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Short Article

mTOR Generates an Auto-Amplification Loop by Triggering the βTrCP- and CK1α-Dependent Degradation of DEPTOR

Shanshan Duan,[1,2] Jeffrey R. Skaar,[1,2] Shafi Kuchay,[1,2] Alfredo Toschi,[1] Naama Kanarek,[3] Yinon Ben-Neriah,[3] and Michele Pagano[1,2,*]
[1]Department of Pathology, NYU Cancer Institute, New York University School of Medicine, 522 First Avenue, SRB 1107, New York, NY 10016, USA
[2]Howard Hughes Medical Institute
[3]The Lautenberg Center for Immunology, Hebrew University, Jerusalem 91120, Israel
*Correspondence: michele.pagano@nyumc.org
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SUMMARY

DEPTOR is a recently identified inhibitor of the mTOR kinase that is highly regulated at the post-translational level. In response to mitogens, we found that DEPTOR was rapidly phosphorylated on three serines in a conserved degron, facilitating binding and ubiquitylation by the F box protein βTrCP, with consequent proteasomal degradation of DEPTOR. Phosphorylation of the βTrCP degron in DEPTOR is executed by CK1α after a priming phosphorylation event mediated by either the mTORC1 or mTORC2 complexes. Blocking the βTrCP-dependent degradation of DEPTOR via βTrCP knockdown or expression of a stable DEPTOR mutant that is unable to bind βTrCP results in mTOR inhibition. Our findings reveal that mTOR cooperates with CK1α and βTrCP to generate an auto-amplification loop to promote its own full activation. Moreover, our results suggest that pharmacologic inhibition of CK1 may be a viable therapeutic option for the treatment of cancers characterized by activation of mTOR-signaling pathways.

INTRODUCTION

The mammalian target of rapamycin (mTOR) kinase controls many aspects of the response to growth factor and nutrient signaling (Laplante and Sabatini, 2009; Zoncu et al., 2011). mTOR complex 1 (TORC1) and TORC2 share the mTOR and mLST8/GβL proteins, but the complexes also feature distinct subunits, with RAPTOR and PRAS40 in TORC1 and RICTOR, PROTOR, and mSin1 in TORC2 (Laplante and Sabatini, 2009). In general, TORC1 controls mRNA translation, ribosome biogenesis, cell growth, and autophagy through substrates such as S6K1 and 4E-BP1, whereas TORC2 controls cell proliferation, cell survival, and the cytoskeleton through substrates such as Akt, SGK1, and PGCα (Dancey, 2010; Guertin and Sabatini, 2007; Sengupta et al., 2010).

The pathways controlled by TORC1 and TORC2 are frequently activated in tumors by mutations in upstream signaling factors (e.g., growth factor receptors, PI3K regulators, or PTEN), and mTOR inhibitors have been used successfully in the treatment of several cancers (Dancey, 2010; Hay, 2005). However, direct activating mutations of mTOR have not been observed in cancer, and in some settings, mTOR has been shown to possess tumor-suppressive properties, likely due to negative feedback loops that control the TORC1 or TORC2 pathways (Laplante and Sabatini, 2009).

Both mTOR complexes are directly inhibited by DEPTOR, which binds and inhibits mTOR through a PDZ domain (Peterson et al., 2009). DEPTOR is downregulated in many tumors, suggesting a tumor suppressor function, which is consistent with the activation of mTOR in many tumors. However, DEPTOR is overexpressed in multiple myeloma via transcription or copy number amplifications, and this overexpression is necessary for Akt activation and cell survival, which is likely mediated through the feedback inhibition of PI3K (Carrasco et al., 2006; Peterson et al., 2009). Notably, despite a general downregulation of DEPTOR across other tumor types, amplification of the genomic region containing the DEPTOR locus is an indicator of poor prognosis or tumor progression in tumor subsets from multiple cancers, including breast cancer, prostate cancer, lung cancer, and CML. DEPTOR is overexpressed in many of these tumors (Chin et al., 2007; Joos et al., 2003; van Duin et al., 2005a, 2005b).

The impact of DEPTOR in cancer makes it vital to understand the regulation of DEPTOR. DEPTOR activity appears to be regulated largely through the control of DEPTOR levels, which are tightly controlled both transcriptionally and posttranslationally in response to growth factor signaling (Peterson et al., 2009). Although DEPTOR levels are high in the absence of serum, in response to serum, transcription of *DEPTOR* decreases and DEPTOR protein is rapidly phosphorylated on as many as 13 sites. Many of these phosphorylations are mTOR dependent, and nonphosphorylated mutants of DEPTOR bind mTOR more efficiently than wild-type DEPTOR, indicating that phosphorylation of DEPTOR inhibits binding to mTOR. mTOR activity also correlates with DEPTOR degradation, suggesting that these two processes are linked. However, the precise mechanisms for this regulation remain unclear.

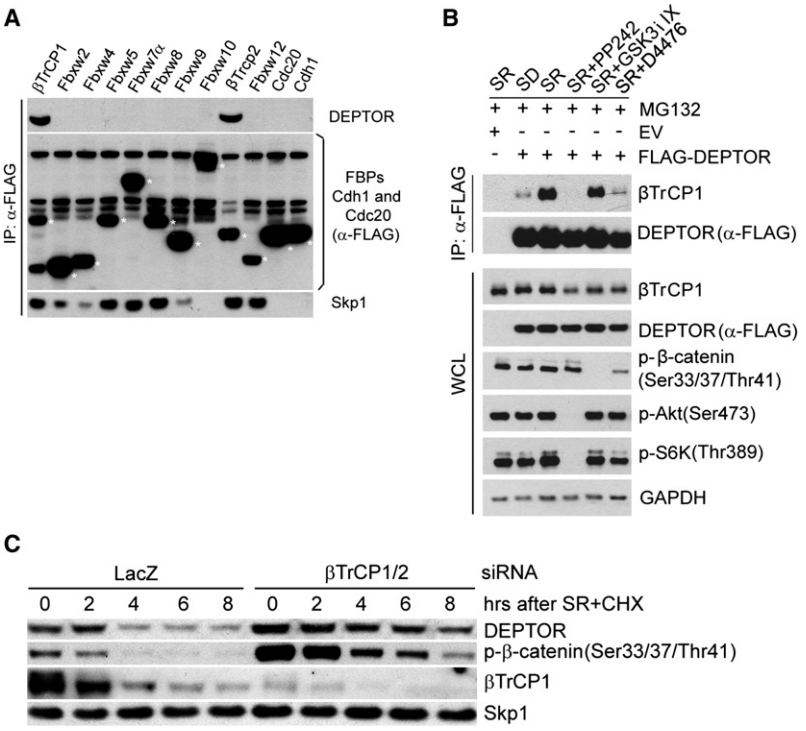

Figure 1. DEPTOR Is a Serum-Dependent Substrate of βTrCP

(A) DEPTOR binds βTrCP1 and βTrCP2. HEK293T cells were transfected with the indicated FLAG-tagged proteins. Forty-eight hours posttransfection, after a serum starvation of 16 hr, cells were restimulated with media containing serum and MG132 for 3 hr prior to harvesting for immunoprecipitations and immunoblotting as indicated. Asterisks indicate the position of exogenously expressed proteins.

(B) HEK293T cells were transfected with an empty vector (EV) or FLAG-tagged DEPTOR. Forty-eight hours posttransfection, after a serum starvation of 24 hr, cells were pretreated with the indicated drugs for 2 hr and then stimulated with serum-containing media (SR) for 3 hr prior to harvesting for immunoprecipitations and immunoblotting as indicated. WCL, whole cell lysate.

(C) During a serum starvation of 72 hr, T98G cells were transfected with siRNAs targeting either LacZ or βTrCP1 and βTrCP2. Cells were subsequently restimulated with media containing serum and cycloheximide (SR+CHX), and samples were harvested at the indicated time points for immunoblotting.

Skp1/Cul1/F box protein (SCF) ubiquitin ligase complexes control the degradation of many important regulatory proteins (Cardozo and Pagano, 2004). In mammals, there are 69 SCF ligases, each characterized by a different F box substrate-targeting subunit (Jin et al., 2004). In this study, we identify SCF$^{\beta TrCP}$ as the ubiquitin ligase for DEPTOR and demonstrate that SCF$^{\beta TrCP}$ mediates the mTOR- and CK1α-dependent degradation of DEPTOR.

RESULTS

The expression of Cul1(1–252), a dominant negative Cul1 mutant that binds Skp1 and F box proteins but cannot recruit an E2 ubiquitin conjugating enzyme, results in the accumulation of SCF substrates (Piva et al., 2002; Yen and Elledge, 2008). To identify new SCF substrates, we transiently transfected Cul1(1–252) into HeLa cells and analyzed cell extracts for the levels of several regulators of cell proliferation by immunoblotting. The level of DEPTOR increased compared to mock transfected controls (Figure S1A available online), suggesting that DEPTOR is an SCF substrate. Therefore, we investigated which F box protein targets DEPTOR to the SCF, using a library of F box protein cDNAs. Screening of the FBXW (F box proteins with WD40 repeats) family proteins, as well as Cdc20 and Cdh1 (WD40 domain-containing subunits of an SCF-like ubiquitin ligase), revealed that endogenous DEPTOR specifically interacts with βTrCP1 and βTrCP2 (Figure 1A), paralogous F box proteins that share identical biochemical properties and substrates. (In this article, βTrCP will refer to both, unless specified.)

Significantly, the binding of DEPTOR to transiently expressed βTrCP was dependent on the substrate-binding domain, as demonstrated by the inability of a previously established substrate-binding point mutant, βTrCP2(R434A), or a WD40 repeat deletion mutant, βTrCP2(ΔWD40) (Suzuki et al., 2000; Wu et al., 2003), to bind endogenous DEPTOR (Figure S1B). Serum starvation of HeLa cells induces accumulation of DEPTOR, whereas serum stimulation results in DEPTOR degradation (Peterson et al., 2009). After confirming these results in T98G cells (Figure S1C), we found that serum stimulation induced a significant increase in the binding of DEPTOR to endogenous βTrCP1 (Figure 1B).

To investigate the hypothesis that βTrCP controls the degradation of DEPTOR in serum-stimulated cells, we reduced the expression of both βTrCP1 and βTrCP2 in T98G and HeLa cells using a validated siRNA (Dehan et al., 2009; Dorrello et al., 2006; Fong and Sun, 2002; Guardavaccaro et al., 2008; Peschiaroli et al., 2006). Figures 1C and S1D show that silencing βTrCP increased the DEPTOR half-life upon stimulation with serum, demonstrating that βTrCP controls DEPTOR stability.

Next, we mapped the βTrCP binding motif in human DEPTOR. Using deletion mutants, the binding motif was mapped to a region of DEPTOR between amino acids 241 and 340 (Figure S2A). βTrCP binds substrates via phosphorylated residues in conserved degradation motifs (degrons), typically including the consensus sequence DpSGXXpS or similar variants, such as pS/TpSGXXpS (Figure S2B). The βTrCP-binding region of DEPTOR contains a conserved ^{286}SSGYFS^{291} motif, matching other βTrCP substrate degrons. To investigate whether DEPTOR binds βTrCP via this motif, we generated serine to alanine mutants and tested their binding to endogenous βTrCP1. Single mutations of Ser286, Ser287, and Ser291 to Ala or a triple mutation of Ser286/287/291 to Ala inhibited the interaction between DEPTOR and βTrCP1, although the mutations did not affect DEPTOR binding to endogenous mTOR (Figure 2A).

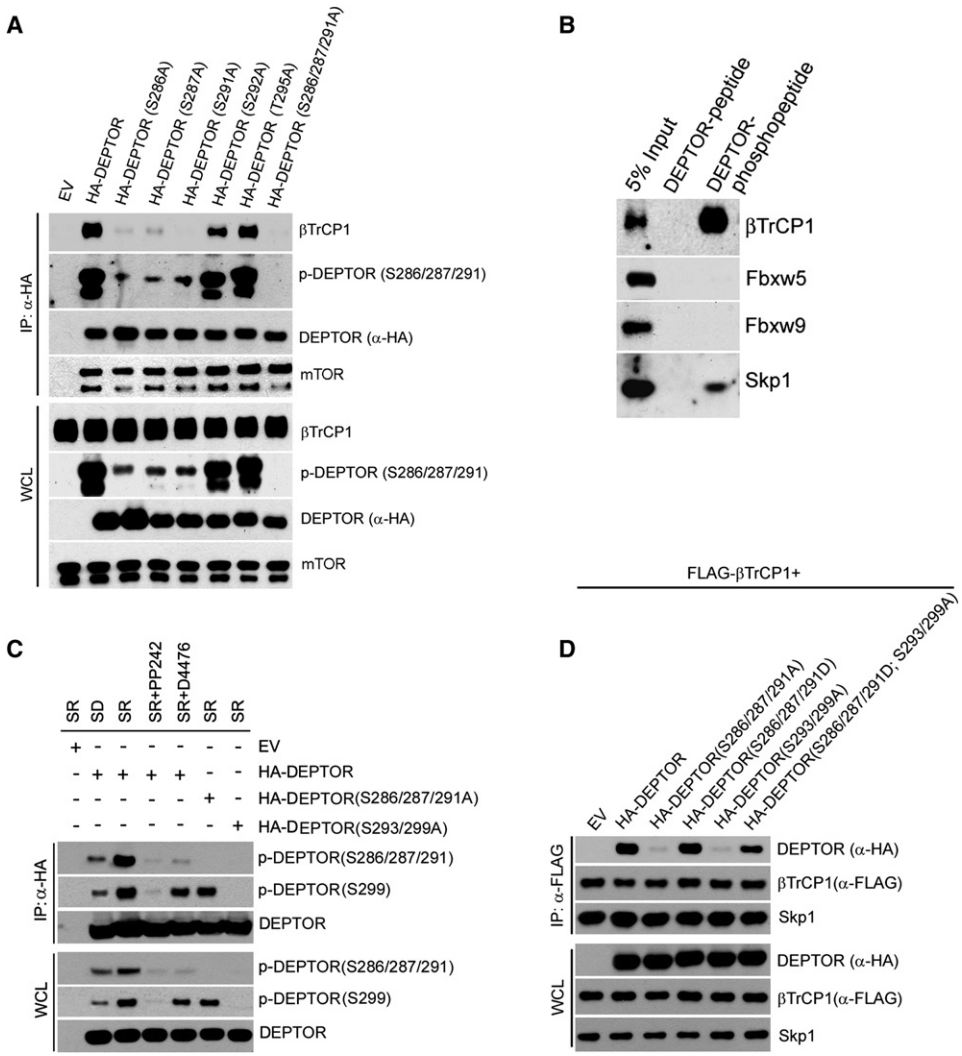

Figure 2. The DEPTOR Degron Is Controlled by Phosphorylation

(A) Ser286, Ser287, and Ser291 are required for the interaction of DEPTOR with βTrCP. HEK293T cells were transfected with an empty vector (EV) or the indicated HA-DEPTOR constructs. Forty-eight hours posttransfection, after a serum starvation of 24 hr, cells were restimulated with media containing serum and MG132 for 3 hr prior to harvesting for HA immunoprecipitation and western blotting as indicated. WCL, whole cell lysate.

(B) The DEPTOR degron requires phosphorylation to bind βTrCP1. HEK293T lysates were used in binding reactions with beads coupled to a peptide containing the sequence SSGYFS (lane 2) or the phosphomotif pSpSGYFpS (lane 3). Beads were washed with lysis buffer, and bound proteins were eluted and subjected to SDS-PAGE and immunoblotting.

(C) In vivo phosphorylation of DEPTOR on Ser286/287/291/299 is induced by mitogens. HEK293T cells were transfected with the indicated HA-tagged DEPTOR constructs. Following a serum deprivation (SD) of 24 hr, cells were stimulated with serum (SR) for 3 hr in the presence or absence of PP242 or D4476 as indicated. Whole cell lysates (WCL) were immunoprecipitated and immunoblotted as indicated.

(D) Ser293 and Ser299 are required for the interaction of DEPTOR with βTrCP. HEK293T cells were transfected with FLAG-βTrCP1 and either an empty vector (EV) or the indicated HA-DEPTOR constructs. Forty-eight hours posttransfection, after a serum starvation of 24 hr, cells were restimulated with media containing serum and MG132 for 3 hr prior to harvesting for immunoprecipitations and immunoblotting as indicated. WCL, whole cell lysate.

To confirm the role of phosphorylation in the interaction of DEPTOR with βTrCP, we used immobilized, synthetic peptides containing the candidate degron sequence to test binding to βTrCP1. Although a peptide containing phosphorylated Ser286, Ser287, and Ser291 efficiently bound βTrCP1 (but not Fbxw5 or Fbxw9), a corresponding nonphosphorylated peptide was unable to bind βTrCP1 (Figure 2B). Accordingly, λ-phosphatase treatment of βTrCP1 immunoprecipitates abolished the interaction with DEPTOR (Figure S2C). These results, together with the analysis of point mutants (Figure 2A), the crystal structure of the βTrCP1-β-catenin complex (Wu et al., 2003), and the modeling of the βTrCP1-BimEL degron interaction (the BimEL and DEPTOR degrons are identical as shown in Figure S2B) (Dehan et al., 2009), indicate that phosphorylation of all three serine residues in the DEPTOR degron (Ser286, Ser287, and Ser291) is necessary for—and directly mediates—the interaction with βTrCP.

To further investigate DEPTOR phosphorylation, we used a phosphospecific antibody against the pSpSGYFpS degron motif. This antibody recognized wild-type DEPTOR, but not a DEPTOR(S286/287/291A) mutant (Figure 2A). Additionally, DEPTOR point mutants displayed decreasing levels of detection, suggesting that all three serines are phosphorylated and contribute to recognition by this antibody. Significantly, we found that DEPTOR was phosphorylated on its degron in HEK293T cells in response to stimulation with serum, but it was poorly phosphorylated in serum-starved HEK293T cells (Figure 2C).

Several βTrCP substrates, such as β-catenin, Cdc25A, Emi1, Snail, Wee1, and YAP, are phosphorylated on their degrons only after an initial phosphorylation event that either allows binding to or exposure of a previously masked site for a second kinase (Frescas and Pagano, 2008; Hunter, 2007). To investigate whether a similar mechanism controls phosphorylation of the DEPTOR degron, we mutated a number of residues flanking the degron. Mutation of Ser279, Ser280, Ser292, Thr295, Ser297, and Ser298 to Ala, singly or in combination, did not inhibit DEPTOR binding to βTrCP1 (Figures 2A and S2D). In contrast, mutation of Ser293 or Ser299, or both, strongly reduced the interaction between βTrCP and DEPTOR, even in serum-stimulated cells (Figures 2D and S2D). Additionally, a DEPTOR phosphomimic mutant, in which Ser286, Ser287, and Ser291 in the degron are mutated to Asp (DEPTOR(S286/287/291D)), retains the ability to bind βTrCP1 even when Ser293 and Ser299 are mutated to Ala (Figure 2D). The ability of the phosphomimic degron mutant of DEPTOR to bind βTrCP, together with the phosphopeptide experiment in Figure 2B, demonstrates that Ser293 and Ser299 are dispensable for binding a prephosphorylated degron and suggests that phosphorylation of Ser293 and Ser299 may function to prime the phosphorylation of Ser286, Ser287, and Ser291.

We also used a phosphospecific antibody generated against a DEPTOR peptide C terminal to the degron, with Ser299 phosphorylated. This antibody recognized wild-type DEPTOR, but not DEPTOR(S299A) or DEPTOR(S293/299A) (Figure 2C and data not shown). We found that DEPTOR was phosphorylated on Ser299 in HEK293T cells stimulated with serum, but this site was poorly phosphorylated in serum-starved cells (Figure 2C). Interestingly, of the five serines in DEPTOR that are important for binding to βTrCP, four (Ser286, Ser287, Ser293, and Ser 299) have been previously identified as phosphorylation sites (Peterson et al., 2009; Villén et al., 2007). Additionally, a different study also identified these four serines as sites of phosphorylation that are enriched after proteasome inhibition (Gao et al., 2011 [this issue of *Molecular Cell*]).

To identify the kinase(s) involved in the phosphorylation and degradation of DEPTOR, we performed a candidate search using pharmacologic inhibition. We found that D4476 (a CK1 inhibitor) and PP242 (an mTOR inhibitor) counteracted the destabilizing effect of serum on DEPTOR, whereas GSK3i IX (a GSK3 inhibitor), U0126 (a MEK inhibitor), and API-2 (an Akt inhibitor) had no effect (Figures S3A and S3B). Importantly, D4476 and PP242, but not U0126 and GSK3i IX, inhibited the interaction between DEPTOR and βTrCP and the phosphorylation of the DEPTOR degron (Figures 1B and 2C and data not shown). In agreement with the involvement of mTOR in DEPTOR degradation, we observed that low doses of rapamycin (an mTORC1 inhibitor) and high doses of wortmannin (a PI3K inhibitor that, at high concentrations, inhibits mTOR) induced DEPTOR stabilization (Figures S3B and S3C). We also found that knockdown of mTOR or CK1α (but not CK1δ or CK1ε) resulted in accumulation of DEPTOR (Figures 3A and 3B). Furthermore, silencing of either RAPTOR or RICTOR inhibited the serum-dependent destabilization of DEPTOR, although to a lesser extent than mTOR depletion (Figure 3A), indicating that both mTORC1 and mTORC2 control DEPTOR turnover.

We then used phosphomimic mutants of DEPTOR to study the hierarchy of mTOR- and CK1α-mediated phosphorylation of DEPTOR. The binding of wild-type DEPTOR to endogenous βTrCP is inhibited by either PP242 or D4476 (Figures 1B and 3C), but DEPTOR phoshomimic mutants are differentially responsive to these inhibitors. The binding of DEPTOR(S286/287/291D) to βTrCP is not inhibited by either D4476 or PP242 (Figure 3C). In contrast, the binding of DEPTOR(S293/299D) to βTrCP is not inhibited by PP242 but is still inhibited by D4476 (Figure 3C). These findings suggest that mTOR phosphorylates Ser293 and Ser299 to promote degron phosphorylation by CK1α. Accordingly, although PP242 inhibited the phosphorylation of DEPTOR on both Ser299 and the degron, D4476 was able to inhibit the phosphorylation of degron, but not Ser299 (Figure 2C). Finally, CK1α-mediated stimulation of the DEPTOR-βTrCP interaction was inhibited by PP242 (Figure S3D).

To test whether CK1 and mTOR can phosphorylate DEPTOR on its degron, we performed in vitro kinase assays using recombinant, bacterially expressed, and purified DEPTOR and kinases. CK1 phosphorylated the degron of DEPTOR as shown by western blotting with the phosphospecific antibody (Figures S3E and S3F). In contrast, mTOR alone was unable to induce phosphorylation of DEPTOR on Ser286, Ser287, and Ser291. Importantly, incubation with mTOR enhanced the CK1-dependent phosphorylation of DEPTOR, likely due to mTOR-dependent phosphorylation of Ser293 and Ser299, as no mTOR-dependent enhancement of phosphorylation was observed with DEPTOR(S293/299A). Finally, mTOR, but not CK1, was able to phosphorylate DEPTOR on Ser299 in vitro (Figures 3D and 3E). Accordingly, Torin, a highly specific mTOR inhibitor, inhibits in vivo phosphorylation of Ser293 and Ser299 (Peterson et al., 2009); mTOR phosphorylates Ser293 and Ser299 in vitro; and prephosphorylation of DEPTOR by mTOR enhances its CK1-dependent in vitro phosphorylation (Gao et al., 2011).

Finally, we reconstituted the ubiquitylation of DEPTOR in vitro. Wild-type DEPTOR, but not DEPTOR(S286/287/291A) or DEPTOR(S293/299A), was ubiquitylated only when both βTrCP1 and CK1 were present in the reaction (Figures 3D and S3G). Moreover, in agreement with the phosphorylation data, mTOR enhanced the βTrCP1- and CK1-dependent ubiquitylation of DEPTOR.

Because the above results indicate that mTOR promotes phosphorylation of the DEPTOR degron by CK1α, we further investigated potential molecular mechanisms. Figure S3D shows that CK1α and DEPTOR bind and that treatment of HEK293T cells with PP242 inhibits this binding. Additionally, purified recombinant mTOR strongly stimulates the in vitro binding of CK1α to

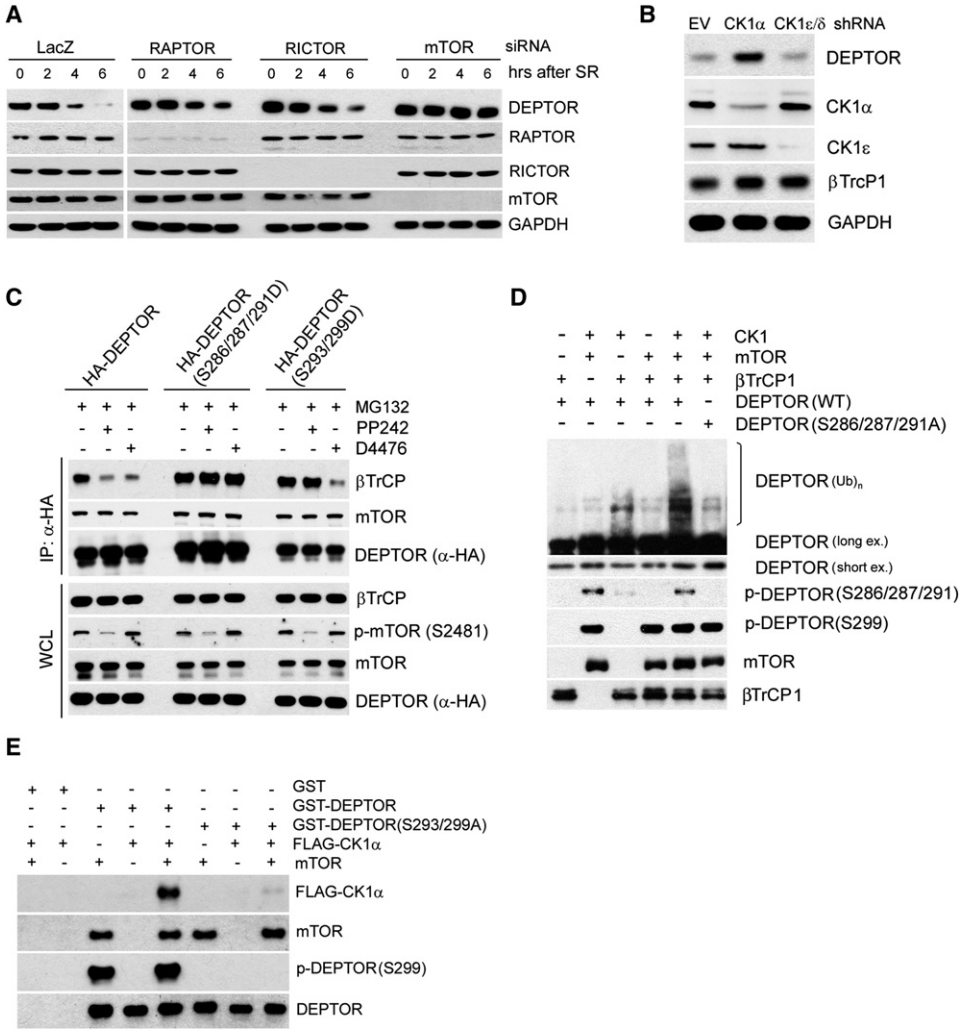

Figure 3. mTOR and CK1α Are Required for DEPTOR Degradation

(A) Inhibition of TORC1 and TORC2 blocks DEPTOR degradation. During a serum starvation of 72 hr, T98G cells were transfected with siRNAs targeting LacZ, RAPTOR, RICTOR, or mTOR. Cells were subsequently restimulated with media containing serum (SR), and samples were harvested at the indicated time points for immunoblotting.

(B) Silencing of CK1α, but not CK1δ or CK1ε, induces DEPTOR accumulation. HeLa cells were infected with an empty lentivirus (EV) or lentiviruses containing shRNA targeting CK1α, CK1δ, or CK1ε. Seven days postinfection, samples were harvested at the indicated time points for immunoblotting.

(C) Differential sensitivity of wild-type DEPTOR and DEPTOR mutants to inhibition of CK1 and mTOR. HEK293T cells were transfected with the indicated HA-DEPTOR constructs. Forty-eight hours posttransfection, after a serum starvation of 24 hr, cells were restimulated with media containing serum and MG132 for 3 hr in the presence or absence of PP242 or D4476 as indicated. Cell lysates were immunoprecipitated and immunoblotted as indicated. WCL, whole cell lysate.

(D) In vitro ubiquitylation assays of recombinant DEPTOR (WT or DEPTOR(S287/287/291A)) were conducted in the presence of the indicated proteins. Samples were incubated at 30°C for 90 min. The bracket (top left) marks a ladder of bands corresponding to polyubiquitylated DEPTOR.

(E) In vitro phosphorylation of DEPTOR by mTOR promotes DEPTOR-CK1α interaction. Recombinant, purified GST-DEPTOR, GST-DEPTOR(S293/299A), or GST alone was incubated with ATP in the presence or absence of purified recombinant mTOR. Reaction products were then diluted, incubated with FLAG-tagged CK1α, purified with GST Sepharose 4B beads, and immunoblotted as indicated.

wild-type DEPTOR, but not to DEPTOR(S293/299A) (Figure 3E), suggesting that phosphorylation of Ser293 and Ser299 in DEPTOR by mTOR generates a binding site for CK1α, thus promoting DEPTOR phosphorylation by CK1α.

Altogether, these data strongly support a model in which, in response to mitogenic stimulation, mTOR phosphorylates DEPTOR on Ser293 and Ser299, thus promoting the CK1α-mediated phosphorylation of DEPTOR on Ser286, Ser287, and Ser291, facilitating βTrCP binding, SCF$^{\beta TrCP}$-mediated ubiquitylation, and consequent degradation. Therefore, inhibition of βTrCP-mediated degradation of DEPTOR should lead to increased DEPTOR levels and decreased mTOR activity. This hypothesis was tested in three ways. First, T98G cells were transfected with siRNAs against LacZ or βTrCP and synchronized in G0/G1 by serum starvation (Figure 4A). Following restimulation with serum, DEPTOR levels rapidly decreased in the

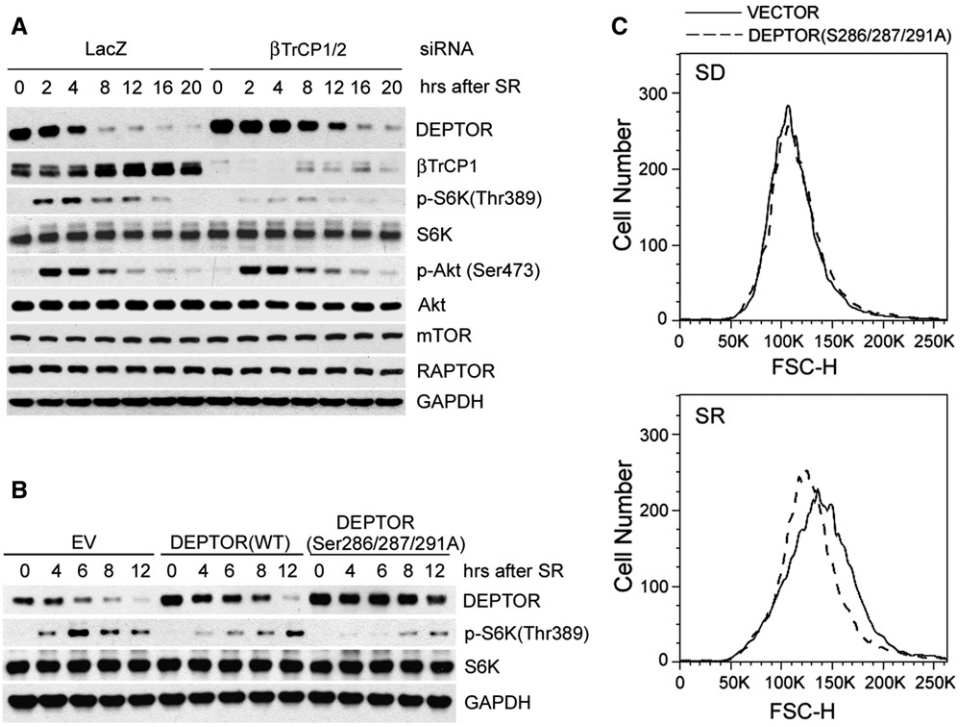

Figure 4. Failure to Degrade DEPTOR Results in mTOR Activation Defects

(A) During a serum starvation of 72 hr, T98G cells were transfected with siRNAs targeting either LacZ or βTrCP1 and βTrCP2. Cells were subsequently restimulated with media containing serum (SR), and samples were harvested at the indicated time points for immunoblotting as indicated.

(B) T98G cells were infected with an empty virus (EV), a retrovirus expressing wild-type DEPTOR, or DEPTOR(S286/287/291A). After a serum deprivation of 72 hr, cells were restimulated with serum (SR) for the indicated times, harvested, and analyzed by immunoblotting.

(C) The experiment was performed as in (B), and cell size was determined by FACS (forward scatter) in cells deprived of serum (SD) and 24 hr after serum addition (SR).

siLacZ-transfected cells but decreased much less in the siβTrCP cells. In accordance with the increased DEPTOR levels in the βTrCP knockdown samples, the induction of phosphorylated S6K1 (Thr389) was severely blunted, demonstrating a decrease in mTOR activation. Second, to confirm that the observed effect of βTrCP knockdown on mTOR activity was mediated through increased DEPTOR levels, we transiently transfected either wild-type DEPTOR or DEPTOR(S286/287/291A) into HeLa cells, which were subsequently serum starved for 24 hr before restimulation with serum. As predicted, in contrast to wild-type DEPTOR, DEPTOR(S286/287/291A) was not degraded when cells were exposed to serum, and the mTOR-mediated phosphorylation of S6K1 in response to serum was strongly inhibited (Figure S4A). Third, virtually identical results were obtained using retroviruses that express DEPTOR(S286/287/291A) at near physiological levels in T98G cells (Figure 4B). Significantly, after serum stimulation, cells expressing DEPTOR(S286/287/291A) displayed reduced cell size relative to cells expressing wild-type DEPTOR or containing an empty virus (Figures 4C and S4B).

DISCUSSION

Proper regulation of mTOR activity is essential to blocking tumorigenesis, and deregulation of the mTOR pathway at the level of DEPTOR appears common (Peterson et al., 2009). Our study demonstrates that DEPTOR is regulated at the posttranslational level by the SCF$^{\beta TrCP}$ ubiquitin ligase in an mTOR- and CK1α-dependent manner, generating a positive feedback loop that facilitates full activation of mTOR. The mTOR dependence of this auto-amplification loop is reminiscent of the SCF^{Skp2}-mediated degradation of the CDK1/2 inhibitor p27 following phosphorylation of p27 by CDK1 or CDK2 (Frescas and Pagano, 2008), suggesting a common mechanism for the regulation of kinase inhibitors. Conversely, the observed effects of CK1α on DEPTOR demonstrate a noncanonical mechanism for CK1, which typically requires priming phosphorylation at the −3 position. The negative charge of phosphorylated Ser293 and Ser299 may function as acidic-like C-terminal residues to prime the phosphorylation of Ser286/287/91, similar to other CK1 substrates (Marin et al., 2003).

Finally, our results show that pharmacologic inhibition of CK1 increases DEPTOR levels and inhibits mTOR signaling, suggesting that CK1 inhibition may be a viable therapeutic option for the treatment of cancers characterized by low DEPTOR levels and activation of mTOR. Paradoxically, although mTOR activity is required for DEPTOR degradation, multiple myelomas appear to retain both high mTOR activity and high DEPTOR levels. It remains undetermined whether the elevated levels of DEPTOR

in multiple myelomas result solely from transcriptional regulation or whether the βTrCP-mediated degradation of DEPTOR is also perturbed.

EXPERIMENTAL PROCEDURES

Extract Generation and Western Blotting

Extract preparation, immunoprecipitation, and western blotting were performed as previously described (Dehan et al., 2009; Dorrello et al., 2006). The rabbit polyclonal phosphospecific antibody was generated using a peptide containing the phosphodegron sequence pSpSGYFpS. Fbxw5, Fbxw9, and cyclin A rabbit polyclonal antibodies were generated/characterized by the Pagano laboratory. Commercial mouse antibodies included S6K(Thr389) (Cell Signaling), CK1ε (BD), β-Catenin (BD), Actin (Sigma), FLAG (Sigma), HA (Covance), GST (Invitrogen), and Claspin (Peschiaroli et al., 2006). Commercial rabbit antibodies included βTrCP1 (Cell Signaling), Akt (Ser473) (Cell Signaling), Akt (Cell Signaling), S6K (Cell Signaling), GAPDH (Cell Signaling), mTOR (Cell Signaling), DEPTOR (Millipore), DEPTOR(Ser299) (Cell Signaling), CK1α (Cell Signaling), RICTOR (Bethyl), RAPTOR (Millipore), β-Catenin (Ser33/37/Thr41) (Cell Signaling), and Skp1 (Invitrogen).

Plasmids, siRNAs, and shRNAs

DEPTOR mutants were generated using QuikChange Site-Directed Mutagenesis Kits (Stratagene). All cDNAs were completely sequenced. Transient transfections of HEK293T cells were performed using polyethylenimine (PEI). Additional cell lines were transfected using Lipofectamine 2000 (Invitrogen). siRNAs were transfected using Metafectene Pro (Biontex). The LacZ and βTrCP siRNAs were previously validated and described (Dehan et al., 2009; Dorrello et al., 2006; Fong and Sun, 2002). RAPTOR, RICTOR, mTOR siRNAs, and CK1α were from Sigma (SASI_Hs02_00366683, SASI_Hs01_00048380, SASI_Hs01_00203144, and TRCN0000006042, respectively). The Precision-LentiORF shRNA vector targeting CK1δ and CK1ε contained the sequence GGGCTTCTCCTATGACTAC. Retrovirus-mediated gene transfer was previously described (Guardavaccaro et al., 2008; Peschiaroli et al., 2006).

Cell Lines, Serum Starvation, and Drug Treatments

Human embryonic kidney 293T (HEK293T), HeLa, and T98G cells (ATCC) were used as indicated. All cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. All cells were starved for the indicated time periods in 0.1% serum. The following drugs were used: MG132 (Peptides International; 10μM), cycloheximide (Sigma; 100 μg/ml, PP242 (Sigma; 2.5 μM), D4476 (Calbiochem; 50 μM), GSK3i (Calbiochem; 5 μM), Rapamycin (LC labs, 200 nM), Akt inhibitor API-2 (Tocris Bioscience, 1 μM), and U0126 (Calbiochem; 10 μM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.molcel.2011.09.005.

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Supplemental Information

mTOR Generates an Auto-Amplification Loop by Triggering the βTrCP- and CK1β-Dependent Degradation of DEPTOR

Shanshan Duan, Jeffrey R. Skaar, Shafi Kuchay, Alfredo Toschi, Naama Kanarek,

Yinon Ben-Neriah, and Michele Pagano

Supplemental Experimental Procedures

In Vitro Kinase and Binding Assay

Purified recombinant, bacterially-expressed, GST-tagged wild type DEPTOR or DEPTOR(S293/299A) (1.5 μg) was incubated at 30°C for 30 minutes with 2 mM ATP and the indicated kinases [1,000 unit CK1 (New England Biolabs) and/or 300ng mTOR (Millipore)] in 30 μl of kinase buffer [10 mM MOPS, pH 7.6, 1x Magnesium/ATP Cocktail (Upstate), 10 mM MnCl2, 1.0 mM DTT). Reaction products were subjected to immunoblotting. In Fig. S3E, the indicated amounts of CK1 were used. For in vitro binding assay, 80 ng GST-DEPTOR were first phosphorylated with mTOR. Reaction products were then diluted, incubated with soluble FLAG-tagged CK1α (purified from HEK293T cells), purified with GST sepharose 4B beads, and finally subjected to immunoblotting.

In Vitro Ubiquitylation Assay

DEPTOR ubiqtuititylation was performed in 30 μl kinase buffer containing 0.1 μM E1 (Boston Biochem), 0.25 μM Ubc3, 0.25 μM Ubc5, 1.5 μg/μl ubiquitin (Boston Biochem), and 500 ng purified, recombinant, bacterially-expressed, GST-tagged DEPTOR. βTrCP1 was immunopurified with anti-FLAG M2 affinity gel (Sigma) from HEK293T cells transfected with FLAG-tagged βTrCP1 construct and eluted by competition with FLAG peptide. Where indicated, 1,000 unit CK1 and/or 300 ng mTOR was used. The samples were then incubated at 30°C for the indicated times and analyzed by SDS-PAGE and immunoblotting.

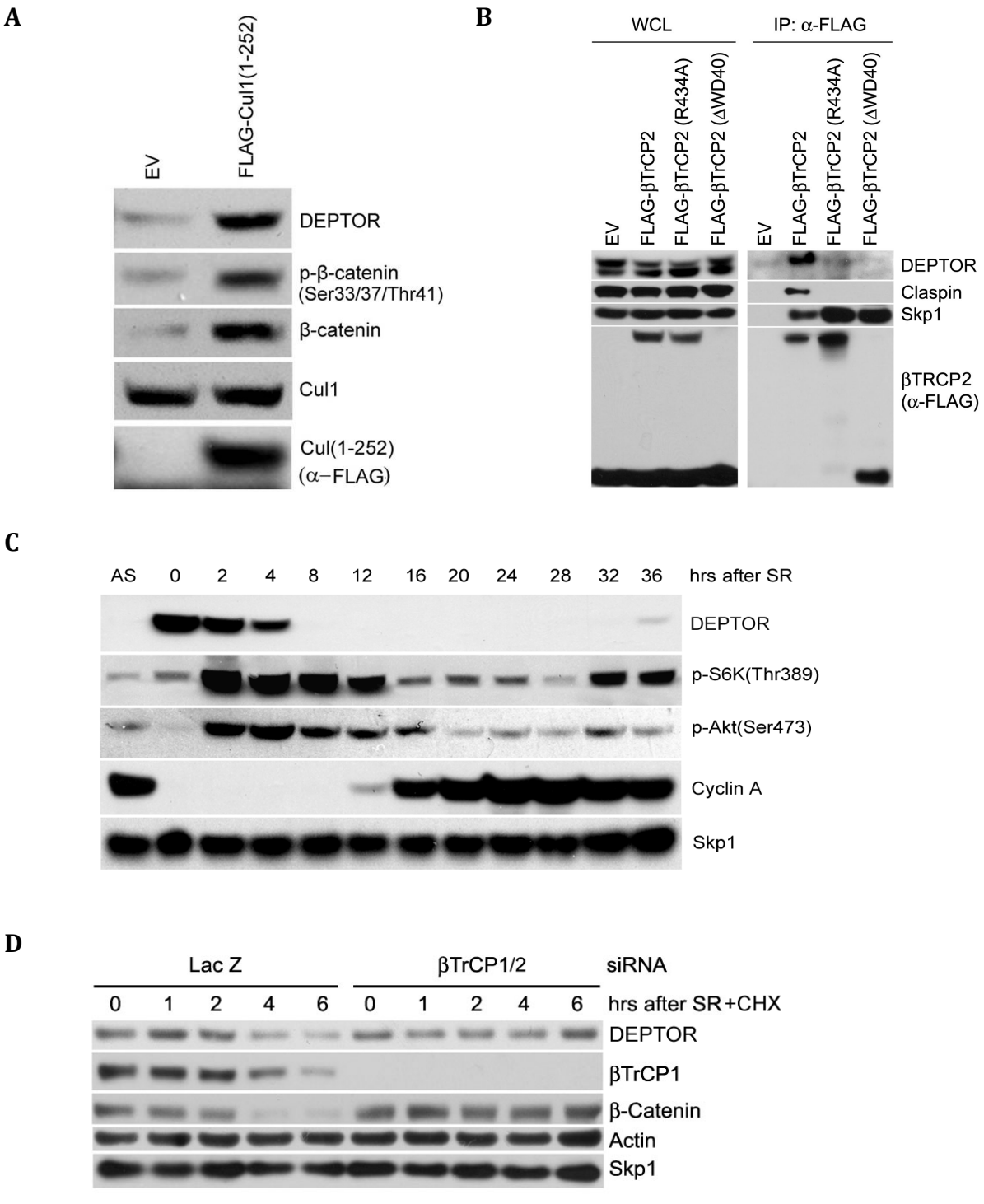

A
EV
FLAG-Cul1(1-252)
DEPTOR
p-β-catenin
(Ser33/37/Thr41)
β-catenin
Cul1
Cul(1-252)
(α−FLAG)
B
WCL
IP: α-FLAG
EV
FLAG-βTrCP2
FLAG-βTrCP2 (R434A)
FLAG-βTrCP2 (ΔWD40)
DEPTOR
Claspin
Skp1
βTRCP2
(α-FLAG)
C
AS 0 2 4 8 12 16 20 24 28 32 36 hrs after SR
DEPTOR
p-S6K(Thr389)
p-Akt(Ser473)
Cyclin A
Skp1
D
Lac Z
βTrCP1/2
siRNA
0 1 2 4 6 0 1 2 4 6 hrs after SR+CHX
DEPTOR
βTrCP1
β-Catenin
Actin
Skp1

Figure S1. DEPTOR Stability Is Controlled by $SCF^{\beta TrCP}$, Related to Figure 1

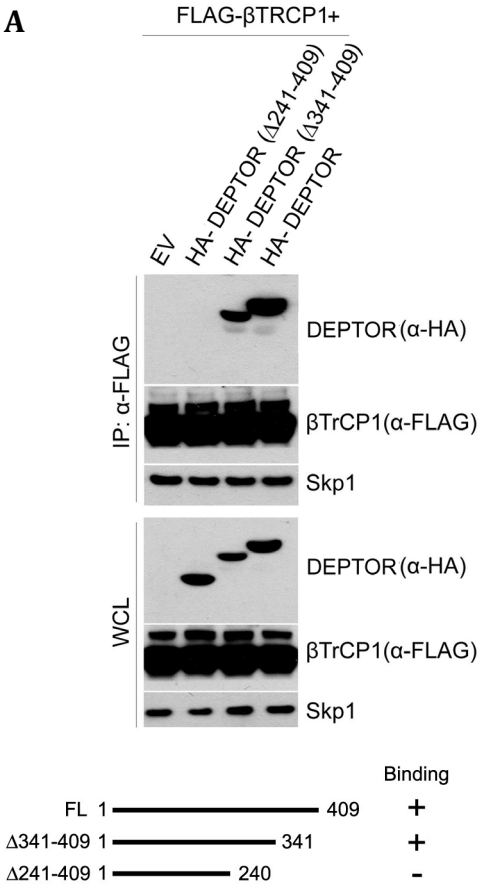
(A) DEPTOR levels increase in response to the expression of dominant negative Cul1. HeLa cells were transfected with an empty vector (EV) or a vector expressing FLAG-tagged Cul1(1-252). Twenty-four hours post-transfection, cells were harvested for western blotting as indicated.

(B) Substrate-binding deficient mutants of βTrCP2 do not bind DEPTOR. HEK293T cells were transfected with either an empty vector (EV) or the indicated βTrCP2 constructs. Forty-eight hours post-transfection, after 16 hours of serum starvation, cells were re-stimulated with media containing serum and MG132 for three hours, prior to harvesting for immunoprecipitations and western blotting as indicated.

(C) DEPTOR levels decrease in response to serum stimulation of T98G cells. T98G cells were serum starved and synchronized in G0/G1 for 72 hours before re-addition of serum (SR). Serum-stimulated cells were harvested at the indicated time points for western blotting. Samples from asynchronous (AS) cells are also included.

(D) βTrCP knockdown increases the half-life of DEPTOR in HeLa cells. HeLa cells were transfected with the indicated siRNAs. Seventy-two hours post-transfection, after 16 hours of serum starvation, cells were re-stimulated with media containing serum and cycloheximide (SR+CHX). Cell lysates were generated at the indicated time points, and western blotting was performed as indicated.

A



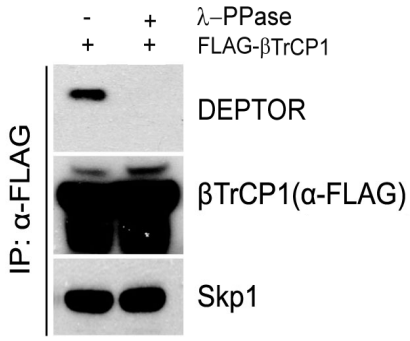
B

βTrCP binding sites:

```
IkBα (Hs)        29-RHDSGLDSMK-38
β-Catenin (Hs)   30-YLDSGIHSGA-40
Claspin (Hs)     27-PSDSGQGSYE-36
Emi1 (Hs)       142-YEDSGYSSFS-152
Per1 (Hs)       119-PSTSGCSSEQ-128
Per2 (Hs)       477-SGSSGYGSLG-486
BimEL (Hs)       91-RSSSGYFSFD-100

Deptor(Hs)      284-CGSSGYFSSS-293
Deptor(Mm)      284-CGSSGYFSSS-293
Deptor(Bt)      302-CGSSGYFSSS-311
Deptor(Cf)      283-CGSSGYFSSS-292
```

C



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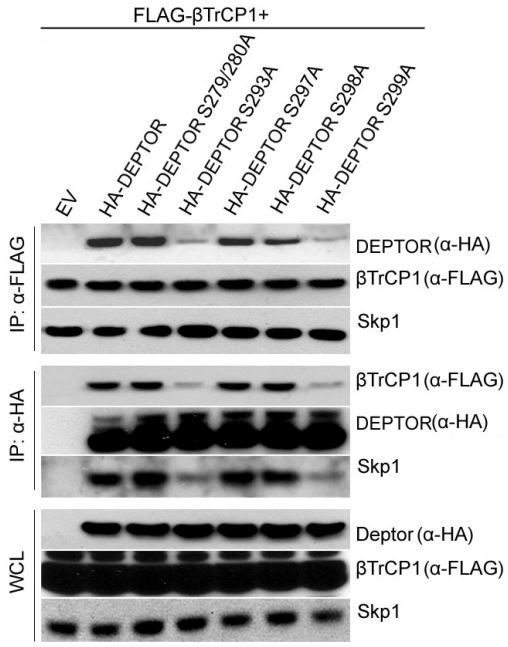


Figure S2. Identification of DEPTOR Phosphodegron, Related to Figure 2

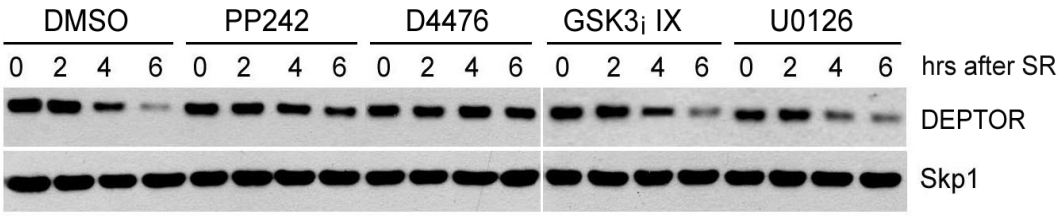
(A) Mapping the βTrCP binding region of DEPTOR. HEK293T cells were transfected with either an empty vector (EV) or the indicated DEPTOR constructs. Forty-eight hours post-transfection, after 16 hours of serum starvation, cells were re-stimulated with media containing serum and MG132 for three hours, prior to harvesting for immunoprecipitations and western blotting as indicated. A schematic of the DEPTOR mutants is shown at the bottom. WCL, whole cell lysate.

(B) DEPTOR contains a conserved, consensus βTrCP degron. An alignment of established βTrCP substrate degrons with the DEPTOR degron from different species is shown.

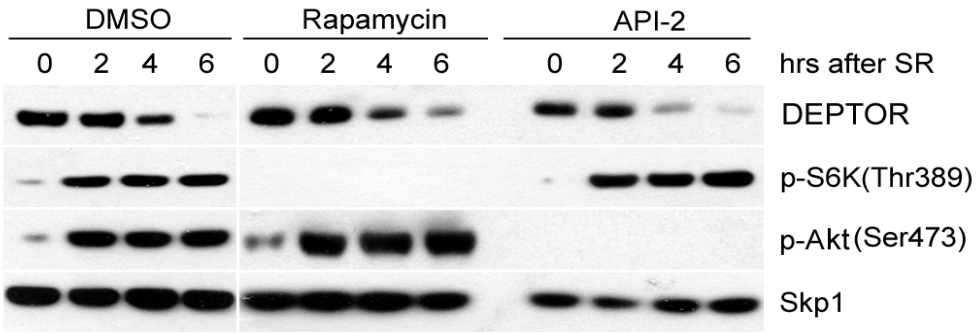
(C) βTrCP binding to DEPTOR is phosphatase sensitive. HEK293T cells were transfected with FLAG-tagged βTrCP1. Forty-eight hours post-transfection, after 16 hours of serum starvation, cells were re-stimulated with media containing serum and MG132 for three hours, prior to harvesting for FLAG immunoprecipitation. FLAG immunoprecipitates were treated with buffer or λ-phosphatase (λ-PPase) for one hour, before extensive washing in lysis buffer. Washed samples were analyzed by western blotting as indicated.

(D) Analysis of serine residues flanking the DEPTOR degron. HEK293T cells were transfected with FLAG-tagged βTrCP1 and either an empty vector (EV) or the indicated HA-tagged DEPTOR constructs. Forty-eight hours post-transfection, after 16 hours of serum starvation, cells were re-stimulated with media containing serum and MG132 for three hours, prior to harvesting for immunoprecipitations and western blotting as indicated. WCL, whole cell lysate.

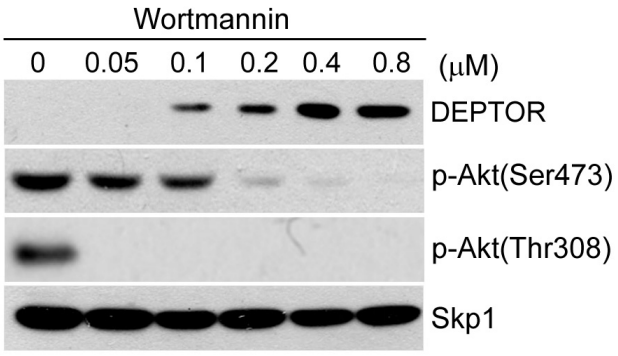
A


DMSO
PP242
D4476
GSK3i IX
U0126
0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
hrs after SR
DEPTOR
Skp1

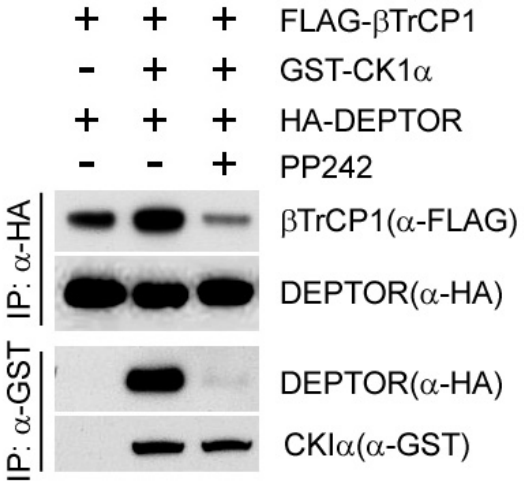
B


DMSO
Rapamycin
API-2
0 2 4 6 0 2 4 6 0 2 4 6
hrs after SR
DEPTOR
p-S6K(Thr389)
p-Akt(Ser473)
Skp1

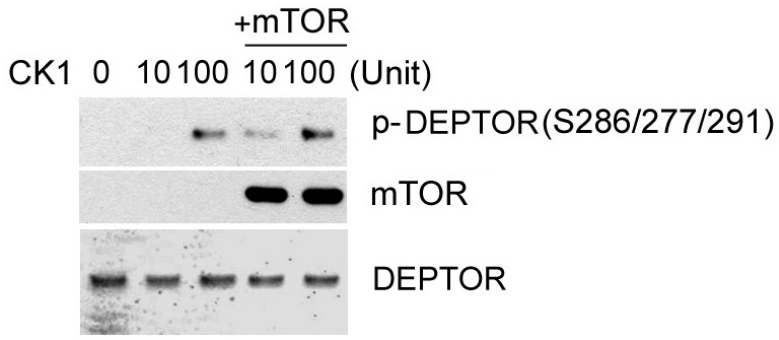
C


Wortmannin
0 0.05 0.1 0.2 0.4 0.8 (μM)
DEPTOR
p-Akt(Ser473)
p-Akt(Thr308)
Skp1

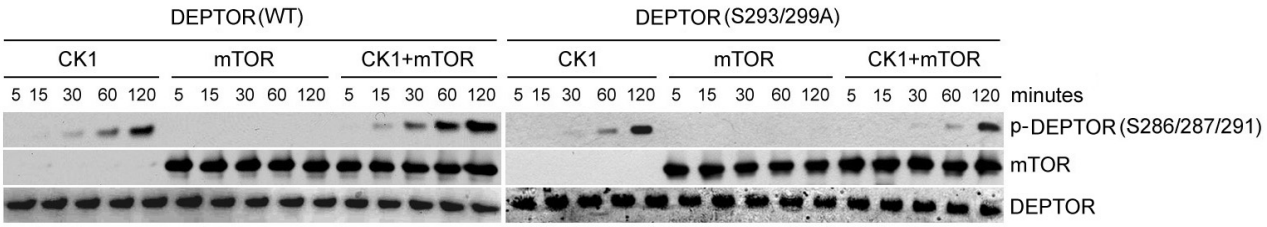
D

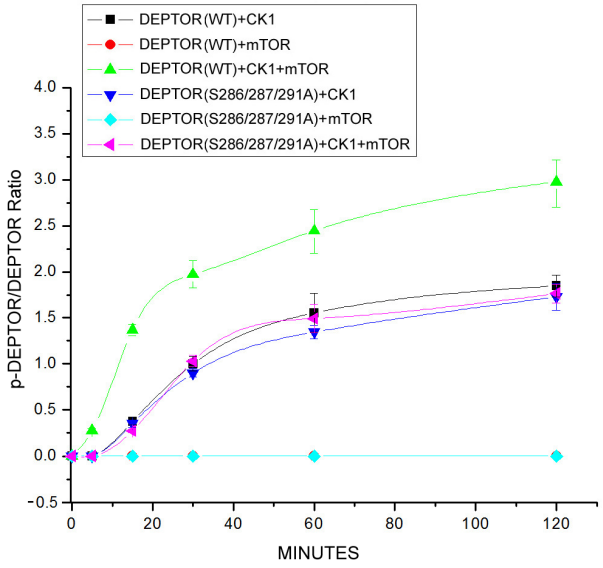

+ + + FLAG-βTrCP1
- + + GST-CK1α
+ + + HA-DEPTOR
- - + PP242
IP: α-HA
βTrCP1(α-FLAG)
DEPTOR(α-HA)
IP: α-GST
DEPTOR(α-HA)
CKIα(α-GST)

E


+mTOR
CK1 0 10 100 10 100 (Unit)
p-DEPTOR(S286/277/291)
mTOR
DEPTOR

F


DEPTOR(WT)
DEPTOR(S293/299A)
CK1
mTOR
CK1+mTOR
5 15 30 60 120
minutes
p-DEPTOR(S286/287/291)
mTOR
DEPTOR


DEPTOR(WT)+CK1
DEPTOR(WT)+mTOR
DEPTOR(WT)+CK1+mTOR
DEPTOR(S286/287/291A)+CK1
DEPTOR(S286/287/291A)+mTOR
DEPTOR(S286/287/291A)+CK1+mTOR
p-DEPTOR/DEPTOR Ratio
MINUTES

G

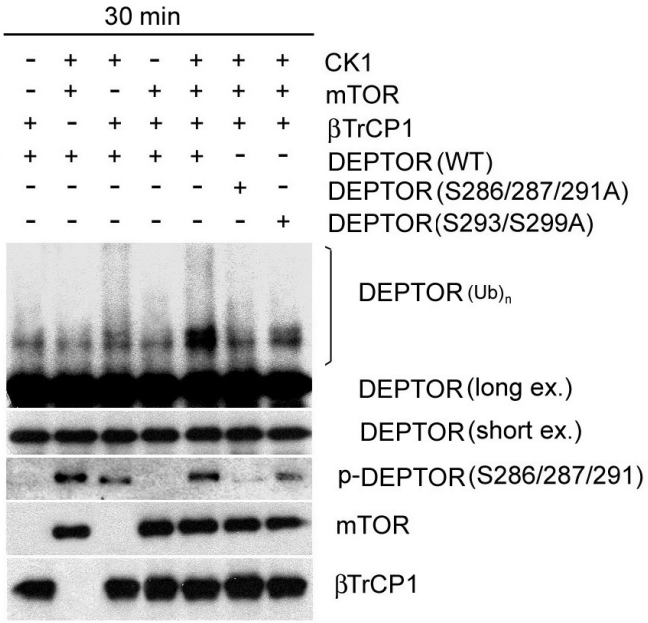

30 min
CK1
mTOR
βTrCP1
DEPTOR(WT)
DEPTOR(S286/287/291A)
DEPTOR(S293/S299A)
DEPTOR (Ub)n
DEPTOR(long ex.)
DEPTOR(short ex.)
p-DEPTOR(S286/287/291)
mTOR
βTrCP1

Figure S3. mTOR and CK1 Cooperate in the Phosphorylation of DEPTOR Degron, Related to Figure 3

(A) Inhibition of mTOR or CK1 blocks DEPTOR degradation. T98G cells were serum starved and synchronized in G0/G1 for 72 hours before re-addition of serum (SR) with DMSO or the indicated kinase inhibitors. Subsequently, serum-stimulated cells were harvested at the indicated time points for western blotting.

(B) Inhibition of mTOR induces DEPTOR stabilization. T98G cells were serum starved and synchronized in G0/G1 for 72 hours before addition of serum (SR) with DMSO or the indicated kinase inhibitors. Subsequently, serum-stimulated cells were harvested at the indicated time points for western blotting.

(C) Asynchronously growing T98G cells were treated with the indicated concentrations of wortmannin. Subsequently, cells were harvested for western blotting.

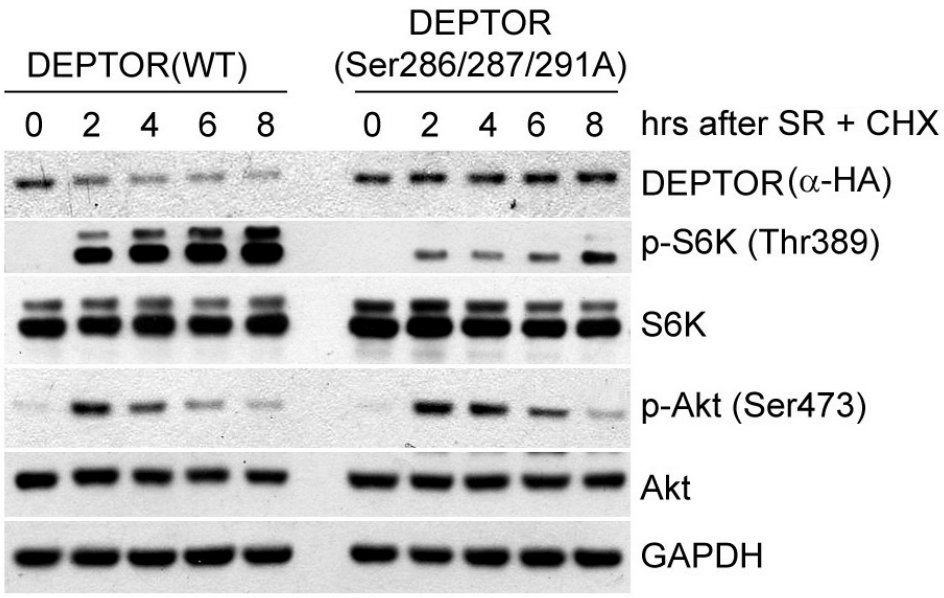
(D) mTOR promotes DEPTOR-CK1α interaction in vivo. HEK293T cells were transfected with HA-DEPTOR, FLAG-βTrCP1, and GST-CK1α constructs as indicated. Forty-eight hours post-transfection, after a 24-hour serum starvation, cells were re-stimulated with media containing serum and MG132 for four hours, in the presence or absence of PP242, as indicated. Lysates were immunoprecipitated and western blotted as indicated. WCL, whole cell lysate.

(E) In vitro phosphorylation of DEPTOR by CK1 is enhanced by mTOR. Recombinant, purified DEPTOR was incubated for 15 minutes with ATP and the indicated, purified kinases. Reaction products were subjected to immunoblotting for the indicated proteins.

(F) In vitro phosphorylation of DEPTOR by CK1 is enhanced by mTOR. Recombinant, purified DEPTOR was incubated for the indicated times with ATP and the indicated purified kinases. Reaction products were immunoblotted for the indicated proteins. DEPTOR phosphorylation was quantified (graph on the bottom) by measuring the intensity of the bands from three different exposures of the experiment shown in the upper blot using Image-Pro Plus 6.0 software (Media Cybernetics). The relative levels were calculated by normalizing the phosphorylated DEPTOR signal versus the total DEPTOR signal for each sample. DEPTOR phosphorylation was quantified (graph on bottom) by measuring the intensity of the bands from three different exposures of the experiment shown in the upper blot using Image-Pro Plus 6.0 software (Media Cybernetics). The error bars represent +/- one standard deviation.

(G) In vitro ubiquitin ligation assays of recombinant DEPTOR [WT, DEPTOR(S287/287/291A) or DEPTOR(S293/299A)] were conducted in the presence of the indicated proteins. Samples were incubated at 30°C for 30 minutes. The bracket on the left side of the top panels marks a ladder of bands corresponding to polyubiquitylated DEPTOR.

A


DEPTOR(WT)
DEPTOR (Ser286/287/291A)
0 2 4 6 8 0 2 4 6 8 hrs after SR + CHX
DEPTOR(α-HA)
p-S6K (Thr389)
S6K
p-Akt (Ser473)
Akt
GAPDH

B

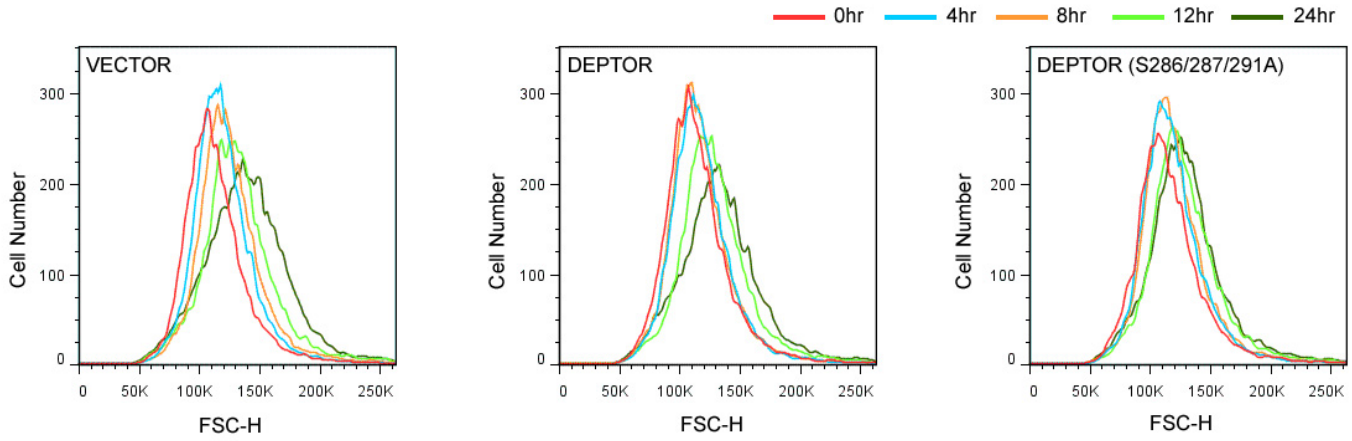

0hr 4hr 8hr 12hr 24hr
VECTOR
DEPTOR
DEPTOR (S286/287/291A)
Cell Number
FSC-H
0 50K 100K 150K 200K 250K
0 100 200 300

Figure S4. Failure to Degrade DEPTOR Results in mTOR Activation Defects, Related to Figure 4

(A) Mutation of the degron blocks DEPTOR degradation and inhibits mTOR. HeLa cells were transfected with HA-tagged wild type DEPTOR or DEPTOR(S286/287/291A). After 24 hours of serum deprivation, cells were re-stimulated with serum (SR) for the indicated times. Cells were then harvested and analyzed by immunoblotting as indicated.

(B) The experiment was performed as in (Fig. 4B), and cell size was determined by FACS (forward scatter) in cells deprived of serum (SD) and at the indicated times after serum addition (SR).