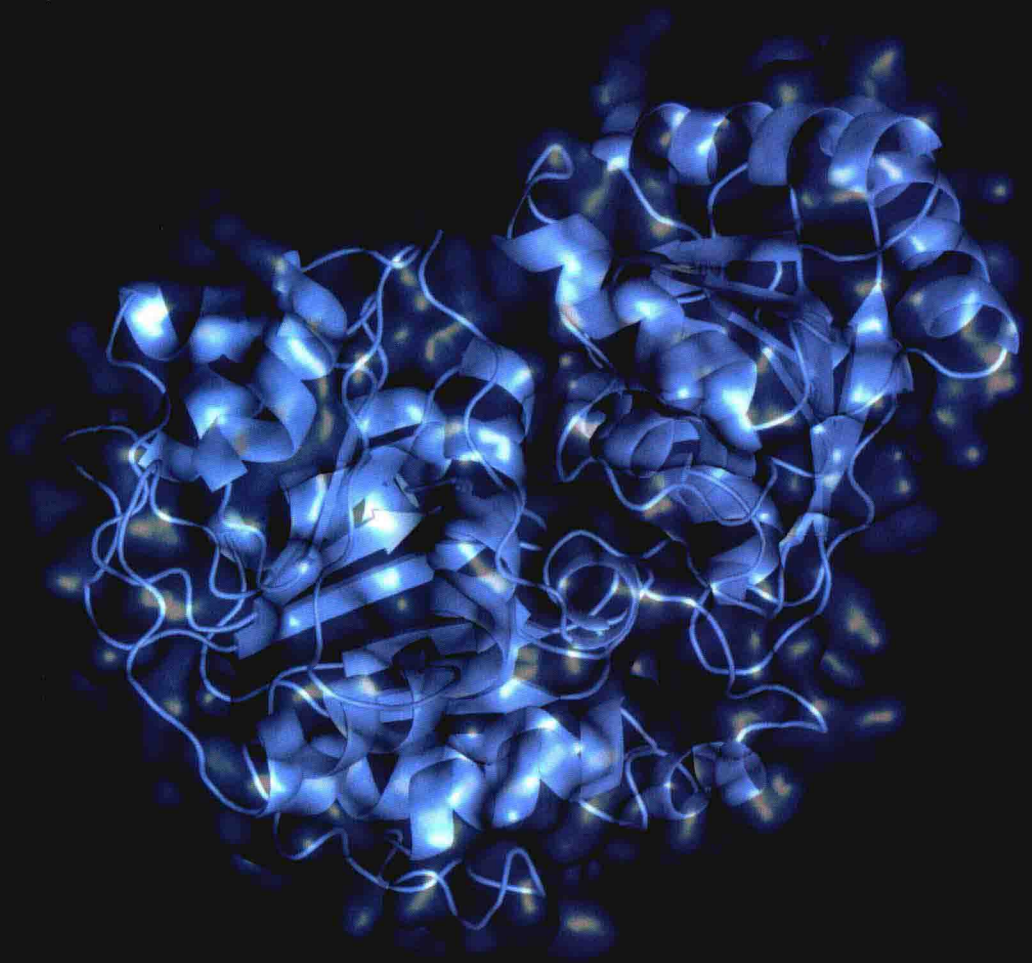
A Practical Approach to

# Protein Phosphorylation



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# A Practical Approach to Protein Phosphorylation

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# Preface

This book aims to highlight the current researches and provides a platform to further the scope of innovations in this area. This book is a product of the combined efforts of many researchers and scientists, after going through thorough studies and analysis from different parts of the world. The objective of this book is to provide the readers with the latest information of the field.

Extensive information regarding protein phosphorylation and human health has been contributed by veteran scientists in this book. The book elucidates the most significant research hot points grouped under two broads sections namely, "AMPK, mTOR, and Akt in cancer & metabolic disorders" and "protein phosphorylation in transcription, pre-mRNA splicing & DNA damage". It connects the basic protein phosphorylation channels with human health and diseases. This book also includes excellent figure illustrations and will be a valuable reference.

I would like to express my sincere thanks to the authors for their dedicated efforts in the completion of this book. I acknowledge the efforts of the publisher for providing constant support. Lastly, I would like to thank my family for their support in all academic endeavors.

**Editor**

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Chapter 1

# Protein Phosphorylation as a Key Mechanism of mTORC1/2 Signaling Pathways

Elena Tchevkina and Andrey Komelkov

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## 1. Introduction

The mammalian target of rapamycin (mTOR) has attracted growing attention during the past decade due to the increase realization of it's extraordinarily significance in cellular life-sustaining activity on the one hand, and because of its crucial role in a variety of diseases, (including cancer, hamartoma syndromes, cardiac hypertrophy, diabetes and obesity) on the other. mTOR is an atypical serine/threonine protein kinase, belonging to the phosphatidylinositol kinase-related kinase (PIKK) family. Cumulative evidence indicates that mTOR acts as a 'master switch' of cellular energy-intensive anabolic processes and energy-producing catabolic activities. It coordinates the rate of cell growth, proliferation and survival in response to extracellular mitogen, energy, nutrient and stress signals [1, 2]. mTOR functions within two distinct multiprotein complexes, mTORC1 and mTORC2, responsible for the different physiological functions. Thus, mTORC1 is considered mostly involved in the regulation of the translation initiation machinery influencing cell growth, proliferation, and survival, while mTORC2 participates in actin cytoskeleton rearrangements and cell survival. mTORC1 and mTORC2 were initially identified in yeast on the basis of their differential sensitivity to the inhibitory effects of rapamycin, mTORC1 being originally considered as rapamycin-sensitive and mTORC2 as rapamycin-insensitive [3-5].

The history of TOR began in the early 1970s when a bacterial strain, *Streptomyces hygroscopicus*, was first isolated from Rapa Nui island during a discovery program for anti-microbial agents. These bacteria secrete a potent anti-fungal macrolide that was named rapamycin after the location of its discovery [6-9]. Later rapamycin was proven to have anti-proliferative and immunosuppressive properties. In the beginning of 1990s, two rapamycin target genes titled TOR1 (the target of rapamycin 1) and TOR2 were discovered through the yeast genetic screens for mutations that counteract the growth inhibitory properties of rapamycin [10, 11]. Further studies revealed that rapamycin forms the complex with its

intracellular receptor, FK506-binding protein 12 kDa (FKBP12), This complex binds a region in the C-terminus of TOR kinase named FRB (FKB12-rapamycin binding) domain, what leads to the inhibition of TOR functions [12-14].

At present, it becomes clear that mTORC1 and mTORC2 activities are mediated through diverse signaling pathways depending on the type of extracellular signal. Thus, signaling from growth factors is mediated predominantly through PI3K-Akt-TSC1/2 pathway and upregulates mTORC1 to stimulate translation initiation, while energy or nutrient depletion and stresses suppress mTORC1 via LKB1–AMPK cascade to trigger off the process of autophagy. In contrast, mTORC2 is insensitive to nutrients or energy conditions. mTORC2 phosphorylates Akt and some other protein kinases regulating actin cytoskeleton and cell survival in response to growth factors and hormones. The physiological functions of mTOR continue to expand. It should be stressed, that the signaling throughout the complicated mTOR network, including branched pathways and feedback loops, is regulated predominantly by phosphorylation and includes myriads of phosphorylation events. Moreover, the complexity of mTOR regulation is amplified by the crosstalk with other signaling pathways, such as MAP kinase- or TNF$\alpha$-dependent cascades, which activity is also determined by vast number of phosphorylations. The complication of mTOR signaling additionally increases due to the hierarchical character of multiple site-specific phosphorylations of the main mTOR targets. Up to date there are no full clarity, concerning which kinase is responsible for each site phosphorylation as well as functional role and precise mechanisms of each phosphorylation event. The better understanding of underlying molecular mechanisms is now especially essential since inhibitors of mTOR signaling are widely used as drugs in the therapy of cancer and neurodegenerative diseases.

## 2. mTOR kinase structural organization

Although mTOR has limited sequence similarities in eukaryotes, it demonstrates a high level of conservation in its key cellular functions. mTOR, also known as FRAP (FKBP12-rapamycin-associated protein), RAFT1 (rapamycin and FKBP12 target), RAPT 1 (rapamycin target 1), or SEP (sirolimus effector protein), is a large 289 kDa atypical serine/threonine (S/T) kinase [15-18] and is considered a member of the phosphatidylinositol 3-kinase (PI3K)-kinase-related kinase (PIKK) superfamily since its C-terminus shares strong homology to the catalytic domain of PI3K [19, 20]. mTOR and yeast TOR proteins share > 65% identity in carboxy-terminal catalytic domains and about 40% identity in overall sequence [21]. At the amino-acid level, human, mouse and rat TOR proteins share a 95% identity [22, 23]. The knockout of mTOR in mice is embryonic lethal, indicating its physiological importance [24, 25].

Structurally, mTOR contains 2549 amino acids and the region of first 1200 N-terminal amino acids contains up to 20 tandem repeated **HEAT** (a protein-protein interaction structure of two tandem anti-parallel $\alpha$-helices found in **H**untingtin, **E**longation factor 3 (EF3), PR65/**A** subunit of protein phosphatase 2A (PP2A), and **T**OR) motifs [26]. Tandem HEAT repeats are present in many proteins and may form an extended superhelical structure responsible for protein-protein interactions. HEAT repeats region is followed by a **FAT** (**F**RAP, **A**TM, and

TRRAP (PIKK family members)) domain and **FRB** (**F**KPB12-**r**apamycin **b**inding domain), which serves as a docking site for the rapamycin -FKBP12 complex formation. Downstream lies a catalytic kinase domain and a **FATC (FAT C**arboxyterminal) domain, located at the C-terminus of the protein (**Figure 1A).** The FAT and FATC domains are always found in combination, so it has been hypothesized that the interactions between FAT and FATC might contribute to the catalytic kinase activity of mTOR via unknown mechanisms [26, 27].

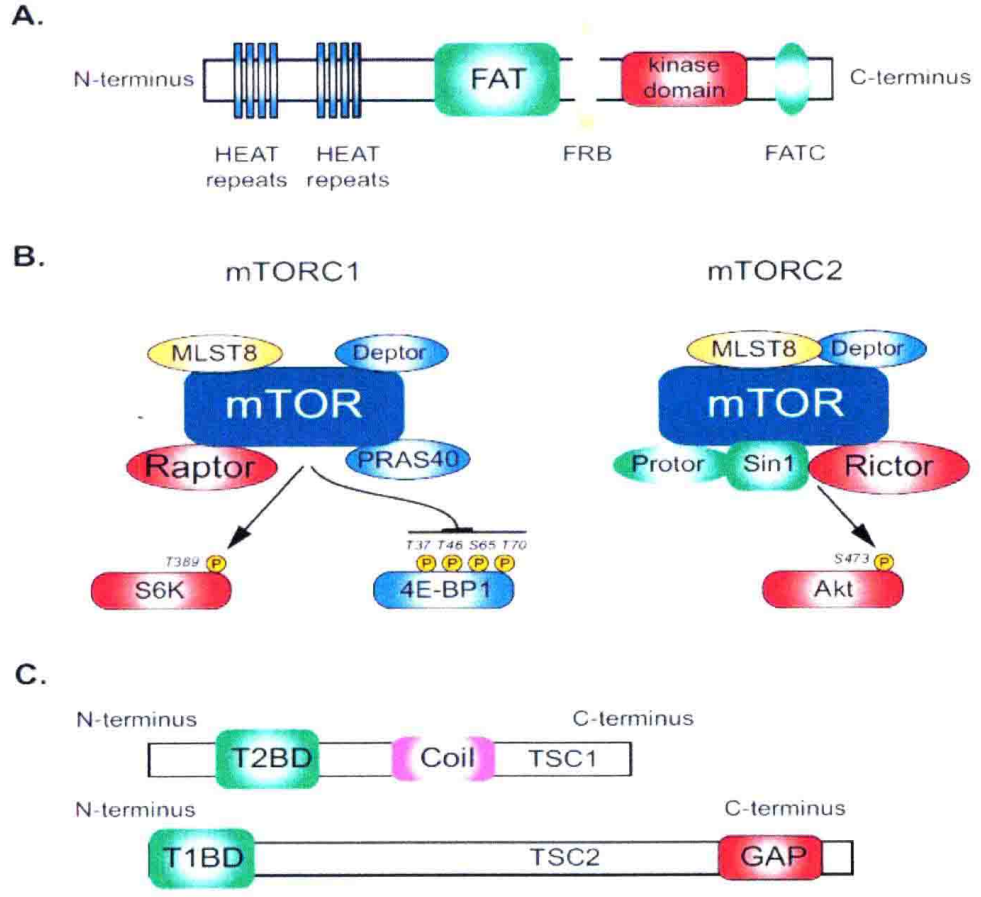


**Figure 1. A. The domain structure of mTOR.** mTOR contains tandem HEAT repeats, central FAT domain, FRB domain, a catalytic kinase domain and the FATC domain. Rapamycin associates with its intracellular receptor, FKBP12, and the resulting complex interacts with the FRB domain of mTOR. Binding of rapamycin–FKBP12 to the FRB domain disrupts the association of mTOR with the mTORC1 specific component Raptor and thus uncouples mTORC1 from its substrates, thereby blocking mTORC1 signaling. **B.** Composition of mTORC1 and mTORC2. mTORC1 consists of mTOR, Raptor, PRAS40, mLST8 and Deptor. mLST8 binds to the mTOR kinase domain in both complexes, where it seems to be crucial for their assembly. Deptor acts as an inhibitor of both complexes. Other protein partners differ between the two complexes. mTORC2 contains Rictor, mSIN1, and Protor1. **C.** Schematic of the TSC1 and TSC2 proteins. The functional domains (including GAP) on TSC1 and TSC2 are represented schematically. T2BD/T1BD — TSC2 and TSC1 binding domains respectively.

Up to date quite a few phosphorylation sites in mTOR have been reported, namely T2446, S2448, S2481 and S1261 and this list will be probably appended. S2481 is considered to be a site of autophosphorylation [28]. S2481 is the only site the phosphorylation of which is well established for regulating mTOR intrinsic activity [29, 30]; the significance of other phosphorylation sites for mTOR activity are not entirely clear. Recently, S1261 has been reported as a novel mTOR phosphorylation site in mammalian cells and the first evidences of this phosphorylation in regulating mTORC1 autokinase activity has been provided [31]. Although phosphorylation at T2446/S2448 was shown to be PI3K/Akt-dependent, mTORC1 downstream kinase S6K has been also reported to phosphorylate these two sites [32]. The significance of this potential feedback loop is unknown, as it is not yet clear whether and how these phosphorylations influence mTOR activity

Binding of rapamycin–FKBP12 to the FRB domain of mTOR disrupts the association of mTOR with mTORC1-specific component Raptor and thus divide mTORC1 from its targets, blocking mTORC1 signaling. However, whether rapamycin directly inhibits mTOR's intrinsic kinase activity is still not entirely clear [3, 33, 34].

**The TOR complexes mTORC1 and mTORC2**

The mammalian mTORC1 and mTORC2 complexes perform non-overlapping functions within the cell. Thus, the best-known function of TORC1 signaling is the promotion of translation. Other mTORC1 functions include autophagy inhibition, promotion of the ribosome biogenesis and of the tRNA production. The main known mTORC2 activity is the phosphorylation and activation of AKT and of the related kinases — serum/glucocorticoid regulated kinase (SGK) and protein kinase C (PKC) [35]. It is also involved in cytoskeletal organization. Although both mTOR complexes exist predominantly in the cytoplasm, some data indicate that they could function in different compartments. Thus, upon nutrients and energy availability mTORC1 is recruited to lysosomes where it could be fully activated [36] and where it functions to suppress autophagy. Unlike mTORC1, mTORC2 according to the most recent data localizes predominantly in ER compartment where it could directly associate with ribosomes [37, 38]. Additionally, some data evidence that mTOR may actually be a cytoplasmic-nuclear shuttling protein. The nuclear shuttling could facilitate the phosphorylation of mTORC1 substrates under the mitogenic stimulation [39]. The unique compositions of mTORC1 and mTORC2 determine the selectivity of their binding partners. Up to date we know more about mTORC1 rather then mTORC2 probably due to the lack of available and wide-spreaded inhibitors of mTORC2 activity.

**TORC1 composition.** Within the mammalian cells, TORC1 functions as a homodimer. Each monomer consists of mTOR, **r**egulatory **a**ssociated **p**rotein of m**TOR** (Raptor), **p**roline-rich **AK**T **s**ubstrate **40** kDa (PRAS40), **DEP** domain **TOR**-binding protein (Deptor) and **m**ammalian **l***ethal* with **S**ec-13 protein **8** (mLST8, also known as GbL) [40, 41]**(Figure 1B).**

**Raptor** is a 150 kDa presumably non-enzymatic subunit of mTORC1 that is essential for the kinase mTORC1 activity *in vitro* and *in vivo* in response to insulin, nutrient and energy level. [42, 43]. It includes a highly conserved N-terminal region followed by 3 HEAT repeats and 7

WD40 (about **40** amino acids with conserved **W** and **D** forming four anti-parallel beta strands) repeats. The Raptor-mTOR interaction is very dynamic, and is thought to require the HEAT repeats of mTOR. It is established that Raptor is indispensable for mTOR to phosphorylate its main effectors p70S6 kinase (S6K1) and **e**ukaryotic **i**nitiation **f**actor **4E** (eIF4E) **b**inding **p**rotein 1 (4E-BP1), but whether Raptor positively or negatively regulates mTOR itself remains controversial [43]. Raptor is essential for mTORC1 complex formation and for the dimerization of TORC1 complexes as it provides direct interaction between TOR proteins from each monomer. Thus it can be considered to be a scaffolding protein that recruits substrates for mTOR thereby demonstrating a stimulating effect on mTOR activity [43]. Alternatively, other study has demonstrated that Raptor negatively regulates mTOR being tightly bound to the kinase [42]. There are also a hypothesis according to which at least two types of interaction exist between Raptor and mTOR depending on nutrients availability. One mTOR-Raptor complex that forms in the absence of nutrients is stable and leads to a repression of the mTOR catalytic activity. In contrast, the other complex that forms under nutrients-rich conditions is unstable, but it is important for *in vivo* mTOR function [42] (reviewed in [44]). Recent studies suggested that the phosphorylation status of Raptor could influence mTORC1 activity [45]. Phosphorylation on S722/792 is mediated by AMPK (AMP-activated protein kinase) and is required for the inhibition of mTORC1 activity induced by energy stress [45], whereas phosphorylation of Raptor on S719/721/722 is mediated by the p90 ribosomal S6 kinases (RSKs) and contributes to the activation of mTORC1 by mitogen stimulation [45, 46]. Most recently, S863 in Raptor was identified as mTOR-mediated phosphorylation site responsible for the insulin-dependent activation of mTORC1 [47].

**PRAS40**, another subunit of mTORC1, has been defined as a direct negative regulator of mTORC1 function [48]. Initially, PRAS40 was identified as a novel substrate of Akt being directly phosphorylated at T246 near its C-terminus [49]. This phosphorylation releases inhibition of mTORC1 by PRAS40. Subsequent studies showed that PRAS40 associates with mTORC1 via Raptor and inhibits mTORC1 activity [48]. A putative TOR signaling motif, FVMDE, has been identified in PRAS40 and shown to be required for interaction with Raptor. Upon binding to Raptor, PRAS40 is phosphorylated on S183 by mTORC1 both *in vivo* and *in vitro* [50] Thus, PRAS40 has been implicated as a physiological substrate of mTORC1. Most recently, two novel sites in PRAS40 phosphorylated by mTORC1, S212 and S221, have been identified [51]. Rapamycin treatment reduced the phosphorylation of S183 and S221 but not S212, indicating that besides mTORC1, other kinases may also regulate the phosphorylation of S212 *in vivo* [51].

**mLST8** has been identified after Raptor as a stable component of both mTOR complexes [52]. It consists almost entirely of seven "sticky" WD40 repeats, and has been initially shown to bind to the kinase domain of mTOR, leading to the hypothesis that mLST8 positively regulates mTOR kinase activity. It was proposed that mLST8 is essential for a nutrient- and rapamycin-sensitive interaction between Raptor and mTOR [52]. However, there is no substantial evidence to support this idea. It has been speculated, that mLST8 may participate in the amino acids mediated activation of TORC1 being insignificant for other

mechanisms of TORC1 activation [52]. Alternatively, recent studies demonstrated functional importance of mLST8 for the Rictor-mTOR interaction, evidencing that mLST8 is involved in mTORC2 rather than in mTORC1 activity.

**Deptor** binds to mTOR at the FAT domain thus originally proposed to be a part of TORC1. Recently it has been identified as mTOR inhibitor that acts on both TORC1 and TORC2. The upstream regulators of Deptor still remain unknown [41].

**mTORC2 composition and distinctions from mTORC1**

In 2004, mTORC2, containing mTOR, mLST8 and Rictor was identified [3, 4]. Since mTORC2 complex was discovered later than mTORC1, its functions and regulatory mechanisms are less understood [3]. TORC2 and TORC1 contain common subunits, as is mTOR itself, mLST8 and Deptor, but instead of Raptor, mTORC2 includes two different subunits, Rictor (**r**apamycin-**i**nsensitive **c**ompanion of m**TOR)** and mSin1 (**m**ammalian **s**tress-activated protein kinase (SAPK)-**in**teracting protein **1**) [3, 4, 53]. In addition, Protor (**pr**otein **o**bserved with Rictor) was also considered a component of mTORC2 (**Figure 1B**) [54, 55]. mTORC2 was originally thought to be rapamycin-insensitive [3], however, further studies demonstrated that prolonged rapamycin treatment inhibits the assembly of mTORC2 as well as its activity towards Akt phosphorylation in certain cell lines [56].

**Rictor** is the first identified TORC2 specific component [3, 4]. It represents a large protein with a predicted molecular weight of about 200 kDa. Although Rictor contains no apparent catalytic domain motifs [4], knockdown of Rictor results in the loss of actin polymerization and cell spreading, the main known mTORC2 functions [4]. It was shown that the Rictor-mTOR complex does not affect the mTORC1 effectors S6K1 and 4E-BP1, but influence the activities of several proteins known as mTORC2 downstream targets, including phosphorylation of Akt, PKC and the focal adhesion proteins.

**mSin1** was recently identified as a novel component of mTORC2, which is important for both the complex assembly and function [57-59]. *Sin1* is conserved among all eukaryotic species especially in the middle part of the sequence [60]. A Ras-binding domain and a C-terminal PH domain have been identified recently [61]. The several experimental techniques showed the importance of Sin1 for mTORC2 function [62]. The interaction *in vivo* between Sin1 and Rictor is more stable than their interactions with mTOR probably due to the ability of Sin1 and Rictor to stabilize each other [59]. Thus knockdown of Sin1 decreases the interaction between mTOR and Rictor, suggesting that Sin1 is important for mTORC2 assembly. Knockdown of Sin1 by RNAi in *Drosophila and* mammals crucially diminishes the Akt phosphorylation on S473 *in vitro*. The same effect was observed in Sin1−/− cells [58].

**Protor-**1 and Protor-2 (also known as Proline rich protein 5 (PRR5) [54, 55] and PRR5-like (PRR5L) [63] are two newly identified mTORC2 interactors which have been identified as Rictor-binding or SIN1 binding proteins [54]. Up to date their functions remain unclear. It is currently accepted that they are dispensable for mTORC2 assembly as well as for its catalytic activity [54], although Protor stability is dependent on the production of other TORC2 components. It is possible that Rictor and Sin1 act as scaffold proteins for various complexes involving different kinases.

**mLST8 and Deptor**, as was mentioned above, are the components of both mTORC1 and mTORC2 complexes.

## 3. Upstream regulation of mTOR signaling

### 3.1. PI3K-AKT-TSC1/2 -"Classical" pathway of mTOR regulation

Although this pathway is still considered to be the main way exerting multi-faceted control over mTORC1 activity which sense insulin and growth factors signals to regulate cell growth, at present it becomes clear that at least some of its components also function to mediate responses on other stimulus, such as energy, stress or nutrients which are provided by discrepant signaling pathways, described below.

#### *3.1.1. TSC1/TSC2 complex and Rheb protein*

The TSC1/TSC2 complex (tuberous sclerosis complex 1/2, TSC1/2) has been established as the major upstream inhibitory regulator of mTORC1 [64, 65]. This complex mediates signals from a large number of distinct signaling pathways to modulate mTORC1 activity predominantly via different phosphorylations of TSC2. Functioning as a molecular switch, TSC1/2 suppresses mTOR's activity to restrict cell growth during the stress, and releases its inhibition under the favorable conditions. The *TSC1* and *TSC2* genes were identified in 1997 and 1993 respectively as the tumor-suppressor genes mutated in the tumor syndrome TSC 1(tuberous sclerosis complex) [66-68]. TSC is a multisystem disorder characterized by the development of numerous benign tumors (e.g. hamartomas) most commonly detected at the brain, kidneys, skin, heart and lungs. Genetic studies of *TSC1* and *TSC2* in humans, mice, *Drosophila* and yeast strongly suggest that these proteins act mainly as a complex. The 140 kDa TSC1 (also known as hamartin) and 200 kDa TSC2 (also known as tuberin) proteins share no homology with each other and very little with other proteins **(Figure 1C)** TSC1 and TSC2 associate through certain regions [69] giving a heterodimeric complex. The only known functional domain throughout these two proteins is a region of homology at the C-terminus of TSC2 to the GAP domain of small G-protein Rap1. Searches for a GTPase target regulated by the TSC2 GAP (GTPase-activating protein) domain revealed the small G-protein Rheb. Mammalian TSC2 was shown to accelerate the rate of GTP hydrolysis of Rheb, converting Rheb from the active GTP-bound to the inactive GDP-bound state [69, 70]. This evidences that Rheb is a direct target of TSC2 GAP activity, and TSC2 suppress Rheb function. While the GAP activity of TSC2 is necessary for the complex functionality, TSC1 is required to stabilize TSC2 and prevent its ubiquitin-mediated degradation [71, 72]. Under growth conditions, the TSC1/2 complex is inactive, thereby allowing Rheb-GTP to activate TORC1.

Rheb is a member of the Ras superfamily that appears to be conserved in all eukaryotes and, despite the term 'brain' in its name, is in fact ubiquitously expressed in mammals. Whether a GEF protein (guanine-nucleotide exchange factor responsible for reverse process, i.e. change GDP-bound to GTP-bound state) for Rheb exists remains unknown. Several evidences demonstrate that Rheb positively regulates mTORC1. In particular, Rheb

overexpression stimulates S6K1 and 4EBP1 phosphorylation, which are indicators of mTORC1 activity. This effect can be reversed by mTOR inactivation or by rapamycin treatment, suggesting that Rheb primarily functions through TORC1 [59]. Although genetic and biochemical studies strongly suggest that GTP-bound Rheb potently activates mTORC1, the molecular mechanism is still unclear. Overexpressed Rheb was shown to bind to mTOR [73, 74]. Associations between endogenous Rheb and mTORC1 components have not been reported. In general, Ras-related small G-proteins bind to their downstream effectors mostly in the GTP-bound state. Surprisingly, Rheb has been found to bind stronger to mTOR in its GDP-bound or nucleotide-free states [74]. At the same time it has been shown that GTP-bound Rheb rather than the GDP-bound stimulates mTOR kinase activity *in vitro* [74]. Although the mechanism by which Rheb-GTP activates mTORC1 has not been fully understood, it needs Rheb farnesylation and can be blocked by farnesyl transferase (FT) inhibitors. Recently, it was found that Rheb can directly interact with the FKBP12 homologue FKBP38 (named also FKBP8), and this binding seems to be tighter with Rheb-GTP [75]. That study suggests that Rheb-GTP binds to FKBP38 and triggers its release from mTORC1, stimulating mTORC1 activity **(Figure 2).** In support of this model, an independent study carried out that decreasing FKBP38 expression with antisense oligonucleotides blocked the growth inhibitory effects of TSC1–TSC2 overexpression [76]. Although more studies are needed, these findings suggest that FKBP38 might be a Rheb effector that regulates mTORC1 and, perhaps, unknown targets downstream of the TSC1/TSC2 complex and Rheb.

### *3.1.2. The PI3K-AKT pathway joins TSC-mTORC1 regulation*

The responsiveness of mTORC1 signaling to growth factors and insulin is provided through activation of PI3K (phosphatidylinositol-3-kinase) and Akt kinase, but the precise mechanism is still not clear. Through PI3K signaling, Akt also termed PKB (serine/threonine protein kinase B) is activated by most growth factors to phosphorylate several downstream substrates [77].

PI3K is a heterodimeric protein containing an 85-kDa regulatory and a 110-kDa catalytic subunits (*PIK3CA*) [78, 79]. PI3K acts to phosphorylate a number of membrane phospholipids to form the lipid second messengers phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2 or PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3 or PIP3). In response to the upstream inputs, PI3K at the cell membrane is activated through the association of a ligand with its receptor, stimulating p85 to bind phosphorylated tyrosine residues of Src-homology 2 (SH2) domain on the receptor. This association promotes the p110 catalytic subunit to transfer phosphate groups to the membrane phospholipids [78, 80]. Consequently these lipids, particularly PtdIns(3,4,5)P3, attract several kinases to the plasmalemma initiating the signaling cascade [78, 80]. PIP3 accumulation is antagonized by the well-known tumor suppressor, lipid phosphatase PTEN (**p**hosphatase and **t**ensin homolog deleted on chromosome 10), which converts PIP3 to PIP2. One important function of PIP3 is to recruit Akt as well as PDK1 (or PDPK1, 3-phosphoinositide-dependent protein kinase-1) [81] via their PH (**p**leckstrin **h**omology) domains to the plasma membrane **(Figure 2).**