Supplemental Materials and Methods

Immunoblot studies

In brief, cells were lysed in NP-40 buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 25mM β-glycerophosphate, 2 mM Na_3VO_4, 1% IGEPAL, and Complete Mini protease inhibitor cocktail tablet +/- EDTA; Roche) or RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM Na_3VO_4, 25 mM β-glycerophosphate, 15 mM NaF, and complete protease inhibitor mix; Roche) and lysate cleared by centrifugation at 13,000 rpm for 10 min at 4˚C. Lysate protein concentration was determined for each sample using BCA assay. Equal amounts of lysate resolved by 10 or 12% SDS-PAGE and transferred to a PVDF transfer membrane. Membrane was blocked in 5% BSA in TBS-T at 4˚C. Primary antibodies were incubated at 4˚C overnight. Secondary ECL antibodies (GE Healthcare) were incubated for 1 h at RT in 5% Milk in TBS-T. Protein was imaged using chemiluminescence method and Genemate autoradiography film. The following antibody dilutions were used: mouse anti-eIF4E (BD Bioscience #610270, 1:5000), rabbit anti-P-eIF4E S209 (Abcam #ab76256, 1:10000), rabbit anti-eEF2 (Cell Signaling #2332, 1:1000), rabbit anti-β-actin (Cell Signaling #4967, 1:1000), rabbit anti-4E-BP1 (Cell Signaling #9644, 1:1000), rabbit polyclonal anti-eIF4G1 (gift from N. Sonenberg, 1:1000), rabbit anti-eIF4A (Cell Signaling #2013, 1:5000), rabbit anti-P-eIF4E-BP1 S65 (Cell Signaling #9451, 1:1000), rabbit anti-S6 ribosomal protein (Cell Signaling #2217, 1:5000), rabbit anti-Akt (Cell Signaling #9272, 1:1000), rabbit anti-P-S6 ribosomal protein S235/236 (Cell Signaling #2211, 1:7000), rabbit anti-mTOR (Cell Signaling #2983, 1:1000), rabbit anti-TSC2 (Cell Signaling #3612, 1:1000), rabbit anti-eIF4B (Cell Signaling #3592, 1:1000), mouse anti-PABP (Abcam #ab6125, 1:1000), rabbit anti-eIF2α (Cell Signaling #9722, 1:1000), rabbit anti-eIF3H (Cell Signaling #3413, 1:1000), rabbit anti-eIF6 (Cell Signaling #3833, 1:1000), mouse anti-Runx2 (Millipore #05-1478, 1:1000), and rabbit anti-β-tubulin (Cell Signaling #2146, 1:1000). Quantitation was performed by determination of the integrated density of the bands using ImageJ software.

^{35}S-Methionine metabolic protein labeling

Global protein synthesis was measured as previously described (Ramírez-Valle et al., 2008). Cells ($2x10^5$) were seeded, in triplicate, into 6-well culture plates and allowed to

adhere for 48-72 h. Cells were labeled with 25 µCi ^{35}S-methionine/cysteine protein labeling mix (PerkinElmer) for 30 min at 37˚C using DMEM without L-Methionine or L-Cysteine supplemented with 5% FBS. Cells were lysed in NP-40 buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM Na_3VO_4, 0.5% IGEPAL, and complete protease inhibitor tablet; Thermo Scientific). Lysates were cleared by centrifugation at maximum speed for 15 min at 4˚C. Proteins were precipitated in 10% TCA followed by scintillation counting. Protein concentration was measured by the BCA assay (Thermo Scientific). For studies involving doxycycline treatment, Dox was added 48 h after plating and maintained throughout the course of the study.

pTripz construct expression

The tetracycline responsive element (TRE) was induced using 1-2 µg/ml of doxycycline was administered to cells for 48-96 h. When possible, RFP expression was analyzed through fluorescent microscopy or FACS analysis.

Generation of Inducible Constructs

shRNA cassettes were cloned into 5'-*XhoI* and 3'-*EcoRI* sites of Inducible TRIPZ vector (pTripz). Constructs were transformed into One Shot Stbl3 cells (Invitrogen) and grown on LB-amp/zeo plates overnight at 37˚C. Plasmids were purified from individual colonies and sequenced. Sequencing primer: (5'-GGAAAGAATCAAGGAGG-3'). Overexpression constructs were generated following a similar method except the ORFs were cloned into 5'-*AgeI* and 3'-*MluI* sites and sequenced with the following primers: Fwd: (5'-CGAGGTTCTAGACGAGTTTA-3'); Rev: (5'-GCCTTAAGAACCCCAGTATCAG-3'). The shRNA cassette sequences are as follows: eIF4E-1 (5'-CACAATAGTCAGAAAACAACT-3'), eIF4E-2 (5'-GCGTCAAGCAATCGAGATTTG-3'), TSC2 (5'-CAGCATTAATCTCTTACCATA-3') and Runx2 (5'-CCACAGAATTTGCATTTAGAG-3'). The eIF4E and 4E-BP1 ORFs were subcloned from the pBABE-puro retrovirus vector using the *AgeI/MluI* cloning sites. Point mutations were made using site-directed mutagenesis (Agilent Technologies).

Generation of stable cells lines

HEK293FT cells were transfected with pTripz lentivirus vector, psPAX2, and PMD2.G using lipofectamine 2000 (Life Technology) for 6-8 h. Media was changed to DMEM

(Corning) supplemented with 5% heat-inactivated FBS (Gibco). Virus was collected 48 and 72 h after transfection. Virus was concentrated and either used immediately to transduce MCF7 cells or stored at -80˚C. Cells were infected in the presence of Polybrene (5-10 μg/ml) and thereafter selected for resistance to puromycin (Sigma) or geneticin (Gibco) for up to one week.

Immunohistochemistry (IHC) and scoring

All kits and developing substrates were obtained from Vector Laboratories. Paraffin-embedded tumor sections (4 μm thickness) were de-paraffinized in xylene and ethanol, rehydrated and subjected to antigen retrieval by microwaving for 30 min in antigen unmasking solution (Vector Laboratories), then 5% H_2O_2 to block endogenous peroxidase activity for 30 min at RT followed by protein blocking of non-specific epitopes with 1.5% normal horse serum (Vector Laboratories). Slides were incubated with primary antibodies for 4E-BP1 and P-4E-BP1 (S65, Cell Signaling), P-eIF4E (S209, Abcam), S6 and P-S6 (S240/244, Cell Signaling), and eIF4E (BD Transduction) overnight at 4°C. After washing with PBS-T, slides were incubated with secondary antibody for 2 h at RT and DAB staining carried out according to manufacturer instructions (Vectastain ABC kit, Vector Laboratories). Slides were counterstained with hematoxylin. Specimens were analyzed by a pathologist blinded to the study and 20% of specimens chosen at random spot scored by a second pathologist also blinded to the study. Specimens were scored as 0, 1, 2, 3+ with a cut-off for staining that had to include at least 5% of the cells in a given population and took into consideration the percentage of positive cells as follows. For quartile scoring: 0 <5%; +1 ≥6% but <33%; +2 >33% but <65%; +3 >66%. For tertile staining: 0 <5%; +1 ≥6% but <50%; +2 ≥50%.