Regulation of the Mammalian Target Of Rapamycin Complex 2 (mTORC2)

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Summary

The growth controlling mammalian Target of Rapamycin (mTOR) is a conserved Ser/Thr kinase found in two structurally and functionally distinct complexes, mTORC1 and mTORC2. The tumor suppressor TSC1-TSC2 complex inhibits mTORC1 by acting on the small GTPase Rheb, but the role of TSC1-TSC2 and Rheb in the regulation of mTORC2 is unclear. Here we examined the role of TSC1-TSC2 in the regulation of mTORC2 in human embryonic kidney 293 cells. Induced knockdown of TSC1 and TSC2 (TSC1/2) stimulated mTORC2-dependent actin cytoskeleton organization and Paxillin phosphorylation. Furthermore, TSC1/2 siRNA increased mTORC2-dependent Ser473 phosphorylation of plasma membrane bound, myristoylated Akt/PKB. This suggests that loss of Akt/PKB Ser473 phosphorylation in TSC mutant cells, as reported previously, is due to inhibition of Akt/PKB localization rather than inhibition of mTORC2 activity. Amino acids and overexpression of Rheb failed to stimulate mTORC2 signaling. Thus, TSC1-TSC2 also inhibits mTORC2, but possibly independently of Rheb. Our results suggest that mTORC2 hyperactivation may contribute to the pathophysiology of diseases such as cancer and Tuberous Sclerosis Complex.

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I. Introduction

I.1 Growth

Cell growth, the increase in size due to accumulation of mass, is a fundamental process for eukaryotic life (Thomas and Hall, 1997). Environmental and genetic determinants, such as nutrients, growth factors and their according sensory signaling pathways control cell growth. The limiting factors for cell growth are amino acids (Dann and Thomas, 2006) and energy, which depends on glucose metabolism and mitochondria (Hardie et al., 2006). A major nutrient sensor in eukaryotes is the serine/threonine kinase target of rapamycin (TOR). Single-cell eukaryotes like the yeast *Saccharomyces cerevisiae* depend on environmental nutrients as their major stimulating source for growth. Metazoans distribute nutrients throughout the organism and specify cell growth via diffusible growth factors. Only the cells that express and present compatible receptors sense growth factors. Upon growth factor binding, the receptor triggers intracellular signaling cascades that induce growth. In higher eukaryotes like flies and mammals, signaling from growth factors converges with signaling from nutrients and energy in the TOR signaling pathway, indicating an evolutionary extension of the functionally conserved TOR signaling pathway (Jacinto and Hall, 2003; Martin and Hall, 2005). Remarkable examples for the importance of TOR signaling in biology include the contribution to human diseases such as cancer or tuberous sclerosis complex (TSC) (Petroulakis et al., 2006) and the embryonic lethality of *tor* deficient mice (Gangloff et al., 2004).

I.2 TOR

TOR was originally identified in yeast as the cellular target of the bacterial macrolide rapamycin (Heitman et al., 1991). Since then it was found in many eukaryotes including fungi, plants, worms, flies and mammals (Jacinto and Hall, 2003). Yeast has two distinct *TOR* genes, *TOR1* and *TOR2*, whereas all other eukaryotes investigated so far have only one *TOR*.

TOR is a typical phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) that exclusively phosphorylates proteins on serine or threonine residues (Abraham, 2004). TOR proteins are highly conserved and consist of N-terminal HEAT repeat stretches (huntingtin, elongation factor 3, A subunit of protein phosphatase 2A (PP2A), and TOR1) (Andrade and Bork, 1995). These stretches are followed by a FAT domain (FRAP, ATM, TTRAP) (Bosotti et al., 2000) that is located next to the FRB (FKBP12-rapamycin binding) domain (Lorenz and Heitman, 1995). The FRB domain is neighbouring the catalytic domain, which is located close to the C-terminal FATC domain.

HEAT repeats are predicted to form α-helical structures. The HEAT repeats in TOR mediate multimerization (Takahara et al., 2006), interaction with other proteins (Kim et al., 2002; Wullschleger et al., 2005) and membrane localization of yeast TOR2 (Kunz et al., 2000). The FAT and FATC domains, which flank the FRB domain and the catalytic domain, are present in all PIKK family members. Based on structural resolution, the FATC domain in TOR forms an α-helix with a C-terminal disulfide bond between two cysteine residues that is sensitive to the cellular redox state and mediates TOR protein stability (Dames et al., 2005). Inhibition of TOR by rapamycin occurs via binding of the FKBP12-rapamycin complex to the bundle of four-α-helices in TOR that are formed by the FRB domain (Leone et al., 2006). Apparently, the FRB domain interacts with the catalytic domain in TOR and together these two TOR domains mediate yeast caffeine-sensitivity (Reinke et al., 2006). The catalytic domain in TOR is highly homologous to the catalytic domains in PI3-kinases but unlike those, the TOR kinase does not phosphorylate lipids, which is the major criterion for TOR's affiliation to the PIKK family (Abraham, 2004). A repressor domain has been identified in mammalian TOR (mTOR) between amino acids 2430 to 2450 as its deletion increases mTOR activity (Edinger and Thompson, 2004; Sekulic et al., 2000). Two proximal phosphorylation sites are located in this domain, Thr2446 and Ser2448. Thr2446 is phosphorylated by the AMP-dependent kinase (AMPK) (Cheng et al., 2004) and Ser2448 is phosphorylated by S6K1 (Chiang and Abraham, 2005; Holz and Blenis, 2005). It is unknown whether phosphorylation of mTOR on these two sites has physiological consequences. In addition, mTOR autophosphorylates on Ser2481 but also for this site the physiological relevance is not determined (Peterson et al., 2000).

I.3 TOR complexes

TOR controls a wide range of cellular processes, of which some are rapamycin-sensitive and others are rapamycin-insensitive. Whether a TOR signal is rapamycin-sensitive or not depends on the two structurally distinct protein hetero-complexes in which TOR is signaling, the rapamycin-sensitive TOR complex 1 (TORC1) and the rapamycin-insensitive TOR complex 2 (TORC2) (Loewith et al., 2002). Both TOR complexes, like TOR itself, are structurally and functionally conserved in eukaryotes including fungi, plants (so far only TORC1), worms (so far only TORC1), flies and mammals (Wullschleger et al., 2005). TOR complexes form active homo-multimers in yeast, flies and mammals (Takahara et al., 2006; Wullschleger et al., 2005; Zhang et al., 2006b).

I.3.1 TORC1

Yeast TORC1 consists of either TOR1 or TOR2, KOG1 (kontroller of growth), LST8 (lethal with sec thirteen) and TCO89 (Loewith et al., 2002; Reinke et al., 2004). TCO89 is the only non-essential component of TORC1 in yeast. Drosophila TORC1 (dTORC1) contains dTOR (Oldham et al., 2000; Zhang et al., 2000), dRaptor (Hara et al., 2002; Sarbassov et al., 2005b) and dLST8 (Yang et al., 2006). In mammalian TORC1 (mTORC1), mTOR (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994) associates with raptor (regulatory associated protein of mTOR; KOG1 ortholog) (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002) and mLST8 (Kim et al., 2003; Loewith et al., 2002). So far, no ortholog was found for TCO89 in mTORC1.

Raptor consists of a highly conserved N-terminal domain of unknown function that locates next to three HEAT repeats, which are followed by seven WD-40 repeats. WD-40 repeats form propeller-like structures that mediate protein/protein interactions (Pickles et al., 2002). Multiple sites in raptor are needed for its interaction with the HEAT repeats in mTOR (Kim et al., 2002). It was reported that the mTOR/raptor interaction is regulated by nutrients and mLST8 (Kim et al., 2002; Kim et al., 2003), but a different report could not reproduce the nutrient sensitivity of

the mTOR/raptor interaction (Oshiro et al., 2004). In yeast, nutrients do not affect the interaction between TOR and KOG1 (Loewith et al., 2002). The FKBP12-rapamycin complex binds TOR exclusively in TORC1 (Loewith et al., 2002). The inhibitory effect of rapamycin on mTOR signaling towards raptor-dependent substrates results from the interference of the FKBP12-rapamycin complex with the mTOR/raptor association (Kim et al., 2002; Oshiro et al., 2004). LST8 consists of seven WD-40 repeats and binds to the catalytic domain of TOR in both complexes, TORC1 and TORC2. In both of these complexes LST8 mediates integrity and is essential for TOR kinase activity (Kim et al., 2003; Loewith et al., 2002; Wullschleger et al., 2005).

I.3.2 TORC2

TORC2 in yeast contains TOR2, AVO1 (adheres voraciously to TOR2), AVO2, AVO3, LST8, and BIT61 (Loewith et al., 2002; Reinke et al., 2004). AVO2 and BIT61 are the only non-essential components of TORC2 in yeast. In *Dictyostelium discoideum*, TORC2 (Dd-TORC2) consists of Dd-TOR, RIP3 (AVO1 ortholog), Pianissimo (AVO3 ortholog) and Dd-LST8 (Lee et al., 2005b). Drosophila TORC2 (dTORC2) is formed by dTOR, dSIN1 (AVO1 ortholog) (Frias et al., 2006), dRictor (AVO3 ortholog) (Sarbassov et al., 2005b) and dLST8. Mammalian TORC2 (mTORC2) consists of mTOR, SIN1 (AVO1 ortholog) (Frias et al., 2006; Jacinto et al., 2006), rictor (rapamycin-insensitive companion of mTOR; AVO3 ortholog) and mLST8 (Jacinto et al., 2004; Sarbassov et al., 2004). So far, no orthologs were found for AVO2 and BIT61 in mTORC2. SIN1 and rictor bind cooperatively to mTOR in mTORC2 (Frias et al., 2006; Jacinto et al., 2006), similar to the cooperative binding of AVO1 and AVO3 in yeast TORC2 (Wullschleger et al., 2005). AVO1 and AVO3 bind multiple sites in TOR2, of which some are located in the HEAT repeats of TOR2. Functional domains in SIN1 and rictor have not been identified. Knockout of either *SIN1* or *rictor* in mice results in embryonic lethality (Jacinto et al., 2006; Shiota et al., 2006).

Although rapamycin does not inhibit TORC2 because the FKBP12-rapamycin complex cannot bind to TORC2, recent work claimed that prolonged rapamycin treatment can inhibit mTORC2 assembly and thereby abrogate mTORC2 signaling

(Sarbassov et al., 2006). As rapamycin could inhibit mTORC2 assembly only in some but not in all tested cell types, the physiological effects of long-term rapamycin treatment await further characterization.

I.4 TOR functions

TOR controls cell growth by regulating transcription, translation, metabolism, survival and the cytoskeleton.

I.4.1 TORC1 functions in yeast

In yeast, TORC1 controls translation initiation by repressing GCN2 from inhibiting the eukaryotic initiation factor eIF2α (Cherkasova and Hinnebusch, 2003), and by repressing the eIF4E associated protein EAP1 (Matsuo et al., 2005). As a consequence from translational control, TORC1 affects G1-phase progression during the cell cycle (Thomas and Hall, 1997). TORC1 controls nutrient- and stress-responsive transcription in yeast by regulating localization of several transcription factors such as GLN3, MSN2, MSN4 and RTG1-RTG3 (Jacinto and Hall, 2003). In concert with PKA, TORC1 controls yeast ribosome biogenesis by acting on transcriptional regulators like the transcription factors RRN3 and FHL1, and the histone deacetylase RPD3 (Martin and Hall, 2005). Type 2A protein phosphatases (PP2As), which dephosphorylate NPR1 and GLN3 in yeast, are controlled by TORC1 via regulation of the phosphatase repressor TAP42 and its regulator TIP41 (Jacinto and Hall, 2003). Nutrient uptake in yeast is also controlled by TORC1 via regulation of the tryptophan permease TAT2 (Beck et al., 1999) and the glucose transporter HXT1 (Schmelzle et al., 2004). TORC1 in yeast controls autophagy and glycogen accumulation via signaling crosstalk with the PKA pathway (Schmelzle et al., 2004). TORC1 inhibits yeast autophagy also via phosphorylation of ATG13, which is a co-activator for the autophagy inducing kinase ATG1 (Miron and Sonenberg, 2001). Inhibition of TORC1 increases life span in yeast (Powers et al., 2006).

I.4.2 TORC2 functions in yeast

Yeast TORC2 controls cell polarity by activating the RHO1 and RHO2 GDP/GTP exchange factor (GEF) ROM2 to regulate actin cytoskeleton organization (Schmidt et al., 1997). Signals from RHO towards the yeast actin cytoskeleton are transduced via the RHO effector PKC1 (Helliwell et al., 1998). In addition, TORC2 signaling maintains yeast viability via direct phosphorylation of the substrates YPK2 (Kamada et al., 2005) and the functionally redundant proteins SLM1 and SLM2 (Audhya et al., 2004). SLM1 and SLM2 mediate TORC2 inhibition towards calcineurin-dependent gene transcription (Mulet et al., 2006).

I.4.3 TORC2 functions in Dictyostelium

In Dictyostelium, Dd-TORC2 controls cell polarity, chemotaxis, activation of adenylyl cyclase and Akt/PKB phosphorylation (Lee et al., 2005b) (see I.4.7 and I.5.3 for further details on Akt/PKB phosphorylation).

I.4.4 TORC1 functions in Drosophila

In *Drosophila melanogaster*, dTOR controls larval development, cell growth and overall body size, which is mediated at least partially via regulation of Drosophila S6 Kinase (dS6K) (Montagne et al., 1999; Oldham et al., 2000; Zhang et al., 2000). Like in mammals, dS6K hyperactivation by dTORC1 exerts negative feedback signaling towards Chico, the Drosophila IRS homolog, to inhibit insulin stimulation of Drosophila protein kinase B (dAkt) (Yang et al., 2006) (see I.4.6 and I.5.2 for further details on IRS-1 regulation). dTORC1 controls translation initiation by regulating dS6K and d4E-BP (eIF4E-binding protein) (Miron et al., 2003; Miron and Sonenberg, 2001; Radimerski et al., 2002; Sarbassov et al., 2004). Like in yeast, dTORC1 affects cell cycle progression (Bjorklund et al., 2006). Furthermore, dTORC1 affects the timing of neuronal differentiation (Bateman and McNeill, 2004). In the Drosophila fat body, dTOR controls a nutrient-sensitive humoral growth mechanism towards peripheral tissue (Colombani et al., 2003), lipid vesicle aggregation (Zhang et al.,

2000) and autophagy (Rusten et al., 2004; Scott et al., 2004). dTORC1 controls autophagy and protein degradation also via inhibition of the clathrin uncoating ATPase Hsc70-4 that mediates a late step in endocytosis (Hennig et al., 2006). dTORC1 activates transcription of genes that are regulated by a hypoxia inducible factor (HIF) responsive element (HRE) (Dekanty et al., 2005). In fruit flies, like in yeast and worms, inhibition of dTORC1 mediates life span extension (Kapahi and Zid, 2004; Martin and Hall, 2005).

I.4.5 TORC2 functions in Drosophila

Similar to mTORC2 signaling, dTORC2 controls activation of dAkt through direct phosphorylation of the hydrophobic motif in dAkt (Sarbassov et al., 2005b) (see I.4.7 and I.5.3 for further details on Akt/PKB phosphorylation).

I.4.6 TORC1 functions in mammals

In mammals, mTORC1 controls cell growth predominantly by stimulating the translational machinery and thus, protein synthesis (see Figure I-1). Translation initiation is activated by mTORC1 via phosphorylation of 4E-BP (eIF4E-binding protein) (Hay and Sonenberg, 2004). mTORC1 also stimulates translation by phosphorylating S6 kinase (S6K) (Ruvinsky and Meyuhas, 2006). Translation is further activated by mTORC1-controlled ribosome biogenesis, which occurs via S6K-regulated translation of ribosomal proteins and transcription of ribosomal RNA (rRNA) (Mayer and Grummt, 2006). Like in Drosophila and yeast, mTORC1 affects cell cycle progression (Fingar and Blenis, 2004).

Learning and memory are enhanced by mTORC1 (Bekinschtein et al., 2006; Dash et al., 2006; Tischmeyer et al., 2003) via regulation of long-term potentiation (LTP) (Cammalleri et al., 2003; Tang et al., 2002) and long-term depression (LTD) (Hou and Klann, 2004) at post-synapses of neuronal dendrites in the hippocampus and the cerebral cortex. LTP and LTD drive synaptic plasticity and are regulated via inhibition of 4E-BP, which contributes locally to the translational competence of

certain stored mRNAs (Banko et al., 2006; Gong et al., 2006). mTORC1 maintains LTP upon stimulation by Brain-Derived Neurotrophic Factor (BDNF) (Hay and Sonenberg, 2004) and upon activation of glutamatergic receptors (Lenz and Avruch, 2005). Changes of dendritic spine and soma morphology in hippocampal neurons, resulting from deficiency in the tuberous sclerosis complex genes *TSC1* and *TSC2*, are mediated by mTORC1 hyperactivation (Tavazoie et al., 2005). Furthermore, mTORC1 signaling in the hypothalamus reduces food intake (Cota et al., 2006).

mTORC1 controls insulin-sensitivity via an S6K-dependent negative feedback loop towards insulin receptor substrate-1 (IRS-1) (Harrington et al., 2005) that limits glucose uptake in skeletal muscle, adipocytes and liver cells during prolonged insulin stimulation (Tremblay et al., 2005). Adipogenesis is supported by mTORC1 via enhancement of rapamycin-sensitive PPARγ (peroxisome proliferator-activated receptor) nuclear receptor activity (Kim and Chen, 2004) and via insulin- and amino acid-stimulated phosphorylation of lipin (Huffman et al., 2002). mTORC1 controls transcription by promoting expression and stability of the transcription factor HIF-1 (hypoxia-inducible factor 1), which increases glucose uptake and glycolysis by raising expression of the glucose transporter GLUT1 (Hudson et al., 2002) and glycolytic enzymes during hypoxia (Nakamura et al., 2005). By regulating HIF-1, hyperactive mTORC1 also induces neovascularisation in cancer cells due to increased expression of VEGF (vascular endothelial growth factor) (Brugarolas et al., 2003; Nakamura et al., 2006). Transcription is further regulated by mTORC1 via phosphorylation of the transcription factor STAT3 (signal transducer and activator of transcription) (Yokogami et al., 2000). Via STAT3 phosphorylation, mTORC1 controls rapamycin-sensitive survival of neural stem cells (Androutsellis-Theotokis et al., 2006). mTOR, most likely in mTORC1, controls clathrin mediated endocytosis that is required for transferrin internalization and thus, iron uptake (Pelkmans et al., 2005). mTORC1 suppresses autophagy in nutrient rich conditions (Meijer and Codogno, 2006).

Since mTORC1 also inhibits the phosphatase PP2A (Peterson et al., 1999) and activates PP5 (Huang et al., 2004) it is important to consider that mTORC1 regulates not only phosphorylation of downstream targets but also dephosphorylation (Gingras et al., 2001b).

Some of the developmental defects observed in *tor-/-* knockout mice rely on impaired mTORC1 signaling, as can be seen in the trophoblast growth defect that appears after rapamycin treatment (Martin and Sutherland, 2001).

I.4.7 TORC2 functions in mammals

In mammals, mTORC2 controls actin cytoskeleton organization but the regulatory mechanism is still unclear and might differ between cell types (see Figure I-1). In HeLa cells, it has been claimed that mTORC2 signals to actin via activating PKCα (Protein Kinase C) to prevent stress fiber formation and alter Paxillin localization (Sarbassov et al., 2004). In NIH3T3 fibroblasts, it was found that mTORC2 is required for Paxillin tyrosine phosphorylation (Tyr118) and activation of the small GTPases Rac and Rho (Jacinto et al., 2004). Paxillin is an adaptor for many proteins and is involved in the regulation of cell shape and motility (Brown and Turner, 2004). Paxillin, like mTORC2, has a fundamental function in mammalian growth and development as illustrated by the embryonic lethality of *paxillin-/-* knockout mice (Hagel et al., 2002). Adhesion or stimulation with either growth factors or phorbol esters induces Paxillin Tyr118 phosphorylation by FAK (focal adhesion kinase) or Src (Sarcoma causing protein). Lamellipodia formation and cell motility depend on Paxillin Tyr118 phosphorylation. Paxillin Tyr118 phosphorylation recruits DOCK180, a GEF for Rac1, to activate Rac1. Rac1 activation induces lamellipodia formation (Nobes and Hall, 1995).

mTORC2 activates Akt/PKB (product of the retrovirus AKT8/Protein Kinase B) by directly phosphorylating Ser473 in the hydrophobic motif of Akt/PKB (Sarbassov et al., 2005b). mTORC2-dependent regulation of Akt/PKB is needed for Akt/PKB-dependent inhibition of the Forkhead box O transcription factors (FoxO) (Jacinto et al., 2006). Although Akt/PKB is an upstream activator of mTORC1, mTORC2 does not regulate mTORC1 signaling (see also I.5.3) (Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004).

I.5 mTOR effectors

mTOR regulates many important steps in growth control by directly phosphorylating 4E-BP1, S6K and Akt/PKB (see Figure I-1).

I.5.1 4E-BP

4E-BP1 (eIF4E-binding protein) represses translation initiation via inhibitory binding to eIF4E (eukaryotic initiation factor 4E) . mTOR-dependent phosphorylation of 4E-BP1 on Thr37, Thr46, Ser65 and Thr70 inhibits 4E-BP1 by decreasing its affinity to eIF4E and releases eIF4E (Burnett et al., 1998; Gingras et al., 2001a; Mothe-Satney et al., 2000b). The rapamycin-sensitive phosphorylation sites Thr37, Thr46, Ser65 and Thr70 in 4E-BP1 share a Ser/Thr-Pro motif and are hierarchically regulated by mTOR (Brunn et al., 1997; Gingras et al., 1999; Mothe-Satney et al., 2000a). mTOR potentially phosphorylates all rapamycin-sensitive sites in 4E-BP1 directly (Brunn et al., 1997; Mothe-Satney et al., 2000a). However, due to differences in rapamycin-sensitivity and in-vitro phosphorylation between the sites Ser65/Thr70 and Thr37/Thr46, it has been proposed that mTOR would directly phosphorylate Thr37/Thr46 but regulate phosphorylation of Ser65 and Thr70 in 4E-BP1 via an indirect mechanism (Gingras et al., 2001b). Upon release from 4E-BP1, eIF4E binds to one of the scaffold proteins eIF4GI or eIF4GII, which additionally recruits the RNA helicase eIF4A to form the protein complex referred to as eIF4F. eIF4F binds the 7-methylguanosine triphosphate capped 5'-end of mRNA and eIF4A together with the co-factor eIF4B melts RNA secondary structures. The ribosome-associated factor eIF3 then tethers the 40S ribosomal subunit to eIF4G. The 5'- and 3'-termini of the mRNA are brought into proximity through the interaction of the poly (A)-binding protein with eIF4G, which increases the rate of re-initiation. The small ribosomal subunit together with a ternary complex composed of eIF2, GTP, and the initiating Met-tRNAi (methionine transfer RNA) are then scanning the mRNA from 5' to 3' for an AUG start codon. At the start codon, the initiation factors are released, the large 60S ribosomal subunit is recruited, and translation elongation continues (Gingras et al., 2001b; Raught et al., 2000; Wang and Proud, 2006).

4E-BP1 is regulated by mTORC1 (Hara et al., 2002) and even mTOR-dependent in-vitro phosphorylation of 4E-BP1 that is bound to eIF4E is sensitive to the FKBP12-rapamycin complex (Wang et al., 2005). A conserved TOR signaling (TOS) motif in 4E-BP1 mediates interaction of 4E-BP1 with the mTORC1-specific component raptor, which is crucial for mTOR-dependent phosphorylation of 4E-BP1 (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). The conserved TOS motif (with the amino acid sequence: FEMDI) is located in the C-terminus of 4E-BP and corresponds in function and sequence to a TOS motif in the N-terminus of S6K (Schalm and Blenis, 2002). Another conserved sequence in the N-terminus of 4E-BP1 is the RAIP motif (termed according to its amino acid sequence) (Tee and Proud, 2002). Although the function of the RAIP motif is controversially debated, this motif is required for phosphorylation of specific mTOR-dependent sites in 4E-BP1 (Beugnet et al., 2003; Choi et al., 2003; Eguchi et al., 2006).

4e-bp1-/- knockout mice do not develop a dramatic phenotype other than a reduction in white adipose tissue. This mild phenotype may be due to the redundant roles of the expressed 4E-BP1 homologs 4E-BP2 and 4E-BP3 (Tsukiyama-Kohara et al., 2001). The physiological relevance of a tight control of translation initiation is reflected by the oncogenic potential of the eIF4F subunits and the tumor suppressing effects of 4E-BP1 (Petroulakis et al., 2006).

I.5.2 S6K

S6K1 belongs to the AGC family of serine/threonine protein kinases (Protein Kinases A, G and C) (Belham et al., 1999; Peterson and Schreiber, 1999) and has two mammalian isoforms out of a single gene, p70S6K1 and p85S6K1. Both isoforms are similarly regulated and differ in their N-terminus by a nuclear localization signal (NLS) that is only present in p85S6K1 (Dufner and Thomas, 1999). Mammals have a second S6K gene *S6K2*, a close homolog of S6K1, which is highly conserved in sequence, function and regulation (Martin et al., 2001). S6K1 activation requires phosphorylation in its activation loop on Thr229 by the phosphoinositide-dependent kinase PDK1 (Alessi et al., 1998). Thr229 phosphorylation depends on prior rapamycin-sensitive phosphorylation of the hydrophobic motif in S6K1 on Thr389 by

mTORC1 (Ali and Sabatini, 2005; Hara et al., 2002; Kim et al., 2002; Pullen et al., 1998). Phosphorylation on Thr389 allows S6K1 to interact with PDK1 (Saitoh et al., 2002). mTOR also phosphorylates the rapamycin-sensitive Ser371 in S6K1 that is required for S6K activation (Saitoh et al., 2002). Furthermore, mTOR phosphorylates the Ser/Thr-Pro sites Ser411, Thr421 and Ser421 (Isotani et al., 1999) in the S6K1 autoinhibitory domain to increase S6K1 catalytic activity (Dufner and Thomas, 1999). The TOS motif in the N-terminus of S6K1 (amino acid sequence: FDIDL), analogous to the TOS motif in 4E-BP1, mediates interaction of S6K1 with raptor and is crucial for mTOR-dependent phosphorylation of S6K1 (Nojima et al., 2003; Schalm and Blenis, 2002). In S6K1, the TOS motif suppresses the inhibitory effect of a short domain in the C-terminus towards the catalytic domain (Schalm et al., 2005).

S6K1 had been suspected to specifically increase translation of 5'-TOP (terminal oligopyrimidine tract) mRNA by phosphorylating the 40S ribosomal protein S6 (Hay and Sonenberg, 2004). 5'-TOP is a highly variable structure near the 5'-cap of mRNA and is overrepresented in transcripts that encode components of the translational machinery (Ruvinsky and Meyuhas, 2006). However, genetic studies in mice revealed that 5'-TOP mRNAs are not regulated by S6K or S6, as translation of 5'-TOP mRNAs is regulated normally in mouse embryonic fibroblasts (MEFs) from *s6k1*-/- and *s6k2*-/- double knockout mice (Pende et al., 2004) and in MEFs where S6 cannot be phosphorylated (Ruvinsky et al., 2005). Therefore it remains unknown how mTOR regulates translation of 5'-TOP mRNA.

S6k1-/- knockout mice are smaller and protected against obesity on a high fat diet, having smaller adipocytes and less white adipose tissue due to increased lipolysis (Um et al., 2004). Furthermore, *s6k1-/-* knockout mice also have a reduced β-cell mass and thus, are limited in glucose uptake due to insufficient insulin secretion. Due to a loss of IRS-1 (insulin receptor substrate) inhibition by S6K1, *s6k1-/-* knockout mice are hypersensitive to insulin. S6K1 induces negative feedback inhibition towards insulin signaling by phosphorylating IRS-1 on Ser302, which blocks the interaction of the insulin receptor (IR) with IRS-1 and inhibits PI3K activation upon insulin stimulation (Harrington et al., 2004; Shah et al., 2004).

By destabilizing PDCD4 (programmed cell death protein 4), a tumor suppressor that prevents the interaction between eIF4A and eIF4G in a rapamycin-sensitive manner, S6K1 activates translation initiation (Dorrello et al., 2006). To promote eIF4B recruitment to eIF4A, S6K1 also phosphorylates eIF4B on Ser 422 (Holz et al., 2005). S6K1 activates translation elongation by phosphorylation of eEF2K, the kinase that inhibits the eukaryotic elongation factor 2 (eEF2) (Wang et al., 2001). S6K1 induces ribosome biogenesis via phosphorylation of the transcription factors UBF and TIF1A that mediate rRNA transcription (Hannan et al., 2003; Mayer et al., 2004). S6K1 phosphorylates mTOR in the repressor domain on Ser2448 (Chiang and Abraham, 2005; Holz and Blenis, 2005).

I.5.3 Akt/PKB

Akt/PKB is an AGC kinase with a N-terminal pleckstrin homology (PH) domain that binds to PI3K-generated phosphatidylinositol (3,4,5) triphosphate (PIP_3) and $PI(3,4)P_2$ (PIP_2) (Scheid and Woodgett, 2003). Adjacent to the PH domain is the catalytic domain in Akt/PKB that contains a regulating T-loop. Near the C-terminus in Akt/PKB lies a hydrophobic motif within a regulatory domain. Mammals have three different Akt/PKB genes: *Akt1*/*PKB*α, *Akt2*/*PKB*β, and *Akt3*/*PKB*γ. The three isoforms differ only slightly in sequence but their tissue-specific expression in mice correlates at least partially with their physiological importance (Yang et al., 2004).

akt1-/- knockout mice are small because of a nutrient shortage during development due to a smaller placenta (Yang et al., 2003). *akt2-/-* deficient mice are diabetic and develop hyperinsulinaemia, insulin resistance and glucose intolerance because insulin action in the liver and skeletal muscle is impaired, which results in defective glucose clearance from the blood (Cho et al., 2001). In *akt3-/-* mutant mice, brain size and weight are reduced by about 25% with preserved brain structures (Easton et al., 2005; Tschopp et al., 2005). Creation of *akt1-/-* and *akt2-/-* double mutant mice and *akt1-/-* and *akt3-/-* double mutant mice revealed functional redundancy for the 3 Akt/PKB isoforms as the resulting double mutant phenotypes are more drastic than the assumed accumulation of the single mutant phenotypes. *akt1-/-* and *akt2-/-* double knockout mice die shortly after birth, are smaller and have

defects in the development of skin, bone, skeletal muscle and adipose tissue (Peng et al., 2003). *akt1-/-* and *akt3-/-* double mutants are embryonically lethal (Yang et al., 2004).

Akt/PKB activation relies on its translocation to the plasma membrane via binding of its PH domain to PI3K-generated PIP_3 and PIP_2 (Andjelkovic et al., 1997). At the membrane, activation of Akt/PKB requires growth factor stimulated phosphorylation in its T-loop on Thr308 and in its hydrophobic motif on Ser473 (Alessi et al., 1996). PDK1 phosphorylates Thr308 in the T-loop of Akt/PKB (Alessi et al., 1997; Stephens et al., 1998; Williams et al., 2000). Like Akt/PKB, PDK1 possesses a PH domain and requires translocation to the plasma membrane via binding to PIP_3 and PIP_2 for the activation of Akt/PKB. Only recently, mTORC2 was identified as the kinase complex that phosphorylates the hydrophobic motif on Ser473 in plasma membrane bound Akt/PKB (Sarbassov et al., 2005b). Phosphorylation of plasma membrane bound Akt/PKB on Ser473 requires additional PI3K activity in a manner that is distinct from Akt/PKB localization (Scheid et al., 2002). Whether phosphorylation in the T-loop of Akt/PKB depends on priming by phosphorylation in its hydrophobic motif, a hierarchy that is found in other AGC kinases, has been debated (Biondi et al., 2001). Recent data from mTORC2-specific knockout mice provided evidence that Akt/PKB phosphorylation in the T-loop on Thr308 does not rely on *a priori* phosphorylation of Ser473 (Jacinto et al., 2006; Shiota et al., 2006). Phosphorylation of the glycogen synthase kinase-3 (GSK-3) and tuberin (TSC2) by Akt/PKB occurs independently of Akt/PKB Ser473 phosphorylation, whereas phosphorylation of FoxO1 and FoxO3a by Akt/PKB was lost upon lack of Ser473 phosphorylation that was due to a defect in mTORC2. Therefore, phosphorylation on Ser473 in Akt/PKB appears to specify Akt/PKB activity towards distinct substrate pools. However, more recent data describes that the Akt/PKB-dependent phosphorylation sites in GSK-3 are phosphorylated by S6K1 in TSC deficient cells (Zhang et al., 2006a). A better understanding of the physiological consequences of Akt/PKB Ser473 phosphorylation requires further examination to determine if GSK-3 is phosphorylated by Akt/PKB or S6K1 in mTORC2 deficient cells which lack Akt/PKB Ser473 phosphorylation.

Akt/PKB controls a wide range of cellular functions, including glycogen synthesis, cell growth, cell cycle, cell survival, and transcription via the phosphorylation of a growing list of direct substrates (Brazil and Hemmings, 2001; Fayard et al., 2005). Highlights of Akt/PKB signaling include inhibition of GSK-3 via direct phosphorylation to regulate transcription and promote glycogen synthesis and protein synthesis (Frame and Cohen, 2001). Akt/PKB regulates cell cycle progression by phosphorylation of FoxO transcription factors and p27 (Brazil et al., 2004). Akt/PKB-dependent phosphorylation of FoxO transcription factors and Bad prevents apoptosis. Akt/PKB phosphorylates and inactivates TSC2, an upstream repressor of mTORC1, thereby promoting cell growth (Martin and Hall, 2005).

Figure I-1.

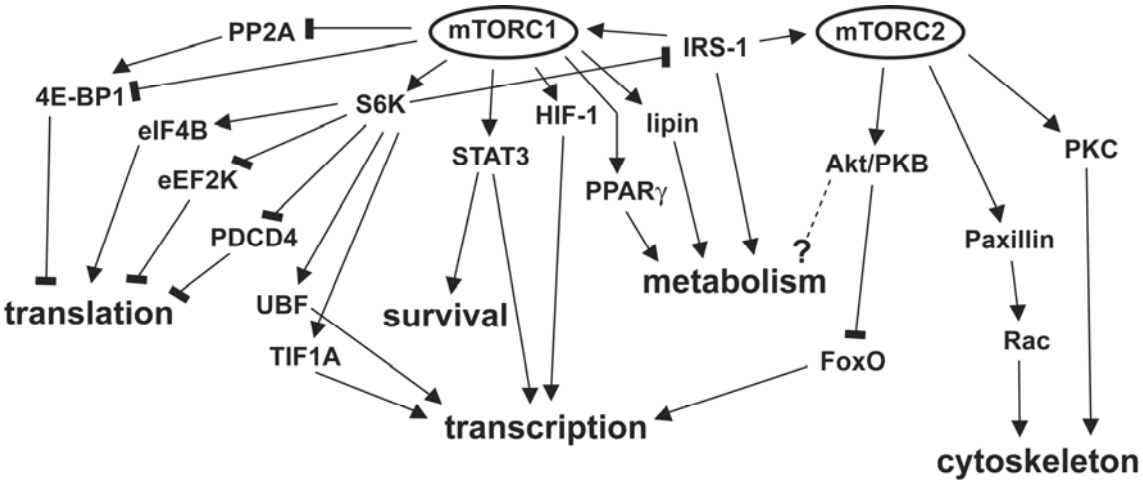

Figure I-1. Signaling network downstream of mTOR (see I.4 and 1.5 for details).
A dashed line with question mark illustrates a putative link without evidence.
An arrowhead represents activation and a bar inhibition.

I.6 Regulation of mTORC1

mTORC1 is activated by growth factors, phorbol esters and amino acids, whereas low cellular energy and hypoxia inhibit mTORC1 (see Figure I-2).

I.6.1 Growth factors activate mTORC1

Growth factors such as insulin induce rapamycin-sensitive phosphorylation of the mTORC1 readouts S6K and 4E-BP via activation of PI3K (Hay and Sonenberg, 2004; Thomas and Hall, 1997). Upon extracellular binding of a growth factor to its receptor tyrosine kinase (RTK) the RTK dimerizes and the cytoplasmic tail of the transmembrane RTK is autophosphorylated (Weiss and Schlessinger, 1998). RTK autophosphorylation recruits and activates the heterodimeric class Ia PI3K, a process that can involve a RTK specific adaptor molecule like the insulin receptor substrate 1 (IRS-1) in the case of the insulin receptor (IR) (Engelman et al., 2006). Upon activation, PI3K generates $PI(3,4,5)P_3$ from $PI(4,5)P_2$ by phosphorylating the D3 position of the lipid. The phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a lipid phosphatase that counteracts PI3K signaling by dephosphorylating the D3 positions of PIP_2 and PIP_3.

Genetic epistasis experiments in the Drosophila eye and wing revealed that dTSC1 (hamartin) and dTSC2 (tuberin) negatively regulate growth and that they signal between activated dPI3K and dS6K, downstream of dAkt and upstream of dTOR (Gao and Pan, 2001; Gao et al., 2002; Li et al., 2004; Potter et al., 2001; Tapon et al., 2001). *Hamartin* (*TSC1*) and *Tuberin* (*TSC2*) are the two genes that were identified to cause the familial autosomal disorder tuberous sclerosis complex (TSC) (Consortium, 1993; van Slegtenhorst et al., 1997). Hamartin has a potential transmembrane domain close to the N-terminus, a tuberin binding region and a coiled coil domain in the N-terminal part. Tuberin has a predicted leucine zipper motive near the N-terminus, which is followed by two small coiled coil domains and a C-terminal GAP domain. The N-terminus of tuberin binds to hamartin (Krymskaya, 2003). The TSC1-TSC2 complex forms rapidly after translation and is predominantly cytosolic (Nellist et al., 1999). Mice deficient for *tsc1-/-* or *tsc2-/-* are embryonically lethal due to defective liver development (Kwiatkowski et al., 2002; Onda et al., 1999). In mammals, it was found that TSC1 and TSC2 interact (van Slegtenhorst et al., 1998) and together form the TSC1-TSC2 complex to inhibit mTORC1 signaling (Tee et al., 2002). PI3K activation of TORC1 is mediated via TSC2 phosphorylation by Akt/PKB in flies and mammals, with the major sites being Ser939 and Thr1462 in human TSC2 (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002).

Phosphorylation of TSC2 by Akt/PKB disrupts the interaction between TSC1 and TSC2, which inhibits the tuberous sclerosis complex (TSC1-TSC2) and leads to ubiquitin-mediated TSC2 degradation (Benvenuto et al., 2000).

TSC1-TSC2 inhibits TOR in mammals and Drosophila by negatively regulating the TOR activator Rheb (Ras homolog enriched in brain) (Manning and Cantley, 2003), a small GTP-binding protein. Via a GAP (GTPase activating protein) domain in TSC2, the TSC1-TSC2 complex activates the GTPase in Rheb (Garami et al., 2003; Inoki et al., 2003a; Tee et al., 2003b; Zhang et al., 2003b), which results in Rheb inactivation due to GDP (guanosyl diphosphate) binding. Insulin stimulates Rheb GTP (guanosyl triphosphate) binding (Garami et al., 2003) and GTP bound Rheb activates the TORC1 targets S6K and 4E-BP in mammals and Drosophila even in the absence of growth factors (Garami et al., 2003; Inoki et al., 2003a; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2003b; Zhang et al., 2003b).

Growth factor binding to RTK also activates the MAPK (mitogen-activated protein kinase) signaling cascade via the kinases Ras and Raf towards MEK1/2 (MAPK/ERK kinases 1 and 2), stimulating ERK1/2 (extracellular-signal related kinases 1 and 2) (Roux and Blenis, 2004; Shaw and Cantley, 2006). The RTK recruits the Ras GEF son-of-sevenless (SOS) for activation of Ras. In addition to RTK activation, phorbol esters strongly stimulate ERK1/2 by recruiting the Ras guanyl nucleotide-releasing protein (RasGRP), which functions as a Ras GEF (Roose and Weiss, 2000). Activation of the MAPK cascade induces phosphorylation of human TSC2 on Ser540 and Ser664 by ERK1/2 and on Ser1798 by the p90 ribosomal S6K1 (RSK1), which is an ERK1/2 substrate (Ma et al., 2005a; Roux et al., 2004). Phosphorylation of TSC2 on Ser540, Ser664 and Ser1798 inactivates the TSC1-TSC2 complex. Besides mTORC1 activation by ERK1/2-dependent phosphorylation of TSC2 there is evidence that ERK1/2 signals also to complement mTORC1 signaling, as it is found for translational regulation of LTP in hippocampal neurons (Kelleher et al., 2004) and for the redundant phosphorylation of the S6K site Ser422 in eIF4B by RSK1 (Shahbazian et al., 2006).

I.6.2 Amino acids activate mTORC1

Amino acids, leucine in particular, stimulate the phosphorylation of the mTORC1 readouts S6K and 4E-BP in a rapamycin-sensitive manner (Anthony et al., 2000; Hara et al., 1998). Upon amino acid starvation, S6K and 4E-BP are dephosphorylated and become unresponsive to insulin stimulation. Amino acids were initially thought to signal via TSC1-TSC2 in Drosophila and mammals because TSC mutant cells show a certain resistance against amino acid deprivation (Gao et al., 2002) and overexpression of TSC1/2 partially inhibits amino acid stimulation towards mTORC1 readouts (Tee et al., 2002). However, several findings suggest that amino acids signal rather directly via Rheb (Ras homolog enriched in brain), and not via TSC1-TSC2. First, amino acid starvation inhibits mTORC1 signaling even in *tsc2* mutant cells (Smith et al., 2005). Second, Rheb overexpression is sufficient to bypass repression from amino acid starvation on mTORC1 readouts (Garami et al., 2003; Inoki et al., 2003a; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2003b). And third, Rheb binds directly to mTOR, independently of nucleotide binding but sensitive to amino acids (Long et al., 2005a; Long et al., 2005b). Rheb GTP charging affects mTOR kinase activity. These findings argue towards two parallel paths for the regulation of Rheb, one induced by growth factors that stimulate Rheb GTP levels via TSC1-TSC2 and the other induced by amino acids that regulate Rheb binding to mTOR. However, our knowledge on how amino acids signal into the TOR pathway is still incomplete and in certain aspects contradictory. Like for amino acids, the interaction between Rheb and mTOR has been reported to be sensitive to TSC1-TSC2 (Smith et al., 2005). Also, it is still not clear whether amino acids affect GTP charging of Rheb or not (Long et al., 2005b; Smith et al., 2005; Zhang et al., 2003b).

Rheb has a mammalian homolog, RhebL1 (Rheb-like 1), that is conserved in structure, function and regulation (Tee et al., 2005). The switch I region and the Ec region of Rheb and RhebL1 have conserved residues that enable specific mTOR interaction and are not found in the according regions in Ras. The switch I region in Ras changes its conformation depending on the nucleotide to which it binds and the Ec (constitutive effector) region is required for Ras activity. Rheb activity towards mTORC1 depends further on farnesylation, and thus requires membrane localization (Tee et al., 2003b).

Recently, it has been described that hVps34, a class III PI3K, activates mTORC1 (Byfield et al., 2005) and mediates the amino acid stimulation towards mTORC1 readouts (Nobukuni et al., 2005). The function of class III PI3Ks is not well understood in mammals but it is known that beclin-1 induces autophagy when bound to hVps34 (Engelman et al., 2006; Meijer and Codogno, 2006). If amino acids generally activate or inhibit Vps34 remains contradictory, and the answer may be that different pools of class III PI3K with different functions do not respond uniformly to amino acids (Dann and Thomas, 2006).

I.6.3 Energy activates mTORC1

Energy activates mTORC1 in a way that low cellular energy levels inhibit mTORC1 via activation of the AMP-activated protein kinase (AMPK) (Hardie, 2005). The regulatory γ-subunit of the heterotrimeric AMPK binds AMP and with lower affinity ATP. Binding of AMP induces allosteric activation of AMPK to allow phosphorylation of AMPK on Thr172 by the tumor suppressor LKB1 that is needed for AMPK activation (Hardie et al., 2006). Activated AMPK phosphorylates human TSC2 on Ser1337, Ser1341 and Ser1345 and thereby activates TSC1-TSC2, which leads to mTORC1 inhibition (Inoki et al., 2003b). Mutations in LKB1 can hyperactivate mTORC1 and cause the Peutz-Jegher syndrome (PJS), a familial autosomal disorder that shows similarity to TSC (Corradetti et al., 2004; Shaw et al., 2004). Glucose deprivation and hypoxia physiologically activate AMPK in glucose sensing and oxygen consumpting tissue, respectively. Furthermore, it has been shown that AMPK activity is regulated by adipokines and that this regulation can be either positive or negative, depending on the tissue (Hardie et al., 2006). In skeletal muscle and liver, the adipokines leptin and adiponectin stimulate AMPK to increase fatty acid oxidation and induce mitochondrial biogenesis. Conversely, leptin inhibits AMPK in the hypothalamus to reduce food intake (Kahn et al., 2005). The mechanism of how AMPK stimulates feeding behaviour is potentially linked to the inhibitory effect of AMPK towards mTORC1 in the hypothalamus (Cota et al., 2006).

AMPK-dependent phosphorylation of TSC2 on Ser1345 primes TSC2 for additional phosphorylation by GSK-3 on Thr1329, Ser1333, Ser1337 and Ser1341 to further activate TSC1-TSC2 (Inoki et al., 2006). Wnt-3a negatively regulates GSK-3 and prevents TSC2 phosphorylation by GSK-3. GSK-3 also phosphorylates TSC1 on Thr357 and Thr390 to stabilize the TSC1-TSC2 complex (Mak et al., 2005).

I.6.4 Hypoxia inhibits mTORC1

Hypoxia inhibits mTORC1 regulated protein synthesis in three ways (Bernardi et al., 2006; Pouyssegur et al., 2006). Hypoxia inhibits mTORC1 as TSC1-TSC2 is activated via stimulation of AMPK that results from increasing AMP levels (Hardie, 2005; Liu et al., 2006). Hypoxia inhibits mTORC1 also by inducing expression of the HIF-1 responsive genes *REDD1* and *REDD2* (Brugarolas et al., 2004), which are orthologs of the redundant growth suppressors *Scylla* and *Charybdis* in Drosophila (Reiling and Hafen, 2004) and activate TSC1-TSC2. Furthermore, hypoxia inhibits mTORC1 via the promyelotic leukaemia (PML) tumor suppressor that interferes with Rheb binding to mTOR, independently of TSC1-TSC2 (Bernardi et al., 2006). As hypoxic inhibition of mTOR is mediated by TSC1-TSC2, it appears that TSC1-TSC2 integrates signals from growth factors and cellular energy into the hypoxic response. Indeed, cells deficient in TSC2 or PTEN show increased mTORC1 activity during hypoxia (Kaper et al., 2006).

The finding that mTORC1 maintains the hypoxic response by positively regulating HIF-1 (see mTORC1 functions) does not fit at first glance with the finding that hypoxia inhibits mTORC1. Nevertheless, it reminds one of a fuse-like control mechanism. An explanation to this confusing signal transduction might be that autophagy, which is induced by mTORC1 inhibition, is required to maintain energy homeostasis during hypoxia but apparently, autophagy needs to be tightly controlled in order to provide a benefit to the cell. Autophagy is a self limited survival strategy, resulting in cell death if not reversed, and the recovery from the catabolic effects takes several days (Lum et al., 2005; Pouyssegur et al., 2006). Hypoxia does not completely shut down mTORC1 activity (Kaper et al., 2006) and a negative feedback signal from mTORC1 to reduce hypoxic stimulation could provide the regulatory

mechanism needed for autophagy control and keep the cells responsive to growth factors during hypoxia.

I.6.5 Further regulation of mTORC1

Recently, it was shown that the intermediate filament keratin 17, which is rapidly induced in wounded epithelia, is required for mTOR-dependent protein synthesis (Kim et al., 2006). Keratin 17 would maintain growth by regulating the cytoplasmic localization of 14-3-3σ.
Furthermore, p53 inhibits mTORC1 upon DNA damage via TSC1-TSC2 (Feng et al., 2005).

I.7 Regulation of mTORC2

If and how mTORC2 signaling is regulated is still unclear (see Figure I-2). Growth factors stimulate the mTORC2 readouts actin organization, cell spreading and Akt/PKB phosphorylation (Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2005b). Growth factors that stimulate mTORC2 readouts also inactivate the TSC1-TSC2 complex but it has been suggested that TSC1-TSC2 would activate mTORC2. Indeed, TSC deficiency or Rheb stimulation exert an inhibitory effect on the insulin pathway, but this is caused indirectly by negative feedback inhibition from hyperactivated mTORC1-S6K1 to IRS-1 (see also I.5.2). Because such negative feedback inhibition blocks insulin stimulation towards the mTORC2 readout Akt/PKB phosphorylation on Ser473, Yang et al. suggested that TSC1-TSC2 activates mTORC2 and Rheb inhibits mTORC2, which would be opposite to the regulation of mTORC1 (Yang et al., 2006). However, even in TSC deficient cells mTORC2 phosphorylates Akt/PKB on Ser473 either upon stimulation with the IRS-1 independent growth factor EGF (epidermal growth factor) or upon insulin stimulation while mTORC1-S6K1 is inhibited (Harrington et al., 2004; Yang et al., 2006). Therefore, TSC1-TSC2 is not a required activator for mTORC2 as Yang et al. proposed. Yet, it may well be that the contrary of what Yang et al. have proposed is true, in that TSC1-TSC2 inhibits mTORC2 activity based on the

coincidence that mTORC2 signals upon stimuli such as insulin that inhibit TSC1-TSC2. The result section in this thesis provides evidence that mTORC2 is indeed inhibited by TSC1-TSC2.

Figure I-2.

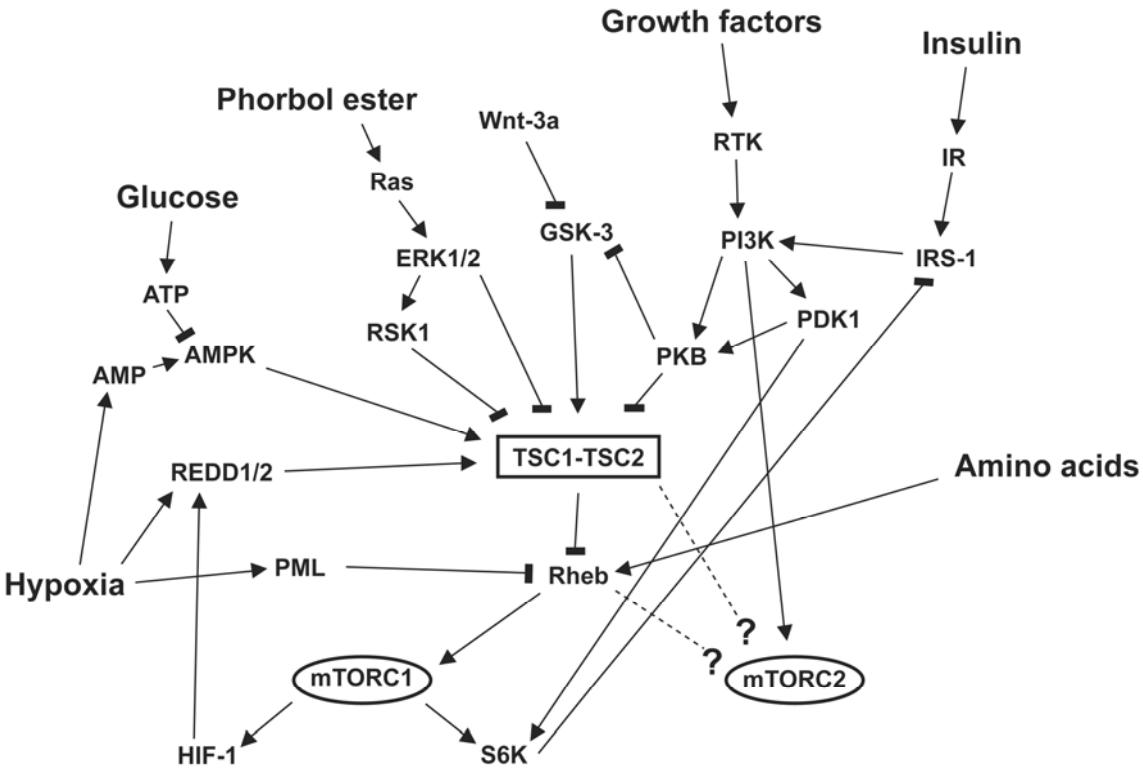

Figure I.2. Network of mTOR regulation (see I.6 and 1.7 for details).
A dashed line with question mark illustrates a putative link without evidence.
An arrowhead represents activation and a bar inhibition.

I.8 mTOR related diseases

Several human diseases result from tissue overgrowth with a link to the mTOR signaling pathway. Typically, mTOR hyperactivation is caused by inactivating mutations of suppressors in the mTOR pathway like the TSC1-TSC2 complex, LKB1 or PTEN, resulting in mTOR-dependent overgrowth. Due to a feedback regulatory mechanism in the mTOR pathway, mutations in TSC1-TSC2 and LKB1 do not necessarily lead to malignant progression. However, combined hyperactivation of mTOR and PI3K due to PTEN deficiency bypass the feedback checkpoint and have been observed in many cancers that show a severe rate of malignancy.

I.8.1 Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC) is an autosomal dominant tumor suppressor gene syndrome with a prevalence of about 1 in 6000 that causes hamartomas (benign tumors) and hamartias (malformations) in multiple tissues with a highly variable clinical manifestation among different patients (Kwiatkowski et al., 2002). Prominent hamartomas in TSC are cortical tubers that appear during embryogenesis and later in life cause seizures, mental retardation and behavioural disorders (Onda et al., 2002). The most frequent tumors in TSC include subependymal giant cell astrocytomas (SEGAs) in the cortex, facial angiofibromas in the skin, cardiac rhabdomyomas in the juvenile heart, and renal angiomyolipomas in the kidney. Inactivating mutations in either *TSC1* or *TSC2* occur sporadic in about two-thirds of TSC patients. In sporadic TSC, *TSC2* is mutated about 5 times more often (4,2 : 1) than *TSC1*, whereas this ratio is 1 : 1 in inherited TSC (Kwiatkowski and Manning, 2005). A broad spectrum of mutations is spanning along the entire *TSC1* and *TSC2* genes that can lead to TSC, including missense mutations, in frame deletions and large deletions. Although there are no dramatic hotspots, several warmspots are apparent, one of them being the GAP domain in *TSC2* (Kwiatkowski, 2003; Maheshwar et al., 1997). Hamartoma development in TSC is often linked to allelic loss of heterozygosity (LOH) but some tubers do not show evidence for LOH or a classical second hit inactivation within the corresponding allele (Kwiatkowski and Manning, 2005). A second hit mechanism that involves other signaling pathways has

been suggested for brain regions in TSC because SEGAs often lack LOH of TSC but instead express high levels of activated Akt/PKB and activated ERK1/2. Such a mechanism is plausible and can experimentally be reproduced by combining heterozygous *tsc2+/-* mice with *pten+/-* heterozygosity, which dramatically increases tumorigenesis in comparison to mice that are only *tsc2+/-* heterozygous (Ma et al., 2005b; Manning et al., 2005). These experiments also provided an explanation for the low rate of progression to malignancy that is observed in TSC patients (Al-Saleem et al., 1998). Activation of Akt/PKB upon stimulation with serum or growth factors is strongly impaired in TSC1-TSC2 deficient cells as well as in tissue from heterozygous *tsc2+/-* mice. The lack in Akt/PKB activation is a consequence of the downregulation of the platelet-derived growth factor receptor (PDGFR) (Zhang et al., 2003a) and the inhibition of IRS-1 in TSC deficient cells (Harrington et al., 2004; Shah et al., 2004). In-vivo, the combination of heterozygous *tsc2+/-* mice with *pten+/-* heterozygosity results in restored Akt/PKB activation and more severe tumor growth.

Hyperactivation of mTORC1 can be observed in TSC lesions and is suspected to contribute in large to the clinical syndromes in TSC (Chan et al., 2004). Apparently, pre-clinical data promises that the use of mTOR inhibitors to treat TSC is beneficial for patients (Lee et al., 2005a). In addition, farnesyltransferase inhibitors promise to be useful drugs against TSC due to Rheb inactivation (Gau et al., 2005).

I.8.2 Autosomal-dominant polycystic kidney disease

TSC patients are prone to develop autosomal-dominant polycystic kidney disease (ADPKD) because large deletion mutations in *TSC2* can affect the adjacent *PKD1* (*Polycystic Kidney Disease 1*, or also called *Polycystin-1* (*PC-1*)) gene on chromosome 16, a major contributor to ADPKD (Sampson et al., 1997). Patients with ADPKD develop renal cysts that lead to renal failure. The clinical use of rapamycin analogs for immunosuppression in kidney transplantation suggested that mTOR inhibition reduces growth of renal sarcomas, which led to the hypothesis that inhibition of mTOR could also be beneficial for the treatment of ADPKD. In animal models for ADPKD, mTOR hyperactivation was observed and mTOR inhibition could

indeed retard cyst formation and progression of renal failure, which suggests that mTOR activity is a molecular mechanism underlying renal cyst formation (Tao et al., 2005; Wahl et al., 2006). Recently, hyperactivated mTOR was found in cyst-lining epithelial cells in human ADPKD patients and it was suggested that Polycystin-1 (PC1) activates mTOR by binding to TSC2 (Shillingford et al., 2006). *PC1* mutations are frequently found in ADPKD.

I.8.3 Lymphangioleiomyomathosis

Pulmonary lymphangioleiomyomathosis (LAM) is a lung lesion caused by infiltrating and proliferating smooth muscle cells and epitheloid cells that form cysts and destroy airways (Kwiatkowski and Manning, 2005). LAM is a gender-specific disease that is rarely detected in men (Aubry et al., 2000). The gender-specificity appears to relate to a high expression of receptors for estrogen and progesterone in LAM cells because estradiol stimulates the growth of *TSC2* mutant cells (Astrinidis and Henske, 2005). LAM occurs in about half of the female TSC patients but clinical syndromes develop only in about 5-10 % of adult females with TSC. Occasionally, LAM occurs sporadic in patients without TSC, but also sporadic LAM is caused by LOH in either *TSC1* or *TSC2* (Carsillo et al., 2000; Sato et al., 2002; Sato et al., 2004). Pulmonary LAM cells are identical in their TSC mutations and LOH pattern with benign angiomyolipoma smooth muscle cells in other organs of LAM patients, suggesting that benign cells with mutations in either *TSC1* or *TSC2* metastasize from outside into the lungs (Henske, 2003). The observation of recurrent recipient LAM cells in donor lungs after lung transplantation clearly demonstrates that TSC deficient cells, which are histologically benign, do metastasize in-vivo (Karbowniczek et al., 2003). In-vitro it has also been found that TSC deficient cells migrate aberrantly (Astrinidis and Henske, 2005) and hamartin and tuberin activate members of the Rho GTPase superfamily that regulate actin polymerization, cell morphology and migration. Reports concerning the role of TSC1 and TSC2 in migration do not completely overlap in their findings and thus the exact mechanism is still not understood. One study found that overexpression of TSC1 activates Rho, which reduces cell migration (Lamb et al., 2000) and a similar result was obtained for overexpression of TSC2 (Astrinidis et al., 2002). However, in LAM cells it was shown that Rho inhibition

decreases cell migration and that TSC1 activates RhoA if TSC2 is deficient in binding TSC1 (Goncharova et al., 2006a).

mTORC1 hyperactivation contributes to the increased growth rate of LAM cells and inhibition of mTORC1 reduces LAM cell growth. Therefore, mTOR inhibition appears as a promising therapeutic strategy for the treatment of LAM (Goncharova et al., 2006b).

I.8.4 Other hamartoma syndromes

Cowden's disease, Lhermitte-Duclos disease, Bannayan-Zonana syndrome and Proteus syndrome are hamartoma syndromes that share similarities to TSC although they result from inactivating mutations in the tumor suppressor PTEN. Because of the similarities to TSC and the fact that PTEN deficiency results in TSC1-TSC2 inhibition and thus mTORC1 hyperactivation, it may be pharmacologically beneficial to consider the inhibition of mTORC1 signaling as therapeutic strategy in the treatment of such diseases (Inoki et al., 2005; Tee and Blenis, 2005).

In Peutz-Jeghers syndrome (PJS), hamartomas form in skin and intestine and the patients are prone to develop tumors. Also in PJS, mTORC1 hyperactivation is thought to contribute to the disease because PJS results from an inactivating mutation in LKB1, which is needed for AMPK activation and thus negative regulation of mTORC1 (Inoki et al., 2005; Tee and Blenis, 2005).

I.8.5 Cancer

PTEN deficiency is associated with the tumorigenesis of many different cancers like endometrial cancer, breast cancer glioblastoma, prostate-, ovarian-, thyroid- and hepatocellular carcinoma. mTOR inhibition promises to be a well tolerated and beneficial therapeutical strategy in PTEN deficient tumors, which is suggested by several clinical studies (Faivre et al., 2006). Since Akt/PKB hyperactivation is one of the molecular hallmarks in PTEN deficient tumors and contributes to cancer growth,

the Akt/PKB activating mTORC2 is also considered as a possible new target in anti-cancer intervention (Guertin and Sabatini, 2005). Patients with renal cell carcinoma already benefit from therapeutic mTORC1 inhibition and the clinical history of rapamycin analogues as immunosuppressants after kidney transplantation (Faivre et al., 2006). Furthermore, rapamycin analogues could be beneficial for the treatment of metastatic tumor growth in general as they inhibit neoangiogenesis by reducing secretion of VEGF (vascular endothelial growth factor) (Guba et al., 2002; Tee and Blenis, 2005). Also in cancers that show elevated 5'-cap-dependent translation due to a hyperactive eIF4F complex, rapamycin analogues are expected to contribute to a better disease prognosis (Mamane et al., 2004; Shaw and Cantley, 2006).

I.8.6 Neurodegeneration

In the brain, autophagy is a protective mechanism against neurodegenerative diseases such as Parkinson, Alzheimer and Huntington because these diseases develop from accumulating defective proteins (Meijer and Codogno, 2006). Neurodegeneration appears as a consequence of deficient autophagy in mouse neurons (Hara et al., 2006; Komatsu et al., 2006). Induction of autophagy by mTOR inhibition in animal models suggest beneficial support in the clearance of protein aggregates that are involved in the development of Huntington's disease and Alzheimer's disease (Berger et al., 2006; Ravikumar and Rubinsztein, 2006; Ravikumar et al., 2004).

II. Materials and Methods

Chemicals and Antibodies. Cell culture reagents, puromycin, tetracycline and insulin were obtained from Sigma; blastocidin and zeocin from Cayla; U0126 and LY294002 from Alexis; rapamycin from LC Laboratories; HRP-coupled anti-mouse and anti-rabbit secondary antibodies from Pierce; HRP-coupled anti-goat secondary antibody from Sigma; anti-Paxillin-pY118, anti-Paxillin, anti-PKB-pS473, anti-S6K-pT389, anti-S6K and anti-Erk1/2-pT202/Y204 were from Cell Signaling Technology; anti-PKB and anti-Rheb from Santa Cruz; anti-4E-BP1 from Zymed; anti-actin (MAB1501) from Chemicon; anti-GFP from Roche. Anti-rictor and anti-raptor were produced as described (Jacinto et al., 2004). We generated polyclonal anti-TSC1 and anti-TSC2 as described (van Slegtenhorst et al., 1998). Plasmids for TSC antigen generation were kindly provided by Dr. Mark Nellist. Anti-HA was from concentrated 12CA5 tissue culture supernatant.

Plasmids. pTER was a kind gift from Dr. Mark van de Wetering (Centre for Biomedical Genetics, Utrecht, The Netherlands) (van de Wetering et al., 2003); pKDM-175 is a pTER-based siRNA expressing plasmid targeting gacacacagaatagctatg in *TSC1* mRNA; pKDM-185 is a pTER-based plasmid targeting gtcctgcagtggatggatg in *TSC2* mRNA; pKDM-176 is a LxSP-based plasmid (with puromycin resistance cassette) constructed by inserting the EcoRI fragment containing the tetracycline inducible siRNA cassette from pKDM-175; pKDM-132 is a pSuper.gfp/neo-based (Oligoengine) siRNA expressing plasmid targeting ctgtgaactagcacttcag in *rictor* mRNA; pKDM-162 is a pSuper.gfp/neo-based (Oligoengine) siRNA expressing plasmid targeting ggacaacggccacaagtac in *raptor* mRNA; pKDM-212 is a pSuper.gfp/neo-based plasmid targeting gcgagttcgtgtcatcgag for non-specific control siRNA in human cells (oligos were kindly provided by Jean Pieters); HA-myr-PKB was a kind gift from Dr. Brian Hemmings (Andjelkovic et al., 1997); myc-Rheb was a kind gift from Dr. George Thomas (Garami et al., 2003). GST-RhebL1 and Flag-RhebL1 were kind gifts from Dr. Andrew Tee (Tee et al., 2005). silent point mutations were inserted into the siRNA target regions of full-length *TSC1* and *TSC2* pcDNA3.1-based constructs (kind gifts from Dr. Mark Nellist) (van

Slegtenhorst et al., 1998) using the QuickChange method (Stratagene) with the primer pairs: 5'-gcccttatgctgatactcaaaatagctatggg-3'/5'-cccatagctattttgagtatcagcataag ggc-3' for *TSC1* and 5'-ggctgactttgtgctccaatggatggatgttggc-3'/5'-gccaacatccatccattgg agcacaaagtcagcc-3' for *TSC2*.

Cell culture and transfections. Cells were maintained in D-MEM containing 10% fetal bovine serum and Penicillin/Streptomycin. TSCsi293 cells were induced for 96 hours with tetracycline (4 µg/ml) to knock down TSC1 and TSC2 protein levels. Cells were stimulated in D-PBS+ (D-PBS, 1x MEM Vitamin mix, 1000 mg/l D-Glucose and Penicillin/Streptomycin) supplemented as indicated with 10% fetal bovine serum, amino acids (1x MEM essential amino acid mix and 1x MEM non-essential amino acid mix), 100 nM insulin or inhibitors (in DMSO as vehicle). For overnight serum deprivation, HEK293T cells were grown for 17 hours in D-MEM containing Penicillin/Streptomycin without serum. For transfection, cells were split in 6-well plates (+/- tetracycline in case of TSCsi293 cells) before transfection 24h later with 8µl Lipofectamine 2000 (Invitrogen) and 3µg DNA per well, following the manufacturer's guidelines. Two hours later, the cells were split again in fresh medium (+/- tetracycline in case of TSCsi293 cells) and cultured for another 48 hours prior to stimulation. For cell lysis, cells were rinsed on ice with PBS before scraping them in ice-cold TNE lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaC, 0,5 mM EDTA, 1% Triton x100) supplemented with protease and phosphatase inhibitors (1x Roche complete protease inhibitor cocktail, 1 µg/ml Aprotinin, 1 µg/ml Pefabloc, 1 mM PMSF, 10 mM NaF, 10 mM NaN_3, 10 mM NaPPi, 10 mM B-glycerophosphate, 10 mM p-nitrophenylphosphate). After 20 minutes of incubation, cell debris was removed by centrifugation for 5 minutes at 7000g. The protein concentration was determined with a Bio-Rad assay and equalized. For Western blotting, SDS-PAGE sample buffer was added and the probes were heated for 5 minutes at 95^0C.

Generation of a stable TSCsi293 cell line. 293T-REx cells which stably express the tetracycline repressor (TetR) (Invitrogen) were a kind gift from Martin Spiess. The cells were transfected with pKDM-176 and pKDM-185 and selected for resistance to blasticidin (10 µg/ml), zeocin (300 µg/ml) and puromycin (1 µg/ml) to generate clones. Individual clones were picked, expanded, and tested for tetracycline-inducible siRNA knockdown of TSC1 and TSC2 by Western blotting. To control that

tetracycline treatment or expression of non-targeted siRNA did not interfere with the studied signalling pathway, we confirmed in control experiments that tetracycline treatment and non-specific siRNA expression in 293T-REx cells did not affect phosphorylation of Paxillin Tyr118, Akt/PKB Ser473 or S6K Thr389.

Immunoprecipitation and Western blotting. To immunoprecipitate HA-tagged protein, 2 µl of anti-HA antibody was added to 300 µl of cell lysate containing 500 µg of total protein and rotated for one hour at 4^0C. 20 µl of protein G-Sepharose slurry (Sigma) was added to the mix and rotation continued for another hour before the beads were washed three times with lysis buffer. Beads were collected by centrifugation, resuspended in 2x SDS-PAGE sample buffer and heated for 5 minutes at 95^0C. The supernatant was transferred to a fresh tube and stored at -20^0C before aliquots were analysed by Western blotting. For Western blotting, 60-100 µg of protein of cell lysate per lane were electrophoresed in SDS-PAGE mini gels and transferred to nitrocellulose. The manufacturer's guidelines were followed for antibody incubations. SuperSignal West Pico and Femto kits (Pierce) were used for ECL-detection.

Quantification of cell spreading. Cells were grown on coverslips and fixed in 4% PFA. Rhodamine-phalloidine and DAPI (Sigma) were used to stain F-actin and nuclei, respectively. Cells were analysed using a Zeiss Axioplan microscope. Only cells that showed an intact nucleus by DAPI staining were counted. Cells were considered spread if there was at least one lamellipodia-like extension with a minimal width of the nuclear radius.

III. TSC1-TSC2 complex inhibits mTORC2 in human cells

III.1 Introduction

The conserved serine/threonine kinase mTOR is a central regulator of eukaryotic cell and organismal growth (Wullschleger et al., 2006). mTOR is found in two structurally and functionally distinct protein complexes, mTORC1 and mTORC2 (Loewith et al., 2002; Sarbassov et al., 2005a). Rapamycin-sensitive mTORC1 consists of mTOR, raptor, and mLST8, and controls a wide range of cellular processes including translation, transcription, nutrient transport, ribosome biogenesis and autophagy. The two best characterized effectors of mTORC1 are S6K and 4E-BP1, which mTORC1 phosphorylates directly to activate translation (Hay and Sonenberg, 2004). Rapamycin-insensitive mTORC2 consists of mTOR, rictor, and mLST8. mTORC2 appears to control organization of the actin cytoskeleton and cell spreading via activation of Paxillin (Tyr118 phosphorylation), Rho, Rac, and PKC (Jacinto et al., 2004; Sarbassov et al., 2004). The mechanism by which mTORC2 activates these effectors is unknown but must be indirect at least in the case of Paxillin which is activated by tyrosine phosphorylation. mTORC2 directly phosphorylates Ser473 in the hydrophobic motif of Akt/PKB and thereby activates Akt/PKB towards a FoxO transcription factor (Sarbassov et al., 2005b).

mTORC1 is regulated in response to nutrients (amino acids), growth factors (insulin/IGF-1), cellular energy status (AMPK), and stress (Wullschleger et al., 2006). Growth factors stimulate mTORC1 via the phosphoinositide 3-kinase (PI3K) pathway (Shaw and Cantley, 2006). PI3K produces PtdIns(3,4,5)P_3 (PIP_3) from PtdIns(4,5)P_2 (PIP_2), which in turn recruits PKB and PDK1 to the plasma membrane (Fayard et al., 2005). At the plasma membrane, PDK1 activates Akt/PKB by phosphorylating Thr308 in the activation loop of Akt/PKB (Lizcano and Alessi, 2002). Akt/PKB phosphorylates TSC2 (Tuberin) and thereby inhibits TSC1-TSC2, a hetero-dimeric GAP (GTPase activating protein) for the small GTPase Rheb (Shaw and Cantley, 2006). Inhibition of TSC1-TSC2 results in increased levels of GTP bound Rheb. Rheb-GTP binds and activates mTORC1. Independent of PI3K, the MAP kinases ERK1 and 2 also phosphorylate TSC2 and inactivate TSC1-TSC2 (Ma et al., 2005a;

Tee et al., 2003a). Loss-of-function mutations in *TSC1* or *TSC2* cause hyperactivation of mTORC1 and lead to various syndromes characterized by cell overgrowth, such as Tuberous Sclerosis Complex (TSC) and Lymphangioleiomyomatosis (LAM) (Kwiatkowski and Manning, 2005; Tee and Blenis, 2005). Amino acids stimulate mTORC1 via an unknown mechanism involving Rheb and possibly TSC1-TSC2 (Wullschleger et al., 2006).

Rapamycin-insensitive mTORC2 was discovered only recently and its upstream regulators are thus largely unknown. mTORC2 readouts such as actin organization, cell spreading, and Akt/PKB-Ser473 phosphorylation are stimulated by growth factors (serum or insulin), suggesting that mTORC2 may be activated by growth factors (Jacinto et al., 2004). This in turn suggests that mTORC2, like mTORC1, is inhibited by TSC1-TSC2. However, Yang et al. (Yang et al., 2006) have reported recently that loss of TSC1/2 results in loss of insulin-stimulated Akt/PKB Ser473 phosphorylation, suggesting that mTORC2, contrary to mTORC1, is activated by TSC1-TSC2. As Yang et al. (Yang et al., 2006) propose, this apparent paradox may be explained by a negative feedback loop from mTORC1-S6K to IRS-1 upstream of PI3K in the insulin signaling pathway. S6K, activated in TSC mutant cells, phosphorylates and inhibits IRS-1 (Harrington et al., 2004; Shah et al., 2004; Um et al., 2004). This results in down regulation of the insulin signaling pathway, including down regulation of PI3K and Akt/PKB Ser473 phosphorylation (Manning, 2004). Loss of Ser473 phosphorylation in this context could be due to loss of mTORC2 kinase activity or, alternatively, due to loss of insulin stimulated recruitment of Akt/PKB to the plasma membrane (Andjelkovic et al., 1997; Inoki and Guan, 2006). The observation that rapamycin treatment, which inhibits mTORC1 and the negative feedback loop to IRS-1, restores Akt/PKB Ser473 phosphorylation in insulin treated TSC mutant cells suggests that loss of TSC does not cause loss of rapamycin-insensitive mTORC2 activity (Harrington et al., 2004; Yang et al., 2006). The observation that stimulation of the PI3K pathway with EGF, which does not involve IRS-1 and is thus not subject to the negative feedback loop, induces Ser473 phosphorylation in TSC mutant cells is further evidence that TSC1-TSC2 is not required for mTORC2 activity (Harrington et al., 2004). It is also worth noting that the experiments of Yang et al. (Yang et al., 2006) were performed largely in *Drosophila* S2 cells which seem to have a particularly robust negative feedback loop. Thus, loss

of TSC may cause loss of insulin stimulated Ser473 phosphorylation due to loss of Akt/PKB localization to the plasma membrane. Although it appears that TSC1-TSC2 is not required for mTORC2 activity, it remains to be determined whether mTORC2, like mTORC1, is inhibited by TSC1-TSC2.

To investigate the regulation of mTORC2 and, in particular, the role of TSC1-TSC2 in this regulation, we created a HEK293T derived cell line (TSCsi293) that allows stable, inducible siRNA mediated knockdown of TSC1 and TSC2. This cell line was used to assay the effect of TSC1/2 knockdown on physiological mTORC2 readouts that are not subject to the confounding effects of the negative feedback loop in the insulin pathway. To circumvent the effect of the negative feedback loop on Akt/PKB localization, we also examined Ser473 phosphorylation of a myristoylated Akt/PKB that is constitutively plasma membrane bound (Andjelkovic et al., 1997). We found that mTORC2, like mTORC1, is activated in the absence of TSC1-TSC2. Our results suggest that mTORC2 is in part responsible for the pathological effects of mutations that inactivate TSC1 and TSC2.

III.2 Results

Figure III-1.

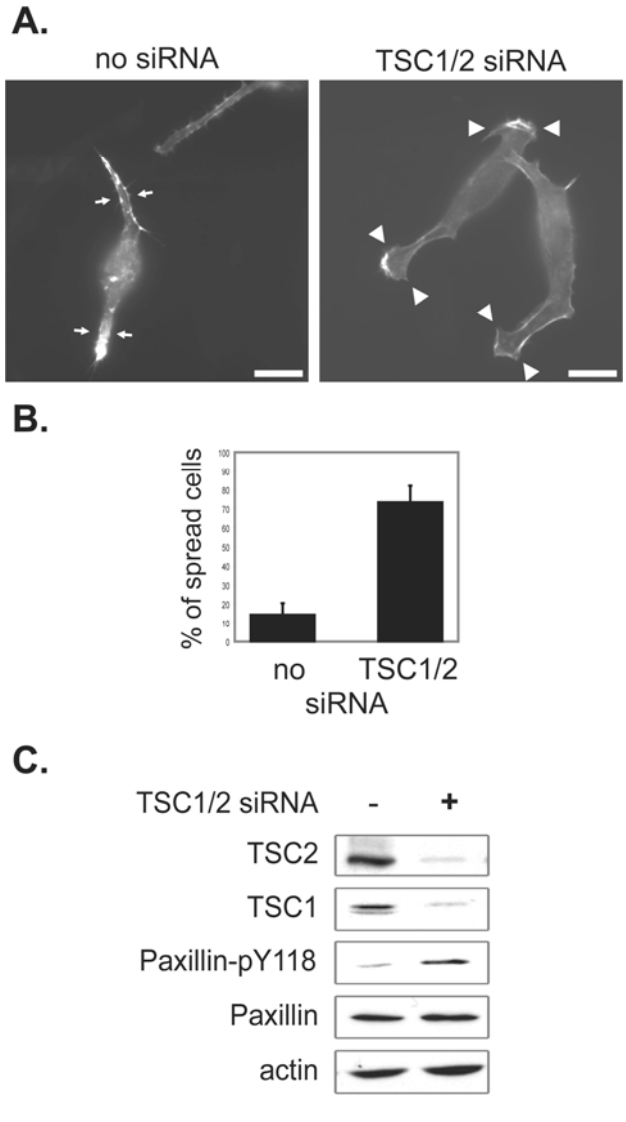

Figure III-1. Knockdown of TSC1 and TSC2 (TSC1/2) by siRNA stimulates cell spreading and Paxillin pY118 phosphorylation.

(A) TSC1/2 siRNA prevents cell retraction during serum starvation. TSCsi293 cells were grown for one hour in D-PBS+ supplemented with amino acids in the absence of serum, fixed, and stained with rhodamine-phalloidine for visualization of F-actin. To induce TSC1/2 siRNA, cells were treated with tetracycline as described in Material

and Methods. Arrowheads indicate lamellipodia-like cell spreading. Thin arrows indicate non-spread cell extensions. Scale bars, 10μm.
(B) Quantification of TSCsi293 cell spreading in (A). 100 cells in each of three independent experiments were analyzed. For cells lacking TSC1/2 siRNA (no siRNA), 16% of cells were spread and 84% were retracted, whereas TSC1/2 siRNA resulted in 73% spread and 27% retracted cells.
(C) Tetracycline induced siRNA against TSC1/2 knocked down TSC1 and TSC2 proteins and stimulated phosphorylation of Paxillin Y118 (Paxillin-pY118). Western analysis of TSCsi293 cells described in (A).

III.2.1 TSC1-TSC2 negatively regulates mTORC2-dependent readouts

To investigate if TSC1-TSC2 is upstream of mTORC2, we created a stable, 293T-REx derived cell line (TSCsi293) expressing TSC1- and TSC2-targeted siRNAs from integrated, tetracycline inducible promoters (van de Wetering et al., 2003). This cell line provided a system to examine mTORC2 readouts under conditions of reproducible and inducible knockdown of both TSC1 and TSC2. TSC1 and TSC2 were efficiently knocked down within 4 days of tetracycline treatment (Figure III-1). We first examined the effect of TSC1/2 knockdown on F-actin organization and cell spreading in serum starved cells (Figure III-1 A. and B.). Serum starved conditions were chosen because TSC1-TSC2 is normally inactive in serum or growth factor treated cells. Organization of the actin cytoskeleton is a previously characterized physiological readout of mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004). Approximately 70% of serum starved cells lacking TSC1/2 (tetracycline treated TSCsi293 cells) were spread with F-actin-based lamellipodia-like extensions (Nobes and Hall, 1995). Conversely, less than 20% of serum starved control cells (tetracycline untreated TSCsi293 cells) exhibited this behavior. To investigate further the effect of TSC1/2 knockdown on the actin cytoskeleton, we examined Paxillin Tyr118 phosphorylation in serum starved cells. Paxillin is a scaffold protein that mediates actin organization in response to growth factors (Brown and Turner, 2004). Paxillin Tyr118 phosphorylation, which promotes lamellipodia formation and cell spreading, is mTORC2-dependent. TSC1/2 knockdown stimulated Tyr118 phosphorylation in serum starved TSCsi293 cells (Figure III-1 C.). Thus, mTORC2

readouts are upregulated in serum starved TSC1/2 knockdown cells, suggesting that TSC1-TSC2 inhibits mTORC2 in the absence of serum.

Figure III-2.

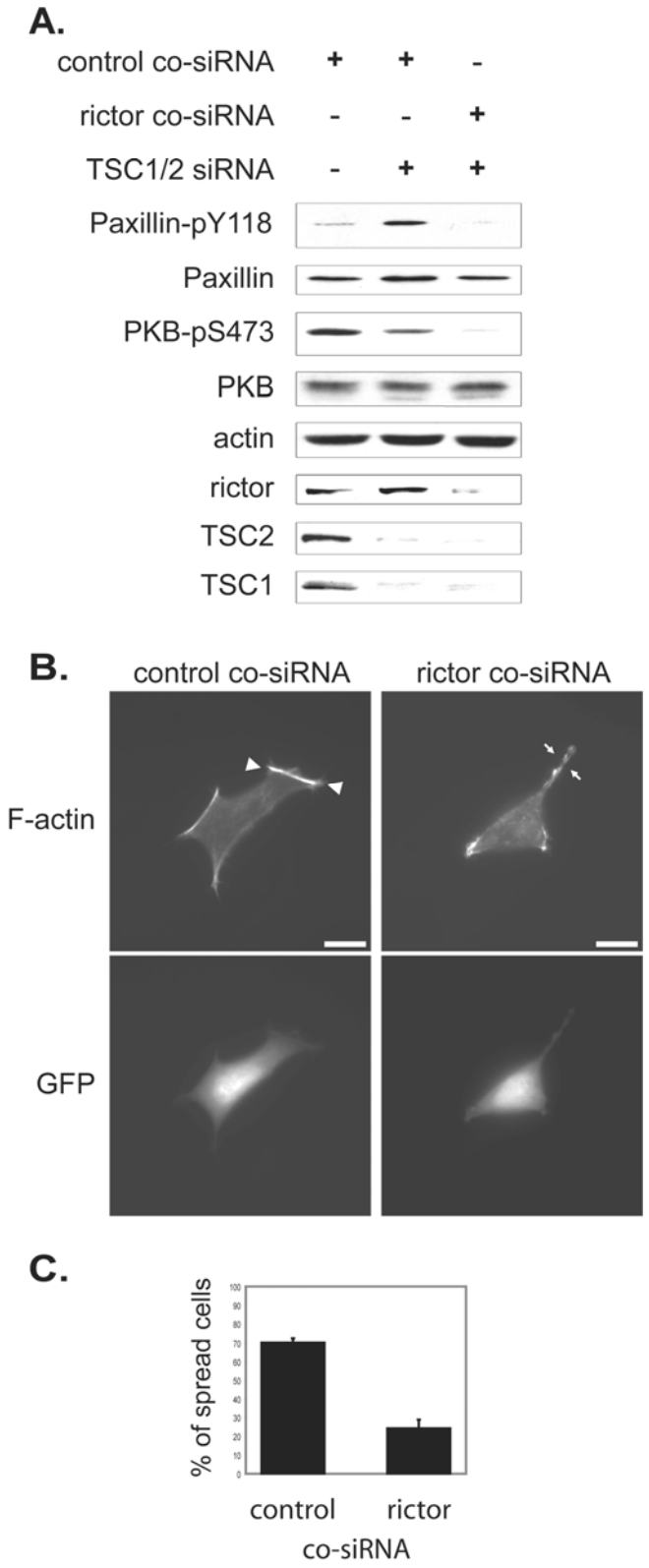

Figure III-2. mTORC2 mediates the effects of TSC1/2 knockdown.
(A) Co-knockdown of mTORC2 (rictor co-siRNA) prevents increased Paxillin Tyr118 phosphorylation (Paxillin-pY118) due to TSC1/2 knockdown and leads to loss of PKB Ser473 phosphorylation (PKB-pS473). Western analysis of TSCsi293 cells (+/- tetracycline induced TSC1/2 siRNA), transiently expressing the indicated co-siRNA. The cells were grown for 30 minutes in D-PBS+ supplemented with amino acids in the absence of serum before lysis.
(B) Co-knockdown of mTORC2 (rictor co-siRNA) prevents cell spreading induced by TSC1/2 knockdown. F-actin and GFP expression in TSCsi293 cells with tetracycline-induced knockdown of TSC1/2 and expressing the indicated co-siRNA. The cells were grown prior to fixation as in (A). Arrowheads indicates lamellipodia-like cell spreading. Thin arrows indicate a non-spread cell extension. Scale bars, 10μm.
(C) Quantification of TSCsi293 cells in (B). For each condition, more than 200 GFP positive cells from three independent experiments were analyzed. For cells expressing a control co-siRNA, 70% of cells were spread and 30% were retracted, whereas upon expression of rictor co-siRNA 24% of the cells were spread and 76% of the cells were retracted.

III.2.2 mTORC2 mediates effects of TSC1/2 knockdown

To investigate if TSC1/2 indeed inhibits mTORC2, we examined whether mTORC2 mediates the effects of TSC1/2 knockdown. To determine the mTORC2-dependence of the effects of TSC1/2 knockdown, we combined knockdown of TSC1-TSC2 with inhibition of mTORC2, in TSCsi293 cells (Figure III-2). mTORC2 was inhibited by transient transfection of TSCsi293 cells with a pSuper plasmid expressing GFP and siRNA (co-siRNA) against the essential mTORC2 component rictor. Knockdown of rictor was confirmed by loss of rictor protein and by loss of Akt/PKB-Ser473 phosphorylation. The rictor co-siRNA prevented the stimulation of Paxillin Tyr118 phosphorylation observed in serum starved TSCsi293 cells upon TSC1/2 knockdown (Figure III-2 A.). Furthermore, the rictor co-siRNA inhibited cell spreading observed upon TSC1/2 knockdown. Less than 25% of TSCsi293 cells with combined knockdown of TSC1/2 and rictor (tetracycline treated, GFP expressing cells) remained spread, compared to 70% for control cells (Figure III-2 B. and C.).

Knockdown of mTORC1 with co-siRNA against the essential mTORC1 component raptor had no effect on Paxillin Tyr118 phosphorylation or cell spreading (data not shown). Thus, inhibition of mTORC2 prevented the effects observed upon TSC1/2 knockdown, confirming that TSC1-TSC2 inhibits mTORC2.

III.2.3 TSC1-TSC2 inhibits mTORC2 activity towards plasma membrane bound Akt/PKB

Yang et al. (Yang et al., 2006), using Akt/PKB Ser473 phosphorylation as a readout for mTORC2 activity, previously suggested that TSC1-TSC2 activates mTORC2 (see Introduction), whereas we find that TSC1-TSC2 inhibits mTORC2 (see above). To investigate this apparent contradiction, we examined the effect of TSC1/2 knockdown on Ser473 phosphorylation of myristoylated, and thus constitutively plasma membrane bound, Akt/PKB (myr-Akt/PKB) (Andjelkovic et al., 1997). This allowed us to determine whether the effect of TSC1/2 knockdown on Akt-PKB Ser473 phosphorylation is due to lack of Akt/PKB localization or, alternatively, due to lack of mTORC2 kinase activity. myr-Akt/PKB Ser473 phosphorylation increased in serum starved TSCsi293 cells upon knockdown of TSC1/2 (Figure III-3). Inhibition of mTORC2 with rictor co-siRNA prevented myr-Akt/PKB Ser473 phosphorylation (Figure III-3), confirming that mTORC2 mediated the phosphorylation of myr-Akt/PKB Ser473. Thus, mTORC2 is able to phosphorylate Akt/PKB in the absence of TSC1-TSC2 if Akt/PKB is at the plasma membrane. Apparently, Akt/PKB that is not plasma membrane bound is not accessible for phosphorylation by mTORC2. These findings suggest that Yang et al. (Yang et al., 2006) failed to detect mTORC2 activity in TSC1/2 mutant cells due to an indirect effect on Akt/PKB localization rather than to an effect on mTORC2 activity itself. These findings also suggest that TSC1-TSC2 is not required to activate mTORC2. Indeed, the observation that mTORC2-dependent myr-Akt/PKB Ser473 phosphorylation, like Paxillin Tyr118 phosphorylation and cell spreading, increased upon TSC1/2 knockdown provides further evidence that TSC1-TSC2 inhibits mTORC2.

Figure III-3.

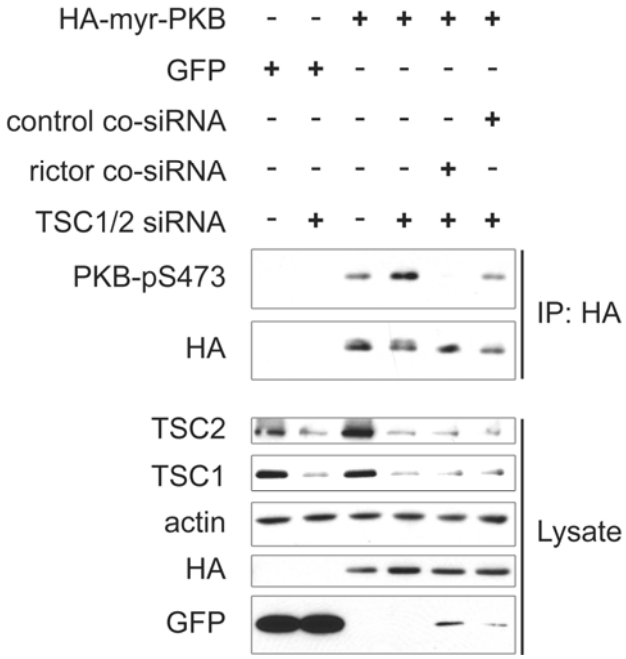

Figure III-3. Knockdown of TSC1/2 increases mTORC2-dependent phosphorylation of membrane bound Akt/PKB (HA-myr-PKB). Ser473 phosphorylated (PKB-pS473) and total (HA) HA-myr-PKB immunoprecipitated with anti-HA (IP: HA) from TSCsi293 cells induced (+) or non-induced (-) for TSC1/2 siRNA, and transiently expressing the indicated constructs. Indicated proteins were probed in total lysate. The cells were grown for 30 minutes in D-PBS+ supplemented with amino acids in the absence of serum before lysis.

III.2.4 Overexpression of Rheb activates mTORC1 but not mTORC2

TSC1-TSC2, a GTPase activating complex, inhibits mTORC1 via downregulation of the small GTPase Rheb. To determine whether TSC1-TSC2 inhibits mTORC2 also via inhibition of Rheb, we investigated if overexpression of Rheb activates mTORC2. The mTORC2 readout Paxillin Tyr118 phosphorylation was examined in starved cells overexpressing Rheb. As a positive control for Rheb function, we also examined the mTORC1 readout S6K Thr389 phosphorylation. Overexpression of Rheb failed to stimulate Paxillin Tyr118 phosphorylation. In contrast, Rheb overexpression stimulated S6K Thr389 phosphorylation (Figure III-4 A.). Similar results (data not

shown) were obtained with overexpression of RhebL1, a functional homolog of Rheb (Tee et al., 2005). Thus, Rheb and RhebL1 activate mTORC1 but not mTORC2. This is consistent with the observation of Yang et al. (Yang et al., 2006) that Rheb knockdown, although inhibiting TORC1, does not prevent activation of TORC2. Taken together, the above findings suggest that TSC1-TSC2 inhibition of mTORC2 is independent of Rheb. TSC1-TSC2 may inhibit mTORC2 via another, unknown small GTPase.

Figure III-4.

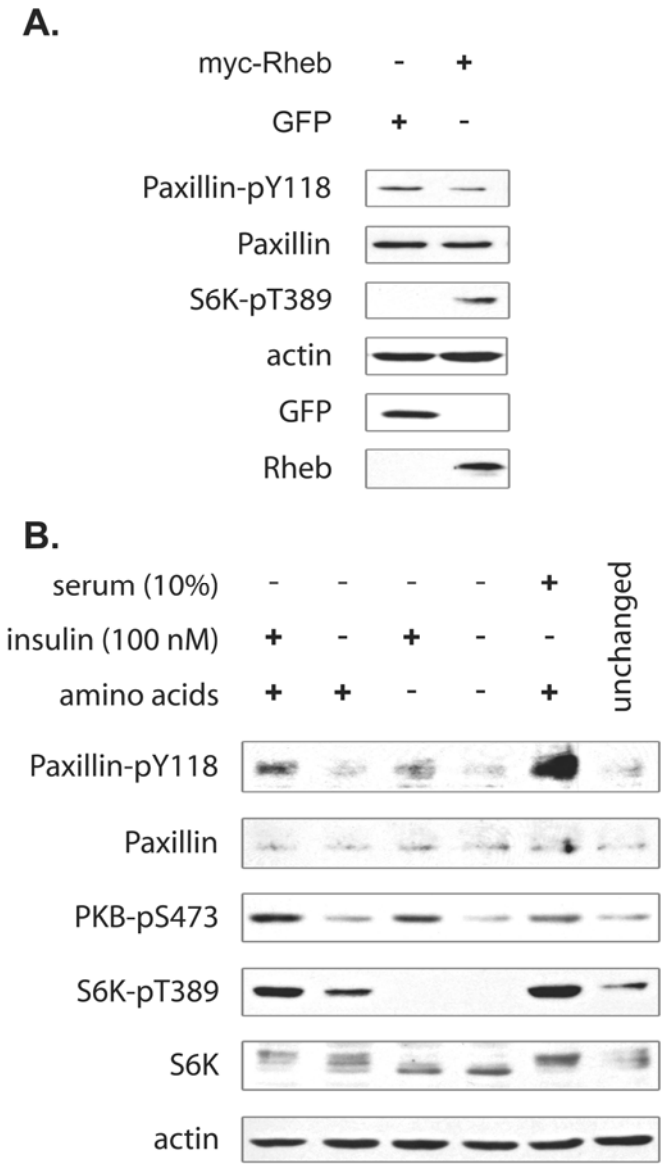

Figure III-4. mTORC1 and mTORC2 respond differently to amino acids and Rheb overexpression but similarly to serum and insulin.

(A) Rheb overexpression stimulates phosphorylation of S6K Thr389 (S6K-pT389) but not of Paxillin Tyr118 (Paxillin-pY118). Western analysis of HEK293T cells expressing the indicated constructs and grown in D-PBS+ lacking amino acids and serum for 30 minutes before lysis.

(B) Amino acids stimulate phosphorylation of S6K Thr389 (S6K-pT389) but not of Paxillin Tyr118 (Paxillin-pY118). Western analysis of HEK293T cells deprived of serum overnight (17 hours) and either grown unchanged in D-MEM or grown in D-PBS+ with the indicated supplements for 30 minutes before lysis. Amino acids strongly induced only S6K-pT389, whereas insulin and serum stimulated PKB Ser473 (PKB-pS473), Paxillin-pY118, and S6K-pT389.

III.2.5 Amino acids activate mTORC1 but not mTORC2

The apparently different requirement for Rheb in the activation of mTORC1 and mTORC2 suggests that the two mTOR complexes may respond differently to extracellular stimuli. To investigate this possibility, we compared the ability of amino acids, insulin and serum to stimulate mTORC1- and mTORC2-dependent readouts in HEK293T cells (Figure III-4 B.). Amino acids, insulin and serum are well-characterized extracellular stimuli for mTORC1. We found that amino acids stimulated phosphorylation of the mTORC1 effector S6K (Thr389) but not of the mTORC2 effector Paxillin (Tyr118). Insulin stimulated phosphorylation of both S6K Thr389 and Paxillin Tyr118, although the stimulation of S6K Thr389 phosphorylation was evident only in the presence of amino acids. Serum stimulated phosphorylation of both Paxillin Tyr118 and S6K Thr389. As a control, we also examined phosphorylation of Akt/PKB Ser473. As expected, insulin and serum but not amino acids stimulated phosphorylation of Ser473. These findings suggest that, unlike mTORC1, mTORC2 does not respond to amino acids. Furthermore, the two mTORCs respond differently to amino acids in the same way they respond differently to Rheb (see above). This correlation suggests that amino acids may impinge on mTORC1 via Rheb. In addition, as both mTORCs are inhibited by TSC1-TSC2, yet only mTORC1 responds to amino acids, the TSC complex appears not to play a role

in signaling amino acid availability. Long et al. (Long et al., 2005b) have reported previously that the GTP loading of Rheb is independent of amino acids, providing further evidence that amino acids do not signal via the TSC complex.

III.3 Discussion

We have presented evidence that mTORC2, like mTORC1, is negatively regulated by TSC1-TSC2. This conclusion appears to contradict Yang et al. (Yang et al., 2006) who suggested that mTORC2, unlike mTORC1, is activated by TSC1-TSC2. This apparent contradiction can be explained by the negative feedback loop in the insulin pathway and the choice of mTORC2 readouts used to study mTORC2 activity. Like Yang et al. (Yang et al., 2006), we observed that loss of TSC1-TSC2 reduced Akt/PKB Ser473 phosphorylation (Figure III-2 A.). However, we also found that loss of TSC1-TSC2 stimulated mTORC2-dependent phosphorylation of Paxillin and constitutively membrane bound myr-Akt/PKB. Thus, loss of TSC1-TSC2 activates mTORC2 but prevents membrane localization of Akt/PKB. In other words, reduced Akt/PKB Ser473 phosphorylation in TSC mutant cells is due to loss of Akt/PKB recruitment to the membrane rather than to loss of mTORC2 activity. Loss of TSC1-TSC2 prevents Akt/PKB recruitment, and thereby accessibility to mTORC2, by triggering the negative feedback loop in the PI3K/insulin pathway. Further support for this explanation is provided by the observation that inhibition of the negative feedback loop (with rapamycin) in insulin treated TSC mutant cells restores Ser473 phosphorylation (Harrington et al., 2004; Yang et al., 2006). Similarly, a PTEN mutation restores Ser473 phosphorylation in TSC mutant cells (Ma et al., 2005b). The negative feedback loop prevents membrane recruitment of Akt/PKB by inhibiting PI3K, which in turn causes reduced PIP_3 levels in the plasma membrane. PIP_3 normally binds the PH domain in Akt/PKB and thereby recruits Akt/PKB to the plasma membrane (Fayard et al., 2005).

We also found that mTORC2 is stimulated by insulin and serum, but not by amino acids. This creates a conundrum when one considers that mTORC2 activates Akt/PKB and that Akt/PKB is required to inhibit TSC1-TSC2 upstream of mTORC2. How does insulin inhibit TSC1-TSC2 and thereby stimulate mTORC2 if mTORC2 is first needed to phosphorylate (Ser473) and activate Akt/PKB? The answer to this question may be found in the observation that knockdown of mTORC2 does not affect mTORC1-dependent S6K activation (Jacinto et al., 2004; Sarbassov et al., 2005b). This observation suggests that mTORC2-mediated phosphorylation of Akt/PKB is not required to activate Akt/PKB towards TSC1-TSC2. PDK1-mediated

phosphorylation of Thr308 in the activation loop of Akt/PKB may be sufficient to activate Akt/PKB towards TSC1-TSC2, but subsequent phosphorylation of Ser473 in the hydrophobic motif of Akt/PKB by mTORC2 may be necessary to activate Akt/PKB towards other substrates such as FoxO (see model in Figure III-5). This model is also supported by recent findings of Jacinto et al. (Jacinto et al., 2006) showing that an mTORC2 knockout eliminates Akt/PKB Ser473 phoshorylation but affects signaling only to a subset of Akt/PKB substrates. In particular, they show that mTORC2-dependent phosphoryaltion of Ser473 is required for signaling to FoxO but not for signaling to TSC1-TSC2 or GSK3. Thus, Ser473 phosphorylation determines Akt/PKB substrate specificity rather than absolute activity, as shown in the model of Figure III-5.

We also observed that overexpression of Rheb activated mTORC1 but not mTORC2. In agreement, Yang et al. (Yang et al., 2006) have reported that knockdown of Rheb prevents activation of TORC1 but not of TORC2. Thus, Rheb is an upstream regulator of mTORC1 but not of mTORC2 (Figure III-5). This correlates with our additional observation that amino acids, although able to activate mTORC1, do not activate mTORC2. Furthermore, recent results have suggested that amino acids activate mTORC1 via Rheb independently of TSC1-TSC2. The binding of Rheb to mTORC1 is regulated by amino acid availability but GTP charging of Rheb is independent of amino acids (Long et al., 2005b). In addition, TSC deficient cells still respond to amino acid starvation (Smith et al., 2005) (data not shown). Together, these findings suggest that TSC1-TSC2 inhibits mTORC2 by acting on a protein other than Rheb. This other protein, possibly a GTPase, would be insensitive to amino acids.

Mutations that inactivate *TSC1* or *TSC2* result in a variety of diseases characterized by cell overgrowth, including Tuberous Sclerosis Complex (TSC) and Lymphangioleiomyomatosis (LAM). Inhibition of mTORC1 with rapamycin analogs is currently being considered as a strategy in the treatment of TSC and LAM (Johnson, 2006; Kwiatkowski and Manning, 2005). Interestingly, deregulation of the actin cytoskeleton via Rac1 and RhoA appears to contribute to the metastatic invasiveness of angiomyolipoma smooth muscle cells in LAM patients (Astrinidis et al., 2002; Goncharova et al., 2006a; Henske, 2003). We found that a TSC1/2 deficiency

stimulates mTORC2-dependent actin polymerization and Paxillin Tyr118 phosphorylation. Paxillin Tyr118 phosphorylation promotes cell migration and regulates Rac1 and RhoA (Brown and Turner, 2004). These findings suggest that mTORC2 may contribute to the invasiveness of TSC and LAM cells. Thus, effective intervention in the treatment of TSC, LAM and related diseases may require inhibition of both mTORC1 and rapamycin-insensitive mTORC2.

Figure III-5.

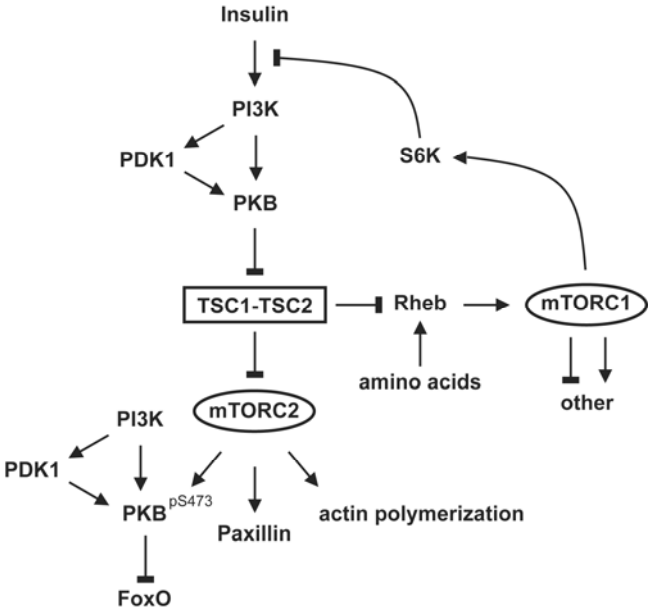

Figure III-5. Model of mTOR signalling network showing negative regulation of mTORC2 by TSC1-TSC2.

TSC1-TSC2 inhibits mTORC2 independently of Rheb. TSC1-TSC2 inhibits mTORC1 via inactivation of Rheb. mTORC2 phoshorylates Ser473 (pS473) in the hydrophobic motif of Akt/PKB (PKB). PDK1 phosphorylates Thr308 (not indicated) in the activation loop of Akt/PKB. Ser473 phosphorylation determines the substrate specificity of Akt/PKB. mTORC1 activates S6K and thereby induces feedback inhibition of the insulin pathway upstream of PI3K. Inhibition of PI3K by the negative feedback loop prevents membrane recruitment of PKB and PDK1. In TSC deficient cells, hyperactive mTORC1 inhibits membrane translocation of PKB, leaving PKB

inaccessible to activated mTORC2. Amino acids stimulate mTORC1 (but not mTORC2) via Rheb.

IV. Additional Results

IV.1.1 Tetracycline treatment of 293T-REx cells does not affect mTOR signaling.

To test if tetracycline treatment interfered with mTOR signaling we examined the effects of tetracycline treatment on readouts for mTORC1 and mTORC2 in 293T-REx cells. We found that phosphorylation of Paxillin Tyr118, Akt/PKB Ser473 or S6K Thr389 did not change due to the tetracycline treatment that we used in TSCsi293 cells to induce knockdown of TSC1/2 (Figure IV-1). This result ensured that the tetracycline inducible system was suitable to study the mTOR pathway.

Figure IV-1.

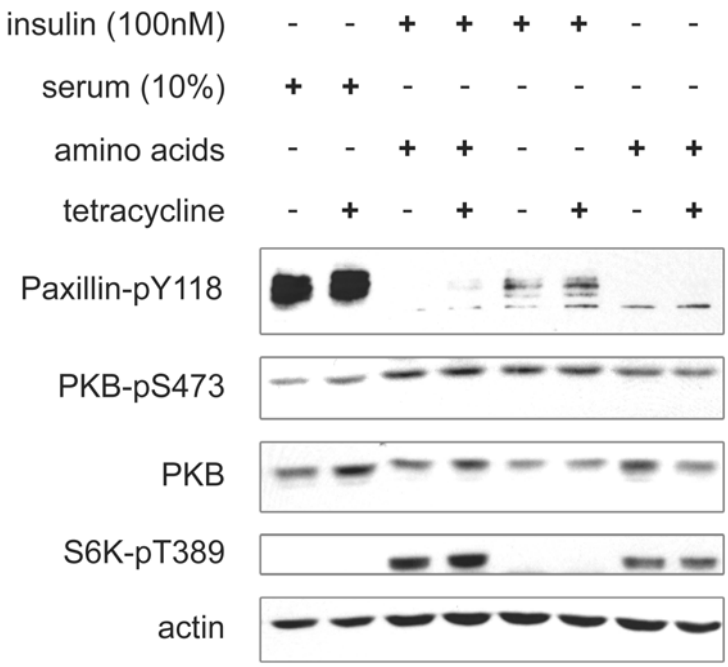

Figure IV-1. Control experiment for tetracycline treatment in 293T-REx cells.
Tetracycline treatment for 72 hours did not affect phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473) or S6K Thr389 (pT389). Western analysis of 293T-REx cells, either treated with tetracycline (4 µg/ml) for 4 days or not and either

stimulated or starved in D-PBS+ supplemented as indicated for 30 minutes before lysis.

IV.1.2 Effects of non-targeting siRNA in 293T-REx on mTOR signaling.

To test if expression of siRNA exhibited off-target effects on mTOR signaling we examined readouts for mTORC1 and mTORC2 in 293T-REx cells that expressed non-targeted control siRNA. We found that phosphorylation of Paxillin Tyr118, Akt/PKB Ser473 or S6K Thr389 was not affected by expression of control siRNA (Figure IV-2). This result ensured that using siRNA was suitable to study mTOR signaling.

Figure IV-2.

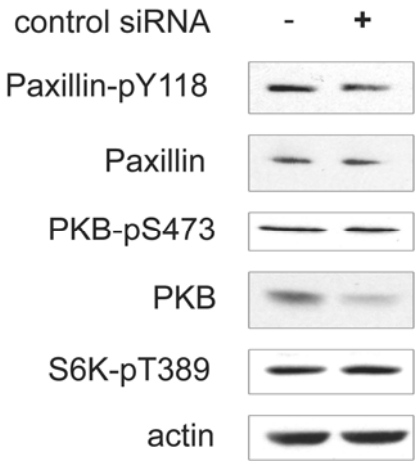

Figure IV-2. Control experiment for non-targeting siRNA in 293T-REx cells.
Transient expression of non-targeted control siRNA did not affect phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473) or S6K Thr389 (pT389). Western analysis of 293T-REx cells either transfected or not with control siRNA. The cells were grown for 60 minutes in D-PBS+ supplemented with amino acids before lysis.

Figure IV-3.

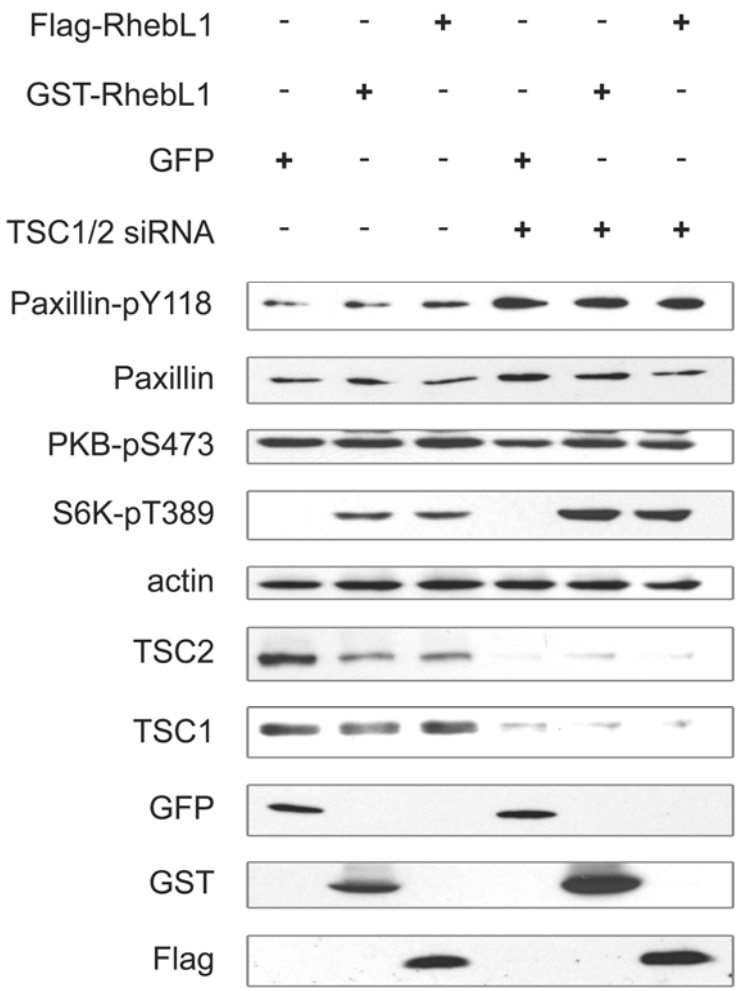

Figure IV-3. RhebL1 overexpression stimulates phosphorylation of S6K Thr389 (pT389) but not of Paxillin Tyr118 (pY118) or Akt/PKB Ser473 (pS473).
Western analysis of TSCsi293 cells, induced for TSC1/2 knockdown or not, that transiently expressed either GFP or RhebL1 tagged with GST or Flag. The cells were grown in D-PBS+ supplemented with serum for 30 minutes before lysis.

IV.1.3 RhebL1 activates mTORC1 but not mTORC2.

The GTPase activating hetero-complex TSC1-TSC2 inhibits mTORC1 via downregulation of the small GTPase Rheb. We found that Rheb did not mediate the stimulation of mTORC2-dependent phosphorylation of Paxillin Tyr118 upon TSC1/2 knockdown (see III.2.4). Alternatively, it was possible that RhebL1 (Tee et al., 2005),

a functional homolog of Rheb, mediated mTORC2 activation upon knockdown of TSC1/2. To determine whether TSC1-TSC2 inhibits mTORC2 via inhibition of RhebL1, we investigated if overexpression of RhebL1 stimulated phosphorylation of Paxillin Tyr118. As a positive control for RhebL1 function, we also examined the mTORC1 readout S6K Thr389 phosphorylation. Overexpression of RhebL1, like overexpression of Rheb, failed to stimulate Paxillin Tyr118 phosphorylation (Figure IV-3). In contrast, RhebL1 overexpression stimulated S6K Thr389 phosphorylation. Thus, RhebL1, just like Rheb, activates mTORC1 but not mTORC2, suggesting that TSC1-TSC2 inhibits mTORC2 independently of Rheb and RhebL1. This finding adds to the conclusion that TSC1-TSC2 inhibits mTORC2 by a mechanism that is different from inhibition of mTORC1.

IV.1.4 Phosphorylation of Paxillin Tyr118 and S6K Thr389 peaks after 30 minutes of serum stimulation.

To determine if serum stimulates phosphorylation of mTOR readouts in TSCsi293 cells in a time dependent manner, we performed a time course for serum stimulation. TSCsi293 cells were stimulated with 10% serum for either 0, 15, 30, 45, 60 or 90 minutes to examine phosphorylation of Paxillin Tyr118, Akt/PKB Ser473 and S6K Thr389. We found that Paxillin Tyr118 phosphorylation peaked after 30 minutes of serum stimulation (Figure IV-4). Phosphorylation of Paxillin decreased again between 30 and 45 minutes after stimulation, and came down to the initial level at 90 minutes post stimulation. We found similar kinetics for serum stimulated Paxillin Tyr118 phosphorylation in TSCsi293 cells if TSC1/2 were knocked down, which suggested the existence of additional regulatory mechanisms for Paxillin phosphorylation in parallel to hyperactivated mTORC2. Phosphorylation of Akt/PKB Ser473 did not change upon serum stimulation. S6K Thr389 phosphorylation increased upon serum stimulation and peaked between 15 and 30 minutes after the stimulation. S6K Thr389 phosphorylation also decreased between 30 and 45 minutes after serum stimulation and came back to the initial level at 90 minutes post stimulation. We cannot exclude that increased S6K phosphorylation upon serum stimulation was due to amino acids present in the serum. In summary, serum stimulation induces a peak in phosphorylation of Paxillin Tyr118 and S6K Thr389

after around 30 minutes. For this reason, we chose 30 minutes as the appropriate period to study serum stimulation in TSCsi293 cells.

Figure IV-4.

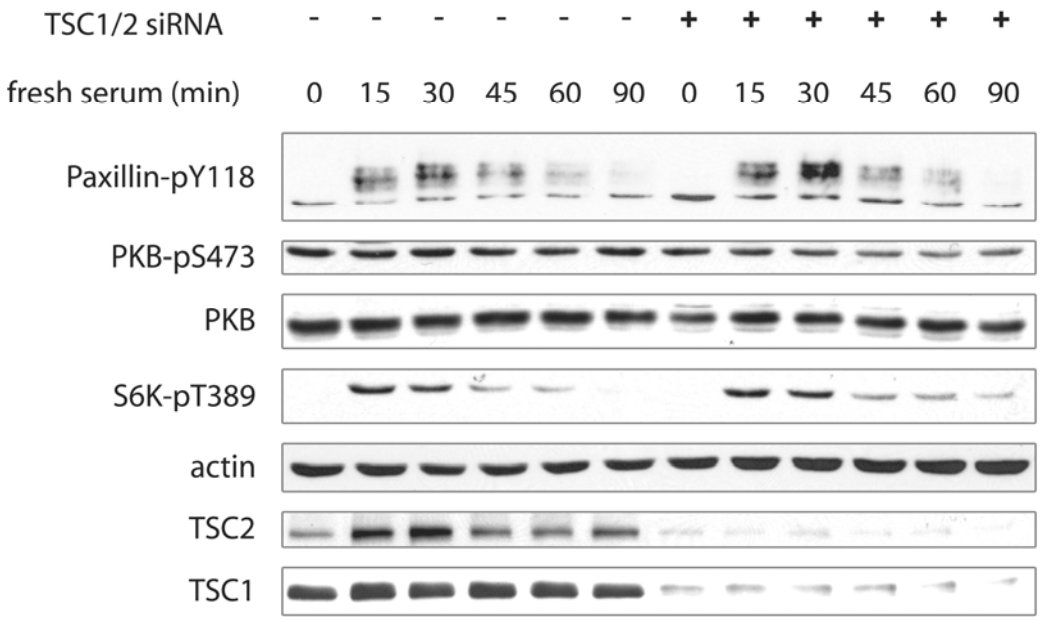

Figure IV-4. Time course for serum stimulation of TSCsi293 cells.
Phosphorylation of Paxillin Tyr118 (pY118), AKT/PKB Ser473 (pS473) and S6K Thr389 (pT389) at indicated times in minutes (min) after stimulation with 10 % serum. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were grown for the according times in D-PBS+ supplemented with serum before lysis.

IV.1.5 Between 5-10 % of serum is required for maximal serum stimulation of Paxillin Tyr118 phosphorylation in TSCsi293 cells.

To determine the optimal serum concentration needed for effective stimulation of mTOR readouts in TSCsi293 cells, we performed a serum concentration gradient for 30 minutes of serum stimulation and examined phosphorylation of Paxillin, PKB and S6K. Between 5-10 % of serum induced maximal phosphorylation of Paxillin Tyr118

in TSCsi293 cells either induced for TSC1/2 knockdown or not (Figure IV-5). Akt/PKB Ser473 phosphorylation did not change upon stimulation with any of the tested serum concentrations. S6K Thr389 phosphorylation was maximal between 5-10 % of serum. Thus, 30 minutes of serum stimulation require 5-10 % of serum for effective stimulation of Paxillin Tyr118 phosphorylation. For this reason, we decided to stimulate TSCsi293 cells with 10 % serum in our experiments.

Figure IV-5.

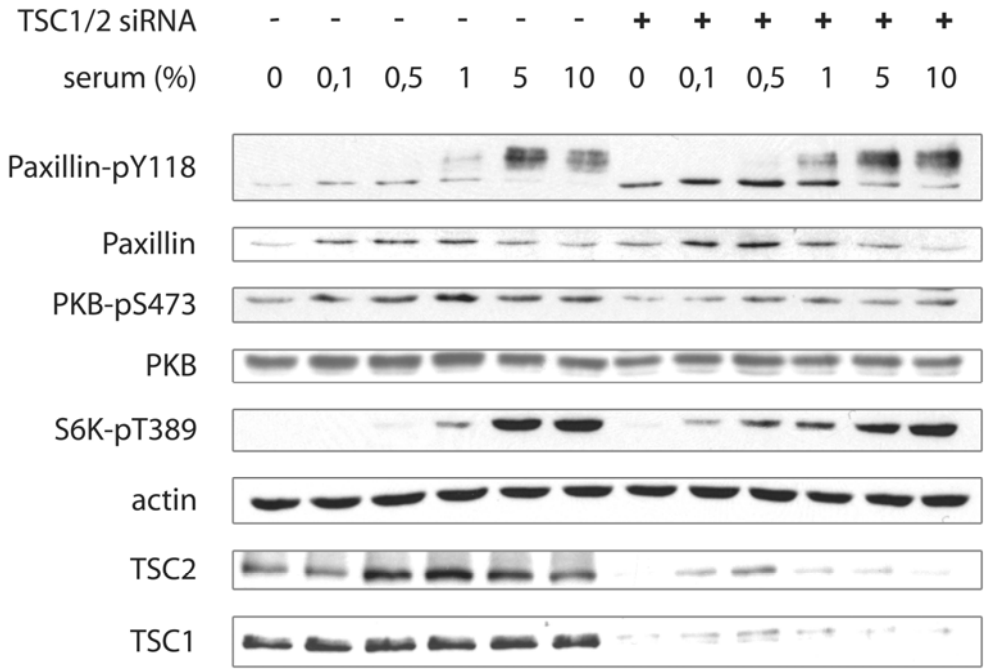

Figure IV-5. Serum gradient for stimulation of TSCsi293 cells.
Phosphorylation of the mTOR readouts Paxillin Tyr118 (Y118), AKT/PKB Ser473 (S473) and S6K Thr389 (T389) after 30 minutes of stimulation with serum at indicated concentrations. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were grown for 30 minutes in D-PBS+ with the indicated concentrations of serum before lysis.

IV.1.6 Serum stimulates mTORC2-dependent Akt/PKB Ser473 phosphorylation.

As serum did not stimulate phosphorylation of Akt/PKB Ser473 in TSCsi293 cells (Figures IV-4 and IV-5), we further analysed the effects of overnight serum deprivation and serum re-stimulation on phosphorylation of mTOR readouts in these cells. We examined the effects of serum deprivation and re-stimulation because similar conditions were chosen in initial studies on Akt/PKB activation by growth factors (Andjelkovic et al., 1997). TSCsi293 cells were serum deprived for 17 hours or not and the deprived cells were then re-stimulated for 30 minutes with serum or not. Phosphorylation of Ser473 in Akt/PKB decreased upon serum deprivation (Figure IV-6), suggesting that the presence of serum maintains Akt/PKB activity. Serum re-stimulation induced phosphorylation of Akt/PKB Ser473 to similar levels as observed in non-deprived cells, which further shows the positive serum effect on Akt/PKB activation. We observed similar kinetics for Akt/PKB activation in TSCsi293 cells having knocked down TSC1/2 and can therefore conclude that TSC1-TSC2 inhibition by siRNA does not induce a negative feedback inhibition towards serum sensing pathways in TSCsi293 cells. Paxillin Tyr118 phosphorylation increased upon serum re-stimulation also if TSC1/2 were knocked down, which further suggests that Paxillin phosphorylation is regulated by other signals in parallel to the regulation by TSC1-TSC2. Phosphorylation of S6K Thr389 increased upon TSC1/2 knockdown. mTORC1-dependent phosphorylation of Ser235/Ser236 in S6 (Pende et al., 2004) increased after changing the medium, which was probably caused by amino acids in the medium (DMEM). S6 Ser235/Ser236 phosphorylation additionally increased upon TSC1/2 knockdown. Furthermore, we found that serum strongly induced phosphorylation of ERK1/2 Thr202/Tyr204 (see also Figures IV-11 and IV-14). Thus, serum not only stimulates phosphorylation of Paxillin Tyr118 but also maintains phosphorylation of Akt/PKB Ser473 and even induces Akt/PKB Ser473 phosphorylation in serum deprived cells. The observation that TSCsi293 cells are still sensitive to serum deprivation and restimulation suggests that more regulators besides TSC1-TSC2 account for phosphorylation of Akt/PKB and Paxillin. The stimulating effect of serum on these mTORC2 readouts also suggests that mTORC2 is activated by serum. Serum may therefore either stimulate mTORC2 activity, or stimulate co-factors that are required for mTORC2-dependent signaling, or both.

Figure IV-6.

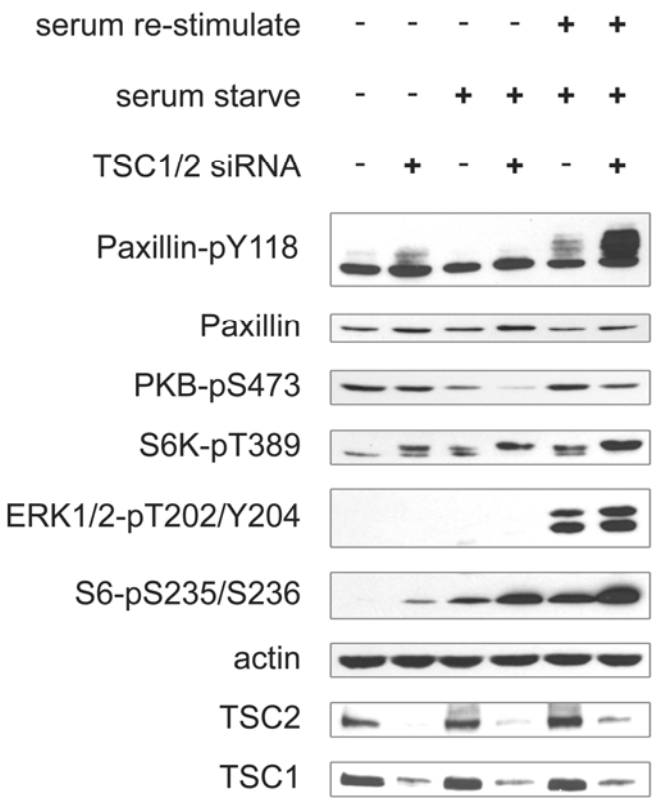

Figure IV-6. Serum starvation and serum re-stimulation of TSCsi293 cells. Phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473), S6K Thr389 (pT389), S6 Ser235/Ser236 (pS235/S236) and ERK1/2 Thr202/Tyr204 (pT202/Y204) were detected after overnight serum deprivation and serum re-stimulation. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were either serum deprived for 17 hours in DMEM (including fresh amino acids) without serum or not and then either re-stimulated with serum for 30 minutes in DMEM (including fresh amino acids) supplemented with serum or not before lysis.

IV.1.7 Insulin stimulation of mTOR readouts reaches a maximum after 30 minutes.

To determine if insulin stimulation of mTOR readouts is time dependent in TSCsi293 cells, we performed a time course for stimulation with 100 nM insulin and examined the phosphorylation of Paxillin Tyr118, Akt/PKB Ser473 and S6K Thr389. If cells were not pre-starved for serum, we detected no strong increase in Paxillin

phosphorylation by insulin stimulation (Figures IV-7, IV-10 and IV-11). Insulin induced Akt/PKB Ser473 phosphorylation already after 15 minutes, resulting in a stable signal over the examined period, independently of TSC1/2 knockdown (Figure IV-7), which suggested that knockdown of TSC1/2 did not inhibit the insulin response in TSCsi293 cells. Phosphorylation of S6K1 Thr389 was only weakly detectable if we knocked down TSC1/2 in TSCsi293 cells, which is a result from the absence of amino acids (IV-7). However, in TSC1/2 knockdown we found S6K Thr389 phosphorylation to peak after 30 minutes of insulin stimulation. We found similar kinetics for Akt/PKB Ser473 phosphorylation in TSCsi293 cells when combining insulin and amino acid stimulation (Figure IV-10). As we found a stable induction over time from insulin stimulation towards Akt/PKB activation while for S6K activation we observed a peak after 30 minutes of insulin stimulation, we decided to study insulin effects after 30 minutes of stimulation.

Figure IV-7.

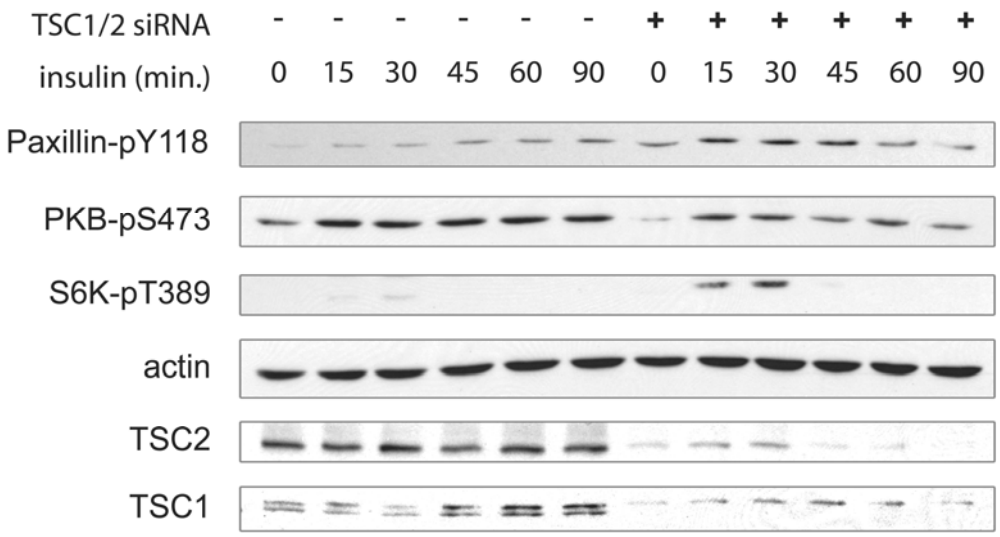

Figure IV-7. Time course for insulin stimulation of TSCsi293 cells.
Phosphorylation of the mTOR readouts Paxillin Tyr118 (pY118), AKT/PKB Ser473 (pS473) and S6K Thr389 (pT389) at indicated times in minutes (min.) after stimulation with 100 nM insulin. Western analysis of TSCsi293 cells either induced

for TSC1/2 knockdown or not. The cells were grown for the according times in D-PBS+ supplemented with insulin before lysis.

IV.1.8 Maximal insulin stimulation of mTORC2 readouts requires 100 nM insulin.

To determine an optimal insulin concentration for effective stimulation of mTORC2-dependent phosphorylation of Paxillin Tyr118 and Akt/PKB Ser473 in serum starved TSCsi293 cells, we performed a concentration gradient for 30 minutes of insulin stimulation and examined phosphorylation of Paxillin and PKB. Phosphorylation of Paxillin Tyr118 and Akt/PKB Ser473 in serum starved TSCsi293 cells was maximal upon 100 nM insulin stimulation (Figure IV-8). We observed similar kinetics when the cells had been knocked down for TSC1/2, again showing that TSC1/2 knockdown did not inhibit insulin signaling. Thus, maximal insulin stimulation of mTORC2 readouts requires 100 nM insulin. For this reason, we stimulated TSCsi293 cells with 100 nM of insulin.

Figure IV-8.

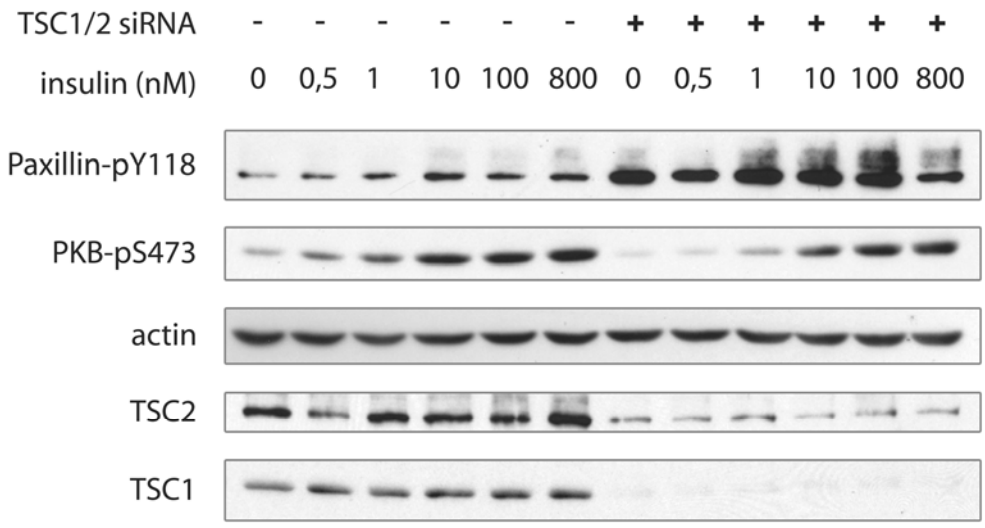

Figure IV-8. Insulin gradient for stimulation of serum deprived TSCsi293 cells.

Phosphorylation of Paxillin Tyr118 (pY118) and AKT/PKB Ser473 (pS473) were detected upon stimulation for 30 minutes with the indicated insulin concentrations. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were serum deprived for 17 hours in DMEM without serum and grown in D-PBS+ supplemented with the according concentrations of insulin before lysis.

IV.1.9 S6K Thr389 phosphorylation peaks after 30 minutes of amino acid stimulation.

To determine if amino acids stimulate phosphorylation of mTOR readouts in TSCsi293 cells in a time dependent manner, we performed a time course for amino acid stimulation and examined migration of 4E-BP1 and phosphorylation of S6K Thr389, Paxillin Tyr118 and Akt/PKB Ser473. Phosphorylation of the mTORC1 readout S6K Thr389 peaked after 30 minutes of stimulation with amino acids (Figure IV-9). Between 45 and 60 minutes post stimulation the phosphorylation of S6K Thr389 declined again. We observed similar kinetics when the cells were knocked down for TSC1/2 or when insulin was present in addition (Figure IV-10). This finding suggests that the TSC deficiency in these cells does not affect the response to amino acid stimulation and provides new evidence for a TSC1-TSC2-independent signal transduction of amino acid stimulation towards mTORC1. Migration speed of 4E-BP1 slowed down after 30 minutes of amino acid stimulation, reflecting complete phosphorylation by mTORC1. 60 minutes post stimulation we found that 4E-BP1 migrated again faster and together with the concomitant decline of S6K Thr389 phosphorylation after an initial peak, this finding suggests that a negative feedback regulatory mechanism limits hyperactivation of mTORC1 after acute amino acid stimulation. The phosphorylation of the mTORC2 readouts Paxillin Tyr118 and Akt/PKB Ser473 did not change upon amino acid stimulation. In summary, amino acid stimulation induced a peak in S6K Thr389 phosphorylation and 4E-BP1 phosphorylation after 30 minutes of stimulation. For this reason, we chose 30 minutes as the appropriate time period to study amino acid stimulation in TSCsi293 cells.

Figure IV-9.

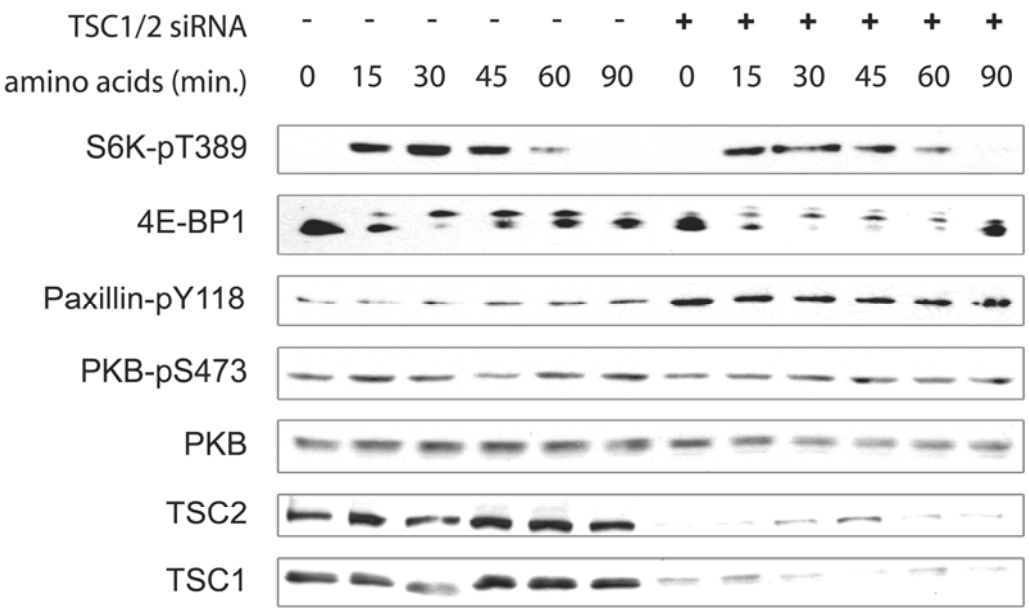

Figure IV-9. Time course for amino acid stimulation of TSCsi293 cells.
Phosphorylation of the mTOR readouts Paxillin Tyr118 (pY118), AKT/PKB Ser473 (pS473) and S6K Thr389 (pT389) and the migration pattern of 4E-BP1 were detected at indicated times in minutes (min.) after stimulation with amino acids. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were grown for the according times in D-PBS+ supplemented with amino acids (1x MEM essential and non- essential amino acids mix) before lysis.

IV.1.10 Combined stimulation of amino acids and insulin.

Combined stimulation with amino acids and insulin reflected the additive signaling response of the two single stimuli on mTOR readouts (compare Figures IV-7 and IV-9).

Figure IV-10.

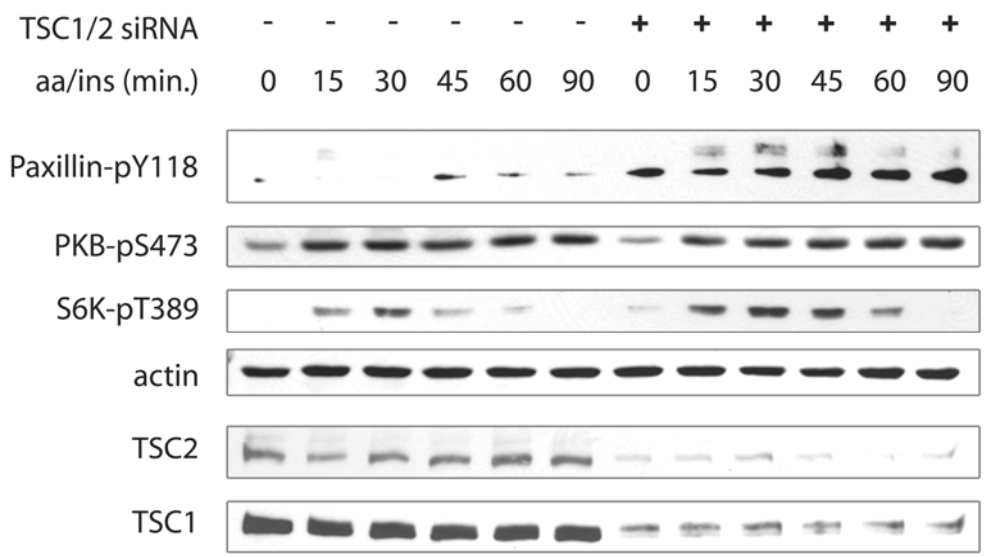

Figure IV-10. Time course for combined amino acid and insulin stimulation of TSCsi293 cells.

Migration of 4E-BP1 and phosphorylation of the mTOR readouts Paxillin Tyr118 (pY118), AKT/PKB Ser473 (pS473) and S6K Thr389 (pT389) was detected at indicated times in minutes (min.) after stimulation with insulin and amino acids. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were serum deprived for 17 hours in DMEM without serum and grown for different times in D-PBS+ supplemented with amino acids (1x MEM essential and non-essential amino acids mix) and 100 nM insulin before lysis.

IV.1.11 Distinct extracellular stimuli or TSC1/2 knockdown exert different effects on phosphorylation of Paxillin Tyr118, PKB Ser473 and S6K Thr389.

We found that stimulation with amino acids, serum or insulin induced phosphorylation of the mTORC1 readout S6K Thr389, whereas only serum and insulin but not amino acids stimulated phosphorylation of the mTORC2 readouts Paxillin Tyr118 and Akt/PKB Ser473 in serum-deprived HEK293T cells (see III.2.5). To determine the role of TSC1-TSC2 in the signal transduction from these extracellular stimuli towards phosphorylation of mTOR readouts, we examined the effects of TSC1/2 knockdown

on phosphorylation of Paxillin, PKB and S6K in TSCsi293 cells that were either stimulated with or starved for amino acids, serum, insulin or a combination of these. Amino acids turned out to be the predominant stimulus for inducing phosphorylation of S6K Thr389 in TSCsi293 cells (Figure IV-11). We found only one condition, in which we could detect a slight increase in S6K Thr389 phosphorylation upon TSC1/2 knockdown that was in the combination of amino acid starvation with insulin stimulation, again illustrating only a very mild effect from TSC1/2 knockdown towards mTORC1 activation in the TSCsi293 cell line. TSC1/2 knockdown did not prevent dephosphorylation of S6K Thr389 upon amino acid starvation, nor did it prevent the responsiveness to amino acid stimulation, illustrating further that amino acids do not primarily signal via TSC1-TSC2.

Independently of TSC1/2 knockdown, mTORC2-dependent phosphorylation of Akt/PKB Ser473 increased upon stimulation with insulin but not upon serum if the TSCsi293 cells had not been serum-starved prior stimulation (Figure IV-11; compare also Figures IV-4, IV-6 and IV-7). TSC1/2 knockdown did not prevent phosphorylation of Akt/PKB Ser473 upon insulin stimulation in TSCsi293 cells, showing once more that TSC1/2 knockdown did not markedly induce feedback inhibition towards the insulin pathway in our system. This was consistent with the mild effects from TSC1/2 knockdown on S6K activation. It appears likely that endogenous Rheb levels are very low in comparison to other cells, in which S6K is strongly activated upon TSC1 or TSC2 deficiency. This assumption would also match with the very faint signal that we received for endogenous levels of Rheb in TSCsi293 cells (data not shown). To determine further if TSC1/2 knockdown did not induce negative feedback inhibition of the insulin pathway, we also examined PDK1-dependent phosphorylation of Akt/PKB Thr308. Efficient phosphorylation of Akt/PKB Thr308 requires membrane recruitment of both, PDK1 and Akt/PKB, via PI3K-dependent PIP_3 generation. Akt/PKB Ser473 phosphorylation did not change upon TSC1/2 knockdown in TSCsi293 cells that were stimulated with growth factors, whereas TSC1/2 knockdown lead to reduced phosphorylation of Akt/PKB Thr308 in TSCsi293 cells that were starved for growth factors (Figure IV-11). This reduction of Akt/PKB Thr308 phosphorylation in TSCsi293 cells upon TSC1/2 knockdown suggests that less PDK1 and Akt/PKB translocated to the membrane. Nevertheless, we found that Akt/PKB Ser473 phosphorylation was not reduced upon TSC1/2

knockdown in growth factor starved cells. The reduction of only Thr308 phosphorylation but not Ser473 phosphorylation upon TSC1/2 knockdown possibly reflects increased mTORC2 activity. Seen it this way, the shifted ratio between Ser473 and Thr308 Akt/PKB phosphorylation towards mTORC2-dependent Ser473 upon TSC knockdown matches with our previous finding that mTORC2-dependent phosphorylation of membrane located Akt/PKB Ser473 (myr-AktPKB Ser473) increases upon knockdown of TSC1/2 and provides further evidence that TSC1-TSC2 negatively regulates mTORC2 activity towards Akt/PKB.

Phosphorylation of Paxillin Tyr118 increased upon TSC1/2 knockdown (Figure IV-11). Serum strongly stimulated Paxillin Tyr118 phosphorylation whereas insulin did not if TSCsi293 cells were not serum-pre-deprived over night. Surprisingly, serum and insulin affected phosphorylation of the mTORC2 readouts Paxillin and Akt/PKB in different ways. Both stimuli induced mTORC2-dependent phosphorylation of Paxillin and Akt/PKB in TSCsi293 cells that were deprived for serum overnight (see III.2.5). However, if the cells were not serum deprived prior stimulation, we found that serum did not induce phosphorylation of PKB Ser473 while efficiently inducing phosphorylation of Paxillin Tyr118, whereas insulin on the other hand did not induce phosphorylation of Paxillin while efficiently inducing PKB phosphorylation (Figure IV-11). This difference between serum and insulin stimulation might result from stronger stimulation of PI3K-dependent PIP_3 levels by insulin in comparison to serum. Serum on the other hand might stimulate a different co-factor stronger than insulin does to allow efficient mTORC2-dependent Paxillin phosphorylation. Putative co-activators for efficient Paxillin phosphorylation could be the MAP kinases ERK1 and ERK2 because ERK1/2 activation, like Akt/PKB activation, inhibits TSC1-TSC2 to activate mTOR. ERK1/2, like Akt/PKB inactivates TSC1-TSC2 by direct phosphorylation of TSC2 on specific residues (Ma et al., 2005a). Furthermore, serum stimulation activates ERK1/2 and ERK1/2 is a known modulator of Paxillin phosphorylation (Brown and Turner, 2004). To determine further if ERK1/2 are indeed co-activators for mTORC2-dependent Paxillin Tyr118 phosphorylation we took a closer look at ERK1/2 activity in our experiment. ERK1/2 activation can be monitored by MEK1/2-dependent phosphorylation of ERK1/2 on Thr202/Tyr204. As for Paxillin Tyr118, serum greatly stimulated phosphorylation of ERK1/2 on Thr202/Tyr204, whereas insulin did not (Figure IV-11). This result argued

in favour of the idea that ERK1/2 could serve as a co-activator for efficient phosphorylation of Paxillin Tyr118 upon serum stimulation (compare also Figure IV-6).

To summarize, we found further evidence that amino acids signal independently of TSC1-TSC2 towards mTORC1. We also found that TSC1/2 knockdown induced only very mild feedback inhibition from S6K to the insulin pathway in TSCsi293 cells and obtained results, which indicate that co-activators like PI3K and ERK1/2 potentially fine tune signal transduction downstream of mTORC2, creating adequate responses to a variable set of extracellular stimuli.

Figure IV-11.

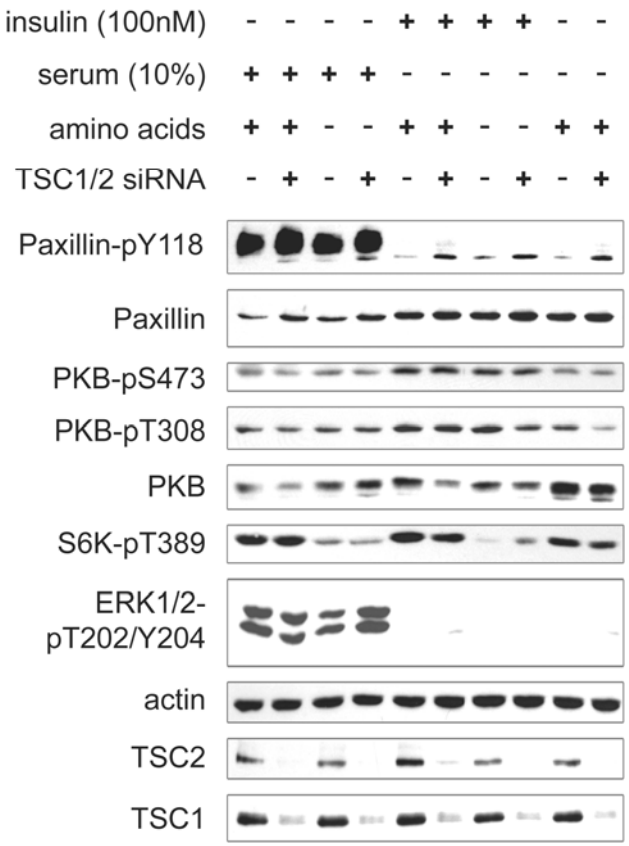

Figure IV-11. Comparison of TSCsi293 cells, stimulated with either amino acids, serum, or insulin or a combination of those.
Phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473), Akt/PKB Thr308 (pT308), S6K Thr389 (T389) and ERK1/2 Thr202/Tyr204 (pT202/Y204) was detected 30 minutes after stimulation with either amino acids and serum, serum alone, amino acids and insulin, or insulin alone. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. The cells were grown for 30 minutes in D-PBS+ supplemented as indicated before lysis.

IV.1.12 Phosphorylation of the mTORC2 readouts Paxillin Tyr118 and Akt/PKB Ser473 is mTORC1-independent.

We expected that knockdown of TSC1/2 would activate mTORC1 in TSCsi293 cells. To determine if hyperactivation of mTORC1 affected our readouts for mTORC2 activity, we inhibited mTORC1 specifically with rapamycin and examined phosphorylation of Paxillin Tyr118 or Akt/PKB Ser473 in tetracycline treated and untreated TSCsi293 cells upon serum stimulation. Rapamycin did not prevent phosphorylation of Paxillin Tyr118 or PKB Ser473 upon serum stimulation, independently of TSC1/2 knockdown, while loss of S6K Thr389 phosphorylation confirmed mTORC1 inhibition by rapamycin (Figure IV-12). This result showed that mTORC1 did not affect Paxillin Tyr118 phosphorylation upon knockdown of TSC1/2 or stimulation with serum.

Figure IV-12.

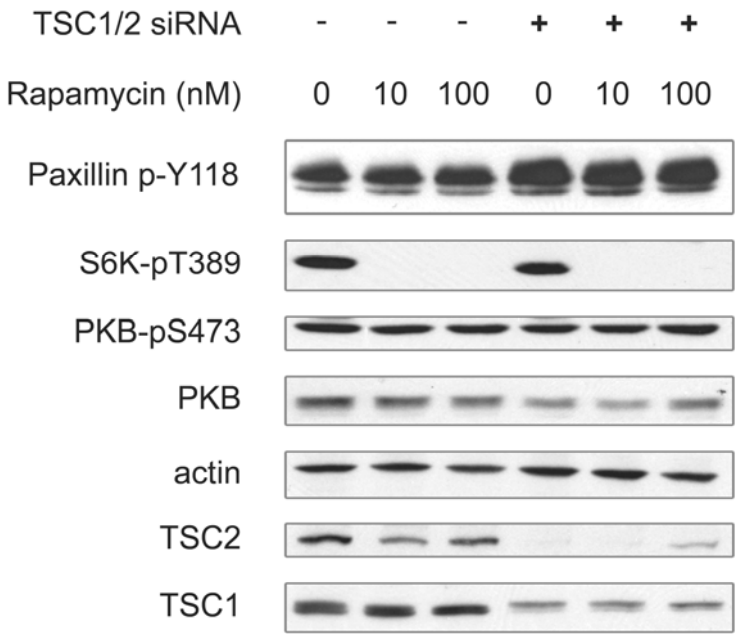

Figure IV-12. mTORC1 inhibition by rapamycin does not block phosphorylation of mTORC2 readouts.

Phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473) and S6K Thr389 (pT389) was detected 30 minutes after stimulation with serum. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. We pre-incubated the cells with rapamycin at indicated concentrations for 30 minutes, using DMSO (1:2000) as vehicle. The cells were then grown for 30 minutes in D-PBS+ supplemented with 10 % serum and rapamycin at the indicated concentrations before lysis.

IV.1.13 Also in serum stimulated cells, mTORC2 mediates increased phosphorylation of Paxillin Tyr118 resulting from TSC1/2 knockdown.

To determine if mTORC2 also mediates increased phosphorylation of Paxillin Tyr118 due to TSC1/2 knockdown in serum stimulated TSCsi293 cells, we inhibited mTORC2 in tetracycline treated TSCsi293 cells, stimulated them with serum and examined phosphorylation of Paxillin and PKB. We inhibited mTORC2 specifically by co-siRNA against rictor using a pSuper-based plasmid. Knockdown of rictor was

confirmed by loss of rictor protein and by loss of Akt/PKB Ser473 phosphorylation. Rictor co-siRNA prevented the increase in Paxillin Tyr118 phosphorylation, resulting from TSC1/2 knockdown also during serum stimulation (Figure IV-13). Thus, mTORC2 activity is required for serum stimulated Paxillin Tyr118 phosphorylation in TSC1/2 knockdown cells.

Figure IV-13.

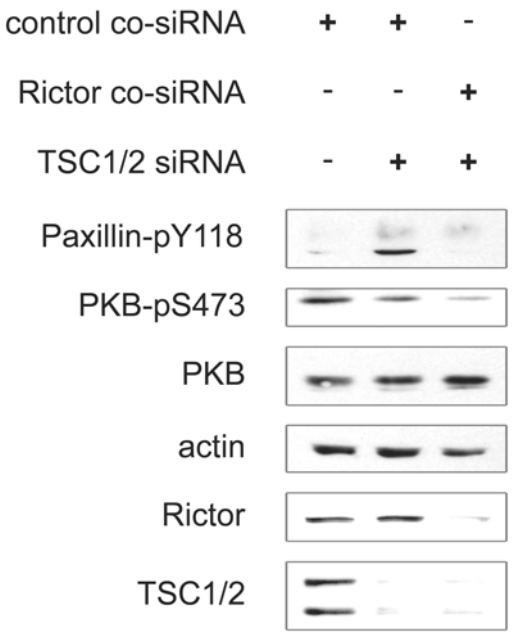

Figure IV-13. mTORC2 mediates the effects of TSC1/2 knockdown also upon serum stimulation.

Also upon serum stimulation, co-knockdown of mTORC2 (rictor co-siRNA) prevents increased Paxillin Tyr118 phosphorylation (Paxillin-pY118) due to TSC1/2 knockdown and leads to loss of PKB Ser473 phosphorylation (PKB-pS473). Western analysis of TSCsi293 cells (+/- tetracycline induced TSC1/2 siRNA), transiently expressing the indicated co-siRNA. The cells were grown for 30 minutes in PBS+ supplemented with 10 % serum before lysis.

IV.1.14 Activities of mTOR and ERK1/2 are both required for efficient Paxillin Tyr118 phosphorylation upon serum stimulation.

To further determine if ERK1/2 signaling is required for efficient mTORC2-dependent phosphorylation of Paxillin Tyr118 upon serum stimulation, we pharmacologically inhibited either ERK1/2 or mTORC2 in TSCsi293 cells before stimulation. For inhibition of ERK1/2 we treated the cells with U0126 and for mTORC2 inhibition we treated the cells with LY294002 at an mTOR inhibiting concentration. Inhibition of either ERK1/2 or mTORC2 prevented serum stimulated phosphorylation of Paxillin Tyr118 (Figure IV-14).

The MEK1/2-specific inhibitor U0126 efficiently blocked ERK1/2 Thr202/Tyr204 phosphorylation, and thus ERK1/2 activity, but not mTORC2 activity as mTORC2-dependent phosphorylation of PKB Ser473 did not change. LY294002 blocked mTORC2-dependent phosphorylation of Akt/PKB Ser473 only at mTOR inhibiting concentrations but not ERK1/2 phosphorylation. These results indicate that serum-induced phosphorylation of Paxillin Tyr118 requires activation of ERK1/2 in addition to activation of mTORC2. The finding that inhibition of ERK1/2 did not block mTORC2-dependent activation of Akt/PKB and that inhibition of mTORC2 did not block ERK1/2 activation additionally showed that ERK1/2 and mTORC2 rather signal in parallel and are not immediately upstream of each other. Thus, the difference between stimulation with serum compared to insulin towards the mTORC2-dependent readouts Paxillin and PKB is likely to rely on differentially activated co-factors by either serum or insulin.

Figure IV-14.

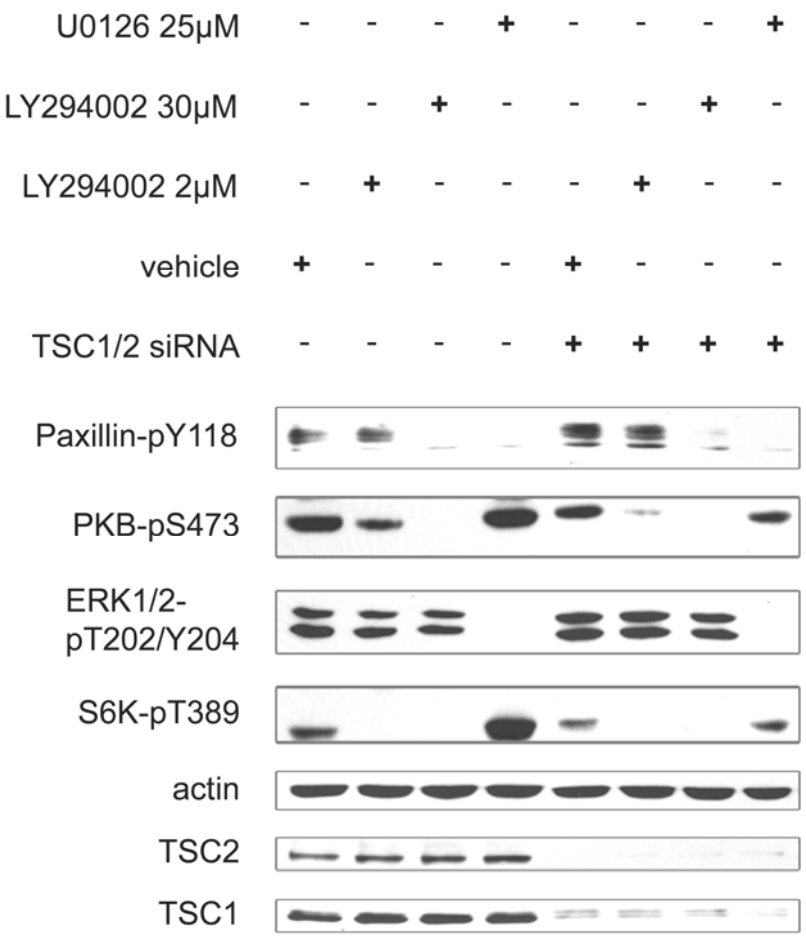

Figure IV-14. Inhibition of either mTOR or ERK1/2 blocks Paxillin Tyr118 phosphorylation upon serum stimulation.

Phosphorylation of Paxillin Tyr118 (pY118), Akt/PKB Ser473 (pS473), S6K Thr389 (pT389) and ERK1/2 Thr202/Tyr204 (pT202/Y204) was detected 30 minutes after stimulation with serum. Western analysis of TSCsi293 cells either induced for TSC1/2 knockdown or not. We pre-incubated the cells for 30 minutes with LY294002 at PI3K inhibiting concentrations (2 μM) or at mTOR inhibiting concentrations (30 μM), or with U0126 at MEK1/2 inhibiting concentrations (25 μM), using DMSO (1:2000) as vehicle. The cells were then grown for 30 minutes in D-PBS+ supplemented with 10 % serum and the indicated inhibitors before lysis.

IV.1.15 Re-expression of TSC1/2 reduced Paxillin Tyr118 phosphorylation upon TSC1/2 knockdown.

To obtain further evidence that the increase in Paxillin Tyr118 phosphorylation upon TSC1/2 knockdown in TSCsi293 cells resulted from reduced levels of TSC1/2 proteins, we transiently re-introduced knockdown resistant versions of TSC1 and TSC2 into TSCsi293 cells while inducing TSC1/2 knockdown. Re-introduction of TSC1/2 in TSCsi293 cells partially reduced phosphorylation of Paxillin Tyr118 upon TSC1/2 knockdown (Figure IV-15). This provided a further control that the increased Paxillin Tyr118 phosphorylation upon TSC1/2 knockdown resulted from the reduction of TSC1/2 protein levels.

Figure IV-15.

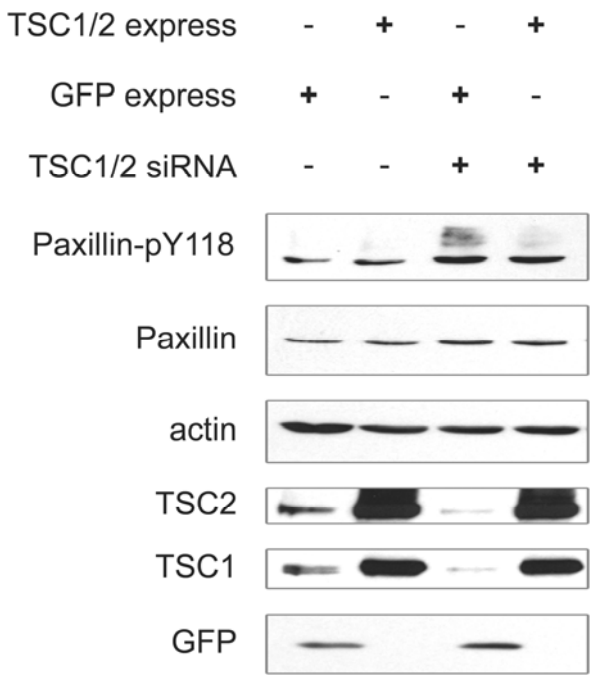

Figure IV-15. TSC1/2 re-expression in tetracycline treated TSCsi293 cells reduced phosphorylation of Paxillin Tyr118 upon serum stimulation.
Phosphorylation of Paxillin Tyr118 (pY118) was detected 30 minutes after serum stimulation. Western analysis of TSCsi293 cells either induced for TSC1/2 siRNA or not. The cells were transfected with either GFP or TSC1 and TSC2, each harbouring

three silent mutations within their target regions for siRNA mediated knockdown. The cells were grown for 30 minutes in D-PBS+ supplemented with 10 % serum before lysis.

IV.2 Discussion

We found that amino acids stimulate the mTORC1 readout S6K1 Thr389 phosphorylation in a transient manner (Figure IV-9). The decline of S6K Thr389 phosphorylation after a short transient peak at about 30 minutes post stimulation suggests that a feedback mechanism exists, which inhibits prolonged hyperactivation of S6K1. It is possible that the reduction in S6K Thr389 phosphorylation following a peak after amino acid stimulation represents reduced mTORC1 activity based on the observation that mTORC1-dependent 4E-BP1 phosphorylation follows the same pattern of S6K Thr389 phosphorylation (Figure IV-9). To further understand if mTORC1 activity is also reduced, it would be interesting to see if Rheb binding to mTORC1 correlates over time with the phosphorylation intensity of the mTORC1 readouts as Rheb binding to mTORC1 has been found to increase after amino acid stimulation. It would also be of interest to determine mTOR phosphorylation on Ser 2448 in mTORC1 by S6K1 in this experiment. Presumably, S6K1 activation upon amino acid stimulation induces mTOR Ser2448 phosphorylation in TSCsi293 cells (Holz and Blenis, 2005). The physiological function of mTOR phosphorylation on Ser2448 is unknown and therefore the speculation is justified that mTOR Ser2448 phosphorylation in mTORC1 by S6K1 might represent a negative feedback mechanism towards amino acid stimulation. Our observation that TSCsi293 cells in which we knocked down TSC1/2 still do respond to amino acid stimulation like normal cells (Figure IV-9) provides additional evidence for the concept that amino acids do not primarily stimulate mTORC1 via inhibition of TSC1-TSC2.

Like stimulation with amino acids induced a transient phosphorylation pattern, serum stimulated phosphorylation of the mTORC2 readout Paxillin Tyr118 also in a transient manner, suggesting that a negative feedback mechanism may exist to limit the effects of serum stimulation (Figure IV-4). This limitation of the response to serum stimulation may have several reasons. As serum contains a non-defined mix of growth factors it is possible that either feedback mechanisms towards growth factor receptors or inhibiting growth factors present in the serum account for this limitation. However, the broad spectrum of possible mechanisms, which could account for such a feedback control, makes it difficult to speculate about the nature of

the transient Paxillin Tyr118 phosphorylation upon serum stimulation. We also found that serum stimulates phosphorylation of Paxillin Tyr118 and Akt/PKB Ser473 in a way that is different to insulin stimulation in our system (see IV.11), which illustrates the capability of cells to fine tune their signaling response to distinct extracellular stimuli. Serum efficiently stimulates Akt/PKB phosphorylation on Ser473 only after the cells were pre-starved for serum (Figures IV-4, IV-6 and IV-11), while Paxillin Tyr118 phosphorylation is found strongly induced upon serum stimulation (Figure IV-4 and IV-11). On the other hand, insulin induces Ser473 phosphorylation in Akt/PKB even without prior serum starvation (Figure IV-7), while insulin does not efficiently induce Paxillin Tyr118 phosphorylation without serum pre-starvation (Figures IV-7, IV-8 and IV-11). We did not measure the difference between the two stimuli towards PI3K activation but presumably, PI3K activity correlates with Akt/PKB Ser473 phosphorylation in our system.

We observed another important difference between insulin and serum stimulation when we measured ERK1/2 activation. ERK1/2 activation is much stronger induced by serum stimulation compared to stimulation with insulin (Figure IV-11). Initial experiments, using siRNA to inhibit mTORC2 (Figure IV-13) and pharmacological inhibitiors for mTOR and ERK1/2 (Figure IV-14) provided encouraging results that the efficiency of mTORC2-dependent phosphorylation of Paxillin Tyr118 arising from serum stimulation might be co-regulated by ERK1/2 activation. In addition to the pharmacological inhibition of ERK1/2 and mTOR during serum starvation (Figure IV-13) it will be necessary to more specifically inhibit ERK1/2 and mTORC2 by siRNA mediated knockdown in combination with serum stimulation to validate our results. If ERK1/2 indeed turns out to co-regulate the mTORC2 readout Paxillin Tyr118 phosphorylation, this would represent the second downstream readout of mTORC2 that is tightly co-regulated next to co-regulated mTORC2-dependent Akt/PKB Ser473 phosphorylation by PI3K. Such tight regulatory mechanism indicate that, if unrestricted, mTORC2 signaling towards the actin cytoskeleton and possibly also towards other readouts has a high potential to promote life threatening steps in disease such as metastasis in cancer. To further determine the role of TSC1-TSC2 in upstream regulation of mTORC2, it will be interesting to test different TSC2 mutant constructs that cannot be inactivated by ERK1/2 (Ma et al., 2005a).

As we could show that mTORC2 is inhibited by TSC1-TSC2, it will be interesting to understand the impact of other stimuli, that are known to regulate mTORC1 via regulation of TSC1-TSC2, towards mTORC2 such as energy and oxygen. Because signal transduction from TSC1-TSC2 towards mTORC2 is apparently different from transduction towards mTORC1, we cannot predict how energy or hypoxia affect mTORC2 activity. The TSCsi293 cell line is a suitable system to address these questions and with more information about the upstream signals that regulate mTORC2 we could widen our understanding of the mTOR signaling network.

We cannot explain the mechanism by which TSC1-TSC2 inhibits mTORC2 as we find that TSC1-TSC2 regulates mTORC2 via an unknown mechanism that is different from mTORC1 regulation by TSC1-TSC2. It will be important to determine if mTORC2 inhibition involves so far unknown proteins or if there is a more direct mechanism that links TSC1-TSC2 with mTORC2 regulation. It will also be important to determine the signaling pathway by which mTORC2 regulates actin organization and presumably the adhesion and migration behaviour of cells to further understand if and how mTORC2 might affect processes in development that rely on tightly regulated cell migration or contribute to diseases that arise from aberrant migration.

Because TORC2 regulates transcription in yeast and mTORC2 apparently regulates the FoxO transcription factors (see I.4.2 and I.4.7), it will be also interesting to investigate if TSC1-TSC2 deficiency induces mTORC2-dependent gene transcription. We believe that the TSCsi293 cell line is also a suitable system to study mTORC2-dependent effects on transcription. In comparison to *tsc1-/-* and *tsc2-/-* knockout cells, induced siRNA-mediated knockdown inhibits TSC1-TSC2 signaling only over a short time period, limiting cellular adaptation and resulting in demonstrated hyperactivation of mTORC2 signaling (Figures III-2 and III-3). This system has additionally the advantage that we already established an efficient epistatic siRNA approach to co-inhibit mTORC2 together with TSC1-TSC2. Increasing knowledge about the signal transduction controlled by mTORC2 promises to provide us with new insights into the biology behind diseases such as tuberous sclerosis complex, lympangioleiomyomathosis and cancer that result from overgrowing and aberrantly migrating cells.

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Peer-reviewed publications

Molle KD and Hall MN. "TSC1-TSC2 complex inhibits mTORC2 in human cells". To be submitted.

Li W, Petrimpol MR, **Molle KD**, Hall MN, Battegay EJ and Humar R. "Hypoxia-induced endothelial proliferation requires both mTORC1 and mTORC2." Circ Res. 2006 Nov 16; [Epub ahead of print].

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International Congresses and poster presentation

2006	Novartis-FMI Meeting: "Targeting the Kinome" in Basel, Switzerland.
2003	Arolla Workshop: "Growth Control in Development and Disease." in Arolla, Switzerland.
2001	MDC Conference: "Cell Migration in Development and Disease." in Berlin, Germany.
2001	EMBO-FMI Conference: "Organizing the Brain: Genes, Neurons and Circuits." in Ascona, Switzerland.