred to as “mTOR” within this manuscript.

The best-characterized effectors of mTOR are S6K1 and 4E-BP1. The catalytic activity of S6K1 is regulated by multiple phosphorylation events, including an mTOR-dependent phosphorylation on Thr371 and Thr389.¹¹¹ Phosphorylation of Thr389 correlates well with the catalytic activity of S6K1.¹¹² *Drosophila* deficient in the *Drosophila* ortholog of S6K (dS6K) display a small-body phenotype secondary to diminished cellular size.¹¹³ Overexpression of dS6K along with dTSC2 and dTSC1 suppresses the aberrant small phenotype of cells overexpressing dTSC2 and dTSC1 in isolation, suggesting that the dTSC2:dTSC1 regulate cell size by decreasing dS6K activity.⁴⁸ Mice harboring homozygous deletions in S6K1 (S6K1^{-/-}) are viable but exhibit a significant reduction in overall body size.¹¹⁴ The S6K1-dependent regulation of cellular size is mediated by ribosomal protein S6, which is phosphorylated by S6K1 and the closely related S6K2 on Ser235/Ser236.^{115,116} Mice harboring serine-to-alanine substitutions in all five phosphorylation sites (rpS6^{P-/-}) are viable but MEFs derived from rpS6^{P-/-} embryos were significantly smaller than controls (rpS6^{P+/+}).¹¹⁵ In addition to serving as an activating kinase toward S6, S6K1 establishes a negative feedback loop on mTOR signaling by negative regulation of insulin receptor substrate-1 (IRS1) function.¹¹⁷ Thus, enhanced activation of the PI3K-AKT pathway leads to activation of the mTOR cascade, which results in enhanced phosphorylation of S6K1, which subsequently leads to attenuated PI3K-AKT signaling.

mTOR also regulates translation by inhibition of 4E-BP1, which in its nonphosphorylated state binds to and inhibits the eukaryotic initiation factor 4E (eIF4E).^{118,119} eIF4E, as part of the eIF4F complex, recognizes and binds to the 7-methylguanosine cap present on the 5' end of mRNAs. 4E-BP1 undergoes consecutive phosphorylation events beginning on Thr37/Thr46 and proceeding to Ser65/Thr70, the phosphorylation of which is greatly enhanced following serum stimulation in a PI3K and rapamycin-sensitive manner.¹¹⁹ Phosphorylation of 4E-BP1 by mTOR results in release of eIF4E from 4E-BP1 and permits the initiation of cap-dependent translation. 4E-BP1 knockout mice are viable and fertile but display several metabolic abnormalities, including hypoglycemia, enhanced metabolic rate and a significant reduction in white adipose tissue, implicating this mTOR effector in regulation of metabolism and adipogenesis.¹²⁰

mTOR signaling plays a pivotal role in promoting angiogenesis. One of the principal players in mTOR-mediated angiogenesis is VEGF. mTOR regulates VEGF expression at both the transcriptional and translational level. Inactivation of TSC2:TSC1 results in enhanced mTOR signaling, which triggers increased intracellular levels of the transcription factor HIF1 α , which in turn turns on expression of VEGF.¹²¹ HIF1 α levels are tightly regulated by oxygen tension, so that during periods of oxygen abundance, oxygen results in hydroxylation of HIF1 α , subsequently leading to its degradation via pVHL-mediated ubiquitination.^{122,123} TSC2-deficient MEFs exhibit increased levels of HIF1 α and VEGF, which normalize following treatment with rapamycin.¹²¹ Reconstitution of TSC2 expression in TSC2^{-/-} MEFs is sufficient to rescue HIF1 α levels.¹²¹ Interestingly, mice heterozygous for the TSC1 gene

(TSC1^{+/-}) or the TSC2 gene (TSC2^{+/-}) display a range of vascular abnormalities, including hepatic hemangiomas and angiosarcomas.^{73,124–126} Hepatic hemangiomas from TSC1^{+/-} and TSC2^{+/-} mice are strongly immunoreactive for VEGF and TSC2, and TSC1-null fibroblasts have been shown to secrete excess VEGF *in vitro*.¹²⁷ Additionally, serum obtained from TSC1^{+/-} animals contains high levels of circulating VEGF, suggesting a potential utilization of VEGF as a biomarker for abnormal TSC2:TSC1 function.¹²⁷ Treatment of TSC1^{+/-} animals with repeated doses of rapamycin attenuates serum VEGF levels, and more importantly, results in readily apparent histological changes in tumor appearance.¹²⁷ In addition to transcriptional regulation, mTOR activation modulates translation of VEGF by inactivating 4E-BP1, which in turn leads to initiation of VEGF mRNA translation.^{128,129} mTOR-mediated initiation of angiogenesis is also regulated by inflammatory pathways that converge on TSC1.⁵⁴ TSC1 is phosphorylated on Ser487 and Ser511 by IKK β , which is one of the principal downstream effectors in the TNF α inflammatory signaling cascade.¹³⁰ Phosphorylation of TSC1 by IKK β results in dissociation of the TSC2:TSC1 complex, enhanced levels of GTP-bound Rheb, and subsequently increased activation of mTOR, ultimately resulting in increased VEGF production and accelerated angiogenesis.⁵⁴

The neurobiology of TSC2:TSC1

The function of TSC2:TSC1 in the brain is currently an active area of investigation. Clearly, both TSC1 and TSC2 play pivotal roles in several processes that are crucial for normal brain development, including regulation of somatic size, dendritogenesis, formation of dendritic spines, axon outgrowth, astrocyte proliferation, and cortical lamination. In addition, because TSC1 and TSC2 are widely expressed throughout the mature brain, these proteins likely serve important homeostatic regulatory functions in neurons during adult life.

Cre-mediated deletion of exons 17 and 18 in the mouse *TSC1* gene (Tsc1^{C/C}) in cultured mouse hippocampal neurons results in enhanced phosphorylation of S6 and increased neuronal soma size.¹³¹ Additionally, Tsc1^{C/C} neurons as well as TSC2-depleted neurons display enhanced dendritic spine length and head width and diminished dendritic spine density.¹³¹ These morphological changes appear to be regulated by phosphorylation of the actin-binding protein cofilin on Ser3, as this phosphorylated isoform is increased in TSC2-deficient neurons and, more importantly, expression of a Ser-to-Ala mutant cofilin (S3A) rescues soma size, dendritic spine length and head width. Most interestingly, neurons harboring a single deleted copy of the *TSC1* gene (Tsc1^{C/+}) exhibit morphological changes characteristic of TSC1- and TSC2-depleted neurons, suggesting that haploinsufficiency rather than LOH may contribute to the neuropathogenesis of TSC.¹³¹ Tsc1^{C/C} neurons also exhibit increased amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSC), suggesting enhanced neurotransmitter sensitivity that may contribute to the epileptogenic phenotype of patients with TSC.

The TSC2:TSC1 complex has been recently implicated in the regulation of axonogenesis.¹³² Exogenous expression of TSC2 and TSC1 in rodent hippocampal neurons inhibits axon formation, whereas shRNA-mediated knockdown of TSC2 and TSC1 results in supernumerary axons in a rapamycin-dependent manner.¹³² Moreover, mice lacking TSC1 in postmitotic neurons (Syn-Cre; Tsc1^{flox/flox}) display ectopic axonal localization throughout the developing brain.¹³² Interestingly, knockdown of TSC2:TSC1 results in increased expression of synapses of amphids defective-A (SAD-A), which has been demonstrated to be required for axonogenesis *in vivo*.¹³³ shRNA-mediated knockdown of SAD-A and SAD-B (SAD-A/B) kinases prevents the formation of supernumerous axons in TSC2-deficient hippocampal neurons.¹³² SAD-A and SAD-B kinases belong to the AMPK-related kinase family and are activated by LKB1-mediated phosphorylation.¹³⁴ Conditional knockout of LKB1 in dorsal telencephalic progenitors (Emx1^{Cre/+};LKB1^{F/F}) results in a global suppression of axon

formation *in vivo* in a SAD-A/B-dependent manner.¹³⁵ Thus, LKB1 seems to regulate axonogenesis by multiple pathways, promoting axonal differentiation by phosphorylation of SAD-A/B kinases and inhibiting axonogenesis during periods of cellular energy deficiency by way of AMPK-TSC2:TSC1 activation.

Several animal models of TSC have been generated by knockout of either *TSC1* or *TSC2* genes. The original reported TSC1-knockout mouse (*Tsc1*^{-/-}) was constructed by gene targeting in embryonic stem cells (ESCs), replacing exons 6 through 8 with an IRES-EGFP-pA sequence and a neo-expression cassette.¹³⁶ These homozygous *Tsc1*^{-/-} mice exhibit embryonic lethality, with the majority of embryos dying between embryonic day 10.5 and 11.5, although several *Tsc1*^{-/-} animals lived until E13.5. *Tsc1*^{-/-} embryos are all smaller compared to age-matched controls, and histological analysis reveals abnormal closure of the neural tube in approximately one-third of the *Tsc1*^{-/-} embryos. Additional developmental defects include aberrant appearance of the cardiac myocytes and liver hypoplasia. All mice heterozygous for the deletion in the *TSC1* allele (*Tsc1*^{+/-}) developed renal cysts by 15–18 months of age. Renal tumor formation was noted in several animals, the genetic analysis of which revealed LOH in two out of six samples. Tumor development in several other organs was also reported, including liver, tail, and uterus. There was no increase in mortality at 18 months of *Tsc1*^{+/-} animals when compared with controls, although sudden death of several animals older than 18 months was noted.¹³⁶

Another TSC1 knockout mouse was subsequently engineered by deletion of exons 17 and 18, which is virtually identical in phenotype to the original *Tsc1*^{-/-} knockout mouse with several notable exceptions.⁷³ Namely, neural tube defects were not detected and impressive vascular dilation was evident in several internal organs. Unlike the original *Tsc1*^{+/-} mice, these heterozygotes (*Tsc1*^{+/-}), exhibited enhanced incidence of mortality with a clear female predominance (45% of female *Tsc1*^{+/-} compared to 10% of male *Tsc1*^{+/-} and 10% of wild types) probably secondary to rupture of liver hemangiomas with subsequent hemorrhage. Similar to the original *Tsc1*^{+/-} animals, all *Tsc1*^{+/-} mice exhibited renal cystadenomas, the histological examination of which revealed high expression of gelsolin. MEFs developed from E10.5 *Tsc1*^{-/-} embryos fail to attenuate phosphorylation of S6K and S6 following serum starvation and do not enhance phosphorylation of AKT following serum stimulation in a rapamycin-dependent manner.⁷³

A third TSC1-knockout was recently developed by deletion of exons 6 through 8 in ES cells by substitution with a β -galactoside reporter/neomycin selection cassette, which unexpectedly produced an aberrantly spliced construct with fusion of exons 5 and 9, resulting in creation of a premature stop codon within exon 9.¹²⁵ Homozygous *Tsc1*^{-/-} mice exhibited a lethal phenotype that was similar to the original *Tsc1*^{-/-} mice, including exencephaly and myocyte anomalies, although liver hypoplasia was not noted. Interestingly, several abnormalities of the heterozygous animals proved to be background specific. For example, the authors report a 27% increase in postnatal mortality of *Tsc1*^{+/-} mice that were backcrossed onto a C57BL/6 background when compared to *Tsc1*^{+/-} mice backcrossed onto Balb/c and C3H backgrounds. Additionally, *Tsc1*^{+/-} on a C3H background developed significantly more renal lesions, both microscopic and macroscopic, when compared with those on a C57BL/6 and Balb/c backgrounds by 15–18 months. Genetic analysis revealed loss of heterozygosity in 42% of renal regions and western blot analysis showed enhanced phosphorylation of mTOR and S6 in all lesions examined when compared to adjacent normal kidney tissue, consistent with hyperactivation of mTOR. Thus, disparate genetic modifiers may contribute to the phenotypic expression of TSC.

Several conditional knockout animals have also been generated. An astrocyte-specific TSC1 conditional knockout mouse was designed by interbreeding mice harboring two LoxP sites

flanking exons 17 and 18 (*Tsc1^{c/c}*) with transgenic mice that express glial fibrillary acidic protein (GFAP)-driven Cre recombinase (GFP-Cre) to produce *Tsc1^{c/c}; GFP-Cre* mice.¹³⁷ By 2 months of age, these mice exhibit spontaneous EEG-confirmed seizures and 50% of *Tsc1^{c/c}; GFP-Cre* mice die by 3 months of age. Histological analysis of cortical and hippocampal tissue revealed enhanced astrocyte proliferation, as evidenced by increased immunostaining for GFAP and PCNA, and aberrant organization of the hippocampus, owing primarily to abnormal positioning of pyramidal neurons in the dentate hilar region. Tubers, however, were not identified in this mouse model. Further analysis demonstrated reduced expression of astrocytic glutamate transporters, GLT-1 and GLAST, and decreased density of glutamate transport currents.¹³⁸ This may disturb glutamate homeostasis and contribute to the epileptogenic phenotype characteristic of this mouse models and patients with TSC.

Recently, a neuronal-specific TSC1 knockout mouse was engineered by crossing *Tsc1^{c/c}* with mice expressing Cre recombinase under the Synapsin1 promoter (*Tsc1^{c/c}; Syn1-Cre*).¹³⁹ Histological analysis of *Tsc1^{c/c}; Syn1-Cre* brains revealed normal hexalaminar cortical architecture absence of tubers. However, electrophysiological recordings from nontuberous cortical tissue slices revealed bicuculline-induced α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-mediated long-duration polyspike responses and periodical epileptiform discharge. A recently reported conditional knockout mouse in which *TSC1* was deleted from a subset of postnatal forebrain neurons was constructed by crossing *Tsc1^{c/c}* with a transgenic mouse expressing Cre recombinase under a α CAMPKII promoter.¹⁴⁰ A small percentage of the *Tsc1^{c/c}; α CAMPKII-Cre* mice that survived past the first postnatal week exhibited macrocephaly secondary to neuronal hypertrophy and astrogliosis. Seizures were not reported in this strain.

Mutations in the *TSC2* gene have also been utilized to model TSC. The Eker rat has a naturally occurring inactivating germline mutation in the *TSC2* gene (*Tsc2^{+/-}*)^{124,141} resulting from retro-transposition of a rat intracisternal-A particle.¹⁴² The functionally heterozygous Eker rats spontaneously develop myriad tumors, most notably renal cell carcinomas¹⁴³ and uterine and pituitary tumors.¹⁴⁴ The majority of nonbrain lesions found in the Eker rat model exhibit LOH.¹⁴⁵ Although no neurological abnormalities have been documented, including spontaneous seizures, several groups documented neurohistopathological findings that approximate those found in human brain samples from patients with TSC. One group reported the occurrence of subependymal hamartomas and subcortical hamartomas in Eker rats between the ages of 18 and 24 months that were mainly composed of GFAP immunoreactive glia, although no classical cortical tubers were noted.¹⁴⁶ Only one study to date of the Eker model reported the finding of a cortical tuber (in one rat only) that closely resembles human tubers in TSC patients.¹⁴⁷ Immunohistological analysis of renal tumors in Eker rats revealed abnormally enhanced phosphorylation of S6 and 4E-BP1, lending credence to the notion that TSC-specific lesions exhibit hyperactivated mTOR signaling.¹⁴⁸ An interesting recent study of Eker rats suggests that tuber formation may not be necessary for manifestation of TSC-associated cognitive dysfunction and epilepsy since Eker rats were found to exhibit increased episodic-like memory and augmented responses to pharmacologically induced plasticity.¹⁴⁹

Since most TSC-associated lesions are postulated to result from either LOH or a “second hit” event, some investigators have employed various methods to enhance the probability of rate-limiting mutagenesis to study the progression of TSC-associated neuropathology. For example, one group irradiated Eker rats at 3 days of age and analyzed their brain tissue at 3 months of age.¹⁵⁰ Irradiated Eker brains contained NeuN/NF immunoreactive large DNPs within layers II–VI of the cortical plate, giant GFAP-positive astrocytes, and subependymal and subcortical hamartomas composed primarily of glial cells. Others have utilized *in utero* exposure to carcinogens, such as hydroquinone (HQ), as well as the natural aging process.¹⁵¹ Although intraperitoneal injections of HQ did not result in tuber formation or any measurable histological

anomalies, analysis of aged (18–24 months) Eker rats demonstrated the presence of NeuN positive cytomegalic neurons and GFAP/vimentin/nestin immunoreactive GCs.

The earliest reported *TSC2* knockout mouse was engineered by deletion of exons 2 through 5 by homologous recombination in ES cells, with subsequent chimeras bred onto a C57BL/6J background.¹⁵² The *Tsc2*^{-/-} phenotype was embryonic lethal around E10.5, with exencephaly noted in 50% of embryos. Some embryos exhibited abnormally thickened myocardia. All heterozygous animals developed renal cell carcinoma by 10 months of age, and 80% of *Tsc2*^{+/-} had hepatic hemangiomas by 1.5 years of age. The second *TSC2*-null mouse was constructed by insertion of a neomycin resistance cassette into exon 2 of the *TSC2* gene.¹²⁶ Three different strains of transgenic mice were produced by backcrossing onto C57BL/6, BALB/c, and Black Swiss backgrounds. Homozygous *Tsc2*^{-/-} exhibited an embryonic lethal phenotype—no viable embryos were observed after E12.5—and had pronounced developmental delay, including exencephaly, hepatic hypoplasia, absence of the diaphragm, and severe vasodilatation. Embryos exhibited pallor, and the cause of death was attributed to liver hypoplasia with secondary anemia. Heterozygous animals (*Tsc2*^{+/-}) developed normally but incurred a substantial burden of cyst and tumor development in several organs, including kidneys, liver, lungs, and extremities. Renal cysts and adenomas, occurring in all mice by 15 months of age, were confined to the renal cortex, and the intercalated cell was defined as the cell of origin by virtue of intense staining for gelsolin. Progression to overt carcinoma was noted in fewer than 10% of cases. Half of *Tsc2*^{+/-} animals developed liver hemangiomas by 15 months of age. Some strain-specific differences were noted, most notably with respect to renal cystadenoma size and frequency of angiosarcomas. LOH was observed in 30% of the examined cysts and tumors. A population of nestin-positive neuroepithelial cells that were morphologically similar to GCs in human tubers was derived from the *Tsc2* null mice by growth factor stimulation.²⁴ These cells exhibited high levels of S6 phosphorylation and expression profiles of a variety of candidate genes including neurotransmitter receptors approximated human GCs.

To mimic the genotype of TSC patients, a novel conditional knockout mouse was recently created that harbors a heterozygous deletion of *TSC2* in all cells and a homozygous deletion of *TSC2* in radial glia cells (RGCs) beginning at E12 in the hippocampus and E13.5–E14.0 in the cortex (*Tsc2*^{flox/ko}; hGFAP-Cre).¹⁵³ This mouse model phenocopies many aspects of the neurological pathology associated with TSC, including macrocephaly, cellular cytomegaly, lamination defects, astrogliosis, hypomyelination, and hyperphosphorylated S6.¹⁵³ Additionally, the mutant brains exhibit abnormal distribution of the layer II–IV marker Cux1 and a reduction in the layer VI-specific FoxP2-positive cells, which is accompanied by an increased population of Trb2-positive progenitor cells.¹⁵³ These results indicate that loss of *TSC2* in RGCs results in expansion of progenitor cells that give rise to later-born neurons designated for superficial layers of the cortical plate at the expense of earlier-born neurons, which are specified for deeper layers. Additionally, this mouse model lends support to the LOH hypothesis that requires a second hit to occur prior to development of TSC-associated lesions.

Recently, Ehniger and colleagues described several hippocampal-dependent learning deficits in *TSC2*^{+/-} mice.¹⁴⁰ Specifically, *TSC2*^{+/-} animals exhibited spatial learning deficits in the Morris water maze, working memory deficits in the win-shift version of the eight-arm radial maze, and abnormal context discrimination in a context-conditioning paradigm. These behavioral changes correlated with extracellular field recordings in hippocampal slices that revealed decreased threshold for late-long term potentiation induction. Most notably, rapamycin treatment prior to induction of hippocampal-dependent learning resulted in substantial reversal of behavioral deficits. Furthermore, the threshold for L-LTP induction of *TSC2*^{+/-} hippocampal slices perfused with rapamycin approximated those of wild-type controls. Thus, inactivation of *TSC2* may result in abnormal learning and memory, contributing

to the cognitive deficits associated with TSC, which may be ameliorated by rapamycin treatment.

TSC as a model disease

The histopathological similarities between tubers and several other types of nonsyndromic focal cortical malformations have provided new insights into the molecular pathogenesis of FCD and HMEG. Unifying features of these disorders are the presence of disorganized cortical lamination, cytomegalic cells, and intractable epilepsy. For example, both FCD type IIB and HMEG exhibit near complete loss of lamination and both contain BCs and cytomegalic neurons. Recent studies have demonstrated that BCs in FCD and HMEG exhibit robust phosphorylation of S6 protein in a pattern similar to GCs in cortical tubers.^{23,154–156} In fact, these groups have suggested that phosphorylation of S6 in FCD and HMEG is consistent with the hypothesis that these focal malformations may form as a result of aberrant mTOR signaling. Indeed, we have described TSC, FCD, and HMEG as “TORopathies” to reflect the abnormal mTOR activation.

However, while mTOR signaling seems to be hyperactive in FCD and HMEG, several challenges remain. For example, while TSC results from identified gene mutations, no genetic association for either FCD or HMEG has been identified. In fact, screening the *TSC1* and *TSC2* genes in FCD and HMEG has not revealed mutations. Thus, if FCD and HMEG result from single gene defects, it is reasonable to propose that the causative gene, at least in some manner, affects mTOR signaling. Since virtually all identified genetic cortical malformation syndromes result from loss of function mutations, it seems logical that gene mutations in FCD and HMEG that lead to enhanced mTOR signaling must occur within negative modulators of mTOR function. Another distinction is that although S6 phosphorylation reflects hyperactive mTOR activity and potentially a unifying molecular event, the histopathology and transcriptional profiles of tubers versus FCD, BCs in FCD versus HMEG, or BCs versus GCs in TSC are not identical. There are well-characterized differences in mRNA and protein expression that distinguish these lesions and cell types. Thus, a comprehensive analysis of genetic, genomic (e.g., SNPs), and transcriptional similarities and differences between TSC, FCD, and HMEG will likely yield new insights.

Rapamycin and clinical trials

In light of the role that the TSC1 and TSC2 proteins play in regulation of mTOR kinase activity, a logical progression is to consider the widely available medication rapamycin in clinical trials for TSC. Rapamycin is a macrolide antibiotic that was identified in the 1970s as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from Easter Island. Rapamycin was initially developed as an antifungal agent but was subsequently shown to have important regulatory effects on cell growth, proliferation, and inflammation via its inhibitory action on mTOR. Rapamycin has been used as an immunomodulatory agent following organ transplantation and has a moderate-risk side effect profile.

The first use of rapamycin in a clinical setting was a case report of four TSC patients with SEGAs and one with pilocytic astrocytoma.¹⁵⁷ All were treated with oral rapamycin (serum levels 5–15 ng/mL) from 2.5 to 20 months and all showed some degree of tumor shrinkage by serial neuroimaging. One patient exhibited tumor recurrence when rapamycin was discontinued, but retreatment led again to tumor reduction. This report demonstrated that rapamycin could be used to treat SEGAs and possibly alleviate the need for resective surgery.

The first formal clinical trial of rapamycin examined a cohort of 25 patients with TSC or sporadic lymphangioleiomyomatosis (LAM) over a 24-month period.¹⁵⁸ Rapamycin was administered for 12 months with a 12-month follow-up period. Outcome measures included

progression of LAM by pulmonary function testing (PFT) and chest computed tomography, change in volume of renal AML and neuroimaging to evaluate cortical tubers. The results were promising in the 18 patients who completed the trial and demonstrated a clear reduction in AML volume and modest improvement in PFT following 12 months of therapy that persisted in some patients at 24 months. There was no change in tuber volume. For some patients, however, renal AML size increased in the 12-month period without rapamycin treatment, echoing previous reports in both preclinical and clinical case series. An accompanying letter¹⁵⁹ reported interim findings at 12 months for 13 patients with TSC-associated LAM or sporadic LAM following rapamycin therapy in a UK-based initiative. All patients exhibited a reduction in AML volume (mean 26%), but none exhibited clear improvement in lung function by PFT. Assessment of memory and executive function in this cohort did not reveal a decrease in cognitive function following rapamycin therapy. In both trials, adverse effects were minimal and included aphthous oral ulcers, hyperlipidemia, peripheral edema, and increase rate of infection.

Both preclinical and clinical trial data suggest that rapamycin may provide a possible therapy for TSC. If so, this would represent a new frontier for therapeutics based on discoveries relating to functional cell cascades. However, several important questions remain to be answered before widespread rapamycin therapy is instituted. First, when should rapamycin therapy be initiated? Should all TSC patients be treated with rapamycin as a prophylaxis or should rapamycin be started for specific symptoms (e.g., dyspnea, seizures, or hematuria) or disease features (e.g., confirmed LAM, tubers, SEGAs, or AMLs)? A benefit of rapamycin in human epilepsy, cognitive disability, or autism has not been shown, yet many clinicians are eager to perform case-control trials. Should rapamycin be initiated if tubers are detected by MRI in the newborn period or even fetal life in a selected group of patients? A particularly devastating problem in TSC is the high incidence of infantile spasms (IS). There is clinical evidence that IS in TSC are linked to subsequent intractable seizures and cognitive disabilities and thus, early rapamycin treatment could be an effective prevention of both. Perhaps more problematic than therapy initiation is when, if ever, to stop rapamycin treatment? Current evidence suggests that the benefits of rapamycin in a preclinical or clinical setting may only be realized during active therapy and that any gains may be lost if the medication is discontinued. Furthermore, rapamycin has side effects that may prevent long-term treatment in some patients.

Summary/future directions

The identification of the *TSC1* and *TSC2* genes provides a unique example of how translational efforts spanning gene cloning in human patients, model generation in *Drosophila*, and extensive *in vitro* and *in vivo* investigation of mTOR signaling have culminated in a new and exciting potential therapy for TSC. Although much has been accomplished, a daunting amount of future work remains. Many fundamental questions regarding disease variability and heterogeneity, genotype-phenotype correlation, and organ-specific pathology are still unanswered.

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References

1. Krsek P, et al. Different features of histopathological subtypes of pediatric focal cortical dysplasia. *Ann Neurol* 2008;63:758–769. [PubMed: 18571798]

2. Mischel PS, et al. Cerebral cortical dysplasia associated with pediatric epilepsy. Review of neuropathologic features and proposal for a grading system. *J Neuropathol Exp Neurol* 1995;54:137–153. [PubMed: 7876884]
3. Andermann F. Cortical dysplasias and epilepsy: a review of the architectonic, clinical, and seizure patterns. *Adv Neurol* 2000;84:479–496. [PubMed: 11091890]
4. Sarnat HB, Flores-Sarnat L. A new classification of malformations of the nervous system: an integration of morphological and molecular genetic criteria as patterns of genetic expression. *Eur J Paediatr Neurol* 2001;5:57–64. [PubMed: 11589314]
5. Palmini A, et al. Terminology and classification of the cortical dysplasias. *Neurology* 2004;62:S2–S8. [PubMed: 15037671]
6. Lee SK, et al. Surgical outcome and prognostic factors of cryptogenic neocortical epilepsy. *Ann Neurol* 2005;58:525–532. [PubMed: 16037972]
7. Crino PB, et al. The tuberous sclerosis complex. *N Engl J Med* 2006;355:1345–1356. [PubMed: 17005952]
8. Osborne JP, et al. Epidemiology of tuberous sclerosis. *Ann NY Acad Sci* 1991;615:125–127. [PubMed: 2039137]
9. Curatolo P, et al. Tuberous sclerosis. *Lancet* 2008;372:657–668. [PubMed: 18722871]
10. Jozwiak S, et al. Usefulness of diagnostic criteria of tuberous sclerosis complex in pediatric patients. *J Child Neurol* 2000;15:652–659. [PubMed: 11063078]
11. Cook JA, et al. A cross sectional study of renal involvement in tuberous sclerosis. *J Med Genet* 1996;33:480–484. [PubMed: 8782048]
12. Rakowski SK, et al. Renal manifestations of tuberous sclerosis complex: Incidence, prognosis, and predictive factors. *Kidney Int* 2006;70:1777–1782. [PubMed: 17003820]
13. O'Callaghan FJ, et al. An epidemiological study of renal pathology in tuberous sclerosis complex. *BJU Int* 2004;94:853–857. [PubMed: 15476522]
14. Henske EP. Tuberous sclerosis and the kidney: from mesenchyme to epithelium, and beyond. *Pediatr Nephrol* 2005;20:854–857. [PubMed: 15856327]
15. Shepherd CW, et al. Causes of death in patients with tuberous sclerosis. *Mayo Clin Proc* 1991;66:792–796. [PubMed: 1861550]
16. Thiele EA. Managing epilepsy in tuberous sclerosis complex. *J Child Neurol* 2004;19:680–686. [PubMed: 15563014]
17. Prather P, de Vries PJ. Behavioral and cognitive aspects of tuberous sclerosis complex. *J Child Neurol* 2004;19:666–674. [PubMed: 15563012]
18. Smalley SL. Autism and tuberous sclerosis. *J Autism Dev Disord* 1998;28:407–414. [PubMed: 9813776]
19. Crino PB, et al. Embryonic neuronal markers in tuberous sclerosis: single-cell molecular pathology. *Proc Natl Acad Sci* 1996;93:14152–14157. [PubMed: 8943076]
20. Park SH, et al. Tuberous sclerosis in a 20-week gestation fetus: immunohistochemical study. *Acta Neuropathol* 1997;94:180–186. [PubMed: 9255394]
21. Goh S, et al. Subependymal giant cell tumors in tuberous sclerosis complex. *Neurology* 2004;63:1457–1461. [PubMed: 15505165]
22. Mizuguchi M, et al. Doublecortin immunoreactivity in giant cells of tuberous sclerosis and focal cortical dysplasia. *Acta Neuropathol* 2002;104:418–424. [PubMed: 12200630]
23. Baybis M, et al. mTOR cascade activation distinguishes tubers from focal cortical dysplasia. *Ann Neurol* 2004;56:478–487. [PubMed: 15455405]
24. Onda H, et al. Tsc2 null murine neuroepithelial cells are a model for human tuber giant cells, and show activation of an mTOR pathway. *Mol Cell Neurosci* 2002;21:561–574. [PubMed: 12504590]
25. Lee A, et al. Markers of cellular proliferation are expressed in cortical tubers. *Ann Neurol* 2003;53:668–673. [PubMed: 12731003]
26. Talos DM, et al. Cell-specific alterations of glutamate receptor expression in tuberous sclerosis complex cortical tubers. *Ann Neurol* 2008;63:454–465. [PubMed: 18350576]

27. Boer K, et al. Cellular localization of metabotropic glutamate receptors in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex. *Neuroscience* 2008;156:203–215. [PubMed: 18706978]
28. Holmes GL, Stafstrom CE. Tuberous sclerosis complex and epilepsy: recent developments and future challenges. *Epilepsia* 2007;48:617–630. [PubMed: 17386056]
29. Koh S, et al. Epilepsy surgery in children with tuberous sclerosis complex: presurgical evaluation and outcome. *Epilepsia* 2000;41:1206–1213. [PubMed: 10999561]
30. Jansen FE, et al. Epilepsy surgery in tuberous sclerosis: a systematic review. *Epilepsia* 2007;48:1477–1484. [PubMed: 17484753]
31. Philippe Major SR, Simon MV, Cheng ML, et al. Are cortical tubers epileptogenic? Evidence from electrocorticography. *Epilepsia* 2009;50:147–154. [PubMed: 19125835]
32. Joinson C, et al. Learning disability and epilepsy in an epidemiological sample of individuals with tuberous sclerosis complex. *Psychol Med* 2003;33:335–344. [PubMed: 12622312]
33. Ryu JH, et al. The NHLBI lymphangiomyomatosis registry: characteristics of 230 patients at enrollment. *Am J Respir Crit Care Med* 2006;173:105–111. [PubMed: 16210669]
34. Smythe JF, et al. Natural history of cardiac rhabdomyoma in infancy and childhood. *Am J Cardiol* 1990;66:1247–1249. [PubMed: 2239731]
35. Mennel S, et al. Current treatment modalities for exudative retinal hamartomas secondary to tuberous sclerosis: review of the literature. *Acta Ophthalmol Scand* 2007;85:127–132. [PubMed: 17305725]
36. Gunduz K, et al. Invasive giant cell astrocytoma of the retina in a patient with tuberous sclerosis. *Ophthalmology* 1999;106:639–642. [PubMed: 10080228]
37. van Slechtenhorst M, et al. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 1997;277:805–808. [PubMed: 9242607]
38. Kandt RS, et al. Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease. *Nat Genet* 1992;2:37–41. [PubMed: 1303246]
39. Maheshwar MM, et al. The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis. *Hum Mol Genet* 1997;6:1991–1996. [PubMed: 9302281]
40. van Slechtenhorst M, et al. Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum Mol Genet* 1998;7:1053–1057. [PubMed: 9580671]
41. Jones AC, et al. Molecular genetic and phenotypic analysis reveals differences between TSC1 and TSC2 associated familial and sporadic tuberous sclerosis. *Hum Mol Genet* 1997;6:2155–2161. [PubMed: 9328481]
42. Benvenuto G, et al. The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. *Oncogene* 2000;19:6306–6316. [PubMed: 11175345]
43. Chong-Kopera H, et al. TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. *J Biol Chem* 2006;281:8313–8316. [PubMed: 16464865]
44. Jones AC, et al. Comprehensive mutation analysis of TSC1 and TSC2 and phenotypic correlations in 150 families with tuberous sclerosis. *Am J Hum Genet* 1999;64:1305–1315. [PubMed: 10205261]
45. Nellist M, et al. TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex. *Hum Mol Genet* 2001;10:2889–2898. [PubMed: 11741832]
46. Ito N, Rubin GM. Gigas, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle. *Cell* 1999;96:529–539. [PubMed: 10052455]
47. Tapon N, et al. The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 2001;105:345–355. [PubMed: 11348591]
48. Potter CJ, et al. *Drosophila* Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* 2001;105:357–368. [PubMed: 11348592]
49. Gao X, Pan D. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev* 2001;15:1383–1392. [PubMed: 11390358]
50. Rosner M, et al. The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners. *Mutat Res* 2008;658:234–246. [PubMed: 18291711]

51. Astrinidis A, et al. Cell cycle-regulated phosphorylation of hamartin, the product of the tuberous sclerosis complex 1 gene, by cyclin-dependent kinase 1/cyclin B. *J Biol Chem* 2003;278:51372–51379. [PubMed: 14551205]
52. Astrinidis A, et al. Hamartin, the tuberous sclerosis complex 1 gene product, interacts with polo-like kinase 1 in a phosphorylation-dependent manner. *Hum Mol Genet* 2006;15:287–297. [PubMed: 16339216]
53. Lamb RF, et al. The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. *Nat Cell Biol* 2000;2:281–287. [PubMed: 10806479]
54. Lee DF, et al. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* 2007;130:440–455. [PubMed: 17693255]
55. Ma L, et al. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* 2005;121:179–193. [PubMed: 15851026]
56. Ma L, et al. Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. *Cancer Res* 2007;67:7106–7112. [PubMed: 17671177]
57. Potter CJ, et al. Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol* 2002;4:658–665. [PubMed: 12172554]
58. Manning BD, et al. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/Akt pathway. *Mol Cell* 2002;10:151–162. [PubMed: 12150915]
59. Inoki K, et al. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002;4:648–657. [PubMed: 12172553]
60. Dan HC, et al. Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberlin. *J Biol Chem* 2002;277:35364–35370. [PubMed: 12167664]
61. Inoki K, et al. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577–590. [PubMed: 14651849]
62. Hahn-Windgassen A, et al. Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem* 2005;280:32081–32089. [PubMed: 16027121]
63. Shaw RJ, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci* 2004;101:3329–3335. [PubMed: 14985505]
64. Inoki K, et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 2006;126:955–968. [PubMed: 16959574]
65. Maldonado M, et al. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. *Neurobiol Dis* 2003;14:279–290. [PubMed: 14572449]
66. Han S, et al. Phosphorylation of tuberlin as a novel mechanism for somatic inactivation of the tuberous sclerosis complex proteins in brain lesions. *Cancer Res* 2004;64:812–816. [PubMed: 14871804]
67. Wullschlegel S, et al. TOR signaling in growth and metabolism. *Cell* 2006;124:471–484. [PubMed: 16469695]
68. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;129:1261–1274. [PubMed: 17604717]
69. Myers MP, et al. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc Natl Acad Sci* 1998;95:13513–13518. [PubMed: 9811831]
70. Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene* 2008;27:5477–5485. [PubMed: 18794882]
71. Gao X, et al. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol* 2002;4:699–704. [PubMed: 12172555]
72. Jaeschke A, et al. Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositolide-3-OH kinase is mTOR independent. *J Cell Biol* 2002;159:217–224. [PubMed: 12403809]

73. Kwiatkowski DJ, et al. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet* 2002;11:525–534. [PubMed: 11875047]
74. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998;273:13375–13378. [PubMed: 9593664]
75. Gao X, et al. *Drosophila* PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Dev Biol* 2000;221:404–418. [PubMed: 10790335]
76. Goberdhan DC, et al. *Drosophila* tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev* 1999;13:3244–3258. [PubMed: 10617573]
77. Di Cristofano A, et al. Pten is essential for embryonic development and tumour suppression. *Nat Genet* 1998;19:348–355. [PubMed: 9697695]
78. Waite KA, Eng C. Protean PTEN: form and function. *Am J Hum Genet* 2002;70:829–844. [PubMed: 11875759]
79. Butler MG, et al. Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. *J Med Genet* 2005;42:318–321. [PubMed: 15805158]
80. Kwon CH, et al. Pten regulates neuronal arborization and social interaction in mice. *Neuron* 2006;50:377–388. [PubMed: 16675393]
81. Stevens C, et al. Peptide combinatorial libraries identify TSC2 as a death-associated protein kinase (DAPK) death domain-binding protein and reveal a stimulatory role for DAPK in mTORC1 signaling. *J Biol Chem* 2009;284:334–344. [PubMed: 18974095]
82. Bialik S, Kimchi A. The death-associated protein kinases: structure, function, and beyond. *Annu Rev Biochem* 2006;75:189–210. [PubMed: 16756490]
83. Schumacher AM, et al. Death-associated protein kinase phosphorylates mammalian ribosomal protein S6 and reduces protein synthesis. *Biochemistry (Mosc)* 2006;45:13614–13621.
84. Cao Y, et al. Interaction of FoxO1 and TSC2 induces insulin resistance through activation of the mammalian target of rapamycin/p70 S6K pathway. *J Biol Chem* 2006;281:40242–40251. [PubMed: 17077083]
85. Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005;24:7410–7425. [PubMed: 16288288]
86. Hardie DG, et al. Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* 2003;546:113–120. [PubMed: 12829246]
87. Shaw RJ, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 2004;6:91–99. [PubMed: 15261145]
88. Corradetti MN, et al. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* 2004;18:1533–1538. [PubMed: 15231735]
89. Hemminki A, et al. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* 1998;391:184–187. [PubMed: 9428765]
90. Dorfman J, Macara IG. STRADalpha regulates LKB1 localization by blocking access to importin-alpha, and by association with Crm1 and exportin-7. *Mol Biol Cell* 2008;19:1614–1626. [PubMed: 18256292]
91. Hawley SA, et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* 2003;2:28. [PubMed: 14511394]
92. Puffenberger EG, et al. Polyhydramnios, megalencephaly and symptomatic epilepsy caused by a homozygous 7-kilobase deletion in LYK5. *Brain* 2007;130:1929–1941. [PubMed: 17522105]
93. Brugarolas J, et al. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* 2004;18:2893–2904. [PubMed: 15545625]
94. Reiling JH, Hafen E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*. *Genes Dev* 2004;18:2879–2892. [PubMed: 15545626]

95. Sofer A, et al. Regulation of mTOR and cell growth in response to energy stress by REDD1. *Mol Cell Biol* 2005;25:5834–5845. [PubMed: 15988001]
96. Moon RT, et al. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004;5:691–701. [PubMed: 15372092]
97. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–331. [PubMed: 9039259]
98. Feng Z, et al. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci* 2005;102:8204–8209. [PubMed: 15928081]
99. Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* 2008;134:451–460. [PubMed: 18692468]
100. Feng Z, et al. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer Res* 2007;67:3043–3053. [PubMed: 17409411]
101. Inoki K, et al. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* 2003;17:1829–1834. [PubMed: 12869586]
102. Garami A, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* 2003;11:1457–1466. [PubMed: 12820960]
103. Long X, et al. Rheb binds and regulates the mTOR kinase. *Curr Biol* 2005;15:702–713. [PubMed: 15854902]
104. Bai X, et al. Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. *Science* 2007;318:977–980. [PubMed: 17991864]
105. Castro AF, et al. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J Biol Chem* 2003;278:32493–32496. [PubMed: 12842888]
106. Kim DH, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 2002;110:163–175. [PubMed: 12150925]
107. Sarbassov DD, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004;14:1296–1313. [PubMed: 15268862]
108. Fadri M, et al. The pleckstrin homology domain proteins Slm1 and Slm2 are required for actin cytoskeleton organization in yeast and bind phosphatidylinositol-4,5-bisphosphate and TORC2. *Mol Biol Cell* 2005;16:1883–1900. [PubMed: 15689497]
109. Jacinto E, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 2004;6:1122–1128. [PubMed: 15467718]
110. Sarbassov DD, et al. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307:1098–1101. [PubMed: 15718470]
111. Dufner A, Thomas G. Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res* 1999;253:100–109. [PubMed: 10579915]
112. Proud CG. Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J* 2007;403:217–234. [PubMed: 17376031]
113. Montagne J, et al. *Drosophila* S6 kinase: a regulator of cell size. *Science* 1999;285:2126–2129. [PubMed: 10497130]
114. Shima H, et al. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J* 1998;17:6649–6659. [PubMed: 9822608]
115. Ruvinsky I, et al. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* 2005;19:2199–2211. [PubMed: 16166381]
116. Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci* 2006;31:342–348. [PubMed: 16679021]
117. Um SH, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 2004;431:200–205. [PubMed: 15306821]
118. Gingras AC, et al. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev* 1998;12:502–513. [PubMed: 9472019]

119. Gingras AC, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev* 2001;15:2852–2864. [PubMed: 11691836]
120. Tsukiyama-Kohara K, et al. Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. *Nat Med* 2001;7:1128–1132. [PubMed: 11590436]
121. Brugarolas JB, et al. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell* 2003;4:147–158. [PubMed: 12957289]
122. Ivan M, et al. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 2001;292:464–468. [PubMed: 11292862]
123. Jaakkola P, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 2001;292:468–472. [PubMed: 11292861]
124. Kobayashi T, et al. A germline insertion in the tuberous sclerosis (Tsc2) gene gives rise to the Eker rat model of dominantly inherited cancer. *Nat Genet* 1995;9:70–74. [PubMed: 7704028]
125. Wilson C, et al. A mouse model of tuberous sclerosis 1 showing background specific early post-natal mortality and metastatic renal cell carcinoma. *Hum Mol Genet* 2005;14:1839–1850. [PubMed: 15888477]
126. Onda H, et al. Tsc2(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J Clin Invest* 1999;104:687–695. [PubMed: 10491404]
127. El-Hashemite N, et al. Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mammalian target of rapamycin. *Cancer Res* 2003;63:5173–5177. [PubMed: 14500340]
128. Chung J, et al. Integrin (α 6 β 4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells. *J Cell Biol* 2002;158:165–174. [PubMed: 12105188]
129. Klos KS, et al. ErbB2 increases vascular endothelial growth factor protein synthesis via activation of mammalian target of rapamycin/p70S6K leading to increased angiogenesis and spontaneous metastasis of human breast cancer cells. *Cancer Res* 2006;66:2028–2037. [PubMed: 16489002]
130. Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science* 2002;296:1634–1635. [PubMed: 12040173]
131. Tavazoie SF, et al. Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. *Nat Neurosci* 2005;8:1727–1734. [PubMed: 16286931]
132. Choi YJ, et al. Tuberous sclerosis complex proteins control axon formation. *Genes Dev* 2008;22:2485–2495. [PubMed: 18794346]
133. Kishi M, et al. Mammalian SAD kinases are required for neuronal polarization. *Science* 2005;307:929–932. [PubMed: 15705853]
134. Lizcano JM, et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J* 2004;23:833–843. [PubMed: 14976552]
135. Barnes AP, et al. LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. *Cell* 2007;129:549–563. [PubMed: 17482548]
136. Kobayashi T, et al. A germ-line Tsc1 mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice. *Proc Natl Acad Sci* 2001;98:8762–8767. [PubMed: 11438694]
137. Uhlmann EJ, et al. Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Ann Neurol* 2002;52:285–296. [PubMed: 12205640]
138. Wong M, et al. Impaired glial glutamate transport in a mouse tuberous sclerosis epilepsy model. *Ann Neurol* 2003;54:251–256. [PubMed: 12891680]
139. Wang Y, et al. Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1. *Ann Neurol* 2007;61:139–152. [PubMed: 17279540]
140. Ehninger D, et al. Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. *Nat Med* 2008;14:843–848. [PubMed: 18568033]
141. Yeung RS, et al. Predisposition to renal carcinoma in the Eker rat is determined by germ-line mutation of the tuberous sclerosis 2 (TSC2) gene. *Proc Natl Acad Sci* 1994;91:11413–11416. [PubMed: 7972075]
142. Xiao GH, et al. Germ-line Tsc2 mutation in a dominantly inherited cancer model defines a novel family of rat intracisternal-A particle elements. *Oncogene* 1995;11:81–87. [PubMed: 7624135]

143. Hino O, et al. Spontaneous and radiation-induced renal tumors in the Eker rat model of dominantly inherited cancer. *Proc Natl Acad Sci* 1993;90:327–331. [PubMed: 8419937]
144. Yeung RS. Lessons from the Eker rat model: from cage to bedside. *Curr Mol Med* 2004;4:799–806. [PubMed: 15579026]
145. Yeung RS, et al. Allelic loss at the tuberous sclerosis 2 locus in spontaneous tumors in the Eker rat. *Mol Carcinog* 1995;14:28–36. [PubMed: 7546222]
146. Yeung RS, et al. Subependymal astrocytic hamartomas in the Eker rat model of tuberous sclerosis. *Am J Pathol* 1997;151:1477–1486. [PubMed: 9358774]
147. Mizuguchi M, et al. Novel cerebral lesions in the Eker rat model of tuberous sclerosis: cortical tuber and anaplastic ganglioglioma. *J Neuropathol Exp Neurol* 2000;59:188–196. [PubMed: 10744057]
148. Kenerson HL, et al. Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. *Cancer Res* 2002;62:5645–5650. [PubMed: 12384518]
149. Waltereit R, et al. Enhanced episodic-like memory and kindling epilepsy in a rat model of tuberous sclerosis. *J Neurochem* 2006;96:407–413. [PubMed: 16300636]
150. Wenzel HJ, et al. Morphology of cerebral lesions in the Eker rat model of tuberous sclerosis. *Acta Neuropathol* 2004;108:97–108. [PubMed: 15185103]
151. Takahashi DK, et al. Abnormal cortical cells and astrocytomas in the Eker rat model of tuberous sclerosis complex. *Epilepsia* 2004;45:1525–1530. [PubMed: 15571510]
152. Kobayashi T, et al. Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line Tsc2 mutation in mice. *Cancer Res* 1999;59:1206–1211. [PubMed: 10096549]
153. Way SW, et al. Loss of Tsc2 in radial glia models the brain pathology of tuberous sclerosis complex in the mouse. *Hum Mol Genet* 2009;18:1252–1265. [PubMed: 19150975]
154. Miyata H, et al. Insulin signaling pathways in cortical dysplasia and TSC-tubers: tissue microarray analysis. *Ann Neurol* 2004;56:510–519. [PubMed: 15455398]
155. Ljungberg MC, et al. Activation of mammalian target of rapamycin in cytomegalic neurons of human cortical dysplasia. *Ann Neurol* 2006;60:420–429. [PubMed: 16912980]
156. Aronica E, et al. Co-expression of cyclin D1 and phosphorylated ribosomal S6 proteins in hemimegalencephaly. *Acta Neuropathol* 2007;114:287–293. [PubMed: 17483958]
157. Franz DN, et al. Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. *Ann Neurol* 2006;59:490–498. [PubMed: 16453317]
158. Bissler JJ, et al. Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N Engl J Med* 2008;358:140–151. [PubMed: 18184959]
159. Davies DM, et al. Sirolimus therapy in tuberous sclerosis or sporadic lymphangioleiomyomatosis. *N Engl J Med* 2008;358:200–203. [PubMed: 18184971]

Figure 1.
TSC2:TSC1 pathway.

Figure 2.

IHC of tubers for P-S6K, P-S6, P-4E-BP1, VEGF. Tubers from TSC patients exhibit hyperactive mTOR signaling. Tuber (*top panel*) and epilepsy-control (*bottom panel*) cortical tissue was probed with antibodies recognizing the phosphorylated isoforms of S6K (A,B), S6 (C,D), 4E-BP1 (E,F), and VEGF (G,H). Large panels were taken at 5 $\times$ (scale bar: 200 μ m), and insets were taken at 40 $\times$ (scale bar: 50 μ m).

Table 1

Diagnostic criteria for tuberous sclerosis complex

Major	Minor
Cardiac rhabdomyoma	Multiple pits in dental enamel
Cortical tuber	Hamartomatous rectal polyps
Facial angiofibroma	Bone cysts
Hypomelanotic macule	Radial migration lines in cerebral white matter
Lymphangiomyomatosis	Gingival fibromas
Renal angiomyolipoma	Retinal achromatic patches
Retinal hamartomas	“Confetti” skin lesions
Shagreen patch	Multiple renal cysts
Subependymal giant-cell tumor	
Subependymal nodule	
Ungual fibromas	

Definite TSC: Two major or one major plus two minor criteria.

Probable TSC: One major plus one minor criteria.

Possible TSC: One major or two minor features.