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Review

Defining the Role of mTOR in Cancer

David A. Guertin[1,2] and David M. Sabatini[1,2,3,*]
[1]Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology Department of Biology, 9 Cambridge Center, Cambridge, MA 02141, USA
[2]The Broad Institute, 7 Cambridge Center, Cambridge, MA 02141, USA
[3]Center for Cancer Research and Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
*Correspondence: sabatini@wi.mit.edu
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The mammalian target of rapamycin (mTOR) has emerged as a critical effector in cell-signaling pathways commonly deregulated in human cancers. This has led to the prediction that mTOR inhibitors may be useful in oncology, and derivatives of one such molecule, rapamycin (from which mTOR derives its name), are currently in clinical development. In this review, we discuss recent progress in understanding mTOR signaling, paying particular attention to its relevance in cancer. We further discuss the use of rapamycin in oncology and conclude with a discussion on the future of mTOR-targeted therapy.

Introduction

The study of rapamycin continues to bring surprises to the signal transduction aficionados, revealing many new signaling molecules and a network increasingly viewed as important in cancer. In the early 1990s, seminal studies in yeast and mammalian systems identified a large 250 kDa protein as the drug's cellular target, which in mammals was named the mammalian target of rapamycin (mTOR). Over the next 10 years, scientists used rapamycin to uncover mTOR-dependent processes showing that mTOR regulates cell growth by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism (Figure 1A) (reviewed in Guertin and Sabatini, 2005; Sarbassov et al., 2005a; Wullschleger et al., 2006).

Growth factors and nutrients regulate mTOR, indicating that mTOR is at the interface of two different growth signals. Nearly a decade after the discovery of mTOR, researchers found that mTOR nucleates a rapamycin- and nutrient-sensitive multiprotein complex (now called mTORC1) (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Following soon after was the unexpected identification of a second growth-factor-sensitive but nutrient-insensitive mTOR-containing complex called mTORC2 (Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004). Unlike its mTORC1 sibling, rapamycin does not bind to mTORC2, and consequently, mTORC2 is often called the "rapamycin-insensitive complex." By outfitting itself with mTORC1- or mTORC2-specific proteins, mTOR acquires different substrate specificities. The discovery of mTORC2 provided conclusive evidence to the lurking suspicions that rapamycin did not inhibit all of mTOR's functions.

A prevailing model suggests that cancers dependent upon activation of the oncoprotein AKT rely on subsequent activation of mTORC1 to drive tumorigenesis. This "addiction" to mTORC1 signaling by some cancers has invigorated the clinical development in oncology of the mTORC1-inhibitor rapamycin. Despite the seemingly clear mechanism of action of rapamycin and sound rationale for its use in cancer therapy, first-generation mTOR inhibitors have had only modest and unpredictable successes in clinical trials. A twist in the mTOR story has emerged with the finding that mTOR, when assembled into mTORC2, directly phosphorylates and activates AKT, perhaps placing mTOR on both sides of the AKT signaling hub. Unexpectedly, rapamycin can inhibit AKT by disrupting mTORC2 assembly, but only in some cell types. These discoveries raise a number of questions regarding the development and application of mTOR inhibitors. In this review, we discuss recent advances in our understanding of mTOR biology and its relevance to the clinical development of mTOR inhibitors for oncology.

mTORC1 Signaling in Cancer

Besides mTOR, mTORC1 contains RAPTOR (regulatory associated protein of mTOR), mLST8 (also known as GβL), and PRAS40 (proline-rich AKT substrate 40 kDa) (Haar et al., 2007; Hara et al., 2002; Kim et al., 2002, 2003; Loewith et al., 2002; Sancak et al., 2007). RAPTOR positively regulates mTOR activity and functions as a scaffold for recruiting mTORC1 substrates (Hara et al., 2002; Kim et al., 2002; Schalm et al., 2003). PRAS40 negatively regulates mTOR activity in a manner that depends upon its phosphorylation state (Haar et al., 2007; Sancak et al., 2007). The molecular function of mLST8 is still ambiguous.

Upstream Regulation of mTORC1 Signaling

A major leap forward in understanding mTORC1 regulation was the discovery that the TSC1 and TSC2 bipartite protein complex negatively controls its activity (Figure 1A) (reviewed in Crino et al., 2006). Mutations in either the *tsc1* or *tsc2* gene cause the hamartomatous syndrome tuberous sclerosis complex (TSC). The discovery of the connection between TSC and the mTORC1 pathway provided the first molecular link between mTOR and cancer.

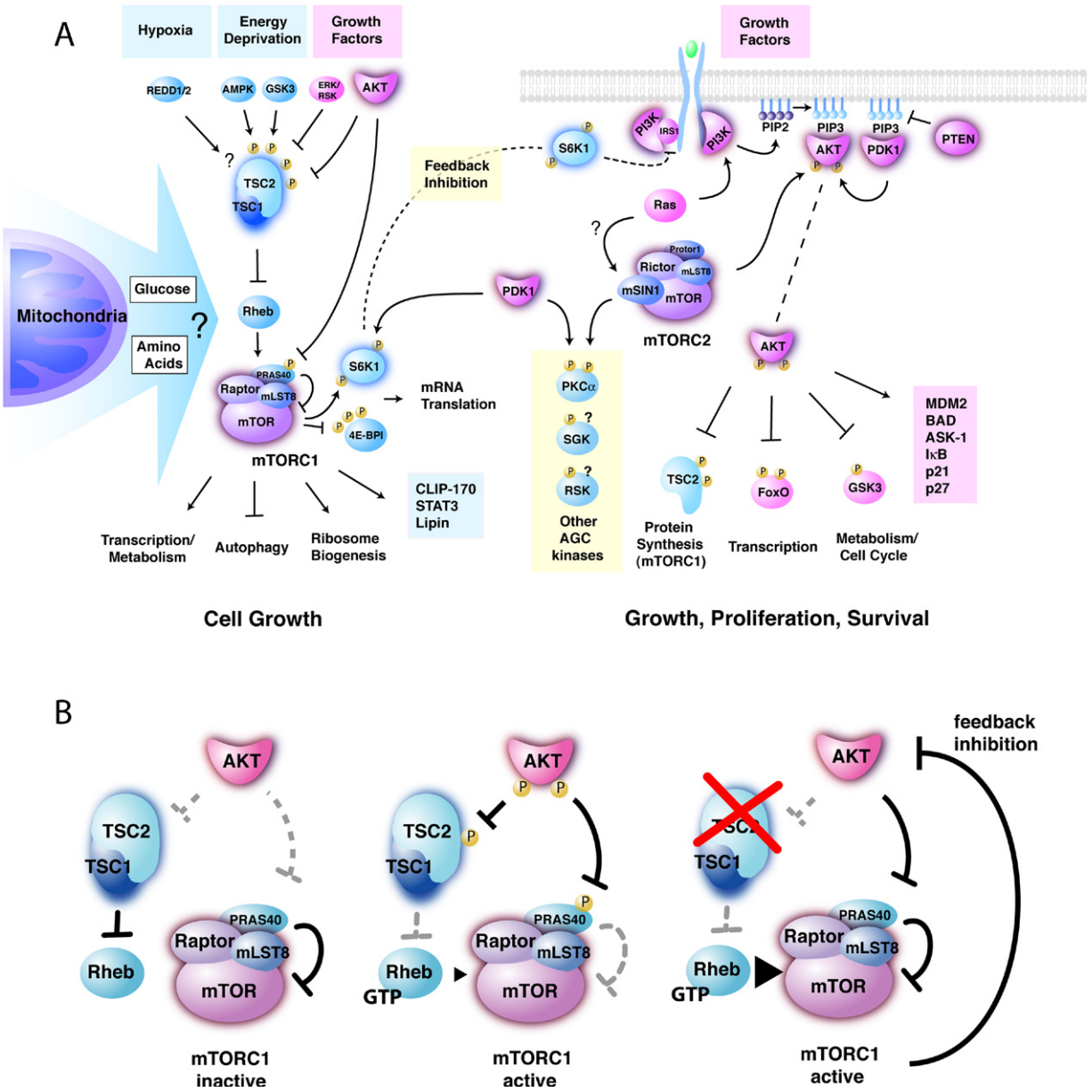

Figure 1. The mTOR Network

(A) The mTOR kinase is the catalytic component of two distinct multiprotein complexes called mTORC1 and mTORC2. (Left) In addition to mTOR, mTORC1 contains RAPTOR, mLST8, and PRAS40. mTORC1 drives cellular growth by controlling numerous processes that regulate protein synthesis and degradation. Diverse positive and negative growth signals influence the activity of mTORC1, many of which converge upon the TSC1/2 complex. (Right) mTORC2 also contains mLST8, but instead of RAPTOR and PRAS40, mTORC2 contains the RICTOR, mSIN1, and PROTOR proteins. Currently, the only characterized substrate of mTORC2 is the AKT kinase, which suggests mTORC2 functions downstream in the PI3K pathway to regulate cell growth, proliferation, and survival. mTORC2 also regulates PKCα phosphorylation, but it is not known if this is direct, or if mTORC2 can regulate other AGC-family kinases. Activation and inhibition induced by direct phosphorylation is indicated by a phosphate (P).

(B) Model of mTORC1 coregulation by RHEB and PRAS40. (Left) When AKT is inactive, TSC1/2 inhibits RHEB while PRAS40 inhibits mTORC1. (Middle) Upon activation, AKT promotes mTORC1 activity by phosphorylating both TSC1/2 and PRAS40. This results in GTP-loading of RHEB, which directly activates mTORC1 and release of mTORC1 from PRAS40 repression. (Right) In *tsc2* null cells, RHEB strongly activates mTORC1. This in turn inhibits AKT by way of the negative feedback loop (described in the text). Even though PRAS40 is dephosphorylated in this state, its ability to repress mTORC1 is overrun by the greatly elevated Rheb activity.

TSC2 possesses GAP (GTPase activating protein) activity and inactivation of the TSC1/2 complex, either by mutation (as in TSC) or by cellular growth signals, leads to the activation of the ras-like GTPase RHEB (reviewed in Crino et al., 2006). A putative RHEB GEF (guanine nucleotide exchange factor) has been described in *Dro-*

sophila, but it has not yet been validated in mammals (Hsu et al., 2007). Although it is difficult to detect a direct biochemical interaction between endogenous RHEB-GTP and mTORC1, in vitro experiments indicate that soluble GTP-loaded RHEB directly activates the kinase activity of mTORC1 (Figure 1B) (Sancak et al., 2007). In contrast, PRAS40 inhibits RHEB-GTP-dependent mTORC1 activation in a dose-dependent manner in an in vitro kinase assay, and it stably associates with mTORC1 in cells (Haar et al., 2007; Sancak et al., 2007). Although RHEB-GTP and PRAS40 are adversaries in a cell free system, in intact normal cells, AKT forces them to cooperate. For instance, AKT (and possibly other kinases) phosphorylates PRAS40 on T246, diminishing its ability to inhibit mTORC1 (Haar et al., 2007; Huang and Porter, 2005; Kovacina et al., 2003; Sancak et al., 2007). Therefore, AKT both promotes RHEB GTP-loading and relieves mTORC1 from PRAS40 repression, although it is not clear if this dual regulatory input functions in all cell types. Other subunits of mTORC1 are also phosphorylated (including mTOR at S2448 [Sekulic et al., 2000], which is commonly used as a biomarker of mTOR activation), but the functional significance of these modifications is unclear.

Diverse signals regulate TSC1/2 suggesting that, like mTORC1, TSC1/2 is a signal integration center. Positive growth signals from the RAS-MAPK pathway inhibit TSC2 (Ballif et al., 2005; Ma et al., 2005a; Roux et al., 2004). However, the phosphorylation and inhibition of TSC2 by AKT is the clearest link between mTORC1 and a pathway deregulated in cancer (Inoki et al., 2002; Manning et al., 2002). Aberrant AKT activation is a widespread oncogenic phenomenon that can result from *pten* deletion, *pik3ca* activating mutations, the *bcr-abl* translocation, and amplification of genes encoding HER-2, EGFR, or AKT itself (reviewed in Guertin and Sabatini, 2005).

A growing body of evidence argues that the connection between AKT and TSC2-RHEB-mTORC1 is a critical step in PI3K-mediated tumorigenesis. For instance, rapamycin slows the proliferation of transformed cells null for *pten* or expressing constitutively active AKT (reviewed in Guertin and Sabatini, 2005). Studies in mouse models support this idea showing that the neoplastic phenotypes induced by *pten* deletion or transgenic activation of AKT are sensitive to rapamycin (Majumder et al., 2004; Neshat et al., 2001; Podsypanina et al., 2001). In mice, depletion of AKT activity can also thwart *pten*-deletion-driven as well as RAS-driven and chemically induced tumorigenesis (Chen et al., 2006; Skeen et al., 2006). Based on in vitro studies in mouse embryo fibroblasts (MEFs), it is argued that the oncogenic activity of AKT in these models depends on mTORC1 (Skeen et al., 2006). The importance of AKT-dependent inhibition of PRAS40 in tumorigenesis is not yet known but, interestingly, the PRAS40 gene is located in a region of chromosome 19 (19q13.33) thought to contain an unidentified tumor suppressor (Hartmann et al., 2002).

In contrast to growth-factor-driven activation of mTORC1, hypoxia, AMPK activation resulting from depletion of cellular energy, WNT-GSK3 signaling, and glucocorticoids all inhibit mTORC1 by promoting TSC1/2 activation (Inoki et al., 2006; Reiling and Sabatini, 2006; Wang et al., 2006). Amino acid deprivation may also activate TSC1/2, although other evidence argues that mTORC1 and S6K1 respond to amino acid availability independently of TSC1/2 (Findlay et al., 2007; Nobukuni et al., 2005; Sarbassov and Sabatini, 2005; Smith et al., 2005) so how nutrients, particularly amino acids, regulate mTORC1 signaling remains a mystery.

Can cancer cells survive by acquiring adaptations that allow mTORC1 to continue signaling in nutrient and oxygen poor environments? Because deprivation for energy, oxygen, and nutrients is common in the microenvironment of tumors, cancer cells insensitive to these stresses may have a selective growth advantage. On the other hand, transformed cells may restrict growth in a suboptimal environment, buying time to acquire other mutations and/or await angiogenesis. The lack of this response could be disadvantageous as it could lead to unrestricted growth signaling in poor nutrient conditions, causing cells to deplete their energy stores and induce apoptosis. Consistent with this idea, *tsc2*-deficient cells undergo apoptosis in glucose-free medium, a response suppressed by rapamycin (Inoki et al., 2003). An additional mechanism tumor cells may use to cope with nutrient deprivation is to temporarily salvage nutrients autonomously by activating autophagy (Liang et al., 2007).

Regulation of mTORC1 by Localization

The consensus from several independent studies is that mTOR is predominantly cytoplasmic but associated with cellular membranes including those of the mitochondria, endoplasmic reticulum, and Golgi (Desai et al., 2002; Drenan et al., 2004; Liu and Zheng, 2007; Sabatini et al., 1999; Tirado et al., 2003; Withers et al., 1997). RHEB also associates with the endomembrane system (Buerger et al., 2006), and collectively, these studies suggest that mTORC1 signaling may emanate from intracellular membranes. Interestingly, a portion of mTOR shuttles between the nucleus and cytoplasm, and, by an unknown mechanism, this may regulate the ability of mTOR to phosphorylate S6K1 (Bachmann et al., 2006; Bernardi et al., 2006; Kim and Chen, 2000; Li et al., 2007).

Effectors of mTORC1

S6K1 and 4E-BP1—both regulators of mRNA translation—are the only extensively described mTORC1 substrates (reviewed in Sarbassov et al., 2005a; Wullschleger et al., 2006). The eIF3 complex facilitates mTORC1-dependent phosphorylation of S6K1 and 4E-BP1 by functioning as a scaffold that mediates the enzyme-substrate interactions (Holz et al., 2005). When activated by mTORC1, S6K1 promotes protein synthesis by phosphorylating PDCD4 and targeting it for degradation (Dorrello et al., 2006). PDCD4 hinders protein

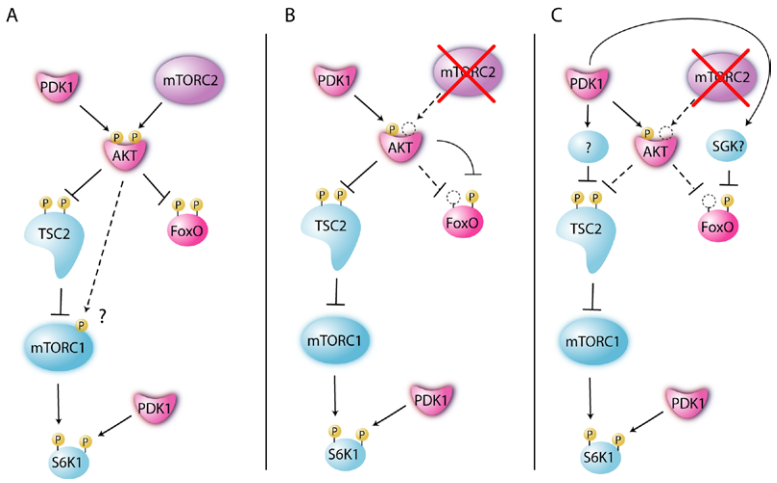

Figure 2. mTOR May Function Both Upstream and Downstream of AKT

(A) Full activation of AKT requires phosphorylation on two sites, T308 by PDK1 and S473 by mTORC2. Activated AKT phosphorylates many substrates including TSC2 and the FOXOs. Akt phosphorylates mTORC1 directly, but this connection is just beginning to be understood.

(B) Eliminating mTORC2 ablates AKT S473 phosphorylation in mouse embryo fibroblasts. This does not affect the phosphorylation of TSC2. In contrast, one AKT phosphorylation site in the FOXO1 and FOXO3 transcription factors is reduced, while phosphorylation at another AKT site is unaffected. One possible model to explain this finding is that S473-unphosphorylated AKT retains enough catalytic activity to phosphorylate some of its targets but not others.

(C) Alternatively, a substitute kinase, which is likely to be regulated by PDK1, can compensate for S473-deficient AKT. In the case of FOXO, it is known that SGK can phosphorylate some of the same sites as AKT.

translation by binding and preventing the eIF4A helicase from unwinding secondary structure in the 5′ untranslated region of mRNA. The ribosomal protein S6 is perhaps the best-known S6K1 substrate. Despite being an indirect measure of mTORC1 activity and lacking a clear function, S6 phosphorylation is widely used in research and in the clinic as a biomarker of mTORC1 activity. In addition to S6K1, mTORC1 phosphorylates 4E-BP1 and releases it from inhibiting the elongation initiation factor 4E (eiF4E).

mTORC1 also drives protein synthesis by regulating ribosome biogenesis. In yeast, TORC1 activity promotes the synthesis of ribosomal proteins, and in higher organisms this may additionally or alternatively involve regulating ribosome assembly (Guertin et al., 2006a; Wullschleger et al., 2006). Other mTORC1 substrates include CLIP-170, LIPIN, and STAT3, but their roles in mTORC1 signaling are less characterized (Choi et al., 2002; Huffman et al., 2002; Yokogami et al., 2000). The dearth of well-known mTORC1 substrates might reflect the fact that the mTORC1 target sites are structurally diverse and difficult to identify bioinformatically (for example, the phosphorylation sites in S6K1 and 4E-BP1 are dissimilar).

Negative Feedback Regulation of PI3K-AKT Signaling

It might seem surprising that patients suffering from TSC do not develop more aggressive tumors, like those linked to *pten* mutations. The reason may be the existence of a potent negative feedback loop. In many cell types, activation of mTORC1 signaling strongly represses PI3K-AKT signaling upstream in the PI3K pathway (reviewed in Manning, 2004). For example, loss of TSC1/2 function results in decreased AKT phosphorylation (Jaeschke et al., 2002; Kwiatkowski et al., 2002). One mechanism by which this occurs appears to be through S6K1-dependent downregulation of IRS-1 (Figure 1A). When mTORC1 is active, S6K1 directly phosphorylates and inhibits IRS-1 (reviewed in Zick, 2005). Mice lacking *s6k1* are viable, but when challenged with a high-fat diet, despite the fact that insulin receptors become desensitized, they are resistant to obesity because loss of the feedback loop enhances their insulin sensitivity (Um et al., 2004). These observations led to the hypothesis that tumors in TSC patients are less aggressive because the feedback loop squelches PI3K-AKT signaling. Genetic evidence in mice supports this hypothesis as inactivation of *pten* in *tsc2*-deficient lesions elevates AKT signaling sufficiently to overcome the feedback loop and results in more severe tumors (Ma et al., 2005b; Manning et al., 2005). Other receptor tyrosine kinase pathways that do not depend on IRS-1 are also likely subject to mTORC1-dependent negative regulation. For instance, *tsc1/2* deletion suppresses PDGFR expression in a rapamycin-sensitive manner (Zhang et al., 2007).

Regulation of mTORC1 by PRAS40 may have an important role in setting the level of feedback inhibition. PRAS40 functions as an mTORC1 inhibitor, but its inhibitory duty is relieved upon being phosphorylated by AKT. Therefore, a decrease in AKT activity—as would occur during negative feedback inhibition—might enhance mTORC1 inhibition by PRAS40 and in turn, decrease the level of feedback inhibition. One study using siRNA to deplete cells of TSC2 or overexpressing recombinant RHEB finds that in each case, simultaneously overexpressing recombinant PRAS40 suppresses mTORC1 activation (Haar et al., 2007). However, in cells deleted for the *tsc2* gene, PRAS40-dependent inhibition of mTORC1 is completely overrun by the greatly elevated level of RHEB activation (Figure 2B) (Sancak et al., 2007). Nevertheless, PRAS40 may influence the magnitude of feedback inhibition in a cell-type-specific manner, although this remains to be seen.

mTORC2 Signaling in Cancer

Regulation of AKT by mTORC2

Study of mTORC2 is in its infancy, but the finding that mTORC2 directly phosphorylates AKT adds a new twist in thinking about the role of mTOR in cancer (Sarbassov

et al., 2005b). mTORC2, like mTORC1, also includes the mLST8 protein, but instead of RAPTOR, mTORC2 contains the RICTOR (rapamycin-insensitive companion of mTOR) and mSIN1 proteins (Frias et al., 2006; Jacinto et al., 2004, 2006; Sarbassov et al., 2004; Yang et al., 2006). mTORC2 additionally contains PROTOR (protein observed with RICTOR), a protein found only in higher eukaryotes that lacks any obvious functional domains (Pearce et al., 2007).

Phosphorylation of S473 in a C-terminal hydrophobic motif is necessary for the full activation of AKT (Alessi et al., 1996). Several kinases have been proposed to fulfill the role of the AKT S473 kinase—the so-called "PDK2 kinase." mTOR was added to the list as a result of loss-of-function RNAi experiments coupled with in vitro biochemistry in *Drosophila* and human cancer cells (Sarbassov et al., 2005b). This study and ensuing work in human adipocytes and *Dictostylium* show that depletion of RICTOR or mTOR, but not RAPTOR, dramatically reduces Akt S473 phosphorylation (Figure 1A) (Hresko and Mueckler, 2005; Lee et al., 2005; Sarbassov et al., 2005b). The discovery that SIN1 functions in TORC2-mediated regulation of AKT emerged from a study in *Dictostylium*, which found that RIP3 and PIA, the orthologs of mSIN1 and RICTOR, respectively, physically interact in a complex, and when mutated, induce similar phenotypes including impaired AKT activation (Lee et al., 2005). Subsequent biochemical studies in mammalian and *Drosophila* cultured cells confirm these observations (Frias et al., 2006; Jacinto et al., 2006; Yang et al., 2006). It has not yet been determined if PROTOR functions in the regulation of AKT.

The recently described PHLPP phosphatases (PHLPP1 and PHLPP2) counteract mTORC2 by dephosphorylating AKT at S473 (Brognard et al., 2007; Gao et al., 2005). Understanding the interplay between these two forces is of obvious importance. In mammals, there are three AKT isoforms, each encoded by a unique gene (Hanada et al., 2004). Interestingly, PHLPP1 and PHLPP2 reportedly have different specificities for the three AKT isoforms (Brognard et al., 2007; Gao et al., 2005). An understanding of how the isoforms of AKT are differentially regulated may have important implications in cancer; however, because most studies of AKT do not differentiate between the different isoforms, it is difficult to speculate on what those implications might be.

Most of the known core mTOR interacting proteins, except for PRAS40 and PROTOR, have been knocked out in mice. Deleting the *mtor* gene results in embryonic lethality around the time of implantation, thus precluding the ability to measure AKT phosphorylation in *mtor* null tissues (Gangloff et al., 2004; Murakami et al., 2004). The early lethality of *mtor* null mice appears to result from a loss of mTORC1 function because *raptor* null mice are phenotypically similar (Guertin et al., 2006b). In contrast, mTORC2-deficient mice survive until midgestation (Guertin et al., 2006b; Jacinto et al., 2006; Shiota et al., 2006; Yang et al., 2006). Importantly, deletion of the genes encoding mTOR interacting proteins that define mTORC2 (*rictor*, *mlst8*, and *msin1*) ablates AKT S473 phosphorylation (Guertin et al., 2006b; Jacinto et al., 2006; Shiota et al., 2006; Yang et al., 2006). These findings provide strong genetic evidence in mammals to substantiate the claim that mTORC2 directly regulates AKT.

An unexpected finding from the genetic knockout studies is that mLST8, a stable component of both mTORCs, is functionally required only for mTORC2 signaling in development (Guertin et al., 2006b). *lst8* null budding yeast and *Dictyostelium* cells are also phenotypically more similar to cells deleted for TORC2-specific components (Lee et al., 2005; Loewith et al., 2002). However, at least one TORC1-specific function is still impaired in *lst8* null budding yeast cells (Loewith et al., 2002). Moreover, depletion of mLST8 in cultured human cancer cells also impairs mTORC1 signaling (Jacinto et al., 2004; Kim et al., 2003) suggesting that mLST8 may have a role, albeit mysterious, in both complexes.

AKT belongs to a family of structurally related kinases called the AGC kinases, which includes the S6Ks, SGKs, RSKs, and PKCs (Hanada et al., 2004). All family members contain the hydrophobic motif phosphorylation site (S473 in AKT; T389 in S6K1), as well as a phosphorylation site for the PDK1 kinase in the kinase domain (T308 in AKT; T229 in S6K1). S6K1 contains an additional C-terminal inhibitory domain that is absent in the other family members, and this domain may preferentially recruit S6K1 to the mTORC1 complex (Ali and Sabatini, 2005). Whether other AGC kinases are targeted by mTOR is an open question. However, knockdown and knockout studies indicate that mTORC2 regulates PKCα phosphorylation and stability, although it is not known if this is direct (Guertin et al., 2006a; Sarbassov et al., 2004).

Growth factors stimulate mTORC2 activity and some mTORC2 subunits are phosphorylated, but the responsible kinases remain unknown (Frias et al., 2006; Sarbassov et al., 2004; Sarbassov et al., 2005b; Yang et al., 2006). In *Dictyostelium*, the mSIN1 homolog (RIP3) interacts with RAS-GTP in a two-hybrid assay and recombinant RIP3 proteins harboring mutations in the Ras binding domain do not fully rescue *rip3*$^-$ cells (Lee et al., 1999, 2005; Schroder et al., 2007). This raises the possibility that RAS regulates mTORC2. Additionally, three mSIN1 isoforms independently interact with mTOR and RICTOR, defining three distinct mTORC2s (Frias et al., 2006; Schroder et al., 2004). All three mTORC2s phosphorylate AKT S473 in vitro, but insulin activates only two of them (Frias et al., 2006). Thus, some mTORC2 complexes may phosphorylate AKT independently of growth factor stimulation. Curiously, the mSIN1 isoform that defines the insulin-independent mTORC2 is truncated at the C terminus and lacks part of the ras-binding domain as well as a putative, but divergent, PH domain (Frias et al., 2006; Schroder et al., 2007). An interesting possibility is that these mSIN1 C-terminal domains link mTORC2 to growth factor stimuli.

Coregulation of AKT by mTORC2 and PDK1

Full AKT activity in vitro requires phosphorylation at both T308 and S473 (Alessi et al., 1996). However, unlike the case for S6K, SGK, and RSK, phosphorylation of AKT at T308 by PDK1 is not contingent upon prior phosphorylation at S473 (Biondi et al., 2001; Collins et al., 2003). Phosphorylation of S473 occurs in *pdk1* null cells in which T308 phosphorylation is abolished (Alessi et al., 1996; McManus et al., 2004). Moreover, ablation of S473 phosphorylation that occurs upon deleting *rictor*, *mlst8*, or *msin1* does not eliminate T308 phosphorylation (Guertin et al., 2006b; Jacinto et al., 2006; Shiota et al., 2006). These results suggest that these two phosphorylation events may occur independently and support a model in which AKT and PDK1 interact as a result of colocalization to the plasma membrane (through their PH domains) (Collins et al., 2003). Once colocalized, PDK1 activates AKT, which is synergistically enhanced by mTORC2. It is not known how the interaction between mTORC2 and AKT is facilitated, but one possibility is that the PH-like domain of mSIN1 localizes mTORC2 with AKT at membranes (Schroder et al., 2007).

In contrast to the results obtained from studying knockout MEFs, reducing mTORC2 activity in cultured cancer cells by depleting RICTOR or mTOR by RNAi simultaneously decreases phosphorylation at both the PDK1 site (T308) and the mTORC2 site (S473) (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). This discrepancy may reflect a fundamental difference between depletion of mTORC2 activity acutely using RNAi versus chronically by gene knockout. However, an interesting possibility is that immortalization might rewire the cellular circuitry such that T308 and S473 phosphorylation become linked. This might suggest that an mTORC2-inhibitor could be more toxic to cancer cells than to normal cells.

mTOR May Function on Both Sides of AKT

In cultured mammalian cells, the expression of a recombinant mutant of TSC2 incapable of being phosphorylated by AKT dramatically reduces mTORC1-dependent phosphorylation of a coexpressed recombinant version of S6K1 (Manning et al., 2002). Combined with the finding that mTORC2 phosphorylates S473 of AKT, this suggests a model in which mTOR may function both upstream and downstream of AKT (Figure 2A).

However, challenging the universality of this model is the surprising finding from mouse genetic studies that deleting *rictor*, *mlst8*, or *msin1* does not affect the phosphorylation of TSC2 on two well-characterized AKT target sites (S939 & T1462), despite the fact that AKT S473 phosphorylation is ablated in MEFs (Guertin et al., 2006b; Inoki et al., 2002; Jacinto et al., 2006; Manning et al., 2002). Similarly, phosphorylation of AKT target sites in GSK3α and GSK3β (S9 and S21, respectively) are also unaffected. In contrast, all three deletions reduce phosphorylation on one predicted AKT phosphorylation site of the Forkhead O (FOXO) transcription factors FOXO1 and FOXO3 (T24 and T32, respectively), while phosphorylation at another (S256) is unimpaired. These findings raise some interesting questions:

How does S473 phosphorylation by mTORC2 regulate AKT activity in vivo? Perhaps S473-unphosphorylated AKT is partially active in vivo. For example, Akt lacking S473 phosphorylation may possess enough intrinsic activity to phosphorylate TSC2, but FOXO1/3 phosphorylation on T24/32 may require a higher threshold of Akt activity (Figure 2B). In addition or alternatively, S473-phosphodeficient Akt may be incapable of making a protein-protein interaction or missing a localization signal that is necessary for phosphorylating FOXO/3. In *Drosophila*, *AKT* is required for viability, but surprisingly, dTORC2 is not (Hietakangas and Cohen, 2007; Staveley et al., 1998). Thus, dAKT lacking the HM phosphorylation (S505 in *Drosophila*) retains all essential biological activities, consistent with a model that not all in vivo functions of AKT require HM phosphorylation. Interestingly, deletion of *drictor* rescues the overgrowth phenotypes associated with overexpressing *dpi3k* or deleting *dpten*, suggesting that dTORC2 becomes limiting only when dPI3K signaling is high (Hietakangas and Cohen, 2007).

Another possibility is that a compensatory kinase may fill in for S473 phosphodeficient AKT in some cases (Figure 2C). For example, phosphorylation sites in GSK3 and mTOR first identified as AKT target sites can also be phosphorylated by S6K1 (Chiang and Abraham, 2005; Holz and Blenis, 2005; Zhang et al., 2006). Moreover, both SGK and AKT can phosphorylate some of the same sites in FOXO1/3 (reviewed in Greer and Brunet, 2005). Thus, one property of AGC kinases appears to be their ability to share substrates in certain circumstances. Importantly, phosphorylation of TSC2, GSK3, and FOXO1/3 at the predicted AKT sites requires PDK1 (McManus et al., 2004). Thus, if an alternative kinase phosphorylates these sites it is likely to be an AGC family member. How other AKT substrates (e.g., PRAS40, BAD, IκB, $p21^{Cip1}$, and $p21^{Kip1}$) respond to losing mTORC2 function still needs to be addressed.

It is possible that TSC2 is not always a critical AKT target. In *Drosophila*, replacement of wild-type TSC2 with a mutant version in which the AKT phosphorylation sites were changed to nonphosphorylatable residues did not impair development (Dong and Pan, 2004). Moreover, the activation of AKT impairs neuronal growth and this phenocopies losing TSC1/2 function, but this effect does not require AKT-dependent phosphorylation of TSC2 at S939 or T1462 (Tavazoie et al., 2005). Since mTORC1 also binds the Akt substrate PRAS40, this raises the possibility that AKT could bypass TSC1/2 and signal directly to mTORC1 (Haar et al., 2007; Sancak et al., 2007). Thus, the role of AKT-dependent phosphorylation of TSC2 in mTORC1 regulation is still uncertain.

Because ablation of mTORC2 activity reduces FOXO1/3 T24/32 phosphorylation in MEFs, this might suggest that FOXOs are important targets of AKT in development. Interestingly, activating mutations in PI3K

promote invasion of human cancer cells by a mechanism that selectively requires AKT signaling to FOXO1/3 (Samuels et al., 2005). Taken together, these findings might further suggest that FOXO1/3 is a critical AKT target in some cancers. FOXO transcription factors, which regulate the cell cycle, apoptosis, and metabolism, and appear to function in angiogenesis among other processes, are inhibited by AKT (Furuyama et al., 2004; Greer and Brunet, 2005; Hosaka et al., 2004; Potente et al., 2005). Mice deleted for the *foxO1*, *foxO3*, and *foxO4* genes develop thymic lymphomas and hemangiomas, unequivocally demonstrating that *foxOs* are tumor suppressors (Paik et al., 2007; Tothova et al., 2007). However, the tumor spectrum in *foxO1/3/4* triple knockout mice is more restricted compared with that seen in *pten*-deficient mice (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998), possibly suggesting that FOXOs are critical AKT targets in some cancers and not others, or that other PIP3-activated pathways contribute to the tumor burden seen in *pten*-deficient mice.

mTOR Inhibitors in Cancer Therapy

Clinical results with three prototype mTOR inhibitors, all rapamycin analogs (CCI-779 [Wyeth], RAD001 [Novartis], AP23573 [Ariad Pharmaceuticals]), have been described (reviewed in Easton and Houghton, 2006; Faivre et al., 2006; Granville et al., 2006; for simplicity, we will refer to all analogs as rapamycin from here on). Unfortunately, clinical updates indicate that rapamycin shows promise against only a few cancers, particularly mantle cell lymphoma, endometrial cancer, and renal cell carcinoma. Overall, the therapeutic response to rapamycin is highly variable, suggesting that biomarkers capable of predicting which cells will respond to rapamycin-therapy are needed. While current results are frustrating, they likely reflect the fact that we do not fully understand the mechanism of action of rapamycin or mTOR circuitry. Unexpectedly, prolonged exposure to rapamycin decreases AKT S473 phosphorylation in a subset of cancer cells (Sarbassov et al., 2006). This appears to result from the capacity of rapamycin to block the assembly of mTORC2 (Sarbassov et al., 2006). A major and problematic question though is why mTORC2 is susceptible to prolonged rapamycin exposure in some cells, partially sensitive in some cells, and resistant in others (Sarbassov et al., 2006). Complicating this question is the fact that no genetic traits predictive of this phenomenon have been identified. Regardless, this finding challenges conventional wisdom that rapamycin is an mTORC1-specific inhibitor, and invites speculation that some of the clinical responses to rapamycin may reflect this dual action of the drug.

Hamartoma Syndromes

The clearest molecular rationale for rapamycin therapy exists for hamartoma syndromes such as tuberous sclerosis complex. There are several hamartoma syndromes that share pathological features with TSC, including Cowden Disease, Peutz-Jeghers Syndrome, neurofibromatosis, and Birt-Hogg-Dube Syndrome. Like *tsc1* and *tsc2*, the tumor suppressor genes linked to these diseases (*pten*, *lkb1*, *nf1*, and *flcn* respectively) encode proteins that restrict mTORC1 signaling (Baba et al., 2006; Corradetti et al., 2004; Eng, 2003; Johannessen et al., 2005; Shaw et al., 2004). Clinical trials with rapamycin are underway for treating some of these conditions and early reports from TSC trials indicate promising successes (Franz et al., 2006).

Angiogenesis

A particularly interesting property of rapamycin is its ability to suppress angiogenesis (Guba et al., 2002). In cells exposed to hypoxia, levels of the HIF1α transcription factor increase and this facilitates expression of *VEGF*, a HIF1α target gene. mTORC1 regulates the translation and activity of HIF1α (Bernardi et al., 2006; Hudson et al., 2002), suggesting that the antiangiogenic properties of rapamycin could result from its ability to disrupt *vegf* expression. A remarkable successful example of treating tumors using rapamycin is in Kaposi's sarcoma (KS), a tumor characterized by high vascularization and increased *vegf* signaling (Campistol et al., 2004; Stallone et al., 2005). Rapamycin may also be particularly effective in treating certain kidney cancers. Many cases of sporadic kidney cancer (50%–60%) exhibit loss of the VHL (Von Hippel-Lindau) tumor suppressor, which encodes a negative regulator of HIF1α (Kim and Kaelin, 2004). In a xenograft model using human kidney cancer cells, loss of VHL expression correlates with elevated HIF1α levels, an increased vascular network, and importantly, with rapamycin sensitivity (Thomas et al., 2006). In this model, rapamycin appears to function by inhibiting the translation of HIF1α, which correlates with a drop in VEGF expression and reduced angiogenesis. VHL expression might therefore be predictive of which kidney cancer patients will respond favorably to rapamycin therapy.

Interestingly, endothelial cells are one of the clearest examples of a cell type in which AKT phosphorylation is susceptible to mTORC2 inhibition by prolonged rapamycin treatment (Sarbassov et al., 2006). AKT is important for VEGF-mediated angiogenesis and KS requires AKT hyperactivation in endothelial cells (Ackah et al., 2005; Sodhi et al., 2004). Rapamycin treatment blocks pathological angiogenesis and decreases tumor growth in a xenograft model by decreasing AKT S473 phosphorylation in the endothelial cells surrounding the tumor (Phung et al., 2006). The inhibition of mTORC2-AKT signaling by rapamycin in endothelial cells suggests an alternative explanation for the antiangiogenic properties of the drug and emphasizes the potential importance of the mTORC2-AKT-FOXO circuit in these cells. A notable characteristic of mice deficient for mTORC2 or AKT activity is an underdeveloped fetal vascular system (Guertin et al., 2006b; Shiota et al., 2006; Yang et al., 2003, 2005). Given the demonstrated role of mTORC2 in FOXO1/3 phosphorylation and the fact that *foxO1/3/4* triple knockout mice are predisposed to developing

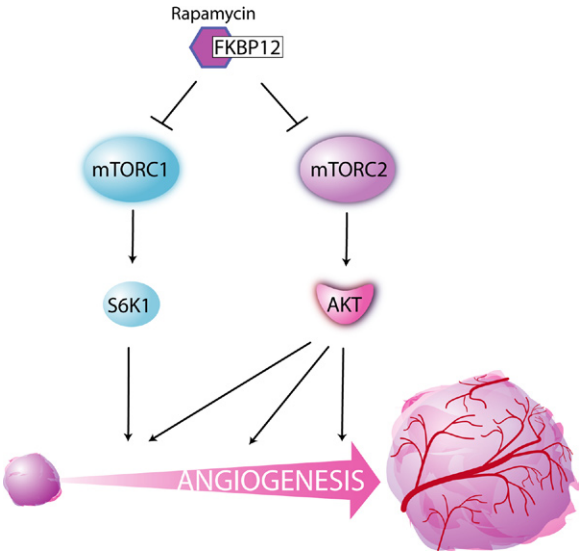

Figure 3. Rapamycin May Block Pathological Angiogenesis by Inhibiting Both mTORC1 and mTORC2

Studies in the laboratory and in the clinic indicate that rapamycin blocks pathological angiogenesis. The dual inhibitory action of rapamycin on both mTORC1 and mTORC2 may be the key to its antiangiogenic properties. Rapamycin, which is pharmacologically active only when bound to the immunophilin (FKBP12), is a universal mTORC1 inhibitor. In endothelial cells, rapamycin additionally blocks mTORC2 assembly and inhibits full AKT activation. In cultured endothelial cells, mTORC1 is required early and transiently for hypoxia-induced proliferation, but the requirement for mTORC2 is sustained and more critical.

hemangiomas, it is tempting to speculate that a rapamycin-sensitive mTORC2-AKT-FOXO circuit may be essential for endothelial cell function. However, the causative agent in KS (a G protein coupled receptor) can stimulate TSC2 phosphorylation and mTORC1 activation (Sodhi et al., 2006). Thus, a role for the mTORC1 pathway in pathological angiogenesis, at least in KS, cannot be ruled out. In fact, depleting endothelial cells of RAPTOR (mTORC1) or RICTOR (mTORC2) by RNAi reveals that both mTORCs function in hypoxia-induced proliferation, but the role of mTORC1 is early and transient, while mTORC2-AKT signaling is sustained and critical (Li et al., 2007). Therefore, the dual inhibitory action of rapamycin on both mTORCs may be the key to its antiangiogenic properties (Figure 3). A direct mTOR kinase domain inhibitor may therefore be an effective angiogenesis inhibitor.

Hematopoietic Cancers

Patients suffering from mantle-cell lymphoma (MCL) exhibit one of the best clinical responses to rapamycin (Witzig et al., 2005; Witzig and Kaufmann, 2006). MCL is a non-Hodgkin's lymphoma with the poorest prognosis among the non-Hodgkin's lymphomas (Williams and Densmore, 2005). The disease is characterized by high CYCLIN D1 expression and currently there is no standard treatment. Treatment of MCL cells with rapamycin in vitro induces cell cycle arrest, but unexpectedly, without affecting CYCLIN D1 levels (Hipp et al., 2005). AKT signaling is also enhanced in many mantle-cell lymphoma lines, although rapamycin's affect on AKT in these cells has not been investigated (Rudelius et al., 2006). Rapamycin is also a potential treatment for acute myelogenous leukemia (AML). The PI3K-AKT-mTOR pathway is hyperactive in patient-derived AML cells, and in vitro, AML cells respond favorably to the drug (Recher et al., 2005). Like in endothelial cells, rapamycin inhibits both the mTORC1-S6K1 and mTORC2-AKT pathways in AML cells (Zeng et al., 2007), suggesting that a positive clinical response in AML may correspond with the drug's ability to additionally inhibit mTORC2.

Deletion of *pten* in adult mouse hematopoietic cells induces hematopoietic stem cell (HSC) proliferation and the generation of leukemia-initiating cells, leading to depletion of normal HSCs and causing a myoproliferative disease that eventually progresses to leukemia (Yilmaz et al., 2006). Rapamycin treatment reverses this effect by both diminishing the number of leukemia-initiating cells and restoring normal HSC function. Deletion of *foxO1/3/4* in adult mouse hematopoietic cells results in a HSC defect phenotypically similar to the *pten*-deficient HSC model (Tothova et al., 2007). Thus, losing FOXO function rather than activating the mTORC1 pathway may be the reason for the HSC defects in these mice. Perhaps HSCs, like endothelial cells, have a critical rapamycin-sensitive mTORC2-AKT-FOXO circuit, which could explain the effects of rapamycin in the *pten*-deficient HSC model. If the effect of rapamycin in the *pten*-deficient HSC model results from inhibiting mTORC2, then rapamycin should not revert the HSC phenotype in the *foxO1/3/4*-deletion model because it would function upstream of FOXO. It will be interesting to see if mTORC1, and in particular mTORC2, have roles in maintaining other adult or embryonic stem cell populations.

The Future of Targeting mTOR in Cancer

Rapamycin derivatives will likely be the first mTOR inhibitors to reach the market as cancer therapeutics. Rapamycin is a universal inhibitor of mTORC1-dependent S6K1 phosphorylation, but the existence of the strong negative feedback loop from S6K1 to AKT signaling presents a potential therapeutic problem as losing feedback inhibition of AKT could promote cell survival and chemoresistance. Clearly an undesirable response! Some studies suggest that sometimes this may be the case. For instance, rapamycin protects Jurkat cells from FAS/APO-1 death receptor activation or mitochondrial stress induced apoptosis (Fumarola et al., 2005). Furthermore, rapamycin restores AKT signaling and NK-κB activation in *tsc2*-deficient cells, protecting them from DNA-damaged induced cell death (Ghosh et al., 2006). Although the release of AKT from feedback inhibition by an mTORC1 inhibitor is a legitimate concern, there is currently no clinical data corroborating this suspicion.

The finding that prolonged rapamycin treatment inhibits mTORC2 assembly and AKT phosphorylation in some cell types suggests the intriguing and perhaps provocative idea that some clinical responses

to rapamycin result from inhibiting both mTORCs. A recent study finds that rapamycin curtails progression of tobacco carcinogen-induced tumors in mice, and this too correlates with reduced AKT S473 phosphorylation (Granville et al., 2007). The implications of this and aforementioned observations are important since current rapamycin trials are based on the premise that rapamycin is an mTORC1-specific inhibitor. The absence of biomarkers to predict in which cells mTORC2 is sensitive to prolonged rapamycin exposure, and the fact that mTORC2 inhibition by rapamycin is inseparable from mTORC1 inhibition, currently complicates our understanding of this phenomenon. While mysterious, this idiosyncrasy in the mechanism of function of rapamycin may provide a valuable clue to finding cancers that will respond to the drug.

Dual inhibition of the PI3K pathway or other signaling pathways and mTOR could be an effective strategy (Fan and Weiss, 2006; Wan et al., 2007). This strategy avoids the potential consequences of disengaging the feedback loop. Drugs currently being considered for combination therapy include gefitinib (Iressa, an EGFR inhibitor), imatinib mesylate (Gleevec, a BCR-ABL inhibitor), tamoxifen (estrogen receptor modulator), cisplatin (DNA damaging agent), and paclitaxel (microtubule stabilizer) (reviewed in Faivre et al., 2006; Granville et al., 2006). Perhaps a more versatile drug would be an ATP-competitive mTOR inhibitor. A molecule of this nature would have the distinct advantage of inhibiting the full gamut of mTOR catalytic activities, although it is unclear if such a drug could be tolerated. Another strategy is to obstruct the binding of mTOR-interacting proteins, but the lack of mTORC structural information is a major challenge to developing this class of inhibitors.

Because AKT activation is widespread in cancer, there is also a rationale for developing an mTORC2-specific inhibitor. Such a molecule might be well tolerated since decreasing mTORC2 activity seems to inhibit transformed cells more severely than MEFs (Guertin et al., 2006b; Hresko and Mueckler, 2005; Jacinto et al., 2006; Sarbassov et al., 2005b). The finding that TORC2 is not essential in *Drosophila*, but becomes essential for phenotypes dependent on elevated dPI3K activity, further suggests the possibility that mTORC2 inhibitors might have therapeutic potential, particularly in cancers "addicted" to elevated PI3K signaling (Hietakangas and Cohen, 2007). While it is exciting to speculate on strategies to target mTOR in cancer, it is clear from several recent studies that many mysteries must be solved so that these strategies can be rationally designed.

The Evolution of mTOR Signaling

As judged by the role of TORC1 in yeast, mTORC1 is an ancient controller of cell growth that is regulated by nutrients. Interestingly, TSC1/2-dependent regulation of TOR is intact in fission yeast (*S. pombe*) but not in budding yeast (*S. cerevisiae*), suggesting *S. pombe*

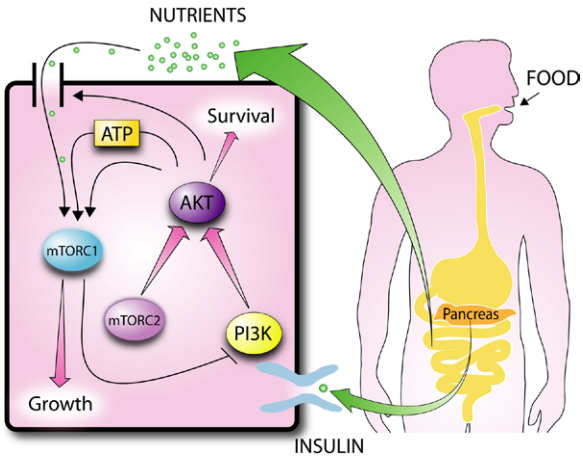

Figure 4. Linking Cell Autonomous and Systemic Nutrient Sensing by mTOR

mTORC1 is an ancient regulator of cell growth that is activated by intracellular nutrients. The ancient function of mTORC2 is unclear, but it may have evolved to indirectly sense nutrients by way of insulin signaling. Circulating glucose triggers the release of insulin into the bloodstream. In peripheral tissues harboring growth factor responsive cells, insulin activates the PI3K-mTORC2-AKT pathway. In individual cells, activation of AKT promotes survival, nutrient influx, and energy (ATP) generation. Signals from intracellular nutrients, energy, and from AKT itself subsequently activate mTORC1, which drives protein synthesis and promotes cell growth. Negative feedback mechanisms modulate PI3K-AKT activity, which may serve to balance nutrient intake with expenditure. Since all cells are not equally response to insulin or nutrients, cells originating from diverse tissues may have differential requirements for each mTOR complex.

may hold important clues to how TOR signaling evolved (Urano et al., 2005; Uritani et al., 2006). Although the ancient function of mTORC2 is unclear, it too may have evolved from a nutrient-sensing pathway that became rewired through evolution to indirectly sense nutrients by way of growth factors. When elevated glucose levels are detected in the bloodstream, the pancreas secretes insulin, which activates the PI3K-mTORC2-AKT pathway in peripheral tissues. AKT, in turn, suppresses apoptosis, promotes an influx of glucose and amino acids into the cell, stimulates ATP production, and perhaps relieves mTORC1 from TSC1/2- and/or PRAS40-dependent inhibition. Incoming nutrients subsequently activate the mTORC1-S6K1 growth engine. By way of negative feedback mechanisms, mTORC1 inhibits the insulin receptor-PI3K pathway, balancing nutrient intake with expenditure. The mTORC2-AKT circuit could therefore be an important link between cell autonomous and systemic nutrient sensing by mTOR (Figure 4). Of course, not all cell types are equally responsive to growth factor stimulation or nutrient uptake and there could be different tissue-specific requirements for mTORC2 signaling. It would be interesting to compare the role of the mTORCs in cancer cells originating from tissues with different sensitivities to insulin and nutrients, or in cancer cells with mutations in different growth factor signaling pathways.

Conclusion

Despite knowing about mTOR for nearly 15 years, we are just beginning to appreciate the complexity of the mTOR network. Since AKT activates mTORC1 by phosphorylating and inhibiting TSC1/2, and mTORC2 phosphorylates and activates AKT, mTOR may function both upstream and downstream of AKT. Defining these complex and perhaps cell-type-specific connections between mTORC1 and mTORC2 is an important challenge for the future. It is also becoming clear that the mTORC1 inhibitor rapamycin has an unforeseen capability to inhibit mTORC2, but only in a subset of cells. The dual sensitivity of the mTORCs to rapamycin is particularly evident in endothelial cells, which is emphasized by the antiangiogenic property of rapamycin. Collectively, these findings are changing the view of the pathological role that mTOR plays in cancer and opening the door to new therapeutic strategies.

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