

Supporting Information

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SI Methods

Cell Culture. Cell lines were grown in 5% CO₂ at 37 °C. All cell lines were purchased from American Type Culture Collection, except for the HCT-116 cell line and its knockout derivatives, which were a generous gift from Bert Vogelstein (The Johns Hopkins University). The breast epithelial cell line MCF-10A (1) and its derivatives were propagated in DMEM/F12 (1:1) supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0.1 μg/ml cholera toxin. All supplements were purchased from Sigma–Aldrich, unless otherwise noted. Cancer cell lines used in this study were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% FBS except for colon cancer cell lines for which McCoy's 5A Medium was used.

Targeted Knockin of the PIK3CA Oncogene and Characterization of Clones. Targeting vectors were transduced into cells, with resultant G418-resistant colonies screened using a unique sib-selection strategy (2). We subsequently isolated 3 independently derived exon 9 knockin clones, 2 independently derived exon 20 knockin clones, and 1 randomly integrated control clone for each of the exon 9 and 20 targeting vectors. The knockin clones were determined to be single targeted homologous integrants and to have equal expression of mutant and wild-type PIK3CA through PCR and direct sequencing of gDNA and cDNA, respectively, as previously described (supporting information (SI) Fig. S1B and C) (3). Of note, all mutant *PIK3CA* clones behaved identically in all assays, suggesting similar effects between exon 9 and exon 20 hotspot mutations. The promoterless gene-targeting vector pSEPT was a generous gift from Fred Bunz (The Johns Hopkins University) and was cloned into an AAV backbone as previously described (4). Briefly, the targeting vector was transduced into cells and antibiotic selection was performed with 120 μg/ml of G418 (Invitrogen) in multiple 96-well plates. Neomycin-resistant colonies were expanded, replicated, and then pooled. PCR-based screening was performed to identify cells that had undergone homologous integration of the targeting vectors, followed by further PCR screening of individual colonies from positive pools. Targeted cells were infected with an adenovirus encoding Cre recombinase to remove the selection cassette, followed by single-cell dilution and screening by PCR for successful Cre recombination. Primer sequences for PCR are available upon request. Genomic DNA and total RNA were prepared from cells using QIAamp DNA Blood Kit and RNeasy Kit (QIAGEN), respectively. cDNA was synthesized with First Strand cDNA Synthesis Kit (Amersham Biosciences). PCR amplification was performed using GeneAmp 9700 (Applied Biosystems) and Platinum Taq (Invitrogen). PCR primers to amplify cDNA were designed so that forward and reverse primers were located at distinct exons. Automated direct sequencing of PCR products was carried out by The Johns Hopkins DNA Analysis Facility. Primer sequences for PCR amplification and sequencing are available upon request.

Colony Formation Assay in Semisolid Medium and Acinar Morphogenesis Assay. For colony formation assays, 3 × 10⁴ exponentially growing cells were cast in 3 ml of top-layer medium comprised of supplemented DMEM/F12 and 0.4% UltraPure Agarose (Invitrogen) and poured on top of a 2 ml bottom layer containing 0.6% agarose in 6-well tissue culture plates. Supplemented DMEM/F12 was added to the wells once a week. After 3 weeks of incubation, the colonies were stained with iodinitrotetrazo-

lium chloride (Sigma–Aldrich) and photographed the next day. Two independent experiments were done in triplicate. Morphogenesis assays were carried out as previously described (5). Photographs were taken under phase contrast microscopy after 2 weeks of incubation.

Immunoblotting. Cells were seeded in media using conditions for proliferation assays as noted (no EGF). After 24 h, the media was then changed so that cells were incubated for an additional 24 h with or without drugs at various concentrations of EGF. Cells were then harvested for protein lysates, and immunoblotting was performed as previously described (3). Briefly, whole-cell protein extracts prepared in Laemmli sample buffer were resolved by SDS-PAGE using NuPAGE gels (Invitrogen), transferred to Invitrolon PVDF membranes (Invitrogen), and probed with primary and horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used in this study are anti-p70 S6Kinase rabbit antibody (9202; Cell Signaling Technology), anti-phospho p70 S6Kinase rabbit antibody (9205S; Cell Signaling Technology), anti-p44/p42 MAP kinase rabbit antibody (9102; Cell Signaling Technology), anti-phospho p44/p42 MAP kinase (Thr-202/Tyr-204) mouse antibody (9106; Cell Signaling Technology), anti-AKT rabbit antibody (9272; Cell Signaling Technology), anti-phospho AKT (Ser 473) rabbit antibody (9271; Cell Signaling Technology), anti-cyclin D1 rabbit antibody (2922; Cell Signaling Technology), anti-GSK3β rabbit antibody (9315; Cell Signaling Technology), anti-phospho GSK3β rabbit antibody (9336S; Cell Signaling Technology), and anti-GAPDH mouse antibody (6C5) (ab8245; Abcam).

Cell Proliferation Assays. Exponentially growing cells were washed with HBSS 3 times and seeded in DMEM:F12 medium without phenol red, using 1% charcoal dextran-treated serum, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0.1 μg/ml cholera toxin, without EGF or with 0.2 ng/ml EGF as indicated, at a density of 2 × 10⁴ cells/well of a 6-well tissue culture dish at day 0. For experiments using 20 ng/ml EGF, 5 × 10³ cells/well were seeded. Medium was changed on days 1 and 4 unless otherwise indicated. Cells were harvested on day 6, and cell numbers were then counted using a Z1 Cell and Particle Counter (Beckman Coulter). All assays and growth conditions were performed in triplicate and repeated at least 3 times. For colon cancer and breast cancer cell lines, proliferation assays were performed similarly, but with 17 beta-estradiol supplementation (5 nM) for breast cancer cell lines. In some experiments, cells were grown in T25cm2 flasks and stained with crystal violet for visualization.

Pathway Inhibitors. LY294002, wortmannin, U0126, and rapamycin were purchased from Cell Signaling Technology. Lithium chloride, SB216763, and SB415286 were purchased from Sigma–Aldrich. Compounds were dissolved in DMSO, which was also used as a vehicle-only control in proliferation assays and used at the concentrations as described in the text. Lithium chloride was dissolved in water. Drugs were added to cells with media changes as described previously for proliferation assays. For preliminary experiments, a range of concentrations for each drug was tested on cell lines to determine doses and potential differences in toxicity between mutant *PIK3CA* knockin clones and control cell lines.

Xenograft Assays. For each group, five 8- to 10-week-old female athymic nude mice (Taconic) were injected s.c. in the bilateral

flanks with 100 μ l PBS containing 5×10^6 HCT-116 cancer cell-line derivatives (mutant or wild-type *PIK3CA* knockout). After the appearance of palpable tumors, tumor volumes (10 per each group) were analyzed biweekly and calculated by multiplying length, width, and height for each individual tumor. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was followed in all experiments.

1. Soule HD, et al. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 50:6075–6086.
2. Konishi H, et al. (2007) A PCR-based high-throughput screen with multiround sample pooling: Application to somatic cell gene targeting. *Nat Protoc* 2:2865–2874.
3. Konishi H, et al. (2007) Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. *Cancer Res* 67:8460–8467.

Statistical Analyses. For xenograft and proliferation assays, average tumor volume and SEM was calculated for each group. Statistical analysis was performed using a two-tailed Student's *t* test and ANOVA with 2 factor designs between groups, calculated with Excel (Microsoft) and ezANOVA. A *P* value <0.05 was considered statistically significant.

4. Topaloglu O, et al. (2005) Improved methods for the generation of human gene knockout and knockin cell lines. *Nucleic Acids Res* 33:e158.
5. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30:256–268.

