Supporting Information

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SI Materials and Methods

Genetically Engineered Mice. *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$; Pdx-1-Cre* (KPC) mice develop advanced and metastatic pancreatic ductal adenocarcinoma (PDA) with 100% penetrance at an early age, recapitulating the full spectrum of histopathological and clinical features of human PDA (1). Mice were housed in a 12-h light, 12-h dark cycle. All procedures were conducted in accordance with the Home Office licence and the Cambridge Cancer Research Institute Ethics committee.

Therapeutic Intervention Study. For the purpose of study enrollment, all KPC mice were subjected to high-contrast ultrasound screening using the Vevo 2100 System with an MS250, 13–24 MHz scanhead (VisualSonics) as described (2). Mice with tumor diameters of 6–9 mm were randomized and enrolled in both therapeutic intervention studies: (*i*) connective tissue growth factor (CTGF) inhibition (four treatment arms: IgG; gemcitabine/IgG; FG-3019; or FG-3019/gemcitabine), and (*ii*) cytidine deaminase inhibition [two treatment arms: gemcitabine and gemcitabine/3,4,5,6-tetrahydrouridine (THU)]. Gemcitabine and THU were administered at 100 mg/kg by i.p. injections every 3–4 d; neutralizing mAb FG-3019 was dosed at 30 mg/kg by i.p. every 3 d. During the study, tumor growth was quantified on day 4 and day 7 by measuring tumor volumes using reconstructed 3D ultrasonography with the integrated Vevo 2100 software package. Mice were terminally perfused on day 9, 2 h after the last gemcitabine administration with PBS. One half of the tumor was fixed in 10% (vol/vol) neutral buffered formalin (Sigma) for histology, and the other half was sliced in four to six pieces and snap-frozen in liquid nitrogen and subsequently stored at −80 °C.

Survival Study. For survival studies, tumor-bearing KPC mice (tumor diameter 6–9 mm) were assigned to one of three arms—gemcitabine, FG-3019, or FG-3019/gemcitabine combination—and treatments were administered as described above. Upon enrollment, tumor growth was continuously monitored by 3D ultrasonography once per week. End-point criteria were defined as 20% body weight loss, general morbidity, lethargy, lack of social interaction, or development of ascites by animal technicians who were blinded to the study cohorts. Where possible, mice received a final dose of gemcitabine 2 h before sacrifice.

Drug Preparations. Gemcitabine powder (a 48% preparation of difluorodeoxycytidine) was provided by Addenbrooke's Hospital pharmacy and resuspended in sterile normal saline at 10.2 mg/mL. Neutralizing mAb FG-3019 is a fully human IgG (FibroGen), and human IgG (Jackson ImmunoResearch) was used in the control cohort. THU (Calbiochem), a cytidine deaminase inhibitor, was diluted in 0.85% saline (stock 15 mg/mL).

Cell Culture. Cell lines were derived from murine KPC tumors as previously described (1) and maintained in DMEM (41966029; Invitrogen) + 10% FBS (SH30070.03; HyClone). Cancer-associated fibroblasts (CAFs) were obtained by serial trypsinization of small tumor pieces originating from *LSL-Kras$^{G12D/+}$;Ptf1a-Cre* (KC) mice. Protein lysates were obtained using radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors (3).

CTGF ELISAs. ***ELISA for mouse plasma samples.*** ELISA plates were incubated with anti–N-terminal CTGF capture antibody [5 μg/mL in PBS without divalent cations (PBS−)] overnight at 2–8 °C. After washing with wash buffer (PBS−, 0.1% Tween 20), the plates were blocked with assay buffer (1% BSA, 0.2% Triton X-100 in PBS−) for 2 h at room temperature (RT). Subsequently, 50 μL of recombinant mouse N-fragment CTGF standards, mouse recombinant N-fragment CTGF quality control samples (three concentrations), and diluted sodium EDTA plasma samples were added to each well. All samples contained 20% mouse plasma to control for matrix effects. Next, 50 μL anti–N-terminal CTGF detection antibody (1 μg/mL) was added and plates were incubated for 2 h at RT on a shaker. After washing, 100 μL of 1:2,000 diluted goat anti-mouse IgG2a-AP (Southern Biotech) was added to all wells, and plates were sealed and incubated for 45 min at RT on a shaker. After washing, 100 μL per well of 1 mg/mL alkaline phosphatase yellow substrate (Sigma-Aldrich) was added and incubated for 5–10 min at RT. Color intensity was measured in an ELISA plate reader at a wavelength of 405 nm. The lower limit of quantitation (LLOQ) for the assay was 9.4 ng/mL.

ELISA for mouse pancreas tissue lysates. Pancreas tissue samples were sonicated in lysis buffer (20 mM Tris, pH 7.5, 10% glycerol, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 150 mM sodium chloride, 0.5% sodium deoxycholate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 5% protease inhibitor mixture) on ice until all samples were homogenized. Tissue lysates were centrifuged at 16,000 × *g* for 10 min, and the supernatants were collected. Pancreatic tissue lysates were incubated with coated ELISA plates as described above for plasma samples up until the wash step following the addition of anti–N-CTGF detection antibody. The final detection was performed by adding 100 μL of 1:20,000 diluted goat anti-human κ HRP (Southern Biotech) to all wells, and the plates were sealed and incubated for 1 h at RT on a shaker. After the plates were washed, 100 μL of SureBlue Substrate (Kirkegaard & Perry Laboratories) was added to each well, and the plates were incubated at RT on a shaker for 7 min. The reaction was stopped by adding 100 μL per well of stop solution (2 N H_2SO_4). Plates were read in an ELISA plate reader at a wavelength of 450 nm. The LLOQ for the assay was 11 ng/mL.

RNA Preparation. Pancreatic tissue samples were immediately placed in RNA*later* solution (Qiagen) and stored for at least 24 h at 4 °C and then snap-frozen until processing. Total RNA was isolated using a TissueLyser (Qiagen) and RNeasy Kit (Qiagen). cDNA was synthesized from 1 μg of RNA using a QPCR cDNA Synthesis Kit (Applied Biosystems) and analyzed by quantitative real-time PCR.

Western Blot Analysis. The following primary antibodies were used: Hsp90 (Cell Signaling; E289, 1:5,000), E-cadherin (Becton Dickinson; 610181, 1:2,500), CTGF (FibroGen; FG-3149, 1:10,000), α-smooth muscle actin (SMA) (Dako; 1A4, 1:1,000), X-linked inhibitor of apoptosis antibody (Abcam; 21278, 1:2,000), and goat anti-human IgG (Jackson ImmunoResearch; 109-001-003, 1:5,000). Membranes were incubated with secondary HRP antibodies (Jackson ImmunoResearch; 1:5,000) and developed using the ECL detection system (GE Healthcare).

Histological Examination. Tissues were fixed in 10% neutral buffered formalin (Sigma) for 24 h and transferred to 70% ethanol. Tissues were embedded in paraffin, and 3- to 5-μm sections were processed for H&E staining, immunohistochemistry, and immunofluorescence using standard protocols as previously described

(4). The following antibodies and kits were used: CTGF (FibroGen; FG-3019, 1:1,000), secreted protein acidic and rich in cysteine (SPARC) (R&D Systems; AF942, 1:500), α-SMA (Dako; 1A4, 1:250), E-cadherin (Becton Dickinson; 610181, 1:1,000), cleaved caspase 3 (CC3) (Cell Signaling; 5A1E, 1:100), phosphohistone H3 (PH3) (Upstate; 06-570), fibronectin (Santa Cruz; sc-6952, 1:100), and CD31 (BD Pharmingen; 553370, 1:100). The following reagents were used for special extracellular matrix stains: Picrosirius Red Stain Kit (Polysciences), Herovici (Sigma), and Masson Trichrome (Sigma). Stainings were performed according to standard protocols. Images were acquired on an Olympus BX51 microscope.

Methods of Quantification. ***Automated quantification.*** Automated quantification of immunohistochemistry for CC3 was performed on 30 randomly chosen fields using the Ariol SL-50 imaging system and analysis software (Genetix). For α-SMA, SPARC, Picrosirius, and Herovici stainings, positive areas and total tumor area were assessed by the Ariol SL-50 imaging system using a low threshold to account for background staining of necrotic tumor areas. For mean vessel density, CD31 immunoreactivity was automatically quantified in a complete tumor cross-section using the Aperio XT automated scanning system and ImageScope 10 software (Aperio).

Manual quantification of apoptosis. Tumor cell apoptosis as assessed by coimmunofluorescence of CC3 and E-cadherin was determined by the number of CC3- and E-cadherin–positive cells per 20× field. At least five fields were quantified per tumor.

Metastasis quantification. Two lobes of liver were sliced into a total of four to six strips and embedded in paraffin. For each mouse, 5 sections at 30- to 50-μm distance were H&E-stained and microscopically analyzed for metastases. Metastatic lesions were classified as micro, small, and large, and burden was calculated by the sum of all metastatic lesions multiplied 1× (micro), 2× (small), and 3× (large).

LC-MS/MS of Gemcitabine. LC-MS/MS was performed on a TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific) fitted with a heated electrospray ionization (HESI-II) probe operated in positive and negative mode at a spray voltage of 2.5 KV, capillary temperature 150 °C. Fresh frozen tumor samples were processed and analyzed by LC-MS/MS as previously described by our group (5). Quantitative data acquisition was done using LC Quan2.5.6 (Thermo Fisher Scientific).

1. Hingorani SR, et al. (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 7(5):469–483.
2. Cook N, Olive KP, Frese K, Tuveson DA (2008) K-Ras-driven pancreatic cancer mouse model for anticancer inhibitor analyses. *Methods Enzymol* 439:73–85.
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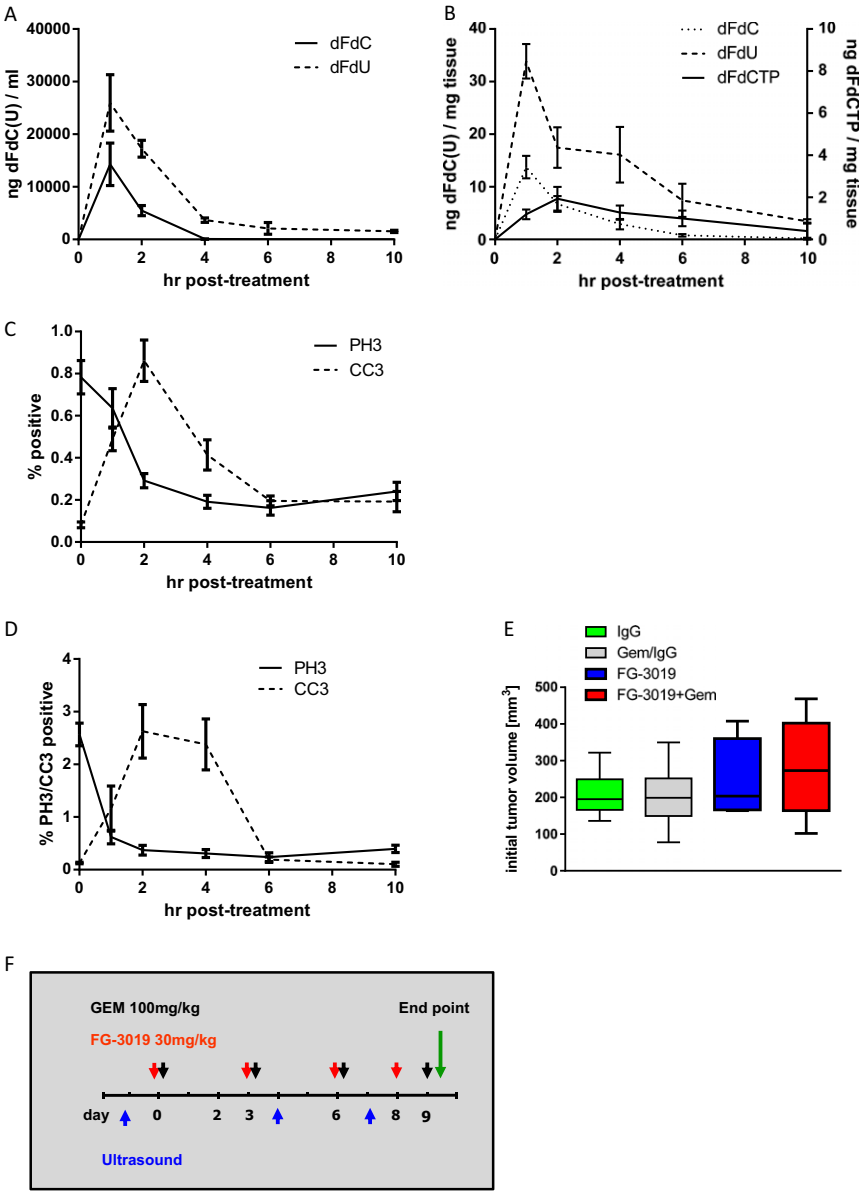

Fig. S1. Pharmacokinetic and pharmacodynamic profile of gemcitabine in KPC mice; error bars represent standard error of the mean (SEM). (*A*) Pharmacokinetic profile of native (2′,2′-difluorodeoxycytidine; dFdC) and inactivated (2′,2′-difluorodeoxyuridine; dFdU) gemcitabine metabolites in plasma of KPC mice ($n = 38$ in total) following a single i.p. injection of 100 mg/kg dFdC. The following time points were analyzed: 0 h ($n = 5$), 1 h ($n = 7$), 2 h ($n = 7$), 4 h ($n = 7$), 6 h ($n = 7$), and 10 h ($n = 5$). (*B*) Pharmacokinetic profile of native (dFdC), inactivated (dFdU), and active (2′,2′-difluorodeoxycytidine-5′-triphosphate; dFdCTP) gemcitabine metabolites in tumor biopsies of KPC mice ($n = 65$ in total) corresponding to the same samples displayed in *A* for 0 h ($n = 4$), 1 h ($n = 21$), 2 h ($n = 15$), 4 h ($n = 19$), 6 h ($n = 7$), and 10 h ($n = 5$). (*C*) Pharmacodynamic profile of cleaved caspase 3 and phosphohistone H3 immunohistochemistry in tumor samples from KPC mice ($n = 65$ in total) following a single i.p. injection of 100 mg/kg dFdC. (*D*) Quantification of proliferation (PH3) and apoptosis (CC3) in small intestinal cells following a single dose of 100 mg/kg i.p. of gemcitabine. Each time point represents ≥5 mice. (*E*) Preenrollment tumor volumes of the 9-d short-term study ($n \geq 6$ each cohort) showing no significant differences before enrollment. (*F*) Schematic of the short-term 9-d trial for the combination therapy of FG-3019 and gemcitabine in tumor-bearing KPC mice.

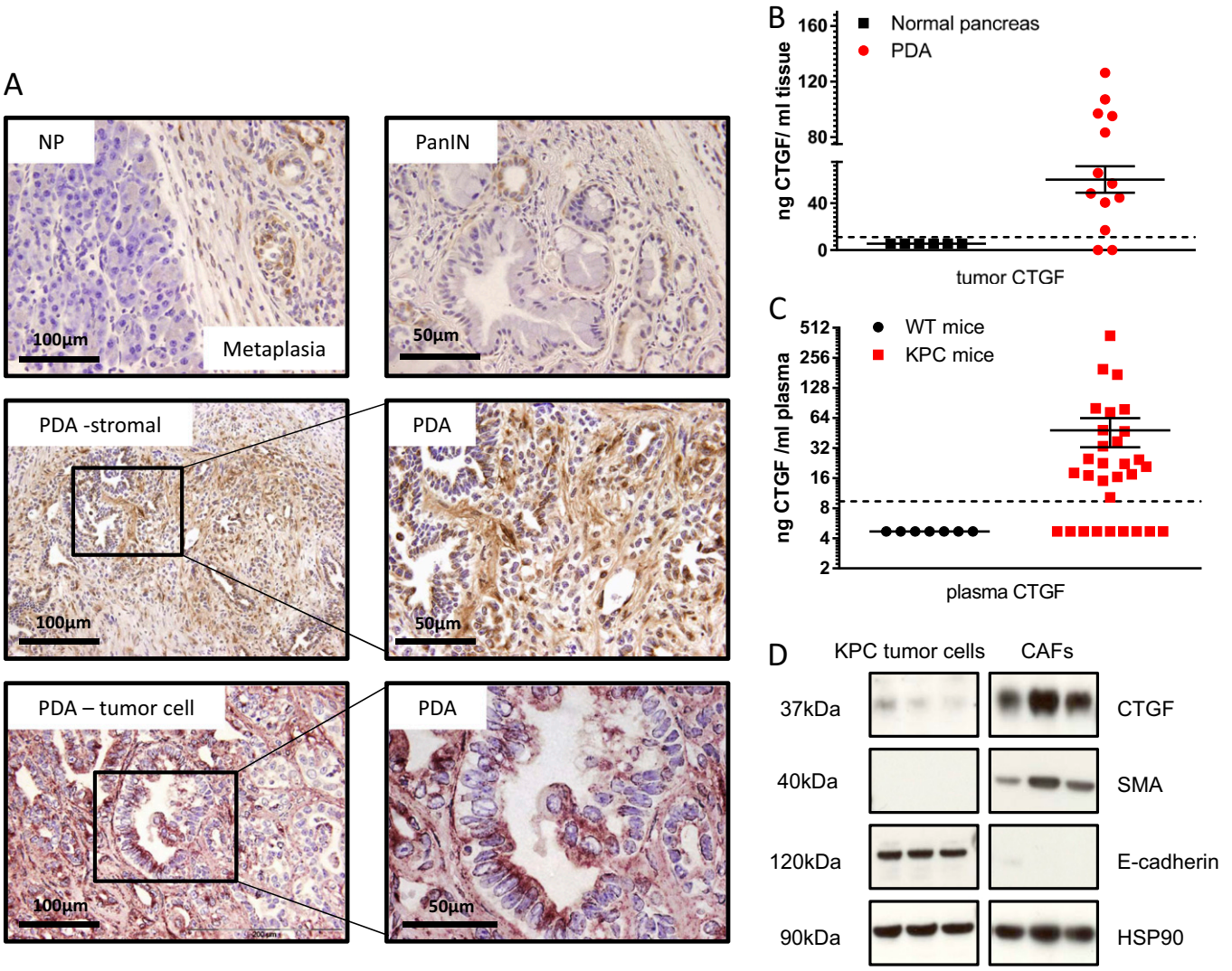

Fig. S2. CTGF is present in KPC tumors and plasma. (*A*) Representative CTGF immunohistochemistry in normal pancreas (NP) tissue ($n = 3$; *Top Left*), pancreatic intraepithelial neoplasia (PanIN) ($n = 6$; *Top Right*), and a murine pancreatic cancer specimen ($n = 11$) with stromal (*Middle*) and tumor cell (*Bottom*) expression patterns. (*B*) CTGF concentration in murine pancreas ($n = 6$, dotted line; LLOQ, 11 ng/mL), murine PDA lysates ($n = 13$; Wilcoxon signed-rank test; $P = 0.001$), and (*C*) plasma of WT ($n = 7$) and tumor-bearing KPC mice ($n = 30$; $P < 0.0001$; LLOQ, 9.4 ng/mL) by ELISA. (*D*) Western blot analysis of CTGF, α-SMA, and E-cadherin in primary murine tumor and cancer-associated fibroblast cell lines.

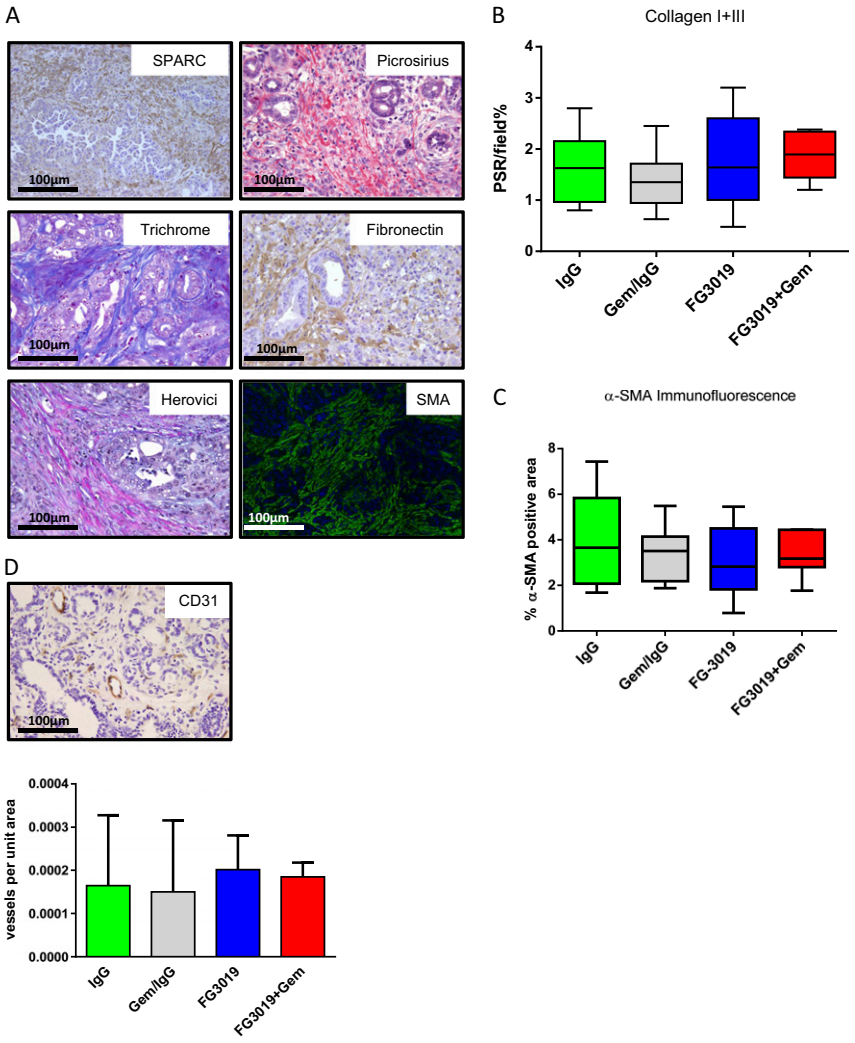

Fig. S3. (Continued)

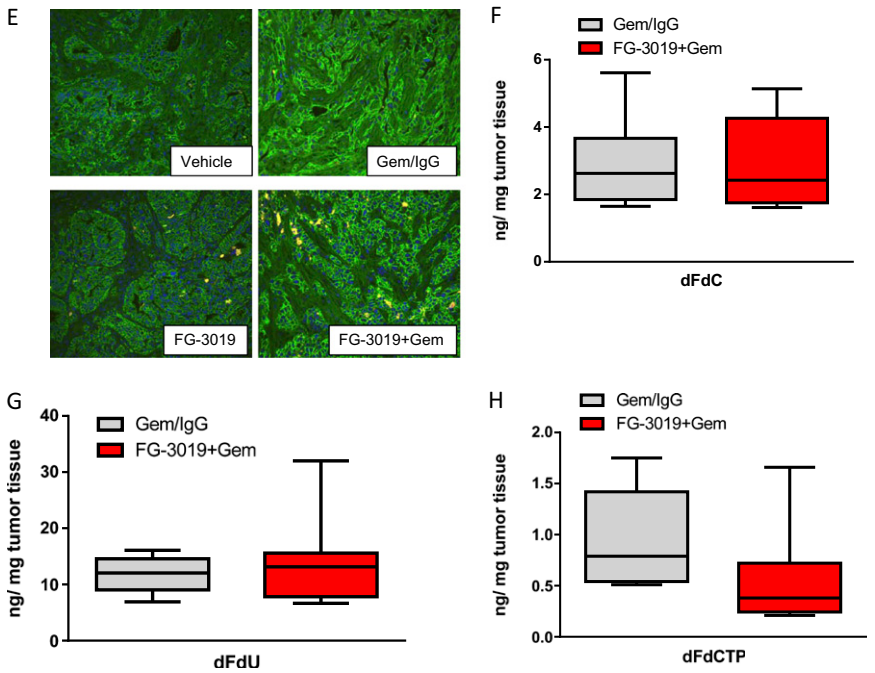

Fig. S3. FG-3019 induces neoplastic apoptosis but does not alter tumor stroma, vasculature, and gemcitabine pharmacology over a 9-d course. (*A*) Representative immunohistochemical and immunofluorescent images of FG-3019/gemcitabine–treated KPC tumors reveal normal structure and composition of the tumor microenvironment. Chemical dyes [Picrosirius Red (PSR), Trichrome, Herovici] and immunohistochemical detection of specific proteins (SPARC, α-SMA, fibronectin) designate the stromal compartment. (*B* and *C*) Automated quantification of collagen I+III by PSR (*B*) and α-SMA (*C*) using polarized light microscopy and immunofluorescence stains ($n \geq 6$ per cohort). (*D*) Tumor vasculature as examined by CD31 immunohistochemistry did not show significant changes among the cohorts upon treatment ($n \geq 5$ each cohort; error bars represent standard deviation). (*E*) Representative coimmunofluorescence images of DAPI (blue), E-cadherin (green), and CC3 (orange) in each of the 9 d-treated cohorts. (*F–H*) Concentration of native gemcitabine (dFdC) and metabolites dFdU and dFdCTP in PDA tumor samples from KPC mice treated with gemcitabine/IgG and FG-3019/gemcitabine ($n \geq 6$).

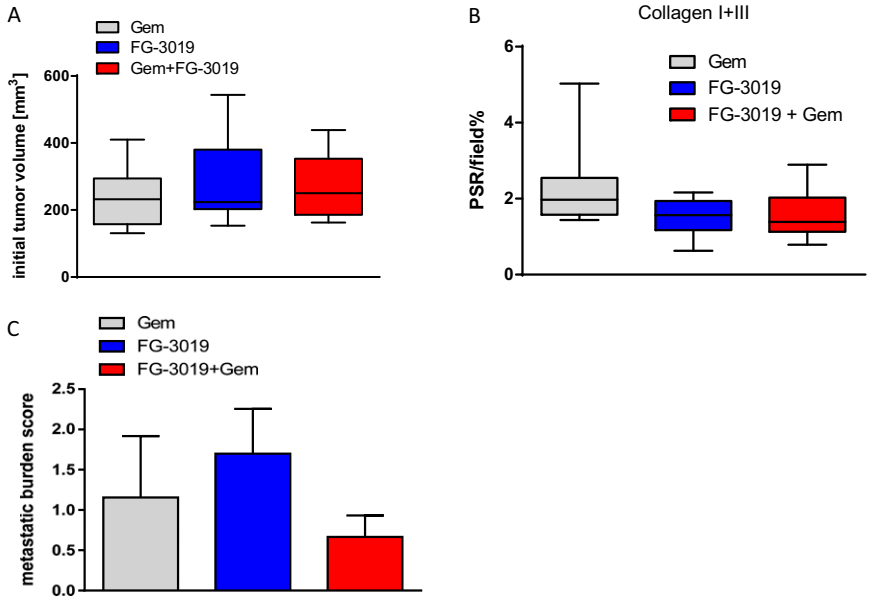

Fig. S4. Long-term treatment with FG-3019/gemcitabine reduces liver metastasis. (*A*) Preenrollment ultrasound PDA tumor volumes of the survival study (gemcitabine, $n = 16$; FG-3019, $n = 12$; FG-3019/gemcitabine, $n = 11$) reveal no significant differences before enrollment. (*B*) Quantification of collagen I+III using polarized light microscopy ($n \geq 11$ each cohort) revealed no significant differences at survival end point. (*C*) Quantification of metastatic burden in the survival study in five consecutive liver serial sections reveals a nonsignificant decrease in FG-3019/gemcitabine–treated mice (mean metastasis score, 0.66) compared with FG-3019– (mean, 1.69; $P = 0.2$) and gemcitabine–treated mice (mean, 1.15; $P = 0.6$) ($n \geq 11$ each cohort; error bars represent SEM).