Supplementary Data

Supplementary Materials and Methods

siRNA Transfection

Cells were plated in 6 well plates for 24 h, transfected with 20 nM siRNA using HiPerFect (Qiagen, Basel, Switzerland), and cell lysates were prepared 72 h later. The following siRNA target sequences were used: raptor #3 (used for all other experiments in this manuscript) = *5′-TAT TTG GTC GTC CAA TCT CGT-3′*; raptor #4 = *5′-TTC TCC ACC GAG TAC ACG GAG-3′;* raptor #6 = *5′-ATA AAG AGT GCG GAG ACC CTT-3′*. rictor #9 (used for all other experiments in this manuscript) = *5′-TTA ATT GTA GCA ATA GAG GGT-3′*; rictor #8 = *5′-TTC ACA GTA ATC ATC TTT CTG-3′;* rictor SmartPool siRNA was purchased from Dharmacon (Lafayette, CO, USA). Luciferase = *5′-AAC GTA CGC GGA ATA CTT CGA-3′*. IRS1 SmartPool siRNA was purchased from Dharmacon (Lafayette, CO, USA).

Supplementary Legends

Supplementary Figure 1. 20 nM RAD001 efficiently inhibits the mTORC1 pathway. Human cancer cell lines were treated for the indicated times with vehicle control (DMSO) or 20 nM RAD001. Cell lines are listed according to ascending IC_{50} values for RAD001 (Table 1). Total protein lysate was prepared and subjected to western blot analysis. RAD001-induced effects are shown for S6 S235/236 phosphorylation.

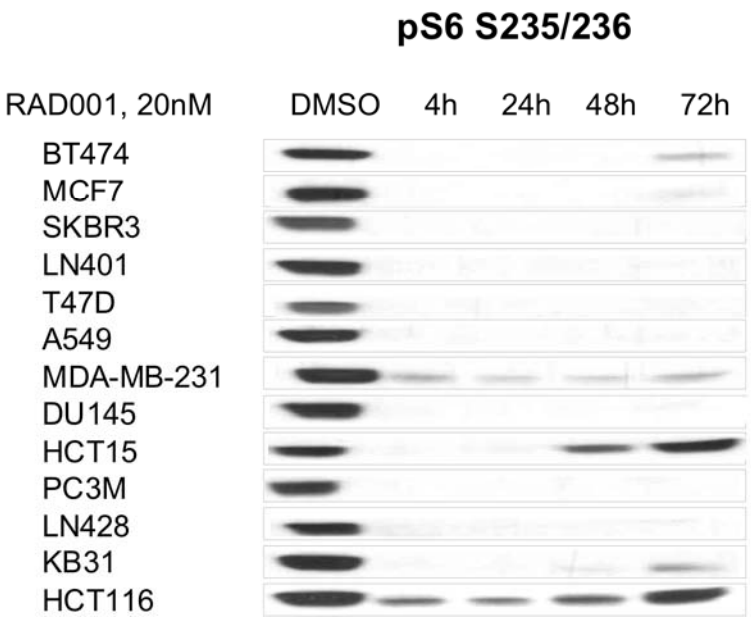
Supplementary Figure 2. Effect of RAD001 and IRS1 siRNA treatment on IRS1 protein levels and AKT S473/T308 phosphorylation. Human cancer cell lines were treated for the indicated times with vehicle control (DMSO) or 20 nM RAD001 (**A**). Cell lines are listed according to ascending IC_{50} values for RAD001 (anti-proliferative IC_{50}: listed in Table 1). Total protein lysates were subjected to immunoblot analysis. RAD001-induced effects are shown for IRS1 protein expression level. (**B**) Cells were transfected with IRS1 siRNA (20 nM) for 72 h and treated with RAD001 (20 nM) or DMSO for the last 24 h. Effects of IRS1 down-regulation on RAD001-induced AKT S473 and T308 phosphorylation are shown for HCT116, SKBR3, and A549 cells.

Supplementary Figure 3. siRNA analysis of the role of raptor and rictor in the regulation of basal AKT phosphorylation. Tumor cells were treated with RAD001 (20 nM) for 48 h or transfected with siRNA (20 nM) for 72 h prior to protein extraction and immunoblot analysis. Increased AKT phosphorylation after RAD001 treatment (**A**) is recapitulated by down-regulation of raptor protein expression (**B**) in cell lines representing a wide range of IC_{50} values for RAD001 (see Table 1). Down-regulation of rictor (**C**) slightly reduces AKT S473 phosphorylation with variable effects on AKT T308 phosphorylation.

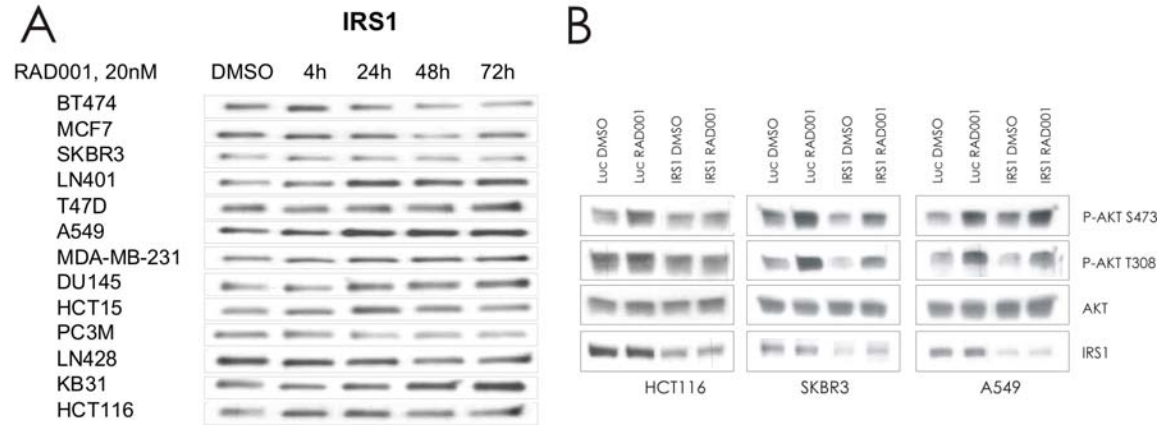
Supplementary Figure 4. Specificity of raptor and rictor siRNA-mediated response on AKT S473 phosphorylation. A549 cells were transfected with 20 nM control luciferase siRNA or siRNA against raptor (#3, #4, #6) or rictor (#9, #8, Dharmacon rictor siRNA) prior to treatment with vehicle control (DMSO) or 20 nM RAD001 for 24 h.

Efficient down-regulation of rictor and raptor protein expression was observed with all siRNA sequences. Effects of raptor siRNA on basal AKT S473 and RAD001-induced AKT S473 phosphorylation was confirmed using three different siRNA sequences. Attenuation of RAD001-induced AKT S473 phosphorylation was achieved using three different rictor siRNA sequences. De-phosphorylation of S6 on S235/236 is shown as a control for RAD001-induced inhibition of mTORC1 signaling. AKT protein levels serve as loading controls.

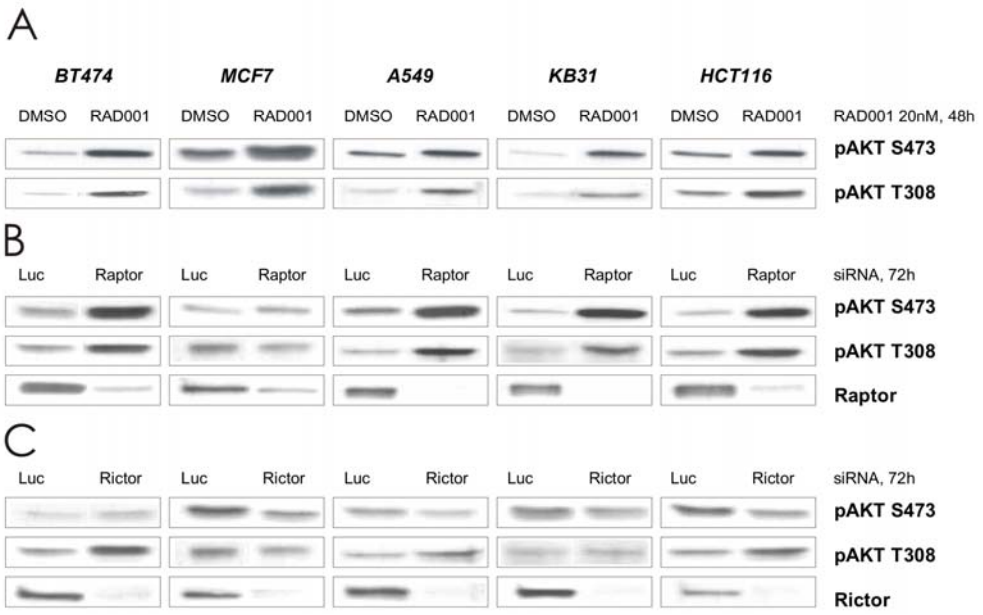
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4