

Supplemental Data

DEPTOR Is an mTOR Inhibitor Frequently

Overexpressed in Multiple Myeloma

Cells and Required for Their Survival

Timothy R. Peterson, Mathieu Laplante, Carson C. Thoreen, Yasemin Sancak, Seong A. Kang, W. Michael Kuehl, Nathanael S. Gray, and David M. Sabatini

Supplemental Experimental Procedures

Materials and Cell Lines

Reagents were obtained from the following sources: rabbit polyclonal antibodies to DEPTOR (09-463), raptor (09-217), Akt (05-591), and phospho-S877 raptor (09-107) from Upstate/Millipore; mouse monoclonal antibody to DEPTOR from Novus Biologicals; mouse monoclonal antibodies to rictor and raptor from Assay Designs; antibodies to mTOR, β -catenin, actin, S6K1, c-MAF, as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, phospho-T37/T46 4E-BP1, phospho-S473 Akt/PKB, phospho-T308-Akt, phospho-T346-NDRG1, phospho-S307-IRS-1, IRS-1, PTEN, TSC2, 4E-BP1, cleaved caspase-3, PARP, PDGFR- β , and the c-MYC epitope from Cell Signaling Technology; antibodies to rictor, HA, c-MYC from Bethyl Laboratories; Flag M2 affinity gel, Flag M2 antibody and SGK1 antibodies, ATP, and SYBR Green JumpStart Taq ReadyMix from Sigma Aldrich; mouse monoclonal antibody to mTOR and recombinant IL-6 from BD Pharmingen; protein G Sepharose and anti-sheep secondary antibody from Pierce; DMEM from SAFC Biosciences; rapamycin from LC Labs; MG-132 from Biomol; PreScission protease from Amersham Biosciences; pTREQ Tet-On vector from Clontech; Adenoviral CRE and GFP from University of Iowa Gene Transfer Vector Core; FuGENE 6 and Complete Protease Cocktail from Roche; 4E-BP1 from A.G. Scientific; SuperScript II Reverse Transcriptase, Platinum Pfx Polymerase, SimplyBlue Coomassie G, Silverquest Staining kit, and inactivated fetal calf serum (IFS) from Invitrogen. An antibody to NDRG1 was kindly provided by Dario Alessi (University of Dundee, UK). We have found that the phospho-S877 raptor antibody also recognizes immunoprecipitated phosphorylated DEPTOR and can be used to read out the DEPTOR phosphorylation state.

HEK-293E cells were kindly provided by John Blenis (Harvard Medical School). HeLa, HEK-293E, HEK-293T, HT-29, U87, PC3, MD-MBA-435, and MEFs were cultured in DMEM with 10% Inactivated Fetal Bovine Serum (IFS). The Human Multiple Myeloma cell lines: FR4, XG-7, U266, KMS-12BM, KMS-12PE, PE2, 8226, OCI-MY5, KMS-28BM were provided by the Kuehl lab. The EJM, MM-1S, JJN-3, and Δ 47 Human Multiple Myeloma cell lines were kindly provided by Ken Anderson (Dana Farber Cancer Institute). Human Multiple Myeloma cell lines were cultured in RPMI-1640 with 10% Fetal Bovine Serum (FBS) supplemented with 2 mM glutamine. XG-7 cells were additionally supplemented with 2 ng/ml IL-6. TSC2^{-/-}, p53^{-/-} and TSC2^{+/+}, p53^{-/-} MEFs were kindly provided by David Kwitakowski (Harvard Medical School). PTEN LoxP/LoxP MEFs were generated from PTEN LoxP/LoxP mice kindly provided by Hong Wu (UCLA). To produce PTEN ^{-/-} and PTEN ^{+/+} MEFs, 1 μ l of Adenoviral CRE or Adenoviral GFP at a titer of 1×10^{10} PFU/mL was added to 500,000 PTEN LoxP/LoxP MEFs. Cell lysates were generated 5 days post-infection. The HeLa cell line with doxycycline-inducible DEPTOR

expression was generated by retroviral transduction of HeLa that were previously modified to express rtTA with an inducible DEPTOR cDNA.

Mammalian Lentiviral shRNAs

Lentiviral shRNAs to human raptor, rictor, and mTOR were previously described (Sarbasov et al., 2005). All other shRNAs were obtained from the collection of The RNAi Consortium (TRC) at the Broad Institute (Moffat et al., 2006). These shRNAs are named with the numbers found at the TRC public website:

(http://www.broad.mit.edu/genome_bio/trc/publicSearchForHairpinsForm.php)

Human DEPTOR_1 shRNA: TRC candidate; NM_022783.1-877s1c1

Human DEPTOR_2 shRNA: TRC candidate; NM_022783.1-1101s1c1

Human TSC2_1 shRNA: TRCN0000040178; NM_000548.2-4551s1c1

Human PTEN_1 shRNA: TRCN0000002746; NM_000314.x-1320s1c1

Mouse DEPTOR_1 shRNA: TRCN0000110157; NM_145470.1-1164s1c1

Mouse DEPTOR_2 shRNA: TRCN0000110159; NM_145470.1-1165s1c1

Mouse TSC2_1 shRNA: TRCN0000042727; NM_011647.1-1843s1c1

Human SGK1_1 shRNA: TRCN0000040175; NM_005627.2-964s1c1

Human SGK1_2 shRNA: TRCN0000040176; NM_005627.2-252s1c1

Human c-MAF_1 shRNA: TRCN0000000255; NM_005360.x-1839s1c1

Human c-MAF_2 shRNA: TRCN0000000257; NM_005360.x-1067s1c1

shRNA-encoding plasmids were co-transfected with the Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T using FuGENE 6 transfection reagent as previously described (Sarbasov et al., 2005). Virus containing supernatants were collected at 48 hours after transfection, filtered to eliminate cells, and target cells (e.g., 300,000 HeLa cells or 500,000 8226 cells) infected in the presence of 8 μ g/ml polybrene. For 8226 cells, infected cells were spun at 300g for 1.5 hours before incubating at 37°C for 24 hours. For all cell types, 24 hours after infection, the cells were split into fresh media (e.g., DMEM/10%IFS for HeLa/MEFs; RPMI/10%FBS for 8226/OCI-MY5), selected with 1 μ g/ml puromycin. Five days post-infection, shRNA-expressing cells were analyzed or split again and analyzed 2-3 days later. For adherent cell lines, shRNA-expressing cells were analyzed at 50-75% confluence.

Gene Expression and Mutation Analysis in Human Cancers and Cancer Cell Lines

For quantification of DEPTOR, Integrin β 7, and GAPDH mRNA expression in HeLa, PC3, or 8226 cell lines, total RNA was isolated from cells grown in the indicated conditions and reverse-transcription was performed. The resulting cDNA was diluted in DNase-free water (1:25) before quantification by real-time PCR. mRNA transcript levels were measured using Applied Biosystems 7900HT Sequence Detection System v2.3 software. Data are expressed as the ratio between the expression of DEPTOR or Integrin β 7 and the housekeeping gene GAPDH. The following primers were used for quantitative real-time PCR:

DEPTOR (H. sapiens):

Forward: TTTGTGGTGCAGGAAGTAA Reverse: CATTGCTTTGTGTCATTCTGG

GAPDH (H. sapiens):

Forward: CTCTCTGCTCCTCCTGTTTCGAC Reverse: TGAGCGATGTGGCTCGGCT

Integrin β 7 (H. sapiens):

Forward: TGGAGCGCTGCCAGTCACCATT

Reverse: CGTCTGAAGTGAACACCAGCAGC

For meta-analysis of DEPTOR mRNA expression in human cancers, “DEPDC6” was searched in NCBI GEO and Oncomine gene expression data repositories. Only those studies where data from primary human tumors could be compared with matched unaffected tissue were considered further. Fold change in DEPTOR mRNA was measured by taking the quotient of the mean level of DEPTOR mRNA in unaffected tissue versus that of the tumor sample. Statistical significance was measured by one-tailed, unequal variance T test. Only those studies with $p < 0.05$ were included in the final analysis.

RNA isolation from primary Multiple Myelomas has been described (Zhan et al., 2002). Normalized DEPTOR mRNA expression was clustered according to the translocation/Cyclin D groups classified in (Bergsagel et al., 2005). The Multiple Myeloma gene expression data used in this study was generated on an Affymetrix U133_Plus_2 platform and can be found in its entirety in the NCBI GEO database with the following identifiers: GSE2658 for 559 newly diagnosed, untreated tumors and GSE5900 for 22 normal plasma cells, 12 smoldering myeloma, and 44 MGUS. DEPTOR mRNA expression in 37 relapse tumors was kindly provided by John Shaughnessy (University of Arkansas). Human Myeloma cell line mRNA data have been deposited in an MMRC genomics portal website that is sponsored by the MMRF (www.broad.mit.edu/mmcp).

PTEN mutation status in human cancer cell lines can be found at the following URL: <http://www.sanger.ac.uk/genetics/CGP/CellLines/>.

Supplemental References

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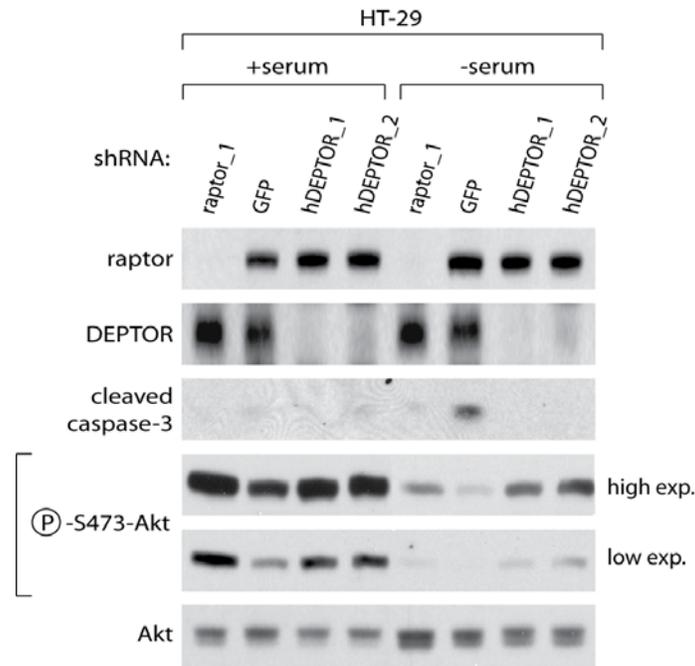


Figure S1. Similar to a Reduction in Raptor Expression, a Reduction in DEPTOR Expression Protects Cells from Apoptosis Induced by Serum Withdrawal

HT-29 cells expressing shRNAs targeting DEPTOR, raptor, or luciferase were serum starved for 6 hours. Cell lysates were analyzed by immunoblotting for the levels of indicated proteins and phosphorylation states.

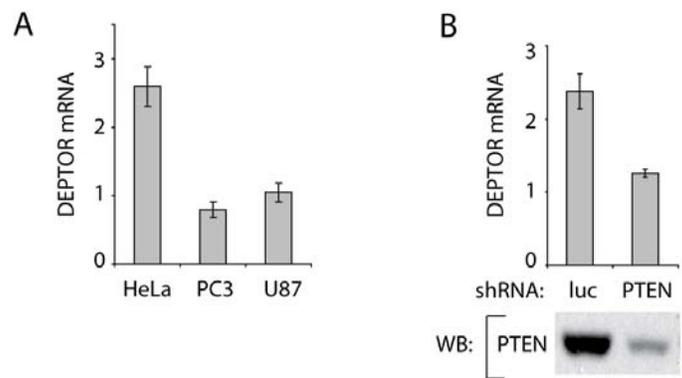


Figure S2. PTEN Positively Regulates DEPTOR mRNA Expression

(A) PTEN Loss Reduces DEPTOR mRNA Expression. HeLa, PC3, and U87 cells were seeded at equal density. 48 hours after seeding DEPTOR mRNA was determined by qRT-PCR from total RNA samples and normalized to GADPH mRNA levels. Error bars indicate mean \pm standard deviation for n=3 per condition.

(B) Expression of an shRNA targeting PTEN in HeLa cells reduces DEPTOR mRNA expression. Five days after transductions with an shRNA-expressing lentivirus, cells were lysed and analyzed by immunoblotting for PTEN levels. DEPTOR mRNA was prepared and analyzed as in (A).

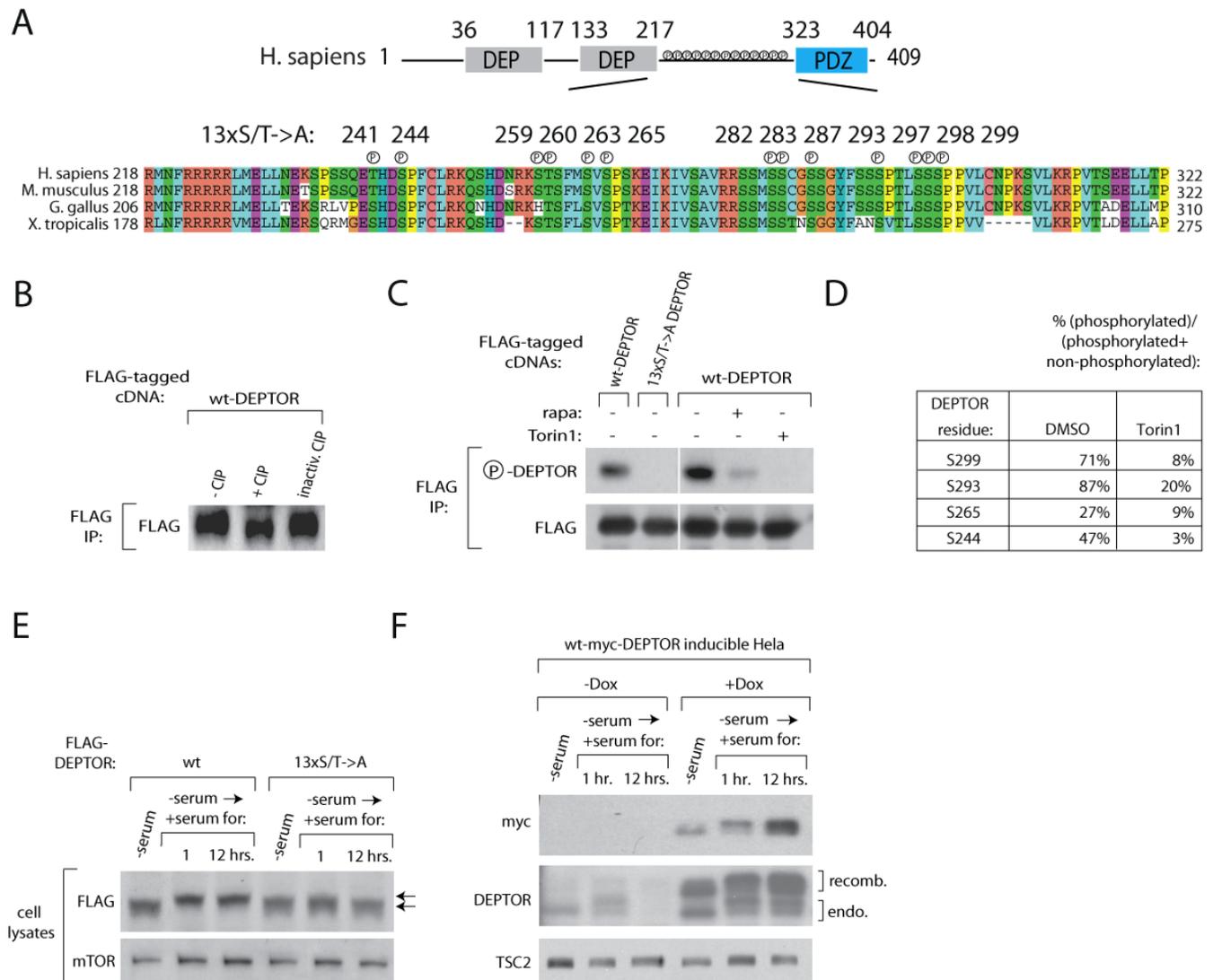


Figure S3. Further Characterization of DEPTOR Phosphorylation and its Functions

(A) Schematic representation of the location of phosphorylation sites identified in DEPTOR by mass spectrometry. All 13 phosphorylation sites were mutated to alanine as described in the methods.

(B) DEPTOR gel mobility is increased by Calf Intestinal Phosphatase (CIP) treatment. Immunoprecipitated wild-type recombinant DEPTOR was incubated without CIP or with active or heat-inactivated CIP for 30 minutes and analyzed by SDS-PAGE followed by immunoblotting.

(C) mTOR inhibitors decrease the recognition of DEPTOR by a phospho-specific antibody. Serum-replete HEK-293T cells expressing FLAG-DEPTOR were treated with vehicle, 50 nM rapamycin, or 250 nM Torin1 for 16 hours. Immunoblotting was performed for the indicated proteins and phosphorylation states. A phospho-specific antibody designed against phospho-S877 raptor cross-reacts with DEPTOR and was used to detect phospho-DEPTOR.

(D) Proline-directed DEPTOR phosphorylation sites serines 244, 265, 293, and 299 are dephosphorylated in the presence of Torin1. Serum-replete HEK-293T cells expressing FLAG-

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DEPTOR were treated with vehicle or 250nM Torin1 for 16 hours. After immunopurification from cell lysates, the extent of phosphorylation at each of the indicated sites was measured using mass spectrometry as described in the methods.

(E) Elimination of the DEPTOR phosphorylation sites impairs the serum-induced mobility shift seen in SDS-PAGE analyses of wild-type DEPTOR. HeLa cells were transfected with 50 ng of the indicated plasmids expressing FLAG-DEPTOR. Three days later, cells were starved for serum for 30 hours and, where indicated, stimulated with serum for the specified times. Cell lysates were analyzed by immunoblotting for levels of recombinant DEPTOR and, as a loading control, mTOR. Arrows indicate serum-induced mobility shifts of wild-type and 13xS/T->A mutant FLAG-DEPTOR.

(F) Overexpression of recombinant wild-type DEPTOR blocks serum-induced degradation of recombinant and endogenous DEPTOR. Myc-tagged DEPTOR was induced by treatment with 10 ng/ml doxycycline in Tet-On HeLa cells. Two days post-induction, non-induced, and induced cells were serum starved for 30 hours and stimulated with serum for the specified times. Cell lysates were analyzed by immunoblotting for the levels of the indicated proteins.

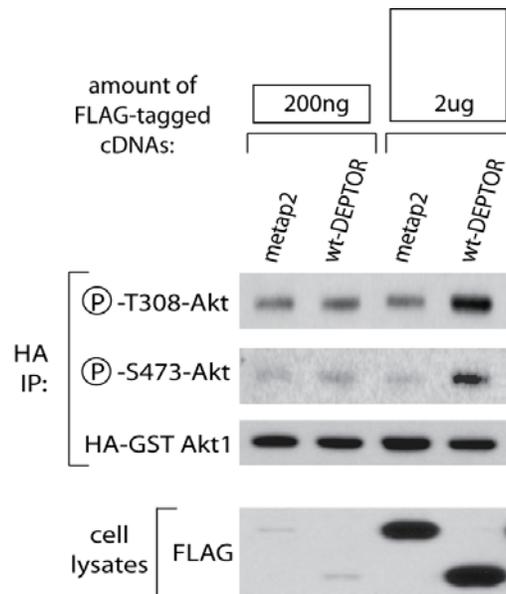


Figure S4. Overexpression of DEPTOR Activates Phosphorylation of T308 and S473 of Akt1 in a Dose-Dependent Manner

p53^{-/-} MEFs cells were cotransfected with expression plasmids encoding HA-GST-Akt1 (200 ng) as well as 200 ng or 2 µg of the indicated FLAG-tagged proteins. Cell lysates were prepared 24 hours after transfection and were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states.

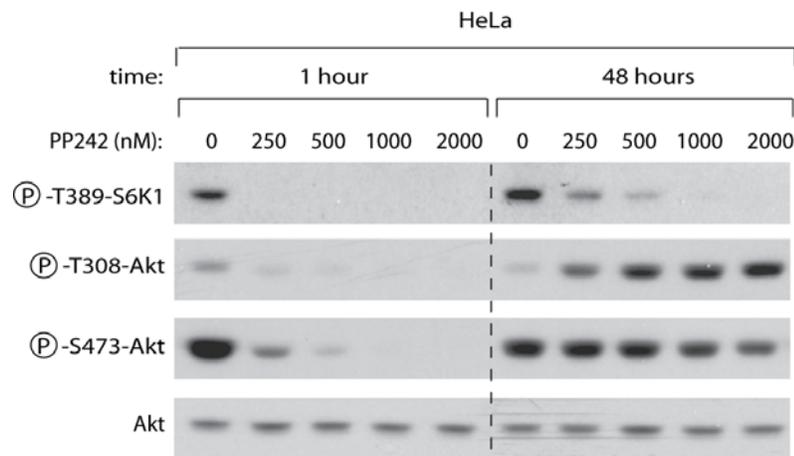


Figure S5. Effects of Prolonged Inhibition of mTOR with the ATP-Competitive Inhibitor PP242 on mTORC1 and PI3K/mTORC2/Akt Signaling

HeLa cells were treated with the specified concentrations of PP242 or vehicle for either 1 hour or 48 hours. Cell lysates were prepared and analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states.

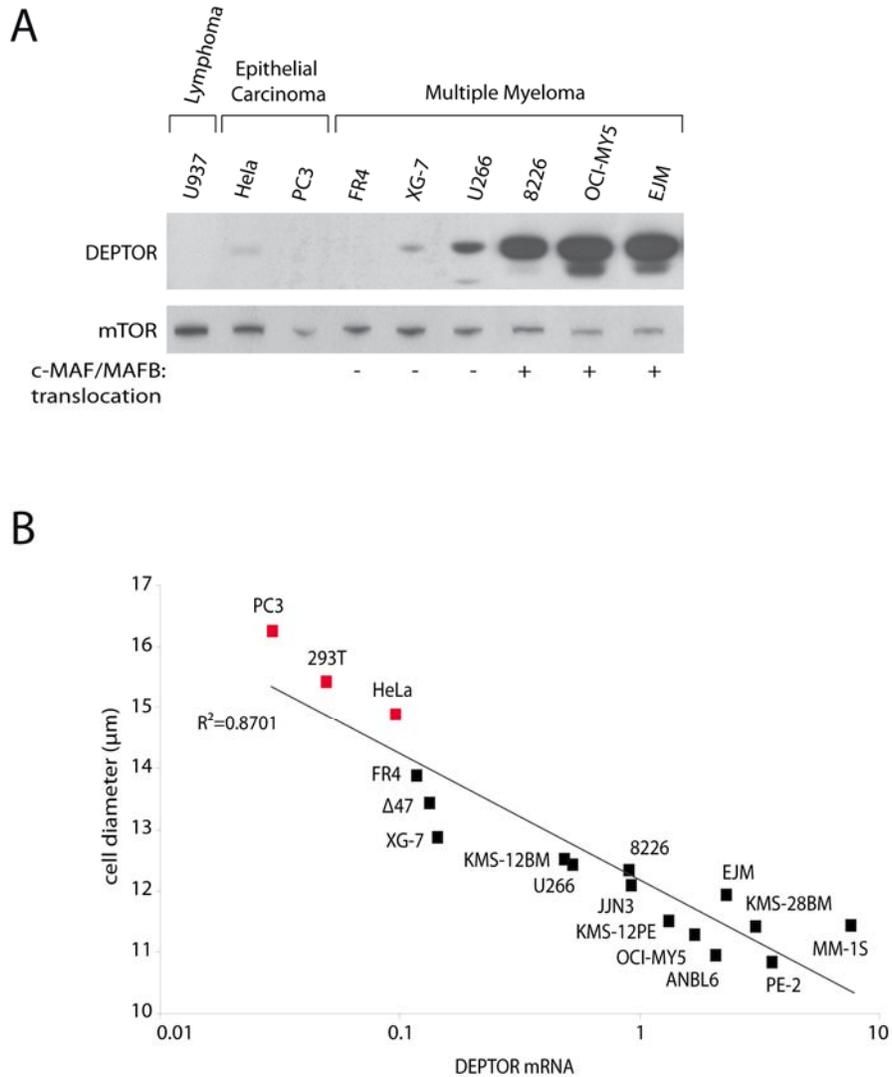


Figure S6. DEPTOR Expression across Human Cancer Cell lines Anti-Correlates with Cell Size.

(A) Indicated cell lines were seeded at equal density and 48 hours later cell lysates were prepared and analyzed by immunoblotting for DEPTOR. mTOR expression was used as a loading control. Multiple Myeloma cell lines with or without c-MAF/MAFB translocations are indicated where known.

(B) DEPTOR mRNA levels anti-correlate with cell size across a variety of human cell lines. Mean values of DEPTOR mRNA levels and cell diameter are shown in Table S1. Cell size was measured using a Coulter Counter. Non-Multiple Myeloma cell lines (PC3, HEK-293T, and HeLa) are indicated by red squares. Multiple Myeloma cell lines are indicated by black squares.

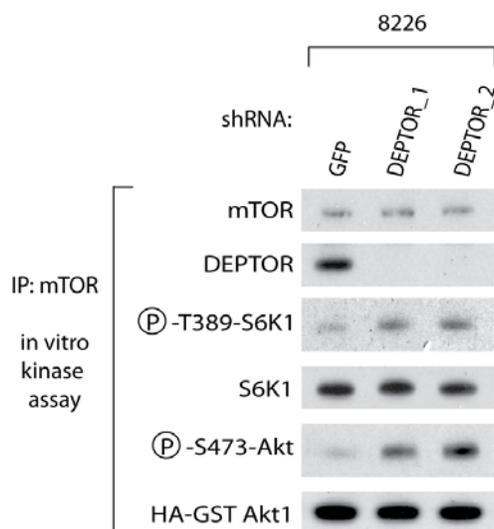


Figure S7. A Reduction in DEPTOR Expression in 8226 Cells Activates the In Vitro Kinase Activity of mTORC2 Despite Decreasing Akt S473 Phosphorylation in Cells

8226 cells were infected with lentiviruses expressing shRNAs targeting GFP or DEPTOR. Six days post-infection, mTOR immunoprecipitates were prepared from cell lysates (0.2 mg total protein) and analyzed for mTORC1/2 kinase activities toward S6K1 and Akt1 and for levels of mTOR and DEPTOR.

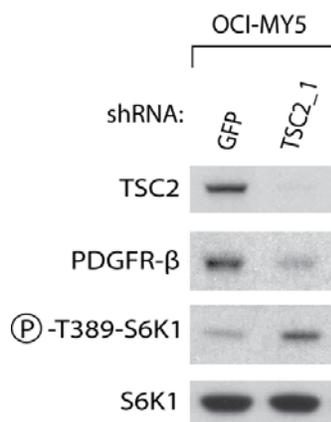


Figure S8. A Reduction in TSC2 Expression Increases mTORC1 Signaling but Represses PDGFR Protein Levels

OCI-MY5 cells were infected with a control shRNA or an shRNA targeting TSC2. Five days after infection, cell lysates were analyzed for the indicated protein levels and phosphorylation states.

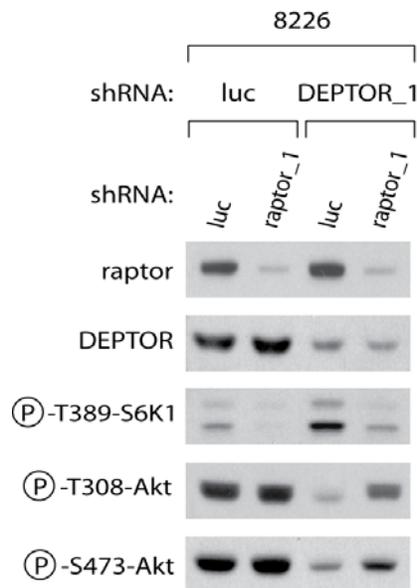


Figure S9. A Raptor Knockdown Restores PI3K Signaling in 8226 Cells with a DEPTOR knockdown

8226 cells co-expressing shRNAs targeting luciferase or DEPTOR along with shRNAs targeting luciferase or raptor were lysed five days after infection and cell lysates were analyzed for the indicated protein levels and phosphorylation states.