Supplemental Information

EMT-induced gemcitabine resistance in pancreatic cancer involves the functional loss of equilibrative nucleoside transporter 1

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Supplemental Materials and Methods

Plasmids

N-cadherin in pCCL-c-MNDU3c-PGK-EGFP was a gift from Nora Heisterkamp (RRID:Addgene_38153) (1), and mouse E-cadherin GFP was a gift from Alpha Yap (RRID:Addgene_67937). EpCAM pLenti-GIII-CMV-RFP-2A-Puro was procured from Applied Biological Materials Inc. (Richmond, BC). For lentiviral production, psPAX2 packaging plasmid (RRID:Addgene_12260) and the pMD2.G envelope plasmid (RRID:Addgene_12259) were gifts from Didier Trono.

Antibodies

Flow cytometry antibodies for mouse anti-human N-cadherin (BD Biosciences Cat# 740327, RRID:AB_2740062) and mouse anti-human E-cadherin (BD Biosciences Cat# 743713, RRID:AB_2741691) were obtained from BD Biosciences (San Jose, CA). The anti-N-cadherin mouse monoclonal antibody was purchased from BD Biosciences (Cat# 610920, RRID:AB_2077527) (San Jose, CA). E-cadherin (Cat# 13-1700, RRID:AB_2533003) mouse monoclonal antibody, goat anti-rabbit Alexa Flour 594 secondary antibody (Cat# A-11037, RRID:AB_2534095) and goat-anti mouse Alexa Flour 488 (Cat# A-11029, RRID:AB_2534088) secondary antibody were purchased from Thermo Fisher Scientific (Waltham, MA). Anti-hENT1 rabbit polyclonal antibody was described previously (2, 3). Immunohistochemical staining utilized Pan-cytokeratin (Cat# 914202, RRID:AB_2565153) mouse monoclonal antibody, BioLegend (San Diego, CA). EpCAM (Cat# 2929, RRID:AB_2098657) mouse monoclonal antibody, α-tubulin (11H10) rabbit monoclonal antibody, Na^+/K^+ ATPase rabbit antibody, rabbit monoclonal anti-ZEB1 (Cat #3396, D80D3), rabbit monoclonal anti-SLUG (Cat# 9585, RRID:AB_2239535), rabbit monoclonal anti-vimentin (Cat# 5741, RRID:AB_10695459), rabbit monoclonal anti-β-catenin (Cat# 8480, RRID:AB_11127855), and rabbit monoclonal anti-ZO-1 (Cat# 8193, RRID:AB_10898025) were obtained from Cell Signaling Technology (Danvers, MA).

Reagents

Prolong Gold anti-fade with DAPI was purchased from Invitrogen, Life Technologies (Carlsbad, CA). Pierce BCA protein assay kit, SuperSignal West Pico chemiluminescent substrate, and Lipofectamine LTX transfection reagent were obtained from Thermo Fisher (Waltham, MA). EDTA-free protease inhibitor cocktail tablets were purchased from Sigma Aldrich (St. Louis, MO). Gemcitabine [5-^{3}H] was procured from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Minute™ Plasma Membrane Protein Isolation and Cell Fractionation Kit was purchased from Invent Biotechnologies (Plymouth, MN). For multiplexed immunohistochemistry staining, Opal 4-Color Manual IHC Kit was purchased from PerkinElmer (Waltham, MA).

Flow Cytometry

PANC-1 cells were costained with antibodies against E-cadherin (Alexa Fluor 488 Mouse Anti-Human-CD324) and N-cadherin (Alexa Fluor 647 Mouse Anti-Human-CD325) in stain buffer (BD Biosciences, Bedford, MA) at passage number 2. The LIVE/DEAD Fixable Violet Dead Cell Stain Kit (ThermoFisher Scientific, Waltham, MA)

was used to exclude dead cells. Cell staining was performed on ice and analyzed using a BD FACSAria III Cell Sorter equipped with four lasers (405, 488, 561, and 633 nm). Color compensation was performed using BD FACSDIVA Software (RRID:SCR_001456) (Fig. S1), and FACS data analysis was performed using FlowJo (RRID:SCR_008520). E-cadherin-N-cadherin- (E-/N-), E-cadherin+N-cadherin- (E+/N-), E-cadherin-N-cadherin+ (E-/N+), and E-cadherin+N-cadherin+ (E+/N+) subpopulations were sorted and quantified. Briefly, cells were gated based on high and low expression of E-cadherin and N-cadherin after the exclusion of dead cells and cellular aggregates.

Colony Formation Assay

Cells were counted and seeded in equivalent number (500 cells/well) in 6-well plates. After 24 hours, gemcitabine treatment was started (10 nM) and maintained for 3 weeks with fresh media added every three days. After 21 days, cells were fixed in methanol and stained with 0.5% crystal violet solution. Colonies were quantified by capturing images of stained wells and counting the number of colonies greater than 1 mm in size per each quadrant. The number of colonies in each quadrant was averaged for individual cell types.

Expression of E-cadherin, N-cadherin and EpCAM

E-cadherin transient transfection was performed using Lipofectamine LTX transfection reagent according to manufacturer's protocol. Immunocytochemistry and transport studies were conducted 48 h post E-cadherin transfection. N-cadherin in pCCL-c-MNDU3c-PGK-EGFP and EpCAM in pLenti-GIII-CMV-RFP-2A-Puro plasmids were individually transfected into HEK293T lentiviral packaging cells with packaging plasmid psPAX2 and envelope plasmid pMD2.G with Lipofectamine LTX reagent. After 48 hours viral particles were harvested, filtered with a 0.45 µM sterile filter, and added to target cells for infection with 10 µg/mL polybrene. Following infection, cells were selected with 1 µg/mL puromycin for two weeks to obtain cells overexpressing the gene of interest. Overexpression cells were maintained in media containing 0.5 µg/mL puromycin.

[5-^{3}H] Gemcitabine Transport Assay

Cells were seeded at a density of 1.0 x 10^{5} cells per well in triplicate in 24-well plates. After 36 hours at 80-90% confluence, media was removed and transport buffer (20 mM Tris-HCl, 3 mM dipotassium phosphate, 1 mM magnesium, 2 mM calcium chloride, 5 mM glucose, 130 mM sodium chloride) containing gemcitabine (0.0338 µM [5-^{3}H] gemcitabine + 1 µM cold gemcitabine) was added to each well for 1 minute. Transport buffer was removed and replaced with ice-cold buffer containing 20 mM uridine to halt gemcitabine transport activity. Buffer was aspirated from each well and 10% SDS was added. Plates were placed on a shaker plate for 30 minutes to ensure complete cell lysis, and then placed in scintillation cocktail for counting with Beckman Coulter LS6500 liquid scintillation counter. Protein was quantified for each cell type and dpm value was normalized to protein content and converted to pmol based on the specific activity of [5-^{3}H] gemcitabine.

Western Blotting

Western blot analysis was performed as previously described with the following exception of an alternative cell lysis buffer (4). Whole cell lysates were prepared for Western blot with lysis buffer containing 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl, 2 mM PMSF, 1 mM NaF, and protease inhibitor cocktail.

Transwell Migration and Invasion Assays

The procedure was followed as described previously (5) with minor modifications. For the migration assay, tissue culture treated transwell permeable support with polycarbonate membrane inserts were used. For the invasion assay, BioCoat cell culture inserts with polyethylene terephthalate (PET) membranes, which are pre-coated with extracellular matrix proteins, (Corning Life Sciences, MA, USA) were used. Cells from moderately confluent petri dishes were trypsinized and 1×10^5 cells in 250 µL of serum free DMEM were seeded into each well of the upper inserts. Next, 600 µL of complete growth medium containing 10% FBS was added into the lower chamber of the plates as a chemoattractant. Plates were incubated at 37 ºC and 5% CO_2 for 48 hours. The media along with the non-invading/non-migrating cells were gently wiped-out from the upper surface of the transwell with the help of a cotton-tipped applicator. The invaded/migrated cells on the lower surface of the membrane were fixed with 4% paraformaldehyde, air-dried, and stained with 0.2% crystal violet. Cells attached on the lower side of the membrane were viewed underneath an inverted microscope at 10x magnification, counted from at least three random fields, and photographed.

Pathway and gene set enrichment analysis of deposited RNA-sequencing data

RNA sequencing datasets for PANC-1 and PANC-1 liver metastasis were deposited by Takahashi *et al.* (6), GEO accession number: GSE107960. Differentially expressed genes (DEGs; FDR < 0.1, FC > 1.3)) were identified using a standard edgeR pipeline (RRID:SCR_012802) (7). Functional annotation of gene sets: Pathway enrichment analysis and gene set enrichment analysis (clusterProfiler, RRID:SCR_016884) (8) were performed using gene sets from the Molecular signatures database (MSigDB) in PANC-1 liver metastasis compared to parental PANC-1 cells.

Multiplexed Fluorescent Immunohistochemistry

Multiplexed staining procedure was established based on Opal Assay Development Guide (Perkin Elmer). In brief, paraffin was removed from TMA samples by baking slides at 60°C for one hour followed by treatment with xylene and rehydrated by ethanol gradient followed by fixation in 10% neutral-buffered formalin for 20 minutes. Each sequential antigen retrieval step was performed by microwaving slides in AR 6 buffer (Perkin Elmer) at 100% power for 45 seconds to bring to a boil, followed by an additional 15 minutes at 10% power. Slides were allowed to cool at room temperature for 30 minutes before proceeding to blocking for 10 minutes and primary antibody incubation for 1 hour at room temperature with N-cadherin, or EpCAM antibody diluted 1:100. Then, secondary antibody containing HRP was incubated for 10 minutes, followed by addition of the Opal 520 fluorophore diluted 1:100 for 10 minutes at room temperature. A second microwave treatment was performed as described above to strip

any unbound fluorophore, followed by blocking and incubation with pan Cytokeratin antibody (1:75) for 1 hour. Secondary antibody and Opal 690 fluorophore were applied to slides for 10 minutes each respectively. Microwave treatment was performed to strip any unbound antibody and fluorophore. Slides were cooled overnight in antigen retrieval buffer. After rinsing in TBST (0.1 M TRIS-HCl, 0.15 M NaCl, 0.05% Tween®20) and water, slides were incubated for 1 hour with ENT1 primary antibody (1:500). Secondary antibody and Opal 570 fluorophore (1:100) were applied to each slide, and a final microwave treatment was performed with antigen retrieval pH 6 buffer. DAPI was applied for 2 minutes and slides were rinsed with TBST and water, then allowed to air dry. Slides were mounted with fluorescent mounting media and coverslips were applied.

TMA Quantification

Multiplexed TMA slides were imaged with the Vectra 2.0 multispectral imaging system (Perkin Elmer) with random fields collected for each core. InForm analysis software was used to obtain percent positive cells for ENT1 and EpCAM in the plasma membrane compartment in an unbiased and blinded manner. In brief, Pan- Cytokeratin positivity was used differentiate epithelial pancreatic cancer tumor cells from stroma using trainable tissue segmentation. Cell segmentation was performed based on nuclear counterstain DAPI to give nuclear, cytoplasmic, and plasma membrane compartments. EpCAM and N-cadherin were quantified in epithelial tumor cells for percent positive cells both singly and for double positive cells. Intensity of membrane ENT1 was quantified by scoring of the fluorescent signal into 4 bins (0+, 1+, 2+, and 3+) based on user- defined threshold values. Each image received a membrane ENT1 H-score based on the formula (1 x (% cells 1+) +2 x (% cells 2+) +3 × (% cells 3+)). TMA samples were provided for each patient in duplicate, so the two H-scores for each patient were averaged to yield one value for each patient.

Statistical Analysis

Demographic and clinical characteristics of patients were analyzed using descriptive statistics. Briefly, continuous variables were summarized as mean/standard deviation (SD) or median/range; categorical data were presented as count (n) and frequency (%). Continuous variables were compared using Student's t tests or one-way ANOVA where appropriate.

Correlation between percentage of EpCAM plasma membrane-positive cells and ENT1 plasma membrane-positive cells, between percentage of N-cadherin plasma membrane-positive cells and ENT1 cytoplasmic-positive cells, between percentage of ENT1 and EpCAM double positive cells and ENT1 membrane expression were analyzed using Pearson's correlation tests.

Median overall survival time in days was calculated from Kaplan-Meier curves with the difference between the curves analyzed using the log-rank test. Multivariate analysis was conducted using the proportional hazard regression model of Cox (Cox PH regression), initially including all factors with a P-value of less than 0.15 in the univariate analyses and using a stepwise backward approach for model reduction. The proportional hazards assumption of a Cox regression was tested as previously described (9).

Statistical analyses for TMA survival analysis were conducted using R3.5.0 (R Foundation for Statistical Computing, RRID:SCR_001905). P values were two-sided, and values of $P < 0.05$ were regarded as statistically significant, with the exception of the multivariate survival analysis for which $P < 0.10$ was set as the significance level to account for the relatively small sample size.

All other statistical analyses were conducted with Graphpad Prism 7 software (RRID:SCR_002798). Two-tailed unpaired Student's t-tests were performed for comparisons between two values unless otherwise specified. Results for in-vitro transport studies are reported at mean ± standard deviation, and in-vivo experiments are reported as mean ± standard error of the mean. $P< 0.05$ was considered as statistically significant, and asterisks defined in figure legends denote the level of significance.

Immunocytochemistry

Immunocytochemistry was performed as previously described (10) except for the following modifications. At ~80% confluency, cells were fixed with ice-cold acetone for 10 minutes, and rinsed three times with PBS followed by incubation with 1% goat serum and 0.25% Triton X-100. Primary antibodies were diluted 1:1000 and incubated overnight at 4°C, and secondary antibodies diluted 1:1000 were applied for 1 hour at room temperature. Coverslips were mounted with Prolong Gold antifade with DAPI. Confocal images of random fields for single slice and Z-stacks were captured with the Olympus BX61F upright microscope system using 60x oil super-corrected objective PLAPONSC. Images were acquired using FV10-ASW software. The Imaris image analysis software (Imaris, RRID:SCR_007370) was utilized to produce orthogonal perspective views of Z-projections and 3D animations. Contrast and brightness were adjusted in PowerPoint using equivalent parameters for each experiment.

References

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concentrative and equilibrative nucleoside transporters in sandwich-cultured human hepatocytes. *Am J Physiol Gastrointest Liver Physiol***. 2008**;295(3):G570-80.

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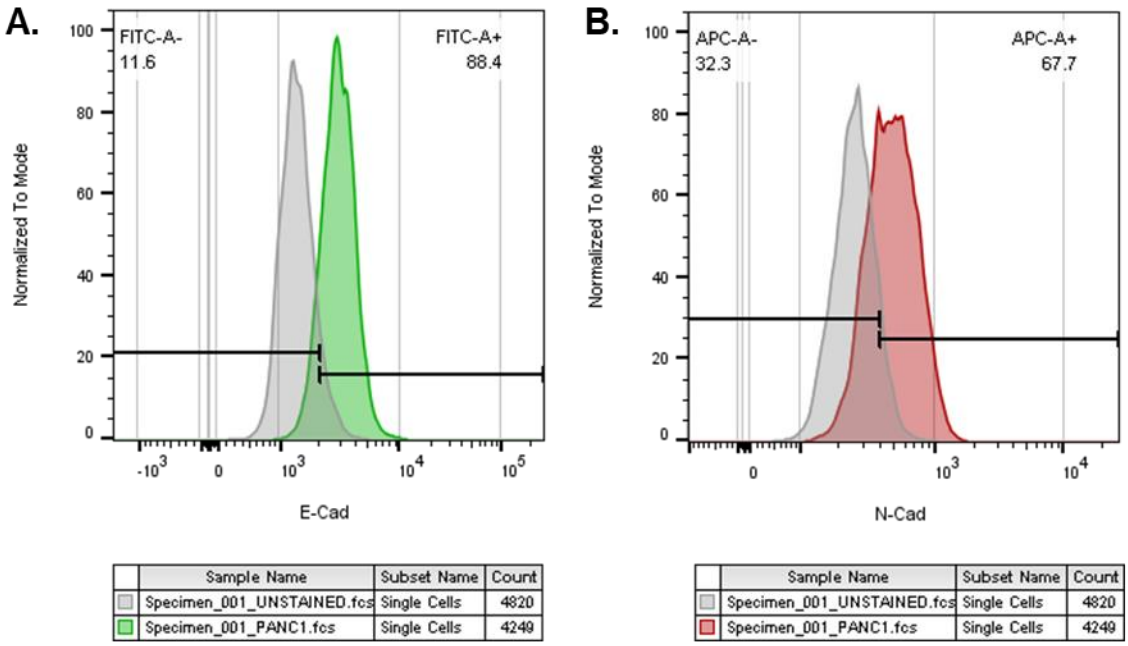
Supplemental Figures and Table



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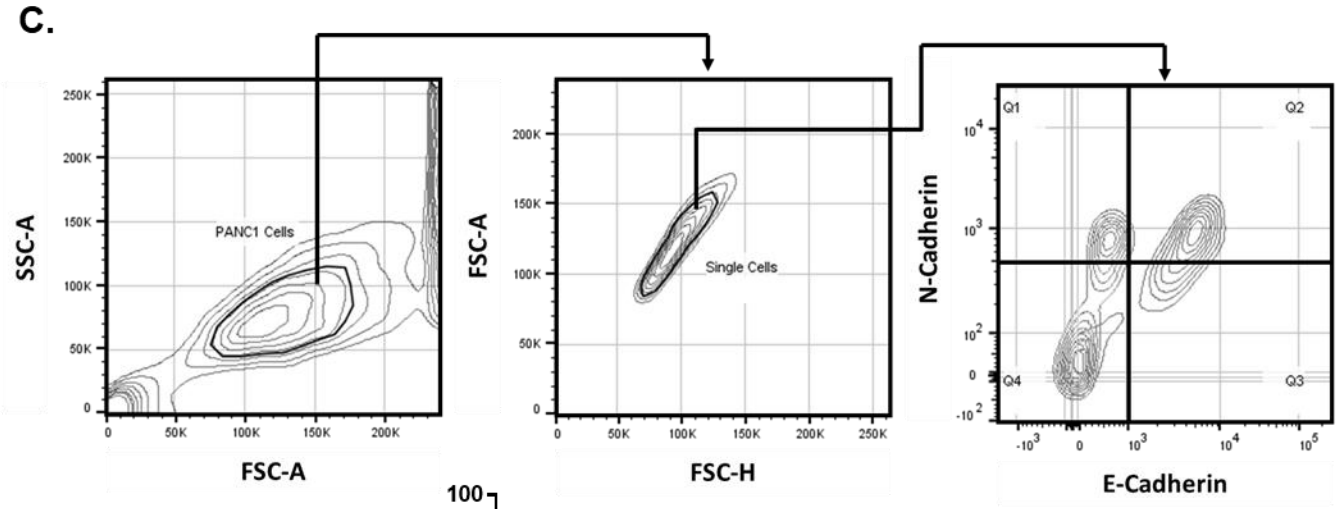
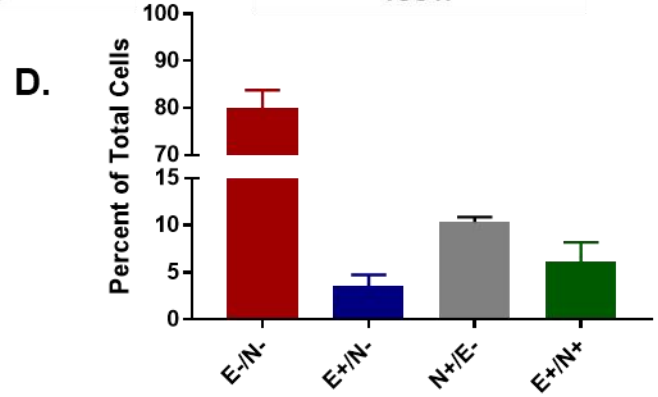
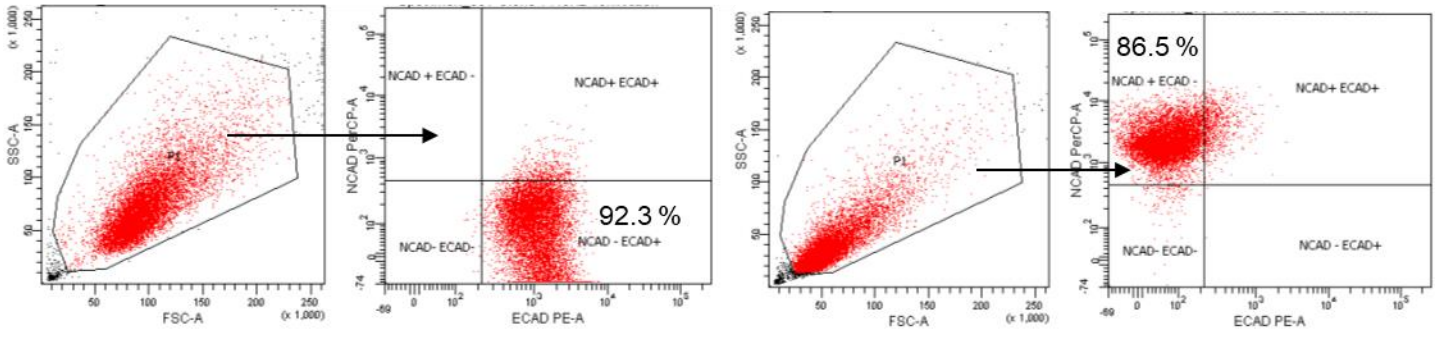
	Specimen_001_PANC1.fcs	Single Cells	4249

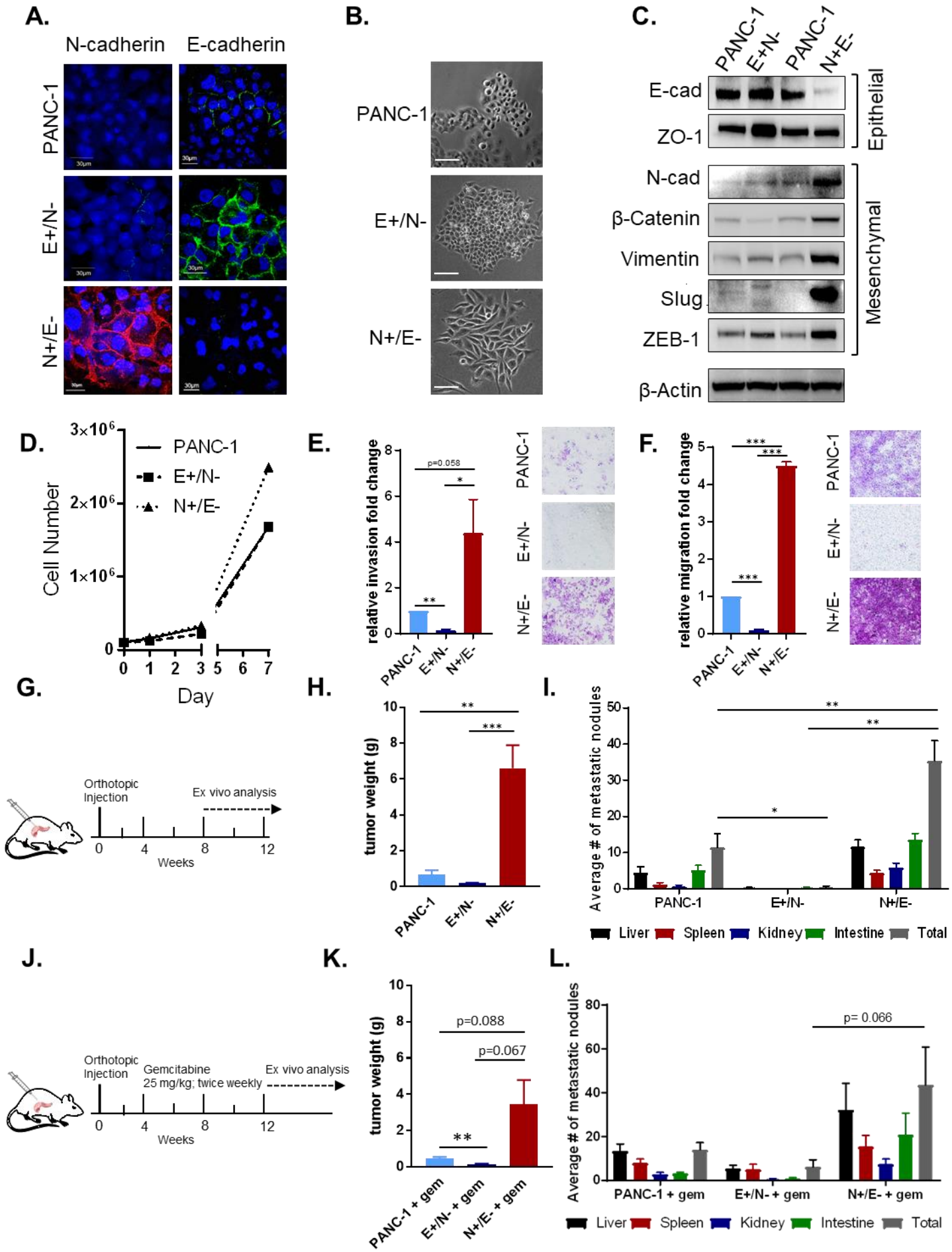






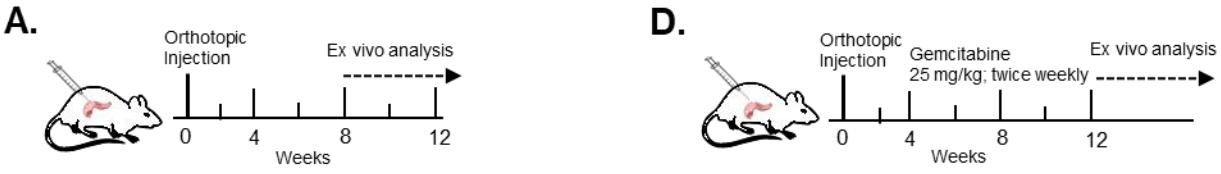
Supplemental Figure 1: Isolation of epithelial and mesenchymal subclones from parental PANC-1 cell line

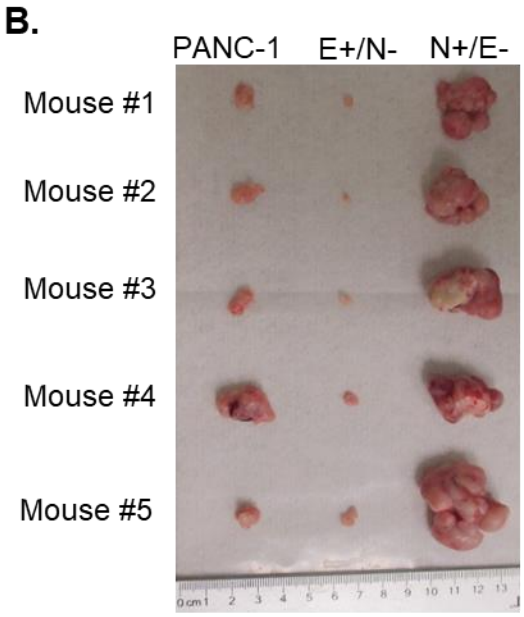
A. Single parameter histogram demonstrating E-cadherin positivity in parental PANC-1 cells compared to unstained control. **B.** Single parameter histogram demonstrating N-cadherin positivity in parental PANC-1 cells compared to unstained control. **C.** Contour plots illustrating the gating strategy used to isolate four distinct subpopulations based on E-cadherin (E) and N-cadherin (N) expression, designated E+/N+, E-/N-, E+/N-, and N+/E-. D. PANC-1 cells at passage 1 were stained for E-cadherin and N-cadherin. **D.** Bar graph shows average percentage of each population of cells isolated from parental PANC-1; n=2, error bars=sd. **E.** Post-sort purity analysis was performed for representative epithelial (E+/N-) and mesenchymal (N+/E-) cells following clonal expansion and 3 passages

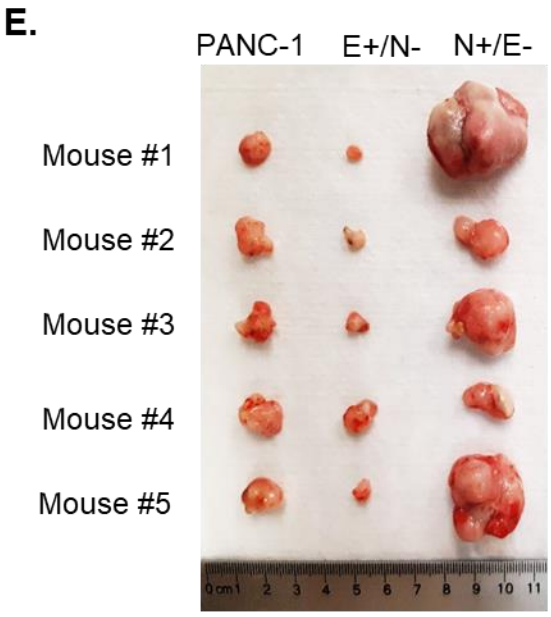

A.
N-cadherin
E-cadherin
PANC-1
E+/N-
N+/E-
B.
PANC-1
E+/N-
N+/E-
C.
PANC-1
E+N-
PANC-1
N+E-
E-cad
ZO-1
Epithelial
N-cad
β-Catenin
Vimentin
Slug
ZEB-1
Mesenchymal
β-Actin
D.
Cell Number
3×10^6
2×10^6
1×10^6
0
PANC-1
E+/N-
N+/E-
0 1 2 3 5 6 7
Day
E.
relative invasion fold change
p=0.058
*
**
PANC-1
E+/N-
N+/E-
F.
relative migration fold change
***
***
***
PANC-1
E+/N-
N+/E-
G.
Orthotopic Injection
Ex vivo analysis
0 4 8 12
Weeks
H.
tumor weight (g)
**
***
PANC-1
E+/N-
N+/E-
I.
Average # of metastatic nodules
**
**
*
PANC-1
E+/N-
N+/E-
Liver
Spleen
Kidney
Intestine
Total
J.
Orthotopic Injection
Gemcitabine 25 mg/kg; twice weekly
Ex vivo analysis
0 4 8 12
Weeks
K.
tumor weight (g)
p=0.088
p=0.067
**
PANC-1 + gem
E+/N- + gem
N+/E- + gem
L.
Average # of metastatic nodules
p= 0.066
PANC-1 + gem
E+/N- + gem
N+/E- + gem
Liver
Spleen
Kidney
Intestine
Total

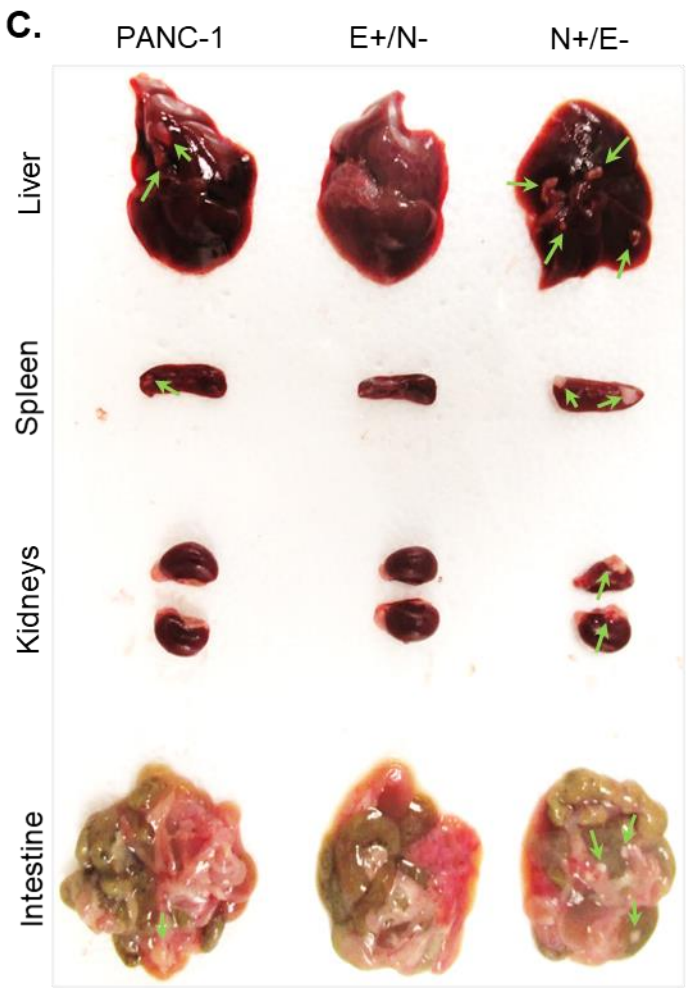
Supplemental Figure 2: Characterization of epithelial and mesenchymal human pancreatic cancer cells

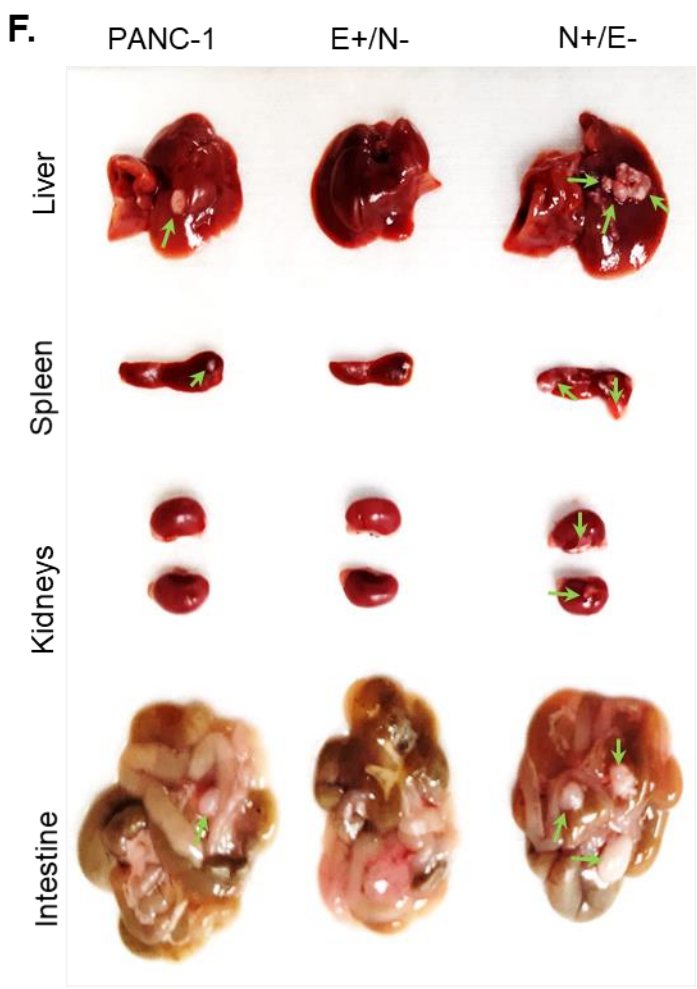
A. Confocal micrographs of cells immunostained for N-cadherin (red), E-cadherin (green) and nuclear counterstain DAPI (blue). Original magnification, x60 type **B.** Differential interference contrast (DIC) micrographs depict the morphology of parental PANC-1 cells and representative E+/N- and N+/E- clones; bar, 50 µm **C.** Biochemical characterization of cells for epithelial and mesenchymal markers with β-actin as loading control **D.** Cell number estimations over the course of seven days demonstrate differences in the proliferation ability of each cell **E.** Transwell migration assay depicting the cells migrated through a polycarbonate membrane towards a chemoattractant. Bar graph displays fold change in invading cells relative to PANC-1 control. **F.** Relative fold change in migration by epithelial and mesenchymal subclones compared to PANC-1. Images of crystal violet staining show migration ability for each cell type. **G**. Schematic representation of *in vivo* mouse tumor study beginning at time of orthotopic injection with PANC-1, E+/N-, or N+/E- through the 12 week course of the experiment (n=5 mice per group). *Ex vivo* analysis occurred starting at week 8 for 3 mice in the N+/E- group that met early removal criteria before 12 week endpoint, shown by the dashed line between weeks 8-12. **H**. Graph shows comparison of average tumor weight between each group of orthotopic tumor-bearing mice. (Bars, mean ± SEM. n=5 mice/group) **I.** Average number of metastatic nodules was graphed by site of secondary tumor formation and are grouped by cell type that generated primary tumor. Bars, mean ± SEM; n=5 mice/group **J.** Schematic representation of *in-vivo* mouse tumor drug sensitivity study. After orthotopic injection with PANC-1, E+/N-, or N+/E- cells at day 0, gemcitabine treatment was started 2 weeks after orthotopic tumor cell engraftment and administered twice weekly by IP injection (25 mg/kg) until the end of the study at 12 weeks **K.** Results of ex-vivo analysis show average tumor weight. Bars, mean ± SEM; n=5 mice/group **L.** Average number of secondary tumor sites graphed by location and categorized by cell type of primary tumor. Bars, mean ± SEM; n=5 mice/group. Statistical analyses were performed using two-tailed Student's t-test. *P≤0.05, **P≤0.01, ***P≤0.001


A.
Orthotopic Injection
Ex vivo analysis
0
4
8
12
Weeks
D.
Orthotopic Injection
Gemcitabine 25 mg/kg; twice weekly
Ex vivo analysis
0
4
8
12
Weeks


B.
PANC-1
E+/N-
N+/E-
Mouse #1
Mouse #2
Mouse #3
Mouse #4
Mouse #5


E.
PANC-1
E+/N-
N+/E-
Mouse #1
Mouse #2
Mouse #3
Mouse #4
Mouse #5

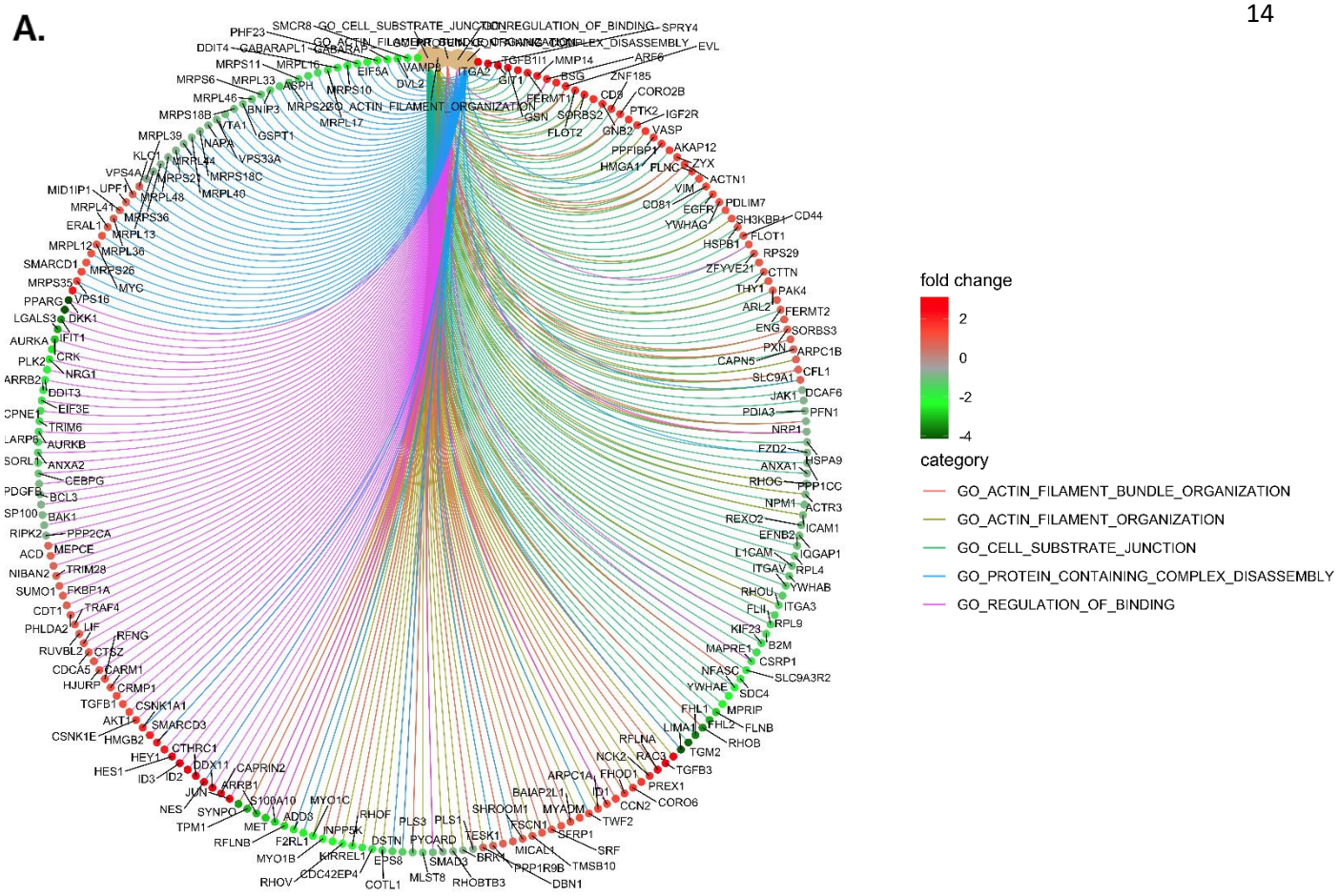

C.
PANC-1
E+/N-
N+/E-
Liver
Spleen
Kidneys
Intestine


F.
PANC-1
E+/N-
N+/E-
Liver
Spleen
Kidneys
Intestine

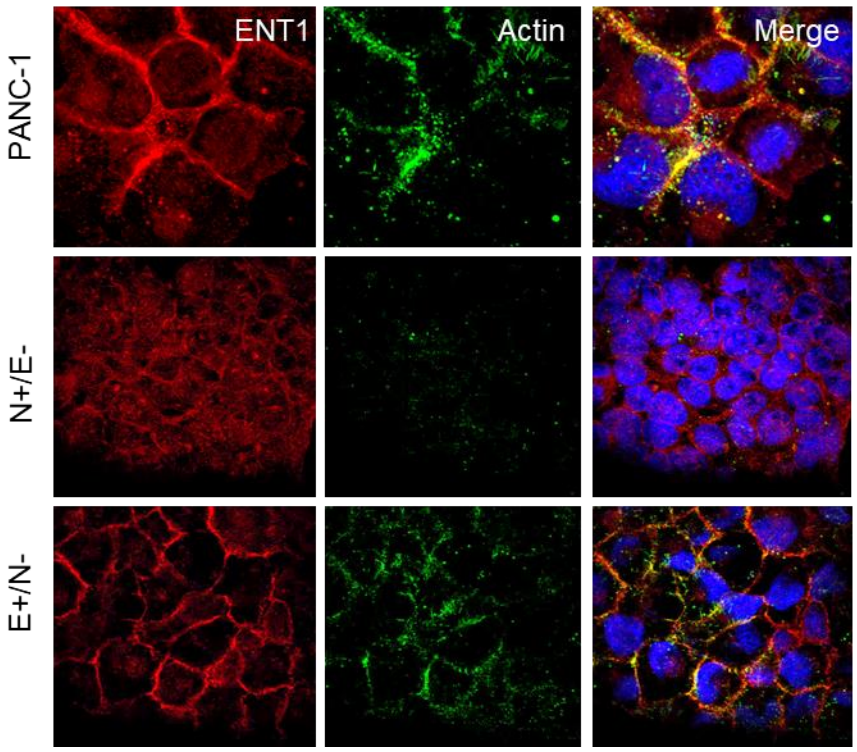
Supplemental Figure 3: *Ex vivo* analysis of orthotopic tumor-bearing mice

A. Schematic representation of *in vivo* mouse tumor study beginning at the time of orthotopic injection with PANC-1, E+/N-, or N+/E- through 12 weeks (n=5 mice per group). *Ex vivo* analysis occurred starting at week 8 for 3 mice in the N+/E- group that met early removal criteria before the 12 week endpoint, indicated by the dashed line between weeks 8-12. **B.** Primary tumor specimens isolated from mice at 12 weeks following orthotopic injection **C.** Representative abdominal organs that are common sites of pancreatic cancer metastasis analyzed for the number metastatic nodules **D.** Schematic representation of *in vivo* mouse tumor drug sensitivity study. After orthotopic injection with PANC-1, E+/N-, or N+/E- cells at day 0, gemcitabine treatment was started 2 weeks after orthotopic tumor cell engraftment and administered twice weekly by IP injection (25 mg/kg) until the end of the study at 12 weeks **E.** Gemcitabine-treated orthotopic mouse study primary tumors aligned by cell type used to generate tumor **F.** Liver, spleen, kidney, and intestines shown from a representative animal from each group of gemcitabine-treated mice

A.

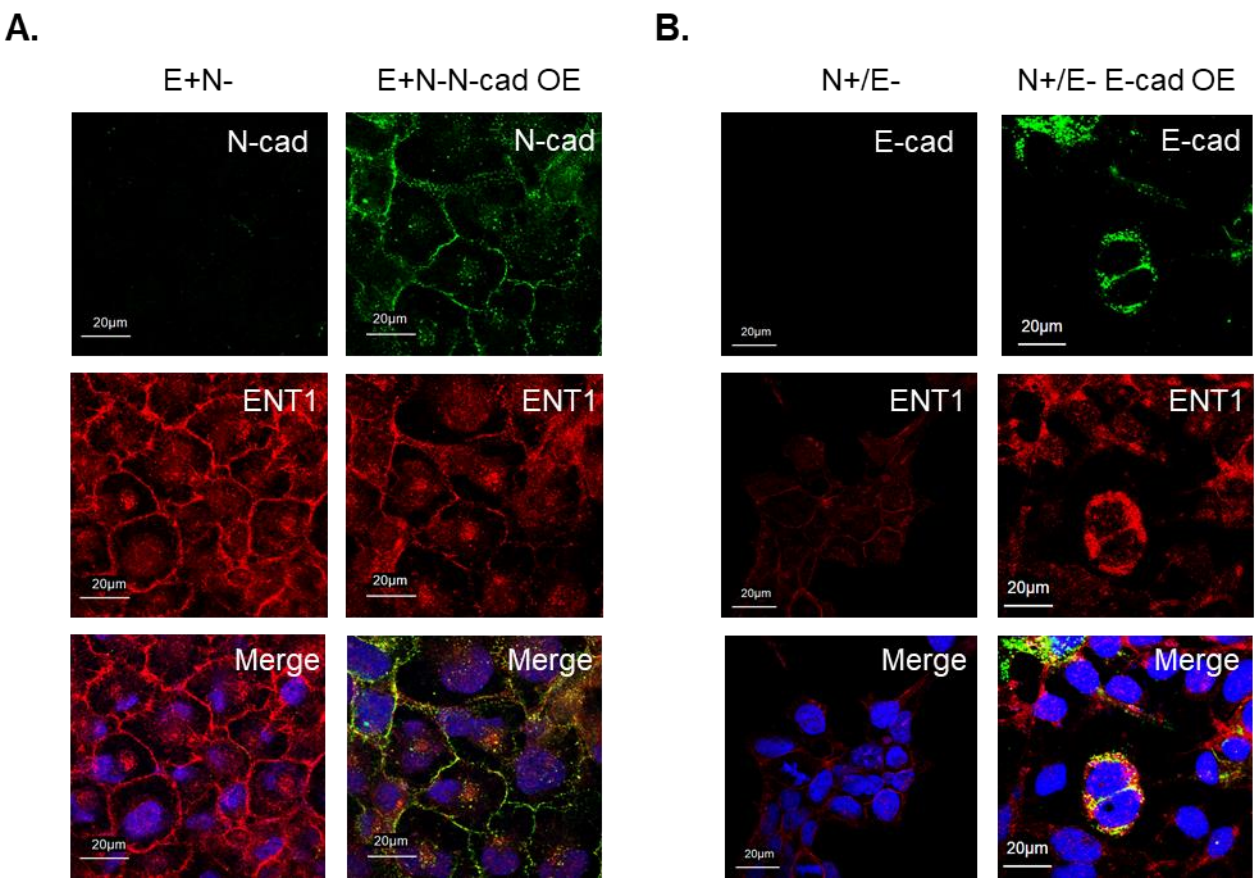

fold change
2
0
-2
-4
category
GO_ACTIN_FILAMENT_BUNDLE_ORGANIZATION
GO_ACTIN_FILAMENT_ORGANIZATION
GO_CELL_SUBSTRATE_JUNCTION
GO_PROTEIN_CONTAINING_COMPLEX_DISASSEMBLY
GO_REGULATION_OF_BINDING

B.


ENT1
Actin
Merge
PANC-1
N+/E-
E+/N-

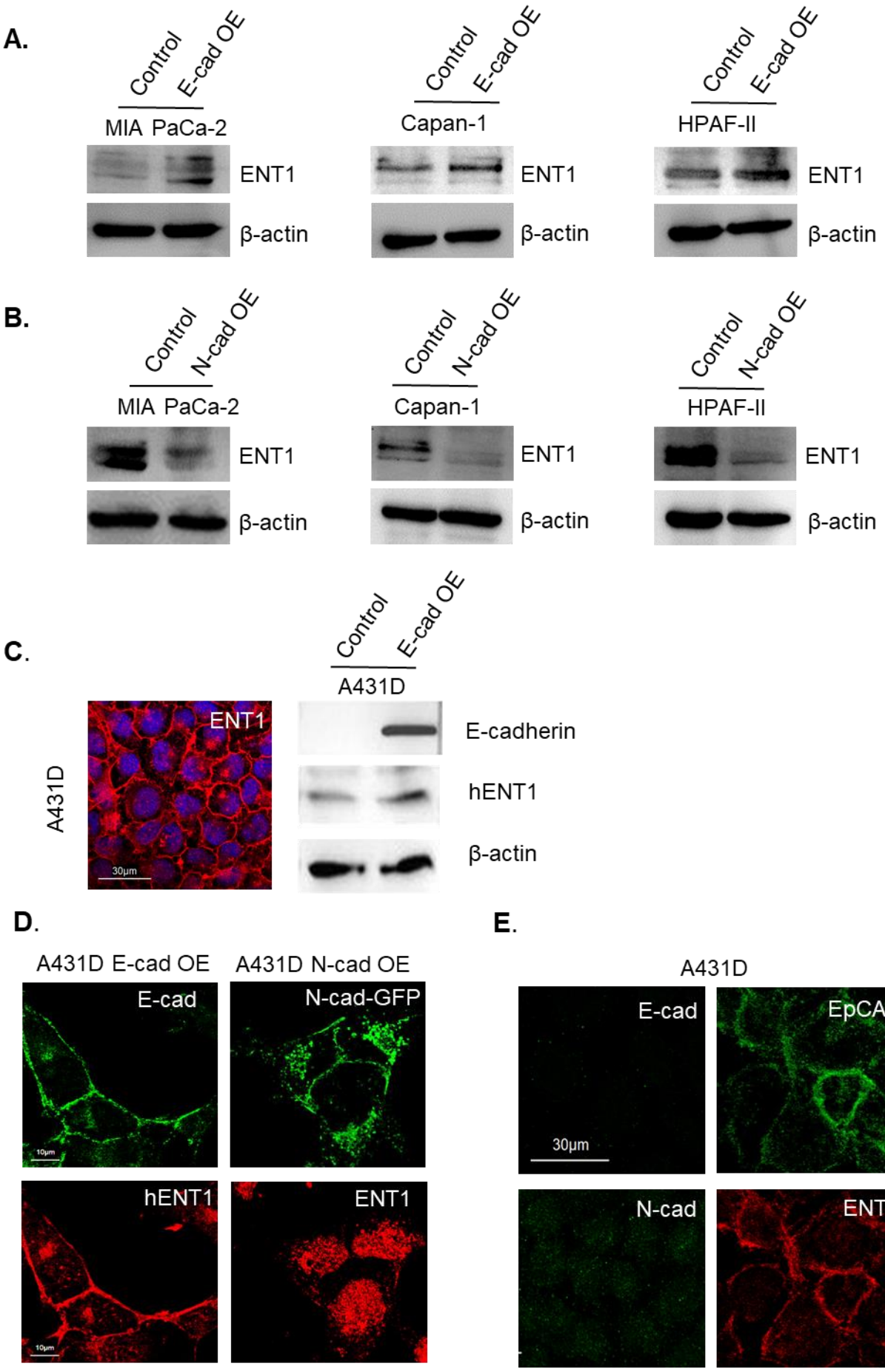
Supplemental Figure 4: F-actin supports ENT1 at the cell surface and is disrupted during EMT

A. Gene net diagram displays top most significant gene ontology enrichment pathways altered between parental PANC-1 cells and PANC-1 cells derived from liver metastasis; schematic diagram shows how cell lines compared in gene ontology analysis were derived **B.** Immunostaining for ENT1 (red) and actin (green) in control PANC-1 cells and sublines representing EMT


A.
E+N-
E+N-N-cad OE
N-cad
N-cad
ENT1
ENT1
Merge
Merge
20μm
B.
N+/E-
N+/E- E-cad OE
E-cad
E-cad
ENT1
ENT1
Merge
Merge
20μm

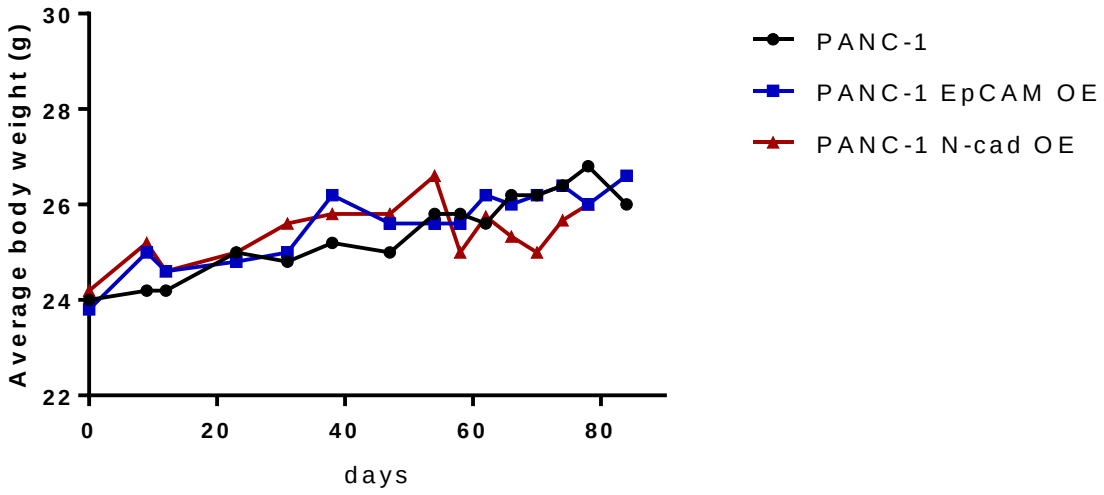
Supplemental Figure 5: N-cadherin diminishes membranous ENT1 and E-cadherin enhances membranous ENT1 expression in epithelial and mesenchymal PANC-1 derived subclones

A./B. Confocal micrographs display immunostaining for N-cadherin (A.) or E-cadherin (B.) shown in green (top), ENT1 in red (middle), and both channels merged with DAPI (bottom). Original magnification, x60


A.
Control
E-cad OE
MIA PaCa-2
Capan-1
HPAF-II
ENT1
β-actin
B.
Control
N-cad OE
MIA PaCa-2
Capan-1
HPAF-II
ENT1
β-actin
C.
Control
E-cad OE
A431D
A431D
ENT1
30μm
E-cadherin
hENT1
β-actin
D.
A431D E-cad OE
A431D N-cad OE
E-cad
N-cad-GFP
hENT1
ENT1
10μm
E.
A431D
E-cad
EpCAM
30μm
N-cad
ENT1

Supplemental Figure 6: E to N-Cadherin switching modulates ENT1 expression, yet E-cadherin is not required for ENT1 cell surface localization

A./ B. Overexpression of E-cadherin or N-cadherin was performed in three pancreatic cancer cell lines followed by Western blot analysis to examine ENT1 protein expression; β-actin was used as a loading control **C.** A431D epidermoid cancer cell line which lacks E-cadherin and N-cadherin expression was immunostained for ENT1 (red) and nucleus (blue). E-cadherin was overexpressed in A431D cells and Western blot analysis assessed ENT1 protein levels **D.** Confocal micrographs show forced expression of E-cadherin and N-cadherin in A431D cells (green) co-stained for ENT1 **E.** Immunostaining for E-cadherin and N-cadherin in A431D cells confirms the absence of classical cadherin expression; although EpCAM (green) is expressed and shows colocalization with ENT1 (red) at the plasma membrane


Average body weight (g)
30
28
26
24
22
0
20
40
60
80
days
PANC-1
PANC-1 EpCAM OE
PANC-1 N-cad OE

Supplemental Figure 7: Body weight measurements reflect increased tumor burden

Orthotopic tumors were generated by PANC-1 control, PANC-1 N-cadherin overexpression, and PANC-1 EpCAM overexpression cells by injecting an equivalent cell number (n=5 mice/cell type) and treated with gemcitabine (25 mg/kg) twice a week as shown in Figure 6. The graph shows body weight measurements that were recorded biweekly from time of orthotopic injection to termination of experiment 12 weeks after injection. Statistical analysis was performed using one-way ANOVA for repeated measurements, and no statistically significant differences were found between groups.

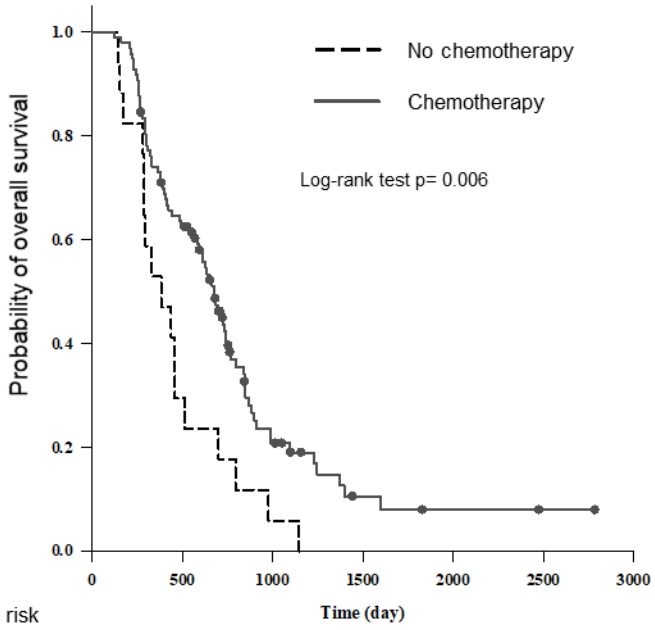
Supplemental Table I: Demographic and clinical characteristics of PDAC patients (n=114)

Characteristics		Values
Age (years)	mean (sd)	63.4 (10.1)
	median (range)	62.5 (39-88)
	< 65	64 (56.1%)
Gender	female	51 (44.7%)
	male	63 (55.3%)
Tumor grade	1	4 (3.5%)
	2	71 (62.3%)
	3	39 (34.2%)
T stage	1	2 (1.8%)
	2	7 (6.1%)
	3	103 (90.4%)
	4	2 (1.8%)
N stage	0	20 (17.5%)
	1	94 (82.5%)
Surgical margin status	negative	72 (63.2%)
	positive	42 (36.8%)
Lymphovascular space invasion (LVSI)	negative	63 (55.3%)
	positive	43 (37.7%)
	N/A	8 (7%)
Perineural invasion (PNI)	negative	86 (75.4%)
	positive	27 (23.7%)
	N/A	1 (0.9%)
Chemotherapy treatment	no	17 (14.9%)
	yes	97 (85.1%)
Type of chemotherapy	gemcitabine	91 (79.8%)
	5-fluorouracil	1 (0.9%)
	capecitabine	1 (0.9%)
	gemcitabine + abraxane	2 (1.8%)
	gemcitabine + 5-fluorouracil	1 (0.9%)
	FOLFIRINOX and gemcitabine + oxaliplatin	1 (0.9%)
ENT1 membrane H-score	mean (sd)	96.1 (48.8)
	median (range)	95.3 (8.5-257)
	< 100	66 (57.9%)

Supplemental Table 1: Pancreatic cancer patient demographics and clinical parameters

Demographic and clinical characteristics of pancreatic cancer patients comprising tissue microarray samples

sd=standard deviation; n=114

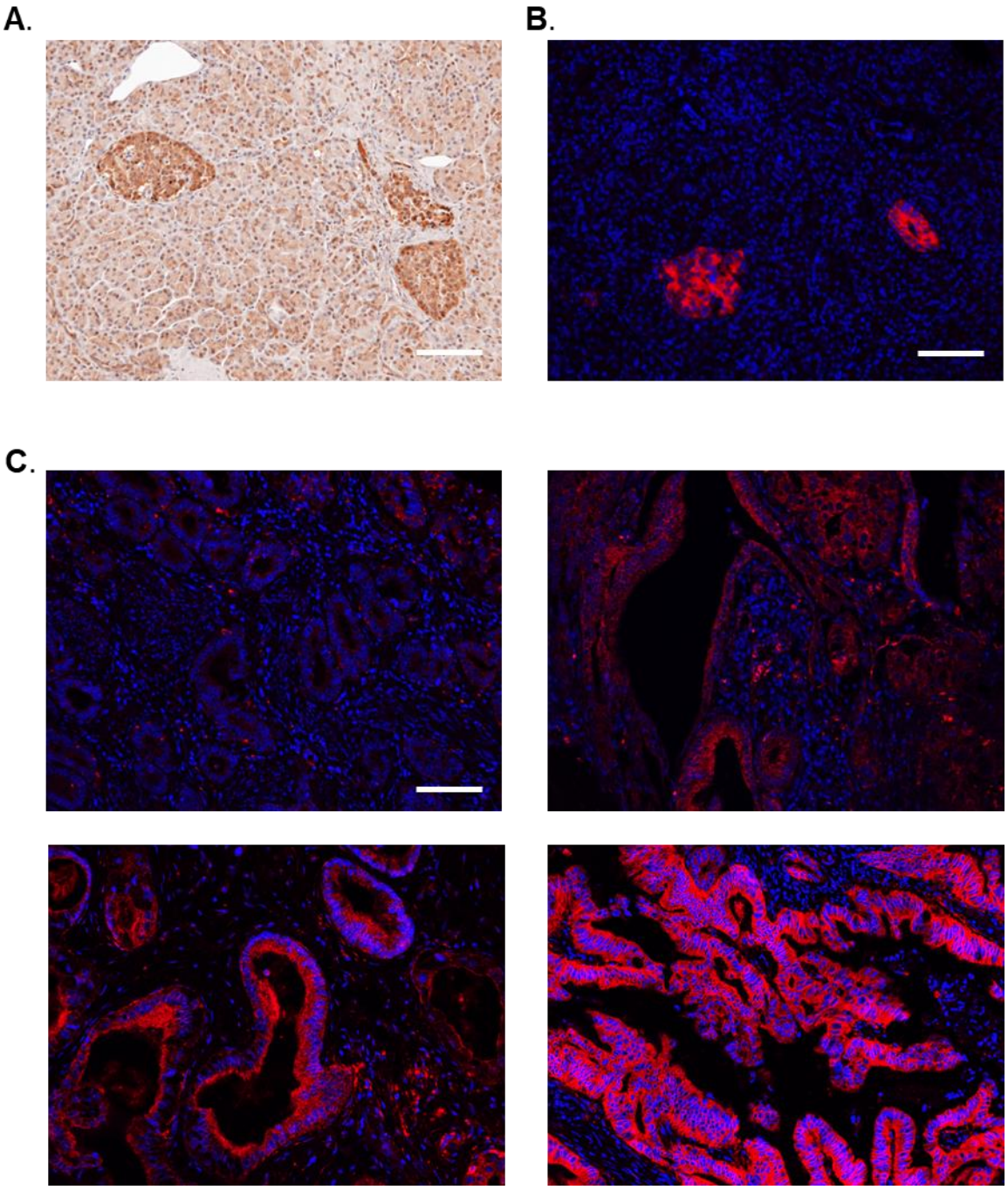


Number at risk

Chemo	97	60	14	4	3	2	2
No chemo	17	5	1	0	0	0	0

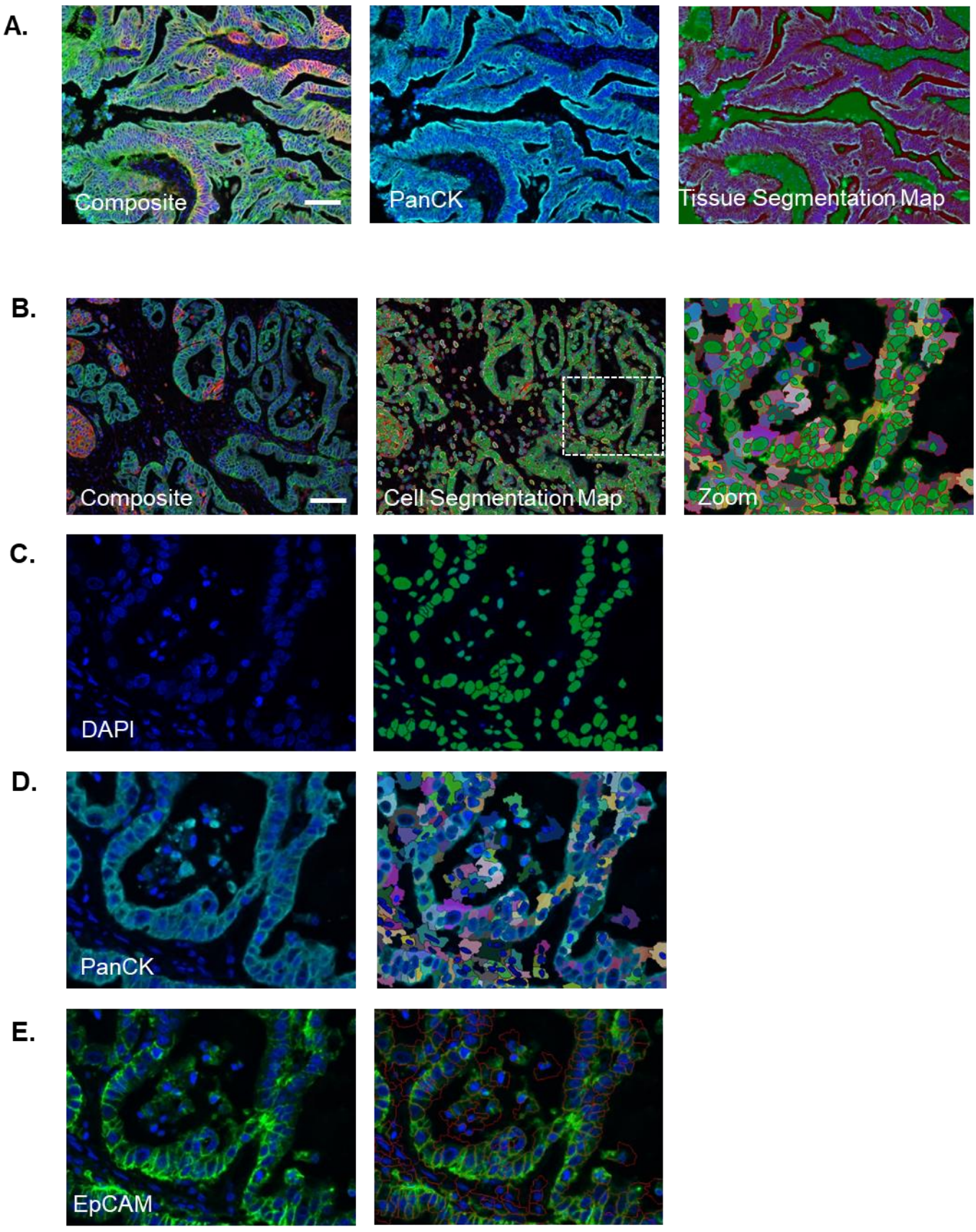
Supplemental Figure 8: Adjuvant chemotherapy treatment favors longer median overall survival

Kaplan-Meier curve shows median overall survival time for patients that were not treated with adjuvant chemotherapy (n=17) and treated patients (n=97).


A.
B.
C.

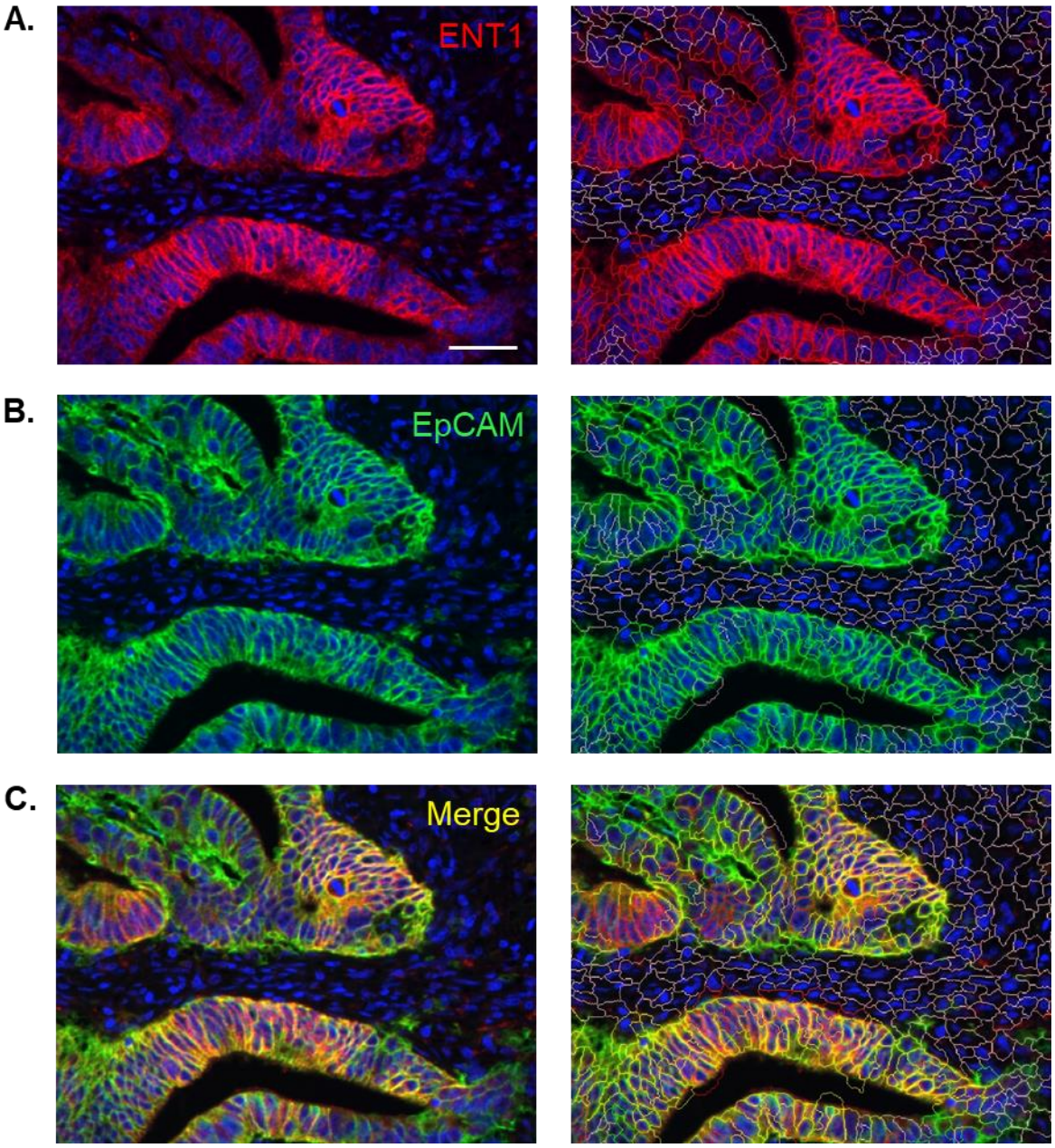
Supplemental Figure 9: ENT1 Immunohistochemical Staining Validation

A. Brightfield micrograph displays 3,3'-diaminobenzidine (DAB) staining for ENT1 in human normal pancreas tissue and hematoxylin nuclear counterstain **B.** ENT1 fluorescent IHC staining (red) and DAPI nuclear counterstain (blue) of normal human pancreas tissue section **C.** ENT1 staining in four representative human pancreatic cancer primary tumors shows a variety of staining intensities. Original magnification, x20; bar= 100 μm


A.
Composite
PanCK
Tissue Segmentation Map
B.
Composite
Cell Segmentation Map
Zoom
C.
DAPI
D.
PanCK
E.
EpCAM

Supplemental Figure 10: Multiplexed immunohistochemical analysis tissue and cell segmentation

A. Example of TMA tissue segmentation method by inForm software. From left to right, composite image of TMA patient sample immunostained for PanCK (cyan), ENT1 (red), EpCAM (green) and DAPI (blue), Pan-CK (cyan) staining pattern; expression of Pan-CK was used for trainable tissue segmentation. Regions shaded in purple represent tumor cells, and green regions are stroma. Original magnification x20; bar= 100 µm **B.** Representation of tumor cell segmentation performed by inForm software. Composite example TMA image overlaid with cell segmentation map. Zoom image shows closer view of outlined region of tumor sample **C.** Single channel DAPI nuclear stain overlaid with nuclear segmentation map **D.** Cytosolic staining for Pan-Cytokeratin overlaid with cytoplasmic compartment segmentation map **E.** Membrane staining for EpCAM overlaid with membrane segmentation map, shown in red.


A.
ENT1
B.
EpCAM
C.
Merge

Supplemental Figure 11: Multiplexed immunohistochemical analysis scoring methodology

A. ENT1 single channel staining overlaid with plasma membrane positivity scoring map. ENT1 plasma membrane positive scoring is outlined in red, and negative cells are pink. Original magnification x20; bar= 100 µm **B.** EpCAM staining overlaid with plasma membrane scoring map; cells positive for EpCAM plasma membrane staining are outlined in green, and negative cells are shown in pink **C.** Merged composite image for ENT1 (red) and EpCAM (green) overlaid with single and double positivity scoring map. Cells that are single positive for plasma membrane expression of ENT1 and EpCAM are outlined in red and green, respectively. Double positive cells are shown in yellow and double negative cells are shown in pink.

Supplemental Files 1-5: ENT1 localization shown by animation of Z-projection confocal images

Video 1. PANC-1 cells stably overexpressing E-cadherin are shown in this animation of a 3D reconstruction of compiled z-stack images. ENT1 (red), E-cadherin (green), DAPI (blue).
Video 2. Animation displays 3D reconstruction of PANC-1 cells grown in monolayer immunostained for ENT1 (red) and DAPI (blue)
Video 3. N+/E- cells 3D reconstruction animation shows localization of ENT1 (red) and DAPI (blue)
Video 4. E+/N- cells animation displays 3D reconstruction of ENT1 (red) and DAPI (blue)
Video 5. PANC-1 cells stably overexpressing EpCAM are shown in this animation of a 3D reconstruction of compiled z-stack images. ENT1 (red), EpCAM (green), DAPI (blue).
For each of the supplemental files described above, the animation illustrates Z-stacks compiled in Imaris software as 3D-view animations; 360° horizontal rotation followed by 360° vertical rotation are shown. Z-stack images were captured by confocal microscopy. Original magnification, x100