MTORC2 is a physiological hydrophobic motif kinase of S6 Kinase 1.

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Abstract

Ribosomal protein S6 kinase 1 (S6K1), a major downstream effector molecule of mTORC1, regulates cell growth and proliferation by modulating protein translation and ribosome biogenesis. We have recently identified eIF4E as an intermediate in transducing signals from mTORC1 to S6K1 and further demonstrated that the role of mTORC1 is restricted to inducing eIF4E phosphorylation and interaction with S6K1.This interaction relieves S6K1 auto-inhibition and facilitates its hydrophobic motif (HM) phosphorylation and activation as a consequence. These observations underscore a possible involvement of mTORC1independent kinase in mediating HM phosphorylation. Here, we report mTORC2 as an *in-vivo* HM kinase of S6K1. We show that rapamycin resistant S6K1 truncation mutant ΔNHΔCT, continues to display HM phosphorylation with selective sensitivity toward torin-1. We also show that HM phosphorylation of wildtype S6K1and ΔNHΔCT depends on the presence of mTORC2 regulatory subunit-rictor. Furthermore, truncation mutagenesis analysis highlights the involvement of a conserved 19 amino acid stretch of S6K1 in mediating mTORC2 dependent HM phosphorylation. We finally show that deletion of this amino acid stretch leads to complete loss of HM phosphorylation regardless of the presence of functional TOS motif. Our data identifies mTORC2 as a physiological HM kinase that can activate S6K1 after its auto-inhibition is overcome by mTORC1. We, therefore, propose a novel mechanism for S6K1 regulation where mTOR complex 1 and 2 act in tandem to activate the enzyme.

Introduction

The mechanistic target of rapamycin (mTOR) is a master regulator of cell growth and proliferation, whose deregulation is implicated in various pathological conditions including cancer, diabetes, arthritis and osteoporosis [1,2]. mTOR forms two structurally and functionally distinct complexes called the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [3,4] . mTORC1 comprises of three core components viz the catalytic subunit-mTOR, the regulatory subunit-raptor and GβL (also known as mLst8). In addition, PRAS40 and Deptor

constitute the inhibitory subunits of mTORC1. On the other hand, mTORC2 also comprises of mTOR and GβL. However, instead of raptor, the regulatory component of mTORC2 is a 200 KDa protein rictor. The other components of mTORC2 are PRR5, Deptor, and SIN1[5]. Among the two complexes, mTORC1 is extensively studied and implicated in integrating signals from multiple growth factors, nutrients, and energy supply to regulate cell growth and proliferation. One of the major downstream effector molecules of mTORC1 is Ribosomal protein S6 kinase 1 (S6K1)--a member of AGC family of protein kinases [6,7]. S6K1 is shown to use special amino terminal sequence motif TOS to interact with mTORC1 regulatory subunit-raptor [8]. This interaction is believed to induce S6K1 (α1variant) phosphorylation at Thr-412 (also referred to as Thr-389 in smaller isoform of S6K1), situated in a hydrophobic motif (HM) C-terminal to the catalytic domain [7,9,10]. Accordingly, loss of Thr-412 phosphorylation, either due to mTORC1 inhibition or its impeded recruitment due to TOS mutation/disruption, endorses the dependence of hydrophobic motif (HM) phosphorylation on the activation state of mTORC1.Contrarily, however, continued display of HM phosphorylation in S6K1 mutants harbouring mutations disrupting TOS function, suggest that mTORC1 regulates S6K1 in a manner that is distinct from the one that influences HM phosphorylation [11,12]. The observations are consistent with our earlier data that highlight disconnect between HM phosphorylation and rapamycin response by S6K1, to foster the argument that loss of phosphorylation may be a consequence rather than a mechanistic basis of mTORC1 inhibition [13,14]. In addition, identification of new mTORC1 substrates without a consensus TOS motif also undermine the centrality of TOS in mediating raptor binding [15–17]. The observations are further endorsed by recent data that demonstrate occurrence of S6K1 phosphorylation even in absence of raptor [18]. Furthermore, we have recently identified eukaryotic translation initiation factor 4E (eIF4E) as an intermediate in transducing signals from mTORC1 onto S6K1 [19]. Therein, we demonstrate that the role of mTORC1 is restricted to engaging eIF4E with S6K1-TOS motif for relieving the auto-inhibition, due to carboxy terminal auto-inhibitory domain (CAD), to facilitate consequential HM phosphorylation and activation of S6K1. Altogether, these observations suggest that relieving auto-inhibition should render S6K1 active and independent of the regulation by mTORC1 and further point towards involvement of mTORC1 independent kinase in phosphorylating S6K1 at hydrophobic motif site [19].

In this study, we report mTORC2 as a physiological HM kinase of S6K1. We identify a 19 amino acid sequence in S6K1 responsible for mediating mTORC2 dependent HM phosphorylation. We conclude that mTORC2 mediated phosphorylation and activation of S6K1 is subservient to

mTORC1 mediated release of S6K1 auto-inhibition. Our data, therefore, proposes a novel mechanism describing a stepwise action of mTOR complex 1 and 2 in regulating S6K1 activation.

Materials and Methods

Cell lines and Culture Conditions

HEK293 and HEK293T cells, described previously [19], were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (Invitrogen), 50 µg/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) at 37°C with 5% CO2.

Reagents and Antibodies

PVDF membrane (GE Healthcare/Millipore), Rapamycin (Sigma Aldrich), Torin-1 (Sigma Aldrich) and Protein G-Agarose beads (Genscript), Polyetheleneimine reagent (Polysciences, Inc.), Radioactive ATP (BRIT, Hyderabad-India). Antibodies against p-S6K1(T389), Raptor, Rictor, S6 and Flag- tag were purchased from Cell Signaling Technologies (Beverly MA); HA-tag, myc-tag and GAPDH (Sigma- Aldrich); S6K1 (GenScript); rabbit and mouse secondary antibodies conjugated to IR Dye 800CW (LI-COR Biotechnology, Lincoln, Nebraska).

Expression Vectors and Transfections

A pMT2-based cDNA encoding an amino-terminal HA-tagged S6K1 (αI variant) and S6K1 truncation mutant ∆NH∆CT S6K1 were gifted by Prof. Joseph Avruch, Harvard Medical School Boston, USA. Truncation mutants of S6K1 *viz* 147-421, 110-421, 91-421 and 77-421 were constructed in pKMYC mammalian expression vector. A common reverse primer 5' GCCGAATTCCTAACTTTCAAGTACAGATGGAG3' and specific forward primers 5'GAGGATCCATGCTGGAGGAAGTAAAGCAT3'; 5'GCGGATCCATGAAAGTAACAGGAGCAAATACT3'; 5'GCGGATCCATGTTTGAGCTACTTCGGGTACTTG3'and 5' GCGGATCCATGACTAGTGTGAACAGAGGGCCA 3' were used for PCR amplification of the respective mutants. cDNA HA-Raptor (#8513), HA-Rictor ( ) were purchased from addgene. HA tagged S6K1 truncation mutant S6K1∆91-109 was generated using primers (1) 5'Phos-AAAGTAACAGGAGCAAATACTGGGAAGATA3' and (2)5'Phos-ACATTCTGGTCTGATTTTTTCTGGCCC3'. The mutations were verified by DNA sequence and restriction analysis. For transient transfection of cells, 1□×□106 cells were plated onto a 60-mm

dish 24 hr prior to transfection. 1-2 µg of Plasmid DNA along with transfection agents Lipofectamine (Invitrogen) or Polyetheleneimine, PEI (Polysciences, Inc.) were used to transfect the cells. Standard protocol was followed for cell transfection.

Stable cell lines

Stable cell lines of HEK293 overexpressing S6K1 were generated by retroviral transduction of pWZL-Neo-Myr-Flag-RPS6KB1 expression plasmid (addgene#20624). Stable cell lines of HEK293 overexpressing S6K1 mutant ∆NHT2 were generated by retroviral transduction of pWZL-Neo-Myr-Flag-∆NHT2 S6K1 plasmid, generated by gateway cloning [20]. Briefly, the PCR amplified ∆NHT2 S6K1 was sub-cloned into TOPO adapted entry vector using pENTR/TEV/D-TOPO Cloning Kit (Invitrogen). The entry vector was then recombined with pWZL-Neo-Myr-Flag-DEST Gateway-compatible retroviral destination vector (addgene# 15300) using LR Clonase™ II Enzyme mix (Invitrogen 11791020). The infected cells were selected and maintained with neomycin (500 µg/ml).

Gene Knock Down using shRNA

Non-target scrambled shRNA (SHC002) was purchased from Sigma Aldrich. shRNA to human raptor (plasmid#1857) and rictor (plasmid#1853) were purchased from Addgene. The preparation of shRNA infected HEK293 cells have been described previously [19]

Immuno-precipitations and Western blotting

48 h post transfection, cells were starved overnight in serum-free DMEM. Cells were serum stimulated for 30 minutes in presence or absence of 50 nM of rapamycin or torin-1 as per the experimental requirements before lysis with ice cold NP40 lysis buffer [21]. Centrifugation (micro centrifuge, 13,000 rpm at 4°C for 20 min) was carried out to remove the precipitated material to obtain a clear lysate. After clearing, the supernatant was added to 2µg each of either anti-HA, anti-Myc or anti- Flag antibodies (as per the experimental requirements) immobilized on 20 µl of a 50% slurry of protein G Agarose and rotated for 4 hours at 4°C. Immunoprecipitates were washed five times with lysis buffer. 2X Laemmli sample buffer was added to the immunoprecipitates. The samples were boiled at 100°C, centrifuged at 13,000 rpm for 2 minutes and resolved by SDS-PAGE. Proteins were transferred on PVDF membrane, probed with different antibodies at indicated concentrations and analysed using Odyssey infrared imager (LI-COR).

In vitro kinase assay

In Vitro Kinase assay has been described previously [14,22] . Briefly, Proteins immobilized on either HA or Myc- beads were incubated with 1µg of the substrate and 5µCi 32PATP in a kinase reaction buffer containing 50mM Tris-Cl (pH 7.0), 10mM MgCl2, 0.5mM DTT, 50mM β-Glycero-phosphate, and 1mM ATP for 15 minutes at 37°C. Reaction was stopped by adding 5X loading buffer, run on an SDS-PAGE gel. Proteins were transferred on PVDF membrane, auto-radiographed and activity determined by standard protocol.

Statistical Analysis

Statistical analysis was performed using GraphPad software (Prism 8). All error bars represent SEM. For multiple group comparisons all groups from each experimental repeat were compared using 2way ANOVA. If the ANOVA test was significant ($p< 0.05$), Tukey's test was performed. The asterisks denote a significant p value defined as ** for $P <0.005$.

RESULTS AND DISCUSSION

MTORC1 is not a hydrophobic motif kinase of S6K1

To determine whether or not TOS motif is indispensable for mediating Thr-412 phosphorylation of S6K1 and whether or not relieving –NH2 and –COOH termini mediated auto-inhibition renders S6K1 active and independent of-the-regulation by mTORC1, we used HEK293 cells stably expressing Flag-tagged wild type S6K1 (described previously,[19]) and furthermore created lines of HEK293 cells stably expressing Flag-tagged S6K1 truncation mutant ∆NH∆CT to observe their state of Thr-412 phosphorylation in response to mTORC1 inhibition. We chose ∆NH∆CT S6K1 for the purpose because it bears truncations at TOS motif bearing -NH2 terminal domain and -COOH terminus auto-inhibitory domain (Fig 1a). mTORC1 inhibition was achieved either by treating the cells with glycolytic inhibitor-2 deoxy glucose (2DG) or rapamycin or by growing in Earl's Balanced Salt Solution (EBSS) [11]. As seen, ∆NH∆CT HEK293 stable cells display Thr-412 phosphorylation to an extent equivalent to their wild type counterpart that also correspond with its ability to phosphorylate GST-S6 *in vitro* (Fig 1b). Furthermore, it was interesting to observe that, unlike wildtype S6K1, ∆NH∆CT S6K1 HEK293 cells exhibit complete resistance to mTORC1 inhibition (Fig 1b).Taken together, the data indicate that mTORC1 by

itself does not appear to be the HM kinase and may only serve as a priming step for the occurrence of HM phosphorylation of S6K1. However, it could still be argued that ΔNHΔCT S6K1, being an unnatural variant, may support raptor recruitment co-incidentally by virtue of some unknown sequence to facilitate HM-phosphorylation. We, therefore, downregulated the expression of raptor by lentivirally transducing HEK293 cells using raptor shRNA and transfected them with HA tagged ΔNHΔCT S6K1 or wild type S6K1 to assess whether ΔNHΔCT S6K1 still sustains HM phosphorylation. Fig 1c clearly demonstrates that raptor knock down induces loss of HM phosphorylation in wild type S6K1 only and no such loss was observed in ΔNHΔCT S6K1. Altogether, the data substantiates that the kinase other than mTORC1 was responsible for mediating HM phosphorylation in S6K1. Pertinently, the Sabatini group has reported the involvement of mTORC2 in mediating HM phosphorylation of ΔNHΔCT S6K1 [12]. However, their proposition that mTORC2 mediated HM phosphorylation is a non-physiological/random event determined by the structure of S6K1 does not reconcile with the data, including ours, that highlight the redundancy of TOS motif in mediating mTORC1 specific phosphorylation of S6K1 or other bonafide mTORC1 substrates, and therefore point towards alternative mechanism for S6K1 regulation [15–17,19,22]. To address this conundrum, we first ascertained the involvement of mTORC2 in mediating HM phosphorylation of ΔNHΔCTS6K1. We, therefore, used HEK293 cells stably expressing Flag-tagged ΔNHΔCT- S6K1. Since ΔNHΔCT-S6K1 is rapamycin resistant variant [11,12,19], we used torin-1, a specific inhibitor of mTOR kinase, to observe the Thr-412 phosphorylation levels. Selective sensitivity of ΔNHΔCT S6K1 towards torin-1 that was also reflected through *in vitro* kinase assay of ΔNHΔCT S6K1 stable HEK293 cells using GST-S6 as substrate, confirmed the prospect of mTORC2 as potential HM kinase (Fig 1c, 1d).

MTORC2 phosphorylates S6K1 at Thr-412 site present in hydrophobic motif

In light of the above results that point towards the involvement of mTORC2 in mediating HM phosphorylation, we examined the state of HM phosphorylation of S6K1 and activation in backdrop of mTORC2 compromise, achieved in rictor shRNA knockdown HEK293 cells. Accordingly, we transfected wild type S6K1 in rictor shRNA or, alternatively, in control shRNA infected HEK293 cells to detect any change in the magnitude of Thr-412 phosphorylation present in HM. As seen, Thr-412 phosphorylation in rictor shRNA infected HEK293 cells decreases as compared to control shRNA HEK293 cells (Fig 2A). Interestingly, the decrease in Thr-412 phosphorylation was more prominent upon treatment with increasing concentration of mTORC1 specific inhibitor-rapamycin (Fig 2A). We have previously demonstrated that the role

of mTORC1 is restricted to inducing eIF4E interaction with TOS motif of S6K1. In the same study, we show ectopic expression of phosphomimetic eIF4E overrides rapamycin mediated mTORC1 inhibition and continues to sustain S6K1 activation [19]. We, therefore, explored whether phosphomimetic eIF4E could sustain S6K1 activation in backdrop of mTORC2 compromise in rictor knockdown HEK293 cells. Accordingly, we transfected rictor or scrambled (control) shRNA infected HEK293 cells with phosphomimetic eIF4E (S209E) to observe S6K1 Thr-412 levels in presence of rapamycin or torin-1. As seen, control shRNA infected cells transfected with myc-eIF4E (S209E) retains Thr-412 phosphorylation levels even in presence of rapamycin, whereas torin-1 brings about significant loss of Thr-412 phosphorylation, suggesting the involvement of mTORC1 in priming of the enzyme and that of mTORC2 in mediating HM phosphorylation (Fig 2B). Interestingly, phosphomimetic eIF4E does not recover Thr-412 phosphorylation in rictor shRNA infected HEK293 cells. However, when co-transfected with myc-rictor, which was used to rescue rictor knockdown effect, HEK293 recovered Thr-412 phosphorylation (Fig 2B). Taken together, the data on one hand confirms the involvement of mTORC2 in mediating Thr-412 phosphorylation of S6K1, while on the other it suggests that rapamycin induced mTORC1 inhibition fails to release S6K1 auto-inhibition and as a result prevents mTORC2 mediated HM phosphorylation of the enzyme. Although, the observation appears in tune with our previous finding that demonstrates the role of mTORC1 in relieving S6K1 auto-inhibition and priming it for HM phosphorylation and subsequent activation[19], we wanted to further substantiate the role of mTORC2 in mediating HM phosphorylation. Therefore, we used two different rapamycin resistant mutants of S6K1 viz. ΔNHΔCT and F28AΔCT for observing their state of Thr-412 phosphorylation in rictor knock down HEK293 cells[12]. While ΔNHΔCT mutant is described above, F28AΔCT shares the same –COOH terminal truncation as ΔNHΔCT but bears an inactivating mutation in the TOS motif instead of the -NH2 terminal truncation (Fig 1A) [12,13]. Both the mutants, despite being rapamycin resistant, exhibit significant loss of Thr-412 phosphorylation in rictor knock down state, indicating the involvement of mTORC2 in mediating Thr-412 phosphorylation (Fig 2C). The result further strengthens our view point that the loss of Thr-412 phosphorylation by rapamycin mediated mTORC1 inactivation in wild type S6K1 is due to the persistent auto-inhibition, caused by CAD, which disallows mTORC2 the access to phosphorylate S6K1 at Thr-412 site. We also evaluated the impact of serum stimulation on Thr-412 phosphorylation in S6K1 transfected rictor shRNA HEK293 cells and observed that addition of serum does not induce Thr-412 phosphorylation in rictor shRNA cells as compared to control shRNA cells (Fig 2D). Altogether, the data

demonstrates that Thr-412 phosphorylation is indeed mediated by mTORC2 after disinhibition or priming by mTORC1.

A 19 amino-acid region of S6K1 mediates mTORC2 phosphorylation.

Although the data presented above validates mTORC2 as a physiological Thr-412 kinase, we further wanted to ensure the specificity of mTORC2 in mediating this phosphorylation. We therefore argued, if mTORC2 mediated T412 phosphorylation in above discussed truncation mutants of S6K1 is a non-physiological/ random event as proposed by the Sabatini group [12], then a smaller mutant of S6K1 should also be phosphorylated in a similar manner by mTORC2. To address this, we examined the sequence of ΔNHΔCT S6K1, which comprises a total of 375 amino acids starting from amino acid 47 to 421 (see Fig 1A). A careful examination of the sequence revealed conserved TOS like motif spanning the region from amino acid 154 to 158 (Fig 3A).Therefore, we truncated 100 amino acids from -NH2 terminal end of ΔNHΔCT to generate a smaller mutant 147-421, which behaves like a TOS swap variant of S6K1 (Fig 3B). This mutant was important in two aspects, as it would tell whether the presence of TOS like motif enhances its prospect of phosphorylation by mTORC1, and whether the smaller size of the mutant better facilitates its non-physiological/random phosphorylation at Thr-412 site by mTORC2. Therefore, we transfected 147-421 S6K1 or wild type S6K1 or ΔNHΔCT S6K1 in HEK293 cells and observed the Thr-412 phosphorylation levels. Unlike wild type S6K1 and ΔNHΔCT S6K1, the mutant 147-421 failed to display any detectable phosphorylation at Thr-412 (Fig 3C), indicating redundancy of TOS motif in mediating this phosphorylation on one hand as well as dispelling the notion that mTORC2 mediated T412 phosphorylation is a non-physiological event on the other. Instead, the results suggest involvement of a truncated region 47-146 in regulating Thr-412 phosphorylation. Therefore, to identify the sequence imparting T412 regulation, we generated a series of smaller deletions *viz* 77-421 S6K1, 91-421 S6K1 and 110-421 S6K1 across this amino acid stretch and transfected them in HEK293 cells. Whereas the mutants 77-421 S6K1 and 91-421 S6K1 exhibit the presence of T412 phosphorylation, no such phosphorylation was observed in 110-421 S6K1 mutant (Fig 3D). Also, addition of torin-1 resulted in loss of the Thr-412 phosphorylation in both 77-421 S6K1 and 91-421 S6K1 mutants (Fig 3D).Together, the data highlights the involvement of this 19 amino acid stretch, starting from amino acid 91 to 109, in imparting Thr-412 regulation. Although we furthered our attempt to examine smaller deletions i.e., 91- 99 and 100-109 with in this stretch, but failed to gather any information due to the unreliable expression of these mutants. Next, we introduced this 19 amino acid deletion in wildtype S6K1 to address whether the presence of TOS bearing amino

and carboxy termini would help the mutant recover its Thr-412 phosphorylation. As seen in Fig 3E, the mutant fails to display any detectable Thr-412 phosphorylation, which was corroborated with the inability of the mutant to phosphorylate GST-S6 substrate in an *in vitro* kinase assay. Taken together, the data suggests that this 19 amino acid stretch is responsible for mediating mTORC2 dependent HM phosphorylation of S6K1.

The proposal that mTORC1 is HM kinase of S6K1 while mTORC2 mediated HM phosphorylation (Thr-412) is only a non-physiological/random event determined by the structure of S6K1, does not satisfactorily reconcile with the data that demonstrate T412 phosphorylation in TOS deficient S6K1 mutants [11,12,19]. The findings contest the preposition that interaction between raptor and TOS motif of S6K1 is the basis for mTORC1 mediated T412 phosphorylation. Furthermore, absence of a consensus TOS motif in other mTORC1 substrates question the centrality of TOS in mediating mTORC1 specific substrate phosphorylation. The observation is further endorsed by our recent findings that identify eIF4E as mTORC1 substrate and an intermediate in transducing signals downstream of mTORC1 onto S6K1[19]. The data, therein, demonstrates that the role of mTORC1 is restricted to engaging eIF4E with TOS motif of S6K1, required for relieving auto-inhibition and subsequently priming S6K1 for Thr-412 phosphorylation in mTORC1 independent manner [19]. Based on these observations and the data presented here, mTORC2 appears to be the physiological Thr-412 kinase of S6K1. The experiments conducted in rictor shRNA infected cells, under serum-starved or serum-stimulated conditions, demonstrate that mTORC2 mediates Thr-412 phosphorylation, subject to the priming by mTORC1. Therefore our proposal, described in Fig 4, that S6K1 requires priming by mTORC1 dependent TOS-eIF4E engagement before it is phosphorylated at Thr-412 by mTORC2 addresses the shortcomings that otherwise discredit mTORC2 as a physiological Thr-412 kinase. Notably, deletion analysis reveals 19 amino acid stretch of S6K1 responsible for mediating Thr-412 phosphorylation by a torin-1 sensitive kinase and therefore associates physiological specificity with mTORC2 mediated Thr-412 phosphorylation. Furthermore, loss of Thr-412 phosphorylation in S6K1 mutant deleted of this 19 amino acid stretch, despite the presence of a consensus TOS motif, unequivocally implicates the region in mediating T412 phosphorylation.

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Author Contributions

S.T.M. designed and performed the experiments, generated S6K1 stable cell lines, constructed S6K1 mutants, analysed and interpreted the data and wrote the manuscript. R.M. performed experiments, constructed rictor, raptor knockdown cell lines. M.A.Z. supervised the project. K.I.A conceived and supervised the project, designed the experiments, interpreted the data and wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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Legends

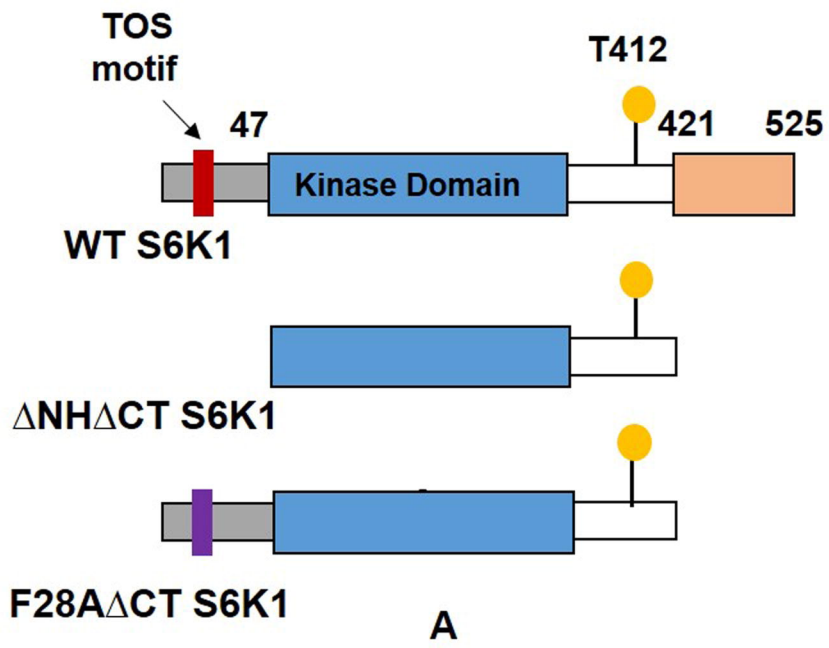
Fig 1 mTORC1 does not phosphorylate hydrophobic motif of S6K1. (A) S6 Kinase 1 domain structure. Representative picture demonstrates the structure full length S6K1 along with the location of TOS motif. Also shown are the truncation mutant ΔNHT2 and F28AΔCT used in the study. (B) Release of auto-inhibition renders S6K1 independent of mTORC1 regulation. Flag-WTS6K1 and Flag-ΔNHT2 S6K1 stable HEK293 cells were either lysed directly or after incubation with agents known to inhibit mTORC1 input (100 mM 2-Deoxy Glucose or 50 nM rapamycin for 30 minutes). Alternatively, mTORC1 inhibition was achieved by growing cells in amino acid free media (i.e., Earl's balanced Salt Solution-EBSS).The lysates obtained were Flag immunoprecipitated and probed for T412 levels of S6K1. Furthermore, S6K1 kinase activity was monitored using GST-S6 as a substrate in an *in vitro* kinase assay. Quantitation represents average results of three independent experimental series. Error bars denote SEM. (C) Raptor is not involved in mediating hydrophobic motif phosphorylation of S6K1. Raptor or scrambled shRNA infected HEK293 cells were transfected with wild type S6K1 or truncation mutant ΔNHT2. Before lysis, the cells were treated with increased concentration of torin in indicated manner for 30 minutes. The lysates obtained were HA immunoprecipitated and probed for T412 levels of S6K1. Additionally, 2 µg of HA-rictor encoding plasmid was transfected in rictor shRNA cell line to rescue its knock down effect. Quantitation showingT412 phosphorylation levels represents average result of two independent experimental series normalized to the respective loading control protein content. Error bars denote SEM. p value denoted as * indicates P<0.05, ** as P< 0.005 and *** as P<0.0005 (D) S6K1 amino and carboxy termini truncation mutant ΔNHT2 is sensitive to torin. Flag-WTS6K1 and Flag-ΔNHT2 S6K1 stable HEK293 cells were either lysed directly or after 30 minutes incubation with 50nM torin. The lysates obtained were Flag immunoprecipitated and probed for T412 levels of S6K1 and S6K1 activity as described in (B).Quantitation represents average results of three independent experimental series. Error bars denote SEM. p value denoted as ** indicates P<0.005.

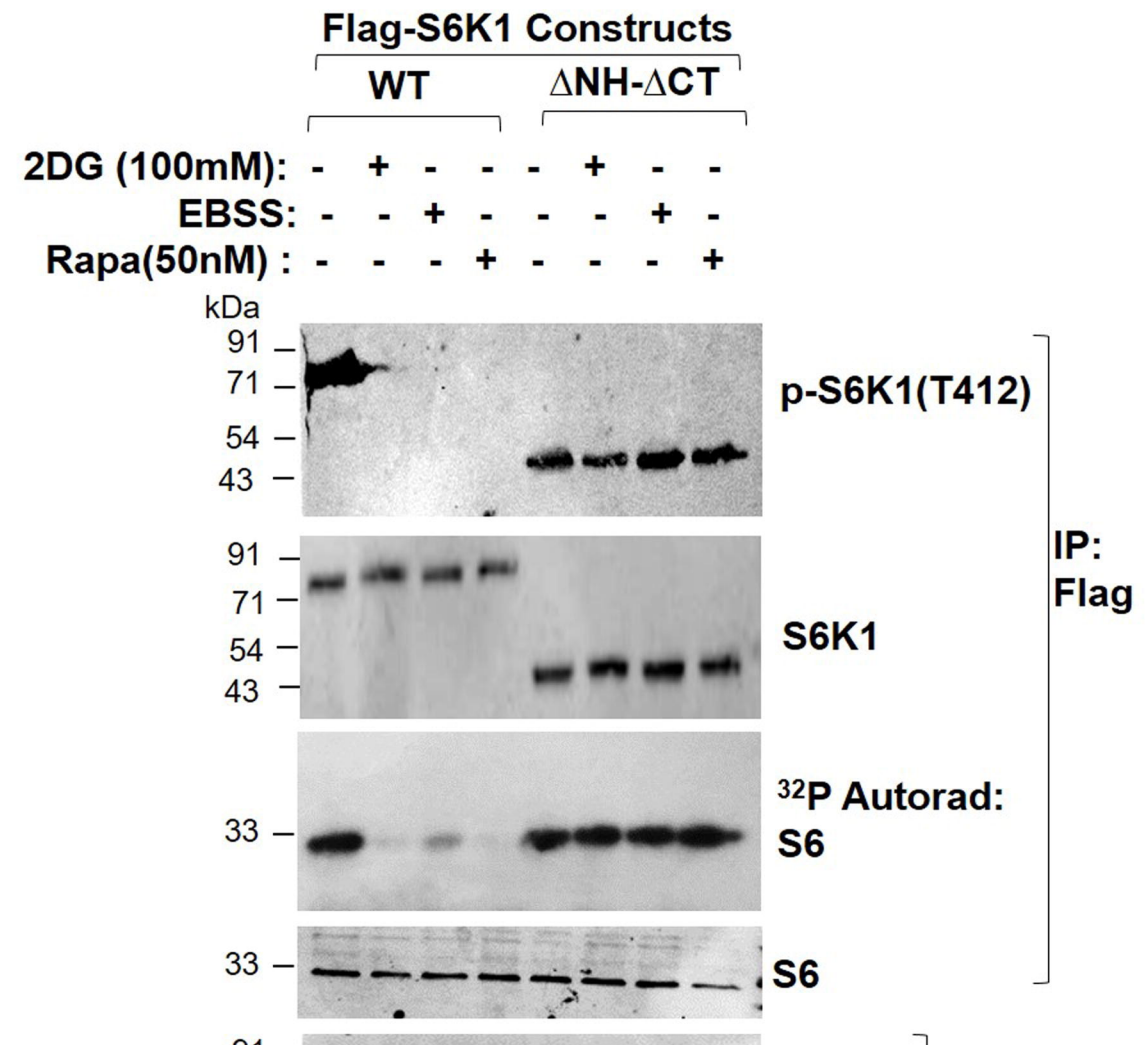
Fig 2 mTORC2 is an *in vivo* T412 kinase of S6K1. (A)S6K1 loses T412 phosphorylation in rictor knock down cells. HEK293 cells were infected with rictor shRNA to generate rictor knockdown cell line. Scrambled shRNA was used as control. The cells were transfected with HA tagged WT-S6K1. Before lysis, the cells were treated with increased concentration of rapamycin for 30 minutes in indicated manner. The lysates obtained were HA immunoprecipitated and probed for T412 levels of S6K1. Quantitation showingT412 phosphorylation levels represents average result of three independent experimental series normalized to their respective protein content. Error bars denote SEM. p value denoted as ** indicates P < 0.005. (B) Phosphomimetic eIF4E does not sustain T412 phosphorylation in rictor knockdown cells. Myc-tagged eIF4E was transfected in rictor or scrambled shRNA infected HEK293 cells in indicated manner. Additionally, 2 µg of myc-rictor encoding plasmid was transfected in rictor shRNA cell line to rescue its knock down effect. Prior to lysis, the cells were treated with 50nM of rapamycin or torin-1 for 30 minutes in indicated manner. The lysates obtained were probed for T412 levels. (C) HA-tagged rapamycin resistant S6K1 mutants F28AΔCTand ΔNHΔCT were transfected in

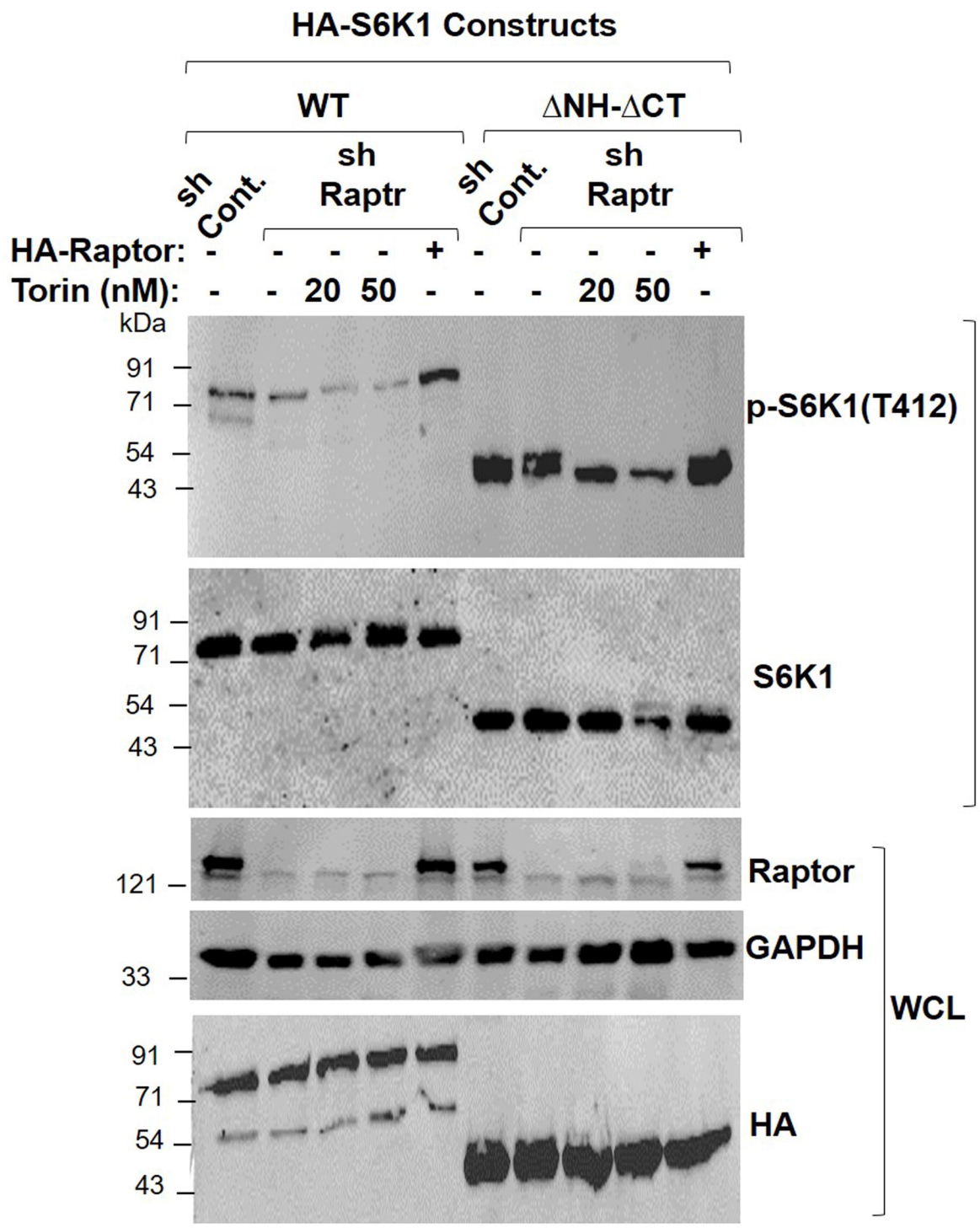
rictor or scrambled shRNA infected HEK293 cells in indicated manner. Additionally, 2 µg of myc-rictor encoding plasmid was transfected in rictor shRNA cell line to rescue its knock down effect. Prior to lysis, the cells were treated with 50nM of rapamycin for 30 minutes. The lysates obtained were HA immunoprecipitated and probed for T412 levels. (D) S6K1 T412 phosphorylation does not respond to serum stimulation in rictor knock down cells. HA tagged wild type S6K1 was transfected in rictor or scrambled shRNA infected HEK293 cells in indicated manner. Additionally, 2 µg of myc-rictor encoding plasmid was transfected in rictor shRNA cell line to rescue its knock down effect. The cells were grown in serum supplemented DMEM for 48 hours and then serum starved for 12 hours. Prior to lysis, cells were serum stimulated for 30 minutes in indicated manner. The lysates were HA immunoprecipitated and probed for T412 levels.

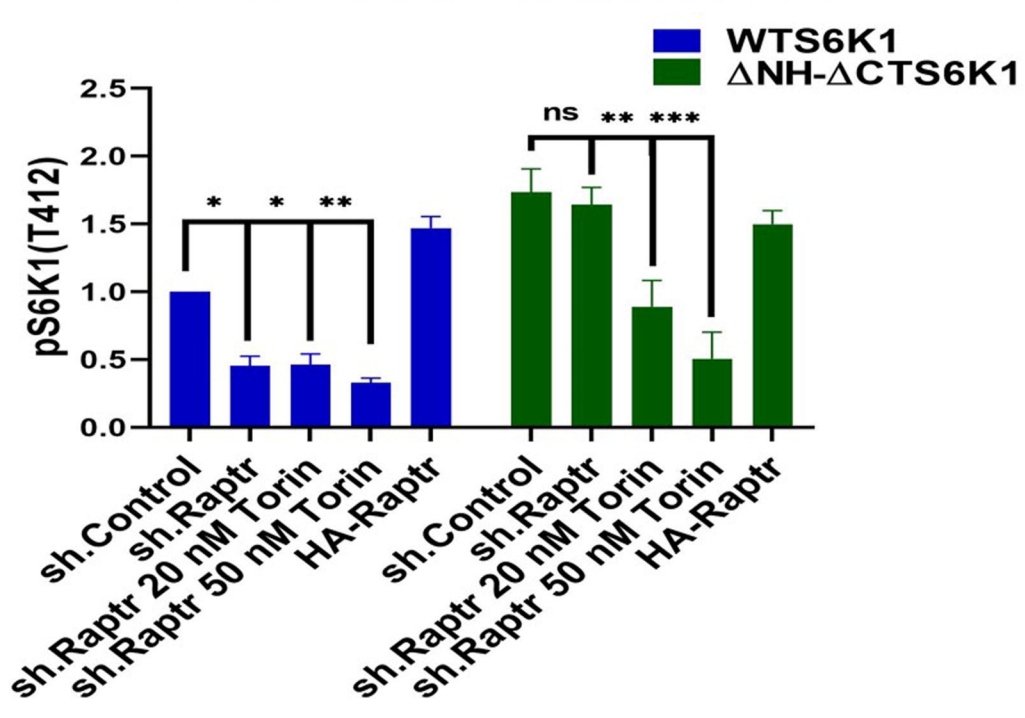
Fig 3 S6K1 harbors mTORC2 responsive site. (A) S6K1 harbors a TOS like motif in kinase domain. Sequence alignment shows a TOS like motif in catalytic kinase domain of S6K1 and its conserved nature across the species. (B) Structure of truncation mutants of S6K1 used in the study. (C-E) A 19 amino acid region is responsible for mediating mTORC2 dependent T412 phosphorylation of S6K1. HEK293 cells were transfected with HA tagged wild type S6K1 or truncation mutant ∆NH∆CT or myc tagged 147-421 S6K1 as indicated (C) or with myc tagged S6K1 truncation mutants 77-421, 91-421 and 110-421 (D) or with HA tagged wild type S6K1 and ∆91-109 (E). Prior to lysis, cells were treated with 50nM of torin-1 (C and D) or with 50 nM of rapamycin (E) for 30 minutes. The lysates obtained were epitope immunoprecipitated and probed for T412 levels. Furthermore, S6K1 kinase activity was monitored using GST-S6 as a substrate in an *in vitro* kinase assay (E). Quantitation represents average results of three independent experimental series. Error bars denote SEM. p value denoted as ** indicates $P < 0.005$.

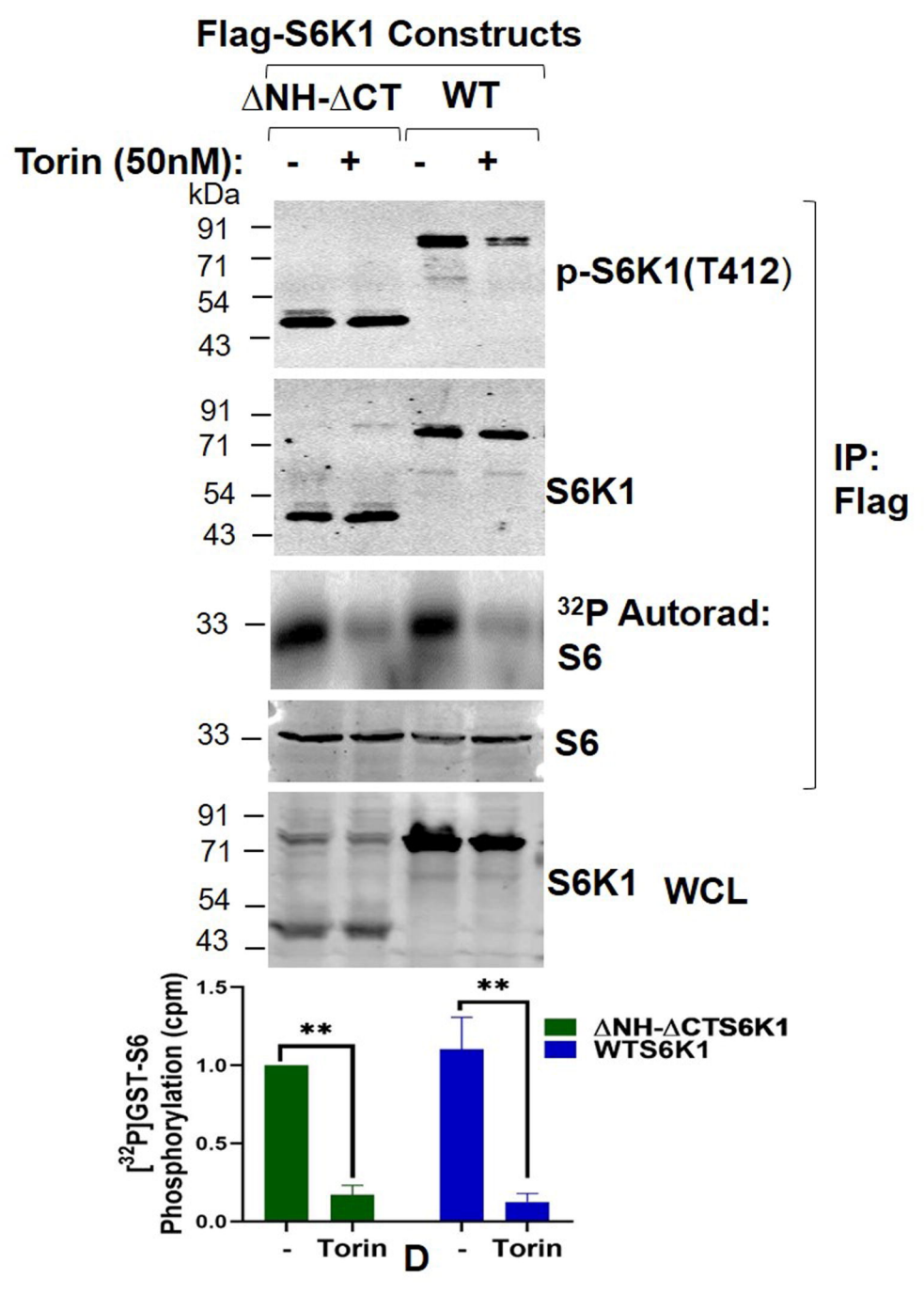
Fig 4 Illustration describing a two-step activation mechanism for S6K1. Step 1. Active mTORC1 induces eIF4E to interact with TOS motif of S6K1 for releasing the S6K1 auto-inhibition and thereby exposing the critical Thr-412 site in HM for phosphorylation and subsequent activation (described previously [19]). Step2. MTORC2 phosphorylates S6K1 at Thr-412 site in HM and leads to complete activation of the enzyme.

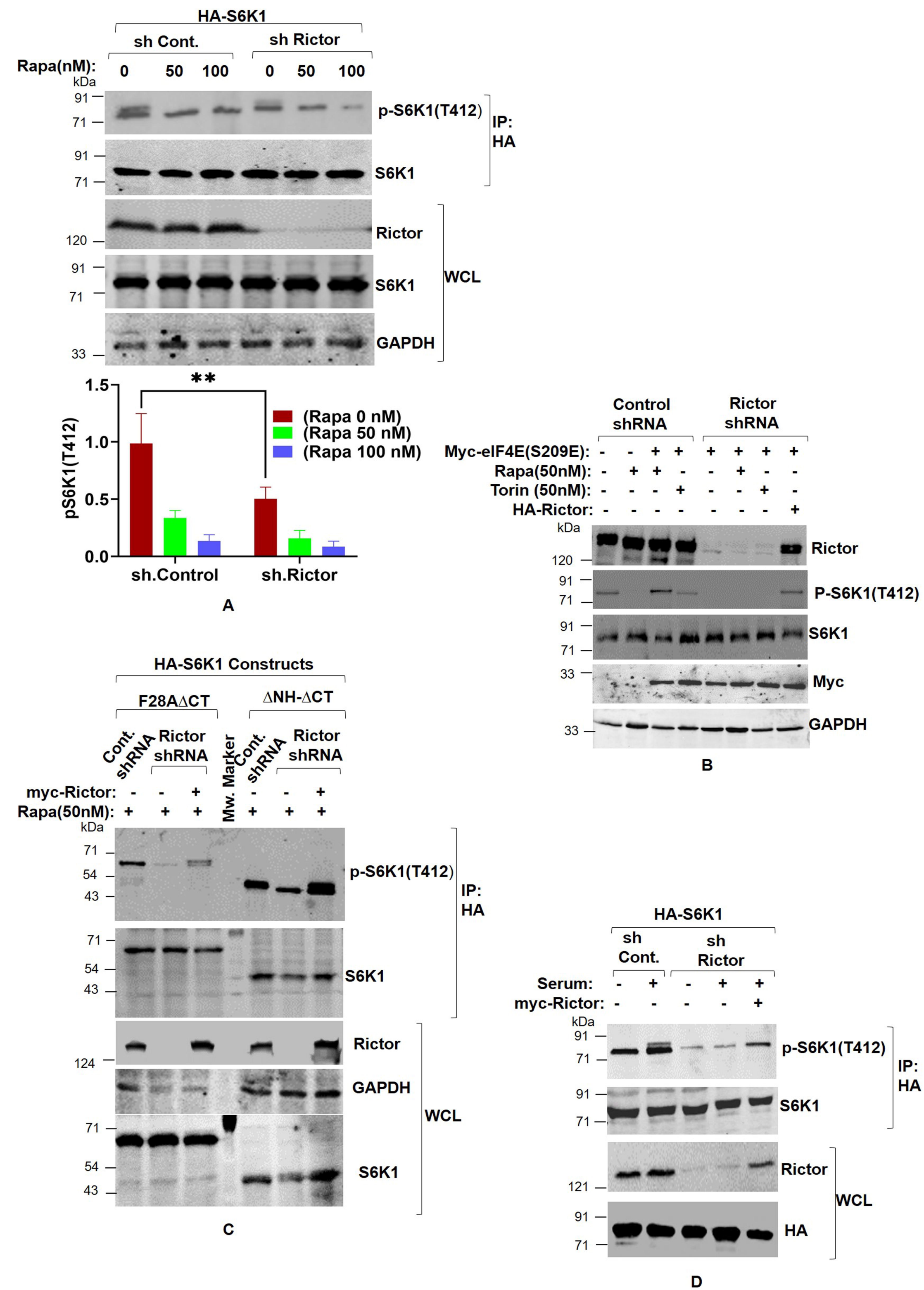

TOS motif
47
T412
421
525
Kinase Domain
WT S6K1
ΔNHΔCT S6K1
F28AΔCT S6K1
A


Flag-S6K1 Constructs
WT
ΔNH-ΔCT
2DG (100mM): - + - - - + - -
EBSS: - - + - - - + -
Rapa(50nM) : - - - + - - - +
kDa
91
71
54
43
p-S6K1(T412)
S6K1
33
32P Autorad: S6
S6
IP: Flag
WCL
GAPDH
WTS6K1
ΔNH-ΔCTS6K1
[32P]GST-S6 Phosphorylation (cpm)
1.5
1.0
0.5
0.0
-
2DG
EBSS
Rapa
B


HA-S6K1 Constructs
WT
ΔNH-ΔCT
sh Cont.
sh Raptr
HA-Raptor: - - - - + - - - - +
Torin (nM): - - 20 50 - - - 20 50 -
kDa
91
71
54
43
p-S6K1(T412)
S6K1
121
Raptr
GAPDH
33
HA
IP: HA
WCL


WTS6K1
ΔNH-ΔCTS6K1
pS6K1(T412)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
ns
**
***
sh.Control
sh.Raptr
sh.Raptr 20 nM Torin
sh.Raptr 50 nM Torin
HA-Raptr
C


Flag-S6K1 Constructs
ΔNH-ΔCT
WT
Torin (50nM): - + - +
kDa
91
71
54
43
p-S6K1(T412)
S6K1
33
32P Autorad: S6
S6
IP: Flag
WCL
ΔNH-ΔCTS6K1
WTS6K1
[32P]GST-S6 Phosphorylation (cpm)
1.5
1.0
0.5
0.0
**
**
-
Torin
D

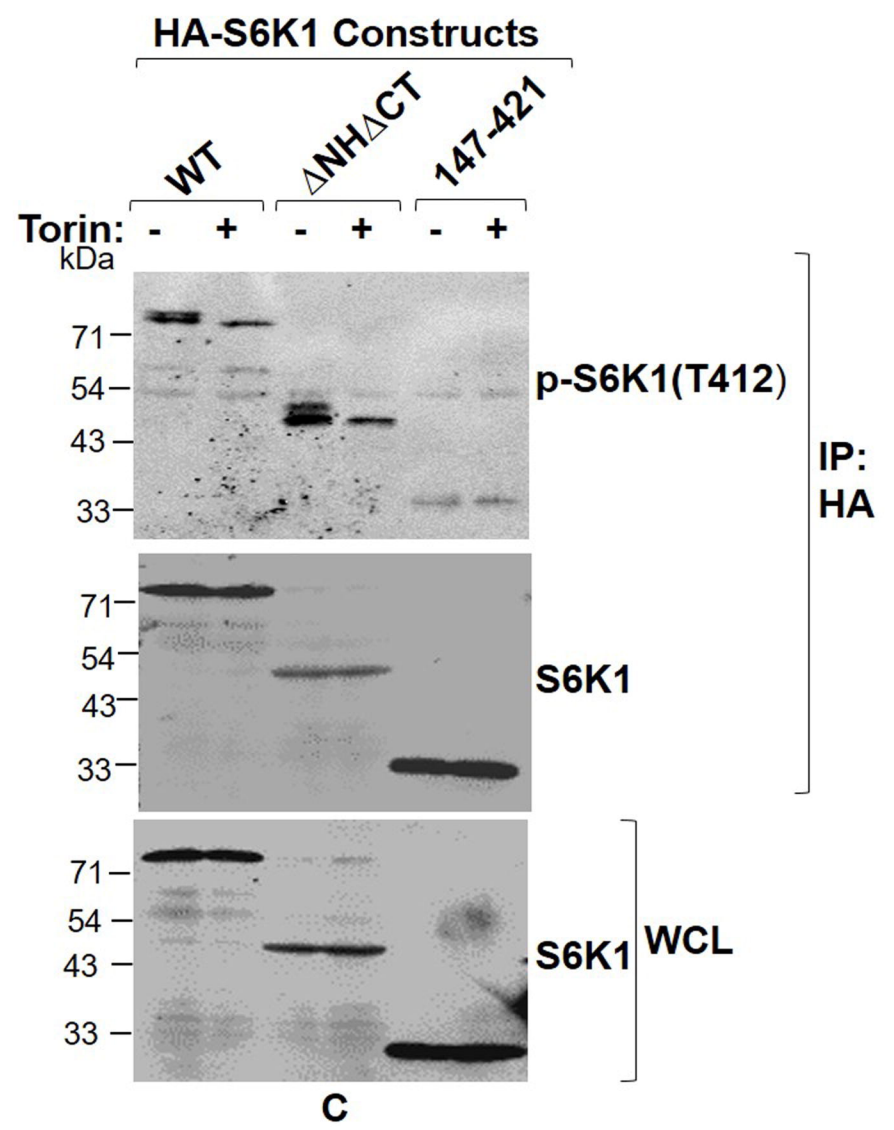

HA-S6K1
sh Cont.
sh Rictor
Rapa(nM):
0 50 100 0 50 100
kDa
91
71
p-S6K1(T412)
S6K1
IP: HA
120
Rictor
GAPDH
33
WCL
**
1.5
1.0
0.5
0.0
pS6K1(T412)
(Rapa 0 nM)
(Rapa 50 nM)
(Rapa 100 nM)
sh.Control
sh.Rictor
A
Control shRNA
Rictor shRNA
Myc-eIF4E(S209E):
Rapa(50nM):
Torin (50nM):
HA-Rictor:
P-S6K1(T412)
Myc
B
HA-S6K1 Constructs
F28AΔCT
ΔNH-ΔCT
Cont. shRNA
Rictor shRNA
Mw. Marker
myc-Rictor:
Rapa(50nM):
54
43
124
C
Serum:
myc-Rictor:
121
HA
D

TOS Consensus Sequence
Φ hydrophobic; X any residue **F X φ D φ**

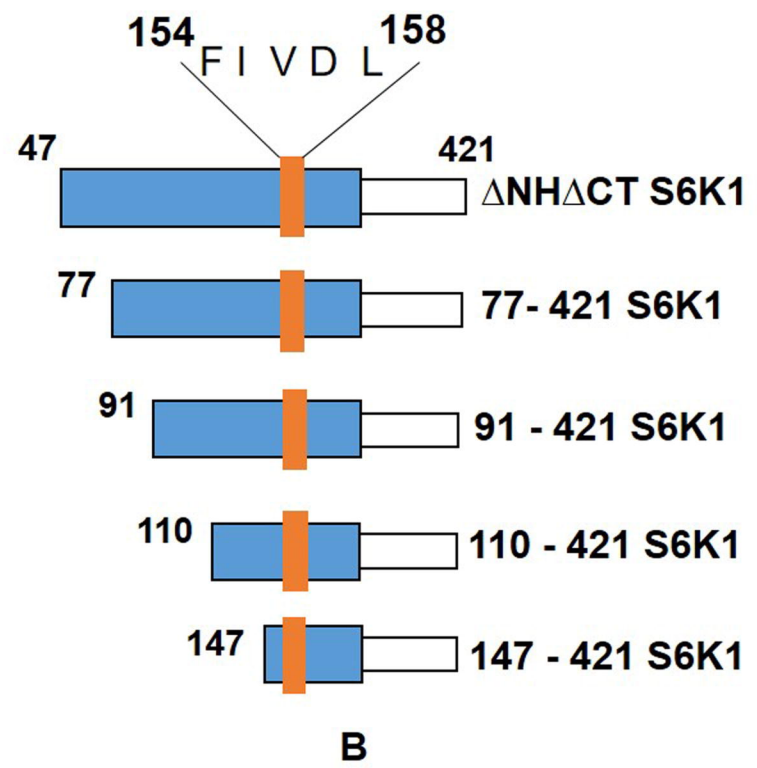
S6K1 TOS motif	F D I D L
S6K2 TOS motif	F D L D L
4EBP1 TOS motif	F E M D I
PRAS40 TOS motif	F V M D E
TOS like motif in S6K1	F I V D L

Homo	154	F I V D L 158
Macaca	154	F I V D L 158
Rattus	154	F I V D L 158
Mus	154	F I V D L 158
Gallus	131	F I V D L 135

A



C



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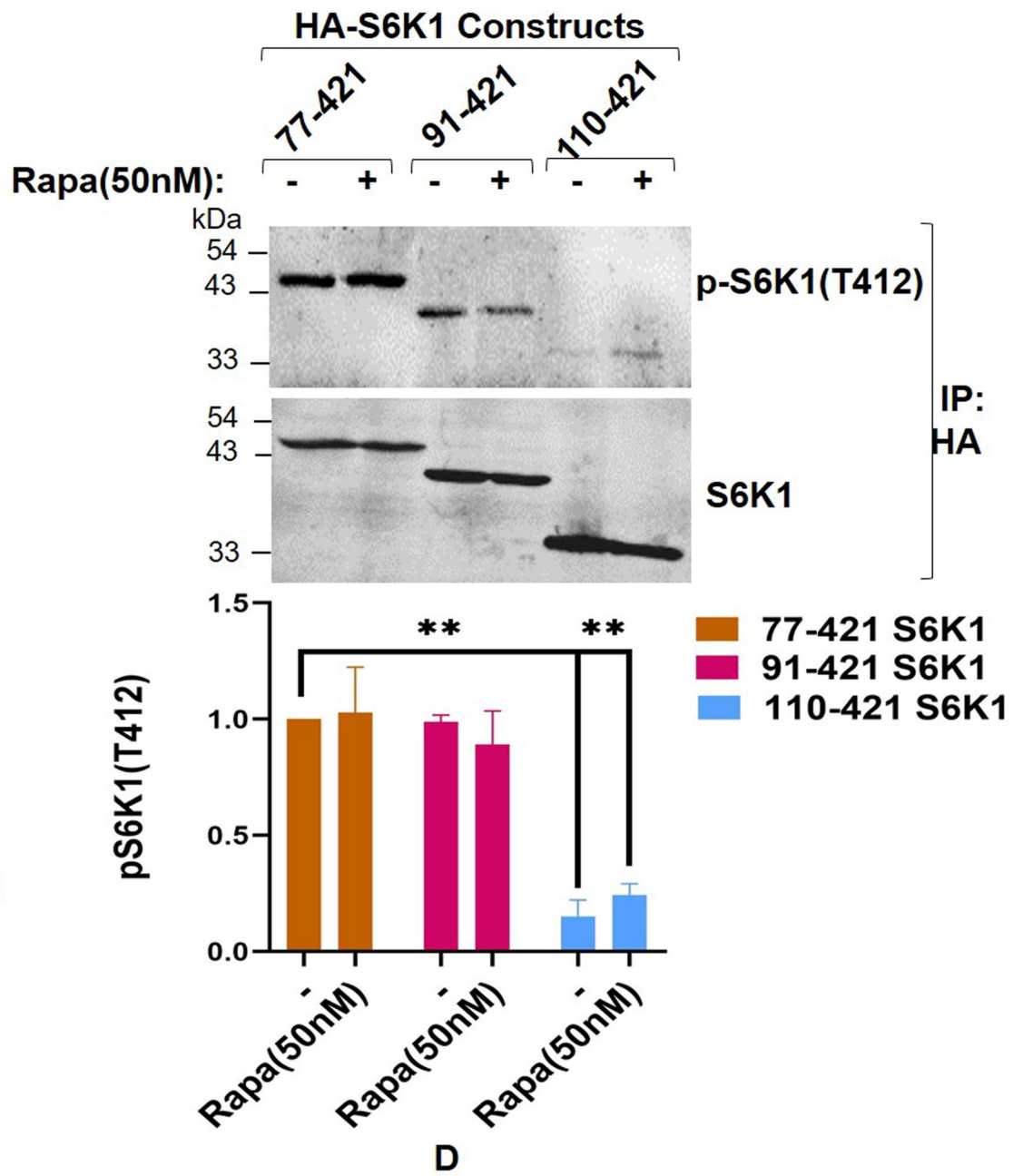

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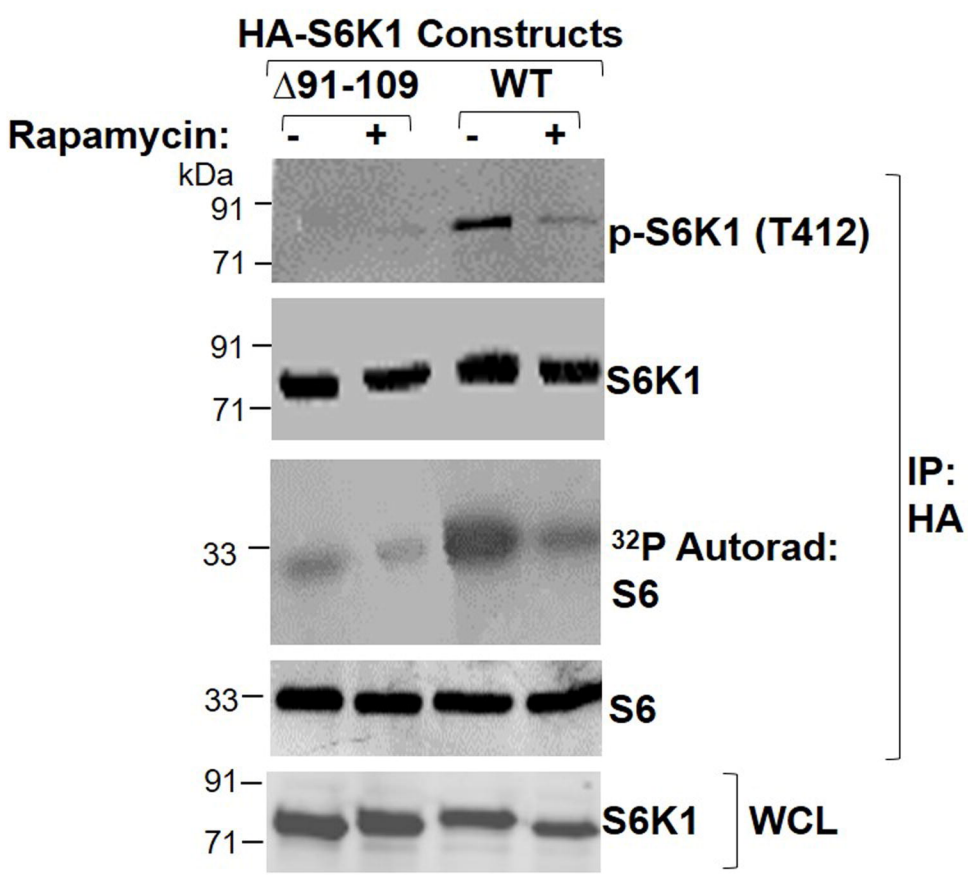
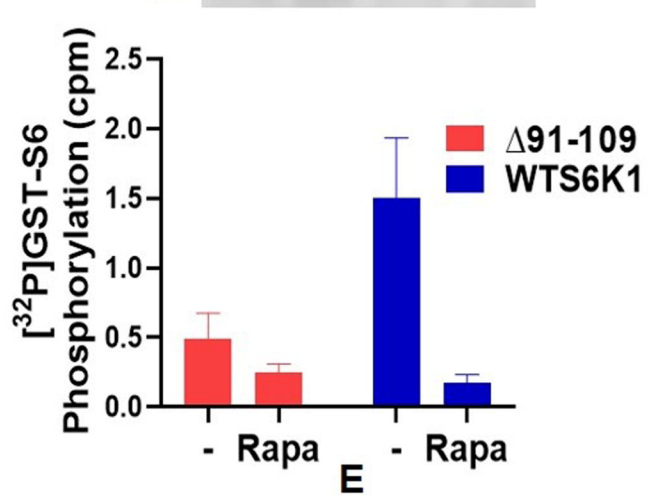
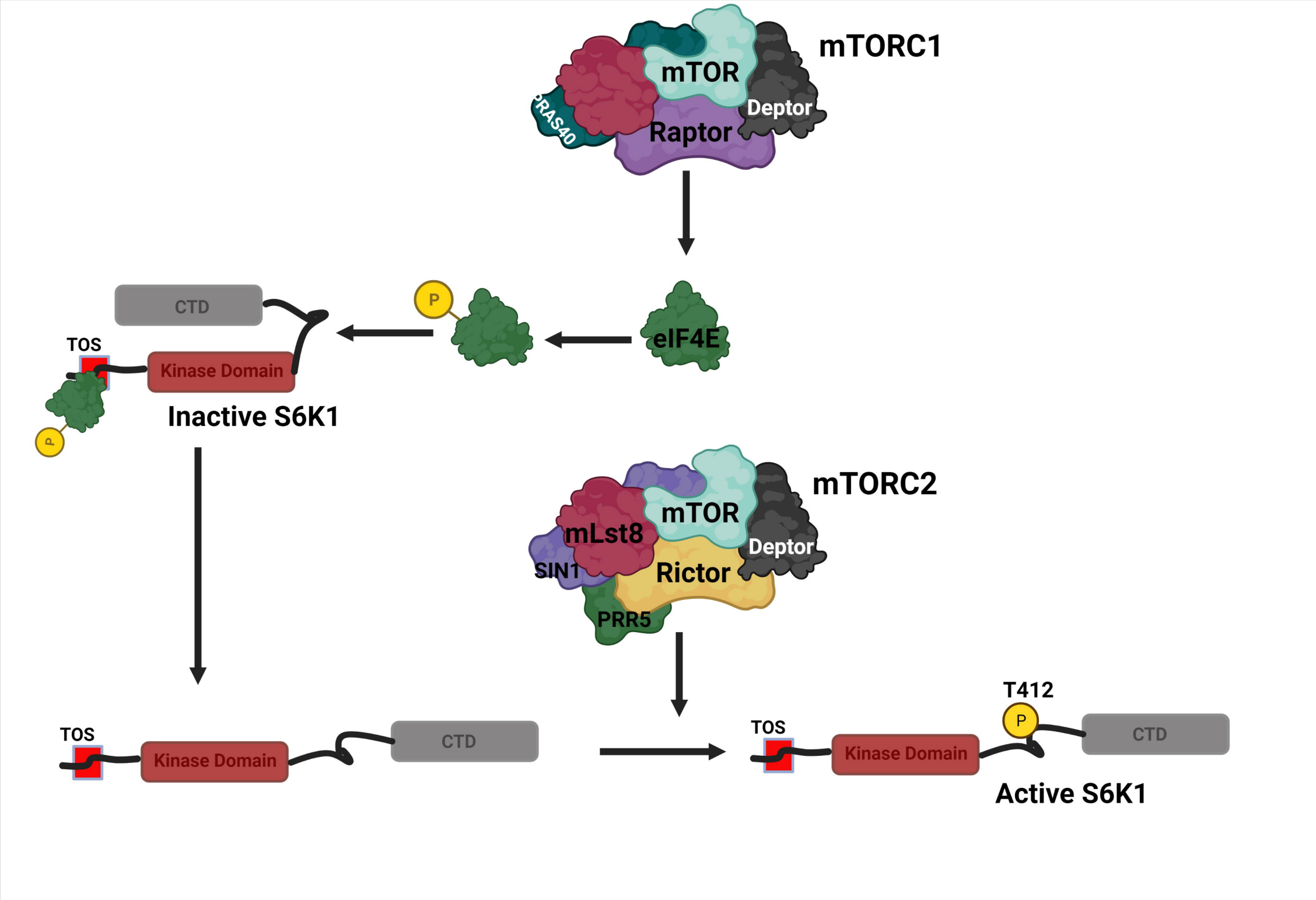




E


mTORC1
mTOR
Deptor
Raptor
PRAS40
eIF4E
P
CTD
TOS
Kinase Domain
Inactive S6K1
P
mTORC2
mTOR
mLst8
Deptor
SIN1
Rictor
PRR5
TOS
Kinase Domain
CTD
T412
P
TOS
Kinase Domain
CTD
Active S6K1