Oncogene (2006) 25, 6373–6383

www.nature.com/onc

REVIEW

Stress and mTORture signaling

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The TOR (target of rapamycin) pathway is an evolutionarily conserved signaling module regulating cell growth (accumulation of mass) in response to a variety of environmental cues such as nutrient availability, hypoxia, DNA damage and osmotic stress. Its pivotal role in cellular and organismal homeostasis is reflected in the fact that unrestrained signaling activity in mammals is associated with the occurrence of disease states including inflammation, cancer and diabetes. The existence of TOR homologs in unicellular organisms whose growth is affected by environmental factors, such as temperature, nutrients and osmolarity, suggests an ancient role for the TOR signaling network in the surveillance of stress conditions. Here, we will summarize recent advances in the TOR signaling field with special emphasis on how stress conditions impinge on insulin/insulin-like growth factor signaling/TOR signaling.
Oncogene (2006) **25,** 6373–6383. doi:10.1038/sj.onc.1209889

Keywords: mTOR; raptor; rictor; growth; cancer; hypoxia

Introduction

A great deal of knowledge about mTOR (mammalian target of rapamycin), a ~290 kDa Ser/Thr kinase of the phosphatidylinositol 3-kinase related protein kinase (PIKK) family, stems from the discovery of the antifungal and immunosuppressant drug rapamycin, a macrocyclic lactone. Rapamycin forms an intracellular complex with the peptidyl-prolyl *cis–trans* isomerase FKBP12 (FK506-binding protein). This drug/receptor complex binds to the FRB (FKBP12-rapamycin) domain located N-terminal to the kinase domain of mTOR (Fingar and Blenis, 2004). Binding of FKBP12-rapamycin leads to the inhibition of mTOR function in a poorly understood manner, but might be caused by weakening/dissociation of the raptor–mTOR interaction (Kim *et al*., 2002; Murakami *et al*., 2004). Transcriptional profiling of rapamycin treatment of yeast, *Drosophila* and mammalian cells shows that the drug affects expression of approximately 5% of all genes in the genome, indicating that TOR has a broad impact on cellular function (Hardwick *et al*., 1999; Peng *et al*., 2002; Guertin *et al*., 2006).

mTOR exists in two functionally distinct complexes dubbed mTOR complex 1 (mTORC1) and mTORC2 (Figure 1). These complexes may act as multimers (Wullschleger *et al*., 2005; Zhang *et al*., 2006). Unlike the situation in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, mammals, *Drosophila*, *Caenorhabditis elegans* and *Arabidopsis thaliana* possess only one *TOR* gene (*AtTOR* in *Arabidopsis*). The rapamycin-sensitive mTORC1 contains the core components mTOR, mLST8/GβL (G protein β-subunit-like protein) and raptor (raptor1 and raptor2 in *A. thaliana*), whereas mTORC2 contains mTOR, mLST8/GβL and Rictor (AVO3 in *S. cerevisiae*), and is believed to be rapamycin insensitive. However, recent findings show that prolonged (>24 h) rapamycin treatment can disrupt mTORC2 assembly and function by sequestering newly synthesized mTOR molecules (Sarbassov *et al*., 2006). In addition to TOR2, LST8 and AVO3, TORC2 in *S. cerevisiae* also contains AVO1, AVO2, and BIT61 (Wullschleger *et al*., 2006). Despite a previous report failing to detect an interaction between the mammalian AVO1 homolog mSin1 (mammalian SAPK interacting protein) and mTOR (Loewith *et al*., 2002), our lab has recently identified three mSin1 isoforms as critical mTORC2 but not mTORC1 components defining three distinct mTORC2s. mSin1 has an essential and evolutionarily conserved role in phosphorylating the critical Serine residue (Ser473 in mammals, Ser505 in *Drosophila*) in the hydrophobic motif of Akt/PKB (Frias *et al*., 2006). Besides the FRB and kinase motifs, mTOR bears N-terminally located HEAT repeats mediating protein–protein interactions and two FAT domains inherent to all PIKK family members. The 36 kDa protein mLST8/GβL interacts with the TOR kinase domain but has no intrinsic catalytic activity. It consists almost entirely of seven WD40 repeats (Kim *et al*., 2003), a motif known to facilitate protein–protein interaction and signal transduction (Smith *et al*., 1999). Seven WD40 repeats can also be found in raptor in addition to a novel RNC domain and three HEAT repeats (Hara *et al*., 2002; Kim *et al*., 2002). Rictor shares conserved domains among eukaryotes, but their functions have so far not been elucidated (Jacinto *et al*., 2004; Sarbassov *et al*., 2004). The richness in domain architecture of the TOR complexes suggests that further proteins are likely to interact transiently with one of the mTORCs.

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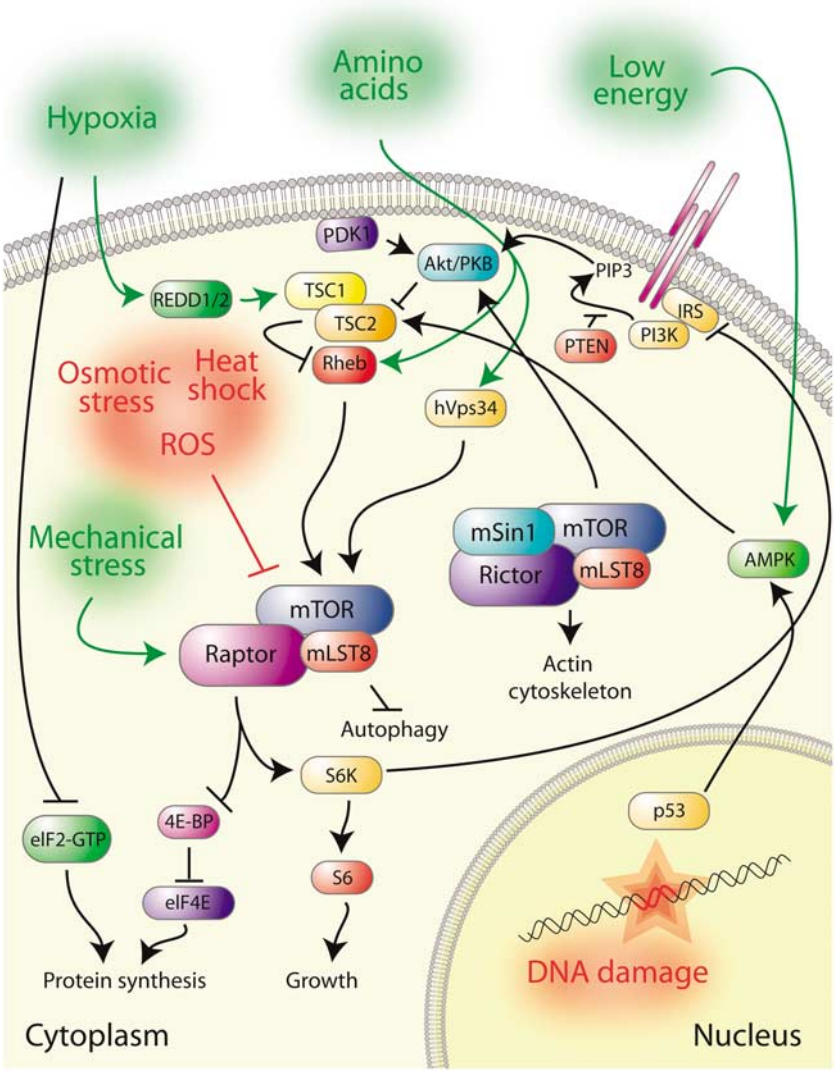

Figure 1 mTOR signaling and stress. mTORC1 promotes growth and protein synthesis through the regulation of S6K and 4E-BP while inhibiting autophagy when sufficient nutrients (e.g. amino acids) are available. Amino acids may feed into the mTOR pathway at the level or upstream of Rheb and hVps34. The mTOR pathway also responds to mechanical stimuli (e.g. muscle exercise, shear and fluid stress) by upregulation of S6K activity. Osmotic stress, heat shock, ROS and DNA damage decrease S6K activity. How the individual stressors signal to mTOR is not known. It is conceivable that the different stress conditions might also affect mTORC2. Low oxygen and energy conditions also diminish protein synthesis. Hypoxia requires the TSC complex to downregulate S6K activity. In addition, translation initiation is inhibited under hypoxic conditions by the eIF2-α-phosphorylating kinase PERK. Low cellular energy level activates AMPK, which inhibits growth by stimulating TSC2 function. Note that the arrows indicate the flow of information and are not necessarily meant to indicate direct interactions. See text for a more detailed description.

Spurred by genetic studies carried out in *Drosophila*, the TSC1 (tuberous sclerosis complex) (Hamartin)/TSC2 (Tuberin) tumor suppressor complex was identified as a negative growth regulator upstream of TOR (Gao and Pan, 2001; Potter *et al*., 2001; Tapon *et al*., 2001; Kwiatkowski and Manning, 2005). Mutations in either gene causes hypertrophic and hyperplastic phenotypes leading to hamartoma syndromes (Kwiatkowski and Manning, 2005). Loss of TSC1/2 leads to unrestrained TOR activity contributing to an enhanced proliferation rate and enlarged size of *TSC1/2* mutant cells. TSC1 contains a potential transmembrane in addition to a coiled-coil domain. The latter mediates binding to TSC2 (van Slegtenhorst *et al*., 1998). *TSC2* encodes a GTPase-activating protein (GAP) that is thought to hydrolyse guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the small GTPase Rheb (Ras homolog enriched in brain). Rheb (Rheb1 and Rheb2 in mouse and human, other model organisms have only one isoform; Patel *et al*., 2003) was identified both in genetic screens and biochemical studies as target of the GAP activity of TSC2 (Castro *et al*., 2003; Garami *et al*., 2003; Inoki *et al*., 2003a; Patel *et al*., 2003; Saucedo *et al*., 2003; Stocker *et al*., 2003;

Tee *et al*., 2003). dRheb overexpression recapitulates *dTSC1/2* mutant phenotypes, and reduction in dRheb function rescues larval lethality caused by loss of dTSC1/2 in the fly (Stocker *et al*., 2003). Furthermore, Rheb has recently been shown to bind to mTOR independent of its guanyl nucleotide charging, but GTP loading determines TOR kinase activity (Long *et al*., 2005).

mTOR substrates include the inhibitory eIF4E-binding proteins (4E-BP1-3, collectively referred to as 4E-BPs) and the ribosomal kinases S6K1/S6K2 (henceforth referred to as S6K). Active mTOR promotes protein synthesis by phosphorylating 4E-BPs on several sites relieving their binding to eIF4E. eIF4E mediates binding of eIF4F, a large protein complex, to the 5′ cap structure of mRNAs when prevented from associating with 4E-BP. 4E-BPs in their hypophosphorylated state bind to eIF4E competitively inhibiting the association of eIF4E and eIF4G and leading to a block in translation.

The other *bona fide* target of mTOR is S6K. mTOR-mediated phosphorylation of S6K (Thr389 in the hydrophobic motif) triggers its activation. In turn, S6K phosphorylates the ribosomal protein (RP) S6. Specific transcripts with polypyrimidine tracts at their 5′ end named 5′ terminal oligopyrimidine tract (TOP) mRNAs largely encode for RPs and elongation factors (Meyuhas, 2000; Thomas, 2002). The original assumption that S6Ks stimulate protein synthesis via S6 phosphorylation by upregulating the translation of 5′TOP mRNAs has lost attraction, because in cells lacking both S6K1 and S6K2, translation of these types of mRNAs was not attenuated (Pende *et al*., 2004). Replacement of the endogenous S6 protein with an S6 mutant form where all five S6K phosphorylation sites were mutated to unphosphorylatable residues ($rpS6^{P-/-}$) showed that phosphorylation of S6 positively regulates cell size but not translation. Rapamycin treatment did not further decrease cell size in the $rpS6^{P-/-}$ mouse embryonic fibroblasts (MEFs), indicating that S6 is a critical determinant of cell size downstream of mTOR (Ruvinsky *et al*., 2005). Furthermore, S6K stimulates translation by phosphorylating several other substrates involved in protein synthesis such as eIF4B, eEF2K and SKAR (Ruvinsky and Meyuhas, 2006). In nutrient-rich conditions, mTOR/raptor are recruited to the eIF3-preinitiation complex to phosphorylate and concurrently displace S6K from eIF3, a multisubunit complex bridging the eIF4G scaffold and the 40S small ribosomal subunit (Holz *et al*., 2005).

The importance of mTOR as key regulator of translation can also be inferred from studies showing that some viruses co-opt insulin/insulin-like growth factor signaling (IIS)/TOR signaling to support translation of viral mRNAs (Frese *et al*., 2003; Mannova and Beretta, 2005; Moody *et al*., 2005; O'Shea *et al*., 2005).

In yeast, translation of RPs is directly controlled by the forkhead transcription factor FHL1, which binds constitutively to ribosomal promoters, in conjunction with either IFH1 or CRF1, a co-activator and co-repressor, respectively. Under nutrient-poor conditions, when TOR activity is low YAK1 is no longer inhibited by the TOR–RAS–protein kinase A (PKA) pathway and phosphorylates CRF1, thereby inducing its nuclear translocation and binding to RP promoters (Martin *et al*., 2004). The TOR–RAS–PKA branch also regulates the localization of the stress-responsive transcription factor Sfp1, which activates the RP and ribosome biogenesis regulons to promote ribosome synthesis in parallel to Sch9. Sfp1 is mainly nuclear under normal growth conditions but dislodges nuclear FHL1 and IFH1 under different stress treatments to repress cell growth (Jorgensen *et al*., 2004; Marion *et al*., 2004). Whether a similar mechanism operates in mammals is not known.

The mTOR signaling network is wired to growth factor signaling via the IIS system. Ligand-binding-induced activation of the insulin/IGF receptor creates binding sites for SH2-containing adaptor proteins including the insulin receptor substrate (IRS) proteins 1–4 binding to phosphorylated tyrosine residues on the intracellular tails of the receptors. Subsequently, receptor-mediated phosphorylation of the IRS proteins recruits and activates class I phosphoinositide 3-kinase (PI3K) by virtue of its regulatory p85 subunit, leading to the production of phosphatidylinositol (PtdIns-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate (PIP2 and PIP3)) lipid second messengers in the membrane. PIP3 serves as substrate for the protein and lipid phosphatase activity-bearing tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten), which specifically removes the phosphate group in the D3 position of the inositol ring and thereby attenuates IIS pathway activity (Vanhaesebroeck and Alessi, 2000). Upon PIP3 production, Akt/protein kinase B (PKB) translocates to the membrane mediated by binding of PIP3 to its PH domain and is fully activated by mTORC2 phosphorylating Ser473 in the C-terminal located hydrophobic motif (Hresko and Mueckler, 2005; Sarbassov *et al*., 2005) and by 3′-phosphoinositide-dependent kinase-1 (PDK1) phosphorylating Thr308 in the activation/T-loop (Alessi *et al*., 1997; Stokoe *et al*., 1997; Stephens *et al*., 1998). Numerous downstream effectors of Akt/PKB have been described, among many others forkhead transcription factors, GSK3β, Bad and the cell cycle inhibitor $p27^{KIP1}$ (Brazil *et al*., 2004). In addition, Akt/PKB is able to phosphorylate TSC2, inhibiting the TSC complex and thereby indirectly stimulating growth (Dan *et al*., 2002; Manning *et al*., 2002; Potter *et al*., 2002). The physiological significance of this modification in *Drosophila* is not clear, as *dTSC2* mutant flies expressing engineered TSC2 constructs where the PKB phosphorylation sites (Ser924 and Thr1518) were mutated to nonphosphorylatable alanine- or phospho-mimicking aspartate/glutamate residues completely rescued lethality of TSC2 mutants (Dong and Pan, 2004). Hahn-Windgassen *et al*. (2005) proposed that Akt/PKB exerts its effect on TSC not only by phosphorylation but also by regulating ATP levels (high when Akt/PKB is active and low in *PKB1/2* double knockout (DKO) cells). Cells with elevated Akt/PKB activity showed lowered AMP-activated protein kinase (AMPK) activity, blunting its

stimulating effect on TSC. Conversely, in the *PKB1*/*PKB2* DKO cells, AMPK activity was significantly elevated, contributing to enhanced TSC2 function (Hahn-Windgassen *et al*., 2005). Furthermore, it is possible that the regulation of the Akt/PKB phosphorylation sites in TSC2 – although dispensable for normal development – becomes important in a context of aberrantly elevated insulin pathway activity as in *PTEN* mutant situations. Another way for Akt/PKB-mediated regulation of TSC1/2 is the sequestration of a 14-3-3–TSC2 complex (phosphorylated on Ser939 and Ser981) from the membrane to the cytosol disabling the formation of a functional TSC1–TSC2 complex (Cai *et al*., 2006).

Finally, the interrelation between IIS and TOR signaling is further complicated by a negative feedback loop from S6K to the PI3K/PKB branch via serine phosphorylation in IRS1 and subsequent downregulation of IIS (Zhang *et al*., 2003; Harrington *et al*., 2004; Shah *et al*., 2004; O'Reilly *et al*., 2006). Therefore, an increase in mTOR activity might lead to insulin resistance and ultimately contribute to the development of diabetes. On the other hand, tumors with an overactivated TOR pathway – for instance caused by loss of TSC – have reduced IIS strength. This might partly explain the more benign nature of *TSC* hamartomas in comparison to the elevated aggressiveness of tumors mutant for *PTEN*, a finding corroborated *in vivo* (Ma *et al*., 2005; Manning *et al*., 2005). Rapamycin treatment of tumors may elicit the undesired effect of increased IIS, thereby aggravating rather than attenuating tumor growth, underscoring the need for appropriate biomarkers for a successful cancer treatment (Guertin and Sabatini, 2005).

Stress and mTOR

mTOR not only controls the rate of protein synthesis but also regulates transcriptional changes in response to a variety of conditions, cell cycle progression, actin organization, autophagy, synaptic plasticity, memory and learning (Fingar and Blenis, 2004). Given this plethora of processes modulated by mTOR, it might not come as a surprise that a variety of extrinsic and intrinsic factors have a direct impact on the mTOR pathway. A cell has to integrate multiple signals to promote growth, progress through the cell cycle or apoptosis. In recent years, numerous stimuli have been shown to cause changes in the activity of the TOR/IIS cascades. Studies in yeast and higher eukaryotes have shown that lowered TOR (and/or decreased IIS in higher eukaryotes) signaling pathway activity increases lifespan (Kenyon *et al*., 1993; Guarente and Kenyon, 2000; Vellai *et al*., 2003; Hwangbo *et al*., 2004; Kapahi *et al*., 2004; Kaeberlein *et al*., 2005; Powers *et al*., 2006). In addition, decreased IIS/TOR signaling activity is associated with increased resistance to some types of stress, suggesting that this pathway plays also an important role in the adaption to different stress conditions (Scott *et al*., 2002; Holzenberger *et al*., 2003; Broughton *et al*., 2005; Teleman *et al*., 2005; Tettweiler *et al*., 2005; Powers *et al*., 2006). In the following section, we will review extra- and intracellular cues that modulate TOR signaling.

Energy and nutrient availability

Adverse nutrient conditions cause a rapid decline in protein synthesis in order to preserve cellular energy levels. mTOR inhibits the induction of autophagy, a process whereby intracellular proteins and organelles are resorbed in vacuole-like structures to retrieve cellular nutrients (Klionsky and Emr, 2000). The autophagic response initiated upon nutrient insufficiency serves to help survive nutritional stress. Autophagy may reflect (1) a protective mechanism for cell survival acting as safeguard to remove damaged polypeptides and organelles or (2) a proapoptotic process to get rid of compromised cells. The finding that *beclin1*, an essential gene for autophagy, is deleted in a large number of cancer cell lines (Aita *et al*., 1999; Liang *et al*., 1999) and that heterozygosity for the *beclin1* locus caused a high number of spontaneous tumors in mice suggests that it acts as a tumor suppressor (Qu *et al*., 2003; Yue *et al*., 2003). However, the dual role for autophagy both as a cell-protective and cell-destructive mechanism hampers to draw a clearcut conclusion about its tumor-suppressing role (Lum *et al*., 2005). Moreover, the antiapoptotic Bcl-2 binds Beclin1 and thereby inhibits autophagy. Beclin1 mutants that cannot bind Bcl-2 induce excessive autophagy and subsequent cell death (Pattingre *et al*., 2005).

What are the actual nutrient components that affect mTOR signaling activity? At which level are nutrients sensed? Which organ within the organism has a critical role for sensing nutrient availability and to systemically relay a signal to the rest of the body? Glucose and amino acids, especially leucine, positively affect TOR activity as evidenced by the fact that amino-acid/glucose depletion downregulates S6/S6K and 4E-BP phosphorylation, which correlates with mTOR activity (Kim *et al*., 2002; Proud, 2002). Moreover, mitochondrial (e.g., oligomycin; Figure 2c) and glycolytic (e.g., 2-deoxyglucose; Figure 2d) inhibitors attenuate mTOR signaling, indicating that glucose and ATP availability positively regulate mTOR. mTOR itself has been proposed to act as an ATP sensor (Dennis *et al*., 2001). However, it seems more likely that AMPK fulfills this job as it senses the AMP:ATP ratio, a more sensitive indicator of the energy status within the cell (Hardie *et al*., 2006). AMPK is activated by virtually any kind of stress that impinges on the cellular energy balance – inhibition of mitochondrial function, hypoxia, low glucose and osmotic stress to name just a few – and in turn shuts down energy-consuming (anabolic) while stimulating energy-producing (catabolic) processes including fatty acid oxidation and glucose uptake (for a more complete list of stress conditions, see Hardie, 2004). Thus, AMPK occupies a central node as a sensor for chronic stress conditions that deplete cellular energy.

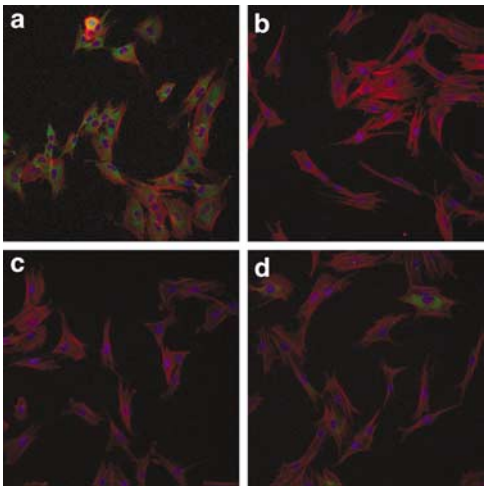

Figure 2 RP S6 phosphorylation in response to different stress treatments. BJ-hTERT cells were exposed to following chemicals for 2 h: (**a**) untreated, (**b**) 20 nM rapamycin, (**c**) 10 μg/μl oligomycin and (**d**) 80 mM 2-deoxyglucose. Staining for phospho-S6 (green), actin (red) and DNA (blue).

The connection to mTOR became apparent when it was found that 5′-aminoimidazole-4-carboxamide riboside (ALCAR), a synthetic AMP analog, could activate AMPK, leading to decreased S6K Thr389 phosphorylation (Bolster *et al*., 2002) and that AMPK phosphorylated TSC2 on at least two residues leading to an enhanced activity of the latter (Inoki *et al*., 2003b). AMPK via TSC2 downregulates TOR/S6K activity in response to energy deprivation (Inoki *et al*., 2003b). As most of the stress conditions to be described below will at some point decrease cellular energy levels, AMPK is likely to contribute to the regulation of mTOR pathway activity in many of these conditions.

Until recently, it was believed that the amino-acid input occurs upstream of the TSC complex, because *TSC1/2* mutant cells sustained high S6K activity even in the absence of amino acids (Gao *et al*., 2002). This finding has been challenged recently. Amino-acid withdrawal in different mammalian TSC2-depleted cells did not prevent S6K1 Thr389 dephosphorylation (Byfield *et al*., 2005; Nobukuni *et al*., 2005; Smith *et al*., 2005). hVps34, a class III PI3K producing PtdIns(3)- phosphate (PI3P), appears to play a role in regulating amino-acid signaling to mTOR, because hVps34 function is regulated by amino acids (Byfield *et al*., 2005; Nobukuni *et al*., 2005). Amino acids also signal in parallel through Rheb. In cells without Rheb function and stimulated with amino acids, Thr389 phosphorylation was mostly prevented (Nobukuni *et al*., 2005). More work is needed to understand whether there are multiple amino-acid/nutrient sensors acting in parallel. For instance, a recent study found that FIP200 (focal adhesion kinase family interacting protein of 200 kDa) binds to TSC1. FIP200 overexpression stimulated S6K activity and disrupted the TSC complex. Cells devoid of FIP200 partially blocked nutrient-stimulated S6K phosphorylation, suggesting that it is required to transduce a nutrient-derived signal to the mTOR complex (Gan *et al*., 2005). Another unanswered question is why hVps34 activity is inhibited upon amino-acid depletion despite being an important positive regulator of autophagy.

When nutrients are scarce, TOR activity is low and accordingly, the cells grow poorly. This phenotype is also recapitulated at the organismal level, as for instance, *Drosophila* or mouse mutants of positive signaling components like the *insulin receptor* (*Inr*), *IRS/chico*, *Akt/PKB*, *PDK1*, *PI3K* and *S6K* have a decreased body size owing to reductions in cell size and cell number (with the exception of *dS6K* mutant flies, which display only the cell size reduction) (Oldham and Hafen, 2003). Pattern formation in these animals is mostly normal, indicating that these gene products are primarily required for growth (Hafen and Stocker, 2003).

Hypomorphic *dTOR* mutant females display a defective oogenesis leading to their sterility (Zhang *et al*., 2006). *mTOR* disruption in mice is incompatible with passage through embryogenesis, and mutant animals die in early postimplantation stages (Gangloff *et al*., 2004). Along these lines, yolk protein synthesis in *Aedes aegypti* stimulated after a bloodmeal is critically dependent on TOR/S6K, as interference with their function lowers Vitellogenin levels and severely disrupts egg development (Hansen *et al*., 2005). Together, these findings suggest that IIS/TOR signaling conveys nutritional information also in the germ line to regulate oogenesis. Indeed, the *Drosophila* ovary dramatically responds to nutrition (Drummond-Barbosa and Spradling, 2001).

In *Drosophila*, TOR signaling has non-autonomous growth effects when the pathway is specifically overactivated or lowered in the fat body, a tissue considered to be the liver analog. Similarly, ablation of insulin-producing neurons in the *Drosophila* brain engenders a systemic growth defect, resulting in the generation of small flies with elevated carbohydrate levels (Ikeya *et al*., 2002; Rulifson *et al*., 2002). Colombani *et al*. (2003) found that reducing dTOR signaling in the fat body led to a pronounced downregulation of insulin pathway activity in peripheral tissues generating smaller-sized flies. Non-autonomous mTOR-dependent effects also exist in mammals. mTOR signaling in the mammalian CNS is linked to the regulation of energy balance, as hypothalamic mTOR stimulation suppressed food intake. Moreover, leptin-mediated anorexia and weight loss could be attenuated by the administration of rapamycin, suggesting that leptin signals through mTOR to regulate feeding behavior (Cota *et al*., 2006). A similar neuronal function for TOR in signaling hunger behavior may operate in the fly (Wu *et al*., 2005). It will be interesting to see whether mammals possess a nutrient-responsive tissue/organ analogous to the *Drosophila* fat body, which could regulate growth in a non-autonomous manner.

Hypoxia

Ambient oxygen concentration has a profound impact on overall body growth, regulating cellular metabolism, energy homeostasis and processes such as cell migration,

angiogenesis and apoptosis, which all are largely HIF-controlled, the master regulators of oxygen homeostasis (Pouyssegur *et al*., 2006). Oxygen concentrations in the atmosphere have been far from being constant during metazoan evolution. Widespread gigantism can be found in insects and other arthropods in the hyperoxic (up to 35% O_2) Carboniferous atmosphere (300 million years ago) generating insect wingspans even larger than 70 cm. The various insect taxa that attained exceptionally large body sizes during the late Paleozoic atmosphere did not persist after the Permian (290–248 million years ago), in which the oxygen concentrations declined to as low as 15% O_2 (Dudley, 1998). The evolution of gigantism may therefore have been caused by increased oxygen fluxes in hyperoxic conditions with direct impact on oxidative metabolism, but other explanations for the effects on size are not excluded. In *Drosophila*, reductions below the physiological oxygen concentrations (hypoxia) go along with developmental delay and decreases in cell size and numbers (Frazier *et al*., 2001; Peck and Chapelle, 2003). These findings suggest that oxygen availability regulates the activity of growth signaling pathways.

Hypoxia in mammals limits protein synthesis by at least two distinct mechanisms regulated in a temporally biphasic manner (Koritzinsky *et al*., 2006; Liu *et al*., 2006). One involves the eIF2α kinase PERK (PKR-like ER kinase) phosphorylating eIF2α on Ser51 (Koumenis *et al*., 2002). eIF2α is part of the ternary complex composed of eIF2α-tRNA^{Met}-GTP and recruits the 40S ribosomal subunit to initiate translation. eIF2α phosphorylated on Ser51 impairs the ability of the eIF2α-guanine-nucleotide exchange factor eIF2B to exchange GDP for GTP, resulting in a decrease of translation initiation. This early response dominates the first phase in response to hypoxia. Chronic hypoxia leads to the accumulation of hypophosphorylated 4E-BP and hence increased binding to eIF4E (Tinton and Buc-Calderon, 1999; Connolly *et al*., 2006). mTOR activity is downregulated under acute low oxygen regimes in an HIF-1- and ATP level-independent manner, as shown by a reduction in phospho-S6K/S6 and phospho-4E-BP phosphorylation (Arsham *et al*., 2003; Brugarolas *et al*., 2004; Liu *et al*., 2006). Non-transformed cells generally reduce translation rates to a much higher extent than fully transformed cancer cell lines, arguing that the latter ones uncouple the growth-inhibitory effects of hypoxia from translation (Connolly *et al*., 2006). Moreover, the effects of hypoxia may vary dependent on the cell line and its constituent active signaling pathways (pathway addiction).

The hypoxia-input to mTOR genetically lies upstream of the TSC complex, as *TSC* mutant cells show no decrease in S6K activity upon oxygen deprivation. Accordingly, lack of TSC function in low-oxygen conditions results in higher translation rates, increased proliferation and/or increased survival (Brugarolas *et al*., 2004; Kaper *et al*., 2006; Liu *et al*., 2006). The hypoxia-inducible RTP801/REDD1/2 proteins and their *Drosophila* homologs Scylla and Charybdis have been identified as negative growth modulators upstream of TSC1/2. Like their *Drosophila* counterparts, *RTP801/REDD1* overexpression decreases S6K activity in a TSC-dependent manner, and RTP801/REDD1 loss prevents S6K downregulation in hypoxia (Brugarolas *et al*., 2004; Reiling and Hafen, 2004).

Whereas short-term hypoxia does not seem to affect mTOR activity by transcriptional changes or energy depletion, a prolonged drop in oxygen levels stimulates HIF-α-dependent as well as HIF-α-independent transcriptional recovery mechanisms (e.g. triggering of the unfolded protein response, nuclear factor-κB induction) and promotes the activation of AMPK. AMPK, as a result of low energy levels, phosphorylates and thereby activates TSC2, resulting in TOR inhibition (Inoki *et al*., 2003a). Additional AMPK/eEF2 kinase-mediated stimulation of eEF2 phosphorylation by hypoxia (Horman *et al*., 2002) further contributes to inhibition of protein synthesis (Connolly *et al*., 2006; Liu *et al*., 2006).

Growth factor signaling and mTOR appear to have an important role in certain settings for HIF accumulation in both normoxia and, surprisingly, hypoxia (Hudson *et al*., 2002; Brugarolas *et al*., 2003; Majumder *et al*., 2004). Most likely, mTOR activity is not completely inhibited in most of the hypoxia studies performed, warranting residual mTOR activity sufficient to impact on HIF-α (Kaper *et al*., 2006). The beneficial effects of mTOR inhibitors may be partly related to the negative effects on HIF-1 and its associated downstream signaling. TOR inhibition in kidney cancer cells mutant for the von Hippel–Lindau (VHL) tumor suppressor (recognizing and earmarking the HIF-αs for degradation in normoxia) using the rapamycin analog CCl-779 led to a marked growth decrease of these cells, an effect that could be attributed to the inhibition of *HIF-1*α mRNA translation containing a 5′TOP motif (Thomas *et al*., 2006).

Tumor hypoxia is a well-known obstacle in the treatment of solid tumors with conventional chemotherapeutics and irradiation (Harris, 2002). Understanding cancer metabolism in the context of a hypoxic tumor microenvironment is therefore important for improving current treatments.

Osmotic stress

When two aequous solutions separated by a semipermeable membrane meet, water flows down its concentration gradient from the solution with lower osmolyte concentration to the one with higher osmolarity. This process is extremely challenging for unicellular organisms like yeast being directly exposed to varying extracellular milieu. It is therefore not surprising that osmotic stress influences cell growth. TOR1 in *S. cerevisiae* is required for growth under high salt concentrations via regulation of *GLN3* and *GAT1* (Crespo *et al*., 2001). Likewise, fission yeast defective for *Tor1* do not form colonies on medium containing high salt concentrations (Weisman and Choder, 2001). Hyperosmotic stress in mammalian cells causes cell shrinkage with a decline in water content, and rapid (<5 min) and reversible dephosphorylation of active

S6K1 and 4EBP-1 independent of the stress-activated p38/c-Jun NH_2-terminal kinase (JNK) MAPK pathways (Parrott and Templeton, 1999; Fumarola *et al*., 2005). S6K activity is also decreased in *TSC2*$^{-/-}$ MEFs upon osmotic stress, suggesting that the regulation of S6K activity occurs downstream of TSC2 (Smith *et al*., 2005). Hyperosmotic stress (600 mM sorbitol for 40 min) in 3T3-L1 adipocytes led to insulin desensitization through mTOR-dependent IRS-1 phosphorylation on Ser307 (Gual *et al*., 2003). Using different inhibitors for mTOR, PI3K, MEK1 and JNK, the authors found that IRS-1 Thr307 phosphorylation is strictly TOR but not S6K-dependent, which was shown to be completely dephosphorylated on Thr389. This argues in favor of an unknown phosphatase that inactivates S6K upon sorbitol treatment, or mTOR directly phosphorylating IRS-1 Thr307. Hyperosmolarity might specifically affect a pool of mTOR complexes that localize to mitochondria, as the mitochondrial proton gradient is perturbed and mitochondrial fragmentation ensues after addition of hypertonic solutions to cells (Desai *et al*., 2002; Copp *et al*., 2005). Mannitol treatment of tobacco or *Arabidopsis* leaves reduced S6K1 but not PDK1 activity by more than 70%, an effect that could be mitigated by overexpression of raptor1 (Mahfouz *et al*., 2006). Downregulation of S6K activity appears to have a protective effect to sustain osmotic stress, as *Arabidopsis* overexpressing S6K are hypersensitive to osmotic stress (Mahfouz *et al*., 2006).

Although hyperosmotic stress reduces S6K phosphorylation, suggesting that the signal is relayed by mTORC1, it also triggers adaptive changes of the cytoskeleton to withstand physical challenges (e.g. cell shrinkage). Therefore, mTORC2 might also sense osmotic stress.

p53 and DNA damage

Proliferating cells exposed to chemicals or harmful conditions that damage DNA halt the cell cycle to ensure faithful transmission of genetic information. If the DNA damage cannot be corrected, cells initiate an apoptotic program. The tumor suppressor p53 functions in cell cycle arrest, induction of DNA repair proteins and initiation of apoptosis (Harris and Levine, 2005). p53 can be induced and stabilized by a variety of different stressors. It is plausible that a damaged cell strives to preserve energy by attenuating growth in order to safeguard repair mechanisms and survival. Intriguingly, cytoplasmic p53 has been found to be covalently linked to 5.8S rRNA and associated with ribosomes (Fontoura *et al*., 1992, 1997). p53 induction decreases protein synthesis and S6K activity (Horton *et al*., 2002), but translational inhibition by p53 may occur also independently of mTOR (Constantinou and Clemens, 2005). In accordance with the former finding, Feng *et al*. (2005) found that etoposide addition to cells resulted in downregulation of S6K activity mediated by AMPK and TSC. Concomitantly, autophagic vesicles and markers were increased by etoposide treatment (Feng *et al*., 2005). AMPK is also involved in regulating a G1/S cell cycle checkpoint in human cells via p53-Ser15 (Ser18 in mouse) phosphorylation in response to glucose deprivation. The physiological importance of this phosphorylation can be inferred from the finding that MEFs containing a p53$^{S18/S18A}$ knock-in mutation fail to arrest the cell cycle upon glucose deprivation. These cells proliferate similar to cells lacking p53 that are more susceptible to undergo apoptosis upon glucose withdrawal (Jones *et al*., 2005). Moreover, the AMPK kinase (AMPKK) LKB1, a Ser/Thr kinase mutated in Peutz–Jegher syndrome characterized by hamartomatous polyps in the gastrointestinal tract, can form a complex with p53 to influence p53-dependent apoptosis pathways. LKB1 overexpression induces caspase activation and ensuing cell death in a p53-dependent manner (Karuman *et al*., 2001). It is therefore likely that in Peutz–Jegher patients, polyps arise not only because of mTOR overactivation through AMPK inhibition but additionally by a failure of mutant cells to undergo apoptosis. Of note, the tumor suppressor genes *PTEN* and *TSC2* have been reported to be transcriptionally induced by p53, which adds another level of keeping the PI3K/TOR signaling in check (Stambolic *et al*., 2001; Feng *et al*., 2005).

Ultraviolet and ROS

The same stress condition can sometimes elicit opposite responses contingent on the cell type (Smith *et al*., 2005). Depending on exposure length, dose/concentration of the stressor and time between stress stimulus and assay, stress conditions may have distinct effects on a particular readout as is for instance the case for ultraviolet (UV) light. UV light exposure initially stimulates S6K activity and within the first half an hour to 7 h after which it decreases and reaches background levels. The stimulating effect upon UV exposure on S6K activity is rapamycin sensitive (Parrott and Templeton, 1999; Brenneisen *et al*., 2000; Ding *et al*., 2002; Huang *et al*., 2002). Interestingly, additional pathways (p38, ERK1/2, JNK and PI3K) may also affect S6K phosphorylation (Zhang *et al*., 2001). The activating effect can be attributed to the generation of reactive oxygen (ROS) species that accumulate in response to UV exposure, whereas pretreatment with ROS scavengers or antioxidants prevents S6K activation (Huang *et al*., 2002). S6K activity upon H_2O_2 treatment of cells is also dose- and time-dependent (Bae *et al*., 1999; Huang *et al*., 2002; Kim *et al*., 2002; Patel *et al*., 2002). Bae *et al*. (1999) found that H_2O_2 addition to mouse epidermal JB6 cells led to an initial rise in phospho-S6K signal within minutes, but dropped to background levels after 1 h (Bae *et al*., 1999). In another study, 1 mM H_2O_2 addition for 10 min in HEK293T cells severely decreased mTOR activity with almost completely wiped out S6K phosphorylation (Kim *et al*., 2002). Using H_2O_2 at a concentration of 200 μM, another group found maximal S6K phosphorylation after 2 h, which was still significantly increased after 7 h (Huang *et al*., 2002). Finally, arsenite, a human carcinogen and a by-product of metal smelting, has been found to initially result in S6K1

activation. Arsenite, similar to UV light, leads to elevated H_2O_2 production, explaining the growth-promoting effect (Jung *et al*., 2003). The importance of the initial upregulation of TOR/S6K activity upon impostion of a life-threatening insult is not readily apparent. Whether this simply reflects the fact that a cell misinterprets the initial stress signals (e.g. ROS) as endogenous stimuli or whether augmented TOR activity provides a short-term survival advantage is not known.

The concept that ROS are not simply a by-product of metabolism but act as secondary messengers with active roles in signaling processes has gained momentum. ROS production is often elevated in tumors (Benhar *et al*., 2002). PTEN is inactivated by oxidative stress (e.g. H_2O_2) via oxidation of a critical cysteine residue in the active site to form a disulfide bond with a neighboring cysteine residue (Denu and Tanner, 1998; Tonks and Neel, 2001; Clancy *et al*., 2002). Inactive PTEN is coupled to increased PIP3 levels and increased downstream signaling. Inactivation of PTEN function occurs in physiological settings as, for instance, macrophages protect themselves from their endogenous high ROS levels by PTEN inactivation and subsequent stimulation of the Akt/PKB-dependent survival pathway (Leslie *et al*., 2003). Redox regulation of PTEN might contribute to cancerogenesis by inactivating PTEN function and thus leading to increased PI3K signaling (Valko *et al*., 2006).

mTOR activity is also influenced by the cellular redox state. Oxidant addition to cells decreased raptor binding to mTOR but increased S6K activity, which is dominant over leucine deprivation and inhibition of mitochondrial or glycolytic function (Sarbassov and Sabatini, 2005). The redox-dependent mTOR regulation is likely to occur through vicinal thiol groups in the FATC domain (Dames *et al*., 2005).

Mechanostress

IIS/TOR signaling positively affects muscle growth. Muscle atrophy can be caused by inactivity, disease or aging processes and is accompanied by reduced protein synthesis (Kandarian and Jackman, 2006). As prominent regulators of cell growth, the IIS and mTOR pathways have a major impact on hypertrophic muscle accumulation (Bodine *et al*., 2001; Rommel *et al*., 2001). In a poorly understood manner, mechanical stimuli elicited by exercise are transduced into muscle cells to regulate protein synthesis. Muscle cells are mechano-sensitive and convey a physical stimulus into a biochemical output (Hornberger and Chien, 2006). mTOR Ser2448 phosphorylation, a site phosphorylated by S6K, is increased upon muscle overload (Reynolds *et al*., 2002). Conversely, in a rat model for skeletal muscle atrophy, hindlimb unloading resulted in S6K dephosphorylation paralleling muscle wasting (Hornberger *et al*., 2001). A recent study showed that mTOR activation by mechanical stimulation of skeletal muscle involved phospholipase D producing the lipid messenger phosphatidic acid (PA) (Hornberger *et al*., 2006). PA binds the FRB domain of TOR and activates S6K1 activity (Fang *et al*., 2003). S6K activity is also regulated by other exercise-induced stress conditions such as cyclic strain imposed on smooth muscle cells (SMC). Repeated strain increased proliferation rates of SMC concomitant with increases in S6K phosphorylation (Li *et al*., 2003). Similar results were obtained by mechanical stretching of porcine trabecular meshwork (TM) cells (Bradley *et al*., 2003).

Endothelial cells lining the interior of blood vessels are constantly exposed to moving blood. These cells endure fluid shear stress, which contributes to the inhibition of cell death. Shear stress led to a time-dependent increase in PKB phosphorylation and increased survival of endothelial cells in response to serum depletion under shear stress conditions (Dimmeler *et al*., 1998). Fluid flow also activated S6K1 in a rapamycin-dependent manner. Increase in S6K phosphorylation occurred rapidly within minutes, suggestive of a direct effect of shear stress on S6K (Kraiss *et al*., 2001). Thus, mechanical forces applied to cells can have direct effects on intracellular signaling and ultimately modify the growth response of cells.

Heat shock

Temperatures above the physiological level trigger an adaptive gene expression program for cytoprotective purposes controlled by the family of heat shock proteins (HSPs). In spite of their name, HSPs also respond to other stress conditions and function during normal metabolism (Santoro, 2000). Members of the Hsp90 family have been identified as playing a role in tyrosine kinase regulation. Hsp90 binds to raptor in mammalian cells and positively regulates S6K activity. Geldanamycin, an Hsp90 inhibitor in clinical trials for cancer treatment, suppressed binding of raptor to Hsp90 and reduced S6K phosphorylation (Ohji *et al*., 2006). Some years ago, the Blenis group found that heat shock unexpectedly increased phosphorylation of S6K1 in fibroblasts (Jurivich *et al*., 1991), a finding also made by another group (Lin *et al*., 1997). The importance of this finding is unclear given the internal ribosomal entry sites (IRES)-mediated translation of HSPs upon heat stress, and because heat shock is known to attenuate translation by increased binding of 4EBP-1 to eIF4E. Nonetheless, inhibition of protein synthesis is also partly brought about by eIF2α phosphorylation (Lamphear and Panniers, 1991; Zapata *et al*., 1991; Feigenblum and Schneider, 1996; Vries *et al*., 1997). Vries *et al*. (1997) did not observe a decrease in S6K activity upon heat stress despite a decrease in 4EBP-1 phosphorylation, suggesting that the effects on S6K are cell type-dependent or that an unknown phosphatase might dephosphorylate 4EBP-1. Furthermore, heat shock in plants inactivates AtS6K and induce dephosphorylation of S6 (Scharf and Nover, 1982; Turck *et al*., 1998). On the other hand, PKB1 has been reported to become activated within minutes upon heat shock, presumably reflecting the pro-survival effects of Akt/PKB (Konishi *et al*., 1997). *S. pombe* devoid of tor1p function are

hypersensitive to cold stress, indicating that TOR might play a more general role in response to temperature (Weisman and Choder, 2001).

Concluding remarks

Multiple stress conditions have been shown to impact growth and mTOR pathway activity. How seemingly disparate stress conditions can elicit the same response is an unanswered question. Interestingly, the nucleolus of a cell has been ascribed a stress-sensing function (Rubbi and Milner, 2003). Many stress conditions including hypoxia, UV and heat shock disrupt nucleolar integrity. Disruption of nucleolar function was found to stabilize p53 (Rubbi and Milner, 2003). Thus, in cells with wild-type p53 function, some stress conditions might employ the p53→AMPK→TSC→mTOR axis to downregulate the mTOR pathway. However, the effects of the stressor on S6K activity can occur within minutes and hence acute treatment is unlikely to signal via p53.

An important issue for the future will be elucidating at which level the different stress conditions feed into the mTOR pathway. The recent identification of two distinct TOR complexes in mammals may suggest that the two TORCs could be regulated by distinct upstream signals. Genome-wide screens for genes whose function affects TOR activity in response to different stresses might uncover novel modulators of this pathway that have so far escaped detection. Gaining more insights into mTOR regulation under stress conditions that are also encountered in tumors will add critical information to advance current therapies to treat cancer.

Acknowledgements

We thank Jason Moffat for critical reading of the manuscript and for the contribution of the immunofluorescent pictures. We also thank Tom DiCesare for the preparation of Figure 1. JHR received financial support from the Human Frontier Science Program Organization (HFSPO), the European Molecular Biology Organization (EMBO) and the Swiss National Science Foundation (SNF).

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