

# Non-canonical functions of the tuberous sclerosis complex-Rheb signalling axis

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## Introduction

Tuberous sclerosis complex (TSC) is a rare genetic disease, which was initially described in 1835 (Whittemore, 2010). The *TSC1* and *TSC2* genes, which are mutated in affected individuals, were cloned in 1997 and 1993, respectively (Consortium ECTS, 1993; van Sleight-horst et al, 1997). Progress in understanding the pathogenesis of TSC was propelled in 2002 by the discovery that the TSC proteins inhibit the mammalian Target of Rapamycin (mTOR), a serine/threonine protein kinase, which regulates a wealth of cellular functions including cell growth and survival (Gao et al, 2002; Jaeschke et al, 2002; Tee et al, 2002). Subsequently, Ras homolog enriched in brain (Rheb) was identified as the mediator through which the TSC proteins regulate the mTOR Complex 1 (mTORC1), and the sequence of the 'canonical' TSC/Rheb/mTORC1 pathway was established (Castro et al, 2003; Garami et al, 2003; Inoki et al, 2003a; Saucedo et al, 2003; Stocker et al, 2003; Zhang et al, 2003). This opened the floodgates of TSC research on basic, translational and clinical levels. It was quickly determined that mTOR is constitutively active in tumour cells from individuals with TSC and in cells from women with the related disorder lymphangi leiomyomatosis (LAM; Goncharova et al, 2006; Kenerson et al, 2002). Studies using the mTORC1 inhibitor Rapamycin and its analogs revealed partial tumour regression responses in the brain and kidney of virtually all TSC animal models and in clinical trials in patients with TSC or LAM (Birca et al, 2010; Bissler et al, 2008; Davies et al, 2008;

The protein products of the tuberous sclerosis complex (TSC) genes, *TSC1* and *TSC2*, form a complex, which inhibits the small G-protein, Ras homolog enriched in brain (Rheb). The vast majority of research regarding these proteins has focused on mammalian Target of Rapamycin (mTOR), a target of Rheb. Here, we propose that there are clinically relevant functions and targets of *TSC1*, *TSC2* and Rheb, which are independent of mTOR. We present evidence that such non-canonical functions of the TSC-Rheb signalling network exist, propose a standard of evidence for these non-canonical functions, and discuss their potential clinical and therapeutic implications for patients with TSC and lymphangi leiomyomatosis (LAM).

Yalon et al, 2010). Thus, there is no doubt that hyperactivation of mTORC1 is a critical component of tumourigenesis in TSC.

The intense focus on the canonical TSC/Rheb/TORC1 network has left a fundamental question largely unanswered: Do the TSC proteins and/or Rheb have other disease-relevant targets? Here, we will discuss the compelling evidence for non-canonical signalling pathways in which *TSC1*, *TSC2* and Rheb function independently of TORC1. These non-canonical pathways may be the cause of some of the fascinating clinical manifestations of TSC and LAM, and may contribute to the fact that TORC1 inhibition alone is not sufficient to induce complete regression of tumours in individuals with TSC.

## Tuberous sclerosis complex

Few, if any, human diseases rival the diversity of clinical manifestations of TSC. TSC can impact nearly every organ system in humans with potentially life-threatening consequences in the brain, heart, lung and kidney (Fig 1). In addition to the development of multiple tumours, most individuals with TSC have seizures during childhood (often with onset in infancy), and about 50% of TSC patients have cognitive defects including autism and intellectual disability. The tumours in TSC are historically classified as hamartomas. Hamartomas are benign focal malformations composed of tissue elements normally found at the site of growth, but developing in a disorganized mass. While some of the lesions in TSC seem to fit

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this definition, such as cerebral cortical **tubers**, cardiac rhabdomyomas and epithelial renal cysts, some of the other manifestations of TSC, do not seem to arise from normal tissue elements. For example, renal **angiomyolipomas** are composed of tri-lineage mesenchymal cells that do not have an obvious relationship to the normal cellular elements of the kidney (Fig 2), and pulmonary LAM cells express smooth muscle and neuronal markers in contrast to the lung epithelium in which they reside. Furthermore, all three lineages within angiomyolipomas arise from a common precursor cell, suggesting that tumours in TSC exhibit cell fate plasticity and, therefore, do not fit the classic definition of a hamartoma. Finally, while the vast majority of tumours in TSC are histologically benign and do not generally metastasize, there are two notable exceptions. First, the smooth muscle-like cells of pulmonary LAM, while histologically benign, are believed to metastasize to the lungs through an as-yet-unknown mechanism. Second, children and adults with TSC can develop renal cell carcinomas, malignant angiomyolipomas and mesenchymal lesions, termed PEComas

(Crino et al, 2006; Folpe & Kwiatkowski, 2010; Henske, 2004; Linehan et al, 2010; Yu & Henske, 2010). While these clearly malignant lesions are rare, they underscore the diversity of clinical manifestations of TSC and further distinguish TSC from a true 'hamartomatous' disorder.

### The canonical TSC-Rheb-TORC1 pathway

The TSC1 and TSC2 proteins (also known as hamartin and tuberlin, respectively), act as a heterodimer to inhibit the activity of the small **GTPase** Rheb through TSC2's evolutionarily conserved GTPase activating protein (GAP) domain located near its carboxy-terminus (Inoki et al, 2003a; Plank et al, 1998; van Slegtenhorst et al, 1998; Zhang et al, 2003). Rheb-GTP activates the mTORC1, which consists minimally of mTOR, Raptor and mLST8 (Hara et al, 2002; Kim et al, 2002, 2003; Loewith et al, 2002). Downstream of activated mTORC1, protein translation is promoted and autophagy is suppressed through mechanisms involving direct phosphorylation of p70S6Kinase, 4E-BP and Ulk1/Atg13 by mTOR (Fig 3; Burnett et al, 1998;

## Glossary

### Aggresome

A proteinaceous inclusion body that forms as a consequence of ubiquitin-proteasome system overload or impairment.

### Angiomyolipoma

A benign tumour composed of fat cells, smooth muscle cells and blood vessels that is usually found in the kidney.

### Centrosome

Small region of the cytoplasm, which is adjacent to the nucleus, contains the centrioles and serves to organize the microtubules.

### DAPT

A  $\gamma$ -secretase inhibitor, which antagonizes Notch signalling through inhibition of Notch processing. The chemical name is *N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-L-phenylglycine-1,1-dimethylethyl ester.

### Eker rat

These rats have a spontaneous, naturally occurring mutation in the *Tsc2* gene.

### GTPase

Guanine triphosphatases hydrolyse guanine triphosphate (GTP) to guanine diphosphate (GDP) and phosphate (Pi).

### Hamartoma

A benign growth, which consists of an abnormal mixture of cells and tissues normally found in the area of the body where the growth occurs.

### Haemangioma

A benign tumour, which consists of a dense mass of dilated blood vessels.

### Hypomorphic allele

A type of allele from which either the gene product is expressed at reduced levels relative to the WT or the gene product displays a reduced level of activity.

### Imaginal disc

A group of undifferentiated cells in the larvae of some insects, which differentiates into adult epidermal structures such as wings and legs.

### LAM

LAM is a progressive disorder of women characterized by nodular and diffuse interstitial proliferation of smooth muscle cells in the lungs and lymph nodes, often accompanied by renal angiomyolipomas.

### MMP

Matrix metalloproteinases are zinc-dependent endopeptidases, which degrade various extracellular matrix and cell surface proteins.

### Primary cilium

A microtubular, non-motile organelle that projects from the cell body. The primary cilium is anchored to the cell body by a centriole present at its base.

### Signalling endosome

A specialized endocytic vesicle that harbours ligand-bound receptors within its membrane and mediates their coordinated transport into the cell body.

### TIMP

Tissue inhibitors of metalloproteinases are a family of secreted proteins, which modulate the activity of MMPs.

### TORC1

TOR Complex 1 is a protein complex that contains the TOR (target of rapamycin) serine/threonine kinase and mediates control of cell growth and autophagy.

### TSC

TSC is an autosomal dominant genetic disorder characterized by benign tumours in many parts of the body and neurologic symptoms including seizures, autism and cognitive disability.

### Tuber

Also known as a cortical tuber. A region of the brain that develops abnormally and contains characteristic 'giant cells'. Tubers can disrupt the cortical architecture and thus lead to neurological problems.

### VEGF-A

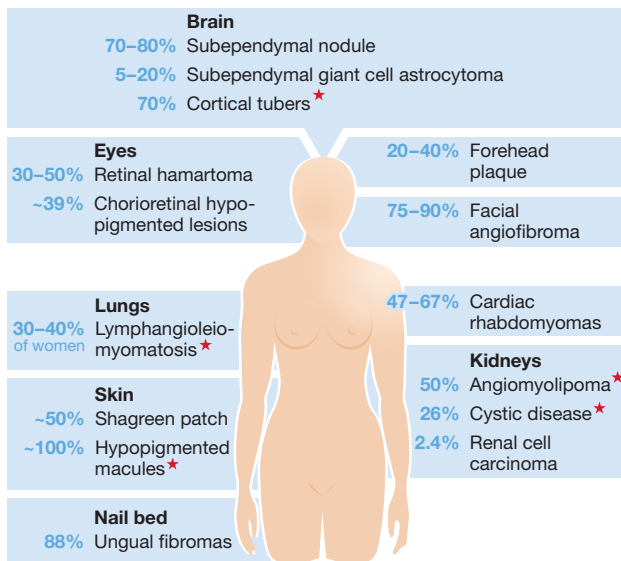
The VEGF-A is a member of a growth factor family and specifically acts on endothelial cells. Among other effects, it increases vascular permeability, induces endothelial cell growth and inhibits apoptosis.

### Xenograft

A graft in which the donor and recipient are of different species.

### $\gamma$ -secretase

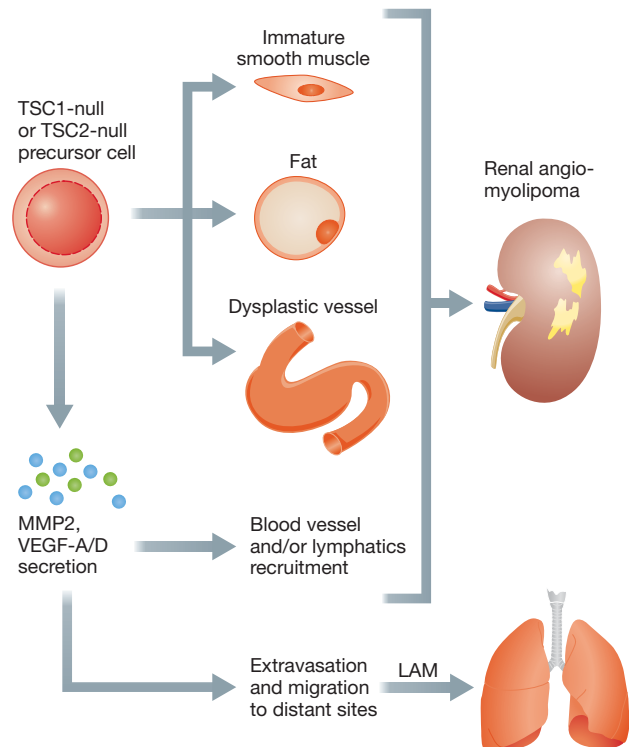
A protease, which processes Notch and contributes to Notch signalling. The authors declare that they have no conflict of interest.



**Figure 1. Clinical manifestations of TSC.** Many organ systems are affected in TSC. The most commonly affected systems and their associated lesions are shown. Percentages shown in blue represent the incidence in individuals with TSC (Kwiatkowski et al, 2010). Red stars indicate manifestations, which may involve non-canonical TSC-Rheb signalling networks. Cortical tubers, LAM, hypopigmented macules and renal angiomyolipomas all reveal defects in differentiation and/or cell fate specification, and may, therefore, involve non-canonical pathways. Renal cystic disease in TSC is suspected to be non-canonical as primary cilia regulation and early cystogenesis is Rapamycin-insensitive.

Ganley et al, 2009; Hosokawa et al, 2009; Jung et al, 2009). The activity of the TSC1–TSC2 complex towards Rheb and mTORC1 is tightly regulated through phosphorylation of both proteins. At least six kinases (Akt, MK2, Erk, GSK3, RSK1 and AMPK) directly phosphorylate TSC2 while at least two kinases (CDK1 and IKK $\beta$ ) directly phosphorylate TSC1; all eight have been shown to regulate the activity of TORC1 (Astrinidis et al, 2003; Catania et al, 2001; Dan et al, 2002; Inoki et al, 2006, 2003b; Lee et al, 2007b; Li et al, 2003; Ma et al, 2005; Manning et al, 2002; Roux et al, 2004). Importantly, there is a wide consensus on the clinical and translational importance of TORC1 activation in TSC tumours because evidence of TORC1 hyperactivity has been documented in yeast, *Drosophila* and rodent models of TSC and in virtually every human TSC tumour that has been studied.

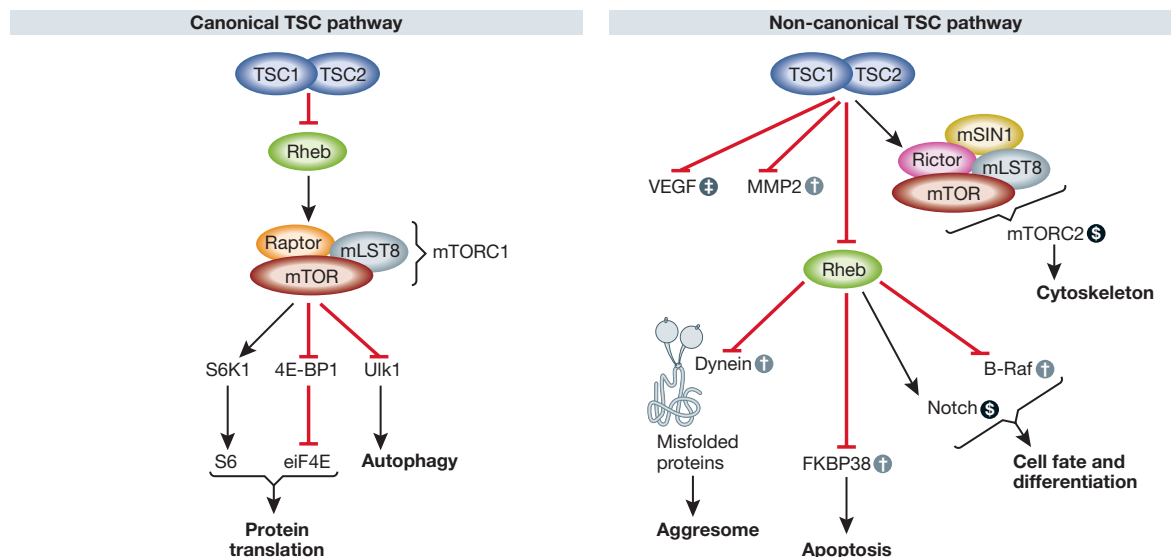
What remains relatively understudied, and is the subject of this *In Focus*, is whether and how TSC1, TSC2 or Rheb impact cellular pathways other than TORC1 signalling. Evidence for these non-canonical TSC-Rheb functions includes reports of TSC2-independent functions of TSC1, Rheb-independent functions of the TSC1–TSC2 complex and TORC1-independent functions of Rheb. Here, we will summarize and highlight data that point towards three emergent non-canonical functions of TSC proteins and Rheb: (1) centrosome-regulated functions, (2) transport, signalling and secretion across the cellular plasma membrane and (3) regulation of the actin cytoskeleton.



**Figure 2. Model for TSC-related renal angiomyolipoma development.** At least two potentially TORC1-independent mechanisms contribute to the development of renal angiomyolipomas from a TSC1-null or TSC2-null mesenchymal precursor cell. First, faulty cell fate specification leads to a tri-lineage differentiation, resulting in the presence of ectopic immature smooth muscle cells, fat and dysplastic vessels all within a single tumour. As noted in the text, TSC/Rheb-dependent regulation of two relevant differentiation/cell fate specification pathways, Notch and B-Raf, may include non-canonical components. Secondly, loss of TSC1 or TSC2 leads to the upregulation of matrix metalloproteinases such as MMP2 and VEGFA/D, which is at least partially independently of mTORC1 and (in the case of MMP2) Rheb. This may promote blood vessel and/or lymphatics recruitment to the tumour, as well as extravasation of TSC1/TSC2-null cells, a mechanism, which may potentially contribute to the development of pulmonary LAM.

Notably, while there is a large body of work studying the brain manifestations of TSC, there is currently virtually no evidence that any of these phenotypes are TORC1-independent. The abnormal cell fate differentiation patterns observed in cerebral cortical tubers and subependymal giant cell astrocytomas (SEGAs) could involve mTORC1-independent activation of the Notch signalling network (see ‘Activation of the Notch Signalling Pathway Section’ below) but this has not yet been tested.

The strength of molecular and physiological evidence that these described functions are TORC1-independent, and thus non-canonical, varies between studies. In fact, the ‘gold standard’ for establishing TORC1-independence is itself not clear. While Rapamycin-insensitivity is suggestive of TORC1-independence, future studies need to combine genetic evidence as well as mTOR-kinase inhibition to prove independence from TORC1.



**Figure 3. Canonical and non-canonical TSC-Rheb signalling pathways.** Several cellular functions, including aggresome formation, apoptosis, differentiation and regulation of the actin cytoskeleton are modulated by TSC1 and TSC2, potentially through the non-canonical mechanisms shown here. Simplified versions of both canonical and non-canonical TSC/Rheb signalling are shown. The strength of evidence for mTORC1-independent regulatory mechanism is indicated:  $\ddagger$ : Regulation is partially Rapamycin-insensitive.  $\ddagger$ : Regulation is completely Rapamycin-insensitive.  $\$$ : Regulation has been shown to be independent of mTORC1 using the mTOR kinase inhibitor TORIN1 and/or siRNA against the mTORC1 component Raptor.

### Non-canonical, centrosome-related functions of TSC1, TSC2 and Rheb

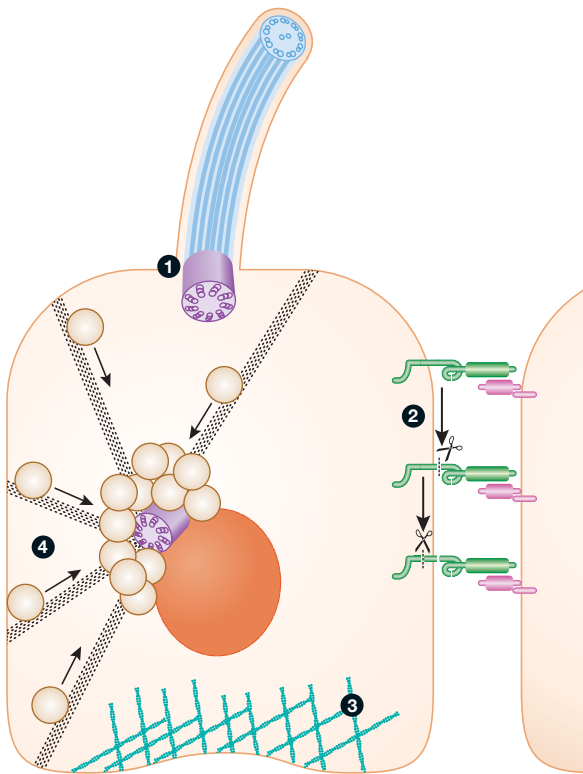
TSC1 localizes to the centrosome in dividing and non-dividing cells (Astrinidis et al, 2006). TSC-dependent, TORC1-independent mechanisms have been implicated in the formation of two centrosome-dependent cellular structures: the **primary cilium** and the **aggresome**.

The primary cilium consists of a finger-like plasma membrane projection, reinforced by an internal stalk of microtubule bundles with a centriole anchoring its base (Goetz & Anderson, 2010). These cilia transmit signals about extracellular flow and are epicenters for certain signal transduction pathways such as Hedgehog signalling. TSC1 is present at the base of the primary cilium in cultured human retinal pigmented and kidney epithelial cells (Fig 4; Astrinidis et al, 2006; Hartman et al, 2009). Interestingly, *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) have longer cilia, and a greater percentage of cells in culture are single- or multi-ciliated compared to wild-type (WT) MEFs, suggesting that the TSC1/TSC2 complex suppresses cilia formation (Hartman et al, 2009). Importantly, Rapamycin treatment restores cilia length in some, but not all, cell lines tested, and it does not reduce the frequency of ciliation in MEFs. Taken together, this could indicate that non-canonical functions of TSC1 and TSC2 are important for ciliary phenotypes.

Primary cilia of the epithelial cells lining renal cysts from *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> mice are twice as long as primary cilia in the tubule cells of WT mice, suggesting a link between primary cilia and cyst pathogenesis in TSC (Bonnet et al, 2009). Importantly, patients with TSC also have kidney cysts, and renal

cysts are a hallmark of ciliopathies in general (Goetz & Anderson, 2010). Consistent with this link between Rapamycin-insensitive ciliary abnormalities and cyst pathogenesis in TSC, some of the small, early stage renal cysts in *Tsc2*<sup>+/-</sup> mice did not display phosphorylation of S6, the target of S6K1, suggesting that cystogenesis may be independent of mTOR activation (Bonnet et al, 2009). Previously, the same group showed that some renal cysts in *Tsc1*<sup>+/-</sup> mice did not lose heterozygosity (an indicator of selective pressure) or have evidence of overt mTOR activation, implicating a non-canonical pathway (Wilson et al, 2006). Interestingly, Rapamycin treatment of the **Eker rat** model of TSC showed no effect on the number of microscopic kidney lesions (Kenerson et al, 2005). Collectively, these data suggest that TSC1/TSC2 may play a role in regulation of the primary cilia, and, thereby, renal cyst pathogenesis, independently of TORC1.

The aggresome is a perinuclear inclusion body that is composed of aggregated, misfolded proteins, which have exceeded the capacity of autophagy- and proteasome-mediated degradation. It forms in a microtubule- and centriole-dependent process (Kopito, 2000). Recently, Zhou et al made the surprising discovery that despite low levels of autophagy in proteasome-inhibited *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> MEFs, aggresome formation was also suppressed (Zhou et al, 2009). Unexpectedly, suppression of aggresome formation in these cells was Rapamycin-insensitive, but Rheb-dependent. In addition, defective aggresome formation caused by loss of *Tsc1* or *Tsc2* sensitized these cells to apoptosis in response to misfolded proteins; a finding that could be exploited in the development of novel therapies for individuals with TSC. As aggresome formation is a centriole-dependent process, an exciting possibility is that both cilia and



**Figure 4. Cell physiological functions of non-canonical TSC-Rheb signalling.** TSC1- and TSC2-mutant cells display increased primary cilium number and length (1) that is not restored by Rapamycin treatment. As TSC1 localizes to the centrosome at the basal body of the cilium (shown in purple), it is possible that TSC1 regulates primary cilium formation through a centrosome-dependent process. Active Rheb induces Notch signalling (2) through a Rapamycin- and TOR kinase inhibitor-insensitive mechanism. TSC1 and TSC2 have also been shown to regulate the actin cytoskeleton (3) through Rho-dependent, TORC1-independent mechanisms, likely involving both TORC2 and other mediators. Active Rheb also suppresses formation of the aggresome (4), another centrosome-dependent process, through a TORC1-independent process.

aggresome defects in TSC-null cells may have a common basis (Fig 4). An important caveat, however, is that insensitivity to Rapamycin is the only evidence to date that these functions are TORC1-independent. While intriguing, these phenotypes could potentially also represent Rapamycin-insensitive functions of TORC1, which have recently been demonstrated to exist in other systems (Thoreen et al, 2009). Further studies, perhaps involving TOR-kinase inhibitors, are needed to determine whether these are true TORC1-independent functions, and if they are mechanistically linked.

### Regulation of transport, secretion and signalling across the plasma membrane by non-canonical TSC-Rheb signalling

In multiple organisms, loss of the TSC genes results in TORC1-independent changes in signalling, transport and secretion across the plasma membrane. Importantly, some of the consequences of TSC1/TSC2 loss appear to reflect non-canonical functions of the proteins (Fig 3). It is unclear if a common mechanism underlies these changes; however, they share a common theme of communication between the cell and the environment.

#### Uptake of basic amino acids

In the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus fumigatus*, activation of the Rheb homolog results in suppression of uptake of basic amino acids

such as arginine (Matsumoto et al, 2002; Panepinto et al, 2003; Tsao et al, 2009; Urano et al, 2000; van Slegtenhorst et al, 2004). In *S. cerevisiae* and *S. pombe*, the defect has been attributed to defective expression and trafficking of the Can1 and Cat1 basic amino acid transporters (Aspuria & Tamanoi, 2008; Urano et al, 2000). Regulation of amino acid transporters appears Rheb-dependent in all three fungal species; however, the involvement of the mTOR homologs in this process is less clear. In *S. pombe*, a constitutively active Rheb (rhb1-K120R) can suppress arginine uptake even in cells deficient in the TORC1-specific mTOR homolog, Tor2 (Urano et al, 2005). Similarly, a kinase-active Tor2 mutant cannot suppress arginine uptake in the absence of Rheb, even though it is sufficient to confer other TORC1-active phenotypes (Urano et al, 2007). Furthermore, Rapamycin does not restore arginine uptake in *tsc1*- and *tsc2*-null *S. pombe*, even though it activates transcription of amino acid permeases and effectively inhibits TORC1 (Nakashima et al, 2010; Weisman et al, 2007). This suggests that the role of TSC and Rheb in regulating permease trafficking to the cell membrane may be TORC1-independent. Interestingly, in *Drosophila* S2 cells, knockdown of dRheb or dTOR by siRNA increases the uptake of arginine, but not global amino acid uptake, suggesting that this might be a conserved function of the pathway (Hall et al, 2007).

#### Secretion of vascular endothelial growth factor-A (VEGF-A)

Since 2000, it has been recognized that the TSC-Rheb-mTOR pathway regulates VEGF-A expression (Hudson et al, 2002; Treins et al, 2002; Zhong et al, 2000). One level of regulation occurs through the TORC1 complex, which indirectly upregulates VEGF-A through stabilization of HIF1- $\alpha$  transcripts. However, at least one group has shown that, while Rapamycin can block upregulation of HIF1- $\alpha$ , it does not entirely block the increased VEGF-A secretion observed in *Tsc2*<sup>-/-</sup> MEFs (Brugarolas et al, 2003). These data suggest that the TSC1-TSC2 complex may regulate VEGF-A secretion, at least partially, independently of TORC1. While it is possible that TORC1 regulates VEGF-A through a HIF1- $\alpha$ -independent, Rapamycin-insensitive mechanism, an intriguing possibility is that secretion of VEGF-A is regulated in a TORC1-independent fashion through a broader protein trafficking mechanism. If this is the case, VEGF-A secretion may be regulated through the same trafficking mechanism that results in enhanced Notch signalling in TSC2-deficient angiomyolipoma cells and decreased amino acid transport in yeast models of TSC.

### Secretion and activation of matrix metalloproteinases

Using microarray matrix metalloproteinase (MMP)-2, -9 and tissue inhibitor of metalloproteinase (TIMP)-4 were shown to be upregulated in the absence of TSC2 using a patient-derived, TSC2-null angiomyolipoma cell line (Lee et al, 2010; Yu et al, 2004). MMP-2 activity was increased in both *Tsc2*<sup>-/-</sup> and *Tsc1*<sup>-/-</sup> MEFs, and this induction was unaffected by either Rapamycin treatment or Rheb knockdown, suggesting this regulation is a Rheb-independent function of the TSC1/TSC2 complex. The role of upregulation of metalloproteinases in TSC tumour-derived cell migration or invasion, however, has yet to be tested.

### Activation of the Notch signalling pathway

Two groups, including ours, showed that Rheb can activate the Notch signalling pathway (Karbowiczek et al, 2010; Ma et al, 2010b). We found evidence of elevated Notch activity in *Drosophila* sensory organ development, in angiomyolipomas and in an angiomyolipoma-derived cell line (Karbowiczek et al, 2010). Treatment with the  $\gamma$ -secretase inhibitor DAPT or knockdown of Rheb in a TSC2-null, angiomyolipoma-derived cell line decreased Notch activity as well as the level of cleaved Notch, suggesting that Notch activation occurs downstream of Rheb and upstream of Notch receptor cleavage. Importantly, Notch activation was not blocked by Rapamycin, the ATP-competitive mTOR inhibitor TORIN1 or by siRNA-mediated downregulation of the TORC1 component Raptor, indicating that in TSC tumour-derived cells, Notch activation is Rheb-dependent but TORC1-independent (Thoreen et al, 2009). Ma et al also found elevated Notch signalling in TSC2-null tumours, which was associated with upregulation of the Notch ligand Jagged (Ma et al, 2010b). However, in this study, induction of Notch was sensitive to Rapamycin and siRNA knockdown of mTOR in cancer cell lines such as MCF7 and HepG2, raising the possibility that Notch regulation by the TSC proteins is cell type-specific. Interestingly, both groups found that the  $\gamma$ -secretase inhibitor DAPT could suppress growth of xenograft tumours with overt activation of the Rheb-mTOR pathway, suggesting that Notch inhibitors might be a viable alternative to established treatments or could be part of a combinatorial therapy for TSC patients. More work is needed to understand the mechanisms by which Rheb activates Notch in a cell type-specific manner, and to resolve the apparent discrepancy between TORC1-dependent and -independent mechanisms (Pear, 2010). Notably, in flies, Notch activity is regulated by trafficking of a 'signalling endosome' containing the receptor-ligand complex, raising the possibility that TSC-dependent activation of Notch signalling might also be part of a TORC1-independent trafficking function (Fortini, 2009).

### Non-canonical TSC/Rheb regulation of actin cytoskeleton-related functions

Several lines of evidence, in organisms ranging from yeast to fly to humans, suggest that TSC2 and TSC1 regulate the actin cytoskeleton through multiple TORC1-independent mechanisms that are likely both Rheb-dependent and -independent.

### Rho-dependent leg morphogenesis in *Drosophila*

It was recently shown that Rheb loss-of-function mutations enhanced a *D. melanogaster* leg morphogenesis phenotype in a hypomorphic Rho allele background (Patch et al, 2009). Additionally, overexpression of Rheb in the leg imaginal disc resulted in short, fat leg segments that were frequently kinked or curved. This is in stark contrast to overexpression of a constitutively active phosphoinositide 3-kinase (PI3K), which resulted in enlarged segments that had no other malformations. Importantly, in contrast to loss of Rheb, loss of dTor did not synergize with loss-of-function Rho mutations. These data suggest that Rheb may play roles in *D. melanogaster* leg morphogenesis that are independent of the PI3K-Rheb-dTor cascade.

### TSC1/TSC2 activation of TORC2, a cytoskeletal regulator

The TOR Complex 2 (TORC2) is evolutionarily conserved from yeast to mammals. In mammals, TORC2 consists of mTOR, Rictor, mSIN1 and mLst8 (Fig 3; Cybulski & Hall, 2009). In contrast to TORC1, TORC2 is relatively insensitive to Rapamycin. However, TORC2 can be dissociated and, thereby, inhibited by long-term Rapamycin treatment, and the degree to which Rapamycin affects TORC1 versus TORC2 may be dependent upon the relative expression levels of different TOR complex members (Rosner & Hengstsclager, 2008; Sarbassov et al, 2005). The primary known target protein of TORC2 is Akt. Significantly, TORC2 also promotes cell spreading and F-actin polymerization through an incompletely understood mechanism involving Rho-type GTPases (Jacinto et al, 2004). Importantly, TORC2 activity is decreased in *Tsc2*<sup>-/-</sup> cells and inhibition of TORC1 activity through downregulation of Raptor or Rheb did not restore TORC2 activity (Huang et al, 2008, 2009). Furthermore, the TSC1/TSC2 complex directly and specifically interacts with TORC2 and not TORC1. Thus, TORC2 may represent a non-canonical arm of the TSC pathway. Consistent with this, it has been shown that *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> MEFs are rounded and display actin cytoskeletal defects (Gau et al, 2005). Moreover, farnesyl transferase inhibitors affecting Rheb, but not inhibition of TORC1 with Rapamycin, could reverse these cytoskeletal rearrangements. Interestingly, TORC2 activity appears lower in tissues from patients with TSC (Huang et al, 2009). It is still unclear what the functional consequences of the apparently lower TORC2 activity in TSC tissues are, and what the cellular consequences of further reduction of TORC2 activity with drugs like the ATP-competitive mTOR inhibitors will be.

### Haematopoietic stem cell mobilization

In 2008, Gan et al showed that somatic deletion of *Tsc1* in the mouse haematopoietic lineage caused fatal defects in haematopoietic stem cell (HSC) mobilization, which could not be rescued by Rapamycin treatment (Gan et al, 2008). It is possible that cytoskeletal-dependent defects in cell migration may underlie the deficiency in HSC mobilization in *Tsc1*<sup>-/-</sup> mice.

### Neuronal morphology

Surprisingly, both knockdown of *Tsc2* in cultured rat neurons and Rapamycin treatment of WT neurons resulted in enhanced

dendritic spine length (Tavazoie et al, 2005). Considering the central role of actin dynamics in dendritic spine morphology, it is likely that actin regulation is involved in this TSC-mutant phenotype, perhaps reflecting a function of TORC2 downstream of TSC1/TSC2. In addition, overexpression of Rheb in *D. melanogaster* motoneurons resulted in Rapamycin-insensitive increases in the number of Boutons per muscle area and the number of branches per synapse, which have also been shown to be regulated by actin dynamics (Knox et al, 2007). Certain TSC-related brain manifestations, including the cortical tubers, involve neuronal differentiation and migration defects, which could be caused by cytoskeletal defects, Notch dysregulation or perhaps a currently unidentified mechanism. However, there is not yet any direct evidence that non-canonical pathways are involved in the brain pathophysiology of TSC patients.

### TSC1 Functions that may be TSC2-independent

TSC1 and TSC2 physically interact to form a heterodimer in mammalian cells and in model organisms including *S. pombe* (Matsumoto et al, 2002; Plank et al, 1998; van Slegtenhorst et al, 1998). Molecular data suggests that TSC2 is the ‘business end’ of the complex, with GAP activity towards Rheb, while TSC1 functions solely as a regulatable stabilizer of TSC2 protein. However, despite the similarities between the phenotypes with loss of heterozygosity of either TSC1 or TSC2 across various species, there are some potentially important differences.

Using Tsc2-null Eker rat cells, Miloloza et al showed that overexpression of TSC1 alone, in the absence of TSC2, could suppress cell proliferation (Miloloza et al, 2002). However, co-overexpression of TSC1 and a dominant-negative TSC2 mutant in HeLa cells only modestly increased the percentage of cells in G0/G1. Interestingly, TSC1 expression downregulated cyclin E expression in Tsc2-null cells, suggesting a possible mechanism through which TSC1 regulates the cell cycle.

While patients with mutations in either *TSC1* or *TSC2* have indistinguishable phenotypic features, there is a clear trend for increased severity of the symptoms in *TSC2* patients (Dabora et al, 2001; Kwiatkowski et al, 2010). The features that tend to be more severe in *TSC2* patients include subependymal nodules, mental retardation, seizures, facial angiofibromas, fibrous forehead plaques, renal angiomyolipomas, renal cysts and retinal hamartomas. We hypothesize that *TSC1* patients have less severe phenotypes compared to *TSC2* patients because of residual TSC2 expression in *TSC1* patients. Interestingly, in mouse models of TSC, liver haemangioma appear to be more prevalent in female Tsc1<sup>+/-</sup> mice compared to Tsc2<sup>+/-</sup> mice of the same genetic background (Kwiatkowski et al, 2002). Though male Tsc1<sup>+/-</sup> and Tsc2<sup>+/-</sup> mice had the same incidence of liver haemangioma formation (~50%), female Tsc1<sup>+/-</sup> mice had a 93% incidence of liver haemangioma compared to 50% of female Tsc2<sup>+/-</sup> mice. This suggests a tissue type (liver)- and sex-specific role for TSC1 in which TSC1 may be the more critical component of the complex.

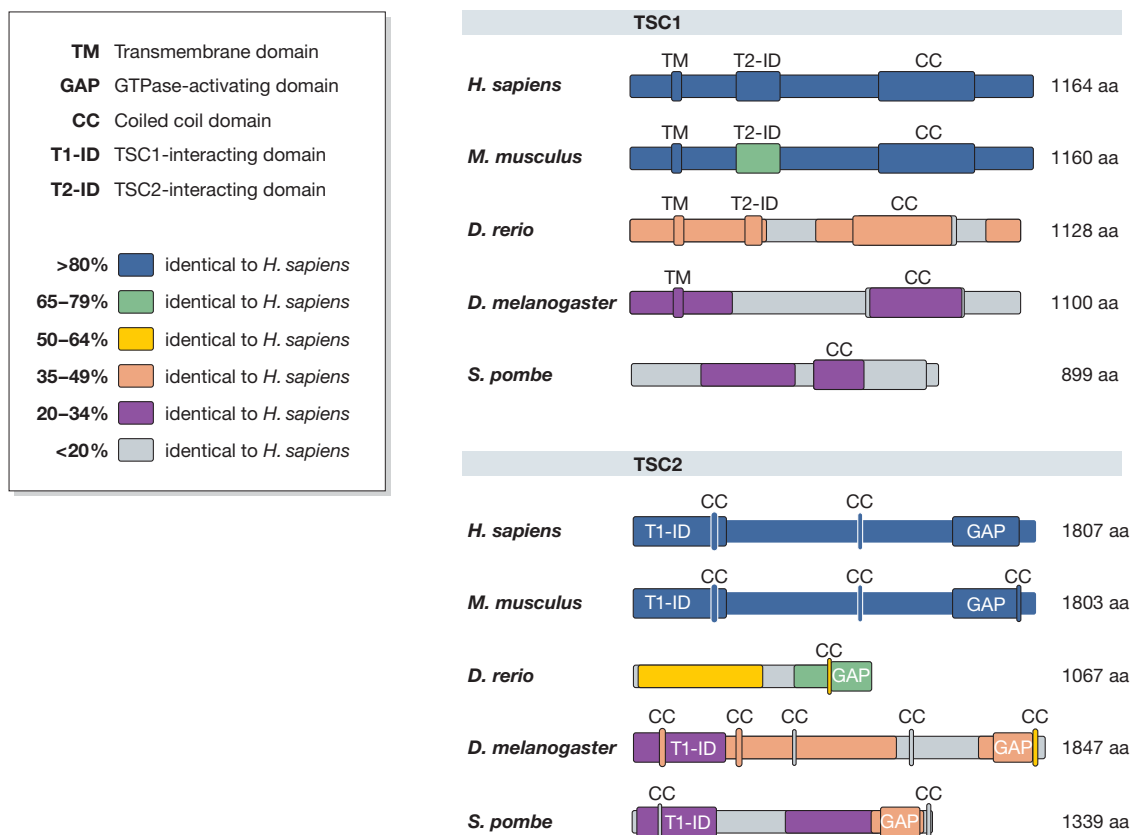
One of the most interesting pieces of evidence suggesting TSC1 may have TSC2-independent functions comes from the examination of the evolutionary conservation of the proteins (Fig 5). The GAP and TSC1-interaction domains of TSC2 are extremely well conserved down to *S. pombe*, supporting current models of TSC2’s primary role as a Rheb-GAP and the importance of the TSC2/TSC1 interaction. However, TSC1’s conserved domains do not support current models as well. The reported ‘TSC2-interacting’ domain is poorly conserved. In contrast, an N-terminal region containing a potential transmembrane domain and a C-terminal coiled-coil domain are well conserved. This suggests that there is likely an unidentified TSC2-interacting domain in TSC1, and that TSC1 possesses other essential, and possibly TSC2-independent, functions.

### Rheb molecular functions that may be TORC1-independent

Rheb is a member of the Ras family of small GTPases. The Rheb subfamily consists of two members in mammals: Rheb1 and Rheb2 (also called RhebL1). In other species, only one Rheb has been identified (Aspuria & Tamanoi, 2004). Most research has focused on Rheb1, which can functionally replace the *S. pombe* Rheb homolog. However, evidence exists that Rheb2 can also activate mTOR (Campbell et al, 2009; Tee et al, 2005; Yuan et al, 2005). Interestingly, other Ras-family small GTPases have multiple targets, while Rheb has only a single generally accepted target, TORC1. However, there are compelling data indicating that Rheb has TORC1-independent targets (Fig 3).

Our group and others have found that Rheb interacts with B-Raf in a GTP-dependent but farnesylation-independent manner, suppressing B-Raf phosphorylation at S446, B-Raf interaction with H-Ras and B-Raf kinase activity (Im et al, 2002; Karbowniczek et al, 2004; Yee & Worley, 1997). Interaction of Rheb with B-Raf also indirectly inhibits C-Raf activity by inhibiting B-Raf/C-Raf dimerization (Karbowniczek et al, 2006). Consistent with these data, loss of TSC2 suppresses p42/44 MAPK phosphorylation. Importantly, the effects of Rheb on B-Raf kinase activity and B-Raf/C-Raf heterodimerization are Rapamycin-insensitive (Karbowniczek et al, 2006). The role of decreased B-Raf and C-Raf activity in TSC-associated manifestations is unclear (Karbowniczek & Henske, 2005). Interestingly, *C-Raf*<sup>-/-</sup> mice die *in utero* from massive haemorrhaging of enlarged blood vessels, which is reminiscent of the enlarged and dysplastic blood vessels in TSC-associated renal angiomyolipomas (Wojnowski et al, 1998). B-Raf, like Rheb, is highly enriched in the brain, and knockout of B-Raf in neural precursors resulted in severe neurological defects, perhaps suggesting a role for B-Raf in the neurological manifestations of TSC (Galabova-Kovacs et al, 2008).

Several reports suggest that there are pro-apoptotic effects of Rheb activation, which are mTOR-dependent (Di Nardo et al, 2009; Freilinger et al, 2006; Inoki et al, 2003b; Kang et al, 2010; Karassek et al, 2010; Lee et al, 2007a; Ozcan et al, 2008). In addition, there may be anti-apoptotic functions of Rheb, which are mTOR-independent. In particular, Rheb interaction with



**Figure 5. Evolutionary conservation of TSC2 (tuberin) and TSC1 (hamartin).** Regions and levels of homology, relative to human TSC2 (A) and TSC1 (B) proteins, are shown by colour coding. Blue  $\geq 80\%$  amino acid identity, green 65–79% amino acid identity, yellow 50–64% amino acid identity, pink 35–49% amino acid identity and purple 20–34% amino acid identity. Regions shown in grey do not have significant homology to TSC2 and TSC1. TM, transmembrane domain; GAP, GTPase activating domain; CC, coiled-coil domain; T1-ID, TSC1-interacting domain; T2-ID, TSC2-interacting domain. *D. rerio* = *Danio rerio* or zebrafish.

FKBP38 regulates apoptosis in a Rapamycin-insensitive, but amino acid- and serum-sensitive manner (Ma et al, 2010a). Rheb directly inhibits FKBP38 interaction with Bcl2 and Bcl-XL, thereby, freeing Bcl2 and Bcl-XL to interact with and suppress the pro-apoptotic proteins Bax and Bak.

## Future Directions

A comprehensive understanding of the dysregulated pathways and cellular consequences of *TSC1* or *TSC2* mutations, including both the canonical TORC1-dependent and the non-canonical TORC1-independent functions of TSC1/TSC2 and Rheb, is an essential step towards the development of effective long-term therapeutic strategies for individuals with TSC and LAM. The most clinically important of the non-canonical pathways are likely to be those regulated by Rheb independently of TORC1. Identifying these pathways may be facilitated by the availability of model organisms in which the canonical pathway is conserved, including *S. pombe*, *Drosophila* and rodents. Proving that pathways are TORC1-independent will be an ongoing challenge because of the existence of multiple and complex feedback loops mediated by TORC1, the existence of TORC1-

dependent pathways that are not inhibited by Rapamycin and other TORC1-specific agents and the cell type-specific and time point-dependent targets of TORC1. Despite the challenges, identifying non-canonical TSC-Rheb-mediated pathways will lead to substantial advances in our understanding of the pathogenesis of not only TSC and LAM, but also the many other human diseases and tumours in which the TSC-Rheb signalling axis is dysregulated. Notably, the TSC-Rheb axis is an important target of PI3K/Akt signalling, which is dysregulated in many types of cancer, including lung, kidney and breast. It is tantalizing to speculate on the therapeutic potential that mTORC1-independent targets of TSC-Rheb might have for these cancers. As yet, though, no studies have been published that establish a role for TSC/Rheb, independently of mTORC1, in these cancers.

The recent development of TOR-kinase inhibitors will help to distinguish true TOR-independent functions from Rapamycin-insensitive functions. Unfortunately, much of the evidence for TOR-independent functions is provided through the observation of phenotypes or effects that are insensitive to Rapamycin. For instance, of the potentially TORC1-independent functions reviewed in this *In Focus*, only Rheb activation of Notch and TSC2/SC1 activation of TORC2 have been shown



to be TORC1-independent through knockdown of TORC1-components. It is now apparent that mTOR has functions, both within the TORC1 complex and outside of it (as in the case of TORC2), which are Rapamycin-insensitive. Greater experimental use of TOR-kinase inhibitors, and genetic ablation of TORC1 and TORC2 components, should help to identify true TOR-independent functions of TSC1, TSC2 and Rheb.

The identification of potentially TORC1-independent functions of TSC1/TSC2 and Rheb in organisms such as *D. melanogaster* and *S. pombe* puts powerful genetic tools at the disposal of those who wish to study these mechanisms. Genome-wide experiments using these organisms, which are still not practical in mice, could enable rapid identification of non-canonical TSC-Rheb pathway members.

The most likely candidates for non-canonical cell biological functions of the TSC-Rheb axis are those involving centriole regulation (e.g. the primary cilia), trafficking (potentially Notch signalling) and secretion. If further research can solidify that these are indeed TORC1-independent functions, they could serve as a surrogate or read-out to probe the mechanisms of the non-canonical pathway.

Finally, some of the non-canonical functions of TSC1, TSC2 and Rheb may be tissue-specific. For instance, despite a two fold increase in cilia length in the epithelial lining of kidney cysts in *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> mice, no defects have been observed in primary cilia in other areas. Indeed, the only ciliopathy of note in individuals with TSC is their kidney cysts (Bonnet et al, 2009). Similarly, while Rheb activates Notch in *Drosophila* sensory organ development, it does not appear to regulate Notch in other settings such as the *Drosophila* wing (Karbowiczek et al, 2010). Notch is activated in TSC2-null human angiomyolipoma cells, and *Tsc2*<sup>-/-</sup> MEFs display enhanced Notch-dependent differentiation to adipocytes and myoblasts, two cell types found in angiomyolipoma, suggesting that non-canonical TSC/Rheb signalling may regulate cell fate specification perhaps only in specific lineages (Karbowiczek et al, 2010; Ma et al, 2010b).

The presence of tissue-specific phenotypes with loss of TSC1 or TSC2 suggests that the efficacy of therapies may also be tissue-dependent.

In conclusion, there is evidence from yeast, flies, zebrafish and mammals for non-canonical functions of TSC1, TSC2 and Rheb. Part of the difficulty in establishing a unified non-canonical TSC/Rheb pathway is the lack of a 'gold standard' for TORC1-independence. We propose that such a standard should include knock-down of TORC1 and TORC2 components, the use of TOR-kinase inhibitors and Rapamycin treatment. We strongly believe that the efficacy of therapies for individuals with TSC and LAM will rely upon understanding and targeting of both the canonical and non-canonical functions of TSC1, TSC2 and Rheb.

## For more information

OMIM link for TSC1

<http://www.ncbi.nlm.nih.gov/omim/191100>

OMIM link for TSC2

<http://www.ncbi.nlm.nih.gov/omim/613254>

OMIM link for LAM

<http://www.ncbi.nlm.nih.gov/omim/606690>

Wikipedia entry 'Tuberous sclerosis'

[http://en.wikipedia.org/wiki/Tuberous\\_sclerosis\\_complex](http://en.wikipedia.org/wiki/Tuberous_sclerosis_complex)

Tuberous Sclerosis Alliance

[www.tsalliance.org](http://www.tsalliance.org)

National Organization for Rare Disorders

[www.rarediseases.org](http://www.rarediseases.org)

The LAM Foundation

[www.thelamfoundation.org/](http://www.thelamfoundation.org/)

LAM Treatment Alliance

<http://lamtreatmentalliance.org/>

## Pending issues

**Distinguishing Rapamycin-insensitive from TORC1-independent functions.** Previous and future studies that identify Rapamycin-insensitive functions of the TSC-Rheb pathway should be followed by genetic and pharmacological confirmation that these are true TORC1-independent functions.

**Unification of TORC1-independent functions of TSC/Rheb by a common mechanism(s).** There is currently not enough experimental evidence to ascertain the underlying link between the disparate reports of possible TORC1-independent functions of TSC and Rheb. Future studies should focus on testing whether there is a common mechanism and what it might be.

**The role of TSC1 in the TSC1–TSC2 complex.** The role of TSC1 is very unclear. Elucidation of the functions of this protein may also help illuminate the Rheb- and TORC1-independent functions of the TSC1–TSC2 complex.

**The role of TORC1-independent functions of TSC-Rheb in PI3K/Akt-dysregulated cancers.** The TSC/Rheb axis is dysregulated in a large number of cancers as a consequence of upstream activation of receptor tyrosine kinase signalling. Targeting TORC1-independent functions of TSC/Rheb in these cancers may represent a novel therapeutic strategy.

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