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A complex interplay between Akt, TSC2, and the two mTOR complexes

Jingxiang Huang and **Brendan D. Manning***
Department of Genetics and Complex Diseases, Harvard School of Public Health Boston, MA 02115, USA

Abstract

Akt/PKB both regulates and is regulated by the TSC1-TSC2 complex. Downstream of phosphoinositide 3-kinase (PI3K), Akt directly phosphorylates TSC2 on multiple sites. While the molecular mechanism is not well understood, these phosphorylation events relieve the inhibitory effects of the TSC1-TSC2 complex on Rheb and mTOR complex 1 (mTORC1), thereby activating mTORC1 in response to growth factors. Through negative feedback mechanisms, mTORC1 activity inhibits growth factor stimulation of PI3K. This is particularly evident in cells and tumors lacking the TSC1-TSC2 complex, where Akt signalling is severely attenuated due, at least in part, to constitutive activation of mTORC1. An additional level of complexity in the relationship between Akt and the TSC1-TSC2 complex has recently been uncovered. The growth factor-stimulated kinase activity of mTOR complex 2 (mTORC2 or the mTOR-Rictor complex), which normally enhances Akt signalling by phosphorylating its hydrophobic motif (Ser473), was found to be defective in cells lacking the TSC1-TSC2 complex. This effect on mTORC2 can be separated from the inhibitory effects of the TSC1-TSC2 complex on Rheb and mTORC1. This review discusses our current understanding of the increasingly complex functional interactions between Akt, the TSC1-TSC2 complex and mTOR, which are fundamentally important players in a large variety of human diseases.

Introduction

Dysregulation of the Ser/Thr protein kinase Akt (or PKB) underlies the pathology of a number of common human diseases, such as cancer and diabetes. Akt activation is highly dependent on the class I phosphatidylinositol 3-kinases (PI3K), which are activated downstream of receptor tyrosine kinases or G-protein-coupled receptors [1]. PI3K generates the lipid second messenger phosphatidylinositol-3,4,5-bisphosphate (PIP_3), which binds directly to the pleckstrin homology domains of Akt and PDK1, thereby recruiting these kinases to the plasma membrane. PDK1 phosphorylates the activation loop of Akt at Thr-308, which is essential for Akt activation [2,3]. While the timing relative to Thr-308 phosphorylation is not fully understood, phosphorylation of Akt on a conserved residue (Ser-473) within its regulatory hydrophobic motif results in a further increase in Akt kinase activity [2]. Once active, Akt phosphorylates a number of substrates involved in regulating cell survival, growth, proliferation, and metabolism [4].

Of the many pathways and processes downstream of Akt, we focus here on the regulation of the mammalian target of rapamycin (mTOR) and cell growth. mTOR is a member of the phosphatidylinositol kinase-related kinase (PIKK) family that assembles into two functionally distinct complexes [5]. In mTOR complex 1 (mTORC1), mTOR associates with two highly conserved proteins Raptor and mLST8. Akt signaling stimulates mTOR within this complex

*Correspondence to: bmanning@hsph.harvard.edu Tel: +1 617 432 5614 Fax: +1 617 432 5236.

to directly phosphorylate the ribosomal S6 kinases, S6K1 and S6K2, and the eIF4E-binding proteins, 4EBP1 and 4EBP2. Interestingly, the residue on the S6Ks phosphorylated by mTORC1 (Thr-389 on the 70-kD isoform of human S6K1) lies within a hydrophobic regulatory motif homologous to that surrounding Ser-473 on Akt. mTORC1-mediated phosphorylation of S6K1/2 and 4EBP1/2 stimulates mRNA translation and, ultimately, cell growth and proliferation [6,7].

The second mTOR-containing complex, mTORC2, is comprised of mTOR, Rictor, mSIN1 and mLST8. mTORC2 functions can be distinguished from mTORC1 through use of the mTOR inhibitor, rapamycin. Rapamycin acutely and specifically inhibits mTORC1, whereas its effects on mTORC2 are more variable and generally require prolonged treatment [8]. By far, the best-characterized function of mTORC2 is in the phosphorylation of Akt on Ser-473 [9], but it is also involved in the phosphorylation of a similar motif of PKCα [10,11]. These kinases, along with S6K, belong to the AGC kinase family. Therefore, within these two complexes, mTOR phosphorylates the hydrophobic motif on several members of this kinase family. The phosphorylation of a second conserved motif on Akt and PKCα, referred to as the turn motif (Thr-450 on Akt1), is also dependent on mTORC2 [12,13]. Therefore, mTOR lies both upstream (mTORC2) and downstream (mTORC1) of Akt. Here, we discuss the molecular relationship between Akt and the mTOR complexes, with a focus on the critical regulatory role of the TSC1-TSC2 complex (summarized in Figure 1).

Akt regulates mTORC1 through phosphorylation of TSC2

TSC1 and TSC2 are encoded by the tumor suppressor genes mutated in the tuberous sclerosis complex (TSC) disease [14]. These two proteins form a heterodimeric complex that acts as a functional unit in the suppression of mTORC1 activity. TSC2 contains a GTPase-activating protein (GAP) domain that stimulates the intrinsic GTPase activity of the small G-protein Rheb, thereby enhancing the conversion of Rheb to its GDP-bound inactive state [15]. While the molecular mechanism is not fully understood, in its GTP-bound form, Rheb is a potent activator of mTORC1.

Through distinct phosphorylation events, many signaling pathways converge on the TSC1-TSC2 complex to regulate mTORC1 [16]. In response to growth factors, Akt directly phosphorylates TSC2 on 4 or 5 distinct residues [17,18], two of which (Ser-939 and Thr-1462 on full-length human TSC2) are conserved and phosphorylated in *Drosophila* TSC2 [19]. Whether individual Akt sites on TSC2 differentially affect downstream regulation of Rheb is currently unknown. However, overexpression of TSC2 containing Ala-substitution mutations at different combinations of these sites can dominantly block Akt-mediated activation of mTORC1 [17,18,20], supporting the idea that phosphorylation of TSC2 by Akt is important in the regulation of mTORC1. We have recently confirmed the critical nature of TSC2 phosphorylation by Akt in the regulation of mTORC1 by reconstituting $Tsc2^{-/-}$ mouse embryo fibroblasts (MEFs) with wild-type versus phopshorylation-site mutants of *TSC2*. We find that, when expressed at near endogenous levels, TSC2 lacking all five identified Akt sites (TSC2-5A) suppresses insulin-stimulated mTORC1 signaling to S6K1 and 4E-BP1 more completely than the TSC2-2A mutant, which lacks just the two conserved phosphorylation sites (JH, unpublished). Furthermore, TSC2-5A-expressing MEFs were significantly smaller than those expressing either wild-type TSC2 or TSC2-2A, which were nearly identical in size (JH, unpublished). Therefore, in this more physiological paradigm, a clear regulatory role is seen for the additional Akt phosphorylation sites (Ser-981, Ser-1130, and Ser-1132), which are conserved in vertebrate but not *Drosophila* TSC2.

These findings demonstrate that Akt-mediated phosphorylation impairs the ability of TSC2 to inhibit Rheb and mTORC1. However, the molecular mechanism is not fully understood. In the

majority of published experiments, phosphorylation of these sites does not affect the association between TSC1 and TSC2 (reviewed in [16]. Furthermore, these phosphorylation sites do not grossly affect the GAP activity of TSC2 towards recombinant Rheb in *in vitro* GAP assays (BDM, unpublished, [20]). While it is possible that such assays are not sensitive enough to detect biologically significant changes in GAP activity, it appears that an alternative molecular mechanism hinders the ability of TSC2 to regulate Rheb upon Akt-mediated phosphorylation. One enticing, but under-studied, mechanism entails 14-3-3-binding to multiple Akt phosphorylation sites on TSC2 [20,21], perhaps leading to changes in its subcellular localization and/or disruption of its association with Rheb.

Feedback regulation of PI3K-Akt signaling by mTORC1

It has been recognized for some time that rapamycin treatment of cells, such as 3T3-L1 adipocytes, increases the sensitivity of the PI3K-Akt pathway to insulin, suggesting that mTORC1 signaling normally inhibits insulin signaling. The molecular effects of mTORC1 on insulin signaling have been reviewed elsewhere [22,23]. This inhibitory effect is particularly evident in cells lacking the TSC1-TSC2 complex, where elevated mTORC1-S6K1 signaling leads to a strong attenuation of PI3K activation downstream of insulin and the insulin receptor substrate proteins, IRS1 and IRS2 [24,25]. In normal cells, this effect of mTORC1 signaling on PI3K and Akt serves, in its simplest terms, as a negative feedback loop to monitor and limit Akt signaling. However, in cells with chronic activation of mTORC1, such as those lacking a functional TSC1-TSC2 complex, this “feedback” mechanism constitutively inhibits Akt signaling to its many downstream substrates involved in cell survival and proliferation. For instance, the FOXO family of transcription factors, which can promote apoptosis or cell-cycle arrest in a context-dependent manner, are normally inhibited by Akt-mediated phosphorylation and sequestration from the nucleus [26]. However, FOXO proteins are not phosphorylated and remain nuclear, even in the presence of growth factors, in cells, tumors, and tissues lacking the TSC1-TSC2 complex [25,27–29]. In most of the settings in which *TSC* gene disruption were examined, prolonged rapamycin treatment, at least partially, restores the ability of insulin to stimulate PI3K-Akt signaling [24,25,28], and siRNA-mediated knockdown of Raptor does the same [30], demonstrating the specificity for mTORC1.

The TSC1-TSC2 complex promotes mTORC2 activity

As currently understood, the mTORC1-dependent feedback mechanism affecting PI3K-Akt signaling acts upstream of PI3K on the IRS proteins. However, attenuation of growth-factor stimulated Akt phosphorylation is seen more generally upon disruption of the TSC1-TSC2 complex, such as under full serum growth conditions [27,28,31], where the IRS proteins should play only a minor role. In studies of MEFs lacking *Tsc1* or *Tsc2*, we observe loss of Akt phosphorylation on Thr-308, Ser-473, and, to a lesser extent, the turn motif at Thr-450 (JH and BDM, unpublished), suggesting that multiple inputs into Akt activation are impaired.

Given the complexity of Akt activation and the strong mTORC1-driven feedback inhibition upstream of PI3K in these cells, we decided to directly assay mTORC2 kinase activity following its isolation via immunoprecipitation of Rictor [30]. Using an exogenous Akt substrate to measure mTORC2 kinase activity, we found that mTORC2 activity was severely blunted in *Tsc1*- or *Tsc2*-deficient MEFs or HeLa cells with stable shRNA-mediated knockdown of *TSC2*. Reciprocally, overexpression of TSC2 in wild-type cells increased mTORC2 kinase activity in these assays. Importantly, insulin-stimulated mTORC2 activity was not restored to *Tsc2*-deficient MEFs after prolonged rapamycin treatment or siRNA-mediated knockdown of Rheb or Raptor, despite all of these treatments blocking mTORC1 activity and restoring insulin signaling to PI3K. Furthermore, phosphorylation of Akt on Ser-473 was also reduced in *Tsc2* null cells, relative to reconstituted cells, following expression

of a variety of constitutively active PI3K alleles, which should bypass feedback mechanisms acting upstream. Finally, stable expression of a GAP-dead mutant of TSC2 partially restored mTORC2 activity and Akt phosphorylation to *Tsc2*$^{-/-}$ MEFs, without detectable effects on the constitutive mTORC1 signaling in these cells. Collectively, these data demonstrate that the TSC1-TSC2 complex promotes mTORC2 activity in a manner that is independent of Rheb, mTORC1, and mTORC1-mediated feedback effects on PI3K.

In further support of the TSC1-TSC2 complex being involved in mTORC2 activation, we have recently found reduced levels of hydrophobic motif phosphorylation on PKCα (Ser-657) and total PKCα levels in *Tsc2*$^{-/-}$ MEFs (JH and BDM, unpublished). While not as severe, these effects on PKCα are similar to that described for MEFs lacking the mTORC2 components Rictor, Sin1, or mLST8 [10,12,13]. Unlike Akt, PKCα does not appear to be affected by feedback loops stemming from mTORC1 activation, and in fact, we found that prolonged rapamycin treatment further decreased PKCα phosphorylation in the *Tsc2*$^{-/-}$ cells (JH and BDM, unpublished). These results provide further support that mTORC2 activity is defective in *Tsc2*−/− cells through mechanisms other than mTORC1-dependent feedback or cross-talk with mTORC2.

Do cellular levels of Akt-Ser473 phosphorylation accurately reflect levels of mTORC2 activity?

One observation that we have had difficulty resolving in the *Tsc2*-deficient MEFs is the finding that prolonged rapamycin and Raptor siRNAs both partially restored insulin-stimulated phosphorylation of endogenous Akt under the same experimental conditions where mTORC2 kinase activity remained unstimulated [30]. In fact, in the case of rapamycin treatment, basal mTORC2 activity was further reduced due to disruption of the interaction between Rictor and mTOR. A similar disconnect can be seen in most cell types, where phosphorylation of Akt-Ser473 is increased in response to prolonged rapamycin despite severe disruption of mTORC2 stability [8]. From these studies, it is safe to conclude that the phosphorylation level of Akt at Ser-473 in cell lysates does not necessarily reflect, nor should be considered an accurate reading of, the activation status of mTORC2. While mTORC2 is primarily responsible for this phosphorylation event, Ser473 phosphorylation is a more accurate marker of PI3K activity than mTORC2.

It is clear that the primary event in Akt activation is its recruitment to the plasma membrane downstream of PI3K. Blocking mTORC1 activity, in both TSC-deficient and wild-type cells, greatly enhances PI3K activation, leading to recruitment of more Akt molecules to the plasma membrane. Once at this site, it appears that very low levels of mTORC2 kinase activity are sufficient to phosphorylate Akt. In support of this model, we have found that targeting Akt to the plasma membrane independent of PI3K signaling, via a myristoylation sequence (myr-Akt), in *Tsc2*-deficient cells leads to robust phosphorylation on Ser-473 (JH and BDM, unpublished). Interestingly, a similar finding has been reported for *Sin1*$^{-/-}$ MEFs, which completely lack mTORC2 activity [12]. Therefore, it appears that recruiting Akt to the plasma membrane through increased PI3K activity, or artificially with membrane-targeting sequences, is sufficient to stimulate phosphorylation at Ser-473 under conditions of low or even no mTORC2 activity. While mTORC2 appears to be the primary Ser-473 kinase downstream of growth factor receptors, the findings in mTORC2-deficient cells [12] suggest that alternative mechanisms, such as autophosphorylation [32] or other kinases [33], might also contribute under conditions of robust Akt membrane recruitment.

How does the TSC1-TSC2 complex affect mTORC2 activity?

The mechanism by which the TSC1-TSC2 complex promotes mTORC2 activity toward Akt and, perhaps, PKCα is currently unknown. However, we have detected a physical interaction between these two complexes [30]. Endogenous TSC1 and TSC2 can be immunoprecipitated with epitope-tagged versions of mTORC2 components (mTOR, Rictor, Sin1, and mLST8) but not Raptor, a component unique to mTORC1. Reciprocally, endogenous mTORC2 components, but not Raptor, can be immunoprecipitated with epitope-tagged TSC1 or TSC2. While the interaction is difficult to detect between endogenous proteins, we can reproducibly immunoprecipitate Rictor with anti-TSC1 antibodies, but not control antibodies, in a number of different cell lines. In general, the TSC1-TSC2 complex and mTORC2 appear to interact substoichiometrically in a somewhat weak, perhaps transient, manner, rather than forming a stable association as part of a larger complex. The molecular details of these interactions are not currently known, but we find that TSC2 is both necessary and sufficient for mTORC2 association.

Whether the physical association between the TSC1-TSC2 complex and mTORC2 plays some regulatory role for one or both complexes is not currently known. We find that the mTORC2 associated with the TSC1-TSC2 complex is active and can phosphorylate Akt on Ser-473 *in vitro* [30]. However, to date, we have yet to identify growth conditions that regulate the interaction between these two complexes. We have recently screened a panel of TSC2 missense mutants representative of those found in TSC patients for their ability to bind mTORC2. While many of these mutations disrupt binding to TSC1 without affecting the association with mTORC2, we did find one TSC2 mutant that bound TSC1 but not mTORC2. Interestingly, unlike those that retain mTORC2 binding, stable reconstitution of $Tsc2^{-/-}$ MEFs with this particular mutant fails to restore mTORC2 kinase activity (JH and BDM, unpublished). Therefore, there is a correlation between the ability of the TSC1-TSC2 complex to bind mTORC2 and the ability of growth factors to stimulate mTORC2 activity. We are actively pursuing the molecular mechanism of this regulation, but based on our previous results [30], it appears to be independent of TSC2 GAP activity and Rheb.

Future directions

Many unanswered questions remain regarding the regulation and functions of the TSC1-TSC2 complex and mTORC2. For instance, it will be important to determine whether any of the multiple regulatory inputs into the TSC1-TSC2 complex, which are known to modulate mTORC1 activity [16], also affect mTORC2 activation. This is particularly interesting with respect to Akt-mediated phosphorylation of TSC2. If this phosphorylation inhibits the ability of the TSC1-TSC2 to promote mTORC2 activity, then it would serve as a negative feedback loop affecting all three signaling components, Akt, the TSC1-TSC2 complex, and mTORC2. It is also essential to determine the extent to which the TSC1-TSC2 complex is required for mTORC2 signaling to substrates other than Akt-Ser473. It is clear from our studies and others that mTORC2 activity is stimulated by growth factors [9,30,34]. However, mTORC2 has also been implicated in the phosphorylation of sites that are not regulated by growth factors, such as Akt-Thr450, PKCa-Ser638, and PKCa-Ser657 [10,12,13]. As described above, the phosphorylation of these sites is also decreased in cells lacking the *TSC* genes (JH and BDM, unpublished), suggesting that the TSC1-TSC2 complex might also regulate growth factor-independent functions of mTORC2. Future studies should uncover the spatial and temporal nature of these and other mTORC2 functions.

Importantly, the contributions of mTORC2 dysregulation to the pathology and progression of the TSC and LAM diseases have yet to be defined. Our studies demonstrate that TSC patient-derived missense mutations on TSC2 can differentially affect the ability of the TSC1-TSC2

complex to inhibit mTORC1 and activate mTORC2 ([30] and JH, unpublished). It seems likely that differences in the regulation of the two mTOR complexes could account for significant variation in clinical presentation and course of disease. Future studies should take mTORC2 binding and regulation into account when examining TSC genotype-phenotype correlations at the molecular level. Finally, it is now clear that the role of the TSC1-TSC2 complex in normal cell physiology extends beyond cell growth suppression through inhibition of mTORC1. By contributing to mTORC2 activation, and through its inhibition of mTORC1-dependent feedback mechanisms, the TSC1-TSC2 complex acts to divert Akt signaling from mTORC1 activation into its many other downstream branches [4]. While incredible progress has been made in recent years, there is still much we do not know regarding the molecular regulation and functions of Akt, the TSC1-TSC2 complex and mTOR, which collectively contribute to an enormous variety of human diseases.

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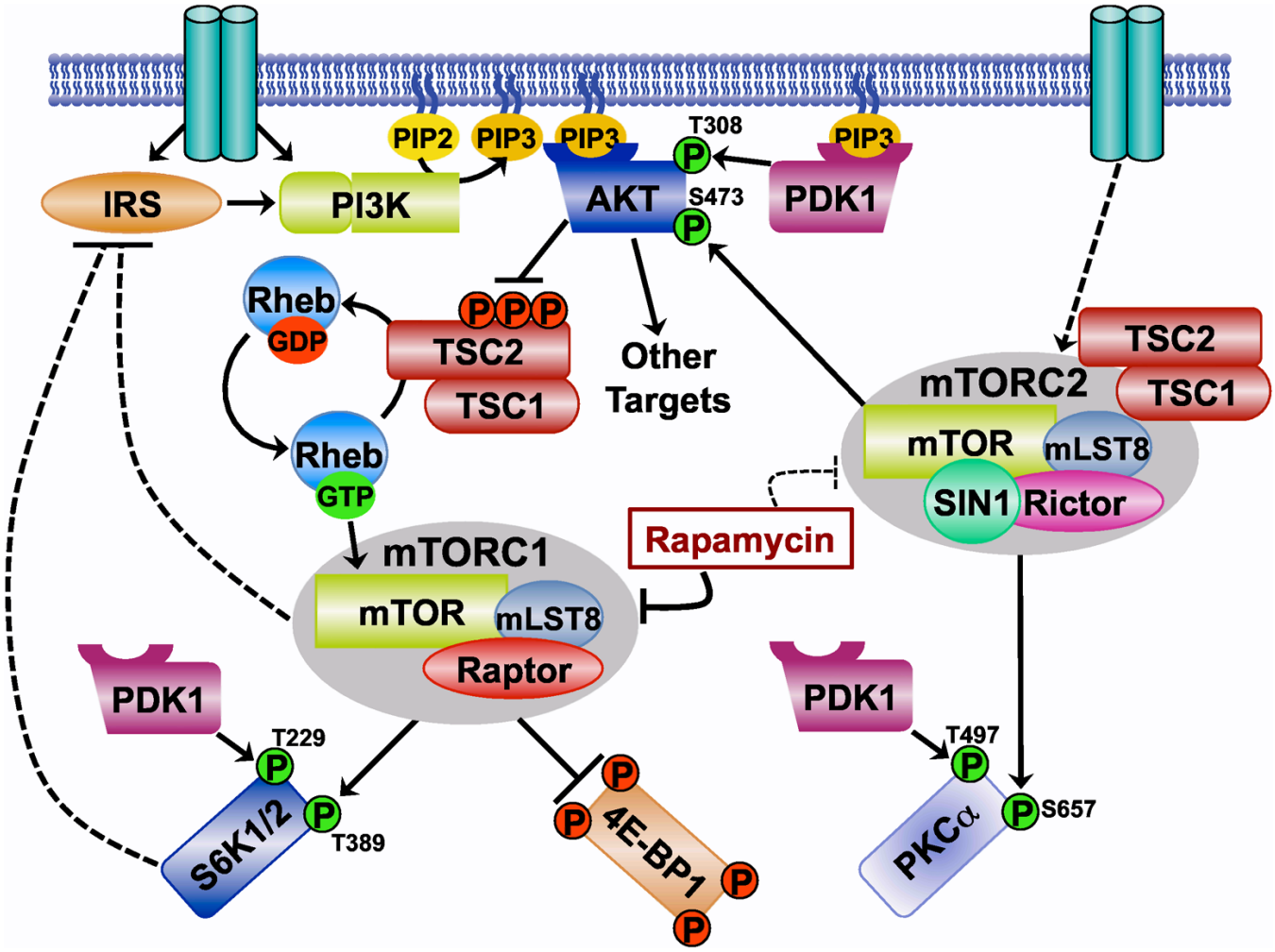

1. . A model of our current understanding of the relationship between Akt, the TSC1-TSC2 complex and the mTOR complexes

PI3K is activated by growth factors through direct interaction with receptors or through interaction with scaffolding adaptors, such as the IRS proteins. These interactions recruit PI3K to its substrate phosphatidylinositol-4,5-bisphosphate (PIP2) allowing generation of the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). Akt and PDK1 are recruited to the plasma membrane through association with PIP3. This allows Akt to be activated through phosphorylation on Thr-308 by PDK1 and Ser-473 by mTORC2. Once active, Akt phosphorylates many downstream targets, including multiple sites on TSC2, which forms a functional complex with TSC1. Phosphorylation of TSC2 impairs the ability of the TSC1-TSC2 complex to act as a GAP toward the small GTPase Rheb, allowing Rheb-GTP to accumulate. Through poorly defined mechanism, Rheb-GTP potently activates mTORC1, which phosphorylates and inhibits 4E-BP1 and activates S6K1 and S6K2. A negative feedback loop exists in which mTORC1 and S6K1 directly phosphorylate IRS-1 and block insulin or IGF-1 signaling to PI3K. Growth factors also increase mTORC2 activity, albeit through unknown signaling events. Through a mechanism distinct from its regulation of Rheb and mTORC1, the TSC1-TSC2 complex can bind to mTORC2 and is required for mTORC2 activation. In addition, the TSC1-TSC2 complex appears to be involved in the phosphorylation of PKCα by mTORC2. Unlike Akt, PDK1 phosphorylates both S6K1/2 and PKCα in a manner

independent of PIP3 binding. Finally, the drug rapamycin strongly and acutely inhibits mTORC1, while it only affects mTORC2 assembly and activity after prolonged exposure.