S2 cell culture, expression constructs and immunoprecipitation. S2 cells were propagated in *Drosophila* serum-free medium (Invitrogen, Carlsbad, CA), as described previously[6]. Myc–Rheb was made in pAc5.1/V5-HisB vector (Invitrogen) by fusing the Myc epitope (MEQKLISEEDLNE) to the amino terminus of full-length Rheb. Point mutations in Tsc2 were introduced using QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). Constructs for epitope-tagged Tsc1 and Tsc2, and immunoprecipitation procedures have been described previously[3]. Antibodies against Myc, V5 and Flag epitopes were from Santa Cruz Biotechnology (Santa Cruz, CA), Invitrogen and Sigma (St Louis, MO), respectively.

***In vivo* labelling of Rheb.** *In vivo* labelling of Rheb was adapted from ref. 17. Briefly, 3×10^6 S2 cells per 60-mm plate were transfected with Myc–Rheb together with indicated combination of Tsc1 and Tsc2 plasmids using the Effectene reagent (Qiagen, Valencia, CA). After 48 h, cells were rinsed once and incubated overnight in phosphate-depleted Schneider's *Drosophila* Medium containing 250 μCi ml^{-1} ^{32}P-orthophosphate (HCl-free; Amersham Pharmacia Biotech, Piscataway, NJ). Labelled cells were washed once with PBS and lysed for 30 min in 0.5 ml ice-cold lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 0.2 mM EDTA, 0.2 mM EGTA, 10 mM magnesium chloride, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM GDP, 1 mM GTP and 1 mM phenyl methylsulphonyl fluoride (PMSF)). Cell lysates were centrifuged and one-tenth of each supernatant was taken out for western blotting. The remaining lysates were incubated with 2 μg of anti-Myc monoclonal antibody (Santa Cruz) and 20 μl of Protein G–agarose beads (Amersham Pharmacia Biotech) at 4 °C for 4 h. Immunoprecipitates were washed three times with lysis buffer and eluted with 20 μl of elution buffer (75 mM KH_2PO_4 at pH 3.4, 5 mM EDTA, 0.5 mM GTP and 0.5 mM GDP) at 85 °C for 3 min. Elutions were spotted on a PEI cellulose TLC plate (Selecto Scientific, Suwanee, GA), resolved in 1 M KH_2PO_4 at pH 3.4 for 90 min and detected by autoradiography.

Experiments involving amino-acid starvation were performed as above, except that ^{32}P-orthophosphate-labelled cells were transferred to complete Schneider's *Drosophila* Medium or amino-acid-free medium[6] for an additional 5 h before cell lysis. Experiments involving RNAi were also performed as above, except that S2 cells were cotransfected with Myc–Rheb and *Tsc2* dsRNA. dsRNA of the mammalian *CYP7A1* gene was used as controls in RNAi experiments. It should be noted that the GTP:GDP ratio of Rheb under normal conditions (that is, without Tsc1/Tsc2 overexpression or RNAi) varies between 5.1 and 7.2 in different experiments (Figs. 1a, c, d and 2c). This variability might be caused by the varying amounts of Rheb expressed in different experiments. It is known that Rheb expression levels affect its activation state[16].

***In vitro* GAP assay.** pGEX-4T-1 vector was used to express GST fusion proteins containing Rheb, Tsc2-GAP and Tsc2-GAP point mutants. The GST fusion of *Drosophila* Ras1 was a gift of M. White. GST–Tsc2-GAP and point mutants included amino acid 1384–1847 of Tsc2. GST fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using standard procedures (Amersham Pharmacia Biotech). A nitrocellulose filter assay[20] was used to measure *in vitro* GAP activity. Briefly, 5 μg of GST–Rheb or GST–Ras1 was incubated with 10 μCi of γ-^{32}P-GTP (or α-^{32}P-GTP if indicated) in 40 μl of loading buffer (20 mM Tris-HCl at pH 7.5, 25 mM sodium chloride, 0.1 M dithiothreitol and 5 mM EDTA) for 30 min at 30 °C. The reaction was stopped by adding ice-cold magnesium chloride to a final concentration of 5 mM and incubating on ice for 3 min. GTP-loaded protein (10 μl) was diluted in 50 μl GAP assay buffer (loading buffer containing 1 mM GTP and 5 mM magnesium chloride) containing 2 μg GST–Tsc2-GAP protein. At 0, 10, 20 and 30 min, 10-μl aliquots were diluted in 1 ml of ice-cold washing buffer (50 mM Tris-HCl at pH 7.5, 50 mM sodium chloride and 5 mM magnesium chloride), passed through nitrocellulose filters and washed with 12 ml washing buffer. The filters were dried and quantified by scintillation counting.