Supplemental Data

Amino acid activation of mTORC1 by a PB1-domain-driven kinase complex cascade

Juan F. Linares, Angeles Duran, Miguel Reina-Campos, Pedro Aza-Blanc, Alex Campos, Jorge Moscat and Maria T. Diaz-Meco

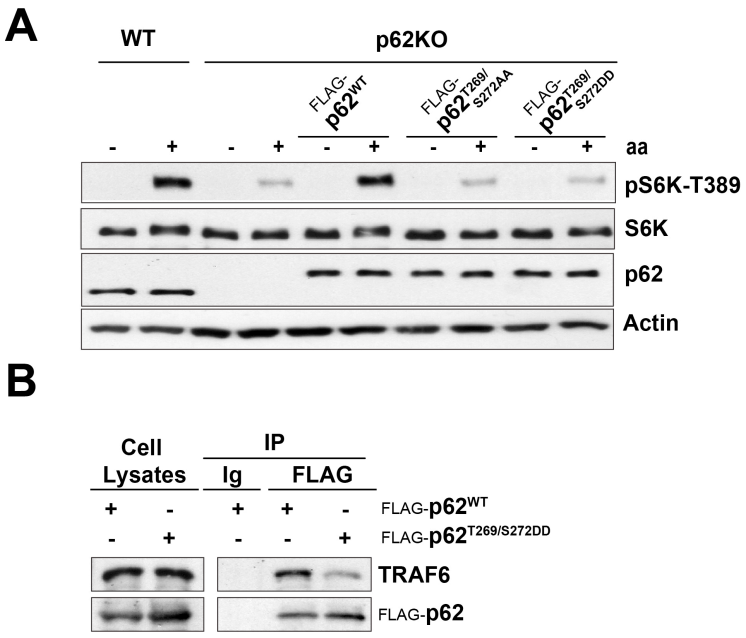


Figure S1. The Mutation of p62 Phosphorylation Sites to Aspartic Acid ,p62$^{T269/S272DD}$, Does Not Mimic p62 Phosphorylation for mTOR Activation and TRAF6 Recruitment in Response to Amino Acids, Related to Figure 1.

(A) The p62$^{T269/S272DD}$ mutant was not able to reconstitute mTOR activation in p62KO MEFs. WT and p62KO MEFs, reconstituted with p62^{WT}, p62$^{T269/S272AA}$, or p62$^{T269/S272DD}$ were starved of amino acids for 50 min and restimulated with amino acids for 20 min. Cell lysates were analyzed by western blot.

(B) The p62$^{T269/S272DD}$ mutant was not able to interact with TRAF6. HEK293T cells, transfected with the indicated plasmid were treated as in (A). Cell lysates and FLAG-tagged immunoprecipitates were immunoblotted to detect the indicated proteins.

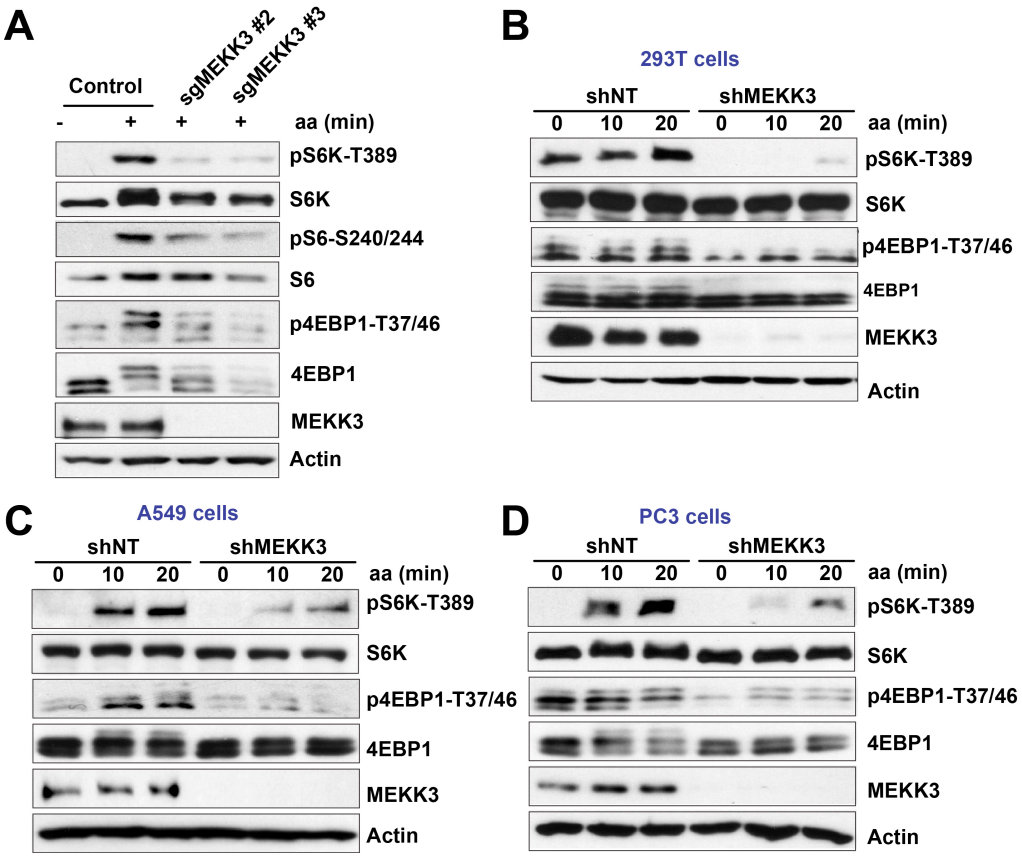


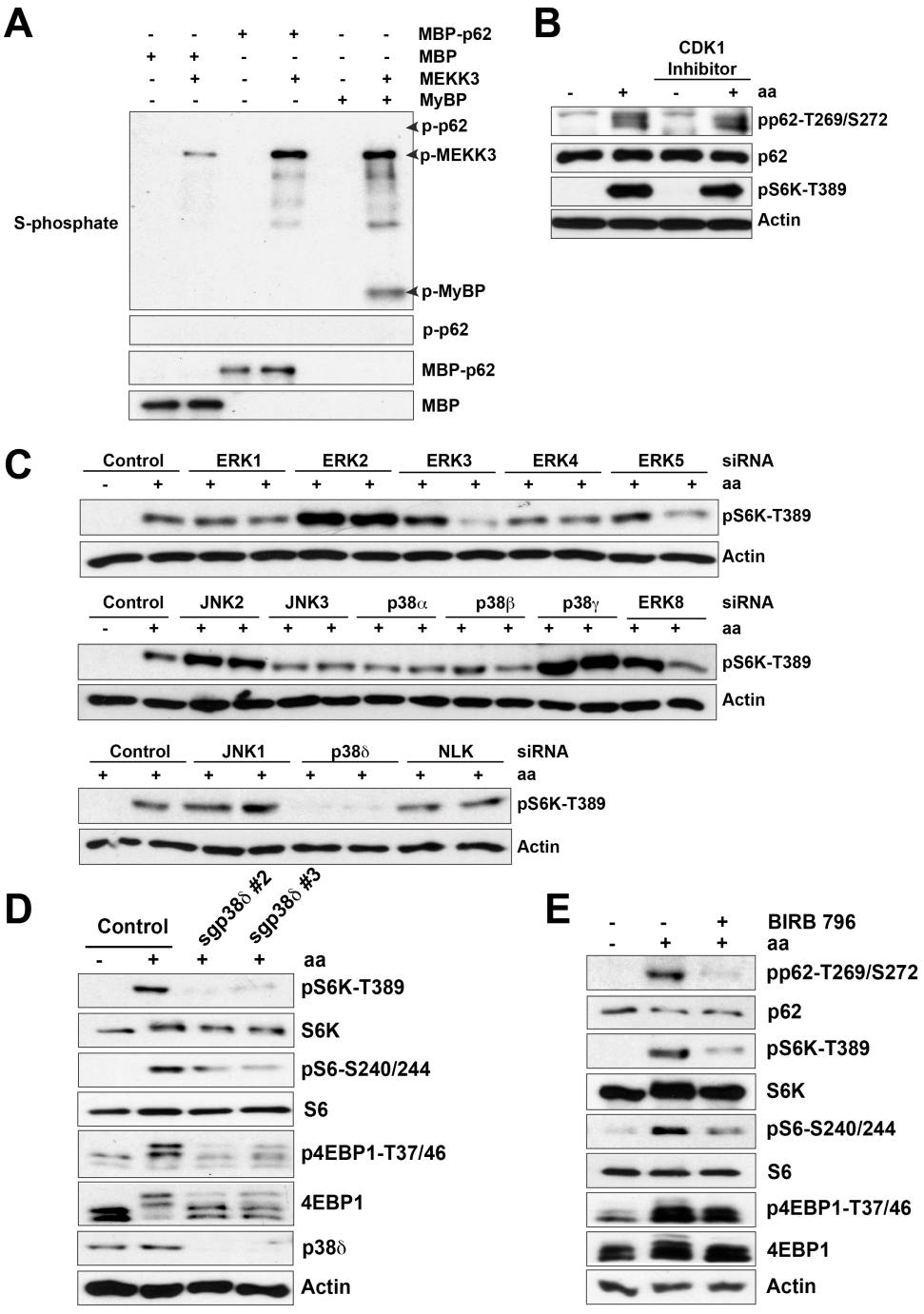
Figure S2. MEKK3 Is Required for mTORC1 Activation in Response to Amino Acids ,Related to Figure 2.

(A) MEKK3 is required for mTOR activation by amino acids. Control or MEKK3-deficient HEK293T cells were deprived of amino acids and serum for 50 min and then stimulated with amino acids for 15 min. Cell lysates were immunoblotted for the specified proteins.

(B) MEKK3 is required for mTOR activation by amino acids. shNT or shMEKK3 HEK293T cells were treated as in (A) and cell lysates were immunoblotted for the specified proteins.

(C-D) MEKK3 is required for mTOR activation by amino acids in different cell lines. shNT or shMEKK3 A549 cells and PC3 cells were treated as in (A), and immunoblotted for the specified proteins.

Results are representative of three experiments.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MBP-p62
MBP
MEKK3
MyBP
p-p62
p-MEKK3
S-phosphate
p-MyBP
p-p62
MBP-p62
MBP
B
CDK1 Inhibitor
aa
pp62-T269/S272
p62
pS6K-T389
Actin
C
Control
ERK1
ERK2
ERK3
ERK4
ERK5
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JNK2
JNK3
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ERK8
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Actin
Control
JNK1
p38δ
NLK
siRNA
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pS6K-T389
Actin
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Control
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sgp38δ #3
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S6
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Actin
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aa
pp62-T269/S272
p62
pS6K-T389
S6K
pS6-S240/244
S6
p4EBP1-T37/46
4EBP1
Actin

Figure S3. p38δ Is Required for mTORC1 Activation and p62 Phosphorylation in Response to Amino Acids, Related to Figure 3.

(A) MEKK3 does not phosphorylate p62 in an in vitro phosphorylation assay with ATPγS.

(B) CDK1 does not phosphorylate p62 in response to amino acids. HEK293T cells, in the presence or absence of purvalanol, were deprived of amino acids for 50 min and then stimulated with amino acids for 20 min. Cell lysates were immunoblotted for the specified proteins.

(C) p38δ is required for mTOR activation by amino acids. HEK293T cells transfected with scramble siRNA or the different MAPK siRNAs were treated as in (B) and cell lysates were then immunoblotted for the specified proteins.

(D) p38δ is required for mTOR activation by amino acids. Control or p38δ-deficient HEK293T cells were treated as in (B) and cell lysates were immunoblotted for the specified proteins.

E) Inhibition of p38 enzymatic activity blocks p62 phosphorylation and mTOR activation by amino acids. HEK293T cells, in the presence or absence of the p38 inhibitor BIRB 796 (10 μM), were treated as in (B). Cell lysates were immunoblotted for the specified proteins

Results are representative of three experiments.

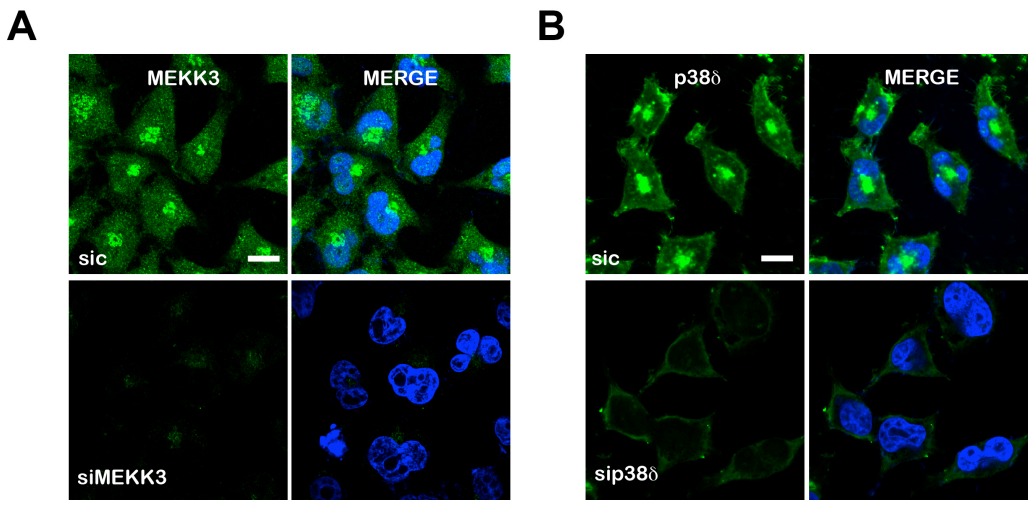

Figure S4. Antibodies Validation for Immunofluorescence, Related to Figure 4.

(A-B) Images of HeLA cells, transfected with scramble siRNA or MEKK3 or p38δ siRNA, coimmunostained for MEKK3 or p38δ and DAPI. Cells were starved for 50 min before processing. Scale bars= 10μm.

Images are representative of two independent experiments.

Supplemental Experimental Procedures

Generation of Knockout Cell Lines

The guide sequences targeting Exon 1 of human MEKK3, p38δ, and p62 are shown below.

MEKK3: 5'- GAACTCAATCATGAACGATC

p38δ: 5'- G TACGTGTCCCCGACGCACGT

p62: 5'-GAAGATCGCCTTGGAGTCCG

The single guide RNAs in the PX458 vector (4μg) were transfected into HEK293T cells using Lipofectamine 2000 according to manufacturer's instructions. 24 hours post transfection, the cells were trypsinized, washed with PBS, and re-suspended in DMEM with 2% FBS and penicillin/streptomycin. GFP-positive cells were single-sorted by FACS (Sanford-Burnham Medical Research Institute FACS core, FACS ARIA) into 96-well plates in DMEM containing 20% FBS and 50μg ml^{-1} penicillin/streptomycin. Single clones were expanded and screened for MEKK3, p38δ, and p62 by protein immunoblotting.

Antibodies and Reagents

Reagents were obtained from the following sources: primary antibodies to HA tag (sc-805), GST tag (sc-138), and Myc tag (sc-40), S6K1 (sc-230), MEKK3 (sc-28769), p38δ (sc-7585), ubiquitin (sc-8017), TRAF6 (sc-7221), and actin (sc-1616); HRP-labeled anti-mouse, anti-mouse IgG1, and anti-goat secondary antibodies were from Santa Cruz Biotechnology. Antibodies to raptor (#2280), phospho-T389 S6K1 (#9205), mTOR (#2983), phospho-T37/46 4EBP1 (#2855), 4EBP1 (#9644), AKT (#9272), phospho-S473 AKT (#4058), LC3 (#4108), phosho-Ser240/244 S6 (#5364), S6 (#2317), and HRP-labeled anti-rabbit secondary antibody were from Cell Signaling Technology. Antibody to

phospho-T269/S272 p62 was from Phospho Solutions. Antibody to LAMP2 (ab25631) was from Abcam, and to human p62 (#610833) from BD biosciences. FLAG antibody (F1804), human recombinant insulin, bafilomycin A1, purvalanol, protein A-sepharose, bovine insulin, RPMI 1640 medium, Y-27632, leucine, and 50x amino acid solution were from Sigma Aldrich. BIRB 796 was from Millipore. DMEM and fetal bovine serum were from Hyclone; FuGENE 6 and Complete Protease Cocktail were from Roche. Alexa 488-, Alexa 555-, and Alexa 568-conjugated secondary antibodies, Lipofectamine 2000, and tyramide signal amplification kits, B27, and Glutamax were from Life Technologies. RPMI 1640 medium modified to be without amino acids was from US Biological; protein G-Sepharose was from Amersham; collagenase type II, DMEM, ADMEM/F12 and TrypLE from GIBCO; EGF 5, R-spondin1, recombinant Noggin from Preprotech; growth factor-reduced Matrigel from Corning; and A83-01 from Tocris.

Isolation and Culture of Prostate Epithelial Cells

Prostate epithelial cells were prepared as previously described (Karthaus et al., 2014) with a few modifications**.** Murine prostates were isolated from 8-week-old $PTEN^{fl/fl}$-PBcre male mice and were placed in 5 mg ml^{-1} collagenase type II in ADMEM/F12 and digested for 1 to 2 h at 37°C. Glandular structures were washed with ADMEM/F12 and centrifuged at 100 G. Subsequently, structures were digested in 5 ml TrypLE with the addition of Y-27632 to 10 µM for 15 min at 37°C. Trypsinized cells were washed and seeded in growth factor-reduced Matrigel. Murine prostate epithelial cells were cultured in ADMEM/F12 supplemented with B27, 10 mM HEPES, Glutamax, and penicillin/streptomycin and containing the following growth factors: EGF 50 ng/ml, R-spondin1-conditioned medium or 500 ng/ml recombinant R-spondin1, 100 ng/ml recombinant Noggin, and the TGF-β/Alk inhibitor A83-01. Murine prostate organoids were passaged either via trituration with a glass Pasteur pipet or trypsinization with

TrypLE for 5 min at 37°C. Lentiviral infections were performed as described previously (Koo et al., 2012) using pLKO.1-puro targeting p62, MEKK3, p38δ, or control scramble. In short, 100,000 single cells were infected with an MOI $1*10^3$. Infection was done during centrifugation for 1 h at 600 G RT. Cells were subsequently placed at 37°C, 5% CO_2 for 3 h to recover. Cells were plated in Matrigel and, 24 h post seeding, 1 µg/ml puromycin was applied for 2 days to ensure only infected cells remained.

Cell Culture

HEK293T, PC3, and A549 cells were from ATCC. p62 KO MEFs were previously described (Duran et al., 2011). Cells were tested for mycoplasma contamination. Cells were cultured in DMEM with 10% FBS. For co-transfection experiments, 0.9 million HEK293T cells were plated in 6 cm culture dishes. 24 hours later, cells were transfected with 500 ng of the expression plasmids. Empty vector was added to transfection mixes to bring the total DNA quantity up to 2 µg. For amino acid starvation, HEK293T cells in 10 cm culture dishes or on coated glass cover slips were rinsed with PBS and incubated in serum and amino acid-free RPMI for 50 minutes. MEFs, PC3, and A549 cells were treated similarly, but starved for 4 hours. Cells were stimulated with a 1X amino acid mixture for different durations, as indicated. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. For insulin stimulation, HEK293T cells were deprived of serum for 24 h and stimulated with 150 nM of insulin. Cells were processed for biochemical or immunofluorescence assays as described below. Cell viability was determined by Trypan Blue exclusion at the indicated times.

Plasmids

pCMV-FLAG-p62, pWZL-Hygro-p62, and pCDNA3-myc-p62 vectors have been described previously (Duran et al., 2011). pCMV-FLAG-p62 T269A/S272A, pCMV-FLAG-p62 T269D/S272D, pWZL-Hygro-p62 T269A/S272A, pCDNA3-myc-p62 D69A/D73A, pCDNA3-FLAG-p38δ T180A/Y182F plasmids were generated by in vitro mutagenesis. The following plasmids were from Addgene: Addgene plasmid 19301, pRK5-HA GST RagBGTP (Sancak et al., 2008); Addgene plasmid 20785, pCDNA3 FLAG-p38δ (Enslen et al., 2000), Addgene plasmid 12186, pCMV5 HA-MEKK3 (Blank et al., 1996); Addgene plasmid 14671, pRc/RSV FLAG MKK3 (Derijard et al., 1995), and Addgene plasmid 22418: mCherry-EGFP-LC3 (Pankiv et al., 2007).

Mammalian Lentiviral shRNAs, siRNAs, and Retroviral Transduction

TRC lentiviral shRNAs targeting human MEKK3 (TRCN0000010692, TRCN0000002305), human p38δ (TRCN0000055428), mouse MEKK3 (TRCN0000025250), mouse p62 (TRCN0000098616) and mouse p38δ (TRCN0000023092) were obtained from Sigma. shRNA-encoding plasmids were co-transfected with psPAX2 (Addgene; plasmid 12260) and pMD2.G (Addgene; plasmid 12259) packaging plasmids into actively growing HEK293T cells by using FuGENE 6 transfection reagent. Virus-containing supernatants were collected 48 hours after transfection, filtered to eliminate cells, and then used to infect target cells in the presence of 8 μg/ml polybrene. Cells were analyzed on the third day after infection. For MAPK siRNA screening, two pools of four siRNAs against each target from two different sources (Dharmacon and Ambion) were used. siRNAs were co-transfected into actively growing cells by using Lipofectamine transfection reagent. Cells were analyzed on the second day after transfection. Retroviruses were produced in Phoenix cells by transient

transfection using Lipofectamine. Culture supernatants were collected 24, 48, and 72 h post-transfection, filtered, and supplemented with 8 μg/ml polybrene. Cells were infected with three rounds of viral supernatants and selected with hygromycin (75 μg/ml).

Cell Lysis, Immunoprecipitation, Fractionation, and Immunoblotting

Cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, and 0.3% CHAPS, and one tablet of EDTA-free protease inhibitors [Roche] per 25 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 15 minutes. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation overnight at 4°C. 40 μl of a 50% slurry of protein G-sepharose or protein A-sepharose was then added and the incubation was continued for an additional 1 hour. Immunoprecipitates were washed three times with lysis buffer. Fractionation of lysates into heavy membrane and light membrane/cytosolic fractions was performed as described (Menon et al., 2014), In brief, HEK293T cells from two near-confluent 15-cm dishes per treatment were washed with cold PBS, scraped into cold PBS, pelleted by centrifugation at 800 g for 2 min at 4°C, and re-suspended in 300 μl cold hypotonic lysis buffer (10 mM HEPES, pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 20 mM NaF, 100 μM sodium orthovanadate, 250 mM sucrose, with freshly added protease inhibitors). Cells were mechanically lysed by drawing 4 times through a 23G needle and then centrifuged at 500 g for 10 min at 4°C, yielding a post-nuclear supernatant (PNS). The PNS was centrifuged at 20,000 g for 2 hr to separate the soluble supernatant (light membrane/cytosolic fraction) from the insoluble pellet (heavy membrane fraction). The pellet was resuspended in RIPA buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF,

0.5 mM sodium orthovanadate, and 1:100 protease inhibitors). Cell extracts or immunoprecipitated proteins were denatured by the addition of 20 μl of sample buffer and boiling for 5 minutes, resolved by 8%–14% SDS-PAGE, and then transferred to nitrocellulose-ECL membranes (GE Healthcare). The immune complex was detected by chemiluminescence (Thermo Scientific).

In Vitro Kinase-Assay and MS/MS Phosphopeptide Identification

For in vitro phosphorylation assays, 1 μg of recombinant MBP-p62 was incubated at 30 ºC for 60 min in kinase assay buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, and 100 μM ATP in the presence of recombinant MEKK3 or p38δ. For ATP analog-based phosphorylation detection, the protocol described previously (Allen et al., 2007) was followed with minor modifications. Briefly, 100 μM of ATPγS (Biolog) was added to the reaction, after which PNBM (Abcam) and EDTA were added to a final concentration of 2.5 mM and 20 mM, respectively, and incubated for 1 h at room temperature. Immunoblotting detection was performed with anti-thiophosphate ester antibody from Cell Signaling. Protein digestion, TiO2-based phosphopeptide enrichment, electrospray ionization-liquid chromatography tandem mass spectrometry, and MS/MS analysis were performed as described previously (Ma et al., 2013).

Ubiquitin Detection Assay

Detection of endogenous in vivo mTOR ubiquitination was performed as described (Xiong et al., 2009). In brief, HEK293T cells were lysed with cell lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, with 2mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitors). Cell lysates were boiled for 10 min to dissociate protein-protein interactions. The samples were diluted with dilution buffer (10

mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton). The diluted samples were incubated at 4°C for 60 min with rotation and then centrifuged for 30 min. Cell lysate was incubated with mTOR antibody overnight, after which Protein A beads were added for an additional 1 h. Immunoprecipitates were washed with washing buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% NP-40). Proteins were eluted in SDS-sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-ubiquitin.

Histological Analysis

Prostate organoids and prostates from 10-month-old $PTEN^{+/-}$ male mice were isolated, rinsed in ice-cold PBS, fixed in 10% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were deparaffinized, rehydrated, and then treated for antigen retrieval. After blocking in avidin/biotin solutions (Vector Laboratories), tissues were incubated with primary antibody overnight at 4 °C, followed by incubation with biotinylated secondary antibody. Endogenous peroxidase was quenched in 3% H_2O_2 in water at room temperature. Antibodies were visualized with avidin/biotin complex (Vectastain Elite; Vector Laboratories) using diaminobenzidine as the chromagen. Human prostate tissue microarray (TMA) slides were obtained from US Biomax. Stained TMA slides were scanned by using the Scanscope XT system (Aperio) and images were captured using the Aperio ImageScope software (v11.1.2.760). For the quantitative analysis, a Histo-score (H score) was calculated based on the staining intensity and percentage of stained cells using the Aperio ScaScope systems.

Immunofluorescence Assays and Colocalization Measurements

HeLa, HEK293T, and A549 cells were plated on fibronectin-coated glass coverslips in 24-well tissue culture plates. 24 hours later, cells were amino acid starved, and then stimulated with amino acids as described above, rinsed with PBS once, fixed with warmed 4% formaldehyde, and permeabilized with 0.1% Triton X-100. Fixed cells on cover slips or sections from human prostate samples, previously deparaffinized, were blocked for one hour in blocking buffer (0.3% BSA in PBS) and then incubated with primary antibody in blocking buffer overnight at 4°C. Samples were then rinsed twice with blocking buffer and incubated with secondary antibodies for one hour at room temperature in the dark, followed by tyramide signal amplification. Slides were mounted on Mowiol and examined with a FluoView 1000 Olympus Laser Point Scanning Confocal Microscope. The colocalization plugin in ImageJ (NIH) was applied to measure co-localization between two channels of confocal z stacks (a constant threshold for all the images within each experiment was applied). A maximum-intensity projection was generated, and the area of co-localizing pixels was quantified using the JACoP plugin in ImageJ, and expressed as the total area of colocalization per cell. Quantification was carried out on at least 15 cells per condition from two independent experiments.

Cell-Size Determinations

For measurement of cell size, triplicates of each sample were analyzed using Countess Automated cell counter (Invitrogen).

Supplemental References

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