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Supplemental Information

**mTOR Drives Its Own Activation
via SCF ^{β TrCP}-Dependent Degradation
of the mTOR Inhibitor DEPTOR**

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Figure S1, related to Figure 1.

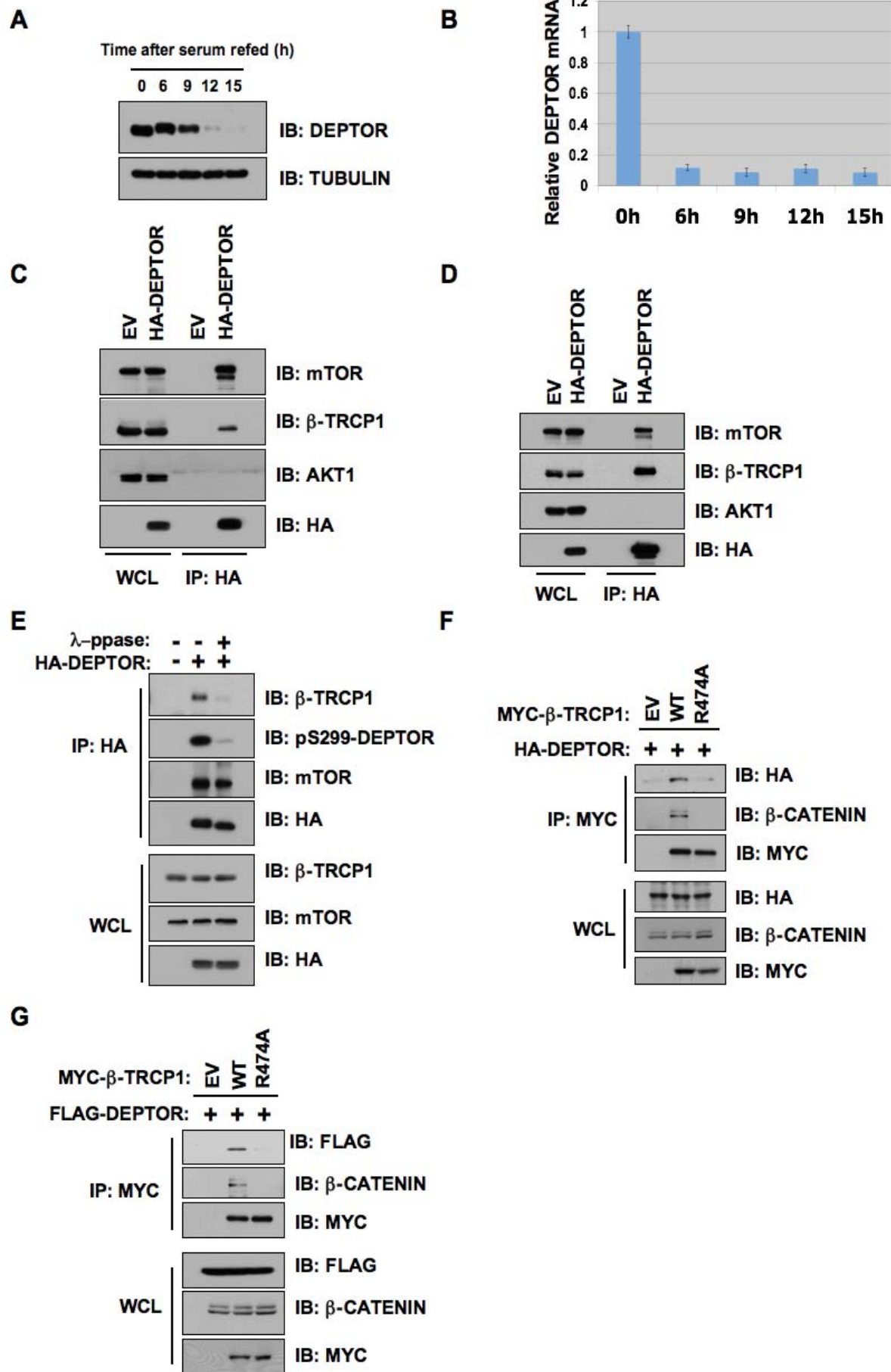


Figure S1. Decreased DEPTOR mRNA Levels Also Contribute to the Rapid Clearance of DEPTOR upon Growth Stimulation in Serum-Starved T98G Cells (Related to Figure 1)

- (A)** Immunoblot (IB) analysis of whole cell lysates derived from T98G cells that were serum-starved for 72 hours and then collected at the indicated time periods following serum re-addition.
- (B)** Real-time RT-PCR analysis to examine the relative DEPTOR mRNA levels in T98G cells that were processed under the same conditions as described in **(A)**. Three independent sets of experiments were performed to generate the error bars. The error bars represent means \pm SD.
- (C and D)** Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa **(C)** or 293T **(D)** cells transfected with HA-DEPTOR. Thirty hours post-transfection, cells were pretreated with 15 μ M MG132 for 10 hours to block the proteasome pathway before harvest.
- (E)** Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with HA-DEPTOR. Thirty hours post-transfection, cells were pretreated with 15 μ M MG132 for 10 hours to block the proteasome pathway before harvest. Where indicated, cell lysate was treated with λ -phosphatase for 30 minutes before immunoprecipitation process.
- (F and G)** Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with the indicated HA-DEPTOR **(F)** or FLAG-DEPTOR **(G)** together with the MYC- β -TRCP1 constructs. Thirty hours post-transfection, cells were pretreated with 15 μ M MG132 for 10 hours to block the proteasome pathway before harvest.

Figure S2, related to Figure 2.

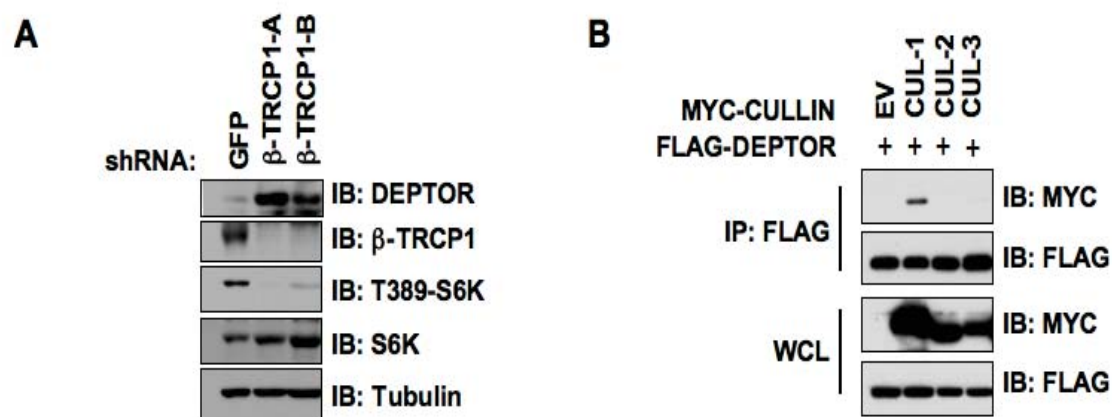


Figure S2. DEPTOR Binds CUL1 (Related to Figure 2)

(A) Immunoblot analysis of whole cell lysates derived from early passage primary human foreskin fibroblast cells infected with the indicated lentiviral shRNA vectors. The infected cells were selected by 2 $\mu\text{g/ml}$ puromycin for 48 hours before harvesting for immunoblot analysis.

(B) Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with FLAG-DEPTOR and the indicated MYC-CULLIN constructs. Thirty hours post-transfection, cells were pretreated with 15 μM MG132 for 10 hours to block the proteasome pathway before harvest.

Figure S3, related to Figure 4.

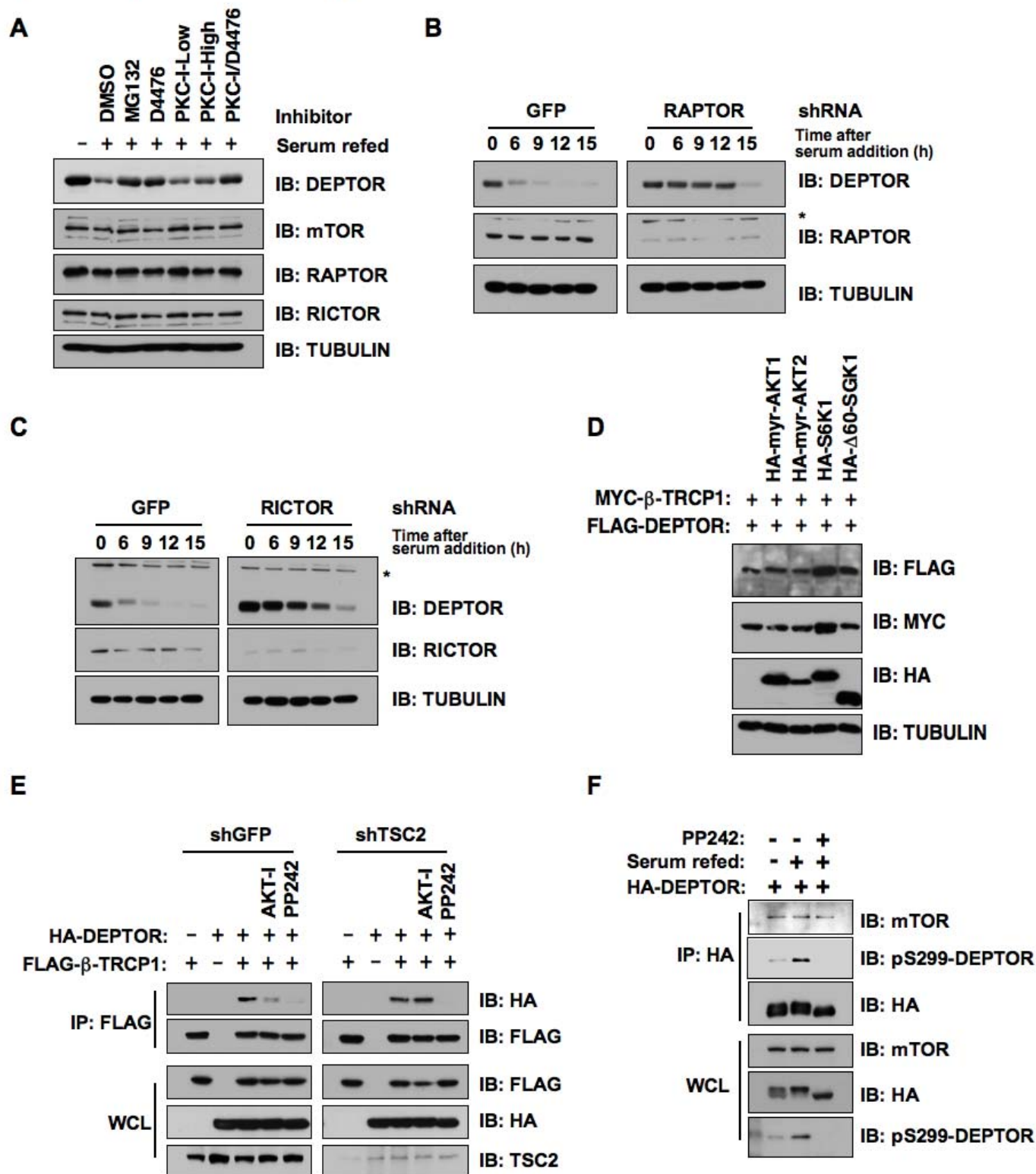


Figure S3. DEPTOR Abundance Is Negatively Regulated by the mTOR Signaling Pathway

(Related to Figure 4)

- (A)** Immunoblot (IB) analysis of whole cell lysates derived from HeLa cells that were serum-starved for 24 hours and then collected 16 hours following serum re-addition. Where indicated, proteasome inhibitor MG132 or the indicated kinase inhibitor was added together with serum. DMSO was used as a negative control. As indicated, two concentrations of PKC inhibitor (PKC-I) were used in the assay. PKC-I-Low: 1 μ M, PKC-I-High: 10 μ M.
- (B and C)** T98G cells were infected with the indicated lentiviral shRNA constructs for 24 hours. Non-infected cells were then eliminated by selection with 2 μ g/ml puromycin for 48 hours. The resulting cell lines were serum-starved for 72 hours and 10% FBS was added to the serum-starved cells for the indicated time period before harvesting. Equal amounts of whole cell lysates were immunoblotted with the indicated antibodies. * indicates the appearance of non-specific bands.
- (D)** Immunoblot analysis of whole cell lysates derived from HeLa cells transfected with the FLAG-DEPTOR, MYC- β -TRCP1 and the indicated HA-tagged AGC kinase constructs.
- (E)** Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from shGFP or shTSC2 HeLa cells transfected with FLAG- β -TRCP1 and HA-DEPTOR constructs. Thirty hours post-transfection, cells were pretreated with 15 μ M MG132 for 10 hours to block the proteasome pathway before harvest. Where indicated, cells were treated with the indicated kinase inhibitors as well for 10 hours before harvest.
- (F)** Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with HA-DEPTOR. Where indicated, cells were serum starved for 24 hours. Starved cells were re-fed with 10% FBS containing medium in the presence or absence of 1 μ M PP242 for 1 hours before harvesting.

Figure S4, related to Figure 6.

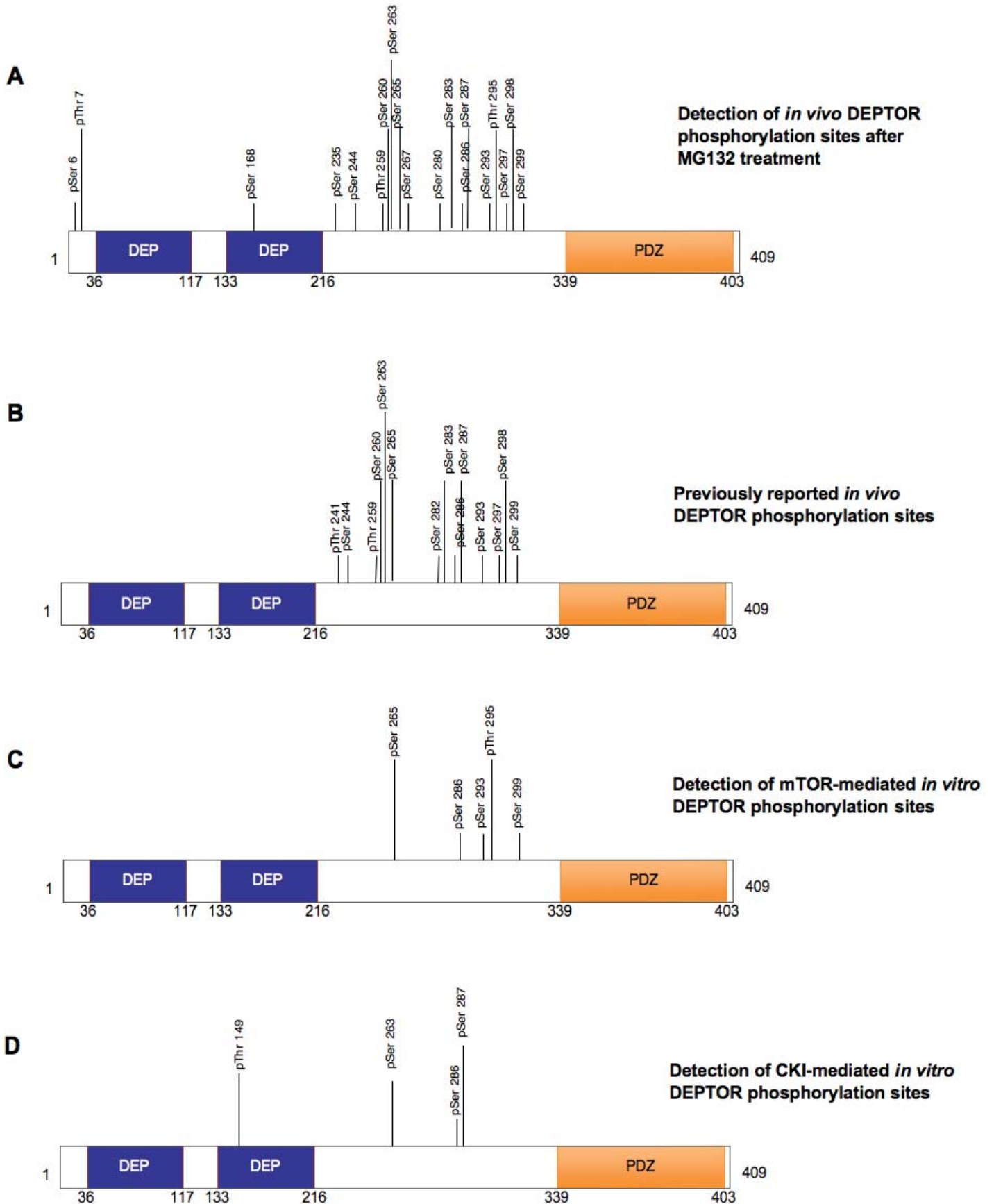


Figure S4, related to Figure 6.

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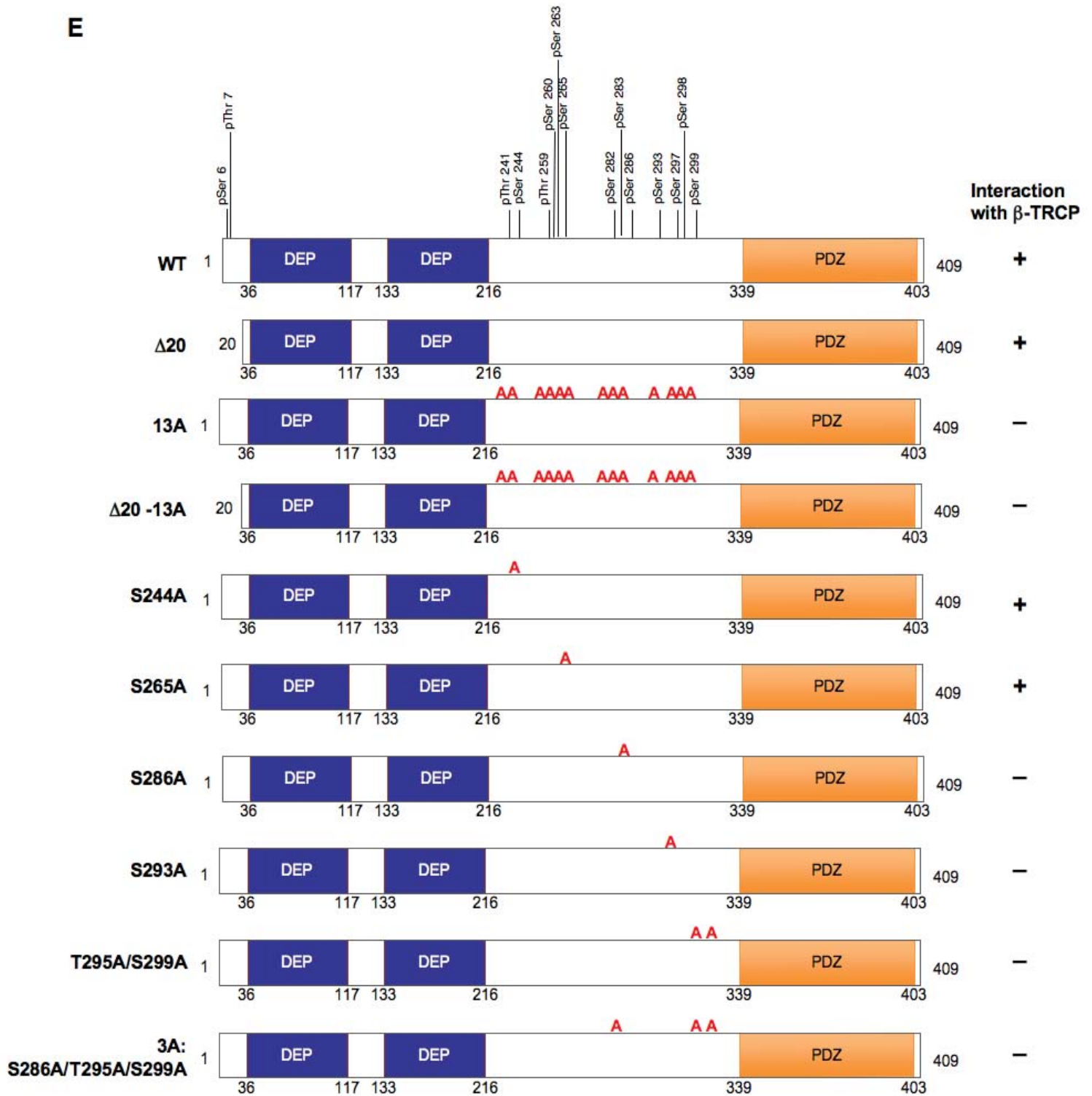


Figure S4, related to Figure 6.

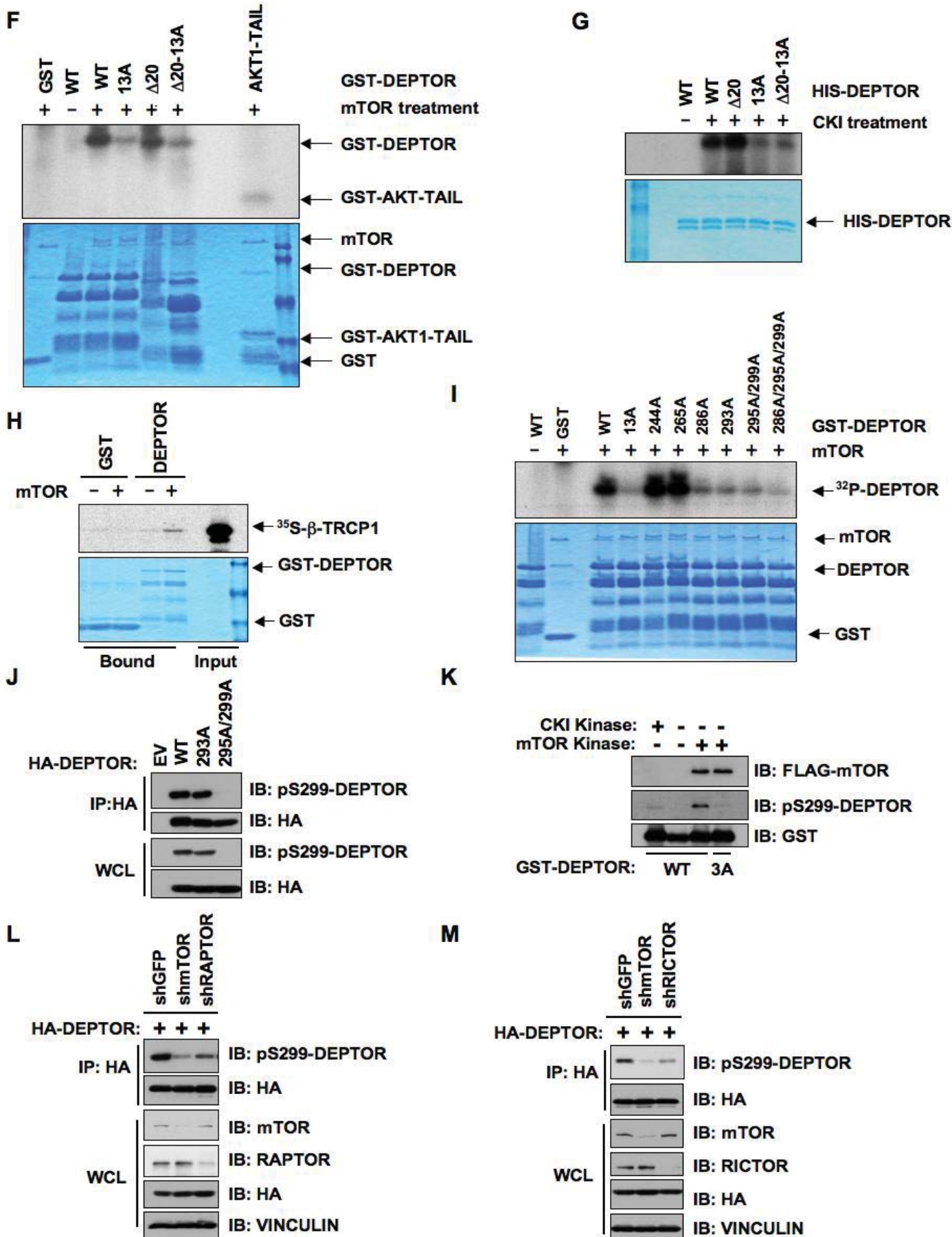


Figure S4, related to Figure 6.

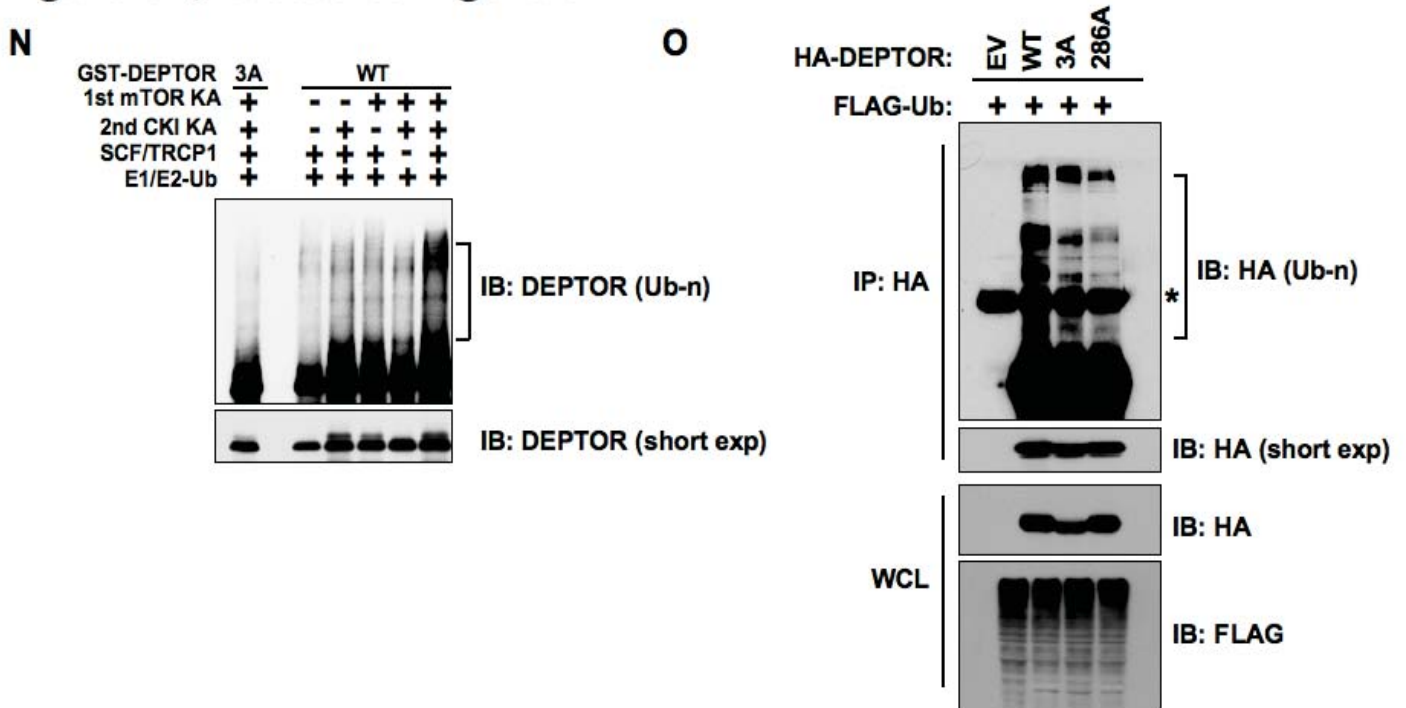


Figure S4. Multisite Phosphorylation of DEPTOR by mTOR and CKI (Related to Figure 6)

- (A) Schematic representation of *in vivo* DEPTOR phosphorylation sites in 293T cells treated with the proteasomal inhibitor MG132 (with DMSO as a negative control) detected by mass spectrometry analysis.
- (B) Schematic representation of the reported phosphorylation sites in DEPTOR.
- (C) Schematic representation of *in vitro* phosphorylation of GST-DEPTOR by mTOR detected by mass spectrometry analysis.
- (D) Schematic representation of *in vitro* phosphorylation of GST-DEPTOR by CKI detected by mass spectrometry analysis.
- (E) Schematic representation of the various DEPTOR mutants used in this study.
- (F) Purified mTOR kinase was incubated with 3 μ g of the indicated GST-DEPTOR proteins in the presence of γ - 32 P-ATP. The kinase reaction products were separated by SDS-PAGE, and phosphorylation was detected by autoradiography. GST was used as a negative control while GST-AKT1 was used as a positive control in this assay.

- (G) Purified CKI kinase was incubated with 3 μg of the indicated His-DEPTOR proteins in the presence of $\gamma\text{-}^{32}\text{P}\text{-ATP}$. The kinase reaction products were separated by SDS-PAGE, and phosphorylation was detected by autoradiography. GST was used as a negative control.
- (H) Autoradiograms showing the recovery of ^{35}S -labelled $\beta\text{-TRCP1}$ protein bound to GST-DEPTOR (with GST as a negative control) incubated with mTOR before the pull-down assay.
- (I) Purified mTOR kinase was incubated with 3 μg of the indicated GST-DEPTOR proteins in the presence of $\gamma\text{-}^{32}\text{P}\text{-ATP}$. The kinase reaction products were separated by SDS-PAGE, and phosphorylation was detected by autoradiography. GST was used as a negative control in this assay.
- (J) Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with the indicated HA-DEPTOR constructs to validate the specificity of pS299-DEPTOR antibody.
- (K) Purified mTOR or CKI kinase were incubated with 1 μg of the indicated GST-DEPTOR proteins in the presence of ATP. The kinase reaction products were separated by SDS-PAGE, and phosphorylation was detected by p-S299-DEPTOR antibody.
- (L) Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with HA-DEPTOR together with the indicated lentiviral shRNA constructs. The cells were selected with 1 $\mu\text{g}/\text{ml}$ puromycin for 48 hours before harvesting.
- (M) Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with HA-DEPTOR together with the indicated lentiviral shRNA constructs. The cells were selected with 1 $\mu\text{g}/\text{ml}$ puromycin for 48 hours before harvesting.

(N) Purified GST-DEPTOR protein was incubated with mTOR (or kinase reaction buffer as a negative control) in the presence of cold ATP for 30 minutes. The reaction product from the first step was then incubated with CKI kinase for 30 minutes. To eliminate mTOR activity in the second step kinase reaction, mTOR inhibitor PP242 (0.2 μ M) was added to the reaction. The kinase reaction products were then incubated with purified SCF ^{β -TRCP} E3 complex together with E1, E2 (UbcH5a and UbcH3) and ubiquitin. The ubiquitination reactions were stopped by addition of SDS sample buffer and resolved by SDS-PAGE for immunoblotting.

(O) Immunoblot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293 cells transfected with FLAG-Ubiquitin together with the various indicated HA-DEPTOR constructs. Thirty hours post-transfection, cells were pretreated with 15 μ M MG132 for 10 hours to block the proteasome pathway before harvest. *: the non-specific band detected by the anti-HA antibody.

Figure S5, related to Figure 7.

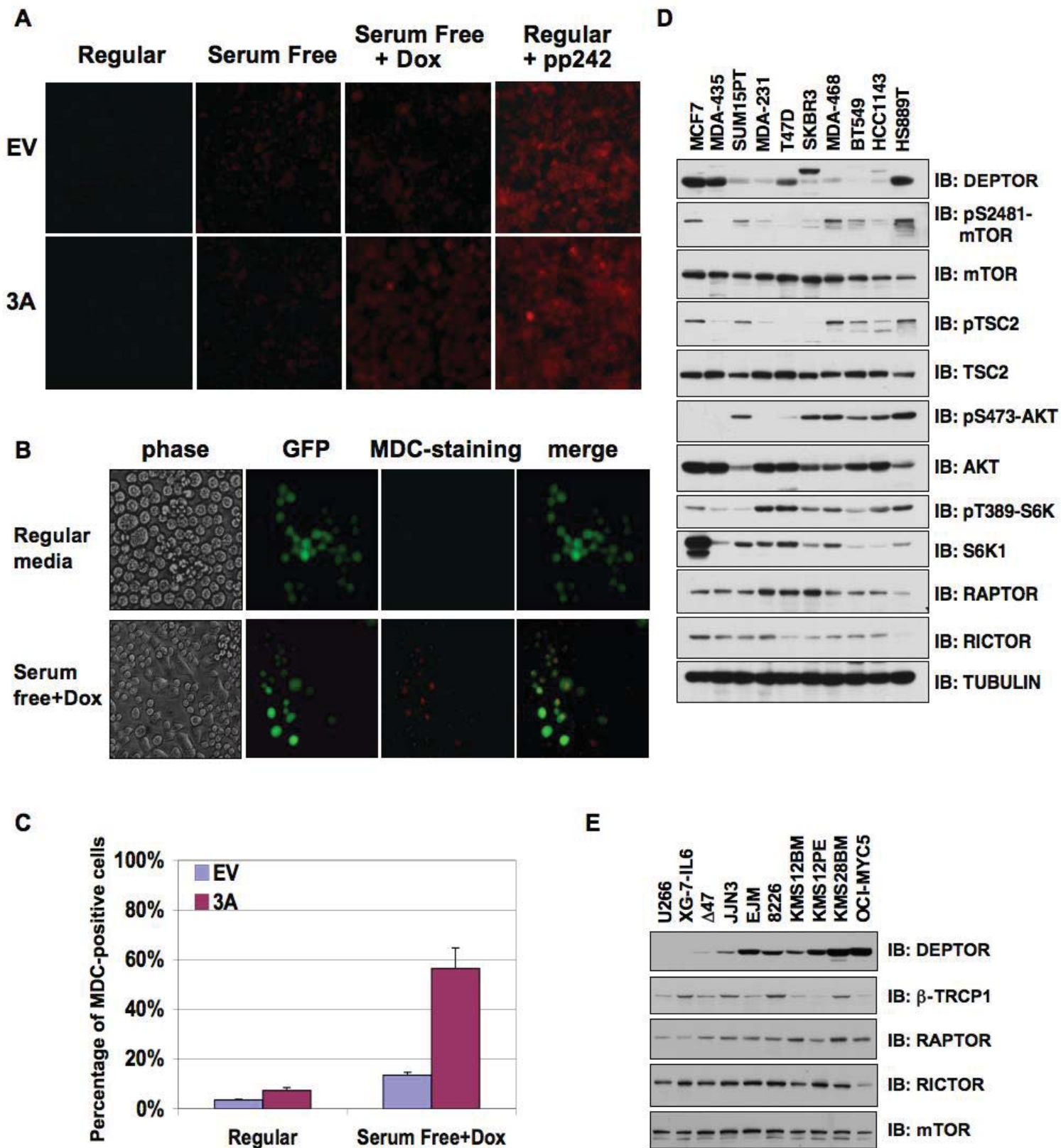


Figure S5, related to Figure 7.

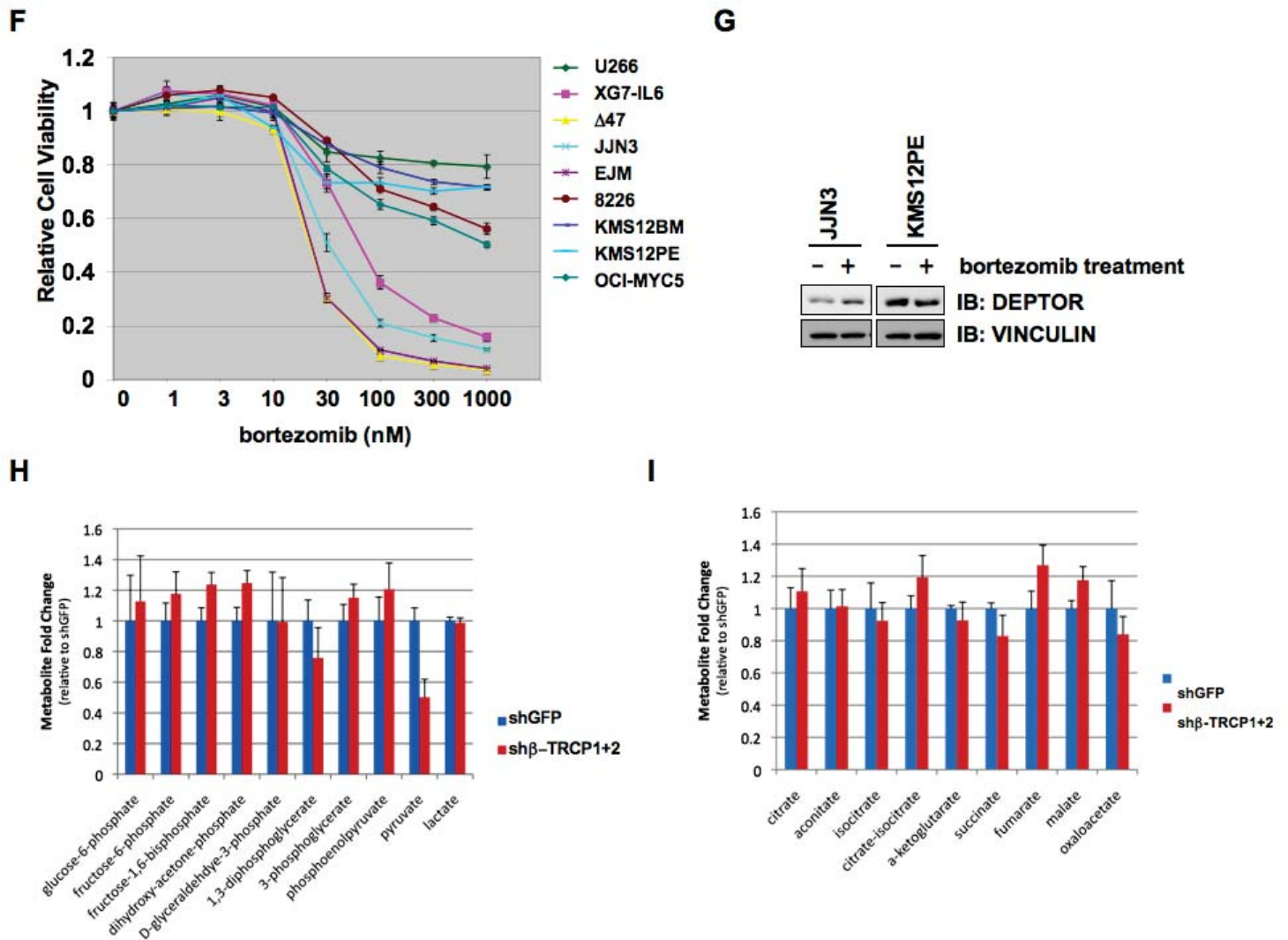


Figure S5. SCF^{β-TRCP}-Mediated Degradation of DEPTOR Governs Cell Growth, Metabolism and Cell Survival Through Modulating the Activity of the mTOR Signaling Pathway (Related to Figure 7)

(A) Engineered Tet-inducible 3A DEPTOR-expressing HeLa cells (with Tet-EV-expressing cells as negative controls) were incubated in normal medium or in serum-free medium for 12 hours to induce autophagy. Where indicated, 1 μM PP242 was added to inhibit mTOR kinase activity to induce autophagy, and 1 μg/ml doxycycline was used to induce the expression of 3A DEPTOR. The resulting cells were then used for monodansylcadaverine (MDC) staining (according to the manufacturer's instruction from Cayman Chemical Company).

- (B)** Engineered Tet-inducible 3A DEPTOR-expressing HeLa cells were infected with pLenti-Hygro-GFP and then selected with 200 µg/ml Hygromycin for 72 hours to eliminate the non-infected cells. The resulting cell line was mixed with the Tet-EV-expressing negative control cells, and the mixed cell population was incubated in either normal medium or in serum-free medium for 12 hours to induce autophagy. Where indicated, 1 µg/ml doxycycline was used to induce the expression of 3A DEPTOR. The resulting cells were then used for monodansylcadaverine (MDC) staining (according to the manufacture's instruction from Cayman Chemical Company).
- (C)** Quantification of the MDC staining positive cells in EV- or 3A-DEPTOR-expressing cells (infected with lentivirus encoding GFP to distinguish from the EV-expressing cells) under the indicated experimental conditions in **B**. Three microscopy fields with over 500 cells per field were counted to generate the data point for each column. The error bars represent means \pm SD.
- (D)** Immunoblot analysis of whole cell lysates derived from a panel of breast cancer cell lines.
- (E)** Immunoblot analysis of whole cell lysates derived from a panel of multiple myeloma cancer cell lines.
- (F)** Cell viability assays showing that low-DEPTOR-expressing multiple myeloma cells were more sensitive to Bortezomib treatment. The indicated multiple myeloma cancer cell lines were cultured in 10% FBS-containing medium with the indicated concentrations of bortezomib for 16 hours before performing cell viability assays. Data was shown as means \pm SD for three independent experiments.
- (G)** Immunoblot analysis of whole cell lysates derived from multiple myeloma cancer cell lines, where indicated, cells were treated with 50 nM bortezomib for 4 hours before harvesting.
- (H and I)** Effect of β -TRCP depletion on the status of metabolites in the glycolysis (**H**) or the TCA (**I**) metabolic pathway.

Supplemental Experimental Procedures

Plasmids

Plasmids to express FLAG-DEPTOR (both wild type and 13A mutant) and pLKO-shRNA against TSC2 (Vander Haar et al., 2007) were obtained from Addgene. To generate the HA- and GST-tagged DEPTOR constructs, DEPTOR cDNA was subcloned into the pcDNA3-HA and pGEX-4T1 vectors, respectively. Plasmids expressing MYC- β TrCP1, MYC-R474A- β TrCP1, FLAG- β TrCP1 and HA tagged AGC kinases (AKT, S6K, SGK) were described previously (Gao et al., 2010; Inuzuka et al., 2010). Point mutations were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. To generate the inducible DEPTOR expression construct, 3A-DEPTOR cDNA was cloned into pTRIZ vector (Open Biosystems). The pBabe-puro-mCherry-GFP-LC3B construct was described previously (Pankiv et al., 2007).

Antibodies and Reagents

Polyclonal and monoclonal DEPTOR antibodies were purchased from Millipore (09-463) and Novus (H00064798-M01). Anti c-MYC antibody (SC-40), polyclonal anti-HA antibody (SC-805), anti-CKI α antibody (sc-6477) and anti-CKI δ antibody (sc-20709) were purchased from Santa Cruz. Anti-mTOR antibody (2972), anti-mTOR-pS2481 antibody (2974), anti-G β L antibody (3274), anti-pERK antibody (4370), anti-pS6-240/244 antibody (4838), anti-TSC2 antibody (3990), anti-pT1462-TSC2 antibody (3617), anti-RAPTOR antibody (2280), anti-S6K antibody (9202), anti-phospho-Ser473-AKT antibody (4051), anti-phospho-Thr389-S6K antibody (9205), anti-LC3B (3868) antibody, anti- β TrCP antibody (4394) were purchased from Cell Signaling. Anti-RICTOR antibody (A300-459A) and anti-SIN1 antibody (A300-910A) were purchased from Bethyl. Anti-SGK1 antibody (KAP-PK015) was from Stressgen. Anti-phospho-Thr1135-RICTOR antibody was a kind gift from Cell Signaling. Anti-

TUBULIN antibody (T-5168), polyclonal anti-FLAG antibody (F2425), monoclonal anti-FLAG antibody (F-3165), anti-VINCULIN antibody (V4505), peroxidase-conjugated anti-mouse secondary antibody (A4416) and peroxidase-conjugated anti-rabbit secondary antibody (A4914) were purchased from Sigma. Monoclonal anti-HA antibody (MMS-101P) was purchased from Covance. Anti-GFP antibody (632380), monoclonal anti-CUL1 antibody (32-2400), anti- β TrCP1 antibody (37-3400) was purchased from Invitrogen. Anti-p27 antibody (610241) was purchased from BD Bioscience. Anti-pS299-DEPTOR antibody was developed in a collaboration with Cell Signaling Technology. Anti-p62/SQSTM1 (PM045) antibody was purchased from MBL International.

Immunoblots and Immunoprecipitation

Cells were lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail set I and II, Calbiochem). Protein concentrations of the whole cell lysates were measured using the Bio-Rad protein assay reagent on a Beckman Coulter DU-800 spectrophotometer. The lysates were then resolved by SDS-PAGE and immunoblotted with indicated antibodies. For immunoprecipitation, 800 μ g lysates were incubated with the appropriate antibody (1-2 μ g) for 3-4 hours at 4 °C followed by an one-hour incubation with Protein A sepharose beads (GE Healthcare). Immuno-complexes were washed five times with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) before being resolved by SDS-PAGE and immunoblotted with indicated antibodies. Cell lysate was treated with λ -phosphatase (New England Biolabs) for 30 minutes as indicated before immunoprecipitation process.

Inhibitors and Kinases

Proteasome inhibitor MG132 (working concentration: 10 μ M), CKI inhibitor D4476 (working concentration: 50 μ M), CKI inhibitor IC261 (working concentration: 50 μ M), general mTOR inhibitor rapamycin (working concentration: 20 nM for mTORC1 and 100 nM for mTORC2/mTORC1), PI3K inhibitor LY294002 (working concentration: 20 μ M), GSK3 inhibitor (working concentration: 10 μ M) and AKT inhibitor VIII (working concentration: 10 μ M) were purchased from Calbiochem. ERK inhibitor U0126 (working concentration: 10 μ M) was purchased from Cell Signaling. mTOR specific inhibitor PP242 (Feldman et al., 2009) (working concentration: 1 μ M) was purchased from Sigma-Aldrich. S6K inhibitor PF-4708671 (Pearce et al., 2010) (working concentration: 10 μ M) and PKC inhibitor Go 6983 (working concentration: 10 μ M) were purchased from Tocris Bioscience. mTOR kinase (475987) was purchased from EMD Chemicals. CKI kinase was obtained from New England Biolabs.

siRNAs and shRNAs

E2F-1 scramble siRNA oligos were purchased from Dharmacon. siRNA oligos targeting SKP2 and CDH1 have been described previously (Wei et al., 2004). siRNA oligos were transfected into sub-confluent cells using Oligofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (Wei et al., 2004; Wei et al., 2005).

Lentiviral shRNA vectors for depletion of RICTOR and RAPTOR were purchased from Addgene. To generate the Hygromycin-pLKO-shDEPTOR construct, the following sequence was cloned into the pLKO Hygro-lentiviral vector (DEPTOR: sense, 5'-GCCATGACAATCGGAAATCTA-3'). pLKO lentiviral expression vectors for depletion of CDH1 (Gao et al., 2009a), S6K, and SGK were kind gifts from Dr. William Hahn at the Dana-Farber Cancer Institute. The pLKO lentiviral vector to knock

down AKT1 and a control shRNA targeting GFP were described previously (Gao et al., 2009b). The pLKO lentiviral vectors to deplete β TrCP1, β TrCP2 and CUL1 were obtained from the Dana Farber/Harvard Cancer Center DNA Resource Core. β TrCP1-A: sense, 5'-GCGTTGTATTTCGATTTGATAA-3'; β TrCP1-B: sense, 5'-GCTGAACTTGTGTGCAAGGAA-3'; β TrCP1-C: sense, 5'-GCGTTTCAATAATGGCATGAT-3'; β TrCP1-D: sense, 5'-CCATTAAAGTTGCGGTATTTA-3'; β TrCP2-A: sense, 5'-TCGTA CTCTCAATGGGCACAA-3'; β TrCP2-B: sense, 5'-GAACGAATGGTACGCACTGAT-3'; β TrCP2-C: sense, 5'-GTCCAGTAAATTGCTAAGTAA-3'; β TrCP2-D: sense, 5'-CCATCAGAAGGAACTATCAA-3'; CUL1-A: sense, 5'-GATTTGATGGATGAGAGTGTA-3'; CUL1-B: sense, 5'-GCCAGCATGATCTCCAAGTTA-3'; CUL1-C: sense, 5'-CGTGGTTATATCAGTTGTCTA-3'; CUL1-D: sense, 5'-GCACACAAGATGAATTAGCAA-3'. To generate the lentiviral shRNA constructs to specifically deplete endogenous SKP2, FBW7, and β TrCP1+2, the following sequences were cloned into the pLKO lentiviral vector (SKP2-A: Sense, 5'-AAGGTCTCTGGTGTGGTTGTAAG-3'; SKP2-B: Sense, 5'-AAGCATGTACAGGTGGCTGTT-3'; FBW7: 5'-AACCTTCTCTCTGGAGAGAGAAA-3'; β TrCP1+2: 5'-AAGTGGAATTTGTGGAACATC-3'). The corresponding lentiviruses were packaged and generated in 293T cells for infection as described before (Irie et al., 2005).

Detection of DEPTOR Phosphorylation Sites In Vivo

To map DEPTOR phosphorylation sites *in vivo*, 293T cells were transfected with HA-DEPTOR using the calcium phosphate method. Thirty hours post-transfection, 293T cells were treated with 10 μ M MG132 for 16 hours to block the 26S proteasome pathway prior to collecting the whole cell lysates for HA-immunoprecipitation. After extensive washing with NETN buffer, the HA-immunoprecipitates were separated by SDS-PAGE and visualized by colloidal Coomassie Blue. The band containing HA-

DEPTOR was excised and treated with DTT to reduce disulfide bonds and iodoacetamide to derivatize cysteine residues. In-gel digestion of the protein was done using trypsin. The resulting peptides were extracted from the gel and analyzed by nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Dibble et al., 2009; Inuzuka et al., 2010).

In Vitro mTOR Kinase Assay

Three μg of GST-DEPTOR proteins (wild type or mutant) was incubated with 150 ng of mTOR kinase (EMD Chemicals) in the presence of 5 μCi [γ - ^{32}P] ATP and 200 μM cold ATP for 30 minutes. The reaction was stopped by the addition of SDS-containing lysis buffer, resolved on SDS-PAGE, and detected by autoradiography. Alternatively, the kinase reaction was performed with cold ATP and the reaction products were separated by SDS-PAGE and visualized by colloidal Coomassie Blue. The band containing GST-DEPTOR was excised and treated with DTT to reduce disulfide bonds and iodoacetamide to derivatize cysteine residues. In-gel digestion of the protein was done using trypsin. The resulting peptides were extracted from the gel and analyzed by nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Dibble et al., 2009; Inuzuka et al., 2010).

Proteomic Analysis of DEPTOR and βTrCP2 Complexes

For proteomic analysis, 293T cells stably expressing the indicated proteins (as HA-fusions) using an MSCV retroviral vectors were employed for immunoprecipitation, and complexes analyzed by mass spectrometry on a Thermo LTQ-Velos instrument (Sowa et al., 2009). Briefly, $\sim 10^7$ cells were lysed in 4 ml of lysis buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA) with

protease inhibitors (ROCHE), and phosphatase inhibitors. Cleared lysates were filtered through 0.45 μm spin filters (Millipore Ultrafree®-CL) and immunoprecipitated with 30 μl of anti-HA resin (Sigma). Complexes were washed with lysis buffer, exchanged into PBS, eluted with HA peptide and precipitated with 10% TCA. Processing of samples for mass spectrometry as well as analysis of proteomic data using a modified version of *CompPASS* (Comparative Proteomics Analysis Software Suite) were described elsewhere (Behrends et al., 2010; Sowa et al., 2009). For HA- βTrCP complexes cells were either left untreated or treated for 4 h with 1 μM bortezomib (generously provided by Millennium Pharmaceuticals) prior to cell lysis in order to stabilize βTrCP targets.

Metabolic Profiling Assay

Metabolites were analyzed by liquid chromatography/mass spectrometry as described previously (Vander Heiden et al., 2010). 10^6 cells exponentially growing in basal growth media were harvested in 3 mL 80% v/v methanol at dry ice temperatures. Fresh media was added 24 hours and 2 hours prior to the experiment. Insoluble material in lysates was centrifuged at 4000 RPM for 15 minutes and resulting supernatant was evaporated using a refrigerated speed-vac. Samples were resuspended using 20 μL LC/MS grade water for mass spectrometry. 10 μL samples were injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/Sciex) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 251 endogenous water soluble metabolites for analyses of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 300 SRM transitions and were indicated as (posi) or (nega) as appropriate. ESI voltage was 5000V in positive ion mode and -4500V in negative ion mode. The dwell time was 3 msec per SRM transition and the total cycle time was 1.70 seconds. Samples were delivered to the MS via normal phase chromatography using a 2.0 mm i.d x 15 cm Amide XBridge column (Waters) at 285

$\mu\text{L}/\text{min}$. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-3 minutes; 42% B to 0% B from 3-12 minutes; 0% B was held from 12-18 minutes; 0% B to 85% B from 18-22 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate in 95:5 water : acetonitrile (pH=9.0). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (Applied Biosystems).

Autophagy Analysis

HeLa cells that were engineered for either Tet-induced overexpression of DEPTOR or shRNA-mediated depletion of DEPTOR, or expression of mCherry-GFP-LC3B, were cultured in DMEM (high glucose) supplemented with 10% FBS for 24 hours before changing into nutrition deprivation (serum-free or glucose-free) media to induce autophagy. To induce the expression of a non-degradable DEPTOR (3A-DEPTOR), indicated doses of doxycycline were added into the serum free media. Cells were then applied to monodansylcadaverine staining (from Cayman Chemical Company) or fixed with paraformaldehyde for confocal microscopy analysis of the mCherry-GFP-LC3B-expressing cells.

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