

1 **Collagen-VI expression is negatively mechanosensitive in pancreatic cancer  
2 cells and supports the metastatic niche**

3 Vasileios Papalazarou<sup>1,2,3\*</sup>, James Drew<sup>1\*</sup>, Amelie Juin<sup>1</sup>, Heather J. Spence<sup>1</sup>, Colin Nixon<sup>1</sup>, Manuel  
4 Salmeron-Sanchez<sup>2</sup>, Laura M. Machesky<sup>1,3</sup>

5 \*Authors contributed equally

6 1. CRUK Beatson Institute, Switchback Road, Bearsden, Glasgow

7 2. University of Glasgow Centre for the Cellular Microenvironment, Glasgow

8 3. Institute of Cancer Sciences, University of Glasgow

9 Email for correspondence: [laura.machesky@glasgow.ac.uk](mailto:laura.machesky@glasgow.ac.uk)

10

11 **Summary Statement:** Collagen-VI is expressed by pancreatic tumors and metastases in a  
12 mechanosensitive way to promote niche colonisation.

13

14 **Abstract**

15 Pancreatic cancer is a deadly disease with high rates of metastasis, though how tumor cells establish  
16 metastatic lesions is not fully understood. A key feature of primary pancreatic tumors is extensive  
17 fibrosis due to deposition of extracellular matrix. While pancreatic cancer cells are programmed by  
18 stimuli derived from a stiff ECM, metastasis requires loss of attachment as well as adaptation to a softer  
19 microenvironment upon reaching distant sites. Growing evidence suggests that stiff ECM influences  
20 pancreatic cancer cell behaviour. Here we argue that this influence is reversible and that pancreatic  
21 cancer cells can be reprogrammed upon sensing of soft substrates. Through use of engineered  
22 polyacrylamide hydrogels with tuneable mechanical properties, we show that Collagen-VI is specifically  
23 upregulated on soft substrates, due to a lack of integrin engagement and low YAP1 activity. Collagen-  
24 VI supports migration *in vitro* and metastasis formation *in vivo*. Metastatic nodules formed by pancreatic  
25 cancer cells lacking *Col6a1* expression, were characterised by stromal cell-derived collagen-VI  
26 deposition, suggesting that collagen-VI, either cancer or stroma derived, is an essential component of  
27 the metastatic niche.

28

29 **Introduction**

30 The dissemination of malignant cells from primary tumors to distant sites and the formation of metastatic  
31 nodules is a key step in cancer progression and aggressiveness. An essential component of the  
32 metastatic niche is the extracellular matrix (ECM) – the collection of extracellular proteins that provide  
33 the three-dimensional (3D) scaffold within which cells organise to form complex structures. It is  
34 emerging that cancer cell-ECM interactions have important roles in the establishment of metastatic  
35 tumors at all stages of the metastatic cascade (Drew and Machesky, 2021). These interactions depend  
36 on not only the biological components of the ECM but also its physical and mechanical properties.

37

38 The ability of cancer cells to respond to the physical properties of their surroundings is increasingly  
39 recognised as crucial to disease progression (Broders-Bondon et al., 2018). In solid tumors such as  
40 pancreatic ductal adenocarcinoma (PDAC), deposition of extracellular matrix (ECM) leads to extensive

41 matricellular fibrosis that is linked to cancer aggressiveness (Mahadevan and Von Hoff, 2007; Perez et  
42 al., 2021) and contributes to its dismal 5-year survival rates (Siegel et al., 2021). Increased tissue  
43 stiffness has been shown to drive extravasation from the primary tumor through initiating epithelial-  
44 mesenchymal transition (EMT) in PDAC cells and stimulating invasion (Rice et al., 2017). However,  
45 cells escaping the primary tumor must survive and grow in low-adhesion and soft tissue environments  
46 such as the liver and lung to form metastatic lesions (Yachida and Iacobuzio-Donahue, 2009). Given  
47 that metastatic dissemination is the leading cause of death in pancreatic cancer (Balaban et al., 2017),  
48 understanding the adaptations that PDAC cells undergo during these transitions is of significant  
49 importance. Specifically, whether sensing of stiff ECM irreversibly modulates cancer cell behaviour or  
50 whether cancer cells can actively respond to low stiffness stimuli remains unclear.

51  
52 Increased tissue stiffness and tension impacts cancer cell identity and behaviour through complex  
53 signalling pathways collectively referred to as “mechanosensation” (Papalazarou et al., 2018). The  
54 fundamental process of mechanosensation involves integrin receptor engagement of extracellular  
55 substrates, actin cytoskeleton remodelling and activation of key transcriptional regulators such as Yes-  
56 associated protein-1 (YAP1) (Panciera et al., 2017). PDAC cells use these mechanosensitive pathways  
57 to direct migration (Laklai et al., 2016), control metabolism (Papalazarou et al., 2020) and  
58 chemoresistance (Rice et al., 2017). Although some fundamental components of these pathways have  
59 been elucidated, much is still unknown about how mechanosensitive pathways are utilised by  
60 pancreatic cancer cells at different stages of disease progression.

61  
62 In primary PDAC, the majority of ECM is deposited by stromal cells (in particular cancer-associated  
63 fibroblasts (CAFs)) that secrete large amount of fibrillar ECM components such as fibronectin and  
64 collagen-I, increasing tissue stiffness (Elyada et al., 2019). Unfortunately, approaches aimed at  
65 targeting ECM deposition in mouse models of PDAC (Ozdemir et al., 2014) and clinical trials (Doherty  
66 et al., 2018) have proven unsuccessful, likely due to the loss of ECM as a physical barrier to restrain  
67 the tumor (Bhattacharjee et al., 2021; Jiang et al., 2020). Therefore, a more nuanced understanding of  
68 the relationship between ECM deposition, tissue stiffness, and PDAC development is required.

69  
70 Recent proteomic studies have revealed that alongside CAFs, cancer cells themselves produce a wide  
71 range of ECM proteins that contribute to the TME (Tian et al., 2019). Indeed, expression of cancer-cell  
72 derived ECM proteins correlate with poor survival in PDAC (Tian et al., 2019) suggesting they exert  
73 important roles in tumor progression. These functions may have relevance in metastatic dissemination,  
74 where cancer cells must quickly form a microenvironment to support their survival and growth (Drew  
75 and Machesky, 2021; Jiang et al., 2020). Indeed, a recent study showed that various PDAC-derived  
76 ECM components had roles in supporting metastatic dissemination (Tian et al., 2020); though our  
77 understanding of the functional roles of PDAC-derived ECM, and the mechanisms controlling its  
78 expression, remains limited. Given the intimate relationship between ECM and tissue stiffness, it is also  
79 unclear whether PDAC cells regulate their own ECM production in response to changes in their physical  
80 environment.

81  
82 In this study, we define a link between substrate stiffness, PDAC ECM expression, and metastatic  
83 potential. By combining the use of mechanically tuneable hydrogels with RNA sequencing  
84 transcriptomic analyses, we observed that in environments of reduced stiffness pancreatic cancer cells  
85 upregulate the expression of genes involved in ECM production. This was prominently manifested by  
86 upregulation of three genes involved in Collagen-type VI synthesis: *Col6a1*, *Col6a2* and *Col6a3*.  
87 Focusing on Collagen-VI, we show that Collagen-VI upregulation is a response to a lack of integrin-  
88 based mechanosensation and diminished YAP nuclear localisation on soft substrates. Further, we find  
89 that PDAC cell-derived Collagen-VI supports invasive behaviour *in vitro* and metastatic potential of  
90 pancreatic cancer cells *in vivo*.

91  
92 **Materials and methods**  
93 **Cell culture**  
94 All cell lines used in this study were cultured at 37°C under 5% CO<sub>2</sub> in a humidified incubator and were  
95 tested regularly for mycoplasma contamination. Primary murine KPC PDAC and human PANC-1 cells  
96 were cultured in high-glucose DMEM supplemented with FCS (10%), glutamine (2mM), sodium  
97 pyruvate (0.11 g L<sup>-1</sup>), penicillin (10,000 units ml<sup>-1</sup>) and streptomycin (10,000 units ml<sup>-1</sup>). PDAC-A and  
98 PDAC-B cell lines were a gift from Jennifer Morton and Saadia Karim, and were isolated from the tumors  
99 of *Pdx1-cre;LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R172H/+</sup>* (KPC) mice either with a mixed or pure C57BL/J  
100 background.

101  
102 **Reagents and Oligos**  
103 Human Fibronectin Protein (1918-FN-02M, R&D Systems), Fibronectin bovine plasma (F1141, Sigma-  
104 Aldrich), Concanavalin A from *Canavalia ensiformis* (L7647, Sigma-Aldrich), Poly-L-Lysine (P4707,  
105 Sigma-Aldrich), Aphidicolin from *Nigrospora spaherica* (A4487, Sigma-Aldrich), Corning Matrigel  
106 Basement Membrane Matrix (354234, Corning), Rat tail Collagen I (354249, Corning), Sigmacote (SL2,  
107 Sigma-Aldrich), sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH; 22589,  
108 Thermo Fisher Scientific), 3-(Acryloyloxy)propyltrimethoxysilane (L16400, Alfa Aesar), Acrylamide 40%  
109 Solution (A4058, Sigma-Aldrich), N,N'-methylene-bis-acrylamide 2% solution (M1533, Sigma-Aldrich),  
110 N,N,N',N'-Tetramethylethylenediamine (TEMED; T9281, Sigma-Aldrich), Ammonium persulfate  
111 (A3678, Sigma-Aldrich), Ammonium hydroxide (221228, Sigma-Aldrich), Hydrogen peroxide (30%w/w;  
112 31642, Sigma-Aldrich), Puromycin dihydrochloride (A1113803, Thermo Fisher Scientific),  
113 Lipofectamine RNAiMAX (13778150, Thermo Fisher Scientific), Lipofectamine 2000 (11668019,  
114 Thermo Fisher Scientific), Lullaby (FLL73000, OZ Biosciences), Amaxa Cell Line Nucleofector Kit V  
115 (VCA-1003, Lonza), Precision Red Advanced Protein Assay (ADV02-A; Cytoskeleton), calcein AM  
116 (C1430, Thermo Fisher Scientific).

117 For CRISPR/Cas9-mediated genome editing, the following sequences were cloned into pSpCas9(BB)-  
118 2A-Puro (PX459) V2.0 (gift from Feng Zhang, Addgene #62988) targeting mouse *Col6a1*: 5'- GTA CTT  
119 GAC CGC ATC CAC GC -3'(*Col6a1*<sup>CRISPR#01</sup>), 5'- TTG AGC TCA TCG CGG CCA C -3'  
120 (*Col6a1*<sup>CRISPR#02</sup>), 5'- CTT GAT CGT GGT GAC CGA C -3' (*Col6a1*<sup>CRISPR#03</sup>), 5'- ACT TGA TCG TGG

121 TGA CCG A -3' (Col6a1<sup>CRISPR#04</sup>) and mouse *Yap1*: 5'- ACT TGA TCG TGG TGA CCG A -3'  
122 (YAP<sup>CRISPR#01</sup>). For siRNA mediated silencing the following sequences were used targeting mouse YAP,  
123 5'- ACC AGG TCG TGC ACG TCC GC -3' (YAP<sup>siRNA#01</sup>), 5'-ATGGAGAAGTTACTACATAA-3'  
124 (YAP<sup>siRNA#02</sup>) and mouse ILK, 5'- CCG CAG TGT AAT GAT CGA TGA -3' (ILK<sup>siRNA#01</sup>) and 5'- CTC TAC  
125 AAT GTT CTA CAT GAA -3' (ILK<sup>siRNA#02</sup>).

126 The following DNA constructs were used, pEGFP-C1 (host lab), pEGFPC1/GgVcl1-258 (VD1, a gift  
127 from Susan Craig, Addgene #46270 (Cohen et al., 2006)), EGFP-talin1 head (a gift from Anna  
128 Huttenlocher, Addgene plasmid #32856, (Simonson et al., 2006)). The L325R mutation was introduced  
129 to the EGFP-talin1 head construct using the Q5-site directed mutagenesis kit (NEB, E0554).

130

### 131 **Polyacrylamide hydrogel preparation**

132 Polyacrylamide hydrogels were prepared at 0.7 kPa, 7 kPa and 38 kPa stiffness values as previously  
133 described (Papalazarou et al., 2020). Polyacrylamide hydrogels were treated with 0.2 mg ml<sup>-1</sup> sulfo-  
134 SANPAH solution in MilliQ water (Thermo Fisher Scientific, 22589) followed by UV irradiation (365 nm)  
135 for 10 min. Hydrogels were extensively washed with 50 mM HEPES buffer (pH 8.5), incubated overnight  
136 with fibronectin (10 µg ml<sup>-1</sup>) and washed extensively in PBS before use.

137

### 138 **ECM coatings**

139 Glass coverslips were washed with Ethanol, oven-dried and then coated with either fibronectin (10µg  
140 mL<sup>-1</sup>) for 60min at room temperature, Concanavalin A (ConA; 10 µg mL<sup>-1</sup>) or Poly-L-Lysine (PLL; 0.5  
141 mg mL<sup>-1</sup>) for 16 hours at 4°C. Coated coverslips were washed in PBS and cells (2x10<sup>4</sup> cells per mL)  
142 were plated and cultured for 16 hours in DMEM-10% FBS.

143

### 144 **RNA extraction, RNA sequencing and qRT-PCR**

145 Total RNA extraction and purification from cells was performed using the RNeasy Mini Kit (Qiagen)  
146 combined with RNaseFree DNase (QIAGEN) treatment, according to the manufacturer's instructions.  
147 Measurements of RNA concentration and purity were routinely performed using NanoDrop2000C  
148 (Thermo Fisher Scientific) before downstream processing. For RNA sequencing, RNA quality was  
149 tested using the Agilent Technologies 2200 TapeStation instrument according to the manufacturer's  
150 instructions. Briefly, 5 µL of RNA sample buffer were mixed with 1 µL of RNA ladder and added to the  
151 first tube of a RNase-free mini-tube strip. Then, 5 µL of RNA sample buffer were mixed with 1 µL of  
152 RNA sample and loaded to the strip. Samples were vortexed and centrifuged for 1 min at 2000 rpm.  
153 Samples were heated to 72°C for 3 min and place on ice for 2 min. Samples were centrifuged for 1 min  
154 at 2000 rpm and loaded into the Agilent 2200 TapeStation instrument.

155 Libraries were prepared using the Library Prep kit (Illumina TruSeq RNA Sample prep kit v2) and were  
156 run on an Illumina NextSeq500 platform using the High Output v2 75cycles (2x36cycle Paired End,  
157 single index) sequencing Kit. Quality checks on the raw RNASeq data files were done using FastQC  
158 (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>) and FASTQ screen  
159 ([http://www.bioinformatics.babraham.ac.uk/projects/fastq\\_screen/](http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/)) tools. RNASeq reads were aligned  
160 to the GRCm38 version of the mouse genome using tophat2 version 2.0.13 with Bowtie version 2.2.4.0.

161 Expression levels were determined and statistically analysed by a combination of HTSeq version 0.7.2  
162 (<http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html>), the R 3.3.3 environment, utilizing  
163 packages from the Bioconductor data analysis suite and differential gene expression analysis based on  
164 a generalized linear model using the DESeq2. Significantly changed genes ( $p_{adj}<0.05$ ) were submitted  
165 to DAVID for gene ontology (GO) analysis. KEGG pathway analysis was performed for genes  
166 demonstrating an increase (Up) or decrease (Down) in RNA expression between conditions. Significant  
167 KEGG GO Terms were identified ( $p_{adj}<0.05$ ). Hierarchical clustering of  $\log_2$  Fold Changes in gene  
168 expression was performed on the basis of Euclidean Distance using complete linkage and visualised  
169 using the Rstudio, v.1.1.453 environment. We also performed DESeq analysis as well as Category  
170 netplot (CNET) analysis using the Bioconductor data analysis suite and the Rstudio, v.1.1.453  
171 environment. The matrisome geneset source was taken from: [https://www.gsea-msigdb.org/gsea/msigdb/cards/NABA\\_MATRISOME](https://www.gsea-msigdb.org/gsea/msigdb/cards/NABA_MATRISOME) (accessed 02.2022)  
172  
173 cDNA synthesis with either DyNAmo cDNA synthesis kit (F-470L, Thermo Fisher Scientific) or Maxima  
174 First Strand cDNA synthesis kit (K1641, Thermo Fisher Scientific). Then qRT-PCR was performed using  
175 the DyNAmo HS SYBR Green qPCR kit (F410L, Thermo Fisher Scientific). PCR was performed on a  
176 C1000 Thermal Cycler (CFX96 Real time system, BioRad) as follows: 3min at 95°C, 40-cycles of 20s  
177 95°C, 30s 57°C, 30s 72°C and final 5min 72°C. Relative mRNA quantification was performed using the  
178 2<sup>Δ</sup>-ddCT method for multiple genes. Cdk2-Fw: TGAAATGCACCTAGTGTGTACC; Cdk2-Rv:  
179 TCCTTGTGATGCAGCCACTT; Col6a1-Fw: CGTGGAGAGAAGGGTCCAG; Col6a1-Rv:  
180 GTCTCTCCCTTCATGCCGTC.  
181

## 182 **Western blotting**

183 Proteins were isolated in radioimmunoprecipitation (RIPA) lysis buffer (150mM NaCl, 10mM Tris-HCl  
184 pH7.5, 1mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1% SDS, 1X protease and  
185 phosphatase inhibitors (Roche)). Protein concentration was determined using Precision Red Advanced  
186 Protein Assay (Cytoskeleton, Inc.). 20ug protein were loaded onto 8-12% SDS-PAGE gels and  
187 transferred onto nitrocellulose membranes. Membranes were blocked (3%BSA / TBST) and incubated  
188 for 16h in 4°C with one of the following antibodies: anti-Collagen VI (1:1000; ab182744, Abcam), anti-  
189 YAP (1:1000; 14074, Cell Signaling Technology), anti-ILK (1:1000; 3856S, Cell Signaling Technology),  
190 anti-ERK1/2 (1:1000; 9102, Cell Signaling Technology), anti-GAPDH (1:1000; MAB374, Millipore) and  
191 anti-αTubulin (1:3000; T6199, Sigma). Protein detection was achieved using Alexa-Fluor conjugated  
192 secondary antibodies and signal was imaged using the LI-COR Odyssey CLx (LI-COR Biosciences)  
193 system. All images were analysed using Image Studio Lite software, version 5.2.5.  
194

## 195 **Cell transfection and genetic modifications**

196 Cells were typically transfected in suspension. 5 µg of DNA was used to transfect the cells using the  
197 Amaxa Cell Line Nucleofector kit (Lonza Bioscience) according to the manufacturer's instructions. To  
198 generate stable cell lines, stably transfected cells were selected with G-418 (G418S, Formedium)  
199 followed by fluorescence activated cell sorting (FACS). For FACS, gating was performed by cell size,  
200 live/dead and GFP-positive signal.

201 For transfection of adhered cells, 1 µg of DNA was used with Lipofectamine 2000 reagent (Thermo  
202 Fisher Scientific) according to the manufacturer's instructions. For siRNA-mediated YAP silencing, Yap  
203 targeting siRNA oligos (25nM; Qiagen) or control siRNA (25nM; AllStars Negative Control siRNA,  
204 Qiagen) were used to transfect the cells using the Lipofectamine RNAiMAX transfection reagent  
205 (Thermo Fisher Scientific) according to the manufacturer's instructions. YAP-silenced cells were  
206 analysed 48h post-transfection. For siRNA-mediated ILK silencing, ILK targeting oligos (15nM; Qiagen)  
207 or control siRNA (15nM; AllStars Negative Control siRNA, Qiagen) were used with the Lullaby  
208 transfection reagent (OZ Biosciences) according to the manufacturer's instructions. Two-step siRNA  
209 delivery with 48h interval was performed and ILK-silenced cells were analysed 24h post-transfection.  
210 For CRISPR/Cas9-mediated knockout, cells were transfected with 5µg of selected plasmid (control,  
211 empty vector (EV) or containing guide RNAs using Amaxa Cell Line Nucleofector kit (Lonza Bioscience)  
212 according to the manufacturer's instructions. Stably transfected cells were selected using puromycin (2  
213 µg mL<sup>-1</sup>).

214

### 215 **Immunofluorescence**

216 Typically, 2x10<sup>4</sup> cells per cm<sup>2</sup> were seeded on 19-mm diameter coverslips or PAAm hydrogels for 16  
217 hours. Cells were washed 1x in PBS and fixed in 4% PFA for 10 min. Then they were permeabilised  
218 with 0.1% Triton X-100 for 5 min followed by 30 min incubation in blocking buffer (1%BSA / PBS). Cells  
219 were incubated for 60 min with the following primary antibodies: anti-YAP (1:100; 14074, Cell Signaling  
220 Technology), anti-phosphoPaxillin (1:400; 2541, Cell Signaling Technology) and anti-Collagen VI  
221 (1:200; ab182744, Abcam). Detection was performed using the following secondary antibodies: Alexa  
222 Fluor 488 donkey anti rabbit (1:500 dilution; A21206, Invitrogen), Alexa Fluor 594 donkey anti mouse  
223 (1:500 dilution; A21203, Invitrogen). Nuclei were visualised with DAPI (0.5µg mL<sup>-1</sup>; D1306, Invitrogen)  
224 and F-actin with Alexa Fluor 647 Phalloidin (1:100 dilution; A22287, Invitrogen) incubation along with  
225 secondary antibodies. Coverslips were mounted using ProLong Diamond antifade reagent (P36965,  
226 Invitrogen).

227 Images were acquired using a Zeiss 880 Laser Scanning Microscope with Airyscan equipped with a  
228 Plan-Apochromat 63x/1.4 oil DIC M27 objective) or a Zeiss 710 confocal microscope equipped with Ec  
229 Plan Neofluar 40x/1.30 and Plan-apochromat 63x/1.40 oil objectives. All images were processed with  
230 Fiji software (ImageJ v2.0.0).

231 Focal adhesion analysis on Fig.3B was performed using the Fiji software (ImageJ v2.0.0) and the  
232 Analyze particles function applying a minimum size threshold of 0.25µm<sup>2</sup>. Cell shape analysis on  
233 Fig.S4A was performed by manually drawing around the cell perimeter based on F-actin staining and  
234 measuring the area and the shape descriptors using the Fiji software (ImageJ v2.0.0). Cell shape  
235 analysis on Fig.S4C was performed using Cell Profiler software (v3.0.0; CellProfiler), applying a mask  
236 for cell area based on F-actin staining and a mask for nuclei. YAP nuclear to cytosolic ratios on Fig.S1B  
237 were calculated using the Fiji software (ImageJ v2.0.0) to quantify mean YAP fluorescence intensity on  
238 similar rectangular areas over and adjacent to the nucleus.

239

### 240 **Immunohistochemistry (IHC)**

241 All Haematoxylin & Eosin (H&E), Immunohistochemistry (IHC) and Picro-Sirius red (PSR) staining was  
242 performed on 4µm formalin fixed paraffin embedded sections (FFPE) which had previously been oven  
243 baked at 60°C for 2 hours.

244 The following antibodies were stained on an Agilent AutostainerLink48,  $\alpha$ SMA (A2547, Sigma-Aldrich),  
245 Collagen VI (ab182744, Abcam), Fibronectin (A0245, Agilent) and p53 CM-5 (P53-CM5P-L). FFPE  
246 sections were loaded into an Agilent pre-treatment module to be dewaxed and undergo heat induced  
247 epitope retrieval (HIER) either using low or high pH target retrieval solution (TRS) (K8005, K8004,  
248 Agilent). Sections for  $\alpha$ SMA staining underwent antigen retrieval using low pH TRS. Sections for  
249 Collagen VI, Fibronectin and p53 staining underwent antigen retrieval using high pH TRS. All sections  
250 were heated to 97°C for 20 minutes in the appropriate TRS. After HIER all sections were rinsed in flex  
251 wash buffer (K8007, Agilent) prior to being loaded onto the autostainer. The sections then underwent  
252 peroxidase blocking (S2023, Agilent) for 5 minutes, then washed with flex wash buffer. Sections for  
253  $\alpha$ SMA were blocked using mouse-on-mouse kit (MKB2213-1, Vector Lab) before primary antibody  
254 application for 35 minutes at a previously optimised dilution ( $\alpha$ SMA, 1/25000; Collagen VI, 1/1000;  
255 Fibronectin, 1/750; p53, 1/250). The sections were then washed with flex wash buffer before application  
256 of appropriate secondary antibody for 30 minutes. Sections for  $\alpha$ SMA had Mouse Envision applied  
257 (K4001, Agilent) and Collagen VI, Fibronectin, and p53 sections had Rabbit Envision (K4003, Agilent)  
258 applied for 30 minutes. Sections were rinsed with flex wash buffer before applying Liquid DAB (K3468,  
259 Agilent) for 10 minutes. The sections were then washed in water, counterstained with haematoxylin z  
260 (RBA-4201-001 CellPath).

261 The following antibodies were stained on a Leica Bond Rx autostainer, F4/80 (ab6640, Abcam, and  
262 Ly6G (BE0075-1, BioXcell). All FFPE sections underwent on-board dewaxing (AR9222, Leica) and  
263 antigen retrieval using appropriate retrieval solution. Sections for F4/80 staining were retrieved using  
264 enzyme 1 solution (AR9551, Leica) for 10 minutes at 37°C. Sections for Ly6G underwent antigen  
265 retrieval using ER2 solution (AR9640, Leica) for 20 minutes at 95°C. Sections were rinsed with Leica  
266 wash buffer (AR9590, Leica) before peroxidase block was performed using an Intense R kit (DS9263,  
267 Leica) for 5 minutes. Sections were rinsed with wash buffer before all sections had the blocking solution  
268 applied from the Rat ImmPRESS kit (MP7444-15, Vector Labs) for 20 minutes. Sections were rinsed  
269 with wash buffer (AR9590, Leica) and then primary antibody applied at an optimal dilution (F4/80, 1/100;  
270 Ly6G, 1/60000). The sections were rinsed with wash buffer and had Rat ImmPRESS secondary  
271 antibody applied for 30 minutes. The sections were rinsed with wash buffer and visualised using DAB  
272 in the Intense R kit.

273 H&E staining was performed on a Leica autostainer (ST5020). Sections were dewaxed, taken through  
274 graded alcohols and then stained with Haem Z (CellPath, UK) for 13 mins. Sections were washed in  
275 water, differentiated in 1% acid alcohol, washed and the nuclei blu'd in scotts tap water substitute (in-  
276 house). After washing sections were placed in Putt's Eosin (in-house) for 3 minutes.

277 Staining for PSR was performed manually on FFPE sections that were dewaxed and rehydrated through  
278 xylene and a graded ethanol series before washing in water. Rehydrated slides were stained for 2 hours

279 in PSR staining solution (equal volumes of 0.1% Direct red 80 (Sigma Aldrich) and 0.1% Fast green  
280 (Raymond A Lamb) (both in distilled water) combined in a 1:9 dilution with aqueous Picric acid solution  
281 (VWR).

282 To complete H&E, IHC and PSR staining sections were rinsed in tap water, dehydrated through graded  
283 ethanol's and placed in xylene. The stained sections were coverslipped in xylene using DPX mountant  
284 (SEA-1300-00A, CellPath).

285 Slides were scanned with a Leica Aperio AT2 slide scanner at 20x magnification and all analyses were  
286 performed with HALO software (Indica labs).

#### 287 **Random migration assay**

288 Cells were plated at 20,000 cells per cm<sup>2</sup> onto fibronectin coated dishes and imaged for 16h using a  
289 Nikon TE2000 microscope with a Plan Fluor 10x/0.30 objective and equipped with a heated CO<sub>2</sub>  
290 chamber. Images were analysed with Fiji software (ImageJ v2.0.0).

291

#### 292 **Inverted invasion assay**

293 100 µL of 50% Matrigel in PBS solution were allowed to polymerise per transwell for 30 min at 37°C.  
294 Cells were trypsinized, resuspended in medium, counted and 4x10<sup>4</sup> cells were plated on the underside  
295 of the transwell filter. Transwells were then placed inside 24-well plates in a way that the cell suspension  
296 droplets are in contact with the base of the 24-well plates and were incubated for 2 hours at 37°C  
297 allowing cell attachment to the bottom side of the filter. Transwells were then washed 3x with 1 mL of  
298 serum-free medium. Chemotactic gradients were created by filling the upper chambers with medium  
299 containing 10% FBS and the bottom chambers were kept in FBS-free medium. Cells were allowed to  
300 invade the Matrigel plug for 3.5 days and stained for 60 min at 37°C with 4 µM of calcein AM. Serial  
301 optical sections at 15 µM interval were obtained using an Olympus FV1000 confocal microscope  
302 equipped with a UplanSApo 20x/0.74 objective. Images were analysed using Fiji software (ImageJ  
303 v2.0.0).

304

#### 305 **Wound invasion assay**

306 96-well Incucyte Imagelock plates were coated for 16h with Matrigel (100µg mL<sup>-1</sup>) at 37°C. PDAC cell  
307 monolayers were generated by plating 7x10<sup>5</sup> cells per mL for 4h. Monolayers were wounded using the  
308 96-pin WoundMaker (Essen Bioscience) and embedded in Matrigel (2.15 mg mL<sup>-1</sup>, diluted in DMEM-  
309 10%FBS). Following 60 min incubation at 37°C, fully supplemented media were added to each well and  
310 images were acquired at 60 min intervals by the IncuCyte Zoom system (Essen Bioscience). Wound  
311 recovery was analysed with the automated Incucyte Zoom software (Essen Bioscience) providing  
312 relative wound density over time.

313

#### 314 **Intraperitoneal and intrasplenic transplantation assays and KPC mice**

315 A list of mice used for IHC staining is provided in Supplementary Table 1. Mice were maintained in the  
316 Biological Services Unit of the Beatson Institute according to UK Home Office regulations and in  
317 compliance with EU Directive 2010/63 and the UK Animals (Scientific Procedures) Act 1986. All

318 protocols and experiments were previously approved by the Animal Welfare and Ethical Review Body  
319 (AWERB) of the University of Glasgow and were accompanied by a UK Home Office project licence to  
320 J. Morton (70/8375, intrasplenic) and to L. Machesky PE494BE48 (intraperitoneal and KPC mice).  
321 Intraperitoneal (IP) transplantation assay of PDAC cells was performed as previously described (Juin  
322 et al., 2019). PDAC cells ( $1 \times 10^6$ ) were resuspended in 100  $\mu$ L PBS and introduced into each nude  
323 mouse (CD-1nu, females, 6-week old; Charles River Laboratories, Wilmington, MA) by intraperitoneal  
324 injection. Tumor nodules were quantified in the mesenterium and the diaphragm following 14 days from  
325 injection.  
326 Intrasplenic assay was performed as previously described (Papalazarou et al., 2020). Following  
327 anaesthesia and transverse incision exposing the spleen, PDAC cells ( $1 \times 10^6$ ) in 100  $\mu$ L PBS were  
328 injected in the spleen of nude mice (CD-1nu, females, 6-week old; Charles River Laboratories,  
329 Wilmington, MA). Surgical clips were typically removed 7 days after surgery and mice were sacrificed 21  
330 days after inoculation. Liver tumour burden was calculated as the percentage of tumour-bearing liver  
331 lobes over the total number of liver lobes.  
332

### 333 **Statistics and reproducibility**

334 All datasets were analysed and plotted using Prism 8 (v8.2.0; GraphPad Software) unless otherwise  
335 stated. Differences between groups were tested for normal distribution and analysed using the  
336 appropriate statistical test, as mentioned in each figure legend. Error bars represent SD, unless  
337 otherwise stated.  
338

### 339 **Acknowledgements**

340  
341 We acknowledge CRUK Beatson Institute Core Services and Advanced Technologies (C596/A17196),  
342 CRUK Core grant A24452 to LMM, CRUK Centre Studentship A18076 to LMM, MSS and VP, EPSRC  
343 Programme Funding to MSS (EP/P001114/1). We especially thank Ann Hedley, Peter Bailey, and Billy  
344 Clark (CRUK Beatson Institute, Glasgow) for their assistance with preparation and analysis of RNA  
345 sequencing experiments; Saadia Karim and Jennifer P. Morton (CRUK Beatson Institute, Glasgow) for  
346 providing the two KPC-derived cell lines (PDAC-A and PDAC-B) used throughout; Jennifer P. Morton  
347 for advice about intrasplenic transplants. T. Hamilton, C. Baxter and E. Onwubiko (CRUK Beatson  
348 Institute, Glasgow) for helping with intrasplenic surgery. Feng Zhang (Massachusetts Institute of  
349 Technology, Cambridge), Anna Huttenlocher (Harvard Medical School, Cambridge) and Susan Craig  
350 (Johns Hopkins School of Medicine, Baltimore) for provision and advice on use of plasmids.  
351

### 352 **Results**

#### 353 **Matrix stiffness alters pancreatic cancer cell expression of extracellular matrix proteins**

354 To understand how pancreatic cancer cells respond to substrate stiffness, we cultured two independent  
355 murine KPC cell lines (pancreas tumor cells cultured from *Pdx1-cre;LSL-Kras<sup>G12D</sup>*; *LSL-Trp53<sup>R172H</sup>*  
356 mice (Morton et al., 2010)) on fibronectin-coated polyacrylamide hydrogels of three defined stiffnesses,  
357 as reported previously (Papalazarou et al., 2020) and investigated gene expression profiles by

358 performing RNA sequencing (Fig. 1A). We chose 0.7kPa, 7kPa, 38kPa to model soft, intermediate and  
359 stiff tissue contexts respectively (Butcher et al., 2009), and included a glass coverslip condition (~2-4  
360 GPa) as a reference for typical cell culture conditions. As stiffness of healthy pancreas is typically <  
361 1kPa and PDAC tissue can reach 7-10 kPa these stiffness values have physiological relevance to  
362 PDAC (Rice et al., 2017). Immunofluorescent (IF) imaging revealed clear morphological differences  
363 between different conditions, with cells on soft substrate appearing round and clustered compared to  
364 the elongated cells with visible protrusions and stress fibres seen on stiff substrate and glass (Fig.1B).  
365 Further, we observed a dramatic redistribution of the mechanosensitive transcriptional regulator YAP1  
366 (Zanconato et al., 2019) from the nucleus to the cytoplasm on soft substrate (Fig.S1A-B), indicating that  
367 the system is indeed driving mechanosensitive pathways in PDAC cells.

368  
369 To better understand the global changes in gene expression caused by different substrate stiffnesses,  
370 we conducted RNA sequencing of PDAC-A and PDAC-B cell lines on soft and stiff hydrogels, and glass.  
371 Hierarchical clustering of replicates revealed markedly distinct transcriptional profiles of the two cell  
372 lines regardless of ECM stiffness (Fig.S1C), but that within cell lines replicates from soft conditions  
373 tended to cluster separately (Fig.S1D). Indeed, analysis of differentially-expressed genes (DEGs)  
374 showed substantial changes in gene expression between soft substrate and both stiff hydrogels and  
375 glass conditions (Fig.1C-D). The observation that gene expression changes are consistent between  
376 stiff and glass conditions supports the idea that PDAC cells are mechanosensitive within a physiological  
377 range of substrate stiffnesses. Pathway analysis of genes upregulated on soft substrates revealed a  
378 surprising enrichment in genes associated with the ECM and substrate-interacting pathways (Fig.1E).  
379 Gene-level analysis of the 'ECM-Receptor Interaction' pathway showed a mix of ECM molecules  
380 (collagens, fibronectin) and integrins were being upregulated on soft substrates (Fig.S1E). To confirm  
381 this, a gene-set enrichment analysis between soft and stiff conditions was performed using the  
382 matrisome geneset from Naba et al. (Naba et al., 2012). Both cell lines showed a significant enrichment  
383 in matrisome gene expression on soft hydrogels (Fig.1F). Thus, PDAC cells show an orchestrated  
384 response to changes in mechanical stiffness of substrate characterised by increased expression of  
385 ECM-related genes.

386  
387 **Collagen-VI is upregulated by pancreatic cancer cells on soft substrates downstream of ECM  
388 adhesion and YAP**

389 Comparing the gene-level changes in PDAC-A and PDAC-B cell lines, we noticed that several subunits  
390 of Collagen-VI were upregulated on soft matrix in both lines (Fig.S1E). Collagen-VI is a fibrillar collagen  
391 that exists as a tetrameric assembly of  $\alpha 1$  and  $\alpha 2$  subunits, with possible inclusion of  $\alpha 3/4/5/6$  (Fig.2A).  
392 We first sought to confirm upregulation of Collagen-VI on soft substrates at the RNA and protein level.  
393 qPCR of *Col6a1* showed a gradual increase in mRNA levels across progressively softer substrates,  
394 which was significant when comparing the 0.7kPa hydrogel and glass conditions (Fig.2B). Increases in  
395 mRNA were shown to translate to protein expression via Western blots for Collagen-VI in both cell lines  
396 (Fig.2C-D). Again, a gradual increase in protein expression was observed across the substrate  
397 stiffnesses, highlighting the sensitivity of Collagen-VI expression in PDAC cells to physical properties

398 of the substrate. IF of Collagen-VI in PDAC-A and a separate human PDAC cell line (PANC1) showed  
399 a clear increase in intracellular Collagen-VI staining as substrate stiffness was reduced (Fig.S2A-B).  
400 Finally, to assess whether Collagen-VI expression could be an adaptive response to slower proliferation  
401 levels of PDAC cells upon low substrate stiffness (Papalazarou et al., 2020), we treated cells with  
402 aphidicolin, an inhibitor of DNA replication (Fig.S3A). Upon aphidicolin treatment there was a trend for  
403 reduced Collagen VI expression, suggesting that the increase of Collagen-VI expression upon soft  
404 matrices is not a direct adaptation in response to lower proliferative capacity on soft matrix.

405  
406 To better understand the mechanism driving increased expression of Collagen-VI on soft hydrogels, we  
407 conducted a range of experiments disrupting key steps in the integrin and YAP-linked  
408 mechanosensation pathway (Fig.2E). Firstly, to assess the requirement of integrin receptor  
409 engagement, PDAC-A cells were cultured on plastic coated with fibronectin, or two substrates known  
410 to allow cell attachment without engagement of integrin receptors, concanavalin A (conA) and poly-L-  
411 lysine (PLL) (Fig.2F). Collagen-VI expression was upregulated in both PDAC-A cells grown on conA  
412 and PLL, suggesting that loss of integrin receptor engagement drives Collagen-VI expression. Integrin-  
413 linked kinase (ILK) is a component of focal adhesion complexes that translate integrin engagement to  
414 intracellular signalling (Hannigan et al., 2005). Transfection of PDAC-A cells with two different siRNAs  
415 targeting *Ilk* led to 2-3 fold increase in collagen-VI expression (Fig.2G), that was also observed in PDAC-  
416 B cells (Fig.S3B). Overexpression of a dominant-negative Talin-head domain mutant that disrupts  
417 binding to integrins (L325R) also increased Collagen-VI expression compared to cells overexpressing  
418 a wild-type Talin-head domain or the vinculin head domain (VD1) in PDAC-A cells (Fig.S3C,D). Finally,  
419 we asked if Collagen-VI expression was directly altered by disruption of YAP, a major mechanosensitive  
420 transcriptional regulator that lies downstream of integrin-focal adhesion complex signalling. Silencing  
421 YAP expression with two siRNAs led to ~2-fold increase in Collagen-VI expression (Fig.2H). These  
422 results were confirmed using a CRISPR KO of *Yap1* in PDAC-A cells, additionally showing that  
423 Collagen-VI expression was higher in *Yap1* KO cells regardless of substrate stiffness (Fig.S3E-F).  
424 Taken together, these results show that upregulation of Collagen-VI expression on soft substrata is  
425 likely driven by loss of integrin receptor engagement and YAP activity.

426  
427 **Cancer cell-derived collagen-VI supports migration invasive behaviours in PDAC cells**  
428 Prior studies have found a role for Collagen-VI in supporting cancer cell migration and invasion, though  
429 these studies did not investigate the influence of cancer cell-derived collagen-VI (Iyengar et al., 2005;  
430 Wishart et al., 2020). To ask if Collagen-VI altered pancreatic cancer cell morphology and migration,  
431 we cultured PDAC-A cells on either fibronectin, Collagen-VI or a 50:50 mix of the two (Fig.3A-B, S4A-  
432 B). Cells plated on Collagen-VI produced significantly less mature focal adhesions, indicating a less  
433 stable association with the substrate (Fig.3B). Supporting this, timelapse imaging over a 16-hour period  
434 revealed an approximate 2-fold increase in migration speed on collagen-VI (Fig.3B, S4B).

435  
436 As these experiments utilise exogenous recombinant protein, it is unclear if cancer cell-derived  
437 Collagen-VI can support migratory behaviour in the same manner. To test this, we selected two of four

438 CRISPR lines in PDAC-A cells in which a critical exon of *Col6a1* was deleted (Fig.3C). Cells appeared  
439 morphologically normal, though we did observe a slight increase in cell area in the *Col6a1.03* line (Fig.  
440 3D, S4C). We used two complementary experiments to investigate the invasive potential of these cells.  
441 Firstly, invasion into a Matrigel plug in response to a serum chemotactic gradient was used (Fig.3E).  
442 Analysis of the depth that cells invaded into the plug after 72 hours revealed a reduction in both CRISPR  
443 KO lines that was significant for *Col6a1.04* (Fig.3F-G, S4D). Secondly, we utilised a 3D wound healing  
444 assay in which a Matrigel-embedded cell monolayer invades into wounded ECM (Fig.3H). We again  
445 observed a reduction in wound closure over a 50-hour period that was significant for the *Col6a1.04* line  
446 (Fig.3I-K). Together, these results indicate that cell-derived Collagen-VI in pancreatic cancer cells  
447 supports invasive behaviours in a range of contexts.

448

449 **Collagen-VI deposition increases during PDAC progression and is associated with poor survival**

450 Having shown that pancreatic cancer cells display mechanosensitive expression of Collagen-VI, and  
451 that Collagen-VI supports their motility in 2D and 3D settings, we investigated whether cancer cell-  
452 derived Collagen-VI supported pancreatic cancer progression *in vivo*. Collagen-VI has well documented  
453 roles in certain cancers including breast (Wishart et al., 2020), ovarian (Sherman-Baust et al., 2003)  
454 and lung (Voiles et al., 2014). We began by investigating whether Collagen-VI expression is altered in  
455 the KPC mouse model of PDAC. We stained pancreata of normal (*Pdx1-Cre<sup>+/−</sup>:Kras<sup>+/+</sup>:Trp53<sup>+/+</sup>*) or KPC  
456 (*Pdx1-Cre<sup>+/−</sup>:Kras<sup>G12D/+</sup>:Trp53<sup>R172H/+</sup>*) mice at different stages of PDAC progression from PanIN I-III (10-  
457 and 15-week) to endpoint PDAC (Fig.4A). We noticed an upregulation of Collagen-VI expression with  
458 PDAC progression (Fig.4B). Further, Collagen-VI was mainly localised in the stroma, suggesting that  
459 stromal cells (particularly cancer associated fibroblasts) may be the primary depositors of this type of  
460 Collagen-VI in primary PDAC tissue, in agreement with a recent proteomic study (Tian et al., 2019). As  
461 primary PDAC tissue is typically stiff, it is unsurprising that cancer cells are not the major source of  
462 Collagen-VI in primary tissue. Indeed, Collagen-VI expression in liver metastasis samples of KPC mice  
463 (where cells will be experiencing softer substrate) showed markedly stronger staining that also  
464 appeared more cytoplasmic and PDAC cell-derived (Fig.4C).

465

466 To confirm that these results were representative of human disease, we consulted the TCGA database  
467 of primary human PDAC tissue (Fig.4D-E). RNA levels of *COL6A1* were significantly increased in  
468 primary tumor vs normal pancreas samples (Fig.4D). Furthermore, high *COL6A1* expression was  
469 associated with reduced disease-free survival in these patients (Fig.4E).

470

471 **Collagen-VI expression supports establishment of pancreatic metastasis *in vivo***

472 We hypothesised that PDAC cells may upregulate Collagen-VI upon extravasation in the liver,  
473 contributing to the establishment of the metastatic niche. To test this hypothesis, we initially performed  
474 intraperitoneal injection (IP) experiments using a KPC mouse-derived cell line (KPC #127445) that we  
475 have used previously for such experiments (Juin et al., 2019) (Fig.5A). Two *Col6a1* CRISPR KO lines  
476 were used alongside an empty vector (EV) control to generate abdominal cavity and diaphragm tumors  
477 (Fig.5A, S5A). Interestingly, both *Col6a1* KO lines showed a trend for decreased tumor formation on

478 the diaphragm (Fig.5B) and the peritoneum (Fig.5C) that was significant for the Col6a1.04 line, despite  
479 no changes in mouse weights (Fig.S5B). Histological analysis of these tumors confirmed the high levels  
480 of Collagen-VI expression in abdominal cavity tumors from EV cells and the significant loss of Col6a1  
481 staining in both KO lines (Fig.5D). What Collagen-VI staining remained in *Col6a1* KO tumors appeared  
482 to colocalise with  $\alpha$ SMA+ cells, which likely represent myofibroblast-like resident cells (Fig.S5C).

483

484 The liver is a common site of metastasis in pancreatic cancer. To further validate these observations  
485 and since IP injections do not represent the metastatic cascade, we decided to assess the role of PDAC-  
486 derived Collagen-VI in formation of liver metastases using the model of intrasplenic (IS) transplantations  
487 (Fig.5E). Interestingly, Collagen-VI KO cells (Col6a1.04) displayed a reduced ability to form metastases  
488 in the liver compared with control cells (EV) following IS transplantation (Fig.5F-G). Histological staining  
489 again showed that Collagen-VI was deposited primarily in areas with high  $\alpha$ SMA+ cells in KO conditions  
490 and was more diffuse in EV tumors (Fig.5H). Further, KO metastases typically had strong Collagen-VI  
491 deposition surrounding tumor nodules, likely of stromal origin, suggesting that presence of Collagen-VI  
492 is required to support metastasis formation - regardless of its source. We validated these histological  
493 results by generating liver metastases with PDAC-B CRISPR control (EV) or *Col6a1* KO (Col6a1.03)  
494 cell lines (Fig.S5D-E). Analysis of common ECM markers and stromal cell types showed no significant  
495 differences between EV and *Col6a1.03* conditions, though there was a trend for 1.5-2-fold more  
496 abundance of fibroblasts ( $\alpha$ SMA+), neutrophils (Ly6G+) and macrophages (F4/80+) in Collagen-VI KO  
497 tumors. Thus, while cancer cell-derived Collagen-VI could be important for the formation of metastasis  
498 at multiple organ sites, other cell types in the metastatic microenvironment may be able also deposit it.

499

## 500 **Discussion**

501 Pancreatic tumors are hallmark by their stiff collagen-rich stroma. However, the metastatic cascade  
502 requires cells to adapt to softer environments, raising the question of how this plasticity is achieved.  
503 Culturing PDAC cells on hydrogels of varied stiffnesses showed substantial reprogramming of cells,  
504 which was accompanied by morphological changes that have been reported previously (Papalazarou  
505 et al., 2020). Despite having the same driver mutations (KRas<sup>G12D</sup> and p53<sup>R172H</sup>), the two PDAC cell  
506 lines isolated from KPC mice showed unique responses to culture on soft vs stiff hydrogels, both at the  
507 individual gene expression and gene-program level. This reflects the heterogeneity of PDAC cell identity  
508 also observed in the clinical setting, that contributes to pancreatic cancer resistance to therapeutic  
509 intervention (Carstens et al., 2021). Given this, it is noteworthy that an upregulation of Collagen-VI, and  
510 matrisome genes more broadly, was a shared response of both PDAC lines to culture on soft substrate.  
511 ECM secretion by cancer cells in low-adhesion environments has been suggested previously, such as  
512 increased expression of SPARC, MPG and SPON2 in circulating tumor cells (CTCs) from patients (Ting  
513 et al., 2014). However, direct evidence for specific stiffness-dependent expression of ECM proteins by  
514 PDAC cells, as presented here, has been lacking. Our data adds to a growing repertoire of  
515 mechanosensitive mechanisms that PDAC cells use to survive and grow in different niches.

516

517 Our data disrupting various components of the mechanosensation pathway through plating cells on  
518 substrates that do not engage integrins, disruption of vinculin or talin interactions or knockdown of ILK  
519 (Figure 2) strongly suggests that increased Collagen-VI expression on soft substrates is due to a lack  
520 of integrin receptor engagement and downstream YAP nuclear translocation. How reduced YAP-driven  
521 transcription leads to increased Collagen-VI transcription is currently unclear. YAP coordinates  
522 expression of large gene networks, including those that can actively repress expression of other genes  
523 (Kim et al., 2015). Thus, increased Collagen-VI on soft substrata could be a consequence of relieved  
524 inhibition of another factor. Alternatively, inactivation of YAP may free up TEAD transcription factors to  
525 interact with other co-factor TFs such as VGLLs (Zhang et al., 2017) to promote expression. The  
526 differing responses of PDAC-A and PDAC-B cells to soft hydrogels highlights the complexity of  
527 mechanosensitive expression and suggest that exact pathways activated may differ depending on other  
528 properties or cell states.

529  
530 An increasingly important question in relation to cancer ECM is the cellular origin and mechanism of  
531 action of specific ECM components. Along with most collagens, Collagen-VI expression is upregulated  
532 in primary PDAC and mostly derives from myofibroblasts (Tian et al., 2019). This is supported by data  
533 here showing that PDAC cells downregulate their own expression of Collagen-VI in stiff environment  
534 such as primary PDAC tumors. Stromal-derived Collagen-VI may still play important roles in PDAC  
535 progression, such as in promoting motility as has been reported for breast cancer (Wishart et al., 2020).  
536 Indeed, our data showing increased motility of PDAC cells on Collagen-VI coated coverslips supports  
537 this. Comparing metastatic tumors formed by PDAC cells with or without *Col6a1* KO, it is clear that  
538 cancer cells are a significant source of Collagen-VI in this context.

539  
540 Our data shows that cancer cell-derived Collagen-VI has cell autonomous roles in determining  
541 metastatic potential of PDAC cells. Using a number of approaches, we show that Collagen-VI supports  
542 invasive potential of PDAC cells, *in vitro* and *in vivo*. This aligns with other recent studies reporting  
543 promotion of migration and invasion by Collagen-VI in other cancers (Chen et al., 2013; Wishart et al.,  
544 2020). Given that we see enhanced migration of PDAC cells on Collagen-VI coated coverslips, our data  
545 support a mechanism whereby motility effects are due to secreted Collagen-VI providing a local  
546 substrate for cancer cells. However, we cannot discount the possibility that Collagen-VI may have  
547 additional intracellular functions or that Collagen-VI may accumulate in cells on soft substrates due to  
548 inability to be secreted. Additionally, the COL6A3 fragment, endotrophin (ETP), has been suggested to  
549 enhance EMT in cancer cells (Park and Scherer, 2012), although it was not responsible for migration  
550 effects in breast cancer cells (Wishart et al., 2020). Finally, Collagen-VI may also support establishment  
551 of metastases by providing ECM peptides for attachment to the liver vasculature during early seeding,  
552 as has been shown for PDAC cell-derived matrisome proteins SERPINB5 and CSTB (Tian et al., 2020).  
553  
554 In summary, this study uncovers a mechanosensitive mechanism whereby pancreatic cancer cells alter  
555 their own extracellular matrix environment to support metastatic colonisation. Using a combination of  
556 gene expression profiling, *in vitro* characterisation and *in vivo* data we show how Collagen-VI

557 expression is dependent on substrate stiffness. Our *in vivo* data also suggest that Collagen-VI is an  
558 important factor in the liver metastatic niche and can be provided either by the tumor cells or the liver  
559 resident cells. Our study highlights the dual nature of mechanosensation – as a response to soft as well  
560 as stiff environments – and illustrates the importance of the former to metastatic dissemination.

561

562 **References**

563 **Balaban, E. P., Mangu, P. B. and Yee, N. S.** (2017). Locally Advanced Unresectable  
564 Pancreatic Cancer: American Society of Clinical Oncology Clinical Practice Guideline  
565 Summary. *J Oncol Pract* **13**, 265-269.

566 **Bhattacharjee, S., Hamberger, F., Ravichandra, A., Miller, M., Nair, A., Affo, S.,  
567 Filiol, A., Chin, L., Savage, T. M., Yin, D. et al.** (2021). Tumor restriction by type I collagen  
568 opposes tumor-promoting effects of cancer-associated fibroblasts. *J Clin Invest* **131**.

569 **Broders-Bondon, F., Nguyen Ho-Boulard, T. H., Fernandez-Sanchez, M. E. and  
570 Farge, E.** (2018). Mechanotransduction in tumor progression: The dark side of the force. *J Cell  
571 Biol* **217**, 1571-1587.

572 **Butcher, D. T., Alliston, T. and Weaver, V. M.** (2009). A tense situation: forcing  
573 tumour progression. *Nat Rev Cancer* **9**, 108-22.

574 **Carstens, J. L., Yang, S., Correa de Sampaio, P., Zheng, X., Barua, S.,  
575 McAndrews, K. M., Rao, A., Burks, J. K., Rhim, A. D. and Kalluri, R.** (2021). Stabilized  
576 epithelial phenotype of cancer cells in primary tumors leads to increased colonization of liver  
577 metastasis in pancreatic cancer. *Cell Rep* **35**, 108990.

578 **Chen, P., Cescon, M. and Bonaldo, P.** (2013). Collagen VI in cancer and its biological  
579 mechanisms. *Trends Mol Med* **19**, 410-7.

580 **Cohen, D. M., Kutscher, B., Chen, H., Murphy, D. B. and Craig, S. W.** (2006). A  
581 conformational switch in vinculin drives formation and dynamics of a talin-vinculin complex  
582 at focal adhesions. *J Biol Chem* **281**, 16006-15.

583 **Doherty, G. J., Temporo, M. and Corrie, P. G.** (2018). HALO-109-301: a Phase III  
584 trial of PEGPH20 (with gemcitabine and nab-paclitaxel) in hyaluronic acid-high stage IV  
585 pancreatic cancer. *Future Oncol* **14**, 13-22.

586 **Drew, J. and Machesky, L. M.** (2021). The liver metastatic niche: modelling the  
587 extracellular matrix in metastasis. *Dis Model Mech* **14**.

588 **Elyada, E., Bolisetty, M., Laise, P., Flynn, W. F., Courtois, E. T., Burkhardt, R. A.,  
589 Teinor, J. A., Belleau, P., Biffi, G., Lucito, M. S. et al.** (2019). Cross-Species Single-Cell  
590 Analysis of Pancreatic Ductal Adenocarcinoma Reveals Antigen-Presenting Cancer-  
591 Associated Fibroblasts. *Cancer Discov* **9**, 1102-1123.

592 **Hannigan, G., Troussard, A. A. and Dedhar, S.** (2005). Integrin-linked kinase: a  
593 cancer therapeutic target unique among its ILK. *Nat Rev Cancer* **5**, 51-63.

594 **Iyengar, P., Espina, V., Williams, T. W., Lin, Y., Berry, D., Jelicks, L. A., Lee, H.,  
595 Temple, K., Graves, R., Pollard, J. et al.** (2005). Adipocyte-derived collagen VI affects early  
596 mammary tumor progression *in vivo*, demonstrating a critical interaction in the tumor/stroma  
597 microenvironment. *J Clin Invest* **115**, 1163-76.

598 **Jiang, H., Torphy, R. J., Steiger, K., Hongo, H., Ritchie, A. J., Kriegsmann, M.,  
599 Horst, D., Umetsu, S. E., Joseph, N. M., McGregor, K. et al.** (2020). Pancreatic ductal  
600 adenocarcinoma progression is restrained by stromal matrix. *J Clin Invest* **130**, 4704-4709.

601 **Juin, A., Spence, H. J., Martin, K. J., McGhee, E., Neilson, M., Cutiongco, M. F. F.,  
602 Gadegaard, N., Mackay, G., Fort, L., Lilla, S. et al.** (2019). N-WASP Control of LPAR1  
603 Trafficking Establishes Response to Self-Generated LPA Gradients to Promote Pancreatic  
604 Cancer Cell Metastasis. *Dev Cell* **51**, 431-445 e7.

605                   **Kim, M., Kim, T., Johnson, R. L. and Lim, D. S.** (2015). Transcriptional co-repressor  
606                   function of the hippo pathway transducers YAP and TAZ. *Cell Rep* **11**, 270-82.

607                   **Laklai, H., Miroshnikova, Y. A., Pickup, M. W., Collisson, E. A., Kim, G. E.,**

608                   Barrett, A. S., Hill, R. C., Lakins, J. N., Schlaepfer, D. D., Mouw, J. K. et al.

609                   (2016). Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular  
610                   fibrosis and tumor progression. *Nat Med* **22**, 497-505.

611                   **Mahadevan, D. and Von Hoff, D. D.** (2007). Tumor-stroma interactions in pancreatic  
612                   ductal adenocarcinoma. *Mol Cancer Ther* **6**, 1186-97.

613                   **Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B.,**

614                   Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G. et al.

615                   (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proc Natl Acad Sci*  
616                   U S A

617                   **Naba, A., Clauser, K. R., Hoersch, S., Liu, H., Carr, S. A. and Hynes, R. O.** (2012).  
618                   The matrisome: in silico definition and in vivo characterization by proteomics of normal and  
619                   tumor extracellular matrices. *Mol Cell Proteomics* **11**, M111 014647.

620                   **Ozdemir, B. C., Pentcheva-Hoang, T., Carstens, J. L., Zheng, X., Wu, C. C.,**

621                   Simpson, T. R., Laklai, H., Sugimoto, H., Kahlert, C., Novitskiy, S. V. et al.

622                   (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and  
623                   accelerates pancreas cancer with reduced survival. *Cancer Cell* **25**, 719-34.

624                   **Panciera, T., Azzolin, L., Cordenonsi, M. and Piccolo, S.** (2017). Mechanobiology  
625                   of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol* **18**, 758-770.

626                   **Papalazarou, V., Salmeron-Sanchez, M. and Machesky, L. M.** (2018). Tissue  
627                   engineering the cancer microenvironment-challenges and opportunities. *Biophys Rev* **10**, 1695-  
628                   1711.

629                   **Papalazarou, V., Zhang, T., Paul, N. R., Juin, A., Cantini, M., Maddocks, O. D.**  
630                   **K., Salmeron-Sanchez, M. and Machesky, L. M.** (2020). The creatine-phosphagen system is  
631                   mechanoresponsive in pancreatic adenocarcinoma and fuels invasion and metastasis. *Nat*  
632                   *Metab* **2**, 62-80.

633                   **Park, J. and Scherer, P. E.** (2012). Adipocyte-derived endotrophin promotes  
634                   malignant tumor progression. *J Clin Invest* **122**, 4243-56.

635                   **Perez, V. M., Kearney, J. F. and Yeh, J. J.** (2021). The PDAC Extracellular Matrix:  
636                   A Review of the ECM Protein Composition, Tumor Cell Interaction, and Therapeutic  
637                   Strategies. *Frontiers in Oncology* **11**.

638                   **Rice, A. J., Cortes, E., Lachowski, D., Cheung, B. C. H., Karim, S. A., Morton, J.**  
639                   **P. and Del Río Hernández, A.** (2017). Matrix stiffness induces epithelial-mesenchymal  
640                   transition and promotes chemoresistance in pancreatic cancer cells. *Oncogenesis* **6**, e352-e352.

641                   **Sherman-Baust, C. A., Weeraratna, A. T., Rangel, L. B., Pizer, E. S., Cho, K. R.,**  
642                   **Schwartz, D. R., Shock, T. and Morin, P. J.** (2003). Remodeling of the extracellular matrix  
643                   through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer  
644                   cells. *Cancer Cell* **3**, 377-86.

645                   **Siegel, R. L., Miller, K. D., Fuchs, H. E. and Jemal, A.** (2021). Cancer Statistics,  
646                   2021. *CA Cancer J Clin* **71**, 7-33.

647                   **Simonson, W. T., Franco, S. J. and Huttenlocher, A.** (2006). Talin1 regulates TCR-  
648                   mediated LFA-1 function. *J Immunol* **177**, 7707-14.

649                   **Tian, C., Clauser, K. R., Ohlund, D., Rickelt, S., Huang, Y., Gupta, M., Mani, D.**  
650                   **R., Carr, S. A., Tuveson, D. A. and Hynes, R. O.** (2019). Proteomic analyses of ECM during  
651                   pancreatic ductal adenocarcinoma progression reveal different contributions by tumor and  
652                   stromal cells. *Proc Natl Acad Sci U S A* **116**, 19609-19618.

653                   **Tian, C., Ohlund, D., Rickelt, S., Lidstrom, T., Huang, Y., Hao, L., Zhao, R. T.,**  
654                   **Franklin, O., Bhatia, S. N., Tuveson, D. A. et al.** (2020). Cancer Cell-Derived Matrisome

655 Proteins Promote Metastasis in Pancreatic Ductal Adenocarcinoma. *Cancer Res* **80**, 1461-  
656 1474.

657 **Ting, D. T., Wittner, B. S., Ligorio, M., Vincent Jordan, N., Shah, A. M.,**  
658 **Miyamoto, D. T., Aceto, N., Bersani, F., Brannigan, B. W., Xega, K. et al.** (2014). Single-  
659 cell RNA sequencing identifies extracellular matrix gene expression by pancreatic circulating  
660 tumor cells. *Cell Rep* **8**, 1905-1918.

661 **Voiles, L., Lewis, D. E., Han, L., Lupov, I. P., Lin, T. L., Robertson, M. J.,**  
662 **Petrache, I. and Chang, H. C.** (2014). Overexpression of type VI collagen in neoplastic lung  
663 tissues. *Oncol Rep* **32**, 1897-904.

664 **Wishart, A. L., Conner, S. J., Guarin, J. R., Fatherree, J. P., Peng, Y., McGinn, R.**  
665 **A., Crews, R., Naber, S. P., Hunter, M., Greenberg, A. S. et al.** (2020). Decellularized  
666 extracellular matrix scaffolds identify full-length collagen VI as a driver of breast cancer cell  
667 invasion in obesity and metastasis. *Sci Adv* **6**.

668 **Yachida, S. and Iacobuzio-Donahue, C. A.** (2009). The pathology and genetics of  
669 metastatic pancreatic cancer. *Arch Pathol Lab Med* **133**, 413-22.

670 **Zanconato, F., Cordenonsi, M. and Piccolo, S.** (2019). YAP and TAZ: a signalling  
671 hub of the tumour microenvironment. *Nat Rev Cancer* **19**, 454-464.

672 **Zhang, Y., Shen, H., Withers, H. G., Yang, N., Denson, K. E., Mussell, A. L.,**  
673 **Truskinovsky, A., Fan, Q., Gelman, I. H., Frangou, C. et al.** (2017). VGLL4 Selectively  
674 Represses YAP-Dependent Gene Induction and Tumorigenic Phenotypes in Breast Cancer. *Sci*  
675 *Rep* **7**, 6190.

676

677

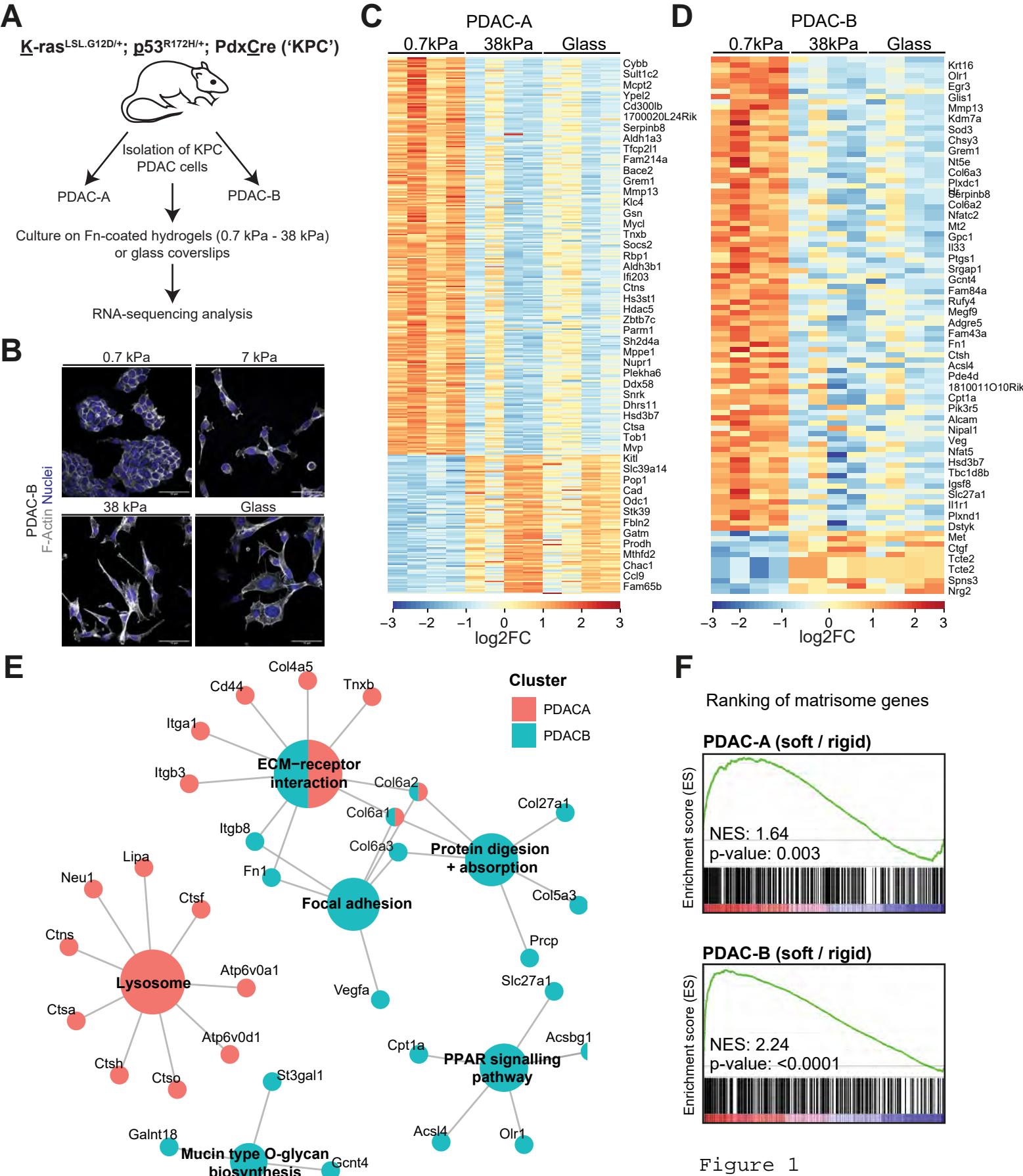


Figure 1

**Figure 1 – Low substrate stiffness promotes the expression of matrisome-related genes in PDAC cells.**

**A:** Schematic representation of RNA sequencing strategy of PDAC cells. Two KPC PDAC cell lines isolated from the *Kras*<sup>LSL.G12D/+</sup>; *Trp53*<sup>R172H/+</sup>; *Pdx-Cre* ('KPC') mouse model were cultured atop of 0.7 kPa, 7 kPa or 38 kPa Fibronectin (Fn)-coated polyacrylamide hydrogels or Fn coated glass coverslips for 24 hours. Total RNA was extracted and RNA sequencing was performed.

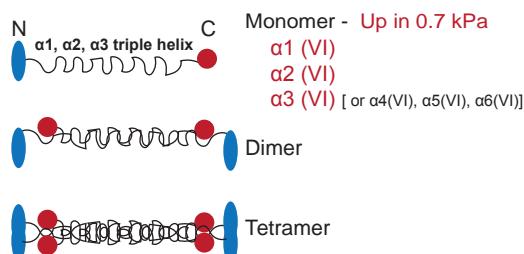
**B:** Immunofluorescence of PDAC-B cells cultured atop of 0.7 kPa, 7 kPa or 38 kPa fibronectin-coated polyacrylamide hydrogels or fibronectin-coated glass coverships showing F-Actin (phalloidin, grey) or nuclei (Hoechst, blue). Scale bars, 50  $\mu$ m.

**C-D:** Expression heatmaps of differentially expressed genes of cells cultured on 0.7 kPa, 38 kPa hydrogels and glass coverslips. In PDAC-A cells (**C**), 258 genes were upregulated and 92 downregulated at 0.7 kPa. In PDAC-B cells (**D**), 90 genes were upregulated and 10 downregulated at 0.7 kPa ( $p_{adj} < 0.05$ ; log2 fold change  $> 1$ ). In both cell lines, glass culture conditions show similarity in expression pattern with cells cultured on 38 kPa hydrogels. Data is from  $n = 4$  independent replicates per condition for each cell line.

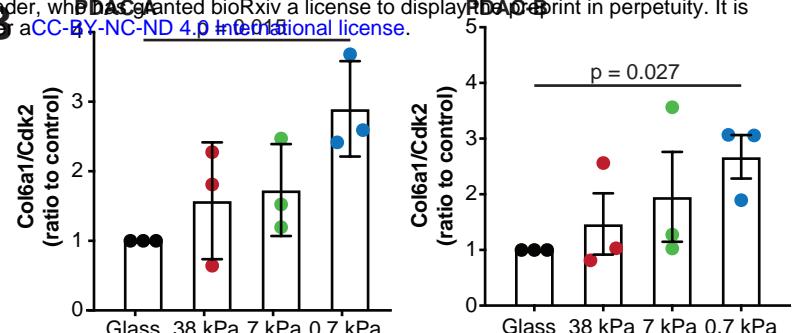
**E:** CNET plot of KEGG pathway analysis based on upregulated genes in PDAC-A and B cells identified in **C-D**. Individual genes important to enrichment of these functional nodes are shown. Colours represent genes upregulated in either PDAC-A (red), PDAC-B (blue) or both (split).

**F:** GWAS plot showing enrichment of matrisome geneset in ranked gene lists of both PDAC-A (top) and PDAC-B (bottom) cell lines.

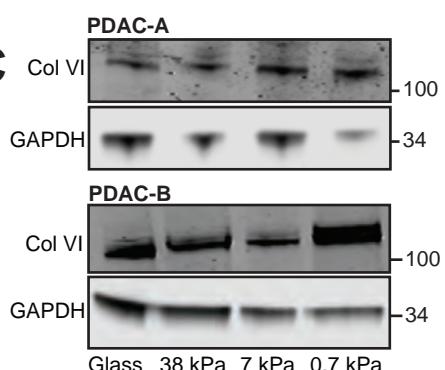
**A**



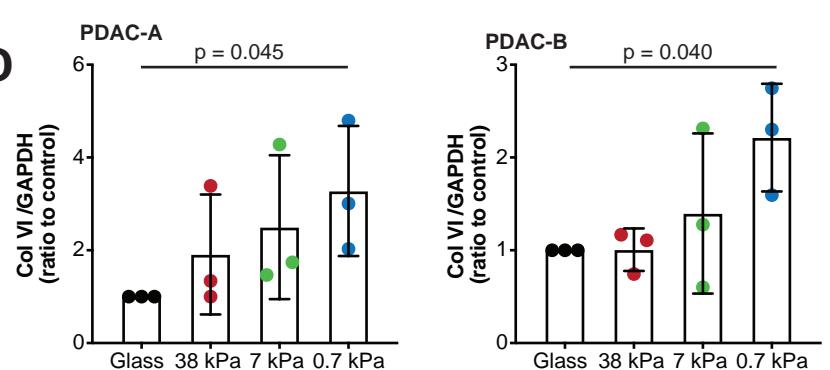
**B** PDAC-A



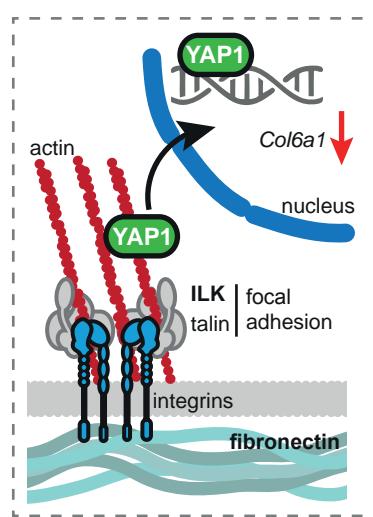
**C**



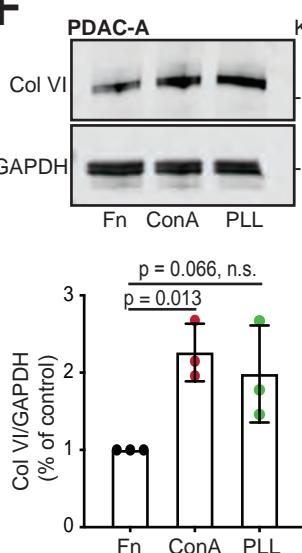
**D**



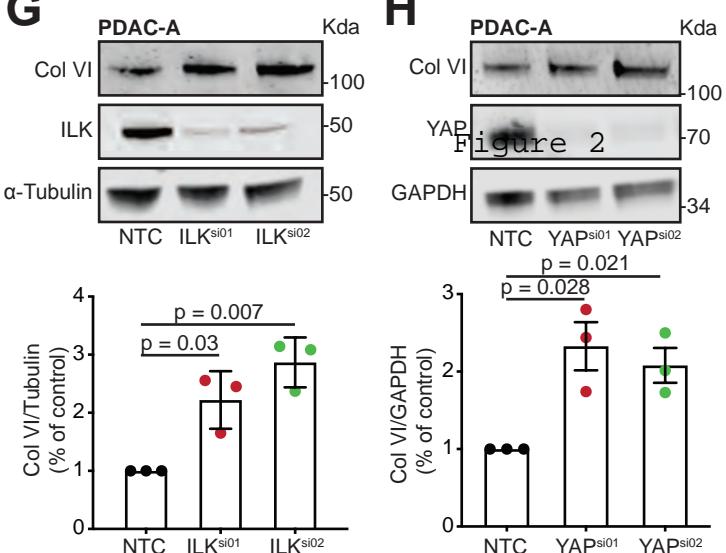
**E**



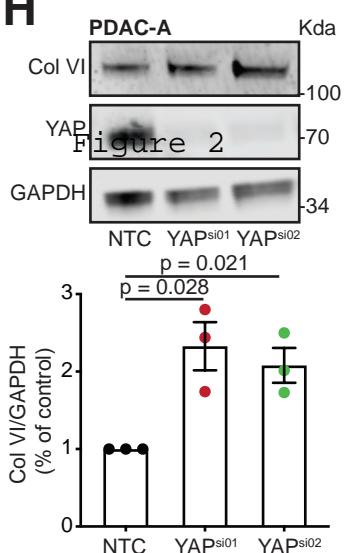
**F**



**G**



**H**



**Figure 2 – Collagen VI is upregulated in PDAC cells upon low substrate stiffness**

**A:** Schematic representation of the collagen VI assembly process in cells. The collagen VI monomer is a triple helix encoded by 3 genes: *Col6a1*, *Col6a2* and *Col6a3*. In certain cases, the *Col6a3* chain can be replaced by *Col6a4* (not functional in humans), *Col6a5* or *Col6a6*.

In B-D, PDAC-A and PDAC-B cells were cultured on 0.7, 7- and 38-kPa fibronectin-coated hydrogels and glass coverslips.

**B:** *Col6a1* mRNA expression was measured with qRT-PCR. Values are mean  $\pm$  SD and relative to control expression (*Cdk2*) from 3 independent experiments.

**C:** Collagen VI protein expression was measured by immunoblotting for Collagen VI (Col VI) and GAPDH (loading control). Blots are representative of three independent experiments.

**D:** Densitometric quantification of protein in C. Values are mean  $\pm$  s.d. of three independent experiments.

**E:** Schematic representation of main adhesion-linked pathways involved in sensing and responding to substrate stiffness by pancreatic cancer cells.

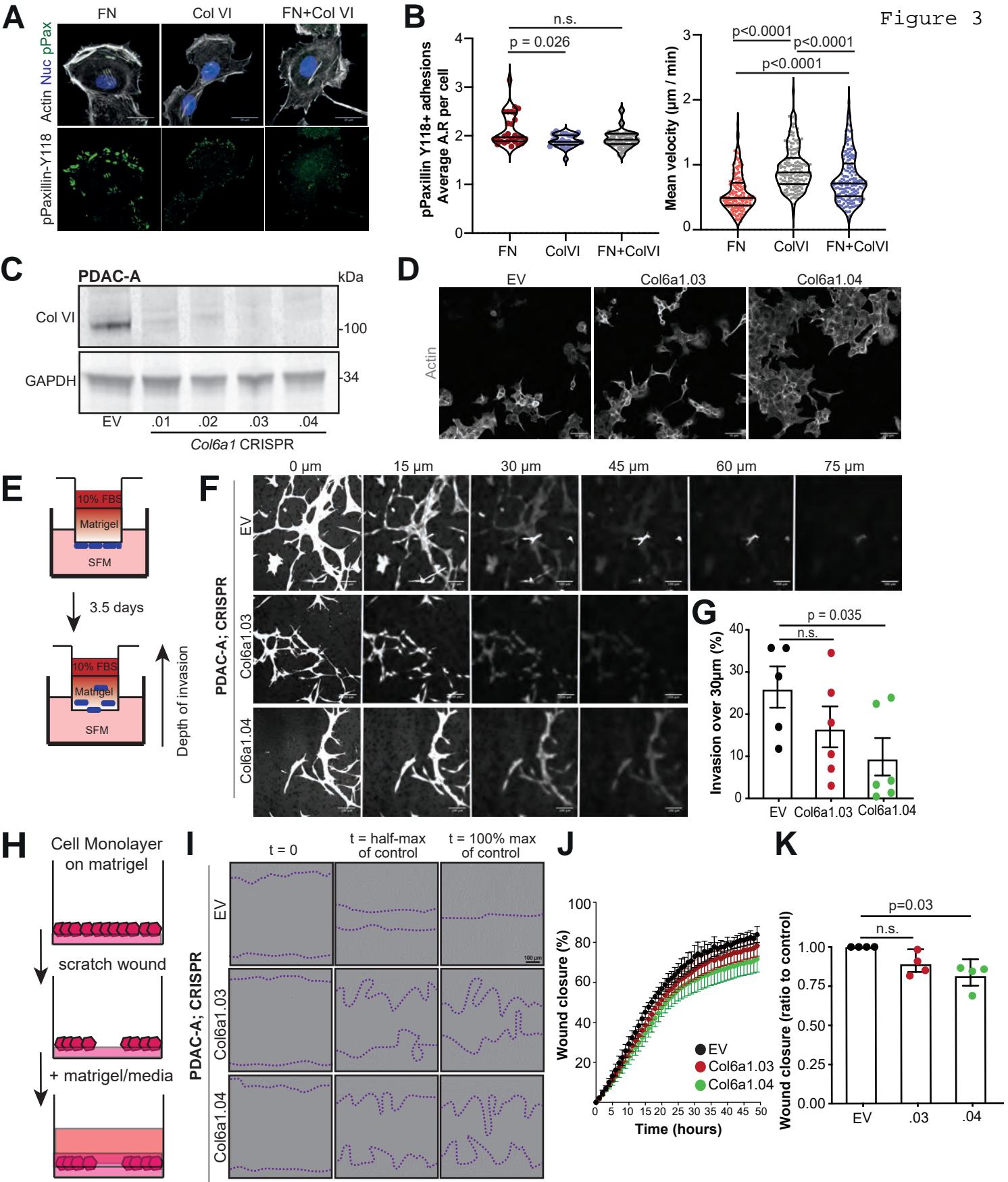
**F:** Top; PDAC-A cells were cultured on fibronectin (Fn, control), concanavalin A (ConA) or poly-L-lysine (PLL) coated glass coverslips and immunoblotting for Collagen VI and GAPDH (loading control) was performed. Bottom; Densitometric quantification of protein expression. Values are mean  $\pm$  s.d. of three independent experiments.

**G:** Top; Control (NTC) or ILK silenced (*Ilk<sup>si01</sup>*, *Ilk<sup>si02</sup>*) PDAC-A cells were immunoblotted for Collagen VI, ILK and  $\alpha$ -Tubulin (loading control). Bottom; Densitometric quantification of protein expression. Values are mean  $\pm$  s.d. of three independent experiments.

**H:** Top; Control (NTC) or YAP silenced (*Yap<sup>si01</sup>*, *Yap<sup>si02</sup>*) PDAC-A cells were immunoblotted for Collagen VI, YAP and GAPDH (loading control). Bottom; Densitometric quantification of protein expression.

All data in B-D and F-H are from 3 independent experiments. B, D, F, G, H: Statistical significance was assessed by two-tailed one-sample *t*-test on natural log-transformed values.

Figure 3



**Figure 3 – Collagen VI ECM supports migratory behaviour of PDAC cells *in vitro* and loss of Col6a1 expression delays invasion through recombinant basement membrane ECM.**

**A:** Top; immunofluorescence of PDAC-A cells cultured on fibronectin (FN), collagen VI (ColVI) or fibronectin and collagen VI (FN+ColVI) glass coverslips showing F-Actin (grey), phospho-Paxillin<sup>Y118</sup> (green) and nuclei (blue). Bottom; Individual phospho-Paxillin<sup>Y118</sup> channel (green). Scale bars, 20μm.

**B:** Left; Quantification of p-Paxillin-positive particles from A showing average aspect ratio of focal adhesions per cell. Values are from  $n = 27$  FN;  $n = 24$ , ColVI;  $n = 25$ , Fn+ColVI cells. Cells are from three independent experiments. Statistical significance was assessed by Kruskal-Wallis with Dunn's multiple comparisons test. Right; Cell speed from Fig. S4B. Values are from  $n = 155$  FN cells;  $n = 163$ , ColVI;  $n = 166$ , Fn+ColVI cells. Cells are from three independent experiments. Statistical significance was assessed by Kruskal-Wallis with Dunn's multiple comparisons test.

**C:** Control (EV) or Collagen VI depleted (Col6a1.01 – .04) mouse PDAC-A cell lines were immunoblotted for Collagen VI and GAPDH (loading control). Pictures are representative of 3 independent experiments.

**D:** Immunofluorescence of control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) PDAC-A cell lines showing Actin (grey). Scale bars, 50μm.

**E:** Schematic representation of the inverted invasion assay setup.

**F:** Representative pictures from z-stack acquisitions of control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) PDAC-A cell lines invading Matrigel plugs showing Calcein staining after 72 hours of invasion. Scale bars, 100μm.

**G:** Invasion over 30μm from F. Values are mean ± s.e.m. from 3 independent experiments. Statistical significance assessed with two-way Welch's t-test.

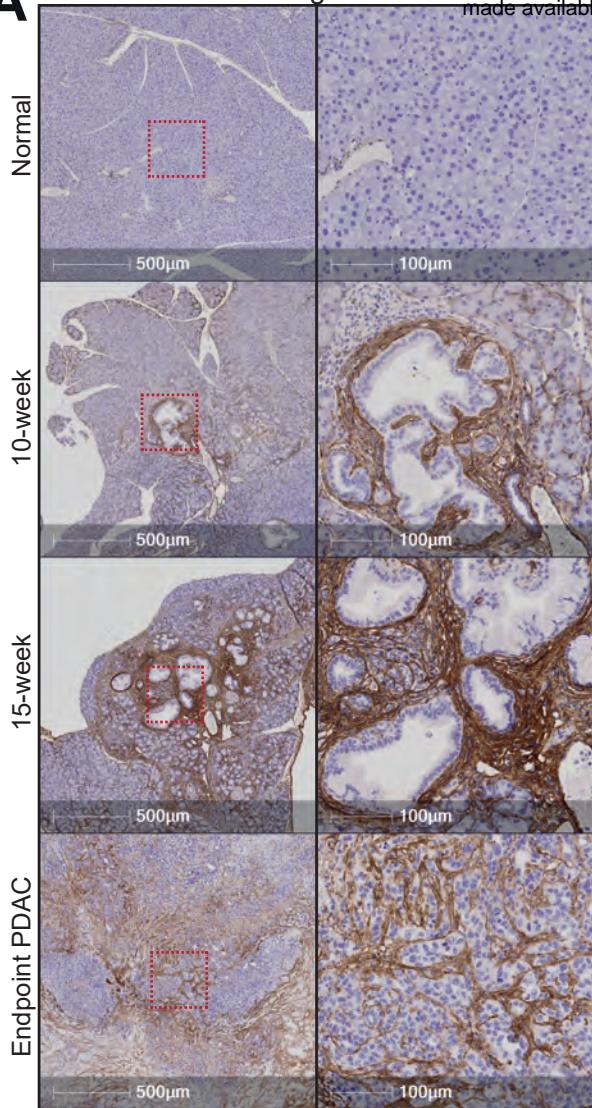
**H:** Schematic representation of the 3D ECM wound invasion assay setup.

**I:** Representative pictures of control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) PDAC-A cells invading 3D ECM. Scale bar, 100 μm.

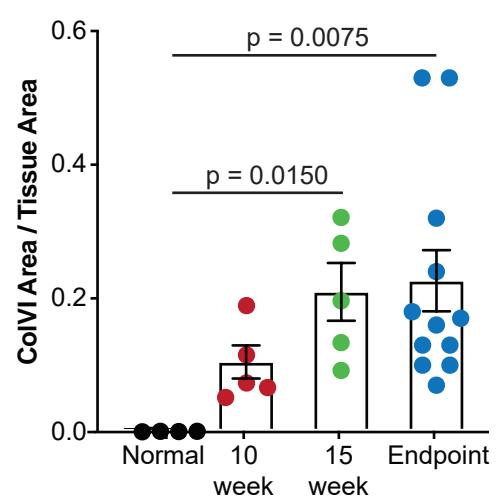
**J:** Wound closure over time of control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) PDAC-A cells invading 3D ECM. Values are mean ± SD from 4 independent experiments.

**K:** Relative wound closure of J normalised at  $t_{1/2}$  wound closure of control. Values are mean ± SD from 4 independent experiments. Statistical significance assessed by one-sample t-test on LN transformed values.

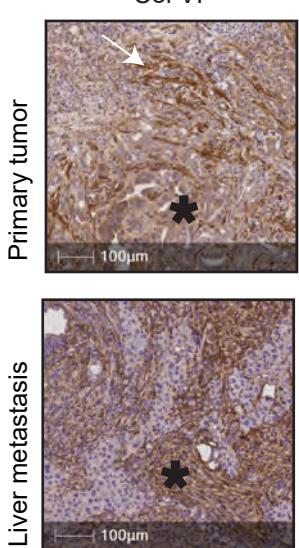
**A**



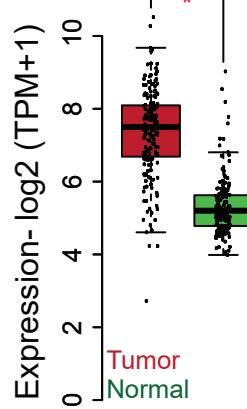
**B**



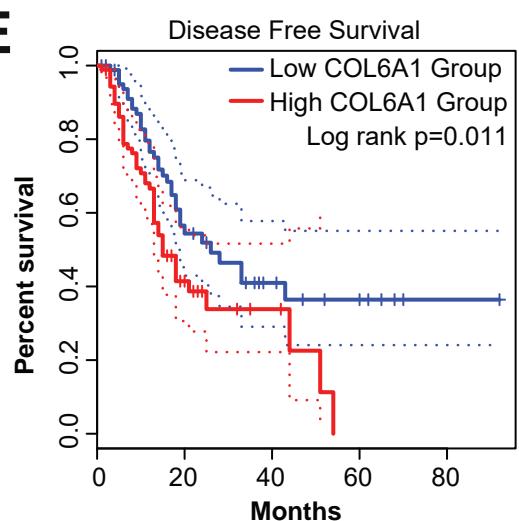
**C**



**D**



**E**



**Figure 4 – Collagen VI is upregulated during PDAC progression and correlates with reduction in disease-free survival.**

**A:** Immunohistochemistry of normal mouse pancreas (top panel), 10-week, 15-week and endpoint PDAC pancreas from KPC mice showing Collagen VI expression. Red boxes on left panel indicate magnified area (right panel). Scale bars, 500  $\mu$ m (left) and 100  $\mu$ m (right).

**B:** Quantification of Collagen VI positive area per tissue area from (A). Values are mean  $\pm$  s.e.m. from n = 4 normal, n = 5 10-week, n = 5 15-week and n = 12 mice. Statistical significance assessed by Kruskal-Wallis with Dunn's multiple comparisons test.

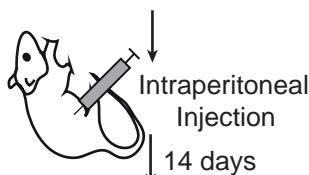
**C:** Representative images of immunohistochemistry from endpoint PDAC pancreas (primary tumor) and metastatic nodules in the liver from KPC mice showing Collagen VI expression. Scale bars, 100  $\mu$ m. Asterisks indicate pancreatic tumor cells and are representative from n = 3 mice with matched primary tumor and liver metastasis samples.

**D:** Expression of COL6A1 is upregulated in the tumors of pancreatic cancer patients compared to normal pancreas tissue specimens. Data taken from TCGA/GTEx (Tumor: n=179 patients; Normal: n=171 patients). Statistical significance assessed by two-sample t-test \* p<= 0.05.

**E:** Kaplan-Meier plot of disease-free survival stratifying patients based on COL6A1 expression in pancreatic tumors. High COL6A1 expression is associated with a significant reduction in disease free survival (Log rank p < 0.012). n = 89 patients with high COL6A1 expression and n = 89 patients with low COL6A1 expression.

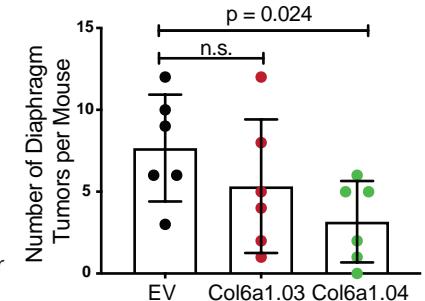
**A**

**KPC #127445**  
EV, Col6a1.03 or Col6a1.04

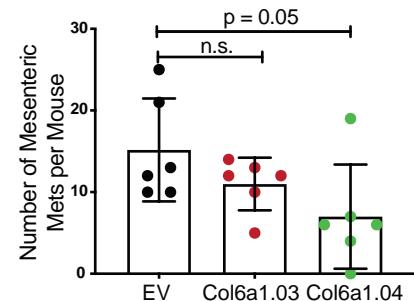


Abdominal Cavity and Diaphragm tumor formation assessment

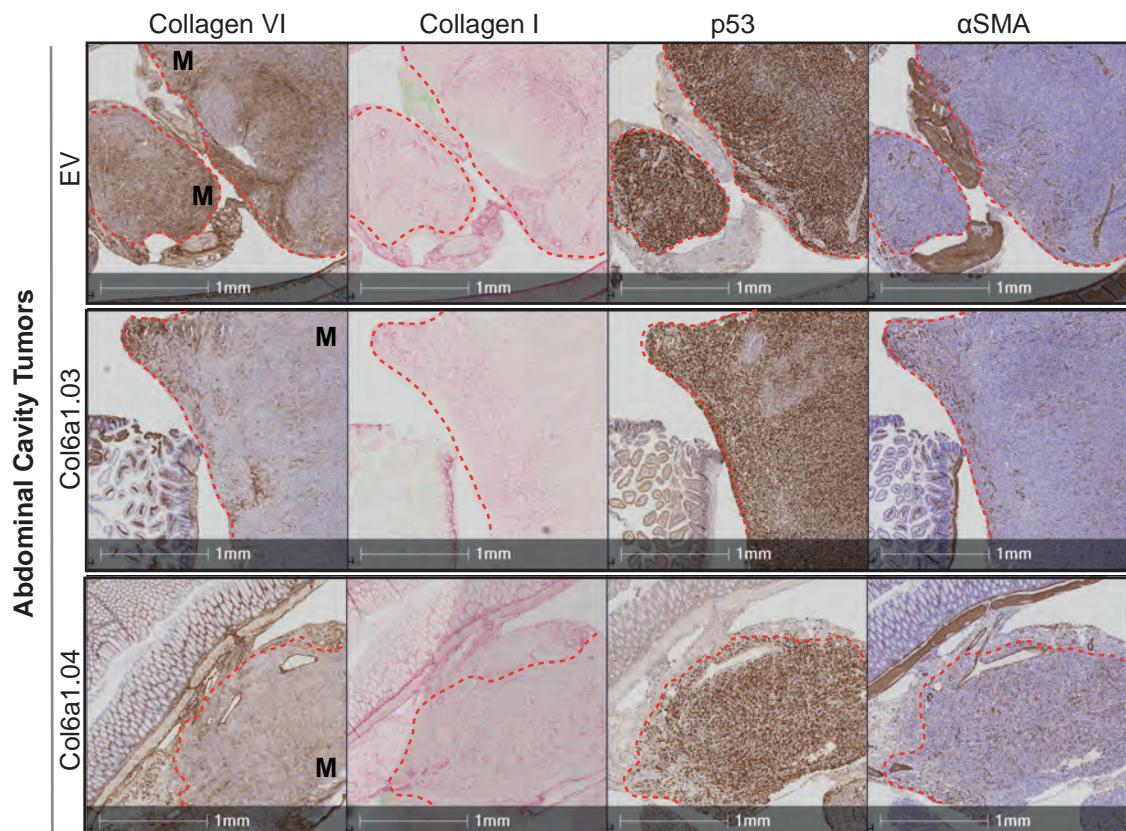
**B**



**C**



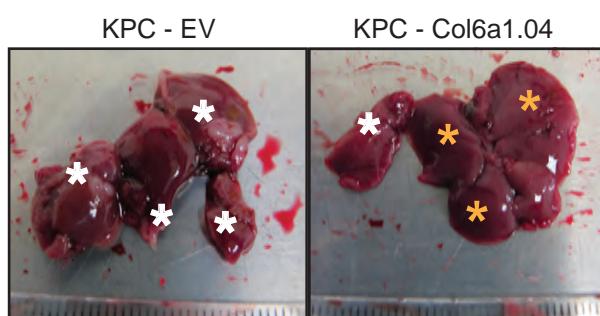
**D**



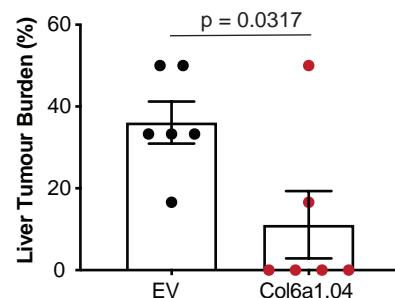
**E**

**KPC #127445**  
EV or Col6a1.04  
Intrasplenic administration  
14 days  
Liver Metastasis

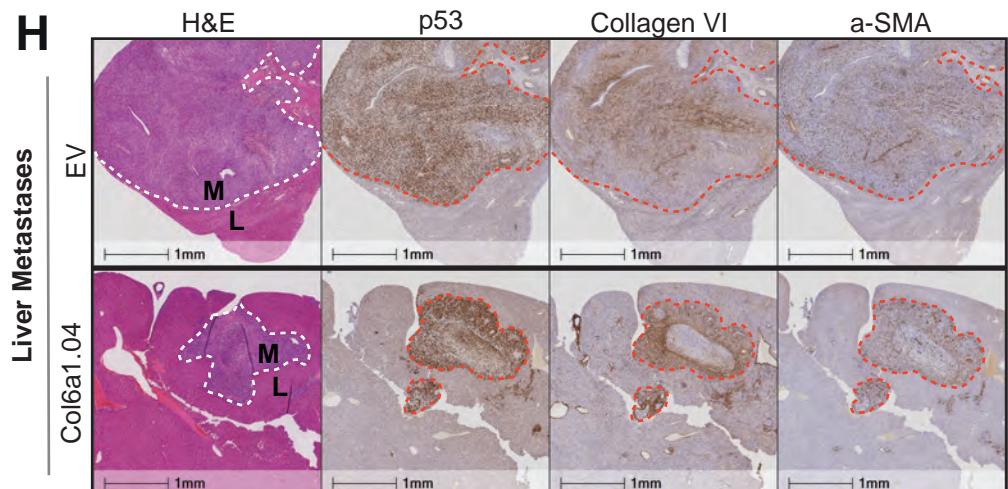
**F**



**G**



**H**



**Figure 5 – Collagen VI expression supports establishment of pancreatic metastasis in vivo.**

**A:** Schematic of intraperitoneal injection model. Control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) KPC cells were injected in the intraperitoneal cavity of nude (Cd1-*nu*) mice. After 14 days the mice were sacrificed, and the number of tumors was quantified before fixation of isolated tissues.

**B:** Quantification of diaphragm tumors per mouse as indicated. Values are mean  $\pm$  SD from n=6 EV, n=6 Col6a1.03 and n=6 Col6a1.04 mice. Statistical significance was assessed with Welch's t-test.

**C:** Quantification of abdominal cavity tumors per mouse as indicated. Values are mean  $\pm$  s.d. from mice receiving control or CRISPR cells n=6 EV, n=6 Col6a1.03 and n=6 Col6a1.04 mice. Statistical significance assessed with Welch's t-test.

**D:** Representative immunohistochemistry images showing Collagen VI, Collagen I, p53 (marks tumor, with mutant p53 accumulation) and  $\alpha$ SMA (marks fibroblasts) expression in abdominal cavity tumor nodules formed by intraperitoneal injection of control (EV) or Collagen VI depleted cells (Col6a1.03 and Col6a1.04). Red line denotes boundary between normal tissue and metastases (M). Scale bars, 1mm.

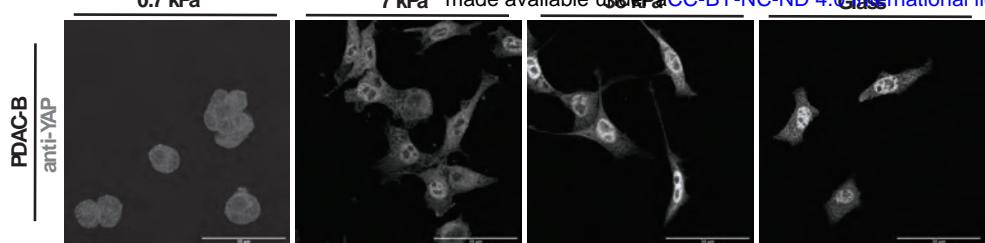
**E:** Schematic of intrasplenic injection model. Control (EV) or Collagen VI depleted (Col6a1.04) KPC cells were transplanted into the spleen of nude (CD1-*nu*) mice and 14 days later formation of metastatic tumors in the liver was assessed.

**F:** Representative liver pictures at the time of dissection. White asterisks indicate lobes with metastatic tumors and yellow asterisks indicate tumor-free liver areas.

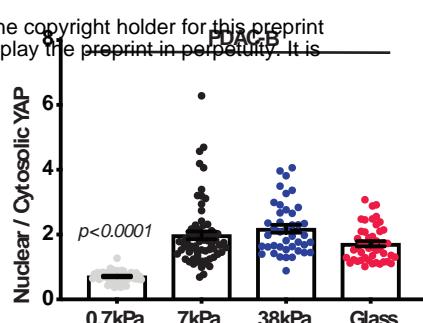
**G:** Liver tumor burden (% of liver lobes affected) of animals treated as indicated. Values are mean  $\pm$  s.e.m. from n = 6 EV and n = 6 Col6a1.04 mice. Statistical significance was assessed with Welch's t-test.

**H:** Representative immunohistochemistry images showing H&E, p53, Collagen VI and  $\alpha$ -SMA expression in liver sections from F as indicated. White or red dotted line denotes boundary between normal liver (L) tissue and metastases (M). Scale bars, 1mm.

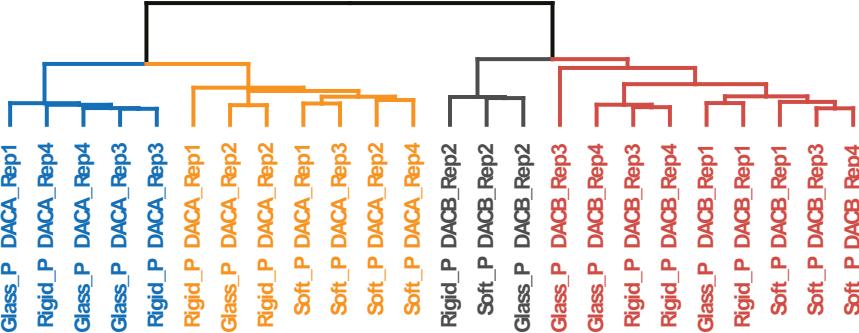
**A**



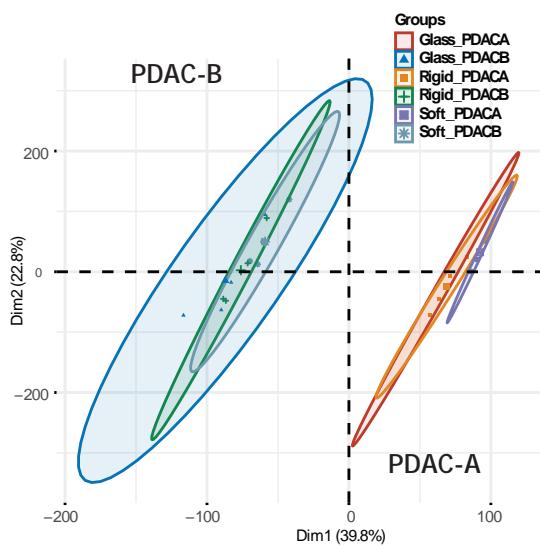
**B**



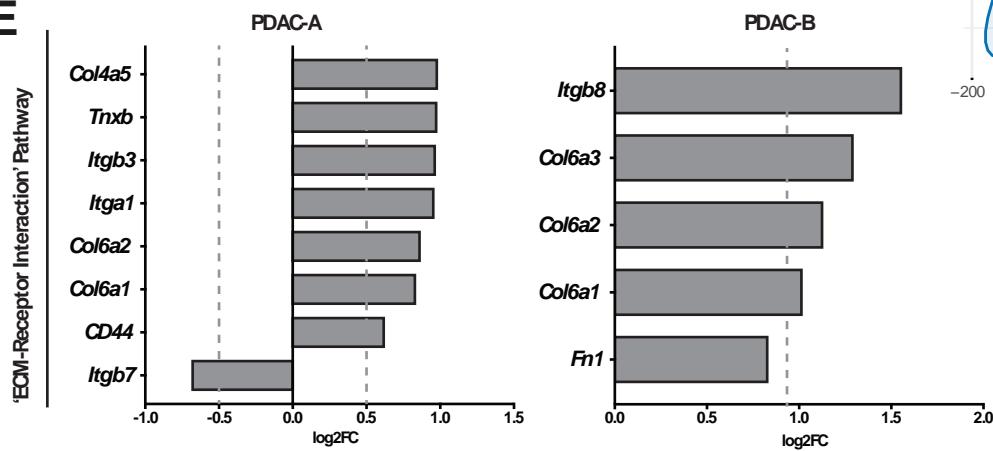
**C**



**D**



**E**



**Supplementary Figure 1 – Low substrate stiffness alters the expression of matrisome-related genes in PDAC cells.**

**A:** Immunofluorescence of PDAC-B cells cultured atop of 0.7 kPa, 7 kPa or 38 kPa fibronectin-coated polyacrylamide hydrogels for 24 hours, showing YAP1 (grey). Scale bars, 50  $\mu$ m.

**B:** Quantification of 'nuclear YAP1'/'cytosolic YAP1' intensity ratio for cells in (A). Values are mean  $\pm$  s.e.m. from n= 52 cells, 0.7kPa; n= 68, 7kPa; n= 42, 38kPa; n= 46, glass. Cells are from 3 independent experiments. Statistical significance was assessed by Kruskal-Wallis test with Dunn's multiple comparisons test.

**C:** Hierarchical clustering of RNA sequencing signatures from PDAC-A and PDAC-B cells cultured on fibronectin-coated 0.7 kPa, 38 PAAm hydrogels and glass coverslips for 24 hours.

**D:** PCA clustering of cells from (C).

**E:** Bar plot displaying differentially expressed genes ( $p_{adj} < 0.05$ ; log2 fold change  $>1$ ) from the 'ECM-Receptor Interaction' KEGG pathway that were enriched in cells from (C). Data is organised by log2 fold change.

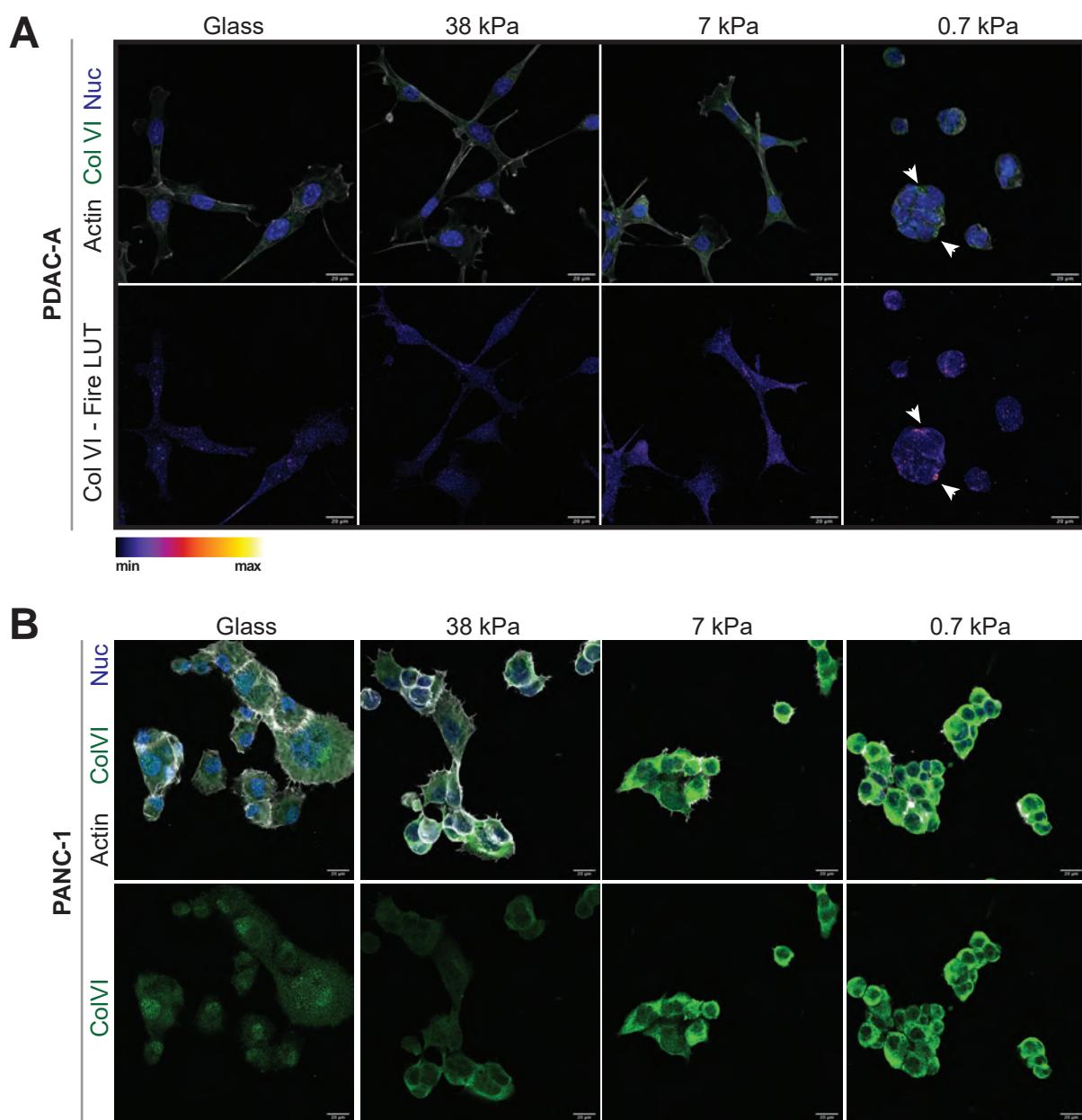
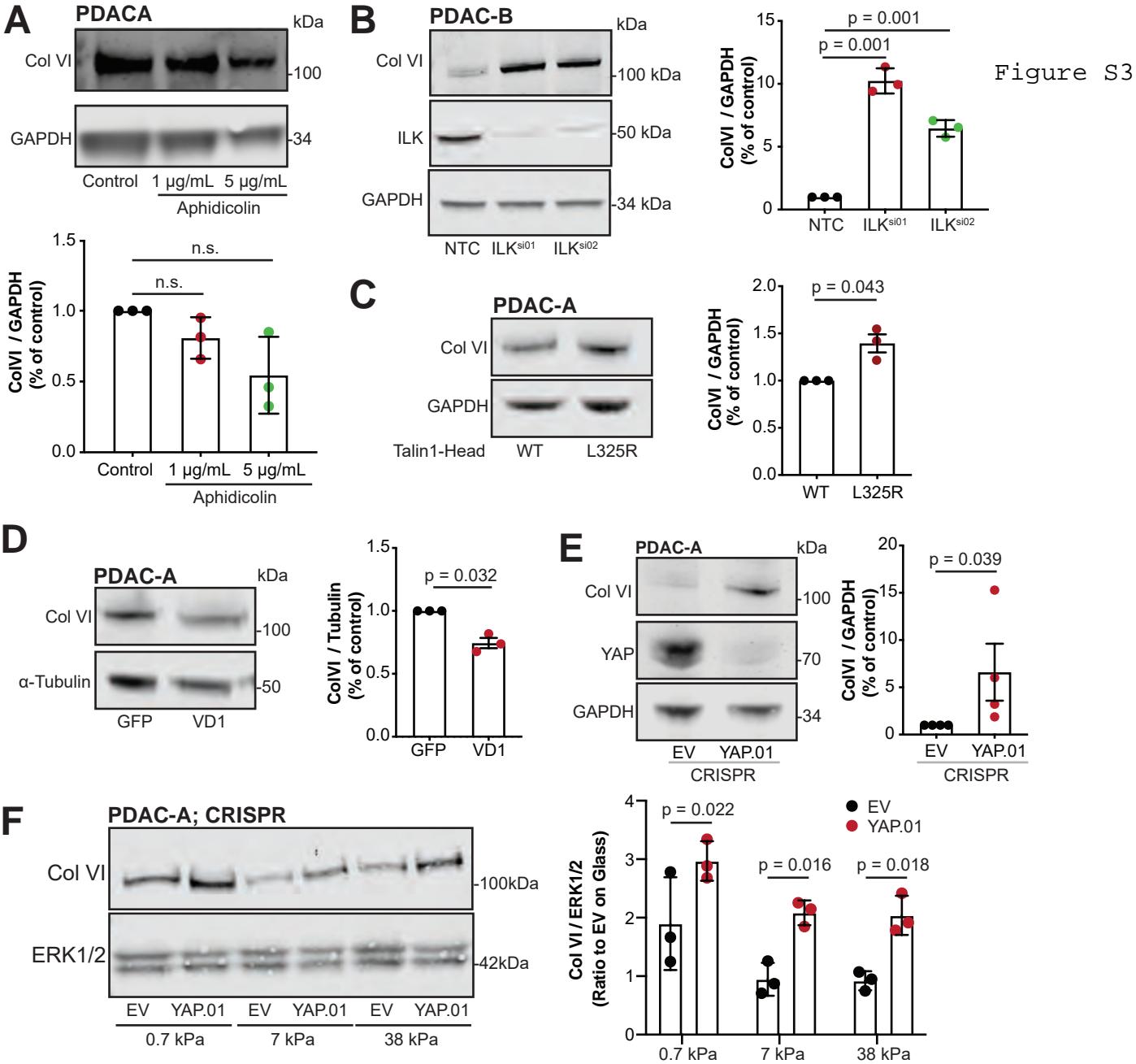


Figure S2

**Supplementary Figure 2 – Collagen VI is upregulated in PDAC cells upon low substrate stiffness**

**A:** Top; Immunofluorescence of PDAC-A cells cultured on glass coverslips, 0.7-, 7- and 38-kPa fibronectin-coated hydrogels, showing Collagen VI (green), Actin (grey) and nuclei (blue). Representative pictures from 3 independent experiments. Bottom; Individual Collagen VI channel (Fire LUT). Scale bars, 20 $\mu$ m. Arrowheads indicate Collagen VI enrichment.

**B:** Top; Immunofluorescence of PANC-1 cells cultured on glass coverslips, 0.7-, 7- and 38-kPa fibronectin-coated hydrogels, showing Collagen VI (green), Actin (grey) and nuclei (blue). Representative pictures from 3 independent experiments. Bottom; Individual Collagen VI channel (Green). Scale bars, 20 $\mu$ m.



**Supplementary Figure 3 – Loss of ECM adhesion and mechanosensing upregulates Collagen VI expression in PDAC cells.**

**A:** Top; PDAC-A cells were treated with 1  $\mu$ g/mL and 5  $\mu$ g/mL aphidicolin for 24 hours and immunoblotted for Collagen VI and GAPDH (loading control). Pictures are representative of 3 independent experiments. Bottom; Densitometric quantification of protein expression. Values are mean  $\pm$  s.d.

**B:** Left; Control (NTC) or ILK silenced ( $Ilk^{si01}$ ,  $Ilk^{si02}$ ) PDAC-B cells were immunoblotted for Collagen VI, ILK and  $\alpha$ -Tubulin (loading control). Right; Densitometric quantification of protein expression. Values are mean  $\pm$  s.d.

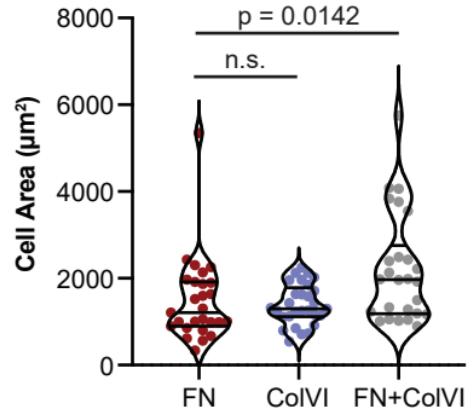
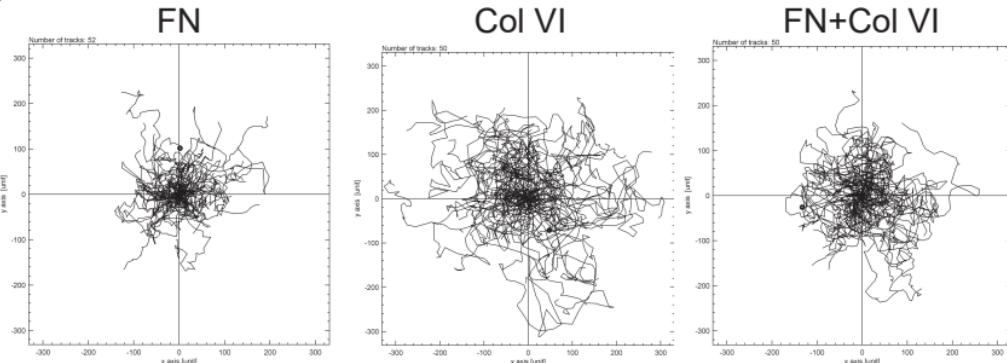
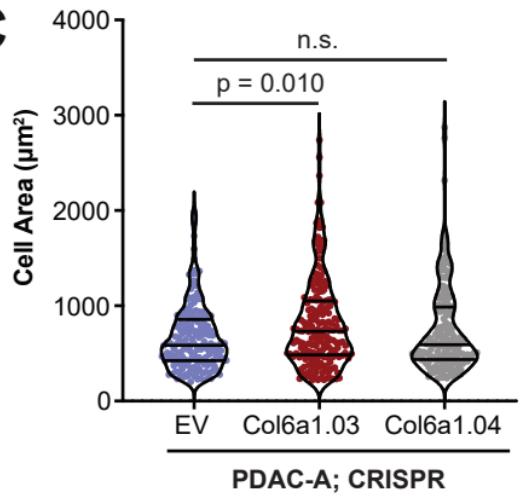
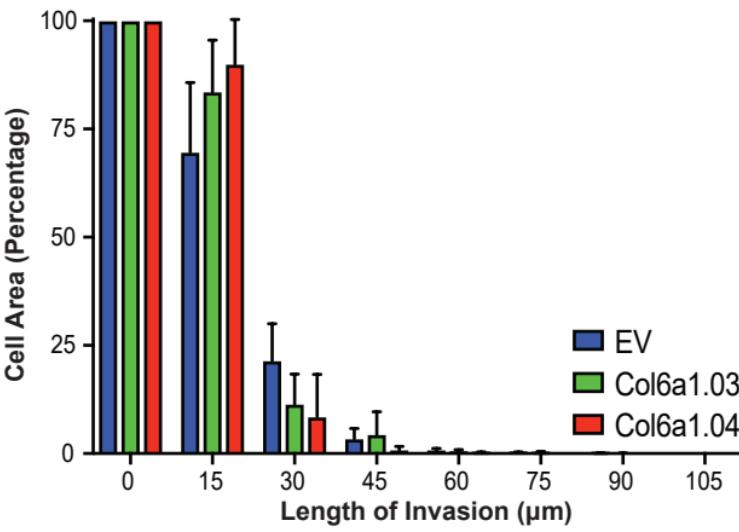
**C:** Left; PDAC-A cells expressing either Talin 1-head domain (WT, control) or Talin-head L325R mutant (L325R) were immunoblotted for Collagen VI and GAPDH (loading control). Right; Densitometric quantification of protein expression. Values are mean  $\pm$  s.d.

**D:** Left; PDAC-A cells expressing either GFP (control) or GFP-tagged Vinculin Domain 1 (VD1) were immunoblotted for Collagen VI and  $\alpha$ -Tubulin (loading control). Blots are representative of three independent experiments. Right; Densitometric quantification of protein in C. Values are mean  $\pm$  s.d.

**E:** Left; Control (EV) or YAP-depleted (YAP.01) PDAC-A cells were immunoblotted for Collagen VI, YAP and ERK1/2 (loading control). Pictures are representative of 4 independent experiments. Right; Densitometric quantification of ColVI protein. Values are mean  $\pm$  s.d.

**F:** Left; Control (EV) or YAP-depleted (YAP.01) PDAC-A cells were cultured on fibronectin-coated 0.7-, 7- and 38-kPa hydrogels and were immunoblotted for Collagen VI and ERK1/2 (loading control). Right; Densitometric quantification of protein. Values are mean  $\pm$  s.d. and representative from 3 independent experiments. Statistical significance was assessed by two-way ANOVA and p-values were corrected for multiple comparisons by Šídák's test.

All data in A-D are from 3 independent experiments. Statistical significance was assessed by two-tailed one-sample *t*-test on natural log-transformed values.

**A****B****C****D**Figure  
S4

**Supplementary Figure 4 – Collagen VI ECM supports migratory behaviour of PDAC cells *in vitro* and loss of Col6a1 expression delays invasion through recombinant basement membrane ECM.**

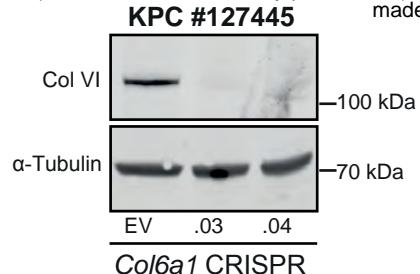
**A:** Cell area ( $\mu\text{m}^2$ ) quantification of PDAC-A cells cultured on fibronectin (FN), collagen VI (ColVI) or fibronectin and collagen VI (FN+ColVI) glass coverslips. Values are from  $n=27$  FN cells;  $n=29$ , ColVI;  $n=26$ , Fn+ColVI cells. Cells are from three independent experiments. Statistical significance was assessed by Kruskal-Wallis with Dunn's multiple comparisons test.

**B:** Tracks (spider plots) of PDAC-A cells migrating on fibronectin (FN), collagen VI (ColVI) or fibronectin and collagen VI (FN+ColVI) glass coverslips for 16 hours.

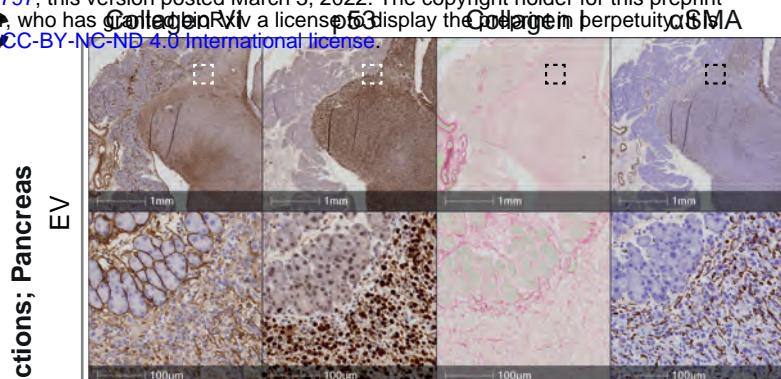
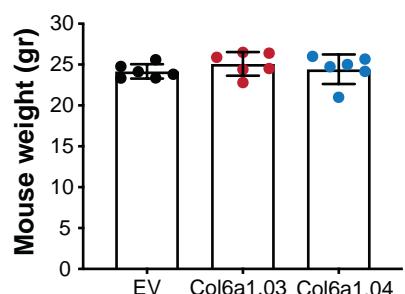
**C:** Cell area ( $\mu\text{m}^2$ ) quantification of Control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) mouse PDAC-A cells. Values are mean  $\pm$  s.d. from  $n=170$  EV,  $n=191$  Col6a1.03 and  $n=183$  Col6a1.04 cells from 3 independent experiments. Statistical significance was assessed by Kruskal-Wallis with Dunn's multiple comparisons test.

**D:** Quantification of invaded area of Control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) mouse PDAC-A cells invading through the inverted invasion assay setup. Intensity for each depth is reported as a percentage of intensity at 0  $\mu\text{m}$ . Values are mean  $\pm$  s.d. from 3 independent experiments.

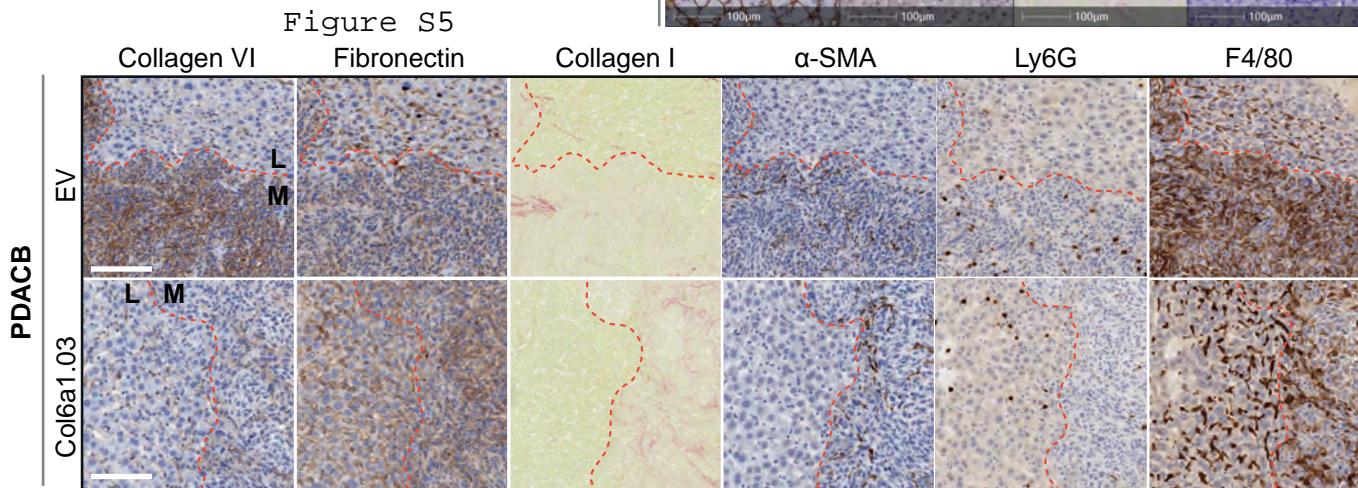
**A**



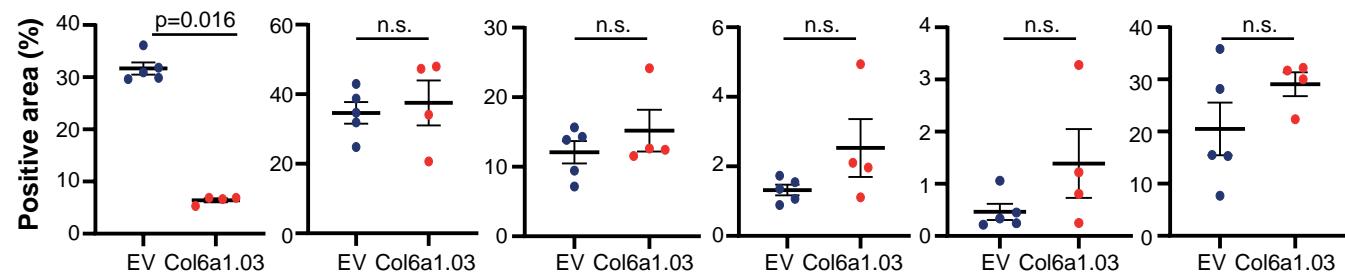
**B**



**D**



**E**



**Supplementary Figure 5 – Collagen VI expression supports establishment of pancreatic metastasis in vivo.**

**A:** Control (EV) or Collagen VI depleted (Col6a1.01-04) KPC cells were immunoblotted for Collagen VI and  $\alpha$ -Tubulin (loading control).

**B:** Weight (gr) per mouse as indicated from intraperitoneal injection of control (EV) or Collagen VI depleted (Col6a1.03 and Col6a1.04) KPC cells after sacrifice. Values are mean  $\pm$  SD from n = 6 EV, n = 6 Col6a1.03 and n = 6 Col6a1.04 mice.

**C:** Representative immunohistochemistry images showing Collagen VI, p53, Collagen I and  $\alpha$ -SMA expression in tumors formed in the pancreas by intraperitoneal injection of control (EV) (top 2 panels) or Collagen VI depleted (bottom 2 panels) KPC cells. Scale bars, 1mm and 100 $\mu$ m.

**D:** Representative immunohistochemistry images showing Collagen VI, Fibronectin, Collagen I,  $\alpha$ -SMA, Ly6G and F4/80 expression in liver metastatic nodules formed by intrasplenic injection of control (EV; top) or Collagen VI depleted (Col6a1.03; bottom) KPC cells. Red line denotes liver (L) and metastasis (M) boundary. Scale bars, 100 $\mu$ m.

**E:** Quantification of positively stained regions over tumor area (%) from D. Values are mean  $\pm$  s.e.m. from n = 5 control (EV) and n = 4 Col6a1.03 mice. Statistical significance was assessed by Mann-Whitney test (Collagen VI) and unpaired t-test (Fibronectin, Collagen I,  $\alpha$ SMA, Ly6G and F4/80).