1 Introduction

1.1 Cancer and Cell growth

Cancerous cells have escaped normal growth constraints and often contain invasive properties. The function of signaling molecules important for cell growth and cell division is often dysregulated in cancer cells, which leads to their aberrant proliferation. Thus, components of the cell signaling machinery that regulate cell growth and cell division are potential therapeutics targets for drug design.

Rapamycin, a bacterially derived macrolide, is a promising anti-cancer drug (Huang and Houghton 2002). Rapamycin completely blocks proliferation in lymphocytes, while delaying proliferation in most other cell types (Abraham and Wiederrecht 1996; Schmelzle and Hall 2000). Rapamycin was originally identified as a potent antifungal and immunosuppressive agent (Abraham and Wiederrecht 1996). Both rapamycin and its soluble ester analog CCI-779 are now in several clinical trials as anti-tumor agents (Hidalgo and Rowinsky 2000; Elit 2002). Moreover, rapamycin and CCI-779 are currently in use for the prevention of allograft rejection following organ transplantations (Sehgal 1995). Both drugs display remarkable efficiency in the prevention of restenosis after coronary artery interventions with stent implantations (Marx, Gaburjakova et al. 2001; Serruys, Regar et al. 2002). The broad clinical application of rapamycin makes it important to elucidate the molecular mechanism of its action.

1.2 Identification of TOR

Rapamycin was first observed to potently inhibit cell proliferation in *S.cerevisiae* (Heitman, Movva et al. 1991). Genetic screens for mutations that rescue the inhibitory effect of rapamycin on proliferation in *S. cerevisiae* identified the cellular binding partners of rapamycin: the FK506-binding protein 12kDa (FKBP12) and the targets of rapamycin, TOR1 and TOR2 (Heitman, Movva et al. 1991; Cafferkey, Young et al. 1993; Kunz, Henriquez et al. 1993). FKBP12 is an abundant and ubiquitously expressed peptidyl-prolyl cis/trans isomerase that might

play a role in protein folding (Heitman, Movva et al. 1991; Schreiber 1991; Gothel and Marahiel 1999). *S.cerevisae* mutants lacking FKBP12 are viable and resistant to rapamycin toxicity (Heitman, Movva et al. 1991; Koltin, Faucette et al. 1991; Dolinski, Muir et al. 1997), indicating FKBP12 is not the target through which rapamycin inhibits proliferation. Rapamycin binds to FKBP12 to form a tight complex, which interacts with the FKBP12 rapamycin-binding (FRB) domain of TOR (Figure 1.1; Heitman, Movva et al. 1991; Schmelzle and Hall 2000). Expression of TOR point mutants (TOR1 (Ser1972Arg) or TOR2 (Ser1975Ile)) that cannot bind to this rapamycin/FKBP12 complex rescues the inhibition of proliferation by rapamycin (Stan, McLaughlin et al. 1994; Chen, Zheng et al. 1995). Therefore, rapamycin blocks cell proliferation by inhibiting the function of TOR.

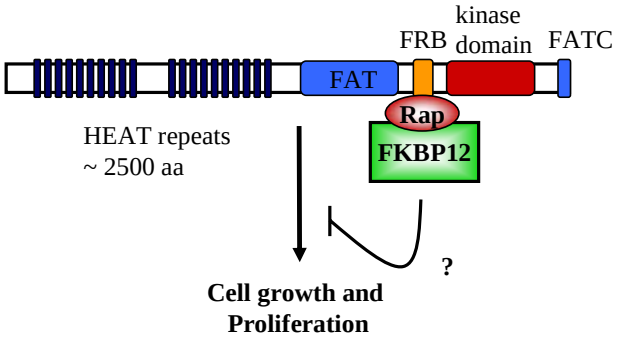

Figure 1.1 Structure of the TOR kinases. Functional domains conserved in TOR proteins are depicted, including the N-terminal HEAT repeats, the central FAT domain, FKBP12-rapamycin binding domain (FRB), the kinase domain and the FATC domain. The figure also shows binding of Rap/FKBP12 complex to FRB domain. aa, amino acids

Subsequent biochemical studies in mammalian cells led to the identification of the mammalian target of rapamycin, mTOR (also referred to as FRAP, RAFT and RAPT, (Brown, Albers et al. 1994; Chiu, Katz et al. 1994; Sabatini, Erdjument-Bromage et al. 1994; Sabers, Martin et al. 1995). TOR is

conserved from yeast to fly and human. In contrast to yeast, which contains two TOR homologs (TOR1 and TOR2) (Helliwell, Wagner et al. 1994), only one TOR ortholog has been identified in higher eukaryotes. TOR belongs to a superfamily of protein serine/threonine kinases termed phosphoinositol (PI) 3-kinase related kinases (PIKK) (Keith and Schreiber 1995). PIKK family members, are large polypeptides (280-300 kDa) containing a C-terminal domain (Figure 1.1), which shares homology with protein and lipid kinases, but has only been demonstrated to phosphorylate Ser/Thr residues on protein substrates rather than lipids. Many PIKK family members modulate key cellular functions, such as DNA damage response, DNA repair, and DNA recombination. The catalytic activity of TOR has been shown to be necessary for the regulation of cell growth and cell proliferation in response to nutrients and mitogens (Figure 1.2; Schmelzle and Hall 2000; Gingras, Raught et al. 2001).

1.3 Regulation of cell growth by TOR signaling

Cell growth and division are usually coupled but distinct processes (Hartwell 1967) that coordinately regulate the proper development of an organ or whole organism (Conlon and Raff 1999). Cells have to grow to a certain size before they can progress through the cell cycle to maintain their characteristic size (Hartwell 1967). It is known that cellular size may vary depending on environmental conditions, such as the levels of nutrients and growth factors (Conlon and Raff 1999). mTOR is thought to be a central regulator of cell growth that integrates signals for mitogen, energy and nutrient sufficiency (Figure 1.2; Schmelzle and Hall 2000). Inhibition of TOR's function in yeast, flies, mice and humans reduces cell size significantly (Montagne, Stewart et al. 1999; Zhang, Stallock et al. 2000; Miron and Sonenberg 2001; Fingar, Salama et al. 2002; Oldham and Hafen 2003). Inhibition of TOR signaling also decreases the proliferative rate of cells (Schmelzle and Hall 2000). However, it is unclear whether TOR directly regulates

proliferation or inhibits the proliferative rate as a secondary consequence caused by its inhibition of cell growth.

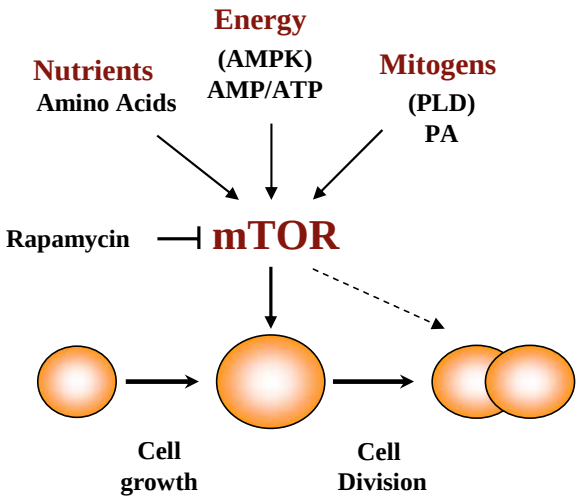

Figure 1.2 mTOR regulates cell growth and proliferation in response to nutrients, energy, and mitogens. TOR might directly regulate cell proliferation (division) or as secondary consequence reduce cell growth. PLD, phospholipase D, AMPK, AMP-dependent kinase.

1.4 Regulation of mTOR

While the function of mTOR as a nutrient sensor has recently been well accepted, the concept that mTOR senses the energy and mitogen status of the cell is just emerging.

1.4.1 mTOR is a conserved nutrient sensor

The nutrient sensing function in yeast, fly and human TOR is conserved (Rohde, Heitman et al. 2001). In *S. cerevisiae*, TOR signaling is sensitive to changes in nitrogen, glucose, and amino acids, while inhibition of TOR signaling by rapamycin or TOR deletion triggers a stress response program similar to nutrient starvation (Rohde, Heitman et al. 2001). To further support the role of TOR as a

nutrient sensor, null mutations in the *Drosophila* TOR (dTOR) impair larval growth and mimic the phenotype of amino acid withdrawal (Kunz, Henriquez et al. 1993; Barbet, Schneider et al. 1996; Oldham, Montagne et al. 2000; Zhang, Stallock et al. 2000). The mammalian TOR also functions as a nutrient sensor (Figure 1.2), as phosphorylation of its best characterized downstream targets, the ribosomal S6 kinase 1 (S6K1), and the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), are sensitive to changes in amino acid levels (Shah, Anthony et al. 2000). 4E-BP1 and S6K1 are important regulators of translation initiation (see more details paragraph 1.5.2). The phosphorylation of S6K1 and 4E-BP1 is particularly responsive to changes in leucine levels and to a lesser extend to levels of other branched-chain amino acids, such as isoleucine and valine (Hara, Yonezawa et al. 1998). Amino acid depletion inhibits, while re-addition of amino acids stimulates the mTOR-dependent phosphorylation of both S6K1 and 4E-BP1.

The mechanism by which mTOR senses amino acid levels is unclear. Several models have been proposed. Iboshi et al. (Iiboshi, Papst et al. 1999) postulated that mTOR might sense the charging of amino-acetylated tRNA. The authors found that amino acid alcohols, which are competitive inhibitors of amino acyl-tRNA synthetases, inhibit mTOR regulation of S6K1. However, these data have been questioned by others (Pham, Heydrick et al. 2000; Dennis, Jaeschke et al. 2001; Beugnet, Tee et al. 2003). In contrast, recently Beugnet et al. (Beugnet, Tee et al. 2003) suggested that mTOR might be regulated by free intracellular amino acids, but did not describe a mechanism by which this regulation may occur.

1.4.2 mTOR senses the energy status

mTOR has also been suggested to sense the energy status of the cell through monitoring the levels of ATP (Figure 1.2; Dennis, Jaeschke et al. 2001). Reduction of cellular ATP levels by the glycolytic inhibitor 2-deoxyglucose (2-DG) specifically inhibits mTOR-dependent phosphorylation of S6K1 and 4E-BP1. Reduction in ATP levels by 2-deoxyglucose leads to an increase in the AMP/ATP ratio. Interestingly, the AMP-dependent kinase (AMPK), which is allosterically activated by 5' AMP, was found to regulate S6K1 activity in a mTOR-dependent manner (Kimura, Tokunaga et al. 2003). AMPK reduces ATP expenditure by inhibiting key enzymes of biosynthetic pathways, and increases the ATP supply by activating pathways producing ATP and stimulating glucose uptake (Hardie and Hawley 2001). Therefore, mTOR might not directly sense ATP levels, as postulated in Dennis *et al.* (Dennis, Jaeschke et al. 2001), but could be regulated by the AMP/ATP ratio via AMPK (Figure 1.2). However, AMPK does not regulate mTOR-dependent signaling in all mammalian cell types (HCE-T epithelial cell and H411E hepatoma cells but not CHO-IR or HEK293 cells) (Kimura, Tokunaga et al. 2003), suggesting that mTOR may also sense AMP or ATP levels via another mechanism.

1.4.3 Mitogen-dependent mTOR regulation

Phosphatidic acid (PA) has been recently shown to stimulate S6K1 and 4E-BP1 phosphorylation in an amino acid-dependent manner (Figure 1.2; Fang, Vilella-Bach et al. 2001). PA is generated by mitogen-activated Phospholipase D (PLD) 1 and 2 and has been found to directly bind to the FRB domain of mTOR *in vitro* (Fang, Vilella-Bach et al. 2001). These observations provide the first evidence that mitogens directly regulate mTOR. Therefore, mTOR requires both nutrient and mitogen sufficiency for downstream signaling.

1.5 m TOR downstream signaling

The best known function of mTOR is the regulation of translation initiation via the S6 kinases 1 and 2 (S6K1 and S6K2), and the eIF4E-binding proteins (4E-BP1/2/3) (Figure 1.3; Gingras, Raught et al. 2001). Protein synthesis is one of the most energetically demanding functions of the cell (Schmidt 1999), and therefore tightly coordinated with both nutrient and energy availability.

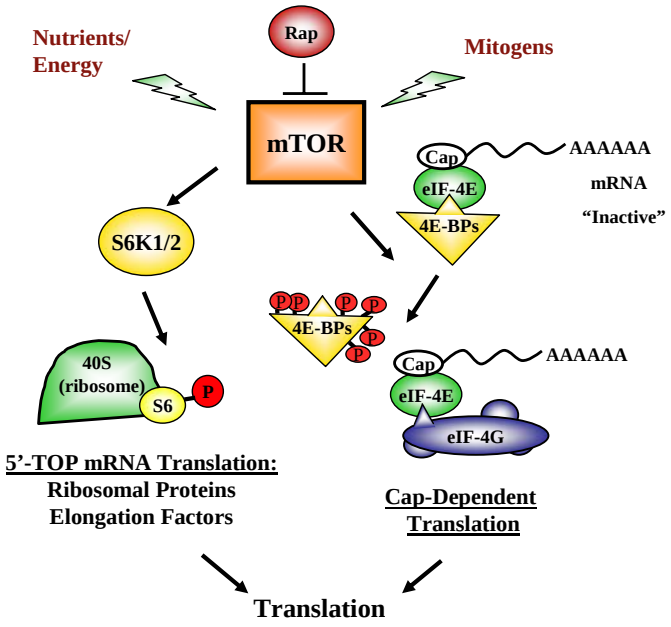

Figure 1.3 mTOR regulates translation initiation via S6K1/2 and 4E-BPs. S6K1/2 phosphorylates the ribosomal S6 protein, phosphorylation of which is thought to be required for 5'TOP mRNA translation. Phosphorylation of 4E-BP1 allows initiation of Cap-dependent translation.

1.5.1 Regulation of Cap-dependent translation by 4E-BPs

eIF4E binds to the Cap structure (m7GpppN, where N is any nucleotide) at the 5' end of mRNAs, which is found in the vast majority of cellular mRNAs, and plays an important role in translation initiation (Raught and Gingras 1999). When hypophosphorylated in the absence of nutrients or growth factors, 4E-BP1

associates with eIF4E and inhibits its function (Figure 1.4; Pause, Belsham et al. 1994; Lin, Kong et al. 1994).Growth factor stimulation in the presence of sufficient nutrients leads to phosphorylation of 4E-BP1 and its dissociation from eIF4E. Free eIF4E then binds to the scaffolding protein eIF4G, which assembles the initiation complex, also containing the RNA helicase eIF4A, which is required to unwind mRNA secondary structure (Figure 1.4; Raught and Gingras 1999), and Figure4). eIF4G further interacts with eIF3, which recruits the 40S ribosome to the 5' end of the mRNA (Sachs, Sarnow et al. 1997) and the poly(A)-binding protein PABP, which brings the 3' termini of the mRNAs into close proximity to the initiation complex, potentially increasing the likelihood of reinitiation. 4E-BP1 and eIF4G have overlapping binding site in eIF4E and therefore compete for binding to eIF4E (Marcotrigiano, Gingras et al. 1999).

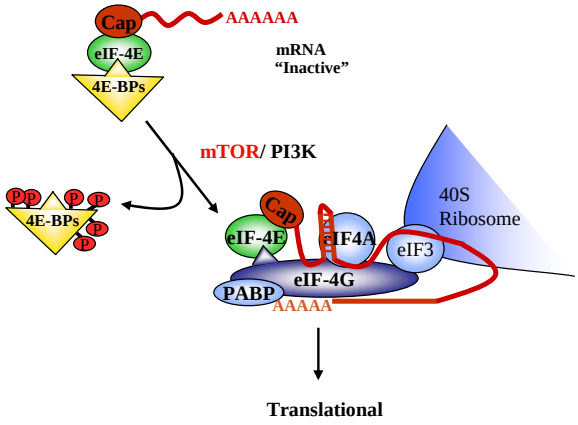

Figure 1.4 Mechanism of translation initiation. Hypophosphorylated 4E-BPs sequester eIF4E bound to the Cap structure found on the extreme 5'-terminus of the mRNA. Hyperphosphorylation of 4E-BPs (indicated by 'P') results in the dissociation of the 4E-BPs from eIF4E and the subsequent association of eIF4E with eIF4G. eIF4G functions as a scaffolding protein that assembles translation factors required for efficient translation initiation. These include the RNA helicase eIF4A, the poly(A)-binding protein PABP, and eIF3, which recruits the 40S ribosome to the 5' end of the mRNA (Hentze97, Hershey, merick2000). 4E-BP1 and eIF4G have overlapping binding site in eIF4E and therefore compete for binding to eIF4E (Marcotrigiano99).

The translation initiation rates can differ dramatically depending on the structure of 5’ untranslated region of mRNA (reviewed in (Gingras, Raught et al. 2001). 4E-BP1 phosphorylation is particularly required for translation initiation of mRNA containing a highly structured 5’UTR, often encoding proteins that regulate cell growth and cell proliferation (Kozak 1991). eIF4E is the least abundant translation initiation factor and most likely limiting for translation initiation (Duncan, Milburn et al. 1987; De Benedetti, Joshi-Barve et al. 1991). Indeed, overexpression of eIF4E transforms rodent fibroblasts (Lazaris-Karatzas, Montine et al. 1990), while co-expression with 4E-BP1 reverses eIF4E-driven transformation (Rousseau, Gingras 1996). In addition, upregulation of both eIF4E and eIF4G proteins has been noted in several tumor types underscoring the importance of regulated translation initiation in the control of cell growth. Overexpression of a d4E-BP1 mutant that binds eIF4E more efficiently decreases cell size in *Drosophila* (Miron and Sonenberg 2001), suggesting that d4E-BP1 along with dS6K1 mediates the function of dTOR to control cell size.

1.5.2 S6K1 signaling

S6K1 phosphorylates the 40S ribosomal protein S6, which may enable the efficient translation of a subset of mRNAs containing a terminal oligopolypyrimidine (TOP) track at the 5’end (Jeno, Ballou et al. 1988; Jefferies, Fumagalli et al. 1997). Many of these 5’TOP mRNAs encode for components of translation machinery, such as ribosomal proteins, elongation factors, and the poly (A)-binding protein (PABP) (Jefferies, Fumagalli et al. 1997) (Hornstein, Git et al. 1999). Therefore, S6K1 stimulation is thought to upregulate the general translation capacity of the cell by enhancing the translation of components required for protein synthesis. However, recent reports reveal that S6K1-mediated S6 phosphorylation may not directly regulate 5’TOP mRNA translation (Tang, Hornstein et al. 2001).

Mice lacking S6K1 retain normal S6 phosphorylation and 5'TOP mRNA translation that is likely due to the upregulation of the S6K1 homolog S6K2, which compensates for the loss of S6K1 (Shima, Pende et al. 1998). Furthermore, research from our laboratory showed that stable expression of a rapamycin-resistant S6K2 mutant rescues rapamycin-inhibited S6 phosphorylation, strengthening the notion that S6K2 is an *in vivo* S6 protein kinase (Kathy Martin and John Blenis, unpublished results). However, these S6K1 null mice show a specific reduction of pancreatic beta-cell size (Pende, Kozma et al. 2000), which leads to a decrease in insulin secretion. Therefore, targets which are unique to S6K1 regulate beta-cell size in mice.

Several other downstream targets of S6K1, such as the transcription factor CREMτ, the RNA splicing and export factor CBP80 (Groot, Ballou et al. 1994) (Wilson, Wu et al. 2000), the apoptotic protein BAD (Harada, Andersen et al. 2001), and the eukaryotic elongation factor 2 kinase (eEF2K) (Wang, Li et al. 2001) have been reported, but their regulation by S6K1 has been not fully characterized. Furthermore, it is unknown whether S6K2 can phosphorylate these S6K1 targets.

1.5.3 Other actions of mTOR signaling

mTOR is thought to control the cell cycle by regulating levels of cyclins and cyclin dependent kinase inhibitors. Rapamycin increases the turnover of both mRNA and protein levels of cyclinD1 (Hashemolhosseini, Nagamine et al. 1998) and has been reported to upregulate the cyclin dependent kinase inhibitor p27 through both the mRNA and protein levels (Kawamata, Sakaida et al. 1998). Furthermore, mTOR has been reported to directly phosphorylate the signal transducer and activator of transcription STAT3 in response to the neuropoietic cytokine "ciliary neurotrophic factor" (CNTF) at Ser727. Phosphorylation of Ser727 in STAT3 is rapamycin sensitive and thought to stimulate the transcriptional activity of STAT3 (Yokogami, Wakisaka et al. 2000). The broad

spectrum of TOR function and substrates in *S.cerevisiae* suggest that there are many more targets of the mammalian TOR that are presently unidentified.

1.6 Coordination of mTOR and PI3K-dependent signaling

1.6.1 PI3K signaling

The mTOR- and PI3K (phosphatidyl inositol 3 kinase)-dependent signaling pathways coordinately regulate cell growth via the phosphorylation of S6K1 and 4E-BP1 (Figure 1.5). PI3K signaling is regulated by a wide variety of extracellular signals and plays a central role for a range of cellular processes (Rameh and Cantley 1999). Cell signaling through the PI3K pathway tightly controls cell growth and proliferation (Oldham and Hafen 2003). Overexpression of dominant negative PI3K alleles decreases cell size, whereas activated forms enlarge cell size (Oldham and Hafen 2003)**.** Furthermore, mutations of any of the components of the PI3K pathway has a striking effect on cell size and cell number, with the exception of dS6K that only affects cell size (Oldham and Hafen 2003).

PI3K generates the lipid second messenger phosphatidylinositol-3,4,5-triphosphate that induces activation of downstream targets, such as Akt and PDK1. PI3K and Akt are considered as proto-oncogenes and their signaling pathways are upregulated in various cancer types. The lipid phosphatase PTEN antagonizes PI3K signaling by dephosphorylating lipids generated by PI3K. PTEN is a tumor suppressor gene and one of the most frequent targets for mutations in cancer (Wu, Senechal et al. 1998; Cantley and Neel 1999). Rapamycin and its analog CCI-779 were found to antagonize the transforming phenotype caused by dysregulation of PI3K pathway on the level of PI3K, Akt, or PTEN (Gingras, Raught et al. 2001), suggesting a link between PI3K and mTOR-dependent signaling.

The level of convergence of nutrient-dependent mTOR signaling and mitogen-dependent PI3K signaling is controversial. Other than PA-mediated mTOR regulation the direct mitogen regulation of mTOR activity is unclear (Fang, Vilella-Bach et al. 2001). The mTOR pathway and the PI3K pathway appear to

regulate in parallel S6K1 activation and 4E-BP1 phosphorylation. In *S.cerevisiae* TOR-dependent growth regulation in *S.cerevisiae* is dependent on nitrogen, carbohydrate and amino acid levels rather than mitogen inputs. In contrast, TOR signaling in multicellular organisms is more complex where cell growth and proliferation are regulated by the integration of both nutrients and growth factors.

1.6.2 Regulation of mTOR signaling by the TSC1/TSC2 complex

Akt has been suggested to regulate mTOR signaling by directly phosphorylating mTOR at Ser2448 within its C-terminal repressor domain *in vivo* (Nave, Ouwens et al. 1999; Sekulic, Hudson et al. 2000). However, mutating Ser2448 to Ala to prevent phosphorylation at this site does not affect mTOR downstream signaling, questioning the importance of ser2448 phosphorylation in mTOR regulation (Sekulic, Hudson et al. 2000).

Many recent publications suggest a different mechanism by which Akt regulates mTOR signaling. Mutation in one of the conserved tumor suppressor genes *TSC1* (hamartin) or *TSC2* (tuberin) was found to cause Tuberous Sclerosis, an autosomal dominant disorder that leads to widespread development of benign tumors in the brain, eyes, skin, and kidney that can lead to mental retardation, seizures, or autism (Montagne, Radimerski et al. 2001). Akt has been shown to suppress the function of the TSC1/TSC2 by directly phosphorylating TSC2 at Ser939 and Thr1462. The TSC1/TSC2 complex specifically inhibits mTOR-dependent signaling, as overexpression of the TSC1/TSC2 complex inhibits nutrient-dependent S6K1 stimulation and 4E-BP1 phosphorylation, but does not affect the activity of a S6K1 mutant that is unresponsive to mTOR-mediated signaling (Tee, Fingar et al. 2002). Furthermore, the activity of S6K1 and phosphorylation of 4E-BP1 are upregulated in mammalian cells with disease mutations in either TSC1 or TSC2 (Goncharova, Goncharov et al. 2002; Kwiatkowski, Zhang et al. 2002), and amino acid dependency of S6K1 is partially overcome in TSC knockout cells (Gao, Zhang et al. 2002; Inoki, Li et al. 2002).

Thus, the TSC1/TSC2 complex is thought to block mTOR signaling during mitogen deprivation, and this inhibition is relieved by Akt phosphorylation of TSC2 upon mitogen stimulation (Manning, Tee et al. 2002; Gao, Zhang et al. 2002). TSC2 has been shown to co-immunoprecipitate with TOR (Gao, Zhang et al. 2002), but the mechanism of how TSC1/2 inhibits mTOR-dependent S6K1 and 4E-BP1 phosphorylation is poorly understood.

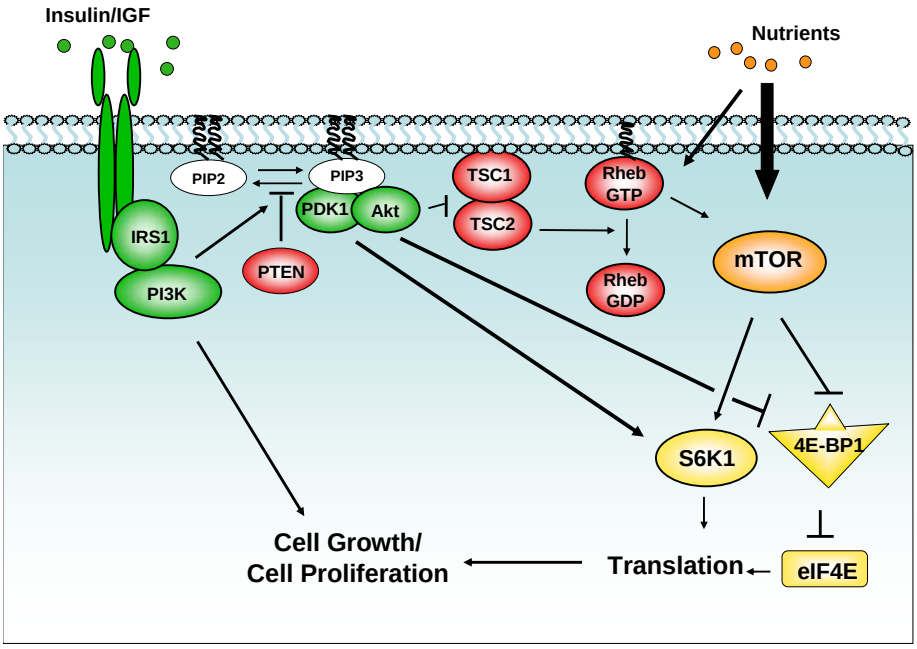

Figure 1.5 Coordination of mTOR and PI3K-dependent signaling pathways. S6 kinases and 4E-BPs are regulated by multiple phosphorylations. Both growth factor and nutrient/energy sufficiency signals are required for the regulation of S6 kinases and 4E-BPs. Arrows indicate activation: bars indicate repression, Rap, rapamycin, Wort, wortmannin.

TSC2 possesses a domain that shares homology with the GTPase-activating protein (GAP) domain of Rap1-GAP (Tuberous Sclerosis Consortium, 1993) and is critical for the tumor suppressor function of TSC2 (Potter, Huang et al. 2001). Recently, the small GTPase Rheb (ras homologue enriched in brain) was identified as a promoter of cell growth in a genetic screen in *Drosophila* (Saucedo,

Gao et al. 2003; Stocker, Radimerski et al. 2003). Subsequent genetic and biochemical analysis suggest that Rheb functions downstream of the TSC1 and TSC2 tumor suppressors in the TOR/dS6K1 signaling pathway to control cell growth. Furthermore, Rheb was shown to be a direct target of TSC2 GAP activity *in vivo* and *in vitro* (Tee 2003; Zhang, Gao et al. 2003). These data support a model where Rheb enhances mTOR signaling when in a GTP-bound form, and TSC2 inhibits TOR signaling by stimulating the intrinsic GTPase activity of Rheb that converts Rheb to an inactive GDP-bound state. The function of Rheb to control TSC1/TSC2-dependent mTOR signaling seems to be conserved from flies to mammals, as overexpression of Rheb within mammalian cell lines activates mTOR-dependent S6K1 and 4E-BP1 phosphorylation and stimulates S6K1 activity during amino acid insufficiency (Tee, Manning et al. 2003). TSC1/TSC2 potently inhibits Rheb while TSC-patient derived mutations within the GAP domain of TSC2 prevented the tumor suppressor complex to inhibit Rheb. Furthermore, it was shown that TSC2 functions as a RhebGAP when bound to its binding partner, TSC1 (Tee, Manning et al. 2003), suggesting that Rheb is a molecular target of TSC1/TSC2 in mammals.

1.6.3 Mechanism of S6K1 and 4E-BP1 regulation

1.6.3.1 Regulation of 4E-BPs

mTOR and PI3K-dependent pathways coordinately regulate 4E-BP1 phosphorylation at least six proline-directed phosphorylation sites (Thr37, Thr46, Ser65, Thr70, Ser83, Ser112) (Figure 1.6). Many external stimuli, including growth factors, hormones, mitogens, and cytokines stimulate 4E-BP1 phosphorylation (Gingras, Raught et al. 2001). 4E-BP1 phosphorylation at both Thr37 and Thr46 is thought to be a priming event, absolutely required for mitogen-induced Ser65 and Thr70 phosphorylation (Figure 1.6), which results in the release of eIF4E (e.g. (Lin, Kong et al. 1994; Pause, Methot et al. 1994; Fadden, Haystead et al. 1997). Thr70 has also been reported to be required for subsequent Ser65

phosphorylation (Mothe-Satney, Brunn et al. 2000; Mothe-Satney, Yang et al. 2000; Gingras, Raught et al. 2001).

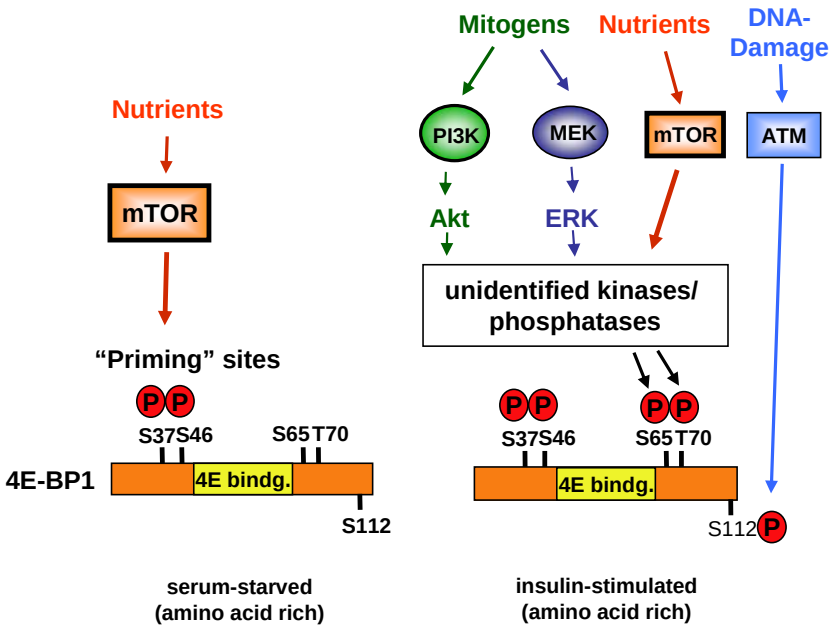

Figure 1.6 Regulation of 4E-BP1 phosphorylation. 4E-BP1 exists in two forms under amino acid rich conditions. In serum-starved cells, phosphorylates the 4E-BP1 priming sites Thr37/46 is regulated by mTOR signaling. Insulin/serum stimulation leads to phosphorylation of Ser65 and Thr70 by PI3K-, ERK-, or/and mTOR-regulated kinases or phosphatases. Phosphorylation of Thr37/46 under insulin/serum stimulation might be mediated by a mitogen-stimulated kinase, which is not mTOR.

Thr37 and Thr46 are basally phosphorylated when cells are deprived of serum and only slightly stimulated by serum (Gingras, Gygi et al. 1999). Rapamycin potently inhibits Thr37 and Thr46 phosphorylation under serum-starved conditions, indicating mTOR to be especially required for Thr37 and Thr46 phosphorylation under serum-starved but nutrient-rich conditions (Figure 1.6; Gingras, Raught et al. 2001). Immunoprecipitated mTOR phosphorylates 4E-BP1 on Thr37 and Thr46 *in vitro*, suggesting that mTOR can directly

phosphorylate these sites (Brunn, Hudson et al. 1997; Burnett, Barrow et al. 1998; Heesom, Avison et al. 1998; Gingras, Gygi et al. 1999). Mitogen-stimulated phosphorylation of Thr37 and Thr46 is mainly resistant to rapamycin, implying that mTOR-independent kinases can also phosphorylate these sites upon mitogen stimulation (Gingras, Raught et al. 2001).

Ser65 and Thr70 are potently phosphorylated upon mitogen stimulation. Rapamycin causes a fast and potent dephosphorylation of Ser65 and Thr70 in the presence of serum (Gingras, Raught et al. 2001), indicating that mTOR plays a critical role in maintaining Ser65 and Thr70 phosphorylation (Figure 1.6). It is unlikely that mTOR phosphorylates Ser65 and Thr70 directly, given that mTOR does not phosphorylate these sites in *vitro* (Burnett, Barrow et al. 1998). Therefore phosphorylation of Ser65 and Thr70 has been proposed to be regulated by a mTOR-dependent kinase or phosphatase (Figure 1.6; Brunn, Hudson et al. 1997; Heesom and Denton 1999; Mothe-Satney, Yang et al. 2000). Treatment of cells with Calyculin A, which inhibits type 1 and -2 phosphatases prevents rapamycin induced dephosphorylation of 4E-BP1 (Peterson, Beal et al. 2000), underscoring the possibility that Ser65 and Thr70 are dephosphorylated by a mTOR-regulated phosphatase.

The PI3K/Akt pathway is required for 4E-BP1 phosphorylation under many cellular conditions (Figure 1.6; von Manteuffel, Gingras et al. 1996; Gingras, Kennedy et al. 1998; Kohn, Barthel et al. 1998; Wu, Senechal et al. 1998; Takata, Ogawa et al. 1999). 4E-BP1 phosphorylation is stimulated by expression of an activated PI3K catalytic subunit or an activated Akt mutant and inhibited by PI3K inhibitors, wortmannin and LY294002 (Gingras, Kennedy et al. 1998; Dufner, Andjelkovic et al. 1999; Takata, Ogawa et al. 1999). However, Akt does not directly phosphorylate 4E-BP1 *in vitro* (Gingras, Kennedy et al. 1998), which implies that an undetermined Akt-regulated kinase(s) phosphorylates 4E-BP1. 4E-BP1 can be phosphorylated *in vitro* by the MAPK and casein kinase (Diggle, Schmitz-Peiffer et al. 1991; Haystead, Haystead et al. 1994; Lin, Kong et al. 1995)

and has been demonstrated to be regulated by the MEK/ERK pathway under some cellular conditions (Figure 1.6; Herbert, Tee et al. 2002). Another mitogen-stimulated phosphorylation site in 4E-BP1, Ser112, has been reported to be phosphorylated by ATM in response to DNA-damage *in vitro* and *in vivo* (Figure 1.6). Phosphorylation of Ser112 is thought to contribute to the dissociation of 4E-BP1 from eIF4E (Yang and Kastan 2000).

Two additional mammalian 4E-BP family members, 4E-BP2 and 4E-BP3, have been identified (Tsukiyama-Kohara, Vidal et al. 1996; Poulin, Gingras et al. 1998), but 4E-BP1 has been characterized the most. 4E-BP1/2/3 are highly conserved in their eIF4E-binding sites as well as their C-termini. Most of phosphorylation site motifs are conserved in 4E-BP2 and 4E-BP3, suggesting similar regulated phosphorylation to 4E-BP1. Slight differences in the regulation of phosphorylation of 4E-BP1/2/3 might lead to different kinetics in eIF4E association. 4E-BP2 phosphorylation is rapamycin and LY294002 sensitive, but seems to occur at fewer sites and leads to a slower release from eIF4E (Gingras, Raught et al. 2001).

1.6.3.2 Regulation of S6K1

S6K1 is ubiquitously expressed, and its activity is stimulated by most mitogens, which include growth factors, proto-oncogenic products, phorbol esters, and cytokines (Dufner and Thomas 1999). S6K1 regulation is very complex, involving regulated phosphorylation of at-least 8 phosphorylation sites by mitogen- and mTOR-dependent pathways (Figure 1.7). There are two isoforms that arise from the same S6 kinase gene, a predominantly cytoplasmatic isoform, called S6K1 (α2) (Coffer and Woodgett 1994; Reinhard, Fernandez et al. 1994), and a nuclear localized isoform p85S6K1 (α1) that contains a nuclear localization motif within a N-terminal 23 aa extension (Grammer, Cheatham et al. 1996). The two S6K1 isoforms appear to be regulated similarly in all systems examined (Laser, Kasi et al. 1998).

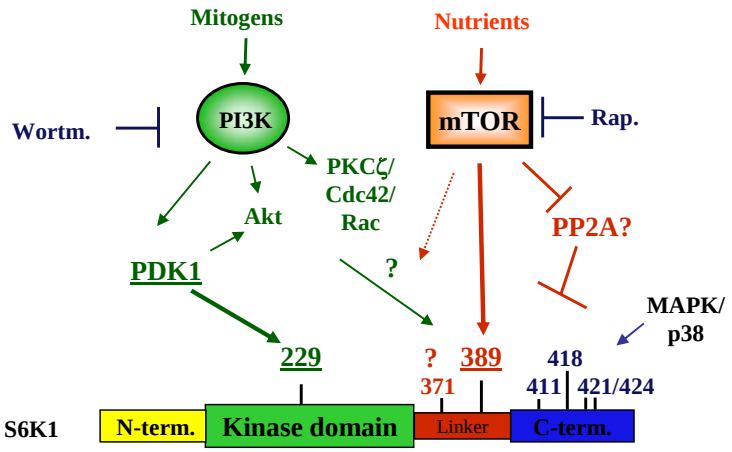

Figure 1.7 Phosphorylation of S6K1 is coordinately regulated by PI3K-, mTOR-, and MAPK-dependent inputs. Schematic diagram of S6K1 structure**.** Mitogen-stimulated phosphorylation sites are indicated by amino acid numbers. Thr229 is directly phosphorylated by PDK1, phosphorylation of Thr389 is regulated by mTOR, and the proline-directed C-terminal sites are possible phosphorylated by MAPK-dependent inputs.

PI3K-dependent activation of S6K1 is mediated by a variety of different effectors, including Akt, PDK1 (3-Phosphinositide-dependent kinase1), PKCζ and the small G proteins Cdc42 and Rac1 (Chou and Blenis 1996; Grammer, Cheatham et al. 1996; Alessi, Kozlowski et al. 1998; Downward 1998; Pullen, Dennis et al. 1998; Romanelli et al. 1999). While PDK1 directly phosphorylates S6K1 at Thr229, the mechanism of how other mitogen-dependent kinases regulate S6K1 phosphorylation has still to be determined. Treatment of cells with rapamycin causes the rapid decrease of S6K1 phosphorylation at Thr389, Thr229 and Ser404 (Pearson and Thomas 1995; Weng, Kozlowski et al. 1998), indicating that mTOR is a key regulator of S6K1 phosphorylation (Burnett, Barrow et al. 1998; Dufner and Thomas 1999; Isotani, Hara et al. 1999; Gingras, Raught et al. 2001). These phosphorylations may be regulated directly by mTOR or via an mTOR-regulated phosphatase.

S6K1 can be divided into four functionally significant structural parts, an acidic N-terminus, followed by a catalytic domain, a C-terminus containing a basic autoinhibitory pseudosubstrate domain (moderately similar to the substrate of S6K1, S6), and a linker region located between the C-terminal autoinhibitory region and catalytic domain (Figure 1.7). The acidic N-terminus is thought to interact with the basic C-terminal region and thereby stabilize the autoinhibitory effect of the pseudosubstrate domain occupying the catalytic site (Cheatham, Monfar et al. 1995). Phosphorylation of at least four C-terminal proline-directed Ser/Thr phosphorylation sites, Ser411, Ser418, Thr421 and Ser424 by mitogen-activated kinases is considered to open the conformation of S6K1, allowing subsequent phosphorylation of other sites that leads to full S6K1 activation. Thus, changing four of these C-terminal sites to acidic residues to mimic phosphorylation, (S6K1D_3E) increases the basal activity of S6K1 (Pearson, Dennis et al. 1995). The MAP kinases, ERK1/2 and p38, are potential candidates that may phosphorylate the C-terminal sites (Grammer, Cheatham et al. 1996; Mukhopadhyay, Price et al. 1992). At least two phosphorylation sites (Ser371 and Thr389) in the linker-region are essential for S6K1 activation (Pearson, Dennis et al. 1995; Moser, Dennis et al. 1997). The kinase that is responsible for phosphorylation of the proline-directed site Ser371 has not been identified.

Thr389 undergoes amino acid- and insulin-induced phosphorylation, which is completely sensitive to rapamycin and partially sensitive to the PI3K inhibitor wortmannin, indicating that Thr389 phosphorylation is regulated by both mTOR- and PI3K-dependent pathways (Pearson, Dennis et al. 1995; Han, Pearson et al. 1995; Weng, Andrabi et al. 1995). Different models have been suggested for the mTOR-dependent regulation of Thr389 phosphorylation. One model proposes direct phosphorylation of Thr389 by mTOR. This model is based on the fact that Thr389 is phosphorylated by mTOR *in vitro* (Burnett, Barrow et al. 1998; Isotani, Hara et al. 1999). In contrast, rapid dephosphorylation of Thr389 in the presence of rapamycin (Pearson, Dennis et al. 1995) supports a second model in which a

mTOR-inhibited phosphatase dephosphorylates Thr389. The kinase activity and Thr389 phosphorylation of a N-and C-terminal truncated S6K1 mutant (S6K1-NT/CT) is rapamycin-resistant but still insulin-stimulated, and sensitive to wortmannin. These data indicate that both PI3K- and mTOR-dependent signaling events can lead to Thr389 phosphorylation of S6K1 (Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995). However, the "mTOR-independent" phosphorylation of Thr389 in S6K1-NT/CT is much lower than phosphorylation of S6K1 wild-type (only 10% of wild-type) (Schalm and Blenis, unpublished results). These findings demonstrate that mTOR-signaling is absolutely required for optimal Thr389 phosphorylation and explain why rapamycin treatment potently inhibits S6K1 activation.

Phosphorylation of Thr229 within the kinase domain by PDK1 enhances the catalytic activity of S6K1 (Weng, Kozlowski et al. 1998). The hydrophobic sequence motif surrounding T389 and T229 and the distance between these sites is highly conserved in a family of Ser/Thr kinases kinases, called AGC kinases (protein kinase A, G, and C) (Pearson, Dennis et al. 1995). The sequence similarity suggests that the regulation of these sites is involved in a general activating mechanism of these class of kinases. Phosphorylation of Thr229 and Thr389 are dependent on each other. Optimal phosphorylation of Thr389 is dependent on Thr229 phosphorylation, as absence of Thr229 phosphorylation in PDK1 null cells (Williams, Arthur et al. 2000) causes inhibition of Thr389 phosphorylation (Weng, Kozlowski et al. 1998). Interestingly, the PI3K downstream effectors PDK1 and PKCξ have been shown to cooperatively stimulate S6K1 phosphorylation at Thr389 and Thr229. Furthermore, the cooperative effect of PDK1 and PKCξ on Thr389 phosphorylation depends on the kinase activity of S6K1 (Romanelli, Martin et al. 1999; Romanelli, Dreisbach et al. 2002). In addition, growth factor-induced Thr389 phosphorylation persisted up to 120 min in wild-type S6K1 but was reduced and more transient in a kinase inactive S6K1 mutant (Romanelli, Dreisbach et al. 2002). These data suggest that PI3K-dependent phosphorylation of

Thr389 is at least partially mediated by autophosphorylation of Thr389 (Romanelli, Dreisbach et al. 2002). Mutation of Thr389 to an alanine (to block phosphorylation) leads to impaired Thr229 phosphorylation. Phosphorylated Thr389 has been suggested to provide a docking site for PDK1 and to facilitate efficient phosphorylation of Thr229 by PDK1 (Biondi, Kieloch et al. 2001).

1.6.3.3 Regulation of S6K2

S6K2 was recently identified as a S6K1 homolog. S6K2 is activated by the same stimuli that activate S6K1 and potently inhibited by both wortmannin and rapamycin, suggesting that S6K2 is also regulated by PI3K and mTOR signaling pathways (Gout, Minami et al. 1998; Shima, Pende et al. 1998; Koh, Jee et al. 1999; Lee-Fruman, Kuo et al. 1999; Martin, Schalm et al. 2001). Similar to S6K1 regulation, S6K2 is regulated by the PI3K effectors PDK1, PKCξ and the small G proteins, Cdc42 and Rac1 (Martin, Schalm et al. 2001) and Akt (Koh, Jee et al. 1999).

Two isoforms (β1 and β2) of S6K2 result from alternative splicing. The longer splice variant, S6K β1, contains an extremely basic sequence in a N-terminal 13-amino acid extension (Gout, Minami et al. 1998). A nuclear localization signal (KKSKRGR) located in the extreme C-terminus causes S6K2 to predominantly reside in the nucleus (Koh, Jee et al. 1999). The general domain structure of S6K2 is similar to S6K1 with highest homology found in their catalytic and linker domains. Phosphorylation sites important for S6K1 activation, except for the proline-directed Thr421 site, are conserved in S6K2. Similar to S6K1, S6K2 contains an acidic region in the N-terminus and a basic pseudosubstrate domain in its C-terminus. The extreme C-terminus of S6K2 has low homology to the corresponding part of S6K1, and contains a poly-proline stretch that is completely distinct from S6K1 (Gout, Minami et al. 1998; Shima, Pende et al. 1998; Lee-Fruman, Kuo et al. 1999). The proline-rich region of S6K2

may interact with an SH3-domain containing protein and cause differential regulation of S6K2.

The major difference in the regulation of both S6 kinases is the lower mitogen-stimulated kinase activity of S6K2, when compared to S6K1. C-terminal truncation of S6K2 increases its specific activity *in vitro* and dramatically enhances S6K2 activation when co-expressed with PI3K effectors, such as Cdc42, PKCξ and PDK1 (Martin, Schalm et al. 2001). These data suggest an additional inhibitory effect of the S6K2 C-terminus not present in S6K1. Additionally, the MAPK pathway may play a more important role for the activation of S6K2 than S6K1, as full length S6K2 is more sensitive to the MEK inhibitor UO126 than S6K1 (Martin, Schalm et al. 2001). Identification of the unique C-terminal regulator of S6K2 might reveal the basis for the distinct regulation and therefore the physiological role of S6K2.

1.7 TOR regulation of phosphatases

The mTOR-dependent phosphorylation of S6K1 and 4E-BP1 at several sites with different kinetics suggest the involvement of various mTOR inputs (Dennis, Pullen et al. 1996; Weng, Kozlowski et al. 1998; Gingras, Gygi et al. 1999). As well as direct phosphorylation of its substrates, mTOR may also inhibit a phosphatase that dephosphorylates 4E-BP1 and S6K1, thereby allowing very rapid regulation by TOR signaling. Evidence already exists for regulation of 4E-BP1 and S6K1 phosphorylation by mTOR-inhibited phosphatases (Peterson, Desai et al. 1999). Treatment of cells with the phosphatase inhibitor Calyculin A prevents 4E-BP1 dephosphorylation, while inhibition of S6K1 activity by rapamycin or amino acid deprivation requires phosphatase activity (Peterson, Desai et al. 1999). The Ser/Thr phosphatase PP2A is a good candidate for such a hypothetical mTOR-dependent phosphatase, as PP2A dephosphorylates S6K1 *in vitro* and associates with full-length S6K1, rather than the rapamycin resistant N-and C-terminal truncated S6K1 mutant (Peterson, Desai et al. 1999).

A role for phosphatases in TOR signaling has been well defined in *S.cerevisiae*, where TOR regulates several of it downstream targets by inhibiting the specific activity of catalytic subunits from PP2A type phosphatses, like Pph21, Pph22, and Sit4 (Figure 1.8; Rohde, Heitman et al. 2001). The substrate specificity and localization of the catalytic PP2A-like phophatase subunit is defined by which regulatory subunits the catalytic PP2A subunit binds to. One such regulatory subunit, TAP42, binds to the catalytic subunits of PP2A and Sit4 under nutrient-rich conditions in a rapamycin-sensitive manner, and inhibits their phophatase function by competing with other regulatory subunits. Mutation of TAP42 confer rapamycin resistance, providing additional evidence that TAP42 is an important component of the TOR pathway (Di Como and Arndt 1996; Jiang and Broach 1999). Recently, the TAP42 interacting protein, TIP41, was identified as a novel regulator of Tap42 and Sit4 (Jacinto, Guo et al. 2001). TIP41 interacts with TAP42 during conditions of nutrient deficiency and therefore promotes PP2A activity. TOR directly phosphorylates TIP41 leading to the release of TAP42, which can now bind to and inhibit PP2A or Sit41 (Jacinto, Guo et al. 2001). The B cell receptor-binding protein Alpha 4 is human ortholog of TAP42 and has been shown to interact with the catalytic subunit of PP2A, but the rapamycin sensitivity of interaction is controversial (Murata, Wu et al. 1997; Nanahoshi, Nishiuma et al. 1998). In addition, TAP42 and alpha 4 interfere with PP2A induced *in vitro* dephosphorylation of 4E-BP1 (Nanahoshi, Nishiuma et al. 1998).

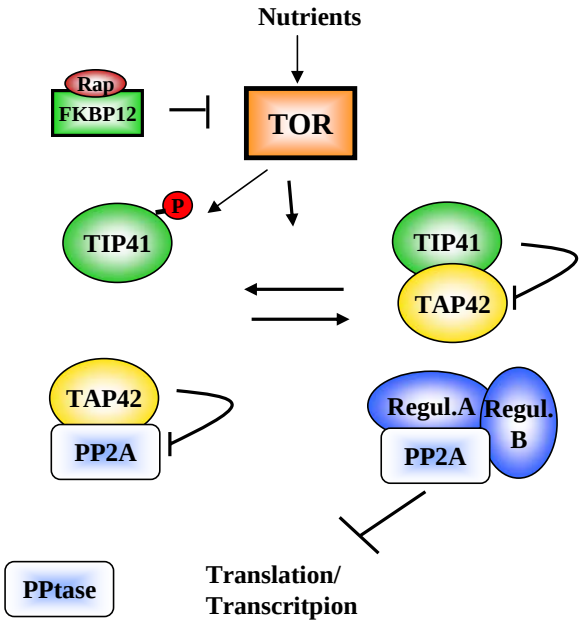

Figure 1.8 Model for TOR regulation of PP2A type phosphatases in yeast. In the presence of sufficient nutrients, TOR directly phosphorylates TIP41, which causes release of TAP42, TAP42 can now bind to the catalytic subunit of PP2A type phosphatases (Sit4, Pph21/22). Association of TAP42 with PP2A prevents its binding to the regulatory subunits A and B (Regul.A, Regul.B), and thus inhibits phosphorylation of downstream targets of PP2A type phosphatses. In the absence of nutrients or in the presence of rapamycin (Rap),TOR does not phosphorylate TIP41. TIP41 can now bind to TAP42, and this binding prevents the association of TAP42 with PP2A type phosphatases. PP2A can now bind to its regulatorysubunits A and B and dephosphorylate downstream targets, resulting in inhibition ofTranslation and Transcritpion (reviewed in Rhode 2001**).**

1.8 Limitations in the study of TOR signaling

mTOR activity is difficult to determine in an *in vitro* mTOR kinase assay and may not reflect the activity of mTOR *in vivo*. Changes of *in vitro* mTOR kinase activity do not correlate with effects seen on mTOR downstream targets, e.g. changes in amino acid levels or mitogens dramatically affect S6K1 activity or 4E-BP1

phosphorylation but do not significantly affect mTOR *in vitro* kinase activity (Scott, Brunn et al. 1998; Sekulic, Hudson et al. 2000). Thus, the mTOR activity measured *in vitro* may not reflect the *in vivo* situation of mTOR regulation. For that reason, activity and phosphorylation of mTOR targets, such as S6K1 and 4E-BP1, are typically used as readouts for mTOR regulation. Since S6K1 and 4E-BP1 can be also regulated by mTOR-independent pathways, it is often difficult to judge if the change of activities or phosphorylation of S6K1 and 4E-BP1 is indeed caused by differences in mTOR-mediated signaling. As a consequence, the mechanisms by which the downstream targets of mTOR as well as TOR activity are regulated remain elusive.

1.9 Identification of TOR binding proteins

The multi-domain structure of mTOR, including several known or putative protein-protein interaction domains, implies that mTOR may interact with several binding partners (Figure 1.1). However, until recently direct TOR binding partners and regulators have not been well characterized. 20 HEAT repeats, located in a large 2000 amino acids N-terminal region of TOR may form an alpha helical structure that provides a hydrophobic structure to which other proteins can bind to (Andrade and Bork 1995; Groves and Barford 1999). In addition, mTOR contains a 500 amino acid spanning FAT domain (Figure 1.1; Bosotti, Isacchi et al. 2000), which is found only in members of PIK-related kinase family, and might also serve as protein-protein interaction domain. The FATC domain at mTOR's extreme C-terminus is only found in combination with FAT domains, and has been therefore suggested to interact with the FAT domain (Figure 1.1; Keith and Schreiber 1995; Bosotti, Isacchi et al. 2000).

Recently, the purification of TOR complexes in yeast and mammals has revealed more insights into the regulation of TOR's function. Two studies described the identification of the regulatory associated protein of mTOR, raptor, in mammalian cells (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002). A

subsequent publication presented the purification of two distinct TOR complexes in *S.cerevisiae*. Both complexes contained the homolog of raptor KOG-1 (Kontroller of growth-1) (Loewith, Jacinto et al. 2002). Thus, raptor/KOG-1 seems to be a conserved binding partner of TOR from yeast to mammals.

The association of raptor and mTOR is very sensitive to non-ionic detergents, such as Triton-X 100 or NP-40, which are in general used in the preparation of cell lysates. The sensitivity of the mTOR/raptor complex to detergents may explain why raptor was not previously identified. Kim et al. (Kim, Sarbassov et al. 2002) initially preserved the mTOR-raptor complex by adding a reversible cross-linker to the lysis buffer, while Hara *et al*. (Hara, Maruki et al. 2002) maintained the mTOR/raptor complex by extracting cells in the absence of detergent. Raptor is an approximately 150 kDa protein that contains no apparent enzymatic activity but several potential protein-protein interaction domains. Raptor may thus function as scaffold protein. A unique N-terminal "raptor N-conserved" (RNC) region (Kim, Sarbassov et al. 2002) is followed by three HEAT repeats and seven WD40 domains.

Kim *et al*. (Kim, Sarbassov et al. 2002) proposes that raptor functions as a bi-directional regulator of mTOR signaling in a nutrient-sensing complex (NSC). Under amino acids or glucose poor conditions, raptor binds tightly to mTOR and inhibits mTOR signaling as measured by mTOR's *in vitro* kinase activity. However, in the presence of sufficient nutrients (amino acids and glucose) raptor enhances mTOR signaling. Indeed, in the presence of raptor, mTOR *in vitro* kinase activity is stimulated by leucine (Kim, Sarbassov et al. 2002). Rapamycin was found to destabilize the interaction of raptor with mTOR under nutrient-rich conditions to induce the dissociation more potently. Thus, rapamycin and amino acids seem to have opposite effects on the mTOR-raptor interaction. Importantly, Kim *et al.* (Kim, Sarbassov et al. 2002) detected no regulation of the raptor-mTOR complex by mitogens, suggesting that raptor specifically mediates the nutrient input for mTOR signaling.

In contrast, Hara *et al.* (Hara, Maruki et al. 2002) found no change in the stability of the raptor-mTOR complex under different nutrient conditions or in the presence of rapamycin. The differences in the nutrient sensitivity described by the two groups might be caused by different lysis conditions used. Furthermore Kim *et al.* (Kim, Sarbassov et al. 2002) mainly studied the regulation of the endogenous raptor/mTOR complex, while Hara *et al.* (Hara, Maruki et al. 2002) mostly analyzed overexpressed raptor/mTOR complexes. Hara *et al.* (Hara, Maruki et al. 2002) described raptor as a scaffold protein that was required to recruit mTOR to its substrates. The authors also found that raptor associated with 4E-BP1 and S6K1 and that raptor association with mTOR was required for efficient S6K1 and 4E-BP1 phosphorylation *in vitro* (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002). Consistently, Kim *et al*. (Kim, Sarbassov et al. 2002) demonstrated that the reduction of raptor expression by siRNA inhibited mTOR-dependent phosphorylation of S6K1 *in vivo.* This was similar to the inhibition of S6K1 activity following reduction of the mTOR protein or treatment of the cells with rapamycin, suggesting that raptor association with mTOR is necessary for efficient mTOR-mediated signaling (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002). The knock down of raptor expression also decreases cell size similar to rapamycin treatment or mTOR knock down, indicating that raptor is required for growth regulation.

Lowith *et al*. (Loewith, Jacinto et al. 2002) identified two functionally distinct TOR complexes, called TORC1 and TORC2 in *S. cerevisiae*. TORC1 consists of TOR1 or TOR2, the raptor homolog KOG1 and LST8 and seems to mediate the nutrient and rapamycin sensitive function of TOR that is required for the regulation of cell growth (Kim, Sarbassov dos et al. 2003). LST8 consists of seven WD40 repeats and is therefore thought to function as a scaffolding protein. LST8 has already been described to regulate nutrient-sensitive functions in yeast (Roberg, Bickel et al. 1997; Liu, Sekito et al. 2001). The second TOR complex, TORC2, contains TOR2 and not TOR1. The TORC2 complex also contains AVO

(adheres voraciously to TOR2)-1, AVO-2, AVO-3 and LST8. TORC2 seems to regulate cytoskeletal reorganization in a rapamycin-independent fashion (Loewith, Jacinto et al. 2002). AVO1 (SIN1) is conserved in mammals, suggesting the existence of another possible rapamycin-independent TOR complex in mammals (Loewith, Jacinto et al. 2002).

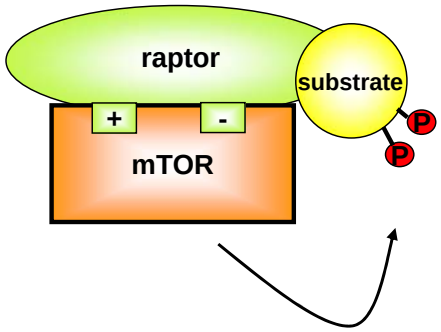

Figure 1.9 Model of mTOR regulation by raptor. Under nutrient rich conditions, raptor binds tightly to mTOR and inhibits mTOR signaling, but in the presence of sufficient nutrients raptor enhances mTOR signaling. In addition, raptor functions as a scaffolding protein that recruits mTOR to its substrates.