Crosstalk between tumor acidosis, p53, and extracellular matrix regulates pancreatic cancer aggressiveness

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Table of contents: Supplementary materials

1. Supplementary methods
    a. IncuCyte cell proliferation assay
    b. Western blot analysis
    c. Immunohistochemistry
    d. RT-qPCR
    e. 3D growth of AsPC-1 and Panc-1 cells in Matrigel and Collagen-I
2. Supplementary table 1. Antibodies used in this study
3. Supplementary Figure 1. Adaptation to lower pH does not enhance Panc02 viability in 2D.
4. Supplementary Figure 2. Immunofluorescence analysis of organotypic Panc02 cultures.
5. Supplementary Figure 3. Organotypic growth of AsPC1 and Panc1 cells.
6. Supplementary Figure 4. Wound healing and Boyden chamber invasion assays.
7. Supplementary Figure 5. Western blot densitometry.
8. Supplementary Figure 6. Immunohistochemical staining of Panc02 spheroids.
9. Supplementary Figure 7. Validation of Akt and TGFβ pathway interventions.

SUPPLEMENTARY MATERIALS

Supplementary methods

IncuCyte cell proliferation assay

Panc02 WT and p53KO cells proliferation rate was determined using IncuCyte live cell analysis system (Essen Bioscience). Cells were seeded in 96-well plate (Falcon, 353072) at 1,000 cells/well in 100 µL of media and placed immediately in the IncuCyte for 14 days in a standard humidified cell incubator. All images were acquired under 10x magnification. After two weeks, confluence was calculated with IncuCyte analysis software (Essen Bioscience).

Western blot analysis

Cells were cultured up to 80-100% confluency and lysed with Tris-SDS buffer (1 %SDS, 10 mM Tris-HCl, 1 mM Na_3VO_4). Samples were sonicated and debris removed by discarding pellet after centrifugation at 4°C and stored at -20°C until further use. BenchMark Protein ladder (ThermoFisher Scientific, 10747012) and equal amount of protein (ranging between 10-15 µg) were loaded onto 10% SDS polyacrylamide gels and transferred onto nitrocellulose membrane using BioRad Trans-Blot®Turbo™ transfer system. Membranes were stained with Ponceau S (Sigma, #P7170-1L), incubated in 5 % skimmed milk in TBST at 37°C for 1 h, and probed with primary antibodies (Suppl. Table 1) overnight at 4°C, followed by 1 h incubation with HRP-conjugated secondary antibodies. Proteins were visualized with a Fusion FX (Vilber) according to manufacturer's instructions. Densitometric quantification was performed using ImageJ software. Data were normalized to p150 or β-actin loading controls.

Immunohistochemistry

Spheroids were collected in Eppendorf tubes, washed in ice-cold PBS, and fixed in 4% PFA (VWR, #9713.1) for 24 h at room temperature, followed by two washes in PBS. After resuspension in a small amount of PBS, spheroids were injected into a drop of 60°C warm 2% agarose solution (Bactoagar; BD, #214050) which was embedded in paraffin and sliced using a microtome (ZEISS, MIKROM HM 200). 5 µm thick paraffin sections containing the spheroids were deparaffinized by passing through decreasing alcohol concentrations for 3 min each: Xylene (2 x 3 min), 1:1 mix of xylene and 99.9% ethanol, 99.9% ethanol, 96% ethanol, 70% ethanol, 50% ethanol. Sections were washed in cold water, placed in citrate buffer (0.21% citric acid monohydrate (Sigma, #C1909) in ddH_2O, pH 6) and irradiated in a microwave oven for 3 × 5 min at 1 min intervals. After cooling, sections were encircled using a PAP pen and washed once in PBS prior to addition of Tris-glycine (0.1 M glycine, AppliChem, #A3707 in ddH_2O, pH 7.4) for 15 min. After washing in PBS, sections were blocked in 5% BSA in PBST

(0.1% Tween) for 30-60 min at room temperature and incubated overnight at 4°C with primary antibodies (Suppl. Table 1) diluted in 1% BSA in PBST. Sections were washed in PBST (3 × 5 min), incubated with 1% BSA in PBST for 15 min and with fluorophore-conjugated secondary antibodies diluted 1:600 in 1% BSA in PBST for 30 min at room temperature, and finally washed in PBST (4 × 5 min) with DAPI (1:1000), mounted with N-propyl gallate and sealed. Fluorescent detection was used to visualize antibody binding to proteins in an Olympus IX83 microscope with a Yokogawa scanning unit, using a PLANAPO 60X/1.4 NA oil immersion objective and the CellSens Dimension software (358 nm, 568 nm, 647 nm). Subsequent image adjustments (overlays and intensity only) were performed in ImageJ.

RT-qPCR

Total RNA from monolayer cells was isolated using RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's protocol. One µg of RNA was used as a template to obtain cDNA by reverse transcription using: a) Master Mix I (1 µl Random Primer (Invitrogen 48190-011) + 1 µl 10 mM dNTP mix (Invitrogen 10297-018) - 5 min at 65ºC, b) Master Mix II (4 µL 5x first strand buffer (Invitrogen 18064-014) + 2 µL 0.1 M DTT (Invitrogen 18064-014) + 0.5 µL Rnasin Promega N351B) + 0.5 µL RNAse-free H_2O) - 2 min at 25ºC, c) 1 ul Superscriptase III (Invitrogen 18064-014) - 10 min at 25ºC, 50 min at 42ºC, 15 min at 70ºC. qPCR amplification was done in triplicates in 384 well reaction plates (Thermo Scientific #4309849) using an ABI7900 qPCR machine, and 10 µL master mix PER SAMPLE (5 µL SYBR Green reagent (Thermo Scientific #3409155), 1 µL of forward and reverse primers (2µM each), 2 µL nuclease free water and 1 µL cDNA). Cycling conditions: 95°C, 10 min, [95°C, 30 s, 55°C, 1 min, 72°C, 30 s] x 40, 95°C, 1 min. Controls without cDNA and SYBR Green were included. Data was analysed with MS Excel and the Pfaffl method was used to calculate relative gene expression. Primer sequences: Actb_Fw CACTGTCGAGTCGCGT, Actb_Rv GTTCAATGGGGTACTTCAGG, Gapdh_Fw CCAGCTTAGGTTCATCAGG, Gapdh_Rv GATGACAAGCTTCCCATTCT, Tgfb1_Fw GAGCCCTGGATACCAACTAT, Tgfb1_Rv GTCCAGGCTCCAAATATAGG, Tgfb2_Fw CTCCGAAAATGCCATCCC, Tgfb2_Rv CAGGGGCAGTGTAAACTTAT, Tgfbr3l_Fw CGGTATTCAATGCCTCAGTA, Tgfbr3l_Rv GAGACTCTTGGATGGCCTA, Serpine1_Fw GACCGATCCTTTCTCTTTGT, Serpine1_Rv GAGGAGGGAGTTAGACTCTT, Mmp2_Fw CCATGAAGCCTTGTTTACCA, Mmp2_Rv AGTGAAGGGGAAGACACAT.

3D growth of AsPC-1 and Panc-1 cells in Matrigel and Collagen-I

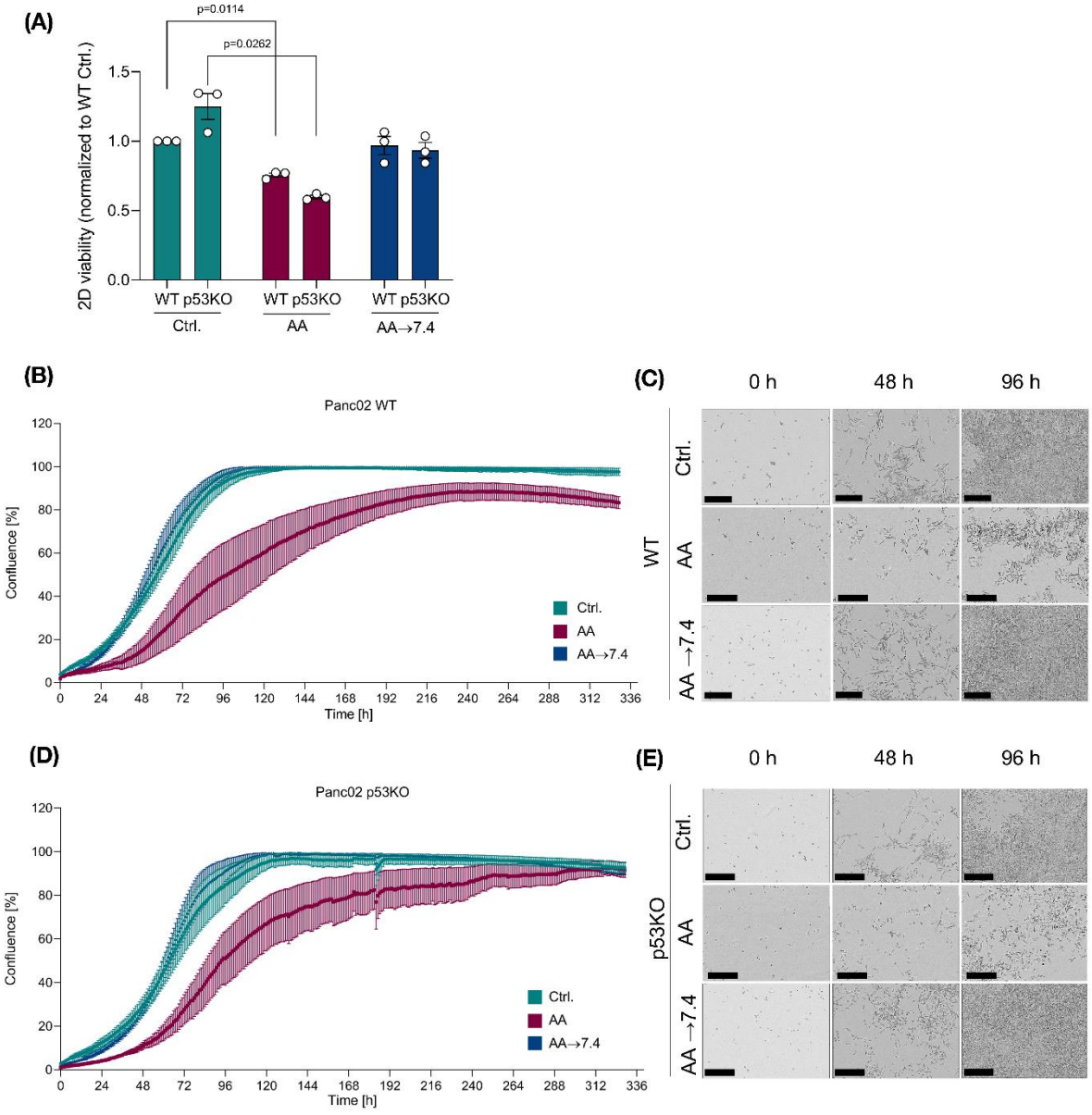
Single AsPC-1 and Panc-1 cell suspensions (in four-five independent biological replicates) were seeded in 50 µL domes of matrigel or collagen-I (10,000 cells/dome), as above, in 24-well-plates and cultured at 5% CO2/37ºC. Solidified 3D domes were subsequently over-laid

with 0,5 ml of appropriate culture media. Media were replaced every third day. Brightfield images were acquired after 5 (for Matrigel) and 7(for Collagen-I) with an Olympus IX83 microscope, 10X objectives and CellSens software. Viability was assessed using the CellTiter GLO kit, as mentioned above.

Antibody/size in kDa	Species/clonality	Catalog no./company	Dilution for WB	Dilution for IF/IHC
α-SMA	Mouse/monoclonal	Sigma Aldrich A2547	1:1000	-
α-tubulin	Mouse/monoclonal	Sigma Aldrich T5168	-	1:100
β-actin	Mouse/monoclonal	Sigma Aldrich A5441	1:40.000	-
Akt	Rabbit/monoclonal	Cell signaling #9272	1:1000	-
Akt (Ser473)	Rabbit/monoclonal	Cell signaling #4060	1:2000	-
ERK1/2	Rabbit/monoclonal	Cell signaling #9102	1:1000	
ERK1/2 (Thr202/Tyr204)	Rabbit/monoclonal	Cell signaling #9101	1:1000	
Histone H2A.X	Rabbit/monoclonal	Cell signaling #2577	1:1000	-
NBCn1	Rabbit/polyclonal	Abcam ab82335	1:1000	1:300
NHE1 (54)	Mouse/monoclonal	Santa Cruz Biotechnology SC-136239	1:500	1:300
p21 Waf1/Cip1	Mouse/monoclonal	Santa Cruz Biotechnology SC-6246	1:200	-
p53 (1C12)	Mouse/monoclonal	Cell signaling #2524	1:1000	-
p150-Glued	Mouse/monoclonal	BD 610473	1:1000	-
PCNA	Mouse/monoclonal	Cell signaling #2586	-	1:100
S6 (Ser235/236)	Rabbit/monoclonal	Cell signaling #4856	1:1000	-
Smad 2/3	Rabbit/monoclonal	Cell signaling #3102	1:1000	-
Smad2 (Ser465/467)	Rabbit/monoclonal	Cell signaling #3101	1:1000	-
TXNIP	Rabbit/monoclonal	NBP2-75692	1:1000	-

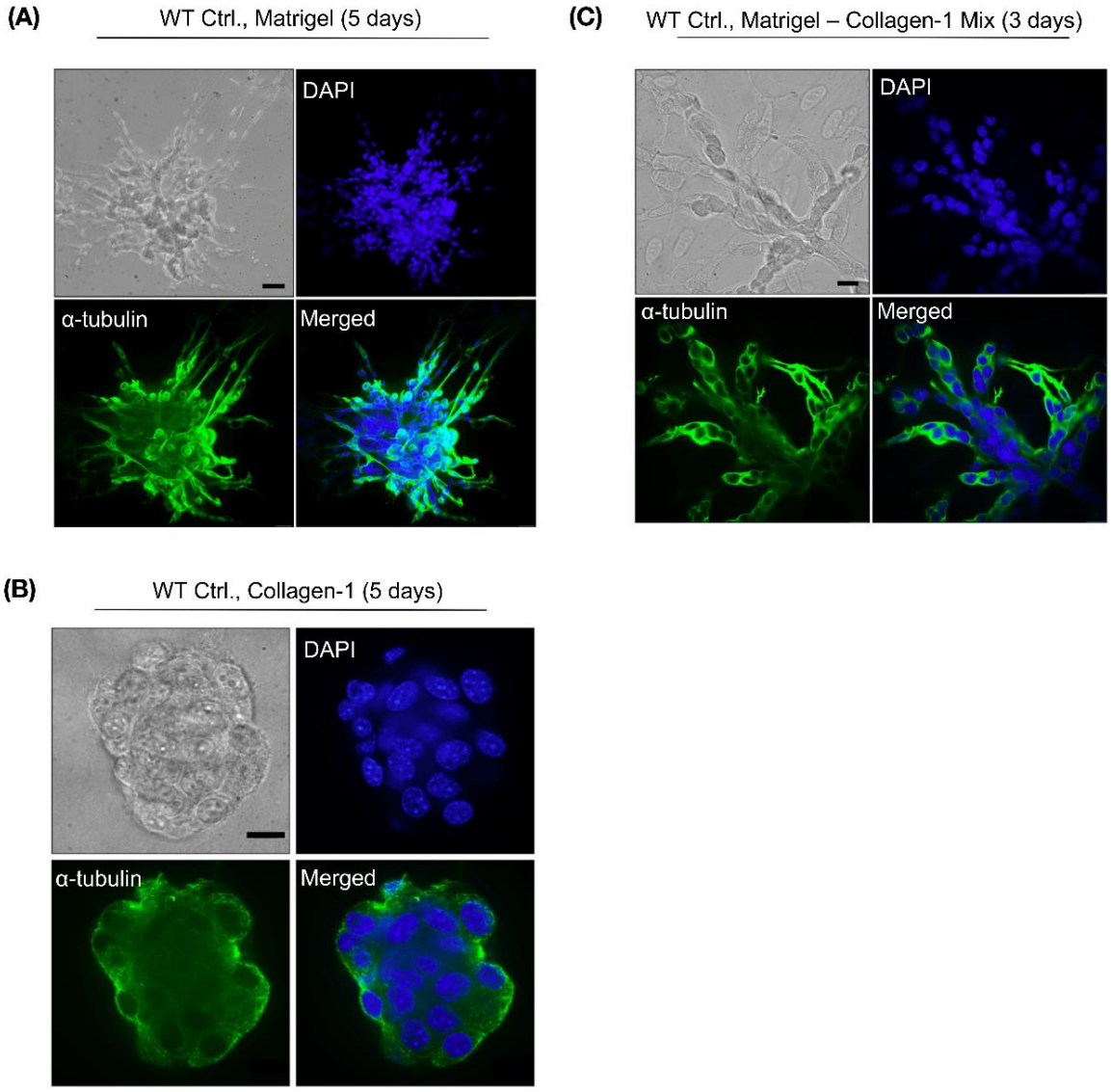
Supplementary table 1. Antibodies used in this study

Supplementary figures

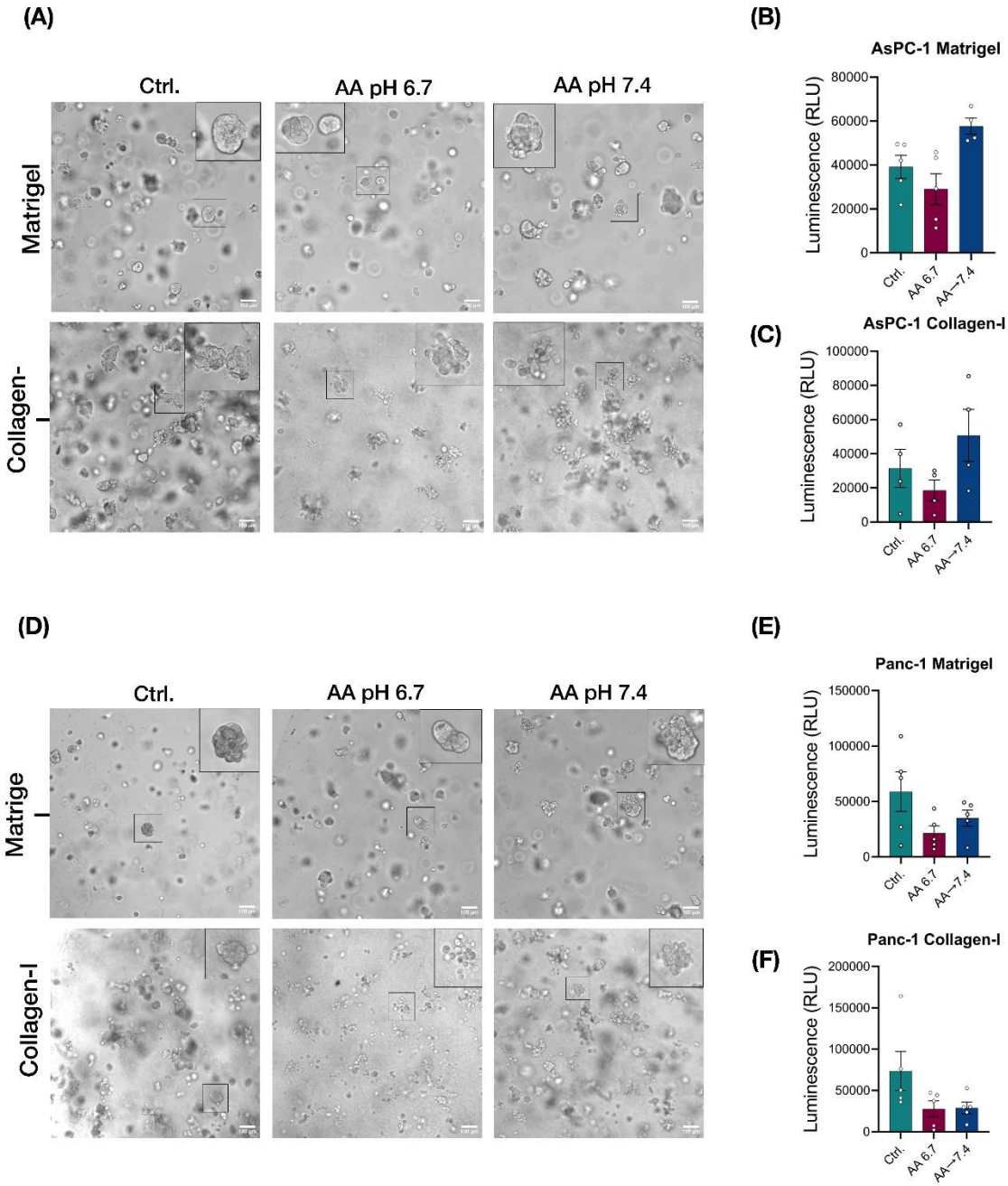


Supplementary Figure 1. Adaptation to lower pH does not enhance Panc02 viability in 2D.

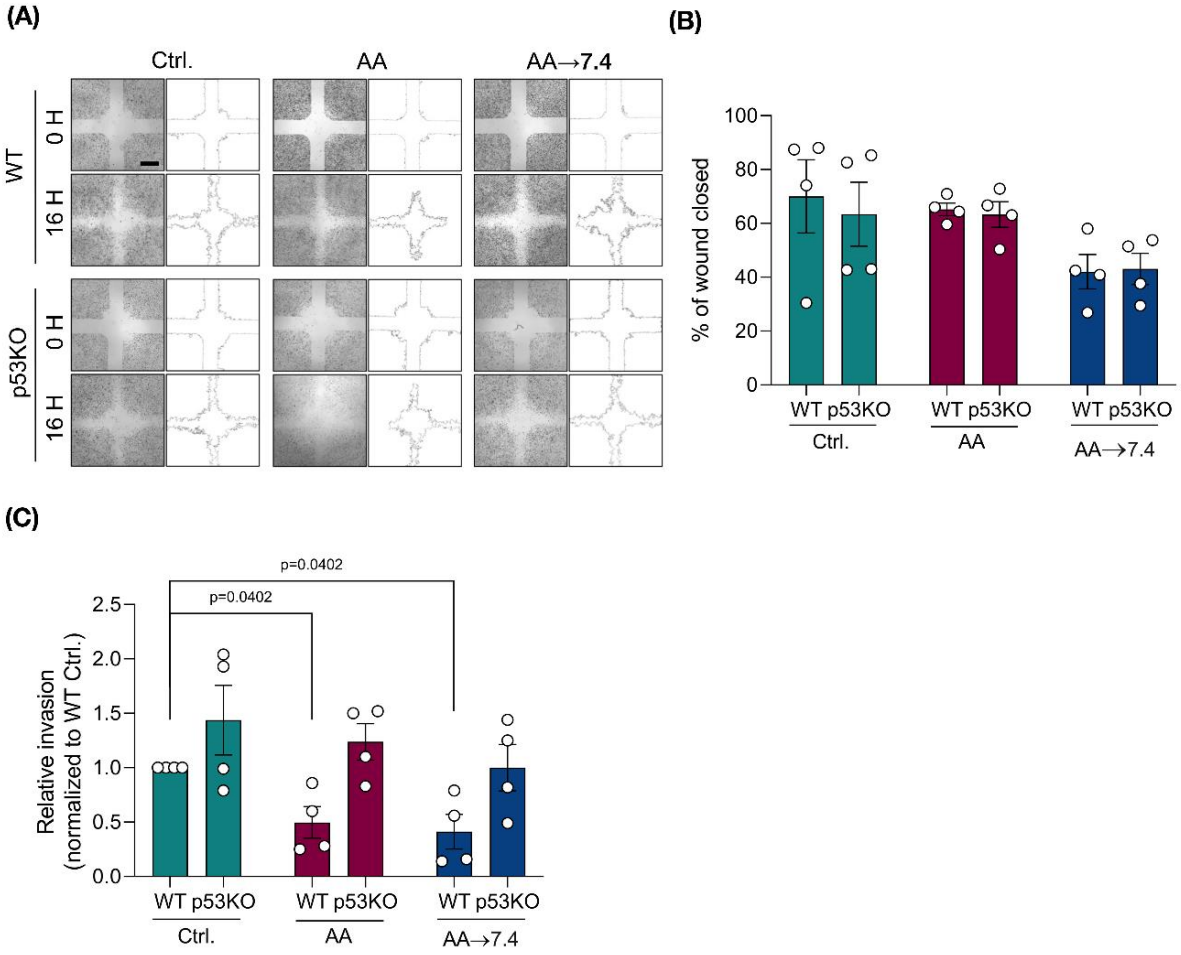
(A) Viability of Panc02 cells cultured as 2D monolayer for 48 hours. Ctrl. cells were cultured at pH 7.4, while their acid-adapted counterparts (AA) were cultured at pH 6.7 or 7.4. n=3. Error bars denote SEM. **(B)** Proliferation curves for Panc02 WT Ctrl., AA and AA→7.4 cells. Cells were seeded in 96-well plate at appropriate pH and proliferation was determined using IncuCyte live cell analysis system (Essen bioscience) in standard humidified cell incubator. n=6. Error bars denote SEM. **(C)** Representative images of WT Ctrl., AA and AA→7.4 cells cultures as monolayers using the IncuCyte live cell analysis system (Essen bioscience). Scale bar: 400 µm. **(D)** Proliferation curves for Panc02 p53KO Ctrl., AA and AA→7.4 cells. Cells were seeded in 96-well plate at appropriate pH and proliferation was determined using IncuCyte live cell analysis system (Essen bioscience) in standard humidified cell incubator. n=6. Error bars denote SEM. **(E)** Representative pictures of Panc02 p53KO Ctrl., AA and AA→7.4 cells cultures as monolayers with IncuCyte live cell analysis system (Essen bioscience). Scale bar: 400 µm.



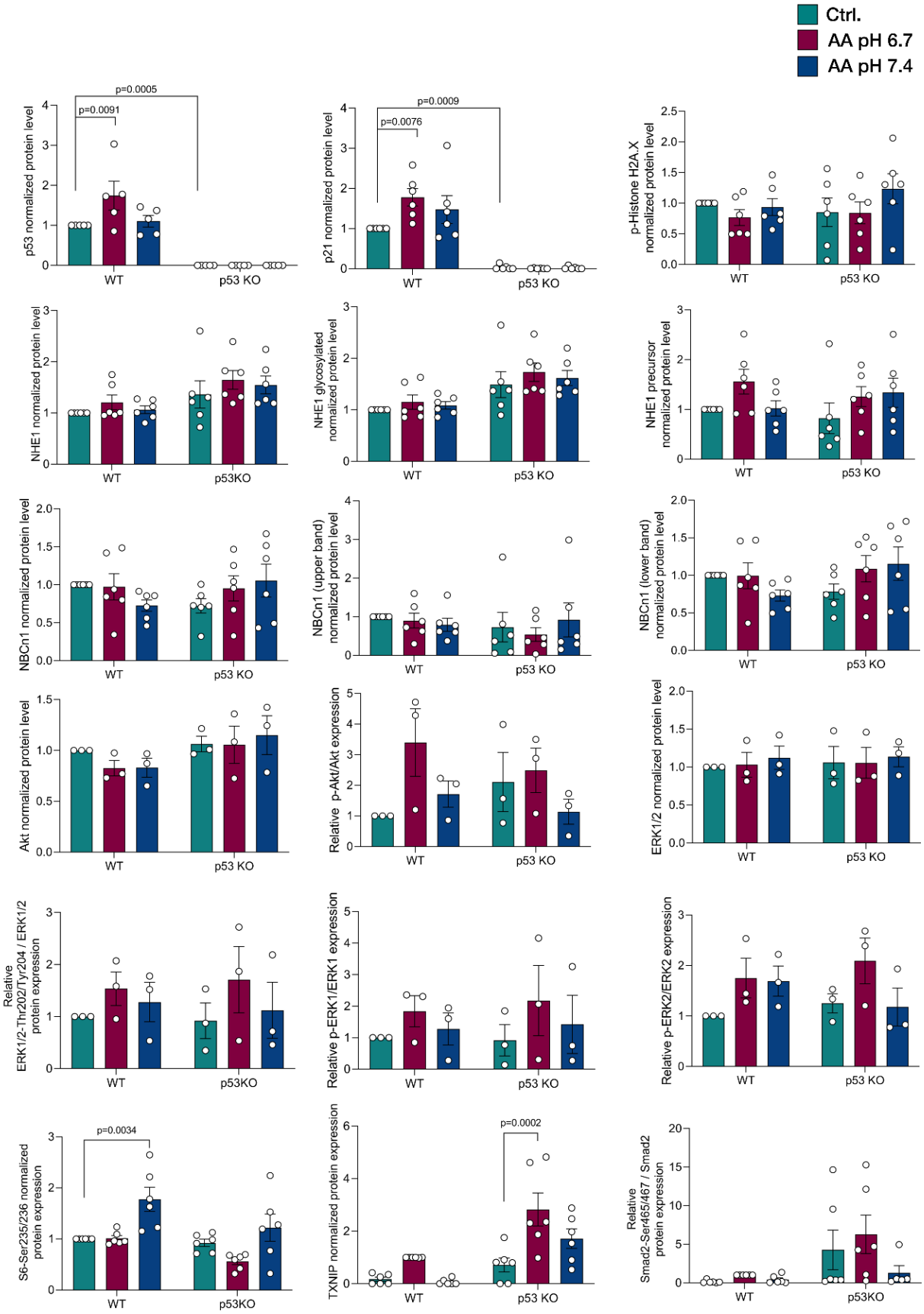
Supplementary Figure 2. Immunofluorescence analysis of organotypic Panc02 cultures.
(A) Immunofluorescence analysis of nuclear (DAPI, blue) and cytoskeletal (α-tubulin, green) markers in Panc02 WT Ctrl. cells cultured in 3D matrigel domes for 5 days. Arrows indicate invasive protrusions developed by the colonies representing rows of migrating cells. Scale bar: 30 μm. **(B)** Immunofluorescence analysis of nuclear (DAPI, blue) and cytoskeletal (α-tubulin, green) markers in Panc02 WT Ctrl. cells cultured in 3D collagen I. Scale bar: 10 μm. **(C)** Immunofluorescence analysis of nuclear (DAPI, blue) and cytoskeletal (α-tubulin, green) markers in Panc02 WT Ctrl. cells cultured in 1:1 matrigel:collagen-I. Scale bar: 10 μm.



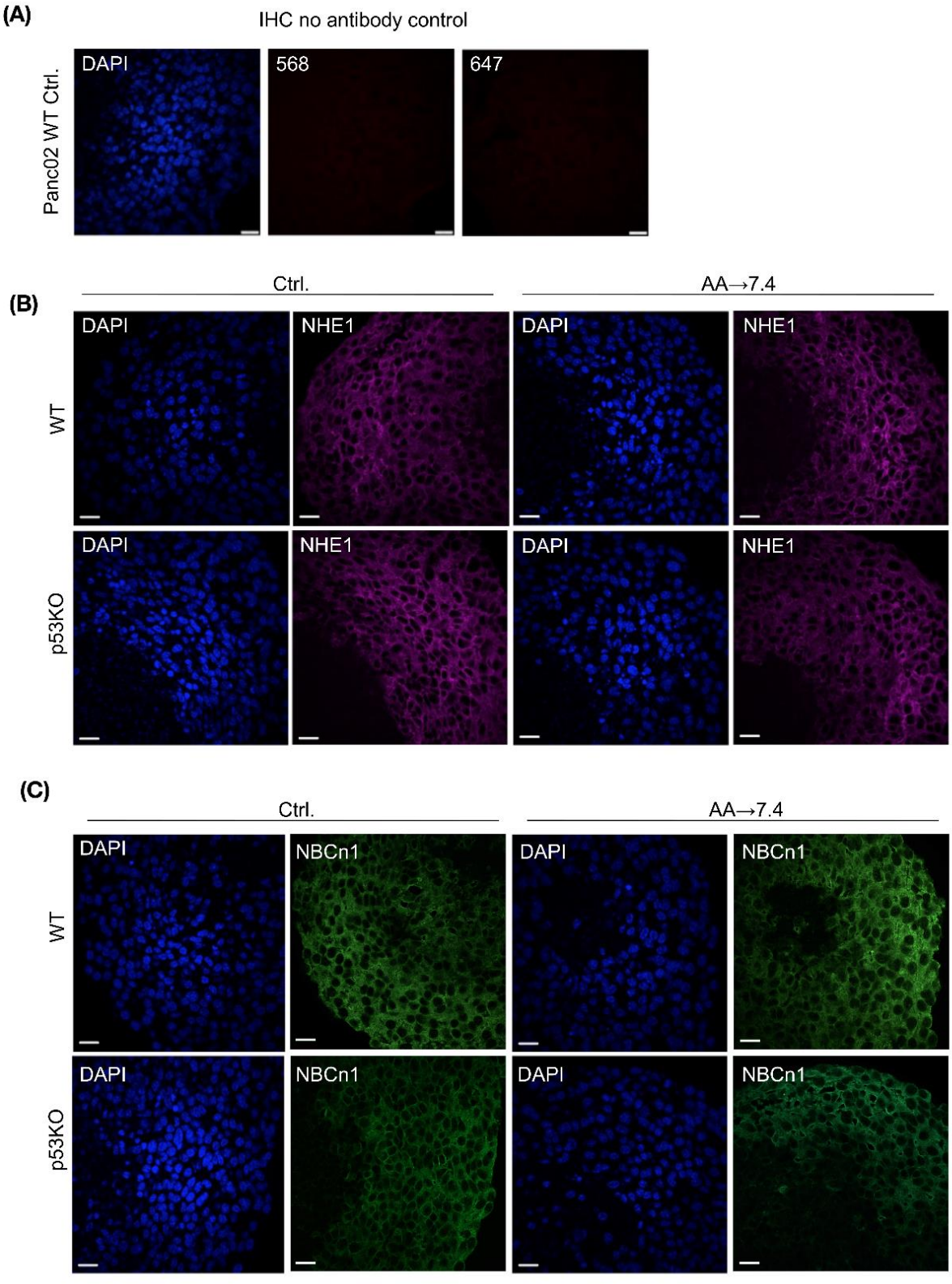
Supplementary Figure 3. Organotypic growth of AsPC1 and Panc1 cells.
(A) AsPC1 human pancreatic cancer cells were grown in matrigel or collagen-I as shown for 5 and 7 days, respectively. **(B)** AsPC1 viability in matrigel culture. **(C)** AsPC1 viability in collagen-I culture**.** n=4-5 independent experiments. Scale bar: 100 µm **(D)** Panc1 human pancreatic cancer cells were grown in matrigel or collagen-I as shown for 5 and 7 days, respectively. **(E)** Panc1 viability in matrigel culture. **(F)** Panc1 viability in collagen-I culture. n=5 independent experiments. Scale bar: 100 µm.



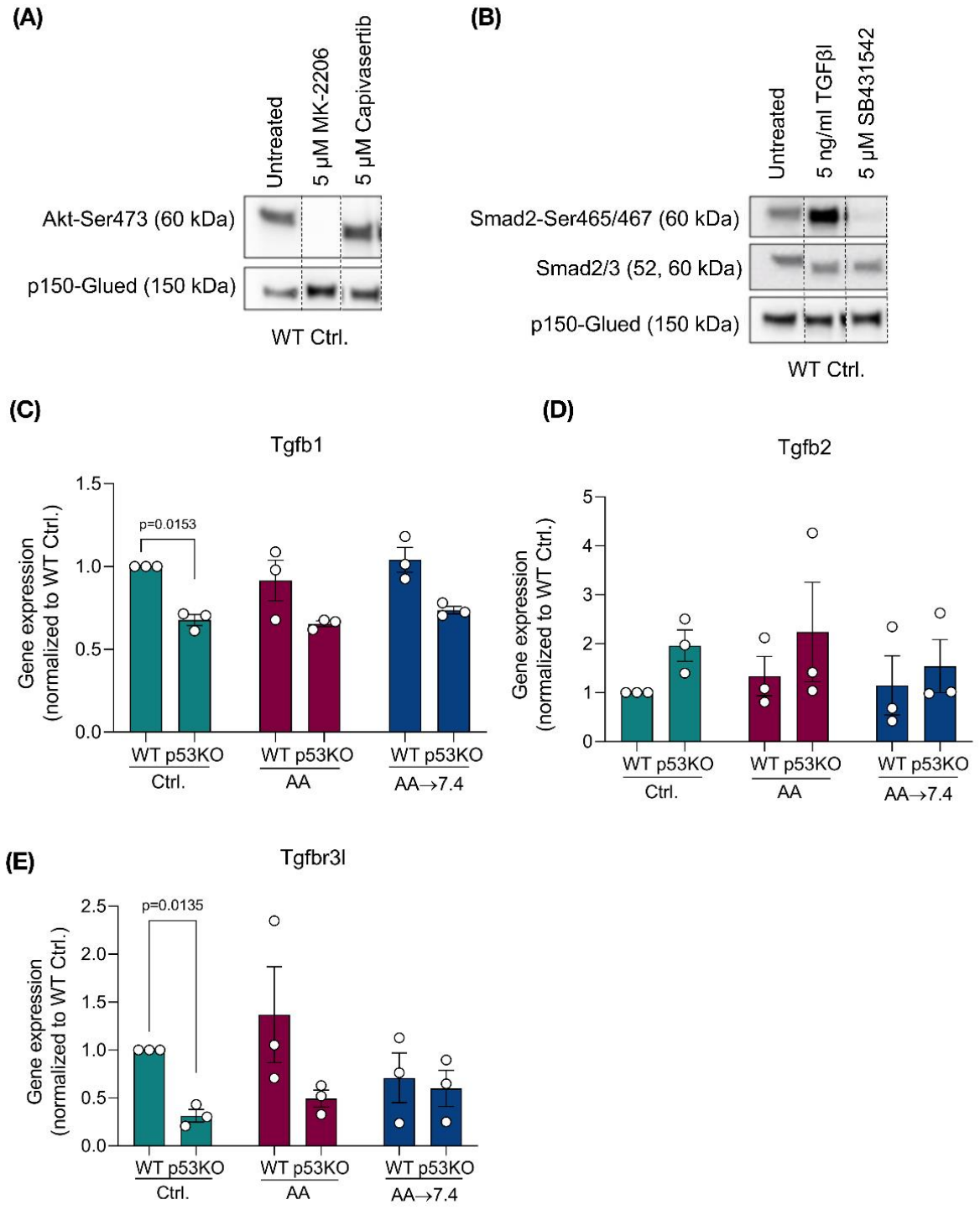
Supplementary Figure 4. Wound healing and Boyden chamber invasion assays.
(A) Panc02 cells were cultured for 24 h in 35 mm dish with 4-well removable silicone insert. Figure shows representative pictures taken right after removing the insert and 16 h later and respective wound areas generated in ImageJ software. Scale bar: 400 μm. **(B)** Acid adaptation does not promote Panc02 cell migration in 2D. Bar graphs showing wound closure percent 16 h after removing the silicone insert. n=4. Error bars denote SEM. **(C)** Panc02 cells starved for 24 h in 2% FBS media migrated to 10% FBS media through 8 μm pores in Boyden chamber inserts coated with matrigel. Each dot represents mean from one independent experiment where 2-3 inserts were used per condition. n=4. Error bars denote SEM.



Supplementary Figure 5. Western blot densitometry.
Quantitative densitometry for all western blots from Fig. 4. Data are shown as mean with SEM error bars. n=3-6 independent replicates per condition, as shown by the data points.



Supplementary Figure 6. Immunohistochemical staining of Panc02 spheroids.
(A) Representative pictures of slides stained only with DAPI (no primary antibody, no secondary antibody) at 568 nm and 647 nm. **(B)** Representative pictures of Panc02 slides stained with NHE1 antibody and DAPI. Scalebar: 20 µm. **(C)** Representative pictures of Panc02 slides stained with NBCn1 antibody and DAPI. Scalebar: 20 µm.



Supplementary Figure 7. Validation of Akt and TGFβ pathway interventions.
(A) Western blotting analysis of Akt-Ser473 in Panc02 WT Ctrl. 2D cells treated with 5 μM MK-2206 or 5 μM Capivasertib. Capivasertib is an ATP-competitive inhibitor and does not affect the Akt phosphorylation. **(B)** Western blotting analysis of Smad2-Ser465/467, a downstream effector of TGFβ pathway in Panc02 WT Ctrl. 2D cells treated with 5 μM SB431542 and 5 ng/ml TGFβI. **(C-E)** RNA isolated from Panc02 WT and p53KO cells cultured as 2D monolayers were subjected to RT-qPCR analysis. Bar charts show relative expression of TGFβ signaling pathway genes Tgfb1, Tgfb2, and Tgfbr3l. Relative expression was normalized to the average of *Actb* and *Gapdh* (housekeeping genes) expression. n=3 Error bars denote SEM.