Supporting Information

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SI Materials and Methods

Plasmids and Reagents. Full-length human RNF43 cDNA (NM_017763.4) was purchased from OpenBiosystems and tagged with a C-terminal HA epitope by PCR. RNF43 cDNA resistant to RNF43 siRNA, RNF43 ΔRING (missing amino acids 272–312), and F69C mutant were generated by two-step mutagenesis PCR and cloned into various mammalian expression vectors. Details of plasmids are available upon request. LGK974 and IWP-2 were synthesized at Novartis.

Cell Culture, Infection, Transfection, and RNA Interference. Pancreatic cancer cells and HEK293 cells were grown in medium recommended by ATCC supplemented with 10% (vol/vol) FBS. Retrovirus or lentivirus was produced from HEK293 cells by standard virus packaging procedure by using FuGENE 6 (Roche) transfection reagent. Pancreatic cell lines expressing SuperTopFlash (STF) reporter, RNF43 constructs, or Doxycycline (DOX)-inducible β-catenin shRNA were generated by viral infection and drug selection.

siRNA transfection was performed by using Dharmafect 1 transfection reagent (Dharmacon) according to manufacturer's instruction. Sequences of siRNAs are listed as followed:

pGL2 (Dharmacon D-001100-01), target sequence,
5′ -CGTACGCGGAATACTTCGA -3′.
RNF43-1 (Dharmacon J-007004-12), target sequence,
5′ -GGUGGAGUCUGAAAGAUCA -3′.
RNF43-2 (Dharmacon J-007004-11), target sequence,
5′ -GGAGAAAGCUAUUGCACAG -3′.
CTNNB1-1 (Dharmacon J-003482-10), target sequence,
5′ -UAAUGAGGACCUAUACUUA -3′.
CTNNB1-2 (Dharmacon J-003482-12), target sequence,
5′ -GGUACGAGCUGCUAUGUUC -3′.

Sequencing Analysis. Genomic DNA was extracted by using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Exons of RNF43 were amplified by PCR and sequenced using the Applied Biosystems platform.

Reverse Transcription and Quantitative PCR. Total RNA was extracted from cells or tumors by using the RNeasy Plus Mini Kit (Qiagen). One microgram of RNA for cells or 200 ng of RNA for tumors was reverse transcribed with Taqman Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instruction. Quantitative PCR was performed in 12-μL reactions consisting of 0.6 μL of 20× Taqman probe and PCR primer mix, 6 μL of 2× Taqman FAST Advanced Master Mix (Applied Biosystems), and 5.4 μL of diluted cDNA template. The thermocycling conditions used were 20 s at 95 °C, followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. All experiments were performed in quadruplicates. Gene expression analysis was performed by using the comparative ΔΔCT method and normalized with the housekeeping gene *GUSB* or *18S*. The Taqman probes were purchased from Applied Biosystems.

Luciferase Reporter Assay. YAPC-STF reporter cells were transfected with siRNA and treated with Wnt3a conditioned medium or compound where applicable. STF luciferase assays were performed by using BrightGlo Luciferase Assay kits (Promega) according to the manufacturer's instructions.

Immunoblotting. Total cell lysates were prepared by lysing cells using RIPA buffer (50 mM Tris·HCl at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors, followed by centrifugation at 16,000 × *g* for 10 min at 4 °C. For cytosolic β-catenin extraction, cell pellets were resuspended in hypotonic buffer (10 mM Tris·HCl at pH 7.5 and 10 mM KCl, supplemented with protease/phosphatase inhibitors) and lysed by three freeze-thaw cycles. Equal amount of proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with primary antibodies for overnight at 4 °C. Secondary antibodies conjugated with either HRP or infrared dyes were used for signal visualization by ECL film or LI-COR Odyssey scanner, respectively. For coimmunoprecipitation experiments, cells were lysed in buffer containing 50 mM Tris·HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.8% Nonidet P-40, phosphatase inhibitors, and protease inhibitors. Cleared cell lysates were incubated with the indicated antibodies and Protein G-Sepharose beads (Amersham) overnight at 4 °C. Beads were washed four times with lysis buffer, and the bound proteins were eluted in SDS sample buffer for immunoblotting analysis. Sources of primary antibodies are as follows: anti-DVL 2, anti-Myc Tag, anti-Frizzled 6, and anti-LRP6 (Cell Signaling Technology); anti-MYC (Abcam); anti-p21(Millipore); anti-HA (Roche); anti-β-catenin (BD Pharmingen); anti-tubulin (Sigma); and anti-Ki67 (SP6) from Vector Laboratories.

Flow Cytometric Analysis. Cells were harvested by using trypsin-free cell dissociation buffer (Invitrogen) and resuspended in FACS buffer (PBS with 1% BSA and 0.02% sodium azide). After blocking, cells were incubated with anti-Myc-Alexa Fluor 647 (Cell Signaling Technology), anti-pan-Frizzled (18R5) antibody for 1 h at 4 °C, followed by incubation with allophycocyanin (APC)-conjugated goat anti-human IgG secondary antibody. After extensive washes using FACS buffer, cells were stained with propidium iodide (PI) and subjected to multichannel analysis by using BD LSR II flow cytometer. Fluorescence signals from PI-negative cells were displayed in histogram plots.

Foci Formation and Soft Agar Assay. For foci formation assay, 6,000~12,000 cells of indicated cell lines were seeded in six-well tissue culture plate in 2 mL of growth media. After overnight culture for cell attachment, media was replaced with fresh growth media containing 1 μM LGK974 in the absence and presence of recombinant Wnt3a. For DOX-inducible β-catenin shRNA experiment, cells were treated 5 ng/mL DOX. When cell colonies reached desirable size, cells were fixed with 10% (vol/vol) formalin in PBS and stained with crystal violet solution. After a few washes, plates were dried and imaged.

For soft agar assay, cells were suspended in 250 μL of 0.3% low melting agarose (Lonza) in DMEM containing 10% (vol/vol) FBS and plated onto 250 μL of solidified 0.8% agarose containing DMEM in 48-well culture plates at a density of 5,000–10,000 cells per well. Growth medium (250 μL) was added on top of the cells after the plate was cooled at room temperature for 30 min. The plate was then incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were treated with fresh growth medium containing DMSO or 1 μM LGK974 every 3–4 d. When colonies reached desirable size, photographs were taken and colonies were stained with AlamarBlue (Invitrogen) according to the manufacturer's instruction. These experiments were repeated three times, and at least four wells were replicated each time for each condition.

EdU Proliferation Assay. Cells were plated in growth medium in a 96-well plate at a density of 6,000–12,000 cells per well and treated with DMSO or 1 μM LGK974. After 3 d, the cells were treated with fresh growth medium containing 20 μM EdU, which was included in the Click-iT EdU Alexa Fluor 488 HCS assay kit (Invitrogen), and the plate was incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were fixed with final 4% (mass/vol) paraformaldehyde for 30 min, washed with PBS, permeabilized, and stained with 50 μg/mL Hoechst in PBS for 30 min. After wash, the cells were proceeded to EdU detection according to the instruction of Click-iT EdU assay kit. Triplet wells were performed for each condition.

Alcian Blue Staining for Mucin. Pancreatic cancer cell lines were plated onto 225 cm^2 tissue culture flasks at various densities and were treated with DMSO or LGK974 (100 nM) for 72 h. Cell pellets were harvested by removing media, washing with 1× PBS, and adding 10 mL of 10% (vol/vol) buffered formalin. Cells were then scraped from the bottom of the flask and placed in a 50-mL conical tube, filled to ∼50 mL with 10% (vol/vol) buffered formalin, and allowed to fix for 1–2 h. The conical tube containing the fixed cells was then centrifuged at 314 × *g* for 5 min, and the pelleted cells were wrapped in lens paper, placed in a histology cassette, processed, and paraffin embedded. Formalin-fixed paraffin-embedded sections were cut at 5 μm, mounted on slides, baked at 60 °C for at least 30 min, and deparaffinized. Slides were then rinsed two times in H_2O, transferred to Acetic Acid 3% (vol/vol) Aqueous for 3 min, and moved directly to Alcian Blue 1% in 3% acetic acid at pH 1 for 30 min. Slides were then placed in running water for 10 min after which they were rinsed in distilled H_2O before being placed in Nuclear Fast Red 0.1% (Kernechtrot) for 5 min. Slides were again washed in running water and then dehydrated. Lastly, the slides were coverslipped with Permaslip.

Immunohistochemistry and Image Analysis. Xenograft tumor samples were fixed in 10% (vol/vol) neutral-buffered formalin for 6–24 h, processed, and paraffin embedded. Immunohistochemical staining was performed on the Ventana Discovery System. The Ki67 antibody was from Vector Laboratories (SP6, VP-RM04). Images were captured by using Aperio Scanscope. Images of whole sections of mouse pancreata and xenograft tumors were analyzed with Visiopharm. Stromal tissue was automatically excluded by using the Section Assembler module, and necrotic regions were manually excluded by using the drawing tools provided by the analysis software. Tissues were segmented by using the TissuemorphDP module. Diaminobenzadine intensity was quantified as the percent of positive nuclei, and Alcian blue staining was quantified as the percent of positive pixels.

In Vivo Efficacy and Pharmacodynamic Studies. Cells were harvested at a density of 95–110% confluency. Ten million cells (HPAF-II) or 3 million cells (Capan-2) mixed 50:50 with BD matrigel matrix basement membrane (Matrigel) (BD Biosciences) were subcutaneously implanted into the upper right supra-axillary region of nu/nu (Harlan) (HPAF-II) or scid.bg (Harlan) (Capan-2) mice. Tumors were monitored twice weekly by calipering, and tumor volumes (TV) were calculated by using the ellipsoid formula: $TV(mm^3) = [(length \times width^2) \times 3.14159]/6$. Xenograft tumor-bearing mice were randomized to treatment groups ($n = 8$ per group) either 14 d after tumor implant (Capan-2), when the mean tumor volume reached 223 mm^3 (range 200–250 mm^3) or 11 d after implant (HPAF-II), when the mean tumor volume reached 341 mm^3 (range 272–418 mm^3). Mice were treated by oral gavage twice daily at a dosing volume of 10 mL/kg with either vehicle [0.5% Methylcelluose (MC)/0.5% Tween 80] or a 0.65 mg/mL suspension of the fumarate salt form of LGK974 (NVP-LGK974-AE-4, free base molecular weight conversion factor 1.293) in 0.5% MC/0.5% Tween 80. In vivo doses are reported as free base equivalents. Following treatment for 14 d (HPAF-II) or 35 d (Capan-2), antitumor activity was reported as percent treatment/control (%T/C) or %Regression (% REG) values. Antitumor activity was calculated by using the following formula: $\%T/C = 100 \times \Delta T_t/\Delta C_t$ if $\Delta T_t \geq 0$; or $\%REG = 100 \times \Delta T_t/T_0$ if $\Delta T_t < 0$; where: T_0 = mean tumor volume (TV) of the drug-treated group on the day of randomization; T_t = mean TV of the drug-treated group at study end; $\Delta T_t = T_0 - T_t$; C_t = mean TV of the control group on the final day of the study; C_0 = mean TV of the control group on the day of randomization; and $\Delta C_t = C_0 - C_t$. %T/C values in the range of 100–42% are interpreted to have no antitumor activity; %T/C values ≤ 42% and >10% are interpreted to have antitumor activity, %T/C values ≤ 10% or %REG ≥ −10% are interpreted to be tumor stasis. %REG values < −10% are interpreted as regressions. Separate, parallel cohorts of animals ($n = 3$ per treatment and time point) were treated with vehicle or LGK974 as above to obtain tumor tissue for ex vivo analysis of pharmacodynamic markers.

Statistical Analysis. Unpaired *t* tests were used to determine statistical significance. Symbols used were as follows: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

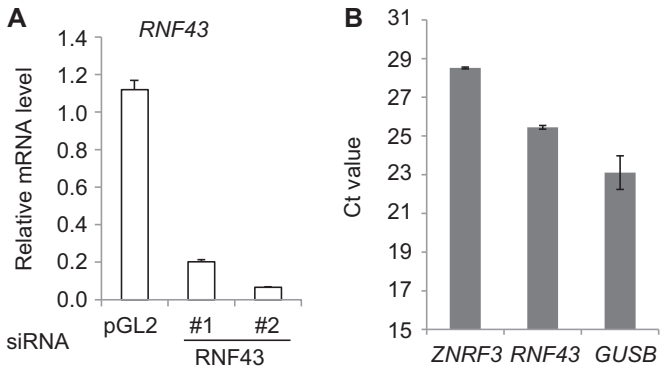

Fig. S1. Relative expression of *RNF43* and *ZNRF3* in YAPC cells. (*A*) Knockdown efficiency of RNF43 siRNA in YAPC cells. YAPC cells were transfected with indicated siRNA. The mRNA expression of *RNF43* was measured by quantitative RT-PCR. (*B*) *RNF43* is expressed at a higher level than *ZNRF3* in YAPC cells. Relative expression of *RNF43* and *ZNRF3* was determined by quantitative RT-PCR.

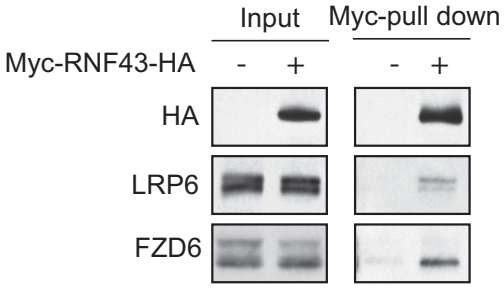

Fig. S2. Coimmunoprecipitation between RNF43 and LRP6 and FZD6. Cells transfected with empty vector or plasmid encoding Myc-RNF43-HA were lysed and immunoprecipitated with anti-Myc antibody. Immunoprecipitated proteins were resolved by SDS/PAGE and blotted with indicated antibodies.

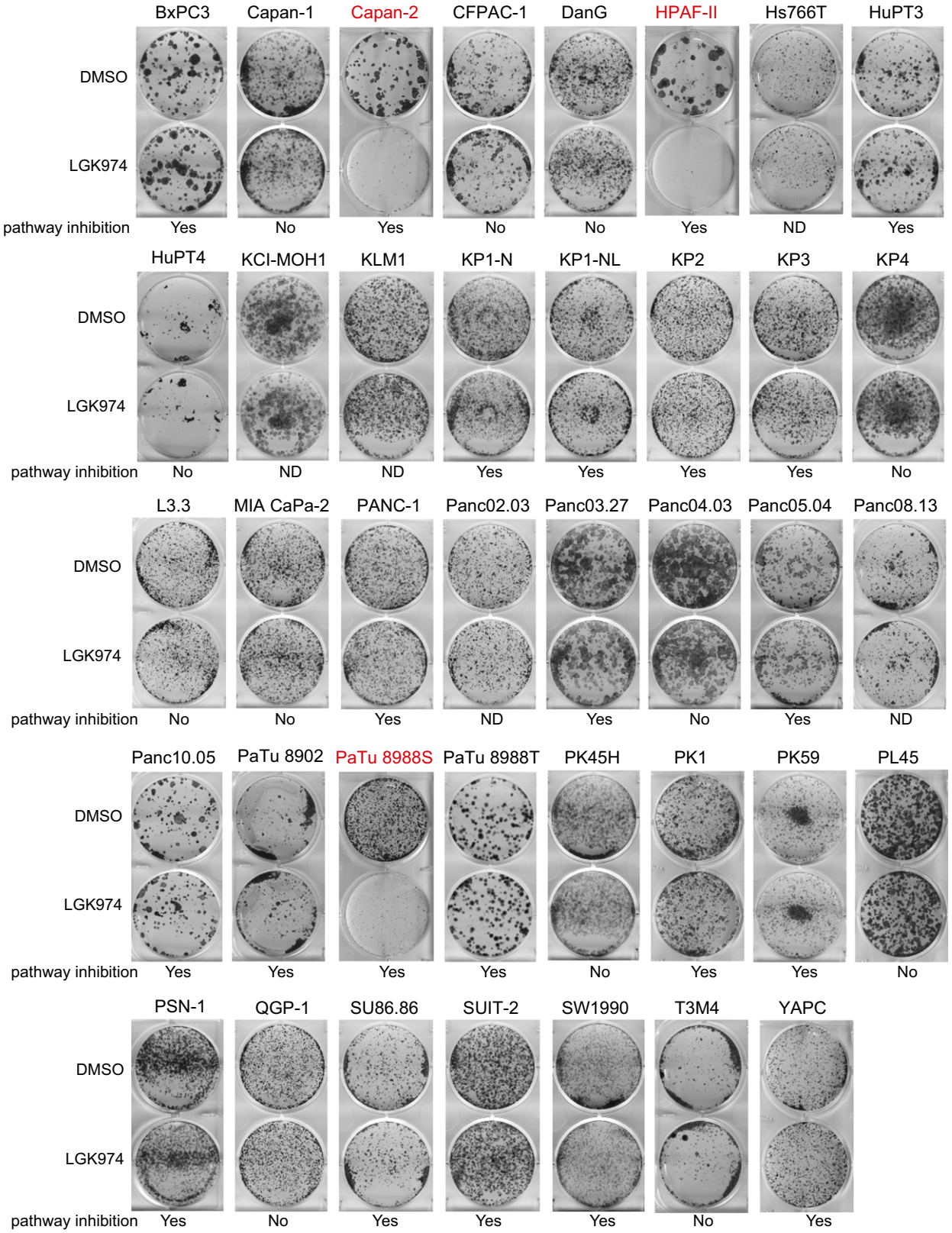

Fig. S3. Sensitivity of pancreatic cell lines to LGK974 in foci formation assay. Wnt dependency of pancreatic cell lines was examined by treating cells with DMSO or 1 μM LGK974 in foci formation assay. Inhibition of Wnt/β-catenin pathway was determined by checking the effect of LGK974 on the mRNA expression of β-catenin target gene *AXIN2*. ND, not determined.

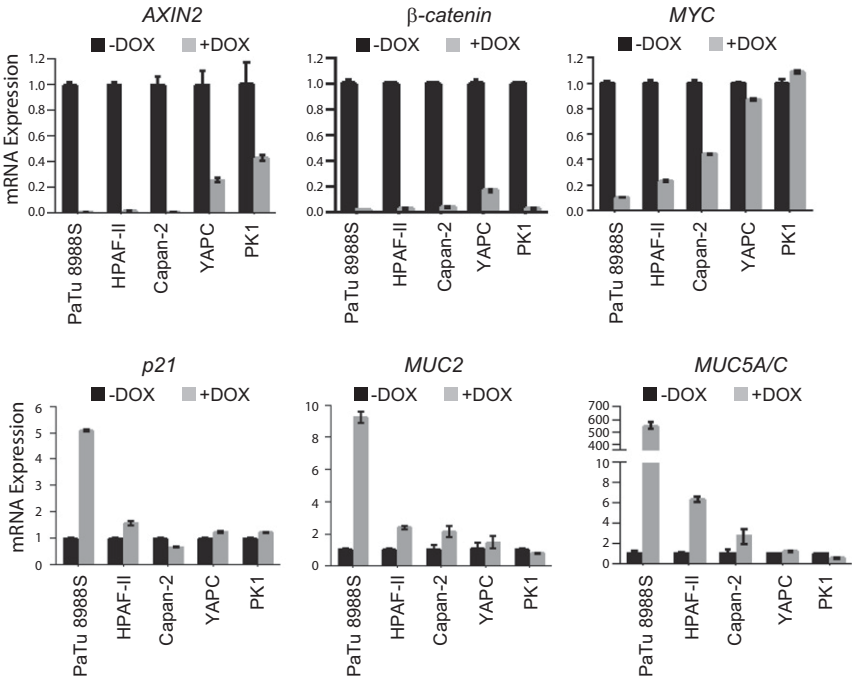

Fig. S4. Effect of β-catenin depletion on gene expression in pancreatic cell lines. Pancreatic cell lines expressing DOX-inducible β-catenin shRNA were generated. The effect of β-catenin shRNA on expression of various genes was determined by quantitative RT-PCR.