Title: High-fat diet imprints a macrophage-tumor CAMP-P2RX7 axis driving pancreatic cancer initiation, plasticity and metastasis

Running title: High-fat diet-trained macrophages induce pancreatic cancer

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Statement of significance

High-fat diet induces pancreatic tumorigenesis in adult tissue, driving metastatic competence and a plastic state, and ultimately engages a macrophage-derived CAMP-P2RX7 circuit that reinforces immune evasion and accelerates PDAC progression.

Abstract

High-fat diet (HFD) and obesity are increasingly recognized as risk factors of pancreatic ductal adenocarcinoma (PDAC), yet the mechanisms by which dietary fat contribute to oncogenic transformation remain elusive. Using an inducible acinar-specific *Kras*^{G12V}/*Trp53*-loss genetically-engineered mouse model of PDAC, we established early- and late-onset protocols to assess age-dependent susceptibility to HFD. Specifically, HFD accelerated tumorigenesis with poorer prognosis in early-onset mice and, strikingly, enabled full PDAC development in late-onset adult mice otherwise resistant to oncogenic transformation. Tumors arising under HFD activated a distinct transcriptional and epigenetic state enriched in pathways or genes related to stemness, plasticity, and metastatic competence, which was maintained even in tumor-derived cell lines. Mechanistically, fatty acid-educated macrophages secreted the cathelicidin antimicrobial peptide (CAMP), activating P2X purinoceptor 7 (P2RX7) signaling in tumor cells to drive a highly plastic, immune-evasive phenotype reinforced by the expression of the peptidoglycan recognition protein 1 (PGLYRP1), further shielding tumor cells from macrophage phagocytosis. Functionally, HFD-induced tumors displayed enhanced metastatic potential independent of host context. Analysis of 164 human PDAC samples revealed that elevated body-mass index (BMI) was associated to a conserved CAMP-P2RX7-CXCR4 signature, maintained despite weight loss during disease progression. Together, these findings uncover a diet-imprinted macrophage-tumor cell circuit that promotes transformation and accelerates PDAC progression, positioning it as a therapeutic vulnerability in obesity-associated pancreatic cancer.

63 **Introduction**

64 High-fat diet (HFD) consumption and obesity are well-established risk factors for pancreatic ductal
65 adenocarcinoma (PDAC) (1). As processed food intake has dramatically increased worldwide over
66 the past decades in parallel with PDAC incidence, this association gains particular relevance in the
67 context of a disease with a dismal 5-year survival rate of approximately 13% in the US (2). Beyond
68 general PDAC cases, the rising incidence of “early-onset” patients (diagnosed before the age of 50)
69 underscores the contribution of exposome-related factors to disease initiation in younger populations
70 (3). However, traditional endogenous mouse models of PDAC, although invaluable over the past 20
71 years, are limited by the presence of oncogenic driver mutations from embryogenesis. In contrast,
72 patients typically acquire these mutations during adulthood, and many harbor preneoplastic lesions
73 that never progress to PDAC (4). Thus, the role of environmental risk factors and their ability to trigger
74 malignant transformation requires investigation in more biologically relevant models.

75 Our group previously demonstrated that genetically engineered mouse models (GEMMs) harboring
76 *Kras*^{G12V} mutations and *Trp53* loss, a hallmark of advanced PDAC, in the adult pancreas were
77 resistant to oncogenic transformation (5, 6). Only upon induction of chronic pancreatitis with cerulein
78 did mice develop pancreatic intraepithelial neoplasias (PanINs) and PDAC lesions. Acinar cell
79 resistance to transformation appeared reversible once inflammation was established, suggesting that
80 inflammatory events beyond pancreatitis could also drive tumorigenesis. Indeed, *Kras*-mutant acinar
81 cells that escape environmental clearance adopt a stem-like/quiescent state (7), and cancer stem
82 cells (CSCs) expand during the earliest stages of tumor development once inflammation-induced
83 metaplasia arises (8). These findings highlight that inflammatory cues fuel transformed cell plasticity
84 and tumor initiation, yet the impact of dietary habits on this process in adulthood remains poorly
85 defined.

86 Although some studies have shown that HFD accelerates tumorigenesis in embryonic *Kras*-mutated
87 GEMMs (9, 10), mechanistic insights into how diet promotes PDAC progression and metastasis in
88 adult models remain scarce. In this work, we evaluated the transformation capacity of adult acinar
89 cells upon *Kras*^{G12V} activation and *Trp53* loss using an inducible GEMM for PDAC that enables

90 temporal control of oncogenic expression, allowing direct comparison between early- and late-onset
91 protocols. We found that HFD induces systemic inflammation that drives PanINs and PDAC formation.
92 Notably, HFD-induced tumors exhibited a distinct transcriptomic program associated with enhanced
93 plasticity and metastatic potential. Mechanistically, fatty acid-exposed macrophages promoted
94 inflammatory signaling through the cathelicidin antimicrobial peptide (CAMP)-P2X purinoceptor 7
95 (P2RX7) axis, ultimately inducing this phenotype in tumor cells and revealing a potential therapeutic
96 opportunity to target HFD-driven PDAC.

97 **Materials and methods**

98 ***Mice.*** The *Kras*$^{+/LSLG12Vgeo}$;*Elas-tTA*/*tetO-Cre* (11) and the *Trp53*$^{lox/lox}$ (12) strains have been previously
99 described. The *Elas-tTA/tetO-Cre* transgenes drive expression of the bacterial Cre recombinase from
100 the *Elastase* promoter under the negative control of doxycycline (DOX) (Tet-off system). For the
101 postnatal adult protocols, DOX (2 mg/mL, Sigma Cat no. D3447) was provided in the drinking water
102 as a sucrose (5% w/v) solution to pregnant mothers (from the time of conception) and to their offspring
103 until the time required to allow expression of the Cre recombinase. For the early onset adulthood
104 protocol, mice were exposed to doxycycline until 3 weeks of age and then fed a HFD 60%
105 (Brogaarden, Cat no. D12492I) *ad libitum* or normal chow diet until 1 year or humane endpoint. For
106 the late onset adulthood protocol, mice were exposed to doxycycline until 8 weeks and at 12 weeks
107 of age were fed a HFD or normal chow diet until 1 year or humane endpoint (**Figure 1A**). Both female
108 and male mice were used for all experiments and body weight was monitored weekly. Necropsies
109 were performed in the dissection laboratory of the CNIO Animal Facility. Mice were euthanized in a
110 CO_2 chamber and tissue samples (pancreas, tumor, metastases, liver, lungs, intestine, white and
111 brown adipose tissue, spleen, kidney) were collected in 10% buffered formalin for histopathology. All
112 animal experiments were carried out in the animal facility of the CNIO and were approved by the
113 Ethical Committee of the CNIO and the Institute of Health Carlos III (PROEX 286.8/23). Mice were
114 genotyped by Transnetyx (Tennessee, USA).

115 ***Intrasplenic metastasis assay.*** For intrasplenic implantation, a total of 250,000 cells were
116 resuspended in 50 µL of phosphate-buffered saline (PBS; Gibco, USA). Mice were anesthetized with

117 4% isoflurane (Braun Vetcare, Germany) in 100% oxygen at a flow rate of 1.5 L/min and received
118 preoperative analgesia with buprenorphine as well as ophthalmic protection with Lacryvisc gel (Alcon,
119 Switzerland). Body temperature was maintained using heated beds throughout the procedure.
120 Following removal of abdominal hair, a small incision was made on the left flank to expose the spleen.
121 The cell suspension was injected directly into the splenic parenchyma using 30-gauge insulin
122 syringes. The peritoneal wall was closed with absorbable Vicryl sutures (Ethicon Inc., USA), and the
123 skin was sealed with wound clips (CellPoint Scientific Inc., USA). Tumor progression was monitored
124 weekly by abdominal ultrasound. After 21 days, mice were euthanized and tissue samples collected
125 for the analysis of liver metastases.

126 ***Tumor monitorization.*** Tumor development was assessed by weekly palpation and by ultrasound
127 imaging performed once per month. After tumor detection, ultrasound monitoring was conducted
128 weekly at the Molecular Imaging Core Unit of CNIO. For imaging, mice were anesthetized with 3%
129 isoflurane (Braun Vetcare, Germany) delivered in 100% oxygen at a flow rate of 0.5 L/min. Bed
130 heaters were used to avoid hypothermia due to the anesthesia and abdominal hair was locally
131 removed. Tumor measurements were obtained using a high-resolution Vevo 770 ultrasound system
132 (VisualSonics, Canada) equipped with a 40 MHz RMV704 probe (VisualSonics, Canada). Tumor sizes
133 were calculated as (Length*Width*Height)/2.

134 ***Primary cell lines.*** Murine pancreatic tumors obtained from genetically engineered mouse models
135 were excised, segmented into smaller fragments, and enzymatically digested with collagenase P (0.2
136 mg/mL, Merck, Cat no. 11213857001c) and DNAse (0.5 mg/mL, Roche, Cat no. 776785) at 37°C for
137 15 minutes. This was followed by a 3-minute incubation at 37°C in 0.25% trypsin. The resulting cell
138 suspensions were cultured in RPMI 1640 medium (Cat. no. R8758), supplemented with 10% fetal
139 bovine serum and 50 units/mL penicillin-streptomycin. Epithelial clones were subsequently isolated,
140 pooled, and expanded to establish a heterogeneous cancer cell line. Human PDAC PDX-derived cell
141 cultures were prepared as previously described from patient-derived xenografts (21). The original
142 PDXs were generously provided by Dr. Manuel Hidalgo through a Material Transfer Agreement with

143 the Spanish National Cancer Centre (CNIO), Madrid, Spain (Reference no. I409181220BSMH). All
144 primary cultures underwent routine Mycoplasma testing at intervals of no more than four weeks.

145 ***PBMC isolation.*** Peripheral blood was collected in 4 mL EDTA Vacutainer tubes (BD, USA) and
146 centrifuged at 1200xg for 10 minutes at room temperature. Plasma was then collected, aliquoted and
147 stored at -80°C for future use. The remaining cell pellet was resuspended in 2 mL PBS, laid on top of
148 3 mL Ficoll-Paque (Cytiva, UK) and centrifuged at 400xg for 30 minutes with the brakes off. The PBMC
149 buffy coat was collected, washed with 2 mL PBS mixed with 1mL red blood cell lysis buffer (Invitrogen,
150 USA) and centrifuged at 260xg for 5 minutes. Additional washes with PBS were performed until the
151 supernatant was clear. Patient blood samples were obtained from the Accelerated Detection of
152 neuroEndocrine and Pancreatic TumourS (ADEPTS) biobank led by Professor Stephen Pereira.
153 Ethical approval for this study was granted by the NHS Research Ethics Committee at Queen's
154 Square, London, UK (REC reference: 06/Q0512/106). Written informed consent was provided by all
155 participants who donated samples.

156 ***Flow cytometry.*** Tumor samples underwent enzymatic digestion to produce single-cell suspensions.
157 In brief, tissues were minced using a scalpel and forceps, transferred into 50 mL conical tubes, and
158 each tube received 5 mL of serum-free medium plus 50 µL of a digestion solution consisting of
159 Collagenase P (0.2 mg/mL) and DNase (0.5 mg/mL). Samples were incubated at 37°C for 30 minutes
160 under continuous agitation. To halt digestion, 1 mL RPMI supplemented with 10% FBS was added.
161 After centrifuging at 1500 rpm for 5 minutes, supernatants were discarded. ACK buffer-mediated
162 erythrocyte lysis was carried out by incubation at room temperature for 5 minutes. Following this, cells
163 were washed in DMEM, centrifuged again, the supernatant removed, and resuspended in flow
164 cytometry buffer (PBS with 2% FBS and 3 mM EDTA).

165 For cell lines, PBS 1X washing steps preceded trypsinization with Trypsin/EDTA 0.25% (Lonza, Cat
166 no. CC-5012). Post-trypsinization, the cells were resuspended in complete RPMI medium. When
167 analyzing murine cells, cell suspensions were treated with purified anti-mouse CD16/CD32 antibodies
168 (eBioscience, Cat no. 14-9161-73) to block Fc receptor binding. Human cell preparations were

169 incubated for 15 minutes at 4°C with blocking buffer (PBS with 5% FBS and 3 mM EDTA) to reduce
170 non-specific binding.

171 Antibodies used for surface marker staining are detailed in **Supplementary Table 1**. For excluding
172 non-viable cells, samples were stained with DAPI at 2 µg/mL (4',6-diamidino-2-phenylindole; Sigma,
173 Cat no. D9542-5MG). Cells were then suspended in flow cytometry buffer [1X PBS, 3 mM EDTA (v/v)]
174 prior to analysis. Flow cytometry was performed on an Attune™ NxT Acoustic Focusing Cytometer
175 equipped with four lasers (Thermo Fisher Scientific), and data analysis was completed using FlowJo
176 software (Tree Star Inc., Ashland, OR).

177 ***Histopathology.*** For histopathological analyses, tissues were serially sectioned (3 µm thick), and
178 every 10th section was stained with hematoxylin and eosin (H&E) by the CNIO Histopathology unit.
179 Representative sections were chosen for the grading and enumeration of lesions and quantification
180 of tissue damage. For H&E-stained slides of pancreata, areas were marked as normal tissue, ADM,
181 low grade and high grade PanINs or PDAC tumor tissue. Moreover, tumors were classified as
182 differentiated or poorly differentiated using the guidelines from the College of American Pathologists
183 (https://documents.cap.org/protocols/cp-pancreas-exocrine-17protocol-4001.pdf).

184 ***Immunohistochemistry.*** Specimens were fixed in 10% formalin (Sigma) and embedded in paraffin.
185 Immunohistochemistry staining was performed by the Histopathology Unit of CNIO. For
186 histopathological analysis, specimens were serially sectioned (3 µm thick) and stained with H&E.
187 Antibodies used for mouse tissue are included in **Supplementary Table 1.** Images were analyzed
188 and exported using QuPath software (v0.5.0). For human tissue, patients have been selected from a
189 cohort already published (13). Immunohistochemistry has been performed as detailed before (14).
190 Antibodies used for human samples are included in **Supplementary Table 1**. For evaluating the
191 expression, a combined semiquantitative score was adopted, taking into account both the percentage
192 score, which is obtained based on the percentage of stained cells (score 0 = 0%, 1 = 1-25%, 2 = 26-
193 50%, 3 = 51-75%, 4 = 76-100%) and the intensity score, which is based on the intensity of the staining
194 (score 0 = no stained cells, 1 = weak staining, 2 = moderate staining, 3 = strong staining). For
195 obtaining the value of combined semiquantitative score, the value of percentage score and the value

196 of intensity score are multiplied. Thus, the combined / semiquantitative score ranges from 0 to 12.
197 The evaluation of the scores has been performed on neoplastic cells and immune cells.

198 ***Immunofluorescence and microscopy.*** Microscopy brightfield images were captured with an EVOS
199 FL Microscope (Invitrogen, Cat no. AMF4300). For immunofluorescence, FFPE sections were
200 prepared at the CNIO Histopathology unit, deparaffinized in graded ethanol, and underwent antigen
201 retrieval using 1 mM EDTA. After blocking (PBS 1x, 1% BSA, 2% FBS), primary antibodies were
202 incubated overnight at 4°C, followed by 1 hour at 37°C with secondary antibodies and DAPI. Samples
203 were mounted in ProLong (Invitrogen, Cat no. P36970) and imaged with a Confocal Stellaris 8 STED
204 microscope (Leica). Image analysis was performed using ImageJ.

205 ***RNA extraction and RT-qPCR.*** For qPCR analysis, RNA was extracted using guanidine thiocyanate
206 as described before (15) and cDNA synthesized with the SS II cDNA synthesis kit (Cat no. 18064022,
207 Invitrogen) followed by RT-qPCR analysis using SYBR green and a StepOne Plus real-time thermo-
208 cycler (Applied Biosystems). The thermal cycle consisted of an initial denaturation step of 10 min at
209 95°C followed by 40 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C). The results
210 obtained for each gene were normalized with the β-actin levels. Primers are listed in **Supplementary**
211 **Table 2**.

212 ***Library preparation for RNA-seq***. RNA concentration was determined using a NanoDrop
213 spectrophotometer, and RNA quality was evaluated based on the RNA Integrity Number (RIN) using
214 the LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer). RNA quality control, library preparation,
215 and sequencing were performed at the CNIO Genomics Unit. For library generation, 1 µg of total RNA
216 was processed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760),
217 following the manufacturer's recommendations. Briefly, the poly(A)+ RNA fraction was isolated and
218 fragmented, followed by synthesis of double-stranded cDNA. Subsequent enzymatic steps included
219 end-repair, dA-tailing, and adapter ligation. The adapter-ligated products were amplified by PCR using
220 Illumina paired-end primers. Final libraries were loaded onto an Illumina flow cell for cluster generation
221 and sequenced on an Illumina NextSeq 500 system according to the manufacturer's protocol.

222 ***DNA extraction and Illumina Mouse Methylation microarrays.*** Genomic DNA was extracted at the
223 Genomics Unit of the Josep Carreras Leukemia Research Institute. Briefly, DNA was isolated from
224 cell pellets using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and quantified with a
225 Qubit fluorometer. DNA integrity was assessed by agarose gel electrophoresis, and samples (≥600
226 ng per sample) were bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research Corp.,
227 CA, USA) according to the manufacturer's instructions. Bisulfite-converted DNA was then hybridized
228 to Infinium Mouse Methylation microarrays as previously described by García-Prieto *et al.* (16).

229 ***Endoscopic ultrasound database generation.*** Upon approval from the Internal Review Board (IRB
230 BIO-PANCREAS, version 3, 2021), specimens were collected from patients diagnosed with
231 pancreatic ductal adenocarcinoma (PDAC) at San Raffaele Research Hospital. Eligible subjects
232 included treatment-naïve individuals with clinical histories and radiological findings indicative of
233 PDAC. Patients were not involved in the design, implementation, analysis, or dissemination of the
234 study. Informed consent was obtained prior to conducting diagnostic EUS procedures under deep
235 intravenous sedation with Propofol (Diprivan, Zeneca, Germany). Procedures utilized a Pentax
236 therapeutic linear echoendoscope (EG3870UTK, EG38J10UT) in conjunction with Hitachi ultrasound
237 systems (Arietta 850, Arietta V70). EUS-guided tissue acquisition (TA) was performed using a 25G
238 fine-needle aspiration (FNA) needle (Expect SlimLine, Boston Scientific) employing the slow-pull
239 technique. RNA was subsequently isolated, quantified, and evaluated according to established
240 protocols (17).

241 ***RNA-sequencing analysis.*** FASTQ file integrity was verified using MD5 checksum comparison, and
242 read quality was assessed with FastQC, with optional contamination screening via FastQ Screen; all
243 quality metrics were summarized in a MultiQC report. Reads from different lanes were concatenated,
244 optionally downsampled to a fixed number of reads using a defined seed and trimmed for adapters
245 and low-quality bases using bbduk (BBTools). Reads were then aligned using STAR, HISAT2, or
246 Salmon as specified in the configuration; STAR and HISAT2 used genome indices generated from a
247 reference FASTA and GTF annotation (with HISAT2 outputs subsequently sorted using samtools),
248 while Salmon performed pseudo-alignment using a transcriptome-based index. For STAR and

249 HISAT2 alignments, gene-level quantification was performed with htseq-count or featureCounts after
250 indexing BAM files with samtools, using the same GTF annotation employed for alignment.
251 Per-sample count files were merged into a final counts matrix for downstream differential expression
252 analysis. Differentially expressed genes (DEGs) were identified from count matrices using DESeq2 in
253 R, excluding genes with fewer than five counts. The Wald test was employed to determine significant
254 changes in expression between conditions, while Principal Component Analysis (PCA) was used to
255 evaluate variability among samples. DEGs for each condition were selected considering Log2FC ≠ 0
256 and p ≤0.05 and subsequently ranked according to their Log2FC value. Gene set enrichment analysis
257 (GSEA) was performed using annotations from Reactome, KEGG, and MSigDB
258 (www.broadinstitute.org/gsea/index.jsp), as well as custom gene sets described in **Supplementary**
259 **Table 3**. In addition, genes were ranked using moderated t statistics from limma. After applying the
260 Kolmogorov-Smirnov test, gene sets with FDR < 0.25 were considered significantly enriched.

261 For the EUS dataset, we performed two kinds of analyses. For the differentially expressed genes in
262 patients with body mass index (BMI) <25 or ≥25, we employed limma package (R/Bioconductor). A
263 design matrix was constructed including BMI group as the primary variable of interest and adjusting
264 for relevant covariates (sex, age, smoking status, batch, and RNA extraction methodology). Linear
265 models were fitted to the expression matrix using lmFit, followed by empirical Bayes moderation with
266 eBayes. Differentially expressed genes for the *Obese vs. reference BMI group* contrast were
267 extracted using topTable. To assess global transcriptional patterns and sample structure, a principal
268 component analysis (PCA) was conducted, and samples were visualized according to BMI group.
269 Differential expression results were further summarized using a volcano plot highlighting genes with
270 nominal $p < 0.05$. Significant genes were filtered based on nominal significance and exported with
271 their log fold changes, *p*-values, and adjusted *p*-values for downstream interpretation. For a set of
272 predefined stem-related genes, we generated violin plots to visualize their expression distribution
273 across BMI groups. For each gene present in the expression matrix, a sample-level dataset was
274 constructed by pairing log-RPKM expression values with the corresponding BMI group. Violin plots
275 were generated using ggplot2, combining kernel density estimates with overlaid boxplots to

276 summarize central tendency and dispersion. Group differences in gene expression were statistically
277 evaluated using the Wilcoxon rank-sum test, and the resulting *p*-values were annotated directly on
278 each plot.

279 For the organoid implantation assay dataset (GSE148135), Raw counts were normalized and
280 converted in logCPM using edgeR (R package). For the figure, the p values refer to Dunn_test that
281 was used post Kruskall-Wallis to test difference in expression.

282 For the PBMC RNA-seq, RNA was extracted from isolated PBMC samples using the RNeasy Mini Kit
283 (Qiagen, Germany), according to the manufacturer's recommendations. RNA concentration and
284 integrity were measured using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher, UK) and
285 TapeStation RNA High sensitivity assay (Agilent, USA), respectively. In collaboration with UCL
286 Genomics (UCLG), cDNA libraries were prepared using the Watchmaker RNA Library Prep Kit with
287 Polaris rRNA and Globin Depletion (Watchmaker Genomics, USA). Libraries were indexed using IDT
288 xGen unique dual index (UDI) adapters incorporating unique molecular identifiers (UMIs) and
289 sequenced with the NovaSeq 6000 (S4 v1.5 flow cell, 300 cycles, XP workflow), to generate paired-
290 end 150 bp reads, yielding approximately 40 million read pairs per sample. Demultiplexing, quality
291 control (FastQC and MultiQC), and transcript quantification using Salmon (pseudo-mapping) were
292 performed by UCLG to generate gene-level raw count matrices for downstream analysis. Data
293 normalization and differential gene expression analysis were conducted using DESeq2 via the
294 SARTools package, developed by Hugo Varet *et al.* (17), and analysis quality was assessed using
295 principal component analysis (PCA) and additional diagnostic visualizations. Significantly differentially
296 expressed genes were identified as those with an adjusted p value <0.05 and log2 fold-change<1
297 between comparison groups.

298 ***DNA methylation analysis.*** Raw signal intensity files (IDATs) were processed using standard settings
299 in the SeSAMe R package. Unsupervised analyses of the global methylome were performed using t-
300 SNE. Supervised analyses were conducted by calculating mean methylation values per condition and
301 per CpG site, and CpG sites with absolute methylation differences between conditions ≥0.33 were
302 considered biologically meaningful. Differential methylation was assessed using a linear regression

303 model implemented in the limma R package, and CpG sites with an adjusted P-value ≤0.05 were
304 considered significant. CpG sites were annotated using the annotation described in García-Prieto *et*
305 *al*., and downstream analyses included gene set enrichment analysis of gene-associated CpG sites
306 using the EnrichR R package and the Panther database. Pathways were considered significant if they
307 had adjusted P-values ≤0.05.

308 ***hCAP-18/LL-37 ELISA.*** Patients with suspected or confirmed pancreatic ductal adenocarcinoma
309 (PDAC) were recruited from the General Surgery, Gastroenterology, and Medical Oncology
310 departments of Ramón y Cajal University Hospital (Madrid, Spain). The study was approved by the
311 local ethics committee, and all participants provided written informed consent. Blood samples were
312 collected at study entry, prior to any oncological treatment, for serum isolation. Serum samples from
313 healthy individuals without a history of digestive disease or cancer were obtained from the Ramón y
314 Cajal-IRYCIS Biobank (PT20/0045; PT23/00098), part of the Spanish National Biobanks Network
315 (ISCIII Biobank Register No. B.0000678). Serum LL-37 levels were quantified using a commercial
316 ELISA kit (ELabScience, E-EL-H2438) according to the manufacturer's instructions. Samples were
317 diluted 1:10 and incubated on plates pre-coated with LL-37-specific capture antibodies. Following
318 incubation with detection antibodies and chromogenic substrate, absorbance was measured at 450
319 nm with correction at 540 nm. Concentrations were calculated by interpolation from a standard curve
320 and expressed as ng/mL. All samples were analyzed in duplicate, and those with intra-assay
321 coefficients of variation >15% were excluded.

322 ***Macrophage isolation.*** Murine bone marrow-derived cells (BMDCs) were harvested from the femurs
323 and tibiae of a minimum of three mice using centrifugation. The resulting BMDC pellets were
324 resuspended in RPMI 1640 medium (Invitrogen, Cat. No. 61870044) and incubated for 24 h to
325 facilitate monocyte attachment. Subsequently, monocytes were differentiated into macrophages
326 under adherent conditions for 5-7 days on non-tissue culture-treated 100 mm dishes in RPMI
327 supplemented with 10% FBS and 10 ng/mL murine M-CSF (PeproTech, Cat. No. 315-02). Additionally,
328 certain experiments utilized immortalized murine bone marrow-derived macrophages generously
329 provided by Dr. Antonio Castrillo.

330 ***Macrophage conditioning and co-culture assays.*** After differentiation, for macrophage
331 polarization, they were cultured without stimulation to obtain untrained conditioned medium or with
332 stimulation (fatty acids) for 24 h to obtain a lipid loaded state. Then, polarization medium was removed
333 and RPMI was left to be conditioned for 48 h to obtain fatty acid trained-macrophage conditioned
334 medium.

335 ***Phagocytosis assay.*** Five days after isolation of macrophages, tumor fluorescent cells were
336 harvested and seeded together with macrophages during 24 h. Afterwards, cells were detached using
337 scrapers and macrophages phagocytosing fluorescent tumor cells were measured by flow cytometry,
338 as described above, gating on the double-positive population: F4/80 and reporter genes (i.e., YFP).

339 ***Stimulation assays.*** For stimulation assays, tumor or immune cells were seeded in 6-well plates and
340 treated with 1 mL of complete RPMI medium plus recombinant CAMP (5 µg/mL, Cat. no. HY-P4855,
341 MedChemExpress) during 24h/48h, fatty acids (50 µM, oleic acid; 50 µM sodium palmitate; 200 µM
342 linoleic acid; Cat no. O3008, P9767, L9530) or MØ-derived conditioned media. After indicated time
343 points, cells were trypsinized or scraped and harvested for flow cytometry of the indicated markers or
344 RNA-seq analysis.

345 ***Spheroid formation assay.*** Cancer stem cell-enriched spheroids were cultured employing 10,000
346 cells per well in ultra-low attachment plates (Corning) during 4-7 days using serum-free DMEM/F12
347 medium (Invitrogen, Cat no. 21331046) with B27 (1:50; Invitrogen, Cat no. 17504044), 20 ng/mL
348 bFGF (PAN-Biotech, Sigma, Cat no. GF446-10UG), L-Glutamine (Invitrogen, Cat no. 25030081) and
349 50 units/mL penicillin-streptomycin (Invitrogen, Cat no. 11548876).

350 ***Transwell invasion assay.*** Cell invasion assays were performed using 24-well Transwell chambers
351 (Corning, NY) equipped with polycarbonate inserts containing 8 µm pores. Inserts were coated with
352 50 µL of Matrigel (Corning, NY) diluted 1:3 in culture medium. A total of 50,000 cells suspended in
353 serum-free medium were seeded into the upper chambers, while the lower wells were filled with
354 medium containing 1% FBS as a chemoattractant. After 24 h of incubation, non-invading cells on the
355 upper surface of the membrane were gently removed with a cotton swab. Cells that had migrated to

356 the lower membrane surface were fixed with 0.1% glutaraldehyde, stained with 0.5% crystal violet,
357 and visualized under a light microscope. The number of invaded cells was quantified by counting five
358 randomLy selected microscopic fields.

359 ***Wound healing assay.*** Cells were seeded (100,000) into 24-well plates and grown until reaching
360 approximately 95% of confluent monolayers. A straight line was scratched through the monolayer with
361 a 10 μL pipette tip to create a wound. Then, the closure of the wound was visualized using an EVOS
362 phase-contrast microscopy and the gap distance between the edges was calculated using ImageJ
363 software.

364 ***Statistical analysis.*** Results are reported as means ± standard error of the mean (SEM) or standard
365 deviation (STDEV), as specified. Differences between group means were assessed using Student's
366 t-test, unless otherwise indicated in the figure legends. P values less than 0.05 were deemed
367 statistically significant, while non-significant outcomes are denoted as "ns". All statistical analyses
368 were conducted using GraphPad Prism version 8.0 (San Diego, California, USA).

369 **DATA AVAILABILITY**

370 **Materials availability**

371 All data related to the results are available as part of the article. Additional information will be available
372 on request.

373 **Data and code availability**

374 Transcriptional and methylation data generated in this study have been deposited in NCBI-SRA
375 BioProject ID PRJNA1422783 (HFD RNA-seq) and in GEO ID GSE319337 (methylation data). Unique
376 identifiers for publicly available datasets are indicated, a list of figures that have associated raw data
377 can be provided, and there are no restrictions on data availability. Data from the EUS dataset can be
378 provided upon reasonable request and DTA signing with the San Raffaele Hospital. This paper does
379 not report any original code. Further information if required is available from the lead contact upon
380 request.

381 **Results**

382 **HFD promotes pancreatic tumorigenic transformation in an early adulthood onset model of**
383 **PDAC.** To study the contribution of high-fat diet in pancreatic tumorigenesis we took advantage of an
384 inducible GEMM of PDAC with KRAS$^{G12V/+}$ and P53^{KO}, which mimics the mutational characteristics of
385 advanced PDAC (*Elas*-tTA/*tetO*-Cre; *Kras*^{LSL}G12V/+; *Trp53*$^{lox/lox}$). We developed two risk factors
386 exposure protocols to model the early and late onset development of the disease, in addition to the
387 classical embryonic setting as a reference. For the early onset (P21), we maintained mice on
388 doxycycline (DOX) for three weeks and then DOX was removed and exposure to high-fat diet (HFD)
389 was initiated. As DOX requires approximately one month for complete clearance, HFD exposure prior
390 to full induction of the mutations repressed by DOX resembles adolescents with HFD-like habits
391 before mutation acquisition (**Figure 1A**). Mice were kept on HFD until one year of age or humane
392 endpoint. For the embryonic setting, *Elas*-tTA/*tetO*-Cre; *Kras*^{LSL}G12V/+; *Trp53*$^{lox/lox}$ mice were kept
393 without DOX allowing for transgenes recombination during embryonic development leading to 100%
394 disease development. As expected, both female and male mice gained weight after HFD-exposure
395 (**Figure S1A**) and the expected systemic effects caused by the diet were assessed, including fatty
396 liver (**Figure S1B**), acinar-to-adipose replacement in the pancreas, intra-acinar lipid droplets
397 accumulation (**Figure S1C**), and brown to white adipose tissue transformation (**Figure S1D**). We
398 further confirmed that HFD exposure alone does not lead to preneoplastic lesions (PanINs) formation
399 in wild-type (WT) mice, demonstrating that the diet by itself does not trigger neoplastic transformation
400 (**Figure S1E**).

401 As reported in previous studies from the laboratory, adult acinar cells present an oncogenic
402 transformation resistance that increases with age, with no tumor formation when mutations are
403 induced at 60 days post-birth (5, 6) (**Figure 1B**). Interestingly, P21 mice exhibited an intermediate
404 phenotype, showing preneoplastic lesions and full PDAC, but with an increased resistance to tumor
405 development compared with the embryonic model (**Figure 1B**). Notably, P21 mice exposed to HFD
406 developed tumors earlier than P21 control mice, although later than the embryonic model (**Figure 1C-**
407 **D**). Despite these differences, survival was comparable between the P21 HFD protocol and the

408 embryonic model, and in both cases significantly shorter than in the P21 control group (**Figure 1D**).
409 Histological analysis of these tumors revealed distinct tumor phenotypes, with control tumors
410 displaying a more differentiated epithelial morphology characterized by ductal-like structures,
411 whereas tumors arising under HFD conditions mainly exhibited a squamous/mesenchymal-like
412 phenotype (**Figure 1E**). These results suggest that HFD promotes early onset of PDAC tumors,
413 affecting survival in an early adulthood context.

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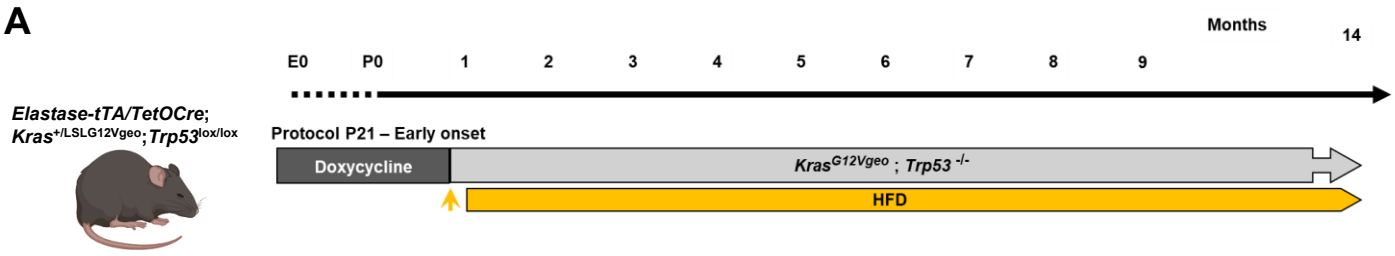
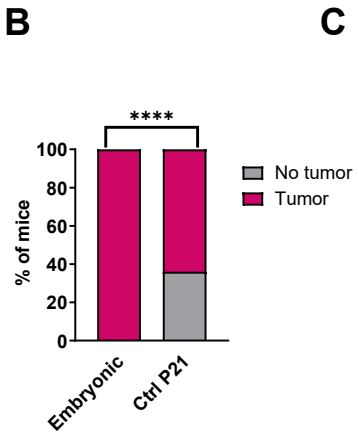
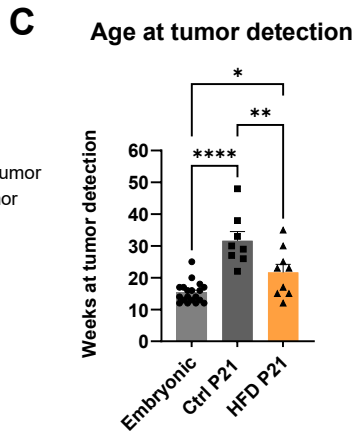
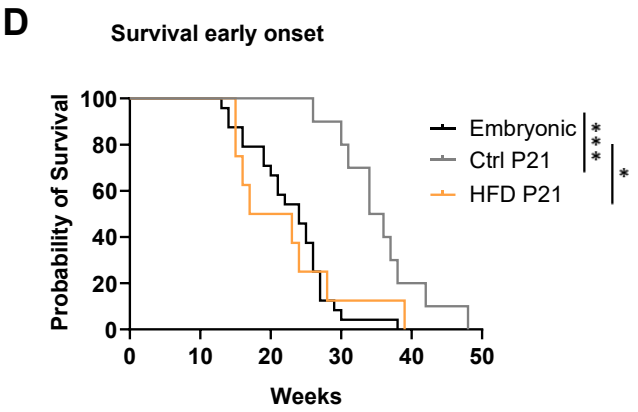
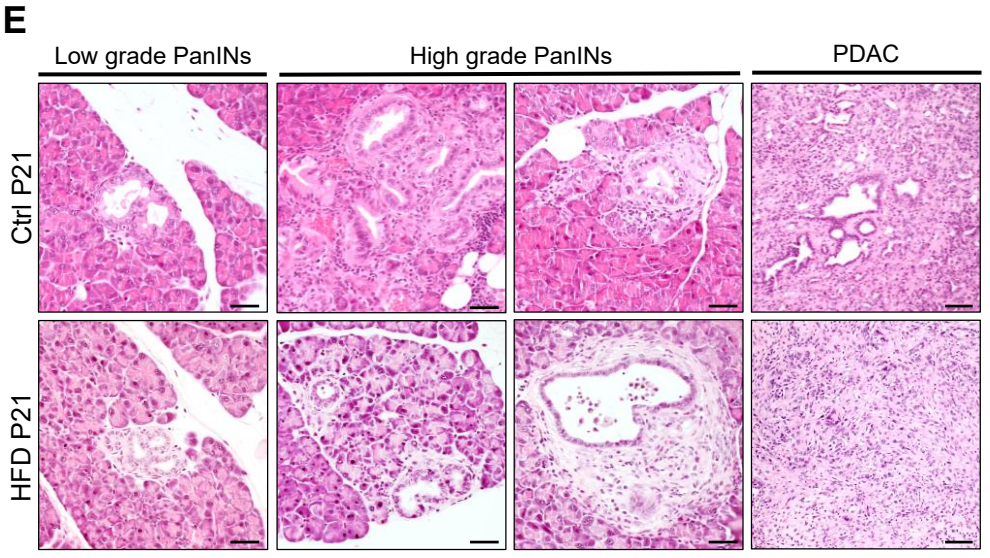
417


A
Months
14
E0
P0
1
2
3
4
5
6
7
8
9
Elastase-tTA/TetOCre;
Kras+/LSLG12Vgeo;Trp53lox/lox
Protocol P21 – Early onset
Doxycycline
KrasG12Vgeo ; Trp53 -/-
HFD


B
****
% of mice
100
80
60
40
20
0
No tumor
Tumor
Embryonic
Ctrl P21


C
Age at tumor detection
Weeks at tumor detection
60
50
40
30
20
10
0
*
**
****
Embryonic
Ctrl P21
HFD P21


D
Survival early onset
Probability of Survival
100
80
60
40
20
0
0
10
20
30
40
50
Weeks
Embryonic
Ctrl P21
HFD P21
***
*

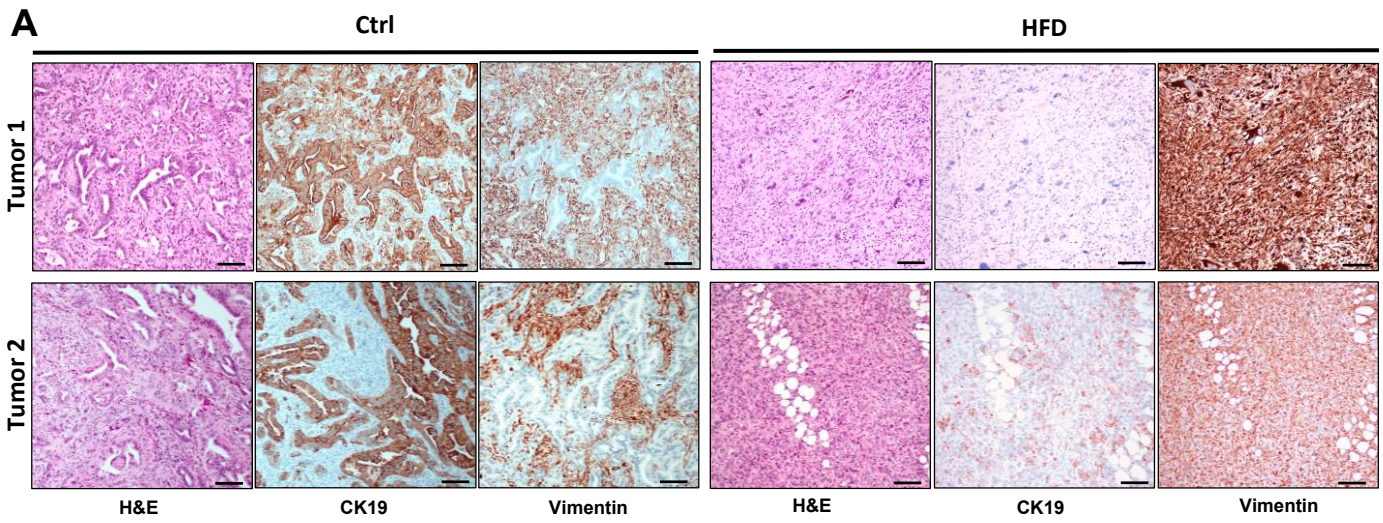

E
Low grade PanINs
High grade PanINs
PDAC
Ctrl P21
HFD P21

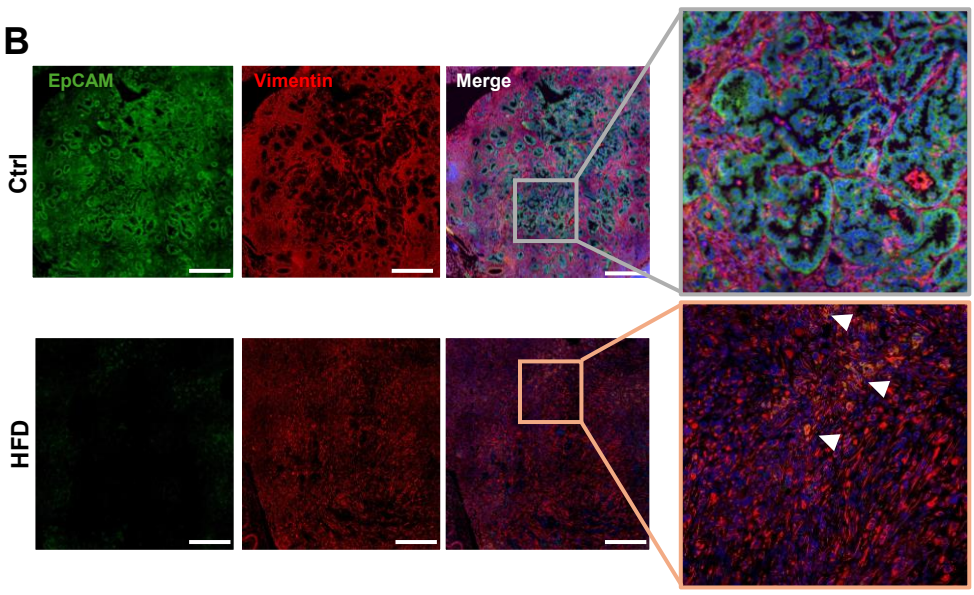
425 ***Figure 1. High-fat diet promotes tumorigenesis in early-onset adult PDAC mouse models**. **A)***
426 *Representative scheme of the protocol employed to study the effect of high-fat diet (HFD) in early onset*
427 *pancreatic cancer (P21).* ***B)*** *Percentage of mice from the embryonic and P21 arms in which tumors were*
428 *detected (embryonic n=19, controls (Ctrl) P21 n=14). P values as determined by Chi-square test and Fisher´s*
429 *exact test.* ***C)*** *Histogram showing the weeks at tumor detection for the embryonic, Ctrl and HFD-exposed mice*
430 *from the P21 protocol. Shown is the mean ± SEM (embryonic n=19, Ctrl P21 n=8, HFD n=9; P values as*
431 *determined by One-way ANOVA, Tukey test).* ***D)*** *Kaplan-Meier plot showing the overall survival of mice from*
432 *the embryonic and P21 (both Ctrl and HFD) settings (embryonic n=24, Ctrl P21 n=10, HFD n=8, P values as*
433 *determined by Mantel-Cox test).* ***E)*** *Representative brightfield microscopy images of hematoxylin and eosin-*
434 *stained pancreata at different stages of tumor development in the P21 fed with Ctrl diet (top panel) or HFD*
435 *(bottom panel). Scale bar= 50 µm. P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

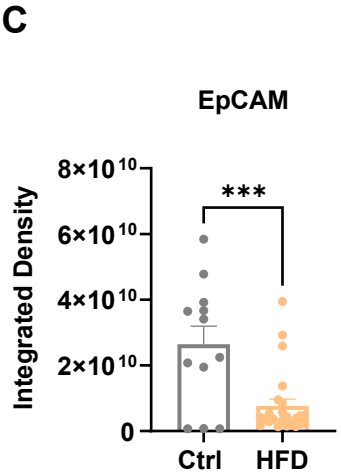
436

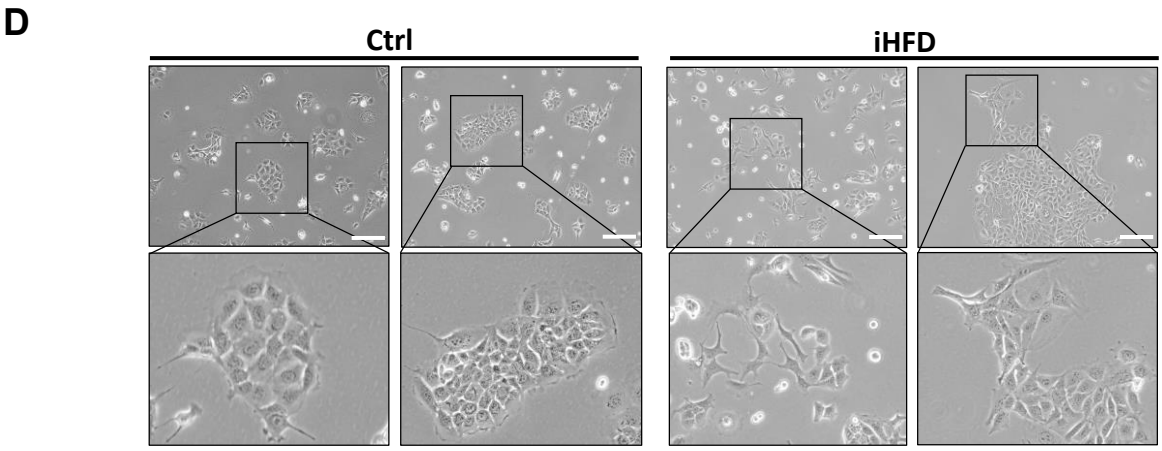
437 **HFD-promoted pancreatic tumors displayed a mesenchymal-like phenotype.** Based on the
438 histological differences observed before, we conducted a marker-based characterization of the P21
439 control and HFD-promoted tumors. We found that control tumors displayed high levels of cytokeratin
440 19, a common marker of epithelial tumors, while HFD-promoted tumors expressed markedly lower
441 levels (**Figure 2A**). On the other hand, vimentin, a marker of mesenchymal tumors, was
442 overexpressed in HFD tumor cells, while in control tumors it was mainly restricted to fibroblasts
443 (**Figure 2A**). Consistently, the epithelial marker EpCAM, was also highly reduced in HFD-promoted
444 tumors (**Figure 2B-C**). Interestingly, in the HFD-promoted tumors we observed a population of cells
445 that expressed both EpCAM and vimentin, suggesting that HFD could induce a hybrid
446 epithelial/mesenchymal phenotype (**Figure 2B-C**).

447 To better understand the molecular changes produced by HFD in tumor cells, we derived primary cell
448 cultures from both P21 control and HFD-induced tumors. As expected, and consistent with the
449 phenotype observed *in vivo*, control cell lines exhibited an epithelial morphology in culture, whereas
450 HFD-induced (iHFD) tumor cell lines displayed a mesenchymal morphology (**Figure 2D**). In line with
451 this, iHFD cell lines showed a reduction of *Krt19, Cdh1 and Gata6*, genes associated with an
452 epithelial/classical-like phenotype. Likewise, we found an upregulation of *Gata2*¸ which regulates
453 *Gata6* expression in an opposite manner (18, 19). Unexpectedly, we observed a decrease in *Cdh2*
454 and an increase in *Tff2,* mesenchymal and epithelial-associated markers respectively (**Figure 2E**).
455 These results indicated that HFD induced a hybrid phenotype between classical/epithelial and
456 basal/mesenchymal states and suggested that HFD promotes a plastic state in tumor cells.


A
Ctrl
HFD
Tumor 1
Tumor 2
H&E
CK19
Vimentin
H&E
CK19
Vimentin


B
EpCAM
Vimentin
Merge
Ctrl
HFD


C
EpCAM
Integrated Density
8×10 10
6×10 10
4×10 10
2×10 10
0
***
Ctrl
HFD


D
Ctrl
iHFD

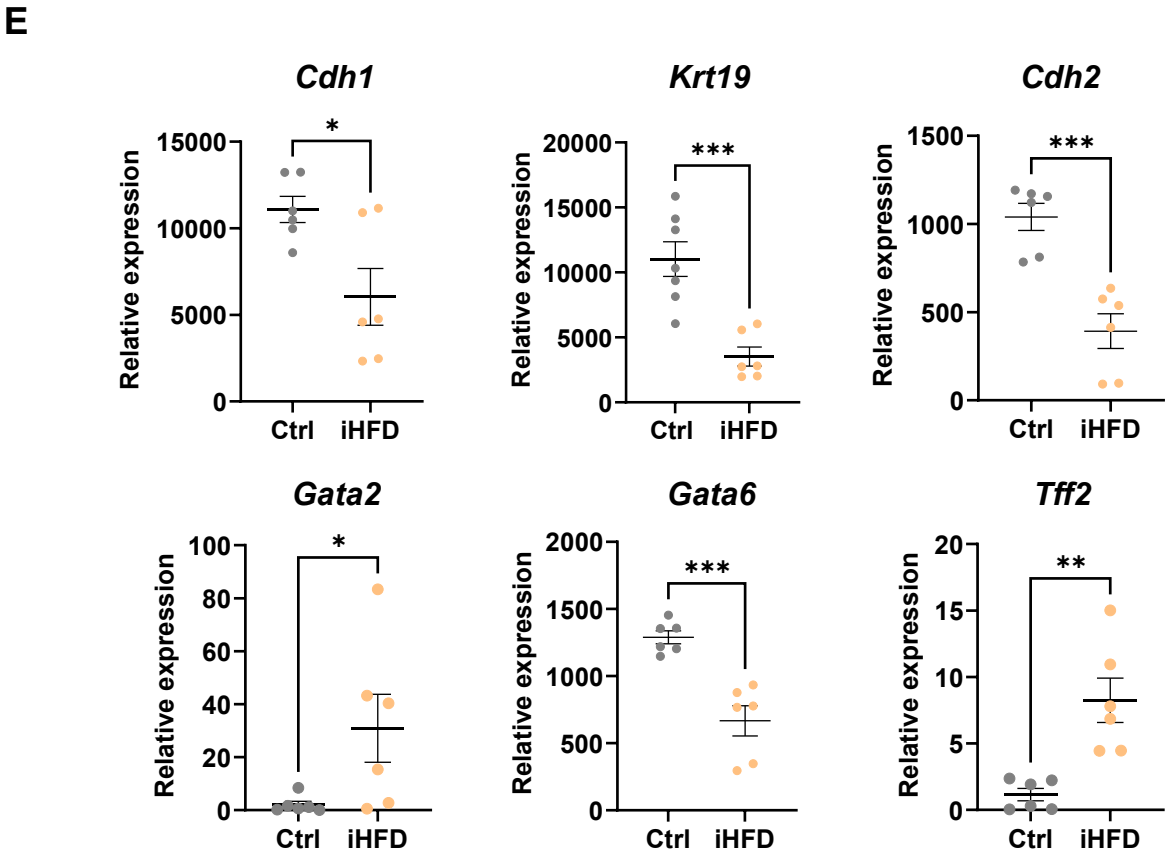

E
Cdh1
Krt19
Cdh2
Gata2
Gata6
Tff2
Relative expression
Ctrl
iHFD
*
***
**

Figure 2. Histology and morphological characterization of control and high-fat diet-promoted tumors and primary tumor-derived cells. A) *Representative brightfield microscopy images of hematoxylin and eosin (H&E), along with cytokeratin 19 (CK19) and vimentin immunohistochemistry from two tumors generated in P21 control (Ctrl) and HFD-fed mice. Scale bar= 50 µm.* ***B)*** *Representative IF confocal microscopy images from Ctrl and HFD tumors stained with EpCAM (green, epithelial tumor cells), Vimentin (red, mesenchymal tumor cells and fibroblasts) and DAPI (blue, nuclei). Arrowheads indicate double-positive EpCAM/Vimentin tumor cells. Scale bar= 500 µm.* ***C)*** *Quantification of the integrated density for EpCAM staining in Ctrl and HFD tumors. Shown is the mean ± SEM for each measured field (n=7 mice, 3 for Ctrl and 4 for HFD; P values as determined by unpaired T-test).* ***D)*** *Representative brightfield microscopy images of P21 Ctrl and HFD-induced (iHFD) primary tumor-derived cell lines. Scale=50µm.* ***E)*** *Relative expression of mRNA levels measured by RT-qPCR of the indicated genes in Ctrl and iHFD primary tumor cell lines. Shown is the mean ± SEM (n=6, P values as determined by unpaired T-test). P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

HFD induces transcriptomic and epigenetic changes in tumor cell state. To interrogate in-depth the transcriptional state of iHFD tumor cells, we performed RNA sequencing analysis of six control and six iHFD P21 cell lines. We first found that iHFD cells exhibited higher transcriptomic differences among themselves than control cells (**Figure 3A**). We next performed a differential gene expression analysis, and found significantly upregulated genes associated with extracellular matrix remodeling (i.e., *Fgfr1, Loxl1, Mmp7, Has2*), stemness (i.e., *Prom2, Pglyrp1, Isg15, Agr2, Fn1*), mesenchymal identity and metastatic behavior (i.e., *Snai1, Zeb2, Grem1, Ngfr, Plau, Foxd1, Foxc1*), and immune evasion (i.e., *Csf1r, Cxcl5, Thbs1, Ccl7, Ccl9, Pglyrp1*) (**Figure 3B**). Among the downregulated genes in iHFD we found mainly genes associated with mitochondria activity (*mt-Co3, mt-Nd3, Ndufb4*). In addition, we found that *Gata2* was upregulated, consistent with the aforementioned data, sustaining the idea of the extinction of the classical subtype program (**Figure 3B**). To better characterize these transcriptomic changes, we performed Gene Set Enrichment Analysis (GSEA) employing Molecular Signature Database and custom gene sets. Interestingly, we found upregulated a gene signature related to increased tumorigenesis by chemical induction (**Figure 3C)**, as expected from a tumor induced by an external agent such as diet. Apart from this, we found pathways mainly associated to epithelial-to-mesenchymal transition (EMT) and the acquisition of mesenchymal identity and trajectory (20), stemness and plasticity, inflammation and immune system modulation (IL1β and interferon response, and antimicrobial response), ECM organization (FGF-response and collagen organization), and, as expected under HFD-induced nutrient overload, nutrient response (**Figure 3C, S2A-E**). Based on this, we also compared the expression of marker genes associated with plasticity and stemness in PDAC (8, 21-33), and we found that iHFD cells presented a general increase in the

492 expression of some (but not all) genes including *P2rx7, Pglyrp1, Agr2, Sox9,* and *Ror2* (**Figure 3D**).
493 As this hybrid/plastic phenotype remains stable in the iHFD cell lines, we evaluated epigenomic
494 changes at the DNA methylation level for these cell lines. Interestingly, this analysis suggested that
495 the global methylome clusters the samples per group (**Figure S2F**). We calculated the mean
496 methylation value per CpG sites per group, and the difference among groups. We selected the CpG
497 sites that had an absolute methylation difference higher than 0.33 between groups, and then, we
498 applied a linear regression model, obtaining 2,526 CpG sites differentially methylated (adjusted p-
499 value<0.05). From them, 92.12% (n=2,327) were hypomethylated in iHFD in comparison to the control
500 group (**Figure S2G**), suggesting that HFD is likely to promote gene upregulation. Moreover, most of
501 these CpG sites that were differentially methylated were located at the 5' regulatory region of the
502 genes (42.91%; n=1,084) (**Figure S2H**). Then, we used the differentially methylated CpG sites to
503 cluster the samples (**Figure S2I**) and we performed an enrichment analysis with EnrichR using the
504 genes that had at least one CpG site differentially methylated. Using the Panther database, we found
505 2 differentially regulated pathways, the WNT signaling pathway and the cadherin pathway (**Figure**
506 **S2J**), supporting that HFD influences the plastic-like EMT state of tumor cells.

507 To cross validate the transcriptomic results with human data, we took advantage of a database
508 including 164 endoscopy-guided ultrasound (EUS) biopsies of PDAC patients of all stages sequenced
509 by bulk RNA-seq (34). First, we divided patients according to their BMI at the time of diagnosis,
510 considering equal to or higher than 25, patients with overweight or obesity; and patients with less than
511 25 as normal weight or lean (**Figure 3E**). When we compared both groups and performed differential
512 gene expression analysis, we found upregulated several genes associated with inflammation (i.e.,
513 *IL1B, TNF, OSM*), antimicrobial response associated with stemness (i.e., *CAMP, PGLYRP1, LTF*),
514 CSCs markers related also to metastases (i.e., *P2RX7, ALK, CXCR4*) and genes involved in immune
515 evasion (*CXCL8, SPP1, S100A9, CD86, CCL7, CCL2*) (**Figure 3F**); some of them also upregulated
516 in our murine dataset. Interestingly, GSEA analysis revealed a significant enrichment in pathways
517 related to macrophages, including those associated with lipids, as well as inflammatory responses
518 mediated by IL1β, TNFα, IL8, or interferons. We also observed enhanced antimicrobial activity via

519 antimicrobial peptides (**Figure 3F, S3A**); as well as several pathways associated with oncogenic
520 KRAS activity, ERK1/2 and MAPK signaling pathways (**Figure S3B**). Likewise, patients with higher
521 BMI presented an enrichment in genes associated with increased blood vessel formation and
522 enhanced migration (**Figure S3C**), as well as response to lipids (**Figure 3G**). Additionally, we
523 generated a gene signature from our murine dataset based on the upregulated genes in iHFD cells
524 (GALVAN_HFD) and assessed its enrichment in the human cohort. As expected, patients with high
525 BMI were significantly enriched for this murine-derived gene signature (**Figure 3G**). Notably, both
526 mouse and human datasets mainly showed downregulation of pathways associated with oxidative
527 phosphorylation (**Figure 3H**). As most of these pathways were common between our human and
528 murine datasets, we compared the genes driving these processes and found 79 commonly
529 upregulated and 36 downregulated (**Figure S4A-B**). Remarkably, we identified P2RX7, an ATP-gated
530 calcium channel and known CSC marker, among the common upregulated genes. P2RX7 is
531 associated to EMT and stemness promotion acting as a receptor for the cathelicidin antimicrobial
532 peptide (*Camp*/CAMP in mice, *CAMP*/hCAP-18/LL-37 in humans), also upregulated in patients with
533 high BMI (**Figure 3F**) (26). These data, along with the enrichment in gene signatures associated to
534 antimicrobial peptides and calcium signaling, led us to hypothesize that HFD could be driving the
535 P2RX7-CAMP axis, promoting a highly plastic, stem-like state in tumor cells driving aggressiveness
536 and tumor progression.

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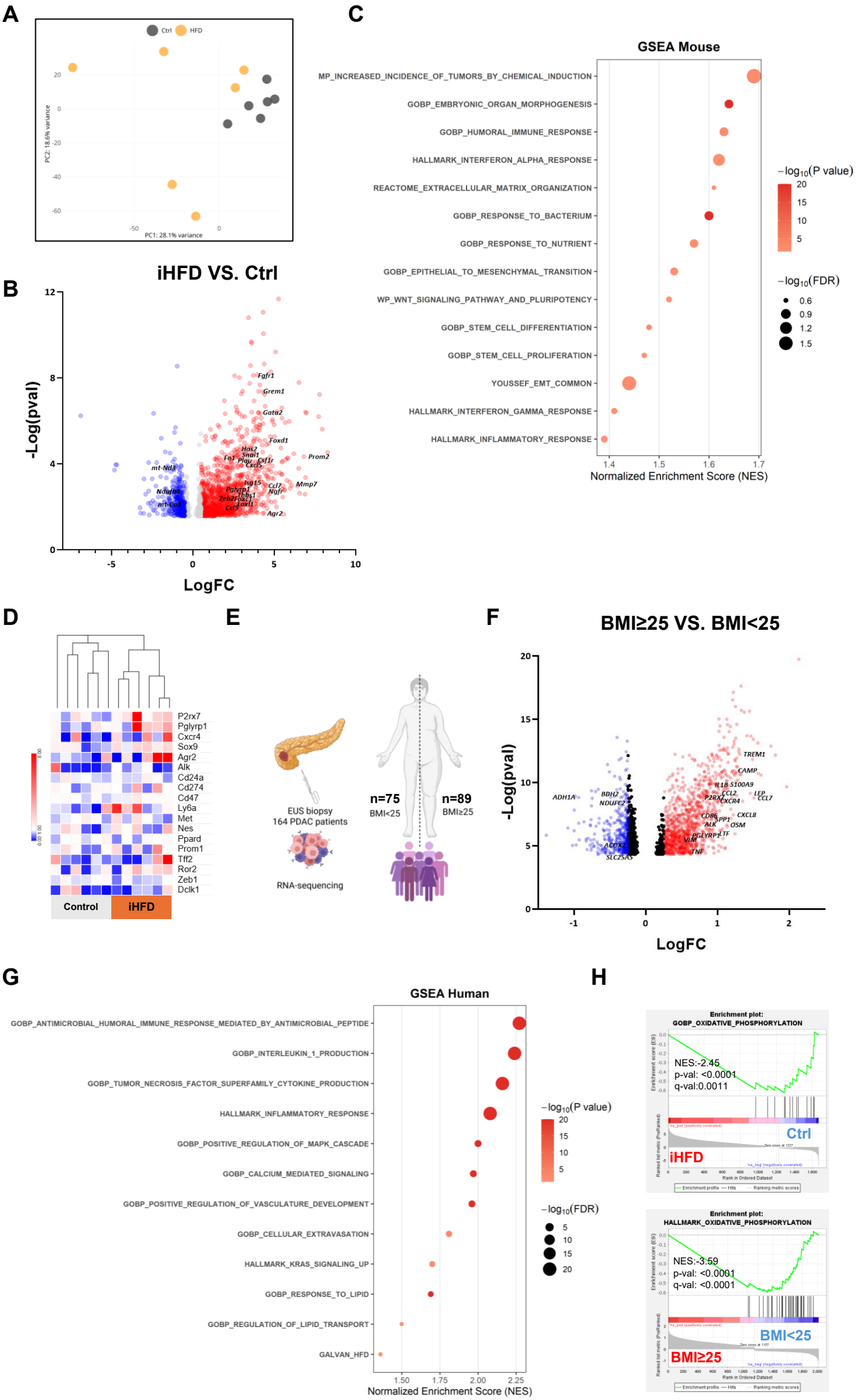
A
Ctrl
HFD
PC2: 18.6% variance
PC1: 28.1% variance
B
iHFD VS. Ctrl
-Log(pval)
LogFC
Fgfr1
Grem1
Gata2
Foxd1
Has2
Snai1
Fn1
Plau
Csf1r
Cxcl5
Prom2
mt-Nd3
Isg15
Ccl7
Mmp7
Ndufb4
Pglyrp1
Ngfr
Thbs1
Zeb2
Foxc1
mt-Co3
Ccl9
Loxl1
Agr2
C
GSEA Mouse
MP_INCREASED_INCIDENCE_OF_TUMORS_BY_CHEMICAL_INDUCTION
GOBP_EMBRYONIC_ORGAN_MORPHOGENESIS
GOBP_HUMORAL_IMMUNE_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION
GOBP_RESPONSE_TO_BACTERIUM
GOBP_RESPONSE_TO_NUTRIENT
GOBP_EPITHELIAL_TO_MESENCHYMAL_TRANSITION
WP_WNT_SIGNALING_PATHWAY_AND_PLURIPOTENCY
GOBP_STEM_CELL_DIFFERENTIATION
GOBP_STEM_CELL_PROLIFERATION
YOUSSEF_EMT_COMMON
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INFLAMMATORY_RESPONSE
Normalized Enrichment Score (NES)
−log10(P value)
−log10(FDR)
D
P2rx7
Pglyrp1
Cxcr4
Sox9
Agr2
Alk
Cd24a
Cd274
Cd47
Ly6a
Met
Nes
Ppard
Prom1
Tff2
Ror2
Zeb1
Dclk1
Control
iHFD
E
EUS biopsy
164 PDAC patients
RNA-sequencing
n=75
BMI<25
n=89
BMI≥25
F
BMI≥25 VS. BMI<25
-Log(pval)
LogFC
ADH1A
BDH2
NDUFC2
ACOX2
SLC25A5
TREM1
CAMP
IL1B
S100A9
CCL2
LEP
CCL7
P2RX7
CXCR4
CD86
CXCL8
SPP1
ALK
OSM
PGLYRP1
TF
VIM
TNF
G
GSEA Human
GOBP_ANTIMICROBIAL_HUMORAL_IMMUNE_RESPONSE_MEDIATED_BY_ANTIMICROBIAL_PEPTIDE
GOBP_INTERLEUKIN_1_PRODUCTION
GOBP_TUMOR_NECROSIS_FACTOR_SUPERFAMILY_CYTOKINE_PRODUCTION
HALLMARK_INFLAMMATORY_RESPONSE
GOBP_POSITIVE_REGULATION_OF_MAPK_CASCADE
GOBP_CALCIUM_MEDIATED_SIGNALING
GOBP_POSITIVE_REGULATION_OF_VASCULATURE_DEVELOPMENT
GOBP_CELLULAR_EXTRAVASATION
HALLMARK_KRAS_SIGNALING_UP
GOBP_RESPONSE_TO_LIPID
GOBP_REGULATION_OF_LIPID_TRANSPORT
GALVAN_HFD
Normalized Enrichment Score (NES)
−log10(P value)
−log10(FDR)
H
Enrichment plot:
GOBP_OXIDATIVE_PHOSPHORYLATION
NES:-2.45
p-val: <0.0001
q-val:0.0011
Ctrl
iHFD
Enrichment plot:
HALLMARK_OXIDATIVE_PHOSPHORYLATION
NES:-3.59
p-val: <0.0001
q-val: <0.0001
BMI<25
BMI≥25

544 ***Figure 3. Transcriptomic profile of high-fat diet-induced cell lines and PDAC patients with high body***
545 ***mass index (BMI). A)*** *Principal Component Analysis (PCA) for P21 control (Ctrl) and high-fat diet-induced*
546 *(iHFD) primary tumor cell lines analyzed by RNA-seq.* ***B)*** *Volcano plot illustrating genes significantly up- (red)*
547 *and downregulated (blue) in iHFD cell lines.* ***C)*** *Gene Set Enrichment Analysis (GSEA) plot showing the*
548 *enrichment in the indicated pathways.* ***D)*** *Heat map showing the levels of expression as fold change of*
549 *normalized counts for the indicated genes in Ctrl and HFD cell lines included in the RNA-seq analyses. The*
550 *indicated genes are linked to stemness, plasticity and immune evasive phenotypes.* ***E)*** *Schematic*
551 *representation of a patient comparison study involving 164 endoscopy-guided ultrasound (EUS) biopsy tumor*
552 *samples, correlating BMI with tumor transcriptomic profiles. BMI cutoff has been set as 25 (n for BMI<25=75, n*
553 *for BMI≥25=89).* ***F)*** *Volcano plot showing the genes significantly up- (red) and downregulated (blue) in patients*
554 *with a BMI higher or equal to 25.* ***G)*** *GSEA plot showing the enrichment of the indicated pathways.* ***H)*** *GSEA*
555 *plots depicting the enrichment of the oxidative phosphorylation pathway in both murine (top) and human*
556 *(bottom) RNA-seq datasets.*

557

558 **HFD-induced tumor cell lines present enhanced plasticity and stemness characterized by**
559 **P2RX7 overexpression.** To test our hypothesis regarding enriched stemness, we cultured control
560 and iHFD cell lines as CSC-enriched spheroids. Plating an equal number of cells, iHFD cell lines
561 formed significantly more spheroids, supporting the plastic/stem-like nature of iHFD tumor cells
562 (**Figure 4A-B**). Next, we evaluated the expression of P2RX7 in our control and iHFD cells by RNA-
563 seq data, qPCR and flow cytometry. P2RX7 was found consistently and significantly overexpressed
564 in iHFD cells (**Figure 4C-F**). Moreover, we found overexpression of P2RX7 in orthotopic tumors
565 generated in HFD-fed C57Bl/6 mice (**Figure 4G**). Given that diet-induced alterations may partially
566 recapitulate the molecular effects of obesity, we analyzed *P2rx7* expression in an RNA-seq dataset
567 of murine orthotopic PDAC organoids grown in HFD-fed and in leptin-deficient (ob/ob) genetic obese
568 mice (35). Interestingly, in both models there was a significant and consistent enrichment in *P2rx7*
569 expression compared to controls (**Figure S5A-B**), suggesting that our HFD-fed model could mimic
570 what occurs in patients with obesity. Thus, aiming to translate these results to patients, we evaluated
571 the expression of *P2RX7* in our cohort at the time of PDAC diagnosis, comparing patients with high
572 and low BMI one year prior to diagnosis and at the time of diagnosis (**Figure S6A**). We found that
573 patients with high BMI one year before PDAC diagnosis exhibited a significant enrichment of *P2RX7*
574 expression (**Figure 4H**), but this difference disappeared at the time of the diagnosis (**Figure 4I**). This
575 effect could be likely due to the cachexia developed during the course of the disease. Therefore, we
576 evaluated those patients that at the time of the diagnosis were obese but lost weight, versus those
577 that were always lean. Interestingly, those patients with high BMI one year before diagnosis retained
578 elevated levels of *P2RX7* expression at the time of diagnosis (**Figure 4J**), even though they were

579 “lean” at that point. This finding suggests that P2RX7 may serve as a risk factor marker linking high
580 BMI to PDAC.

581 As P2RX7 is considered a CSC-associated marker, we evaluated the presence of other stem-related
582 surface markers in these cell lines. Specifically, we assessed the expression of the “highly metastatic
583 CSC population” marker CXCR4 (36) in iHFD cells and found it to be upregulated at the transcriptomic
584 and protein levels, supporting the link between HFD-induced tumors with a more invasive phenotype
585 (**Figure 4K-L**). Notably, most P2RX7-positive cells in our cultures also co-expressed CXCR4 (**Figure**
586 **4M**). We also found an increase abundance of the triple-positive (i.e., EpCAM^{+}, SCA1^{+}, CD133^{+})
587 stem-like cell population (8) in our iHFD cultures (**Figure 4N-O**). Altogether, these results position
588 HFD as an activator of plasticity and stemness in mouse PDAC. Stem-related markers such as
589 *EPCAM*, *PROM1* or *ISG15* were not differentially expressed between patients with high versus low
590 BMI one year before diagnosis (**Figure S6B-D**). However, *CXCR4* mirrored the expression pattern of
591 *P2RX7* (**Figure S6E-G**), strongly suggesting that HFD may also induce cellular plasticity and a
592 potentially metastatic phenotype in PDAC tumors.

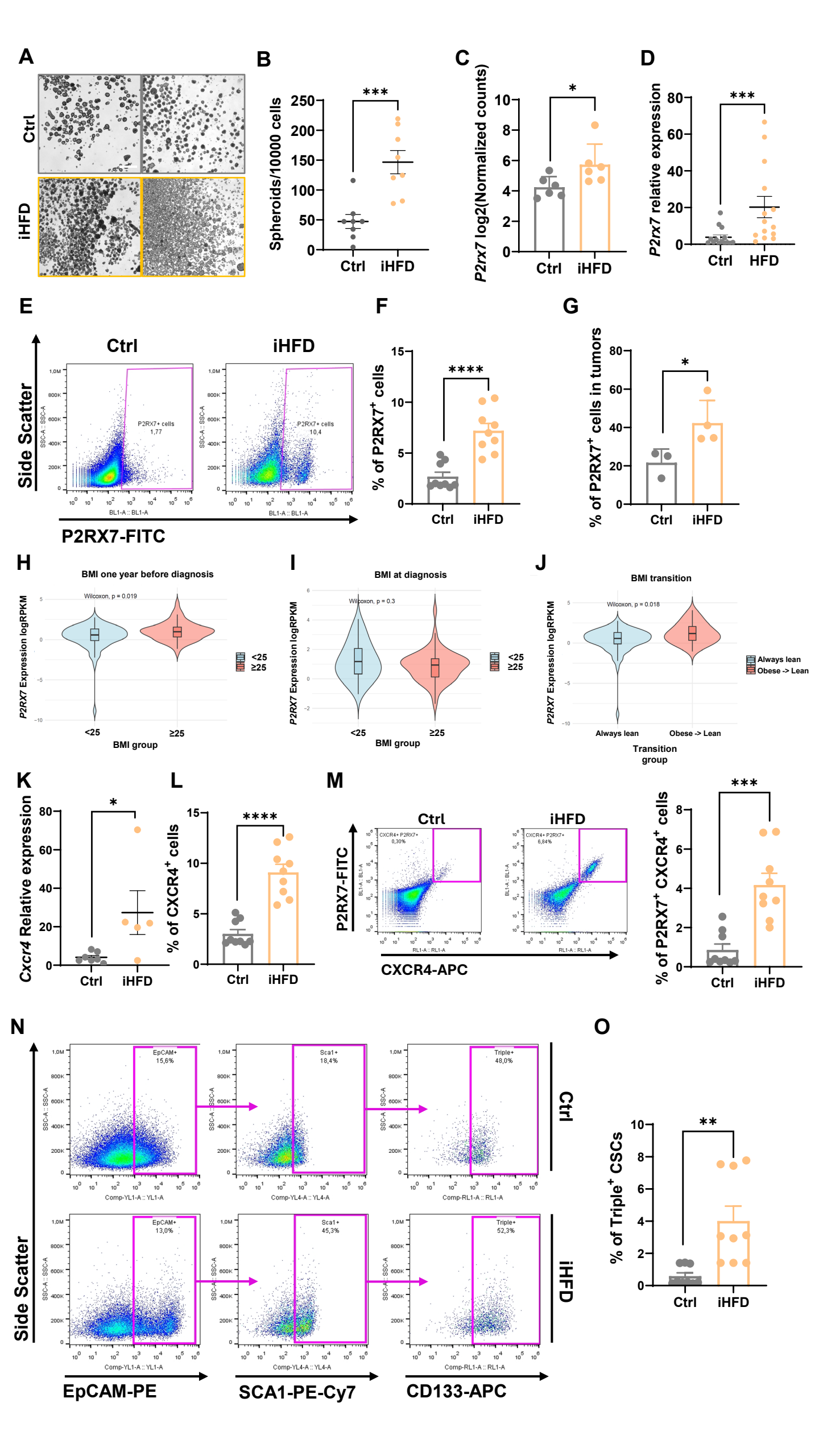
A
Ctrl
iHFD
B
Spheroids/10000 cells
***
Ctrl
iHFD
C
P2rx7 log2(Normalized counts)
*
Ctrl
iHFD
D
P2rx7 relative expression
***
Ctrl
HFD
E
Ctrl
iHFD
Side Scatter
P2RX7+ cells
1,77
P2RX7+ cells
10,4
P2RX7-FITC
F
% of P2RX7+ cells
****
Ctrl
iHFD
G
% of P2RX7+ cells in tumors
*
Ctrl
iHFD
H
BMI one year before diagnosis
Wilcoxon, p = 0.019
P2RX7 Expression logRPKM
<25
≥25
BMI group
I
BMI at diagnosis
Wilcoxon, p = 0.3
P2RX7 Expression logRPKM
<25
≥25
BMI group
J
BMI transition
Wilcoxon, p = 0.018
P2RX7 Expression logRPKM
Always lean
Obese -> Lean
Transition group
K
Cxcr4 Relative expression
*
Ctrl
iHFD
L
% of CXCR4+ cells
****
Ctrl
iHFD
M
Ctrl
iHFD
P2RX7-FITC
CXCR4-APC
% of P2RX7+ CXCR4+ cells
***
Ctrl
iHFD
N
Side Scatter
EpCAM+
15,6%
Sca1+
18,4%
Triple+
48,0%
Ctrl
EpCAM+
13,0%
Sca1+
45,3%
Triple+
52,3%
iHFD
EpCAM-PE
SCA1-PE-Cy7
CD133-APC
O
% of Triple+ CSCs
**
Ctrl
iHFD

Figure 4. HFD-induced cells present an enrichment in CSC markers and enhanced plasticity. A) *Representative brightfield microscopy images of spheroids generated from control (Ctrl) and high-fat diet-induced (iHFD) primary tumor cell lines after 5 days in culture. Scale=750 µm.* ***B)*** *Quantification of the number of spheroids generated from 10000 cells. Shown is the mean ± SEM (n=8, P values as determined by unpaired T-test).* ***C)*** *Quantification of P2rx7 expression in Ctrl and iHFD primary cell lines from the RNA-seq analyses. Shown is the mean ± SEM of log2(normalized counts) (n=6, P values as determined by unpaired T-test).* ***D)*** *Relative expression of P2rx7 mRNA levels measured by RT-qPCR in Ctrl and iHFD primary tumor cell lines. Shown is the mean ± SEM (n=14, P values as determined by unpaired T-test).* ***E-F)*** *Representative flow cytometry plots showing the levels of P2RX7 surface expression in Ctrl and iHFD cell lines (**E**) and quantification of the expression (**F**), shown as the mean ± SEM (n=3, P values as determined by unpaired T-test).* ***G)*** *Quantification of the percentage of P2RX7 positive cells in tumors grown in Ctrl or iHFD-fed mice. Shown is the mean ± STDEV (n=3, P values as determined by unpaired T-test).* ***H)*** *Violin plot showing the quantification of P2RX7 expression in PDAC patients based on their BMI 1 year before diagnosis (n for <25=75, n for ≥25=89). P value as determined by Wilcoxon test.* ***I)*** *Violin plot showing the quantification of P2RX7 expression in PDAC patients based on their BMI at time of diagnosis (n for <25=35, n for ≥25=54). P value as determined by Wilcoxon test.* ***J)*** *Violin plot showing the quantification of P2RX7 expression in PDAC patients based on their BMI transition (patients previously obese that lost their weight versus those that always present a BMI<25) (n for always <25=75, n for obese → lean =35). P value as determined by Wilcoxon test.* ***K)*** *Relative expression of Cxcr4 mRNA levels measured by RT-qPCR in Ctrl and iHFD primary tumor cell lines. Shown is the mean ± SEM (n=5, P values as determined by unpaired T-test).* ***L)*** *Quantification of CXCR4 expression by flow cytometry in Ctrl and iHFD primary tumor cell lines. Shown is the mean ± SEM (n=3 in triplicates, P values as determined by unpaired T-test).* ***M)*** *Representative flow cytometry plots showing the levels of P2RX7/CXCR4 double positive cells in Ctrl and iHFD cell lines (left panel) and quantification of the expression (right panel), shown as the mean ± SEM (n=3, P values as determined by unpaired T-test).* ***N-O)*** *Representative flow cytometry plots showing the levels of EpCAM/SCA1/CD133 triple positive cells in Ctrl and iHFD cell lines (**N**) and quantification of the expression (**O**), shown as the mean ± SEM (n=3, P values as determined by unpaired T-test). P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

HFD-induced PDAC in a late adulthood model shows histological and molecular concordance with early adulthood disease. The HFD exposure in an early-adulthood onset revealed an important role of HFD in tumor progression; however, in this model we could not address if the HFD had a role in tumor induction (5, 6). As such, we next investigated whether HFD exposure could play a role in tumor induction in a model resistant to tumor development. To this end, mice were kept with DOX for two months, left for an additional month for DOX clearance before HFD exposure and monitored until one year on a HFD diet or humane endpoint (P60 protocol). This protocol resembles adults that gain mutations due to ageing or other factors and that acquire HFD-like habits later in life (**Figure 5A**). Using this approach, and as expected from our previous work, control mice were resistant to tumor formation (**Figure 5B-E**) (5, 6). Importantly, we found that the HFD intake led to the development of lesions in 63% of the cohort, with 38% of the total mice presenting full PDAC (**Figure 5E**). Next, we performed immunohistopathological analyses of these tumors and found a strong parallelism with the P21 setting, including a highly mesenchymal tumor phenotype characterized by most of the cells expressing vimentin and a very low proportion of cells expressing cytokeratin 19 (**Figure 5F**). iHFD primary tumor-derived cell lines from the P60 protocol shared common features with iHFD P21 cell

636 lines, including a mesenchymal morphology (**Figure 5G**) and a similar expression profile, both at the
637 mRNA and protein levels for P2RX7 and CXCR4 upregulation, as well as a reduction in *Krt19* and
638 *Cdh1* expression (**Figure 5H-I**). Therefore, we found shared common features in the induction and
639 progression of a molecular mechanism in both HFD-exposed P21 and P60 protocols. Furthermore,
640 these results strongly support a critical role for HFD in two important steps of PDAC development,
641 tumor initiation and in tumor progression.

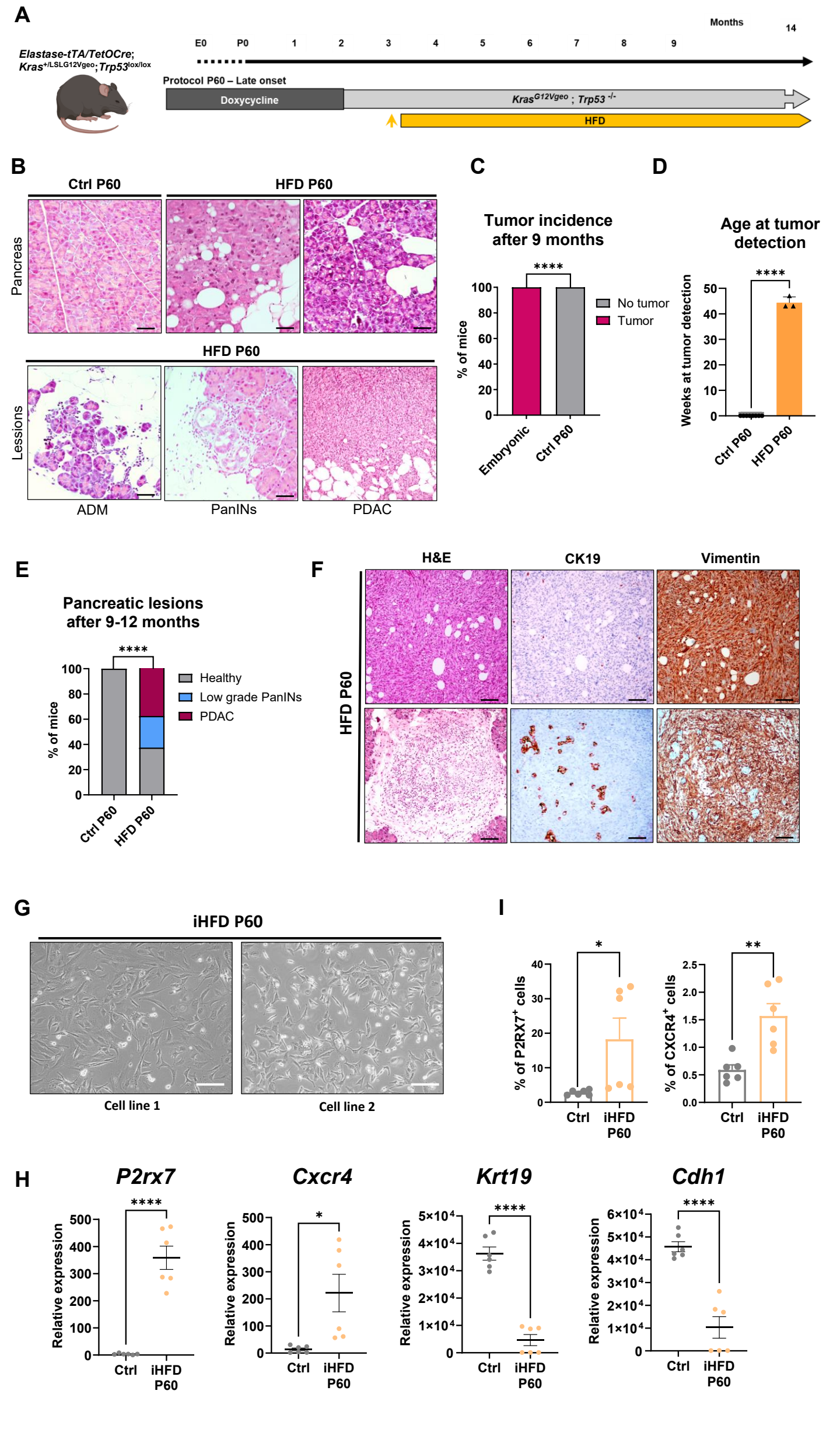
A
Elastase-tTA/TetOCre; Kras+/LSLG12Vgeo; Trp53lox/lox
Months
E0 P0 1 2 3 4 5 6 7 8 9 14
Protocol P60 – Late onset
Doxycycline
KrasG12Vgeo ; Trp53 -/-
HFD
B
Ctrl P60
HFD P60
Pancreas
HFD P60
Lesions
ADM
PanINs
PDAC
C
Tumor incidence after 9 months
****
% of mice
No tumor
Tumor
Embryonic
Ctrl P60
D
Age at tumor detection
****
Weeks at tumor detection
Ctrl P60
HFD P60
E
Pancreatic lesions after 9-12 months
****
% of mice
Healthy
Low grade PanINs
PDAC
Ctrl P60
HFD P60
F
H&E
CK19
Vimentin
HFD P60
G
iHFD P60
Cell line 1
Cell line 2
I
*
% of P2RX7+ cells
Ctrl
iHFD P60
**
% of CXCR4+ cells
Ctrl
iHFD P60
H
P2rx7
****
Relative expression
Ctrl
iHFD P60
Cxcr4
*
Relative expression
Ctrl
iHFD P60
Krt19
****
Relative expression
Ctrl
iHFD P60
Cdh1
****
Relative expression
Ctrl
iHFD P60

Figure 5. High-fat diet induces tumorigenesis in a late-onset adult PDAC mouse model and presents similar characteristics to the early-onset model. A) *Representative scheme of the protocol employed to study the effect of high-fat diet (HFD) in late onset pancreatic cancer.* ***B)*** *Representative brightfield microscopy images of H&E staining from pancreata at different stages of tumor development in the P60 protocol (top panel), as well as the identified pancreatic lesions in the P60 protocol mice fed with HFD. Scale bar= 50 µm.* ***C)*** *Percentage of mice from embryonic (n=19) and P60 controls (Ctrl) (n=8) in which tumors were detected. P values as determined by Chi-square test and Fisher´s exact test.* ***D)*** *Histogram showing the weeks at tumor detection for the Ctrl (n=8) and HFD-fed (n=3) mice from the P60 protocol. Shown is the mean ± SEM (n=8 mice, P values as determined by One-way ANOVA, Tukey test).* ***E)*** *Percentage of control and HFD P60 mice presenting healthy tissue, low grade PanINs or PDAC (n=8). P values as determined by Chi-square test and Fisher's exact test.* ***F)*** *Representative brightfield microscopy images of hematoxylin and eosin (H&E) staining, along with cytokeratin 19 (CK19) and vimentin IHC from two tumors generated in P60 HFD mice. Scale bar= 50 µm.* ***G)*** *Representative brightfield microscopy images of primary tumor-derived cell lines from P60 high-fat diet-induced (iHFD) mice. Scale=300µm.* ***H)*** *Relative expression of mRNA levels measured by RT-qPCR of the indicated genes in P21 Ctrl and P60 iHFD primary tumor cell lines. Shown is the mean ± SEM (n=2 in triplicates, P values as determined by unpaired T-test).* ***I)*** *Quantification of P2RX7 (left panel) and CXCR4 (right panel) expression by flow cytometry in P21 Ctrl and iHFD P60 primary tumor cell lines. Shown is the mean ± SEM (n=2 in triplicates, P values as determined by unpaired T-test). P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

HFD-derived tumor cells present enhanced metastatic capacity. Considering the more plastic-like/mesenchymal phenotype of iHFD cell lines, as well as the expression of pro-metastatic-related markers (i.e., CXCR4), we evaluated the capacity of these cells to migrate and invade *in vitro* and *in vivo.* First, we performed wound healing and transwell invasion assays comparing the migratory capacity and invasiveness of control and iHFD cells. In both cases, iHFD cell lines presented an enhanced ability to close wounds and to migrate through Matrigel™ (**Figure 6A-D**). We next evaluated the presence of spontaneous metastases in our GEMMs. As P60 control mice did not form primary tumors or lesions, we focused on the embryonic and P21 protocols. We found that in the P21 protocol, 22.22% of control mice presented liver metastases, while 71.43% of the HFD mice presented both liver and/or lung metastases, showing a pro-metastatic impact of the HFD (**Figure 6E, G**). Due to the aggressive nature and rapid development of primary PDAC tumors in the embryonic model, none of the control mice presented metastases, but in the HFD-exposed embryonic model, we found that 54.54% of mice presented metastases in the liver and lung at humane end point (**Figure 6F-G**). Thus, in general, for both protocols, we found that HFD induced more metastatic burden and primed cells to both the lung and liver (**Figure 6H**).

To further validate that the pro-metastatic effect of HFD is imprinted in tumor cells, we performed an intrasplenic experimental metastasis assay in C57Bl/6 mice with control and iHFD cell lines to evaluate their capacity to generate liver metastasis (**Figure 6I**). After three weeks, we sacrificed the

mice and found that all iHFD cell lines colonized the liver and produced metastasis, while the controls cell lines did not (**Figure 6J**). Considering that HFD-induced fatty liver could be a better soil for metastatic seeding (37), we evaluated the capacity of control cell lines to metastasize in mice fed with normal diet or HFD (**Figure S7A-B**). Although we did not find an increased metastatic burden in HFD-fed mice (**Figure S7C-D**), we observed that the EpCAM^{+} cells found in the liver were mainly P2RX7^{+}, suggesting the implication of this receptor in metastatic seeding (**Figure S7E**). To validate this observation in a human setting, we interrogated an already annotated single cell RNA-seq dataset comprising 12 matched samples from primary tumors and liver metastases (38) (**Figure 6K**). *P2RX7* expression was mainly restricted to the immune compartment both in primary tumor and metastasis (**Figure 6L**); however, we found a small proportion of cells in the epithelial compartment expressing the gene, as expected from for CSC marker (**Figure 6M**). In line with our results in mouse models, we found that metastasis presented a higher proportion of P2RX7-expressing tumor cells compared to primary tumors (**Figure 6N**). Altogether, these results support that HFD induces a metastatic phenotype in tumor cells characterized by P2RX7-expression during tumor evolution.

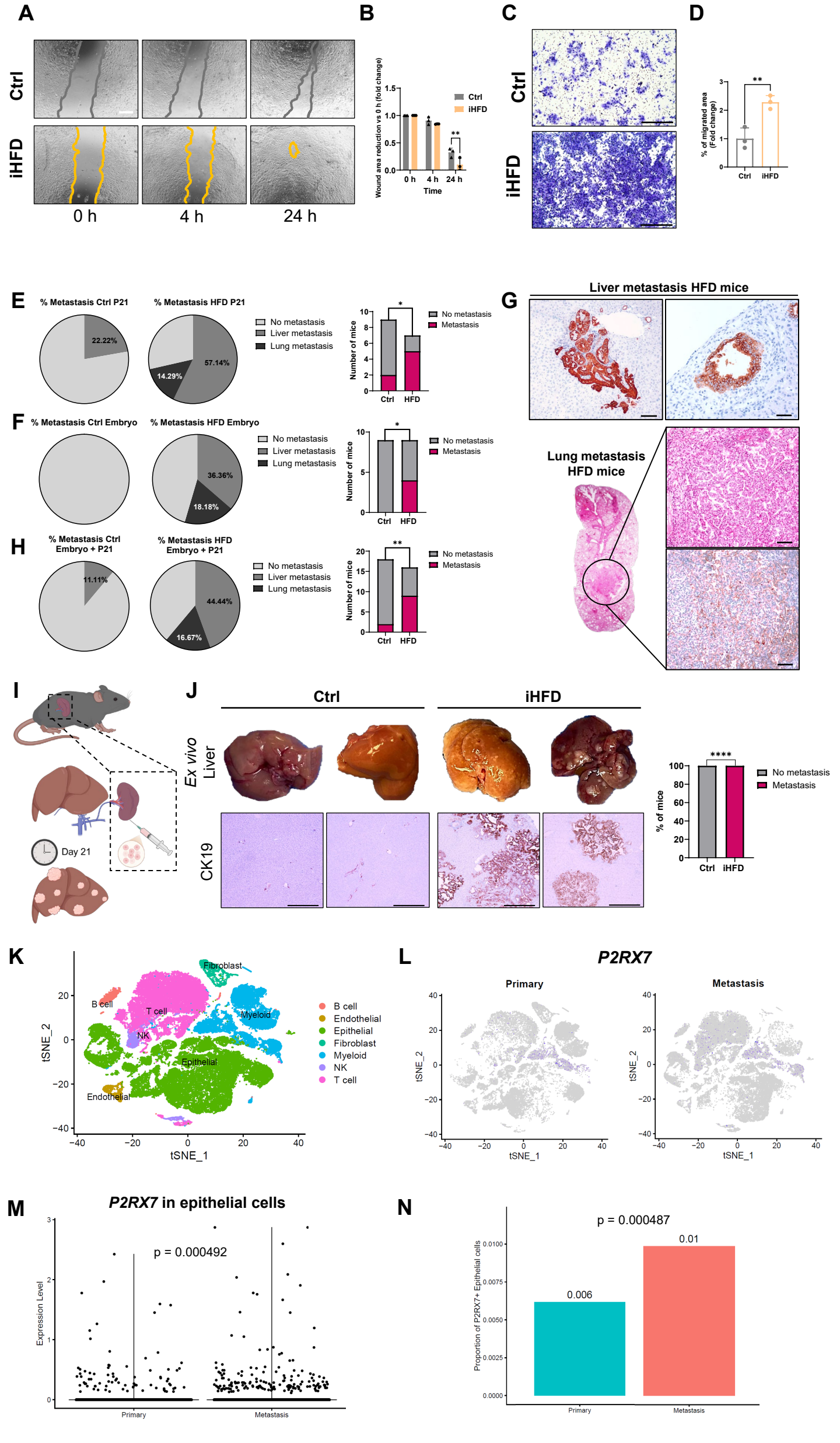
A
Ctrl
iHFD
0 h
4 h
24 h
B
Wound area reduction vs 0 h (fold change)
Ctrl
iHFD
Time
C
D
% of migrated area (Fold change)
E
% Metastasis Ctrl P21
% Metastasis HFD P21
22.22%
57.14%
14.29%
No metastasis
Liver metastasis
Lung metastasis
Number of mice
F
% Metastasis Ctrl Embryo
% Metastasis HFD Embryo
36.36%
18.18%
G
Liver metastasis HFD mice
Lung metastasis HFD mice
H
% Metastasis Ctrl Embryo + P21
% Metastasis HFD Embryo + P21
11.11%
44.44%
16.67%
I
Day 21
J
Ctrl
iHFD
Ex vivo Liver
CK19
% of mice
K
Fibroblast
B cell
T cell
Myeloid
NK
Epithelial
Endothelial
tSNE_1
tSNE_2
L
P2RX7
Primary
Metastasis
M
P2RX7 in epithelial cells
p = 0.000492
Expression Level
N
p = 0.000487
0.006
0.01
Proportion of P2RX7+ Epithelial cells

Figure 6. High-fat diet induces a pro-metastatic phenotype. A) *Representative brightfield microscopy images of wound healing assays at 0-, 4- and 24-hours post-scratch in both control (Ctrl) and high-fat diet-induced (iHFD) cell lines. Scale=750 µm.* ***B)*** *Quantification of wound area reduction shown in A represented as the mean fold change ± STDEV with time 0h set as 1.0 (n=3, P values as determined by unpaired T-test).* ***C)*** *Representative brightfield microscopy images of crystal violet-stained transwell migration assays at 24 hours post-seeding in both Ctrl and iHFD cell lines.* ***D)*** *Quantification of migration area shown as the mean fold change ± STDEV with Ctrl cell lines set as 1.0 (n=3, P values as determined by unpaired T-test).* ***E)*** *Left panel: Proportion plots showing the percentage of Ctrl and iHFD mice with metastasis from the P21 protocol. Right panel: Quantification of the number of mice with metastasis in both conditions (n=9 in Ctrl and 7 in HFD, P values as determined by Chi-square test).* ***F)*** *Left panel: Proportion plots showing the percentage of Ctrl and HFD mice with metastasis from the embryonic (Embryo) model. Right panel: Quantification of the number of mice presenting metastasis in both conditions (n=9 in Ctrl and 9 in HFD, P values as determined by Chi-square test).* ***G)*** *Representative brightfield microscopy images from cytokeratin 19 immunohistochemistry-stained and H&E-stained samples from metastatic disease in HFD mice from the P21 protocol in liver (top panel) and lung (bottom panel). Scale bar= 500 µm.* ***H)*** *Left panel: Proportion plots showing the percentage of Ctrl and HFD mice from both models (embryonic and adult P21), which present metastasis. Right panel: Quantification of the number of mice presenting metastasis in both conditions (n=18 in Ctrl and 16 in HFD, P values as determined by Chi-square and Fisher´s exact test).* ***I)*** *Graphical scheme showing the basis of the intrasplenic assay to evaluate the liver metastatic capacity of a cell line.* ***J)*** *Left panel: Representative pictures of livers from the intrasplenic assay injected with Ctrl and iHFD cell lines (top) and representative brightfield microscopy images from cytokeratin 19 (CK19) immunohistochemistry of Ctrl and HFD livers (bottom). Right panel: Quantification of the percentage of mice that showed macroscopic metastasis in the intrasplenic assay (n=3 cell lines per condition, P values as determined by Chi-square and Fisher´s exact test).* ***K)*** *tSNE plot showing the different annotated cell types from the Liu et al. (38) single cell RNA-seq dataset of human primary tumors and metastasis.* ***L)*** *tSNE plots showing the P2RX7-expressing (blue) cells in the Liu et al. dataset separated by condition (primary for primary tumor and metastasis for liver metastasis).* ***M)*** *Quantification of P2RX7 expression in the epithelial compartment in both primary tumor and metastasis. P value indicated and determined by Wilcoxon test.* ***N)*** *Bar plot of P2RX7-expressing cells in primary tumors and metastasis. P value indicated and determined by Fisher test. P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

Lipid-conditioned macrophages drive plasticity in tumor cells through a CAMP-P2RX7 axis. We next studied the mechanisms by which HFD induces the mesenchymal/plastic phenotype of tumor cells. Since fatty acids (FAs) have been reported to induce a pro-CSC phenotype in human tumor cells (39), we asked whether increase exposure to FAs could cause the observed phenotype. Regarding P2RX7 expression, none of the FAs we tested (oleic acid, monounsaturated; linoleic acid, polyunsaturated; palmitic acid, saturated) could increase P2RX7 levels in mouse control cell lines (**Figure 7A**) or in PDX-derived human primary cell lines (**Figure 7B**). Thus, we hypothesized that the increase in P2RX7 and the phenotype induction could be driven by components of the tumor microenvironment such as adipose tissue or neutrophils (9). To test this hypothesis, we cultured adipose tissue from regular diet and HFD-fed C57Bl/6 mice and obtained adipose tissue conditioned medium (ACM). Exposure of control cell lines to ACM did not result in an increase in P2RX7 (**Figure 7C**). Also, we found that neutrophils were poorly infiltrated or almost absent in HFD tumors (**Figure S8A-B**). Next, we assessed highly tumor infiltrating macrophages, although they were not significantly

745 different in both settings (**Figure S8A, C**). Importantly, since the human RNA-seq data revealed an
746 enrichment in gene signatures for lipid-conditioned macrophages and antimicrobial response, we
747 theorized that macrophages might influence *P2RX7* expression in tumor cells and activate it via its
748 ligand, CAMP. Indeed, *P2RX7* and *CAMP* positively correlated in human PDAC (**Figure 7D**) and
749 CAMP expression has been linked to metabolic diseases related to obesity such as atherosclerosis
750 or diabetes (40, 41). To address this, we cultured immortalized primary bone marrow-derived
751 macrophages (IBMDMs) with FAs, and measured *Camp* expression at the transcriptomic level. We
752 found that naive IBMDMs did not express *Camp*, whereas FAs stimulation robustly induced its
753 expression (**Figure 7E**). Importantly, mice fed with HFD presented increase CAMP-expressing
754 macrophages in the bone marrow (BM) and intratumorally compared to control mice (**Figure 7F-G**).
755 As it was described that macrophage-derived hCAP-18/LL-37 can induce P2RX7 expression in
756 human cell lines (26), we next cultured control cells with naive macrophages conditioned medium
757 (MCM) or FAs-treated macrophage conditioned medium (F-MCM). Interestingly, F-MCM induced
758 more the expression levels of *P2rx7* compared to control cell lines treated with MCM (**Figure 7H**).
759 Furthermore, F-MCM decreased *Epcam* expression levels (**Figure 7I**) and induced a more
760 mesenchymal phenotype (**Figure 7J**), supporting that FAs derived from the diet conditioned
761 macrophages to produce molecules, such as CAMP, that induce P2RX7 expression and changes in
762 the cellular phenotype. Notably, stimulation of control cell lines with recombinant CAMP (rCAMP)
763 increased the levels of P2RX7 and CXCR4 (**Figure 7K-L**). In addition, we evaluated whether rCAMP
764 could also account for the elevated expression of PGLYRP1 observed in iHFD cells (**Figure S9A**).
765 We found that rCAMP significantly increased PGLYRP1 expression levels in control cell lines (**Figure**
766 **S9B**). Due to the protective role of PGLYRP1 blocking macrophage phagocytosis in PDAC cells (8),
767 we next performed a phagocytosis assay to determine whether iHFD cells exhibit enhanced protection
768 and immune evasive features. Consistent with the overexpression of PGLYRP1, iHFD cell lines were
769 phagocytosed less efficiently by primary macrophages than control cell lines (**Figure S9C-D**).

770 After finding an increase in CAMP expression in murine BM macrophages, we evaluated its
771 expression in patients' PBMCs as a possible proxy marker for HFD-induced PDAC. Although

macrophages are not present in circulation, we detected *CAMP* expression in PBMCs from both patients and non PDAC controls (**Figure S10A-B**). As this circulating CAMP is unlikely to be macrophage derived, no significant differences were observed between groups stratified by BMI. However, in both groups (BMI<25 and BMI>25), PDAC patients exhibited higher *CAMP* expression in PBMCs compared with non-PDAC controls (**Figure S10C-D**). As a complementary approach, we assessed the serum levels of the hCAP-18-derived peptide LL-37 in a cohort of healthy donors and PDAC patients. We found that PDAC patients with locally advanced and metastatic disease presented increased levels of sera LL-37, compared with healthy donors (**Figure 7M**). When we divided PDAC patients according to their BMI and compared the highest and lowest quartiles of the cohort, we found higher levels in those with high BMI (**Figure 7N**). These distinctions became more evident upon assessing *CAMP* expression at the transcriptomic level in patients, where we observed a parallel trend with *P2RX7* and *CXCR4* expression; specifically, increased levels among individuals with high BMI one year prior to diagnosis (**Figure 7O-Q**). Finally, we also evaluated EpCAM and hCAP-18/LL-37 levels by immunohistochemistry in a small patient cohort divided by BMI. In line with our results, patients with BMI>25 displayed lower EpCAM expression in tumor cells and higher levels of LL-37 within the immune compartment compared to patients with BMI<25 (**Figure S11A-C**). Altogether, these results support that HFD-induced obesity induces the systemic expression of CAMP by macrophages, which may contribute to the induction of an immune evasive and more mesenchymal plastic tumor cell state within the tumor.

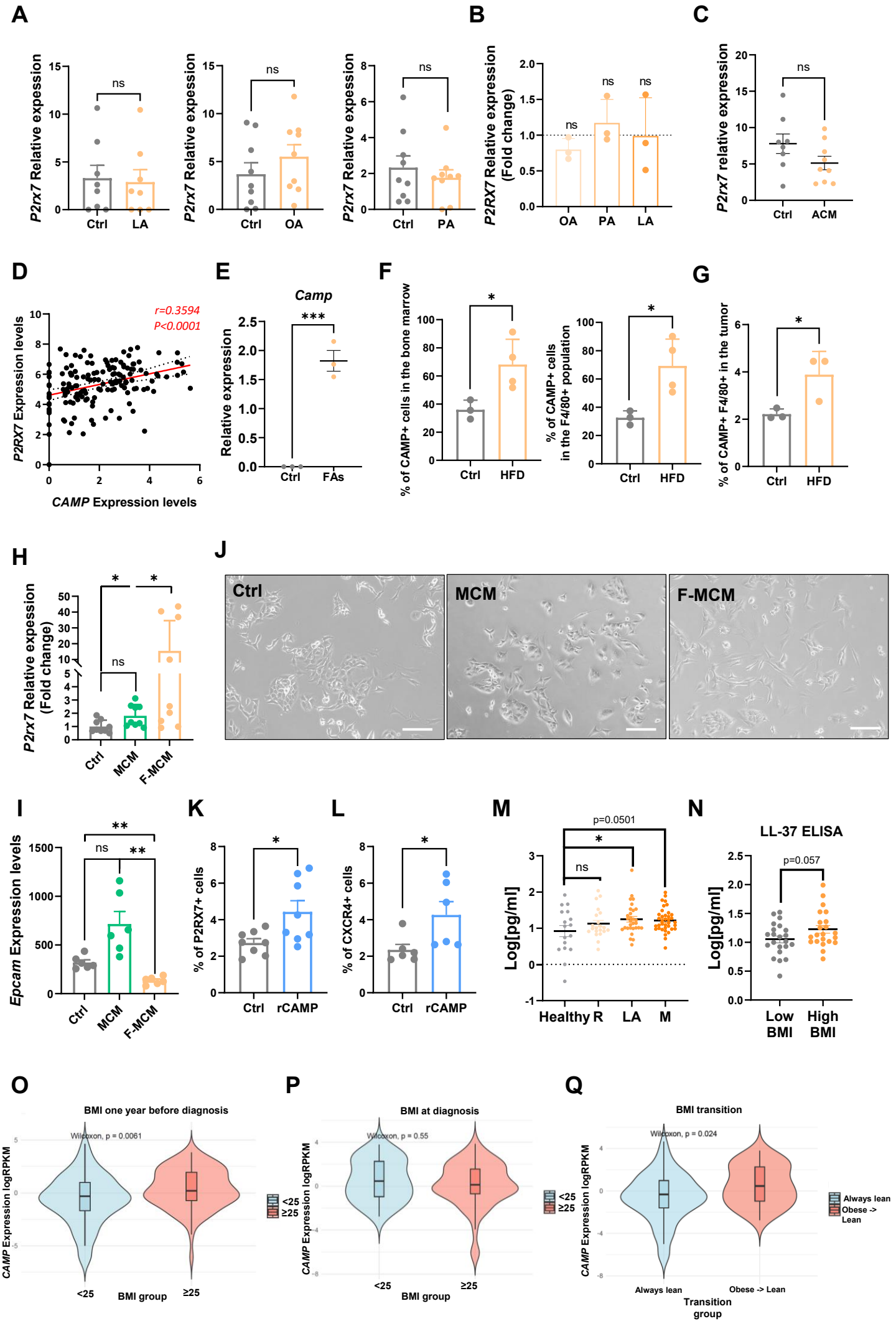
A
P2rx7 Relative expression
ns
Ctrl
LA
OA
PA
B
P2RX7 Relative expression (Fold change)
OA
PA
LA
C
P2rx7 relative expression
Ctrl
ACM
D
P2RX7 Expression levels
CAMP Expression levels
r=0.3594
P<0.0001
E
Camp
Relative expression
***
Ctrl
FAs
F
% of CAMP+ cells in the bone marrow
% of CAMP+ cells in the F4/80+ population
*
Ctrl
HFD
G
% of CAMP+ F4/80+ in the tumor
H
P2rx7 Relative expression (Fold change)
Ctrl
MCM
F-MCM
J
Ctrl
MCM
F-MCM
I
Epcam Expression levels
**
K
% of P2RX7+ cells
rCAMP
L
% of CXCR4+ cells
M
p=0.0501
Log[pg/ml]
Healthy R
LA
M
N
LL-37 ELISA
p=0.057
Low BMI
High BMI
O
BMI one year before diagnosis
Wilcoxon, p = 0.0061
CAMP Expression logRPKM
<25
≥25
BMI group
P
BMI at diagnosis
Wilcoxon, p = 0.55
Q
BMI transition
Wilcoxon, p = 0.024
Always lean
Obese -> Lean
Transition group

Figure 7. P2RX7-CAMP axis is induced by macrophages. A) *Quantification of P2rx7 expression by RT-qPCR in control (Ctrl) murine cell lines treated with fatty acids (FAs) (linolenic acid, LA [200µM]; oleic acid, OA [50µM]; palmitic acid, PA [50µM sodium palmitate]). Shown is the mean ± SEM (n=4 cell lines in duplicates for LA, and 3 cell lines in triplicate for OA and PA, P values as determined by unpaired T-test).* ***B)*** *Quantification of P2RX7 expression by RT-qPCR in PDX-derived primary human cell lines treated with FAs. Shown is the mean fold change ± SEM with controls set as 1.0 (n=3 cell lines, P values as determined by unpaired T-test).* ***C)*** *Quantification of P2rx7 expression by RT-qPCR in Ctrl murine cell lines treated with adipose tissue conditioned medium (ACM) for 48 hours. Shown is the mean ± SEM (n=4 cell lines in duplicates, P values as determined by unpaired T-test).* ***D)*** *Dot plot showing the correlation between P2RX7 and CAMP expression in TCGA patients. P values and r were calculated by Pearson's correlation.* ***E)*** *Quantification of Camp expression by RT-qPCR in immortalized bone marrow-derived macrophages treated with FAs (linolenic acid, LA; oleic acid, OA; palmitic acid, PA). Shown is the mean ± STDEV (n=3, P values as determined by unpaired T-test).* ***F)*** *Quantification of CAMP positive total cells (left panel) and CAMP positive cells in macrophages (F4/80) (right panel) from the bone marrow determined by flow cytometry in mice fed with Ctrl diet and HFD. Shown is the mean ± STDEV (n=3 mice for control group, 4 for HFD group, P values as determined by unpaired T-test).* ***G)*** *Quantification of CAMP positive tumor-associated macrophages determined by flow cytometry in mice fed with Ctrl diet and HFD. Shown is the mean ± STDEV (n=3 mice, P values as determined by unpaired T-test).* ***H)*** *Quantification of P2rx7 expression by RT-qPCR in Ctrl murine cell lines treated with macrophage conditioned medium (MCM) or fatty acid-trained macrophage conditioned medium (F-MCM) for 48 hours. Shown is the mean fold change ± SEM with Ctrl set as 1.0 (n=3 cell lines in triplicates, P values as determined by One Way ANOVA).* ***I)*** *Quantification of Epcam expression by RT-qPCR in Ctrl murine cell lines treated with MCM or F-MCM for 48 hours. Shown is the mean fold change ± SEM with Ctrl set as 1.0 (n=3 cell lines in duplicates, P values as determined by One Way ANOVA).* ***J)*** *Representative brightfield microscopy images of Ctrl cells treated with MCM and F-MCM. Scale bar= 50µm.* ***K)*** *Quantification of P2RX7 positive cells determined by flow cytometry in a Ctrl cell culture treated with recombinant (r) CAMP (100 µg/mL) for 48 hours. Shown is the mean ± SEM (n=8, P values as determined by unpaired T-test).* ***L)*** *Quantification of CXCR4 positive cells determined by flow cytometry in a Ctrl cell culture treated with rCAMP (100 µg/mL) for 48 hours. Shown is the mean ± SEM (n=6, P values as determined by unpaired T-test).* ***M)*** *Quantification by ELISA of soluble LL-37 levels in sera from healthy donors (Healthy) (n=17) and PDAC patients with resectable (R) (n=21), locally advanced (LA) (n=30) or metastatic disease (M) (n=37). Shown is the mean of log normalized concentration ± SEM (P values as determined by One-Way ANOVA, Dunnet's test).* ***N)*** *Quantification by ELISA of soluble LL-37 levels in sera from PDAC patients from the highest and lowest quartiles of BMI (n=22 patients per condition). Shown is the mean of log normalized concentration ± SEM (P values as determined by unpaired T-test).* ***O)*** *Violin plot showing the quantification of CAMP expression in PDAC patients based on their BMI 1 year before diagnosis (n for <25=75, n for ≥25=89). P value as determined by Wilcoxon test.* ***P)*** *Violin plot showing the quantification of CAMP expression in PDAC patients based on their BMI at time of diagnosis (n for <25=35, n for ≥25=54). P value as determined by Wilcoxon test.* ***Q)*** *Violin plot showing the quantification of CAMP expression in PDAC patients based on their BMI transition (patients previously obese that lost their weight versus those that always present a BMI<25) (n for always <25=75, n for obese → lean =35). P value as determined by Wilcoxon test. P values: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.*

Discussion

Mouse models of PDAC have been instrumental over the past decade for preclinical studies, enabling the field to capture the complexity of a disease in which the tumor microenvironment plays a central role. Among them, the KPC model (*Pdx1/Ptf1a*-Cre; *Kras*$^{+/G12D}$; *Trp53*$^{mut\ or\ KO}$) has been widely used (42), yet, like all models, it presents inherent limitations. The acquisition of oncogenic mutations during embryonic development generates tumors that mimic key features of human PDAC. However, in humans, these mutations are not acquired during embryonic development and this could be the

842 reason why it has been suggested that mutations are not sufficient to drive PDAC (43-45). Indeed,
843 Carpenter *et al.* recently demonstrated that healthy individuals frequently harbor *KRAS*-mutant
844 PanINs (4), and the prevalence of these lesions far exceeds the incidence of PDAC. This strongly
845 suggests that oncogenic potential does not reside solely in mutations but requires additional events,
846 particularly chronic inflammation, to initiate tumorigenesis.

847 Multiple studies over the past two decades have linked inflammatory cues to PDAC initiation and
848 progression, whether through epigenetic priming (46, 47), microenvironmental remodeling (48, 49) or,
849 as our group previously showed, through tissue damage that propagates oncogenic signaling and
850 suppresses senescence (5, 6). To dissect these processes, we developed inducible mouse models
851 in which mutations arise specifically in the acinar compartment of adult mice using a Tet-Off system.
852 This KPeC model (*Elas-tTA/tetO-Cre*; *Kras*$^{+/LSL}$G12Vgeo; *Trp53*$^{lox/lox}$) revealed that the adult pancreas is
853 intrinsically resistant to oncogenic transformation and that chronic pancreatitis is required to overcome
854 this barrier, a concept now widely accepted in the human setting. Importantly, this model provides a
855 unique opportunity to interrogate “early-onset” and “late-onset” PDAC, which we modelled through
856 our P21 and P60 protocols.

857 The rising incidence of PDAC in individuals under 50 years of age (3) suggests that environmental
858 exposures, including diet, may play a major role. By inducing mutations at different ages, we observed
859 that younger hosts were more permissive to tumor formation than the adult pancreas, which was
860 completely resistant to transformation by the *Kras* oncogene expression and *Trp53* loss, as previously
861 reported (5). This may reflect the retention of plasticity or stem-like features within subsets of acinar
862 cells, traits that become progressively restricted with age (50-52).

863 Strikingly, we found that HFD consumption increased tumor aggressiveness, metastatic behavior, and
864 reduced survival in the P21 protocol. These findings led us to hypothesize that chronic HFD exposure,
865 particularly during childhood or early adulthood, creates a systemic inflammatory landscape that
866 facilitates oncogenic transformation once mutations arise. Supporting this idea, we observed that
867 HFD-induced systemic signaling imprints stable transcriptional changes in tumor cells that persist in
868 culture, likely through epigenetic mechanisms. The acquisition of a plastic, stem-like phenotype,

869 robustly preserved in iHFD cell lines, has important clinical implications, as such states are strongly
870 associated with therapy resistance and metastatic behavior (53-56). Notably, this phenotype has not
871 been previously described in HFD-fed PDAC models.

872 Noteworthy, in our late-onset protocol (P60) we could induce preneoplastic lesions and full tumors in
873 a model that is normally resistant to the disease. This shows for the first time a link between specific
874 dietary habits and tumor induction in a model that presents oncogenic mutations at this age stage. In
875 alignment with our results with the P21 protocol, HFD-induced tumors and tumor cell lines both
876 presented a mesenchymal histology and morphology, as well as an enrichment in plastic-like cells
877 characterized by the expression of P2RX7 and CXCR4. These results strongly support the effect of
878 diet in the molecular characteristics and dynamics of PDAC formation and evolution despite the age
879 of mutation acquisition.

880 Although other inducible KRAS models have been used to study the intersection between HFD and
881 PDAC, they often rely on protocols that do not account for age-dependent differences in acinar cell
882 susceptibility to transformation (9, 10). These studies attributed the tumor-promoting effects of HFD
883 to enhanced desmoplasia, collagen deposition, and inflammation mediated by COX2 and IL-1β,
884 implicating pancreatic stellate cells, neutrophils, and adipocytes as key drivers. In contrast, our
885 models did not support a major role for neutrophils or adipose tissue. Instead, macrophages emerged
886 as central regulators of the tumor cell state observed both *in vitro* and *in vivo*. These discrepancies
887 may reflect differences in model design or the extended tracking period in our study, which spanned
888 up to one year. Importantly, the morphological and transcriptional reprogramming toward a
889 mesenchymal/plastic-like state had not been previously reported. Moreover, our analysis of real-world
890 patient data corroborated these findings, reinforcing their clinical relevance. Collectively, these
891 observations highlight HFD-induced obesity as a critical variable that should be considered when
892 studying interpatient PDAC heterogeneity and raises the question of whether dietary interventions
893 could reverse or mitigate this acquired phenotype.

894 Mechanistically, we show that FAs systemically condition macrophages to produce CAMP. In humans,
895 macrophage-derived hCAP-18 (the human homolog of murine CAMP) and its active peptide LL-37

896 have been shown to activate the CSC compartment in PDAC and contribute to tumorigenesis (26).
897 We found that both CAMP and its receptor P2RX7 were upregulated in patients with high BMI and in
898 HFD-derived tumors. Furthermore, CAMP induced the expression of PGLYRP1, another antimicrobial
899 protein produced by neutrophils and CSCs that protects tumor cells from macrophage- and
900 T-cell-mediated elimination (8). This CAMP-P2RX7-PGLYRP1 axis not only reinforces the plastic,
901 stem-like state of HFD-induced tumor cells but also shields them from immune clearance and
902 promotes metastatic behavior.

903 Consistent with this, we found that HFD enhances metastasis in a tumor cell-intrinsic manner. In this
904 context, the "seed" acquires the capacity to colonize distinct environments (lung and liver)
905 independently of the host, as HFD-fed mice did not exhibit increased metastasis when injected with
906 control cell lines in an intrasplenic assay. Notably, metastatic EpCAM^{+} tumor cells consistently
907 expressed high levels of P2RX7, underscoring its importance for metastatic success, a role previously
908 described in melanoma and prostate cancer (57, 58). Interestingly, in colorectal cancer the P2RX7-
909 CAMP axis appears to function in the opposite direction (59), highlighting the context-specific nature
910 of this pathway.

911 Although further research is needed, our findings position P2RX7 as a central driver of HFD- and
912 obesity-related PDAC, opening the door to more personalized therapeutic strategies for this patient
913 subgroup. Circulating hCAP-18/LL-37 may be a potential biomarker for early PDAC detection and
914 identifying candidates for targeted therapy. However, our analysis showed minor differences in LL-37
915 serum levels compared to RNA-seq results. This phenomenon may be attributed to the possibility that
916 patients with elevated BMI one year prior to diagnosis are misclassified as having low BMI at the time
917 of diagnosis due to disease-related weight loss. Thus, proper clinical data management seems to be
918 crucial to properly assess these changes.

919 In conclusion, here we describe the effects of HFD in tumor development in novel early onset and late
920 onset protocols for PDAC GEMMs. Our results show that diet could influence the transcriptional state
921 of tumor cells, rendering them a more stem/plastic-like state, which favors the metastatic disease.
922 HFD induces the expression of P2RX7, which is activated via macrophage-derived CAMP and favors

923 this characteristic tumor cell state. In patients, high BMI correlates with *P2RX7* and *CAMP* expression,
924 as well as the metastatic marker *CXCR4*, and it is preserved despite weight loss during disease
925 evolution. Altogether, these findings not only reveal a diet- and obesity-driven mechanism that shapes
926 tumor cell plasticity and metastatic potential but also highlights P2RX7 and CAMP as actionable
927 biomarkers and therapeutic targets, paving the way for early detection, risk stratification, and
928 personalized interventions in PDAC patients.

929

930 **Lead contact**

931 Requests for further information and resources of this study should be directed to the lead contact,
932 Dr. Carmen Guerra (mcguerra@cnio.es).

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972 Conceptualization of the study: C.G. and M.B. *In vitro* experimental work: A.G.-d-R., J.C.L.-G., S.A.,
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