Macrophages direct cancer cells through a LOXL2-mediated metastatic cascade in pancreatic ductal adenocarcinoma

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Gene expression datasets, GSEA analyses, Kaplan–Meier analysis and Pearson Correlation

LOXL2 expression levels in indicated datasets were compared in adjacent normal tissue, PDAC tumor tissue and metastatic tissue (if available), and Box Whisker plots were generated, where rectangles show the first quartile, the median, and the third quartile. The two whiskers indicate the minimum and maximum values, and outliers are depicted as circles. For Gene Set Enrichment Analysis (GSEA) analysis, tumor samples were subdivided into top and bottom quartiles based on *LOXL2* expression and compared using GSEA, v4.0.3 (Broad Institute). Fragments Per Kilobase of transcript per Million (FPKM) mapped reads were analyzed using the Gene signatures (Hallmark gene sets, h.all.v7.1.symbold.gmt) from GSEA - Molecular Signature Database for Gene set enrichment analysis was used for pathway enrichment analysis with GSEA, with 1000 permutations and FDR<25% was considered statistically significant.

The dataset, patient subtypes and survival data from Bailey *et al.* were included in Supplementary Tables 14-16 of their published work[1]. To analyze the prognostic value of *LOXL2* mRNA expression, patients were stratified based on the median value of *LOXL2* and a survival analysis (Kaplan–Meier survival plot) was performed with R. LogRank test was used to determine statistical significance. Cox regression was used to calculate the hazard ratio.

A Pearson correlation of epithelial/mesenchymal specific gene expression with *LOXL2* expression was computed for the 179 PDAC patients included in the PDAC TCGA RNAseq database[2]. The correlation matrix was subjected to supervised hierarchical clustering and Euclidean distance measurement; average linkage clustering was applied. The heat map was generated using Morpheus provided by the Broad Institute.

Human cell cultures

To establish low-passage primary PDAC PDX-derived *in vitro* cultures, the indicated xenografts were minced, enzymatically digested with collagenase (Cat no. 07416, Stem Cell Technologies) for 60 min at 37°C and after centrifugation for 5 min at 500 × g, the cell pellets were resuspended and cultured in RPMI 1640 media (Invitrogen, Cat no. 61870044) containing 10% FBS (Invitrogen), 50 units/ml penicillin/streptomycin (Invitrogen, Cat no. 11548876) and fungizone (Invitrogen, Cat no. 15290018). Primary cultures were tested for Mycoplasma at least every 4 weeks. EGFP-labelled human PDAC cells were established by lentivirus transduction with an EGFP-expressing reporter lentiviral vector backbone (pRRL sin CMV IRES eGFP). sh*LOXL2*- and shSrc-expressing cells were established by lentivirus transduction with the previously-described[3] GFP-expressing lentiviral vectors from SuperArray. All lentiviruses used in this study were propagated as previously described in HEK293T

cells[4]. For lentivirus transduction of human PDAC cells, Panc354 or Panc253 cells were seeded on 6-well plates at a concentration of 3 to 5×10^4 cells/well. One ml of virus was directly overlaid on cells and polybrene (Cat no. 638133, Sigma) was added at a final concentration of 8 µg/ml. After 16 h, medium was changed. Stably transduced cells were obtained after GFP-positive cell sorting using a FACS Vantage SE Flow Cytometer and analyzed by BD FACSDiva software (BD Biosciences).

HEK293T were obtained from American Type Culture Collection (ATCC) and cultured as described above. To over express LOXL2, cells were transfected with a pcDNA3-flag vector encoding the mouse (NM_033325.2) Loxl2 or human (NM_002318.3) LOXL2 coding sequence (cds) as described[5]. These cells served as a positive control for the protein analyses described below.

Blood samples from healthy donors were provided by the BioBank Hospital Ramón y Cajal-IRYCIS (PT13/0010/0002), integrated in the Spanish National Biobanks Network (ISCIII Biobank Register No. B.0000678). Samples were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees (Control no. No. Control: DE-BIOB-73 AC65, RG.BIOB-57, and RG.BIOB-54), with informed consent and according to Declaration of Helsinki principles. Blood samples were diluted with PBS (Cat no.10010023, Gibco), overlayed on Ficoll (Cat no. L6115, Merck), and centrifuged at 400 × g for 40 min to isolate peripheral blood mononuclear cells (PBMC). PBMCs were cultured in the absence of serum for 24 h to allow monocytes to attach. Adherent monocytes were then cultured with RPMI 1640 media (Invitrogen, Cat no. 61870044) containing 10% FBS (Invitrogen), 50 units/ml penicillin/streptomycin (Invitrogen, Cat no. 11548876) and 1000U/ml M-CSF (R&D, Minneapolis, MN) for 7 to 10 days to allow for differentiation into M2 macrophages as previously described[6]. For macrophage conditioned media (MCM), polarized M2 macrophages were washed with 1X PBS and complete medium lacking M-CSF was added to the cultures and harvested 48 h later.

Microscopy, histology, histopathology and immunohistochemistry

For histopathological analysis, formalin-fixed, paraffin embedded (FFPE) blocks of human and/or mouse samples were serially sectioned (3 µm thick) and stained with hematoxylin and eosin (H&E). Additional serial sections were used for immunohistochemical (IHC) studies or for Picrosirious red staining. IHC primary antibodies, secondary antibodies and dilutions are detailed in Table S1. Surgically resected human PDAC tumors shown in Figure S1 have been previously described[6]. For IHC, antigens were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+). Counterstaining was performed with hematoxylin. Human LOXL2 protein expression by IHC was performed on 2 µm FFPE tissue sections. After deparaffinization (60 °C for 40 min) and antigen-retrieval (Leica Bond ER Solution 2, pH 8.9-9.1 for 20 min), the LOXL2 primary antibody (1:200) was incubated for 20 min at RT followed the secondary-HRP antibody incubation. The staining was revealed by DAB standard Leica procedure.

In negative controls, the primary antibody was omitted. The rabbit polyclonal antisera against human LOXL2 was kindly provided by Dr. Csiszar and previously described [3 7 8]. The LOXL2 quantification was defined as positive when it was detected in at least 10% of tumor cells when compared with non-expression in normal tissue. To assess fibrillar collagen, FFPE sections of tumor tissues were stained with 0.1% picrosirious red (Sigma, Cat no. 141048.1610) and counterstained with Weigert's hematoxylin. Slides were digitalized with the Slide Scanner Axio Scan.Z1 (Carl Zeiss) and a 20X PlanApo objective (0.22 µm/pixel resolution) and analyzed with ZEN light (Carl Zeiss). Picrosirious red-stained sections were captured using polarized light and analyzed with Fiji package[9]. Fourier component analysis for directionality was performed on digitalized images using the Fiji plug-in "Directionality" (http://pacific.mpi-cbg.de/wiki/index.php/Directionality) to generate directionality plots. Analyses shown in Figure 7C were performed comparing wild-type [(+/+) KPC and HET KPC (KPC;Loxl2$^{+/-}$)] mice to KO KPC (KPC;Loxl2$^{-/-}$) mice or wild (+/+) KPC mice to KPCL2^{KI} [KPC;R26L2$^{+/KI}$ and KPC;R26L2$^{KI/KI}$] mice. Groups were only merged when no significant differences were observed between them.

For H&E-stained slides of pancreata, an in-house pathologist marked areas as normal tissue, ADM, PanIN or PDAC tumor tissue, which were compared to the total area to determine the percent of each. Moreover, tumors were classified as differentiated or poorly differentiated using the guidelines from the College of American Pathologists (https://documents.cap.org/protocols/cp-pancreas-exocrine-17protocol-4001.pdf). For macroscopic images of tumors or metastatic tissue, a Leica Stereomicroscope (MZ10F, Germany) with fluorescence was used, and images were captured with Leica LAS V4.8 software.

For IHC images acquired and analyzed in Figures 5D-E, S10, S11, S15 and S16A-B, sections (3-µm) were incubated with primary antibodies overnight at 4 °C followed by biotinylated secondary antibodies for 1h at room temperature (RT). Avidin-biotin peroxidase complex was applied according to the manufacturer's protocol (Vector Laboratories, CA). Slides were counterstained with hematoxylin and eosin. Quantification of stained slides was done by QuPath 0.2.3 (Bankhead, P. et al. (2017). QuPath: Open-source software for digital pathology image analysis. *Scientific Reports*. https://doi.org/10.1038/s41598-017-17204-5).

Primary mouse cell cultures

Established KPCL2^{KO} and KPCL2^{KI} cell lines were tested for Mycoplasma at least every 4 weeks. H2b-mCherry-labelled KPCL2^{KO} cells were established by lentivirus transduction of KPCL2^{KO} cells with a H2B-mCherry reporter lentiviral vector backbone, propagated as previously described in HEK293T cells[10]. For lentivirus transduction, KPCL2$^{-/-}$ ID90 cells were seeded on 6-well plates at a concentration of 3 to 5×10^4 cells/well. One ml of virus was directly overlaid on cells and polybrene (Cat no. 638133, Sigma)

was added at a final concentration of 8 µg/ml. After 16 h, medium was changed. Stably transduced cells were obtained after mCherry-positive cell sorting using a FACS Vantage SE Flow Cytometer and analyzed by BD FACSDiva software (BD Biosciences).

Murine bone-marrow-derived cells (BMDC) were isolated from the femur and tibiae of at least two mice by centrifugation. BMDC pellets were resuspended in RPMI 1640 media (Invitrogen, Cat no. 61870044) and cultured in the absence of serum for 24h to allow monocytes to attach. Adherent monocytes were then differentiated into macrophages under adherent conditions on non-tissue culture-treated 100mm dishes in RPMI supplemented with 10% FBS and 10ng/ml of murine M-CSF (Cat no. 315-02, PeproTech, London, UK). To polarize macrophages to M2, cultures were treated with 10 ng/ml of IL-4 (Cat no. 214-14, PeproTech) for 24h. For MCM, polarized M2 macrophages were washed with 1X PBS and complete medium lacking M-CSF was added to the cultures and harvested 48h later.

Patient serum samples and Enzyme linked immunosorbent assay (ELISA)

OSM levels in serum from PDAC patients and healthy donors and in conditioned media samples were determined using the OSM DuoSet Development kit (R&D, Cat no. DY295) as per the manufacturer's instructions.

MCM, recombinant TGFß and OSM experiments

For induction of EMT, indicated cells were seeded in 6-well plates and treated with 100 ng/ml of recombinant human OSM (no. PHC5015, Thermo Fisher Scientific), 200 ng/ml of recombinant mouse Osm (Cat. no 495-MO-025; R&D), 5 ng/ml of recombinant human TGFß1 (Cat no. 100-21, Peprotech) or 2ml of MCM. Treatments and MCM were replaced daily for the duration of the experiment. After indicated time points, cells were photographed with an EVOS FL microscope (Thermo Fisher Scientific) using a 10X objective with phase contrast and cells and/or media were harvested for further analyses, including WB analysis, RT-qPCR, or ELISA.

RNA Preparation and Real-Time quantitative PCR

Total RNA from human PDX-derived cell lines and murine PDAC tumors and cell lines was isolated by the guanidine thiocyanate (GTC, VWR AMRESCO Chemicals, Cat no. K965-250ML) method using standard protocols[11]. RNA from the surgically resected human PDAC tumors (n=30) shown in Figure 1B have been previously described[6]. RNA from normal pancreas was purchased from ThermoFisher Scientific (Cat no. QS0621) and Quimigen (Cat no. R1234188-P). RNA extraction from formalin-fixed paraffin-embedded (FFPE) sections was performed using the PureLink™ FFPE RNA Isolation Kit (ThermoFisher Scientific, Cat. no. K156002). Total RNA was extracted from 10 slices of 10 µm-thick FFPE sections.

For cDNA synthesis, 1 microgram of purified RNA was reverse-transcribed using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Cat no. K1672) according to manufacturer´s instructions, followed by SYBR green RT-qPCR (PowerUp™ SYBR™ Green Master Mix, ThermoFisher Scientific, Cat no. A25777) using an Applied Biosystems StepOnePlus™ real-time thermocycler (ThermoFisher Scientific). Thermal cycling consisted of an initial 10 min denaturation step at 95 °C followed by 40 cycles of denaturation (15 sec at 95 °C) and annealing/extension (1 min at 60 °C). mRNA copy numbers were determined relative to standard curves comprised of serial dilutions of plasmids containing the target coding sequences and normalized to ß-actin or HPRT levels, as indicated. Species-specific primers used are listed in Table S2.

Western blot analysis

Tumors, PDXs or cells were harvested in RIPA buffer (Sigma, Cat no. R0278-50ML) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Tumors and PDXs were mechanically digested. Cell supernatants were concentrated using the Millipore Centriplus centrifugal concentrator 50kDA (Cat. no. 4423), as per the manufacturer's instructions. Fifty to 80 µg of protein or 30 µl of concentrated media were resolved by SDS-PAGE and transferred to Amersham™ Hybond® P Western blotting PVDF membranes (Sigma, Cat no. 10600021). Membranes were sequentially blocked with 1X TBS containing 5% BSA (w/v) and 0.5% Tween20 (v/v), incubated with a 1:500-1:5000 dilution of indicated antibodies (see Table S1) overnight at 4ºC, washed 5 times with 1X TBS containing 0.5% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Amersham), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Pierce, Cat no. 34580) and images were obtained using the MyECL or iBright Imager (Thermo Fisher Scientific). Densitometric ratios were determined using ImageJ software and by measuring the intensity of each band and normalizing to total protein (i.e., Ponceau S, Sigma, Cat. no. P7170) or a specific housekeeping loading control.

The specificity of the anti-human/mouse-LOXL2 antibody for WB was validated using lysates from HEK239T cells transiently transfected with a plasmid (pcDNA3-Flag) expressing the full cds of murine or human LOXL2.

Migration assay

Invasion assays were performed using 8.0 µm transparent PET membrane trans-well chambers (Cat no. 353097, Falcon). 1×10^5 human PDX-derived cells or mouse PDAC-derived cells, cultured with control media, MCM for 48h or pretreated with OSM or TGFß1 (concentrations detailed above), were added to the top of the insert, and 750 µl of complete DMEM/F12 media supplemented with 20% FBS, or RPMI media supplemented with 2%, 10% or 50% FBS was added to the lower chamber. The assay

chambers were incubated for 18h at 37°C. Invaded cells were fixed in 4% PFA and stained with Diff Quik (Cat. no. WS32-1L, Sigma) or Crystal violet (Sigma, Cat no. C3886-100G), and the number of cells that migrated through to the lower chamber were counted for Diff Quik-stained cells or optically measured (i.e., optical density) following incubation with 1X PBS containing 1% SDS.

Wound healing assay

Human PDX-derived cells were cultured until confluence and then wounded using a 200 µl yellow pipette tip. Cells were treated with 100 ng/ml of rOSM 24h prior to wounding. Three wounds were made for each sample, and migration distance was photographed and measured at time 0h and 12h.

Sphere formation assay

Pancreatic CSC spheres were generated by culturing primary pancreatic cancer cells (2,000-10,000 cells/ml) in ultra-low attachment plates (Corning) using serum-free DMEM/F12 (Invitrogen, Cat no. 21331046) supplemented with B27 1:50 (Invitrogen, Cat no. 17504044), 20 ng/mL bFGF (PAN-Biotech, Sigma, Cat no. GF446-10UG), L-Glutamine (Invitrogen, Cat no. 25030081), 50 units/ml penicillin/streptomycin (Invitrogen, Cat no. 11548876) and fungizone (Invitrogen, Cat no. 15290018). Seven days later, spheres were counted with an inverted EVOS FL microscope (Thermo Fisher Scientific) using a 10X objective with phase contrast. Sphere counts are represented as the no. of spheres/ml.

Colony assay

For colony formation assays, 500 cells were seeded in 24-well plates. Cells were cultured in RPMI 1640 containing 10% FBS at 37°C, 5% CO2. After 5-10 days, cells were fixed with PFA 4% (Paraformaldehyde, 16% w/v aq. soln., methanol free, Alfa Aesar™, Cat no. 11400580) for 10min, washed with PBS and stained with Crystal violet (Sigma, Cat no. C3886-100G) for 1h. Wells were digitalized and the percent of colonies/total areas was determined with Fiji package.

Flow cytometry

Cells were resuspended in Flow buffer [1X PBS; 3% FBS (v/v); 3mM EDTA (v/v)] before analysis with a 4-laser Attune™ NxT Acoustic Cytometer (Thermo Fisher Scientific). Explanted lungs and livers were digested with Collagenase P (11213857001, Roche), to obtain single cell suspensions. Briefly, tissues were minced using a scalpel and forceps and transferred to a 50ml conical tube. 500ml of FBS-free medium and 500 µl of Collagenase P 1x (0.2mg/ml) (diluted in HBSS) were added to the tubes. Samples were incubated for 15 min at 37°C, vortexing every 5 min. After incubation, digestions were stopped by adding 1 mL of HBSS containing 10% FBS. Tubes were centrifuged for 5 min at 1500 rpm and supernatants were removed. ACK lysis buffer was used to remove erythrocytes by incubating samples for 5 min at room temperature HBSS was then added, and samples were centrifuged, supernatant

removed and cell pellets resuspended in flow cytometry buffer (PBS with 2%FBS and 3 mM EDTA). For analysis of murine cells, cell suspensions were blocked with anti-mouse CD16/CD32 Purified (Cat no. 14-0161, Thermo Fisher Scientific) and normal mouse serum (Cat no. 24-5544, Thermo Fisher Scientific) to block Fc receptors. For cell surface marker expression, refer to antibodies listed in Table S1. For all assays, 2mg/ml DAPI (4',6-diamidino-2-phenylindole, Sigma, Cat no. D9542-5MG) was used to exclude dead cells. Data were analyzed with FlowJo 9.3 software (Tree Star Inc., Ashland, OR). For the MAM cytometry experiments shown in Figure 7A, analyses were performed comparing [wild-type (+/+) KPC and HET KPC (KPC;Loxl2$^{+/-}$)] mice to KO KPC (KPC;Loxl2$^{-/-}$) mice or wild (+/+) KPC mice to KPCL2^{KI} [KPC;R26L2$^{+/KI}$ and KPC;R26L2$^{KI/KI}$] mice. Groups were only merged when no significant differences were observed between them.

For the detection of H2B-mCherry KPCL2^{KO} and EGFP KPCL2^{KI} cells in blood samples, the Attune™ NxT No-Wash No-Lyse Filter Kit (Cat no. 100022776, Thermo Fisher Scientific) was used to efficiently detect nucleated mCherry or EGFP in blood samples without RBC lysing or washing. Data were analyzed with FlowJo 9.3 software (Tree Star Inc., Ashland, OR). For the detection of H2B-mCherry KPCL2^{KO} and EGFP KPCL2^{KI} in indicated organs, tissues were processed as described above.

In vivo tumorigenicity and metastasis assays

NOD-SCID and C57Bl/6 mice were purchased from our in-house breeding facility (Instituto de Investigaciones Biomédicas **"**Alberto Sols**"** CSIC-UAM). For liver metastasis assays using human PDX-derived cells, 8-week-old NOD-SCID mice (Instituto de Investigaciones Biomédicas **"**Alberto Sols**"** CSIC-UAM) were injected intrasplenically with 1×10^5 Panc354 cells pretreated in vitro for 48h with Ctl or M2-polarized macrophage conditioned media (MCM). Mice were sacrificed 10 weeks post injection and macroscopic liver metastases were determined. For lung colonization assays, 8-week-old NOD-SCID mice were injected intravenously via the tail vein with 2.5×10^5 EGFP-expressing Panc354 or Panc253 cells pretreated 5 days with control or MCM, or 1×10^6 KPCL2^{KO} or KPCL2^{KI} cells (resuspended in 100 µl 0.9% physiological saline solution) using a 27-G needle. Lungs were harvested 2- or 4-weeks post injection (as indicated), photographed, weighed, fixed in 4% PFA overnight at 4 °C and subsequently paraffin embedded. For images presented in Figure 6A-B, when tumor cells where present in a vascular space lined with endothelium it was deemed intravasation. To determine the percentage of EGFP positive Panc354 or Panc253 cells, lungs were digested with Collagenase P (11213857001, Roche) and EGFP was determined by flow cytometry using an Attune NxT Acoustic Cytometer (ThermoFisher Scientific).

For in vivo tumorigenicity assays using murine PDAC cells, serial dilutions of KPCL2^{KO} or KPCL2^{KI} cell lines in Matrigel^{TM} (Cat no. 356234, Corning) were subcutaneously injected into 8-week-old female

nude mice (Hsd:Athymic Nude-Foxn1nu/Foxn1+; Envigo) and tracked for 14 weeks for tumor formation. At the time of sacrifice, tumors were extracted, photographed and weighed.

For orthotopic experiments, 2.5×10^5 H2b-mCherry-labelled KPCL2^{KO} or EGFP+ KPCL2^{KI} cells, alone or at a 1:1 ratio, were resuspended in 50 µl Matrigel™ (Cat no. 356234, Corning) and slowly injected into the pancreas of 8-week-old NOD-SCID mice. Mice were sacrificed 4 weeks post infection, blood was collected in Vacutest® EDTA tubes (VWR, Cat no. KIMA135300) and analyzed as described above, and pancreata and livers were excised, photographed, weighed, digested as detailed above using Collagenase P (11213857001, Roche) and the percentage of mCherry and/or EGFP-positive cells were determined by flow cytometry using an Attune NxT Acoustic Cytometer (ThermoFisher Scientific).

Syngeneic orthotopic experiments were performed in C57Bl/6 mice as described above; however, murine PDAC tumor pieces [derived from a backcrossed KPC mouse[12]] of approximately 20 mm^3 were orthotopically implanted into the pancreas in lieu of a cell suspension. For macrophage depletion, mice received 150 µl of clodronate-encapsulated liposomes (ClodronateLiposomes, Amsterdam, Netherlands) via retro-orbital injection twice per week, beginning 24h prior to orthotopic tumor implantation and lasting throughout the course of the experiment. At sacrifice, pancreata, livers and lungs were excised, photographed, weighed, fixed in 4% PFA overnight at 4°C and subsequently paraffin embedded. A macroscopic inspection for metastases was performed.

SUPPLEMENTARY TABLES

Table S1. Antibodies

1ª Abs-Epitope	Source	Dilution	Application	Manufacturer (Catalog no.)
α-hu/mu-β-ACTIN	Mouse monoclonal	1:5000	WB	ThermoFisher (MA1-140)
α-hu-TUBULIN	Mouse monoclonal	1:5000	WB	Elabscience (E-AB-20033)
α-hu/mu-LOXL2	Rabbit	1:1000	WB	Abcam (ab179810)
α-hu-phospho-STAT3	Rabbit	1:500	WB	Cell Signalling (9145)
α-hu-STAT3	Mouse monoclonal	1:500	WB	BD (610189)
α-hu-LOXL2	Rabbit polyclonal antisera	1:200	IHC	kindly provided by Dr. Csiszar and previously described [3 7 8]
α-ms-GAPDH	Mouse monoclonal	1:5000	WB	Abcam (ab8245)
α-ms-EpCAM-FITC; or -EpCAM-PE	Rat	1:100; 1:50	FC	FITC (BioLegend, 118208, BioLegend 118202; PE (Miltenyi, 130-102-265)
α-ms-CD133 APC	Rat	1:10	FC	Miltenyi (130-102-19)
α-ms-CD11b PerCP Cy5.5	Rat	1:200	FC	TONBO Bioscience (65-0112)
α-ms-F4/80-PE	Mouse monoclonal	1:100	FC	MIltenyi (130-102-422)
α-ms-Vimentin	Mouse	1:200	IHC	Abcam (ab8978)
α-ms-Twist	Rabbit	1:100	IHC	Abcam (ab49254)
α-ms-ß-Catenin	Rabbit	1:200	IHC	Cell Signalling (9562)
α-ms-Snail1	Rabbit	1:100	IHC	Abcam (ab180714)
α-ms-Zeb1	Rabbit	1:500	IHC	Bethyl (A301-922A-T)
α-ms-CD3	Rabbit	1:100	IHC	DAKO (A0452)
α-ms-F4-80	Rat	1:100	IHC	Invitrogen (MF-48000)
α-ms-CD206	Goat	1:150	IHC	R&D (AF2535)
α-ms-E-cadherin	Mouse	1:100	IHC	BD Pharmingen (610181)
α-ms-pStat3	Rabbit	1:100	IHC	Cell Signaling (CS9145)
α-ms-pFAK	Rabbit	1:200	IHC	Thermo (900255)
α-ms-pMLC2	Rabbit	1:200	IHC	Cell Signaling (CS3674)
α-ms-CD3	Rabbit	1:100	IHC	DAKO (A0452)

2ª Abs-Epitope	Source	Dilution	Application	Manufacturer (Catalog no.)
Anti-mouse-HRP	Sheep	1:5000	WB	Amersham (NA931)
Anti-rabbit-HRP	Donkey	1:5000	WB	Amersham (NA934)

Table S2. RTqPCR primer sequences

Gene	Species	Primer sense	Primer antisense
β-ACTIN	human	GCGAGCACACGAGCCTCGCCTT	CATCATCCATGGTGAGCTGGCGG
SNAIL1	human	CTCCCTGTCAGATGAGGAC	CCAGGCTGAGGTATTCCTTG
SNAIL2	human	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG
VIMENTIN	human	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
ZEB1	human	CCAGGTGTAAGCGCAGAAA	CCACAATATGCAGTTTGTCTTCA
CDH1	human	TGCCCAGAAAATGAAAAAGGC	GTGTATGTGGCAATGCGTTC
ZEB2	human	GAGCAGGTAATCGCAAGTTCAA	CACTCGTAAGGTTTTTCACCACTGT
LOX	human	GCATACAGGGCAGATGTCAG	GTGTTGGCATCAAGCAGGTC
LOXL1	human	AGCATCCACTTATGTGCAGAGA	GAGGAAGTCTGCTGTGCCCT
LOXL2	human	GGCACCGTGTGCGATGACGA	GCTGCAAGGGTCGCCTCGTT
LOXL3	human	AGAGGCCACAGGCTGGACCC	GCCCCGGGAGGCACATTCAG
LOXL4	human	TGACTTTCGTCCAAAGACTGG	TTGGAGCCATTGAGAGTGAG
β-actin	mouse	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG
Hprt	mouse	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
Lox	mouse	CCTTCAGCCACTCTCCTCTG	GCACAGCTGTCACCAACATT
Loxl1	mouse	GCCAGTGGATCGACATAACTG	ACAATGTACTTGGGGTTCACG
Loxl2	mouse	TGACTGCCAGTGGATAGACATC	GTTGGGGTTAATGACAACCTG
Loxl3	mouse	ATCCACAACCTAGGAAGAGCTG	TGGTAATGCCCATGACACTC
Loxl4	mouse	GGTTGCACAACTGCCACA	GGGAGTGCAGTAATGGCTT
Zeb1	mouse	TGAGCACACAGGTAAGAGGCC	GGCTTTTCCCCAGAGTGCA
Snail1	mouse	CTTGTGTCTGCACGACCTGT	CTTCACATCCGAGTGGGTTT
Twist	mouse	AGCTACGCCTTCTCCGTCT	TCCTTCTCTGGAAACAATGACA
Pdgfb	mouse	GCGTATCTATATCTTTGTGCCAGA	ACAGGTCCTCGGAGTCCAT
Sparc	mouse	AGAGGAAACGGTCGAGGAG	CTCACACACACCTTGCCATGTT
S100a4	mouse	GGAGCTGCCTAGCTTCCTG	TCCTGGAAGTCAACTTCATTGTC
MCol6A	mouse	GCAAGGATGAGCTGGTCAA	GTCCACGTGCTCTTGCATC
Cdh1	mouse	CCCGGGACAATGTGTATTACTATGA	GCAGCTGGCTCAAATCAAAGTCC

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SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

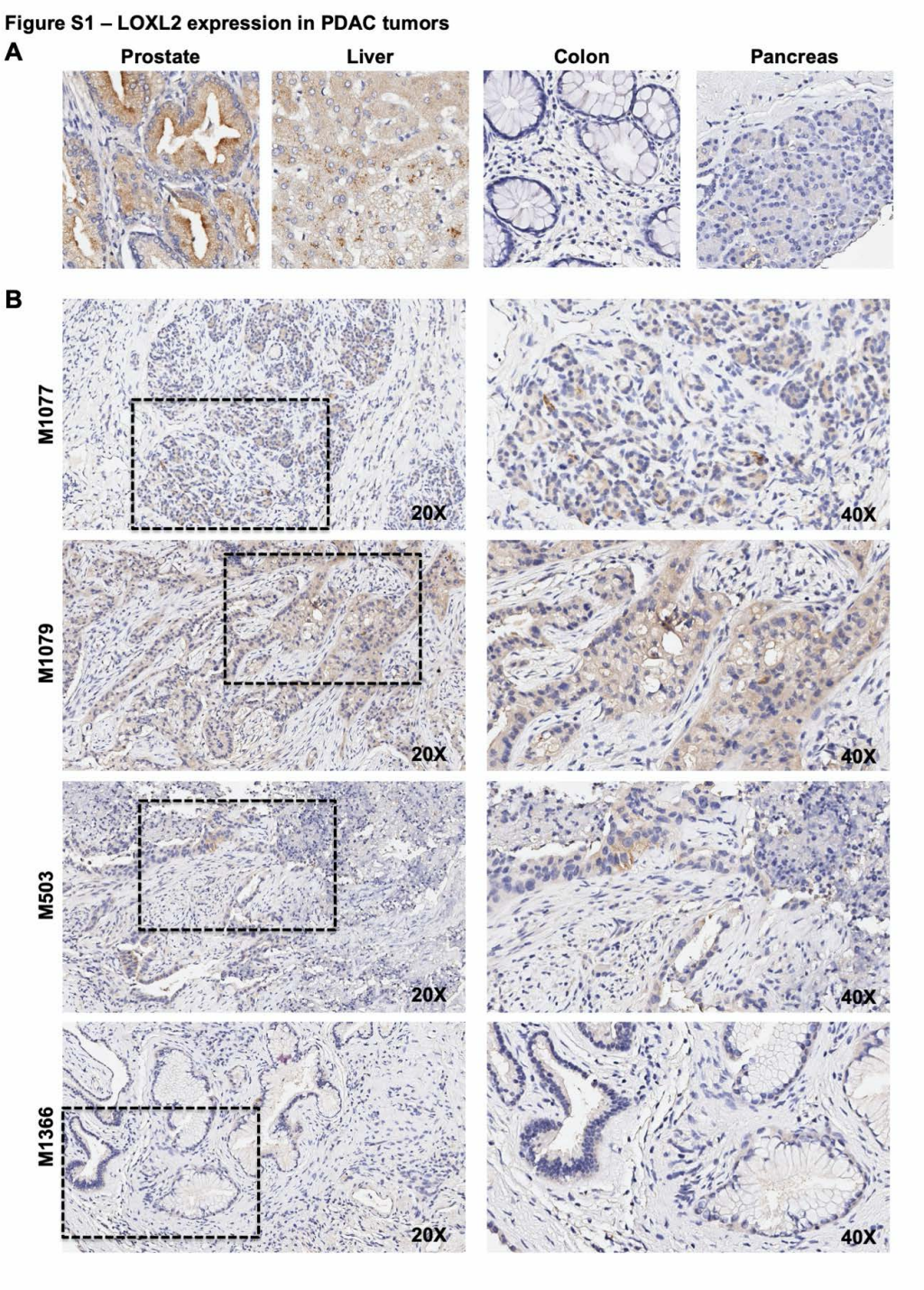


Figure S1: LOXL2 expression in PDAC tumors.
(A-B) Representative immunohistochemical staining for LOXL2 expression in sections from (A) normal prostate, liver, colon or pancreatic tissue or (B) PDAC samples analyzed in Fig 1B. 20X magnification. Insets indicate area that is shown on the right at 40X.

Figure S2 – GSEA of pathways enriched in patients with high *LOXL2* mRNA levels

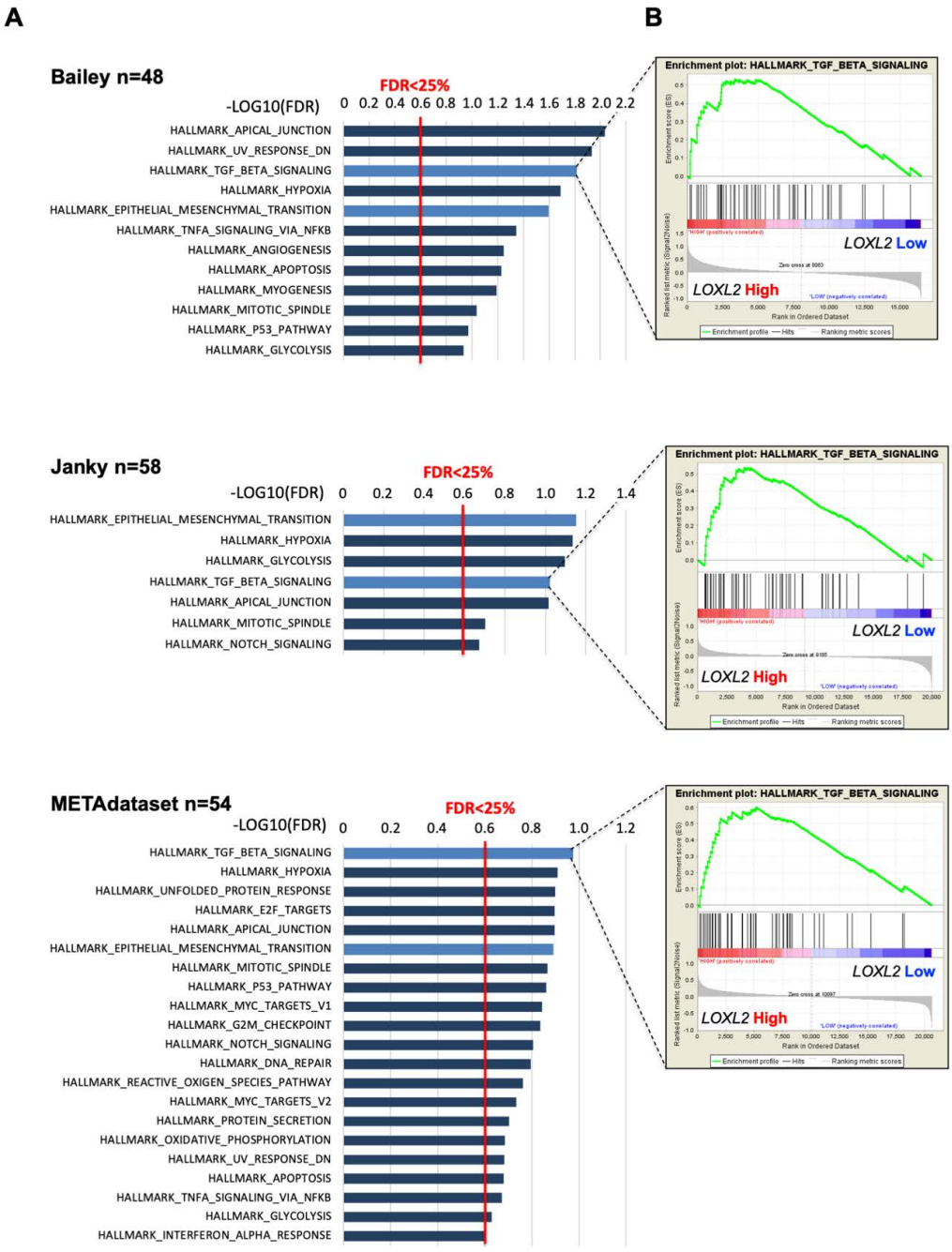


Figure S2: GSEA of pathways enriched in patients with high *LOXL2* mRNA levels.
(A) Pathways enriched in the transcriptional profiles of tumors belonging to the top *LOXL2* high expression quartile group, compared with the bottom expression quartile group in the Bailey, Janky or META datasets. A nominal p value of <0.05, FDR<25% is considered statistically significant. Shown are the –log10 (FDR) values for each pathway using the Hallmark genesets.
(B) Example enrichment plots for TGFß signaling pathways from the indicated datasets.

Figure S3 – *LOXL2* mRNA levels correlate with EMT, disease stage and survival parameters

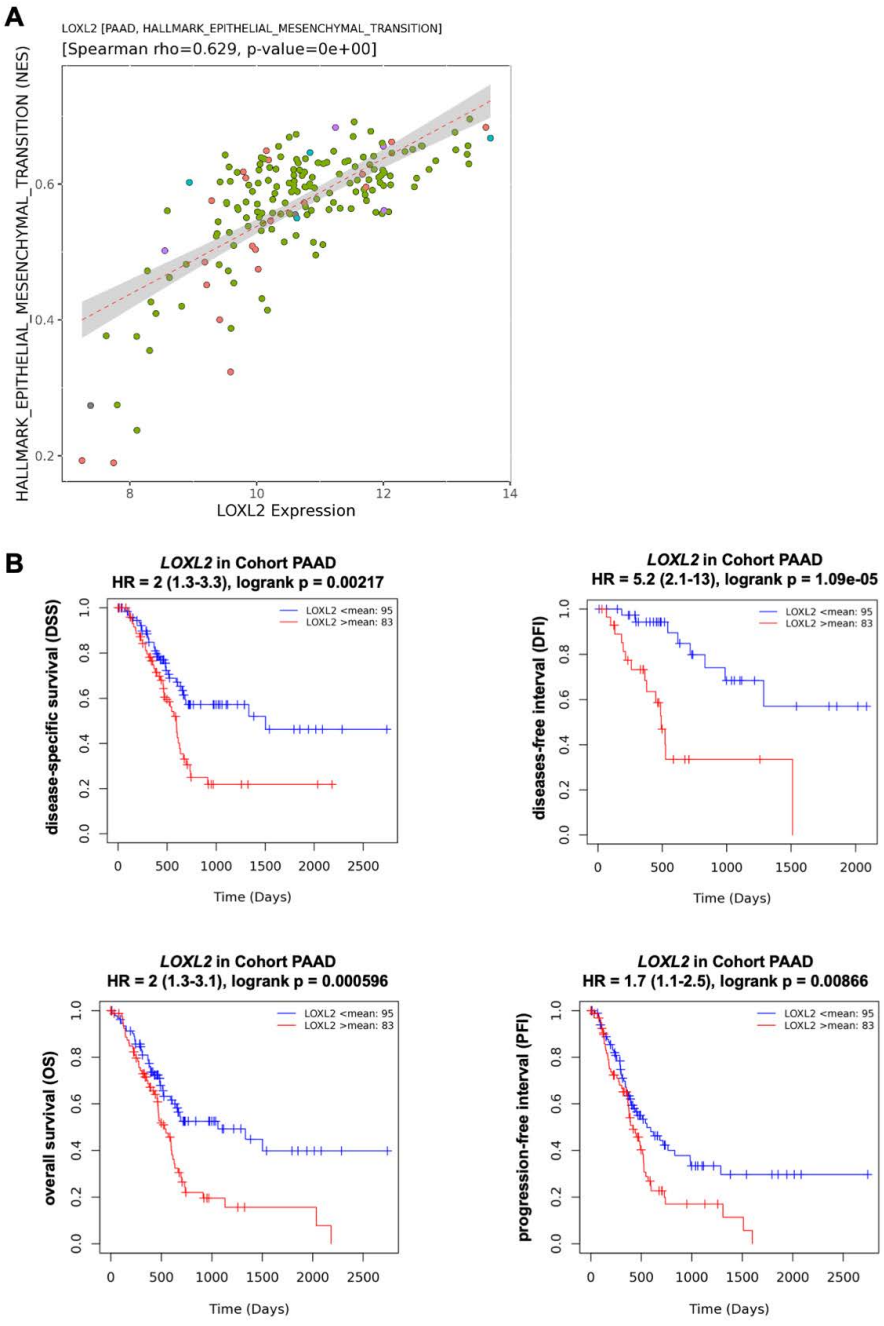


Figure S3: *LOXL2* mRNA levels correlate with EMT, disease stage and survival parameters.
(A) Scatter Plot showing correlation between *LOXL2* mRNA levels and EMT-related genes (Hallmarks EMT) in TCGA-PAAD dataset (n=185) curated by EMTome. (Spearman's ρ=0.629, p=0.000)
(B) *LOXL2* mRNA levels correlate with disease-specific survival (DSS) (HR=2, logrank p=0.00217), diseases-free interval (DFI) (Hazard ratio (HR)=5.2, logrank p=1.09e-05), overall survival (OS) (HR=2, logrank p=0.000596) and progression-free interval (PFI) (HR=1.7, logrank p=0.00866), curated by EMTome (http://www.emtome.org).

Figure S4 – MCM or OSM promote metastasis and EMT in PDAC PDX-derived cells

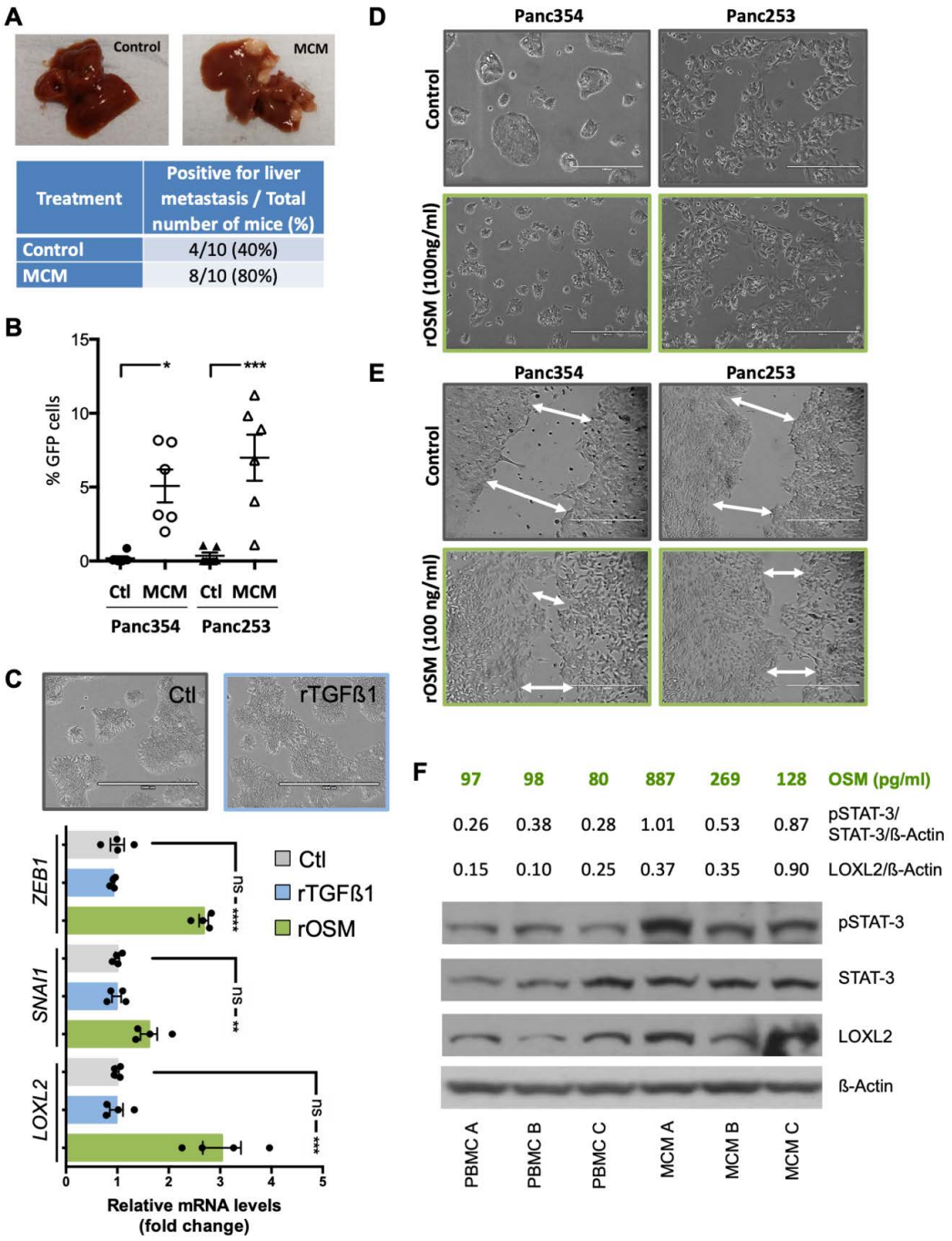


Figure S4: MCM or OSM promote metastasis and EMT in PDAC PDX-derived cells.

(A) Representative images of liver metastasis in NOD-SCID mice injected intrasplenically with 1×10^5 Panc354 cells pre-treated for 48 h with Ctl or M2-polarized macrophage conditioned media (MCM) in vitro and then injected in vivo. (top) Livers were collected and imaged for macrometastases. (bottom) Summary of the number of mice with macrometastases / number of mice injected.

(B) GFP-expressing Panc354 and Panc253 cells were pre-treated for 5 days with control media or MCM, and then 2×10^5 pre-treated cells were resuspended in 100µl physiological saline and tail vein injected into NOD-SCID mice (*n* = 6 mice per group). Lungs were harvested 2 weeks post injection and the percentage of GFP-positive cells

was determined by flow cytometry. Shown are the mean percent of GFP positive cells ± SEM within the single cell, live and debris-free population for each digested lung (* p<0.05, *** p<0.001, as determined by two-sided t test with Mann Whitney u test).

(C) Human Panc354 PDX-derived cells were cultured for 72h with control media (Ctl), recombinant (r) TGFß1 (5ng/ml) or rOSM (100ng/ml). (top) Representative images of control- and rTGFß1-treated cells. Scale bar = 400µm. (bottom) Mean fold-change ± SD of relative mRNA levels for the indicated target genes in Ctl-, rTGFß1 or rOSM-treated PDX-derived cells. Values were normalized to ß-actin levels and Ctl-treated samples were set as 1.0. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant, determined by one-way ANOVA with Dunnett post-test, comparing rTGFß1 and rOSM to Ctl).

(D-E) Human Panc354 and Panc253 PDX-derived cells were cultured for 72h with control media or rOSM (100ng/ml). (D) Representative images of control- and rOSM-treated cells. (E) Representative images from wound healing assay for Control-treated or rOSM-treated cells 12 h following wound initiation. Scale bar = 400µm.

(F) Western blot analysis of pSTAT3, total STAT-3 and LOXL2 in human Panc354 PDX-derived cells following treatment for 72h with media from unpolarized PBMCs or conditioned media (CM) from M2-polarized macrophages, from 3 donors (A, B and C). Levels of OSM present in each CM was determined by ELISA and is indicated above as pg/ml. Densitometric ratios for indicated proteins are shown. ß-actin served as a loading and densitometry normalization control.

Figure S5 – *LOXL2* is not necessary for OSM- or MCM-mediated EMT

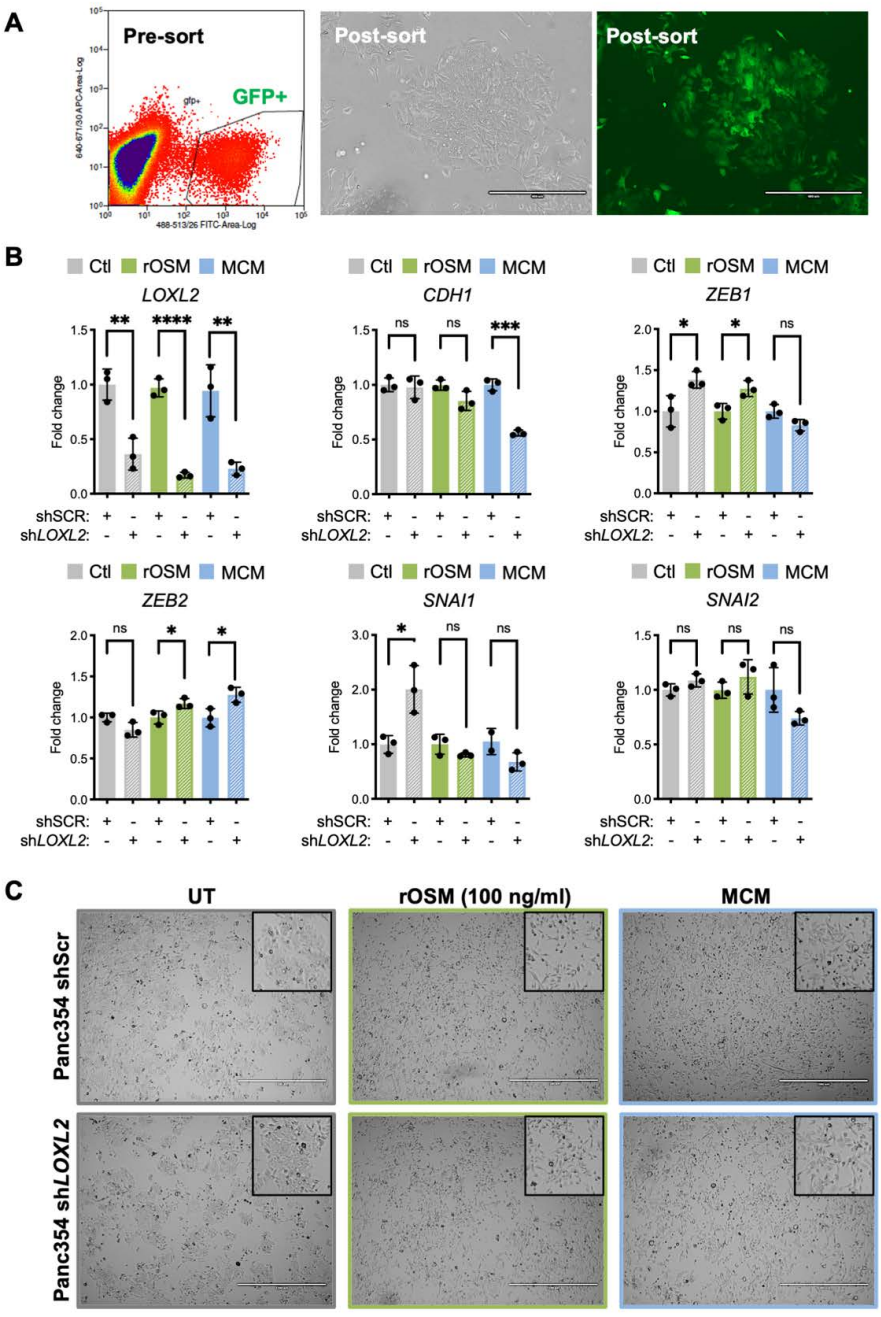


Figure S5: *LOXL2* is not necessary for OSM- or MCM-mediated EMT.
(A) Flow cytometry dot plot analysis of human Panc354 cells 48h post-infection with an sh*LOXL2* GFP-expressing lentiviral vector and prior to sorting for GFP (left), and the same cells 5 days post-sorting: light field (middle) and fluorescence (right). Scale bar = 400µm.
(B-C) Human Panc354 cells, stably transfected with an shScr GFP- or sh*LOXL2* GFP-expressing lentiviral vector were cultured for 72h with control media (Ctl), recombinant (r) rOSM (100ng/ml) or MCM. (B) Mean fold-change ± SD in relative mRNA levels for the indicated target genes in Ctl-, rOSM- or MCM-treated shScr or sh*LOXL2* cells. Values were normalized to ß-actin levels and shSrc cultures were set as 1.0 for all treatments (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, as determined by unpaired Student's t test). (C) Representative images of cells 72h post treatment. Scale bar = 1000µm. Inset = 4X amplification.

Figure S6 – MCM, TGFß1 and OSM promote Loxl2 expression in murine PDAC-derived cells

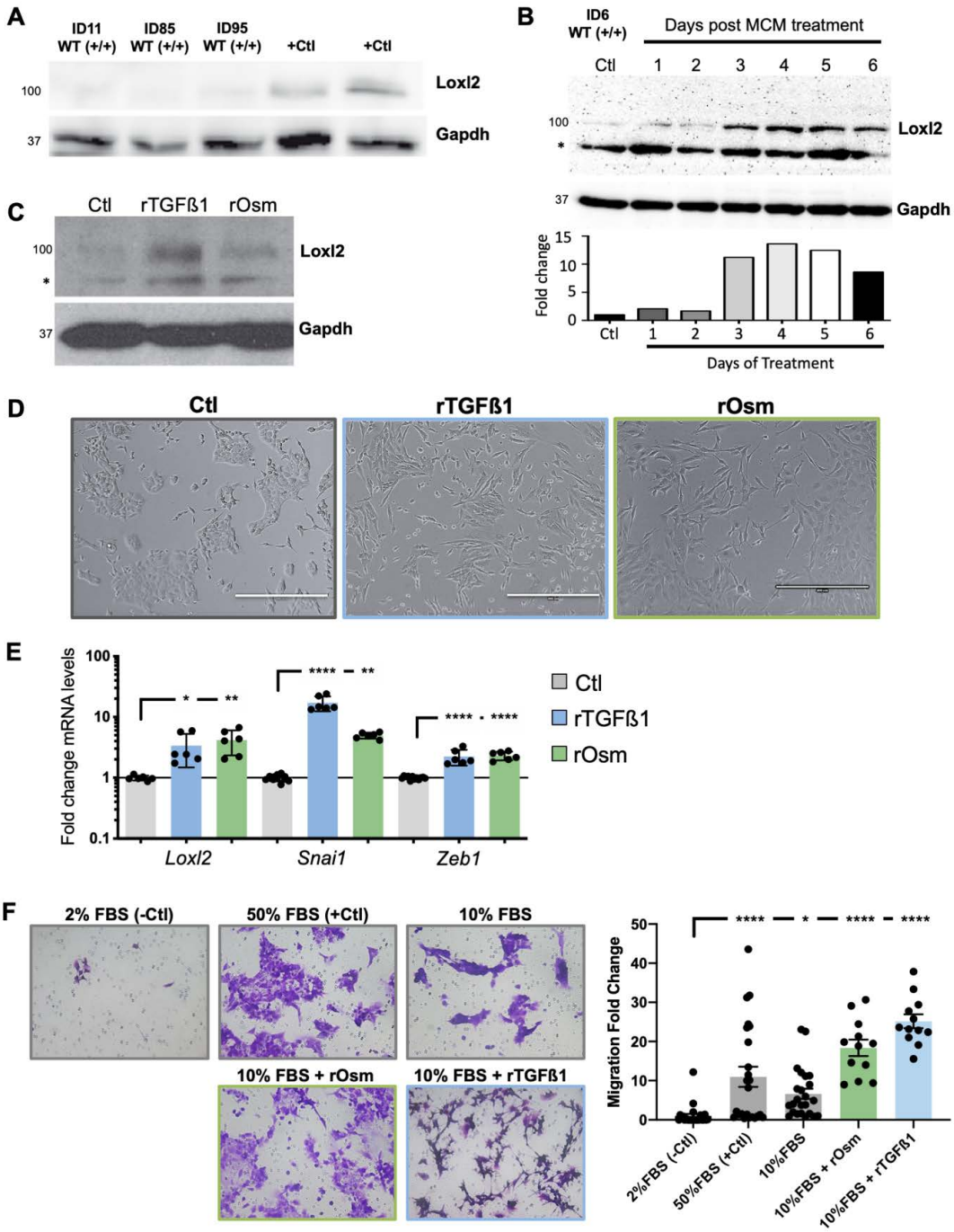


Figure S6: MCM, TGFß1 and Osm promote Loxl2 expression in murine PDAC-derived cells.
(A) Western blot analysis of Loxl2 expression in cell lysates from the indicated three murine KPC PDAC-derived wt (+/+) cell lines (ID11, ID85 and ID95). Gapdh, loading control. Positive control (+Ctl) = cell lysate from murine Loxl2 overexpressing 293T cells.
(B) Murine KPC PDAC-derived ID6 cells were untreated (Ctl) or cultured for 6 days with M2-polarized murine macrophage conditioned media (MCM). (top) Western blot analysis of murine Loxl2 expression over the course of 6 days. Gapdh served as a loading and densitometry normalization control (bottom). * = non-specific band.

(C-E) Murine KPC PDAC-derived cells were untreated (Ctl) or cultured for 72h with rTGFß1 (5ng/ml) or rOsm (100-200ng/ml). (C) Western blot analysis of murine Loxl2. Gapdh served as a loading control. * = non-specific band. (D) Representative images of Ctl and treated cells. Scale bar = 400µm. (E) Mean fold-change ± SD of relative mRNA levels for the indicated target genes in untreated (Ctl), rTGFß1- or rOsm-treated murine KPC PDAC-derived cells (ID11, ID85 and ID95). Values were normalized to ß-actin levels and Ctl-treated samples were set as 1.0. (*n*=2 technical replicate values per cell line, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, as determined by one-way ANOVA with Dunnett post-test, comparing rTGFß1 and rOsm to Ctl).

(F) Murine KPC PDAC-derived ID95 cells were untreated or cultured for 72h with rTGFß1 (5ng/ml) or rOsm (200ng/ml). Left, representative images of migrating cells through a 0.8 micron trans-well towards the indicated attractants (2% FBS, -Ctl; 50% FBS, +Ctl; or 10% FBS, experimental). Right, mean fold-change ± SEM of invading cells compared to 2% FBS (-Ctl) set as 1.0. (* $p < 0.05$, **** $p < 0.0001$, as determined by one-way ANOVA with Dunnett post-test, comparing all groups to 2% FBS (-Ctl)).

Figure S7 – Pancreas area analysis and classifications

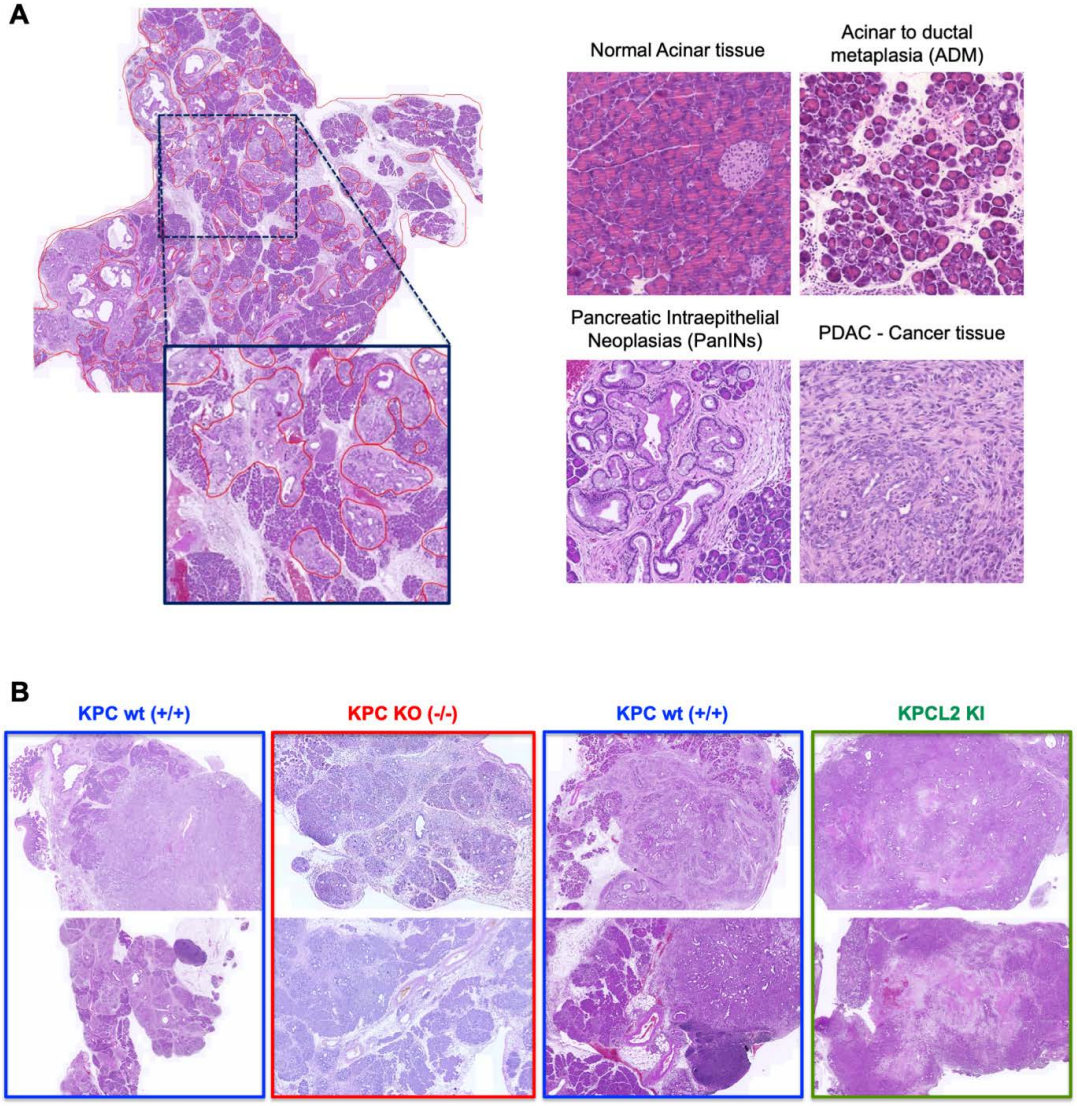


Figure S7: Pancreas area analysis and classifications.
(A) Representative hematoxylin and eosin (HE)-stained section of a KPC tumor. Total area and sub areas were selected and categorized as severely altered tissue (acinar-to-ductal metaplasia (ADM) and inflammation), pancreatic intraepithelial neoplasias (PanINs I–III), cancer tissue (PDAC) or normal acinar tissue.
(B) Representative H&E images of pancreata from indicated genotypes.

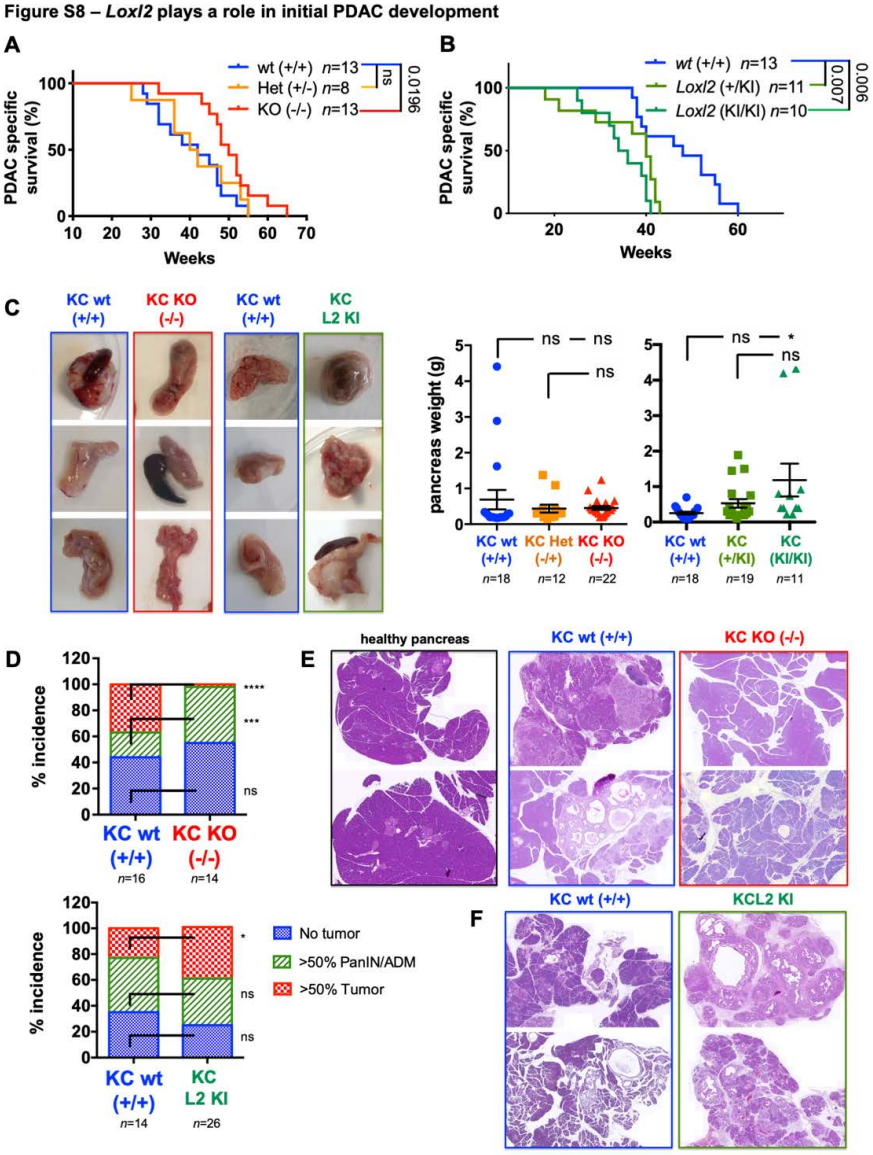


Figure S8: *Loxl2* plays a role in initial PDAC development.

(A) Survival of KC wild-type (+/+), HET KC (KC;*Loxl2*$^{+/-}$), and KO KC (KC;*Loxl2*$^{-/-}$) mice. All mice died of PDAC associated disease at the indicated times (p value is shown, ns = not significant, log-rank (Mantel–Cox) test). Calculated median survivals are: wild-type (+/+): 42; HET KC (KC;*Loxl2*$^{+/-}$): 41; and KO KC (KC;*Loxl2*$^{-/-}$): 50 weeks.

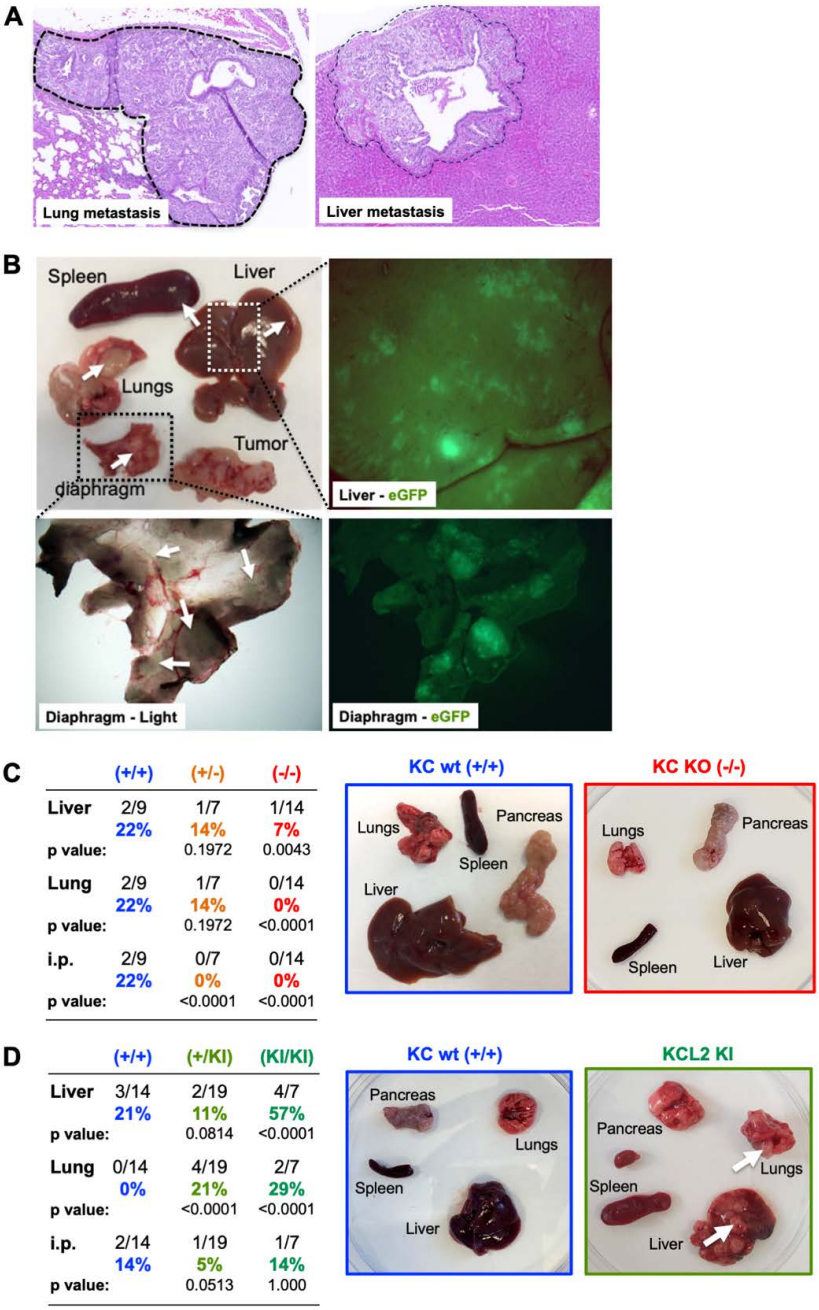
(B) Survival of KC wild-type (+/+), KC;R26L2$^{+/KI}$ and KC;R26L2$^{KI/KI}$ mice. All mice died of PDAC associated disease at the indicated times (p value is shown, log-rank (Mantel–Cox) test). Calculated median survivals are: wild-type (+/+): 48; KC;R26L2$^{+/KI}$: 40; and KC;R26L2$^{KI/KI}$: 35 weeks.

(C) Representative images (left) and mean pancreas weight ± SD (right) in wild-type (+/+), HET KC (KC;*Loxl2*$^{+/-}$), KO KC (KC;*Loxl2*$^{-/-}$), and wild-type (+/+), KC;R26L2$^{+/KI}$ and KC;R26L2$^{KI/KI}$ mice, determined at 37-40 weeks post birth. (* $p < 0.05$, ns = not significant, as determined by one-way ANOVA with Tukey post-test).

(D) Quantification of tissue area in mouse pancreata from wild-type (+/+) KC and HET KC (KC;*Loxl2*$^{+/-}$) mice (blue, n = 16) and KO KC (KC;*Loxl2*$^{-/-}$) mice (red, n = 14) (top) and in wild-type (+/+) KC mice (blue, n = 14) and KCL2^{KI} (KC;R26L2$^{+/KI}$ and KC;R26L2$^{KI/KI}$) mice (green, n = 26) (bottom), determined at 37-40 weeks post birth, categorized as normal acinar tissue (No tumor), cancer tissue (Tumor) or Pancreatic intraepithelial neoplasias (PanINs I–III) / acinar-to-ductal metaplasia (ADM). (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant, as determined by contingency analysis, two-sided Fisher's exact test).

(E-F) Representative H&E images of healthy pancreata or pancreata of the indicated genotypes.

Figure S9 – Overexpression of *Loxl2* enhances tumor metastasis



	(+/+)	(+/-)	(-/-)
Liver	2/9	1/7	1/14
	22%	14%	7%
p value:		0.1972	0.0043
Lung	2/9	1/7	0/14
	22%	14%	0%
p value:		0.1972	<0.0001
i.p.	2/9	0/7	0/14
	22%	0%	0%
p value:		<0.0001	<0.0001

	(+/+)	(+/KI)	(KI/KI)
Liver	3/14	2/19	4/7
	21%	11%	57%
p value:		0.0814	<0.0001
Lung	0/14	4/19	2/7
	0%	21%	29%
p value:		<0.0001	<0.0001
i.p.	2/14	1/19	1/7
	14%	5%	14%
p value:		0.0513	1.000

Figure S9: Overexpression of *Loxl2* enhances tumor metastasis

(A) Representative hematoxylin and eosin (HE)-stained section of PDAC metastatic lesions in the lung or liver of a 24-week-old wt KPC mouse.

(B) Representative images of PDAC tumors and metastases from a KPC;R26L2$^{KI/KI}$ mouse. Shown are photographs of explanted organs (white arrows indicate metastases), and light and fluorescent images of liver and diaphragm taken with a fluorescent dissecting microscope.

(C-D) Percent incidence of liver, lung and i.p. metastasis in (C) wild-type (+/+) KC, HET (KC;*Loxl2*$^{+/-}$) and KO (KC;*Loxl2*$^{-/-}$) mice or (D) wild-type (+/+) KC, KC;R26L2$^{+/KI}$ and KC;R26L2$^{KI/KI}$ mice determined at 37-40 weeks post birth. (p values are indicated, as determined by contingency analysis, two-sided Fisher's exact test). (right) Images of explanted organs from mice of the indicated genotypes (white arrows indicate metastases).

Figure S10 – Expression of EMT-associated proteins in tumor samples

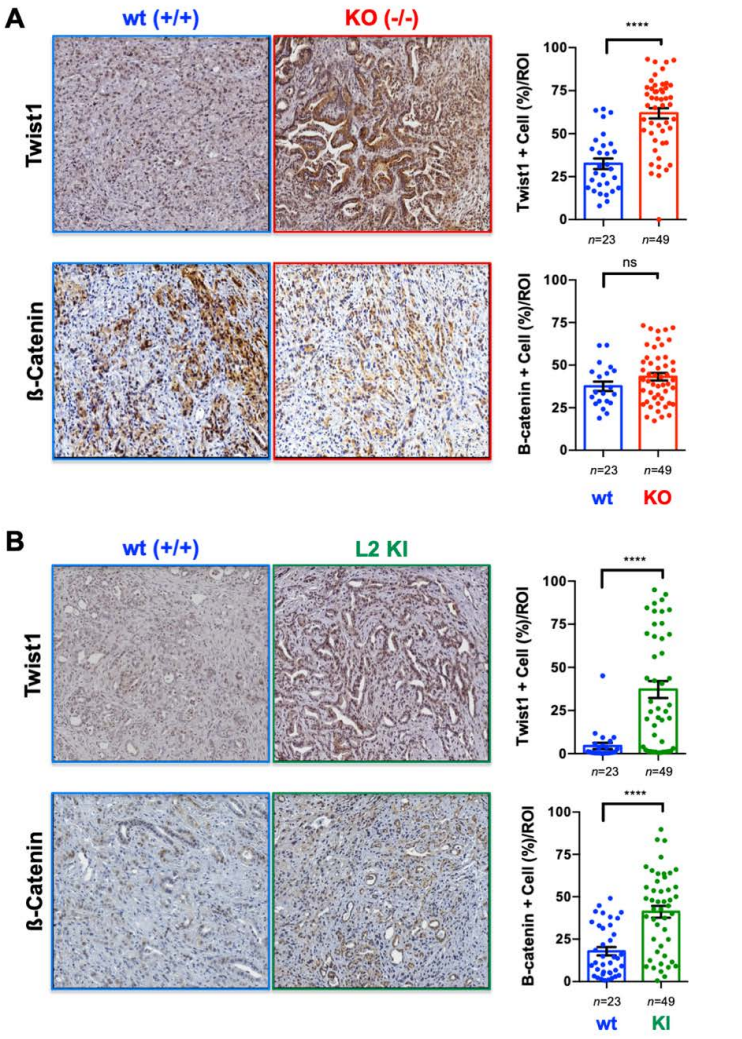


Figure S10: Expression of EMT-associated proteins in tumor samples

(A) Left: Representative immunohistochemical stainings for Twist1 and ß-catenin expression in tumor sections from wild-type (+/+) KPC and KO KPC (KPC;*Loxl2*$^{-/-}$) mice. Scale bar = 250µm. Right: Quantification of percent positive (+) cells/ROI. (**** $p < 0.0001$, ns = not significant, as determined by unpaired Student's t test).

(B) Left: Representative immunohistochemical stainings for Twist1 and ß-catenin expression in tumor sections from wild-type (+/+) KPC and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) mice. Scale bar = 250µm. Right: Quantification of percent positive (+) cells/ROI. (**** $p < 0.0001$, as determined by unpaired Student's t test).

Figure S11 – Expression of E-cadherin in tumor samples

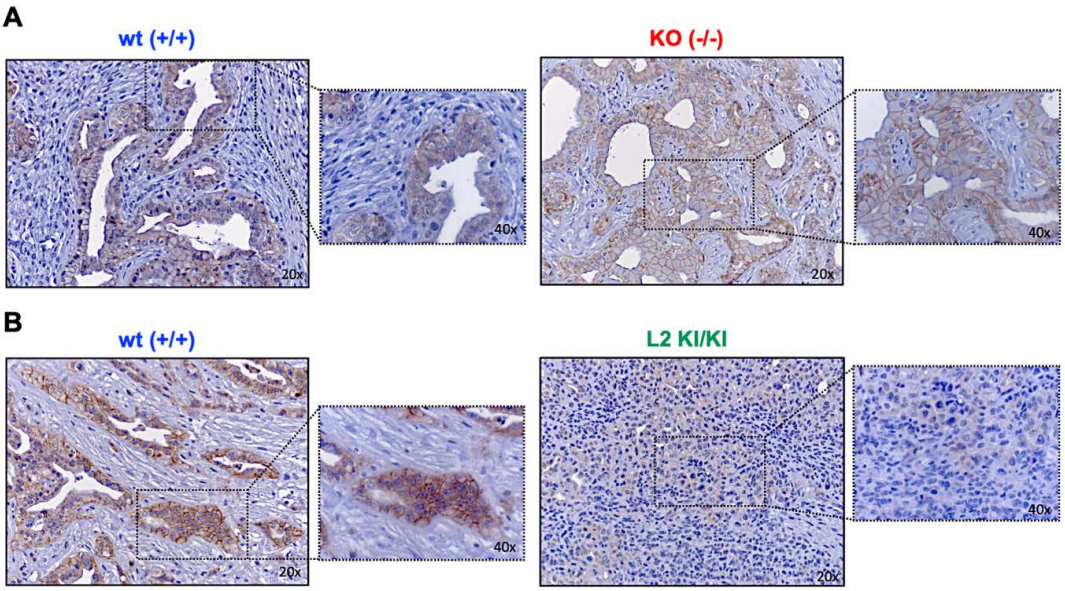


Figure S11: Expression of E-cadherin in tumor samples.
(A-B) Representative immunohistochemical staining of E-cadherin in tumor sections from (A) wild-type (+/+) KPC and KO KPC (KPC;*Loxl2*$^{-/-}$) mice or (B) wild-type (+/+) KPC and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) mice. 20X magnification. Insets indicate area that is shown on the right at 40X.

Figure S12 – Loxl2 levels do not affect TGFß responsiveness

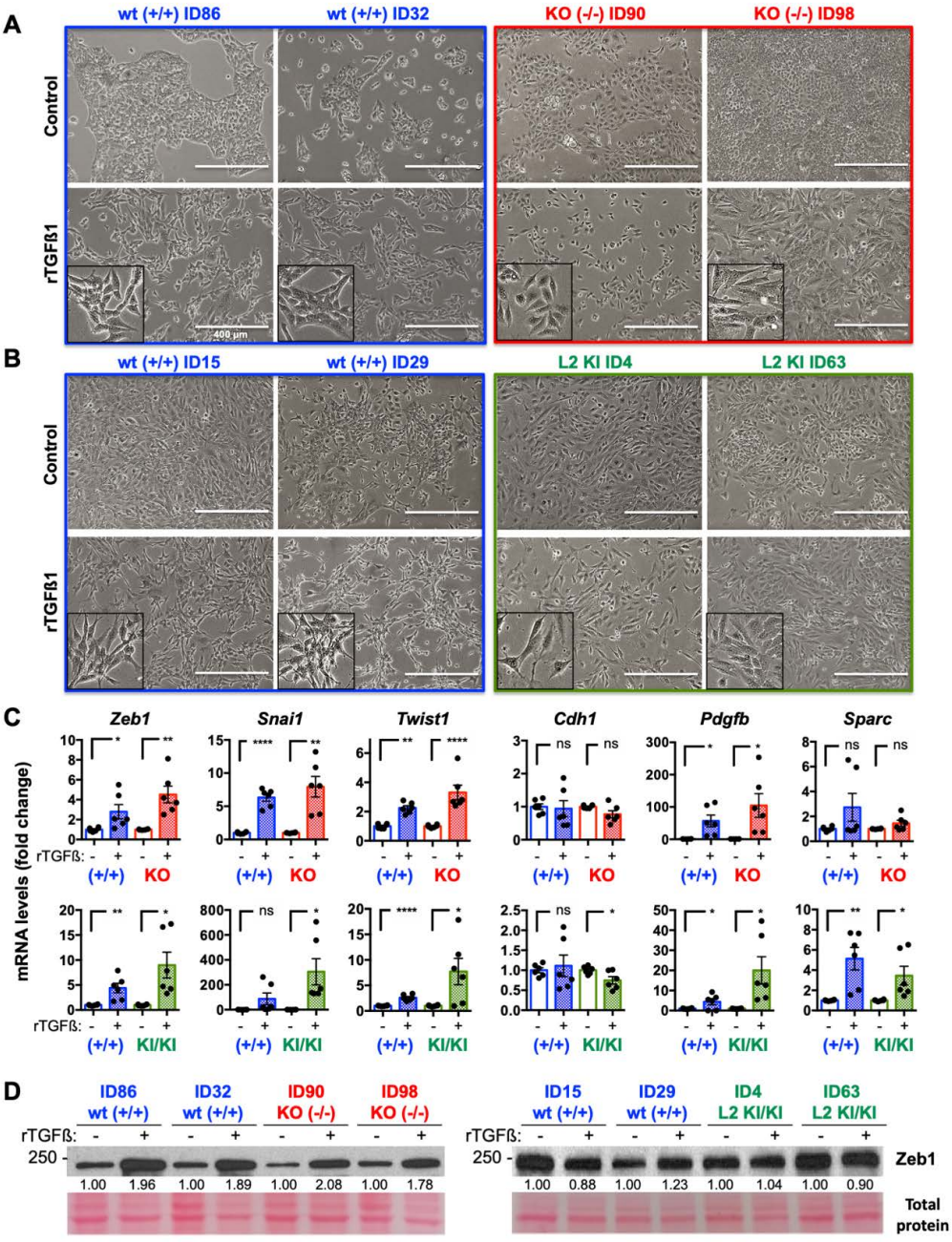


Fig S12: Loxl2 levels do not affect TGFß responsiveness

(A-B) Representative light micrographs of tumor cell lines derived from (A) wild-type (+/+) KPC tumors (ID86 and ID32, blue) and KO KPC (KPC;*Loxl2*$^{-/-}$) tumors (ID90 and ID98, red) or (B) wild-type (+/+) KPC tumors (ID15 and ID29, blue) and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) tumors (ID4 and ID63, green). Scale bar = 400µm. Inset = 4X amplification.
(C) RT-qPCR analysis of mean fold change ± SEM of indicated EMT- or metastasis-related genes in tumor cell lines derived from the indicated genotypes following treatment with rTGFß1 (5ng/ml) daily for 3 days (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns = not significant, as determined by unpaired Student's t test).
(D) WB analysis of Zeb1 in samples from (A-C). Ponceau S staining served as a normalization control. Zeb1 densitometric values, normalized to total protein, are indicated.

Figure S13 – Loxl2 levels do not affect Osm responsiveness

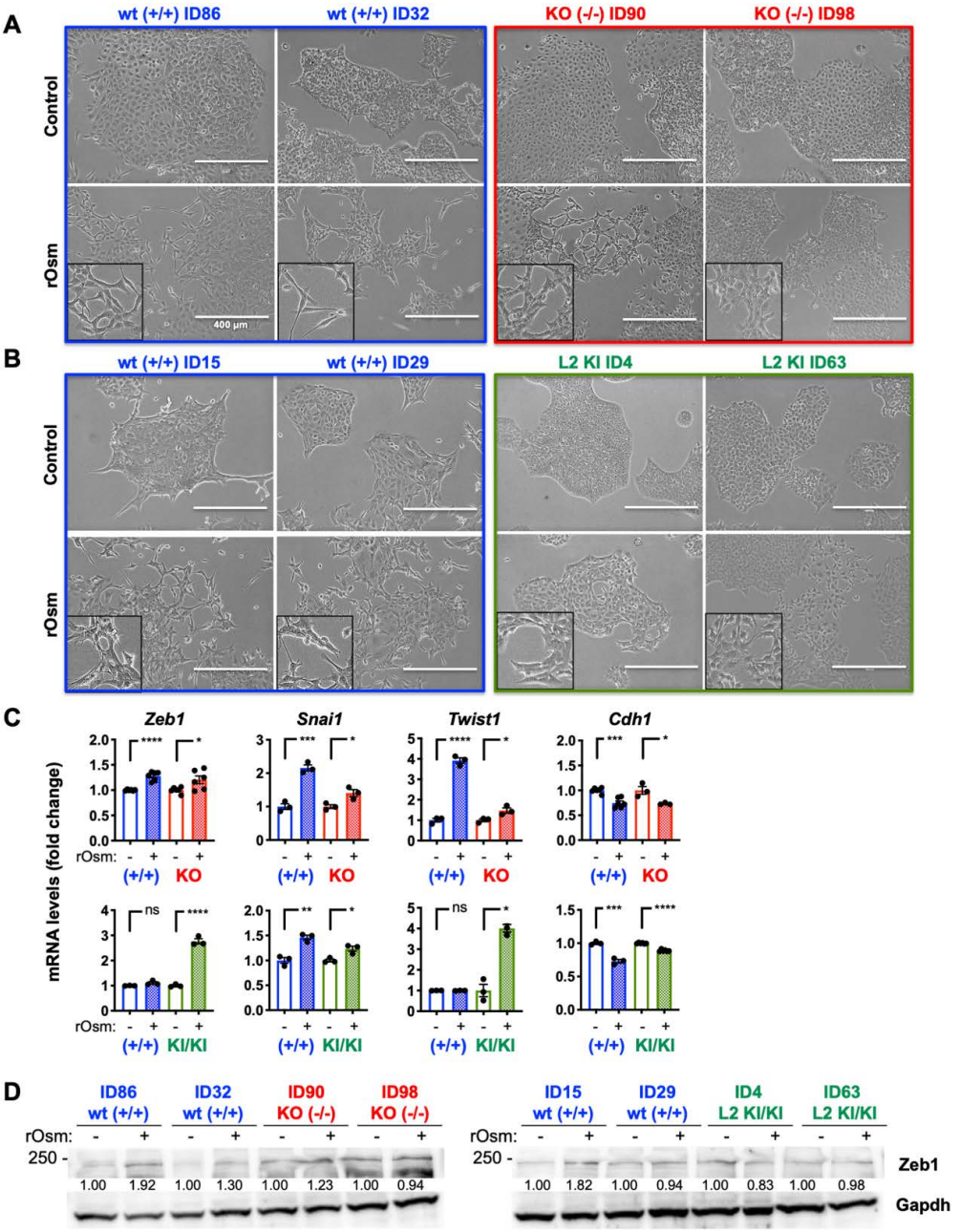


Fig S13: Loxl2 levels do not affect Osm responsiveness

(A-B) Representative light micrographs of tumor cell lines derived from (A) wild-type (+/+) KPC tumors (ID86 and ID32, blue) and KO KPC (KPC;*Loxl2*$^{-/-}$) tumors (ID90 and ID98, red) or (B) wild-type (+/+) KPC tumors (ID15 and ID29, blue) and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) tumors (ID4 and ID63, green). Scale bar = 400µm. Inset = 4X amplification.
(C) RT-qPCR analysis of mean fold change ± SEM of indicated EMT-related genes in tumor cell lines derived from the indicated genotypes following treatment with rOsm (200ng/ml) daily for 3 days (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant, as determined by unpaired Student's t test).
(D) WB analysis of Zeb1 in samples from (A-C). Gapdh served as a normalization control. Zeb1 densitometric values, normalized to Gapdh values, are indicated.

Figure S14 – Loxl2 and tumor formation

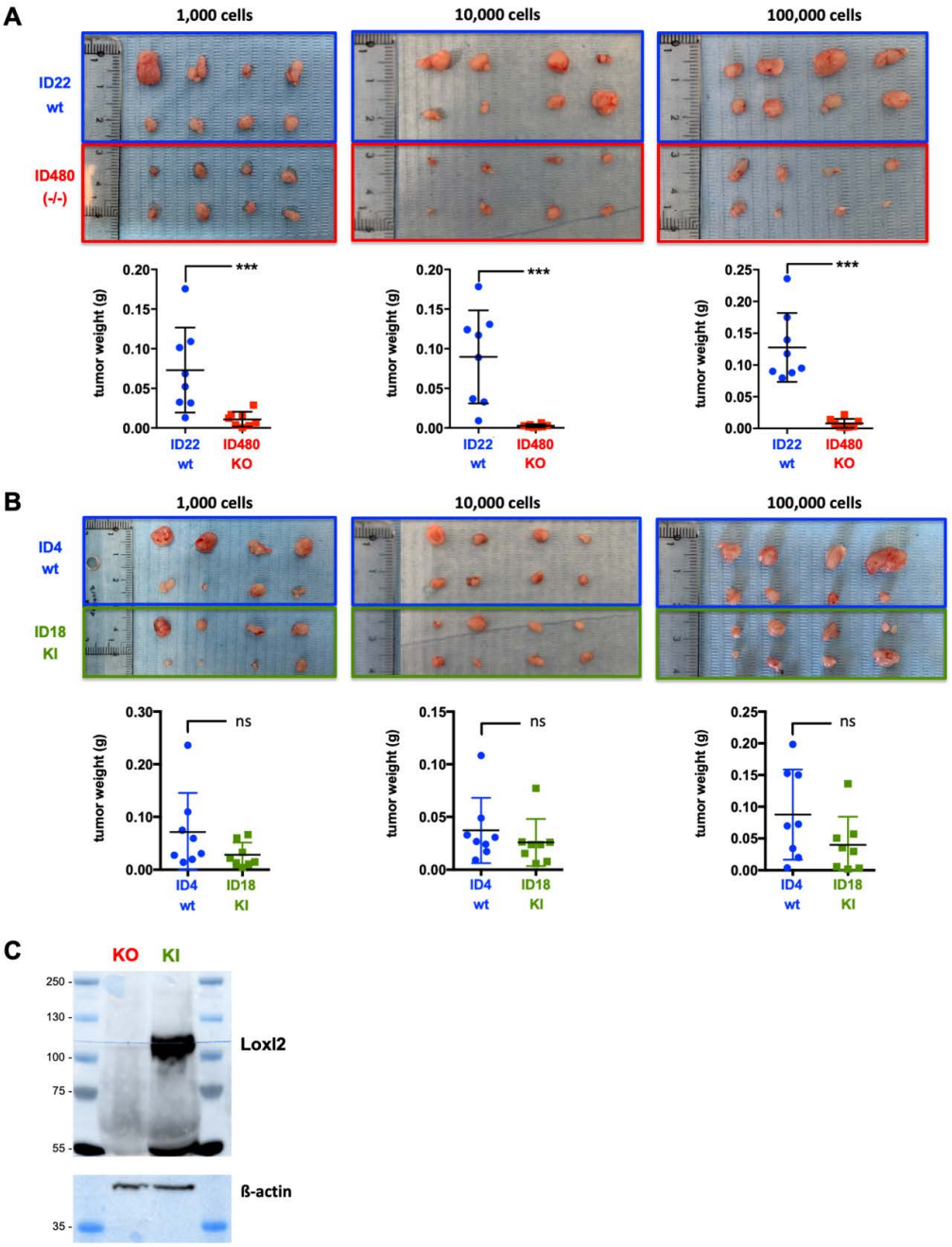


Figure S14: Loxl2 and tumor formation.

(A) Images of tumors formed (top) and mean tumor weight ± SD (bottom) for wild-type (+/+) KPC cells (ID22, blue) and KO KPC (KPC;*Loxl2*$^{-/-}$) cells (ID80, red), determined at 14 weeks post injection in immunocompromised nude mice at the indicated dilutions.

(B) Images of tumors formed (top) and mean tumor weight ± SD (bottom) for wild-type (+/+) KPC cells (ID4, blue) and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) cells (ID18, green), determined at 14 weeks post injection in immunocompromised nude mice at the indicated dilutions.

(C) Western blot analysis of Loxl2 in concentrated cell supernatants from KPCL2^{KO} and KPCL2^{KI} cells cultured for 48h in the absence of serum. ß-actin served as a loading control. The membrane was cut.

Figure S15 – Expression of mechanosignaling-associated proteins in tumor samples

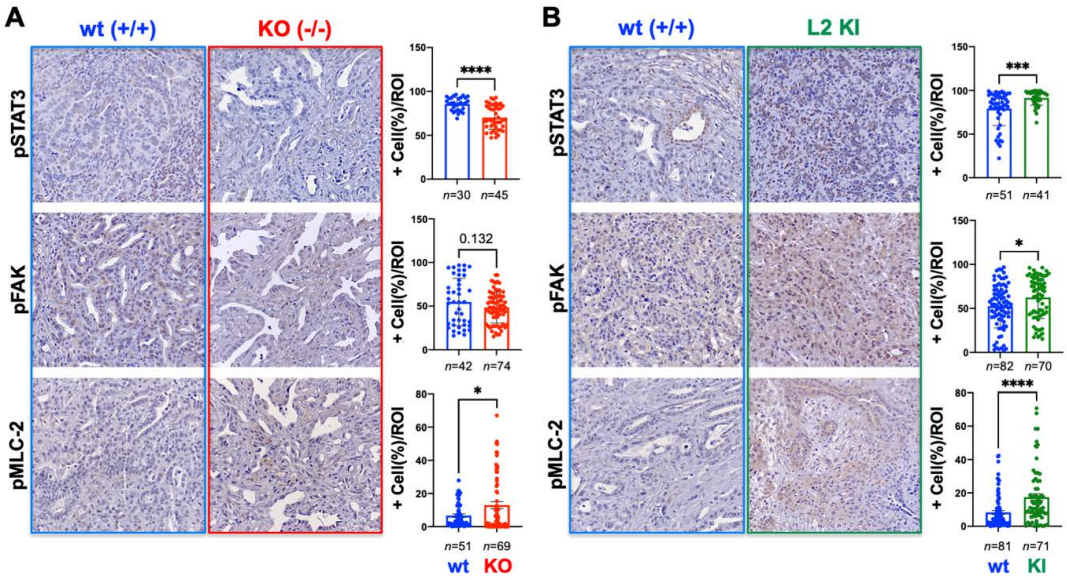


Figure S15: Expression of mechanosignaling-associated proteins in tumor samples.
(A) Left: Representative immunohistochemical stainings of pSTAT3, pFAK, pMLC-2 expression in tumor sections from wild-type (+/+) KPC and KO KPC (KPC;*Loxl2*$^{-/-}$) mice. Right: Quantification of percent positive (+) cells/ROI. (* $p < 0.05$, **** $p < 0.0001$, as determined by unpaired Student's t test).
(B) Left: Representative immunohistochemical stainings of pSTAT3, pFAK, pMLC-2 expression in tumor sections from wild-type (+/+) KPC and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) mice. Right: Quantification of percent positive (+) cells/ROI. (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, as determined by unpaired Student's t test).

Figure S16 – Immune cell profile in tumors with different levels of Loxl2

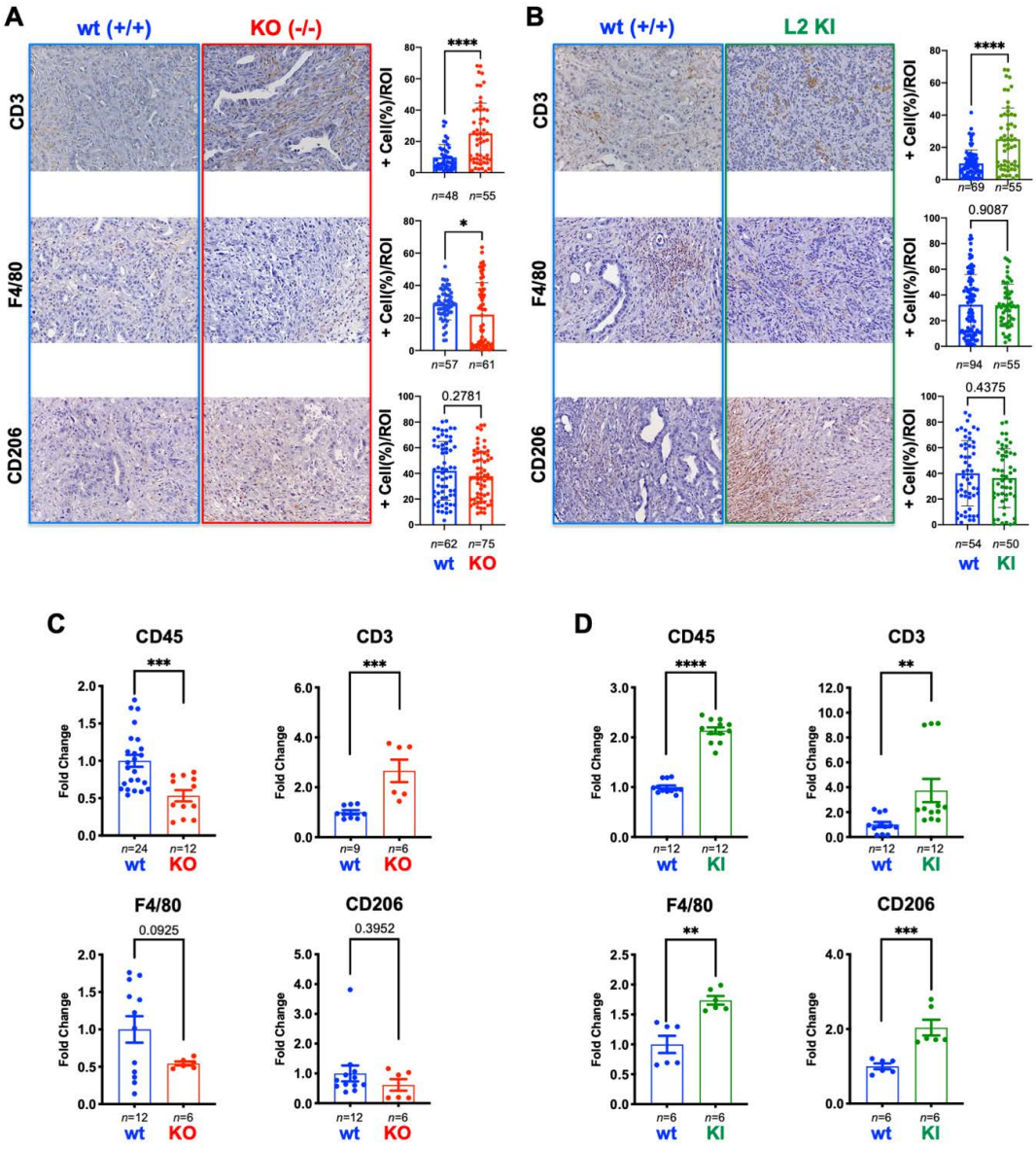


Figure S16: Immune cell profile in tumors with different levels of Loxl2.

(A) Left: Representative immunohistochemical stainings of CD3, F4/80 and CD206 expression in tumor sections from wild-type (+/+) KPC and KO KPC (KPC;*Loxl2*$^{-/-}$) mice. Scale bar = 250µm. Right: Quantification of percent positive (+) cells/ROI. (* $p < 0.05$, **** $p < 0.0001$, as determined by unpaired Student's t test).

(B) Left: Representative immunohistochemical stainings of CD3, F4/80 and CD206 expression in tumor sections from wild-type (+/+) KPC and KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) mice. Scale bar = 250µm. Right: Quantification of percent positive (+) cells/ROI. (**** $p < 0.0001$, as determined by unpaired Student's t test).

(C-D) Mean fold-change ± SD of the percentage of CD45+, CD3+, F4/80+ or CD206+ cells in freshly digested PDAC tumors from 18-week old (C) wild-type (+/+) KPC or KO KPC (KPC;*Loxl2*$^{-/-}$) mice, or (D) wild-type (+/+) KPC or KPCL2^{KI} (KPC;R26L2$^{KI/KI}$) mice. (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, as determined by unpaired Student's t test).

SUPPLEMENTARY VIDEOS

Supplementary Video 1: Movement and cell scattering of Panc354 cells in cultured with control medium

Supplementary Video 2: Movement and cell scattering of Panc354 cells in cultured with macrophage-conditioned medium