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mTOR Pathway and mTOR Inhibitors in Cancer Therapy

Humana Press

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A theory is more impressive the greater the simplicity of its premises, the more different kinds of things it relates, and the more extended its area of applicability

Albert Einstein

Preface

The main objective of this book is to provide an up-to-date survey of the rapidly advancing field of cancer therapy. Moreover, since our knowledge in this area rapidly evolves, some data have got obsolete during the process of book editing. Our understanding of the mechanisms involved in cancer genesis and progression underwent unprecedented expansion during the last decade, opening a new era of cancer treatment – targeted therapy. The surge in this area results in no small part from studies conducted jointly by basic health scientists and clinical investigators. It is our hope that this book will help foster even further collaboration between investigators in these two disciplines.

The target of rapamycin (TOR) was first identified in *Saccharomyces cerevisiae* and subsequently in mammals (mTOR) as a conserved atypical serine/threonine kinase. In mammalian cells, mTOR exists in at least two multi-protein complexes that have critical roles in regulating cellular homeostasis and survival. As with many other areas of science, discovery of TOR signaling was fortuitous. Rapamycin was isolated as a product of the soil bacteria *Streptomyces hygroscopicus*, identified in a soil sample taken from the island of Rapa Nui (Easter Island). Rapamycin was first discovered to be a potent antifungal agent and next as an immune suppressive drug. It was only later that it was found to be active as an antitumor agent in non-clinical models; although it was not developed for this indication.

The history of rapamycin presents one of the first examples of chemical genetics. TOR was identified in a yeast screen designed to find genes conferring rapamycin resistance. Identification of mammalian TOR (mTOR) activation pathways and their roles in regulating cap-dependent translation, transcription, growth, proliferation, and survival continues to be a dynamic field of research. Dysregulation of mTOR is associated with several human diseases including cancer-prone syndromes, such as tuberous sclerosis and Peutz–Jegher, Cowden's, and Lhermitte–Duclos disease; most adult human malignancies; and potentially with autism. Undoubtedly, the list is not complete.

Rapamycin or derivatives have been approved for use as immunosuppressive agents for organ transplantation, for treatment of both renal cell carcinoma and mantle cell lymphoma, and have shown glimpses of activity in a broad range of human cancers. Critical to optimizing the use of these *rapalogs* as pharmacological agents will be a more comprehensive understanding of pathways that activate

mTOR; which of these pathways are critical for cancer cell genesis, maintenance, and progression survival; and how the cellular consequences of inhibiting mTOR signaling, either in the mTORC1 or mTORC2 complex, interact with transforming events that characterize human neoplasias.

In this work, experts in TOR signaling have contributed in two thematic areas: mTOR signaling and cancer therapy (chapters "mTORC1: A Signaling Integration Node Involved in Cell Growth", "The Regulation of the IGF-1/mTOR Pathway by the p53 Tumor Suppressor Gene Functions", "mTOR Signaling in Angiogenesis", "mTORC1 Signaling and Hypoxia", "mTOR Signaling in Glioblastoma: Lessons Learned from Bench to Bedside", "mTOR and Cancer Therapy: General Principles", "mTOR and Cancer Therapy: Clinical Development and Novel Prospects", and "Drug Combinations as a Therapeutic Approach for mTORC1 Inhibitors in Human Cancer") and therapeutic targeting downstream of mTOR (chapters "Downstream Targets of mTORC1", "Downstream of mTOR: Translational Control of Cancer", "Genome-Wide Analysis of Translational Control", "Translational Control of Cancer: Implications for Targeted Therapy", and "Downstream from mTOR: Therapeutic Approaches to Targeting the eIF4F Translation Initiation Complex"). All chapters are completely new or have been extensively updated by their authors and we are indebted to all authors who have exemplified the links between these two thematic areas.

We hope that this book will attract a diverse audience, reflecting the broad range of scientific and clinical disciplines focused on current problems in cancer etiology and therapy – and future perspectives of drug development. Consequently, we have brought together biochemists, cancer biologists, and clinicians to share their unique perspectives on the role of mTOR signaling pathway in cancer genesis and contemporary therapeutic approaches.

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mTORC1: A Signaling Integration Node Involved in Cell Growth

Neil Kubica and John Blenis

Abstract The mammalian target of rapamycin (mTOR) is an atypical serine /threonine kinase that plays an indispensable role in the control of cell growth. When localized with the interacting proteins raptor and mLST8 in the mammalian target of rapamycin complex 1 (mTORC1), mTOR serves as an integrator of cellular signals to control the balance between cellular anabolism and cellular catabolism. Under conditions that promote cell growth, or in the presence of common genetic lesions associated with cancer, mTORC1 signals to the effectors 4E-BP1 and S6K1 resulting in ribosomal biogenesis and enhanced mRNA translation. The positive effects of mTORC1 on mRNA translation involve a dynamic molecular process that results in an increase in bulk protein synthesis, including more dramatic effects on a subset of mRNA species encoding pro-growth, anti-apoptotic proteins. Recent data also suggest a role of mTORC1 in the "pioneer" round of mRNA translation, in addition to the more established effects on "steady-state" protein biosynthesis. Growth control by mTORC1 is required in physiological and developmental settings for proper maintenance of cellular homeostasis, cell survival, and embryonic development, while inappropriate regulation of mTORC1 signaling is observed in the overwhelming majority of human cancers. This review will discuss the current view of the signaling network upstream of mTORC1 and the regulation of protein biosynthesis by this evolutionarily conserved, clinically relevant cell signaling node.

Keywords mTOR · mTORC1 · S6K1 · 4E-BP1 · Protein metabolism · mRNA translation · Ribosomal biogenesis · Cell growth · Cell proliferation · Cancer

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1 Introduction

Since the discovery of the mammalian target of rapamycin (mTOR) [1–5], considerable progress has been made toward understanding the signaling network that feeds into and emanates from this evolutionarily conserved serine/threonine kinase. As is true in yeast model systems, mTOR is now recognized to be a member of two distinct protein complexes termed mTORC1 and mTORC2. mTORC1 serves as an integration hub for a variety of upstream growth factor, nutrient, and stress signals and modulates a variety of anabolic (e.g., protein biosynthesis) and catabolic (e.g., autophagy) processes to suit the status of the cellular environment. mTORC1 plays a critical role in determining progression of the intimately linked processes of cell growth and cell division through the action of its downstream targets 4E-BP1 and S6K1. Careful control of these fundamental biological processes is required for maintenance of cellular homeostasis, cell survival, embryonic development, and tissue/body patterning, while aberrant mTORC1 signaling is associated with a number of pathophysiological conditions, including human cancer. A plethora of known oncogenes (e.g., EGFR, PDGFR, PI3K, Akt, Ras, Raf, Rheb, S6K1, and eIF4E) and tumor suppressors (e.g., PTEN, NF1, LKB1, REDD1, TSC1/2, PDCD4) are members of the mTORC1 signaling network. Mutations in several of these network components are causative in a number of inherited tumor pre-disposition syndromes, such as tuberous sclerosis complex (TSC), lymphangioleiomyomatosis (LAM), neurofibromatosis (NF), Cowden's disease (CD), and Peutz–Jeghers syndrome (PJS), that share the common clinical feature of non-metastatic large-celled hamartomas. Hyperactivation of mTORC1 signaling is observed in nearly all sporadic human cancers and results in a variety of cellular phenotypes that confer a selective growth advantage to cancer cells compared to their non-malignant counterparts. The importance of mTORC1 in cancer biology is further reinforced by the ability of the specific mTORC1 inhibitor rapamycin to inhibit some tumorigenic phenotypes. Several rapamycin analogues are currently approved, or being evaluated in clinical trials, for treatment of numerous cancer subtypes. This chapter will focus on signaling upstream of mTORC1 and the role of mTORC1 in protein biosynthesis, cell growth, and proliferation.

2 The Domain Structure and Protein Complex Assembly of mTOR

Yeast TOR1 (yTOR1) and TOR2 (yTOR2) are the founding members of the PI3K-related kinase (PIKK) superfamily that includes mTOR, ATM, ATR, and DNA-PK [6–9]. Members of this atypical protein kinase family are characterized by their high molecular weight (mTOR ~289 kDa) and an unusual C-terminal kinase domain with significant homology to the PI3K lipid kinase domain. Despite the obvious homology to lipid kinases, mTOR functions as a protein serine/threonine kinase. The N-terminus of mTOR contains a series of 20 HEAT repeats, domains that

are composed of tandem anti-parallel α-helices known to mediate protein–protein interactions. C-terminal to these HEAT repeats is a FAT domain of unknown function found in all PIKK family members [10], followed by an FRB domain that binds the FKBP12–rapamycin complex [4], the atypical kinase domain, a putative NRD (*n*egative *r*egulatory *d*omain) [11], and finally an FATC domain, another FAT domain at the extreme C-terminus of the protein. The mTORC1 inhibitor rapamycin acts through a novel mechanism that requires interaction of the small molecule with an endogenous protein known as an immunophilin (FKBP12) in order to interact with and repress its protein target. This molecular arrangement confers a high degree of specificity, such that TOR is the only described target of the FKBP12–rapamycin complex. Interestingly, the serine residue associated with the dominant point mutations (TOR1 S1972A and TOR2 S1975I) that allowed for the genetic identification of yTOR isoforms [12, 13] is conserved in the mTOR (Ser-2035) FRB domain and mutation of this residue ablates the interaction between TOR and the FKBP12–rapamycin complex [14, 15]. A 2.7 Å crystal structure of the FKBP12–rapamycin–FRB domain ternary complex reveals that interactions rely on the ability of rapamycin to bind hydrophobic pockets on both proteins simultaneously [16]. The FATC domain is required for mTOR kinase activity [17, 18] and, although the mechanism is not formally established, it is proposed that the FAT and FATC domains interact in a manner that exposes the catalytic region in the kinase domain.

In yeast, the two TOR genes localize to distinct protein complexes termed yTORC1 and yTORC2 [19]. yTORC1 is a rapamycin-sensitive complex composed of TOR1 or TOR2 and the interacting proteins KOG1, LST8, and Tco89p. Despite the fact that mammals have only a single TOR gene, mTOR protein complex architecture appears to be conserved. mTOR forms two distinct protein complexes termed mTORC1 and mTORC2. mTORC1 is a heterotrimeric protein complex that contains mTOR bound to two associated proteins, namely raptor (regulatory-associated protein of mTOR; KOG1 ortholog) and mLST8 (LST8 ortholog) [20–22]. Tco89p does not appear to have a mammalian ortholog. The cellular functions of mTORC1 seem to be largely conserved as this complex, similar to yTORC1, regulates cell growth by modulation of protein synthesis, ribosomal biogenesis, autophagy, and metabolism [23–25]. Raptor is a 150 kDa protein with a conserved N-terminal domain, three HEAT domains, and seven WD40 motifs. The spatial expression pattern of mTOR and raptor shows a striking correlation and evidence exists to support the notion that raptor is involved in mTOR protein stability [20, 21]. Raptor is also involved in mediating the ability of mTOR to phosphorylate the well-described downstream targets 4E-BP1 and S6K1. Overexpression of exogenous raptor results in increased mTORC1 signaling in vitro [20], while knockdown of raptor expression leads to decreased S6K1 phosphorylation and a reduction in cell size in vivo [21]. These effects seem to be explained by a model in which raptor serves as a scaffold to recruit 4E-BP1 and S6K1 to mTORC1 where they can be efficiently phosphorylated by mTOR. 4E-BP1 and S6K1 physically interact with raptor [20] via a TOS (TOR signaling) motif found in both downstream effectors [26]. Mutations in the TOS motif decrease 4E-BP1 and S6K1 binding to raptor and subsequent phosphorylation of these downstream effectors by mTOR [26–29]. Interestingly, raptor

knockout mice exhibit early embryonic lethality at ~E6.5 days [30], a result that phenocopies timing of prenatal death observed in mTOR$^{-/-}$ mice [31, 32]. Taken together, these results suggest the interdependence of raptor and mTOR function in regulating cell growth and embryogenesis. mLST8 binds to the kinase domain of mTOR leading to the hypothesis that mLST8 may play a positive role in regulating mTORC1 signaling. Overexpression of mLST8 enhances mTORC1 signaling [22], while RNAi-mediated knockdown of mLST8 represses mTORC1 signaling [19, 22, 33] and results in reduced cell size [22]. The mechanism by which mLST8 positively effects mTORC1 signaling is less clear, although Kim et al. propose a model by which mLST8 interprets cellular inputs and regulates the stability of the mTOR–raptor interaction [22]. Surprisingly, mLST8$^{-/-}$ knockout embryos display lethality at a later stage (~E10.5) when compared to mTOR$^{-/-}$ and raptor$^{-/-}$ animals, but similar to knockouts of the mTORC2-specific components rictor and mSin1 [30]. mLST8$^{-/-}$ MEFs show no change in mTORC1 function, as S6K1 and 4E-BP1 phosphorylation levels are unchanged in these cells in vivo and mTORC1 purified from these cells can phosphorylate S6K1 in vitro [30]. Furthermore, mLST8$^{-/-}$ MEFs exhibit a marked reduction in Akt Ser-473 phosphorylation, a biomarker for mTORC2 function. The knockout animal models suggest that mLST8 may be more important in mTORC2 function and dispensable with regard to its role in the mTORC1 complex. The discrepancies between the initial observations in established cell lines and the knockout animal models could be explained by the ability for cellular compensation of mLST8 in early embryonic development that is lost in immortalized cell lines or differences in the genetic background between otherwise normal knockout animals and the highly aberrant genomes of established human cell lines [34]. Despite the nuances of the roles of various mTORC1 components recent work has elucidated a myriad of upstream signaling events that regulate the activity of the mTORC1 complex.

3 Cellular Signaling Upstream of mTORC1: Integration of Anabolic and Catabolic Cues

The activity of yTORC1 is predominantly regulated by the availability of nutrients. In addition to these evolutionarily ancient nutrient inputs, cell signaling networks in higher eukaryotes have developed connections between mTORC1 and inputs from growth factors and stress signaling pathways (Fig. 1).

3.1 Growth Factor Signaling

Growth factors play an important role in mammalian cell growth and proliferation. Growth factor binding (e.g., insulin/IGF-1) to their receptor tyrosine kinases (e.g., IR/IGF-1R) promotes recruitment of adaptor molecules such as IRS-1. IRS-1 recruits PI3K, which then phosphorylates PIP_2 leading to the generation of PIP_3, a

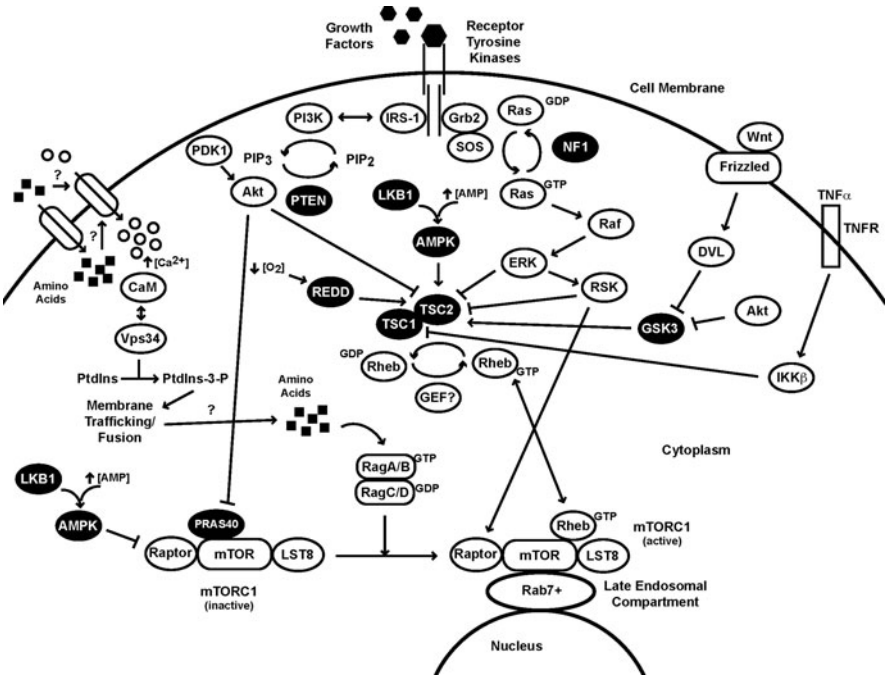

Fig. 1 Cellular signaling pathways upstream of mTORC1. mTORC1 integrates a diverse set of anabolic and catabolic cellular cues to establish appropriate rates of mRNA translation, cell growth, and cell proliferation. Upstream inputs include growth factor signaling pathways (e.g., PI3K/Akt and MAPK), nutrients (e.g., amino acids), and stress signals (e.g., energy deprivation and hypoxia). Many of these signals regulate the GAP activity of the TSC1/2 complex toward the small G protein Rheb. Several lines of evidence suggest that Rheb-GTP serves as the proximal upstream activator of mTORC1 via direct binding. Proper co-localization of mTORC1 and Rheb to late endosomes is maintained by amino acid-mediated regulation of the Rag family of GTPases (RagA-D). Growth factor signaling also promotes mTORC1 signaling via inactivation of the inhibitory protein PRAS40 via Akt and direct phosphorylation of raptor by RSK. Repressive phosphorylation events on raptor are mediated by AMPK subsequent to energetic stress. The upstream inputs to mTORC1 include many known oncogenes and tumor suppressors that play a role in sporadic cancers and a variety of tumor pre-disposition syndromes

process antagonized by the lipid phosphatase PTEN. PIP_3 recruits PDK1 and Akt to the plasma membrane through their respective pleckstrin homology (PH) domains. Based on co-localization at the membrane, PDK1 efficiently phosphorylates Akt on Thr-308 resulting in partial Akt activation, sufficient to promote downstream signaling to numerous Akt target proteins [30, 35]. Knockin mice expressing PDK1 with a mutated PH domain have reduced Akt signaling, a small body phenotype and exhibit insulin resistance [36]. Early efforts to elucidate the role of Akt in promoting mTORC1 signaling focused on direct phosphorylation of mTOR on Ser-2448 [11, 37–39]. While phosphorylation of this residue appears to correlate with mTORC1 signaling, the role of this phosphorylation event was called into question, as substitution of this residue with alanine does not affect mTORC1 signaling

[11]. A key breakthrough in the understanding of PI3K/Akt signaling to mTORC1 was the discovery of the TSC1/TSC2 complex as a negative regulator of mTORC1 signaling.

Genetic linkage studies in families with tuberous sclerosis complex (TSC), an autosomal dominant disorder characterized by the development of non-metastatic tumors with enlarged cells [40], mapped the causative loss-of-function mutations to two genetic loci subsequently named *TSC1* and *TSC2* [41–43]. In the years following this discovery, in seemingly unrelated work, several groups employed classic *Drosophila* genetic screens in an effort to discover genes involved in cell/body size determination. When combined with epistatic analysis, these screens revealed a critical role for numerous components in the insulin/IGF-1 pathway as regulators of cell size. dInr (insulin receptor homolog) [44], chico (IRS1–4 homolog) [45], dPI3K [46, 47], dAkt [47, 48], dTOR [49], and dS6K [50] form a linear genetic axis that promotes cell size, while dPTEN [47, 51] serves as a repressor of cell size lying upstream of dAkt. Subsequent efforts in this experimental system demonstrated that dTSC1 and dTSC2 were also negative regulators of cell size [52–54], functioning downstream of dAkt and upstream of dS6K [53]. The results prompted studies in mammalian systems showing that cells lacking TSC1 or TSC2 exhibit rapamycin-sensitive [55] hyperactivation of mTORC1 [56, 57]. Furthermore, overexpression of TSC1 and TSC2 reduces phosphorylation of 4E-BP1 and S6K1 and RNAi-mediated knockdown of TSC2 increases phosphorylation of S6K1 [58, 59]. Taken together, the evidence supports the conclusion that TSC1/TSC2 serves as a negative regulator of mTORC1 signaling.

TSC1 and TSC2 form a tight heterodimeric complex in which both proteins are required for proper function. TSC1 (also known as hamartin) is a 140 kDa protein that contains several coiled-coil domains. TSC2 (also known as tuberin) is a 200 kDa protein with a coiled-coil loop domain and a C-terminal GTPase-activating protein (GAP) domain. The crucial function of the TSC1/TSC2 complex in inhibiting mTORC1 appears to be GAP activity toward the small G protein Rheb. *Drosophila* genetic epistasis experiments were again employed to place Rheb in the cell size network downstream of dTSC1/dTSC2 but upstream of dTOR [60, 61], and an RNAi screen in *Drosophila* S2 cells demonstrated that Rheb knockdown inhibits S6K1 phosphorylation [62]. Biochemical approaches in mammalian cells show that GTP-loaded Rheb stimulates mTORC1 activity both in vitro and in vivo, while GAP activity of the TSC1/TSC2 complex results in GTP hydrolysis and formation of inactive Rheb-GDP [63–66]. Interestingly, there are several Akt consensus phosphorylation motifs in TSC2 that are conserved between *Drosophila* and *Homo sapiens* and experimental evidence confirms Akt-dependent phosphorylation of Ser-939, Ser-1130, and Thr-1462 in human TSC2 [58, 67, 68]. These phosphorylation events inhibit TSC2 GAP activity leading to derepression of Rheb and activation of mTORC1 signaling. In support of an inhibitory role for Akt phosphorylation of TSC2, several studies demonstrate that exogenous expression of a TSC2 mutant with alanine substitutions at Akt-sensitive serine/threonine residues acts as a dominant-negative inhibitor of growth factor-mediated mTORC1 signaling [58, 67].

Several unresolved issues remain in the current understanding of PI3K/Akt/TSC2/Rheb signaling pathway. First, the mechanism for TSC2 GAP inhibition via Akt-mediated phosphorylation remains unclear. Several studies suggest that phosphorylation by Akt disrupts the integrity of the TSC1/TSC2 complex, leading to subsequent degradation of TSC1 and TSC2 [58, 68]; however, other reports have not supported this conclusion [67, 69]. Binding of 14-3-3 proteins to Akt-dependent phosphorylation sites on TSC2 has also been proposed to inhibit GAP activity [70, 71], but others have demonstrated that 14-3-3 binds to TSC2 via phosphorylated sites other than those targeted by Akt [72, 73]. Discovery of the GAP activity of the TSC1/TSC2 complex has also prompted the search for an activating Rheb-GEF. The initial observation that Rheb has low intrinsic GAP activity and is predominantly found in a GTP-bound form in vivo prompted the hypothesis that a putative Rheb-GEF was either unregulated or entirely absent. A relatively recent publication [74] provides evidence that TCTP serves as the Rheb-GEF in *Drosophila*. Interestingly, exogenous expression of human TCTP appears to rescue the dTCTP mutant phenotypes; however, GEF activity of dTCTP toward dRheb is noticeably low in biochemical exchange assays [74]. Data from mammalian cells strongly question a functional role for TCTP as the GEF for Rheb [75, 76] and Rheb-GEF discovery remains an active area of inquiry in the field. Additionally, it is uncertain whether mTORC1 activation by Rheb is direct or indirect. A search for intermediates between Rheb and mTOR was initially unsuccessful, leading to the hypothesis that Rheb is the immediate upstream activator of mTOR. Long et al. demonstrate that exogenously overexpressed GST-Rheb binds directly to the kinase domain of mTOR; however, binding of Rheb to mTOR is independent of GTP-loading status [77, 78], a surprising result considering Rheb binds to TOR in *Schizosaccharomyces pombe* in a GTP-dependent manner [79]. The authors propose that while nucleotide-free Rheb is capable of binding mTOR, GTP-Rheb is unique in the ability to induce conformational changes in mTORC1 required for activation [77, 78]. Additional work will be required to confirm the nature of the Rheb interaction with mTOR and the mechanism by which this interaction stimulates mTORC1 signaling. Alternatively, several recent publications suggest candidates for the elusive targets downstream of Rheb and upstream of mTORC1. Bai et al. [80] report that Rheb regulates mTOR through an interaction with the FK506-binding protein family member FKBP38. The authors of this study propose a model where FKBP38 inhibits mTORC1 by binding to mTOR in a manner similar to FKBP12–rapamycin, a function antagonized by Rheb-GTP binding to FKBP38. Other groups confirm the FKBP38–Rheb interaction, but do not support the inhibitory role of FKBP38 on mTORC1 function [76]. Sun et al. demonstrate that Rheb directly binds to and activates phospholipase D1 (PLD1). PLD1 is an enzyme that functions to generate the lipid second messenger phosphatidic acid (PA). PA activates mTORC1 signaling [81] via direct binding to the mTOR FRB domain [82] in a manner that is competitive to FKBP12–rapamycin binding [83].

It is also not clear that Akt-mediated repression of TSC2 is sufficient to maximally activate mTORC1 signaling. Mutation of the Akt sites in dTSC2 does not result in the expected effect on *Drosophila* cell growth [84]. Furthermore,

Akt1/Akt2 double-knockout MEFs exhibit a dramatic decrease in 4E-BP1 phosphorylation without a concomitant decrease in TSC2 phosphorylation [85]. This point of controversy may be at least partially explained by the recent discovery of several parallel Akt-dependent and Akt-independent mechanisms for mTORC1 activation. PRAS40 (proline-rich Akt substrate 40 kDa) was recently identified as a novel growth factor-sensitive repressor of mTORC1 [86, 87]. PRAS40 binds directly to the mTOR kinase domain [87] and/or to raptor [86]. High salt concentrations weaken the association of PRAS40 and mTOR resulting in increased mTORC1 kinase activity in vitro. Growth factors inhibit PRAS40 function via activation of Akt, which phosphorylates the C-terminal Thr-246 residue in PRAS40 resulting in mTORC1 derepression. Interestingly, several groups report the presence of a TOS motif in PRAS40 [88, 89], supporting the conclusion in Sancak et al. that PRAS40 interacts with mTORC1 via raptor, but conducive to the alternative hypothesis that PRAS40 may be a novel mTORC1 substrate. Consistent with this idea, mTORC1 was shown to phosphorylate PRAS40 on Ser-183 in a rapamycin-sensitive manner. The interaction between PRAS40 and raptor appears to be highly stable and overexpression of PRAS40 suppresses phosphorylation of 4E-BP1 and S6K1. These data suggest that PRAS40 may serve as a competitive inhibitor of substrate recruitment to raptor. PRAS40 antagonizes mTORC1 activation by Rheb in vitro [86], suggesting that the dynamic interplay between negative regulation by PRAS40 and positive regulation by Rheb can determine mTORC1 signaling strength. Interestingly, Akt has the dual role of inhibiting PRAS40 directly and activating Rheb indirectly via repression of TSC1/2 GAP activity, events that collaborate to activate mTORC1 signaling.

The MAPK pathway has also been shown to directly activate the mTORC1 pathway through Akt-dependent and Akt-independent mechanisms. Growth factors (e.g., EGF) and tumor-promoting phorbol esters (e.g., PMA) activate the potent oncogene Ras. Ras can directly activate PI3K [90, 91] presumably leading to mTORC1 activation via the Akt/TSC2 pathway described earlier. Additionally, Ras activates the canonical MAPK pathway that includes Raf, MEK, ERK, and RSK [92]. PMA activates mTORC1 in a wortmannin-insensitive, UO126-sensitive manner, while overexpression of TSC1 and TSC2 represses PMA stimulation of mTORC1, suggesting a role for MAPK signaling in the regulation of TSC2 GAP activity [93]. Indeed, PMA treatment promotes TSC2 phosphorylation in a PI3K-independent, MAPK-dependent manner [93]. Subsequent investigations revealed that both ERK [94, 95] and RSK [96] directly phosphorylate and inactivate GAP activity of TSC2 against Rheb. ERK appears to preferentially phosphorylate TSC2 on Ser-664 in vitro and in vivo, an event that leads to TSC1/TSC2 complex disassembly and derepression of mTORC1 signaling [94]. Similar to Akt, RSK phosphorylates Ser-939 and Thr-1462 residues on TSC2 along with the unique Ser-1798 site, which also dramatically represses GAP activity of TSC2 [96]. Quantitative analysis of phosphorylation using stable isotope labeling by amino acids in cell culture (SILAC) coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS) confirms the existence of numerous ERK/RSK-sensitive sites on TSC2 [97]. Subsequent work demonstrates that loss-of-function mutations

in the inhibitory Ras-GAP NF1 also lead to repression of TSC1/TSC2 function and hyperactivation of mTORC1 signaling [98]. Recent data suggest that RSK can also promote mTORC1 signaling via direct phosphorylation of raptor [99]. Taken together, the data suggest that the PI3K/Akt and MAPK pathways collaborate to transmit growth factor signals to mTORC1 via TSC2-dependent and TSC2-independent mechanisms.

Finally, the mTORC1/S6K1 signaling axis contains a number of described feedback loops that can influence the pathway. S6K1 phosphorylates the Ser-2448 site on mTOR directly [100, 101], but as mentioned above, the functional consequences of this event are not known. mTORC1/S6K1 signaling also promotes a negative feedback loop that represses insulin signaling. Insulin-mediated Akt activation is repressed by chronic rapamycin treatment [102] and excess amino acid availability can inhibit insulin signaling in a rapamycin-sensitive manner [103]. Furthermore, $TSC2^{-/-}$ *Drosophila* larvae exhibit reduced Akt activity, which can be rescued by deletion of dS6K [104], while loss of TSC1/TSC2 function in mammalian cells leads to decreased Akt phosphorylation [57, 105]. Subsequently, S6K1 was shown to directly phosphorylate IRS-1 on several inhibitory sites, resulting in degradation of the adaptor molecule, explaining loss of PI3K/Akt function in response to elevated mTORC1 signaling [102, 106–108]. $S6K1^{-/-}$ mice have decreased pancreatic β cell mass, but display normal fasting glucose levels due to insulin hypersensitivity as a result of loss of the mTORC1/S6K1 negative feedback loop [109]. These mice are also resistant to obesity when challenged with a high-fat diet due to heightened insulin sensitivity, suggesting that negative feedback loop signaling may contribute to the insulin resistance phenotype associated with type II diabetes and metabolic syndrome [109, 110]. The negative feedback loop may also explain the observation that TSC1/TSC2-null tumors, such as the hamartomas seen in tuberous sclerosis complex, are typically benign (i.e., non-metastatic) in nature. While TSC lesions would be predicted to drive tumor growth via mTORC1, the S6K1 negative feedback loop would simultaneously restrain pro-survival, pro-growth, and pro-metastatic signaling from Akt. Consistent with this hypothesis, feedback loop signaling in $TSC2^{+/-}$ heterozygous mice correlates with limited tumor growth, and crossing these animals with $PTEN^{+/-}$ mice hyperactivates Akt and results in a severe tumorigenic phenotype [111, 112]. Unfortunately, the negative feedback loop may also explain the limited success of rapamycin analogue monotherapy observed in clinical trials for a variety of human cancers. While rapalogs potently inhibit mTORC1 signaling, they also repress the negative feedback loop resulting in Akt activation. At this time it is not clear whether IRS-1 is the sole target of the negative feedback loop. Interestingly, a murine model system expressing a non-phosphorylatable form of ribosomal protein S6, known as $rpS6(P^{-/-})$ knockin mice, also exhibits decreased β cell mass [113], suggesting an undefined role for S6K1 phosphorylation of S6 in this process. TSC1/TSC2 deletion also suppresses PDGFR expression in a rapamycin-sensitive manner through an undefined mechanism [114], suggesting the involvement of other receptor tyrosine kinase pathways in mTORC1-dependent feedback mechanisms. Finally, recent data support a role for the unfolded protein response (UPR) in negative feedback signaling in $TSC1^{-/-}$ and $TSC2^{-/-}$ MEFs [115].

3.2 Nutrients

TORC1 serves as a nutrient sensor in yeast where exogenous supply of metabolic precursors, particularly high-quality nitrogen sources such as glutamine [116], is usually rate limiting for growth and division. Nutrient sensing remains an important input in the regulation of mTORC1 function in mammalian cells which seems advantageous to cellular fitness, as it is not economical for the cell to promote cell growth and proliferation in the absence of an adequate supply of energy and molecular building blocks. Consistent with the critical role of mTORC1 in protein biosynthesis (see below), mTORC1 function appears to be particularly sensitive to amino acid availability. Amino acid inputs to mTORC1 appear to be dominant over growth factor inputs, as growth factors such as insulin are unable to stimulate 4E-BP1 and S6K1 phosphorylation in the absence of amino acids [117].

Amino acid deprivation in mammalian cells results in the rapid dephosphorylation of the mTORC1 targets 4E-BP1 and S6K1, while resupplementation of amino acids stimulates 4E-BP1 and S6K1 phosphorylation in a rapamycin-sensitive manner [117–123]. Mammalian cells appear to be particularly sensitive to deprivation of branch chain amino acids, particularly leucine [121, 124–129]. The mechanism(s) by which amino acid signals are propagated to mTORC1 are not clearly defined; however, roles for amino acid sensing have been ascribed to TSC1/TSC2, Rheb, and signals that appear to be parallel to the TSC1/TSC2–Rheb axis. One study demonstrates that inactivation of TSC2 leads to resistance of mTORC1 signaling to amino acid withdrawal in *Drosophila* and mammalian cells [130]. However, amino acid starvation still results in mTORC1 repression in $TSC2^{-/-}$ cells [131, 132], leading to the hypothesis that Rheb functions as the proximal amino acid sensor for mTORC1. Overexpression of Rheb rescues TORC1 signaling in the face of amino acid withdrawal [60, 64–66] and binding of Rheb to mTOR is regulated by amino acids in some [78] but not all [131] reported studies.

Amino acid withdrawal from $TSC2^{-/-}$ cells leads to mTORC1 repression in the absence of a change in Rheb-GTP levels [132], the Rheb-binding state proposed to be required for mTORC1 activation [77]. Furthermore, *Saccharomyces cerevisiae* lacks TSC1 and TSC2 orthologs, while *S. pombe* lacks homologs of TSC1, TSC2, and Rheb, yet yTORC1 in both species responds to nutrient availability arguing for an evolutionarily conserved mechanism for TORC1 nutrient sensing that does not require these network components. One possible mechanism consistent with this observation may be direct nutrient sensing by the mTORC1 complex. Nutrient deprivation promotes a high-affinity interaction between mTOR and raptor in a manner that inhibits mTORC1 activity [21], likely by preventing recruitment of 4E-BP1 and S6K1 to raptor. mLST8 also plays a positive role in nutrient-dependent activation of mTORC1, possibly by stabilizing the low-affinity mTOR–raptor interaction that promotes substrate recruitment [22]. The mechanism by which amino acids induce these conformational changes in mTORC1 requires further investigation.

Recently published data also support a role for the class III PI3K Vps34 in communicating amino acid levels to mTORC1 independently of TSC1/TSC2 [132, 133].

Amino acid stimulation of mTORC1 signaling is sensitive to treatment with the PI3K inhibitor wortmannin despite the fact that Akt is not activated by amino acids. RNAi-mediated knockdown of class I PI3K, a well-established wortmannin target, has no effect on amino acid stimulation of mTORC1 function [132]. Several approaches demonstrate that amino acids modulate the activity of the class III PI3K hVps34 in a manner required for amino acid stimulation of mTORC1 [132–134]; however, overexpression of hVps34 is not sufficient to prevent reduction in S6K1 phosphorylation following amino acid deprivation. Gulati et al. [134] demonstrate that amino acids induce elevation in intracellular Ca^{2+}, leading to increased binding of Ca^{2+}/calmodulin (CaM) to hVps34, an event required for lipid kinase activity and the subsequent increase in mTORC1 signaling. The molecular events downstream of hVps34 and mTORC1 activation are not clearly defined. hVps34 phosphorylates phosphatidylinositol (PtdIns) generating phosphatidylinositol-3-phosphate (PtdIns-3-P), a regulator of membrane trafficking and membrane fusion. Interestingly, the vacuolar membrane-associated EGO protein complex has been suggested to play a role in yTORC1 activation [135]. The vacuole is the major amino acid reservoir in yeast. Overexpression of the EGO complex constituents GTR2 (RagA-D homolog) or EGO3 promotes rapamycin resistance [136] and transcriptional profiling of the yeast ego3Δ mutant shows similarity to profiles from rapamycin-treated cells [137]. A recent synthetic lethal screen in yeast designed to find interactions with *TOR1* identified EGO proteins, Vps34, Vps15, and components of the class C Vps complex [138]. In yeast, Vps34 and Vps15 are members of the PAS protein complex that regulates autophagy and protein sorting [139, 140], while the class C Vps complex functions in the recognition and fusion of vesicles with vacuolar and secretory membranes. Importantly, supplementation of glutamine or glutamate was sufficient to rescue growth of single class C Vps mutants or the *tor1/pep3* double mutant. Taken together, the results suggest an important interaction between TORC1 signaling and proper vacuolar function. One hypothesis is that these proteins are required to promote autophagy and for proper liberation of amino acids from intracellular vesicles, disruption of which reduces intracellular amino acid levels and inhibits TORC1. Defects in this process may be lethal in the context of TOR1 loss of function where cells are unable to properly downregulate protein biosynthesis in response to reduced supply of amino acid building blocks. Alternatively, recent work shows that the Rag family of GTPases (RagA-D) interacts with mTORC1 in an amino acid-dependent manner, leading to co-localization of mTORC1 with Rheb in Rab7-positive late endosomal and/or lysosomal compartments [141].

Finally, a role for the sterile 20 family protein kinase MAP4K3 as another possible player in amino acid stimulation of mTORC1 was recently reported [142]. Resupplementation of amino acids to starved cells induces an increase in MAP4K3 activity that is required for activation of mTORC1. It is not clear if hVps34 and MAP4K3 are linear members of the same pathway or parallel sensors that propagate amino acid signals to mTORC1. While elucidation of amino acid inputs to mTORC1 remains an elusive goal of the field, apparent discrepancies in the available data may be explained by the existence of multiple layers of intracellular regulation of mTORC1 by amino acids including distal regulation of intracellular amino acid

concentrations from membrane-bound vesicles and proximal interpretation of amino acid levels by TSC1/TSC2, Rheb, and protein–protein interactions within mTORC1. Ongoing investigations in numerous laboratories are currently being undertaken in an effort to present a unifying model for amino acid regulation of mTORC1.

3.3 Stress Signals

An ever-growing number of cellular stresses appear to communicate to the mTORC1 signaling hub including energy stress, hypoxia, glucocorticoid signaling, DNA damage, reactive oxygen species, viral infection, and osmotic stress. In addition to the aforementioned role of amino acids, glucose deprivation also leads to repression in mTORC1 signaling; however, particular glucose breakdown products that specifically mediate this effect have not been identified. Instead, this effect may be mediated through a general reduction in cellular energy status. Treatment of cells with the non-hydrolyzable glucose analogue 2-deoxyglucose (2-DG) leads to depletion of intracellular energy status and recapitulates the effect of glucose deprivation on mTORC1 [143]. Early studies suggested a role of mTOR itself as a sensor of cellular ATP levels [143]; however, repression of mTOR activity requires a drastic reduction in ATP concentrations far below normal physiological levels. The tight homeostatic regulation of cellular ATP suggested the existence of another sensor for energy stress.

An alternative mechanism for transmission of energy signals to mTORC1 involves the 5′-AMP-activated protein kinase (AMPK). AMPK is activated by numerous mechanisms including allosteric regulation via binding by AMP [144, 145] and activation loop phosphorylation by LKB1. Under conditions that promote energy stress, AMP/ATP ratios can be dramatically elevated making AMP sensing a more sensitive readout of energy stress compared to a mechanism that monitors ATP levels. AMPK was initially implicated in regulation of mTORC1 signaling by activation of this kinase by the AMP analogue AICAR, which promotes a reduction in 4E-BP1 and S6K1 phosphorylation [146–148]. Overexpression of an activated allele of AMPK represses S6K1 phosphorylation while exogenous expression of a dominant-negative AMPK construct leads to elevated S6K1 phosphorylation [148]. AMPK directly phosphorylates TSC2 in vitro and in vivo on Thr-1227 and Ser-1345 [149], presumably causing an increase in TSC2 GAP activity and subsequent repression of Rheb/mTORC1 activation. Mutation of AMPK-dependent sites on TSC2 to alanine leads to partial insensitivity of mTORC1 signaling following energy depletion, the same result observed in $TSC2^{-/-}$ cells [149]. Furthermore, $TSC2^{-/-}$ cells and cells overexpressing AMPK phospho-resistant TSC2 mutants undergo apoptosis in response to energy deprivation [149]. AMPK can also directly phosphorylate raptor in response to AICAR treatment, leading to 14-3-3 binding and contributing to repression of mTORC1 signaling in response to severe energy stress [150]. Maximal

activation of AMPK requires phosphorylation of the activation loop residue Thr-172 by LKB1, a serine/threonine kinase that localizes to a protein complex with the adaptor proteins STRAD and MO25 [151, 152]. $LKB1^{-/-}$ MEFs have a deficiency in AMPK activity in response to energy deprivation [153] and exhibit elevated mTORC1 signaling [153–156]. Loss of LKB1 function is causative in Peutz–Jeghers syndrome (PJS), an autosomal dominant disorder with clinical similarities to TSC including the development of non-malignant hamartomas. $LKB1^{+/-}$ mice phenocopy the manifestations of PJS, and S6K1 phosphorylation is elevated in tissue samples from the spontaneous hamartomas that develop in these animals [156]. Liver-specific $LKB1^{-/-}$ mice display diabetic phenotypes such as hyperglycemia [157] and the diabetes drug metformin activates AMPK in an LKB1-dependent manner [157, 158].

AMPK is also involved in regulation of cell growth in response to genotoxic stress. Upon DNA damage, p53 inhibits mTORC1 activity via association with LKB1 and activation of the AMPK–TSC2 signaling pathway [159]. Surprisingly, mTORC1 was subsequently shown to play a positive role in p53 expression in TSC1/TSC2-deficient cells [160], suggesting the possibility of another negative feedback loop in the mTORC1 signaling axis. In addition, AMPK also plays a role in the newly described link between Wnt signaling and mTORC1 regulation [161]. Wnt binds to the Frizzled family of cell surface receptors and plays an important role in cell growth, animal development, and cancer [162, 163]. The canonical Wnt signaling pathway involves repression of GSK-3 followed by stabilization and translocation of β-catenin to the nucleus where it can induce a pro-growth gene program [164–167]. Wnt activates mTORC1 in a GSK-3-dependent β-catenin-independent manner in mammalian cells and mouse models [168]. AMPK phosphorylation of TSC2 on Ser-1345 serves as a priming event that is a prerequisite for subsequent GSK-3β-mediated activating phosphorylation of TSC2 on Ser-1341 and Ser-1337. Wnt repression of GSK-3 results in activation of mTORC1 and rapamycin dramatically reduces tumor formation by Wnt-1-expressing cells in an immunodeficient mouse xenograft model [168].

Interestingly, there appear to be a number of interactions between AMPK and Akt signaling with regard to regulation of mTORC1. Akt appears to play a role in regulating energy levels and repressing AMPK. Akt1/Akt2 double-knockout MEFs exhibit an elevated AMP/ATP ratio and an increase in AMPK activity [169]. Conversely, cells expressing a constitutively activate Akt mutant display a decreased AMP/ATP ratio and a subsequent decline in AMPK activity [169]. The mechanism by which Akt regulates AMP/ATP levels was not formalized in this study, but Akt is known to regulate nutrient uptake in an mTORC1-dependent manner [170] and promotes translocation of the glucose transporter GLUT-4 to the plasma membrane [171–173]. Furthermore, Akt activates the ubiquitin ligase MDM2 resulting in degradation of p53 [174] and is also known to phosphorylate and repress GSK-3 [175]. These data suggest that Akt functions to repress TSC2 GAP activity both by direct inhibitory phosphorylation of TSC2 and via repression of stimulatory signals

to TSC2 by AMPK through modulation of AMP/ATP levels, p53 protein expression, and GSK-3 activity.

In addition to the repressive inputs from energy and genotoxic stress, the culturing of mammalian cells under low oxygen conditions (hypoxia) potently inhibits mTORC1 signaling. Repression of mTORC1 by hypoxia requires TSC1/TSC2 because $TSC1^{-/-}$ and $TSC2^{-/-}$ cells do not exhibit a decrease in S6K1 phosphorylation under low-oxygen conditions [176]. A *Drosophila* genetic screen identified the *Scylla* and *Charybdis* genes as repressors of the dPDK1/dAkt-driven increase in cell size. Epistatic analysis places these genes downstream of dAkt and upstream of dTSC [177]. Importantly, *Scylla* and *Charybdis* are upregulated in response to hypoxia [177]. Similarly, the mammalian orthologs of these proteins, Redd1 and Redd2, negatively regulate mTORC1 downstream of Akt and upstream of TSC1/TSC2 [176, 178]. Redd is transcriptionally upregulated by HIF-1α in response to hypoxia [179] and $Redd1^{-/-}$ MEFs cannot repress mTORC1 signaling in response to hypoxia [176]. Interestingly, Redd proteins also appear to be induced by other stress signals that repress mTORC1, including energy deprivation [180], glucocorticoid treatment [181], DNA damage [182], reactive oxygen species [183], and alcohol intoxication [184]. Redd1 appears to have a very short half-life (~5 min) and rapid turnover of the protein is responsible for activation of mTORC1 observed after inhibition of protein synthesis with cyclohexamide [185]. Redd1 expression is elevated in response to chronic energy deprivation and can repress S6K1 phosphorylation independently of AMPK [180]. Loss of Redd1 function has no effect on AMPK-mediated TSC2 phosphorylation, but it does repress S6K1 activation in response to long-term energy stress. This AMPK-independent input to TSC2 in response to energy deprivation was predicted by the absence of conserved AMPK activation sites in dTSC2 [186] and the aforementioned incomplete nature of S6K1 phosphorylation rescue in response to energy stress in $TSC2^{-/-}$ cells and cells overexpressing TSC2 mutants that cannot be phosphorylated by AMPK [149]. The mechanism by which Redd proteins activate TSC1/TSC2 and repress mTORC1 is not clearly defined. Redd1 lacks any known functional domains with the exception of a C-terminal coiled-coil domain [182]; therefore, it is unlikely to act as a kinase to phosphorylate TSC2 like other known upstream activators/repressors. Recently, DeYoung et al. demonstrated that Redd1 suppresses mTORC1 by releasing TSC2 from growth factor-induced association with inhibitory 14-3-3 proteins in response to hypoxia [187]. In addition to these Redd-dependent mechanisms, the Bcl-2 homology 3 domain-containing protein Bnip3 can also repress mTORC1 signaling in response to hypoxia by directly interacting with Rheb, thus reducing Rheb GTP-loading status [188].

Finally, recent data suggest a role for the pro-inflammatory TNFα signaling pathway in mTORC1 activation via post-translational modification of TSC1. Lee et al. demonstrate that IKKb directly interacts with and phosphorylates TSC1 at Ser-487 and Ser-511 resulting in repression of TSC1/TSC2 function, activation of mTORC1, increased VEGF expression, enhanced angiogenesis, and tumor development [189]. These data support a novel role for the mTORC1 function in inflammation-mediated increases in angiogenesis in human cancer.

4 Downstream Targets of mTORC1 Regulate Cell Growth Control

Under cellular conditions that are conducive to cell growth, mTORC1 modulates a number of downstream effectors that promote cell anabolism while simultaneously repressing catabolic processes. The most well-understood targets of mTORC1 signaling are 4E-BP1 and S6K1. Upon phosphorylation by mTORC1, 4E-BP1 and S6K1 collaborate to promote the intricately controlled process of mRNA translation initiation. While the discussion below will focus on the initiation phase of mRNA translation, it is important to note that mTORC1/S6K1 also appears to affect translation elongation through repressive phosphorylation of eEF2K and subsequent derepression of eEF2 [190–192]. In addition, S6K1 stimulates ribosomal biogenesis and represses the catabolic process macroautophagy. Taken together, regulation of these molecular processes by mTORC1 results in cellular level effects on growth (i.e., cell size) and proliferation. In addition to other pathophysiological outcomes, aberrant regulation of these processes can contribute to a tumorigenic phenotype. The discussion below will focus on pro-anabolic regulation of protein biosynthesis by mTORC1.

4.1 mRNA Translational Control

mRNA translation is a fundamental biological process by which genetic information encoded in messenger RNA is used to manufacture cellular proteins by a massive molecular machine known as the ribosome. mRNA translation is subdivided into three distinct phases: translational initiation, translational elongation, and translational termination. Translational initiation is the process in which the ribosome is recruited to the mRNA and scans the translational start site. This carefully orchestrated operation serves as the rate-limiting process in protein biosynthesis. Translation initiation itself appears to have two rate-limiting steps: delivery of the eIF2-GTP-Met-tRNA$_i$ ternary complex to the 40S ribosomal subunit to form the 43S pre-initiation complex (PIC) and subsequent recruitment of messenger RNA via the eIF4F complex to form the 48S PIC (Fig. 2).

The eIF4F complex is composed of the mRNA 5′-m7GppN cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A. The protein expression level of eIF4E makes this initiation factor limiting for eIF4F complex formation. Additionally, bioavailability of eIF4E is antagonized by the action of a family of inhibitory-binding proteins known as the 4E-BPs. This family is comprised of three members named 4E-BP1, 4E-BP2, and 4E-BP3. The overwhelming majority of the literature is focused on 4E-BP1 and thus we will focus our discussion on this 4E-binding protein. 4E-BP1 binds to eIF4E on a region that overlaps with the binding surface of the eIF4F complex member eIF4G such that these interactions are mutually exclusive [193, 194]. Hypophosphorylated 4E-BP1 binds to eIF4E with high affinity, while ordered, hierarchical phosphorylation of 4E-BP1

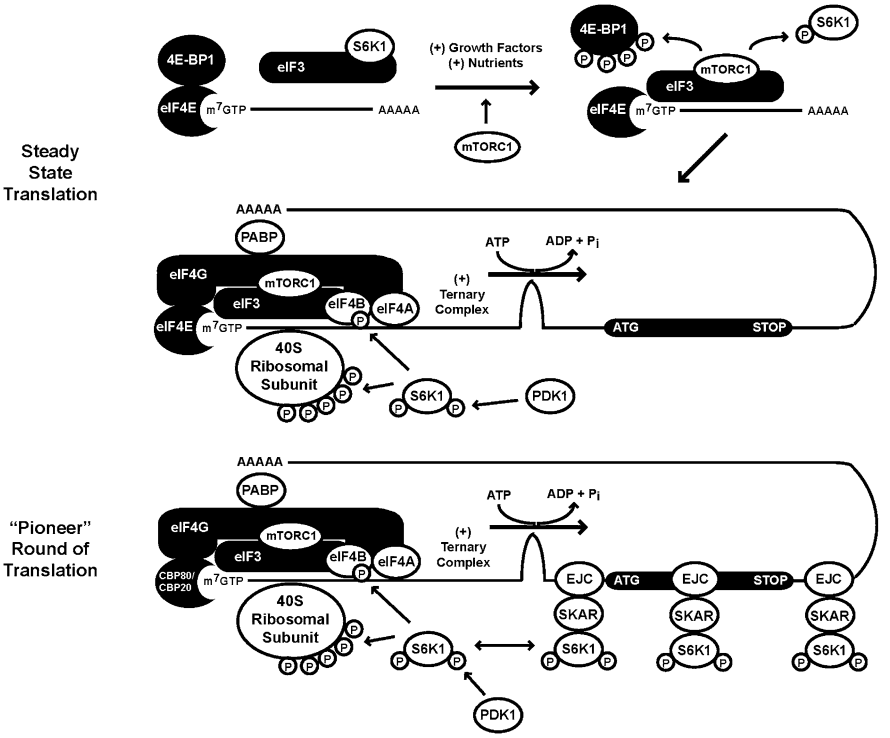

Fig. 2 Control of mRNA translation initiation by mTORC1/S6K1 through dynamic protein–protein interactions and ordered phosphorylation events. Under conditions such as serum starvation and nutrient deprivation mTORC1 signaling is repressed and mRNA translational initiation is downregulated. Hypophosphorylated 4E-BP1 interacts with the cap-binding protein eIF4E, while inactivated S6K1 binds to eIF3. Upon stimulation with growth factors or re-addition of nutrients mTORC1 is activated and binds to eIF3 displacing S6K1. From this platform mTORC1 phosphorylates 4E-BP1 and S6K1. Phosphorylation of 4E-BP1 disrupts the interaction with eIF4E, allowing binding of eIF4G, assembly of the eIF4F complex, and formation of the 48S pre-initiation complex (PIC). Liberated S6K1 is fully activated by PDK1-mediated phosphorylation and acts on local substrates such as the ribosomal protein S6 and eIF4B. S6K1 plays an additional role in the pioneer round of translation on newly synthesized mRNAs. The CBP80/20 complex serves as the cap-binding protein on newly minted mRNAs. These mRNAs are also bound at exon–exon boundaries by the exon junction complex (EJC). The S6K1-specific interacting protein SKAR is a novel EJC-interacting protein required for splicing-dependent enhancement of mRNA translation. S6K1 phosphorylates several proteins in CBP80-bound mRNPs in a SKAR-dependent manner

sequentially on Thr-37, Thr-46, Thr-70, and Ser-65 results in disruption of the interaction between 4E-BP1 and eIF4E [195]. A structural model of the 4E-BP1–eIF4E interaction shows that these 4E-BP1 phosphorylation sites are in proximity to a series of acidic amino acids in eIF4E, suggesting that negatively charged phosphate groups may disrupt the interaction of 4E-BP1 and eIF4E by electrostatic repulsion [196]. mTORC1 can phosphorylate 4E-BP1 on Thr-37 and Thr-46 in vitro [195,

197, 198], events that are required for subsequent phosphorylation of Thr-70 and Ser-65 and disassociation of 4E-BP1 and eIF4E. Thus, mTORC1 action increases the availability of eIF4E and promotes eIF4F complex formation, driving the rate-limiting recruitment of mRNAs to the forming translation pre-initiation complex and subsequent cap-dependent translation. Importantly, one potential mechanism for rapamycin resistance in some cancer cell lines is re-phosphorylation of 4E-BP1 despite the presence of the inhibitor [199].

The other well-studied mTORC1 target, the ribosomal protein S6 kinase (S6K), also plays an important role in translation initiation via phosphorylation of numerous downstream effectors. Mammalian cells have two S6K isoforms, S6K1 [200] and S6K2 [201, 202], that have significant homology (~70% identity) including conservation of functional domains and phosphorylation sites. Based on alternative splicing S6K1 is found in a short predominantly cytoplasmic isoform (70 kDa) and a longer isoform (85 kDa) that contains a nuclear localization signal (NLS). These isoforms appear to be regulated in an identical manner by mTORC1 and the significance of the localization of these variants is not known. The discussion below will primarily focus on S6K1 consistent with availability of information in the published literature; however, it is important to note that S6K2 is the predominant isoform in multiple cell and tissue types [203] and S6K2 can partially compensate for the loss of S6K1$^{-/-}$ knockout mice [201]. Activation of S6K1 is a complicated process and an active area of investigation that is beyond the scope of the current chapter; however, this process seems to require phosphorylation of the C-terminal hydrophobic motif at Thr-389 by mTORC1 and the Thr-229 residue in the T-loop region by PDK1 [8].

Upon activation, S6K1 phosphorylates numerous downstream targets, many of which play a role in mRNA translation and protein biosynthesis. Two known S6K1 targets also influence proper eIF4F complex formation suggesting that S6K1 collaborates with 4E-BP1 in regulation of this critical cellular process. First, S6K1 phosphorylates the tumor suppressor PDCD4. Reminiscent of the competitive binding of eIF4E by 4E-BP1 and eIF4G, PDCD4 binds to eIF4A [204] via two tandem MA3 domains [205–207] preventing interaction of eIF4A with eIF4G, repressing eIF4A helicase activity, and inhibiting cap-dependent translation [204]. Phosphorylation of PDCD4 on Ser-67 by S6K1 promotes the recruitment of the ubiquitin ligase bTRCP and subsequent degradation of PDCD4 [208]. Overexpression of a PDCD4 mutant deficient in binding bTRCP results in translational repression of an mRNA with a highly structured 5′-UTR that would normally inhibit efficient 48S PIC scanning and results in reduced cell size and cell proliferation [208]. Interestingly, recent data show that the oncogenic fusion protein BCR-ABL activates mTORC1/S6K1 and leads to reduction in PDCD4 expression [209]. S6K1 also phosphorylates eIF4B on Ser-422 in vitro [210] and in vivo [210, 211]. eIF4B is an RNA-binding protein that promotes the RNA helicase activity of eIF4A [212]. Phosphorylation of eIF4B by S6K1 promotes its recruitment to the PIC through an interaction with the heteromeric scaffolding complex eIF3 [211], enhances binding of the PIC to mRNAs containing highly structured 5′-UTRs [213], and increases the translation of mRNAs with highly structured 5′-UTRs [214].

Recent work also demonstrates a role for mTORC1/S6K1 in the "pioneer" round of translation, the first passage of the ribosome along the length of a newly minted mRNA [215]. Unlike steady-state translation where eIF4E serves as the 5′-cap-binding protein, the nascent pre-mRNA 5′-m7GppN cap is co-transcriptionally bound by the nuclear cap-binding complex (CBC), a heterodimer of the proteins CBP80 and CBP20. CBC binding is required for efficient mRNA splicing [216]. In turn, splicing imprints the mRNA with several protein complexes, including the exon junction complex (EJC), which is deposited ~20 nt upstream of each exon–exon junction [217]. The EJC is involved in proper nuclear export of mRNA and is only removed by the first passage of the ribosome [218–221], suggesting a potential role for the EJC in regulation of the "pioneer" round of translation [222–224]. Interestingly, the presence of introns enhances subsequent protein expression in a splicing-dependent manner [225–229] and deposition of the EJC is necessary and sufficient to mediate this result at least partially by increasing the association of spliced mRNAs with actively translating polysomes [222, 223]. Rapamycin treatment inhibits the increase in translational efficiency gained by mRNA splicing [215] implicating mTORC1 in this process. The S6K1-specific interacting protein SKAR [230] was recently shown to be a novel EJC-interacting protein that is required for the splicing-dependent increase in protein synthesis [215]. S6K1 appears to phosphorylate several proteins in CBP80-bound mRNPs in a SKAR-dependent manner. Future work will be required to elucidate the identity and function of CBP80-bound S6K1 phospho-proteins in regulating the "pioneer" round of mRNA translation.

In addition to the mTORC1-dependent mechanisms that drive global mRNA translation described earlier, mTORC1 signaling also appears to play a role in preferential regulation of specific RNA species. The first example of such regulation involves phosphorylation of the small ribosomal protein S6 by S6K1. S6K1 phosphorylates S6 on a cluster of C-terminal residues including Ser-235, Ser-236, Ser-240, Ser-244, and Ser-247. Early reports supported a role for S6K1-mediated phosphorylation of S6 in the translation of mRNAs containing a 5′-terminal oligopyrimidine tract (5′-TOP) immediately adjacent to the 5′-m7GppN cap structure. Interestingly, 5′-TOP-containing messages include numerous components of the translational machinery such as ribosomal proteins, elongation factors, and the poly(A)-binding protein (PABP). Rapamycin treatment represses 5′-TOP translation, an effect that is rescued by overexpression of rapamycin-resistant S6K1 [231, 232]. More recently, several publications have called the sufficiency of S6K/S6 in regulation of rapamycin-sensitive 5′-TOP translation into question. Tang et al. demonstrated that overexpression of dominant-negative S6K1 was unable to repress stimulation of 5′-TOP translation by amino acid stimulation, a result repeated in S6K1$^{-/-}$ S6K2$^{-/-}$ double-knockout MEFs [233] and ES cells [203, 234]. Additionally, knockin mice expressing S6 with all five phosphorylation sites mutated to alanine (rpS6(P$^{-/-}$)) have no defect in 5′-TOP translation [113]. MEFs from these animals have a small size phenotype explained by defects in cell growth; however, they had elevated protein synthetic rates and accelerated cell division compared to rpS6(P$^{+/+}$) cells. The search to identify the mTORC1-dependent,

S6K1/S6-independent mechanism for 5′-TOP translational control continues, as do efforts to uncover the physiological role of the first described target of S6K1.

mTORC1 also affects the translation of specific mRNAs through the aforementioned role on eIF4F complex formation and eIF4A/eIF4B activation. While the availability of a functional eIF4F complex is thought to modestly effect global rates of protein synthesis, the effect on individual messages is unequal. As discussed previously, mRNAs that contain highly structured 5′-UTRs have an increased requirement for the helicase function of eIF4A and eIF4B for efficient translation. Koromilas et al. fused the CAT reporter mRNA to artificial 5′-UTRs with increasing degrees of secondary structure to show that eIF4E overexpression results in elevated translation of structured messages [235]. This result is sensible because, as the limiting member of the eIF4F complex that is required for cap binding, overexpression of eIF4E should lead to a general increase in eIF4F function. Similar results might not be expected for eIF4F members that are expressed in excess of stoichiometric levels. The result of Koromilas et al. prompted the discovery of numerous "non-competitive" endogenous mRNAs [236] that are preferentially translated in eIF4E-overexpressing model systems including ornithine decarboxylase (ODC) [214, 237–239], cyclin D1 [240], c-myc [241], fibroblast growth factor (FGF) [242], vascular endothelial growth factor (VEGF) [243], Bcl-2 [244], Pim-1 [245], and ribonucleotide reductase [246]. Interestingly, recently reported data show that eIF2Bϵ mRNA is translationally controlled in a rapamycin-dependent manner in rat skeletal muscle following recovery from resistance exercise [247]. This message encodes the catalytic subunit of the guanine nucleotide exchange factor (GEF) complex for eIF2. eIF2B controls GTP loading on eIF2, thus regulating the delivery of eIF2-GTP-Met-tRNA$_i$ to the 40S ribosomal subunit. This process represents the other rate-limiting step in mRNA translation initiation. These mTORC-dependent mRNAs all contain a long, highly structured 5′-UTR and the proteins they encode are known to have pro-growth and/or anti-apoptotic functions, consistent with known mTORC1 phenotypes. Many of these mRNAs code for proteins that are bona fide oncogenes or are overexpressed in human cancers.

A recent report by Holz et al. [248] provides insights into the spatial and temporal nature of mTORC1 regulation of translation initiation in which eIF3 serves as a central scaffold. eIF3 is the largest eukaryotic initiation factor, composed of 13 unique subunits (eIF3a-m) encoded on distinct genomic loci. eIF3 binds to free 40S ribosomal subunits and is required for assembly of the eIF2-GTP-Met-tRNA$_i$ ternary complex, recruitment of the eIF2-GTP-Met-tRNA$_i$ ternary complex and the other members of the 43S PIC, eIF4F-mRNA recruitment, and scanning of the 48S PIC to the AUG start codon [249]. In addition to these functions, eIF3 also appears to be a scaffold for the mTORC1 translational control signaling axis [248]. Under conditions where mTORC1 signaling is repressed, S6K1 associates with eIF3. Stimulation with growth factors activates mTORC1 promoting S6K1 Thr-389 phosphorylation, which leads to a dynamic interchange whereby S6K1 is released from eIF3 and the mTORC1 complex binds to the eIF3 scaffold. From this vantage point, mTORC1 is in close proximity to 4E-BP1 and can phosphorylate this downstream target promoting eIF4E release and eIF4F complex formation. S6K1

liberation allows further activation of the kinase by PDK1 and subsequent phosphorylation of, or interaction with, the local targets S6, eIF4B, PDCD4, and SKAR promoting translation initiation by the mechanisms described above.

4.2 Ribosomal Biogenesis

In addition to the effects of mTORC1-mediated phosphorylation events on mRNA translation and 5′-TOP-mediated translation of many protein components of the translation apparatus, mTORC1 also plays an established role in transcriptional regulation of ribosome-related genes via all three major DNA polymerases. Indeed, coordinated regulation of Pol I, Pol II, and Pol III is mandatory to economically generate the required amount of rRNA, tRNA, and ribosomal proteins to support ribosome generation. Exponentially growing HeLa cells generate ~7,500 ribosomes/min requiring the transcription of ~200 unique genes and the synthesis of ~300,000 ribosomal proteins [250]. In yeast, the synthesis of rRNA represents ~60% of total cellular transcriptional investment, while production of ribosomal protein encoding mRNAs equals roughly the same percentage of all Pol II-mediated transcriptional events [251]. Based on the massive cellular energy investment in ribosomal biogenesis and protein biosynthesis these processes need to be tightly coupled to amino acid availability, mitogenic status, and ATP concentrations, a role for mTORC1 that is conserved from yeast to man.

rRNA synthesis appears to be the rate-limiting process in ribosomal biogenesis; therefore, sustained Pol I transcription is required for ribosome production, sustained mRNA translation, and subsequent cell growth/proliferation. Deprivation of essential amino acids (especially leucine) [252] and rapamycin treatment [253–256] result in rapid repression of Pol I transcription. Pol I transcription requires a minimum of three basal factors, namely TIF-IA, TIF-IB, and UBF. Rapamycin-mediated repression of Pol I transcription can be rescued by exogenous overexpression of TIF-1A, mTOR, and wild-type S6K1, but not kinase-dead S6K1 [257]. Rapamycin treatment represses TIF-1A activity by decreasing inhibitory Ser-44 phosphorylation and promoting stimulatory Ser-199 phosphorylation [257], events partially regulated by mTORC1-dependent regulation of PP2A. The yeast homolog of TIF-1A (Rrn3p) is also involved in yTORC1-mediated Pol I regulation [258]. Rapamycin treatment results in repression of the interaction between TIF-1A and TIF-1B [257] disrupting assembly of the Pol I transcription initiation complex [258] and promoting translocation of TIF-1A from the nucleolus to the cytoplasm where it is functionally sequestered. UBF is also regulated by the mTORC1/S6K1 signaling pathway. Rapamycin treatment represses protein expression levels of UBF. Growth factor-stimulated C-terminal phosphorylation of UBF, which promotes the interaction between UBF and TIF-1B, is rapamycin sensitive and can be rescued by exogenous expression of constitutively active S6K1 [256]. This work relied on the use of nuclear extracts and did not resolve whether S6K1-dependent UBF phosphorylation was direct or indirect. Subsequent work by Nader et al. [259] shows that