Supplementary Figure Legends

Figure S1: Effects of NVP-BEZ235 and LY294002 on PI3Kα activity using an ADP release (A) or a Maxisorp (B) assay. For both assays (see methods), the activity of the p85/p110α complex is reported as a percentage of inhibition, for which the 0% represents the activity in absence of inhibitors.

Figure S2: Dose-dependent effects on activated Akt levels. PC3M cells were incubated with increasing amounts of NVP-BEZ235 for 30 minutes. Cells were then lyzed and cell extracts analyzed by Western-blotting to assess total-, S473P- or T308P-Akt levels.

Figure S3: NVP-BEZ235 causes rapid relocalization of FKHRL1 into the nucleus A. Kinetic of FKHRL1-GFP relocalization by NVP-BEZ235. U2OS-GFP-FOXO3A cells were imaged as in B, every minute for a period of 20 minutes after administration of the compound. B. Effects on FKHRL1 induced transcription. U2OS-GFP-FKHRL1 cells were transfected with the pGL3-FKHR-LUC gene reporter, exposed to increasing concentrations of either NVP-BEZ235 (left panel) or LY294002 (right panel). Transcription activation was determined as described in the methods section.

Figure S4: NVP-BEZ235 inhibits mTor. Purified mTor from rat brain was used in a kinase reaction using a GST-p70S6K fusion protein as a substrate. Phosphorylated levels are represented as Absorbance Units. B. mTORC2 and mTORC1 phosphorylate 4EBP1 on Serine 46/37 in a time dependent manner. IgG-sepharose-pulldowns of Hela cells stably expressing either TAP-tagged Rictor (500 μg) or TAP-tagged Raptor (30 μg) were subjected to in vitro kinase assays with 4EBP1 (Phas) as a substrate for indicated amounts of time at 30º C. Kinase

reaction were analyzed by western-blotting to assess phosphorylation of 4EBP1 on Serine 46/37.

Materials and Methods for Supplementary Figures

ADP-release and MaxiSorp PI3K assays

For the two PI3Kα kinase assays shown in figures S1, the full-length p110/p85 protein from Upstate (#14-602) was used. The ADP-release assay was adapted from a previously described method (30). PI3Kα was incubated for 60 min at room temperature in 96-well half-area black microplates (Corning #3694) in 50 µl medium containing 10 µM PIP(4,5)diC_8 (Avanti Polar Lipids #850185P), 20 µM ATP (Sigma #A7699), 5 mM $MgCl_2$, 30 mM KCl, 70 mM NaCl, 0.4 mM phosphoenolpyruvate, 10 unit/ml pyruvate kinase (Sigma #P9136), 20 mM Tris-HCl pH7.4, 0.1 mg/ml γ-globulin (Sigma #G5009), 0.1 mM EGTA, and 1% DMSO. The reaction was started by adding PI3Kα (0.5 µg/ml, <2.5 nM) and stopped by adding 50 µl of 160 mM phosphate buffer pH6.0 containing 0.02% Triton X-100, 20 U/ml horseradish peroxidase (Sigma #P2088), 2 U/ml pyruvate oxidase (Sigma #P4591), 0.4 mM TPP, 20 µM FAD, and 20 µM Amplex® Red (Molecular Probes #A12222). Fluorescence was read after 5 min in a GeniosPro™ reader (Tecan, Switzerland) with 540 nm excitation and 590 nm emission filters. For the The MaxiSorp™ assay (31), 50 µl/well of a 1:1 mixture of 100 µg/ml L-α-phosphatidylinositol (Avanti Polar Lipids #840044C) and L-α-phosphatidylserine (Avanti Polar Lipids #840032C) dissolved in chloroform:ethanol (2.2:7.8) was pipetted into 96-well MaxiSorp™ plates (Nunc #436110). The solvents were evaporated at room temperature and plates were washed with Tris-buffered saline (TBS, pH7.4). PI3Kα was incubated for 60 min at room temperature in coated plates in 50 µl medium containing [γ^{33}P]-ATP (~6 kBq/well; Amersham #AH9968), 0.5 µM ATP (or higher as indicated in Fig. 1-2), 5 mM $MgCl_2$, 150

mM NaCl, 25 mM Tris-HCl pH7.4, and 1% DMSO. The reaction was started by adding PI3Kα (0.4 µg/ml, <2 nM) and stopped by adding 50 µl of 50 mM EDTA. Plates were washed twice with TBS and dried; 100 µl/well MicroScint™ PS (Packard) was added, and bound radioactivity was determined using a TopCount™ (Packard) counter. The Michaelis-Menten kinetics of ATP was similar in the ADP release and Maxisorp assays (Km =26 µM; Vmax =110 $nmol.mg^{-1}.min^{-1}$). The inhibitory effect of NVP-BEZ235 and LY294002 was evaluated by nonlinear curve fitting with the 4-parameter logistic equation $y=A+(B-A)/(1+(((10^{C})/x)^{D}))$ where A is the minimum (%), B the maximum (%), C the log IC_{50} and D the Hill coefficient (Model 203 of XLfit™). Independent experiments (n=2-5) were fitted simultaneously with shared parameters using the Global fit wizard of Xlfit.

K-Lisa mTor assay

The K-LISA™ mTOR activity kit was from Calbiochem (ref #CBA055). Briefly, Inhibitors were incubated with mTOR enzyme for 30 minutes on ice. This mixture was then transferred to a glutathione-coated 96-well plate pre-incubated with GST-$p70^{S6K}$ recombinant protein. Kinase reactions were initiated by adding kinase buffer containing 100 µM ATP and were incubated for 30 minutes at 30°C. mTor activity was quantified using an anti-phospho Thr389-HRP conjugate detection system.

mTORC1 and mTORC2 assay

Sub-confluent HeLa cells stably expressing either N-terminally TAP-tagged versions of Rictor or Raptor were lysed (lysis buffer: 40mM Hepes (pH7.5), 120mM NaCl, 1mM EDTA, 0.2% CHAPS, 50mM beta-glycerophosphate, 50mM NaF, 1mM Na_3VO_4, 40mg/ml PMSF, 1mM DTT, EDTA-free protease inhibitors (Roche)) and protein concentrations were determined. 500µg (TAP-Rictor) or 30µg (TAP-Raptor) of total cell lysate / sample were

subjected to a pulldown with 15 µl IgG-sepharose beads (1:1 slurry, Amersham) for 2h at 4ºC. IgG-sepharose pull-downs were washed 3 times with washing buffer (40 mM Hepes (pH7), 120mM NaCl, 0.3% CHAPS, 10 mM beta-glycerophosphate, 10mM NaF, 10 mM NaN_3, 10 mM NaPPi, 20 µg/ml heparin, 0.5 mM benzamidine / HCl, 1.5 mM Na_3VO_4, 1 mg/ml PMSF, 1 mM DTT, EDTA-free protease inhibitors and prior to the kinase assay samples were pretreated with indicated amounts of NVP-BEZ235 at a final concentration of 0.005% DMSO for 15min at 4ºC. Then, samples were subjected to kinase assays in a volume of 50µl reaction buffer (40mM Hepes (pH7), 120mM NaCl, 0.3% CHAPS, 2µg/ml heparin, 1mM DTT, 10mM $MnCl_2$, 100 µM ATP, 1µg/µl 4E-BP1/PHAS (Stratagene), 1µM PKI (Protein kinase A inhibitor – Calbiochem) at 30ºC for 30min (TAP-Rictor) or 20min (TAP-Raptor) if not indicated otherwise. The reaction was stopped with 25µl of 4x SDS-sample buffer. Samples were boiled for 5min and loaded onto 12% SDS gels. Gels were transferred to nitrocellulose membranes and phosphorylation of 4EBP1 on Serine 46/37 was detected with phosphor-specific antibodies from Cell Signaling Technology.

Forkhead Transcription assay

U2OS-GFP-FKHRL1 cells (density of 3.75 x 10^5 cells/mL) were transiently transfected with pGL3-FKHR-LUC reporter gene (8 µg DNA per T175 cell culture flask) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and were seeded into white Greiner 384 well plates. After an incubation period of 18 hours at 37°C and 5% CO_2, 10 µM compound was added for 12 hours. SteadyGlo (Promega, Madison, WI) reagent was dispensed into the plates and after an incubation time of 20 min on a shaker the luminescence was read on the Viewlux (Perkin Elmer, Waltham,MA).