Supporting Information

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SI Materials and Methods

Cell Lines and Reagents. BT474, EFM-192A, HCC1419, AU565, T47D, MCF7, MDA-MB-453, BT-20, and CAL-51 cells were cultured in DMEM (Cellgro; Mediatech Inc.) with 10% (vol/vol) FBS. MDA-MB-361 cells were cultured in RPMI 1640 (Cellgro; Mediatech Inc.) with 5% (vol/vol) FBS. Cell lines were obtained from the Center for Molecular Therapeutics at Massachusetts General Hospital Cancer Center. The mutation status was obtained from the Wellcome Trust Sanger Institute Cancer Genome Project Web site (www.sanger.ac.uk). The following drugs were used: GDC-0941 (supplied by the Targeting PI3K in Women's Cancers Stand Up to Cancer Dream Team), AKT-1/2 inhibitor (Sigma), and MK-2206 (Selleck Chemicals). Compounds were dissolved in DMSO to a final concentration of 10 mmol/L and stored at −20 °C. BYL719 and BKM120 were kindly provided by Novartis and also stored at a final concentration of 10 mmol/L at −20 °C.

Lentiviral shRNA Experiments. The tet-inducible shRNA vector used in this study was kindly provided by Novartis. The sequence encoded by inducible shP-Rex1 is cctatgaaccacagcttacaa. Lentiviral preparation and infections were performed as previously described (1). shRNA transduced cell lines were grown in each medium supplemented with 10% (vol/vol) Tet-approved FBS (Clontech) in the presence of 2 μg/mL puromycin. In the tet-inducible system, expression of shRNA was induced by growing cells in the presence of 10 ng/mL doxycycline (Sigma).

siRNA Knockdown. Cells were seeded into six-well plates at a density of 1.5×10^5 cells per well. Twenty-four hours later, cells were transfected with ON-TARGETplus SMARTpool siRNA against p-21 activated protein 1(PAK1), p-21 activated protein 2 (PAK2), p-21 activated protein 3 (PAK3), small GTPase K-RAS (K-RAS), small GTPase H-RAS (H-RAS) or phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 (P-Rex1) (Dharmacon) or Silencer negative control #1 siRNA (Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Transfected cells were cultured at 37 °C for 72 h before analysis.

Apoptosis Analysis. Cells were treated with media with or without indicated drugs. After 72 h, media was collected. Cells were washed with PBS and trypsinized. PBS wash and trypsinized cells were added to the collected media in a single tube. Cells were pelleted, washed once with PBS, and resuspended in annexin binding buffer (BD Biosciences). Cells were stained with propidium iodide (BD Biosciences) and annexin V Cy5 (Biovision) according to the manufacturer's protocol and assayed on an LSRII flow cytometer (BD Biosciences).

RNA Extraction and Quantitative RT-PCR. For cell lines, RNA was isolated and purified using the Qiagen RNeasy Mini kit and further purified by DNase treatment with Ambion Turbo DNase. After extraction and purification, RNA was reverse transcribed and amplified using superscript first-strand cDNA synthesis (Invitrogen). The amplicon abundance of PAK3 and β-actin was monitored in real time on a Roche Lightcycler 480 (Roche Diagnostics) by measuring the fluorescence increases of SYBR Green. The PAK3 primers used in this study were: forward, 5′-AGTCAGAAGTTCAGTTCGCC-3′, reverse, 5′-CATGATGGAGTTGAAGGTAGTTTCGT-3′. The β-actin primers were: forward, 5′-CTGTGCTATCCCTGTACGCCTC-3′ and reverse, 5′-CATGATGGAGTTGAAGGTAGTTTCGT-3′. Relative PAK3 RNA levels were calculated using the Δ-Δ threshold cycle (C_t) method as previously described.

1. Rothenberg SM, et al. (2008) Modeling oncogene addiction using RNA interference. *Proc Natl Acad Sci USA* 105(34):12480–12484.

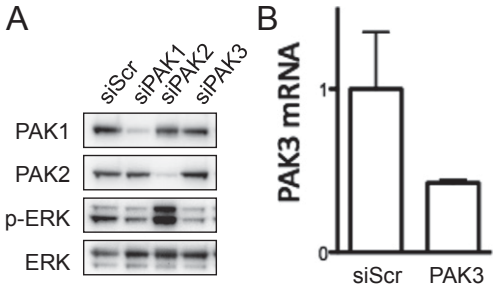

Fig. S1. PAK1 and PAK3 regulate ERK activity. T47D cells were transfected with control, PAK1, PAK2, or PAK3-targeted siRNA for 72 h. (*A*) Cell lysates were probed with the indicated antibodies. Independent experiments were performed at least four times, and a representative result is shown. (*B*) PAK3 mRNA was quantified by quantitative PCR (because PAK3 protein was not detectable using available antibody). The average ± SD of two independent experiments is shown.

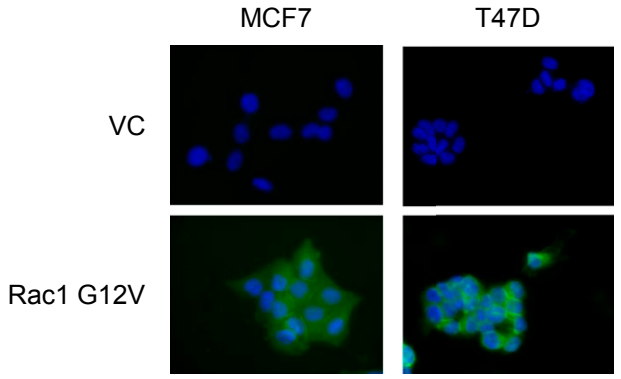

Fig. S2. Expression of constitutively active form of Rac1 (Rac1 G12V) in MCF7 and T47D cells. Cells were transduced with lentiviruses expressing Rac1 G12V fused with GFP or control vector (VC). The expression of GFP (fluorescence) was confirmed directly using fluorescence microscopy.

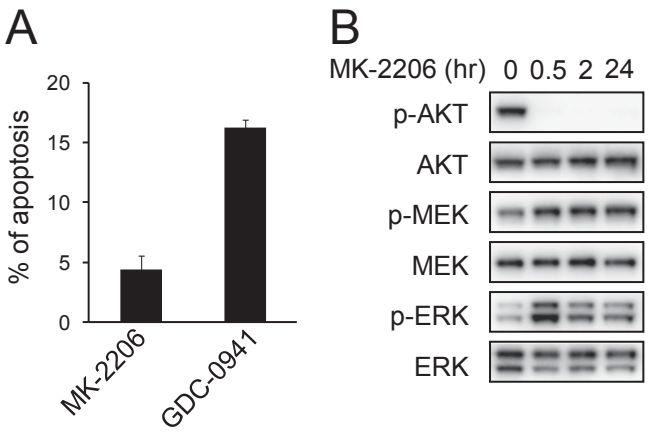

Fig. S3. AKT inhibitor MK-2206 fails to induce apoptosis and down-regulate p-ERK in MDA-MB-361 cells. MDA-MB-361 cells were treated with 1 μM GDC-0941 or 1 μM MK-2206 for 72 h (*A*) or indicated time points (*B*). (*A*) The percentage of cells undergoing apoptosis, as measured by annexin V positivity, is shown relative to DMSO-treated cells. The average ± SD is shown ($n = 3$). (*B*) Cell lysates were probed with the indicated antibodies. Independent experiments were performed three times, and a representative result is shown.

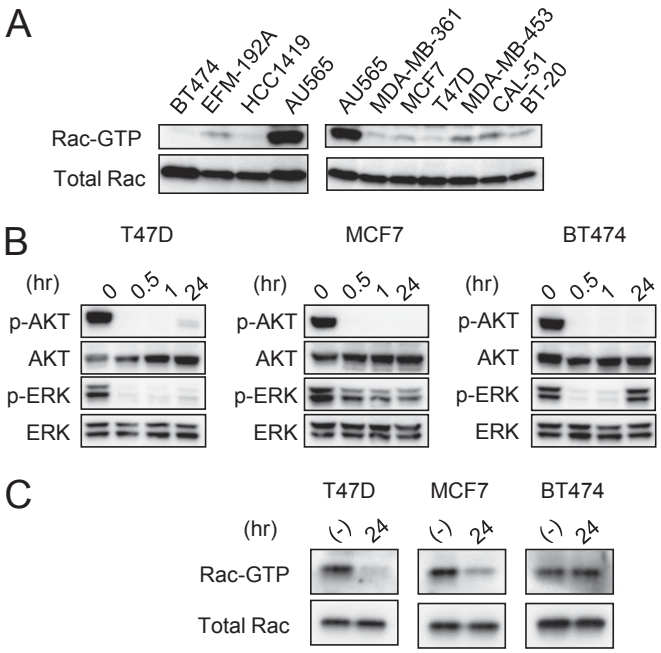

Fig. S4. Cell-type dependent ERK and Rac activation after 24 h treatment with GDC-0941. (*A*) Cells were lysed and Rac-GTP levels were determined with a PAK1-binding domain pull-down assay. (*B*) Cells were treated with 1 μM GDC-0941 for the indicated times, and lysates were probed with the indicated antibodies. (*C*) Cells were serum starved for 16 h, and media containing 10% (vol/vol) FBS with or without 1 μM GDC-0941 (GDC) was added. Cells were lysed after 24 h after treatment with GDC-0941, and Rac-GTP levels were determined with a PAK1-binding domain pull-down assay.

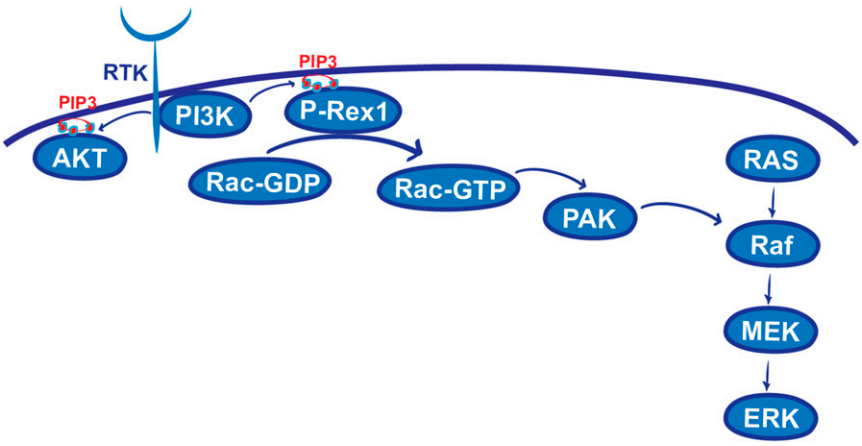

Fig. S5. PI3K controls the MEK/ERK pathway through the Rac-GEF P-Rex1. Schematic representation of how PI3K is proposed to regulate ERK pathway.