Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation

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The p70 ribosomal protein S6 kinase 1 (S6K1) plays a key role in cell growth and proliferation by regulating insulin sensitivity, metabolism, protein synthesis, and cell cycle. Thus, deregulation of S6K contributes to the progression of type 2 diabetes, obesity, aging, and cancer. Considering the biological and clinical importance of S6K1, a complete understanding of its regulation is critical. One of the key motifs in the activation of S6K1 is a turn motif, but its regulation is not well understood. Here we provide evidence for two mechanisms of modulating turn motif phosphorylation and S6K1 activity. First, mammalian target of rapamycin regulates turn motif phosphorylation by inhibiting its dephosphorylation. Second, we unexpectedly found that glycogen synthase kinase (GSK)-3 promotes turn motif phosphorylation. Our studies show that mammalian target of rapamycin and GSK-3 cooperate to control the activity of S6K1, an important regulator of cell proliferation and growth. Our unexpected results provide a clear rationale for the development and use of drugs targeting GSK-3 to treat diseases such as diabetes, cancer, and age-related diseases that are linked to improper regulation of S6K1.

Mammalian target of rapamycin (mTOR) is a central regulator of cell proliferation and growth. mTOR integrates signals from multiple inputs such as growth factors, stress, nutrients, and energy to regulate protein synthesis, cell cycle progression, actin organization, and autophagy (1). Because of the essential roles of mTOR in cell growth and metabolism, deregulation of mTOR is prominent in the development and progression of cancer and in metabolic diseases such as diabetes and obesity. Its deregulation can be caused by overexpression and/or overactivation of upstream effectors, or deletion of negative regulators.

The 40S ribosomal protein S6 kinase (S6K) is a major substrate of mTOR and is a crucial effector of mTOR signaling (2). One of the S6K isoforms, S6K1, plays important roles in cell growth, proliferation, and cell differentiation by regulating ribosome biogenesis, protein synthesis, cell cycle progression, and metabolism (3–5). Recent studies suggest that deletion of S6K1 not only increases lifespan but also reduces the incidence of age-related pathologic processes, including bone, immune, and motor dysfunction and insulin resistance (6, 7). Because of its important role in cell growth and insulin sensitivity, aberrant activation of S6K1 plays a major role in the progression of tumors, diabetes, obesity, and aging (2, 6, 7). Therefore, understanding the mechanism of S6K1 regulation will contribute to the ongoing efforts to develop novel drugs that provide effective treatments to combat diseases that are characterized by deregulation of the S6K signaling pathway.

S6K1 belongs to the AGC kinase family, a subgroup of Ser/Thr protein kinases (PKs) that are related to PKA, PKG, and PKC. This group includes S6K1, Akt, p90 ribosomal S6K (RSK), mitogen- and stress-activated protein kinase, and several members of the PKC family, which regulate cell growth, survival, metabolism, and motility (8). AGC kinases share a common core mechanism of activation that is dependent on the phosphorylation of two highly conserved regulatory motifs: an activation loop and a hydrophobic motif. Several AGC kinases also contain another important phosphorylation site, the turn motif, which has a Ser/Thr-Pro sequence and stabilizes the active conformation of the kinase (9). The turn motif phosphorylation site is the most poorly characterized of the three conserved sites, yet its mutation significantly reduces kinase activity and hydrophobic motif phosphorylation in some AGC kinases such as S6K1. Recently, it was shown that mTOR complex (mTORC) 2 regulates phosphorylation of the turn motif in Akt cotranslationally (10–12).

The activity of S6K is regulated by a wide range of extracellular signals including growth factors, hormones, nutrients (glucose and amino acids), and stress. Work from many research groups has revealed the complexity of S6K1 activation via sequential phosphorylation at multiple sites (3). The best characterized sites are Thr-229 (T229) in the activation loop and Thr-389 (T389) in a conserved hydrophobic motif (2). It is known that PDK1 and mTOR can phosphorylate T229 and T389, respectively. The current model for S6K activation under nutrient and energy sufficient conditions is that PI3-kinase and/or Ras signaling converge to suppress the negative regulator of mTORC1 signaling, the tuberous sclerosis complex (TSC1/2). Inhibition of TSC GAP function results in Rheb-G protein and mTORC1 activation. mTORC1 then phosphorylates T389, creating a docking site for PDK1, which is then able to phosphorylate the activation loop T229 (13, 14). More recently, it has been found that Ser-371 (S371), which resides within a turn motif, is essential for T389 phosphorylation and S6K1 activity (2, 14). However, it remains unclear how S371 phosphorylation is regulated. One report suggested that this site is also regulated by mTOR (15), but did not fully explain how it contributes to the mechanism of S6K1 activation. For example, rapamycin, an mTOR inhibitor, slightly inhibits S371 phosphorylation, whereas it completely inhibits T389 phosphorylation. Serum starvation and insulin treatment also do not substantially affect S371 phosphorylation, whereas T389 phosphorylation is significantly affected by these factors. These examples demonstrate that regulation of these two sites is very different, although it appears that mTOR is involved in regulating both sites through an unknown mechanism. Therefore, we have set out to determine how S371 phosphorylation is regulated. During the course of our investigation, we made the surprising observation that glycogen synthase kinase-3 (GSK-3), a proline-directed kinase, positively

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regulates S6K1 activity by modulating S371 phosphorylation. We also found that mTOR cooperates with GSK-3 to regulate S6K1 activity and cell proliferation.

Results

S371 Phosphorylation Is Essential for Thr-389 Phosphorylation and S6K1 Activity. It is well established that the T389 residue in S6K1, which is regulated by mTORC1, is essential for S6K1 activity. However, little is known about the regulation and role of S371 phosphorylation. We first examined the importance of the S371 phosphorylation site in comparison with the T389 on S6K1 activity. For this, cells were transfected with WT-S6K1, S371A-S6K1, T389A-S6K1, and kinase-inactive K100R-S6K1, and an in vitro kinase assay was performed by using purified GST-S6 as a substrate. As shown in Fig. 1*A*, the single mutation of each residue abolished the kinase activity of S6K1, which confirms that the S371 phosphorylation site is essential for S6K1 activity. To determine whether the S371 site affects T389 phosphorylation, cells were transfected with WT-S6K1 or S371A S6K1, and were stimulated with various agonists. As shown in Fig. 1*B*, S371A S6K1 did not exhibit any increase in T389 phosphorylation upon stimulation, regardless of the agonist. Taken together, these results suggest that S371 phosphorylation is essential for T389 phosphorylation and for the activity of S6K1.

S371 and T389 Phosphorylation Are Differentially Regulated. Despite the important role of S371 phosphorylation, its regulatory mechanism is not well understood. To our knowledge, there are a few reports that describe the regulation of S371 phosphorylation. Saitoh et al. suggested that S371 is regulated by mTOR (15). However, changes in S371 phosphorylation induced by serum starvation or rapamycin, an inhibitor of mTOR, were not dramatic in their studies, which suggests the possibility that another kinase (or kinases) may cooperate with mTOR to regulate this residue or that the phosphatase for this site does not dephosphorylate it under these conditions. To discern the details of the mechanism of S371 phosphorylation, we first treated cells growing in the presence of serum with rapamycin for 1 h. As shown in Fig. 1*C*, rapamycin markedly inhibited T389 phosphorylation. Rapamycin was quite effective in T389 dephosphorylation, as we could see a marked effect between 5 min and 10 min after the treatment (*SI Appendix*, Fig. S1*A*). However, rapamycin did not appreciably decrease S371 phosphorylation (Fig. 1*C*). Similarly, 1 h treatment with an mTOR catalytic inhibitor, Torin1, did not change S371 phosphorylation. In contrast, T389 phosphorylation was completely inhibited by this inhibitor. We also used LY294002, an inhibitor of the PI3-K/Akt pathway, a major upstream regulator of mTOR/S6K1. LY294002 did not decrease S371 phosphorylation, although it showed a potent inhibitory effect on T389 phosphorylation (Fig. 1*C*). We used different cell types to determine if this was a general phenomenon. As shown in *SI Appendix*, Fig. S1*B*, rapamycin and Torin1 treatment did not induce an appreciable decrease in S371 phosphorylation in several other cell lines. Serum starvation and agonist treatment did not appreciably change S371 phosphorylation, whereas T389 phosphorylation was profoundly affected under these conditions (Fig. 1 *D* and *E*, and *SI Appendix*, Fig. S1*C*). In addition, rapamycin did not have much of an effect on S371 phosphorylation under insulin treatment conditions, although T389 phosphorylation was completely inhibited with rapamycin treatment (Fig. 1*F*). Taken together, these results suggest the possibility that S371 and T389 phosphorylation are regulated differentially.

Long-Term Inhibition of mTORC1 Regulates S371 Phosphorylation. Based on our finding that S371 and T389 phosphorylation were differentially regulated, and that acute inhibition of mTOR by chemical inhibitors had little effect on S371 phosphorylation, we wondered if mTOR is a physiological kinase for S371. To investigate this question further, we first asked whether S371 phosphorylation was dependent on the TOS (i.e., TOR signaling) motif, which is necessary to facilitate mTOR signaling to activate S6K1 (16). To do this, we used several TOS-motif mutants of S6K1. As shown in Fig. 2*A*, mutation of the TOS motif reduced phosphorylation of S371 phosphorylation as well as phosphorylation of T389. Interestingly, T389 phosphorylation was markedly decreased by the TOS-motif mutation, and S371 phosphorylation was decreased approximately two or threefold.

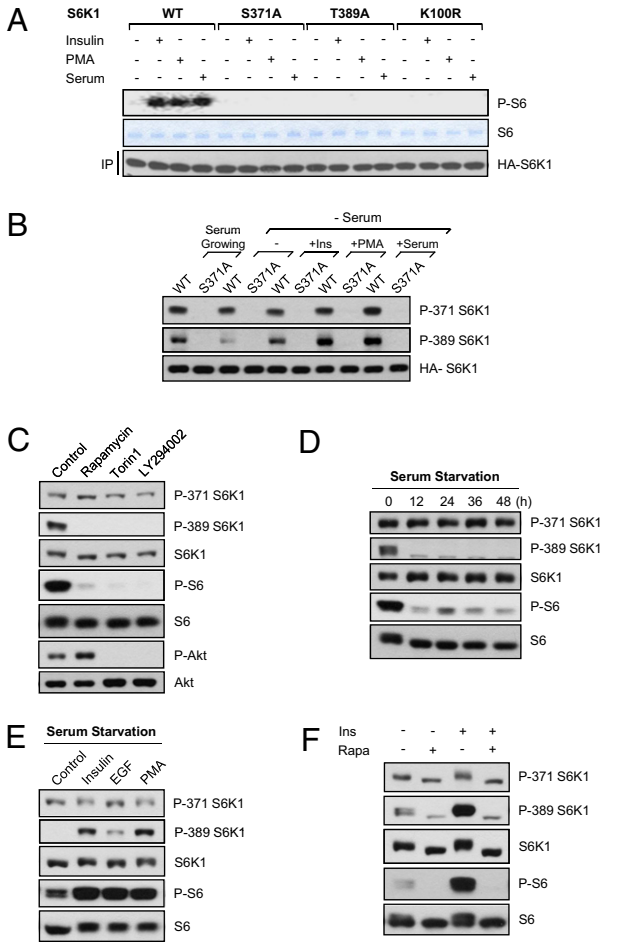

Fig. 1. The S371 residue is essential for S6K1 activity, and is differently regulated compared with T389. Data are representative of at least three independent experiments. (*A*) HEK293 cells were transfected for 24 h with plasmids encoding WT or mutant S6K1 and then incubated in serum-free medium for an additional 24 h. Cells were treated with insulin (50 nM), phorbol myristate acetate (PMA; 50 ng/mL), or serum (10%) for 20 min and then lysed. After immunoprecipitation, phosphotransferase assays were performed by using purified GST-S6. (*B*) HEK293 cells were transfected with WT or S371A S6K1 plasmids for 24 h, and then incubated in 10% serum-containing medium or serum-free medium for an additional 24 h. Serum-starved cells were treated with agonists for 20 min. After cell lysis, immunoprecipitation and immunoblot analyses were performed. (*C*) Mouse fibroblasts growing in the presence of serum were treated with rapamycin (20 nM), torin1 (250 nM), or LY294002 (15 μM) for 1 h. Cells were then lysed, and immunoblot analysis was performed. (*D*) Mouse fibroblasts were incubated in serum-free medium for the indicated time, lysed, and analyzed by immunoblot. (*E*) Mouse fibroblasts were incubated in serum-free medium for 24 h and treated with agonists for 20 min. Cells were then lysed, and immunoblot analysis was performed. (*F*) Mouse fibroblasts were incubated in serum-free medium for 24 h. Cells were pretreated with rapamycin for 30 min, and then treated with insulin for 20 min. Cells were lysed, and immunoblot analysis was performed.

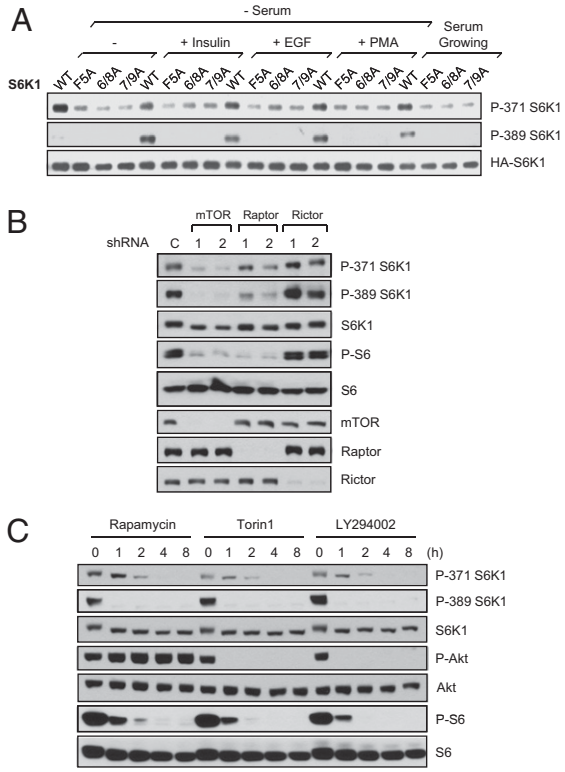

Fig. 2. Long-term inhibition of mTOR decreases S371 phosphorylation. Data are representative of at least three independent experiments. (*A*) HEK293 cells were transfected for 24 h with plasmids encoding WT or TOS-motif mutant (F5A, 7/9A, D6/8A) S6K1. Cells were maintained in serum-containing medium or serum-free medium for an additional 24 h. Serum-starved cells were treated with agonists for 20 min. After cell lysis, immunoprecipitation and immunoblot analyses were performed. (*B*) We generated stable mouse fibroblasts with mTOR, raptor, or rictor shRNA. Cells growing in the presence of serum were lysed, and immunoblot analysis was performed. C, control. (*C*) Growing mouse fibroblasts were treated with rapamycin (20 nM), Torin1 (250 nM), or LY294002 (15 μM) for the indicated times, lysed, and analyzed by immunoblot.

This suggests that S371 phosphorylation is dependent on the mTORC1 pathway, but only partially, compared with T389 phosphorylation. The main components of mTORC1, a direct kinase for S6K1, are mTOR, raptor, mLST8, and PRAS40. mTORC2, a kinase for Akt/SGK/PKCα, consists of mTOR, rictor, and mSIN1. Thus, we used shRNA against mTOR, raptor, and rictor to knock down these proteins. Knockdown of mTOR in cells growing in the presence of serum decreased phosphorylation of both S371 and T389 (Fig. 2B). Interestingly, knockdown of raptor decreased T389 phosphorylation dramatically, but only partially reduced S371 phosphorylation (Fig. 2*B*). Rictor knockdown did not change the phosphorylation of S371 or T389. Taken together, these data suggest that T389 phosphorylation is largely dependent on mTORC1, but that S371 phosphorylation may be only partially dependent on mTORC1. We next asked why S371 phosphorylation showed partial dependence on the TOS motif and was partially inhibited by knockdown of mTORC1 components, but was not affected by treatment with rapamycin, Torin1, or LY294002. These results led us to investigate the effects of acute versus chronic treatment with these inhibitors. Interestingly, we found that long-term rapamycin treatment abolished S371 phosphorylation in cells growing in serum (Fig. 2*C*). Prolonged treatment with LY294002 and Torin1 also abolished S371 phosphorylation (Fig. 2*C*). We observed the same results in other cells (*SI Appendix*, Fig. S2*A*). Long-term treatment with other PI3K inhibitors such as PI-103 and wortmannin also inhibited S371 phosphorylation (*SI Appendix*, Fig. S2*B*). These results suggest that S371 phosphorylation is ultimately dependent on the PI3K/mTOR pathway, although short-term inhibition of this pathway does not inhibit S371 phosphorylation.

mTORC1 Regulates S371 Phosphorylation Through Inhibition of Phosphatases. To understand the details of the relationship between mTOR and S371 phosphorylation, we used an exogenous S6K1 expression system with WT and mutant S6K1 plasmids. The ^{410}RSPRR^{414} motif in the C terminus of S6K1 is responsible for suppression of T389 phosphorylation and S6K1 inactivation (17). Thus, we wondered if this motif was involved in S371 phosphorylation. For this, we used C-terminal truncation mutants of S6K1 (Fig. 3*A*) and measured S371 phosphorylation. As shown in Fig. 3*B*, C-terminal mutants truncated at amino acids 417 (S6K1-ΔCT417) and 422 (S6K1-ΔCT422) showed reduced S371 phosphorylation compared with S6K1-ΔCT401 and S6K1-ΔCT409. This suggests that the protein sequence between amino acids 409 and 417 negatively regulates S371 phosphorylation, which correlates with known suppressive function of ^{410}RSPRR^{414} motif on S6K1 activity. It is known that the TOS motif mediates S6K1 activation by suppression of an inhibitory activity associated with the ^{410}RSPRR^{414} motif of S6K1, and mutation within RSPRR motif partially rescues the kinase activity of the S6K1 TOS motif mutant (16, 17). To determine whether the R5A mutant (mutation of ^{410}RSPRR^{414} to ^{410}AAAAA^{414}) could rescue TOS motif mutant-mediated reduction of S371 phosphorylation, we used the double mutant (TOS-motif mutation and RSPRR mutation). As shown in Fig. 3*C*, mutation of RSPRR motif restored S371 phosphorylation of S6K1-F5A. Taken together, these findings suggest that the C-terminal RSPRR motif is important for the suppression of S371 phosphorylation. It is known that the ^{410}RSPRR^{414} motif in S6K1 is responsible for an mTOR-dependent suppression of S6K1 activation, possibly through the binding of PP2A-like phosphatases to this motif (17). Thus, we first examined the interaction of PP2A with the S6K1 WT or RSPRR mutant. As shown in *SI Appendix*, Fig. S3, PP2A showed a stronger interaction with S6K1 WT compared with the RSPRR mutant. This suggests that the RSPRR motif is important for the binding of PP2A with S6K1. We performed further studies to determine whether mTOR regulates S371 phosphorylation through phosphatases. We treated cells with rapamycin first, and then overexpressed WT and mutant S6K1 to create cells in which mTORC1 was inhibited while exogenous S6K1 was expressed. As shown in Fig. 3*D*, WT and TOS-motif mutants (F5A) S6K1 showed reduced S371 phosphorylation in the presence of rapamycin, which further supports a role for mTORC1 in regulating this phosphorylation. We also used a kinase-inactive mutant of S6K1 (S6K1-K100R), which does not exhibit any S6K1 activity (Fig. 1*A*). This mutant also showed S371 phosphorylation (Fig. 3*D*), suggesting that this site is not an autophosphorylation site and is not dependent on S6K1 activity itself. Interestingly, RSPRR mutants exhibited a relatively strong S371 phosphorylation, which was expressed in the presence of rapamycin. If mTORC1 is the main kinase for S6K1 S371 phosphorylation, WT and all mutant S6K1 would be expected to exhibit only low levels of S371 phosphorylation under these conditions because inactive mTORC1 cannot phosphorylate S6K1 WT and mutants. Based on the facts that (*i*) mTOR suppresses protein phosphatase 2A (PP2A) activity and inhibition of mTOR by rapamycin restores PP2A activity (18), (*ii*) PP2A has been shown to bind to WT-S6K1 but not the C-terminal truncated S6K1 (19), and (*iii*) the RSPRR motif in S6K1 may be the binding site of PP2A-like phosphatases (17), our findings, along with previous reports, suggest that mTOR regulates S371 phosphorylation through inhibition of PP2A-like phosphatases. To test this hypothesis, we

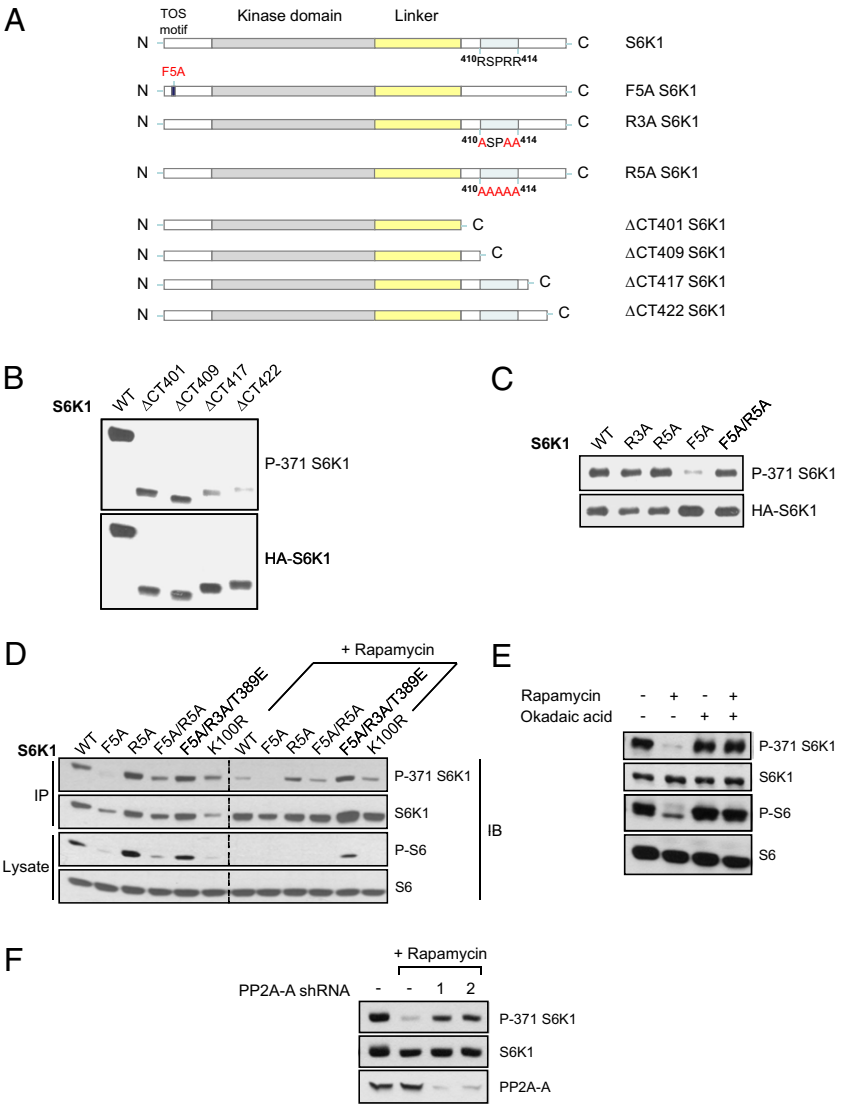

Fig. 3. mTOR modulates S371 phosphorylation by regulating phosphatase activity. Data are representative of at least three independent experiments. (*A*) Schematic representation of the WT and mutant forms of S6K1. (*B*) HEK293 cells were transfected with WT and C terminus–truncated S6K1 mutants and lysed. After immunoprecipitation, immunoblot analysis was performed. (*C*) HEK293 cells were transfected with WT or mutant S6K1, lysed, immunoprecipitated, and analyzed by immunoblot. (*D*) HEK293 cells were pretreated with DMSO or rapamycin (20 nM) for 1 h, and then transfected with WT or mutant S6K1 for 16 h. After cell lysis, immunoprecipitation and immunoblot analyses were performed. (*E*) Mouse fibroblasts were first treated with rapamycin for 2 h, and then treated with DMSO or okadaic acid (200 nM) for an additional 2 h. Cells were lysed and then analyzed by immunoblot. (*F*) Stable PP2A A subunit knockdown mouse fibroblasts were generated using shRNAs. Cells were treated with 5 nM rapamycin for 3 h, lysed, and analyzed by immunoblot analysis.

treated cells with rapamycin and/or phosphatase inhibitors and examined the dephosphorylation of S371. Long-term rapamycin treatment abolished S371 phosphorylation of endogenous S6K1. Importantly, however, at the concentration that inhibits only PP2A activity, okadaic acid prevented dephosphorylation of S371 by rapamycin (Fig. 3*E*). To further confirm this, we used shRNAs specific for the PP2A structural subunit A. As shown in Fig. 3*F*, knockdown of the PP2A A subunit partially inhibited dephosphorylation of S371 by rapamycin. These results suggest that long-term rapamycin treatment activates PP2A, and that PP2A dephosphorylates S371. Taken together, our data indicate that mTORC1 regulates S371 phosphorylation by suppressing a PP2A phosphatase activity.

GSK-3 Positively Regulates S371 Phosphorylation. Our data suggest that mTORC1 regulates S371 phosphorylation by modulating a phospho-S371 phosphatase activity. However, these data do not address the nature of the kinase or kinases that can phosphorylate S371. Thus, we set out to identify the kinase that cooperates with mTOR to regulate S371 phosphorylation. For this, we used prediction programs for phosphorylation motifs. Because S371 is just N-terminal to a proline (i.e., P), ^{371}SPDDS^{375}, proline-directed kinases were identified as potential S371 kinases by these programs. Among the tested proline-directed kinases, GSK-3 exhibited interesting results that were quite unexpected (*SI Appendix*, Fig. S4*A*). The minimal recognition motif for phosphorylation by GSK-3β was identified as S/T-X-X-X-S/T, where the first serine (i.e., S) or threonine (i.e., T) is phosphorylated by GSK-3β (20). Interestingly, many GSK-3β substrates have S/T-P-X-X-S/T, where the first S/T residue is just N-terminal to a proline (Fig. 4*A*). GSK-3β is thus considered to be a proline-directed kinase (21). Comparison of the turn motif of S6K1 with known GSK-3β substrates indicated that they were similar, and suggested that GSK-3β could phosphorylate S371 (Fig. 4*A*). To

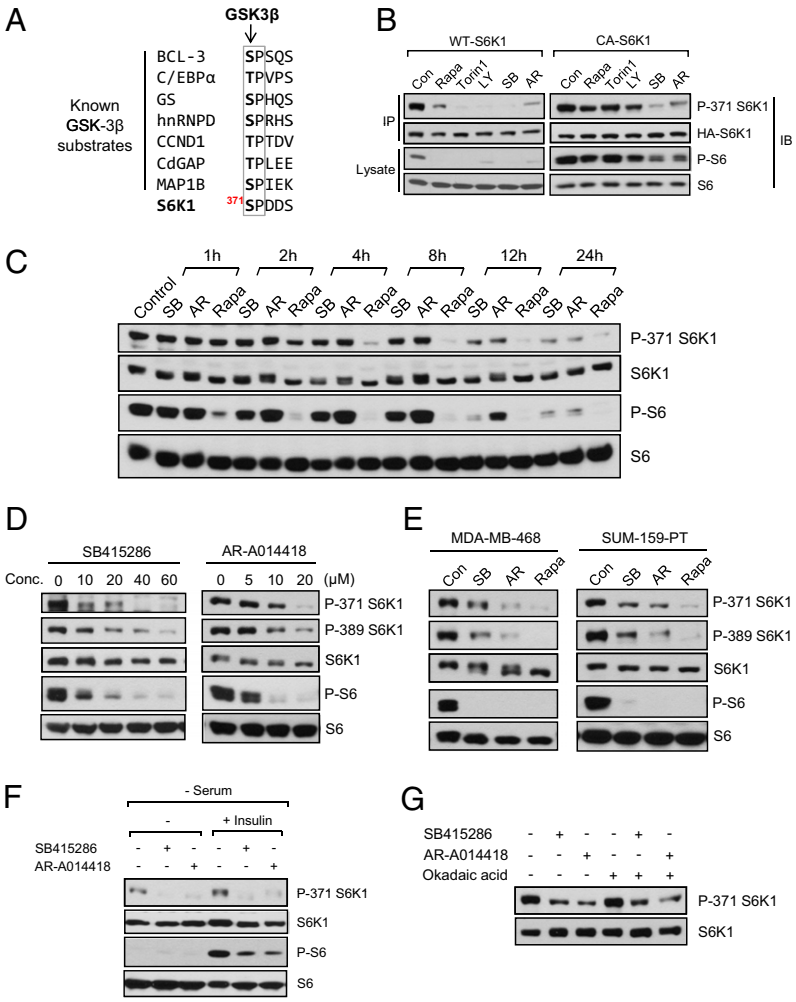

Fig. 4. GSK-3 regulates S371 phosphorylation and S6K1 activity. Data are representative of at least three independent experiments. (*A*) Comparison of GSK-3 substrate phosphorylation sites and S6K1 sequence. (*B*) HEK293 cells were pretreated with rapamycin (20 nM), Torin1 (250 nM), LY294002 (15 μM), SB415286 (20 μM), or AR-A014418 (10 μM) for 1 h, and then transfected with WT S6K1 or F5A-R3A-T389E S6K1 for 16 h. After cell lysis, immunoprecipitation and immunoblot analyses were performed. (*C*) Mouse fibroblasts growing in the presence of serum were treated with SB415286 (40 μM) or AR-A014418 (20 μM) for the indicated times. Cells were then lysed and analyzed by immunoblot. (*D*) Mouse fibroblasts were treated with different concentrations of SB415286 or AR-A014418 for 24 h. Cells were lysed, and immunoblot analysis was performed. (*E*) Cells were treated with SB415286 (40 μM) or AR-A014418 (20 μM) for 24 h, lysed, and analyzed by immunoblot. (*F*) Mouse fibroblasts were incubated in serum-free medium for 24 h in the presence or absence of GSK-3 inhibitors. Cells were treated with insulin for 20 min, lysed, and then analyzed by immunoblot. (*G*) Mouse fibroblasts were treated with SB415286 or AR-A014418 for 8 h and then treated with okadaic acid for an additional 2 h. Cells were then lysed, and immunoblot analysis was performed.

further determine if S371 is a GSK-3 phosphorylation site, we first treated cells with mTOR pathway inhibitors (rapamycin, Torin1, and LY294002) or specific inhibitors of GSK-3 (SB415286 and AR-A014418), and then overexpressed WT S6K1 to create cells in which mTOR or GSK-3 was inhibited while exogenous S6K1 was expressed. Interestingly, GSK-3 inhibition decreased phosphorylation of S371 in WT S6K1, as did rapamycin and Torin1 (Fig. 4*B*). We then used known phosphatase-resistant S6K1 mutant (F5A-T389E-R3A S6K1) (17) under the same conditions. Specific GSK-3 inhibitors also decreased S371 phosphorylation in this mutant, whereas mTOR inhibitors did not alter S371 phosphorylation in this context (Fig. 4*B*). This suggests that GSK-3 may regulate S6K1 phosphorylation at S371 independent of phosphatase. This observation prompted us to further investigate the role of GSK-3 in S6K1 regulation. To determine whether GSK-3 inhibition also affected endogenous S6K1, we used two specific GSK-3 inhibitors, SB415286 and AR-A014418. Short-term treatment with these inhibitors did not result in significant changes in S371 phosphorylation (Fig. 4*C*). However, longer-term inhibition of GSK-3 reduced S371 phosphorylation (Fig. 4*C*). Phosphorylation of a downstream substrate of S6K1, ribosomal protein S6 (rpS6), was also decreased with GSK-3 inhibition (Fig. 4*C*). To determine the concentration-dependent effects of these inhibitors, cells were treated with different concentrations of the inhibitors. As shown in Fig. 4*D*, the inhibitors decreased S6K1 phosphorylation in a concentration-dependent manner. To determine if this positive regulation of S6K1 by GSK-3 was cell type-specific, we tested the effects of GSK-3 inhibitors on many other cell types such as breast cancer cells (MDA-MB-468, MCF7, SUM-159-PT), prostate cancer cells (DU145, PC3), colon cancer cells (HT29), osteosarcoma cells (U2OS), and kidney cells (HEK-293). Inhibition of GSK-3 resulted in inhibition of S6K1 in these cell lines, suggesting that it is a general phenomenon (Fig. 4*E* and *SI Appendix*, Fig. S4*B*). We were interested in determining whether inhibition of GSK-3 blocked S6K1 activation stimulated by insulin. As shown in Fig. 4*F*, GSK-3 inhibitors decreased S6K1 phosphorylation under these conditions. To determine whether the regulation of S371 phos-

phorylation by GSK-3 is dependent on the phosphatase pathway, we used GSK-3 inhibitors and okadaic acid, a PP2A inhibitor. As shown in Fig. 4*G*, okadaic acid did not inhibit the decrease of S371 phosphorylation by GSK-3 inhibitors. Taken together, our findings suggest that GSK-3 positively regulates S6K1 activity by regulating phosphorylation of S371.

GSK-3β Directly Phosphorylates S6K1 at the Ser371 Residue. GSK-3 has two isoforms, GSK-3α and GSK-3β, and it is known that GSK-3 inhibitors block the activities of both isoforms. To determine whether GSK-3α and/or GSK-3β are responsible for S6K1 phosphorylation, we used two different shRNAs specific for GSK-3α or GSK-3β. As shown in Fig. 5*A*, knockdown of GSK-3α did not result in changes in S6K1 phosphorylation. However, GSK-3β knockdown resulted in decreased phosphorylation of S6K1 and S6. We further tested the effect of GSK-3β on S6K1 phosphorylation by using GSK-3 WT and GSK-3$^{-/-}$ mouse embryonic fibroblasts (MEFs). Compared with GSK-3β WT MEFs, GSK-3β$^{-/-}$ MEFs exhibited a significant decrease of S371 phosphorylation (Fig. 5*B*). GSK-3α KO, however, did not have profound effect on S371 phosphorylation (Fig. 5*B*). To determine if this positive regulation of GSK-3β on S6K1 was cell type-specific, we tested the effects of GSK-3β shRNAs on many other cell types. Knockdown of GSK-3β resulted in inhibition of S6K1 in these cell lines, suggesting that it is a general phenomenon (Fig. 5*C* and *SI Appendix*, Fig. S5*A*). Interestingly, knockdown of GSK-3α did result in decreased S6K1 phosphorylation in HEK293 and U2OS cells (*SI Appendix*, Fig. S5*A*), suggesting that GSK-3α contributes to S371 phosphorylation under specific conditions or in certain cells. To follow up on this observation, we knocked down GSK-3α in GSK-3β$^{-/-}$ MEFs and examined S371 phosphorylation. Interestingly, GSK-3α knockdown further decreased S371 phosphorylation as well as S6 phosphorylation (Fig. 5*D*). These results indicate that, although GSK-3β is more dominant in regulating S371 phosphorylation, GSK-3α is also involved in this process. We next asked if GSK-3β directly phosphorylated S6K1 at the S371 residue. For this, we performed an in vitro kinase assay by using recombinant GSK-3β protein (NEB) and immunopurified S6K1, and examined phosphorylation of S6K1 at S371. As shown in Fig. 5*E*, GSK-3β increased Ser371 phosphorylation and GSK-3 inhibitor blocked GSK-3β–mediated S6K1 phosphorylation. This suggests that GSK-3β can directly phosphorylate S6K1 at the S371 residue in vitro. Like the turn motif of S6K1, many GSK-3β substrates have a S^1/T^1-P-X-X-S^2/T^2 motif (Fig. 4*A*), where the first S^1 or T^1 is phosphorylated by GSK-3β and the second S^2 or T^2 is the "priming site." Although not strictly required, priming site phosphorylation facilitates the phosphorylation of the first S^1 or T^1 by GSK-3 (22, 23). To determine if S375 in the turn motif of S6K1 (^{371}SPDDS^{375}) is a priming site for S371 phosphorylation, we first examined if S375 is phosphorylated or not. By using MS analysis, we found that S375 was not phosphorylated whereas S371 was phosphorylated. This suggests that S375 is not a priming site for S371. It is known that many GSK-3 substrates do not require priming site phosphorylation to be phosphorylated by GSK-3 (22, 23), and many of these nonprimed substrates display negatively charged residues at or near the priming position that mimic a phosphoresidue (23). Although the S375 site in S6K1 is not phosphorylated, the S6K1 turn motif contains two negatively charged amino acids, aspartic acid (i.e., D), in the turn motif (^{371}SPDDS^{375}). To determine if D373 and D374 play a role in S371 phosphorylation, we made a double mutant of the aspartic acid positions (D373A/D374A) and found that this mutant displayed less S371 phosphorylation compared with WT S6K1 (Fig. 5*F*). Therefore, these results suggest that the S6K1 turn motif contains negatively charged amino acids that mimic the priming phosphorylation required for the subsequent phosphorylation by GSK-3. Taken together, these observations reveal that GSK-3 positively regulates phosphorylation at S371, the conserved site in various species (*SI Appendix*, Fig. S5*B*).

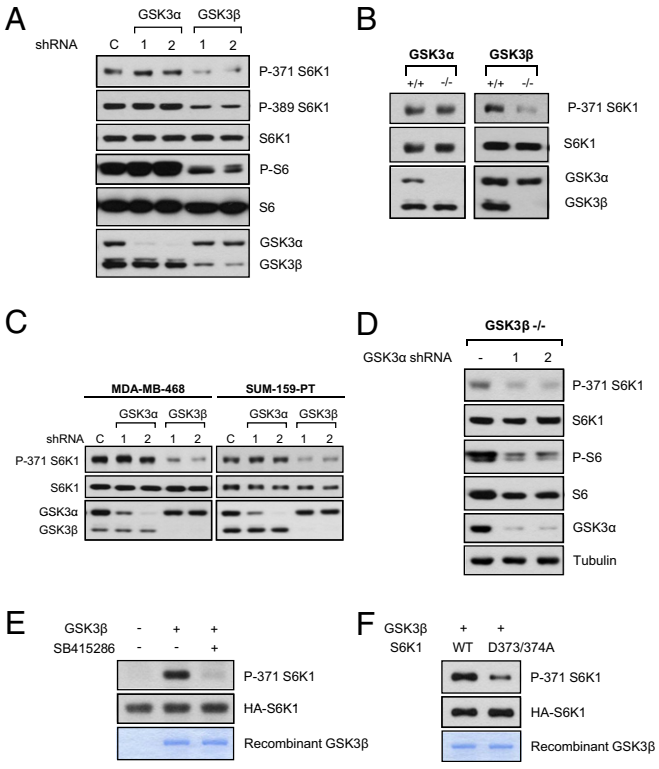

Fig. 5. GSK-3β regulates S371 phosphorylation. Data are representative of at least three independent experiments. (*A*) Immunoblot analysis was performed on GSK-3α or GSK-3β knockdown mouse fibroblasts. C, control. (*B*) GSK-3α$^{+/+}$, GSK-3α$^{-/-}$, GSK-3β$^{+/+}$, or GSK-3β$^{-/-}$ MEFs were lysed and analyzed by immunoblot. (*C*) Growing cells with GSK-3α or GSK-3β knocked down were lysed and analyzed by immunoblot analysis. C, control. (*D*) shRNA was used to knock down GSK-3α in GSK-3β$^{-/-}$ MEFs. After cell lysis, immunoblot analysis was performed. (*E*) In vitro phosphotransferase assays were performed by using recombinant GSK-3β (NEB) and immunopurified HA-S6K1 as described in *Materials and Methods*. For the inhibition of GSK-3β, 10 μM SB415286 was used. S371 phosphorylation was measured by immunoblot analysis. (*F*) In vitro phosphotransferase assays were performed by using recombinant GSK-3β (NEB) and immunopurified HA-S6K1 WT or HA-S6K1 D373/374A mutant as described in *Materials and Methods*. S371 phosphorylation was measured by immunoblot analysis.

GSK-3β Can Modulate S371 Phosphorylation Indirectly by Regulation of Activities Upstream of S6K1. After obtaining the surprising result that GSK-3 positively regulates S6K1, we performed experiments to further define the molecular mechanism to explain this. The activity of S6K1 is regulated by mTORC1, which is mainly regulated by Akt. Akt positively regulates mTORC1 by inhibiting the negative regulators of mTORC1. To determine if Akt is regulated by GSK-3, we examined Akt phosphorylation after treatment with GSK-3 inhibitors. Interestingly, Akt phosphorylation was reduced by these inhibitors (Fig. 6*A*). We also used GSK-3β shRNA and found that knockdown of GSK-3β decreased Akt phosphorylation (Fig. 6*B*). To investigate if GSK-3β is a general regulator of Akt, different cell lines were treated with GSK-3 inhibitors, and Akt phosphorylation was examined. As shown in Fig. 6*C* and *SI Appendix*, Fig. S6, some cells exhibited decreases in Akt phosphorylation, whereas other cells did not show appreciable changes in Akt phosphorylation. These results suggest that Akt, a main regulator of mTOR/S6K1, may not be the general factor that regulates S6K1 phosphorylation

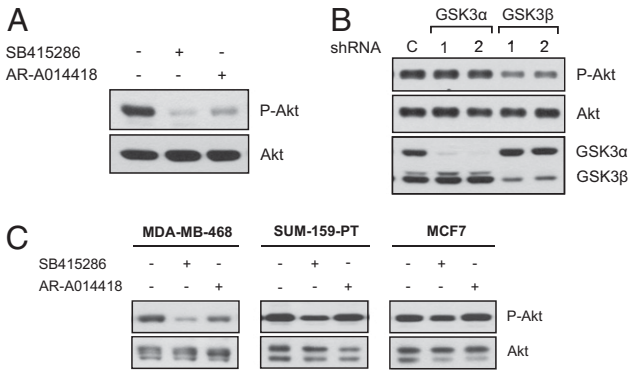

Fig. 6. GSK-3 regulates Akt phosphorylation. Data are representative of at least three independent experiments. (*A*) Mouse fibroblasts were treated with SB415286 (40 μM) or AR-A014418 (20 μM) for 24 h, lysed, and then analyzed by immunoblot. (*B*) GSK-3α or GSK-3β knockdown mouse fibroblasts were lysed and immunoblot analysis was performed. C, control. (*C*) Cells were treated with SB415286 or AR-A014418 for 24 h, lysed, and analyzed by immunoblot.

after treatment with GSK-3 inhibitors. All together, our data indicate that GSK-3 regulates S371 phosphorylation in some cell types by regulating elements upstream of S6K1 but, for the most part, by directly regulating phosphorylation of this residue.

GSK-3–Mediated S6K1 Phosphorylation Regulates Cell Proliferation. After finding that GSK-3 promotes S6K1 phosphorylation and activity, we were interested in determining the cellular functions affected by GSK-3–regulated S6K1 activity. We first investigated GSK-3 function in several cell types. We observed that GSK-3 inhibitors (Fig. 7*A*) and GSK-3β knockdown (Fig. 7*B*) decreased proliferation of mouse fibroblasts. However, knockdown of GSK-3α did not appreciably affect cell proliferation (Fig. 7*B*). We also tested different cells such as MDA-MB-468 and MCF7 cells, and found that GSK-3 inhibitors dramatically decreased proliferation of these cells (*SI Appendix*, Fig. S7 *A* and *B*). To determine the function of GSK-3-regulated S6K1, we overexpressed constitutively active S6K1 (F5A-R5A-T389E) and then treated cells with GSK-3 inhibitors. Under these conditions, this S6K1 mutant remains active upon addition of GSK-inhibitors because S371 is already phosphorylated and this phosphorylation site is resistant to phosphatase-mediated inactivation (Fig. 7*C*). As shown in Fig. 7*D* and *SI Appendix*, Fig. S7 *C* and *D*, overexpression of this constitutively active mutant S6K1 partially rescued the proliferation of cells growing in the presence of GSK-3 inhibitors, which suggests that GSK-3–mediated S6K1 regulation is important for cell proliferation. Taken together, our studies show that mTOR and GSK-3 cooperate to regulate the activity of S6K, an important player in cell proliferation and growth.

Discussion

Considering the important roles of S6K in insulin sensitivity, protein synthesis, cell-size control, metabolism, cell proliferation, and cell survival, it is not surprising that deregulation of S6K is involved in the progression of cancer, type 2 diabetes, obesity, and aging (3–5). Although mTOR is known as a primary regu-

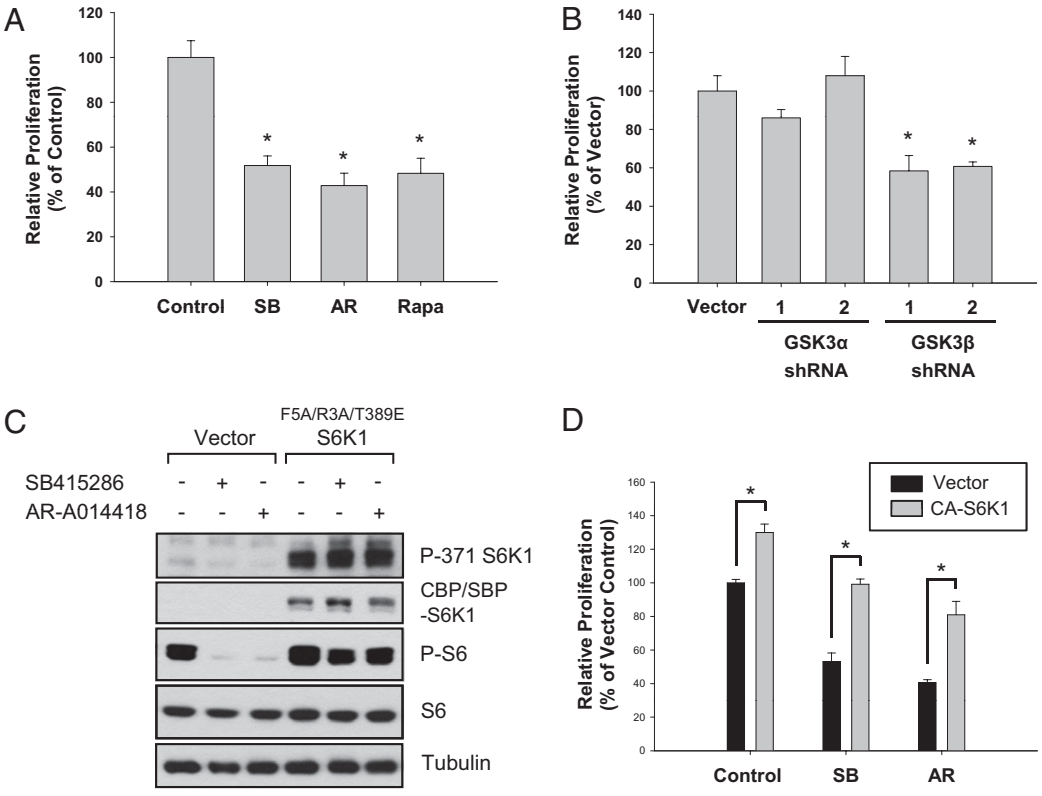

Fig. 7. GSK-3–mediated S6K1 regulation controls cell proliferation. (*A*) Mouse fibroblasts growing in serum were treated with DMSO, SB415286, AR-A014418, or rapamycin for 2 d, after which cell numbers were counted. Data are the mean ± SEM of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) by Student *t* test. (*B*) GSK-3α or GSK-3β knockdown cells were generated, and rate of proliferation was measured by counting cell numbers. Data are the mean ± SEM of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) by Student *t* test. (*C* and *D*) Mouse fibroblasts stably expressing control or constitutively active (F5A-R3A-T389E) S6K1 were treated with DMSO, SB415286, or AR-A014418 for 2 d. Cells were lysed for immunoblot analysis (*C*) or cell proliferation rate was measured (*D*). Data are displayed as the mean ± SEM of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) by Student *t* test.

lator of S6K, the cooperation of upstream molecules is important for full activation and functionality of S6K. Here we provide evidence that GSK-3β cooperates with mTOR to positively regulate S6K1 activity and cell proliferation.

GSK-3β belongs to the proline-directed PK family that phosphorylate serine or threonine residue preceding proline (Ser/Thr-Pro) (21). GSK-3β is involved in a variety of cellular processes such as glycogen metabolism, insulin signaling, cell proliferation, apoptosis, neuronal function, and embryonic development (24). Accordingly, GSK-3β has emerged as a potential target for treating type 2 diabetes mellitus, Alzheimer's disease, mood disorders, and atherosclerosis. Thus, GSK-3 inhibitors are being actively developed as promising drugs. However, their use in clinical trials has been questioned because of a concern that inhibition of GSK-3 may promote oncogenesis by activating pathways stimulating cell survival and proliferation (25). If GSK-3 inhibits cancer progression, GSK-3 inhibitors should not be used to treat diseases such as type 2 diabetes despite findings that these inhibitors clearly show that GSK3 can be a good therapeutic target for the treatment of insulin resistance and type 2 diabetes without any significant side effects in mice (26). Furthermore, type 2 diabetes is associated with elevated risk and increased mortality for breast, liver, pancreatic, colorectal, bladder, and kidney cancer, and non-Hodgkin lymphoma (27, 28), underscoring concerns associated with the use of GSK-3 inhibitors if these inhibitors are indeed promoters of tumorigenesis. However, despite these concerns, no direct in vivo evidence has actually indicated that administration of GSK-3 inhibitors promotes tumor development (25, 29, 30). Moreover, there is growing evidence that supports our finding that GSK-3β can positively regulate cell survival and proliferation, thereby promoting tumorigenesis and tumor progression (30–32), and thus suggesting that GSK-3 inhibitors may be an effective anticancer therapeutic approach. For example, GSK-3β protein overexpression has been found in many tumor types such as ovarian, colon, pancreatic, and liver tumors (31). In addition, GSK-3 inhibition by pharmacological inhibitors and RNAi reduced the survival of various cancer cell types and predisposed them to undergo apoptosis in vitro and in tumor xenografts (30). GSK-3β inhibition also resulted in antitumor effects in prostate, colon, pancreatic, ovarian, and thyroid cancers; melanoma; hematologic malignancies; malignant gliomas; pheochromocytoma; and paraganglioma (30).

Although emerging data show that GSK-3 can promote tumor progression and inhibition of GSK-3 suppresses tumor progression, little is known about the mechanisms by which GSK-3β performs this function. Several reports suggest that GSK-3β plays crucial roles in NF-κB–mediated cell survival and Notch signaling (33, 34). It also has been reported that GSK-3β inhibits the activity of the tumor suppressor protein p53 (35), whereas it activates Maf, an oncoprotein (36). In addition, a recently published report showed that GSK-3 is involved in potentiating leukemia through homeobox gene misregulation (37). Therefore, the overall conclusion from these recent studies is that GSK-3β is likely to be a promising therapeutic target in a range of cancer types, but the molecular basis remains unclear. Here we provide evidence that GSK-3 positively increases cell proliferation through regulation of S6K1.

S6K1 has been shown to be involved in tumor growth and proliferation (38, 39). In many cancers, the mTOR/S6K pathway is highly active because of mutations and/or overexpression of upstream positive regulators such as PI3K, Akt, and ErbB2, or loss of expression or function of negative regulators such as the tumor suppressors PTEN, LKB, NF1, or TSC1/2. Deregulation of the mTOR/S6K pathway plays important roles in tumor development and progression (1, 2). In addition, the chromosomal region containing the *S6K1* gene is frequently amplified in breast cancer cells, which correlates with poor prognosis and reduced survival for patients with breast cancer (40, 41). In addition to the role of S6K1 in cancer development, hyperactivation of S6K1 is also involved in the progression of type 2 diabetes and obesity. In contrast, inhibition or deletion of S6K1 increases lifespan and reduces the incidence of age-related pathologic processes, including bone, immune, and motor dysfunction, and insulin resistance (6, 7). In this regard, our data suggest that pharmacological inhibition of GSK-3 could be beneficial in the treatment of diabetes, obesity, age-related diseases, and cancer through its effect on S6K1.

Although our results clearly showed that GSK-3 positively regulated S6K1 in most cell types we tested, Inoki et al. showed that GSK-3β negatively regulated phosphorylation of S6K1 at T389 by activating TSC, a negative regulator of mTOR (42). Currently, we do not know the reason why there is a discrepancy in the function of GSK-3 on S6K1. However, based on our studies, it seems that the duration and potency of GSK-3 inhibition influences the cellular response. We found that potent and long-term GSK-3 inhibition was effective in the control of S6K1 by regulating S371 phosphorylation in cells growing in the presence of serum. Although, we did not see much change in S6K1 activity with short-term inhibition of GSK-3, it is possible that results may vary with cellular context.

Our findings suggest that S371 phosphorylation levels are regulated by the balance of phosphatase and kinase activities that are dependent on mTOR and GSK-3. Unlike other kinases, GSK-3 is highly active in the absence of serum, and this activity maintains S371 phosphorylation of S6K1 under this condition. Therefore, S6K is in a ready state or poised state, even in the absence of growth factors. Upon growth factor stimulation, S6K1 is rapidly activated by mTOR, and performs its function. Growth factors may inhibit GSK-3 activity. However, under stimulation conditions, activated mTOR also suppresses phosphatase activity toward S371. Therefore, the net result is that there is a reduced requirement for positive regulation of S371 phosphorylation during growth factor stimulation to maintain an active S6K1. However, when mTORC1 inhibition is sustained and the S371 phosphatases no longer inhibited, the S6K1 ready state or poised state cannot be attained. Unlike the transient effects of growth factors, many tumors have constitutively active Akt, a major upstream kinase that directly inhibits GSK-3 activity. This raises the question of how GSK-3 regulates S6K under these conditions. Constitutive Akt activation could inhibit GSK-3 activity, which could lead to S6K inhibition in these cancer cells. However, many studies show that GSK-3 is highly active and overexpressed in many cancers such as ovarian, colon, pancreatic, and liver tumors (31, 43). Moreover, it has been shown that GSK-3 remains highly active in most cancers that have highly active Akt (43–45). Therefore, for reasons that are not clear, Akt activation and GSK-3 inhibition are not always correlated in human cancers, and a pool of GSK-3 remains active in cancers and cells growing in the presence of serum. Considering the role of GSK-3 in cell metabolism, insulin signaling, and neuronal function, this active pool of GSK-3 may contribute to the progression of diabetes, neurological disorders, and cancer.

In conclusion, here we provide evidence supporting the unexpected finding that GSK-3 positively regulates S6K1 activity and cell proliferation. S6K1 is involved in cell survival, growth, and proliferation by regulating protein synthesis, cell metabolism, glucose homeostasis, insulin sensitivity, cell cycle, and gene transcription. Considering its roles, it will be of great interest to explore the novel downstream targets and functions of GSK-3 in these processes, which will provide the rationale for the development of drugs targeting GSK-3 to treat diseases such as diabetes, cancer, and age-related diseases.

Materials and Methods

Cells and Reagents. GSK-3$\alpha^{+/+}$, GSK-3$\alpha^{-/-}$, GSK-3$\beta^{+/+}$, and GSK-3$\beta^{-/-}$ MEFs were provided by James R. Woodgett (University of Toronto, Toronto, ON, Canada). The 293TD cells were generously provided by Andrew L. Kung (Dana-Farber Cancer Institute, Boston MA). MEFs, 293TD cells, HEK 293 cells, human cervical carcinoma HeLa cells, breast cancer cells (MCF-7, MDA-MB-468), prostate cancer cells (DU145, PC3), colon cancer cells (HT29), and osteosarcoma cells (U2OS) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. SUM-159-PT breast carcinoma cells were maintained in Ham F-12 supplemented with 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 100 U/mL penicillin, and 100 μg/mL streptomycin. Anti–phospho-S371-S6K1, anti–phospho-T389-S6K1, anti–phospho-S6, anti–phospho-Akt, anti-tubulin, anti-S6K1, anti-Akt, anti-S6, anti-mTOR, anti-raptor, anti-rictor, and anti-PP2A subunits antibodies were purchased from Cell Signaling Technology. Anti–HA-tag and anti–GSK-3α/β antibodies were from Santa Cruz Biotechnology. Anti–CBP-tag antibodies were purchased from GenScript. SB415286 was from Enzo Life Sciences. AR-A014418 (GSK-3β inhibitor VIII), LY294002, PI-103, wortmannin, PD98059, SB203580, PNU 112455A, rapamycin, and okadaic acid were obtained from Calbiochem. Torin1 was provided by David Sabatini (Whitehead Institute, Cambridge, MA) and Nathanael Gray (Dana-Farber Cancer Institute, Boston, MA). Insulin, EGF, phorbol myristate acetate, and Polybrene were purchased from Sigma. Recombinant GSK-3β was obtained from NEB. Protein A– and G–Sepharose beads were purchased from GE Healthcare. A HA antibody immobilized onto Sepharose matrix was obtained from Covance. MagnaBind protein G beads and dithiobis[succinimidyl propionate] (DSP) were from Thermo Scientific.

Plasmids. Lentiviral packaging (Δ8.9) and envelope plasmids were a gift from Andrew L. Kung (Dana-Farber Cancer Institute, Boston, MA) and David Baltimore (California Institute of Technology, Pasadena, CA). Lentiviral GSK-3α (human and mouse), GSK-3β (human and mouse), mTOR (mouse), raptor (mouse), rictor (mouse), and PP2A A subunit (mouse) shRNA plasmids were purchased from Open Biosystems. pRK7-HA-WT S6K1, pRK7-HA-S371A S6K1, pRK7-HA-T389A S6K1, pRK7-HA-K100R S6K1, pRK7-HA-F5A S6K1, pRK7-HA-7/9A S6K1, pRK7-HA-D6/8A S6K1, pRK7-HA-ΔCT S6K1, pRK7-HA-F5A-ΔCT S6K1, pRK7-HA-R5A, pRK7-HA-F5A-R5A, and pRK7-HA-F5A-R3A-T389E-S6K1 (constitutively active and rapamycin-resistant) have been described (16, 17). pRK7-HA-D373A/D374A S6K1 was generated by Genewiz.

Generation of Stable Knockdown and Overexpression Cells. To generate lentiviruses, GSK-3α, GSK-3β, mTOR, raptor, rictor shRNA plasmids, or control shRNA plasmids were transfected into 293TD cells with the expression plasmids for packaging (Δ8.9) and envelope (VSVG), and medium was changed the next day. After 48 h, viral supernatants were harvested, and new medium was added. Viral supernatants were collected again after 30 h. Cells were infected with viral supernatants in the presence of a serum-containing medium supplemented with 8 μg/mL Polybrene. After 30 h, viral-containing medium was removed and cells were grown in serum-containing medium for 12 h. Cells were treated with puromycin (4 μg/mL puromycin for MEFs and 2 μg/mL puromycin for other cells), and cells that stably expressed shRNA were selected. The decreased level of target protein expression was confirmed by immunoblot analysis.

Transfections. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) in the presence of 10% serum without antibiotics according to the manufacturer's protocol.

Immunoblot Analysis. For immunoblot analysis, the cells were extracted in lysis buffer A (20 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mg/mL pepstatin) containing 1% Triton X-100, and samples were resuspended in reducing buffer (6×, 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). Samples were boiled for 5 min and electrophoresed by SDS/PAGE. Proteins were then transferred to nitrocellulose membranes (Whatman). The membranes were blocked with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 containing 5% nonfat dried milk or 5% BSA, and probed overnight with primary antibodies, followed by 1 h incubation with secondary antibodies coupled to peroxidase. Blots were developed by using enhanced chemiluminescence.

Immunoprecipitation. For the immunoprecipitation of HA-tagged S6K1, cells were extracted with lysis buffer A containing 1% Triton X-100. After centrifugation, supernatants were collected and preabsorbed for 1 h with protein A– and G–Sepharose beads (GE Healthcare Biosciences). After centrifugation at 800 × *g* for 5 min, the supernatants were incubated with antibody at 4 °C for 2 h, and then incubated with protein A– and G–Sepharose for an additional 1 h. Beads were washed four times with a buffer and eluted in 2× reducing sample buffer.

For the immunoprecipitation of PP2A, intracellular proteins were first cross-linked with DSP according to the manufacturer's protocol (Thermo Scientific). Briefly, cells were lysed in buffer (40 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mg/mL pepstatin) containing 0.8 mg/mL DSP. After incubation at room temperature for 30 min, the cross-linking reaction was quenched by adding 1 M Tris (pH 7.5) to a final concentration of 20 mM. After an additional incubation for 20 min and centrifugation, the supernatants were incubated with an anti-PP2A C subunit antibody at 4 °C for 2 h, and then incubated with MagnaBind protein G beads for an additional 1 h. Beads were washed four times with a buffer and eluted in 2× reducing sample buffer.

Protein Phosphotransferase Assays. For S6K1 kinase assay, HA-S6K1 WT or mutants transfected cells were lysed with buffer A containing 1% Triton X-100, and immunoprecipitation was performed by using immobilized HA antibody onto Sepharose matrix (Covance). Beads were washed twice in lysis buffer and twice in kinase reaction buffer, and S6K1 kinase activity was performed as described previously by using recombinant GST-S6 as a substrate (46). The reaction products were subjected to SDS/PAGE, and dried gel was autoradiographed.

For the GSK-3β kinase assay using S6K1 WT, HEK293 cells were pretreated with rapamycin, and pRK7-HA-WT S6K1 was transfected. Cells were lysed with buffer A containing 1% Triton X-100, and immunoprecipitation was performed by using an HA antibody immobilized onto Sepharose matrix (Covance). Beads were washed twice in lysis buffer and three times in calf intestinal phosphatase buffer (NEB) and incubated with calf intestinal phosphatase (NEB) for 1.5 h at 37 °C. Beads were then washed three times with GSK-3 reaction buffer (NEB). Kinase assays were performed for 1 h at 30 °C with recombinant GSK-3β (NEB) and immunoprecipitated HA-S6K1 as the substrate in GSK-3 reaction buffer containing 500 μM ATP. The reaction products were subjected to SDS/PAGE, and immunoblot analysis was performed by using an anti–phospho-S371 S6K1 antibody. For the GSK-3β kinase assay using S6K1 WT and D373A/D374A, the same method was used except kinase assays were performed for 20 min at 30 °C.

MS Analysis. Liquid chromatography/tandem MS characterization of S6K1 phosphorylation was carried out as described previously (47). Briefly, HEK293 cells were transfected with the S6K1 expression plasmid and lysed. Whole-cell lysates were collected to perform immunoprecipitation. Immunoprecipitates were resolved on SDS/PAGE and visualized by colloidal Coomassie blue. The band containing S6K1 was excised, and in-gel digest of the protein was performed with trypsin. The peptides were extracted from the gel and phosphopeptides were enriched by immobilized metal ion affinity chromatography and were analyzed by nanoscale-microcapillary reversed-phase liquid chromatography/tandem MS. Peptides were separated across a 37-min gradient ranging from 4% to 27% (vol/vol) acetonitrile in 0.1% (vol/vol) formic acid in a microcapillary (125 μm × 18 cm) column packed with C18 reverse-phase material (Magic C18AQ, 5 μm particles, 200 Å pore size; Michrom Bioresources), and analyzed online on an LTQ linear ion trap mass spectrometer (Thermo Scientific). MS/MS spectra were searched using the SEQUEST algorithm against a composite human database and its reverse compliment with dynamic modification of methionine oxidation and serine, threonine, and tyrosine phosphorylation. All peptide matches were filtered based on mass deviation, tryptic state, XCorr, and dCn and were confirmed by manual validation.

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1. Efeyan A, Sabatini DM (2010) mTOR and cancer: Many loops in one pathway. *Curr Opin Cell Biol* 22:169–176.

2. Dann SG, Selvaraj A, Thomas G (2007) mTOR Complex1-S6K1 signaling: At the crossroads of obesity, diabetes and cancer. *Trends Mol Med* 13:252–259.

3. Jastrzebski K, Hannan KM, Tchoubrieva EB, Hannan RD, Pearson RB (2007) Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. *Growth Factors* 25:209–226.
4. Carnevalli LS, et al. (2010) S6K1 plays a critical role in early adipocyte differentiation. *Dev Cell* 18:763–774.
5. Düvel K, et al. (2010) Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 39:171–183.
6. Harrison DE, et al. (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460:392–395.
7. Selman C, et al. (2009) Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* 326:140–144.
8. Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* 11:9–22.
9. Alessi DR, Pearce LR, García-Martínez JM (2009) New insights into mTOR signaling: mTORC2 and beyond. *Sci Signal* 2:pe27.
10. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL (2008) Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* 27: 1919–1931.
11. Facchinetti V, et al. (2008) The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* 27:1932–1943.
12. Oh WJ, et al. (2010) mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. *EMBO J* 29:3939–3951.
13. Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10:307–318.
14. Fingar DC, Blenis J (2004) Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 23:3151–3171.
15. Saitoh M, et al. (2002) Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. *J Biol Chem* 277:20104–20112.
16. Schalm SS, Blenis J (2002) Identification of a conserved motif required for mTOR signaling. *Curr Biol* 12:632–639.
17. Schalm SS, Tee AR, Blenis J (2005) Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation. *J Biol Chem* 280:11101–11106.
18. Asnaghi L, Bruno P, Priulla M, Nicolin A (2004) mTOR: A protein kinase switching between life and death. *Pharmacol Res* 50:545–549.
19. Peterson RT, Desai BN, Hardwick JS, Schreiber SL (1999) Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci USA* 96:4438–4442.
20. Fiol CJ, Wang A, Roeske RW, Roach PJ (1990) Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. *J Biol Chem* 265:6061–6065.
21. Hardt SE, Sadoshima J (2002) Glycogen synthase kinase-3beta: A novel regulator of cardiac hypertrophy and development. *Circ Res* 90:1055–1063.
22. Eldar-Finkelman H (2002) Glycogen synthase kinase 3: An emerging therapeutic target. *Trends Mol Med* 8:126–132.
23. Doble BW, Woodgett JR (2003) GSK-3: Tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116:1175–1186.
24. Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A (2009) Glycogen synthase kinase 3: More than a namesake. *Br J Pharmacol* 156:885–898.
25. Patel S, Woodgett J (2008) Glycogen synthase kinase-3 and cancer: Good cop, bad cop? *Cancer Cell* 14:351–353.
26. Lee J, Kim MS (2007) The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* 77(suppl 1):S49–S57.
27. Barone BB, et al. (2008) Long-term all-cause mortality in cancer patients with preexisting diabetes mellitus: A systematic review and meta-analysis. *JAMA* 300: 2754–2764.
28. Wolf I, Sadetzki S, Catane R, Karasik A, Kaufman B (2005) Diabetes mellitus and breast cancer. *Lancet Oncol* 6:103–111.
29. Manoukian AS, Woodgett JR (2002) Role of glycogen synthase kinase-3 in cancer: Regulation by Wnts and other signaling pathways. *Adv Cancer Res* 84:203–229.
30. Miyashita K, et al. (2009) An emerging strategy for cancer treatment targeting aberrant glycogen synthase kinase 3 beta. *Anticancer Agents Med Chem* 9:1114–1122.
31. Luo J (2009) Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett* 273:194–200.
32. Ougolkov AV, Billadeau DD (2006) Targeting GSK-3: A promising approach for cancer therapy? *Future Oncol* 2:91–100.
33. Hoeflich KP, et al. (2000) Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406:86–90.
34. Foltz DR, Santiago MC, Berechid BE, Nye JS (2002) Glycogen synthase kinase-3beta modulates notch signaling and stability. *Curr Biol* 12:1006–1011.
35. Qu L, et al. (2004) Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. *Genes Dev* 18:261–277.
36. Rocques N, et al. (2007) GSK-3-mediated phosphorylation enhances Maf-transforming activity. *Mol Cell* 28:584–597.
37. Wang Z, et al. (2010) GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. *Cancer Cell* 17:597–608.
38. Nakamura JL, Garcia E, Pieper RO (2008) S6K1 plays a key role in glial transformation. *Cancer Res* 68:6516–6523.
39. Akar U, et al. (2010) Targeting p70S6K prevented lung metastasis in a breast cancer xenograft model. *Mol Cancer Ther* 9:1180–1187.
40. Bärlund M, et al. (2000) Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. *J Natl Cancer Inst* 92:1252–1259.
41. Klos KS, et al. (2006) ErbB2 increases vascular endothelial growth factor protein synthesis via activation of mammalian target of rapamycin/p70S6K leading to increased angiogenesis and spontaneous metastasis of human breast cancer cells. *Cancer Res* 66:2028–2037.
42. Inoki K, et al. (2006) TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 126:955–968.
43. Kim GP, Billadeau DD (2008) GSK-3β inhibition in pancreatic cancer. *Pancreatic Cancer*, ed Pollock RE (Springer, Boston), pp 635–646.
44. Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD (2005) Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. *Cancer Res* 65:2076–2081.
45. Shakoori A, et al. (2005) Deregulated GSK3beta activity in colorectal cancer: Its association with tumor cell survival and proliferation. *Biochem Biophys Res Commun* 334:1365–1373.
46. Martin KA, Schalm SS, Romanelli A, Keon KL, Blenis J (2001) Ribosomal S6 kinase 2 inhibition by a potent C-terminal repressor domain is relieved by mitogen-activated protein-extracellular signal-regulated kinase kinase-regulated phosphorylation. *J Biol Chem* 276:7892–7898.
47. Gao D, et al. (2010) Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction. *Mol Cell* 39:797–808.