Research Focus

Rheb fills a GAP between TSC and TOR

Brendan D. Manning and Lewis C. Cantley

Department of Cell Biology, Harvard Medical School, Division of Signal Transduction, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Rm 1028, 4 Blackfan Circle, Boston, MA 02115, USA

There has been much interest in determining the molecular and cellular functions of hamartin and tuberin, which are encoded by the genes *TSC1* and *TSC2* that are mutated in the tuberous sclerosis complex disease. Recently, several laboratories have independently reported a major breakthrough in this field. Together, these genetic, biochemical and cell-biological studies have demonstrated that the tuberin–hamartin complex inhibits target of rapamycin (TOR) signaling by acting as a GTPase-activating protein for the Ras-related small G protein Rheb.

Once a relatively obscure disease, tuberous sclerosis complex (TSC) has gained significant notoriety over the past couple of years. This clinically complicated disorder is estimated to affect 1 in 6000 individuals [1] and most commonly manifests itself in infants and young children. TSC is characterized by the occurrence of different types of benign tumors, often classified as hamartomas, affecting a variety of tissues including the brain, kidneys, lungs, heart and skin. Pathologically, these lesions cause neurological disorders (e.g. epilepsy and autism); lung, heart and kidney failure; and severe skin rashes. Despite the prevalence and severity of this disease, it took the findings of many concordant genetic and biochemical studies to intensify the interest of cancer biologists in TSC. Together, these studies placed hamartin and tuberin, which are the products of the genes *TSC1* and *TSC2* that are mutated in TSC patients, within the cell growth and proliferation pathway leading from phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB/Akt) to activation of the target of rapamycin (TOR) in both *Drosophila* and mammalian systems ([2–7]; reviewed in [8]). These findings demonstrated that the complex formed by tuberin and hamartin regulates growth factor-dependent and nutrient-dependent activation of TOR signaling to its downstream targets ribosomal S6 kinase-1 (S6K1) and eIF-4E binding protein-1 (4E-BP1). However, until recently, the mechanism of TOR inhibition by the tuberin–hamartin complex was unknown. Once again, several distinct, yet complimentary, genetic and biochemical studies have combined to make a very significant breakthrough towards defining the molecular mechanism of TSC by demonstrating that the small G protein Rheb (for 'Ras homolog enriched in brain') is a direct target of the tuberin–hamartin complex. The impact of this discovery on our understanding of mammalian (m)TOR signaling, TSC and cancer in general are discussed below.

Corresponding author: Lewis C. Cantley (lewis_cantley@hms.harvard.edu).

The tuberin–hamartin complex controls TOR proteins through inhibition of Rheb

The molecular function of the tuberin–hamartin complex has been elusive. Tuberin possesses a region of homology to the catalytic domain of Rap-family GTPase-activating proteins (GAPs) at its C terminus and has weak *in vitro* GAP activity towards both Rap1 and Rab5 [9,10], which are two very different small G proteins. However, there is no *in vivo* evidence to support tuberin being a GAP for either of these proteins. Genetic and biochemical data from several groups have now convincingly shown that tuberin is a legitimate GAP for the Ras-related small GTPase Rheb.

In recent studies, the power of *Drosophila* genetics led to the discovery of a link between the TSC genes and Rheb in the control of cell size. Like *Drosophila* hamartin (dTSC1) and tuberin (dTSC2) [11–13], the gene encoding *Drosophila* Rheb was identified in genetic screens for genes that, when overexpressed or mutated, affect an *in vivo* cell growth assay [14,15]. However, unlike dTSC1 and dTSC2, which have growth-suppressing properties [11–13], Rheb was found to enhance cell growth. Genetic analyses demonstrated that Rheb is epistatic to dPI3K, dAkt and dTSC1/dTSC2, and that dTOR and dS6K are epistatic to Rheb [14,15]. The combined data strongly suggest that the dTSC1–dTSC2 complex negatively regulates Rheb and that Rheb is a positive regulator of TOR signaling in *Drosophila*. As GAPs stimulate the intrinsic GTPase activity of small G proteins, thereby converting them from their GTP-bound active state to their GDP-bound inactive state, an attractive model was that Rheb is a direct target of the tuberin GAP domain.

In parallel studies, these groups and others were looking specifically for a small G protein that tuberin might regulate. Some hints that the relevant target could be Rheb came from yeast genetics, specifically from the fission yeast *Schizosaccharomyces pombe*, which, unlike budding yeast, encodes hamartin and tuberin homologs. Loss of *S. pombe* Rheb, Rhb1, results in a growth arrest phenotype analogous to that seen upon nitrogen starvation [16]. This suggests that Rheb, like TOR proteins, might be part of a pathway that senses and signals the availability of nutrients. Interestingly, *S. pombe* mutants of *tsc1* or *tsc2* are defective in nutrient uptake and nitrogen-starvation-induced mating [17], suggesting that the cells might be aberrantly sensing that they have sufficient nutrients (e.g. amino acids). This would be the expected phenotype if the nutrient-sensing Rheb was constitutively active (GTP bound) in these mutants. Given the recent studies in *Drosophila* and mammalian cells on

the relationship between the TSC gene products and Rheb, this model would be interesting to test in *S. pombe*.

Studies by several laboratories have now demonstrated that mammalian Rheb can activate mTOR signaling to its downstream targets S6K1 and 4E-BP1 [18–21]. As in *Drosophila* cells [14,15], *Rheb* overexpression in mammalian cells greatly enhances mTOR signaling and can even activate mTOR in the absence of growth factors or amino acids [18–21]. Importantly, overexpression of *TSC1* and *TSC2*, which has been shown previously to inhibit mTOR signaling [2,3,6], can block the effect of Rheb on S6K1 activation and 4E-BP1 phosphorylation [18,20,21].

Biochemical analyses of the nucleotide-bound state of Rheb, both *in vivo* and *in vitro*, reveal that tuberin stimulates the intrinsic GTPase activity of Rheb. Garami *et al.* found higher levels of GTP-bound Rheb in $TSC2^{-/-}$ mouse embryonic fibroblasts (MEFs) relative to wild-type MEFs [18]. In a reciprocal manner, overexpression of *TSC1* and *TSC2* was shown by several groups to decrease the levels of Rheb–GTP relative to Rheb–GDP within both *Drosophila* [22] and mammalian cells [18,19,21]. Furthermore, treating cells with insulin increases the levels of intracellular Rheb–GTP [18]. This is probably through activation of the PI3K/Akt pathway leading to phosphorylation and inhibition of tuberin [2–4], as the effect of insulin on Rheb is blocked by PI3K inhibitors [18]. Finally, *in vitro* GAP assays have demonstrated that Rheb is a direct target of the tuberin GAP domain [20–22]. Therefore, Rheb is the much sought-after target of the tuberin–hamartin complex that regulates TOR signaling (Figure 1).

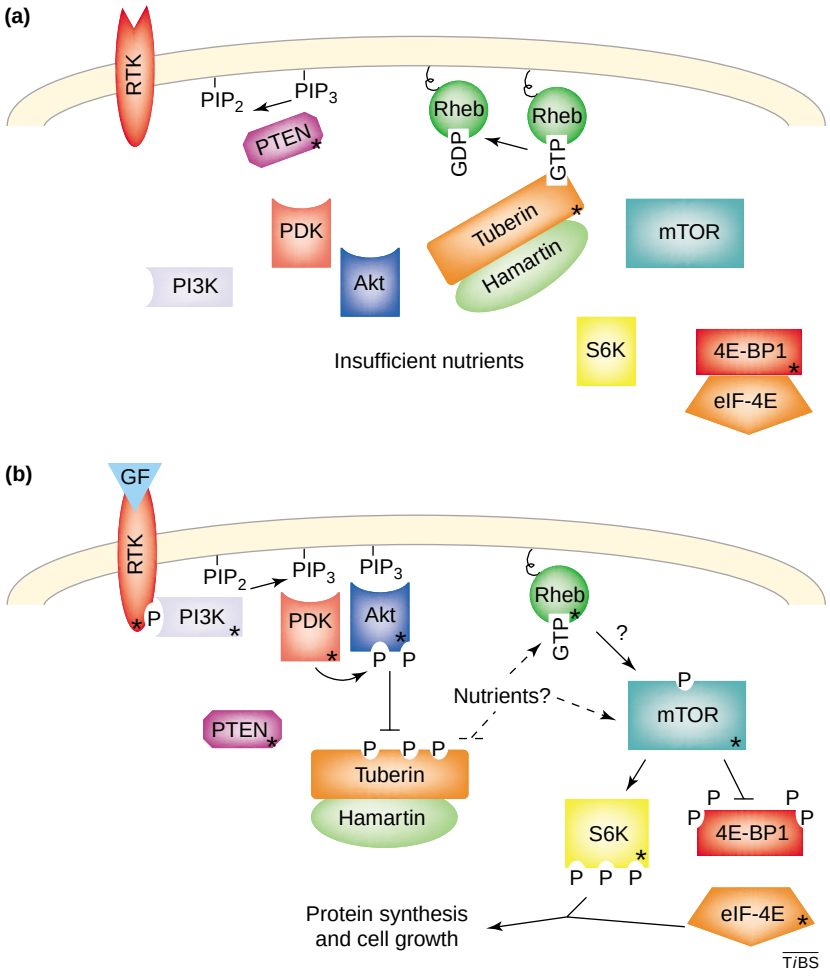

Figure 1. The regulation of, and relationship between, the tuberin–hamartin complex, Rheb and target of rapamycin (TOR) proteins, as supported by recent genetic and biochemical studies [2–6,14,15,18–22]. **(a)** In the absence of growth factors, PTEN keeps phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels low by converting it to phosphatidylinositol (4,5)-bisphosphate (PIP_2). The tuberin–hamartin complex is active as a GTPase-activating protein (GAP) towards Rheb, causing hydrolysis of GTP by Rheb and conversion from the active GTP-bound form to the inactive GDP-bound form. Active proteins are denoted with an asterisk (*). **(b)** Receptor tyrosine kinases (RTKs) are activated and phosphorylated (P) following growth factor (GF) binding. Class Ia phosphoinositide 3-kinases (PI3Ks) are then activated by binding to phosphorylated RTKs, or proteins phosphorylated by RTKs, and convert PIP_2 to PIP_3. The serine/threonine kinases PDK1 and Akt bind to PIP_3 at the membrane through their pleckstrin-homology (PH) domains, and PDK1 activates Akt through phosphorylation of its activation loop. Akt then phosphorylates tuberin on three residues (S939, S1130 and T1462 of full-length human tuberin), and this inhibits the tuberin–hamartin complex through an as-yet-undefined mechanism (reviewed in [8]). Relief of the Rheb GAP activity of tuberin allows Rheb to bind to GTP, perhaps through the activity of an unknown Rheb GEF (not shown). Rheb–GTP then activates mammalian (m)TOR, either directly or indirectly, through an unknown effector (question mark). Additionally, the availability of nutrients (e.g. amino acids) regulates mTOR through an unknown mechanism, perhaps by inhibiting the tuberin–hamartin complex or by activating Rheb or mTOR itself (broken lines). TOR activity leads to phosphorylation and activation of ribosomal S6 kinase-1 (S6K1), and phosphorylation and inhibition of translation initiation factor eIF-4E binding protein-1 (4E-BP1). Phosphorylation of 4E-BP1 relieves its binding to and inhibition of eIF-4E, which, along with S6K1 activity, leads to increased protein synthesis and cell growth.

Unresolved questions: mechanisms of Rheb regulation and activation of TOR proteins

Whereas the identification of Rheb as a downstream target of the tuberin–hamartin complex and an upstream activator of TOR adds a crucial step to this emerging and highly conserved pathway, many important questions remain. For instance, the role of hamartin in the GAP activity of tuberin towards Rheb is still unclear, as the various *in vitro* and *in vivo* studies lead to conflicting results. Some groups found that, in the context of full-length tuberin, complex formation between tuberin and hamartin increases tuberin GAP activity *in vivo* [18,22] and dramatically enhances it *in vitro* [20]. However, others found little to no effect of hamartin on this activity [19,21]. Another important question with regard to Rheb regulation is whether a guanine-nucleotide exchange factor (GEF) exists to counter the GAP activity of tuberin. It has been speculated that Rheb might not require a GEF for its activation [23], as Rheb is found in a highly GTP-bound state within cells and exhibits low intrinsic GTPase activity relative to other Ras-related small G proteins [18–23]. Interestingly, Rheb overexpression bypasses the need for nutrients, in the form of amino acids, to activate TOR signaling [14,15,18,20,21], and overexpression of tuberin and hamartin blocks the ability of both amino acids [3,5,6] and Rheb [18,20,21] to activate TOR. However, the molecular nature of nutrient sensing and its potential regulation of the tuberin–hamartin complex, Rheb and TOR remain poorly understood. Finally, the identification of a Rheb effector that directly or indirectly regulates TOR proteins is essential to close the circuit on this pathway. It seems likely that, considering the number of researchers interested in this area, the mechanism of TOR activation by Rheb–GTP will be elucidated, once again, through complimentary genetic and biochemical approaches.

Impact on the study and potential treatment of TSC

The placement of Rheb directly downstream of the tuberin–hamartin complex has many implications for TSC researchers. The most important question is whether or not constitutive activation of Rheb upon genetic loss of *TSC1* or *TSC2* can account for the entire phenotypic mosaic of the TSC disease. The fact that patient-derived mutations mapped to the GAP domain of tuberin are defective in GAP activity towards Rheb [18,20,22] suggests that Rheb is, at the very least, an important player in the pathology of TSC. It will also be important to determine if other pathways, aside from mTOR, controlled by Rheb–GTP contribute to the development of TSC lesions. However, at this point, no *bona fide* Rheb effector has been identified. Rheb has been suggested to bind to, and inhibit, the Ras effector B-Raf and thereby inhibit mitogen-activated protein kinase (MAPK) signaling [23]. However, two recent studies detected no effect of Rheb on this pathway [18,20]. The identification of Rheb effectors will surely be a priority to those in this field.

The pharmacological ramifications of these Rheb studies could be far reaching. Rheb and any Rheb GEF, if one exists, are now recognized as strong drug targets for the treatment of TSC. Two studies demonstrating that farnesylation of Rheb is important for its activation of mTOR signaling [19,20] suggest that farnesylation inhibitors could be effective against TSC. Interestingly, many oncogenes [e.g. receptor tyrosine kinases (RTKs), PI3K, Akt] and tumor suppressors (e.g. PTEN, TSC1/2) act upstream of Rheb activation. The development of an easy assay to detect levels of Rheb–GTP within tumors could be a valuable diagnostic tool for determining the course of treatment for TSC as well as various cancers. The drug rapamycin, which specifically inhibits TOR proteins, appears to be effective against cell and rodent models of TSC [7,24,25] and is emerging as an anticancer drug for many different types of tumors. Therefore, a Rheb–GTP detection assay might be useful in identifying those human tumors that are most likely to be sensitive to rapamycin treatment. The newly defined TSC–Rheb–mTOR connection has added these and many more exciting insights into this crucial pathway in human disease and has paved the way for further connections to be made.

Acknowledgements

We thank the Tuberous Sclerosis Alliance Rothberg Courage Fund and the American Cancer Society for support, and Nicole Logsdon for discussion and critical comments on the manuscript.

References

1 Gomez, M.R. *et al.* (1999) *Tuberous Sclerosis*, 3rd edn, Oxford University Press
2 Manning, B.D. *et al.* (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/Akt pathway. *Mol. Cell* 10, 151–162
3 Inoki, K. *et al.* (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* 4, 648–657
4 Potter, C.J. *et al.* (2002) Akt regulates growth by directly phosphorylating Tsc2. *Nat. Cell Biol.* 4, 658–665
5 Gao, X. *et al.* (2002) Tsc tumour suppressor proteins antagonize amino acid-TOR signalling. *Nat. Cell Biol.* 4, 699–704
6 Tee, A.R. *et al.* (2002) Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13571–13576
7 Jaeschke, A. *et al.* (2002) Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent. *J. Cell Biol.* 159, 217–224
8 Manning, B.D. and Cantley, L.C. (2003) United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signaling. *Biochem. Soc. Trans.* 31, 573–578
9 Wienecke, R. *et al.* (1995) Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. *J. Biol. Chem.* 270, 16409–16414
10 Xiao, G.H. *et al.* (1997) The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. *J. Biol. Chem.* 272, 6097–6100
11 Gao, X. and Pan, D. (2001) *TSC1* and *TSC2* tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* 15, 1383–1392
12 Tapon, N. *et al.* (2001) The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 105, 345–355
13 Potter, C.J. *et al.* (2001) *Drosophila Tsc1* functions with *Tsc2* to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* 105, 357–368
14 Stocker, H. *et al.* (2003) Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. *Nat. Cell Biol.* 5, 559–566
15 Saucedo, L.J. *et al.* (2003) Rheb promotes cell growth as a component of the insulin/TOR signaling network. *Nat. Cell Biol.* 5, 566–571
16 Mach, K.E. *et al.* (2000) Loss of Rhb1, a Rheb-related GTPase in fission

yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. *Genetics* 155, 611–622
17 Matsumoto, S. *et al.* (2002) Role of the Tsc1–Tsc2 complex in signaling and transport across the cell membrane in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 161, 1053–1063
18 Garami, A. *et al.* (2003) Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell* 11, 1457–1466
19 Castro, A.F. *et al.* (2003) Rheb binds TSC2 and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J. Biol. Chem.* 278, 32493–32496
20 Tee, A.R. *et al.* (2003) Tuberous sclerosis complex gene products, tuberin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* 13, 1259–1268
21 Inoki, K. *et al.* (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17, 1829–1834
22 Zhang, Y. *et al.* (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* 5, 578–581
23 Im, E. *et al.* (2002) Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. *Oncogene* 21, 6356–6365
24 Onda, H. *et al.* (2002) Tsc2 null murine neuroepithelial cells are a model for human tuber giant cells, and show activation of an mTOR pathway. *Mol. Cell. Neurosci.* 21, 561–574
25 Kenerson, H.L. *et al.* (2002) Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. *Cancer Res.* 62, 5645–5650

0968-0004/$ - see front matter
doi:10.1016/j.tibs.2003.09.003

Protein Sequence Motif

The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants

Jason Perry[1,2] and Yunde Zhao[2]

[1]Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA
[2]Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

A previously undetected domain, named CW for its conserved cysteine and tryptophan residues, appears to be a four-cysteine zinc-finger motif found exclusively in vertebrates, vertebrate-infecting parasites and higher plants. Of the twelve distinct nuclear protein families that comprise the CW domain-containing superfamily, only the microrchida (MORC) family has begun to be characterized. However, several families contain other domains suggesting a relationship between the CW domain and either chromatin methylation status or early embryonic development.

The term zinc finger was originally coined to describe a series of zinc-binding modules present in transcription factor IIIA, but its definition has since expanded to include a wide variety of compact domains whose structures are stabilized by the presence of one or more protein-chelated zinc ions [1–3]. Grishin and colleagues have recently undertaken a structural classification of zinc-binding domains, and have found that there are at least eight canonical protein-fold groups that fall under the umbrella of 'zinc finger' with the C_2H_2, the treble-cleft and the zinc-ribbon groups being the most highly represented in the current databases [4]. From a biological perspective, zinc fingers are perhaps even more diverse. Although they are most commonly known for interacting with nucleic-acid templates, such as DNA and RNA, zinc fingers also promote protein–protein interactions and interactions between proteins and small molecules. In these contexts, zinc-finger proteins participate in a host of fundamental biological processes ranging from DNA-based activities, such as transcription, replication and recombination to ubiquitination and the assembly of large protein complexes [3]. Here, we define the CW domain, which is composed of at least four cysteine and two tryptophan residues and is predicted to be another building block in the repertoire of zinc-binding structural modules.

Identification of the CW domain

We first noticed an unusual pattern of cysteine and tryptophan residues upon manual inspection of a predicted protein sequence (CAB83140) that emerged from one of our genetic screens (for auxin mutants) after no conserved domains were found searching the Pfam and SMART databases [5–7]. We next performed iterative PSI–BLAST searches of the non-redundant protein database using the described segment of sequence (of 61 amino acids) as a query with an inclusion cut-off score of 0.005 [8]. From this analysis, 102 sequences were retrieved, 36 of which were above the cut-off score (Figure 1). Of these 36 sequences, there were no false positive identifications. Six additional candidate sequences emerged following manual inspection of the sequences that had been retrieved in the search but had fallen below our prescribed threshold (Figure 1). The search converged after the fourth iteration, and the same group of proteins was retrieved regardless of which newly retrieved sequence was used as the query in subsequent searches. Domain boundaries were delimited by compiling all of the

Corresponding author: Yunde Zhao (yzhao@biomail.ucsd.edu).