Figure S1 - related to Figure 1

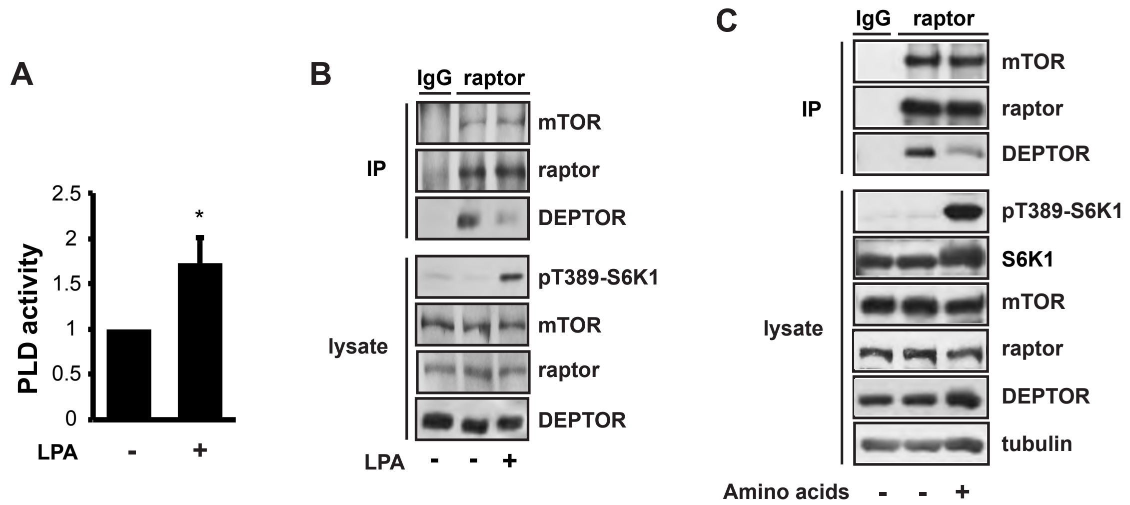
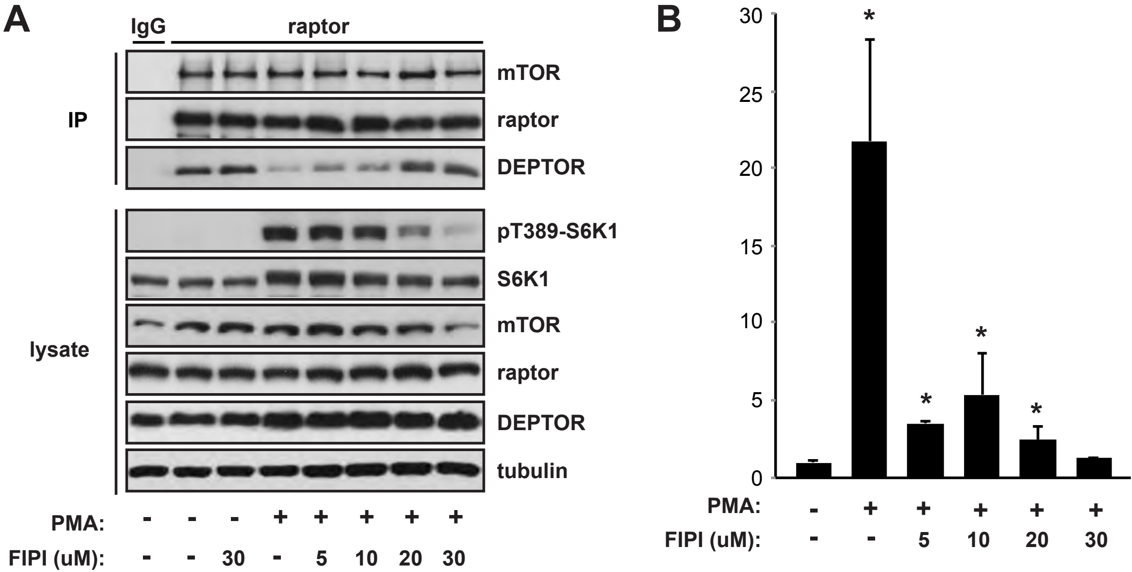


Figure S2 - related to Figure 2



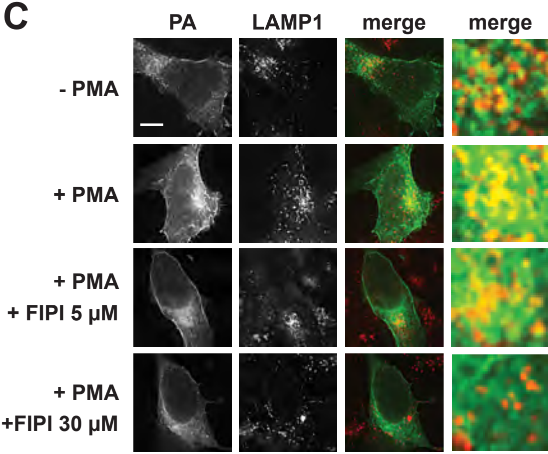
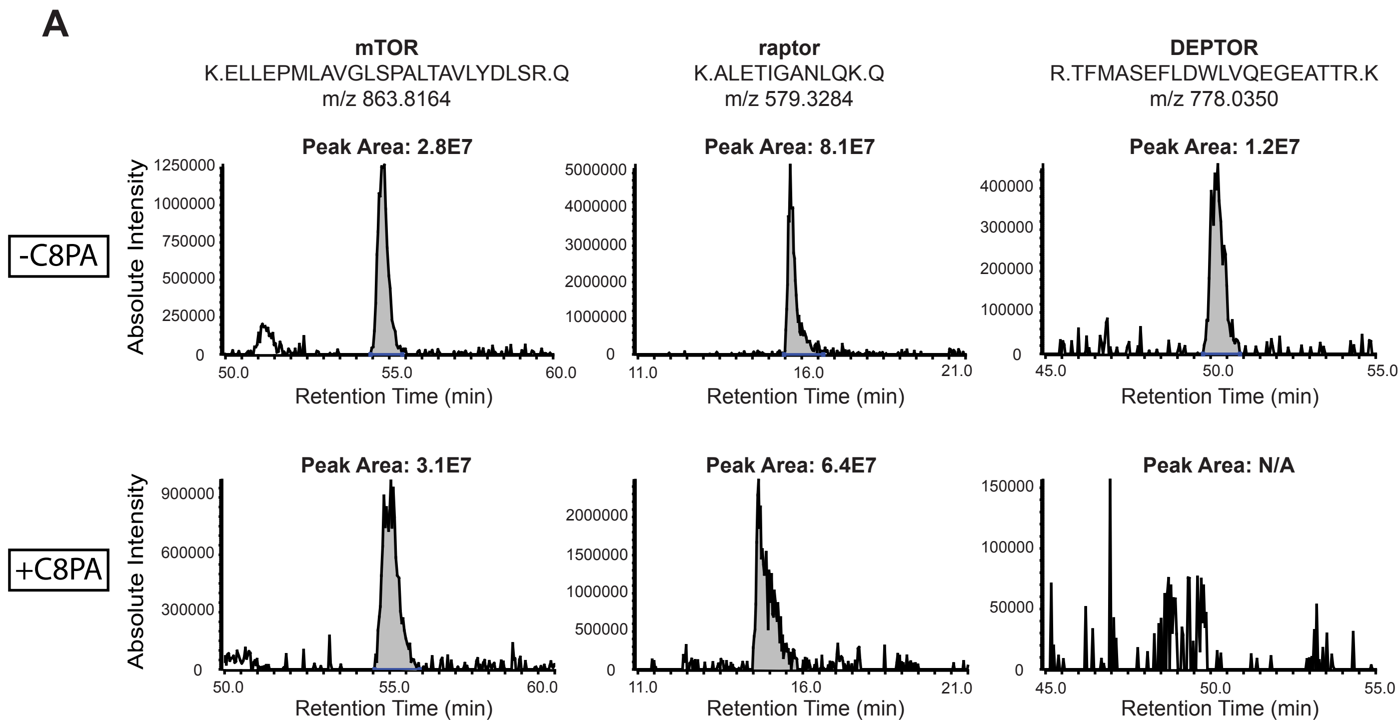


Figure S3 - related to Figure 2



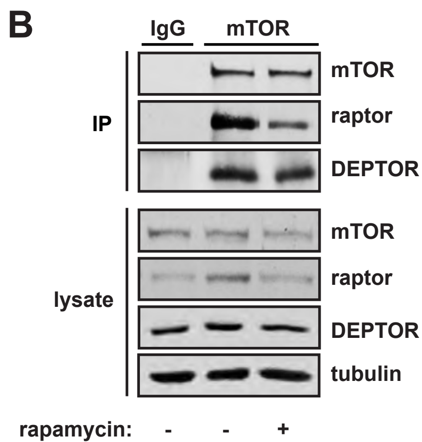
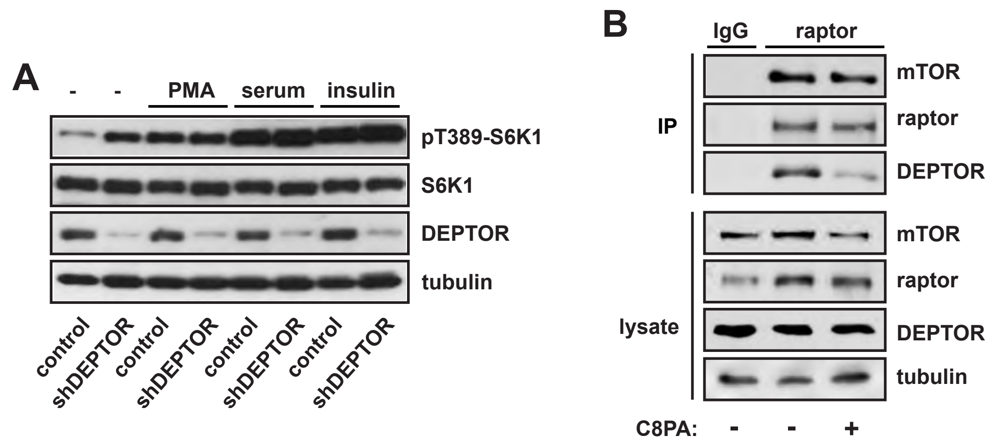


Figure S4 - related to Figure 3



SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1.
Disruption of DEPTOR-mTORC1 Interaction Involves Mitogens.
(A,B) Swiss 3T3 cells were serum starved overnight, and stimulated for 30 min with 10 μM LPA, followed by (A) PLD activity assays and (B) raptor IP and western analysis. (C) HEK293 cells were incubated in amino acids-free medium for 2 hours in the presence of 10% dialyzed FBS, followed by stimulation with amino acids- and 10% dialyzed FBS-containing medium for 30 min. Raptor IP and cell lysates were analyzed by western blotting.

Figure S2, related to Figure 2.
PLD Activity Is Necessary for Mitogenic Disruption of DEPTOR-mTORC1 Interaction.
(A) HEK293 cells were serum starved overnight, then stimulated with 200 nM PMA in the presence of increasing concentrations of FIPI for 30 min. Cell lysates and raptor IP were analyzed by western blotting. (B) Cells treated as above were subjected to PLD assay. Data shown are mean ± standard deviation of triplicates. $*P \leq 0.05$ when compared to "no PMA no FIPI" by t-test. (C) Cells were transfected with a GFP PA biosensor for 24 hrs, serum starved, and then stimulated with 200 nM PMA in the presence of 0, 5, or 30 μM FIPI for 30 min, followed by immunostaining with an anti-LAMP1 antibody. The merged images were pseudo-colored with green for GFP and red for LAMP1. Scale bar: 5 μm. Enlarged (8x) images of the merges are also shown. Results shown are representative of 3 independent experiments with similar outcome.

Figure S3, related to Figure 2.
PA Specifically Disrupts DEPTOR-mTORC1 Interaction
(A) HEK293 cells stably expressing Flag-mTOR were serum starved overnight and stimulated for 30 min with 300 μM C8PA vesicles. Flag-mTOR and associated proteins were isolated by Flag-IP and subjected to trypsin digest. Resulting peptides were analyzed by mass spectrometry. Shown are peaks representing mTOR, raptor, and DEPTOR. (B) HEK293 cells were serum starved overnight and then treated for 30 min with 100 nM rapamycin. Cell lysates and mTOR IP were analyzed by western blotting.

Figure S4, related to Figure 3.
Mitogens Induce Acute mTORC1 Activation by Displacing DEPTOR
(A) HeLa cells were infected with lentivirus expressing a negative control- or DEPTOR-shRNA and selected with puromycin for 4 days, followed by serum starvation overnight and stimulation with various mitogens for 30 min. Cell lysates were analyzed by western blotting. (B) HeLa cells were starved overnight and stimulated for 30 min with 300 μM C8PA. Cell lysates and raptor IP were analyzed by western blotting.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents

All antibodies used in this study were obtained from the following commercial sources: anti-Flag M2 from Sigma-Aldrich, anti-raptor and -rictor for immunoprecipitation (A300-553A and A300-458A, respectively) from Bethyl Laboratory, Inc., anti-DEPTOR (NBP1-49674) from Novus, anti-tubulin and anti-LAMP1 (ab25630) from Abcam, Alexa Fluor 594 anti-mouse from Life Technologies (A11020), all other secondary antibodies from Jackson ImmunoResearch, and all other antibodies from Cell Signaling. Glutathione Sepharose was from GE Healthcare. Protein G-agarose was from Millipore. All lipids were purchased from Avanti Lipids. All other reagents were purchased from Sigma-Aldrich.

Plasmids

Flag-mTOR plasmid was described previously (Vilella-Bach et al., 1999). Human DEPTOR cDNA constructs were obtained from Addgene: plasmid 21334 (pRK5-Flag-DEPTOR) and plasmid 21702 (pRK5-Flag-DEPTOR, 13xS/T→A or 13A) (Peterson et al., 2009). The PASS biosensor for PA was a generous gift from Dr. Guangwei Du (Zhang et al., 2013).

Cell Culture

HEK293 and HeLa cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37 °C with 5.5%. C2C12 cells were cultured similarly as above but with 7.5% CO_2. HEK293 cells stably expressing Flag-mTOR, Flag-DEPTOR-WT, Flag-DEPTOR-13A, Flag-mTOR(RR), or Flag-mTOR(RR/KI) were selected with 1 mg/mL G418. Swiss 3T3 cells were grown in DMEM containing 10% calf serum at 37°C with 5.5% CO_2. Transfections were performed with Polyfect (Qiagen) following the manufacturers' recommendations. Serum starvation was achieved by incubating cells in serum-free DMEM overnight, and 10% FBS was used for serum stimulation.

Cell lysis, Immunoprecipitation (IP) and Western Blotting

For IP, cells were lysed in ice-cold lysis buffer (40 mM HEPES, pH7.4, 120 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 2 mM EDTA, 1x protease inhibitor cocktail (PIC) (Sigma-Aldrich), and 0.05% saponin). To make lysates for western blotting only, cells were lysed in 20 mM Tris-HCl at pH7.5, 0.1 mM Na_3VO_4, 25 mM NaF, 25 mM glycerophosphate, 2 mM EGTA, 0.3% Triton X-100. C2C12 cells were lysed directly in SDS sample buffer and subjected to brief sonication to shear genomic DNA.

Upon cell lysis, the lysate supernatant was collected after centrifugation at 10,000g for 10 min, and subjected to IP at 4 °C with various antibodies and protein G Sepharose. Lysates and IP products were mixed with SDS sample buffer. All samples were boiled for 5 min and proteins were resolved on SDS-PAGE and transferred onto PVDF membranes (Millipore). Antibody incubations were performed following the manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with chemiluminescence solution (100 mM Tris-HCl, 0.009% H_2O_2, 225 μM coumaric acid, 1.25 mM luminol). Images were developed on x-ray films. Quantification of band intensities was performed by densitometry of film images using the Image J software.

Immunofluorescence imaging

HEK293 cells cultured on poly-L-lysine-coated glass coverslips were transfected and treated as indicated in Figure S2 legend, followed by fixation in 3.7% paraformaldehyde and permeabilization with 0.1% Triton X-100. Incubation with anti-LAMP1 antibody was performed in 3% BSA/PBS at room temperature for 2 hrs, followed by incubation with Alexa 594-anti-mouse antibody in 3% BSA/PBS for 30 min at room temperature. A personal deconvolution microscope system (DeltaVision; Applied Precision) was used with a 60× NA 1.4 lens to analyze the fluorescence images as previously described (Yoon et al., 2011a).

Lipid Vesicle Preparation

All lipids were purchased from Avanti Polar Lipids, Inc. in chloroform. For cell stimulation and in vitro kinase assay, dioctyl-phosphatidic acid (C8PA), various forms of long-chain PA, and PC vesicles were prepared as previously described (Yoon et al., 2011b) by water bath sonication, at a final concentration of 3 mM. For in vitro FRB-lipid binding assay, SUVs were prepared by microprobe sonication as described previously (Fang et al., 2001). In this case, each vesicle preparation contained 16.67 mg of lipid (10% PA and 90% PC or 100% PC) in a total volume of 1 mL.

PLD Assay

PLD activity was determined as previously described, by measuring [^{3}H]phosphatidylbutanol (PBt) produced in [^{3}H]oleic acid-labeled cells via PLD-catalyzed transphosphatidylation (Fang et al., 2003; Yoon et al., 2006).

Lentivirus-mediated RNAi

Lentiviral shRNA plasmids used in this study were in the pLKO.1puro vector and obtained from Sigma-Aldrich (Mission® shRNA). The human PLD1 shRNA (TRCN0000001011) and the negative control shRNA (scrambled hairpin sequence) were previously described (Yoon et al., 2011a). The human DEPTOR shRNA (TRCN0000168363) was also reported (Peterson et al., 2009). Viral packaging, infection, and puromycin selection of infected cells were performed as previously described (Yoon et al., 2011a).

Mass Spectrometry

For each sample, Flag bead-associated proteins were washed five times with IP lysis buffer (without PIC) and five times with PBS, and then subjected to trypsin digest. The resulting peptides were loaded onto a PLRP-S trap column and resolved by PLRP-S analytical column using Eksigent 2D nano-liquid chromatography system. The samples after nanoLC separation were electrosprayed into a custom hybrid linear-ion-trap Fourier-transform ion cyclotron resonance mass spectrometer (11-Tesla LTQ-FTUltra mass spectrometer, Thermo Fisher Scientific) as previously described (Wu et al., 2012). Full scans were collected at 25,000 resolution; a data-dependent top-3 method was used for tandem mass spectrometry in FT-ICR cell at 12,500 resolution. The MS data were collected as a .raw file, processed in ProSightPC 2.0 SP1 software (Thermo Fisher Scientific), and searched against a human trypsin database on a multi-core cluster in absolute search mode (previously described, Wu et al., 2012). Specific mTOR-interacting proteins were identified by eliminating proteins that also appeared in the background IP. mTOR-interacting proteins were then examined in Qual Browser for relative abundance comparison between starved and PA-treated samples.

SUPPLEMENTAL REFERENCES

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