Supporting Online Material

Material and Methods

Materials

Reagents were obtained from the following sources: protein G-sepharose from Pierce; ATP-[γ-^{32}P] from NEN; compounds LY294002, wortmannin, and staurosporine were obtained from Calbiochem; DMEM from Life Technologies; mTOR, S6K1, ATM, α-tubulin, and PKCα antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; phospho-T389 S6K1, phospho-S473 and phospho-T308 Akt/PKB, Akt/PKB, phospho-S256 FKHR (also recognizes phospho-S193 of AFX), AFX, phospho-S505 *Drosophila* Akt/PKB, and *Drosophila* Akt/PKB antibodies from Cell Signaling; *Drosophila* S6K antibody from Mary Stewart, North Dakota State University; and the rictor and raptor antibodies were previously described [*1*].

Cell lysis

Cells growing in 10 cm dishes were rinsed once with cold PBS and lysed on ice for 20 min in 1 ml of ice-cold Lysis Buffer (40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors (Roche)) containing 1% Triton X-100. After clearing of Triton X-100 material by centrifugation at 13,000Xg for 10 min, samples containing 50-100 μg of protein were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting as described [*2*]. For experiments with AFX the Triton X-100 insoluble materials were solubilized in 1% SDS in 10 mM Tris-HCl pH 7.4 by heating at 100°C for 3 minutes followed by a brief sonication. Equal protein amounts were then analyzed by immunoblotting.

Immunoprecipitations and kinase assays

For all immunoprecipitation experiments the lysis buffer contained 0.3% CHAPS instead of 1% Triton in order to preserve the integrity of the mTOR complexes [*1, 2*]. 4 μg of the indicated antibodies were added to the cleared cellular lysates and incubated with rotation for 90-min. 25 μl of a 50% slurry of protein G-sepharose was then added and the incubation continued for 1 h. Immunoprecipitates captured with protein G-sepharose were washed four times with the CHAPS Lysis Buffer and once with the rictor-mTOR kinase buffer (25 mM Hepes pH 7.5, 100 mM potassium acetate, 1 mM $MgCl_2$). For kinase reaction immunoprecipitates were incubated in a final volume of 15 μl for 20 min at 37 $^{\circ}$C in the rictor-mTOR kinase buffer containing 500 ng inactive Akt1/PKB1 (Ak1t/PKB1, Upstate Biotechnology, #14-279) and 500 μM ATP. The reaction was stopped by the addition of 200 μl ice-cold Enzyme Dilution buffer (20 mM MOPS, pH 7.0, 1 mM EDTA, 0.01% Brij 35, 5% glycerol, 0.1 % 2-mercaptoethanol, 1 mg/ml BSA). After a quick spin, the supernatant was removed from the protein G-sepharose and analyzed by immunoblotting [*2*]. For experiments involving PDK1, the rictor-mTOR phosphorylation was performed as described above and the second reaction was initiated by adding to the samples 100 ng of PDK1 (Upstate Biotechnology, #14-452) and 5 μl of Mg/ATP Cocktail (220 mM MOPS, pH-7.2, 75 mM $MgCl_2$, 500 μM ATP, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT; Upstate Biotechnology, #20-113). The samples were incubated for a further 20 min at 37 $^{\circ}$C, the reactions stopped by adding 40 μl Enzyme Dilution buffer and the samples quickly spun to pellet the protein G-sepharose. Supernatants were used in the Akt1/PKB1 kinase assay as described below and were also analyzed by immunoblotting. The pelleted G-sepharose beads were also analyzed by immunoblotting to determine the levels of rictor, mTOR, and raptor in the immunoprecipitates. Akt1/PKB1 kinase activity was determined using Crosstide (Upstate Biotechnology, #12-331) as substrate as recommended by the manufacturers protocol. Briefly, supernatant samples containing phosphorylated Akt1/PKB1 were incubated

for 10 min at 30 ˚C in a final volume of 25 μl of Akt/PKB kinase buffer (8 mM MOPS pH 7.0, 0.2 mM EDTA) containing 2.5 μl of Crosstide peptide (30 μM final concentration), 4.5 μl of Mg/ATP Cocktail, and 10 μCi of [γ-^{32}P]ATP. After the incubation the samples were cooled on ice and 20 μl aliquots were transferred onto the center of P81 paper square (Upstate Biotechnology, #20-134). After drying the P81 paper squares were washed 3 times for 5 min each time with 0.75 % phosphoric acid and once for 5 min with acetone. After the washing, the P81 squares were dried and radioactivity read in a scintillation counter.

***Drosophila* RNAi and analysis**

dsRNAs targeting *Drosophila* TOR pathway components were synthesized by in vitro transcription in 20 μl reactions using a T7 MEGAscript™ kit (Ambion). DNA templates for IVT were generated by RT-PCR from total *Drosophila* cellular RNA using the OneStep RT-PCR kit (Qiagen). Primers (which incorporated a 5' and 3' T7 promoter) for dAkt and dPTEN dsRNA synthesis were as follows:
dPTEN forward primer: 5'GAATTAATACGACTCACTATAGGGAGAATTAAGCTATTGGAAGAGAATCATGC.
dPTEN reverse primer:
5'GAATTAATACGACTCACTATAGGGAGAATCGATTTCTGATTTGCTTAAAGTG.
dAkt/dPKB forward primer:
5'GAATTAATACGACTCACTATAGGGAGAGTCAATAAACACAACTTTCGACCT.
dAkt/dPKB reverse primer:
5'GAATTAATACGACTCACTATAGGGAGAGAATATTTGAGTGAAATGAGGAACG.

The underlined region indicates the T7 promoter sequence. Primers for the synthesis of other dsRNAs were previously described [*1*]. dsRNA products were purified by adding 80 μl of RNAse free water to IVT reactions and filter purified with a vacuum manifold using Millipore filter plates (MANU 030 PCR). Final dsRNA concentrations were measured on a Nano-drop spectrophotometer.

Drosophila Kc_{167} cells were prepared for dsRNA addition by diluting an overnight culture seeded at 80 x 10^6 total cells in 12 ml *Drosophila* Schneider's medium to 1 x 10^6 cells/ml in Schneider's. 2 ml of media containing cells was then seeded to each well in 6-well culture dishes. dsRNAs were administered to cells using FuGENE 6 transfection reagent (Roche). Briefly, 3 μl of FuGENE was added to 97 μl of *Drosophila* SFM (Invitrogen), followed by addition of 2 μg of the indicated dsRNA in a sterile eppendorf tube. Tubes were gently mixed and incubated for 15 minutes at room temperature. FuGENE:dsRNA complexes were then administered to cells by adding the entire mix drop-wise around wells and then swirling to ensure even dispersal. For combination dsRNA addition experiments, 1.0 μg of PTEN dsRNA was mixed with 1.0 μg of the indicated dsRNA species (except in the GFP only control which contained 2.0 μg of the GFP dsRNA). Additional FuGENE:dsRNA complexes were added to wells on each of the following 2 days. On the third day of dsRNA addition, the medium was changed to avoid potential negative effects of excessive FuGENE on cell viability. After 4 days total of incubation to allow turnover of the target mRNAs, cell lysates were prepared as described [*1*]. 50 μg of total cellular protein was loaded per lane on 8% SDS-PAGE gels, separated, transferred to nitrocellulose membranes and analyzed by immunoblotting.

Lentiviral shRNA cloning, production, and infection

Desalted oligonucleotides (IDT) were cloned into LKO.1 [*3*] with the Age I/EcoRI sites at the 3' end of the human U6 promoter. The sequences of the oligonucleotides are as follows:

mTOR_shRNA_1 sense: 5'CCGGCCGCATTGTCTCTATCAAGTTCTTCCTGTCAAACTTGATAGAGACAATGC GGTTTTTG.
mTOR_shRNA_1 antisense: 5'AATTCAAAAACCGCATTGTCTCTATCAAGTTTGACAGGAAGAACTTGATAGA GACAATGCGG.
Raptor_sRNA_1 sense: 5'CCGGGGCTAGTCTGTTTCGAAATTTCTTCCTGTCAAAATTTCGAAACAGACTAGC CTTTTTG.
Raptor_sRNA_1 antisense:
5'AATTCAAAAAGGCTAGTCTGTTTCGAAATTTTGACAGGAAGAAATTTCGAAACAGACTAGCC.
Rictor_sRNA_1 sense:
5'CGGGCAGCCTTGAACTGTTTAACTTCCTgTCATTAAACAGTTCAAGGCTGCTTTTTG.
Rictor_sRNA_1 antisense:
5'AATTCAAAAAGCAGCCTTGAACTGTTTAATGACAGGAAGTTAAACAGTTCAAGGCTGC.
mTOR_shRNA_2 sense:
CCGGTTCAGCGTCCCTACCTTCTTCTCTCGAGAGAAGAAGGTAGGGACGCTGATTTTTG.
mTOR_shRNA_2 antisense: AATTCAAAAATCAGCGTCCCTACCTTCTTCTCTCGAGAGAAGAAGGTAGGGA CGCTGAA.
Raptor_shRNA_2 sense: CCGGAGGGCCCTGCTACTCGCTTTTCTCGAGAAAAGCGAGTAGCAGGGCCCTT TTTTG.
Raptor_sRNA_2 antisense: AATTCAAAAAAGGGCCCTGCTACTCGCTTTTCTCGAGAAAAGCGAGTAGCAGG GCCC.
Rictor_sRNA_2 sense: CCGGTACTTGTGAAGAATCGTATCTTCTCGAGAAGATACGATTCTTCACAAGTTTTT TG.
Rictor_sRNA_2 antisense: AATTCAAAAAACTTGTGAAGAATCGTATCTTCTCGAGAAGATACGATTCTTCACA AGTA.

Plasmids were propagated in and purified from Stbl2 bacterial cells (Invitrogen) and co-transfected together with the Delta VPR and CMV VSVG plasmids into actively growing HEK-293T using FuGENE (Roche) as described [*1, 3*]. Virus-containing supernatants were collected at 36 and 60 hours after transfection, and concentrated by ultracentrifugation for 1.5 hrs at 23,000 RPM in an SW28 rotor at 4°C. Pellets were resuspended overnight at 4°C in 1/600th of the original volume. Cells were infected twice in the presence of 6 μg/ml protamine sulfate, selected for puromycin resistance and analyzed on the 7th day after infection. In previous work we noted that an acute knockdown of mTOR expression in HEK-293T cells using siRNAs also partially decreased raptor expression [*2*]. This effect is decreased in magnitude in the chronic mTOR knockdown cell lines made with lentivirally-expressed shRNAs.

References for Materials and Methods

1. D. D. Sarbassov *et al., Curr Biol* **14**, 1296 (2004).
2. D.-H. Kim *et al., Cell* **110**, 163 (2002).
3. S. A. Stewart *et al., RNA* **9**, 493 (2003).

Supplemental Figure S1

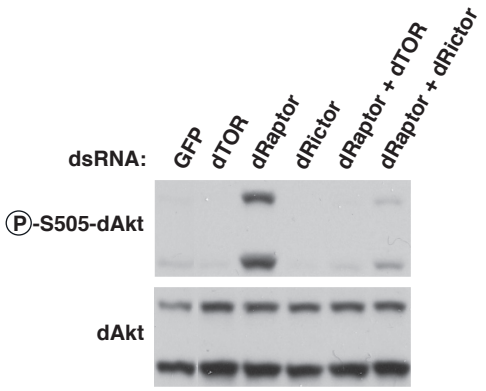

Fig. S1. dRictor and dTOR are required for the increase in dAkt/dPKB phosphorylation caused by a knockdown in dRaptor expression. dsRNAs corresponding to the genes for the indicated proteins were transfected into Kc167 Drosophila cells. A dsRNA corresponding to Green Fluorescent Protein (GFP) served as a negative control. After 4 days lysates were prepared and analyzed by immunoblotting for levels of phospho- and total dAkt/dPKB.

Supplemental Figure S2

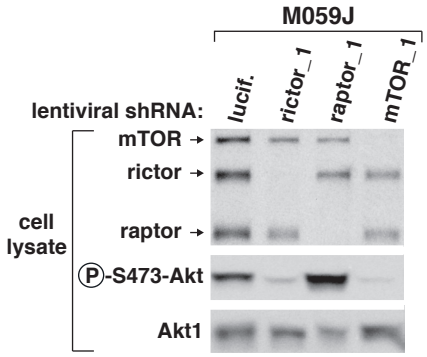

Fig. S2. Rictor and mTOR, but not raptor, positively regulate the phosphorylation of serine 473 of Akt/PKB in a cell line that is null for DNA-PKcs. Immunoblotting was used to analyze the total levels and phosphorylation states of the indicated proteins in M059J glioblastoma cell lines having stable decreases in rictor, raptor, or mTOR expression. The experiment was analyzed as in Figure 2.

Supplemental Figure S3

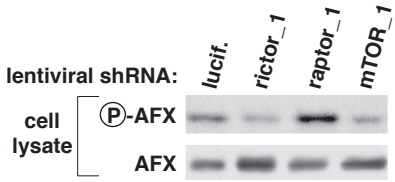

Fig. S3. Decreases in rictor or mTOR expression inhibit AFX phosphorylation. Cell lysates from the stable knockdown HeLa cell lines used in Figure 2B were analyzed by immunoblotting for the phosphorylation states and total levels of the AFX (Foxo4) transcription factor.