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Article

# Oncogenic MAPK Signaling Stimulates mTORC1 Activity by Promoting RSK-Mediated Raptor Phosphorylation

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## Summary

**Background:** The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that controls cell growth in response to mitogens, as well as amino acid and energy sufficiency. The scaffolding protein Raptor binds to mTOR and recruits substrates to the rapamycin-sensitive mTOR complex 1 (mTORC1). Although Raptor has been shown to be essential for mTORC1 activity, the mechanisms regulating Raptor function remain unknown.

**Results:** Here, we demonstrate that Raptor becomes highly phosphorylated on R*X*R*XX*pS/T consensus motifs after activation of the Ras/mitogen-activated protein kinase (MAPK) pathway. Using pharmacological inhibitors and RNA interference, we show that the p90 ribosomal S6 kinases (RSKs) 1 and 2 are required for Raptor phosphorylation in vivo and directly phosphorylate Raptor in vitro. Quantitative mass spectrometry and site-directed mutagenesis revealed that RSK specifically phosphorylates Raptor within an evolutionarily conserved region with no previously known function. Interestingly, expression of oncogenic forms of Ras and MEK that elevate mTORC1 activity induced strong and constitutive phosphorylation of Raptor on these residues. Importantly, we demonstrate that expression of Raptor mutants lacking RSK-dependent phosphorylation sites markedly reduced mTOR phosphotransferase activity, indicating that RSK-mediated phosphorylation of Raptor is important for mTORC1 activation by the Ras/MAPK pathway.

**Conclusions:** We propose a unique mode of mTOR regulation in which RSK-mediated phosphorylation of Raptor regulates mTORC1 activity and thus suggest a means by which the Ras/MAPK pathway might promote rapamycin-sensitive signaling independently of the PI3K/Akt pathway.

## Introduction

The mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase that integrates signals from nutrients, energy sufficiency, and growth factors to regulate mammalian cell growth as well as organ and body size in a variety of organisms [1, 2]. mTOR was discovered as the molecular target of rapamycin, an antifungal agent used clinically as an immunosuppressant and, more recently, as an anti-cancer drug [3]. Emerging evidence indicates that deregulation of the mTOR pathway occurs in many types of cancer [4, 5], underscoring the importance of understanding how this pathway is regulated by oncogenic and mitogenic cues.

mTOR forms two distinct multiprotein complexes, the rapamycin-sensitive and -insensitive mTOR complexes (mTORCs) 1 and 2, respectively [6]. mTORC1 consists of the mTOR catalytic subunit and three associated proteins, Raptor, mLST8, and PRAS40. Activated mTORC1 phosphorylates two main regulators of mRNA translation and ribosome biogenesis, S6K1 and 4E-BP1, and thus stimulates protein synthesis [7, 8]. The molecular functions of most mTOR-associated proteins are not understood, but Raptor has been proposed to work as a scaffolding protein that recruits mTOR substrates through their TOR signaling (TOS) motif [9, 10]. Inhibition of Raptor expression with RNA interference (RNAi) decreases mTORC1 activity and reduces cell size, indicating that Raptor is an essential mediator of mTORC1 action in vivo and plays a crucial role in mTOR activation [11–13]. However, the molecular mechanisms by which Raptor regulates mTORC1 activity and how Raptor function is modulated by growth factors and oncogenes remain unknown.

The mechanisms underlying mTORC1 regulation are not fully understood, but the key upstream player appears to be the small GTPase Rheb, which is negatively regulated by the dimeric TSC1-TSC2 GTPase-activating protein (GAP) complex [14]. It is now appreciated that several pathways in addition to the insulin-stimulated PI3K/Akt pathway signal to mTORC1 by modulating TSC1-TSC2 activity toward Rheb. For example, energy deprivation sensed by AMPK [15] and hypoxia [16] regulate TSC1-TSC2 activity and Rheb GTP loading. Moreover, the Ras/MAPK signaling pathway positively regulates mTORC1 activity through extracellular signal-regulated kinase (ERK)- and p90 ribosomal S6 kinase (RSK)-mediated phosphorylation and inactivation of TSC2 [17–21].

Herein, we describe a novel link between the Ras/MAPK pathway and mTORC1 signaling. We demonstrate that Raptor is phosphorylated mainly on three serine residues by the RSK1 and RSK2 protein kinases in vitro and in vivo. We find that RSK-mediated phosphorylation of Raptor positively regulates mTORC1 kinase activity stimulated by the Ras/MAPK pathway. Our findings suggest a unique mode of mTOR regulation by which Raptor acts as a molecular sensor that integrates growth signals and promotes mTORC1 signaling.

## Results

### An Approach Involving a Phospho-Motif Antibody Identifies Raptor as a Substrate of Ras/MAPK Signaling

We analyzed Raptor phosphorylation by using an antibody that recognizes the phosphorylated consensus motif Arg/Lys-*X*-Arg/Lys-*X*-*X*-pSer/Thr (R*X*R*XX*pS/T, where *X* is any amino acid), which is found in substrates of many AGC family kinases, including RSK [20], Akt, and S6K1 [22]. HEK293 cells transfected with Myc-tagged Raptor were stimulated with different mitogens and growth factors, and immunoprecipitated Raptor

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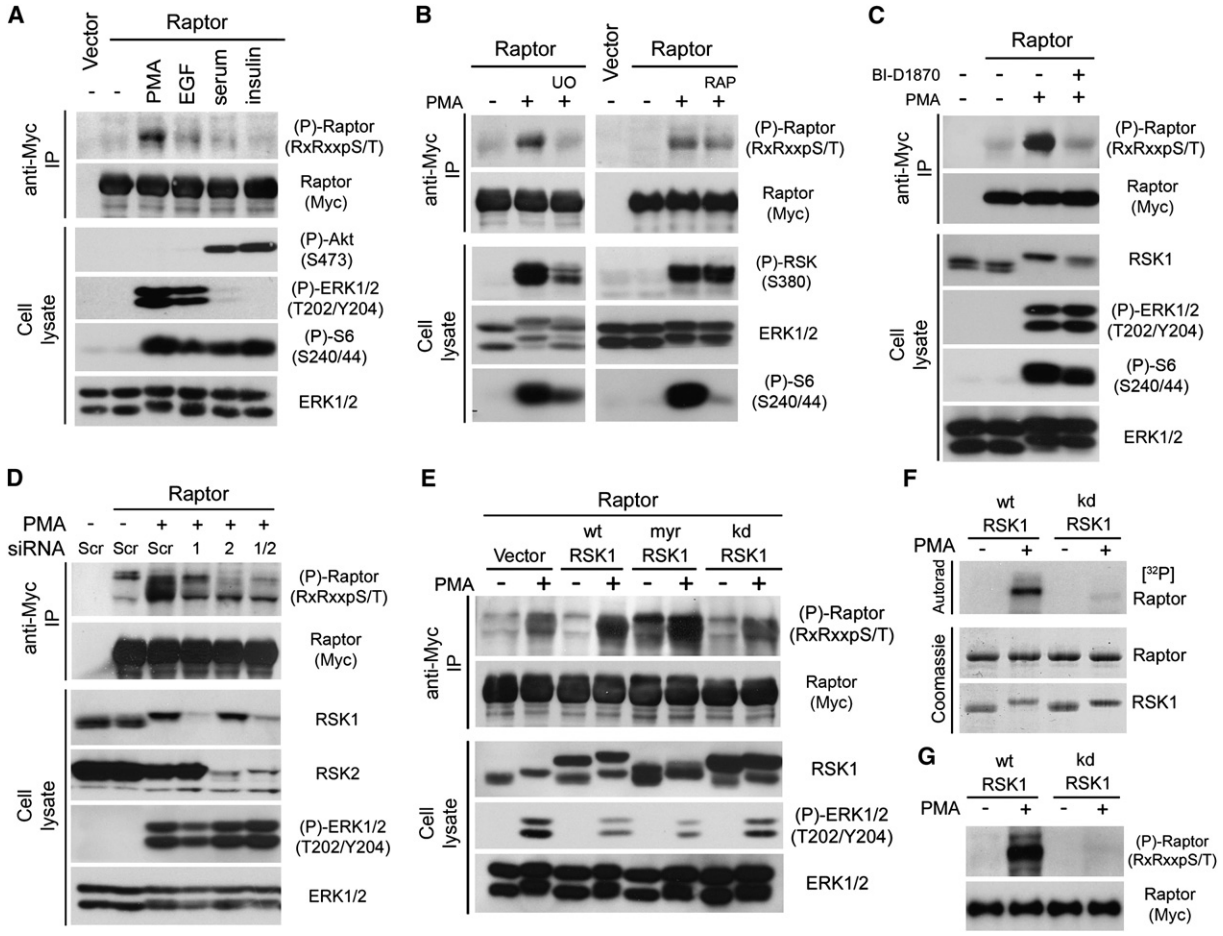


Figure 1. RSK Phosphorylates Raptor In Vitro and in Cells Stimulated with Agonists of the Ras/MAPK Pathway

(A) HEK293 cells were transfected with empty vector or Myc-tagged Raptor, serum-starved, and stimulated for 10 or 20 min with agonists of the Ras/MAPK and PI3K/Akt signaling pathways. Immunoprecipitated Raptor was then assayed for phosphorylation with a phospho-motif antibody that recognizes the R*X*R*XX*pS/T consensus motif.

(B and C) As in (A), but cells were pretreated with U0126 (10 μM), rapamycin (100 nM), or BI-D1870 (10 μM) for 30 min prior to PMA (50 ng/ml) stimulation.

(D) HEK293 cells were cotransfected with control vector or Myc-tagged Raptor, and siRNA duplexes were targeted against a scrambled sequence (Scr), RSK1 or RSK2, serum-starved, and stimulated with PMA (50 ng/ml). Immunoprecipitated Raptor was then assayed as in (A).

(E) HEK293 cells were cotransfected with Raptor and wild-type RSK1, constitutively activated RSK1 (myr), or kinase-inactive RSK1 (kd), serum starved, and stimulated with PMA (50 ng/ml). Immunoprecipitated Raptor was then assayed as in (A).

(F) Immunoprecipitated HA-tagged wild-type or kinase-dead RSK1 (kd) from PMA-stimulated cells was incubated with immunopurified Raptor in a kinase reaction with $\gamma[^{32}P]$ATP. The resulting samples were subjected to SDS-PAGE, and the dried Coomassie-stained gel was autoradiographed.

(G) Samples were treated as in (F), but kinase reactions were performed without radioactivity. The resulting samples were immunoblotted for Raptor phosphorylation on R*X*R*XX*pS/T consensus sites.

was analyzed for phosphorylation via immunoblotting with the R*X*R*XX*pS/T phospho-motif antibody. Using this method, we found that potent Ras/MAPK-pathway agonists, such as the phorbol ester PMA and epidermal growth factor (EGF), robustly stimulated Raptor phosphorylation at R*X*R*XX*pS/T consensus sites in HEK293 cells (Figure 1A), as well as in HeLa and NIH 3T3 cells (data not shown). This antibody specifically recognized phosphorylated Raptor, as demonstrated by the fact that phosphatase treatment of immunoprecipitated Raptor resulted in a complete loss of immunoreactivity (Figure S1A in the Supplemental Data). Because EGF also weakly stimulates the PI3K/Akt pathway, we tested serum and insulin, which are potent stimulators of Akt phosphorylation at Ser473, and found that these agonists did not promote Raptor phosphorylation (Figure 1A). To further assess the role of PI3K signaling in Raptor phosphorylation, we treated cells with increasing concentrations of insulin or transfected them with a constitutively activated form of Akt (myristoylated Akt). As shown in Figure S1B, whereas PMA strongly stimulated the Ras/MAPK pathway and Raptor phosphorylation, no phosphorylation was detected under conditions in which Akt phosphorylation was potently stimulated. Together, these data strongly implicate Raptor as a phosphorylation target of the Ras/MAPK pathway.

### RSK1 and RSK2 Phosphorylate Raptor In Vivo and In Vitro

We next wanted to identify the kinase(s) responsible for Raptor phosphorylation on R*X*R*XX*pS/T consensus motifs after activation of the Ras/MAPK pathway. Cells were pretreated with the inhibitor U0126 to block MEK 1 and 2 downstream signaling. Interestingly, Raptor phosphorylation was almost completely abrogated by U0126 treatment (Figure 1B), suggesting that a kinase located downstream of MEK 1 and 2 is involved in Raptor phosphorylation. RSK and S6K are two kinases stimulated by MEK 1 and 2 signaling that are capable of phosphorylating

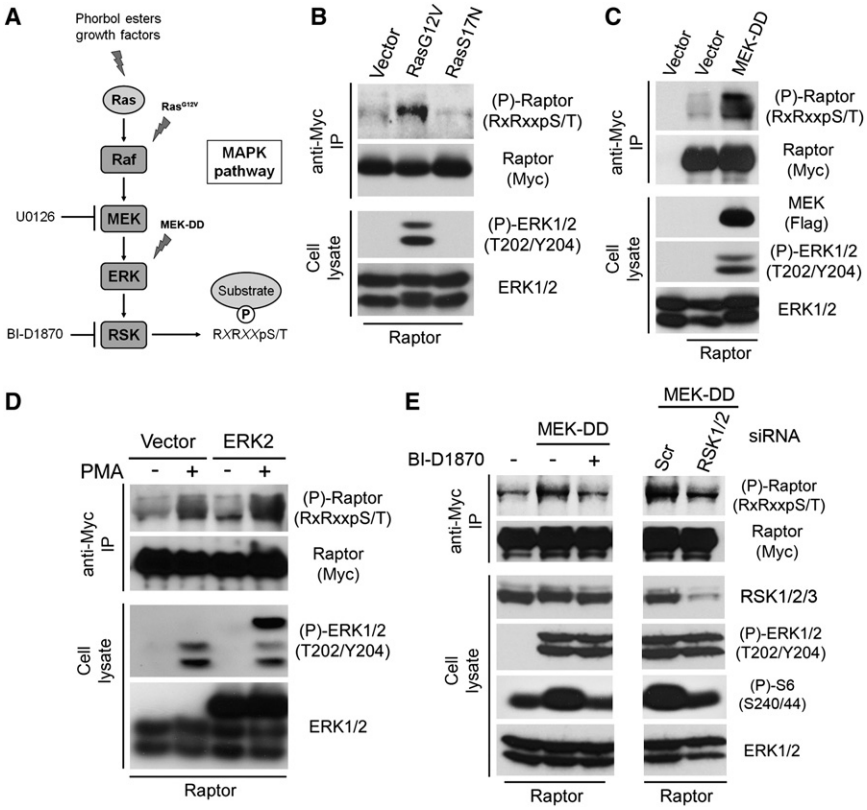


Figure 2. Oncogenic Activation of the Ras/MAPK Pathway Promotes Constitutive Raptor Phosphorylation

(A) Model depicting the Ras/MAPK pathway and inhibitors and activators used in this study.

(B and C) HEK293 cells were cotransfected with Raptor and constructs expressing activated Ras (G12V), dominant-negative Ras (S17N), constitutively activated MEK1 (MEK-DD), or control vector and serum starved. Immunoprecipitated Raptor from unstimulated cells was then assayed for phosphorylation with the R*X*R*XX*pS/T phospho-motif antibody.

(D) HEK293 cells were cotransfected with Raptor and wild-type ERK2, serum starved, stimulated with PMA, and analyzed as in (B).

(E) HEK293 cells were either cotransfected with Raptor and MEK-DD, serum starved, and treated with BI-D1870 for 60 min (left panel) or cotransfected with control siRNA (Scr) or siRNAs targeted against RSK1 and RSK2 and serum starved (right panel). Immunoprecipitated Raptor was then assayed for phosphorylation as in (B).

Raptor at R*X*R*XX*pS/T consensus motifs. To test their implication in Raptor phosphorylation, we pretreated cells with the mTORC1 inhibitor rapamycin to inhibit mTOR-mediated S6K activation. Although rapamycin treatment efficiently inhibited S6K-mediated S6 phosphorylation at Ser240/44 (Figure 1B), Raptor phosphorylation remained unaltered. Consistent with a role for RSK, we found that treatment of cells with BI-D1870, a recently identified pan-RSK inhibitor [23], completely abolished Raptor phosphorylation upon PMA (Figure 1C) and EGF treatments (data not shown). The RSK1–4 ATP antagonist has recently been tested against a panel of more than 60 protein kinases and, remarkably, it was found to be very selective for RSK family members when it was used on cells in the low μM range [24].

To confirm the direct involvement of RSK in Raptor phosphorylation, we used small interfering RNA (siRNA)-mediated RNAi to specifically reduce expression of RSK1 and RSK2, the two predominantly expressed isoforms of RSK in HEK293 cells [25]. Importantly, siRNA-mediated knockdown of RSK1 and RSK2 resulted in complete inhibition of Raptor phosphorylation (Figure 1D), demonstrating that endogenous RSK1 and RSK2 mediate Raptor phosphorylation in cells. To further assess the role of RSK, we cotransfected HEK293 cells with Raptor and different RSK mutants. Compared to the control, expression of wild-type RSK1 (Figure 1E), as well as RSK2, RSK3, and RSK4 (Figure S2A), specifically increased Raptor phosphorylation after PMA stimulation, suggesting that Raptor is a common RSK family substrate. Moreover, expression of a constitutively activated form of RSK1 (myristoylated RSK1) induced Raptor phosphorylation even in the absence of serum and PMA stimulation (Figure 1E), consistent with the idea that RSK is required and sufficient to stimulate Raptor phosphorylation in cells. RSK1 phosphotransferase activity was found to be required for stimulating Raptor phosphorylation: A RSK1 mutant with inactivating mutations in both kinase domains (K112/464R) [18] did not increase Raptor phosphorylation over the level already stimulated by endogenous RSK activity (Figure 1E).

To verify that RSK could directly phosphorylate Raptor, we performed in vitro kinase assays with purified proteins. HEK293 cells were transiently transfected with wild-type or kinase-inactive HA-tagged RSK1, and purified RSK1 from unstimulated or PMA-treated cells was incubated with full-length Raptor immunopurified from serum-deprived cells. Although no incorporation of [$^{32}$P] label was seen in purified Raptor incubated with unstimulated RSK1 or the kinase-inactive RSK1, we found that activated RSK1 robustly increased [$^{32}$P] incorporation in purified Raptor (Figure 1F). In a similar experiment performed without radioactivity, we found that wild-type, but not kinase-inactive, RSK1 robustly stimulated the phosphorylation of purified Raptor at R*X*R*XX*pS/T consensus sites (Figure 1G). These experiments were also performed with recombinant GST-Raptor as a substrate, and consistent with aforementioned data, it was found to be an excellent in vitro substrate for RSK (Figure S1C). Together, our data indicate that RSK directly promotes Raptor phosphorylation in vivo and in vitro at R*X*R*XX*pS/T consensus sites.

### Constitutive Phosphorylation of Raptor in Cells with Oncogenic Activation of the Ras/MAPK Signaling Cascade

Ras proteins are often mutated in human cancers such that they constitutively activate growth signaling cascades, including the MAPK, PI3K and mTOR pathways [26]. To determine whether oncogenic forms of Ras and its downstream effector kinase MEK stimulate Raptor phosphorylation, we transfected cells with active (G12V) and inactive (S17N) mutants of H-Ras or a constitutively activated form of MEK1 (MEK1-DD) (Figure 2A). Interestingly, we found that transient (Figures 2B and 2C) or stable (data not shown) expression of either RasG12V or MEK1-DD strongly stimulated Raptor phosphorylation in the absence of serum or growth factors. Additionally,

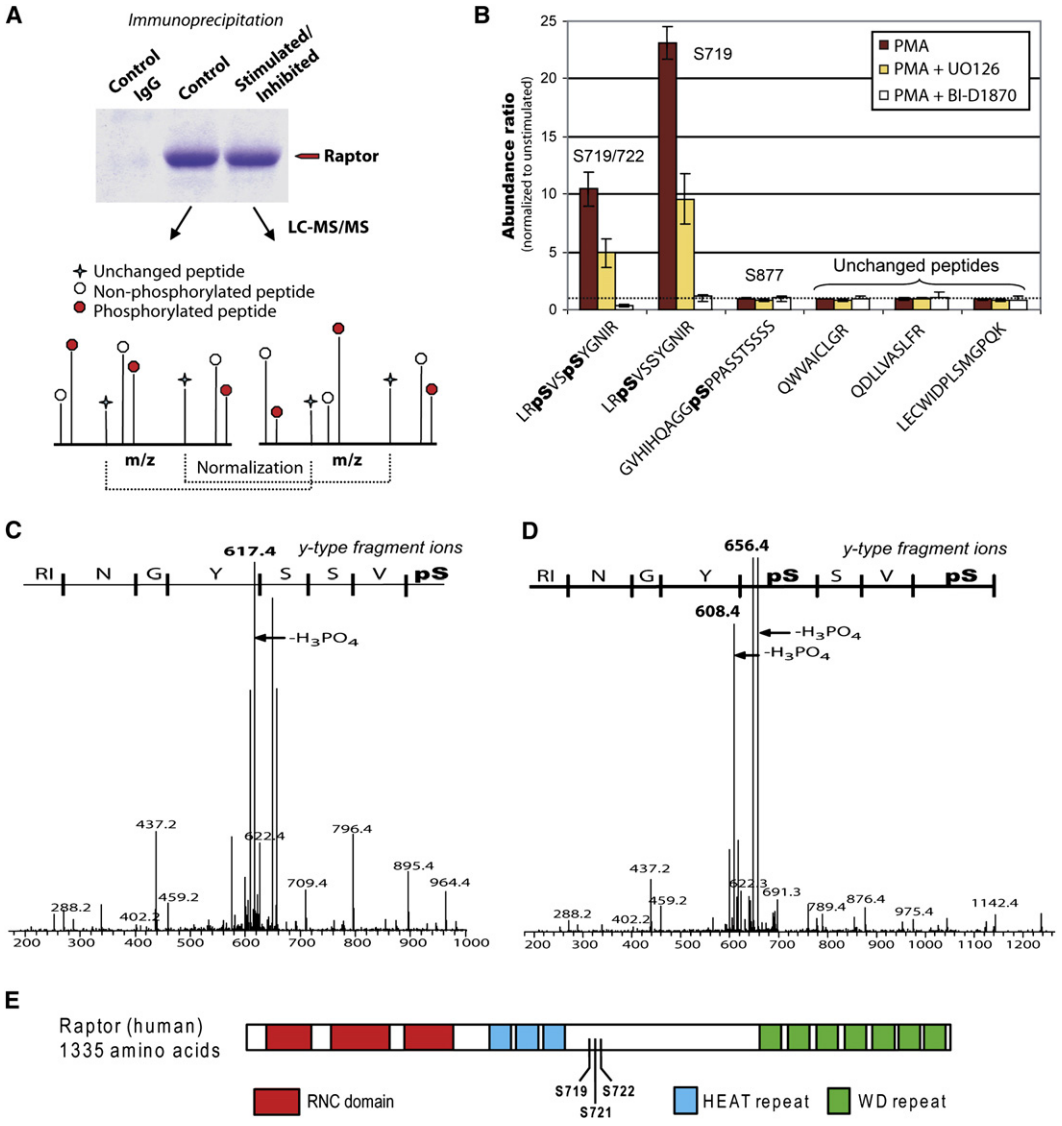


Figure 3. Quantitative Mass Spectrometry Identifies and Profiles the Phosphorylation of Raptor upon Stimulation of the Ras/MAPK Pathway

(A) Schematic representation of ion mapping and intensity normalization for determining changes in phosphorylation upon PMA stimulation and kinase inhibitor treatment.

(B) Abundance ratios of different modified and invariant Raptor tryptic peptides normalized to unstimulated cells. A dotted line indicates no changes in abundance ratio. Results from three independent replicates are represented in a histogram as the means ± standard error.

(C and D) MS/MS spectra of doubly protonated precursor at m/z 666.3 (C) and m/z 706.3 (D) corresponding to tryptic peptides LRpSVSSYGNIR and LRpSVSpSYGNIR, respectively. Fragment ions indicated correspond to y-type cleavages.

(E) Schematic representation of the identified phosphorylation sites within Raptor.

overexpression of wild-type ERK2 increased Raptor phosphorylation upon PMA stimulation (Figure 2D), demonstrating that oncogenic and mitogenic stimulations of the Ras/MAPK pathway promote Raptor phosphorylation. Importantly, we show that activated MEK-induced Raptor phosphorylation was drastically inhibited by both the BI-D1870 inhibitor and RSK 1 and 2 RNAi (Figure 2E), demonstrating the requirement for RSK in Raptor phosphorylation induced by oncogenic Ras/MAPK signaling.

### Ser719, Ser721, and Ser722 Are the Predominant RSK-Dependent Phosphorylation Sites in Raptor

To identify R*X*R*XX*pS/T phosphorylation sites in Raptor, we performed quantitative mass spectrometry (MS) analyses on immunoprecipitated Myc-tagged Raptor derived from unstimulated and PMA-stimulated cells. We performed combined ion profiling and MS/MS peptide sequencing analyses to correlate abundance changes of identified phosphopeptides across the different experimental paradigms (Figure 3A). Using this approach, we identified two phosphopeptides corresponding to phosphorylated Ser719 and Ser722, showing more than a 10-fold change in phosphorylation upon PMA stimulation (Figure 3B). In contrast to these findings, another phosphopeptide (Ser877) identified in this study showed no statistically significant change in phosphorylation upon PMA stimulation. Importantly, treatment of cells with either U0126 or BI-D1870 significantly decreased the relative abundance of the singly and doubly phosphorylated peptides (Figure 3B), thereby

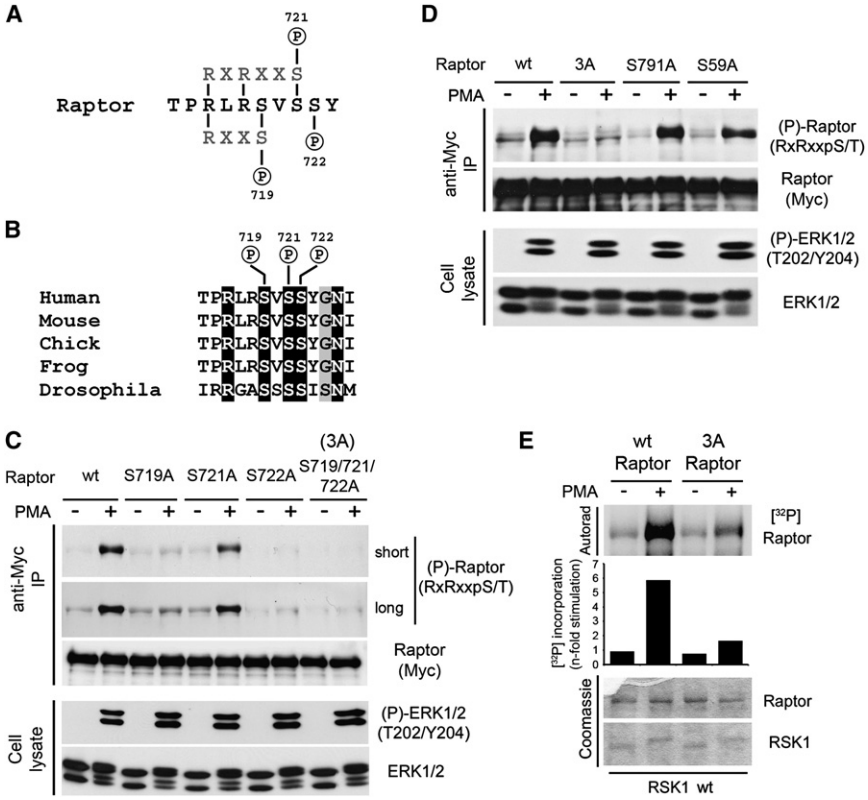


Figure 4. Ser719, Ser721, and Ser722 are the Primary RSK-Dependent Phosphorylation Sites in Raptor

(A) Schematic representation of the consensus motifs encompassing residues Ser719, Ser721, and Ser722 of Raptor.

(B) Primary sequence alignment showing conservation of the three RSK-dependent phosphorylation sites within different Raptor orthologs.

(C) HEK293 cells were transfected with wild-type Raptor or potential RSK-phosphorylation-site mutants S719A, S721A, S722A, and the triple mutant S719/721/722A (3A), serum starved, and stimulated with PMA for 20 min. Immunoprecipitated Raptor was then immunoblotted for phosphorylation at R*X*R*XX*pS/T sites.

(D) As in (C), except cells were transfected with wild-type, 3A, or potential RSK-phosphorylation-site mutants (S59A and S791A) that were not identified by MS analysis. Raptor immunoprecipitates were then immunoblotted for phosphorylation at R*X*R*XX*pS/T sites.

(E) As in Figure 2D, except that HA-tagged wild-type RSK1 immunoprecipitates were incubated with either wild-type Raptor or the 3A mutant in an in vitro kinase reaction.

confirming that phosphorylation of Ser719 and Ser722 requires MEK- and RSK-dependent signaling. Identification of Ser719 and Ser722 as phosphorylation sites was obtained by MS/MS sequencing, and the assignment is shown for these residues in the corresponding tryptic peptides (Figures 3C and 3D). Importantly, similar findings were obtained when endogenous Raptor was immunoprecipitated from cells and analyzed via this method (data not shown). Ser719 and Ser722 are located close to the HEAT repeats of Raptor (Figure 3E), suggesting that this region integrates upstream signals that may regulate Raptor function.

To determine whether RSK targets Ser719 and Ser722 for phosphorylation, we mutated these sites to unphosphorylatable alanine residues (S719A and S722A). Although it was not reproducibly identified by MS, we also mutated nearby Ser721 because this residue lies within an optimal RSK consensus sequence (Figure 4A). Interestingly, all three serine residues are conserved in organisms from *Drosophila* to humans (Figure 4B), suggesting that they play an important and evolutionarily conserved function. Although mutation of Ser721 only slightly but reproducibly reduced Raptor phosphorylation, we found that mutation of either Ser719 or Ser722 drastically decreased the phosphorylation of Raptor after PMA stimulation (Figure 4C). Importantly, when combined into a single Raptor mutant (S719/721/722A, from here on termed 3A), mutation of all three serine residues completely inhibited Raptor phosphorylation upon stimulation of the Ras/MAPK pathway. We also analyzed the potential involvement of additional putative R*X*R*XX*pS/T phosphorylation sites in Raptor. Importantly, mutagenesis of Ser59 and Ser791 did not reduce Raptor phosphorylation after PMA stimulation (Figure 4D), indicating that RSK does not phosphorylate these residues in cells. The specificity of RSK toward Ser719, Ser721, and Ser722 was also confirmed in vitro. Mutation of all three residues almost completely inhibited RSK-mediated [$^{32}$P] incorporation in immunopurified full-length Raptor (Figure 4E) and in recombinant GST-Raptor (Figure S1C), confirming that RSK predominantly phosphorylates Raptor at these residues both in vitro and in vivo.

### Raptor Phosphorylation Regulates mTORC1 Activity

To assess whether Raptor phosphorylation regulates its association with mTOR or the recruitment of mTOR substrates, we transfected cells with wild-type or 3A Raptor and assessed association of mTOR, S6K1, and 4E-BP1 after immunoprecipitation. Interestingly, we found that the Raptor 3A mutant bound as efficiently as wild-type Raptor to 4E-BP1 (Figure 5A) and S6K1 (Figure S3), indicating that Raptor phosphorylation does not regulate TOS-motif-mediated binding. To determine whether Raptor phosphorylation affected its interaction with mTOR, we transfected cells with mTOR and wild-type or 3A Raptor and assessed the presence of mTOR within Raptor immunoprecipitates by immunoblotting. As shown in Figure 5B, we found that the Raptor 3A mutant bound as efficiently as wild-type Raptor to mTOR, indicating that phosphorylation of these residues does not regulate Raptor-mTOR interactions.

Next, we determined whether Raptor phosphorylation regulates mTORC1 activity by using an in vitro mTOR phosphotransferase activity assay and recombinant GST-4E-BP1 as a substrate. Our assay specifically monitored mTORC1 activity given that 4E-BP1 phosphorylation was exclusively detected within Raptor immunoprecipitates (Figure 5C), was sensitive to rapamycin treatment (Figure S4A), and required a functional mTOR kinase domain (Figure S4B). Under these conditions, activation of the Ras/MAPK pathway with PMA robustly stimulated mTORC1 activity (Figure 5D). Importantly, we found that mTOR kinase activity associated with the Raptor 3A mutant was dramatically lower than that associated with wild-type Raptor, suggesting that Raptor phosphorylation by RSK

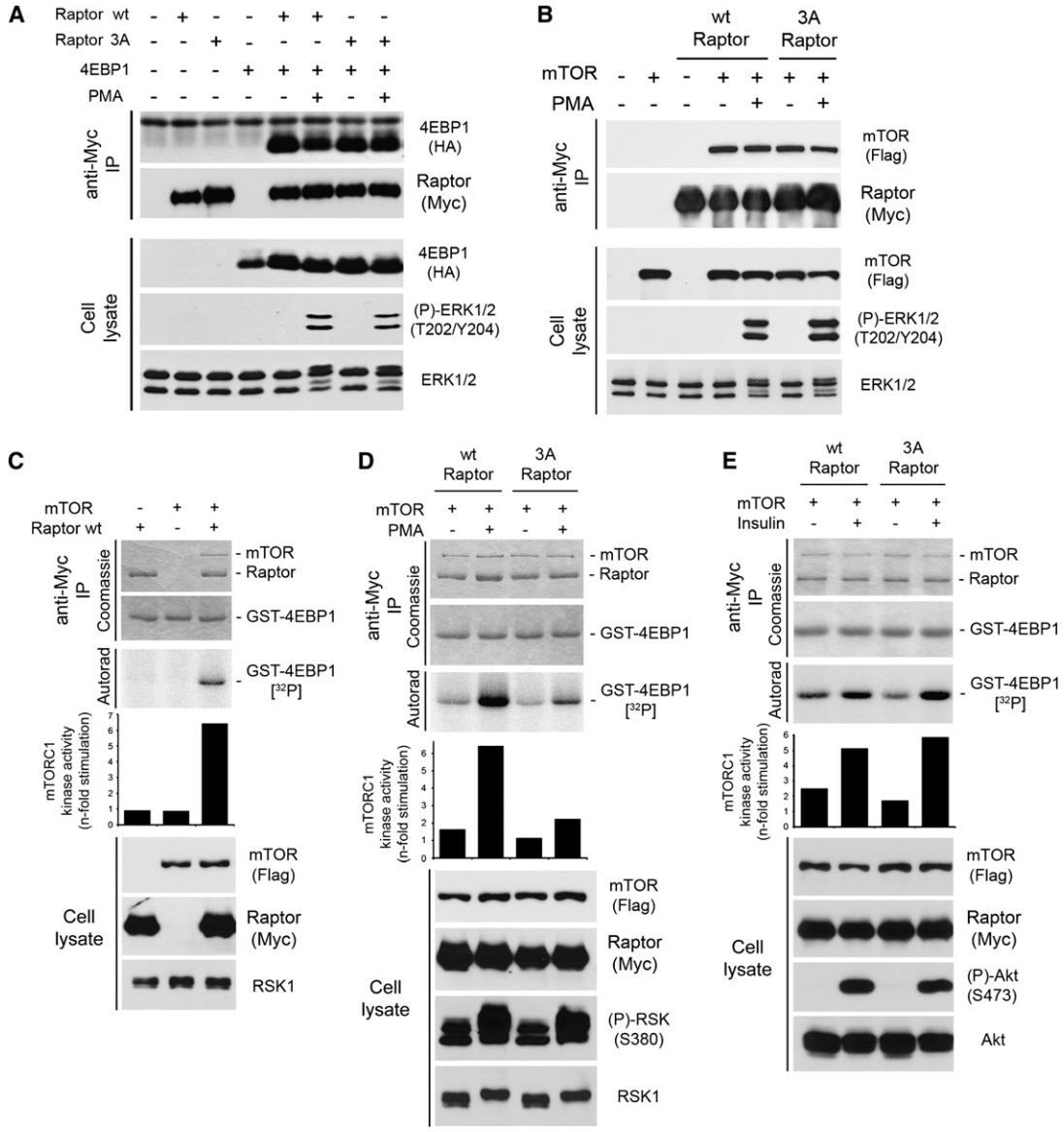


Figure 5. RSK-Mediated Phosphorylation of Raptor Promotes mTORC1 Activity without Altering Raptor Interaction with mTOR or Its Substrates

(A) Cells were cotransfected with HA-tagged 4E-BP1 and either wild-type Raptor or the 3A mutant, serum starved, and stimulated with PMA. Associated 4E-BP1 was assayed within Raptor immunoprecipitates by immunoblotting.

(B) Cells were cotransfected with Flag-tagged mTOR and either wild-type Raptor or the 3A mutant, serum starved, and stimulated with PMA. Associated mTOR was assayed within Raptor immunoprecipitates.

(C) Cells were cotransfected with mTOR and Raptor and serum starved, and mTORC1 kinase activity was assayed within Raptor immunoprecipitates, with GST-4EBP1 as a substrate. The kinase reaction was performed in the presence of $\gamma[^{32}P]$ATP, the resulting samples were subjected to SDS-PAGE, and the dried Coomassie-stained gel was autoradiographed.

(D) Cells were cotransfected with mTOR and wild-type Raptor or the 3A mutant, serum starved, and stimulated with PMA. mTORC1 activity was assayed as described in (A).

(E) As in (D), except that cells were stimulated with insulin (100 nM) for 20 min.

positively stimulates mTORC1 activity. Because we found that insulin did not stimulate Raptor phosphorylation, we were interested in determining whether insulin-stimulated mTORC1 activity was affected by mutation of Raptor. Interestingly, we found that insulin stimulation led to comparable activation of mTORC1 in cells expressing wild-type or 3A Raptor (Figure 5E). The role of RSK in Ras/MAPK-dependent activation of mTORC1 was confirmed by the finding that overexpression of all RSK isoforms increases the phosphorylation of S6K and 4E-BP1 on Thr389 and on Thr37 and Thr46, respectively, sites known to be exclusively phosphorylated by activated mTOR (Figure S2A). Consistent with a positive role for RSK in mTORC1 activation, we found that BI-D1870 treatment decreased PMA-induced activation of mTORC1, as reflected by a reduction in phosphorylation of S6K and 4E-BP1 (Figure S2B), as well as S6 on Ser240/244 (Figures 1C snf 2E; Figure S2B), residues that are exclusively phosphorylated by activated S6K, as previously reported [25].

These data support a novel and specific role for the Ras/MAPK pathway in mTORC1 activation; in this role,

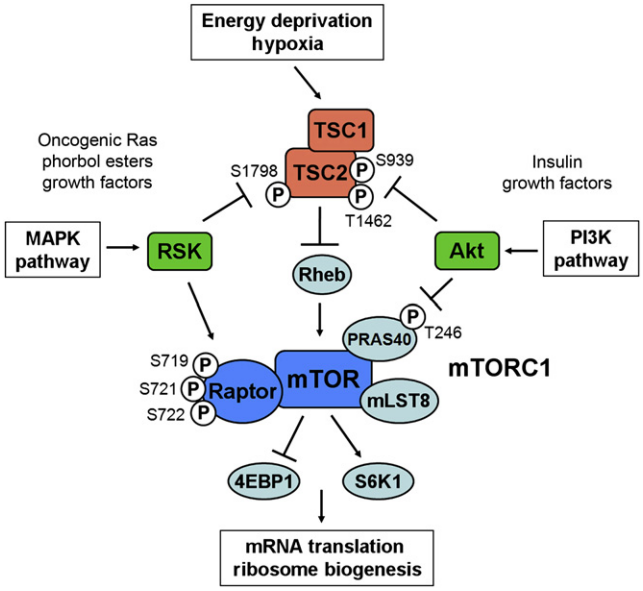


Figure 6. The Ras/MAPK and PI3K/Akt Pathways Regulate mTORC1 Activation by a Twofold Mechanism

Although Akt and RSK inactivate TSC2 and thereby promote Rheb GTP-loading, both kinases also directly regulate mTORC1 via alternative mechanisms. Whereas Akt phosphorylates PRAS40 to suppress its inhibitory function, RSK directly phosphorylates Raptor to promote mTORC1 activity.

RSK-mediated phosphorylation of Raptor promotes mTORC1 activity independently of the PI3K/Akt pathway (Figure 6).

## Discussion

Our findings demonstrate that the Ras/MAPK pathway regulates mTOR signaling via a twofold mechanism. Although previous studies indicated that both ERK and RSK stimulate Rheb GTP-loading through the inactivation of TSC2 [17–19], we show herein that RSK also directly targets the mTORC1 complex by phosphorylating Raptor and thereby promoting mTORC1 kinase activity (Figure 6). RSK phosphorylates three evolutionarily conserved Raptor serine residues that lie within a region with no homology to known functional domains. Our findings suggest that this region might play a novel function in conferring the capacity to respond to upstream signaling. Whereas Ser721 lies within a classical RSK consensus sequence (R*X*R*XX*pS/T), Ser719 is located within a minimal phosphoacceptor sequence (R*XX*pS) that was found to be sufficient in other RSK substrates, such as DAPK, c-Fos, and CREB [20, 21]. Interestingly, Ser722 is located at the +1 position of the classical RSK consensus motif, suggesting that under some circumstances, RSK efficiently phosphorylates residues located just outside of its consensus. We have previously found that RSK promotes phosphorylation of TSC2 at both Ser1798 and Ser1799, and this represents another situation in which RSK specificity can extend to the +1 serine residue (P.P.R. and B. Ballif, unpublished observation) [18].

Whereas mutation of Raptor at RSK-dependent phosphorylation sites did not reduce its ability to interact with mTOR or TOS-motif-containing substrates (4E-BP1 and S6K1), our data indicate that these phosphorylation events strongly and positively regulate mTORC1 kinase activity. These findings suggest that Raptor phosphorylation modulates mTORC1 activity without altering the scaffolding function between mTOR and at least some of its substrates. Recent structural evidence indicates that the C-terminal region of Raptor's yeast analog (named KOG-1), including the HEAT and WD40 repeats, closely interact with the N-terminal portion of TOR [27]. The position of the RSK phosphorylation sites on Raptor suggests that they could lead the mTOR-Raptor complex to undergo a conformational change that would allow activation of the catalytic activity of mTOR. This could result from specific association or dissociation of regulatory proteins to or from the mTORC1 complex, leading to the direct regulation of mTOR activity. Another possibility is that Raptor phosphorylation could suppress the activity or interaction of mTORC1 with a negative regulator and therefore promote mTORC1 activity. Although many studies have investigated the upstream regulation of mTORC1, very few have reported the regulation of mTOR activity through the direct modification of mTORC1 constituents. The PI3K/Akt pathway was shown to directly regulate mTORC1 activity via the phosphorylation of PRAS40, a component of the mTORC1 complex [28, 29]. Although PRAS40 phosphorylation at Thr246 was not found to be regulated by Ras/MAPK signaling [30], we have also determined that PRAS40 binding to Raptor was unaltered by RSK-mediated phosphorylation (data not shown). It has recently been demonstrated that Raptor phosphorylation by AMPK negatively controls mTORC1 activity [31]. Together with the present study, these new findings suggest that, as for TSC2, Raptor appears to be a major signal integrator that interprets cell growth cues as well as energy sufficiency.

Our findings also highlight the interrelation between the Ras/MAPK and mTOR pathways, both frequently deregulated in human cancers. Hyperactivation of mTOR has been described in several cancers harboring oncogenic activation of Ras [4, 5, 32], and mTORC1 activity has been shown to be essential for Ras-mediated proliferation and transformation [33]. Although the molecular mechanisms at the origin of mTOR hyperactivation in cancer cells remain to be fully elucidated, the constitutive Raptor phosphorylation observed in cells expressing oncogenic forms of Ras and MEK suggests that rapamycin may be of therapeutic value for the treatment of cancers with activating mutations in the Ras/MAPK pathway.

### Experimental Procedures

#### Cell Culture and Transfection

HEK293, HeLa, and NIH 3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. HEK293 and NIH 3T3 cells were transfected with calcium-phosphate as described previously [18]. HeLa cells were transfected with Fugene (Roche) according to the manufacturer's instructions. Cells were grown for 24 hr after transfection and starved of serum where indicated for 16–18 hr. Starved cells were pretreated with wortmannin (100 nM), U0126 (10 μM), rapamycin (100 nM) (Biomol, Plymouth Meeting, PA), or BI-D1870 (10 μM) [23] and stimulated with either FBS (10%), insulin (100 nM), epidermal growth factor (EGF; 50 ng/ml) (Invitrogen), or phorbol 13-myristate 12-acetate (PMA; 25-100 ng/ml)(Biomol) before being harvested.

#### Immunoprecipitations and Immunoblotting

Cell lysates were prepared as described previously [18]. For mTOR immunoprecipitations, cells were harvested in CHAPS lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 0.3% CHAPS). Immunoprecipitations were carried out with the indicated antibody for 2 hr followed by 1 hr incubation with Protein A-Sepharose CL-4B beads (GE Healthcare). Immunoprecipitates were washed thrice in lysis buffer and, along with total cell lysates, were subjected to SDS-PAGE and electroblotted onto nitrocellulose. The data presented are representative of at least three independent experiments.

### In Vitro Kinase Reactions

RSK in vitro kinase assays were performed as previously described [18] with immunopurified Myc-Raptor (2 μg per assay) or bacterially purified recombinant GST-Raptor (2 μg per assay). For mTOR kinase assays, beads from immunoprecipitations were washed thrice in CHAPS lysis buffer and then thrice in CHAPS buffer supplemented with 150 mM NaCl. Assays were performed for 30 min at 30°C with bacterially purified recombinant GST-4EBP1 (2 μg per assay) and 10 μCi γ[$^{32}$P]-ATP in mTOR kinase buffer (25 mM HEPES [ph 7.4], 50 mM NaCl, 50 mM β-glycerophosphate, 10 mM $MnCl_2$, 100 μM cold ATP). All samples were subjected to SDS-PAGE, and incorporation of cold or radioactive phosphate [$^{32}$P] was determined by immunoblotting or quantified by use of a Fuji PhosphorImager with ImageQuant software. The data presented are representative of at least three independent experiments.

### RNA Interference

For the small interfering RNA (siRNA) studies, 21 nucleotide complementary RNA with symmetrical 2 nt overhangs were obtained from QIAGEN (Valencia, CA). The DNA sequences against which RNA duplexes were created for RSK1 and RSK2 and the scrambled control were described elsewhere [25, 34, 35]. For transfection of HEK293 cells, calcium-phosphate and 50 nM siRNA per dish were used. Transfection efficiency was determined to be greater than 90% by a fluorescently-labeled mock siRNA.

### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and are available with this article online at http://www.current-biology.com/cgi/content/full/18/17/1269/DC1/.

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