Supplemental Materials and Methods

Antibodies and reagents

Anti-Myc antibody was obtained from Covance. CP-724,714 was purchased from SelleckChem. HRGα, (residues 177-241) and Protein G beads were obtained from Sigma and Invitrogen, respectively.

DNA constructs

Rictor (Addgene plasmid 1860) and Raptor (Addgene plasmid 1859) DNA constructs were obtained from Addgene (20).

Cell culture conditions

HEK 293T (ATCC® CRL-11268™) cells were obtained from the American Type Culture Collection (ATCC). Cells were authenticated by the ATCC for viability (prior to freezing and immediately after thawing), growth, morphology, isoenzymology, and short tandem repeat (STR) analysis. Cells were passaged for less than 3 months after resuscitation of frozen aliquots. HEK 293T cells were maintained in DMEM (Invitrogen) containing 10% FBS at 37°C, 5% CO_2. For inhibitor analysis, SKBR3 cells were pretreated with 5 μM CP-724,714 for 30 min, followed by the addition of 1 nM HRG.

Transfection

For shRNA rescue experiments, SKBR3 cells were infected with control or Rictor shRNA expressing lentiviral particles twice, one day apart. Cells were then selected with

2 μg/mL puromycin for 48 hours. Following selection, cells were transfected with mock (no DNA) or 5 μg of Rictor plasmid using Lipofectamine and Plus Reagent, following the manufacturer's protocol (Invitrogen). HEK 293T cells were seeded at $1x10^6$ cells on 100 mm cell culture plates. Four μgs of DNA were transfected into the cells the next day using Lipofectamine and Plus Reagent.

Immunoprecipitation

HEK 293T cells were lysed with cell lysis buffer (50 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, and 0.3% CHAPS). Lysates were pre-cleared with Protein G beads on a rotator at 4°C for 10 min. The supernatant was collected and incubated with anti-Myc antibody for 2 h, followed by the addition of Protein G beads for 1 h at 4°C. Immuno-precipitates were washed 3 times with the lysis buffer followed by the addition of 2X Laemilli buffer.

Supplemental Figure Legends

Supplemental Figure 1. HRG-dependent stimulation of mTORC1-related signaling activities in SKBR3 cells is blocked by pre-treatment with the ErbB2-specific kinase inhibitor, CP-724,714. SKBR3 cells were serum-starved followed by treatment with or without 5 μM CP-724,714 for 30 minutes prior to stimulation with 1 nM HRG for an additional 30 minutes. Cells were collected and lysed, and the lysates were analyzed by Western blotting using specific antibodies to probe for phospho-ErbB2 (Y1248),

phospho-mTOR (S2448), phospho-TSC2 (T1462), phospho-AKT (T308), phospho-AKT (S473), phospho-ribosomal S6 (S235/236), the corresponding pan-specific antibodies, or actin.

Supplemental Figure 2. HRGα, as well as HRGβ, is capable of stimulating the ability of SKBR3 cells to form colonies and activate components of the mTORC1 signaling pathway. A, SKBR3 cells were seeded in 0.3% agarose-containing complete medium with the addition of 1 nM HRGα or 1 nM HRGβ. Cells were fed every three days with the growth factor-containing medium and colonies were counted on day 13. The experiment was performed in triplicate and the results were averaged and graphed. B, SKBR3 cells were serum-starved for 40-48 h, and were then challenged with HRGα for the indicated times. Cell lysates were then collected and analyzed by Western blotting to determine relative levels of phospho-mTOR (S2448), phospho-TSC2 (T1462), phospho-AKT (T308), phospho-AKT (S473), phospho-ribosomal S6 (S235/236), actin, as well as for the total levels of the respective proteins.

Supplemental Figure 3. Two mechanistically-distinct mTOR inhibitors, rapamycin and INK-128, show differential abilities to inhibit signaling components upstream of mTORC1. SKBR3 cells were serum-starved for 40-48 h. Rapamycin (50 nM) or INK (50 nM) was added to the cells for 30 minutes prior to the additional treatment of cells with or without 1 nM HRG for 30 min. Cells were then harvested, lysed, and then lysates were analyzed by Western blotting for relative levels of phospho-mTOR (S2448),

phospho-TSC2 (T1462), phopsho-AKT (T308), phospho-AKT (S473) and the corresponding total proteins.

Supplemental Figure 4. Attenuation of HRG-stimulated AKT phosphorylation by the knock-down of Rictor can be rescued by the expression of an shRNA-insensitive Rictor construct. SKBR3 cells were infected either with control or Rictor shRNA-containing virus twice, one day apart, and selected with 2 μg/mL puromycin for 48 h. Cells were then subjected to transfection either with mock (no DNA) or 5 μg of Rictor plasmid for 3 hours, followed by serum-starvation for 48 h. On the day of the harvest, cells were treated with or without 1 nM HRG. Whole cell lysates were collected and subjected to Western blotting probing for Rictor, phospho-AKT (T308), phospho-AKT (S473), and total AKT.

Supplemental Figure 5. Ectopically-expressed components of both mTORC1 and mTORC2 in HEK 293T cells can interact with TSC2 as well as mTOR. HEK 293T cells were transfected with either Myc-Raptor or Myc-Rictor. Cells were then lysed in lysis buffer containing 0.3% CHAPS and subjected to immunoprecipitation using anti-Myc antibody to precipitate Raptor or Rictor. The precipitated samples were subjected to Western blotting probing for mTOR, TSC2, and Myc.