Supplementary material

Supplementary methods

Apoptosis assays

KPC cells were seeded at a density of 300,000 cells per well in 6-well plates and allowed to attach for 24 h. Cells were then treated with 30 nM and 50 nM of gemcitabine-hydrochloride or water as control. 24 h after treatment, protein samples were prepared as previously described and cleaved caspase-3 levels were determined by Western blot analysis. Three biological replicates were used.

Conditioned medium MTT cell viability assay

PSCs were seeded at a density of $1*10^6$ cells per 10 cm dish, allowed to attach for 24 hours, and subsequently treated with 25 nM of gemcitabine-hydrochloride for 24 hours. 5000 KPC tumor cells were seeded per well in 96-well plates and also allowed to attach for 24 hours. Conditioned medium was centrifuged briefly and supernatants were added to the tumor cells. PSCs had a confluency of more than 70 % at time of medium collection. Tumor cells were incubated for 72 hours and MTT cell viability assay (1) was performed afterwards. MTT reagent (Thiazolyl blue tetrazolium bromide, Sigma) was added to the media (0.5 mg/ml). Following 2 hours incubation at 37°C the medium was removed, 100 µl of acidified isopropanol added, and absorption measured at 595 nm (no reference wavelength) (Autobio PHOmo Microplate reader, Labtec Instruments Co., LTD). Four biological replicates were used with three technical replicates per experiment.

References

1. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1–2):55–63.

Supplementary figures

Taqman probes	Catalogue number	Supplier	
murine NT5C1A	Mm01192248_m1	Thermo Fisher Scientific	
human NT5C1A	Hs00261369_m1	Thermo Fisher Scientific	
murine β-actin	Mm00607939_s1	Thermo Fisher Scientific	
human β-actin	Hs99999903_m1	Thermo Fisher Scientific	
Antibodies	**Source**		**Dilution**
NT5C1A	Abcam Cat# ab199632		1:1000 (WB)
HSP90	Cell Signaling Technology Cat# 4875, RRID:AB_2233331		1:1000 (WB)
Cleaved Caspase-3	Cell Signaling Technology Cat# 9664, RRID:AB_2070042		1:1000 (WB)
HA-Tag	Cell Signaling Technology Cat# 3724, RRID:AB_1549585		1:1000 (WB) / 1:300 (IHC)
Anti-rabbit IgG/HRP	Dako/ Agilent Technologies Cat# P0217, RRID:AB_2728719		1:2000 (WB)
Alexa Fluor® 555	Invitrogen/ Thermo Fisher Scientific Cat# A-31572, RRID:AB_162543		1:500 (ICC)
NT5C1A	Assay Biotech Cat# C15296, RRID:AB_10687827		1:100 (IHC)
NT5C1A	Atlas Antibodies Cat# HPA050283, RRID:AB_2681072		1:100 (TMA-2)

Table S1: List of used Taqman™ reagents for qRT-PCR, antibodies for Western blot analysis, and immunohistochemistry or immunocytochemistry, respectively**.**

Supplementary Figure 1

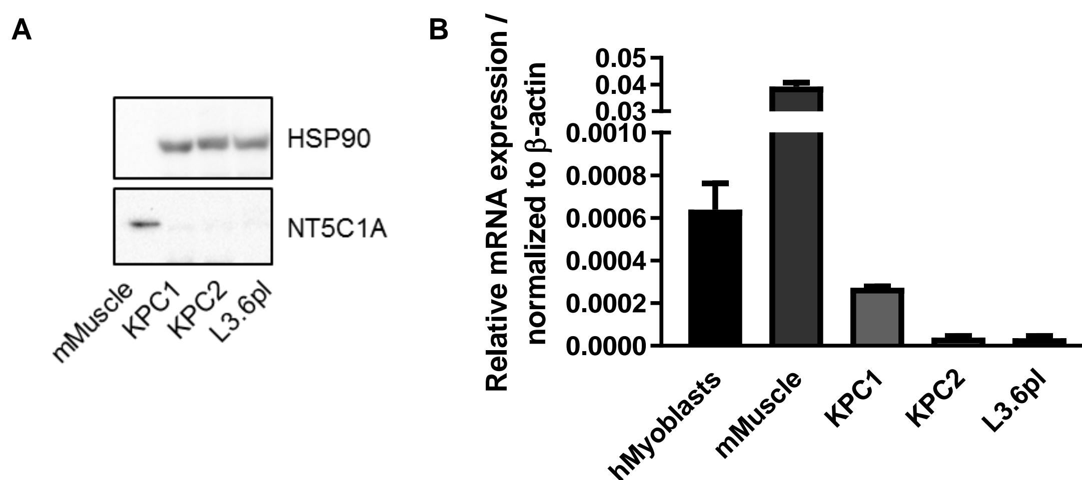

Figure S1: Reduced expression of NT5C1A in murine and human pancreatic cancer cell lines *in vitro.* A) Western blot analysis showing protein expression of untransfected KPC1 and KPC2 cells, as well as of human L3.6pl cells. HSP90 was used as loading control and murine muscle as positive control. B) mRNA-expression of NT5C1A in KPC1, KPC2, and L3.6pl cells. Values were normalized to the housekeeping gene β-actin. Murine muscle extract and human myoblasts were added as positive controls for NT5C1A-expression.

Supplementary Figure 2

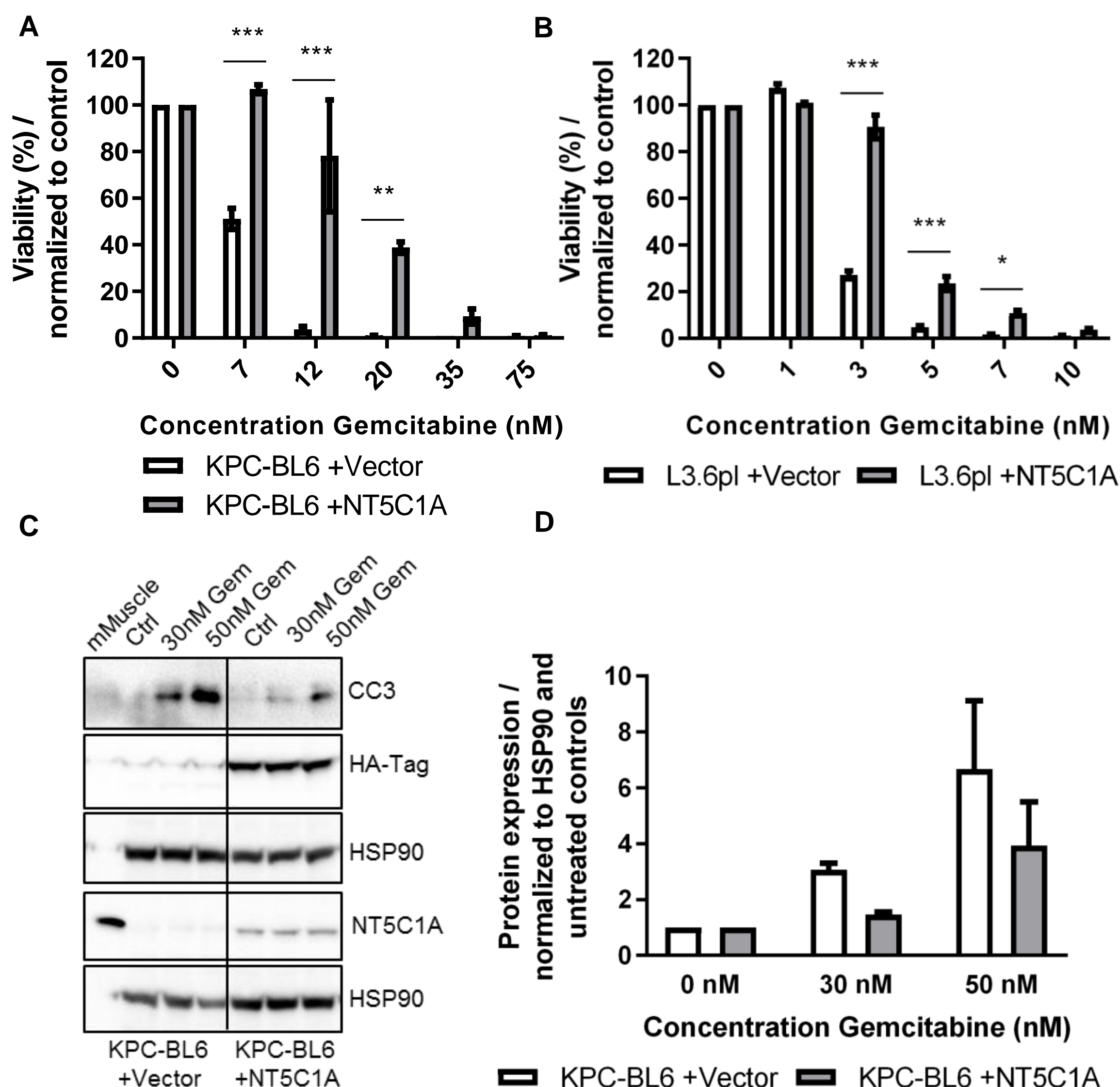

Figure S2: High levels of NT5C1A reduce chemotherapeutic response to gemcitabine in pancreatic cancer cells. A) and B) Crystal violet assay of murine KPC-BL6 cells and human L3.6pl cells. Crystal violet staining was performed following six days of gemcitabine treatment. Crystal violet staining intensity was more pronounced in NT5C1A overexpressing cell lines. Three independent experiments were performed, with each two technical replicates. Quantification of crystal violet assay was performed and results were normalized to untreated control cells. Graphs present mean ± SEM, two-way ANOVA with Sidak's multiple comparisons test was performed (KPC-BL6: 7 nM: $p < 0.0001$, 12 nM: $p < 0.0001$, 20 nM: $p = 0.007$, L3.6pl: 3 nM: $p < 0.0001$, 5 nM: $p < 0.0001$, 7 nM: $p = 0.015$). C) Western blot analysis of murine KPC-BL6 cells showed reduced protein levels of CC3 in NT5C1A-expressing cells following gemcitabine treatment for 24 h. NT5C1A and HA-tag expressions were confirmed in the +NT5C1A-cell line. Three independent experiments were performed. HSP90 was used as loading control and murine muscle as positive control for NT5C1A. D) Quantification of CC3 protein expression in the three Western blot analyses. Graph shows mean ± SEM.

Supplementary Figure 3

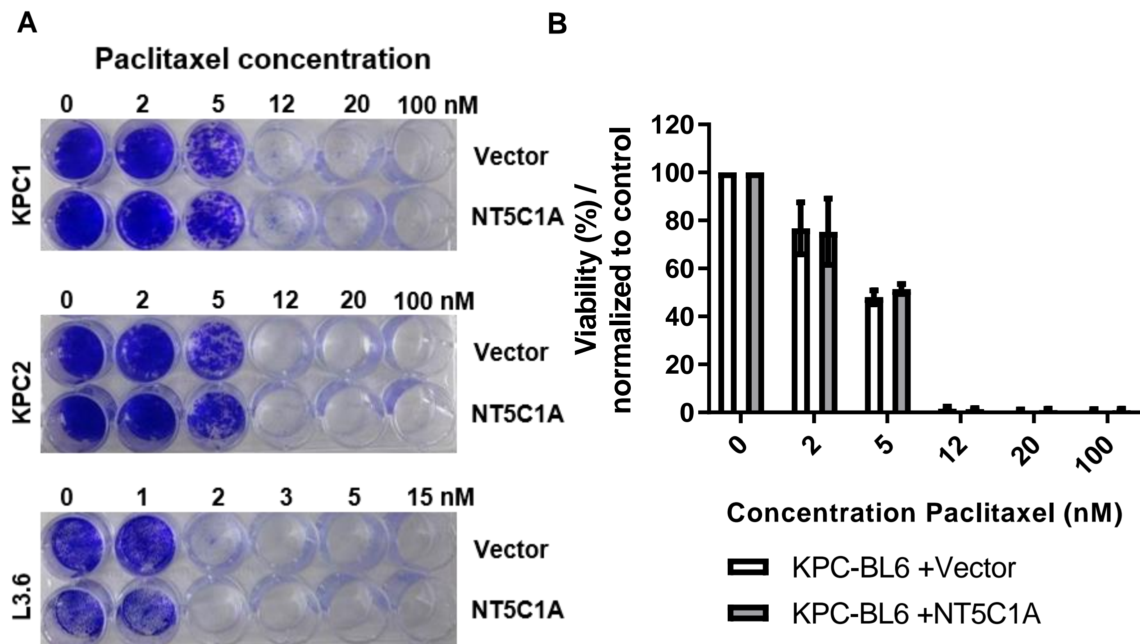

Figure S3: Pancreatic cancer cell lines expressing high levels of NT5C1A are still sensitive to paclitaxel treatment. A) Crystal violet assay was performed with murine KPC cells and human L3.6pl cells that have been treated with increasing concentrations of paclitaxel for six days. The staining intensity was comparable between control cells and cells overexpressing NT5C1A. Two independent experiments were performed, with each two technical replicates. B) Quantification of crystal violet assay was performed. Diagram shows the quantification of murine KPC-BL6 cells. The results were normalized to untreated control cells. Graph presents mean ± SEM, two-way ANOVA with Sidak's multiple comparisons test was performed (ns).

Supplementary Figure 4

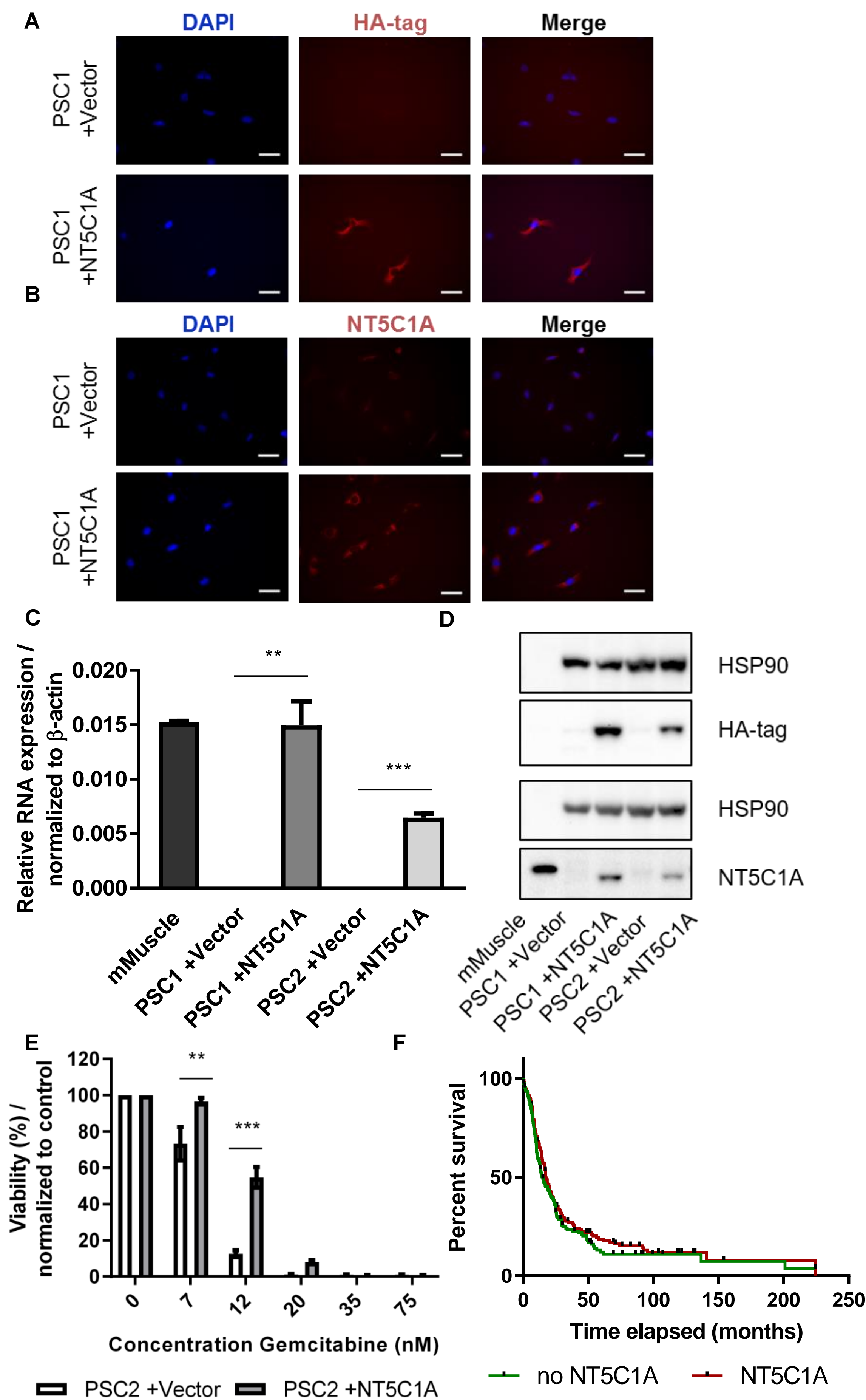

Figure S4: NT5C1A expression in stromal cells does not correlate with overall survival in resected pancreatic cancer patients and increases gemcitabine resistance. A) and B) Representative immunocytochemistry pictures for NT5C1A and HA-tag expression in stably transfected PSCs (red; DAPI nuclear staining: blue). HA-tag expression was exclusively shown for +NT5C1A cells (A, lower panel), control cells were devoid of immunoreactivity (A, upper panel). Very low endogenous levels of NT5C1A were demonstrated in vector control cells (B, upper panel) compared to NT5C1A expression in the NT5C1A-overexpressing cells (B, lower panel). Two technical replicates were analyzed per cell line and antibody. Scale bars 50 µm. C) Strong NT5C1A mRNA expression was shown in both +NT5C1A murine pancreatic stellate cell lines, as analyzed by quantitative RT-PCR (PSC1: $p = 0.003$ and PSC2: $p = 0.0001$). NT5C1A expression was hardly detectable in vector control cells. Diagram indicates mean ± SEM of three biological replicates. Murine muscle sample served as positive control. Values were normalized to β-actin as housekeeping gene. D) Western blot analysis of transfected PSCs showing robust NT5C1A and HA-tag protein expression in cells expressing NT5C1A and no detectable expression in vector control cells. Representative image of three independent experiments is shown with murine muscle lysate as positive control for NT5C1A expression. E) Quantification of crystal violet assay for stably transfected PSC2 cells with and without NT5C1A expression. Two-way ANOVA with Sidak's multiple comparisons test was performed (7 nM: $p = 0.002$, 12 nM: $p < 0.0001$). F) Survival analysis of the Erlangen TMA-2 cohort comparing no NT5C1A expression with any intensity of NT5C1A expression. Median survival was 15.2 months ($n = 156$) for no NT5C1A expression and 17.3 months ($n = 173$) with stromal expression of NT5C1A ($p = 0.3$, log-rank test).

Supplementary Figure 5

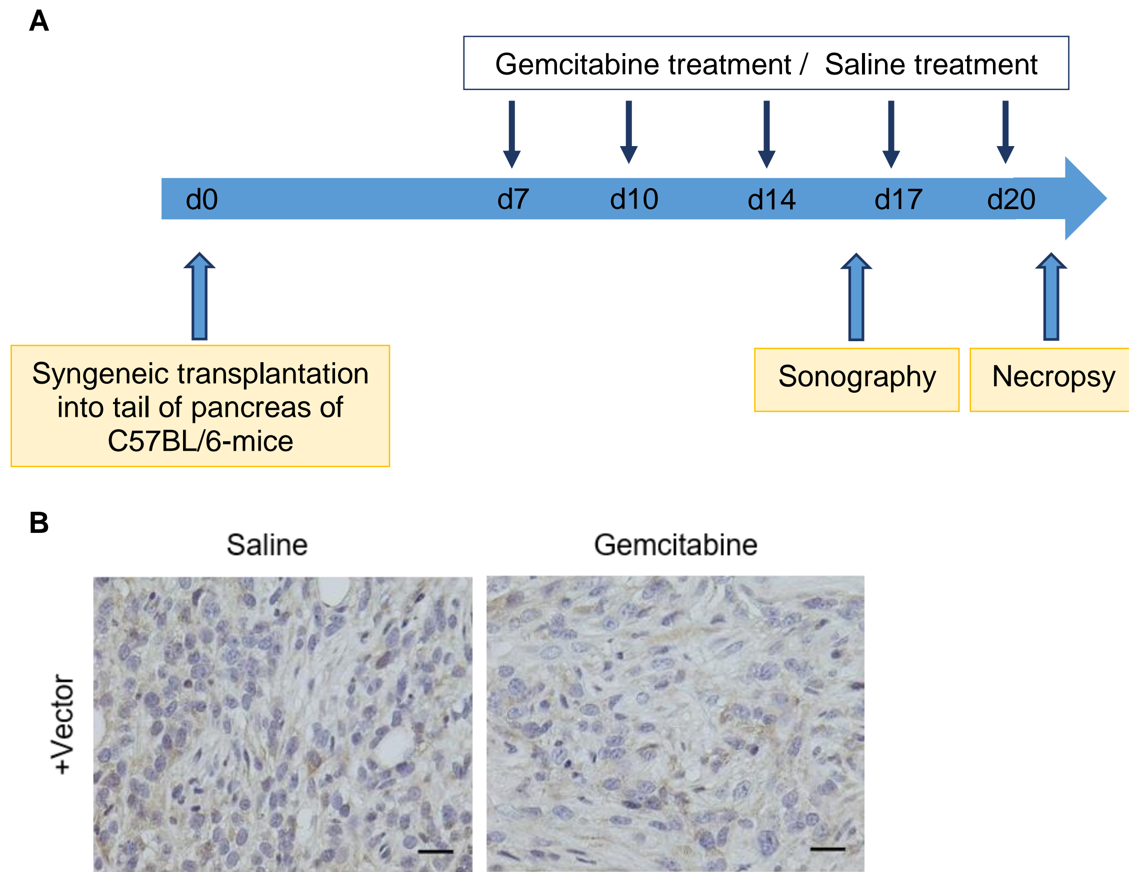

Figure S5: Syngeneic orthotopic transplantation of modified KPC tumor cells. A) Schematic presentation of orthotopic transplantations in C57BL/6-J mice and subsequent treatment schedule. Mice were treated seven days after transplantation with gemcitabine 100 mg/kg or saline, respectively on treatment days 0, 3, 7, 10, and 13. Sonography was performed on treatment day 9. B) Comparison of NT5C1A expression in control tumors treated with saline or gemcitabine, respectively. NT5C1A expression was not induced by gemcitabine treatment. Expression was low in both groups. Representative IHC images are shown. Scale bars 20 µm.