

An emerging role for TOR signaling in mammalian tissue and stem cell physiology

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Summary

The mammalian target of rapamycin (mTOR) is a kinase that responds to a myriad of signals, ranging from nutrient availability and energy status, to cellular stressors, oxygen sensors and growth factors. The finely tuned response of mTOR to these stimuli results in alterations to cell metabolism and cell growth. Recent studies of conditional knockouts of mTOR pathway components in mice have affirmed the role of mTOR signaling in energy balance, both at the cell and whole organism levels. Such studies have also highlighted a role for mTOR in stem cell homeostasis and lifespan determination. Here, we discuss the molecular mechanisms of TOR signaling and review recent *in vitro* and *in vivo* studies of mTOR tissue-specific activities in mammals.

Key words: mTOR, TORC1, Stem cells, Rapamycin, TSC

Introduction

The ability of cells to respond appropriately to nutrient flux is essential for maintaining energy homeostasis. From yeast to humans, nutrient deprivation activates a highly conserved cellular program that acts to prune back energy-intensive processes while promoting energy-producing catabolic activities. Target of rapamycin (TOR) is a key regulatory kinase that acts at the nexus of a vast array of nutrient-sensitive signals to regulate cellular metabolism. TOR exists in two complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2), which are functionally conserved in eukaryotes. TORC1 is the primary effector for the nutrient-sensitive functions of TOR, whereas TORC2 has been implicated in cytoskeletal reorganization and cell survival. During periods of nutrient deprivation, TORC1 activity *in vitro* is drastically reduced, resulting in the modulation of several key processes, including translational initiation, ribosome and tRNA biogenesis, and autophagy. Key upstream regulators of TORC1 include AKT (a serine/threonine protein kinase) and the tuberous sclerosis complex (TSC). More recently, the activity of Rag GTPases in concert with Ras homolog enriched in brain (Rheb) has been described in the promotion of TORC1 activity *in vitro* by amino acids. These studies have filled in a major gap in our understanding of how intracellular nutrients regulate TOR activity. However, our understanding of TOR signaling *in vivo* has progressed more slowly. Deletion of TOR is lethal in all eukaryotic model systems, making the study of TOR signaling *in vivo* more challenging. Deletion of mTOR in mice, for example, is early embryonic lethal; however, conditional knockouts of mTOR or TORC components in mice have begun to reveal exciting functions for mTOR in metabolically sensitive organs. In this review, we

introduce our current understanding of TOR signaling *in vitro* and highlight recent advances in this field. Moreover, we review recent genetic studies in mice that explore perturbation of mTOR pathway components. Finally, we discuss several recent studies that have identified a role for TOR signaling in various stem cell models.

TOR kinase

The discovery of TOR kinases arose from the study of the anti-fungal agent rapamycin. Additional applications for rapamycin were soon discovered, and today rapamycin is used clinically (under the trade name Sirolimus) as an FDA-approved immunosuppressant and chemotherapeutic agent. The potent anti-proliferative properties and exquisite specificity of rapamycin have also proven to be useful characteristics for studying cell growth regulation. In the early 1990s, yeast genetic screens revealed two genes, *TOR1* and *TOR2*, the mutation of which relieved the growth suppressive effects of rapamycin (Heitman et al., 1991; Kunz et al., 1993). Within the cell, rapamycin complexes with FK506-binding protein 12 kDa (FKBP12), and the resulting dimer then binds directly to TOR, causing a potent inhibition of TOR activity (Choi et al., 1996). From *Drosophila melanogaster* to mammals, TOR exists as a single gene product, commonly referred to as *Drosophila* TOR (dTOR) and mammalian TOR (mTOR, also known as mechanistic TOR). TOR kinase has putative orthologs throughout eukaryotes and possesses a striking conservation of its core cellular functions despite limited sequence similarities.

TOR is a large atypical serine-threonine protein kinase with a predicted molecular weight of 289 kDa. The N terminus of mTOR contains numerous HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) repeats that are thought to mediate the majority of interactions between mTOR and other proteins (Fig. 1). The C terminus contains a kinase domain that places it in the phosphatidylinositol 3 kinase (PI3K)-related kinase protein family of kinases. Functionally, TOR kinase acts as a central hub that regulates a diverse array of signals involved in cell growth (increased cell size) and cell proliferation (the rate of cell division). Hyperactivation of TOR activity in both yeast and mammals results in an increase in cell growth, and can cause some cell types to enter the cell cycle (Soucek et al., 1997; Oldham et al., 2000; Soucek et al., 2001).

The TOR complexes and inhibitors

TOR forms two kinase complexes, which perform non-overlapping functions within the cell. TORC1 is responsible for promoting translation, which is the best-known function of TOR signaling. Other functions performed by TORC1 include inhibiting autophagy, promoting ribosome biogenesis and promoting tRNA production. TORC2, by contrast, is responsible for the phosphorylation and activation of AKT and of the related kinases serum/glucocorticoid regulated kinase (SGK) and protein kinase C (PKC); it also regulates cytoskeletal organization. The unique

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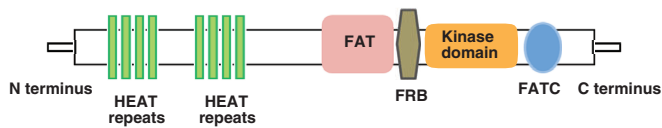


Fig. 1. The domain structure of mTOR. Mammalian target of rapamycin (mTOR) is an atypical serine-threonine protein kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase protein (PIKK) family. Along with other members of the PIKK family, mTOR contains a defining C-terminal kinase domain (orange) that bears similarity to the kinase domain of PI3K. In addition, mTOR possesses numerous N-terminal HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) repeats (green) that are thought to mediate the bulk of protein-protein interactions between mTOR and other signaling proteins. Other domains include a FAT (FRAP, ATM and TTRAP) domain (pink), which is also found in other PIKK family members, a C-terminal FAT (FATC) domain (blue) of unknown function and a FKBP12/rapamycin-binding (FRB) domain (brown), which is adjacent to the kinase domain in TOR.

binding partners of TOR are responsible for the selectivity of these kinases, and the identification of these binding partners has been the focus of intense investigation.

The initial characterization of the mammalian TOR complexes (TORC1 and TORC2) was made in yeast. Yeast TORC1 was shown to be potently sensitive to rapamycin, whereas TORC2 was insensitive to rapamycin treatment (Loewith et al., 2002). Subsequent studies have shown TORC1 and TORC2 to be functionally conserved in mammals (Jacinto et al., 2004; Sarbassov et al., 2004). Recently, it was shown that FKBP12/rapamycin promotes the stepwise dissociation of the mTORC1 complex, and that rapamycin might also be capable of physically blocking the docking of some mTORC1 substrates (Yip et al., 2010). However, in mammalian cells, rapamycin probably does not produce complete inhibition of all mTORC1-dependent functions. For example, TORC1 inhibition in yeast potently reduces global translation and rapidly halts the cell cycle (Barbet et al., 1996), whereas the effects of rapamycin in mammalian cells are more subdued: global translation is modestly reduced and cell cycle inhibition is observed in only a subset of cells (Pedersen et al., 1997; Shor et al., 2008; Thoreen et al., 2009). Moreover, the effects of mTOR loss are often more severe than those elicited by rapamycin treatment on processes that are generally considered to be TORC1 dependent (Murakami et al., 2004; Guertin et al., 2006b). Rapamycin is often used with the assumption that TORC1 is being completely inhibited *in vitro* and *in vivo*; however, secondary disruption of TORC1 can easily be achieved by the use of recently developed inhibitors of the active site of mTOR (Feldman et al., 2009; Garcia-Martinez et al., 2009; Thoreen et al., 2009; Yu et al., 2009). These active site inhibitors also potently inhibit mTORC2; thus, RNAi-mediated knockdown of TORC1- or TORC2-specific components is often used as a follow-up assay. In addition, TORC2 is commonly referred to as being rapamycin insensitive; however, it should be noted that prolonged exposure to rapamycin is also capable of destabilizing TORC2 in some cell types, so care should be taken when describing the effects of extended rapamycin treatment (Sarbassov et al., 2006). If the FKBP12/rapamycin-binding (FRB) site on TOR is not available for binding to FKBP12/rapamycin in TORC2, it is quite possible that the binding of FKBP12/rapamycin to newly synthesized TOR would preclude its incorporation into TORC2, thus resulting in the delayed kinetics of TORC2 destabilization.

TORC1 composition

TORC1 exists as a homodimer in mammals, with each monomer containing mTOR, regulatory associated protein of mTOR (raptor), proline-rich AKT substrate 40 kDa (PRAS40), mammalian lethal with Sec-13 protein 8 (mLST8, also known as GbL) and DEP domain TOR-binding protein (Deptor) (Takahara et al., 2006; Wang et al., 2006; Zhang et al., 2006; Peterson et al., 2009; Yip et al., 2010) (Fig. 2A). The raptor subunit of TORC1 is essential for the kinase activity of TORC1 *in vitro* and *in vivo*, although raptor itself does not possess any enzymatic activity (Hara et al., 2002; Kim et al., 2002; Guertin et al., 2006a). Raptor is also required to promote TORC1 complex formation and to allow TORC1 to interact with some substrates and to bind key regulators at the lysosomal membrane (Hara et al., 2002; Kim et al., 2002; Kim et al., 2008; Sancak et al., 2008; Yip et al., 2010). By contrast, PRAS40 binding to TORC1 *in vitro* inhibits TORC1 activation (Sancak et al., 2007; Vander Haar et al., 2007). Cellularly, growth factor depletion inhibits TORC1, in part, through PRAS40 (Sancak et al., 2007; Vander Haar et al., 2007). PRAS40 itself contains a TOR signaling motif, and overexpression of PRAS40 suggests that it is capable of competing with other TORC1 targets for phosphorylation (Oshiro et al., 2007; Wang et al., 2007). Therefore, although the overall negative impact of PRAS40 on TORC1 target genes has been well established, additional studies are needed to determine whether PRAS40 inhibits TORC1 as a subunit, as a substrate, or as both. mLST8 also binds to mTOR as part of the TORC1 complex, although the role of mLST8 in TORC1 activity has not been fully reconciled (Kim et al., 2003). RNAi-mediated knockdown of mLST8 has been reported to decrease TORC1 activity, as monitored by phosphorylation of downstream substrates; however, the same TORC1 target genes are reported to be phosphorylated normally in *Mlst8*^{-/-} mouse embryo fibroblasts (MEFs) (Guertin et al., 2006b; Kim et al., 2003). Moreover, *Mlst8* knockout mice do not phenocopy *Mtor* knockout mice, indicating that mLST8 is not essential for mTORC1 activity (Guertin et al., 2006b). Interestingly, overexpression of *Mlst8* with *Mtor* and *Rptor* (the gene encoding raptor) restores the amino acid-sensitive interaction between raptor and mTOR that is observed upon endogenous co-immunoprecipitation from cultured cells (Kim et al., 2003). These reports suggest that mLST8 may be involved in the activation of TORC1 by amino acids, but that it is dispensable for other mechanisms of TORC1 activation. Deptor, which also binds mTOR and thus may be part of TORC1, is a recently described inhibitor of mTOR that is capable of inhibiting both TORC1 and TORC2, although the upstream regulators of Deptor remain unknown (Peterson et al., 2009).

TORC2 composition

Owing to the specific inhibition of TORC1 by rapamycin, we know vastly more about the function of TORC1 than we do about TORC2. However, the recently developed TOR active site inhibitors that are capable of inhibiting TORC2 and TORC1 will hopefully enable further characterization of TORC2. TORC2 shares some common subunits with TORC1, including mTOR, Deptor and mLST8 (Fig. 2B). However, several complex members that are unique to TORC2 have been described, including rapamycin-insensitive companion of mTOR (Rictor), stress-activated protein kinase-interacting protein 1 (Sin1) and protein-binding Rictor (protor) (Jacinto et al., 2004; Sarbassov et al., 2004; Yang et al., 2006a; Pearce et al., 2007; Peterson et al., 2009) (Fig. 2B). The binding of Rictor and raptor to mTOR is mutually exclusive. Rictor contains domains that are conserved within

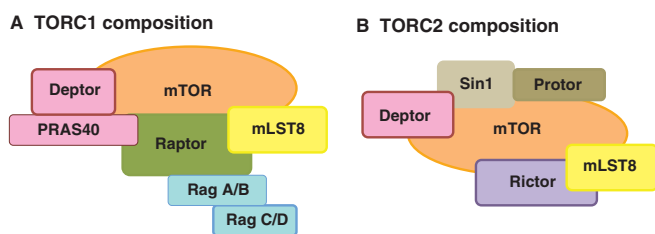


Fig. 2. Composition of TORC1 and TORC2. (A) Target of rapamycin complex 1 (TORC1) (shown as a monomer) consists of mammalian TOR (mTOR), regulatory associated protein of mTOR (raptor), proline-rich AKT substrate 40 KDa (PRAS40), mammalian lethal with Sec-13 protein 8 (mLST8) and DEP domain TOR-binding protein (Deptor). Raptor interacts with some substrates and promotes dimerization of TORC1 complexes by direct interaction with TOR subunits from each monomer. PRAS40 binding to TORC1 is inhibitory and may be mediated by direct interaction with either mTOR or raptor. mLST8, via its multiple WD40 repeats, binds to the kinase domain of mTOR. Deptor binds the FAT (FRAP, ATM and TTRAP) domain of TOR and is capable of inhibiting both TORC1 and TORC2. Rag A/B and Rag C/D bind TORC1 through direct interaction with raptor. (B) TORC2 complex members include mTOR, rapamycin-insensitive companion of mTOR (Rictor), stress-activated protein kinase-interacting protein 1 (Sin1), mLST8, Deptor and protein-binding Rictor (protor). Rictor contains conserved domains that are hypothesized to be important for TORC2 complex formation and substrate recruitment. Sin1 has been described to promote Rictor-mTOR binding and regulate substrate specificity. Protor binds Rictor, although its function is currently unclear. mLST8 also binds mTOR and may be required for TORC2 function in vivo based on knockout data.

eukaryotes and have been postulated to be important for TORC2 complex formation and substrate recruitment, although these functions remain to be verified (Sarbasov et al., 2004). Protor binds TORC2 through Rictor, and protor stability is dependent on the expression of other TORC2 components, although protor has been shown not to be required for TORC2 complex assembly or for catalytic activity (Pearce et al., 2007). Additional studies are needed to uncover the molecular function of protor within the TORC2 complex. Sin1 has been described to promote Rictor-mTOR binding and to regulate substrate specificity (Jacinto et al., 2006); thus, it is thought to be required for TORC2 but not for TORC1 function.

Upstream regulation of TOR signaling

To date, the majority of studies on the signaling events upstream of mTOR have been focused on TORC1 rather than on TORC2. This analysis of TORC1 regulation in vertebrates and invertebrates has provided us with clues into the regulation of TOR signaling and into the mechanisms by which the TOR pathway correctly balances cellular energy consumption (translation and ribosome biogenesis) and production (autophagic production of metabolites). TORC1 is regulated by many extracellular and intracellular cues. Upstream regulators include growth factors, nutrients, energy, oxygen and stress. Importantly, perturbations in the regulation of TORC1 kinase have been implicated in the pathogenesis of diabetes and cancer. Many of the upstream signals regulating TOR that have been discovered to date, with the exception of growth factors, are selective for TORC1 and do not affect TORC2; undoubtedly, key upstream activators of TORC2 must exist, although they remain to be characterized.

TSC1/TSC2 and Rheb

The identification of the tumor suppressors tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2) as upstream regulators of mTORC1 kinase brought mTOR firmly into the field of cancer biology. Mutations in either *TSC1* or *TSC2* result in TSC disease, which is typified by the development of benign tumors or hamartomas in multiple tissue types and the development of neurological problems. Very few genes are capable of causing increases in cell volume; therefore, the discovery of *Drosophila* tuberous sclerosis complex (dTSC) as a regulator of cell size quickly led researchers to make the link to TOR signaling (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). A rapid succession of papers described TSC1 and TSC2 as potent repressors of TORC1 kinase activity in both *Drosophila* and mammals (Fig. 3) (Oldham et al., 2000; Gao et al., 2002; Goncharova et al., 2002; Inoki et al., 2002).

The TSC1/2 complex acts as a GTPase activating protein (GAP), with TSC1 acting to stabilize the complex and TSC2 containing a C-terminal GAP domain. The major target downstream of TSC1/2 is the small GTPase Rheb, which is targeted from its GTP-bound (active) form to the GDP-bound (inactive) form by the GAP activity of TSC1/2 (Garami et al., 2003; Inoki et al., 2003a; Tee et al., 2003). Under growth conditions, the TSC1/2 complex is inactive, thereby allowing Rheb-GTP to activate TORC1. Rheb overexpression is capable of increasing TORC1 target phosphorylation, which can be reversed by mTOR inactivation or by rapamycin treatment, suggesting that Rheb primarily functions through TORC1 (Yang et al., 2006b).

PI3K-AKT

The PI3K-AKT pathway, which lies downstream of insulin and growth factor signaling, exerts multi-faceted control over TORC1 activity. The most prominent effector of PI3K activity is AKT [alternately referred to as protein kinase B (PKB)]. The phosphatase and tensin homolog (PTEN) protein acts as a phosphatase for the phospholipid phosphatidylinositol-3,4,5-trisphosphate [PtdIns(4,5) P_2 or PIP $_3$], a key upstream activator of AKT signaling (Fig. 3). Activated AKT promotes TORC1 signaling by phosphorylating multiple sites on TSC2, thereby relieving inhibition of Rheb and activating TORC1 (Inoki et al., 2002; Potter et al., 2002; Miron et al., 2003). AKT is also capable of activating TORC1 through the direct phosphorylation of PRAS40, resulting in a reduced ability of PRAS40 to inhibit TORC1 (Kovacina et al., 2003; Sancak et al., 2007; Vander Haar et al., 2007). The inhibition of PRAS40 by AKT is conserved; in *Drosophila*, the PRAS40 ortholog Lobe regulates TORC1 signaling (Wang and Huang, 2009). Taken together, these findings highlight AKT as a potent upstream activator of TORC1 activity and provide a mechanistic understanding of the elevated TORC1 activity levels that are observed in the majority of cancers in which PI3K-AKT signaling is elevated.

Amino acids

Mammalian cells convey cues from extracellular amino acids through the actions of amino acid transporters. Branched-chain amino acids (such as leucine) are crucial for TORC1 activity and are internalized in conjunction with the system L amino acid transporter (Hara et al., 1998; Christie et al., 2002). Surprisingly, the sensitivity of TORC1 to amino acids persists in TSC-null cells (Smith et al., 2005). Proteins with a purported role in TORC1 nutrient sensitivity are Ste20-related kinase MAP4K3 (mitogen-activated protein kinase kinase kinase kinase 3) and the VPS34

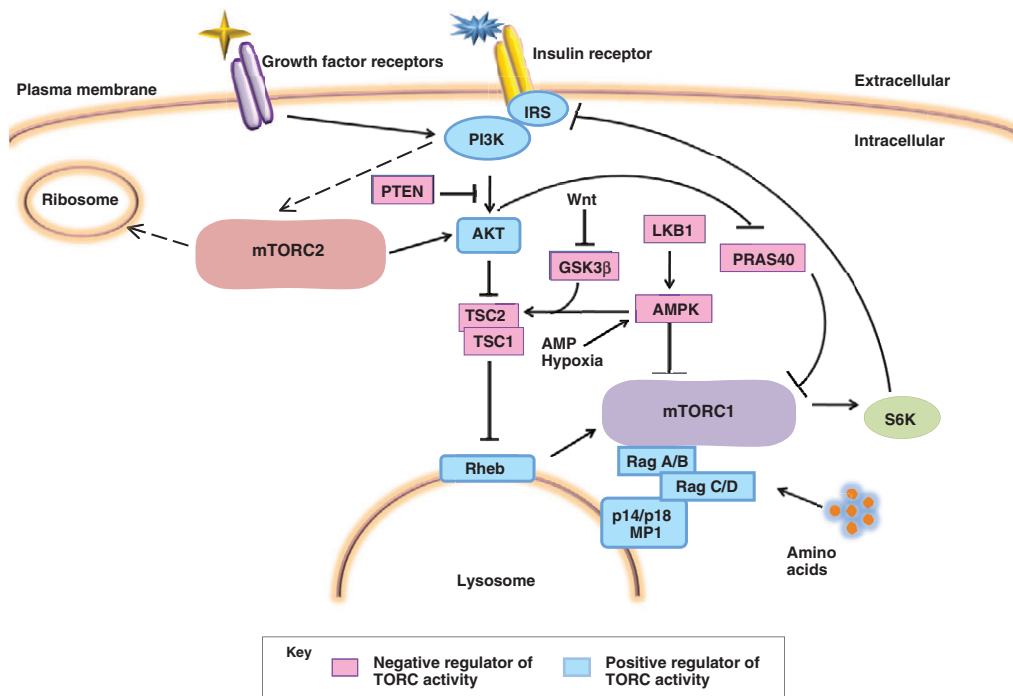


Fig. 3. Upstream regulation of mTORC signaling. Mammalian target of rapamycin complex (mTORC) activity is influenced by a number of positive (shown in blue) and negative (shown in pink) regulators. mTORC1 is activated by growth factors and insulin signaling. This activation is mediated by phosphatidylinositol 3-kinase (PI3K) and AKT (a serine/threonine protein kinase), which inhibit the tuberous sclerosis complex (TSC) 1/2 complex, thereby relieving the TSC1/2-mediated repression of Ras homolog enriched in brain (Rheb) and allowing activation of TORC1. By contrast, low cellular energy levels (conveyed by AMP) and hypoxia activate AMP kinase, which represses mTORC1 both through direct phosphorylation of TSC2 and through regulatory associated protein of mTOR (raptor) inhibition. The TSC1/2 complex thus acts to integrate intracellular cues and extracellular demands, and under unfavorable cellular conditions represses the activity of mTORC1. Intracellularly, mTORC1 can also be activated by amino acids at the lysosomal membrane, which act via the Rag proteins and the Ragulator complex (which consists of p14, p18 and MP1). mTORC2 can also be activated by growth factors through PI3K and through undetermined effectors (indicated by the broken arrow). In turn, mTORC2 can regulate AKT, thereby placing it upstream of TORC1. mTORC2 can also be activated through its association with ribosomes. Abbreviations: GSK3 β , glycogen synthase 3 β ; IRS, insulin receptor substrate; LKB1, liver kinase B1; MP1, MEK partner 1; PRAS40, proline-rich AKT substrate 40 kDa; PTEN, phosphatase and tensin homolog protein; S6K, ribosomal S6 kinase.

kinase (phosphatidylinositol 3-kinase) (Byfield et al., 2005; Nobukuni et al., 2005; Findlay et al., 2007; Juhasz et al., 2008; Yan et al., 2010). However, additional studies are needed to clarify the roles of these proteins in TORC1 activation.

A recent series of studies reporting the discovery of the nutrient-dependent Rag GTPase pathway has offered us the strongest link between amino acids and TORC1 stimulation. Rag family members (Rag A-D) are part of the Ras family of GTPases. They are unique in their ability to dimerize through long C-terminal extensions. In the presence of amino acids, the dimeric Rag complex, which consists of a Rag A/B monomer and a Rag C/D monomer, binds to TORC1 (Kim et al., 2008; Sancak et al., 2008; Sancak et al., 2010). Rag-mediated activation of TORC1 still requires Rheb, indicating that, during amino acid signaling, Rag complexes act upstream of Rheb. Amino acid-mediated activation of TORC1 also depends on the correct subcellular localization of both the Rag activators and the TORC1 components to the lysosomal membrane. Rag complexes are recruited to the lysosomal membrane by the trimeric Ragulator complex (Sancak et al., 2010), which contains the proteins MP1 (MEK partner 1), p14 and p18. The GTP-loading of Rag A/B appears to be regulated by amino acids, and binding to TORC1 is observed most robustly under nutrient-rich conditions – when Rag A/B is in the GTP-bound state and Rag

C/D is in the GDP-bound state (Kim et al., 2008; Sancak et al., 2008). However, there are many key aspects of TORC1 regulation by amino acids that remain to be discovered, such as how branched amino acids are detected by Rag GTPases and the identity of the Rag guanine exchange factor (GEF).

Cellular energy levels

A low cellular ATP level is a potent signal to curtail cell growth. This inhibitory signal is transduced to the TOR pathway by AMP-activated protein kinase (AMPK) (Inoki et al., 2003b). As cellular stores of ATP are depleted, AMP levels rise and activate AMPK, which phosphorylates a wide array of proteins within the cell. These proteins, in turn, act collectively to halt energy-consuming processes, such as ribosome biogenesis and translation, in order to restore energy homeostasis (Hardie, 2007). Within the TOR pathway, AMPK phosphorylates TSC2, thereby enhancing inhibition of Rheb/TORC1 and directly phosphorylating raptor to inhibit substrate phosphorylation (Inoki et al., 2003b; Gwinn et al., 2008). AMPK phosphorylation of TSC2 has also been reported to act as a primer for the phosphorylation and enhancement of TSC2 function by glycogen synthase 3 β (GSK3 β), a serine/threonine kinase reported to phosphorylate the TSC1/2 complex directly (Inoki et al., 2006). Wnt signaling upstream of GSK3 β relieves the inhibition of the TORC1 kinase by TSC1/2 (Inoki et al., 2006).

Activation of AMPK requires activation loop phosphorylation by upstream kinases, including LKB1 (liver kinase B1) (Lizcano et al., 2004). LKB1 is a tumor suppressor, the inactivation of which results in a net activation of TORC1 activity via reduced AMPK-mediated suppression (Shaw et al., 2004).

Oxygen sensing and hypoxia

Limited intracellular oxygen (hypoxia) has been described as a negative regulator of TORC1 activity that can act via multiple proposed mechanisms (for a review, see Wouters and Koritzinsky, 2008). TSC1/2-mediated suppression of TORC1, for example, can be potentiated by hypoxia in one of two ways. First, activation of AMPK by hypoxia can enhance TSC complex function, as described above (Liu et al., 2006). Second, the upregulation of regulated in development and DNA damage responses (REDD1) transcription by hypoxia can activate the TSC1/2 complex by disrupting the inhibitory interactions between TSC2 and 14-3-3 proteins that are observed under nutrient and growth factor sufficiency (Brugarolas et al., 2004; Reiling and Hafen, 2004; DeYoung et al., 2008). Downstream of TSC1/2, the hypoxia-inducible BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) binds to and partially inhibits Rheb function under hypoxic conditions (Li et al., 2007). Finally, the pro-myelocytic leukemia tumor suppressor (PML) protein has been described to bind and inactivate mTOR by sequestering TORC1 in nuclear bodies (Bernardi et al., 2006). The above pathways ensure that limited oxygen levels can curtail growth signals through TORC1, even in the presence of nutrient and growth factors.

Regulation of TORC2

Very little is known about the upstream regulation of TORC2. Like TORC1, TORC2 can be stimulated by growth factors and PI3K (Jacinto et al., 2004). Treatment with PI3K inhibitors can inhibit TORC2-mediated target phosphorylation (Sarbasov et al., 2005). However, there is a lack of mechanistic information to explain how TORC2 receives this signaling information. The best-characterized target of TORC2 is AKT, which is phosphorylated at S473 upon TORC2 activation (Sarbasov et al., 2005). Phosphorylation of AKT on S473 enhances the activation phosphorylation motif at T308, which is absolutely required for AKT activity (Alessi et al., 1997). Interestingly, TORC2 has also been shown to phosphorylate AKT, SGK and PKC α (Garcia-Martinez and Alessi, 2008; Yan et al., 2008; Jones et al., 2009; Soukas et al., 2009). Additional studies are needed to determine whether all these proteins represent direct targets of the TORC2 kinase, and to elucidate their physiological function in TORC2 signaling. Interestingly, the TORC2-mediated activation of AKT places TORC2 upstream of TORC1 in the TOR signaling cascade (Fig. 3). A recent publication has highlighted a role for ribosomes in the activation of TORC2 (Zinzalla et al., 2011). Interaction of TORC2 with NIP7 (nuclear import 7, a protein responsible for ribosome biogenesis and rRNA maturation) was shown to be required for full activation of TORC2 by insulin. This observation raises many interesting questions regarding the regulation of TORC2 and its ribosomal interactions, but it also indicates that additional levels of interplay between TORC2 and TORC1 may exist, as both complexes are linked to the process of ribosome biogenesis. TORC2 is also capable of regulating actin cytoskeletal organization through Rho GTPases, a function conserved with yeast TORC2 (Jacinto et al., 2004). Additionally, TORC2 has been reported to regulate directly or indirectly SGK and PKC α , although much remains to be discovered regarding this aspect of TORC2 activity and regulation.

Downstream effects of TOR signaling

TORC1 has been described to mediate the majority of biological functions attributed to TOR kinase. The primary function of TORC1 activity is to transmit information from insulin, growth factors, energy, amino acids and stress to activate translation and inhibit autophagy (Fig. 4). Inhibition of TORC1 activity thus results in a decrease in cell size and a reduction in proliferation; these phenotypes are most striking in yeast, but are also observed, albeit more subtly, in mammalian models. As discussed above, the major functions of TORC2 include the regulation of cytoskeletal organization and the promotion of cell survival.

TORC1 targets the translation machinery

The collective input of nutrients and growth factors are conveyed through TORC1 to the translation machinery through the two most extensively studied targets of TORC1, eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) and ribosomal S6 kinase (S6K) (Fig. 4). 4E-BP and S6K act on the regulatory complexes bound to the untranslated regions of mRNAs (for a review, see Ma and Blenis, 2009). Under nutrient-poor conditions, 4E-BP negatively regulates the assembly of initiation factors by binding and sequestering eIF4E, thereby hindering the ability of eIF4E to recruit the translation initiation complex to the 5' mRNA cap structure (Holz et al., 2005; Ma and Blenis, 2009). Stimulation of TORC1 by growth factors or nutrients results in phosphorylation of 4E-BP and the release of eIF4E to promote translation (Fingar et al., 2002). Activation of TORC1 also promotes initiation of translation through phosphorylation of S6K (Fingar et al., 2002). S6K phosphorylates multiple substrates involved in translation initiation, including the regulatory subunit (eIF4B) of RNA helicase (eIF4A), resulting in progression of the small ribosomal subunit towards the start codon (Fig. 4) (Holz et al., 2005; Ma and Blenis, 2009). S6K is also involved in a negative-feedback loop that acts to curtail TORC1 activity (Fig. 3); the phosphorylation and inactivation of insulin receptor substrate 1 (IRS1) by S6K results in a decrease in PI3K activity and therefore reduces AKT activation and hence TORC1 signaling (Harrington et al., 2004; Shah et al., 2004). Additionally, TORC1 has been shown to regulate ribosome biogenesis and tRNA production. Ribosome biogenesis is regulated by TORC1 via increased translation of ribosomal protein transcripts and via altered rRNA and tRNA synthesis (Mahajan, 1994; Zaragoza et al., 1998; Cardenas et al., 1999; Powers and Walter, 1999; Hannan et al., 2003; Kantidakis et al., 2010). TORC1-dependent phosphorylation of the key RNA polymerase regulatory subunit TIFIA regulates the production of RNA gene transcripts in a nutrient-sensitive manner (Mayer et al., 2004).

TORC1-mediated repression of autophagy

TORC1 is a potent inhibitor of autophagy, a lysosomal-dependent cellular degradation process that generates nutrients and energy to maintain essential cellular activities upon nutrient starvation. The ability of TORC1 to regulate autophagy is as highly conserved as the process of autophagy itself. Accumulating evidence suggests that ULK1 (Unc-51-like kinase 1), the mammalian homolog of the yeast protein kinase autophagy-specific gene 1 (ATG1), is a key regulator of autophagy initiation. ULK1 is directly phosphorylated by the energy-sensing kinase TORC1 (Chang and Neufeld, 2009; Jung et al., 2009; Kamada et al., 2010). Recently, it has also been shown that TORC1-mediated phosphorylation of ULK1 impairs its activation following energy depletion and results in an overall

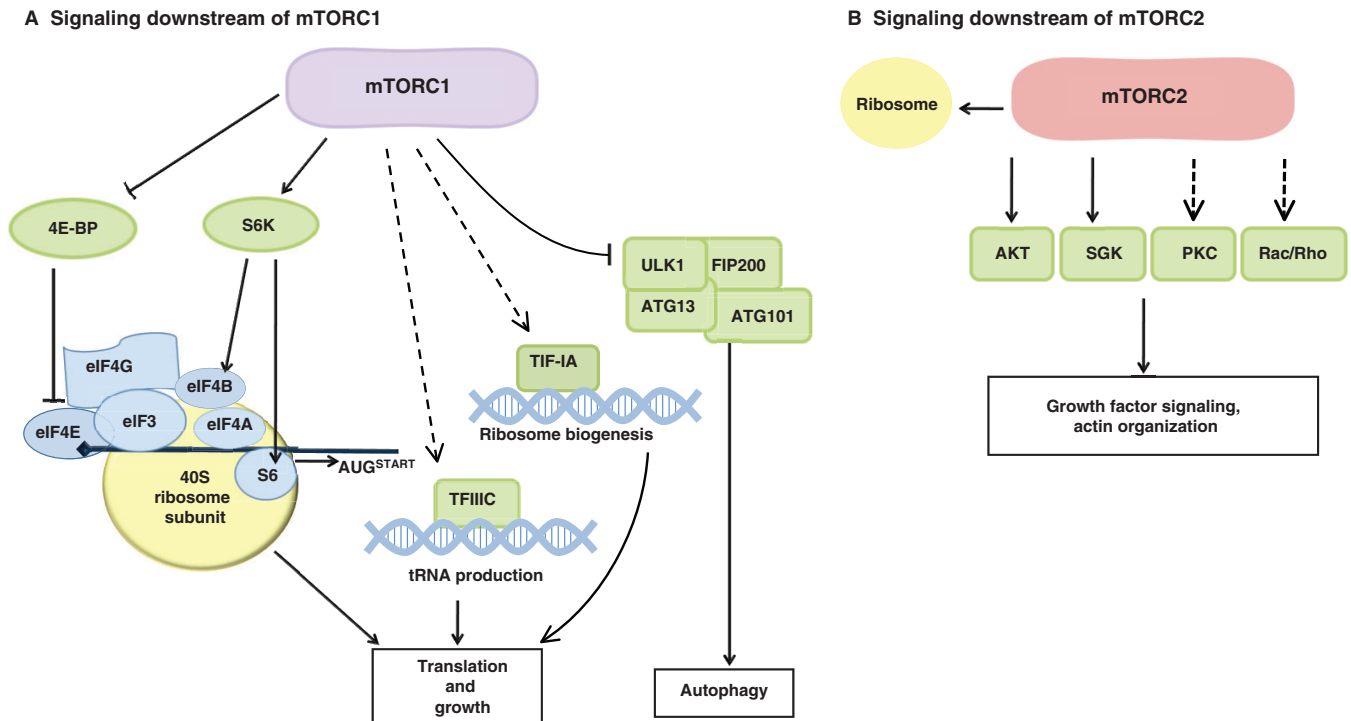


Fig. 4. Downstream effectors of mTORC signaling. (A) Mammalian target of rapamycin complex 1 (mTORC1) phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) disrupts the interaction of 4E-BP with eIF4E, which leaves eIF4A free to promote the binding of ribosomes to the transcriptional start site. mTORC1 also activates ribosomal S6 kinase (S6K), which activates the translational initiation factor eIF4B and the S6 ribosomal protein by direct phosphorylation. mTORC1 can also associate with general transcription factor III C (TFIIC) and relieve its inhibitor Maf1, leading to increased tRNA production. mTORC1 activity also promotes association between transcription initiation factor 1A (TIF-1A) and polymerase I (PolI), thereby promoting rRNA synthesis. Finally, mTORC1 signaling can inhibit autophagy through phosphorylation of Unc-51-like kinase 1 (ULK1) and autophagy related 13 homolog (ATG13). (B) mTORC2 phosphorylates and activates AKT (a serine/threonine protein kinase) and serum/glucocorticoid regulated kinase (SGK), and has been implicated in signaling via protein kinase C (PKC) and Rac/Rho. Collectively, these mTORC2-mediated activities promote growth factor signaling and cytoskeletal reorganization. In response to insulin signaling, mTORC2 can also interact with ribosomes. Abbreviation: ATG101, autophagy-related protein 101; FIP200, FAK family interacting protein of 200 kDa.

decrease in autophagy (Kim et al., 2011). Phosphorylation of ULK1 by mTORC1 disrupts its interaction with, and hence activation by, AMPK (Egan et al., 2011; Kim et al., 2011).

Cell growth and division

Cells of multicellular organisms must balance growth and division with both intracellular nutrient availability and growth cues from energy-sensing organs. TORC1 is a recognized central regulator of growth and division that is capable of correctly integrating these diverse inputs. These *in vivo* functions of TORC1 during cell growth and division are best described in *Drosophila*. Biologically, disruption of *Drosophila* TORC1 activity mimics the reduction of cell size and proliferation induced by nutrient deprivation (Zhang et al., 2000). Deletion of *Drosophila* TOR, or the *Drosophila* TORC1 effectors S6K and 4EB-P1, results in a decrease in cell size, linking increases in translation to overall growth (Oldham et al., 2000; Zhang et al., 2000; Radimerski et al., 2002a; Radimerski et al., 2002b). The ability of TORC1 to regulate cell size in response to extrinsic factors has also been eloquently shown in *Drosophila*. Conditional deletion of the gene encoding the amino acid transporter slimfast in the fat body (see Glossary, Box 1) of *Drosophila* results in a global reduction of cell and organism size, and the starvation response in distal tissues was shown to be mediated by TORC1 (Colombani et al., 2003).

TOR signaling also elicits effects on cell division via regulation of the cell cycle. DNA replication begins in the G1 stage of the cell cycle, and the initiation of DNA replication is sensitive to cell size. Deletion of TOR1 and TOR2 in yeast results in a rapid halt in the cell cycle at G1, mimicking rapamycin treatment (Heitman et al., 1991; Kunz et al., 1993). Inhibition of TORC1 activity is less potent in halting the cell cycle in mammalian cells, and only some types of cells arrest in G1 upon rapamycin treatment (Heitman et al., 1991; Chung et al., 1992; Zhang et al., 2000; Patel et al., 2003). Interestingly, TOR has also been implicated in cell cycle entry following quiescence (G0 to G1). In *Drosophila*, neuroblasts (see Glossary, Box 1) enter a programmed quiescence, which is exited upon nutritional re-activation (Sousa-Nunes et al., 2011). Entry into the cell cycle from senescence requires signaling from the fat body where slimfast and TOR signal to the central nervous system in the presence of amino acids. This signal activates a PI3K- and TOR-dependent signal that promotes cell cycle entry (Sousa-Nunes et al., 2011). The precise mechanism by which mTOR controls the cell cycle remains elusive, and the differences observed in rapamycin potency between yeast and mammalian cells are enigmatic. However, the effects of rapamycin treatment have been attributed to the TORC1 translational effectors S6K and 4E-BP; thus, the identification of the pathways controlling the cell cycle downstream of S6K and 4E-BP will greatly add to our understanding of this crucial function of TOR (Fingar et al., 2002).

Box 1. Glossary

Cortical tubers. A lesion of benign tissue growth in the brain.

Epiblast. One of the layers of cells in the early embryo, which gives rise to all three definitive germ layers of the embryo.

Ethylnitrosourea. A potent mutagen, able to induce point mutations.

Fat body. Secretory organ of adipose tissue distributed throughout *Drosophila*.

Hypothalamus. Region of the brain that is the main control center for the autonomic nervous system and acts as an endocrine gland.

Inner cell mass. A small clump of apparently undifferentiated cells in the blastocyst, which gives rise to the entire fetus plus some of its extra-embryonic membranes.

Ketone bodies. Products of fatty acid breakdown from liver and kidney that are involved in providing energy to essential organs during starvation.

Neuroblast. Neural progenitor cells that give rise to the central nervous system in metazoans.

Ommatidia. Single light-sensitive structures that make up a compound eye.

Pro-opiomelanocortin neurons. Glucose-excited neurons expressing pro-opiomelanocortin that are involved in appetite suppression.

Pyramidal neurons. Neurons present in forebrain structures, such as the cerebral cortex, hippocampus and amygdala, that act as key excitatory units for the prefrontal cortex.

Subependymal nodules. A benign growth in the ependymal lining of the lateral ventricle of the brain.

Telencephalon. The anterior region of the forebrain, including the cerebrum and related structures.

Trophoblast giant cells. The first cells of the embryo to terminally differentiate, these cells form part of the placenta and are essential for establishing a link between the embryo and the maternal blood supply.

Yolk sac. A nutrient-containing membrane sac attached to the embryo before the development of the placenta.

TOR signaling during development

mTOR signaling is essential in early development. As such, disruption of mTOR in mice is embryonically lethal (summarized in Table 1) and early studies of mTOR knockout mice yielded scant clues in to the *in vivo* functions of mTORC1 and mTORC2. More recently, however, conditional knockouts (summarized in Table 2) using essential subunits of either TORC1 or TORC2 have generated interesting insights and questions into the role of mTOR signaling in areas such as homeostatic regulation, neurogenesis and stem cell biology.

Insights from mTOR pathway knockouts

As discussed above, rapid advances have been made in our understanding of the mTOR pathway *in vitro*; however, the *in vivo* functions of TORC kinases have been more difficult to ascertain. The first mouse model with altered mTOR was the flat-top mutant, which was viable to E12.5 and so named because of its altered head morphology resulting from a loss of expansion of the telencephalon (see Glossary, Box 1) in the anterior forebrain (Hentges et al., 2001). Generated by random mutagenesis using ethylnitrosourea (see Glossary, Box 1), flat-top mutants harbor a three amino acid insertion in mTOR that interrupts mRNA splicing and results in a 95% reduction in proper transcript handling. Aside from forebrain defects, flat-top mutants failed to turn around the embryonic body axis and exhibited abnormal limb buds and ventral body walls. Flat-top mutants also exhibited defects in TORC1

target gene phosphorylation, and they phenocopied the developmental delays caused by rapamycin treatment of pregnant females (Hentges et al., 2001). Together, these results indicated that mTOR is dispensable for peri-implantation development but is required for times of rapid cell expansion. These data come with the caveat that the flat-top mouse is not a complete knockout, with biochemical data indicating that, in the flat top background, the TORC1 target S6K can retain up to 17% activity of that seen in wild-type mice (Hentges et al., 2001). As such, the more recent complete knockout of *Mtor* by two independent groups tells a different story. In these knockouts, *Mtor* deletion was shown to be required for embryonic gastrulation, and embryos died shortly after implantation at embryonic day (E) 5.5-E6.5 (Gangloff et al., 2004; Murakami et al., 2004). Explanted blastocysts from these *Mtor* knockout mice appear normal; however, the inner cell mass (ICM, see Glossary, Box 1) and trophoblast giant cells (TGCs, see Glossary, Box 1) failed to proliferate *ex vivo*. The discrepancy between these knockout models and the flat-top mutant are probably the result of the remaining 5% of mTOR wild-type transcript in the flat-top mouse. Complete *Mtor* deletion also results in a phenotype that is more severe than that produced by rapamycin treatment of pregnant females. This could be due to either incomplete inhibition by rapamycin or to the effect of a TORC1-dependent rapamycin-insensitive process. The latter is likely to be true, as proliferation of the ICM is unaffected by rapamycin treatment. Although mammalian TORC2 had not been defined during these studies, TORC2 does not account for these differences in embryogenesis (as discussed below).

In order to dissect the *in vivo* roles of mTORC1 and mTORC2, mice with deletions of *Rptor*, *Rictor* and *Mlst8* were generated (Guertin et al., 2006b; Shiota et al., 2006). Similar to *Mtor* deletion, *Rptor* deletion results in death shortly after implantation (Guertin et al., 2006b). Explanted *Rptor*-null blastocysts were defective in proliferation of the ICM and TGC, phenocopying the deletion of *Mtor*. These data indicate that TORC1 activity is required for the proliferation of cells in early embryonic development and that raptor is indispensable for TORC1 activity *in vivo*. Surprisingly, disruption of the TORC1 component mLST8 exhibited a very different phenotype: *Mlst8* deletion results in embryonic lethality at E10.5 and developing *Mlst8*-null embryos exhibit a thick and disorganized TGC labyrinth (Guertin et al., 2006b). *Mlst8* mutants also exhibit a profound defect in the yolk sac (see Glossary, Box 1) vasculature. These results clearly indicate that mLST8 is dispensable for TORC1 activity *in vivo*, at least in the early stages of embryogenesis. Interestingly, the deletion of *Rictor*, which is specific for TORC2, results in a phenotype very similar to that of *Mlst8* knockout mice, and embryos are viable until E10.5 (Guertin et al., 2006b; Shiota et al., 2006). Specific deletion of *Rictor* in the epiblast (see Glossary, Box 1) extended viability, but viable *Rictor*-null offspring could not be produced by this approach (Shiota et al., 2006). The high expression of *Rictor* in the embryo and the early time point of lethality indicate that Rictor is required for early embryogenesis and that placental defects contributed only partially to the lethality of *Rictor*-null, and presumably *Mlst8*-null, embryos. Moreover, the observation that knockouts of *Mlst8* phenocopy *Rictor* knockouts, provides *in vivo* evidence that mLST8 is a required component of TORC2.

The *in vivo* role of S6K, one of the major downstream effectors of TORC1 activity, has also been analyzed through the creation of a S6K1/2 (*Rps6kb1/Rps6kb2*) double knockout. Deletion of both S6K isoforms resulted in a reduction in viability; however, the defects observed in *Rptor* or *Mtor* knockout embryos indicate that

Table 1. Summary of mouse knockout studies of target of rapamycin pathway components

Target	Gene deleted/perturbed or genotype	Reported phenotype(s)	References
Whole organism	<i>Mtor</i> (mutagenesis, reduced activity)	Embryonic lethal at E12.5; defects in forebrain expansion	(Hentges et al., 2001)
	<i>Mtor</i> ^{-/-}	Embryonic lethal E5.5; explanted blastocysts fail to expand	(Gangloff et al., 2004; Murakami et al., 2004)
	<i>Rptor</i> ^{-/-}	Embryonic lethal at E5.5-6.5; explanted blastocysts fail to expand	(Guertin et al., 2006b)
	<i>Rictor</i> ^{-/-}	Embryonic lethal E10.5; vascular and placental labyrinth defects	(Guertin et al., 2006b; Shiota et al., 2006)
	<i>Mlst8</i> ^{-/-}	Embryonic lethal at E10.5; vascular and placental labyrinth defects	(Guertin et al., 2006b)
	<i>Rps6kb1</i> ^{-/-} , <i>Rps6kb2</i> ^{-/-}	Partial perinatal lethality	(Pende et al., 2004)
	<i>Eif4ebp1</i> ^{-/-} , <i>Eif4ebp2</i> ^{-/-}	Insulin resistance	(Le Bacquer et al., 2007)
Adipose	<i>Rptor</i> ^{-/-}	Reduced weight; protection from insulin resistance	(Polak et al., 2008)
	<i>Rictor</i> ^{-/-}	Increases in organ weight with cellular changes in glucose uptake and insulin-mediated AKT activation	(Cybulski et al., 2009; Kumar et al., 2010)
Muscle	<i>Mtor</i> ^{-/-}	Premature death, altered metabolism and reduced dystrophin levels in muscle	(Risson et al., 2009)
	<i>Rptor</i> ^{-/-}	Premature death, altered metabolism and reduced dystrophin levels in muscle	(Bentzinger et al., 2008)
	<i>Rictor</i> ^{-/-}	Normal; slight weight gain	(Bentzinger et al., 2008)
Neuronal	<i>Rictor</i> ^{-/-}	Schizophrenic-like behavior	(Siuta et al., 2010)
Hypothalamus	<i>Tsc1</i> ^{-/-}	Neuronal projection defects; hyperphagic obesity	(Mori et al., 2009)
Proopiomelanocortin	<i>Tsc1</i> ^{-/-}	Hyperphagic obesity	(Mori et al., 2009)
Liver	<i>Tsc1</i> ^{-/-}	Defects in fasting-induced ketone body production and activation of <i>Ppara</i>	(Sengupta et al., 2010)

S6K is not required for the proliferation induced by TORC1 in early embryogenesis (Pende et al., 2004). In support of this, cultured S6K-null MEFs proliferate and still respond to rapamycin, indicating that S6K-independent pathways are capable of executing TORC1 function in vivo (Pende et al., 2004). Knockouts of 4E-BP, another well-studied effector of TORC1 signaling, have also been generated. Three isoforms of 4E-BP exist in mammals. Deletions of 4E-BP1 and 4E-BP2 (*Eif4ebp1* and *Eif4ebp2*) result in the development of viable fertile offspring; however, these offspring exhibit insulin resistance and sensitivity to diet-induced obesity (Le Bacquer et al., 2007). Interestingly, the growth suppressive effects of TORC1 inhibitors require 4E-BP expression, indicating that TORC1-dependent proliferation in early embryogenesis may require 4E-BP (Dowling et al., 2010). Additional studies will need to be carried out to identify the downstream effectors of TORC1 signaling in embryogenesis.

TORC1 activity in homeostatic tissues

The early embryonic lethality induced by deletion of *Mtor*, *Rictor* or *Rptor* greatly precluded studies of mTOR study in vivo. Interestingly, however, the conditional knockout of *Rptor* in adipose tissue resulted in lean mice that were protected against diet-induced obesity – almost the polar opposite of the *Eif4ebp1/Eif4ebp2* double knockout mice (Polak et al., 2008). Therefore, it was suggested that TORC1 activity in adipose tissue regulates lipid metabolism and metabolic homeostasis at the organismal level. This role extends beyond adipose tissue, with *Tsc1* deletion in the hypothalamus (see Glossary, Box 1) resulting in an elevation in mTOR activity and increased weight gain due to hyperphagia, which highlights a role for mTOR in

the hypothalamus in appetite regulation (Mori et al., 2009). Additionally, the deletion of *Tsc1* in the liver resulted in reduced fasting-induced ketogenesis, a process that is used to distribute energy via the liver-specific production of ketone bodies (see Glossary, Box 1). *Rptor* liver-specific knockouts were also generated and exhibited an inverted phenotype to the *Tsc1* knockout (Sengupta et al., 2010). The production of ketone bodies is impaired in the aging liver and correlates with increased mTOR signaling. These effects are not irreversible, as mTORC1 inhibition resulted in the restoration of ketone production in aged mice.

These studies show that TORC1 signaling is essential in adipose tissue, the hypothalamus and the liver, tissues that are all involved in striking a balance between energy use and energy storage throughout the organism. Recent work has begun to uncover exciting links between mTORC1 and ageing (see Box 2), and continuation of this line of research will undoubtedly yield new in vivo functions of mTORC1 that will hopefully further our understanding of mTOR function beyond the cellular level. Intriguingly, recent studies (Cybulski et al., 2009; Kumar et al., 2010) have shown that adipose-specific *Rictor* deletion results in defective activation of AKT by insulin and an altered uptake in glucose (Kumar et al., 2010). Interestingly, deletion of *Rictor* in the adipose also results in an increase in the size of non-adipose organs, providing evidence for an unexpected role for TORC2 in whole organism energy sensing. Furthermore, single deletion of *Mtor* or *Rptor*, but not *Rictor*, in the muscle results in dystrophy (Bentzinger et al., 2008; Risson et al., 2009). This is probably due to an altered metabolic program in which oxidative metabolism, mitochondrial biogenesis and AKT signaling are dysregulated in

Table 2. Summary of studies perturbing TOR signaling in stem cell models

Stem cell background	TOR manipulation	Reported observation	References
Murine embryonic stem cells	mTOR inactivation	Loss of proliferation	(Murakami et al., 2004)
	Rapamycin	Slight reduction in size, normal proliferation	(Murakami et al., 2004)
Murine hematopoietic stem cells	Rapamycin	Increased differentiation	(Schieke et al., 2008)
	TSC1 deletion	TORC1-dependent depletion of stem cell pool, TORC1-independent defect in stem cell mobilization, increase in proliferation	(Gan et al., 2008; Chen et al., 2009; Chen, C. et al., 2008)
	PTEN inactivation; rapamycin rescue	TORC1 activation decreases stem cell pool	(Yilmaz et al., 2006)
	AKT activation; rapamycin rescue	TORC1 activation increases proliferation, depletes stem cell pool	(Kharas et al., 2010)
<i>Drosophila</i> germline stem cells	Rapamycin	Maintenance of stemness in presence of reactive oxygen species	(Jang and Sharkis, 2007)
	TSC null allele	Precocious differentiation; depletion of stem cell pool	(Sun et al., 2010)
Human embryonic stem cells	TOR loss-of-function mutation	Loss of maintenance, G2 arrest	(LaFever et al., 2010)
	Rapamycin	Induction of osteoblastic differentiation	(Lee et al., 2010)
	Rapamycin	Loss of stemness	(Zhou et al., 2009)

Abbreviations: AKT, a serine/threonine protein kinase; PTEN, mTOR, mammalian target of rapamycin; TORC, target of rapamycin complex; TSC, tuberous sclerosis complex.

these muscle cells. Together, these studies paint a picture of mTOR as a crucial regulator of metabolic homeostasis at the level of both individual tissues and the whole organism.

TORC activity in neuronal tissues

The early development of neural tissue depends on intrinsic cellular programs and input from extracellular cues. Individuals with TSC disease, which arises from mutations in either *TSC1* or *TSC2*, exhibit several neurological architectural abnormalities, including an abundance of astrocytes, abnormal cell morphology, as well as three classic lesions: giant cell astrocytomas, subependymal nodules and cortical tubers (see Glossary, Box 1) (Curatolo et al., 1991). Given the severe neural pathologies associated with TSC disease, perhaps it is not surprising that the AKT-PI3K-TOR pathway is emerging as a crucial regulatory pathway in the establishment and maintenance of correct neural architecture. TORC1 signaling exerts profound changes on the morphology and function of hippocampal neurons in vitro. Inactivation of TSC1 in mouse pro-opiomelanocortin (POMC) neurons (see Glossary, Box 1) results in axon projection defects (Mori et al., 2009). The neuronal defects can be rescued by rapamycin treatment, suggesting a crucial role of mTORC1 in axon path finding. Moreover, inactivation of TSC in mouse or rat pyramidal neurons (see Glossary, Box 1) of the hippocampus results in increased soma size and a reduced number of dendrites and dendritic spines (Tavazoie et al., 2005). Stimulation of the mTOR pathway in hippocampal neurons was shown to be important for the translation of genes downstream of glutamate receptors and for promoting dendritic morphogenesis downstream of RAS (Gong et al., 2006; Kumar et al., 2005). Translational inhibitors are similarly capable of blocking the signaling downstream of glutamate receptors, suggesting that TORC1 probably upregulates the translation of genes essential for neural plasticity. The antidepressant ketamine, a N-methyl-D-aspartic acid (NMDA) receptor antagonist, currently in clinical trials, rapidly activates the TORC1 pathway and is repressed by rapamycin, underscoring a crucial role for TORC1 in regulating appropriate signaling by upstream cellular cues in neuron

function (Li et al., 2010). Post-translational modification of the TORC1 target 4E-BP2 isoform during postnatal development has been shown to alter the signaling kinetics of synaptic signaling (Bidinosti et al., 2010). Several studies have sought to identify which genes mediate these neuronal phenotypes in response to translational control by TORC1 (Schratt et al., 2004; Takei et al., 2004; Gong et al., 2006). However, although many promising candidate genes have been identified (for a review, see Swiech et al., 2008), the crucial mechanisms responsible for the biological effects downstream of TORC1 in the neuron remain unidentified. Using the unique and well-defined system of photoreceptor cell differentiation in *Drosophila*, Bateman and colleagues discovered that mutation of *Drosophila* TSC in undifferentiated photoreceptor cells of the ommatidia (see Glossary, Box 1) results in a profound defect in the timing of differentiation in these cell clusters (Bateman and McNeill, 2004). S6K, but not 4EB-P1, was found to act downstream of TORC1 in this pathway, and the epidermal growth factor receptor (EGFR) was proposed to act downstream of S6K to produce this phenotype (McNeill et al., 2008). It will be interesting to see whether TORC1 signaling has similar effects on the differentiation of other cell types.

Similar to our understanding of TORC2 function in vitro, the role of TORC2 signaling during neural development in vivo remains unclear. Neuronal-specific *Rictor* deletion results in schizophrenic-like behaviors (Siuta et al., 2010). This study indicates a role for TORC2 in the neuron, with it possibly acting through the AKT-dependent regulation of the noradrenaline transporter. However, additional studies in vivo and in vitro will be needed to obtain a clearer picture of TORC2 function.

TORC activity in stem cells

The defective ability of ICMs from *Mtor*-null blastocysts to expand ex vivo suggests that mTOR is crucial for the viability of murine embryonic stem cells (ESCs). Since this initial observation, several studies have highlighted a role for TORC1 activity in regulating stem cell populations. Interestingly, rapamycin has been described to have varying effects on different stem cell populations, raising the issue of how mTOR signaling conveys lineage-specific signals

Box 2. TOR signaling and ageing

A reduction in dietary calories has been shown to prolong lifespan in a variety of model organisms. However, the molecular mechanism(s) underlying this observation are only beginning to be revealed. Recently, it emerged that a reduction in target of rapamycin (TOR) signaling can regulate lifespan in yeast, *C. elegans* and *Drosophila* (Vellai et al., 2003; Kapahi et al., 2004; Kaeberlein et al., 2005). Excitingly, it was then shown that reducing TOR complex 1 (TORC1) activity in mammals can also prolong lifespan (Harrison et al., 2009; Selman et al., 2009). Deletion of S6K1 in mice alters gene expression, mimicking caloric reduction, and results in an overall increase in lifespan and a decrease in age-related pathologies (Selman et al., 2009). The suppression of mammalian TORC1 signaling was also confirmed to affect lifespan by recent studies demonstrating that rapamycin treatment in aged mice increased lifespan and enhanced the function of ageing livers (Harrison et al., 2009; Sengupta et al., 2010). Finally, rapamycin treatment of aged hematopoietic stem cells resulted in an increase in the ability of these stem cells to self-renew, a capability that is slowly lost in ageing stem cells (Chen et al., 2009). The mechanisms responsible for this enhancement in lifespan and tissue function are not clearly outlined. However, the inhibition of translation (Hansen et al., 2007; Pan et al., 2007; Steffen et al., 2008), the promotion of autophagy (Toth et al., 2008) and a reduction in ROS (reactive oxygen species) production, by lowering metabolic demands and clearing out old and damaged mitochondria via autophagy (Artal-Sanz and Tavernarakis, 2009), are all implicated in the regulation of lifespan.

to control differentiation. Although transcriptional profiling of ESCs and their progeny has received extensive attention (Chen, X. et al., 2008; Ng et al., 2008; Chan et al., 2011), the role of translational regulation in stem cell function is only beginning to be examined (Sampath et al., 2008). In particular, the ability of TORC1 signaling to influence stem cell function has been tested in several systems using the readily available inhibitor rapamycin. The manipulation of hematopoietic stem cell (HSC) populations has yielded a fairly harmonious picture of the effects of TORC1 signaling on these cells. For example, the deletion of *Tsc1* in HSCs results in increased TORC1 kinase activity and a decrease in the size of the stem cell pool, coupled with an increase in HSC proliferation (Chen, C. et al., 2008; Gan et al., 2008; Chen et al., 2009). Activation of TORC1 in HSCs, either through constitutively active AKT or through PTEN inactivation, similarly results in a depletion of stem cells associated with an initial increase in proliferation (Yilmaz et al., 2006; Kharas et al., 2010). The generation of reactive oxygen species (ROS) also correlates with an increase in TORC1 activity and a decrease in stem cell maintenance (Jang and Sharkis, 2007). Conversely, the inhibition of HSC TORC1 by rapamycin in response to AKT activation, PTEN or TSC loss, or reactive oxygen species (ROS), acts to rescue the maintenance of the HSC pool (Yilmaz et al., 2006; Jang and Sharkis, 2007; Chen, C. et al., 2008; Gan et al., 2008; Chen et al., 2009; Kharas et al., 2010). Taken together, these studies show that an elevation in TORC1 activity is capable of causing a loss of stemness in HSCs. Interestingly, Chen and colleagues noticed that the deletion of *Tsc1* in HSCs leads to the generation of HSCs that phenocopy those isolated from aged mice (Chen et al., 2009). Rapamycin treatment of both aged HSCs and *Tsc1*^{-/-} HSCs resulted in an increased capacity to self-renew and to reconstitute the hematopoietic system in vivo (Chen et al., 2009). Moreover, older mice treated with rapamycin were more competent in a lethal

challenge with influenza virus, a surprising result considering the known role of rapamycin as an immunosuppressant (Chen et al., 2009); however, rapamycin has been described to increase efficiency of CD8 T-cell function (Araki et al., 2009). Therefore, the alternating roles of mTOR in immune function needs to be clarified before we use TOR inhibitors to promote immune function in the elderly.

Studies in *Drosophila* germline stem cells (GSCs) have yielded similar information; activation of TORC1 in GSCs by conditional deletion of *Drosophila TSC1/2* results in a rise in proliferation and a concomitant reduction of the GSC pool, in agreement with the results obtained from HSC studies (Sun et al., 2010). Interestingly, deletion of *Drosophila TOR* also results in depletion of the GSC pool; however, this observation did not extend to the follicle stem cell pool, indicating that GSCs possess a specific requirement for a minimal level of TORC1 activity for their maintenance (LaFever et al., 2010).

Finally, the effects of TORC1 signaling in human and mouse ESCs has been analyzed in vitro. Interestingly, rapamycin treatment of human ESCs resulted in an increase in differentiation towards the mesoderm and endoderm lineages (Zhou et al., 2009; Lee et al., 2010). Both of these studies found a decrease in octamer-binding transcription factor 4 (Oct4) and NANOG protein levels following rapamycin treatment. Interestingly, siRNA-mediated knockdown of mTOR in human ESCs also reduced sex-determining region Y (Sox2) and Oct4 levels, indicating that TORC1 is required for maintaining stemness in human ESCs (Zhou et al., 2009). However, in murine ESCs, opposing studies have reported that rapamycin can both increase and decrease the rate of differentiation (Schieke et al., 2008; Zhou et al., 2009). The reasons for the discrepancies are not clear; however, it is possible that differences in culture media or other experimental culture variables could be at play. Finally, a total loss of mTOR signaling in murine ESCs, achieved through a null allele, resulted in a loss in ESC viability (Murakami et al., 2004).

Conclusions

Great advances have been made in elucidating the components of the mammalian TOR complexes TORC1 and TORC2. Many of the mechanisms that mediate signaling to TORC1 from extrinsic and intrinsic factors have also been recently clarified. With regards to TORC1 signaling, a number of issues remain unresolved. For example, aside from S6K, 4EBP1 and ULK1, the downstream targets that mediate the cellular effects of TORC1 signaling are largely unaccounted for. In addition, how the specificity of TORC1 signaling is achieved and how multiple signals are integrated are not known. With regards to TORC2, the upstream regulators are ill-defined, although ribosomes have been implicated in mediating the stimulating signal from PI3K to TORC2. Aside from AKT, SGK and PKC, the downstream substrates that mediate the biological function of mTORC2, especially those involved in regulating cell morphology, remain to be clarified. Further studies will presumably give us a better appreciation of the TORC2 complex and provide additional insights into its relationship with TORC1.

The early lethality of *Mtor*-null mouse embryos has hampered our ability to understand the role of mTOR in mammalian biology and development. However, many informative knockouts of mTOR binding partners and downstream targets have provided us with a consistent picture of mTOR as a master regulator of organismal energy sensing. Perturbation of mTORC1 activity in the brain, liver, adipose tissue or muscle affects whole-body

metabolism. Deletions of S6K and 4E-BP have revealed that the role of maintaining whole-body metabolism is, at least partially, managed by translational control. However, the phenotypes of partial 4E-BP gene deletion (*Eif4ebp1* and *Eif4ebp2*, but not *Eif4ebp3*) and *S6K* deletion (both *Rps6kb1* and *Rps6kb2*) are less severe than that observed following *Rptor* deletion. Therefore, either 4E-BP1 and S6K are at least partially functionally redundant, or the primary downstream effectors involved in metabolic regulation lie with another molecular function (or combination thereof) of mTOR. The ability of TORC1 to regulate whole-body metabolism is likely to be intimately related to its recently discovered role in ageing (see Box 2). The conserved role of TOR in nutrient sensing has probably influenced the focused studies of mTOR pathway conditional knockouts in metabolically responsive organs. However, given the contribution of kidney, skin, lungs and heart to TSC disease, we can safely postulate that there are many additional tissue-specific roles for mTOR in mammalian development.

TORC1 signaling is also emerging as an important pathway involved in stem cell differentiation. However, our understanding of the role of TORC1 in specific stem cell populations is far from complete. For example, we are at a loss to explain how TORC1 signaling is essential for the maintenance of some stem cell pools, such as GSCs and HSCs, but not others, such as follicular stem cell pools. Moreover, we cannot explain why rapamycin exhibits contradictory effects in HSCs and ESCs. Obviously, we are dealing with a process that is more complex than a 'general repression' of translation. Likely explanations for the variance observed are: (1) different stem cell populations are differentially sensitive to specific extrinsic factors that signal through TORC1; (2) signaling to or from TORC1 is augmented by lineage-specific factors; or (3) signaling from TORC1 exhibits translational control with a greater degree of subtlety and specificity than we currently appreciate. Other possible scenarios can be imagined; however, more detailed studies need to be performed to gain a clearer picture of TORC1 signaling in the various stem cell pools. The role of TORC1 in conditional knockout models suffers the same quandaries. Before we can claim to have a comprehensive understanding of TORC1 activity *in vivo*, we need to have a more complete picture of TORC1 signaling; for example, one that extends beyond 4E-BP and S6K. Finally, the generation of additional conditional knockout mice in which mTORC1 and mTORC2 function is disrupted will be essential to address the many unanswered questions that remain with regards to mTOR function in development and disease.

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Competing interests statement

The authors declare no competing financial interests.

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