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REVIEW ARTICLE

Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks

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The ribosomal protein S6K (S6 kinase) represents an extensively studied effector of the TORC1 [TOR (target of rapamycin) complex 1], which possesses important yet incompletely defined roles in cellular and organismal physiology. TORC1 functions as an environmental sensor by integrating signals derived from diverse environmental cues to promote anabolic and inhibit catabolic cellular functions. mTORC1 (mammalian TORC1) phosphorylates and activates S6K1 and S6K2, whose first identified substrate was rpS6 (ribosomal protein S6), a component of the 40S ribosome. Studies over the past decade have uncovered a number of additional S6K1 substrates, revealing multiple levels at which the mTORC1–S6K1 axis regulates cell physiology. The results thus far indicate that the mTORC1–S6K1 axis controls fundamental cellular processes, including transcription, translation, protein and lipid synthesis, cell growth/size and cell metabolism. In the present review we summarize the regulation of S6Ks, their cellular substrates and functions, and their integration within rapidly expanding mTOR (mammalian TOR) signalling networks. Although our understanding of the role of mTORC1–S6K1 signalling in physiology remains in its infancy, evidence indicates that this signalling axis controls, at least in part, glucose homoeostasis, insulin sensitivity, adipocyte metabolism, body mass and energy balance, tissue and organ size, learning, memory and aging. As dysregulation of this signalling axis contributes to diverse disease states, improved understanding of S6K regulation and function within mTOR signalling networks may enable the development of novel therapeutics.

Key words: mammalian target of rapamycin complex 1 (mTORC1), mammalian target of rapamycin complex 2 (mTORC2), S6 kinase 1 (S6K1), S6 kinase 2 (S6K2), target of rapamycin (TOR).

INTRODUCTION

Organismal homoeostasis requires that cells, tissues and organs respond appropriately to diverse environmental cues, which operate via tightly controlled signal transduction networks. Indeed, the dysregulation of many signalling pathways underlies numerous human pathological disease states. The protein kinase TOR (target of rapamycin) functions as an evolutionarily conserved environmental sensor. In unicellular eukaryotes, such as yeasts, TOR responds primarily to nutrient levels. Evolution of multicellular organisms provided TOR with the ability to respond to a diverse array of environmental cues, such as hormones, growth factors and mitogens, enabling this kinase to orchestrate a myriad of cellular functions. From yeasts to mammals, TOR forms at least two multi-protein complexes known as TORC1 (TOR complex 1) and TORC2 [1–3]. These complexes exhibit distinct subunit composition, regulation, substrate selectivity and sensitivity to the drug inhibitor rapamycin, clinically known as sirolimus. TORC1, which displays sensitivity to acute rapamycin, promotes a diverse array of anabolic processes and suppresses catabolic processes such as autophagy. The significantly less-well understood TORC2, which displays insensitivity to acute rapamycin, is thought to promote cell proliferation and cell survival. Today, rapamycin or its analogues (rapalogues) are FDA (Food and Drug Administration)-approved in clinical medicine as immunosuppressive agents to blunt organ transplant rejection, in cardiology to reduce restenosis following angioplasty, and in oncology to treat renal cell carcinoma, thus underscoring the

Abbreviations used: AGC, cAMP-dependent protein kinase/cGMP-dependent protein kinase/protein kinase C; AMPK, AMP-activated kinase; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; CBP, cAMP-response-element-binding protein; CCTβ, chaperonin-containing TCP-1 (t-complex protein 1) β; CRB, CBP-binding protein; CREMτ, cAMP-response-element modulator τ; deptor, DEP domain-containing mTOR-interacting protein; dS6K, *Drosophila* S6 kinase; 4EBP, eukaryotic initiation factor 4E-binding protein; eEF, eukaryotic elongation factor; eEF2K, eEF2 kinase; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; FDA, Food and Drug Administration; FKBP, FK506-binding protein; FMRP, fragile X mental retardation protein; FRB, FKBP12–rapamycin-binding; GβL, G-protein β-protein subunit-like; GAP, GTPase-activating protein; GSK3, glycogen synthase kinase 3; HIF, hypoxia-inducible factor; HM, hydrophobic motif; IGF, insulin-like growth factor; IKK, inhibitor of nuclear factor-κB kinase; IRS, insulin receptor substrate; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; Mdm2, murine double minute 2; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; mLST8, mammalian lethal with SEC13 protein; mSin1, mammalian stress-activated MAPK-interacting protein 1; mTOR, mammalian target of rapamycin; mTORC, mammalian TOR complex; NF-κB, nuclear factor-κB; NLS, nuclear localization sequence; PA, phosphatidic acid; PDCD4, programmed cell death 4; PDK, phosphoinositide-dependent kinase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIF, PDK-interacting fragment; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLD, phospholipase D; PP2A, protein phosphatase 2A; PRAS40, proline-rich Akt substrate of 40 kDa; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTM, post-translational modification; REDD1, regulated in development and DNA damage responses 1; raptor, regulatory associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; RNAi, RNA interference; rpS6, ribosomal protein S6; RSK, ribosomal S6 kinase; S6K, S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; SKAR, S6K1 Aly/REF-like substrate; SREBP, sterol-regulatory-element-binding protein; TBK1, tumour necrosis factor-receptor-associated factor-associated NF-κB activator-binding kinase 1; TM, turn motif; TNF, tumour necrosis factor; TOP, terminal oligopyrimidine; TOR, target of rapamycin; TORC, TOR complex; TOS, TOR signalling; TSC/Tsc, tuberous sclerosis complex; ULK, unc-51-like kinase; YY1, Yin-Yang 1.

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important role of mTORC1 (mammalian TORC1) in physiology [4–7].

The ribosomal protein S6K (S6 kinase) and 4EBP [eIF4E (eukaryotic initiation factor 4E)-binding protein] represent the first TOR substrates identified in metazoans and remain the best characterized [8,9]. Since then, only a small set of additional *bona fide* TOR effectors have been identified. Today, we understand that S6K activation absolutely requires TORC1-mediated phosphorylation. S6K phosphorylates its own set of diverse targets, many of which promote protein production [10]. In a parallel pathway, TORC1-mediated phosphorylation of 4EBP1 initiates cap-dependent translation by eIF4E [10]. Thus TORC1 signals along parallel pathways to co-ordinately promote protein synthesis. rpS6 (ribosomal protein S6), a component of the 40S ribosome, represents the most extensively studied S6K substrate, although the biochemical consequence of rpS6 phosphorylation remains poorly understood [9,11].

In mammals, S6K represents a family composed of two distinct genes, S6K1 (*RPS6KB1*; also known as S6Kα) and S6K2 (*RPS6KB2*; also known as S6Kβ) [12–18]. Mice singly null for S6K1 or S6K2 are born at normal Mendelian ratios, whereas mice null for both S6K1 and S6K2 display perinatal lethality [14,19]. Whole-body knockout of mTOR (mammalian TOR) in mice causes early embryonic lethality [e5.5 (embryonic day 5.5)] [20,21]. Taken together, these data reveal that non-S6K substrates mediate essential roles for mTORC1 during embryonic development, with S6Ks controlling physiological homoeostasis post-development. Emerging evidence suggests that aberrant mTORC1–S6K1 signalling contributes to various pathological states, including diabetes, obesity, cancer, organ hypertrophy and aging-related pathology [2,22]. However, a mechanistic understanding of how S6K signalling contributes to many of these pathophysiological settings remains limited.

In the present review, we summarize the regulation and function of the TOR–S6K signalling axis in cellular and organismal physiology. Owing to limited research on S6K2, we will focus almost entirely on S6K1, although we will discuss S6K2 when appropriate. We will describe the complex biochemical mechanisms that govern the activation state of S6K1 within cells and describe how S6K1 integrates within cellular TOR signalling networks. We will review our understanding of the function of the TORC1–S6K1 axis in cellular function as well as physiology. To open, we provide an historical timeline of events that united TOR and S6K1.

HISTORICAL PERSPECTIVE

TOR and S6K share an inextricable biochemical link, yet possess unique histories that merged upon the discovery of the drug rapamycin, a naturally produced macrolide antibiotic. Rapamycin was discovered nearly a half-century ago in soil samples containing the bacterium *Streptomyces hygroscopicus* found on Easter Island, a South Pacific Polynesian island owned today by Chile and known as Rapa Nui to the native population [23]. This island, famous for its mysterious giant stone statues called 'Moai' that dot the coastline, was inhabited by an ancient civilization that precipitously failed prior to the arrival of the first Europeans, possibly due to mismanagement of vital natural resources within a fragile island ecosystem (i.e. deforestation) [24]. The first studies on rapamycin revealed the compound to possess anti-fungal activity due to G_1-phase arrest [6,25]. During the research of the efficacy of treating fungal infections with rapamycin, the compound was found to potently suppress the immune response in rats [26]. Discovery of the immunosuppressive properties of rapamycin was pivotal both in its development as a clinical therapeutic, but also for basic biomedical research aimed at understanding mitogen-driven cellular growth and proliferation.

In mammals, rapamycin blocks the G_1- to S-phase transition in T-lymphocytes and thus inhibits T-cell proliferation by inhibiting signalling via interleukin-2 and its receptor [25]. As rapamycin generally reduces the proliferation of other cell types, to various degrees, rapalogues continue to be tested as anti-cancer chemotherapy drugs [6,7]. To identify genetic components that control rapamycin-induced toxicity in yeast, an elegant genetic screen was performed in 1991 in *Saccharomyces cerevisiae* [27]. Mutations in three genes, *Fpr1* [an orthologue of FKBP12 (FK506-binding protein 12)], *Tor1* and *Tor2*, conferred rapamycin resistance. Today, we understand that rapamycin bound to FKBP12 binds the TOR FRB (FKBP12–rapamycin-binding) domain, a short sequence lying immediately upstream of the C-terminal kinase domain (see Figure 1A). Thus rapamycin functions as an allosteric inhibitor rather than as an ATP-competitive catalytic inhibitor [6]. Soon thereafter in 1994–1995, several groups identified a mammalian counterpart to budding yeast Tor1/2 by immuno-affinity purification of FKBP12–rapamycin-interacting proteins, known today as mTOR (mammalian or mechanistic target of rapamycin) [1]. In an interesting evolutionary twist, higher eukaryotes (i.e. worms, flies and mammals) possess only one *TOR* gene, whereas the budding yeast *S. cerevisiae* and the fission yeast *Saccharomyces pombe* possess two *Tor* genes (*Tor1* and *Tor2*).

Although we know today that TOR, as part of TORC1, phosphorylates and activates the S6Ks in a manner potently sensitive to rapamycin, S6K1 was identified independently of either rapamycin or TOR. In the 1970s, it was discovered that diverse growth factors and mitogens promote phosphorylation of 40S rpS6, a component of the small ribosomal subunit, sparking intensive research efforts aimed at understanding this phenomenon. The 90 kDa RSK (ribosomal S6K) represents the first identified rpS6 kinase [28], now known to represent a family of three genes, RSK1–RSK3 [29]. The second rpS6 kinase, identified shortly thereafter, was the 70 kDa RSK (now called p70-S6K1) [12,13]. Thus two distinct but related kinases, both members of the AGC [PKA (cAMP-dependent protein kinase)/PKG (cGMP-dependent protein kinase)/PKC (protein kinase C)] kinase family, were found to phosphorylate rpS6 *in vitro* and, unfortunately, both were given similar names due to similar substrate preference, engendering significant confusion in the literature over the years. It is important to note that, although S6K1 was believed for many years to represent the physiological kinase for rpS6 in intact cells, persistent phosphorylation of rpS6 upon genetic knockout of S6K1 and S6K2 in mice ultimately revealed that RSK also represents a *bona fide* rpS6 kinase [19].

Since rapamycin was known to inhibit proliferation, the drug became an interesting candidate probe for the rpS6 pathway. Indeed, in 1992 rapamycin was found to potently inhibit mitogen-induced activation of S6K1 and the phosphorylation of rpS6 [30–32]. The rapamycin–FKBP12 complex, however, did not appear to directly interact with S6K1. Thus how rapamycin controlled S6K1 activity remained unclear. In 1995, rapamycin was found to ablate phosphorylation of S6K1 on Thr^{389} [33]. Consistent with a link between rapamycin and protein synthesis, in 1996 rapamycin was found to inhibit the phosphorylation of 4EBP1, an event that suppresses cap-dependent translation initiated by eIF4E [34]. A direct connection between S6K and mTOR was not demonstrated until 1997–1998 when mTOR was shown to directly phosphorylate S6K1 (Thr^{389}) and 4EBP1 (Thr^{37}/Thr^{46}) by *in vitro* kinase assay and other *in vivo* approaches [35,36]. In 1999, mTOR-mediated phosphorylation of S6K1 (Thr^{389}) *in vitro* was

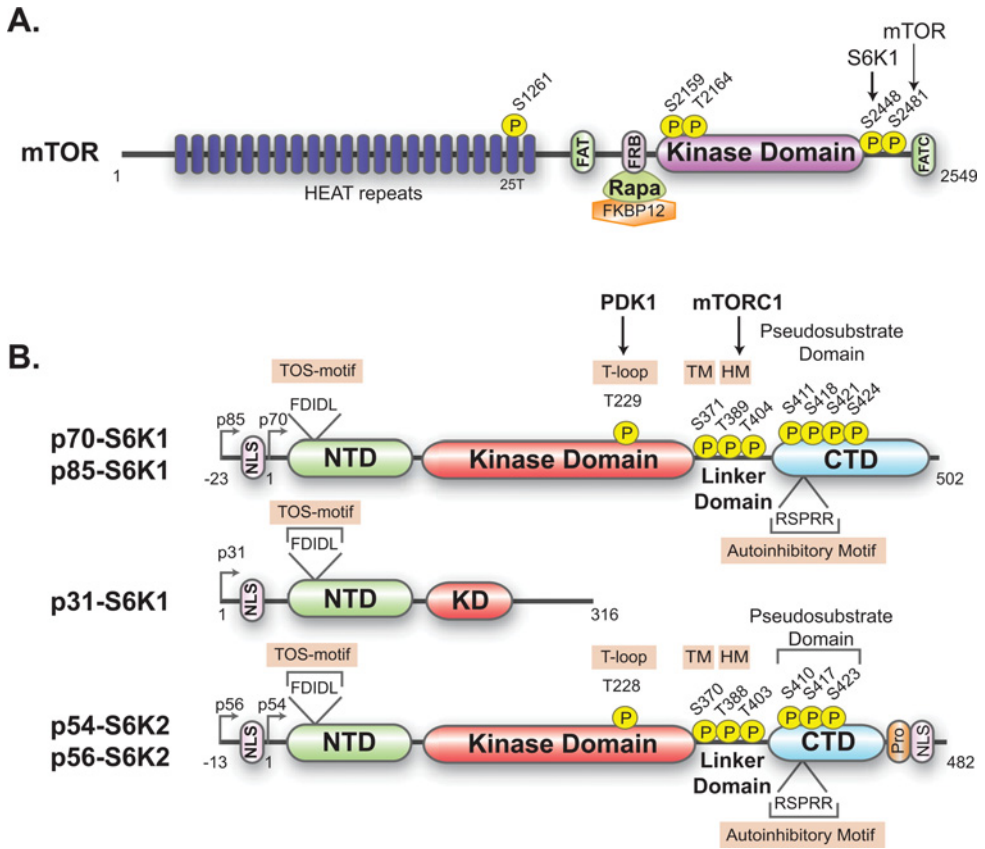

Figure 1 Domain structures of S6K and mTOR

(**A**) mTOR domain structure and phosphorylation sites: mTOR contains a tandem series of HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and TOR) repeats thought to mediate protein–protein interactions. Evolutionarily conserved FAT [FRAP (FKBP–rapamycin-associated protein)/ATM (ataxia telangiectasia mutated)/TRRAP (transformation/transcription domain-associated protein)] and FATC (FATC-terminal) domains flank the kinase domain. The rapamycin (Rapa)–FKBP12 complex binds the FRB domain. Upon activation, mTOR autophosphorylates on Ser^{2481}. Upon activation by mTORC1, S6K1 phosphorylates mTOR Ser^{2448} via a feedback loop. mTOR Ser^{1261}, Ser^{2159} and Thr^{2164} phosphorylation promotes mTORC1 signalling. (**B**) S6K isoforms, domain structure and phosphorylation sites: S6K1 isoforms include p70-, p85- and p31-S6K1; alternative start site usage lengthens the p85- and p31-S6K1 N-termini by 23 amino acids (note that p31-S6K1 lacks most of the kinase domain). S6K2 isoforms include p54- and p56-S6K2; alternative start site usage lengthens the p56-S6K2 N-terminus by 13 amino acids. NLSs lie within the N-terminal extensions of p85-S6K1 and p56-S6K2, whereas S6K2 additionally contains an NLS within the C-terminus as well as a proline-rich domain (Pro). S6Ks contain an acidic N-terminal domain (NTD), kinase domain (KD), linker region and acidic C-terminal domain (CTD). The N-terminal domain contains the TOS motif, whereas the CTD contains the autoinhibitory pseudosubstrate domain and RSPRR motif. mTORC1 phosphorylates the HM site (Thr^{389}) in the linker region and PDK1 phosphorylates the T-loop site (Thr^{229}) within the kinase domain. Other regulatory phosphorylation (P) sites, including the TM site (Ser^{371}), are shown.

found to increase S6K1 activity towards rpS6 [37]. Throughout the 1990s, several other phosphorylation sites on S6K1 were identified that contributed to S6K1 regulation (described in more detail below). Thus S6K1 regulation occurs via complex multi-site phosphorylation. In 1998, it was shown that full activation of S6K1 requires not only phosphorylation of Thr^{389} by mTOR, but also phosphorylation of Thr^{229} by PDK1 (phosphoinositide-dependent kinase 1) [38,39]. The discovery of distinct TOR-containing complexes in 2002 in *S. cerevisiae* and mammals, a rapamycin-sensitive raptor (regulatory associated protein of mTOR)-containing TORC (TORC1) and a rapamycin-insensitive rictor (rapamycin-insensitive companion of mTOR)-containing TORC (TORC2), marked the next major turning point in the field [40–42].

S6K DOMAIN STRUCTURE AND CELLULAR REGULATION

S6K1 and S6K2 belong to the AGC kinase family, named for its three founding members PKA, PKG and PKC [43,44]. Although S6K1 was cloned in 1990 [12,13], S6K2 was not cloned until almost a decade later [14–18]. AGC kinases share several structural features that confer similar modes of regulation. Their kinase domains exhibit a bilobal fold structure in which a small N-terminal lobe and a larger C-terminal lobe co-ordinate ATP binding [43,44]. At the beginning of the C-lobe lies an activation segment or loop (commonly known as the T-loop), phosphorylation of which effects conformational changes important for phosphoryl transfer. PDK1 represents the T-loop kinase for many AGC kinases, including S6K, RSK and Akt [also known as PKB (protein kinase B)]. Substrates engage a groove located near the T-loop. Two other important phosphorylation sites, the TM (turn motif) (so-named due to its location at the cusp of a structural turn in the PKA tail) and HM (hydrophobic motif) sequentially follow the kinase domain. The phosphorylated HM site engages a hydrophobic pocket within the N-lobe. The phosphorylated TM site stabilizes phospho-HM binding to the N-lobe hydrophobic pocket. Together, these three critical phosphorylation events stabilize a catalytically competent conformation [43,44].

S6K domain structure

The *S6K1* and *S6K2* genes each encode two protein isoforms generated by alternate ATG start site utilization [45]. S6K1 and

S6K2 share 84 % identity within their kinase domains, with less homology in their N- and C-terminal regions (43 % and 59 % identity respectively) [15]. The more extensively studied 70 kDa S6K1 isoform (also known as S6Kα2) contains 502 amino acids, whereas the larger 85 kDa isoform (also known as S6Kα1) contains an additional 23 N-terminal amino acids (Figure 1B). Although p70-S6K1 predominantly localizes to the cytosol, the presence of an NLS (nuclear localization sequence) within the N-terminal extension of p85-S6K1 suggests that this isoform shuttles to the nucleus, although this notion has little experimental support. The 54 kDa S6K2 isoform (p54-S6K2; also known as S6Kβ2) contains 482 amino acids, whereas the larger 56 kDa isoform (also known as S6Kβ1) contains an additional 13 N-terminal amino acids (Figure 1B). Although the larger isoforms of S6K1 and S6K2 each contain an NLS in their N-terminal extensions, S6K2 uniquely contains an NLS in the C-terminus, suggesting that both S6K2 isoforms shuttle to the nucleus. A smaller splice variant of S6K1 has been reported, p31-S6K1, that is required for cellular transformation induced by the splicing factor SF2/ASF (splicing factor 2/alternative splicing factor) [46]. This 31 kDa isoform lacks most of the kinase domain, however, suggesting a function independent of kinase activity. Lastly, evidence exists for a 60 kDa splice isoform of S6K1, particularly in breast cancer cell lines [47].

The S6K1 and S6K2 proteins can be subdivided into several important regulatory domains (see Figure 1B): an acidic N-terminus that contains the TOS (TOR signalling) motif; the kinase domain that contains the T-loop; a linker region that contains the TM and HM sites; and a basic C-terminus containing an autoinhibitory pseudosubstrate domain. This C-terminal domain is unique to S6Ks among other AGC family members and experiences phosphorylation on multiple sites. In S6K2, a proline-rich region follows the pseudosubstrate domain, which may facilitate interaction with SH3 (Src homology 3)- and/or WW-domain-containing proteins. Co-ordination between these modular domains via hierarchical multi-site phosphorylation underlies the regulation of S6Ks.

S6K regulation by complex multi-site phosphorylation

Diverse growth factors and mitogens [i.e. serum, insulin/IGF (insulin-like growth factor), epidermal growth factor and PKC-promoting phorbol esters] activate the S6Ks [8,9]. The insulin/IGF pathway, the best-studied activator of S6Ks, signals via PI3K (phosphoinositide 3-kinase)/Akt to activate mTORC1 and thus the S6Ks [1,2]. In a PI3K-independent manner, the Ras/MAPK (mitogen-activated protein kinase) pathway also activates mTORC1. Both pathways, however, co-operate with other inputs to maximally activate the S6Ks. For example, downstream of PI3K, the Rho family G-proteins Cdc42 and Rac and the atypical PKC isoforms PKCζ and PKCλ contribute to S6K1 activation [48,49]. In response to serum or insulin, S6K1 undergoes phosphorylation on at least eight well-mapped sites [8]. S6K1 activity absolutely requires phosphorylation on three critical sites: the T-loop site on the activation loop (Thr^{229} in p70-S6K1; Thr^{252} in p85-S6K1), the TM site in the linker domain (Ser^{371} in p70-S6K1; Thr^{394} in p85-S6K1), and the HM site, also in the linker domain (Thr^{389} in p70-S6K1; Thr^{412} in p85-S6K1), as mutagenic alanine residue substitutions at each of these sites abolishes S6K1 activity [50,51]. Owing to the essential nature of Thr^{389} phosphorylation, the mTORC1 inhibitor rapamycin potently blocks S6K1 activation by all known agonists [30–32], which correlates with dephosphorylation of the HM site, T-loop site and Ser^{404} in the linker region [33,52].

Over the past 20 years, a progressive series of S6K1 structure–function studies have elucidated the molecular steps that govern S6K1 activation by mitogens, revealing roles for complex interactions between specific domains and phosphorylation sites. This research has led to models for stepwise activation of S6K1 via complex multi-site phosphorylation [38,50,53–55]. In these models, S6K1 phosphorylation on multiple C-terminal sites represents an early event that facilitates mTORC1-mediated phosphorylation of the HM site (Thr^{389}) in the linker domain and PDK1-mediated phosphorylation of the T-loop site (Thr^{229}) on the activation loop. Owing to the strong positive co-operativity between the HM and T-loop sites, it has been difficult to determine unequivocally the temporal order of Thr^{389} and Thr^{229} phosphorylation events relative to each other. The current data support two models for activation of S6K via ordered phosphorylation, a conventional widely accepted model (Model 1; Figure 2A), and an alternate model (Model 2; Figure 2B). The temporal occurrence of TM site phosphorylation remains poorly understood, primarily because this phosphorylation event has received relatively scant attention by researchers. A recent study, however, suggests that TM site phosphorylation on Ser^{371} by a constitutive kinase represents one of the first known phosphorylation events, one that may occur co-translationally [55] in a manner similar to the phosphorylation of the Akt TM site Thr^{450} [56]. Below, we first review the data that led to models for the activation of S6K1 by complex multi-site phosphorylation; secondly, we present two models for the temporal ordering of these phosphorylation events.

C-terminal phosphorylation

An early step in S6K1 activation in response to growth factors and mitogens involves priming phosphorylation on four proline-directed sites that lie in the C-terminal autoinhibitory pseudosubstrate domain (Ser^{411}, Ser^{418}, Ser^{421} and Ser^{424}), which bears significant homology to the phosphorylated region of rpS6 [53,54]. It was first proposed in the early 1990s that, in the inactive state, the basic C-terminal pseudosubstrate domain interacts with the acidic N-terminus, which occludes the kinase domain and results in an inactive conformation (see Figure 2) [12,57]. Mitogen-induced C-terminal phosphorylation relieves this inhibition by releasing pseudosubstrate domain binding, thus inducing a conformational change that enables access to the HM and T-loop sites [12,57]. Although phosphorylation of these four C-terminal sites contributes to S6K1 activation, it is not critical. Mutation of these four sites to alanine residues, or deletion of 101 amino acids from the C-terminus (ΔCT), modestly reduces S6K1 activation, whereas substitution with phospho-mimetic residues (D3E) modestly increases basal activity in some reports [52,58,59]. Although the proline-directed mitogen-regulated MAPKs phosphorylate these C-terminal sites *in vitro* [53], the physiological kinases for these sites in intact cells remain unclear.

HM site phosphorylation by mTORC1

In 1998–1999, mTORC1 was shown to directly phosphorylate S6K1 on the HM site Thr^{389} to promote S6K1 activity (see Figure 2) [36,37]. Mutation of Thr^{389} to alanine (T389A) abolishes S6K1 activity, whereas substitution of an acidic glutamate residue for Thr^{389} (T389E) to mimic phosphorylation augments basal S6K1 activity in the absence of mitogens, thus rendering S6K1 partially constitutively active [50,59,60]. In 1995–1996, it was found that deletion of 30 amino acids from the N-terminus of p70-S6K1 (ΔNT) abolishes the serum-stimulated activation of S6K1 and the phosphorylation of the rapamycin-sensitive

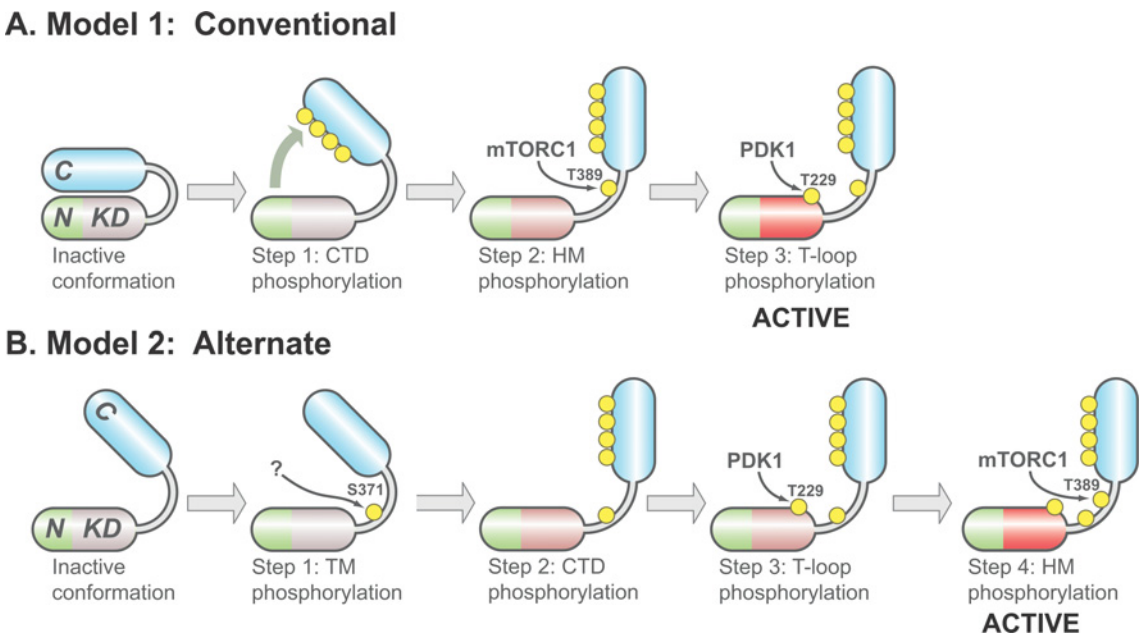

Figure 2 Stepwise activation of S6K1 via multi-site phosphorylation

(**A**) Model 1: conventional model. The interaction of the C- and N-terminal domains results in autoinhibition of S6K1. Step 1: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 2: the release of the autoinhibitory C-terminal domain (CTD) enables mTORC1 access to the HM and thus phosphorylation of Thr^{389}. Step 3: the release of the autoinhibitory CTD and phosphorylation on Thr^{389} enables PDK1-mediated phosphorylation of the T-loop on Thr^{229}, resulting in full activation of S6K1. Phospho-Thr^{389} serves as docking site for PDK1. Owing to insufficient data, the temporal order of TM site phosphorylation (Ser^{371}) is not depicted. (**B**) Model 2: alternative model. Step 1: an unknown kinase phosphorylates the inactive form of S6K on the TM site Ser^{371}. Step 2: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 3: the release of the autoinhibitory C-terminal domain enables PDK1 access to the T-loop. Step 4: PDK1-mediated phosphorylation of Thr^{229} promotes mTORC1-mediated phosphorylation on the HM site, Thr^{389}. KD, kinase domain; N, N-terminal domain. An animation of this Figure is available at http:www.BiochemJ.org/bj/441/0001/bj4410001add.html.

sites Thr^{389}, Thr^{229} and Ser^{404} [61–63]. Strikingly, additional deletion of the C-terminus (ΔNT/ΔCT) restores kinase activity (although to levels significantly less than maximal, 5–15 %) and restores phosphorylation of the rapamycin-sensitive sites. These results indicated that the N-terminus of S6K1 serves two critical functions: first, it functions in the reception of an activating input critical for Thr^{389} and Thr^{229} phosphorylation; and, secondly, it suppresses an inhibitory function mediated by the C-terminus. In 2002, a short sequence at the extreme N-terminus of p70-S6K1 (FDIDL; amino acids 5–9) was identified to be critical for mitogen-stimulated S6K1 activation and phosphorylation of rapamycin-sensitive sites, and was named the TOS motif [59]. Deletion of the TOS motif or mutagenic inactivation of the motif (F5A mutation within the FDIDL sequence) abolishes S6K1 kinase activity as well as Thr^{389} and Thr^{229} phosphorylation, thus mapping the critical regulatory function of the N-terminus to a specific motif. As in ΔNT/ΔCT, deletion of the C-terminus from the F5A mutant (F5A-ΔCT) partially restored kinase activity and Thr^{389} phosphorylation. 4EBP1, the other well-characterized mTOR-regulated target, was also found to contain a TOS motif (FEMDI; amino acids 114–118 at the extreme C-terminus) [59]. In 2003, several groups demonstrated that the S6K1 and 4EBP1 TOS motifs directly bind raptor, a critical mTOR-interacting scaffold protein, thus enabling mTORC1 to engage substrates and to mediate phosphorylation of rapamycin-sensitive sites [64,65].

The discovery that the S6K1 N-terminus also functions to suppress an inhibitory C-terminal function was not elucidated further until 2005, when a motif (RSPRR) was identified in the C-terminus of p70-S6K1 (amino acids 410–414), shortly after the linker region (see Figure 2) [66]. Mutation of the RSPRR motif within the dead ΔNT or TOS motif-mutant (F5A) backbone (ΔNT-R3A or F5A-R3A) rescued insulin-stimulated Thr^{389} phosphorylation and S6K1 activation. Thus, in a mutant lacking an intact TOS motif (i.e. ΔNT or F5A), an intact RSPRR motif suppresses mTOR-mediated Thr^{389} phosphorylation, and thus inactivation of the RSPRR motif rescues activity. The mechanism by which the S6K1 TOS motif suppresses the inhibitory RSPRR motif remains a mystery, although one hypothesis posits that the RSPRR motif functions as a docking site for a negative regulator, such as a phosphatase, that is suppressed by mTORC1 [66]. Indeed, weak evidence suggests the involvement of a phosphatase in the regulation of mTORC1 substrates (described in more detail below).

T-loop site phosphorylation by PDK1

Maximal S6K1 activation in response to growth factors requires the co-ordinate phosphorylation of both Thr^{229} and Thr^{389} [38,39,50]. Using *in vitro* and *in vivo* approaches, in 1998 the constitutive kinase PDK1 was shown to directly phosphorylate S6K1 on the T-loop site Thr^{229} to promote S6K1 activity [38,39], similar to the earlier identified role of PDK1 in phosphorylation of the Akt T-loop site Thr^{308} [67]. As S6K1 is enzymatically dead in *PDK1*$^{-/-}$ embryonic stem cells or when mutated to T229A, S6K1 activation absolutely requires T-loop phosphorylation [50,68]. *In vitro* phosphorylation of S6K1 by PDK1 was found to activate S6K1-ΔCT significantly better than full-length S6K1, but to poorly activate an S6K1 mutant bearing alanine residue substitutions at C-terminal phosphorylation sites [38]. These results suggested that an intact unphosphorylated C-terminus blocks access of PDK1 to the activation loop. Moreover, PDK1 poorly activated S6K1 T389A-ΔCT or T371A-ΔCT *in vitro*, revealing required roles for the HM and TM sites in PDK1-mediated S6K1 activation [38].

S6K1 T-loop phosphorylation (Thr^{229}) relies upon the PIF (PDK-interacting fragment)-binding pocket found within the PDK1 kinase domain, but does not depend on the PDK1 PH (pleckstrin homology) domain [69]. Conversely, Akt T-loop phosphorylation (Thr^{308}) does not require the PIF-binding pocket of PDK1, yet is highly dependent on the PDK1 PH domain [70]. Clearly, a distinction exists among PDK1 substrates in that PtdIns(3,4,5)P_3-dependent activation at the membrane (e.g. Akt) relies upon PH domain function, whereas PtdIns(3,4,5)P_3-independent activation in the cytosol (e.g. S6K1) relies upon the PIF-binding pocket for PDK1 interaction. The observation that Thr^{229} phosphorylation on S6K1 occurs in a PI3K-dependent manner (sensitive to wortmannin) in response to insulin [71] probably reflects co-operativity between Thr^{229} phosphorylation and Thr^{389} phosphorylation, as Thr^{389} phosphorylation occurs in a PtdIns(3,4,5)P_3-dependent manner via Akt/mTORC1. A recent crystal structure has confirmed that S6K1 bearing a PDK1-phosphorylated T-loop relative to an unphosphorylated T-loop induces local ordering of this normally disordered segment [72].

TM site phosphorylation

Although S6K1 activation absolutely requires TM site phosphorylation on Ser^{371} (S6K1-S371A is enzymatically dead), the regulation and function of this phosphorylation event, as well as the identity of the Ser^{371} kinase, remains unclear [51]. In some reports, serum or insulin stimulation modestly increases Ser^{371} phosphorylation ($\sim$2-fold) in a rapamycin- and wortmannin-sensitive manner [59,71]. S6K1 in growth-factor-deprived cells, however, bears significant Ser^{371} phosphorylation that is rapamycin-resistant. As kinase-dead S6K1 displays normal Ser^{371} phosphorylation, this site does not represent a site of autophosphorylation [51]. As addition of a T389E substitution fails to restore any kinase activity to the dead S371A mutant, these data suggest that Ser^{371} phosphorylation plays an important yet independent role in regulating the intrinsic catalytic activity of S6K1 [51]. Lastly, mTOR reportedly phosphorylates Ser^{371} *in vitro*, and overexpression of TOR in intact cells modestly increases Ser^{371} phosphorylation, whereas overexpression of a kinase-dead mTOR allele modestly reduces Ser^{371} phosphorylation [71]. These results suggest that mTOR contributes to the regulation of S6K1 Ser^{371} phosphorylation. As phospho-Ser^{371} does not correlate well with mTORC1 activity, another kinase may co-operate with mTOR to regulate Ser^{371} phosphorylation. By analogy to Akt in which TM site phosphorylation (Thr^{450}) occurs co-translationally and thus represents an early phosphorylation event [56], it is temping to speculate that S6K1 TM site phosphorylation also represents an early event that occurs co-translationally prior to T-loop and HM site phosphorylation. Indeed, a recent report supports such an idea, as Ser^{371} phosphorylation occurs simultaneously with the production of S6K1 protein from a transfected plasmid [55].

Models: stepwise activation of S6K1 via ordered multi-site phosphorylation

Investigation of Thr^{229} and Thr^{389} phosphorylation not only revealed strong positive co-operativity between these sites for S6K1 activation, but also addressed their temporal relationship to each other. The results support two models for the stepwise activation of S6K1 via ordered multi-site phosphorylation (Figure 2). The conventional Model 1 suggests that mTORC1-mediated phosphorylation of Thr^{389} occurs prior to PDK1-mediated phosphorylation of Thr^{229} (Figure 2A) [38,39,60]. In this model, based largely on analogy to PDK1-mediated activation of RSK2, phospho-Thr^{389} becomes a docking site for PDK1, which then phosphorylates Thr^{229} on the activation loop [73]. The alternative Model 2 suggests that PDK1-mediated phosphorylation of Thr^{229} occurs prior to mTORC1-mediated phosphorylation of Thr^{389} (Figure 2B) [50,55].

In support of Model 1, an S6K1-T389A mutant exhibits reduced growth-factor-stimulated Thr^{229} phosphorylation in intact cells [50]. *In vitro*, PDK1 phosphorylates S6K1-T389E-D3E significantly better than wild-type, T389E or D3E, revealing important roles for the HM site and C-terminal phosphorylation in PDK1-mediated S6K1 phosphorylation [39,60]. In support of Model 2, an S6K1-T229A mutant exhibits reduced growth-factor-stimulated Thr^{389} phosphorylation in intact cells; S6K1 bears substantial Thr^{229} but not Thr^{389} phosphorylation in serum-deprived cells, and $PDK1^{-/-}$ embryonic stem cells lack Thr^{389} phosphorylation [50,68,74]. A recent study demonstrates that mTOR-mediated phosphorylation of S6K1 on Thr^{389} *in vitro* (using a C-terminally truncated S6K1 allele) requires prior *in vitro* phosphorylation by PDK1 on Thr^{229} [55]. Owing to insufficient data, the widely accepted Model 1 does not order TM site phosphorylation on Ser^{371} relative to HM and T-loop site phosphorylation. Recent data supporting alternate Model 2, however, suggest that TM site phosphorylation represents an early event that occurs prior to HM and T-loop site phosphorylation [55].

Generation of rapamycin-resistant S6K1 mutants

S6K1 structure–function analysis has generated various rapamycin-resistant mutants, which have proven useful as tools to identify mTORC1-regulated cellular processes mediated by S6K1 [75–77]. ΔNT/ΔCT represents the first rapamycin-resistant mutant [61]. Although possessing low intrinsic catalytic activity, this mutant bears complete rapamycin resistance. Addition of phospho-mimetic T389E to ΔNT/ΔCT (ΔNT-T389E-ΔCT) creates a constitutive kinase with full activity and complete rapamycin resistance [59]. Similarly, targeted inactivation of the C-terminal RSPRR-motif within the F5A-T389E backbone (F5A-T389E-R3A) recapitulates the behaviour of the F5A-T389E-ΔCT mutant [66]. This F5A-T389E-R3A mutant thus represents an improved rapamycin-resistant S6K1 allele due to its full-length nature.

The presence of Thr^{389} phosphorylation on S6K1-ΔNT/ΔCT isolated from rapamycin-treated cells questioned the idea that mTORC1 represents the sole S6K1 Thr^{389} kinase. In ΔNT/ΔCT, serum and insulin promote Thr^{389} phosphorylation and kinase activation in a completely rapamycin-resistant manner, suggesting that a rapamycin-insensitive kinase mediates Thr^{389} phosphorylation [61,63]. This conundrum was resolved in 2005 with the discovery that rapamycin-insensitive mTORC2 mediates non-physiological S6K1 Thr^{389} phosphorylation in S6K1 mutants lacking a C-terminus [78]. A notable feature of S6Ks is their rather atypical C-terminal extension not found in other AGC kinase family members. The absence of this C-terminal extension in Akt may explain why mTORC2 mediates phosphorylation of the Akt HM site Ser^{473}. This knowledge explains the range of sensitivities to rapamycin displayed by various S6K1 mutants. In the dead ΔNT allele, mTORC1 (mTOR/raptor) cannot dock to S6K1 and phosphorylate Thr^{389}, and mTORC2 (mTOR/rictor) cannot phosphorylate Thr^{389} due to steric hindrance imposed by the extended C-terminus [78]. In the partially rapamycin-resistant ΔCT mutant, both rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 co-operatively mediate Thr^{389} phosphorylation. In ΔNT/ΔCT (and F5A-ΔCT), only rapamycin-insensitive mTORC2 mediates Thr^{389} phosphorylation.

S6K1 regulation by other less well-defined modes

Although phosphorylation represents the best understood mechanism underlying S6K1 regulation, roles for other PTMs (post-translational modifications) have been proposed, including phosphatase-mediated dephosphorylation, acetylation, ubiquitination and regulated subcellular localization.

Dephosphorylation

Addition of rapamycin to cycling cells in culture results in rapid dephosphorylation of S6K1 (Thr^{389}), suggesting action by a regulated phosphatase. Indeed, S6Ks have been suspected to represent targets of PP2A (protein phosphatase 2A)-like phosphatases. In *S. cerevisiae*, TOR regulation of several substrates occurs via suppression of PP2A-like phosphatases [79]. In mammals, PP2A reportedly co-immunoprecipitates with S6K1 [80]; moreover, an independent study showed that PP2A binds wild-type but not ΔNT/ΔCT S6K1 [81]. It is important to note, however, that since the late 1990s there has been little follow-up regarding the role of PP2A-like phosphatases in S6K1 regulation. Recent work in *Drosophila melanogaster* demonstrates that genetic ablation of the PP2A regulatory subunit B' (PP2A-B') leads to dS6K (*Drosophila* S6K) deregulation and a variety of metabolic defects [82]. High levels of phosphorylated S6K (on Thr^{389}) were also detected in human cells upon knockdown of PPP2R5C, the human PP2A-B' orthologue [82]. Whether mTOR suppresses a PP2A-like phosphatase to modulate S6K1 Thr^{389} phosphorylation in mammals as in yeast remains unclear at this time.

Acetylation and ubiquitination

Although significantly less well understood relative to protein phosphorylation, acetylation and ubiquitination represent additional PTMs that modify protein function. Two acetyltransferase enzymes, p300/CBP (cAMP-response-element-binding protein-binding protein) and PCAF (p300/CBP-associated factor), reportedly interact with and acetylate S6K1 and S6K2 both *in vitro* and *in vivo* [83]. Acetylation of S6K1 occurs at the extreme C-terminus (Lys^{516}) in response to mitogens, and acetylation and phosphorylation events appear to occur independently of one another [84]. Although the function of acetylation remains unclear, this PTM may serve to stabilize S6Ks, as treatment of cells with the HDAC (histone deacetylase) inhibitor trichostatin A increases S6K2 acetylation and protein abundance [83]. Polyubiquitination of proteins induces their degradation by the 26S proteasome. Both S6K1 and S6K2 appear to experience this PTM in response to mitogen stimulation [85,86]. Identified in a yeast two-hybrid screen as an S6K1-intereacting protein, the ubiquitin ligase ROC1 was shown to interact with and ubiquitinate S6K1 [87]. As RNAi (RNA interference) against ROC1 increases steady-state levels of S6K1, these results suggest that polyubiquitination may destabilize S6K proteins and thus may function as a mechanism for signal attenuation. Indeed, it was reported recently that Akt phosphorylation on its HM site (Ser^{473}) induces Akt polyubiquitination and subsequent degradation as a means to attenuate Akt signalling [88]. Future work will be required to define the roles of acetylation and polyubiquitination in S6K regulation and function.

Subcellular localization

Whether subcellular localization of S6K1 and S6K2 contributes to their regulation and/or function remains an important unresolved question. The lack of antibodies that specifically detect endogenous S6K1 and S6K2 isoforms by immunofluorescence of fixed cells has precluded such a traditional approach. Thus the limited analysis performed so far has relied on either immunofluorescence of tagged exogenously-expressed kinases or cellular fractionation, a challenging biochemical technique. Treatment of cells with the nuclear export inhibitor leptomycin B causes p70-S6K1, p54-S6K2 and mTOR to accumulate in the nucleus (even though p70-S6K1 does not possess an obvious NLS), indicating that these isoforms shuttle between the cytosol and nucleus [89,90]. p54-S6K2 was found to localize predominantly to the nucleus (probably due to the presence of both N- and C-terminal NLS motifs), and thus leptomycin B had little effect [17,90]. Activation of PKCs via the phorbol ester PMA induced the phosphorylation of both S6K2 isoforms within their C-terminal NLS motifs, which promoted the shuttling of p54- but not p56-S6K2 from the nucleus to the cytosol [90]. It was proposed that NLS phosphorylation blunted NLS function. These data support the idea that S6Ks may shuttle between different subcellular compartments. More research will be required, however, to understand whether S6K subcellular localization controls S6K regulation and/or cellular function.

S6K2 regulation

Although the majority of studies aimed at elucidating S6K regulation have focused on S6K1, more limited results suggest that S6K2 regulation occurs via similar, although probably non-identical, mechanisms [91–93]. Insulin, serum and phorbol esters activate S6K2, similar to S6K1, and seven of eight phosphorylation sites found in S6K1 are also conserved in S6K2 (Thr^{228}, Ser^{370}, Thr^{388}, Ser^{403}, Ser^{410}, Ser^{417} and Ser^{423} on p54-S6K2) [15–17,93]. Insulin-stimulated activation of S6K2 requires C-terminal phosphorylation (on Ser^{410}, Ser^{417} and Ser^{423}), yet the C-terminus of S6K2 exerts a more potent inhibitory effect on kinase function than the C-terminus of S6K1 [91,92]. Wortmannin and rapamycin block insulin-stimulated activation of S6K2 [15–17,93], suggesting that mTORC1 phosphorylates the HM site (Thr^{388}) and PDK1 phosphorylates the activation loop site (Thr^{228}), similar to S6K1. Consistent with these data, phosphorylation of S6K2 on the PDK1 site (Thr^{228}) and the mTORC1 site (Thr^{388}) is required for kinase activity, as alanine residue substitution mutants at these sites render S6K2 enzymatically dead [93]. Unlike S6K1, phospho-mimetic substitution at Thr^{388} (T388E) renders S6K2 fully active, as well as wortmannin- and rapamycin-resistant [93]. Lastly, S6K2 localizes predominantly to the nucleus via a C-terminal NLS (KKSK^{474}RGR), disruption of which results in cytosolic localization [17]. Mutation of K474M within the NLS had no effect on S6K2 kinase activity, however, indicating that S6K2 activation does not require its ability to localize to the nucleus [17].

mTOR SIGNALLING NETWORKS

The absolute requirement for mTOR in S6K activation renders understanding of mTOR regulation and mTORC network wiring essential if we hope to fully understand S6K regulation and function. TOR belongs to the PIKK (PI3K-related kinase) superfamily, yet acts as a serine/threonine protein kinase, not a lipid kinase [1,2]. TOR functions as an environmental sensor, as it responds to and integrates diverse cellular signals (e.g. growth factors and mitogens, nutrients, energy, stress) to modulate cell physiology in an appropriate manner. TOR forms the catalytic core of at least two known multi-subunit complexes, TORC1 and TORC2. These complexes contain shared as well as unique partners that confer differential sensitivity to rapamycin, regulation and substrate selectivity. Acute treatment of cells with

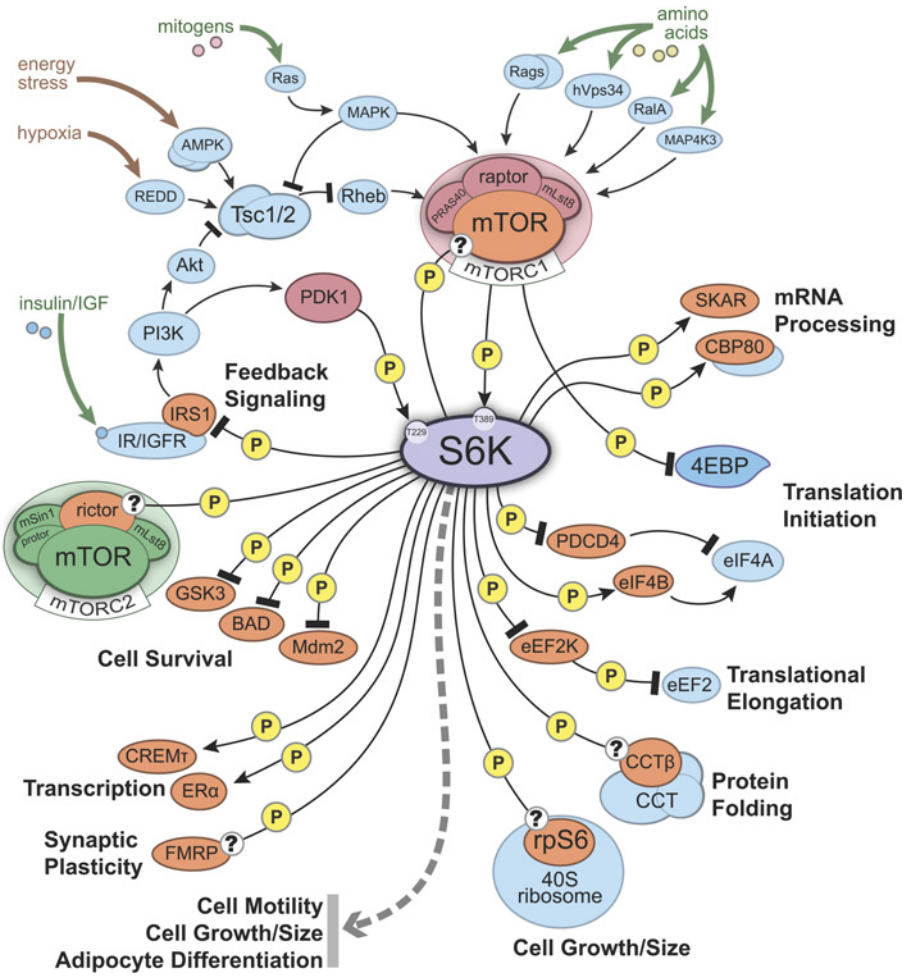

Figure 3 Regulation, substrates and functions of the mTORC1–S6K1 signalling network

S6K1 activation occurs via co-ordinated phosphorylation by mTORC1 and PDK1. Growth factors/mitogens, amino acids and energy activate, whereas cellular stresses such as hypoxia and depleted ATP levels suppress, the mTORC1–S6K1 axis. Through its identified substrates, and other unclear mechanisms, S6K1 promotes several broad cellular processes: protein production, cell growth/size, cell survival, gene transcription, adipocyte differentiation and synaptic plasticity. S6K1 regulates protein production in several ways: mRNA processing (via SKAR and CBP80), cap-dependent translation initiation (via PDCD4, eIF4B and co-ordination with 4EBP1) and translational elongation (via eEF2 kinase); additionally, S6K may regulate ribosome function (via rpS6; function unknown) and nascent protein folding (via CCTβ). S6K1 participates in cell survival signalling [via BAD, Mdm2, GSK3, and possibly through rictor (mTORC2)], regulates two transcription factors [CREMτ and oestrogen receptor α (ERα)], modulates synaptic plasticity (via FMRP) and promotes negative feedback on PI3K signalling (via IRS1, thus suppressing insulin/IGF sensitivity, and rictor). Refer to the main text for more detail. Key: black arrows, activation; black blocks, inhibition; circled question mark, unclear function; grey broken arrow, activates through unknown effectors; yellow circled P, phosphorylates; orange ovals, S6K substrates; green arrows/text, positive mTORC1–S6K1 inputs; brown arrows/text, negative mTORC1–S6K1 inputs. IR, insulin receptor; IGFR, insulin-like growth factor receptor.

rapamycin inhibits mTORC1 but not mTORC2 [6], although chronic rapamycin treatment inhibits mTORC2 by blocking complex assembly [94].

In mammals, both mTORC1 and mTORC2 contain mTOR, mLST8 (mammalian lethal with SEC13 protein)/GβL (G-protein β-protein subunit-like) and deptor (DEP domain-containing mTOR-interacting protein) [3,95] (Figure 3). The presence of raptor and PRAS40 (proline-rich Akt substrate of 40 kDa) defines mTORC1, whereas the presence of rictor, mSin1 (mammalian stress-activated MAPK-interacting protein 1) and protor 1/2 (protein observed with rictor 1/2) defines mTORC2. Raptor and rictor serve as critical scaffolds that control complex assembly, regulation by cellular signals and substrate choice. Deptor functions as a negative regulator of both complexes, whereas the function of mLST8/GβL remains unclear. Within mTORC1, PRAS40 suppresses mTORC1 signalling, either by functioning as a *bona fide* inhibitor or competitive substrate. It is important to note that the roles of the various mTOR-associated proteins in mTORC1/2 function remain incompletely defined. Today, we understand the regulation of mTORC1 significantly better than mTORC2.

mTORC1 regulation

mTORC1 responds to a diverse array of upstream signals, including growth factors, mitogens, and cytokines, amino acids, energy and cell stress. Below, we review several of the best-understand pathways that control mTORC1 signalling, thus enabling mTORC1 to function as an environmental sensor (Figure 3).

Insulin/PI3K signalling

The insulin/PI3K pathway represents the best-characterized activator of mTORC1 [1,95]. Binding of insulin or IGF to its cognate cell-surface receptor leads to tyrosine phosphorylation

of IRS (insulin receptor substrate) proteins, followed by recruitment and activation of PI3K. Generation of PtdIns(3,4,5)P_3 on the plasma membrane by PI3K leads to PDK1-mediated phosphorylation of Akt on its T-loop site (Thr^{308}) and mTORC2-mediated activation on its HM site (Ser^{473}) [67,96]. Activated Akt then phosphorylates Tsc2 (tuberous sclerosis complex 2) on several sites (Ser^{939} and Thr^{1462}) to suppress the inhibitory effect of the Tsc1–Tsc2 complex on mTORC1, thus leading to increased mTORC1 signalling [97]. In addition to phosphorylating Tsc2, Akt phosphorylates PRAS40 (Ser^{246}) to disrupt the inhibitory raptor–PRAS40 interaction, thus promoting mTORC1 signalling [98,99]. Tsc1 and Tsc2 function as tumour suppressors [100]. Loss of either causes the autosomal dominant TSC, a disease characterized by benign tumour formation in various organs, including the brain, kidneys and heart. Tsc2 (also called tuberin) acts as a GAP (GTPase-activating protein) toward the Ras-like small GTP-binding protein Rheb, whereas Tsc1 (also called hamartin) functions to maintain the Tsc1–Tsc2 complex stability [101,102]. Rheb represents the most proximal positive regulator of mTORC1 known to date. Rheb reportedly binds weakly to the mTOR kinase domain to enhance substrate recruitment in a GTP-dependent manner [103,104]. Upon activation by insulin, mTOR within mTORC1 and mTORC2 autophosphorylates (Ser^{2481}); thus mTOR Ser^{2481} autophosphorylation monitors intrinsic catalytic activity of mTOR complexes [105].

The current model posits that insulin/PI3K signalling leads to Akt-mediated inactivation of Tsc1/Tsc2 function, which in turn promotes Rheb-GTP-mediated activation of mTORC1. However, many details implicit to this model remain incomplete. For example, it remains unclear how Akt-mediated phosphorylation of Tsc2 suppresses Tsc1–Tsc2 function. Does Tsc2 phosphorylation inhibit Tsc2 GAP activity, dissociate Tsc2 from Tsc1, and/or induce Tsc2 degradation [100]? The mechanism by which Rheb promotes mTORC1 signalling also remains poorly understood, and the GEF (guanine-nucleotide-exchange factor) that loads Rheb with GTP remains unknown. Emerging data also indicate that phosphorylation of mTOR and its interacting partners (e.g. raptor, rictor, PRAS40 and deptor) contributes to mTORC1 and mTORC2 regulation. For example, phosphorylation of mTOR on several sites (Ser^{1261}, Ser^{2159} and Thr^{2164}) promotes mTORC1 signalling and cell growth [106,107]. In response to the appropriate signals, mTOR, RSK and ERK (extracellular-signal-regulated kinase) phosphorylate raptor, whereas Akt and mTOR phosphorylate PRAS40, events that promote mTORC1 signalling [98,99,108–113]. Additionally, growth factor and nutrient signals promote the phosphorylation and degradation of deptor by the ubiquitin–proteasome system, which results in increased mTORC1 and mTORC2 signalling [114].

Ras/MAPK signalling

Independent of the insulin/PI3K/Akt pathway, the mitogen-activated Ras/MEK (MAPK/ERK kinase)/MAPK signalling cascade activates mTORC1 by converging on Tsc1/Tsc2. Reminiscent of Akt phosphorylation, ERK and its substrate RSK phosphorylate Tsc2 (Ser^{540}, Ser^{644} and Ser^{1798} respectively), which inhibits Tsc1/Tsc2 and thus promotes Rheb-mediated mTORC1 activation [115,116]. The Ras/MAPK pathway also converges on raptor to promote mTORC1 function. Both ERK and RSK phosphorylate raptor (on Ser^{8}/Ser^{696}/Ser^{863} and Ser^{719}/Ser^{721}/Ser^{722} respectively) [110,111]. Thus the PI3K/Akt and Ras/MAPK pathways signal in a parallel manner to regulate Tsc1/Tsc2 and raptor function, indicating a level of functional redundancy between these two mitogen-regulated signalling systems.

PLD (phospholipase D) signalling

PA (phosphatidic acid), a lipid second messenger produced by PLD-mediated hydrolysis of phosphatidylcholine, binds the mTOR FRB domain to promote mTORC1 signalling in a rapamycin-sensitive manner [117]. Growth factors and amino acids activate PLD, which appears to function downstream of Rheb-GTP [118]. More recently, it was reported that PA promotes assembly of both mTORC1 and mTORC2, which accordingly promotes signalling [119]. Interestingly, rapamycin competes for PA binding to mTOR, and much higher concentrations of rapamycin are required for PA–mTORC2 than PA–mTORC1 competition. These results provide an intriguing molecular model for the rapamycin insensitivity of mTORC2 relative to mTORC1.

Cytokine signalling

Downstream of TNFα (tumour necrosis factor α), activated IKKβ [inhibitor of NF-κB (nuclear factor-κB) kinase-β] binds to and phosphorylates Tsc1 (Ser^{487} and Ser^{511}), resulting in Tsc1–Tsc2 dissociation, mTORC1 activation and increased tumour angiogenesis and insulin resistance [120,121]. Additionally, insulin and TNFα promote mTORC1 signalling via a mechanism that involves direct interaction of IKKα with mTORC1 in an Akt-dependent manner [122,123], which promotes NF-κB-dependent transcriptional activity [124]. Moreover, recent studies suggest that the IKK-related kinase TBK1 (TNF-receptor-associated factor-associated NF-κB activator-binding kinase 1) phosphorylates Akt on both its T-loop and HM sites, Thr^{308} and Ser^{473} respectively [125–127]. Taken together, these results reveal novel links between innate immune signalling and mTOR that warrant further exploration.

Nutrient sensing

Sufficient levels of amino acids are absolutely required for mTORC1 function. Thus even in the presence of abundant growth factors, withdrawal of amino acids, particularly the branched-chain amino acids leucine and isoleucine, rapidly inhibits mTORC1 signalling. As amino acid withdrawal suppresses mTORC1 signalling in Tsc-deficient cells [128], amino acid sensing appears to converge on mTORC1 downstream of Tsc1/2. The bidirectional amino acid permease SLC7A5-SLC3A2 (where SLC, solute carrier family), which imports leucine across the plasma membrane into the cell while exporting glutamine out, is essential for mTORC1 activation [129]. Although the mechanism by which cells sense amino acids levels remains a mystery, several biochemical mediators that link amino acid sensing to mTORC1 have been reported, including hVPS34, a class III lipid kinase known to function in vacuolar sorting and autophagy in yeast, the MAP4K3 (MAPK kinase kinase kinase 3), the RalA GTPase and the Rag GTPases [1,2,95].

The Rag family of GTPases represents the best-characterized link between amino acid sensing and mTORC1 [130,131]. Rags function as heterodimers in which RagA or RagB dimerizes with RagC or RagD, with each Rag class bearing opposing nucleotide-bound states. Upon amino acid stimulation, active Rag heterodimers (i.e. $RagB^{GTP}$–$RagD^{GDP}$) bind mTORC1 directly via raptor, which enables mTORC1 to localize to a Rab7-positive late endosomal/lysosomal membrane compartment that has been shown to also contain exogenously expressed Rheb [130]. Endogenous Rheb, however, has not been localized to this compartment. Such a model explains why growth-factor-induced mTORC1 activation absolutely requires amino acids: mTORC1 must reside in the correct subcellular compartment to undergo

activation by Rheb. In a follow-up study, a complex containing three proteins (MP1, p14 and p18) renamed 'Ragulator' was found to reside on lysosomal membranes and to bind to and recruit Rag heterodimers [132]. Thus, upon amino acid stimulation, active Rag heterodimers bound to the Ragulator complex recruit mTORC1 to lysosomes for activation in a Rheb-dependent manner. Interestingly, the MP1–p14–p18 complex was first identified as a scaffold for MEK that is critical for the endosomal localization and activation of a branch of the MAPK pathway [133]. Thus it appears as though the Ragulator complex regulates at least two signalling systems via endosomal-anchored spatial control. Indeed, forced localization of mTORC1 to the lysosomal surface eliminates the requirement of amino acids, Rag GTPases and the Ragulator for mTORC1 activation, but not the requirement for Rheb [132]. Recently, Rab family GTPases, which function in endocytic trafficking (e.g. Rab5 and Rab7), were found to modulate mTORC1 signalling [134]. Additionally, the Rho family GTPase Rac1 was recently reported to positively regulate mTORC1 (and mTORC2) by directly binding mTOR and controlling its localization to specific internal membranes [135]. These results lend support to the idea that subcellular trafficking of mTORC1 contributes to its regulation, with important roles for small G-proteins.

Energy and stress sensing

Diverse forms of cell stress down-regulate mTORC1 signalling, including glucose withdrawal, hypoxia, DNA damage and ER (endoplasmic reticulum) stress [95]. Energy stress induced by glucose withdrawal or by chemical inhibition of glycolysis or mitochondrial respiration leads to a rapid fall in cellular ATP levels. The resulting rise in the cellular AMP/ATP ratio activates AMPK (AMP-activated kinase), a trimeric complex composed of $\alpha/\beta/\gamma$ subunits [136]. Activated AMPK phosphorylates Tsc2 (Thr^{1227} and Ser^{1345}) to augment Tsc1–Tsc2-mediated inhibition of mTORC1 [137]. In Tsc-deficient cells, however, energy stress still induces partial inhibition of mTORC1, suggesting a Tsc-independent mechanism for AMPK regulation of mTORC1. Indeed, AMPK phosphorylates raptor (Ser^{792} and Ser^{722}) to down-regulate mTORC1 in response to energy stress [138]. Independent of AMPK, energy stress inhibits mTORC1 by suppressing Rheb-GTP loading via a mechanism involving PRAK-mediated phosphorylation (Ser^{130}) and inhibition of Rheb in response to p38β MAPK signalling [139]. Thus AMPK-dependent and -independent mechanisms co-operate to down-regulate mTORC1 in response to energy stress.

Hypoxia also reduces ATP levels, thus leading to AMPK-mediated down-regulation of mTORC1 by mechanisms described above. Via a mechanism independent of cellular ATP levels, hypoxia stabilizes the HIF1 (hypoxia-inducible factor 1) transcription factor, which induces expression of a number of survival genes, including REDD1 (regulated in development and DNA damage responses 1) [140,141]. Although the mechanism of action of REDD1 remains poorly understood, REDD1 appears to bind 14-3-3 proteins, inducing their dissociation from Tsc2 and leading to Tsc1–Tsc2 activation and thus mTORC1 inhibition [142]. In response to DNA damage, stabilization and activation of the p53 transcription factor and tumour suppressor leads to induction of sestrins 1 and 2, which bind to and activate AMPK via an unknown mechanism to down-regulate mTORC1 [143]. ER stress results from the accumulation of misfolded proteins, which activates a signal transduction cascade known as the unfolded protein response that slows global protein synthesis. Indeed, ER stress leads to transcriptional up-regulation of REDD1, resulting in down-regulation of mTORC1 signalling [144]. Clearly, diverse signals of cell stress utilize a variety of molecular mechanisms to ensure mTORC1 inhibition during unfavourable conditions.

mTORC2: regulation and function

The regulation and function of mTORC2 remains poorly understood and thus represents an important area for future research. mTORC2 phosphorylates Akt, SGK1 (serum- and glucocorticoid-induced protein kinase 1) and PKCα on their hydrophobic motif sites (Ser^{473}, Ser^{422} and Ser^{657} respectively) [96,145,146]. Indeed, rictor$^{-/-}$, mSin1$^{-/-}$ and mLST8/GβL$^{-/-}$ MEFs (mouse embryonic fibroblasts) display significantly reduced Akt Ser^{473} phosphorylation [147,148]. On the basis of these substrates, mTORC2 probably controls cell proliferation, cell survival and cell metabolism. Additionally, in yeast and mammals, mTORC2 appears to modulate the actin cytoskeleton [42,149,150]. mTORC2 also promotes TM site phosphorylation on Akt (Thr^{450}) and several PKCs (PKCα Thr^{638} and PKCβ Thr^{641}) (directly or indirectly) [151,152], which enhances their stability and folding. In the case of Akt Thr^{450} phosphorylation, mTORC2 associates with ribosomes to promote co-translational TM site phosphorylation and stability of nascent Akt polypeptides [56]. As the mTORC1 substrate S6K1 and the mTORC2 substrates Akt, SGK1 and PKCα all belong to the AGC kinase family, an emerging theme in the mTOR field is that mTORC1 and mTORC2 phosphorylate AGC kinases. It is important to note, however, that additional kinases have been reported to mediate Akt HM site phosphorylation on Ser^{473}, including DNA-PK (DNA-dependent protein kinase) [153], and quite recently the IKK-related kinases TBK1 and IKKε [125–127], indicating that several upstream kinases co-operate to regulate this important survival kinase. As insulin/PI3K signalling promotes Akt Ser^{473} phosphorylation, and as pharmacological inhibition of PI3K reduces mTORC2 kinase activity *in vitro* [154], PI3K presumably lies upstream of mTORC2. Consistent with this idea, insulin/PI3K signalling in cultured adipocytes promotes mTOR phosphorylation (Ser^{1261}) as part of mTORC2 [106]. Interestingly, the Tsc1–Tsc2 complex promotes rather than suppresses mTORC2 activity, the opposite to its effect on mTORC1, hinting that mTORC2 regulation may be quite different from that of mTORC1 [154,155]. As the mTORC2 substrates Akt, SGK1 and PKCα respond to different growth factors, it is likely that several types of growth factor signals converge on mTORC2.

CELLULAR SUBSTRATES AND FUNCTIONS OF THE mTORC1/S6K1 SIGNALLING AXIS

At the cellular level, mTORC1 functions as a critical regulator of translation initiation, the rate-limiting step in protein synthesis in which ribosomes are recruited to mRNA. Increased protein biosynthetic rates are thought to drive cell growth (an increase in cell mass and size), a requirement for cells to progress through the cell division cycle and proliferate (increase in number). Indeed, mTORC1 promotes cell growth and cell proliferation in response to anabolic cues via phosphorylation of S6K1 and 4EBP1, at least in part. Although it has been appreciated that the mTORC1–4EBP1 axis directly controls translation initiation, identifying substrates and functions of the mTORC1–S6K1 axis has proven more challenging.

For at least a decade after the discovery of S6K, rpS6 held ground as its one and only substrate. Since then, several S6K1 substrates have been identified that control protein production (Figure 3). Additional S6K1 substrates participate in

the transcriptional control of ribosome biogenesis, metabolism, lipid synthesis and adipocyte differentiation, as well as cell survival, DNA damage sensing and synaptic plasticity. An S6K1-mediated 'feedback loop' acts on several components of mTOR signalling networks, in some cases to down-regulate insulin signalling, which may contribute to insulin resistance during states of diabetes and obesity. However, detailed understanding of the functional significance of S6K1-mediated phosphorylation for many of these substrates remains limited. It is important to note that many S6K1 substrates {e.g. rpS6, eIF4B, eEF2K [eEF2 (eukaryotic elongation factor 2) kinase], CCTβ [chaperonin containing TCP-1 (t-complex protein 1) β], BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death), GSK3 (glycogen synthase kinase 3)} also serve as RSK substrates, illustrating the convergence of the mTORC1 and MAPK pathways in the common goal of regulating cell physiology.

Does the S6K–rpS6 axis promote mRNA TOP (terminal oligopyrimidine) translation?

S6K1-mediated phosphorylation of rpS6 was believed for quite a while to promote the translation of a class of ~90 transcripts known as TOP mRNAs that encode ribosomal proteins and translation factors [9]. The localization of rpS6 to the boundary between the 40S and 60S subunits in the mature ribosome and thus its potential to interact with mRNA, tRNA and translation initiation factors supported such a notion. In response to growth factors/mitogens or amino acids, the increased translational efficiency of TOP mRNAs correlated with S6K1 activation and rpS6 phosphorylation and occurred in a rapamycin-sensitive manner [156]. Moreover, expression of a rapamycin-resistant mutant of S6K1 (T389E) partially rescued the rapamycin-mediated suppression of TOP translation [157]. Thus increased TOP translation via the action of the mTORC1–S6K1–rpS6 axis was thought to prepare cells for a burst in protein synthesis. A series of subsequent experiments, however, were unable to provide further support for this long-standing model.

First, although inhibition of PI3K strongly inhibits the translational activation of TOP mRNAs in response to growth factors or amino acids, inhibition of mTORC1 with rapamycin only mediates a partial inhibitory effect, while causing complete dephosphorylation of S6K1 and rpS6 [158,159]. Secondly, genetic inactivation of S6K1 in the mouse has no effect on TOP translation; MEFs from these mice, however, still possess rpS6 phosphorylation, which led to the discovery of S6K2 as a second rpS6 kinase [14]. Thirdly, mitogens still promote TOP mRNA translation in a rapamycin-sensitive manner in MEFs lacking both S6K1 and S6K2 [19]. Thus neither of the two S6K genes is required for TOP translation. At this point, a role for rpS6 phosphorylation still remained possible, as rpS6 phosphorylation persisted on two (Ser^{235} and Ser^{236}) of the five (Ser^{235}, Ser^{236}, Ser^{240}, Ser^{244} and Ser^{247}) mitogen-stimulated sites in double-null MEFs [19]. This observation led to the re-identification of RSK as a *bona fide* rpS6 kinase. One final experiment refuted the idea that rpS6 phosphorylation promotes TOP translation. MEFs isolated from mice bearing a genetic knockin of a rpS6 mutant containing alanine residue substitutions at all five mitogen-stimulated sites of phosphorylation ($rpS6^{P-/-}$) exhibit normal TOP translation and, unexpectedly, the rate of global protein synthesis is actually ~2.5-fold higher in these mice [160]. Further analysis confirmed that insulin promotes TOP translation via the PI3K–Tsc–Rheb–mTOR pathway in an S6K–rpS6-independent manner [161]. Knockdown of mTOR strongly reduces the translational efficiency of TOP mRNAs; knockdown of raptor or rictor, however, results in only a modest decrease [161]. These data suggest that, although mTOR regulates TOP translation, it may do so independently of mTORC1 or mTORC2, an intriguing idea that will require further testing.

Protein biosynthesis

Protein biosynthesis represents a major cellular process controlled by mTORC1, which is co-ordinately regulated by the mTORC1–S6K1 and mTORC1–4EBP1 axes (Figure 4). The mTORC1–4EBP1 axis controls critical well-defined steps in the initiation of cap-dependent translation by assembling the eIF4F complex at the m^7-GTP (7-methylguanosine) cap structure found at the 5′-end of mRNA transcripts. Significantly less is known regarding the role of the mTORC1–S6K1 axis in translational control, however (see the reviews by Ma and Blenis [10] and Hershey et al. [162] for greater detail). In the absence of mTORC1-activating stimuli (i.e. growth factors/mitogens, amino acids and energy), hypophosphorylated 4EBP1 binds to and represses eIF4E, which directly interacts with the 5′-cap [34]; additionally, a sub-pool of inactive S6K1 associates with the multi-subunit scaffold eIF3 [163] (Figure 4A). In response to mTORC1-activating stimuli, mTORC1 binds to eIF3, where it is well positioned to phosphorylate S6K1 (on Thr^{389}) and 4EBP1 on several sites (e.g. Thr^{37}, Thr^{46}, Thr^{70} and Ser^{65}). mTORC1-mediated 4EBP1 phosphorylation induces the dissociation of 4EBP1 from eIF4E and the dissociation of S6K1 from eIF3 (Figure 4B). As 4EBP1 and eIF4G (a modular scaffold) bind eIF4E in a mutually exclusive manner, 4EBP1 dissociation enables binding of eIF4G to eIF4E. Once bound to eIF4E, eIF4G recruits eIF4A, a helicase critical for unwinding inhibitory secondary structure in the 5′-untranslated region of mRNA. These events lead to formation of eIF4F, a complex composed of eIF4E, eIF4G and eIF4A, on the 5′-cap (Figure 4C). The assembled eIF4F complex then recruits the 40S ribosome and the ternary complex (composed of eIF2, Met-tRNA and GTP) to the 5′-cap to form the 48S translation pre-initiation complex (Figure 4D).

Upon mTORC1-mediated dissociation of S6K1 from eIF3, active S6K1 phosphorylates several substrates that function in translation initiation as well as other steps that drive protein production, including eIF4B [164,165], PDCD4 (programmed cell death 4) [166], SKAR (S6K1 Aly/REF-like substrate) [167,168], eEF2K [169], CCTβ [170], CBP80 [171] and rpS6, a component of the 40S ribosome (Figures 3 and 4C). S6K1- (and RSK-) mediated phosphorylation of eIF4B (Ser^{422}) induces the recruitment of eIF4B to eIF4A and eIF3 [163–165] (Figures 4C and 4D). eIF4B functions to enhance eIF4A helicase activity, thus alleviating inhibitory secondary structure in the 5′-untranslated region. S6K1-mediated phosphorylation of the eIF4A inhibitor PDCD4 (on Ser^{67}) further enhances eIF4A helicase activity. Phosphorylation of PDCD4, a tumour suppressor, promotes its recognition by the ubiquitination ligase $SCF^{\beta-TRCP}$ and thus its degradation by the proteasome [166]. SKAR, a nuclear mRNA-binding protein of the Aly/REF family, couples transcription with mRNA splicing and nuclear export by interacting with the exon junction complex, which binds to spliced mRNAs [168]. Upon growth-factor-stimulated activation, S6K1 but not S6K2 binds to and phosphorylates SKAR (Ser^{383}/Ser^{385}) to enhance the translational efficiency of newly spliced mRNA [167]. S6K1 (and RSK) also phosphorylates and inactivates eEF2K (Ser^{366}), which phosphorylates and inactivates eEF2 [169]. Thus S6K1 augments the activity of eEF2, a protein that catalyses translocation (codon shifting) during translation elongation. S6K1 (and RSK) phosphorylates CCTβ (Ser^{260}), the β-subunit of chaperonin containing TCP-1 [170]. CCTβ, a large

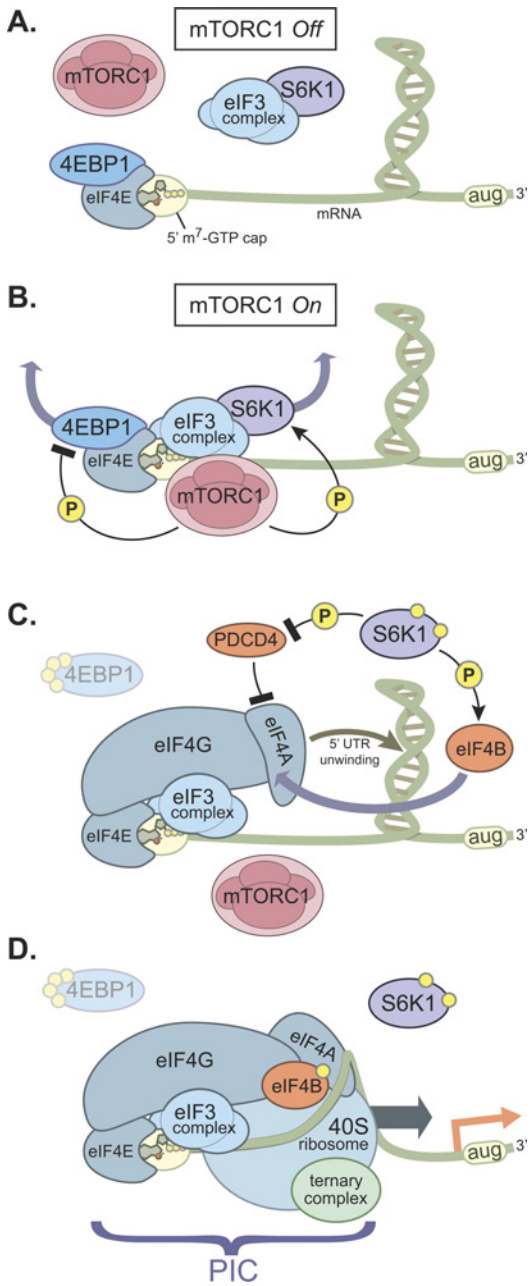

Figure 4 Model for initiation of cap-dependent translation by the mTORC1–4EBP1 and mTORC1–S6K1 axes

(**A**) In the absence of mTORC1-activating stimuli (i.e. growth factors/mitogens, amino acids and energy), hypophosphorylated 4EBP1 binds to eIF4E on the mRNA 5′-cap to suppress assembly of the pre-initiation complex. (**B**) In response to mTORC1-activating stimuli, mTORC1 docks to eIF3, localized at the 5′-cap, whereby it phosphorylates 4EBP1 and S6K1, inducing 4EBP1 release from eIF4E and S6K1 release from eIF3. (**C**) Dissociation of 4EBP1 enables eIF4G to dock to eIF4E, thus initiating assembly of the eIF4F complex (eIF4E, eIF4G and eIF4A). Upon release, S6K1 phosphorylates eIF4B, which induces eIF4B binding to eIF4A, an event that enhances eIF4A helicase activity. S6K also phosphorylates and inactivates PDCD4, which functions as an eIF4A inhibitor. (**D**) Assembly of these factors enables binding of the 40S ribosome and the ternary complex (eIF2, Met-tRNA and GTP) at the 5′-cap and thus formation of the pre-initiation complex (PIC) to initiate cap-dependent translation. See the main text for more details.

multi-subunit complex that interacts with ribosomes and nascent polypeptides, functions in protein folding. Knockdown of CCTβ reduces cell proliferation, a phenotype rescued upon expression of a phospho-mimetic S260D but not a phospho-defective S260A mutant, thus linking CCTβ phosphorylation by S6K1/RSK to CCTβ function [170]. Whether phosphorylation actually affects CCTβ protein folding function will require further study. CBP80, together with CBP20, forms a heterodimeric complex that co-transcriptionally binds the 5′-cap and enhances mRNA splicing. The functional consequence of S6K1-mediated phosphorylation of CBP80 remains unknown [171]. Lastly, S6K1 phosphorylates rpS6 on several sites (Ser^{235}, Ser^{236}, Ser^{240} and Ser^{244}), whereas RSK phosphorylates a subset of these sites (Ser^{235} and Ser^{236}). It is important to note that the functional significance of rpS6 phosphorylation remains unclear [9,11]. Taken together, these data indicate that the mTORC1–4EBP1 and mTORC1–S6K1 axes control several diverse steps in protein biosynthesis.

Cell growth compared with cell proliferation

Cell growth

A plethora of studies in cell culture, flies and mice have revealed cell growth as a major cellular function of the mTORC1–S6K1 axis. The role of S6K in cell growth was first demonstrated with the finding that inactivation of dS6K in *D. melanogaster* results in severe developmental delay and lethality with a marked reduction in body and organ size in surviving animals [172]. Strikingly, this small body/organ phenotype results from a cell autonomous decrease in cell and organ size, without an effect on cell number [172]. Consistently, inhibition of mTORC1 and S6K via rapamycin treatment of cultured mammalian cells reduces cell size, and expression of rapamycin-resistant S6K1 alleles (T389E-D3E and T389E-ΔCT) rescues the rapamycin-induced decrease in cell size [75]. Moreover, acute knockdown of S6K1 with RNAi reduces cell size, and overexpression of S6K1 increases cell size [75,167,173]. As in flies, inactivation of S6K1 in the mouse reduces body and cell size, with pancreatic β-cells and myoblasts showing a prominent decrease in size [14,76,174]. The single knockout of S6K2 in the mouse actually results in larger animals, whereas $S6K1^{-/-}$/$S6K2^{-/-}$ double-knockout animals are similar in size to single $S6K1^{-/-}$ animals [19]. These data indicate that S6K1 but not S6K2 promotes organismal growth.

MEFs and myoblasts isolated from $S6K1^{-/-}$/$S6K2^{-/-}$ double-knockout mice display a reduced cell size relative to wild-type controls, but a similar cell size relative to wild-type MEFs treated with mTOR catalytic inhibitors (e.g. Torin1 and PP242) [76,175]. Moreover, introduction of a constitutively active S6K1 (T389E-D3E) allele into double-knockout MEFs rescues this decrease in cell size in both the absence or presence of mTOR catalytic inhibitors [175]. These results reveal the S6Ks (most likely S6K1) as major downstream effectors of mTORC1-driven cell growth [175]. $rpS6^{P-/-}$ MEFs also display a significantly reduced cell size compared with wild-type controls; moreover, rapamycin fails to decrease their size further [160]. These results reveal an important role for rpS6 phosphorylation in cell growth control. It is important to note, however, that S6K1 can promote cell growth independently of rpS6 phosphorylation, as small $S6K1^{-/-}$ myotubes show normal levels of rpS6 phosphorylation (due to S6K2) [76]. The identification of these additional S6K1 substrates awaits future research. SKAR may represent one of these additional S6K1 effectors that controls cell growth, as its knockdown reduces cell size [167]. Lastly, the ability of the mTORC1–S6K1 axis to promote lipid biosynthesis as well as protein biosynthesis contributes to cell growth control, as knockdown of the lipogenic transcription factor SREBP (sterol-regulatory-element-binding protein) in flies or mammalian cells blunts the increase in cell size driven by PI3K/Akt signalling [176].

Cell proliferation

mTORC1 signalling promotes cell cycle progression and cell proliferation, although the molecular details underlying mTORC1-controlled proliferation remain poorly understood. Although several studies suggest that the mTORC1–S6K1 axis promotes cell cycle progression and cell proliferation, this notion remains somewhat controversial. On the one hand, overexpression of rapamycin-resistant S6K1 confers partial protection from rapamycin-inhibited G_1-/S-phase progression and cell proliferation, and acute knockdown of S6K1 with RNAi reduces G_1-/S-phase progression [76,177]. On the other hand, S6K1$^{-/-}$/S6K2$^{-/-}$ double-knockout MEFs and myoblasts proceed through the cell cycle and proliferate at rates that are similar to wild-type cells [76,175]. How can this apparent paradox be explained? Perhaps S6K1 signalling is sufficient to promote cell proliferation when other mTOR effectors are inactive (as during rapamycin treatment) or perhaps other signalling systems compensate to fully rescue proliferation under chronic inactivation of S6K function (as during S6K1$^{-/-}$/S6K2$^{-/-}$ knockout). Alternatively, rapamycin-resistant S6K1 could signal in a manner different from the wild-type kinase. To confuse matters more, rpS6$^{P-/-}$ MEFs proliferate faster than wild-type controls [160]. Other data suggest that overexpression of S6K1 and S6K2 confers a proliferative advantage on cells in culture [177–179]. Similar to S6K1, overexpression of rapamycin-resistant S6K2 (T388E) partially rescues rapamycin-inhibited G_1-/S-phase progression and cell proliferation [178]. Lastly, S6K2 may play a role in mitosis, as S6K2 but not S6K1 reportedly localizes to the centrosome, and S6K2 kinase activity peaks in G_2- and M-phases [180,181].

The mTORC1–4EBP1 axis appears to promote cell cycle progression and cell proliferation independent of the mTORC1–S6K1 axis. Overexpression of eIF4E under full serum conditions accelerates G_1-/S-phase progression and confers partial protection from rapamycin, whereas dominant inhibitory 4EBP1-F114A (TOS motif) or 4EBP1-AA (phosphorylation site-defective) mutants reduce G_1-/S-phase progression (by blunting mTORC1-mediated 4EBP1 phosphorylation and derepression) [177]. Additionally, MEFs lacking 4EBP1s display resistance to the inhibition of cell cycle progression and cell proliferation caused by mTOR catalytic inhibitors or raptor knockdown [175]. These data reveal the 4EBPs as major cellular effectors of mTORC1-driven cell cycle progression and cell proliferation [175].

Feedback signalling

The mTORC1–S6K1 axis participates in several feedback loops. Chronic mTORC1-mediated activation of S6K1, as in Tsc1$^{-/-}$ or Tsc2$^{-/-}$ cells that lack tumour-suppressive function, induces a state of cellular insulin resistance by a mechanism termed the 'negative-feedback loop' [182,183]. S6K1 signalling represses IRS-1 gene expression and directly phosphorylates IRS-1 on several inhibitory serine residues (i.e. Ser^{307} and Ser^{1101} in humans) (Figure 3) [183–186]. In a co-operative manner, mTOR phosphorylates IRS-1 (Ser^{636}/Ser^{639}) [187]. IRS-1 serine phosphorylation induces IRS-1 degradation via the proteasome and thus uncouples PI3K from the insulin/IGF receptor, leading to reduced signalling to downstream PI3K effectors, including Akt and the Ras/MAPK pathway [184]. This negative-feedback loop may explain in part the insulin resistance common to obesity, a state of chronic nutrient overload and mTORC1 activation. The inactivation of this negative-feedback loop upon mTORC1 inhibition probably also explains in part why rapalogues (e.g. CCI-779 and RAD001) have failed to perform in anti-cancer clinical trials as well as originally hoped [6,7]. Upon mTORC1 activation, S6K1 also participates in other feedback loops of unclear functional significance. S6K1 phosphorylates mTOR (Ser^{2448}) [188,189] and rictor (Thr^{1135}) [190–193]. As an mTOR S2448A mutant exhibits normal mTORC1 signalling, analysis of the mTOR Ser^{2448} phosphorylation state serves only as a read-out for S6K1 activity. Although one report suggested that S6K1-mediated phosphorylation of rictor Thr^{1135} suppresses mTORC2 signalling to Akt [190], other reports did not report such a phenotype [191–193]. In parallel to the mTORC1–S6K1 axis, recent work indicates that mTORC1 phosphorylates Grb10 to mediate negative feedback to insulin/IGF signalling [194,195].

Gene expression

Transcriptional profiling using microarrays has defined both positive and negative roles for mTORC1 signalling in control of gene expression [196–198]. These analyses have revealed mTORC1 signalling to control diverse metabolic genes. mTORC1 signalling up-regulates genes involved in lipid/sterol, nucleotide and protein synthesis, as well as genes involved in mitochondrial oxidative function, glycolysis and the pentose phosphate pathway; conversely, mTORC1 signalling down-regulates genes involved in nutrient breakdown and energy production [196,198]. At the molecular level, how mTORC1 signalling controls gene expression remains poorly understood. Signalling via the mTORC1–S6K1 axis promotes ribosome biogenesis by phosphorylating the rDNA (ribosomal DNA) transcription factor UBF (upstream binding factor) (either directly or indirectly), leading to the activation of 45S ribosomal gene transcription [199]. These data further underscore the dedicated role of mTORC1 in enhancing protein biosynthetic capacity. mTORC1 promotes the expression of genes involved in mitochondrial oxidative function by interacting with the transcription factor YY1 (Yin-Yang 1) [197]. mTORC1 binding to YY1 promotes interaction of YY1 with the co-activator PGC-1α (peroxisome-proliferator-activated receptor γ coactivator-1α). More recently, mTORC1 signalling was shown to modulate gene expression via the transcription factors HIF1α, SREBP1 and SREBP2 [176,198]. Signalling via the mTORC1–4EBP1 axis enhances the translation of HIF1α, which promotes expression of glycolytic genes; signalling via the mTORC1–S6K1 axis promotes the proteolytic processing of SREBP from an inactive precursor to an active transcription factor that rapidly shuttles into the nucleus, which promotes expression of genes in the oxidative pentose phosphate pathway as well as those involved in lipid and sterol biosynthesis [198]. Limited data suggest that S6K1 directly phosphorylates transcription factors to modulate their function. Serum stimulation of S6K1 was shown to phosphorylate and transactivate CREMτ (cAMP-response-element modulator τ) (Ser^{117}) [200]. Additionally, S6K1 (and RSK) phosphorylates oestrogen receptor α (Ser^{167}), leading to its transcriptional activation, which may contribute to breast cancer progression (Figure 3) [201].

Other functions

The mTORC1–S6K1 axis has been linked to a variety of other cellular processes, including GSK3 regulation, adipocyte differentiation, cell survival, cell motility, DNA damage response and synaptic plasticity (Figure 3). In the absence of Tsc1/Tsc2 function, which results in high S6K1 activity but low Akt activity owing to feedback inhibition of PI3K/Akt, S6K1 (rather than Akt) phosphorylates and inactivates the multi-functional kinase

GSK3 [202]. mTORC1 signalling promotes discrete steps in adipocyte differentiation. The mTORC1–S6K1 axis enhances commitment of stem cells to early adipocyte progenitors [203], whereas mTORC1 signalling independent of S6K1 controls terminal adipocyte differentiation [204,205]. These steps in adipogenesis correlate with mTORC1-mediated increases in the expression of several transcription factors, further underscoring the role of mTORC1 signalling in control of gene expression. The mTORC1–S6K1 axis promotes cell survival via S6K1-mediated phosphorylation and inhibition of BAD (Ser^{136}), a pro-apoptotic BH3-only member of the Bcl-2 family [206]. Signalling through mTORC1 promotes cell motility via both the S6K1 and 4EBP1 axes [77]. The mTORC–S6K1 axis has also been reported to participate in the DNA damage response. Upon genotoxic stress, the p38α MAPK pathway activates mTORC1–S6K1, whereby S6K1 binds to and phosphorylates Mdm2 (murine double minute 2) (Ser^{163}), blocking its nuclear import and ability to ubiquitinate the tumour suppressor protein p53 [207]. Thus p53 levels increase, resulting in cell cycle arrest or apoptosis. Lastly, the mTORC1–S6K1 axis participates in protein synthesis-dependent synaptic plasticity. In response to type I mGluR (metabotropic glutamate receptor) stimulation of mouse hippocampus, S6K1 phosphorylates FMRP (fragile X mental retardation protein), a dendritic RNA-binding protein that functions in translational repression and synaptic plasticity [208,209]. Thus S6K1-mediated phosphorylation of FMRP may modulate learning and memory.

THE mTORC1–S6K1 SIGNALLING AXIS IN PHYSIOLOGY AND DISEASE

Abundant evidence indicates important roles for mTORC1 in physiology and myriad disease states, including diabetes, obesity, cancer and benign tumour syndromes, organ hypertrophy, neurological disorders (e.g. autism spectrum disorders and Alzheimer's disease) and aging-related pathology [2,22,209]. These pathological responses probably stem from cellular effects of mTORC1 on protein and lipid synthesis, cell growth, cell proliferation and cellular metabolism. However, our understanding of the mTORC1 effectors that mediate these cellular and physiological responses remains in its infancy. In the present review, we will focus specifically on identified roles for the mTORC1–S6K1 axis in physiology and disease.

Diabetes: glucose homoeostasis, insulin sensitivity and adipocyte metabolism

Deletion of S6K1 in the mouse results in a number of physiological alterations. In addition to having a smaller body and organ size, $S6K1^{-/-}$ mice present with hypoinsulinaemia and glucose intolerance as a result of insufficient insulin production by pancreatic β-cells, which results, at least in part, from a reduction in β-cell size [174]. Strikingly, even though these mice are hypoinsulinaemic, they remain sensitive to insulin due to the elimination of the mTORC1–S6K1-mediated negative-feedback loop and inhibitory IRS-1 phosphorylation (described above). Consistently, the livers of wild-type and obese *db*/*db* (leptin receptor deficient) but not $S6K1^{-/-}$ mice fed on a high-fat diet show increased IRS-1 phosphorylation (Ser^{1101}) and reduced PI3K/Akt signalling [186]. These results demonstrate that up-regulated signalling along the mTORC1–S6K1 axis contributes to insulin resistance *in vivo*. The phenotype of $rpS6^{P-/-}$ knockin mice mimics that of $S6K1^{-/-}$ mice, as they display hypoinsulinaemia with impaired glucose tolerance owing to reduced insulin production by β-cells of reduced size [160]. As these phenotypes reflect the aggregate response of all tissues, conditional knockout of S6K1 in specific tissues (adipose, muscle and liver) will be required to evaluate more precisely the roles of the mTORC1–S6K1 axis in peripheral control of glucose homoeostasis and metabolism and how its dysregulation contributes to diabetes [22].

The mTORC1–S6K1 axis also controls adipocyte metabolism. $S6K1^{-/-}$ mice possess fewer adipocytes owing to decreased differentiation of stem cells into adipogenic precursors (described above) [203]. Additionally, the mice store less fat than wild-type mice, owing to enhanced triacylglycerol (triglyceride) lipolysis, enhanced mitochondrial biogenesis and fatty acid β-oxidation, due to elevated AMPK activity and enhanced metabolic rate, as indicated by increased O_2 consumption [210,211]. Similarly, mTORC1 inhibition in cultured adipocytes via rapamycin or raptor knockdown enhances lipolysis, suppresses lipogenesis and thus reduces fat storage [212,213]. These phenotypes are consistent with the general anabolic role of mTORC1 in metabolism.

Obesity: body mass and energy balance

Whole-body $S6K1^{-/-}$ mice display resistance to age- and diet-induced obesity, revealing a role for the mTORC1–S6K1 axis in control of body mass and energy balance [185]. Modulation of S6K1 activity, specifically in the mediobasal hypothalamus of rat brain, suggests a role for S6K1 in central control of feeding and metabolic responses that maintain energy balance [214]. Injection of adenoviruses expressing constitutively active S6K1 directly into the rat mediobasal hypothalamus decreases food intake and body weight; conversely, injection of adenoviruses expressing dominant-negative S6K1 produces opposite phenotypes [214]. These phenotypes mimic those resulting from mTORC1 activation effected by injection of leptin, an anorexigenic adipocyte-derived hormone, or leucine, an mTORC1-activating signal, into rat hypothalamus [215]. Thus, by these approaches, leptin signalling via mTORC1–S6K1 in the hypothalamus appears to modulate energy balance towards a leaner phenotype. It is important to note that the leaner phenotype produced by increased S6K1 signalling in hypothalamus opposes that produced by whole-body S6K1 knockout, a phenotype that indicates a role for S6K1 in promotion of body mass and adiposity. As many different types of neurons, each with specialized functions, compose the hypothalamus, the regulation and function of mTORC1–S6K1 signalling is probably unique within each type of neuron. Therefore the available data only assess aggregate responses [216]. Indeed, activation of the mTORC1 pathway by inactivation of Tsc1 in one class of neurons (anorexigenic pro-opiomelanocortin neurons) actually increases food intake and body weight, opposite to the results described above [217]. Clearly, more research will be required to unravel the complexities of energy balance control by the mTORC1–S6K1 axis.

Cancer: cell number control

Cancer results from aberrant control of fundamental cellular processes that ultimately control cell number, including cell proliferation, cell growth, cell survival and cell metabolism. The mTORC1 and mTORC2 pathways demonstrate frequent up-regulation in cancer, particularly under cellular conditions of heightened PI3K signalling due to oncogenic activation of PI3K or mutagenic inactivation of the lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) [2,6,7]. As a result, mTOR signalling networks have emerged as attractive targets for novel therapeutic strategies to treat

cancer and various tumour syndromes (e.g. TSC). Indeed, the rapalogues CCI-779 (also known as temsirolimus) and RAD0001 (everolimus) were FDA-approved in 2007 and 2009 respectively to treat renal cell carcinoma. Overall, however, rapalogues have shown a rather disappointing efficacy in anti-cancer clinical trials, probably due to suppression of the mTORC1-mediated negative-feedback loop, which produces the undesired effect of increasing PI3K signalling. Additionally, it has recently become clear that rapamycin and rapalogues do not fully inhibit phosphorylation of all mTORC1 substrates (e.g. 4EBPs) [218]. Thus there exist rapamycin-sensitive (i.e. S6K) and rapamycin-insensitive (i.e. 4EBP1) mTORC1 substrates, which may explain in part the poor clinical efficacy of rapalogues as anti-cancer drugs. The recent development of ATP-competitive mTOR catalytic inhibitors (i.e. Torin1, PP242, Ku-0063794 and WAY600) that inhibit both mTORC1 and mTORC2 offer renewed optimism in clinical oncology [7].

To date, evidence for S6K1 and/or S6K2 signalling in mTOR-mediated tumorigenesis remains limited. S6K1 frequently shows overexpression in certain cancers, particularly breast cancer, due to 17q23 amplification of the *RPS6KB1* gene, and this phenotype correlates with poor prognosis [219]. More recent work suggests that S6K1 indeed contributes to tumorigenesis, although only in tissues bearing low S6K2 expression [220]. At the mRNA level, *S6K1* and *S6K2* show ubiquitous expression to comparable extents across diverse mouse tissues [220]. Although the S6K1 protein shows ubiquitous expression as well, S6K2 protein expression varies greatly in a tissue-specific manner, indicating that post-transcriptional mechanisms fine-tune the protein abundance of S6K2. Low S6K2 expression correlates with the ability of S6K1 deletion to impair tumorigenesis driven by heterozygous PTEN inactivation; in tissues bearing high S6K2 expression, deletion of S6K1 fails to blunt tumorigenesis. These results reveal a role for the mTORC1–S6K1 axis in tumorigenesis driven by PTEN inactivation, at least in tissues in which S6K2 fails to compensate for S6K1 function.

Hypertrophy: organ growth

In addition to promoting physiological cell and organ growth, mTORC1 signalling promotes pathological responses that induce organ hypertrophy, which describes increased organ mass and size primarily due to cellular hypertrophy rather than hyperplasia. In general, chronic organ hypertrophy correlates with impaired organ function and ultimately with patient morbidity and mortality. Indeed, mTORC1 inhibition with rapamycin in mice reduces load-induced cardiac hypertrophy and compensatory renal hypertrophy that occurs upon removal of a kidney (known as 'uninephrectomy') [221,222]. Renal hypertrophy represents an early event in the development of diabetic nephropathy, the leading cause of renal failure in the U.S.A. S6K1-knockout mice show resistance to uninephrectomy- and diabetes-induced renal hypertrophy, thus indicating that the mTORC1–S6K1 axis contributes to renal hypertrophy [223]. However, knockout of S6K1 as well as single S6K2 or double S6K1/S6K2 knockout fails to confer resistance to cardiac hypertrophy induced by physiological (exercise), pathological (aortic banding) or elevated PI3K signalling [224]. These results suggest that the mTORC1–S6K1 axis is not required for cardiac hypertrophy. S6K1 may be sufficient to promote cardiac hypertrophy, however, as transgenic overexpression of S6K1 in the heart induces modest hypertrophy [224]. Additionally, S6K1 contributes to airway smooth muscle hypertrophy, a structural change associated with asthma and airway hyper-responsiveness [173] and contributes to skeletal muscle hypertrophy, as S6K1$^{-/-}$ mice display skeletal muscle atrophy [76].

Learning and memory

Long-lasting synaptic plasticity and the formation of enduring memories requires protein synthesis [209]. Recent work suggests roles for mTORC1 in memory and learning. Although S6K1 or S6K2 knockout in mice has no effect on protein synthesis-dependent L-LTP [late-phase LTP (long-term potentiation)], S6K1, but not S6K2, knockout mice display impaired early-phase LTP and a diverse array of behavioural phenotypes associated with deficits in cognitive processing [225]. S6K2-knockout mice, however, indeed demonstrate memory impairment [225]. These findings suggest that the S6Ks, at least individually, are not required for the *de novo* protein synthesis that is important for enduring LTP. The mTORC1–S6K1 axis, however, is required for early phases of plasticity required for synaptic modifications and ultimately memory.

Aging

Caloric restriction and genetic inactivation of components within the insulin/IGF-1 signal transduction pathway increase lifespan and decrease age-related pathology in diverse model organisms [226]. Strikingly, genetic inactivation of TOR in yeast, worms and flies, which is thought to mimic nutrient restriction, increases longevity. Current thought suggests that TOR signalling promotes aging via mechanisms that may include increased generation of metabolic by-products and thus increased oxidative stress as well as impaired ability to maintain stem cell function [226]. Additionally, mTOR-mediated suppression of autophagy [via mTOR-mediated phosphorylation of ULK1/2 (unc-51-like kinase 1/2) and Atg13, in part] may promote aging due to a decreased ability to manage cellular damage [227–229]. Autophagy represents a cellular process whereby misfolded proteins and/or damaged organelles become sequestered within double-membrane vesicles for degradation in lysosomes. Indeed, many lifespan-extending strategies correlate with enhanced autophagy [228]. A role for mTORC1 in mammalian aging has been demonstrated with the finding that rapamycin-fed mice live longer than controls [230]. Moreover, knockout of S6K1 in mice or knockout of the single S6K gene in *C. elegans* (*rsks-1*) extends lifespan [231]. Strikingly, S6K1-knockout mice show decreased aging-related pathology, including bone, immune and motor dysfunction, and altered gene expression in a manner that resembles caloric restriction [231]. These results demonstrate a role for the mTORC1–S6K1 axis in longevity and aging-related pathology.

CONCLUDING COMMENTS

We have learned a great deal over the past 20 years regarding the cellular regulation and function of S6Ks within mTOR signalling networks; however, many questions remain. Although mTOR provides a critical activating input for S6Ks, other poorly understood phosphorylation events contribute to S6K regulation. It will be important in the future to identify the kinases for C-terminal S6K phosphorylation as well as the kinase(s) for the critical turn-motif site (Ser^{371}). The role that subcellular localization plays in the regulation and function of S6Ks needs to be better defined. Undiscovered S6K substrates probably exist. Identification of shared and unique substrates of S6K1 and S6K2 represents an important area for future research that

will provide improved understanding of the similarities and differences between S6K1 and S6K2 function. The development of a novel pharmacological inhibitor specific for S6K1, PF-4708671, will greatly facilitate the discovery of S6K1-specific cellular functions, similar to the use of rapamycin as a tool to identify mTORC1-controlled functions [232]. Our understanding of the role of the mTORC1–S6K1 axis in organismal physiology and pathophysiology remains in its infancy. Thus future research effort focused on elucidating the tissue-specific functions of S6K1 and S6K2 should prove informative and may identify diseases amenable to treatment with mTORC1- or S6K1-specific inhibitors. Lastly, the limited set of known mTOR substrates (i.e. S6K, 4EBP1, ULK1/2, Atg13, raptor, Akt, SGK, PKC and Grb10) and S6K1 substrates (Figure 3) probably does not account for the myriad cellular and physiological functions controlled by mTOR and S6K1 [1,3,195,194,233]. Thus the identification of the complete set of direct mTOR and S6K1/2 substrates remains an important area for future research. Recent MS-based phosphoproteomic screens in cultured cells identified a plethora of novel as well as known phosphorylation targets controlled directly by mTOR or indirectly via S6K1 or other downstream kinases [194,195]. Indeed, several of the reported S6K1 substrates described in the present review (Figure 3) were identified in these studies, including eIF4B, PDCD4, eEF2K, rpS6, Mdm2, BAD, GSK3β, rictor, IRS and mTOR [194,195]. Hits from these screens probably represent novel mTOR and S6K substrates. The identification of mTOR and S6K targets will enable basic researchers to better define how mTOR controls physiology in S6K-dependent and -independent fashions.

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