

# Integration of Growth Factor and Nutrient Signaling: Implications for Cancer Biology

## Review

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Signaling networks that promote cell growth are frequently dysregulated in cancer. One regulatory network, which converges on effectors such as 4EBP1 and S6K1, leads to growth by promoting protein synthesis. Here, we discuss how this network is regulated by both extracellular signals, such as growth factors, and intracellular signals, such as nutrients. We discuss how mutations amplifying either type of signal can lead to tumor formation. In particular, we focus on the recent discovery that a tumor suppressor complex whose function is lost in tuberous sclerosis patients regulates the nutrient signal carried by the critical signaling protein TOR to the effectors 4EBP1 and S6K1. Finally, we describe how the small molecule rapamycin, which inhibits TOR and thereby the activation of these effectors, could be useful to treat tumors that have become dependent upon this pathway for growth.

### Introduction

The next generation of cancer chemotherapy aims at targeting the oncogenic or tumor suppressor pathway dysregulated in a particular tumor. This approach appears promising given an emerging concept that tumors often become dependent upon dysregulated signaling pathways and hence become hypersensitive to down-regulation of these signals (Weinstein, 2002). For example, tumors resulting from overexpression of the c-Myc protein in mice are highly sensitive to even transient inactivation of the oncogene (Jain et al., 2002). Normal cells, which are not as dependent on the dysregulated pathway, are relatively less affected by such targeted therapy. Early successes exploiting this principle include Gleevec (a small molecule inhibitor of the oncogenic tyrosine kinase BCR-Abl) for the treatment of chronic myelogenous leukemia and Herceptin (an antibody inhibitor of oncogenic tyrosine kinase Her2) for the treatment of certain breast carcinomas that overexpress Her2 (Shawver et al., 2002).

Understanding how different signaling pathways interact to form networks will expand the therapeutic potential of treatments that exploit the dependent phenotype of cancer. In addition to developing drugs targeting

the dysregulated pathway directly, it may be possible to develop drugs targeting parallel pathways that converge on common critical effectors. This principle is becoming evident from studies of the regulation of cell growth (size), which is under the control of both extracellular signals, such as growth factors, and less-understood intracellular signals, such as nutrients. Both types of signals regulate a common set of effectors involved in control of cell growth, including the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP1) and the S6 ribosomal protein kinase 1 (S6K1). Mutations amplifying the growth factor signals upstream of 4EBP1 and S6K1, such as loss of the tumor suppressor PTEN (phosphatase and tensin homolog), have been commonly observed in numerous types of cancer (Vogt, 2001). PTEN is mutated in Cowden's disease, which is characterized by benign sebaceous (oil gland) tumors, as well as in gastrointestinal and breast carcinomas (Cantley and Neel, 1999). Recent evidence now suggests that mutations amplifying the nutrient signal to 4EBP1 and S6K1 can also lead to tumor formation, although the tumors are typically benign.

In this review, we discuss how 4EBP1 and S6K1 are effectors for a growth factor- and nutrient-regulated signaling network containing the signaling proteins phosphatidylinositol-3-kinase (PI3K) and target of rapamycin (TOR). We discuss how dysregulation of either the growth factor signal or the nutrient signal leads to uncontrolled growth. We focus on recent discoveries relating to the nutrient-regulated pathway of growth activation in the onset of tuberous sclerosis, a disease characterized by the appearance of benign tumors called hamartomas. Finally, we discuss how small molecules such as rapamycin that block the PI3K/TOR network from activating its effectors show promise in treating tumors resulting from dysregulation of either the growth factor or the nutrient signal, both of which are characterized by dependence on the PI3K/TOR network for growth.

### 4EBP1 and S6K1 Promote Growth in Response to Both Growth Factors and Nutrients

Mutations in signaling proteins regulating cell growth, in addition to those regulating cell cycle progression, contribute to the formation of tumors. In particular, mutations leading to activation of protein synthesis by affecting factors such as eIF4E/4EBP1 and S6K1 are commonly observed in several types of cancer (Vogt, 2001). When hypophosphorylated, the translational repressor 4EBP1 binds tightly to eIF4E, preventing proper formation of the eIF4 translation initiation complex at the 5' end of cap-bearing mRNAs (Gingras et al., 2001). Following multisite phosphorylation of 4EBP1, eIF4E is released, allowing it to associate with eIF4G and other relevant factors to promote cap-dependent translation. Overexpression of eIF4E in mammalian cell culture is sufficient to transform rodent fibroblasts (Lazaris-Karatzas et al., 1990) and cause increases in cell size (Fingar et al., 2002), which is reversed by cooverexpression

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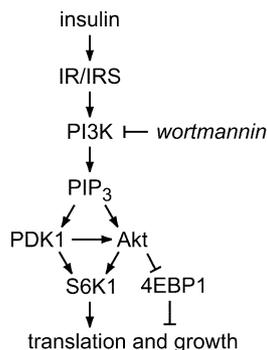


Figure 1. Insulin Signals to 4EBP1/S6K1 via the PI3K Signaling Pathway

Binding of insulin to the insulin receptor leads to phosphorylation of an associated IRS protein. PI3K binds to the phosphorylated IRS via its SH2 domain and phosphorylates PIP<sub>2</sub> in the inner leaflet of the membrane to form PIP<sub>3</sub>. PIP<sub>3</sub> recruits PDK1 and Akt to the membrane via their PH domains. PDK1 both phosphorylates S6K1 directly, and phosphorylates Akt, which signals to 4EBP1/S6K1 through an independent mechanism. This response to insulin is blocked by wortmannin, a potent inhibitor of PI3K.

of 4EBP1. These experiments are consistent with the finding that overexpression of eIF4E is observed in several types of cancer, including lymphomas, cancers of the head and neck, and colon carcinomas (Vogt, 2001).

Overexpression of S6K1 also causes increased cell size in mammalian cell culture, but the precise mechanism for this effect is unclear (Fingar et al., 2002). Upon phosphorylation, S6K1 was initially thought to promote the translation of mRNAs containing a 5' oligopyrimidine tract (TOP) in response to growth factors by phosphorylating the ribosomal S6 protein (Grammer et al., 1996). These mRNAs encode components of the translational machinery, which are presumably required to sustain the increased biosynthesis needed for cell growth in response to growth factors (Grammer et al., 1996). However, recent data suggest that ribosomal S6 phosphorylation by S6K1 is not acting alone in this growth pathway as growth factors are able to stimulate translation of 5' TOP-containing mRNAs even if S6K1 and S6 phosphorylation are inhibited by treatment with a small molecule inhibitor of the pathway (Stolovich et al., 2002). Indeed, several other targets of S6K1 have been identified, such as the antiapoptotic protein BAD (Harada et al., 2001), the transcription factor CREM- $\tau$  (de Groot et al., 1994), and the translational regulator eEF2 kinase (Wang et al., 2001). Further investigation will be required to determine the relative importance of these targets in S6K1-mediated cell growth.

Appropriate regulation of 4EBP1 and S6K1 during normal cell growth is achieved by controlling the levels of extracellular signals such as the hormone insulin (Gingras et al., 2001; Grammer et al., 1996). For example, by binding to the insulin receptor on the cell surface, insulin causes the rapid phosphorylation of both 4EBP1 and S6K1, setting the stage for increased protein synthesis (Figure 1). 4EBP1 contains at least six phosphorylation sites (Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112), two of which (Ser65 and Ser70) are strongly stimulated by serum in many cell types (Gingras et al.,

2001). S6K1 contains at least nine phosphorylation sites (Ser17, Thr229, Ser371, Thr389, Ser404, Ser411, Ser418, Thr421, and Thr424), the latter eight of which are responsive to serum (Grammer et al., 1996; Volarevic and Thomas, 2001). The role for PI3K in mediating signals from insulin to these effectors became evident from a combination of biochemical and chemical genetic experiments. Engagement of insulin receptor by insulin normally leads to recruitment of the IRS-1 protein, which subsequently recruits PI3K (Figure 1). Mutations in IRS-1 that block recruitment of PI3K also block activation of S6K1 by insulin (Grammer et al., 1996). Furthermore, treatment of cells with the small molecule wortmannin, which inhibits PI3K directly at nanomolar concentrations, also blocks the insulin signal to 4EBP1 (Ser65 and Thr70) and S6K1 (Thr229, Thr389, Ser404, and Ser411) (Gingras et al., 2001; Grammer et al., 1996). Active PI3K subsequently converts phosphatidylinositol-4,5-phosphate (PIP<sub>2</sub>) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP<sub>3</sub>), which binds kinases containing a plextrin-homology domain such as phosphoinositide-dependent kinase 1 (PDK1) and Akt, and recruits them to the membrane. PDK1 both phosphorylates S6K1 directly and activates Akt, which signals to 4EBP1 and S6K1 through a mechanism not completely understood (Kozma and Thomas, 2002) (Figure 1).

More than a decade after the connection between growth factors and S6K1 was characterized, recent studies have revealed that the activation of 4EBP1 and S6K1 by insulin is subject to an additional regulatory mechanism, one sensitive to the presence of nutrients. Starvation of cells for amino acids, glucose, or ATP (by inhibition of glycolysis), and the induction of mitochondrial dysfunction by various chemical agents, have both been reported to inhibit the growth factor-induced activation of 4EBP1 and S6K1 (Dennis et al., 2001; Desai et al., 2002; Hara et al., 1998; Xu et al., 2001). That the status of nutrients can impinge on growth-promoting pathways in mammalian cells is consistent with similar findings in yeast (Tapon et al., 2001). Indeed, starvation inhibits both the transcription and translation of *CLN3*, a cyclin that triggers the G1/S transition in *S. cerevisiae* (Tapon et al., 2001). In mammalian cells, such a nutrient-sensing mechanism would serve to prevent activation of cell growth in response to extracellular signals during times when growth could not be sustained due to lack of nutrient resources. Ultimately, our ability to integrate such a nutrient-sensing mechanism into the molecular model of 4EBP1 and S6K1 regulation by growth factors relied heavily on work examining the small molecule rapamycin and its modulation of the mammalian TOR (originally named FRAP or RAFT1), the founding member of the PI3K-related family of kinases (Kuruvilla and Schreiber, 1999).

Previous studies showed that treatment of cells with the small molecule rapamycin causes dephosphorylation of 4EBP1 and S6K1, even in the presence of activating extracellular signals—a dominant inhibitory effect (Kuruvilla and Schreiber, 1999). Rapamycin binds the immunophilin FK506 binding protein (FKBP12) to form the FKBP12-rapamycin complex, which then complexes with TOR to inhibit its function, somehow blocking activation of its downstream effectors by insulin (Grammer et al., 1996). Formation of the FKBP12-rapamycin-TOR

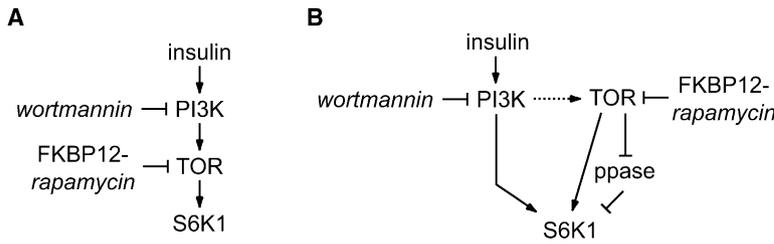


Figure 2. Possible Models for How S6K1 Is Regulated by Both PI3K and TOR

(A) In a linear signaling model, TOR mediates the entire signal from PI3K to S6K1. If this were the case, then any allele of S6K1 should respond identically to treatment with wortmannin and treatment with rapamycin. Experimental evidence is not consistent with such a pure linear model.

(B) In a parallel signaling model, TOR and PI3K mediate independent signals to S6K1.

Experimental evidence suggests the existence of such parallel inputs. The dashed arrow indicates that a combination of the linear and parallel models may exist. Although the dominant mechanism by which TOR signals to S6K1 is unclear, arguments have been presented for both direct regulation (arrow) and signaling via a phosphatase (T bars).

ternary complex results in the dephosphorylation of Ser65 and Thr70 in 4EBP1, and the dephosphorylation of several residues in S6K1 (Thr389, Thr229, and Ser404), though Thr389 is thought to be the crucial residue for regulation by TOR (Gingras et al., 2001; Grammer et al., 1996). Treatment with rapamycin, however, does not interfere with events that occur early after binding of insulin to its receptor, such as activation of PI3K or Akt (Grammer et al., 1996). These data give rise to two possible models for how TOR functions in the network. A simple and prevalent model accounting for these observations places TOR downstream of PI3K/Akt in a linear fashion (Figure 2A). This model is consistent with the existence of an insulin-sensitive phosphorylation site in TOR (Nave et al., 1999). However, an alternative model places insulin and TOR in parallel pathways, both of which are required to activate S6K1. Several detailed studies of S6K1 regulation are consistent with the latter model. One such study showed that an allele of S6K1 truncated at both the N and C termini is resistant to treatment with rapamycin but still sensitive to inhibition of insulin signaling at the level of PI3K by treatment with wortmannin (Grammer et al., 1996). This result is inconsistent with the simple linear model (Figure 2A): if the entire signal from PI3K is carried by TOR, then a rapamycin-resistant mutant of S6K1 should also be resistant to inhibition of PI3K by wortmannin. However, the fact that the mutant is still sensitive to wortmannin implies that PI3K signals to S6K1, at least in part, via a pathway independent of TOR.

Thus, we and others believe that TOR does not carry the complete signal from insulin to S6K1 but rather constitutes a parallel input (Figure 2B) (Kozma and Thomas, 2002). The mechanism by which TOR permits signaling to S6K1 remains uncertain; however, evidence has been presented for two models. (1) TOR may restrain a PP2A-like phosphatase. Evidence supporting this model is that phosphatase inhibitors partially suppress the inhibition of S6K1 by rapamycin and a TOR-regulated and S6K1-associated phosphatase fails to associate with the doubly truncated allele of S6K1, which is rapamycin resistant (Peterson et al., 1999). (2) TOR may directly phosphorylate S6K1, initially suggested by in vitro phosphorylation data (Burnett et al., 1998). Recent work by Schalm and Blenis (2002) suggests that TOR may even signal to S6K1 through a combination of the above two mechanisms (Figure 2B).

A more detailed picture of how TOR may directly phosphorylate its effectors 4EBP1 and S6K1 has emerged

from the study of the TOR-associated protein, raptor. In 2002, several groups identified raptor as a protein coimmunoprecipitating with TOR that is required for proper TOR signaling in both mammalian cells, *C. elegans*, and *S. cerevisiae* (Abraham, 2002; Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Nojima et al., 2003). In vitro, raptor is required for phosphorylation of 4EBP1 by TOR and stimulates TOR-catalyzed phosphorylation of S6K1 ~5-fold (Hara et al., 2002). In cells, depletion of raptor by siRNA blunts the increases in S6K1 activity and cell size normally observed in response to nutrients (Kim et al., 2002). Since raptor interacts directly with 4EBP1 and S6K1, it was proposed that raptor acts as a scaffold bridging TOR and its putative targets for phosphorylation (Abraham, 2002). In another study, Schalm and Blenis (2002) identified a region conserved between 4EBP1 and S6K1 termed the TOR signaling (TOS) motif, mutation of which abrogates the sensitivity of these effectors to signals from TOR. Several groups have now converged on the finding that raptor interacts with the TOS motif of S6K1 and 4EBP1 (and with an additional domain of 4EBP1) in order to bring TOR in close proximity to its targets (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). These findings may explain why the doubly truncated allele of S6K1 is resistant to rapamycin, as the allele lacks the TOS motif found in the N terminus of the protein and is presumably unable to interact with raptor and TOR (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003).

Since its discovery, the doubly truncated allele of S6K1 has served as a tool for determining whether newly identified signals regulating S6K1 could be relayed by TOR. Unlike the wild-type S6K1, the truncated protein was found to be resistant to inhibition by nutrient stresses such as amino acid starvation and mitochondrial dysfunction in the same way that it is resistant to treatment with rapamycin (Desai et al., 2002; Hara et al., 1998). This resistance is consistent with a model in which the nutrient signals regulating S6K1 are carried by TOR, although the formal possibility exists that the signals are mediated by another effector to which the doubly truncated S6K1 allele is also resistant. However, the fact that nutrients modulate a phosphorylation site within TOR (Ser2448) argues that TOR itself receives signals from nutrients, consistent with a model in which it relays the nutrient signal to effectors such as 4EBP1/S6K1 (Nave et al., 1999). Thus, a model emerged in which cell growth effectors such as 4EBP1 and S6K1 lie at the intersection of two regulatory signals: a growth factor

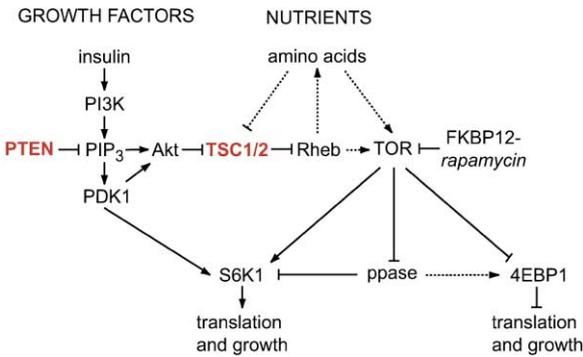


Figure 3. A Model for the Dual Regulation of 4EBP1/S6K1 by Growth Factors and Nutrients

Growth factors signal to 4EBP1/S6K1 via PI3K, as depicted in Figure 1. Nutrients signal to 4EBP1/S6K1 via TOR, independently from PI3K. We refer to this as the PI3K/TOR network. The PTEN tumor suppressor restrains growth factor signals to 4EBP1/S6K1, and loss of PTEN function leads to hyperactivation of S6K1 and tumor formation. The TSC1/2 tumor suppressors restrain nutrient signals to 4EBP1/S6K1, and loss of TSC1/2 function leads to hyperphosphorylation of 4EBP1/S6K1 and tumor formation. The TSC proteins are themselves regulated by growth factor signals, constituting a point of crosstalk between the growth factor-regulated and nutrient-regulated signaling. A curious feature of the network is the predominance of activating steps on the growth factor pathway (symbolized by the arrows) and inhibiting steps on the nutrient pathway (symbolized by the T bars), perhaps reflecting a braking mechanism to permit growth factor signaling under nutrient-rich conditions but to dominantly override growth factor signaling under nutrient-poor conditions.

signal carried by PI3K (and potentially in part by TOR) and a nutrient signal (amino acids, glucose) carried by TOR (Figure 3). Although 4EBP1 and S6K1 have been well characterized, it is possible that the PI3K/TOR network regulates additional effectors to control cell growth.

The importance of the PI3K/TOR network in regulating cell growth and division has been effectively demonstrated in *Drosophila* (Kozma and Thomas, 2002). The PI3K/TOR network is complex in *Drosophila*, and it differs from the mammalian network in some respects. For example, loss of Akt or PI3K does not affect S6K1 in *Drosophila*, although PDK1 signals to S6K1 in both organisms (Kozma and Thomas, 2002; Radimerski et al., 2002). 4EBP1, however, may be controlled by Akt in *Drosophila* as in the mammalian network (Kozma and Thomas, 2002; Miron et al., 2001). Nevertheless, the major function of PI3K/TOR network, integrating signals from both growth factors and nutrients to regulate growth, is conserved in *Drosophila*. Both mutations in components mediating the growth factor signal (Chico [IRS-1 in *Drosophila*], dPI3K, dAkt, dPTEN) and perturbations of nutrient signal (mutations in *Drosophila* TOR or by starvation directly) affect cell size and number (Kozma and Thomas, 2002). Both the loss of S6K1 and overexpression of 4EBP1 variants that more strongly inhibit eIF4E lead to reduction in cell size (Kozma and Thomas, 2002). The conservation of such growth phenotypes in higher organisms (Fingar et al., 2002; Kozma and Thomas, 2002) leaves little surprise that dysregulation of either regulatory network could contribute to the formation of many tumors.

### Dysregulation of the Growth Factor Signal to Growth and Its Role in Disease

Cowden's disease is characterized by the formation of hamartomas in numerous sites, including the breast, skin, thyroid, and intestine (Cantley and Neel, 1999). Those afflicted with Cowden's are also predisposed to develop other cancers, including breast cancer, thyroid carcinoma, and meningiomas (Cantley and Neel, 1999). The disease occurs in patients with a germline mutation in a tumor suppressor called PTEN, which serves to attenuate the growth-promoting signal from insulin to 4EBP1 and S6K1 carried by PI3K (Figure 3). PTEN dephosphorylates the same position of the phosphatidylinositol ring phosphorylated by PI3K, thereby inhibiting the subsequent membrane recruitment and activation of Akt and PDK1 (Figure 3). This mechanism ultimately acts to diminish translation and growth by effectors downstream of PI3K (Figure 3). Loss of PTEN function thus leads to increased activation of this process and uncontrolled growth.

Loss of the PTEN tumor suppressor is not unique to Cowden's disease. Germline mutations in PTEN also lead to Bannayan-Riley-Ruvalcaba syndrome and Proteus syndrome, diseases characterized by aberrant growth and, in some cases, massive hamartoma formation (Cantley and Neel, 1999; Smith et al., 2002). The breadth of tissues affected by loss of PTEN is highly evident in Proteus patients, who often display macrocephaly, lipomatosis, and hemangiomas, as well as enlargement, disfigurement, and asymmetry of the skull, hands, and limbs (Biesecker et al., 1999). In addition to the aforementioned hamartomatous syndromes, PTEN deficiency resulting from somatic mutation is also commonly observed in prostate cancer, glioblastoma, and endometrial tumors, underscoring its importance in restraining the positive signals from growth factors (Mills et al., 2001). As Cantley and Neel (1999) discuss, it is not clear whether different types of PTEN mutations are responsible for the diversity of resulting disease phenotypes; indeed, mouse studies suggest an important role for genetic background in determining the outcome of PTEN mutation.

It is unlikely that the eIF4E/S6K1 activation, resulting from loss of PTEN, is itself oncogenic. Indeed, overexpression of S6K1 leads to changes in size and morphology without affecting proliferation (Vogt, 2001). Rather, 4EBP1 and S6K1 are likely two of several targets of Akt signaling contributing to tumor progression. In addition to promoting growth, Akt promotes cell survival in part by phosphorylating and inhibiting the proapoptotic BAD and FKHR proteins, and promotes cell proliferation in part by phosphorylating and inhibiting GSK3 $\beta$  to prevent degradation of cyclin D1 (Vivanco and Sawyers, 2002). Thus, increased levels of Akt activity, resulting from loss of PTEN function or overexpression of Akt itself, lead to tumor progression by a combination of mechanisms.

Recent studies provide preliminary data demonstrating the hypersensitivity of PTEN-deficient tumors to inhibition of TOR by a rapamycin derivative (CCI-779) currently under examination as an anticancer agent (Mills et al., 2001; Neshat et al., 2001; Podsypanina et al., 2001). The rapamycin derivative selectively inhibited the proliferation and reduced the size of PTEN null cells as compared to PTEN-wild-type cells, even though the

downstream effector S6K1 was efficiently inhibited by the drug in both backgrounds (Neshat et al., 2001). This hypersensitivity of PTEN null cells might be understood in the context of recently proposed models for how cancer cells can become dependent on dysregulated signaling pathways (Weinstein, 2002). Oncogene expression or tumor suppressor loss is not tolerated by all cell types (Weinstein, 2002). In the case of c-Myc, overexpression of the oncogene can actually cause cell death (Pelengaris et al., 2002). One speculative model of cancer that we favor is that certain cells are able to escape potentially deleterious effects and thrive in the environment of the cancer-causing alteration. Such surviving cells, which ultimately give rise to the tumor, have likely undergone additional genetic or epigenetic alterations to balance the effects and potential toxicity of the original cancer-causing alteration. These adapted cells are then hypersensitive relative to wild-type cells to modulation of the original dysregulated pathway as it both disturbs their newly achieved balance and removes their growth advantage.

In the case of PTEN, loss of the tumor suppressor is likely to be tolerated by cells because of the resulting antiapoptotic signals via Akt. However, Mills et al. (2001) provide a useful speculative model for how loss of PTEN can give rise to a situation similar to that described above, wherein additional mutations lead to the hypersensitive state. They suggest that the genomic instability necessary for tumor initiation and progression might lead to the dysregulation of additional signaling pathways. Such events might normally lead to cell death but are tolerated in the environment of the PTEN-deficient cell, or they might affect pathways that perform functions redundant to those of the PI3K/Akt pathway and, hence, would not be detrimental to a cell in which the pathway is hyperactivated. In both of these scenarios, targeting the PTEN pathway with CCI-779 should selectively affect the cancer cells by revealing either the toxicity of additional mutations or the lack of redundant pathways necessary for compensation.

Interestingly, the only PTEN wild-type cell line hypersensitive to the rapamycin variant contains constitutively elevated Akt3 expression and TOR phosphorylation. One would expect these cells to be similar to cells lacking PTEN, which display increased Akt activity. Thus, the cell line could also have become dependent upon elevated signaling via the PI3K/TOR network for growth (Neshat et al., 2001). These studies suggest that rapamycin and its analogs could potentially be useful to inhibit progression of tumors carrying mutations leading to amplification of PI3K or Akt even if PTEN is wild-type and expressed at normal levels. Importantly, such mutations have been found in a variety of cervical, ovarian, and pancreatic cancers, suggesting that rapamycin analogs should be investigated in such malignancies (Mills et al., 2001; Vivanco and Sawyers, 2002; Vogt, 2001).

#### **Dysregulation of the Nutrient Signal to Growth and Its Role in Disease**

Unlike the components mediating the growth factor signal to 4EBP1 and S6K1, the machinery regulating the nutrient signal via TOR has remained largely uncharac-

terized until recently. In 2002, several research groups converged upon the tumor suppressors TSC1 and TSC2 (or hamartin and tuberlin, respectively) as candidate regulators of this key nutrient status signal. TSC2 encodes a putative GTPase-activating protein (GAP), while TSC1 contains coiled-coils but has no known enzymatic activity; the two proteins exist as a heterodimer (Cheadle et al., 2000). Loss-of-function mutations in TSC1 and TSC2 lead to the autosomal dominant disorder tuberous sclerosis, a disease characterized by the formation of hamartomas commonly of the brain, heart, skin, and kidney (Cheadle et al., 2000). Such mutations serve as the first demonstration that dysregulation of nutrient-sensitive pathways, like dysregulation of growth factor-sensitive pathways, can lead to uncontrolled growth and tumor formation.

In certain regards, loss of TSC1/2 function phenocopies loss of PTEN, the tumor suppressor regulating growth factor signals to effectors of growth. For example, in *Drosophila*, both TSC1/2 and PTEN normally antagonize insulin signaling, and mutations in either lead to increased cell size (Kozma and Thomas, 2002); in humans, mutations in either give rise to tumor formation including hamartomas (Cheadle et al., 2000; Mills et al., 2001); in both organisms, mutations in either lead to increased activation of S6K1 while overexpression of either leads to its inhibition. The phenotypes of these mutations diverge under conditions of nutrient starvation, however. Whereas depletion of TSC1/2 function by RNAi in *Drosophila* leads to an activation of S6K1 that is resistant to inhibition by amino acid starvation, the activation of S6K1 observed following similar depletion of PTEN function remains highly sensitive to amino acid starvation (Gao et al., 2002). The resistance of TSC1/2 mutants to the starvation signal was further confirmed in mammalian cells (Gao et al., 2002), and it was additionally shown that overexpression of TSC proteins abrogates nutrient-induced activation of S6K1 (Inoki et al., 2002). Thus, while PTEN functions as a tumor suppressor by attenuating growth-promoting signals from growth factors, the TSC1/2 complex appears to function as a tumor suppressor by attenuating growth-promoting signals from nutrients (Figure 3).

Further biochemical and genetic experiments provide correlative evidence that the TSC proteins act upstream of TOR in the nutrient-regulated network. First of all, the increased 4EBP1 and S6K1 phosphorylation of TSC mutants is still sensitive to rapamycin, implying that TSC1/2 are either upstream of TOR or in a parallel pathway. The dephosphorylation of S6K1 observed following TSC overexpression occurs at the rapamycin-sensitive site Thr389, but not the rapamycin-insensitive sites Thr421 or Ser424, consistent with a role for TOR in the TSC mechanism (Inoki et al., 2002). Furthermore, unlike the wild-type allele of S6K1, rapamycin-resistant mutant alleles are resistant to the inhibition caused by overexpression of the TSC proteins, again suggesting that TSC signaling is mediated by TOR (Inoki et al., 2002; Tee et al., 2002). It is worth noting, however, that although two research groups have reported this latter finding (Inoki et al., 2002; Tee et al., 2002), a third group has reported the opposite result (Jaeschke et al., 2002). As Manning and Cantley (2003) suggest, the discrepancy could be due to differences in S6K1 alleles used in these studies

or the fact that the third group used histone H2B as a substrate for the S6K1 kinase assays, while the other two used recombinant S6 protein. Genetic analysis reveals that phenotypes caused by TSC mutations in *Drosophila* such as increased cell size are alleviated by subsequent deletion of one TOR allele, suggesting that TOR could be downstream of the TSC proteins (Gao et al., 2002). This is further suggested by the fact that overexpression of TSC1/2 leads to reduced phosphorylation of TOR itself at Ser2448 while depletion of TSC2 by RNAi increases TOR phosphorylation (Inoki et al., 2002). This site on TOR is reportedly dephosphorylated by amino acid starvation and phosphorylated by Akt in response to insulin (Nave et al., 1999; Sekulic et al., 2000). And finally, the effects of TSC mutations and PTEN mutations on cell size are additive, suggesting that the TSC complex does not function within a linear pathway from insulin to 4EBP1 and S6K1, but rather regulates a parallel input such as the nutrient signal (Gao and Pan, 2001) (Figure 3).

Although the mechanism by which TSC1/2 actually signals to TOR remains unclear, recent studies have identified the small GTPase protein Rheb (Ras homolog enriched in brain) as a mediator of the signal (Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003) (Figure 3). This finding is particularly exciting as it accounts for the importance of the GAP activity of TSC2, loss of which is observed in mutant alleles of TSC2 known to give rise to tuberous sclerosis (Maheshwar et al., 1997). Additionally, Rheb is known to be overexpressed in several tumor cell lines (Gromov et al., 1995), and overexpression of Rheb can cause the transformation of mouse fibroblasts (Yee and Worley, 1997). Recently, Rheb was isolated in both loss-of-function and gain-of-function screens for genes regulating cell growth in *Drosophila* (Saucedo et al., 2003; Stocker et al., 2003). Loss of Rheb leads to reduced cell size (and number) and to reduced S6K1 activity, while overexpression of Rheb has the opposite effect (Saucedo et al., 2003; Stocker et al., 2003). Of 18 GTPases examined, only depletion of Rheb by RNAi caused loss of S6K1 phosphorylation, further suggesting the physiological relevance and specificity of its effects on the network (Zhang et al., 2003).

Further experiments suggest that Rheb functions downstream of the TSC1/2 complex in the PI3K/TOR signaling network (Figure 3). First, Rheb is genetically epistatic to TSC2: loss of Rheb blocks the increased cell growth and increased S6K1 activity caused by loss of TSC2 (Saucedo et al., 2003; Stocker et al., 2003). Second, biochemical work performed in parallel with the genetic screens suggests that Rheb is a relevant small GTPase target for the GAP activity of TSC2 in both *Drosophila* and mammalian cells. Working in *Drosophila*, Zhang et al. (2003) show that TSC2 promotes the GTPase activity of Rheb in vitro but not that of the close homolog Ras1. This result was dependent on an intact GAP domain of TSC2, as catalytically inactive TSC2 mutants do not affect Rheb. Furthermore, they demonstrate that cooverexpression of TSC1/2 reduced the GTP loading of Rheb, again dependent on a functional TSC2 GAP domain (Zhang et al., 2003). Working in mammalian cells, several groups arrive at similar conclusions (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Thus, Rheb

has emerged as a potential direct target of the TSC complex in the signaling network regulating cell growth.

Like the TSC1/2 proteins, Rheb appears to impinge on the nutrient-regulated signal to growth. The status of Rheb is dominant to the status of nutrients in controlling the activation of S6K1. Even in the absence of the nutrients, overexpression of Rheb is able to promote S6K1 activity and cell growth (Garami et al., 2003; Stocker et al., 2003; Zhang et al., 2003). Further experiments are consistent with a model in which Rheb functions upstream of TOR in controlling the nutrient signal of the network (Figure 3). The increase in cell size caused by Rheb overexpression is abrogated by mutation of TOR, and the increase in S6K1 activity caused by Rheb overexpression remains sensitive to rapamycin (Garami et al., 2003; Inoki et al., 2003; Stocker et al., 2003; Tee et al., 2003; Zhang et al., 2003). Furthermore, overexpression of Rheb does not lead to activation of a rapamycin-resistant allele of S6K1, suggesting that Rheb signals to S6K1 through TOR (Tee et al., 2003). Finally, like depletion of TSC2, overexpression of Rheb increases the phosphorylation of TOR itself at Ser2448 (Inoki et al., 2003). Ultimately, the body of work placing TSC1/2-Rheb upstream of TOR has great implications for the treatment of tuberous sclerosis. It suggests that the hamartomas resulting from tuberous sclerosis will be dependent on the pathway activating TOR and its effectors for growth. Hence, like the tumors arising from mutation of PTEN, the hamartomas should be sensitive to treatment by rapamycin and its analogs. Indeed, preliminary evidence has shown that primary tumors from an established rat model are responsive to rapamycin, displaying increased apoptosis and arrest of proliferation (Kenerson et al., 2002).

#### TSC Proteins as a Point of Crosstalk between Growth Factor- and Nutrient-Regulated Signaling

Although the TSC1/2 proteins appear to modulate nutrient signals to TOR, several researchers have shown that they receive inputs from growth factors as well. A variety of biochemical and bioinformatic studies now point to TSC2 as a direct substrate for Akt and as a primary effector of Akt-mediated signaling to 4EBP1 and S6K1 (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002) (Figure 3). Understanding the nature of this crosstalk would increase our understanding of PTEN-deficient tumors, in which Akt is hyperactivated.

Researchers have established that phosphorylation of TSC2 by Akt leads to activation of 4EBP1 and S6K1 (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Indeed, mutating the phosphorylation sites to alanine blocks the ability of Akt overexpression to activate 4EBP1 and S6K1, whereas phosphomimetic mutations in TSC2 at the Akt-dependent sites lead to increased activation of the effectors (Inoki et al., 2002; Manning et al., 2002). Consistent with this model, tumor-derived cell lines deficient in PTEN tumor suppressor function, which have increased activation of Akt, also have increased phosphorylation of the tumor suppressor TSC2 (Manning et al., 2002). This suggests that PTEN deficiency leads to additional activation of S6K1 by impinging upon TSC2, the tumor suppressor of the nutrient-sensitive pathway containing TOR. This connection

might explain why PTEN-deficient tumors may be hypersensitive to inhibition of TOR by CCI-779, as TOR appears to act downstream of Akt via the TSC complex.

The mechanism by which phosphorylation of TSC2 by Akt leads to activation of S6K1 is unclear. It will be interesting to determine whether this affects its GAP activity and thereby its ability to regulate Rheb. Indeed, Garami et al. (2003) have shown that treatment with insulin increases GTP loading of Rheb. Two groups have shown that phosphorylation of TSC2 disrupts its association with TSC1 (Inoki et al., 2002; Potter et al., 2002). Other studies suggest that dissociation of TSC1 and TSC2 leads to ubiquitination/degradation of TSC2 via the proteasome (Dan et al., 2002; Plas and Thompson, 2003). This would have the effect of removing the inhibitory function of TSC and hence promoting signaling through TOR. However, contradictory data suggesting that the TSC1/2 association is not regulated by this process have also been presented (Dan et al., 2002; Manning et al., 2002). Four groups have shown that phosphorylation of TSC2 leads to association of 14-3-3 proteins with the TSC complex, which could potentially interfere with its function, its ability to be regulated by other signals, and other properties (Li et al., 2002; Liu et al., 2002; Nellist et al., 2002, 2003; Shumway et al., 2003). This model of signaling has been observed previously downstream of Akt, in which phosphorylation of the proapoptotic protein BAD or the FKHR transcription factor promotes their binding to 14-3-3 $\zeta$ , which inhibits apoptosis and transcription of proapoptotic transcripts, respectively (Brunet et al., 1999; Datta et al., 1997, 2000). However, the role for Akt-dependent phosphorylation sites on TSC2 in mediating binding to the 14-3-3 proteins is unclear as conflicting results have been presented (Manning and Cantley, 2003; Nellist et al., 2003). A combination of the above mechanisms likely exists to achieve tight regulation of TOR.

Thus, while the precise mechanism remains controversial, a reasonable model is that phosphorylation of TSC2 by Akt relieves inhibition of TOR by the TSC complex, thereby allowing phosphorylation of 4EBP1 and S6K1. A major question remains: How does the TSC1/2-Rheb branch interact with the nutrient signal? We do not know whether the TSC1/2-Rheb branch (1) senses amino acid starvation itself and signals to TOR in a linear fashion, or (2) simply impinges upon the sensing mechanism, affecting TOR function in a parallel pathway (Gao et al., 2002) (Figure 3). Xia et al. (2003) provided initial support for the first model by showing that glutamine causes a mild increase in TSC2 phosphorylation. However, the experiment should be performed with other amino acids such as leucine and arginine, which also potentially activate S6K1 (Hara et al., 1998). Additionally, it should be examined whether phosphomimetic mutations in TSC2, like depletion of TSC2 by RNAi, abrogate the ability of amino acid starvation to inhibit S6K1. These two experiments could demonstrate that: (1) the phosphorylation state of TSC2 is nutrient sensitive and (2) the phosphorylation state of TSC2 determines the nutrient signal to S6K1. Because starvation does not modulate the activity of Akt, the nutrient signal would have to affect TSC2 phosphorylation through a novel pathway, perhaps by blocking its ability to act as a substrate for Akt or by modulating the activity of a TSC2

phosphatase. It is also possible that amino acid starvation causes posttranslational modification of the TSC complex at a not yet characterized site.

Recent work by Zhang et al. (2003), however, suggests a different model. They reasoned that if indeed TSC1/2 sensed nutrients, it should regulate Rheb function in response to starvation. However, they show that amino acid starvation has no effect on the levels of GTP-loaded Rheb, suggesting that the GAP activity of TSC2 is not modulated by starvation. These data are consistent with the parallel model in which TOR senses nutrients independently of TSC1/2-Rheb. The mechanism by which Rheb controls TOR function remains unclear. Overexpressed TSC1/2 associate with TOR (Gao et al., 2002); Rheb may form part of this complex and affect TOR function either directly or via an associated factor, such as raptor. Another possibility that has been suggested is that Rheb may stimulate TOR by increasing the levels of amino acids within the cell itself (Saucedo et al., 2003). Rheb affects amino acid import in *S. cerevisiae*, although in the opposite direction as one would predict given the growth-promoting function of Rheb—deletion of Rheb (*RHB1*) led to increased import of arginine and lysine (Chattopadhyay and Pearce, 2002). Saucedo et al. (2003) speculate that this discrepancy might be due to compensation by alternative nutrient-gathering pathways in response to Rheb mutation. In *S. pombe*, overexpression of a dominant-negative form of Rheb leads to a state mimicking nitrogen starvation (Mach et al., 2000), and loss of the TSC homologs (which are lacking in *S. cerevisiae*) affects amino acid import (Matsumoto et al., 2002). It will be interesting to test this model by monitoring nutrient levels in response to changes in Rheb function in higher organisms.

In addition to being regulated by growth factors, the TSC1/2-Rheb signaling branch appears to participate in a negative feedback loop to repress growth factor signaling itself. For example, loss of TSC function (Jaeschke et al., 2002; Kwiatkowski et al., 2002) or overexpression of Rheb (Garami et al., 2003; Zhang et al., 2003) leads to reduced activity of Akt, the kinase necessary for activation of TSC2. These data are consistent with the previously suggested model of a negative feedback loop involving S6K1 to regulate signaling via the insulin receptor (Haruta et al., 2000; Radimerski et al., 2002).

Ultimately, the placement of the TSC proteins in the nutrient pathway and downstream of Akt helps to explain the difference in malignancy between tumors arising from tuberous sclerosis and those from diseases of PTEN deficiency, such as Cowden's disease. Tuberous sclerosis rarely leads to malignancy; although tuberous sclerosis patients develop renal cell carcinoma with higher frequency than the average population, it is still not common (<2%) (Cheadle et al., 2000). Cowden's patients, however, are at much higher risk of developing breast cancer (30%–50%) and thyroid carcinoma (10%) (Cantley and Neel, 1999). This might be explained by the fact that disease-causing mutations of TSC2, to our knowledge, activate the pathway promoting cell growth but not those promoting cell proliferation or survival. Although loss of TSC function in cell culture has been shown to affect cell proliferation, several disease-causing mutations of TSC2 do not have the same effects

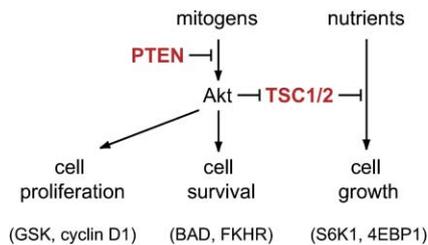


Figure 4. Lack of PTEN Function, but Not Lack of TSC Function, Leads to Malignant Tumor Formation

Lack of PTEN function leads to activation of Akt, which promotes cell growth, proliferation, and survival. Each of these processes, mediated in part by the effectors given in parentheses, would contribute to the progression of the resulting tumor, potentially predisposing it for malignancy. Lack of TSC function, however, seems to promote cell growth without affecting proliferation or survival. Hence, tuberous sclerosis is characterized by the formation of benign tumors called hamartomas, regions of disorganized but nonmalignant tissue.

(Soucek et al., 2001). Additionally, loss of TSC2 has been shown to decrease cell survival in one study, perhaps by negative regulation of Akt and its antiapoptotic effects (Wataya-Kaneda et al., 2001). Loss of PTEN, however, which leads to activation of Akt, causes the concerted activation of all three pathways, sustaining the growth, proliferation, and survival of cells within the tumor (Vivanco and Sawyers, 2002) (Figure 4).

### Conclusion

The organization of the cellular factors regulating translation via 4EBP1 and S6K1 has been the subject of continued study and debate for almost 20 years, motivated in part by the prevalence of mutations in this signaling network that lead to cancer. A model has emerged that places 4EBP1 and S6K1 under regulation of two interacting signals, one extracellular from growth factors and the other intracellular from nutrients. Many mutations in the growth factor-regulated machinery leading to cancer have been described. However, only recently has it been shown that mutations in components mediating the nutrient signal can cause tumor formation, although predominantly benign. Like the PTEN tumor suppressor, which attenuates signaling from growth factors to growth, the TSC proteins appear to act as tumor suppressors to attenuate nutrient signals to growth (Figure 4). Mutating either of these tumor suppressors leads to the formation of tumors that are likely dependent on the same growth network. Thus, one would expect a small molecule such as rapamycin, capable of dominantly inhibiting the growth signal in response to either mutation, to antagonize the growth of such neoplasms.

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