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Supplemental Information

K63 Polyubiquitination and Activation

of mTOR by the p62-TRAF6 Complex

in Nutrient-Activated Cells

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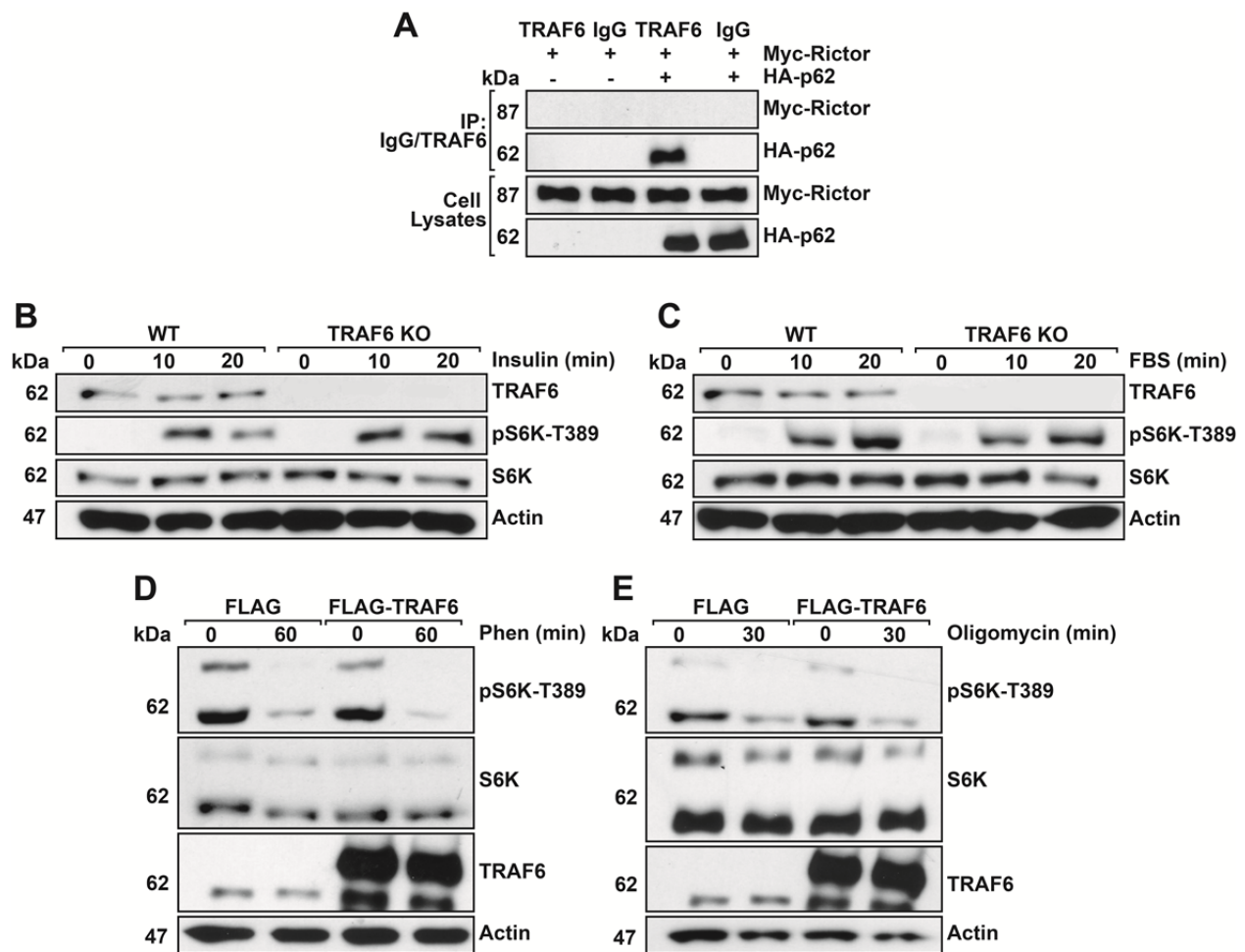


Figure S1. TRAF6 Is an mTORC1 Modulator Selectively in the Nutrient-Response Pathway, Related to Figure 1

(A) HEK293T cells were transfected with the indicated cDNAs in expression vectors, cell lysates were prepared, and lysates and TRAF6 immunoprecipitates were analyzed by immunoblotting to detect the specified proteins. (B and C) WT and TRAF6 KO MEFs were serum starved for 24 h and then stimulated with insulin (B) or serum (C) for the indicated durations. Cell lysates were analyzed by immunoblotting to determine levels of the specified proteins. (D and E) HEK293T cells stably expressing Flag or Flag-TRAF6 were treated with phenformin (D) or oligomycin (E) for the indicated durations, and cell lysates were analyzed by immunoblotting to determine levels of the specified proteins. These results are representative of three experiments.

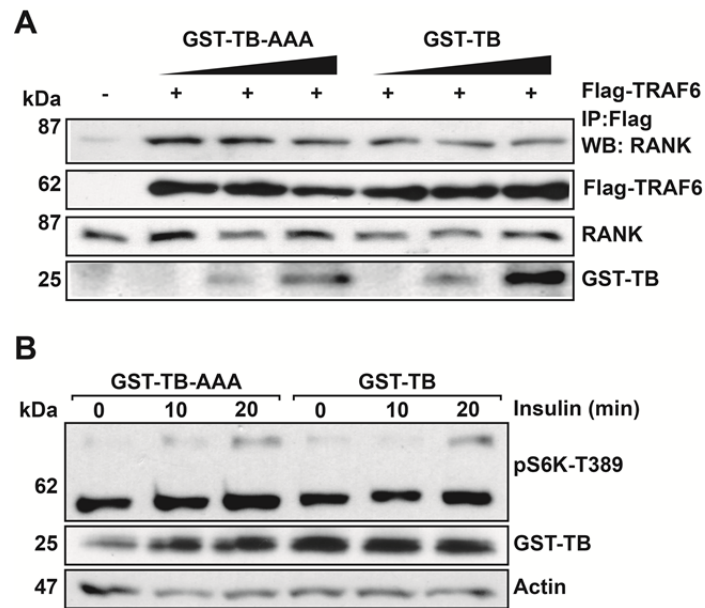


Figure S2. Blocking p62-TRAF6 Interaction Is Selective for Amino Acid-Induced mTORC1 Activation, Related to Figure 2

(A) HEK293T cells were transfected with the indicated cDNAs in expression vectors, cell lysates were prepared, and lysates and Flag immunoprecipitates were analyzed by immunoblotting to detect the specified proteins (B) HEK293T cells transfected with GST-TB-AAA or GST-TB were serum starved for 24 h and then stimulated with insulin for the indicated durations. Cell lysates were analyzed for the indicated proteins. These results are representative of three experiments.

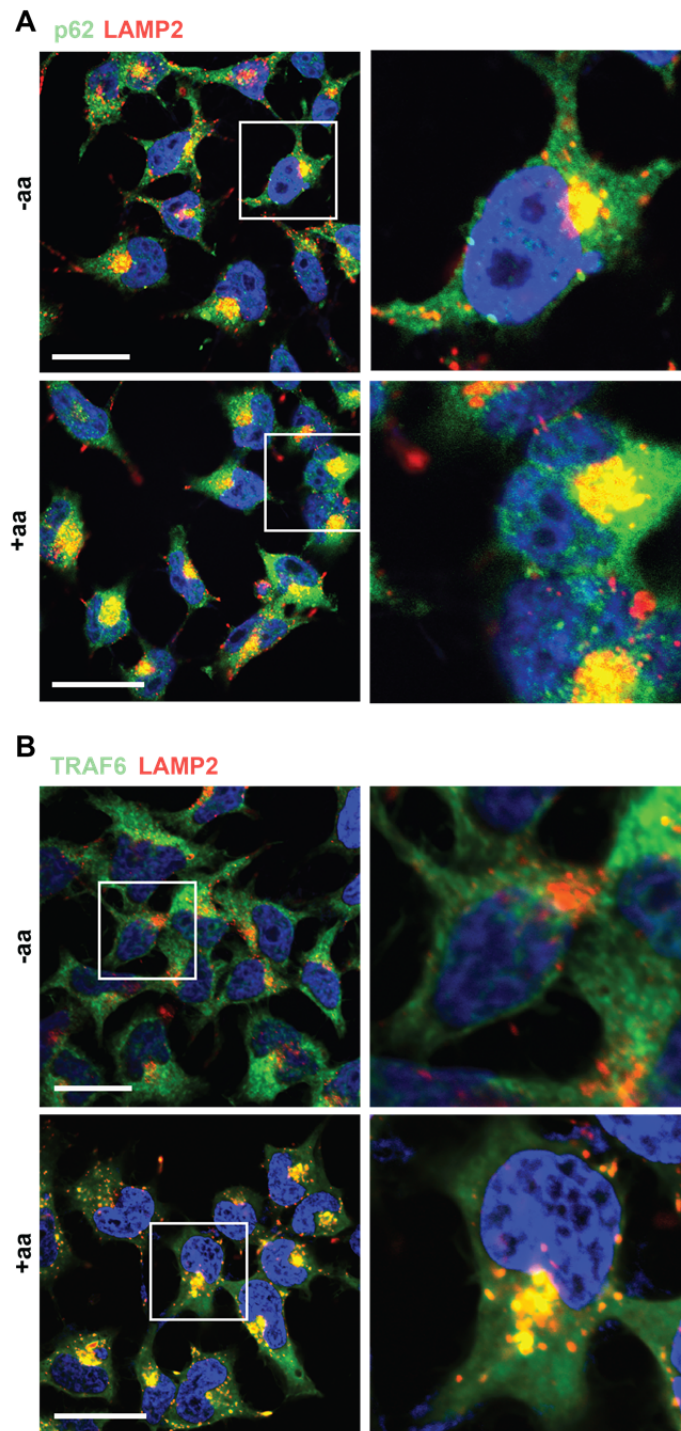


Figure S3. TRAF6 Is Recruited to LAMP2-Positive Lysosomes upon Amino Acid Stimulation, Related to Figure 3

Images of HEK293T cells co-immunostained for (A) p62 (green) and LAMP2 (red); (B) TRAF6 (green) and LAMP2 (red). Scale bars=10 μ m. These images are higher magnification of the merge panels of Figures 3A and 3B.

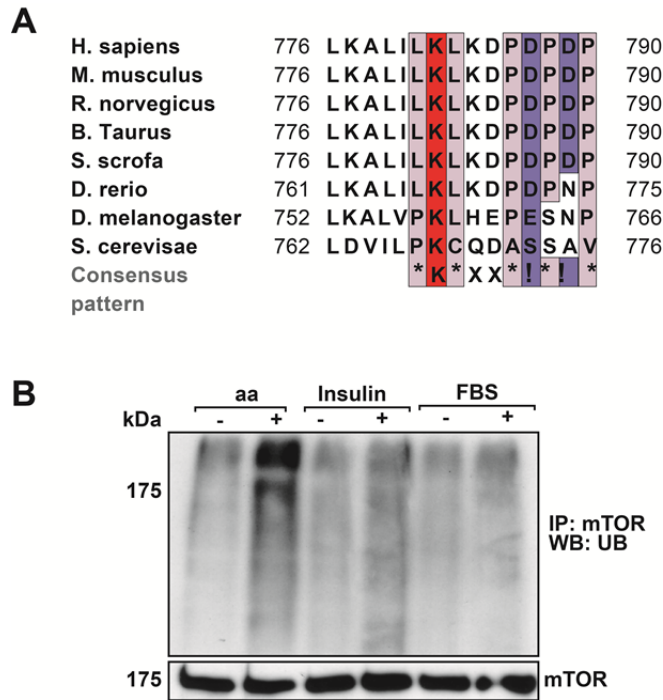


Figure S4. mTOR Ubiquitination in Response to Nutrients, Related to Figure 4

(A) Alignment of the amino acid sequence of the TRAF6/p62 ubiquitin acceptor site in human mTOR (776-790) with orthologs in other species. Amino acids of the same type are marked as (*) hydrophobic, (!) polar, (X) any amino acid residue, and (K) the acceptor lysine residue. (B) HEK293T cells were deprived of amino acids and serum for 50 min and then stimulated with amino acids for 10 min, or serum starved for 24 h and stimulated with either insulin or serum for 10 min. Cell lysates and mTOR immunoprecipitates were analyzed by immunoblotting to quantify the specified proteins. These results are representative of three experiments.

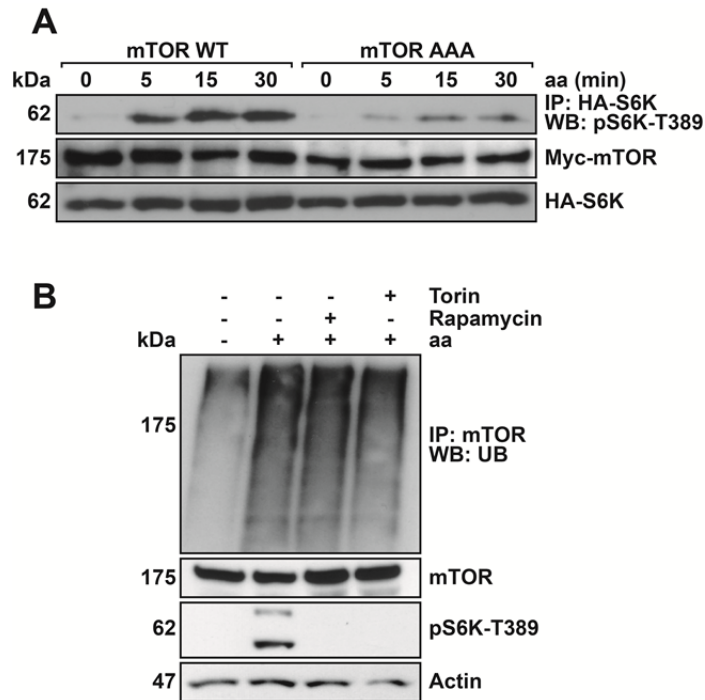


Figure S5. mTOR Ubiquitination Modulates Amino Acid-Induced mTORC1 Activation, Related to Figure 5

(A) HEK293T cells transfected with mTOR WT or mTOR AAA mutant and HA-S6K as reporter. Cells were deprived of amino acids and serum for 50 min and then stimulated with amino acids for the indicated durations. Cell lysates and HA-tagged immunoprecipitates were analyzed by immunoblotting for the specified proteins. (B) HEK293T cells were deprived of amino acids and serum for 50 min and then stimulated with amino acids for 10 min in the presence of mTOR inhibitors, torin or rapamycin. mTOR immunoprecipitates and cell lysates were analyzed by immunoblotting for the specified proteins. These results are representative of three experiments.

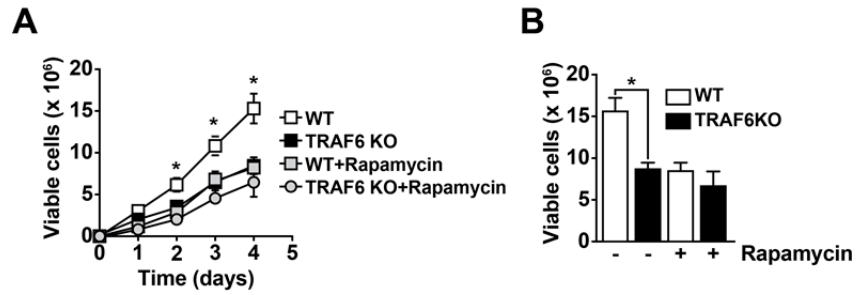


Figure S6. Effect of Rapamycin Treatment on the Growth of WT and TRAF6 KO MEFs, Related to Figure 6

(A) Proliferation curve of WT and TRAF6 KO MEFs growing in the presence or absence of rapamycin. Cell viability was determined by trypan blue exclusion assay at the indicated times
 (B) Cell viability at day 4 of the proliferation curve in (A). Results are the mean \pm SEM ($n=3$). * $p < 0.05$ (WT vs. TRAF6 KO).

Supplemental Experimental Procedures

Mice

Animals were maintained under conditions of controlled temperature (22.5 °C) and illumination (12 h dark/light cycle). All mice were born and maintained under pathogen-free conditions. TRAF6^{fl/fl} mice were described previously (Polykratis et al., 2012). TRAF6^{fl/fl} mice were bred to Alb-cre mice to generate liver-specific TRAF6-KO. All genotyping was done by PCR.

Materials

Reagents were obtained from the following sources: Antibodies to the HA tag, GST tag, and the myc tag; S6K1, RANK, TRAF6, actin; HRP-labeled anti-mouse, anti-mouse IgG1, and anti-goat secondary antibodies were from Santa Cruz Biotechnology. Antibodies to raptor, phospho-S2481 mTOR, phospho-T389 S6K1, RagC, mTOR, phospho-T37/46 4EBP1, AKT, phospho-S473 AKT, LC3, phospho-Ser240/244 S6; HRP-labeled anti-rabbit secondary antibody; and rapamycin were from Cell Signaling Technology. Antibodies to LAMP2 were from Abcam, the antibody to murine p62 was from Progen, antibodies to human p62 were from BD biosciences; and the Flag antibody, Flag peptide, human recombinant insulin, protein A-sepharose, bovine insulin, RPMI 1640 medium, leupeptin, torin, phenformin, oligomycin B leucine, and 50x amino acid solution were from Sigma Aldrich. 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 were from Life Technologies. RPMI 1640 medium modified to be without amino acids was from US Biological; protein G-Sepharose was from Amersham; TRAF6 peptide and control peptide were from American Peptide Company; and recombinant protein Ubch7 (E2), ubiquitin activating enzyme (E1), and recombinant ubiquitin were from Enzo Life Sciences.

Cell Culture

HEK293T and A549 cells were from ATCC. TRAF6^{fl/fl} primary cells MEFs were derived from E13.5 embryos (Polykratis et al., 2012). p62 KO MEFs were previously described (Duran et al.,

2011). Cap2 cells have been previously described (Fernandez-Marcos et al., 2009). 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 and A549 and Cap2 cells were treated similarly, but starved for 4 hours. Cells were stimulated with a 1X amino acid mixture for different durations, as indicated in the figures. 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 or MTT assay (Promega) at the indicated time. For the colony-formation assay, 10^3 cells were plated in triplicate onto 6-well plates in complete medium. Cultures were maintained at 37°C for 7 days. Afterwards, cells were fixed in 4% paraformaldehyde and stained with 0.05% crystal violet. Colonies containing at least 30 cells were counted.

Plasmids

pCDNA3-HA-p62 and pCDNA3-myc-p62 vectors have been described previously (Sanchez et al., 1998). pWZL-Hygro and pWZL-Hygro-H-RasV12 have been described previously (Duran et al., 2008). The p62 TRAF6-binding domain (TB) was subcloned into the pCDNA3-GST vector. The following plasmids were from Addgene: Addgene plasmid 8513, HA-Raptor (Kim et al., 2002); Addgene plasmid 1861, myc-mTOR (Sarbasov et al., 2004); myc-Rictor (Sarbasov et al., 2004); Addgene plasmid 19301, pRK5-HA GST RagB^{GTP} (Sancak et al., 2008); Addgene plasmid 19315, pBJ5-p70-S6K-HA (Sancak et al., 2008); and Addgene plasmid 18712, HA-Ubiquitin (Kamitani et al., 1997). Plasmids myc-mTOR K1777A/K1782A/K1784A, HA-p62 Δ TB, HA-p62 P228A, HA-p62 P228A/E230A, HA-p62 P228A/E230A/P232A, GST-TB

P228A/E230A/P232A, Flag-TRAF6 C70A, HA-Ub K63R, and HA-Ub K48R were generated by in vitro mutagenesis. Plasmid pCR-FLAG-TRAF6 was the generous gift of Dr. Nakano Hiroyasu (Sanz et al., 2000).

Mammalian Lentiviral shRNAs, siRNAs, Retroviral Transduction, and Cre-Recombinase Adenovirus Infection

TRC lentiviral shRNAs targeting murine (TRCN0000040736) and human (TRCN0000007349) TRAF6 were obtained from Open Biosystems. 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. Small interfering RNAs (siRNA) for p62 and TRAF6 were obtained from QUIAGEN. 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 HEK293T 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. MEFs were infected with three rounds of viral supernatants and selected with hygromycin (75 µg/ml). For Cre infection in MEFs, we used adeno-GFP or adeno-Cre (Ad5 CMV and Ad5 CMV Cre, respectively; University of Iowa GTVR) as the control. We incubated MEFs in medium containing adenovirus for 24 h to allow for efficient infection and lox-site excision.

Cell Lysis and Immunoprecipitations

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 continued for an additional 1 hour. Immunoprecipitates were washed three times with lysis buffer. 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).

Ubiquitin Detection and In Vitro Ubiquitination 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. The in vitro ubiquitination assay was performed as previously described (Geetha et al., 2005). HEK293T cells were transfected with myc-mTOR or FLAG-TRAF6 constructs by the Lipofectamine2000 method. Cells were lysed with SDS lysis buffer (1% SDS, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin and aprotinin), and 750 µg of the lysate was then immunoprecipitated with myc or FLAG antibody and collected with agarose-coupled secondary antibody. TRAF6 was purified by elution with FLAG-peptide. Ubiquitination was carried out with mTOR as the substrate in a 50 µl reaction (50 mM Tris [pH

7.5], 2.5 mM MgCl₂, 2 mM DTT, and 2 mM ATP) containing 100 ng E1, 200 ng Ubch7 WT, or dominant-negative (E2), along with 1 µg of TRAF6 (E3) and 5 µg of ubiquitin. Reactions were carried out by continuous shaking at 37°C for 2 hr followed by washing three times with reaction buffer. The proteins were released by boiling in SDS-PAGE sample buffer, electrophoresed on 7.5% SDS-PAGE, and western blotted with anti-ubiquitin and anti-myc.

In Vitro mTOR Activity

In vitro kinase mTOR activity was determined in cell extracts using the K-LISA mTOR (Recombinant) Activity Kit (Calbiochem). Briefly, HEK293 cells were transfected with myc-mTOR or myc-mTOR-AAA and cell extracts were prepared and used immediately to immunoprecipitate mTOR with anti-myc antibody. Equal amounts of protein were immunoprecipitated from transfected and control non-transfected cells. mTOR activity was determined following manufacturer's instructions. The mTOR activity of non-transfected cells was subtracted to control for background activity.

Immunofluorescence Assays and Colocalization Measurements

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. After rinsing twice with PBS, the coverslips 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. Coverslips 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. Glass cover slips 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.

Electron Microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer and, after a brief buffer wash, postfixed in 1% osmium tetroxide. Following an additional buffer wash, cells were treated with 0.5% tannic acid, 1% sodium sulfate, and buffer wash. Cells were then dehydrated in an ethanol series, transitioned in 2-hydroxypropyl methacrylate (HPMA), and embedded in LX112 (Ladd Research, Williston, VT). Pieces of the flat embedded resin containing the cells were glued to a blank block face and 60nm thin sections were cut, mounted on copper slot grids coated with Parlodion, and stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR) at 80kv. Images were collected using a Megaview III CCD camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

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