Beyond controlling cell size: functional analyses of S6K in tumorigenesis

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As a substrate and major effector of the mammalian target of rapamycin complex 1 (mTORC1), the biological functions of ribosomal protein S6 kinase (S6K) have been canonically assigned for cell size control by facilitating mRNA transcription, splicing, and protein synthesis. However, accumulating evidence implies that diverse stimuli and upstream regulators modulate S6K kinase activity, leading to the activation of a plethora of downstream substrates for distinct pathobiological functions. Beyond controlling cell size, S6K simultaneously plays crucial roles in directing cell apoptosis, metabolism, and feedback regulation of its upstream signals. Thus, we comprehensively summarize the emerging upstream regulators, downstream substrates, mouse models, clinical relevance, and candidate inhibitors for S6K and shed light on S6K as a potential therapeutic target for cancers.

Cell Death and Disease (2022) 13:646; https://doi.org/10.1038/s41419-022-05081-4

FACTS

- S6K canonically governs cell size by facilitating mRNA transcription, splicing, and protein synthesis.
- S6K also plays pivotal role in directing cell apoptosis, metabolism, and feedback regulation of its upstream signals.
- Diverse stimuli and upstream regulators modulate S6K kinase activity.
- Targeting S6K individually or in combination is a promising strategy for cancer therapies.

OPEN QUESTIONS

- What are the functional differences between S6K isoforms in mediating tumorigenesis?
- What is the connection between the canonical roles of S6K and its noncanonical roles?
- How can S6K and its upstream kinases be targeted together to overcome negative feedback regulation?

INTRODUCTION

The balance and tight control of cell size and cell numbers are two essential aspects for organ formation biologically and tumor growth pathologically. Of note, cell size has been found to be tightly controlled by mTORC1 by phosphorylating its diverse downstream substrates [1], in particular the S6K kinase, whereas cell numbers have been preliminarily dictated by the Hippo pathway by modulating the key regulator yes-associated protein 1 (YAP) [2]. Interestingly, the cross talk between these two pathways has been recently reported, highlighting the notion that disruption of their balance will result in tumorigenesis [3]. Due to the critical roles of the mTORC1 and Hippo pathways under pathobiological conditions, studies and reviews investigating mTORC1 and YAP have been well established [4, 5]. However, a summary of the upstream regulation and downstream functions of S6K has still not been provided.

As one of the most well-established mTORC1 downstream effectors, S6K is directly phosphorylated by mTORC1 upon diverse stimuli, such as amino acids [6] and insulin [7]. Many studies have indicated that amino acid availability and insulin stimulation are both required for mTORC1 activation [5]. On the one hand, insulin binds its ligand to activate the phosphoinositide-3-kinase (PI3K)-AKT pathway, in turn regulating the tuberous sclerosis complex 1/2 (TSC1/2)-Ras homolog enriched in brain (Rheb) axis to release mTORC1 repression [7]. On the other hand, upon amino acid stimulation, the activated Rag GTPase heterodimer, containing Rag A/B with Rag C/D, binds to the regulatory associated protein of mTOR complex 1 (Raptor) and recruits mTORC1 to the lysosomal membrane, where it colocalizes with Rheb, leading to subsequent activation [8]. Specifically, upon amino acid deficiency, GAP activity toward the rags 2 and 1 (GATOR2-GATOR1) complex was identified to be in response to the suppression of Rag GTPases and subsequent mTORC1 inactivation [6]. Subsequently, activated mTORC1 phosphorylates S6K and 4E-binding protein 1 (4E-BP1), leading to protein synthesis, cell cycle progression, and glucose homeostasis [9, 10]. Consistent with these findings, dysregulation of P13K-AKT-mTOR-S6K components is predominantly linked to a large proportion of human malignancies, including breast, kidney,
AGC kinases are serine/threonine protein kinases that evolutionarily share several conserved posttranslational modifications (PTMs) but also with observed differences among isoforms. C1/C2, conserved domain 1 or 2; HR1, heptad repeat 1; CNMP, cyclic nucleotide monophosphate-binding; RGS, regulator of G protein signaling; PH, pleckstrin homology; KD, kinase domain; RBD, Rho-binding domain; CNH, citron homology; P, G, and A represent phosphorylation, glycosylation, and acetylation, respectively.

Beyond controlling cell size, S6K has recently attracted more attention due to its important roles in cellular homeostasis [15, 16]. Thus, whether and how other stimuli, except via mTORC1, regulate S6K and how S6K plays distinct roles in various pathological conditions, especially within their kinase domains. They also manifest unique posttranslational modifications (PTMs) and regulatory mechanisms.

Members and functional domains of S6K
S6K contains two members, S6K1 and S6K2, encoded by RPS6KB1 on chromosome 17 and RPS6KB2 on chromosome 11 in humans, respectively [22]. Given the alternative translation start sites, transcripts of both genes include two isoforms: p70 S6K (S6KαI, 502 aa) and p85 S6K (S6KαII, 482 aa) for S6K1, as well as p54 S6K (S6KβI, 482 aa) and p56 S6K (S6KβII, 495 aa) for S6K2 (Fig. 1B). Of note, the longer forms of S6K obtain a putative nuclear localization signal (NLS) within additional amino acids in the N-terminus than the shorter forms. The p56 S6K2 isofrom is actually located in the nucleus because of its N-terminal NLS, while the p54 isoform shuttles between the cytoplasm and nucleus in response to growth factor stimuli [23]. However, previous studies have shown that the p85 S6K1 isofrom primarily resides in the cytoplasm and that the p70 isoform is located in both the cytoplasm and nucleus [24–27]. Interestingly, a recent finding indicated that via its N-terminal six-arginine motif, p85 S6K, but not p70 S6K, could be secreted from cancer cells into microenvironments and educate surrounding cells to confer malignant breast cancer behaviors [28]. In addition, alternative splicing of the RPS6KB1 gene generates a shorter isoform of S6K, namely, p31 S6K1 in mice (and two isoforms in humans, namely, h6A and h6C), which resides in the nucleus and may function as oncoproteins [24, 29, 30].

S6K1 and S6K2 share more than 80% amino acid sequence identity, especially within their kinase domains. They also manifest a similar modular structure comprising an N-terminal regulatory region that contains a TOR signaling (TOS) motif, kinase domain (KD), and C-terminal regulatory region (containing a pseudosubstrate domain) (Fig. 1B). However, accumulating studies have revealed that S6K1 and S6K2 might play distinct roles in cells in part due to minor differences [22, 31, 32]. Specifically, the PDZ domain in S6K1 might associate with the cytoskeleton by binding to neurabin [32]; in contrast, an additional NLS and a proline-rich region [26] in S6K2 might facilitate diverse cellular localization and protein interactions [22]. Similar to other AGC kinases, such as AKT and SGK, S6K also recognizes and phosphorylates a conserved motif: (RxR/KxxS/T containing Ser/Thr to be phosphorylated.
preceded by Arg/Lys at position -3 and alternatively another Arg/Lys at position -5. As such, S6K phosphorylates distinct substrates to regulate protein synthesis, transcription, cell proliferation, cell metabolism, and survival [31, 33], which will be further summarized in the following sections.

**Biological functions of S6K in metabolism, aging, and neuro-related diseases**

In metabolic regulation, depletion of S6K1/2 causes activation of AMPK in multiple tissues to control the energy state of the cell and the AMPK-dependent metabolic program [34]. Many studies have revealed that caloric restriction protects against age-related pathologies and increases lifespan across various animal models, such as yeast, C. elegans, and mice [35]. Consistently, depletion of S6K1 resembles gene expression patterns of caloric restriction or prolongs lifespan by pharmacological activation of AMPK in mice [36], indicating a potential function of S6K in aging. Meanwhile, S6K1 also plays an important role in glucose metabolism via feedback regulation of insulin receptor substrate 1 (IRS1) to improve glucose tolerance and insulin sensitivity in liver-specific and systemic S6K1-deficient mice [37, 38]. Given the impacts of mTOR in neurodegenerative diseases, targeting S6K suggests a potent strategy for patients to achieve better outcomes [39].

**Clinical relevance of S6K in tumorigenesis**

Regarding its essential function in cell growth, cell cycle, and cell metabolism, dysregulation of S6K occurs in various malignancies, including but not limited to breast [22], ovarian [40], hepatic [41], and prostate [42] cancers. Previous studies revealed that both RPS6KB1 and RPS6KB2 exhibit amplifications or gene gains in breast cancer tissues [31, 43]. However, S6K1 and S6K2 might display distinct functions in specific breast cancers [22]. In ovarian cancer, S6K1 persists in an active state, which is essential for the initiation and progression of tumors [44]. It has also been reported that dendriplex nanoparticle delivery of siRNA targeting S6K1 could potently diminish the stemness and metastatic properties of ovarian cancer stem cells [40]. Consistent with this finding, increased transcripts of RPS6KB1 are considered an independent prognostic marker for poor prognosis of hepatocellular carcinoma patients [41]. S6K1 also participates in the development of brain tumor pathogenesis [45], and the gene expression patterns of S6Ks have been evaluated in brain and central neuron system tumors, indicating that S6K1 rather than S6K2 exhibits elevated expression in brain tumors and is associated with poor prognosis of patients [41]. Lu et al. reported that the phosphorylation level of S6K1 was closely related to TNM staging and lymph node metastasis of esophageal squamous cell carcinoma [46]. Through IHC staining analyses, Du et al. demonstrated that the expression of S6K1 and phosphorylation of S6 were markedly elevated in human vascular tumors, suggesting mTORC1 inhibitors as promising agents for cutaneous vascular lesions [47].

In contrast to S6K1, S6K2 amplification is a common event in gastric cancer, indicating a poor outcome [48]. S6K2 has also been suggested as an effector under fibroblast growth factor-2 (FGF2) stimulation, inducing the survival of small cell lung cancer cells and chemoresistance via the formation of a complex with B-Raf and PKCs [49]. In hepatocellular carcinoma (HCC), S6K overexpression is positively correlated with tumor nuclear grade and inversely correlated with tumor size [50]. Moreover, S6Ks also play key roles in the progression, survival, migration, and response to chemotherapy drugs of prostate cancer, indicating that S6K is a promising target for advanced prostate cancer treatment [42].

**Genetic mouse models for S6K**

Given that S6K functions as a pivotal regulator in the context of both physiological and pathological conditions, various transgenic or knockout mouse models have been developed to study corresponding diseases (Table 1). Pende et al. showed that mice depleted of S6K1 or S6K2 were viable, while double knockout of S6K1/2 results in a sharp reduction in survival because of perinatal lethality [51]. In addition, compared with WT mice, S6K1−/− mice exhibited a markedly smaller size, while S6K2−/− mice were slightly larger. Although S6K2 may play a dominant role in the process, S6K1 and S6K2 are both required for the maximum phosphorylation of S6. However, S6 phosphorylation at S235 and S236 remained in S6K1−/− cells, which might be maintained by 90-kDa ribosomal protein S6 kinases (RSKs) [51–53].

Recently, ribosome biogenesis has emerged as a promising vulnerability for cancer treatment due to its essential role in cell transformation and tumorigenesis [54]. It has been reported that depletion of both S6K1 and S6K2 in the liver significantly retards the ribosome biogenesis transcriptional program, but has no influence on the recruitment of RNAs into polysomes [55]. Knockdown of S6K1 impedes the self-renewal potential of murine hematopoietic stem cells, and depletion of S6K1 shows prolonged survival in mice, suggesting a potential strategy for myeloid disorders.
malignancy treatment [56]. Implementing xenograft models in S6K1−/− and S6K2−/− mice, Lee et al. observed that depletion of tumor stroma S6K1, but not S6K2, retarded tumor growth and angiogenesis, accompanied by impaired mRNA transcription and protein expression of HIF1α and its target genes [57]. This observation highlights S6K1 as a key regulator in the reconstruction of tumor microenvironments in favor of tumor growth.

In addition to cancer, S6Ks knockout mice have also been employed to investigate several other pathologies. Interestingly, upon high-fat diet, S6K1 KO mice exhibited increased glucose and free fatty acid levels coupled with markedly enhanced β-oxidation and metabolic rates but resist weight gain [38]. For the insulin response, high-fat diet-mediated excess nutrients might potentiate mTORC1-S6K activation, leading to IRS1 phosphoylation and degradation, thus desensitizing insulin signaling [38]. In contrast, S6K2 KO mice exhibited lower IRS1 phosphorylation and a normal response to insulin signaling. Simultaneously, S6K-mediated S6 phosphorylation-deficient knock-in mice (S6β−/−) phenocopied S6Ks KO mice in reduced ribosomal biogenesis transcriptional programs [55]. As reported, S6K1−/− mice exhibited hypoinsulinemia and glucose intolerance accompanied by decreased insulin secretion, which is mildly phenocopied by S6β−/− mice [58, 59]. It has been reported that the gene expression patterns of S6K1 depletion mice resemble those of caloric restriction or pharmacological activation of AMPK to prolong the mouse lifespan [36]. In hepatic steatosis, after high-fat diet feeding, liver-specific S6K1 knockout mice exhibited improved glucose tolerance and systemic insulin sensitivity, with consistent food intake and body weight compared with their wild-type (WT) counterparts [37]. In addition, S6K2−/−, but not S6K1−/− mice, exhibited increased ketogenesis [16]. Mice generated by crossing S6K1−/− mice with the R6/2 mouse model, an acknowledged model for Huntington’s disease, did not show any improvements in behavioral or physiological phenotypes [60]. Caccamo et al. reported that in a mouse model of Alzheimer’s disease (AD), depletion of one copy of S6K1 significantly reduced amyloid-β and tau pathology, two neuro-pathological hallmarks for AD, accompanied by improvements in synaptic plasticity and spatial memory [61]. Moreover, rapamycin suppressed 60–70% hypertrophy in WT mice, but failed to rescue the 10% hypertrophy in S6K1−/− mice, suggesting that S6K1 plays an essential role in the progression of compensatory renal hypertrophy and diabetogenic renal hypertrophy induced by uninephrectomy [62]. Ohanna et al. showed that depletion of S6K1, but not S6K2, evidently reduced the size of myoblasts, as well as rapamycin sensitivity, but left myoblast cell proliferation intact. In the differentiated state, S6K1−/− myotubes also exhibited smaller sizes but normal nuclei, which abolished IGF1-induced hypertrophy [63]. Previous studies also showed that cardiac hypertrophy could be caused by pathological and active forms of PI3K, which might heavily correlate with their regulatory function in non-cardiac tissues [29, 30]. It has been revealed that Sam68, an RNA-binding protein, can bind to mRNA encoded by RPS6KB1 and suppress alternative splicing by SRSF1 [75]. Upon Sam68 deficiency, SRSF1-mediated splicing resulted in the expression of p31 S6K1, which dampened lipogenesis in adipocytes [75].

In terms of S6K mRNA, Ramaiah et al. reported that the miR-15/16 complex was responsible for the RPS6KB1 degradation in MDA-MB-231 cells, regulating cell proliferation and survival [76]. In prostate cancers, upregulated Snail suppressed the transcription of miR-128, a negative regulator of S6K1 mRNA, resulting in increased expression of S6K1, hypoxia-inducible factor 1 subunit alpha (HIF1α), and pyruvate kinase M 2 (PKM2) [77]. In addition, miR-195 was proven to play a suppressive role in prostate cancer development by targeting S6K1. Re-expression and knockdown of S6K1 abolished and resembled the effects of miR-195, respectively, both of which were closely correlated with the progression and prognosis of prostate cancer patients [78]. Yao et al. demonstrated that miR-193a-3p was responsible for the quality control of S6K2 mRNA in non-small cell lung cancer (NSCLC), which might serve as a tumor suppressor and suppress the metastatic traits of NSCLC [79]. Furthermore, miR-557 and miR-3182 were identified as regulators of the degradation of S6K1 mRNAs in TNBC [80]. miR-SO6-3p could bind to the 3′-UTR of S6K1 mRNA, leading to its degradation and subsequently increasing insulin sensitivity [81]. Wang et al. reported that miR-181a downregulated S6K1, resulting in spermatogenesis, an uncommon function of S6K1 [82]. In addition to miRNAs, long noncoding RNAs (lncRNAs) have also been implicated in abrogating the regulation of S6K expression by miRNAs. Of note, lncRNA PCA3 disrupted the binding of miR-106b with its target genes, including S6K1, in ovarian cancer cells; [83] IncRNA CCAT2 was responsible for radiotherapy resistance by downregulating miR-145-S6K1 in esophageal cancer cells [84]. (Fig. 2).

Phosphorylation modification of S6K
The kinase activity and subcellular localization of S6K are predominantly regulated by multiple-site phosphorylation events in response to diverse extracellular stimuli, including growth factors, nutrients and mitogens, which cooperate with each other to fully activate S6Ks (Figs. 1B and 2). Over recent decades, a stepwise activation model of S6Ks has been widely accepted, including a conventional and an alternate model [33]. Unlike other AGC family kinases, S6Ks possess an autoinhibitory domain, which blocks the activation loop from mTORC1 and PDK1, resulting in an inactive form of S6K [33]. The release of the activation loop is achieved by phosphorylation at several sites (S411, S418, T421, and S424) on its C-terminal autoinhibitory domain by members of MAPK and CDK1 under mitogen stimuli [85]. It is worth noting that
phosphorylation of S6K by JNK1 at S411 and S424 might account for its degradation only when IKKβ is inhibited [86].

It is widely known that upon the stimulation of insulin, activation of the PI3K-AKT pathway results in the activation of mTORC1, which is responsible for the phosphorylation of the hydrophobic motif of S6Ks (S6K1 at T389 and S6K2 at T388) [87]. In parallel, activation of PDK1 is responsible for a subsequent phosphorylation event on the activation loop of S6Ks (S6K1 at T229 and S6K2 at T228) [88], finally leading to full S6K activation. Conceivably, pathways and messengers that converge onto mTORC1 or PDK1 lead to the activation of S6Ks to control downstream cellular progression.

In the upstream regulation of mTORC1, the TSC complex, acting as a GTPase, plays the most important repression role as a central hub [89] (Fig. 2). Upon stimulation with growth factors, active AKT directly phosphorylates and inhibits TSC2 [90, 91]. Similarly, following growth factor-induced Ras activation, the MEK-ERK pathway is capable of phosphorylating and suppressing TSC2 [92, 93]. Under stimulation of TNFα, IKKβ directly phosphorylates and suppresses TSC1 in breast cancer [94]. However, hypoxia can enhance TSC GAP activity via the induction of regulated DNA damage and development 1 (REDD1) [95]. In contrast, energy deficiency-mediated activation of AMPK directly phosphorylates the mTORC1 subunit Raptor and indirectly phosphorylates and activates TSC2, jointly abrogating mTORC1 activity [91, 92]. LATS1/2, the key components of the Hippo pathway, can directly phosphorylate the mTORC1 core subunit Raptor to counteract the interaction with Rheb, resulting in the downregulation of mTORC1-S6K signaling [3]. Apart from directly phosphorylating S6K1 by glycogen synthase kinase 3 (GSK3) [96], S6K also regulates GSK3 by inactivating AKT kinase via a feedback loop [97]. Interestingly, Wnt and energy signals also modulate TSC2 via GSK3 and AMPK-mediated phosphorylation [98]. Except for the insulin-mediated PI3K-AKT pathway, amino acid availability also essentially contributes to mTORC1 activation via its sensors, the GATOR2-GATOR1 complex and Rag GTPases [6]. Thus, signals regulating these components will also modulate mTORC1 kinase activity and subsequently S6K phosphorylation [8, 99, 100]. Unlike mTORC1, the upstream regulation of PDK1 has been far behind due to the properties of autophosphorylation and activation [101–104]. In addition to PIP3, assisting PDK1 binding with its substrate AKT, copper has also been identified as an inhibitor for mTORC1 activity, resulting in the activation of oncogenic pathways [105].

It is of great interest that with treatment with IFN-γ for myeloid cells and insulin for adipocytes, CDK5 could directly phosphorylate S6K1 on its C-terminal domain at S424 and S429 along with phosphorylation at T389 by mTORC1. Furthermore, CDK5-mediated phosphorylation causes a conformational change in S6K1 and leads to a higher affinity for targets related to lipid metabolism than its canonical target S6 [106]. In neurons, CDK5 can also phosphorylate S6K1 on the autoinhibitory domain at S411, which facilitates S6K1 activation to impact spine morphology [107]. As we mentioned above, GSK3 can also directly phosphorylate S6K1 on its D371, which is antagonized by phosphatase 2A (PP2A), resulting in the activation of S6K1 [96]. This finding provides evidence for the usage of GSK3 inhibitors for diabetes or cancers harboring dysregulated S6K1.

In addition to influencing kinase activity, phosphorylation could participate in other aspects of the regulation of S6Ks (Figs. 1B and 2). For example, PKC is able to phosphorylate S6K2, but not S6K1, at S486 located within its NLS in the C-terminus, thus abrogating the function of the NLS and leading to retention of active S6K2 in the cytoplasm [108]. Moreover, casein kinase 2 (CK2) phosphorylates S6K1 on its N-terminus at S17, which enhances S6K1 phosphorylation and thus leads to a cytoplasmic pool of S6K1 [109]. S6Ks also undergo tyrosine phosphorylation by platelet-derived growth factor receptor (PDGFR)/Src, yet its mechanism remains unknown [110].

Similar to the regulation of AKT, both phosphatase PP2A and PHLPP are involved in the dephosphorylation of S6K1 [111, 112].
Among them, the B regulatory subunit of PP2A (PP2A-B) in Drosophila and its homolog PPP2R5C in humans have been identified to interact with S6K and specifically target the PP2A holoenzyme to dephosphorylate S6K [113]. The vitamin D receptor, PP1c or PP2Ac, can assemble a ternary complex with S6K1 to dephosphorylate S6K1 [114]. PHLPP dephosphorylates S6K1 independent of the process of AKT dephosphorylation [115].

Other regulatory modifications of S6K1
In addition to phosphorylation, S6Ks are also subject to ubiquitination and acetylation modifications (Fig. 1B). Notably, S6Ks, with ubiquitinated residues in the kinase domain, could be degraded through the 26S proteasomal machine [116]. Later studies revealed that ROC1 was the potential corresponding E3 ligase for S6K1 ubiquitination [117], whereas E3 ligase-mediated S6K2 ubiquitination is currently unknown. Similar to ubiquitination, acetylation also plays a role in the regulation of S6K stability. S6K1 undergoes acetylation at K516 by p300 and p300/CBP-associated factor (PCAF) to stabilize the S6K1 protein under mitogen stimulation [118, 119]. Meanwhile, both HDAC and Sir2tun inhibitors could markedly enhance the acetylation level and protein abundance of S6K [118]. Of note, during calorie restriction, SirT1 deacetylated S6K1 in intestinal stem cells (ISCs), thereby enhancing its phosphorylation via mTORC1, leading to ISC self-renewal [120]. In addition, under high glucose, S6K acetylation was eliminated by HDAC1, contributing to kidney glomerular cell hypertrophy and matrix expansion [121]. In addition, S6K1 was also modified by O-GlyNAc transferase (OGT) at S489 to suppress mTORC1-S6K1 signaling, likely impairing the transcription of proinflammatory genes in macrophages [122].

DOWNSTREAM SUBSTRATES AND BIOLOGICAL ROLES OF S6K
With the accumulation of downstream substrates, the functions of S6Ks have been expanded in many biological processes, including canonical cell size control, cell metabolism regulation, cell growth, and anti-apoptosis, as well as negative regulation of S6K upstream signals (Fig. 3).

Contribution to cell size
As the most well-established substrate of S6K, phosphorylation and activation of S6 play an important role in promoting protein synthesis (Fig. 4). There are two subunits of ribosomes in higher eukaryotes, the 40S (small) and 60S (large) subunits, which consist of rRNA and various ribosomal proteins. Among them, 40S ribosomal protein S6 could be phosphorylated by S6Ks on its C-terminus at five conserved serine residues (S235, S236, S240, S244, and S247) [123]. Of note, S6K2, but not S6K1, has been reported to be a primary kinase for S6 phosphorylation [51]. rpS6<sup>−/−</sup> knock-in mice, substituting these phosphorylatable serine residues into alanine, displayed a smaller cell size of mouse embryo fibroblasts and pancreatic β cells [55]. This might be derived from the effects of S6 phosphorylation on ribosomal biogenesis [55]. rpS6<sup>−/−</sup> mice also exhibited glucose intolerance, reduced insulin secretion, and hypoinsulinemia [59]. It is worth noting that rpS6<sup>−/−</sup> mice presented diminished long-term potentiation and reduced translation of mitochondria-related mRNAs limited to the nucleus. In contrast, rpS6<sup>−/−</sup> mice exhibited unchanged brain size, weight, and morphology, which was consistent with the finding that S6 phosphorylation by S6Ks promotes global protein synthesis [124]. Despite extensive reports about S6 as an S6Ks substrate to regulate protein synthesis, the biological function of S6 phosphorylation still needs to be further characterized. For example, the phosphorylation level of S6 at S235 and S236 was detectable, although with decreased intensity in S6K<sub>−/−</sub> mice, which may suggest that other kinases function in tuning S6 phosphorylation and function [51]. Indeed, RSKs and CK1 were observed to responsive for the phosphorylation of S6235/236 and S237, respectively [53, 125]. Therefore, it is necessary to be cautious when decoding S6K-S6 signaling. Interestingly, eukaryotic initiation factor 3 (eIF3) function may serve as an adaptor protein, where it associates with inactive S6K1 and recruits activated mTORC1 upon growth factor
Stimulation, leading to activation of S6K1 and subsequent phosphorylation of elf4B for translation initiation (Fig. 4) [126]. The elf3 preinitiation complex may guarantee the coordination of dynamic sequences followed by various stimuli [127].

The process of protein synthesis involves the initiation, elongation, termination, and recycling of ribosomes [128]. Initiation of translation is the key process for protein synthesis, which is under strict control via several regulatory mechanisms. Eukaryotic translation initiation factor 4B (eIF-4B) plays a key role not only in recruiting ribosomes to the translation initiation complex but also in promoting the RNA helicase activity of eukaryotic translation initiation factor 4A (eIF4A) [126, 129], which is essential for the sufficient unwinding of mRNA for initiation of codon scanning. eIF4B is phosphorylated by S6Ks at S422, mutating which with Alanine impairs translation efficiency, indicating the important function of elf4B phosphorylation by S6Ks [130]. Programmed cell death 4 (PDCD4), a known tumor suppressor, can bind with elf4A by blocking the binding between elf4A and elf44G or suppressing elf4A helicase activity, resulting in impaired translation [131]. Upon the stimulation of mitogens, S6K1 directly phosphorylates PDCD4, leading to β-TRCP-mediated degradation, which accounts for efficient protein translation and further cell growth [132]. In addition to initiation, the elongation phase is achieved by a cluster of eukaryotic elongation factors (eEFs), which also undergo phosphorylation regulation. S6Ks have been reported to phosphorylate eEF2 kinase (eEF2K) at S366, leading to inhibited elf2 kinase (eEF2K) activity toward eEF2, which accounts for eEF2 dephosphorylation and being more active (Fig. 4) [133]. This S6K-eEF2K-eEF2 axis may serve as an accelerator of protein synthesis and cell growth under S6K activation. Via a proteomic analysis, Pavan et al. showed that the S6K1 interactome was predominantly associated with the “stress response” and “cytoskeleton” [134]. Indeed, under mildly stressful conditions, phosphorylated S6K1 and S6K2 are responsible for the phosphorylation of translation initiation factor 2a (eIF2a) at S51, which favors the assembly of stress granules (SGs). Specifically, S6K1 is the predominant kinase for eIF2a phosphorylation and subsequent SG formation, while S6K2 is vital for SG persistence [135]. Upon recovery from stress, protein synthesis resumes, which might protect cells from stressful insults.

Under insulin stimulation, S6K1 Aly/REF-like target (SKAR), acting as an adaptor protein, could be phosphorylated by S6K1, but not S6K2, at S383 and S385, which functions in recruiting S6K1 to the de novo mRNAs, possibly promoting the translation of specific messengers [136]. Further studies reveal that SKAR is a component of the exon-junction complex (EJC), the phosphorylation of which results in increased efficiency in pre-mRNA splicing and exportation to the cytoplasm, leading to enhanced translational efficiency [137]. Cell division cycle 42 (CDC42) is a GTP-binding protein that coordinates various cellular events through fine-tuned GTP binding and hydrolysis, including transcriptional regulation and cell cycle progression. The function of CDC42 in regulating pre-mRNA splicing is achieved by the nuclear RNA cap-binding complex (CBC) [138]. It is believed that S6K1 directly binds to and phosphorylates the CBC subunit CBP80, leading to enhanced RNA cap-binding activity [139], which possibly stimulates efficient splicing of pre-mRNA and subsequent mRNA translation. Serine/arginine-rich (SR) proteins are a family of RNA-binding proteins that are responsible for RNA processing by directly binding to the exons of pre-mRNAs and subsequently recruiting small nuclear ribonucleoproteins to enhance alternative splicing [140]. SR proteins are phosphorylated by SR protein kinases (SRPKs), which dictate their conformation, localization, and/or interactions with other proteins. Notably, SRPK2 undergoes S6K1-mediated phosphorylation, priming subsequent phosphorylation by CK1, which promotes its nuclear localization and SR protein phosphorylation (Fig. 4) [141]. Consequently, phosphorylated SR protein binds to small nuclear ribonucleoprotein U1 to promote the splicing of lipogenic pre-mRNAs.

Figure 4 S6K controls cell size by directing multiple processes of protein synthesis. Phosphorylation of histones by S6K may dictate specific gene transcription, followed by phosphorylation and activation of CBC and EJC components, which play key roles in pre-mRNA maturity and exportation. S6Ks also function in preinitiation complex formation and translation initiation by phosphorylating elf3, S6, elf4B, and PDCD4, resulting in efficient initiation of protein synthesis. On the other hand, during elongation, phosphorylation of eEF2K by S6Ks favors an active state of eEF2, resulting in efficient ribosome translocation and protein translation.
which controls ~3% of mammalian brain mRNA translation [142]. S6K1 has been identified as the predominant kinase responsible for FMRP phosphorylation in the mouse hippocampus, which contributes to FMRP activation and provides insights for the occurrence and treatment of Fragile X syndrome [143, 144]. In contrast to S6K1, S6K2 also plays a vital role in the nucleus. Ismail et al. identified that S6K2 could bind to chromatin and nuclear matrix cellular fractions and directly phosphorylate H3 at T45, which plays essential roles in the response to mitogens and cell proliferation and/or differentiation of leukemic cells [145]. In addition to S6K2, AKT also directly phosphorylates H3 at T45 upon DNA damage, which facilitates transcriptional termination for DNA damage-inducible genes, guaranteeing maximum transcriptional efficiency to maintain genomic integrity [146]. After transcription, precursor miRNAs need to be processed by the miRNA biogenesis machinery to assemble the RNA-induced silencing complex, which plays pivotal roles in mammalian development. Transactivating response RNA-binding protein 2 (TRBP), along with DROSHA, DGCR8, AGO2, and PACT, comprises the machinery for miRNA processing, which are often targets for phosphorylation regulation [147–149]. S6Ks have been reported to dictate the phosphorylation of TRBP, resulting in its optimal expression and promoting miRNA biogenesis in primary human dermal lymphatic endothelial cells (Fig. 4) [150].

**Contribution to feedback regulation of the PI3K-AKT pathway**

Interestingly, as an mTORC1 inhibitor, rapamycin markedly enhances PI3K/AKT activity in various cell lines, which suggests a potential negative feedback of mTORC1-S6Ks [151]. With this observation, the feedback regulation of S6K to AKT has attracted extensive attention, which may account for a better exploration of the underlying mechanism of rapamycin-induced drug resistance in cancer therapies (Fig. 5A).

Upon insulin stimulation, IRS, an adaptor protein, could be recruited to the insulin receptor via its PH domain, capturing proteins containing the Src-homology (SH) domain, such as the p85 regulatory subunit of PI3K, followed by conversion of colocalized phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3, finally leading to activation of the PI3K-AKT-mTORC1 pathway [152]. The initial observed pathway is S6K1-mediated phosphorylation of IRS1. Interestingly, S6k1-deficient mice sustain a regular level of glucose after fasting, suggesting hypersensitivity to insulin [58]. It has been reported that S6K1 can directly phosphorylate IRS1 at S307 and S636/S639, which abolishes the adaptor function of IRS1, leading to its insensitivity to upstream stimuli [38]. S6K-dependent inactivation of IRS also stems from the suppression of gene expression [153]. Subsequent studies revealed that residues S270, S307, S636, and S1101 within IRS1 are subject to phosphorylation by S6K1 directly under TNFα treatment, among which S270 might be the most important site [154, 155]. Under TNFα treatment, this phosphorylation event shows an increase in adipocytes of obese mice, possibly due to the inhibition of IRS activity and thus suppression of insulin-stimulated glucose uptake. Upon exposure to upstream stimuli, mTOR can be phosphorylated at S2448 by active AKT, which then initiates a series of cellular responses by phosphorylating various mTORC1 substrates. Rapamycin treatment blocks serum-stimulated mTOR phosphorylation, which cannot be explained by repressing AKT. Additionally, after treatment with rapamycin, rapamycin-resistant S6K1 is capable of restoring S2448 phosphorylation and phosphorylating mTOR at S2446 within the reported regulatory repressor domain of mTOR [156, 157]. It is tempting to speculate that mTORC1 undergoes feedback regulation of S6K, but its function needs further investigation. As a key regulator of growth and proliferation in all eukaryotic cells, mTOR, along with LST8, Pictor, and stress-activated protein kinase-interacting protein 1 (Sin1), forms mTOR complex 2 (mTORC2), which perceives various stimuli and
results in the phosphorylation of some AGC kinases, including the well-known phosphorylation and activation of AKT at S473 [129].

Interestingly, based on cellular context, both S6K1 and AKT1 could phosphorylate Sin1, causing the dissociation of Sin1 from other partners of mTORC2, which abolishes AKT activation [158]. This finding reveals a negative feedback to the activation of the PI3K/AKT pathway, provides a possible explanation for hyperactive AKT induced by aberrations of the mTORC1-S6k-Sin1 axis, and suggests corresponding strategies in tissue and cellular contexts. RPTOR independent companion of mTOR complex 2 (Rictor), another unique component of mTORC2, plays an important role in mTORC2-regulated cell growth, metabolism, and survival [92]. Rictor has also been observed to function as an E3 ligase coupled with Cullin1 and Rbx1, accounting for S6K1 ubiquitination and destruction [159]. Furthermore, a group of AGC family kinases, including AKT, S6K1, and S6K, has been validated to phosphorylate Rictor at T1135, which disassociates the Cullin1-Rictor complex but with minimal effect on the kinase activity of mTORC2 [159, 160]. Nevertheless, Rictor T1135A mutant cells show complex but with minimal effect on the kinase activity of mTORC2 [159, 160]. Rictor T1135A mutant cells show complex but with minimal effect on the kinase activity of mTORC2 [159, 160].

Therefore, the specific role of Rictor in the regulation of mTORC2 activity remains to be elucidated. However, it is clear that the phosphorylation of Rictor at T1135, which disassociates the Cullin1-Rictor complex, is essential for the full activation of S6K together with the regulatory loop for mTORC1-S6K1-mTORC2 signaling.

PDK1 is another AGC family kinase and is known as an upstream kinase responsible for full activation of S6K together with mTORC1. Notably, our recent study revealed PDK1 as a bona fide substrate of S6K1 in a phosphorylation-dependent manner [162]. Briefly, S6K1 directly phosphorylates PDK1 on its PH domain at SS49, which impedes its membrane localization by binding with adaptor protein 14-3-3 and then abrogates the interaction with AKT. Conceivably, mutations that abolish S6K1-mediated phosphorylation of 14-3-3 interaction with PDK1 are sufficient to promote AKT hyperactivation and facilitate tumorigenesis [162]. As discussed above, the TSC complex acts as a suppressor of mTORC1 and leads to the inhibition of S6Ks. As a direct substrate of AKT, GSK3 has been detected in persistent phosphorylation in tumor tissues and cells deficient in TSC1/2. Furthermore, phosphorylation of GSK3 is sensitive to rapamycin or deprivation of amino acids [97]. Zhang et al. revealed that S6K1 is responsible for GSK3 phosphorylation and subsequent inhibition in settings of growth factor deficiency. This feedback regulation on S6K for GSK3 enriches the complexity of the regulatory network for PI3K-AKT-mTORC1 signaling, which provides another explanation for a diverse range of diseases.

Control of cell apoptosis

S6K1 also plays an essential role in the regulation of cell growth and survival, partially through the inhibition of cellular apoptosis. BCL-2 family proteins are responsible for the intricate regulation of apoptosis and are frequently subjected to PTMs to dictate cell death. BAD, a pro-apoptotic protein, has been reported to be phosphorylated by S6K1, which inactivated its pro-apoptotic function [163]. Indeed, rapamycin treatment could abolish IGF1-induced BAD phosphorylation and cell survival in S6k-deficient cells [163]. Unconventional prefoldin RBPS interactor (URI) is a chaperone belonging to the prefoldin family that can associate with PP1γ in mitochondria and inhibit its phosphatase activity. Growth factors can dissociate URI/PP1γ and release the dephosphorylation activity of PP1γ, which can be blocked by rapamycin [164]. Indeed, S6K1 is a kinase responsible for URI phosphorylation at S372, after which discharged PP1γ is capable of dephosphorylating S6K1 at T389 [164], consisting of a negative feedback loop for delicate apoptosis control by sensing the status of homeostatic and stimulated conditions (Fig. 5B).

The cellular genome is continuously attacked by various factors, including irradiation and reactive oxygen species (ROS), after which cells employ a network of pathways to maintain genome integrity. p53, a gatekeeper for the genome, has been a “star molecule” for research in cell cycle arrest and apoptosis [165]. Mouse double minute protein 2 (MDM2) has been reported to be a primary E3 ligase for p53 degradation [166, 167]. S6K1 has been reported as a multifaceted modulator of MDM2. On the one hand, S6K1 phosphorlyates MDM2, promoting its nuclear-cytoplasmic shuttling; on the other hand, S6K1 closely binds to MDM2, hinderingsubrination of p53, which leads to p53 stabilization and transcription [168]. This finding bridges the metabolic/energy cues in the cellular response to DNA damage. In the process of DNA repair, the E3 ubiquitin ligase RNF168 can be recruited to DNA damage sites to generate and multiply histone ubiquitination signals, promoting the repair of DNA double-strand breaks. Apart from the regulation of p53 by phosphorylating MDM2, S6Ks directly phosphorylate RNF168, leading to its degradation mediated by TRIP12 and loss of its function in response to DNA damage signals, which finally results in genome instability [169].

In parallel, PDCD4 binds to the mRNA of X chromosome-linked inhibitor of apoptosis (XIAP) and Bcl-xL, suppressing the translation of XIAP and Bcl-xL. In response to FGF2 stimulation, S6K2 directly phosphorylates PDCD4, accounting for its degradation, which releases the translational inhibition of XIAP and Bcl-xL, favoring cell survival by apoptosis inhibition [170]. Heterogeneous nuclear ribonucleoproteins (hnRNPs), comprising at least 20 members, namely, hnRNPA1 to hnRNPAU, are essential for the alternative splicing, metabolism, and transportation of precursor mRNA [171]. FGF2-induced survival is also dependent on S6K2-mediated phosphorylation of hnRNPA1. Upon S6K2-dependent phosphorylation coordinated with other modifications, hnRNPA1 binds to Bcl-xL and XIAP mRNAs, leading to their expression and prefering cell survival [172]. Interestingly, S6K2-dependent phosphorylation of hnRNPA1 is essential for the preferred splicing of the PKM2 isoform, favoring enhanced glucose expenditure and lactate production [173]. FGF2 has also been implicated in the antia apoptotic traits and chemoresistance of small cell lung cancer. It has been reported that S6K2, but not S6K1, forms a multiple protein complex with B-Raf and PKCe and functions in promoting the expression of antia apoptotic proteins, including XIAP and Bcl-xL, resulting in chemoresistance [49].
glucose transportation, promoting glycolysis in the heart [179]. During fasting, adipose tissues release nutrients, such as glycerol, into the circulation to sustain energy homeostasis, which is a process accomplished by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors [180]. It has been proposed that instead of S6K1, S6K2 binds to nuclear receptor corepressor 1 (NCOR1), a corepressor of PPARα, leading to intact transcriptional activity of PPARα, which sustains hepatic energy homeostasis [16].

Upon adipogenic stimuli, S6K1 translocates to the nucleus and leads to the phosphorylation of histone H2B at S36, which accounts for the recruitment of the enhancer of zeste homolog 2 (EZH2) and subsequent trimethylation of H3K27. This epigenetic mark retards the transcription of the Wnt gene, leading to enhanced adipogenesis. Of note, obese humans and high-fat diet-fed mice exhibit detectable pS36 H2B and H3K27me3. In contrast, adipose tissue of mice depleted with S6k1 shows lower levels of pS36 H2B and H3K27me3 [181], highlighting strategies to employ specific S6K1 inhibitors to suppress obesity and overcome insulin resistance, bypassing mTORC2 inhibition. In islet cells, S6K1 is capable of promoting H3K4me3 and inhibiting H3K27me3 to support transcription marker genes of β cells and suppress those of α cells [182]. It is believed that the pancreatic cell transition induced by S6K1 might provide basic clues for type I diabetes.

mTORC1-S6K1-SRPK2 signaling results in phosphorylation and activation of β1 protein, leading to enhanced splicing of lipogenic pre-mRNAs, which suggests SRPK2 as a target for metabolic disorders induced by mTORC1. Under growth factor stimulation, the PIIIK-AKT-mTORC1 signaling pathway is mobilized to dictate cellular anabolic processes and cell growth. It is well established that mTORC1 regulates mRNA translation, ribosomal biogenesis, and de novo lipid and sterol synthesis [55, 141, 183]. Through unbiased metabolomic profiling, Issam et al. revealed that mTORC1 plays an essential role in de novo pyrimidine synthesis via S6K1-mediated phosphorylation of carbamoyl-phosphate synthetase 2 (CAD) at S1859 [184], which is vital for de novo pyrimidine synthesis. Apart from lipid and protein synthesis, cells also adapt pyrimidine synthesis via the mTORC1-S6K pathway to respond to growth factor signals.

Control of cancer proliferation

In addition to changing cell size, metabolic homeostasis, and apoptosis to contribute to cell malignancies, S6K also tethers other signaling pathways to control cell proliferation and migration. For instance, S6K1 directly phosphorylates glioma-associated oncogenes (Gli1), a key effector of the Hedgehog signaling pathway [185], and unleashes Gli1 from its endogenous inhibitor SuFu to enhance its transcriptional activity and tumorigenesis capability [186]. Similarly, Gli2 also goes through S6K1-mediated phosphorylation and dissociation from SuFu, contributing to chondrocyte proliferation and differentiation [187]. These findings provide a rationale for the combined inhibition of the Hedgehog pathway and mTORC1-S6K axis in context-dependent diseases. On the other hand, S6K1 can also directly phosphorylate estrogen receptor alpha (ERα) and promote ERα-related transcription [188–190]. As mentioned above, ERα can transcriptionally promote S6K1 expression [73]. Thus, S6K1-ERα consists of a positive feed-forward relationship in dictating breast cancer cell proliferation, which may be adopted as a therapeutic target for breast cancer with S6K1 amplification (Fig. 5C). Furthermore, histone deacetylase 1 (HDAC1), an essential epigenetic regulator that modulates chromatin structures, has also been identified as a substrate of S6K1 upon mitogen stimuli in breast cancer cells, which accounts for HDAC1 hyperphosphorylation and activation, leading to deacetylase-dependent suppression of ERα transcription. As such, the combination of mTOR and HDAC1 inhibitors exhibits a synergistic effect on the inhibition of breast cancer proliferation [191]. Thus, HDAC1 may function as a node for the S6K1-mediated negative feedback loop in the regulation of cell proliferation (Fig. 5C). Moreover, S6K1 has also been reported to directly phosphorylate MAX interactor 1 (Mxi1), a member of the MAD family capable of competing with MAX binding to c-Myc to suppress c-Myc-induced proliferation [192], and recruit the E3 ligase β-TRCP to degrade Mxi1 to unleash c-Myc oncogenic functions [193].

Control of inflammation and immune response

Transforming growth factor β-activated kinase 1 (TAK1) is essential for the production of inflammatory mediators and plays a key regulatory role in innate immunity signal transduction mediated by cytokines and Toll-like receptors (TLRs). S6K1 has been reported to negatively regulate the TLR signaling pathway by inhibiting TAK1 kinase activity [194]. In detail, S6K1 competes with TAB1, an essential regulator of TAK1 catalytic activity, for binding to TAK1, which dampens TAK1-induced transcription of AP-1 and NF-κB target genes. Moreover, TAK1 could also inhibit S6K1 phosphorylation by disrupting its interaction with the mTORC1 subunit Raptor, thus inducing autophagy [195]. CD4+ T helper (Th) cells are essential for adaptive immune responses by secreting distinct cytokines. Among them, Th17 cells play a vital role in the development of chronic inflammation underlying rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [196]. RORγ and Ghi1 are key regulators of the differentiation of Th17 cells, which account for the promotion and suppression of specific transcription events, respectively [197]. Kurebayashi et al. reported that S6K2, but not S6K1, is capable of binding with RORγ, promoting its nuclear localization and resulting in Th17 differentiation [198].

Other functions

In addition to the above well-established functions, S6K also phosphorylates other substrates and performs certain functions. Upon activation of the adenylate cyclase signaling pathway, CAMP responsive element modulator (CREM) [199] undergoes S6K1-mediated phosphorylation [200]. Chaperonin containing TCP-1 (CCT) is also considered an S6K substrate and functions in protein folding. Mitogen-activated RSK and insulin-activated S6Ks all can converge on CCTβ by phosphorylating it at S260 in various mammalian cells [201]. Although deficiency of S6ks results in a smaller size and prolonged lifespan, the impacts of S6ks on cellular senescence remain an unsolved problem. Recently, through a chemical genetic screen, Barilari et al. proposed that Zuo1-related factor 1 (ZRF1) is a direct substrate of S6K1. Knocking down or expressing nonphosphorylatable ZRF1 markedly impairs the inhibitory effects of S6K1 in senescence, which is evidenced by a sharp alteration of p16 levels, a cell cycle inhibitor and primary modulator of senescence [202]. Moreover, S6K has certain functions that are independent of its kinase activity. Notably, S6K1 directly binds to actin and promotes the activation of Rac1 and CDC42, leading to subsequent actin cytoskeleton reorganization and cell migration independent of its kinase activity [203]. Moreover, upon exposure to mitogen stimuli, S6K2, but not S6K1, binds with Yin Yang 1 (YY1) via its C-terminal region, but the physiological significance is still undefined [204].

INHIBITORS TARGETING S6KS FOR CANCER THERAPIES

Given the multifactorial functions of S6ks in controlling cell size, cell growth, cell apoptosis, and metabolic homeostasis, inhibitors targeting S6ks and their upstream activators, such as mTORC1 and PDK1, hold promise for cancer therapies (Table 2).

S6ks inhibitors

PF-4708671 is a cell-permeable piperezynyl-pyrimidine compound that specifically inhibits p70-S6K. Its inhibitory effects are evidenced by a prominent decrease in the phosphorylation of
| Target(s) | Inhibitor | Inhibition mode(s) | Features | References |
|-----------|-----------|--------------------|----------|------------|
| S6K1      | PF-4708671| Piperazinyl-pyrimidine compound | Decreased phosphorylation of S6 | [205] |
| S6K1      | PF-4708671|                      | Sensitized resistant colorectal cancer cells to treatment of selumetinib | [206] |
| S6K1      | PF-4708671|                      | Suppressed cell invasion and proliferation in human lung cancer cell lines and tumorigenesis in nude mice | [207] |
| S6K1      | PF-4708671|                      | Improved corticospinal tract regeneration and locomotor recovery in central nervous system injury mice | [208] |
| S6K2      | Compound 2| PF-4708671 derivative | Showed a high specificity to S6K2 over other kinases | [210] |
| S6K1      | A77 1726  | Active metabolite of Leflunomide | Released feedback suppression of IRS1, promoting the cell cycle arrest | [211] |
| S6K1      | FS-115    | Synthesized compound | Inhibited colony formation and sphere formation of breast cancer cells, suppressing primary tumor growth and distant metastasis of breast cancer in nude mice | [212] |
| S6K1      | FL772     | ATP competitive organometallic inhibitor | Reduced kinase activity of S6K1 but not of S6K2 | [213] |
| S6K1      | LY2584702 | ATP competitive inhibitor | Multiple-dose study showed no intolerability, a dose-dependent reduction in LDL cholesterol, triglycerides, and Factor V activity in hypercholesterolemia volunteers | [214, 215] |
| S6K1 and AKT | LY2780301 | ATP competitive inhibitor | Phase I/II trials showed a linear PK with minor variability and irrelevant with problems of significant safety and tolerability when orally administered with 200 mg | [216] |
| S6K1      | LY56K2    | Synthesized compound | Reduced cellular triacylglyceride level and apolipoprotein-B100 secretion in TSC-deficient cells | [217, 218] |
| S6K1      | AST       | Natural product | Inhibited phosphorylation of S6 and IRS1, enhancing phosphorylation of AKT and S6K T389 | [219] |
| Ret, Src, Raf, TOR, S6K kinases | AD80 and AD81 | Synthesized compound | As polypharmacological agents, functioning in achieving an optimal balance by targeting various kinases | [220] |
| S6K1, AKT1/3 | M2698     | ATP competitive inhibitor | Dose-dependently impaired tumor growth and prolonged survival of mice with U251 glioblastoma | [221, 222] |
| S6K1, JAK2 | Gingerenone A | Natural compound | Suppressed cell growth and tumor growth in mice | [223, 224] |
| mTOR      | Temsirolimus, Ridaforolimus, Everolimus | Rapalogs | Approved or underwent several clinical trials. Showed preferable water solubility and pharmacokinetic properties; suppressing the activation of S6K1 and 4E-BP1 via mTORC1 inhibition, subsequently resulting in cell cycle arrest and cell death | [225] |
| mTORC1/2  | Streptomyces sp OA293 | Ethyl acetate extracts | Showed potent inhibition of mTORC1 and AKT, suppressing activation of S6K1 and 4E-BP1 | [226] |
| mTORC1/2  | CC-223    | ATP competitive inhibitor | Phase I dose-escalation study exhibited tolerable and manageable toxicities when patients were treated who had advanced cancer | [227] |
| Target(s) | Inhibitor       | Inhibition mode(s)                                                                 | Features                                                                                           | References |
|----------|----------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|------------|
|          | CC-223         |                                                                                | Phase II study revealed highly durable tumor regression, marked reduction in NET carcinoid symptoms among well-differentiated neuroendocrine tumor (NET) patients | [232]      |
| PI3K, AKT, mTOR | AZD8055 GDC0941 Selumetinib      |                                                                              | Reduced cell proliferation and retarded tumor growth in PDX mice models of ovarian clear cell carcinoma | [233]      |
| PDK1     | SBF1           | Allosteric inhibitor of PIF pocket                                             | Predicted IC50 ranging from 2.0 to 10.0 μM                                                        | [234]      |
| PDK1     | OSU-03012      | ATP competitive inhibitor; Celecoxib derivative                                | With IC50 variation in the low μM range                                                          | [235]      |
|          | OSU-03012      |                                                                                | Showed induction of autophagy and ROS levels and subsequent suppression of cell growth in various cancers | [236]      |
| PDK1     | GSK470         | Inhibition of T loop phosphorylation                                           | Suppressed cell growth and enhanced cell death of multiple myeloma cells, which could synergize with proteasome inhibitor MG132 | [237]      |
| PDK1     | JX06           |                                                                                | Inhibited glycolysis and promoted cell apoptosis of multiple myeloma cells; synergized with proteasome inhibitor bortezomib | [238]      |
| PDK1     | BX795 and BX912 | Inhibition of T loop phosphorylation                                           | Abolished PDK1 dependent on Myc induction, resulting in cell death and a reduction in tumor sphere formation of various breast cancer cell lines | [239]      |
| PDK1     | BX795          | Inhibition of T loop phosphorylation                                           | Synergized with cisplatin to markedly reduce the epithelial-mesenchymal transition of oral squamous cell carcinoma | [240]      |
| PDK1     | Dichloroacetate |                                                                                | Synergized with NOS inhibitor T1023, leading to an evident reduction of tumor growth                 | [241]      |
| AKT, PDK1| PHT-427        | Binding with PH domains of AKT and PDK1                                      | Enhanced oxidative stress levels and apoptosis in various cancers                                  | [242, 243] |
S6 after IGF1 stimulation, which also shows excellent selectivity for mitogen- and stress-activated kinases and RSK [205]. The effectiveness of PF-4708671 has also been validated in various settings. For example, S6K1 inhibition induced by PF-4708671 sensitizes colorectal cancer cells to selumetinib (an MEK1/2 inhibitor) treatment [206] to suppress cell invasion and proliferation [207] and improves corticospinal tract regeneration and locomotor recovery in central nervous system injury mice [208] and glucose homeostasis in high-fat diet-induced obese mice [209]. By targeting cysteine 150 of S6K2, but not S6K1, Gerstenecker et al. indicated that Compound 2 generated from PF-4708671 via a nucleophilic aromatic substitution reaction shows a higher specificity for S6K2 than other kinases, which may serve as a model for the development of next-generation S6K2-specific inhibitors [210]. Although the broad preclinical application of PF-4708671 as an S6K1-specific inhibitor has achieved promising results under diverse pathological conditions, no clinical trial regarding PF-4708671 has been conducted. Alternatively, A771726, an active metabolite of leflunomide approved by the FDA for curing rheumatoid arthritis, could inhibit S6K1 activity, release feedback suppression of IRS1, and promote cell cycle arrest in the S phase [211]. Furthermore, F5-115 potently suppressed the activity of S6K1, exhibiting marked selectivity for kinases from the AGC family. Treatment with F5-115 could also inhibit colony formation and sphere formation of breast cancer cells, suppress primary tumor growth, and reduce distant metastasis of breast cancer in nude mice [212].

ATP competitive inhibitors might be a powerful strategy for developing potent and specific suppressors for kinases. As an ATP competitive organometallic inhibitor, FL772 markedly reduces the kinase activity of S6K1 in the nanomolar range and exhibits more than 100-fold selectivity over S6K2 [213]. LY2584702 is another selective ATP competitive inhibitor for S6K1 against 83 other kinases. Phase I/II trials of LY2584702 indicate that the maximum tolerated doses are 75 mg twice daily or 100 mg once daily [214, 215]. Moreover, LY2584702 has been adopted in the treatment of dyslipidemia [216]. These phenomena may derive from the inhibition of S6K1 in the regulation of lipid metabolism. A single-ascending-dose study was performed to evaluate the safety, tolerability and pharmacokinetic (PK) features of LY2780301, an ATP competitive dual inhibitor of S6K1 and AKT, followed by a single oral dose. LY2780301 also shows a linear PK with minor variability and is irrelevant to problems of significant safety and tolerability when orally administered at 200 mg [217, 218]. Most recently, a TAKTIC study revealed that combined treatment with LY2780301 and paclitaxel (weekly) among patients with hormone-resistant HER2-negative advanced breast cancers showed a 6-month objective response rate (ORR) of 63.9% and 55% in all patients and patients with an activated PI3K/AKT pathway, respectively [219].

It has been reported that LYS6K2 could function as a selective inhibitor of S6Ks, resulting in the reduction of cellular triacylglycerol levels and apolipoprotein-B100 secretion in TSC-deficient cells [220]. As a xanthophyll carotenoid, astaxanthin (AST) is a natural product mainly derived from seafoods, functioning as an inhibitor of S6K1, which is evidenced by inhibited phosphorylation of S6 and IRS1. However, administration of AST is also accompanied by enhanced phosphorylation of AKT and S6K at T389 because of abolished negative feedback regulation of S6K1 on the PI3K/AKT pathway [221]. Combining genetics with chemistry, Dar et al. synthesized a series of compounds, among which AD80 and AD81 were identified as polypharmacological agents, possessing optimal balance against Ret, Src, Raf, TOR, and S6K kinase activities [222]. As another dual inhibitor of S6K1 and AKT, M2698, which is capable of penetrating the blood–brain barrier, exhibits marked suppression of the activity of S6K1 and AKT1/3 in vitro and in vivo [223, 224]. In a phase I study, M2698 was well tolerated. In combination with tamoxifen or trastuzumab, M2698 manifests antitumor effects on patients with advanced cancer who are unresponsive to multiple standard therapies [225]. Gingerenone A is a natural compound extracted from ginger that could serve as a dual inhibitor of S6K1 and JAK2, as evidenced by a marked reduction in S6K1 phosphorylation. Gingerenone A functions in suppressing cell and tumor growth in mice [226]. Chen et al. also provided evidence that gingerenone A enhances insulin sensitivity and glucose uptake, possibly by inhibiting S6K1-IR51 negative feedback regulation [227]. Several natural components also show inhibitory effects toward S6K1, which has been well summarized previously [44].

**mTORC1 inhibitors indirectly repress S6K activity**

Due to its essential roles in cell physiological processes, aberrations of mTORC1 are frequently implicated in various diseases, including cancers. Thus, targeting mTORC1 represents a powerful apparatus for combating diseases [92]. The first and most well-studied inhibitor of mTORC1 is rapamycin, whose unpleasant water solubility and chemical stability promote the development of rapalogs to induce apoptosis by heterodimerization with FKBP12, suppressing the activation of S6K1 and 4E-BP1 [228]. However, clinical trials for mTORC1 inhibitors are unsatisfactory, possibly due to the release of AKT activation induced by the negative feedback regulation of S6K1 [152, 154]. Therefore, inhibitors exhibiting dual suppression of mTORC1 and mTORC2 have emerged as a promising strategy alone or in combination with other agents. In addition, the active fraction of Streptomyces sp OA293 shows potent inhibition of mTORC1 and AKT activation induced by active mTORC2, which is accompanied by suppression of S6K1 and 4E-BP1 [229]. In addition to rapalogs, ATP analogs also represent another prospective inhibitor of mTOR by competing with ATP binding to mTOR kinase [230]. As an ATP competitive inhibitor, CC-223 has been reported to exhibit tolerable and manageable toxicities when treating patients who had advanced cancer in a phase I study [231]. A subsequent phase II study among well-differentiated neuroendocrine tumor (NET) patients revealed highly durable tumor regression and a remarkable reduction in NET carcinoid symptoms, suggesting that CC-223 is a promising agent [232]. Combination usage of PI3K, AKT, and mTOR inhibitors (AZD8055, GDC0941, and selumetinib) markedly reduces cell proliferation and retards tumor growth in PDX mouse models of ovarian clear cell carcinoma [233].

**PKD1 inhibitors function indirectly in fine-tuning S6K activity**

As another key regulator of S6Ks, PKD1 inhibition could lead to suppression of S6Ks. Unlike mTOR inhibitors, the development of PKD1 inhibitors is still in a start-up stage. PKD1 possesses an ATP binding pocket, PKD1-interacting fragment (PIF)-binding pocket, and phosphate-binding pocket, which could be targeted to develop specific inhibitors for PKD1. Of note, SBF1 may serve as a specific allosteric inhibitor of PKD1 anchoring the PIF pocket, as a promising scaffold template for future modulation [234]. Derived from celecoxib, OSU-03012 has emerged as a potent inhibitor of PKD1-AKT signaling by targeting the ATP binding pocket of PKD1 [235]. OSU-03012 has been tested in various cancers, such as pancreatic, esophageal, and endometrial carcinoma cells, and is capable of inducing autophagy and increasing ROS levels and subsequently suppressing cell growth [236]. GSK470 has also been developed as a specific PKD1 inhibitor to suppress cell growth and enhance cell death of multiple myeloma cells, synergized with proteasome inhibitor MG132 [237]. JX06 is also considered a prospective agent for suppressing PKD1 in multiple myeloma cells. In combination with the proteasome inhibitor bortezomib, JX06 has shown synergetic effects on cell death in multiple myeloma cells [238]. Tan et al. reported that BX795 and BX912 might function as PKD1 inhibitors to abolish PKD1-induced c-Myc expression, resulting in cell death and a reduction in tumor sphere formation [239]. Moreover, BX795 synergizes with cisplatin.
to markedly reduce the epithelial-mesenchymal transition (EMT) in oral squamous cell carcinoma [240]. Dichloroacetate synergizes with the nitric oxide synthase (NOS) inhibitor T1023, reducing tumor growth [241]. It has been illustrated that PHT-427, a dual inhibitor of AKT and PDK1, exhibits promising inhibitory effects in various tumors by binding the PH domains of AKT and PDK1 [242]. Furthermore, efficiently delivering PHT-427 with nanoparticles leads to high oxidative stress levels and enhanced apoptosis in head and neck squamous cell carcinoma [243].

CONCLUSION AND PERSPECTIVES

It is generally accepted that S6Ks play oncogenic roles in different cancers, although various isoforms and alternative splices exhibit distinct distributions and contributions in cells and tissues. Specifically, due to the different chromatin locations of genes encoding S6K1 and S6K2, genomic alterations or upstream regulation at the epigenetic and transcriptional levels have shown significant differences, which possibly explains the context-dependent specificity for S6K1 and S6K2. To further investigate the physiological or pathological roles of S6K isoforms, tissue-specific conditional knockout mice for different isoforms are valuable tools and should be generated. Although S6K1 and S6K2 share a similar structure and amino acid sequence, different upstream regulators possibly determine the distinct roles for S6Ks. On the other hand, although posttranslational modifications, such as phosphorylation, have been extensively investigated for S6Ks, other PTMs involved in S6K regulation, such as methylation, ubiquitination, palmitoylation, and their cross talk with phosphorylation and their contributions to S6K kinase activity, warrant further exploration. S6K1 and S6K2 share most substrates, suggesting potential redundant functions for these S6Ks and highlighting the limitation of specific inhibitors targeting single S6K. Simultaneously, different substrates have also been identified for the S6K isoforms (Fig. 3), indicating distinct functions between S6Ks. This observation will help identify specific inhibitors of S6K1 or S6K2 for precision therapies in a tissue context-dependent manner. Although accumulating substrates have been identified for S6Ks, characterization of novel and specific substrates is also desired to better dissect the S6K functions. Similar to AKT kinase, S6K also phosphorylates its substrates at the R/KxxS/T motif, but interestingly, there are some substrates, such as Erα, H2B, S6, elf4B, Rictor, and IRS1, which have also been characterized as substrates of AKT or SGK.

As we mentioned above, in addition to controlling cell size by promoting the initiation and elongation processes of protein synthesis, S6Ks also directly contribute to cell proliferation, cell apoptosis, cell metabolic homeostasis, and feedback regulation of their upstream signals by phosphorylating different proteins. These processes integrate to direct S6K functions to mediate mTORC1-dependent or mTORC1-independent tumorigenesis. Thus, it has become promising to target S6K for cancer therapies. However, due to the negative feedback regulation of the PI3K-AKT pathway (Fig. 5A), directly [211, 221] or indirectly [244] targeting S6K will induce elevated AKT kinase activity, which may result in drug resistance. To this end, dual-target inhibitors for S6K and AKT have begun to be developed and display a good response in cancer therapies [219, 225]. Collectively, there is no doubt that S6K plays important roles in tumor cell proliferation, apoptosis, survival, and metastasis by regulating multiple processes, including cell size control and metabolic homeostasis, which highlights a potential strategy to target S6K for cancer therapies in the near future.

DATA AVAILABILITY

The published article includes all data sets generated/analyzed for this study.

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ACKNOWLEDGEMENTS

The authors sincerely apologize to all those colleagues whose important work was not cited in the paper owing to space limitations. They thank the members of the Guo laboratory for critical reading and discussion of the manuscript. The figures were generated with Biorender.com.

AUTHOR CONTRIBUTIONS

XW and JG conceived and wrote this review together with the help of Wei Xie, Wenxuan Xie, and Wenyi Wei.

FUNDING

This work was supported by the National Natural Science Foundation of China (NSFC) to JG (32070767).

COMPETING INTERESTS

WW is a co-founder and consultant for the ReKindle Therapeutics. Other authors declare no competing interests.

ADDITIONAL INFORMATION

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