

KDM6 Histone Demethylases Tune Pancreatic Cancer Plasticity

Edited Running Title: KDM6 histone demethylases tune pancreatic cancer plasticity

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Abstract

Aberrant epigenetic regulation is a central driver of tumorigenesis, and the reversibility of epigenetic modifications underscores their potential as therapeutic targets. Epigenetic factors often function in a highly context-dependent manner, necessitating rigorous study of their dynamic and complex roles. Here, we uncovered an intricate regulation of tumor development and plasticity by the KDM6 family of H3K27me3 histone demethylases in pancreatic ductal adenocarcinoma (PDAC), a disease with sustained high mortality and limited treatment options. Temporally resolving KRAS-driven tumor development revealed a switch from tumor suppressive to oncogenic functions for KDM6B, with implications for tumor aggressiveness in rodents and patients. These oncogenic functions were mediated by regulation of NOTCH signaling, a pathway known to promote PDAC tumorigenesis. Concurrent elimination of KDM6A and KDM6B led to profound phenotypic changes resembling sarcomatoid carcinomas, a rare, poorly characterized subtype of human PDAC. These tumors exhibited altered oncogenic networks, including NOTCH and JNK signaling, that may support development of therapies tailored to molecular subtypes. Given the critical and distinct activities of KDM6 histone demethylases during PDAC development, targeting KDM6 activities may be beneficial in the right cellular and temporal context.

Statement of Significance

Histone demethylases KDM6A and KDM6B dynamically modulate pancreatic cancer development, aggressiveness, and plasticity by altering NOTCH signaling, providing a rationale for developing selective KDM6 inhibitors.

Introduction

Pancreatic cancer continues to carry one of the most dismal prognoses of all malignancies, mostly due to late detection and a scarcity of effective therapeutic options¹. Over the last several years, whole-genome and transcriptome sequencing studies have provided key insights into the underlying diverse mutational landscape and identified several molecular subtypes of pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer^{2–4}. Despite these efforts in characterizing the molecular heterogeneity in PDAC few therapies against novel targets have been advanced⁵. In addition to genomic alterations, aberrant epigenetic regulation of gene expression is a major contributor to PDAC tumorigenesis, cell plasticity and drug resistance^{6–8}. Yet, the mechanistic actions of many targetable epigenetic drivers remain to be elucidated. Recent evidence suggests critical roles of Kdm6a (also known as Utx) and Kdm6b (Jmjd3), closely related histone demethylases that specifically erase repressive trimethylated lysine 27 of histone 3 (H3K27me3), in pancreatic tumorigenesis. While Kdm6a is X-linked and constitutively expressed in many tissues, the expression of autosomally encoded Kdm6b is induced by stress such as inflammatory cytokines, hypoxia and oncogenic stress⁹. The third family member, Kdm6c (Uty), is located on the Y chromosome and has minimal demethylase activity, thus Kdm6c is unlikely to compensate for a loss of Kdm6a and Kdm6b¹⁰. Mutations within the *KDM6A* gene are associated with a subset of PDAC patients and its inactivation has previously been shown to be a driver of PDAC tumorigenesis, resulting in the development of the squamous subtype of pancreatic cancer^{2,3,11–14}. In contrast, Kdm6b functions appear to be more nuanced, as it has been implicated in both tumor promoting and restraining functions in pancreatic and other cancers^{15–18}. Here, we set out to further elucidate the mechanistic underpinnings of Kdm6a and Kdm6b in pancreatic cancer tumorigenesis. This work was further motivated by the translational potential of a readily available, targeted Kdm6 inhibitor with promising chemotherapeutic effects¹⁹.

Our studies establish Kdm6b as a novel oncogenic driver of pancreatic cancer via the epigenetic regulation of Notch ligand Jagged1. Notably, the combined loss of Kdm6a and Kdm6b results in a rare sarcomatoid pancreatic cancer subtype with mesenchymal characteristics, indicating that Kdm6a and Kdm6b have synergistic functions that mediate cell plasticity. Our findings thus point to critical and distinct activities of Kdm6 histone demethylases during PDAC development depending on the precise cellular and temporal context. We provide compelling evidence that developing inhibitors specifically targeting Kdm6b may have therapeutic potential for treating pancreatic cancer and other malignancies where pan-Kdm6 inhibition may have adverse effects.

Materials and Methods

Mice

The following mouse strains were used in this study: *Kdm6a*^{flox} (Jackson #024177, RRID: IMSR_JAX:024177), *Kdm6b*^{flox} (Jackson #029615, RRID: IMSR_JAX:029615), *LSL-Kras*^{G12D} (gift from D. Tuveson, Cold Spring Harbor Laboratory²⁰, RRID: IMSR_JAX:008179), *p48*^{CreER} (gift from C. Wright, Vanderbilt University²¹, RRID: IMSR_JAX:019378), and *Rosa26-mTmG* (Jackson #007676, RRID: IMSR_JAX:007676). Mice were maintained on a mixed genetic background and a balance of males and females was used for all experiments. Tamoxifen citrate (Teva Pharmaceuticals) was given by oral gavage of 3 doses of 6.7mg at 7-9 weeks of age. All animals were housed in the barrier facility according to protocols approved by the University of California San Francisco Institutional Animal Care Use Committee (IACUC).

Histology and Immunohistochemistry

Tissues were fixed in Z-Fix (Anatech Ltd., 174) overnight at 4 °C, processed for paraffin embedding and cut into 5 µm sections. Paraffin-embedded sections were stained with either

hematoxylin (Sigma, MHS32) and eosin (Sigma, HT110132) or Alcian Blue (Sigma, B8438) and Nuclear Fast Red (Vector Laboratories, H-3403) according to standard protocols. Picrosirius Red staining kit (Abcam, ab150681) was used according to manufacturer's instructions and counterstained with Weigert's iron hematoxylin (Sigma, HT1079). For immunohistochemical analysis, antigen retrieval was performed by boiling in Citrate buffer (BioGenex, HK086) for 15 min. Sections were blocked for 1 hour with 10% FBS in PBS at room temperature and incubated with primary antibodies in blocking solution overnight at 4 °C, followed by incubation with either Alexa fluorophore-conjugated secondary antibodies (1:500, Thermo Fisher) or biotin-conjugated secondary antibody (1:400, Jackson ImmunoResearch) for 1 hour at room temperature. For immunofluorescence, slides were mounted with DAPI (Thermo Fisher, P36981). For immunohistochemistry, sections were further incubated for 30 min in ABC solution (Vector Laboratories, PK-6100), developed using the DAB Substrate Kit (Vector Laboratories, SK-4100) as per manufacturer's instructions and counterstained with hematoxylin. For human tissues, IHC for Notch1, Jagged1 and KDM6B was performed using a Bond RXm system with pre-treatment with Epitope Retrieval Solution 2 (Leica Biosystems); IHC for Kdm6a was performed on a Benchmark XT (RRID: SCR_026335) system with pre-treatment with Cell Conditioning Solution 2 (Ventana Medical Systems). Antibody binding was either detected with a polymer refine detection kit without post primary reagent or with an ultraView universal DAB detection kit and visualized with DAB as a dark brown precipitate, followed by hematoxylin counterstaining. All primary antibodies used in this study are included in Supplementary Table S1. For Alcian Blue staining, slides were scanned at 20X or 40X magnification and one section per animal was quantified using QuPath (RRID: SCR_018257). Percentages were recorded as Alcian Blue positive area versus whole section area, excluding lymph nodes. For Picrosirius Red staining, one stained slide per animal was scanned using polarized light microscopy on a Nikon Eclipse TE2000-U (RRID: SCR_023161) and quantified using Fiji (ImageJ, RRID: SCR_002285).

IHC evaluation of patient samples

All studies involving patient specimens were performed with appropriate institutional review board (IRB) or ethics approvals and written informed consent was obtained from patients unless otherwise noted. The UCLA PDAC tissue microarray has been previously described²². PDAC and patient-matched normal pancreas and PanIN lesions were obtained from archival tissue blocks from treatment-naïve patients undergoing surgical resections of stage I/II tumors at UCLA. The study was conducted in accordance with the U.S. Common Rule and the ethical principles of the Belmont Report. The construction and use of TMAs and patient records was performed under UCLA institutional review board approved protocol (IRB-11-0512) with an exemption for patient informed consent approved on the basis of the use of remnant pathology material and deidentified patient data (exempt category 4). The clinicopathological variables used for analysis of the tissue microarray were based on the 7th edition of the AJCC/UICC TNM staging system for pancreatic cancer. KDM6B histoscores are based on a semi-quantitative assessment of nuclear staining (0-300, staining intensity [0-3] multiplied with percentage of tumor cells with that intensity [0-100]). Values shown represent averages of 2-3 cores per tumor and one core per PanIN and adjacent normal tissue.

SCP cases were retrospectively identified, and archival tissue was provided as unstained anonymized or de-identified slides from Johns Hopkins University (JHU) pancreatectomy biobank (6 cases), the Mayo Clinic Biobank (1 case), an Italian institution database (2 cases) and the tissue bank of the University Hospital of the Technical University of Munich (4 cases). Studies were conducted in accordance with the revised US Common Rule, The Declaration of Helsinki, the Belmont Report, and CIOMS. The JHU pancreatectomy biobank (IRB00138853) and database are IRB approved and prospectively maintained institutional tissue collection biobank and patient database. Samples and clinical data were collected from PDAC patients who underwent surgical resection at the Johns Hopkins Hospital. All patients from whom samples were collected for the biobank provided written consent to collect, store, and use tissue samples in

future institutional, multi-institutional and international research. The Mayo Clinic Biobank samples were collected as part of clinical care. Studies are approved under IRB 21-006453 and 18-003263 with an exemption for patient informed consent. The study at the Italian institution was approved by the local ethical committee as a part of a national-based study on PDAC rare histotypes and subtypes (Bari ethical committee, date of approval: 10-01-2018, project n.242, code: 708/CE). The TUM TMA is part of a PDAC cohort that has been described previously. Briefly, it consists of patients who underwent pancreatic resection for PDAC at the Department of Surgery, TUM University Hospital Klinikum rechts der Isar (Germany) and was assembled retrospectively. Usage of this material was approved by the TUM Ethics Committee (1926/2007 and 126/2016). For each tissue the Immunoreactive Score²³ was calculated as a product between the proportion of positive tumor cells (0-4) and staining intensity (0-3).

RNA isolation and Reverse Transcription-qPCR

RNA was isolated using the RNeasy Mini Kit (Qiagen, 74106) according to manufacturer's instructions. cDNA was generated using the SuperScript III First Strand synthesis kit (Thermo Fisher Scientific, 18080085) with 500-1000 ng of RNA and random hexamers. Quantitative PCR (RT-qPCR) was performed using FastStart Universal SYBR Green mix (Sigma, 4913914001). RNA expression of target genes was normalized to *cyclophilin*. RT-qPCR primer sequences are included in Supplementary Table S2.

RNA-sequencing and analysis

For bulk RNA-seq, a stranded mRNA library was prepared by PolyA capture and paired-end sequencing was performed by Novogene, generating 40-50 million reads per sample. Quality of raw FASTQ sequences was assessed using FASTQC (RRID: SCR_014583). To process RNA-seq libraries, adapter sequences were trimmed using Cutadapt version 1.14 (requiring a length of >10 nucleotides after trimming, RRID: SCR_011841) and quality-filtered by requiring all bases

to have a minimum score of 20 (-m 20 -q 20). Only reads that passed the quality or length threshold on both strands were used for mapping. Reads were aligned to the mouse genome GRCm38 (mm10) with the STAR Aligner (version 020201, RRID: SCR_004463). Ensembl reference annotation version 89 was used to define gene models for mapping quantification. Uniquely mapped reads for each gene model were produced using STAR parameter ‘-quant-Mode GeneCounts.’ Differential expression analysis was performed in R using DESeq2 (v.1.16.0, RRID: SCR_015687) with default parameters, including Cook’s distance treatment to remove outliers. The codes are available upon request. Gene Set Enrichment Analysis was performed using the GSEAPreranked option in GSEA 4.1.0 (RRID: SCR_003199) with default parameters. The ranked input file was generated by ordering genes according to signed $\log_2FC \times -\log_{10}(\text{adjusted p-value})$. The analysis was based on predefined gene sets from the Molecular Signatures Database (MSigDB v7.4, RRID: SCR_016863). To calculate the accurate p-values, R package fgsea (v1.31.4, RRID: SCR_020938) was run using the same gene sets.

10X Xenium sequencing and analysis

Formalin fixed, paraffin embedded mouse pancreas tissue sections were processed for spatial transcriptomics data using the Xenium In Situ platform (10x Genomics, RRID: SCR_023910) following the manufacturer’s protocol, using a custom Xenium RNA v1 gene panel consisting of 475 genes. The tissue sections were processed, imaged, and sequenced at the Core Facility Comparative Experimental Pathology at the Institute of Pathology of the Technical University of Munich (RRID: SCR_028771). Raw Xenium output files were obtained from the core facility for downstream bioinformatics analysis. A total of 15 Xenium datasets were processed using InSituPy²⁴ (version 0.11.4, RRID: SCR_028769). For each sample, Xenium cell-level and transcript-level data were imported into an InSituData object using the read_xenium function. Corresponding H&E whole-slide images acquired on the same tissue section were then automatically registered to the Xenium nuclear (DAPI) image using the InSituPy image

registration pipeline. Tissue compartments were manually annotated in QuPath (version 0.7.0) to select only tumor regions. The resulting region masks were exported as GeoJSON files and subsequently imported into the InSituData object as region annotations, enabling histology-guided restriction of downstream transcriptomic analyses to defined pathological compartments. Within this annotated InSituPy object, we computed standard per-cell quality control metrics using `calculate_qc_metrics`, followed by filtering of low-quality cells and lowly detected genes with `filter_cells(xd, min_genes = 20)` and `filter_genes(xd, min_cells = 5)`, respectively. The resulting filtered count matrix was then normalized and log-transformed using the InSituPy `normalize_and_transform` function, yielding a normalized expression matrix that was used for all subsequent downstream analyses. For each inSitu sample, an AnnData object was first exported after subsetting to tumor region only, and these sample-level AnnData objects were then concatenated into a single dataset. The combined AnnData was integrated using the ResolVI model from scvi-tools²⁵ (version 1.4.2, RRID: SCR_026673) to obtain a batch-corrected latent space. Based on this integrated embedding, a standard Scanpy²⁶ (version 1.12.1, RRID: SCR_018139) workflow was applied, including construction of the neighborhood graph, UMAP embedding, and Leiden clustering at a resolution of 1.5 to derive fine-grained clusters. Clusters were subsequently curated into major cell types based on canonical marker gene expression. Specific populations were subsequently reclustered at a resolution of 1.0 to resolve additional cellular subtypes. Palantir²⁷ (version 1.4.4, RRID: SCR_027194) pseudotime analysis was performed on ductal and sarcomatoid clusters by choosing a start cell from within the ductal cluster. For visualization of dynamic gene expression changes over pseudotime, MAGIC-imputed values from the `MAGIC_imputed_data` layer were used. Spatial co-occurrence of cell populations were quantified using the co-occurrence probability module implemented in Squidpy²⁸ (version 1.8.1, RRID: SCR_026157) tool. Distance-resolved co-occurrence profiles were computed between the ductal/sarcomatoid cluster and all other annotated cell types.

Western blotting

Cells were lysed in Pierce RIPA Buffer (Thermo Scientific, 89901) containing 1X PhosSTOP phosphatase inhibitor (Sigma Aldrich, 4906845001), 1X complete protease inhibitor cocktail (Roche, 11697498001) and 100 mM PMSF. The insoluble fraction was separated from the lysate by centrifugation at 20,817 x g. PVDF membranes were incubated with the primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 1 hour at RT. Membranes were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, 34580). Antibodies are listed in Supplementary Table S1.

Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR)

ChIP was performed as previously described^{29,30}. Briefly, cells were fixed using 1% formaldehyde (Thermo Scientific, 28908). Nuclei were isolated using lysis buffer [167.5 mM NaCl, 5 mM EDTA pH 7.5, 50 mM Tris pH 7.5, 0.5% NP40, 1% Triton X-100, and protease inhibitors] and chromatin was sonicated in 0.1% SDS sonication buffer using a Bioruptor® Pico sonicator (Diagenode, RRID: SCR_028774). Sheared chromatin was then immunoprecipitated with antibodies against IgG (Cell Signalling #2729, RRID: AB_1031062), H3K4me3 (Millipore #05-745R, RRID: AB_1587134), or H3K27me3 (Abcam #ab6002, RRID: AB_305237). DNA was purified using MinElute PCR Purification Kits (QIAGEN) and quantitative PCR (qPCR) was performed to measure DNA enrichment, using Power SYBR Green Master Mixes on a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems). Primer sequences are detailed in Supplementary Table S3.

Flow cytometry

Cells were dissociated to single cells using 0.25% Trypsin-EDTA and filtered through a 40 µm strainer. After blocking for 15 min with anti-mouse CD16/CD32 FcγR block (BioXCell #BE0307, RRID: AB_2736987), cells were incubated with primary antibody or isotype controls for 1 hour on

ice. If appropriate, cells were incubated with fluorophore-conjugated secondary antibodies (1:500, Thermo Fisher) for 30 min on ice. For compensation, UltraComp™ eBeads Compensation Beads (Invitrogen, 01-2222) were incubated with 1 µl of each primary antibody for 15 min on ice. Cells were analyzed on a BD LSRFortessa (RRID: SCR_018655) with FACSDiva software (RRID: SCR_001456). Antibodies are listed in Supplementary Table S1.

Acinar cell isolation and culture

Mouse pancreata were cut into small pieces and digested with 0.4 mg/ml Collagenase P (Sigma, 11213865001) at 37 °C for 15 min. Tissue was washed twice with 5% FBS in HBSS/- and filtered through a 100 µm strainer. The flow-through was layered over 30% FBS in HBSS/- and centrifuged for 2 min at 300 x g to pellet the intact acini. Acini were let rest for 4-6 hours in acinar media (RPMI 1640 containing 10% FBS, 1X Penicillin/Streptomycin, 100 µg/ml soybean trypsin inhibitor, 1 µg/ml dexamethasone) before embedding in a 1:2 ratio of acinar media to growth factor-reduced Matrigel (Corning, 356231) on collagen I-coated 24-well plates (Corning, 354408). Acinar cells were grown for 4 days and images taken daily of 3 wells, 2 images per well. Quantification was performed on all images by counting the number of acini and ductal clusters present (at least 60 per time point). On day 4, cells were extracted by resuspending contents of wells in Cell Recovery Solution (Corning, 354253) and incubation at 4 °C for 1 hour, followed by subsequent RNA isolation.

Generation of murine cancer cell lines

A piece of the mouse tumor was minced into small pieces and digested in a solution containing 0.5 mg/ml Collagenase Type I (Fisher Scientific, 17-018-029) for 25-30 min at 37 °C with occasional vigorous shaking of the tube. The cell suspension was filtered through a 100 µm strainer and the cells were plated on a collagen I-coated tissue culture dish (Corning, 354450). Experiments were performed after reaching passage 5 with no fibroblasts present in the culture

any more. Cell lines were authenticated via genomic PCR to confirm recombination. Genomic DNA was isolated from cell pellets using QuickExtract DNA Extraction Solution (Epicentre, QE09050) according to manufacturer's instructions followed by genotyping PCR using GoTaq Green Master Mix (Promega, M7123). The following primers were used to amplify the *Kdm6b* locus: *Kdm6b_flox_fw* 5'-CAGCGATCCTGACTTGTTCA-3' or *Kdm6b_Intron_fw* 5'-GTCAACCAGCCTTGAGGTGT-3', *Kdm6b_flox_rv* 5'-GTGCCAAGGCTGGAGGA-3'. In case of incomplete recombination of the *Kdm6b* floxed allele, cells were transduced with Ad5CMVCre-eGFP (University of Iowa, VVC-U of Iowa-1174), sorted for GFP+ cells three days later via FACS, subcloned and clones with successful recombination mixed together to obtain a new heterogenous cell line. All cell lines were regularly tested for Mycoplasma using the PCR-based Venor GeM Mycoplasma Detection Kit (Sigma, MP0025) with the last testing performed on 01/14/22.

Cell culture assays

Murine cancer cell lines were cultured in DMEM supplemented with 10% FBS and 1X Penicillin/Streptomycin at 37 °C, 5% CO₂. For cell proliferation assays, cells were seeded in quadruplicates and grown for 72 hours. For treatment experiments, cells were seeded in triplicates and the agent added after 4-5 hours of letting cells attach. Relative cell numbers were evaluated using CellTiter-Glo reagent (Promega, G7571) according to manufacturer's instructions. The following inhibitors were used in this study: GSK-J4 (MedChemExpress, HY-15648B), JNK-IN-8 (MedChemExpress, HY-13319), γ-Secretase Inhibitor XX - CAS 209984-56-5 (Millipore, 565789). For tumorsphere assays, 1500 single cells were seeded in triplicates in ultra-low attachment 6-well plates (Corning, 3471) in DMEM/F12 supplemented with 1X Penicillin/Streptomycin, 2% B27 (Stem Cell Technologies, 05711), 1% N2 Supplement (Fisher Scientific, 17502048), 20 ng/ml EGF and 20 ng/ml FGF2. After 4 or 6 days, tumorspheres were manually counted using a stereo microscope or harvested for flow cytometry. For transwell

migration assay, cells were serum-starved for 16h in DMEM supplemented with 0.5% FBS prior to seeding 7×10^4 cells in triplicates into the upper chamber of 8- μ m pore membranes (Merck Millipore, Cat. No. PCEP24H48), while DMEM supplemented with 10% FBS was added to the lower chamber. After incubation for 30h, non-migrated cells were removed and migrated cells at the lower surface were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet solution. 5 randomly selected fields were acquired per well using bright-field microscopy. The crystal violet-positive area was quantified using a custom-written macro in ImageJ (NIH). Transient transfections were performed using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, L3000008) and P3000™ Enhancer Reagent according to the manufacturer's instructions. DNA–lipid complexes were prepared in Opti-MEM and incubated for 10–15 min at room temperature. Cells were harvested and added directly to the DNA–lipid complexes in suspension (reverse transfection). 2.5 μ g (6-well) or 500 ng (24-well) total plasmid DNA was used per well. Mouse Jagged1 (OriGene, NM_013822; MR212152) was cloned into a pCMV6-Entry vector containing a C-terminal Myc-DDK-tag. A pmaxGFP plasmid (Lonza) was used as transfection control. Following transfection, cells were cultured under standard conditions (37°C, 5% CO₂) and harvested 48h post-transfection for downstream analyses.

Patient-derived pancreatic cancer organoids

Pancreatic tumor specimens were obtained from patients undergoing surgical resection at the Medical Center of the University of California at San Francisco. Samples were collected under approved protocol CC#134510 from the UCSF Molecular Profiling in Gastrointestinal Malignancies and Registry Oversight Committee. PDO1 line was derived from a fine needle aspirate sample, minced and digested in digestion buffer (DMEM/F12, 5 mg/ml Collagenase II, 1X Penicillin/Streptomycin, 10 mM HEPES and 100 μ g/ml Primocin) for 10 min shaking at 37 °C. Digestion procedure was repeated 2 additional times with fresh digestion buffer after letting undigested tissue fragments settle and removing the supernatant. All supernatant fractions were

pooled and centrifuged at 200 x g for 5 min at 4 °C. The pellet was resuspended in growth factor-reduced Matrigel (Corning, 356231) and 50 µl drops plated in 24-well plates. Matrigel was allowed to solidify in a CO₂ incubator (5% CO₂, 37 °C) and complete human organoid media added to each well. PDO2 line was derived from pleural effusion fluid, centrifuged at 530 x g for 10 min, washed in PBS and centrifuged again. The cell pellet was resuspended in Matrigel and plated as described above. The complete human organoid media for each line is listed in Supplementary Table S4. For viability assays, 1x10⁴ cells were seeded in triplicates in 15 µl Matrigel on 96-well plates. Treatments were started after letting organoids recover for 3-7 days, for the duration indicated in the figure legends. Relative cell numbers were determined using 3D CellTiter-Glo reagent (Promega, G9681) according to manufacturer's instructions. For RNA isolation, Matrigel was dissolved using Cell Recovery Solution (Corning, 354253) for 1 hour at 4 °C with occasional manual agitation and proceeded with the RNeasy Micro Kit (Qiagen, 74004) according to manufacturer's instructions.

Statistical analysis

The number of biological replicates and independent experiments performed, as well as the statistical tests used to determine statistical significance are indicated in the figure legends. All tests were performed using the GraphPad Prism (versions 9.1.2 and 11.0.0, RRID: SCR_002798) software. If appropriate, outliers were determined using the ROUT test with Q = 10%.

Data availability

Bulk RNA-sequencing datasets generated for this study have been deposited at NCBI Gene Expression Omnibus (GEO, RRID: SCR_005012) under GSE202300. Spatial transcriptomics datasets have been deposited under GSE341984. Processed files and all other raw data generated in this study are available upon request from the corresponding author.

Results

KDM6B negative PDAC has a better prognosis

In contrast to the putative driver mutations occurring in the known tumor suppressor gene *KDM6A*, genetic alterations in its related family member *KDM6B* are less frequent in PDAC based on cBioPortal data (1.4%, 13/910 cases, Supplementary Fig. S1A). Deletions of *KDM6B* were coincidental with those of *TP53*, likely due to their close genetic proximity of ~100 kilobases. These observations point to potentially non-redundant functions of *KDM6A* and *KDM6B* in PDAC and challenges in assigning *KDM6B* activities in humans due to its fate being closely tied to *TP53* loss. We assessed *KDM6B* levels on tissue microarrays (TMAs) of treatment naïve resectable human PDAC cases with matching pancreatic intraepithelial neoplasia (PanIN) and adjacent normal tissue of a previously described cohort from the University of California, Los Angeles (UCLA cohort)²². *KDM6B* histoscores were significantly increased for PanIN and PDAC epithelial cells compared to normal ducts, with variable levels in PDAC cases (Fig. 1A, B). Patients with *KDM6B* negative tumors (histoscore <40) had a longer median survival (Fig. 1C, $p=0.0344$) despite the fact that no significant difference in lymph node status or tumor grade, stage or size was observed between *KDM6B* positive and negative tumors (Supplementary Table S5). Similarly, *KDM6B* expression levels were not correlated with any of these clinicopathological variables (Supplementary Fig. S1B). Furthermore, in a PDAC cohort from the Technical University of Munich (TUM cohort) consisting of partially matched primary and metastatic tumors we detected robust *KDM6B* staining in metastases with a trend towards higher Immunoreactive Scores compared to primary tumors (Fig. 1D), indicating sustained expression during PDAC progression. Summarily, these findings show that *KDM6B* expression is observed in human PDAC and that higher expression levels indeed correlate with shortened survival, suggesting an oncogenic role for this histone demethylase in pancreatic cancer.

Loss of Kdm6b impairs acinar identity and accelerates Kras driven neoplasia

While human histology provides valuable insights into cancer morphology and subtypes, it does not reveal insights into gene requirements during disease formation. Kdm6a and Kdm6b are robustly expressed in the healthy mouse pancreas in both exocrine and endocrine cells (Supplementary Fig. S2A), indicating that genetically modified transgenic mice could illuminate Kdm6 functions during disease progression. Since Kdm6b has a vital role during the development of the endocrine pancreas³¹, we utilized the inducible *p48^{CreER}* knock-in allele (Supplementary Fig. S2B) crossed to a conditional *Kdm6b^{fllox}* allele and administered tamoxifen to adult mice (Supplementary Fig. S2C) to determine the function of Kdm6b in mature acinar cells. *Kdm6b* expression was significantly reduced in acinar cells of *p48^{CreER/+}; Kdm6b^{fl/fl}* (*Kdm6b^{KO}*) compared to control *p48^{CreER/+}; Kdm6b^{+/+}* mice, while expression levels of *Kdm6a* and *Kdm6c*, the third member of the Kdm6 family, were not significantly changed (Supplementary Fig. S2D). 10 weeks post tamoxifen treatment the acinar architecture in *Kdm6b^{KO}* mice was disrupted compared to controls, with individual acini appearing smaller and more condensed and being marked by a reduction in amylase staining (Supplementary Fig. S2E). Several genes expressed in pancreatic progenitors (*Rbpj*, *Pdx1*, *Notch1*, *Sox9*) were upregulated in *Kdm6b^{KO}*, indicating a more immature state of the acinar cells (Supplementary Fig. S2F). These data suggest that Kdm6b is critical for maintaining the mature acinar differentiation state.

To assess a potential role for Kdm6b in oncogenic Kras-driven neoplasia we next generated cohorts of *p48^{CreER/+}; LSL-Kras^{G12D/+}; Kdm6b^{fl/fl}* (*KCB^{KO}*) as well as control *p48^{CreER/+}; LSL-Kras^{G12D/+}; Kdm6b^{+/+}* (*KC*) mice (Fig. 2A). 10 and 25 weeks after tamoxifen treatment, *KCB^{KO}* mice exhibited an increased pancreas weight to body weight ratio (PW/BW) compared to *KC* (Fig. 2B), indicative of cellular and stromal expansion. Indeed, histologically *KCB^{KO}* pancreata at 10 weeks post tamoxifen displayed more widespread areas of neoplasia compared to *KC*, including Alcian blue-positive acinar-to-ductal metaplasia (ADM) and low-grade PanIN accompanied by stromal remodeling, which was further increased at the 25 weeks time point (Fig. 2C, D). *KCB^{KO}* further

showed a greater fibrillar collagen content compared to *KC* 10 weeks post tamoxifen, as also shown by increased Picrosirius red staining (Fig. 2E, F). To summarize, these results indicate that *Kdm6b* deficiency accelerates *Kras*-driven neoplasia derived from acinar cells.

***Kdm6b* impedes PDAC progression and aggressiveness**

To interrogate the role of *Kdm6b* in later stages of PDAC tumorigenesis, we aged out *KC* and *KCB^{KO}* mice until their humane endpoints. Surprisingly, despite the acceleration of neoplasia in *KCB^{KO}* mice these animals did not exhibit a reduced median survival compared to *KC* mice (69.0 vs. 73.1 weeks, Fig. 3A). Further, we did not observe a difference in PW/BW or PDAC incidence between genotypes (Fig. 3B, C) and histological examination of the tumors at end-stage revealed a similar range from mostly moderately to partially undifferentiated PDAC (Fig. 3C, D). However, similar to the early neoplasia stage, *KCB^{KO}* tumors presented with higher collagen content compared to *KC* (Fig. 3D, E). Strikingly, there was a pronounced decrease in metastatic incidence in *KCB^{KO}* mice compared to *KC* (16.7% vs. 64.3% of tumor-bearing mice, Fig. 3F), a finding that coincided with *KCB^{KO}* tumors containing significantly fewer proliferating Ki-67⁺/CK19⁺ cells (Fig. 3G, H). To gain insights into the cellular characteristics, we next generated cell lines from tumors of both genotypes (Supplementary Fig. S3A-C). Congruent with the *in vivo* data (Fig. 3G, H), *KCB^{KO}* cells had a reduced proliferative capacity than *KC* cells when grown in 2D (Fig. 3I). *Kdm6b* loss further strongly impaired tumorsphere formation and expression of cancer stem cell (CSC) markers CD24 and EpCAM in *KCB^{KO}* compared to *KC* cells, indicating that *Kdm6b* loss interferes with CSC formation (Fig. 3J, K). Despite the marked decrease in the occurrence of metastases *in vivo*, *KCB^{KO}* cells did not exhibit an altered migratory phenotype *in vitro* as assessed by transwell migration assay (Supplementary Fig. S3D), suggesting that niche factors and stromal cells present *in vivo* are required to support cell migration. Furthermore, the loss of stem cell properties may also hinder effective metastatic dissemination in *KCB^{KO}*. Summarily, our data indicate that *Kdm6b* has stage-specific and opposing effects on tumor development, impairing early neoplastic

transition while promoting oncogenic functions in PDAC cells to assert a more aggressive phenotype. The oncogenic activity in established PDAC is consistent with the higher rates of survival observed in human patients with PDAC lacking KDM6B expression (Fig. 1C).

Oncogenic activities of Kdm6b are mediated by modulation of Notch signaling

To understand the molecular underpinnings of the cellular changes induced by Kdm6b deficiency, we performed bulk RNA-sequencing on *KC* and *KCB*^{KO} tumor-derived cell lines. 640 genes were significantly differentially expressed between the genotypes (Fig. 4A, Supplementary Fig. S3E) with 229 and 160 Gene Set Enrichment Analysis (GSEA) pathways significantly enriched in *KC* and *KCB*^{KO}, respectively (padj<0.05, Fig. 4B). The top enriched pathways in *KC* cells were mostly related to cholesterol metabolism, DNA replication and Notch signaling, while *KCB*^{KO} cells were enriched in invasiveness and epithelial-mesenchymal-transition (EMT) gene sets. The latter was surprising given their reduced metastatic burden *in vivo* and the unchanged migratory phenotype *in vitro*. A deeper look into the pathway genes revealed that those differentially expressed in *KCB*^{KO} were largely involved in desmoplasia and stromal remodeling (e.g. *MME*, *PLAU*, *MMP2*, *SERPINF1*, *LGALS1*, *PCOLCE*, *PDGFRA*) rather than intrinsic drivers of EMT, possibly contributing to the increased fibrosis seen at 10 weeks post tamoxifen (Fig. 2E-F). Thus, Kdm6b elimination in the context of oncogenic Kras modulates signaling of PDAC to niche cells without promoting metastatic growth.

We decided to further follow up on the Notch pathway as this signaling cascade has previously been implicated in promoting proliferation, invasiveness and cancer stem cell properties^{32,33}. Several major pathway components, most notably the ligands *Jagged1* and *Jagged2* as well as *Neur11b*, the ligand processing ubiquitin ligase, were downregulated in *KCB*^{KO} cells (Fig. 4C-E, Supplementary Fig. S3F). Immunostaining of end-stage tumors confirmed the reduced Jagged1 expression in *KCB*^{KO} compared to *KC*, while Notch1 levels remained unchanged between genotypes (Fig. 4F). To test whether the reduction of Notch signaling might act as a potential

driver of the *KCB^{KO}* phenotype we blocked the pathway with the well-described γ -secretase inhibitor XX which prevents cleavage of the receptor and thereby downstream pathway activation. Importantly, treating KC cells with XX reduced proliferation, with rates approaching those of *KCB^{KO}* (Fig. 4G). Conversely, the inhibitor only had a minor effect on *KCB^{KO}* cells presenting with already reduced Notch signaling. Ectopic expression of the ligand Jag1 in *KCB^{KO}* cells via transient transfection led to a moderate increase in proliferation and formation of tumorspheres, trending toward values observed for control transfected KC lines. In contrast, overexpression of Jag1 in KC lines did not elevate proliferation or tumorsphere formation (Fig. 4H, I, Supplementary Fig. S3G). This partial rescue of Kdm6b-deficient PDAC cells would indicate that despite the requirement for Notch signaling in maintaining aggressive PDAC features, other factors contribute to assert the *KCB^{KO}* phenotype.

We then performed chromatin immunoprecipitation (ChIP) studies of H3K27me3 (repressive) and H3K4me3 (activating) histone marks at the promoter regions of select Notch genes to test whether Kdm6b H3K27me3 demethylase activity directly regulates their expression. Indeed, we found an increase in repressive H3K27me3 marks specifically at promoters of ligands *Jag1*, *Jag2* as well as *Neur11b* in *KCB^{KO}* tumors, thus providing molecular evidence for their reduced expression, while levels were unchanged for Notch1 and Notch2 receptors (Fig. 4J). Activating H3K4me3 marks were not affected at any target gene (Fig. 4K). These findings support our expression data and indicate that Kdm6b histone demethylase activity upregulates the expression of Notch pathway components specifically on the side of the signal sending cell, thereby likely reducing downstream signaling output in the receiving cell. Summarily, our data point to a direct and positive regulation of key Notch pathway genes by Kdm6b, which in turn drive tumor cell proliferation, stemness and metastasis *in vivo*.

Combined Kdm6 deficiency modulates pancreatic cancer development and tumor subtype

Considering the homology and similar functions of Kdm6a and Kdm6b, we next investigated the effect of their combined loss on PDAC tumorigenesis. Due to its Y-linked nature and weak demethylase activity a compensatory effect of Kdm6c was unlikely, and thus it was not included in this study. Interestingly, Kdm6a/b double-KO ($p48^{CreER/+}; Kdm6a^{fl/fl}$ or $fl/Y; Kdm6b^{fl/fl}$) acinar cells were largely protected from undergoing ADM *ex vivo*, which was not observed with loss of *Kdm6b* alone (Supplementary Fig. S4A-D). In support of the stabilizing effect on cell identity, expression of critical acinar as well as other lineage genes was largely unchanged in freshly isolated Kdm6a/b double-KO acinar cells (Supplementary Fig. S4E). To assess the roles of the demethylases in the context of oncogenic Kras we generated compound $p48^{CreER/+}; LSL-Kras^{G12D/+}; Kdm6a^{fl/fl}$ or $fl/Y; Kdm6b^{fl/fl}$ ($KCA^{KO}B^{KO}$) mice (Supplementary Fig. S4F). In support of the impaired ADM phenotype *in vitro*, $KCA^{KO}B^{KO}$ mice also exhibited a reduced neoplastic burden compared to *KC* at 10 weeks post tamoxifen (Supplementary Fig. S4G-I). Thus, while the loss of Kdm6b alone perturbs acinar identity and promotes ADM formation *in vivo*, the combined loss of both demethylases impairs such dedifferentiation. Notably, at 25 weeks post tamoxifen a switch toward increased neoplasia had occurred in $KCA^{KO}B^{KO}$ mice (Supplementary Fig. S4H, J). Further aging out $KCA^{KO}B^{KO}$ mice resulted in an initial, slightly increased median survival compared to *KC* controls (80.9 vs. 73.1 weeks, Fig. 5A), which was mainly observed in female mice (Supplementary Fig. S5A). However, the curves converged later with $KCA^{KO}B^{KO}$ rapidly succumbing to disease with advanced age displaying an overall impaired life span. Upon histological examination we found that the majority (62.5%, vs. 7.7% in *KC*) of PDAC tumors arising from $KCA^{KO}B^{KO}$ mice could be classified as sarcomatoid carcinoma of the pancreas (SCP, Fig. 5B, C, $p=0.0139$). SCP is an undifferentiated tumor type that also presents itself in human patients and has been reported as highly aggressive³⁴. Another 25% of tumors presented as a mix of adeno- and sarcomatoid carcinoma, a phenotype that was observed in 30.1% of *KC* tumors as well. The SCPs exhibited spindle-shaped neoplastic cells and an absence of gland formation, except for entrapped benign PanINs (Supplementary SFig. 5B, asterisk). Local invasiveness into the wall of blood vessels was also

observed for $KCA^{KO}B^{KO}$ (Supplementary SFig. 5B, arrowheads), although there was no significant difference in the occurrence of macroscopic metastases compared to KC (Fig. 5D). An increase in PW/BW suggests that $KCA^{KO}B^{KO}$ tumors were larger than those from KC (Fig. 5E). In addition, they were characterized by the absence of classical epithelial markers such as E-Cadherin and CK19 that were strongly present in KC tumors (Fig. 5F). Both KC and $KCA^{KO}B^{KO}$ SCPs were positive for CK8, indicative of their epithelial origin, while $KCA^{KO}B^{KO}$ tumor epithelial cells also expressed the mesenchymal marker vimentin. This partial EMT phenotype was indicated further in areas of $KCA^{KO}B^{KO}$ tumors resembling transition zones in which E-Cadherin- and CK19-positive PDAC niches were adjacent to spindle-shaped cells negative for both markers (Supplementary Fig. S5C). Of note, only PanIN, but no other precursor lesions such as intraductal papillary mucinous neoplasm (IPMN) or mucinous cystic neoplasm (MCN) were found adjacent to tumors for both genotypes at end-stage (Supplementary Fig. S5D).

To address the potential contribution of loss of *Kdm6a* to these phenotypes we further analyzed $p48^{CreER/+}; LSL-Kras^{G12D/+}; Kdm6a^{fl/fl \text{ or } fl/Y}$ (KCA^{KO}) mice (Supplementary Fig. S5E). ADM and PanIN formation was almost completely abrogated 10 weeks post tamoxifen in KCA^{KO} mice (Supplementary Fig. S5F, G), suggesting a requirement for Kdm6a activity for the transition from acinar to metaplastic duct-like cells. The loss of *Kdm6a* may therefore be the main cause for the reduced early neoplasia formation observed in $KCA^{KO}B^{KO}$ (Supplementary Fig. S4H, I). Female, but not male, KCA^{KO} mice were trending towards having an increased median survival compared to KC controls (93.3 vs. 73.1 weeks, Supplementary Fig. S5H). Unlike $KCA^{KO}B^{KO}$, KCA^{KO} tumors were largely poorly differentiated or undifferentiated PDAC with no sarcomatoid component except for one animal (Supplementary Fig. S5I, J). Taken together, single loss of Kdm6a or combined loss of Kdm6a and Kdm6b in pancreatic acinar cells inhibits initiation of early neoplasia but only deficiency in both Kdm6a and Kdm6b promotes later formation of undifferentiated SCP in the context of oncogenic *Kras*.

Kdm6-deficient sarcomatoid tumors have distinct cellular signatures

We subsequently derived cell lines from $KCA^{KO}B^{KO}$ tumors (Supplementary Fig. S6A, B), which maintained their spindle-shaped morphology *in vitro* and did not grow in colonies (Fig. 5G). Despite the increased PW/BW (Fig. 5E) there was no significant difference in proliferation compared to KC (Supplementary Fig. S6C). Bulk RNA-sequencing of $KCA^{KO}B^{KO}$ vs KC cells resulted in 2,316 differentially expressed genes (DEGs, Fig. 5H, Supplementary Fig. S6D) and GSEA returned a large number of significantly enriched pathways, 670 in KC and 886 in $KCA^{KO}B^{KO}$ (padj<0.05, Fig. 5I). In support of the observed switch from epithelial to mesenchymal markers (Fig. 5F), EMT pathways were enriched in $KCA^{KO}B^{KO}$ cells while those related to epithelial differentiation were downregulated (Supplementary Fig. S6E, F). However, there was no difference in the migratory behavior of $KCA^{KO}B^{KO}$ cells (Supplementary Fig. S6G), suggesting that the partial EMT phenotype in SCP cells as defined by co-expression of epithelial/mesenchymal markers (Fig. 5F) does not correlate with increased invasiveness and, ultimately, metastasis. Despite the phenotypic differences with regards to PDAC subtypes, we observed an even more profound downregulation of the same critical Notch components *Jag1*, *Jag2* and *Neur11b*, and to a lower extent *Notch1* and *Maml2* in $KCA^{KO}B^{KO}$ cancer cells that we had identified in KCB^{KO} mice (Suppl. Fig. 6H-J). These findings indicate that general loss of Kdm6 histone demethylase activity perturbs Notch signaling, shapes PDAC plasticity and may serve as an underlying driver towards the development of undifferentiated sarcomatoid pancreatic tumors. We then asked whether $KCA^{KO}B^{KO}$ SCP cells exhibit differences in their molecular signaling networks and indeed, GSEA analysis revealed profound global changes in the expression levels of signaling pathway components compared to PDAC cells derived from KC mice. Several 'interferon response' as well as 'JNK signaling' pathways were enriched in $KCA^{KO}B^{KO}$ combined with a simultaneous downregulation of Myc targets (Supplementary Fig. S6K). The increase in interferon and c-Jun N-terminal kinase (JNK) signaling was further confirmed on the RNA and protein level of the tumor-derived cell lines as well as in the primary tumors (Fig. 5J, K,

Supplementary Fig. S6L). Treating the cell lines with a specific JNK inhibitor (JNK-IN-8) revealed an increased sensitivity of $KCA^{KO}B^{KO}$ cancer cells to JNK inhibition (Fig. 5L-N). These observations suggest overall altered oncogenic and metabolic networks in $KCA^{KO}B^{KO}$ SCPs that may present novel vulnerabilities for targeted treatment.

Spatial transcriptomics analysis reveals Kdm6-mediated plasticity in PDAC

To further assess tumor composition, we performed spatial transcriptomics on end-stage tumor sections (n=5 for each KC , KCB^{KO} , $KCA^{KO}B^{KO}$) using 10X Xenium technology. Processing and filtering resulted in 34 clusters that were assigned to the major cell types using known marker expression (Fig. 6A, B, Supplementary Fig. S7A). $KCA^{KO}B^{KO}$ tumors displayed a reduced proportion of ductal cancer cells (from here on referred to as “ductal”) and a major proportion of sarcomatoid cancer cells (“sarcomatoid”) scarcely found in tumors from KC and KCB^{KO} (Fig. 6C, Supplementary Fig. S7B-C). Other cell types were similarly distributed between genotypes except for a slight decrease in immune cells in $KCA^{KO}B^{KO}$. Sarcomatoid cells exhibited more transcriptional similarity with fibroblasts (e.g. high expression levels of *Vim*, *Lox*, *Sparc*) while simultaneously expressing epithelial markers *Krt8* and *Krt18* (Fig. 6D), as previously shown by IHC (Fig. 5F). They were also characterized by the expression of plasticity markers such as *Spp1*, *Axl*, *Prrx1*, *Grem1* and *Id3*, as well as EMT markers (*Twist1*, *Zeb1*, *Cdh2*, *S100a16*). Particularly *Spp1*/osteopontin and the BMP inhibitor *Grem1* have been reported as critical regulators of cellular heterogeneity and mesenchymal cell fate in PDAC^{35,36}. Pseudotime analysis on the subset of malignant cells comprising only ductal and sarcomatoid clusters using Palantir²⁷ revealed that sarcomatoid cells are positioned at the end of the inferred trajectory (Fig. 6E, F), suggesting that SCP might arise from bona fide existing adenocarcinomas.

We next analyzed the stromal compartments of KC , KCB^{KO} and $KCA^{KO}B^{KO}$ tumors. When compared to KC , KCB^{KO} tumors had increased levels of neutrophils while NK cells and lymphocytes were less abundant, indicating a potentially slightly increased immunosuppressive

TME (Supplementary Fig. S7D, E). In contrast, $KCA^{KO}B^{KO}$ tumors exhibited a higher proportion of NK cells at the expense of pericytes and dendritic cells. However, these differences in infiltrating immune cell types were generally minor. Reclustering of fibroblasts identified two inflammatory (iCAF), two myofibroblast (myCAF) and one antigen-presenting (apCAF) cancer-associated fibroblast (CAF) clusters based on published gene signatures³⁷, as well as a cluster of cells with strong expression of proliferation-associated genes thus labeled proliferative CAFs (pCAF, Fig. 6G-I, Supplementary Fig. S7F, G). Notably, the iCAF2 cluster was expanded in both KCB^{KO} and $KCA^{KO}B^{KO}$ compared to KC tumors, while the myCAF1 cluster was reduced. iCAF2 cells were characterized by a profound loss of both pan-CAF markers, as well as some iCAF markers, particularly cytokines *Il6*, *Ccl7*, *Ccl21a* and *Cxcl12*, as well as *Il1r1*, pointing to a potentially impaired polarization of iCAF2 cells by *Il1*. Furthermore, iCAF2 cells expressed high levels of decorin (*Dcn*), a secreted ECM proteoglycan that has been proposed to limit cancer cell growth and reduce metastasis, with quiescent CAFs (qCAFs) being a major source in PDAC³⁸. Despite the increased fibrosis seen at 10 weeks post tamoxifen (Fig. 2E-F), KC and KCB^{KO} displayed a similar abundance of alpha-SMA positive CAFs in the TME (Supplementary Fig. S7H).

We then investigated the co-occurrence of stromal cells with ductal (KC , KCB^{KO}) or sarcomatoid ($KCA^{KO}B^{KO}$) cells. When compared to KC tumors, the vicinity of KCB^{KO} ductal cells was more strongly depleted in iCAFs, apCAFs, NK cells and M2 macrophages, while being enriched in neutrophils and MDSCs (Fig. 6J). Whereas lymphocytes were strongly excluded from KC duct cells, they were infiltrating closer to KCB^{KO} ductal cells. These observations suggest that *Kdm6b*-deficient PDAC alters the immune landscape by modulating the local infiltration of immune cells, rather than their numbers, toward both apparent tumor-restraining (neutrophils, MDSCs) and tumor-promoting (lymphocytes) features. In summary, the changes in CAF abundance and localization, in particular iCAFs, together with the expression of stroma-modulating factors by PDAC cells (Fig. 4B) suggest a profoundly changed ECM and inflammatory environment that may work in tandem with impaired Notch signaling support the reduced proliferation and metastasis

observed in *KCB^{KO}*. *KCA^{KO}B^{KO}* tumors exhibited an exclusion of most stromal cell types in the vicinity of sarcomatoid cells (Fig. 6J), pointing to an overall low stromal content in these tumors. Together with the high abundance of decorin-positive iCAFs this may have implications on the invasiveness of SCP by restraining the partial EMT phenotype, ultimately resulting in similar migratory rates and metastasis observed for PDAC.

Evaluation of KDM6 activities in human PDAC

To determine whether molecular changes observed in the rodent PDAC models were mirrored in human samples, we obtained 13 human cases of the very rare SCP for immunohistological assessment. While KDM6B and NOTCH1 levels were comparable between an independent PDAC cohort and the SCP cases, JAG1 expression was significantly reduced in SCP tumors, further supporting the hypothesis that Notch signaling is altered in this subtype (Fig. 7A, B). Additional analysis in both PDAC and SCP cohorts revealed that expression of KDM6B and JAG1 was trending towards being positively correlated ($p=0.0695$ and $p=0.0696$, respectively), suggesting a potential direct regulation between the genes (Fig. 7C). To further parse out the relationship between Kdm6 histone demethylases and the Notch signaling pathway, we then treated *KC* tumor cells with the selective Kdm6 inhibitor GSK-J4 targeting all Kdm6 family members. All cell lines were sensitive to GSK-J4 (IC_{50} values of 1.5-2.2 μ M, Fig. 7D) and treatment led to a reduction of Jag1, but not Notch1, protein levels (Fig. 7E, F). This further indicates that Kdm6 activity positively regulates Notch signaling specifically via inducing the expression of its ligand Jagged1. Finally, we assessed whether KDM6 inhibition in patient-derived pancreatic cancer organoid (PDO) lines had therapeutically relevant effects. GSK-J4 treatment led to significantly reduced viability in two metastatic PDO lines (Fig. 7G-J). GSK-J4 in combination with Gemcitabine had an even more pronounced effect on reducing organoid viability in one PDO line compared to either Gemcitabine or GSK-J4 alone (Fig. 7H, I), suggesting a potential use of KDM6 inhibition together with conventional chemotherapy. Summarily, our data

raise the possibility of including KDM6 inhibition as another potential treatment option for PDAC patients in certain context.

Discussion

Despite a growing body of literature, much remains to be learned about the epigenetic control of pancreatic tumorigenesis and the implications for targeted therapies. We have focused our attention on the histone demethylases Kdm6a and Kdm6b essential for removing di-methylated and tri-methylated marks from histone H3 lysine 27 (H3K27me2/me3). Despite their close relationship these demethylases have distinct properties in cancer, with Kdm6a generally known for its tumor suppressor activity while we now show that Kdm6b displays context dependent oncogenic and tumor suppressor functions. A key finding of our study is the observation that Kdm6b tunes PDAC development and phenotype depending on the temporal context. Loss of Kdm6b accelerates ADM and PanIN formation, supporting a tumor suppressive role during early neoplasia. Despite this initial acceleration, subsequent PDAC formation and median survival are comparable in both *KCB*^{KO} and *KC* cohorts. Together with more aggressive features of Kdm6b-deficient tumors these findings are suggestive of an oncogenic requirement of Kdm6b in PDAC after the initial stages of neoplasia. This hypothesis is further supported by the observation that patients with Kdm6b-negative tumors display a better prognosis. The increased fibrosis and changes in CAF populations, most prominently the expansion of tumor-restraining decorin-positive iCAFs, in *KCB*^{KO} point to complex, cell non-autonomous functions of Kdm6b on the tumor environment in addition to the intrinsic functions on proliferation and stemness. Our data further support the notion that Notch signaling is a required component for the *KCB*^{KO} phenotype, but to what extent this pathway is sufficient to drive tumor traits warrants further investigation. Our studies further reveal unanticipated synergy between Kdm6a and Kdm6b in regulating formation of pancreatic cancer subtypes. In the context of concomitant deletion of Kdm6a, the

resulting $KCA^{KO}B^{KO}$ tumors display a sarcomatoid phenotype of PDAC, indicating a tumor cell-intrinsic regulation of cellular plasticity by Kdm6 demethylases. The origins and molecular characteristics of the rare sarcomatoid carcinoma of the pancreas (SCP) are still poorly understood^{34,39}. This type of undifferentiated cancer lacks a specific genetic signature compared to adenocarcinomas, thus pointing to epigenetic events or influences from the tumor microenvironment underlying this phenotypic variation⁴⁰. Our findings clearly demonstrate that SCP caused by the loss of both Kdm6a and Kdm6b in the presence of oncogenic Kras preferentially forms via ADM and PanIN precursors. The occurrence of PDAC-to-SCP transition zones in a subset of $KCA^{KO}B^{KO}$ tumors in conjunction with the trajectory analysis from spatial transcriptomic data suggest that SCP emerges via dedifferentiation of existing adenocarcinomas. These data further raise the question of whether SCP represents an independent subtype or rather the end of a spectrum of neoplastic plasticity from fully differentiated to undifferentiated pancreatic cancer. While loss of E-Cadherin and EMT characteristics have been previously reported for undifferentiated pancreatic carcinomas and were observed in $KCA^{KO}B^{KO}$ SCP as well, our findings point toward a partial EMT phenotype that is not sufficient to drive the formation of metastasis, possibly due to a lack of permissive stromal elements as indicated by our spatial transcriptomics analysis. The current notion of SCP as a more advanced, invasive cancer state^{41,42} stems from a small number of patients and thus requires further validation. Few genetic mouse models have been described to develop sarcomatoid pancreatic tumors, for example those with *Arid1a* or *Cdkn2a* deletion^{43–45}, however, SCP in these animals only occurs sporadically. Furthermore, loss of *Hnf1a* or *Kdm6a* resulted in tumors with sarcomatoid features yet without comprehensive SCP characteristics^{12,46}. Notably, ablation of the Notch2 receptor in the context of oncogenic Kras caused a phenotypical switch from PDAC to sarcomatoid tumors with hallmarks of EMT and impaired Myc signaling, thus partially phenocopying our $KCA^{KO}B^{KO}$ mouse model³³. Of note, Kdm6b has previously been reported as both a co-activator of Notch

target genes and an inducer of Notch receptor expression in T-cell acute lymphoblastic leukemia (T-ALL)^{47–49}. In other cellular context, Kdm6b was shown to directly activate transcription of *Notch1* and *Jagged1* by demethylation of H3K27me3 at the gene promoters^{50–52}. To our knowledge, data presented here provide the first evidence linking Kdm6b activity to Notch signaling in PDAC and other solid cancers, manifesting mainly in de-repression of its ligand Jagged1 in tumor epithelial cells and thereby promoting downstream pathway activation in neighboring epithelial or stromal cells that respond to ligand-mediated activation.

The downregulation of Notch components seen in *KCB*^{KO} is further exacerbated in *KCA*^{KO}*B*^{KO} tumors, suggesting redundant functions of Kdm6a and Kdm6b in regulating Notch signaling and raising the question of a threshold of Notch pathway activity needed to maintain PDAC differentiation. Indeed, we found Jagged1 to be strongly downregulated in SCP cases compared to PDAC. This contrasts with a recent study indicating high Jagged1 levels in SCP, yet the number of cases assessed was smaller and lacked direct comparison to PDAC⁵³. Conversely, NOTCH1 loss of heterozygosity was reported in 1 out of 10 patients with SCP⁵⁴. Taken together, these results corroborate aberrant Notch signaling as a characteristic of SCP. Whether sustained impaired Notch signaling is required to maintain the SCP phenotype remains to be elucidated. Given the profound changes in cell differentiation and the surrounding stroma, particularly CAF populations, it is likely that more complex mechanisms are at play. KDM6B levels however did not appear to have changed between PDAC and SCP, for which a larger cohort of SCP cases may be necessary to uncover potential differences.

Despite a delay in cancer initiation, *KCA*^{KO}*B*^{KO} mice ultimately succumb to pancreatic tumors, likely having acquired alternative oncogenic mechanisms as a consequence of perturbed Notch and Myc signaling. One such example is the activation of a tumor-cell intrinsic interferon transcriptional program in *KCA*^{KO}*B*^{KO} SCP cells, which is normally suppressed by combined actions of Myc and Kras yet found enriched in a subset of human pancreatic tumors^{55,56}.

Furthermore, *KCA^{KO}B^{KO}* cells exhibit increased JNK signaling and transcriptional upregulation of c-Jun, suggesting a previously unknown potential vulnerability of sarcomatoid tumors to JNK inhibition, which is already being explored as a potential treatment for pancreatic cancer^{57,58}.

In conclusion, our findings add Kdm6 demethylases as pivotal and upstream gatekeepers of cellular identity and regulators of Notch signaling in pancreatic cancer. Targeting Kdm6a/b using the selective inhibitor GSK-J4 in rodent PDAC and human PDO cells showed promising anti-tumorigenic effects, which could be potentiated by synergistic effects of GSK-J4 and established chemotherapy^{57,59}. Since simultaneous loss of Kdm6a and Kdm6b however leads to SCP in our mouse model, long-term dual Kdm6a/b inhibition may drive an undifferentiated phenotype with ultimately worse outcome. In light of our observations of primarily oncogenic activities of Kdm6b, the development of a Kdm6b-specific inhibitor to treat PDAC in a (neo)adjuvant setting may have significant translational potential. Further research is warranted to explore whether inclusion of such an inhibitor in therapeutic regimens for PDAC would lead to tumor regression and delayed metastasis, which would have significant implications on other cancers driven by oncogenic Kdm6b.

There are several limitations of the study that need to be addressed in future work. The autochthonous mouse models utilized here may not fully recapitulate tumor development in patients and the mixed genetic background of the mice may have an influence on metastatic rates. The small size of the available SCP cohort limits generalization to the human disease and requires further validation using a larger SCP cohort, once more tissues become available. Lastly, the contribution of known off-target effects of GSK-J4 such as inhibition of H3K4 demethylases KDM5B/C cannot be excluded at this moment. Development of a more selective inhibitor, ideally specific to KDM6A and KDM6B, respectively, is required to precisely delineate their individual contributions and targetability during PDAC development.

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Figure legends

Figure 1: KDM6B expression during PDAC development. (A) KDM6B histoscores of the UCLA cohort tissue microarrays of PDAC cases with corresponding adjacent normal and PanIN tissues. For PDAC values represent the average of 2-3 cores. (B) Representative images of IHC staining for KDM6B quantified in A. (C) Kaplan-Meier survival analysis of UCLA cohort patients with tumors negative (histoscore <40, n=23) or positive (histoscore >40, n=120) for KDM6B. Ticks represent censored patients. Median survival is 43.68 months (negative) vs. 21.48 months (positive), Log-rank test. (D) Average KDM6B Immunoreactive Scores of primary tumor and partially matched metastasis tissues of a TUM PDAC cohort. Unless otherwise indicated, mean is shown with unpaired t-test, * $p<0.05$, *** $p<0.001$. All scale bars are 50 μm .

Figure 2: Acinar-specific loss of Kdm6b promotes Kras-driven neoplasia. (A) Schematic of the genetic strategy for the conditional deletion of *Kdm6b* and activation of oncogenic Kras in acinar cells. Arrowheads denote loxP sites. (B) Pancreas weight over body weight ratio (PW/BW) at indicated time points. (C) Representative images of hematoxylin and eosin (H&E) and Alcian Blue stained pancreata at 10 and 25 weeks post TAM. (D) Quantification of Alcian Blue positive area at 10 and 25 weeks post TAM, with representative images shown in C. Dots indicate individual animals. (E) Representative images and (F) quantification of Picrosirius Red stained pancreata at 10 weeks post TAM. For all plots mean $\pm$ SEM is shown with unpaired t-test, * $p<0.05$ and ** $p<0.01$. All scale bars are 100 μm . TAM: tamoxifen, wks: weeks.

Figure 3: Loss of Kdm6b modulates PDAC progression. (A) Kaplan-Meier survival curve of indicated genotypes. Ticks represent censored animals. Median survival is 73.14 wks for *KC* (n=23) and 69.00 wks for *KCB^{KO}* (n=17, $p=0.1874$, Log-rank test). (B) Pancreas weight over body weight ratio (PW/BW) of animals at end-stage. (C) Quantification of PDAC incidence and

classification of end-stage tumors determined by a pathologist in a blinded manner. (D) Representative H&E and Picrosirius Red images of end-stage tumors. (E) Quantification of Picrosirius Red staining of end-stage tumors with representative images shown in D. (F) Percentage of animals presenting with macroscopically visible metastasis upon necropsy at end-stage with numbers of animals indicated. Fisher's exact test, $p=0.0214$. (G) Representative images of immunostaining of end-stage tumors for cytokeratin 19 (CK19) and Ki-67. (H) ImageJ quantification of Ki-67⁺/CK19⁺ co-positive cells as a percentage of all CK19⁺ epithelial cells. Values are average of at least 4 independent high-power fields per animal. (I) CellTiter-Glo assay with tumor-derived cell lines, normalized to respective day 0 values. Mean $\pm$ SEM of $n=7$ KC and $n=5$ KCB^{KO} lines shown with 2-way ANOVA. Each value is the average of at least 3 independent experiments. (J) Tumorsphere formation assay of tumor-derived cell lines with representative brightfield images (left) and quantification of tumorspheres per cell line (right) on day 4. Values are the average of 3 wells per independent cell line. (K) Flow cytometry analysis of day 4 tumorspheres and quantification of percent CD24⁺/EpCAM⁺ co-positive cells pre-gated on DAPI- population. Each value is an independent cell line. Unless otherwise indicated, mean $\pm$ SEM is shown with unpaired t-test, * $p<0.05$, ** $p<0.01$. All scale bars are 100 μ m. n.s.: not significant.

Figure 4: Kdm6b is a critical regulator of Notch signaling in PDAC. (A) Volcano plot visualizing differentially expressed genes from bulk RNA-sequencing of KC ($n=5$) and KCB^{KO} ($n=4$) cell lines. Dotted lines denote ± 0.75 for $\log_2$ fold change (FC) and adjusted p-value (padj) <0.05 . (B) Selection of top pathways enriched in KC or KCB^{KO} by Gene Set Enrichment Analysis. (C) GSEA enrichment plot for indicated pathway. (D) RT-qPCR analysis for Notch pathway genes with dots denoting individual tumor-derived cell lines. (E) Western blot analysis of tumor-derived cell lines for Jagged1 (Jag1) and quantification, with intensities of Jag1 bands normalized to corresponding β -actin bands. (F) Representative images of immunostaining of end-stage tumors

for Notch1 and Jagged1 (Jag1). CK19: cytokeratin 19. Scale bars are 100 μ m. (G) CellTiter-Glo assay with tumor-derived cell lines treated with DMSO or 10 μ M γ -secretase inhibitor XX (NOTCHI) for 72 hours. Values are normalized to respective day 0 values. (H) CellTiter-Glo assay with tumor-derived cell lines transfected with GFPmax or mJag1 plasmid with readout of luminescence 48h after seeding. Each value represents the average of 2-3 independent experiments per cell line. (I) Quantification of tumorsphere formation assay of tumor-derived cell lines on day 6. Values are the average of 3 wells per cell line. (J), (K) ChIP-qPCR against H3K27me3 and H3K4me3 using tumor-derived cell lines and primers directed against the transcription start site (TSS) region of the indicated genes. Values represent individual cell lines or independent replicates thereof. For all plots mean $\pm$ SEM is shown with unpaired t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. n.s.: not significant.

Figure 5: Combined loss of Kdm6a and Kdm6b delays neoplasia yet leads to sarcomatoid carcinoma with an altered oncogenic signaling network. (A) Kaplan-Meier survival curve of indicated genotypes. Ticks represent censored animals. Median survival is 73.14 wks for *KC* (n=23) and 80.86 wks for *KCA^{KO}B^{KO}* (n=17, $p=0.8197$, Gehan-Breslow-Wilcoxon test). (B) Quantification of PDAC incidence and classification of end-stage tumors determined by a pathologist in a blinded manner. Fisher's exact test for occurrence of pure sarcomatoid carcinomas compared to all other tumor types, $p=0.0139$. (C) Representative H&E images of end-stage tumors. (D) Percent animals presenting with macroscopically visible metastasis upon necropsy at end-stage with numbers of animals indicated. Fisher's exact test, $p=0.3496$. (E) PW/BW of animals at end-stage. (F) Representative images of immunostaining of end-stage tumors for E-Cadherin (E-Cad), cytokeratin 19 (CK19), cytokeratin 8 (CK8) and vimentin (Vim). (G) Brightfield images of tumor-derived cell lines grown in adherent, low density condition. (H) Volcano plot visualizing differentially expressed genes from bulk RNA-sequencing of *KC* (n=5) and *KCA^{KO}B^{KO}* (n=4) cell lines. Dotted lines denote ± 0.75 for $\log_2$ fold change (FC) and adjusted

p-value (padj) <0.05. (I) Selection of top pathways enriched in *KC* or *KCA^{KO}B^{KO}* by Gene Set Enrichment Analysis. (J) Representative images of immunostaining of end-stage tumors for Stat1 and phospho-c-Jun (p-c-Jun). (K) Western blot analysis of tumor-derived cell lines. (L) CellTiter-Glo assay with tumor-derived cell lines treated with different concentrations of JNK inhibitor JNK-IN-8 for 72 hours. Values are normalized to respective DMSO control and curves obtained by nonlinear regression. Data points are mean +/- SEM of 3 wells per cell line. (M) Area under the curve (AUC) calculated from graph in L for individual cell lines. (N) Viability for individual cell lines for 10 μ M JNK-IN-8 relative to DMSO control, as shown in L. Unless otherwise indicated, mean +/- SEM is shown with unpaired t-test, * p <0.05 and ** p <0.01. All scale bars are 100 μ m. TAM: tamoxifen, wks: weeks, n.s.: not significant.

Figure 6: Spatial transcriptomics uncovers plasticity and changes in stromal composition

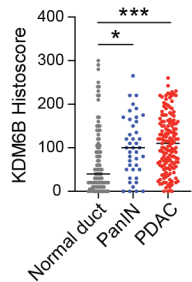
of Kdm6-deficient tumors. (A) UMAP plot of 399,021 cells from 10X Xenium sequencing of 15 tumor samples (n=5 for each *KC*, *KCB^{KO}*, *KCA^{KO}B^{KO}*). Each dot is a single cell colored by major cell lineage. (B) Dot plot of cell lineage genes used for annotation of major cell type clusters shown in A. (C) UMAP plots depicting the contribution of each genotype to the major cell type clusters. (D) Heatmap of select top genes enriched in ductal, sarcomatoid or fibroblast clusters shown as z-score normalized values. (E) UMAP plot of subclustering of ductal and sarcomatoid cells and overlaid trajectory from ductal to sarcomatoid cells colored by inferred pseudotime using Palantir. A start cell was selected from the ductal cluster, and all cells were ordered along the inferred trajectory toward the sarcomatoid state. (F) Heatmap of MAGIC-imputed gene expression values for select genes along the Palantir pseudotime. (G) UMAP plot of subclustering of fibroblasts, colored by subtype. iCAF: inflammatory CAF, myCAF: myofibroblast CAF, apCAF: antigen-presenting CAF, pCAF: proliferative CAF. (H) Cell proportions for each fibroblast subtype by genotype. Values are the average of 5 samples per genotype. (I) UMAP plots of cumulative iCAF and pan-CAF scores calculated from the individual expression of previously reported marker

genes. (J) Cluster co-occurrence score computed for all clusters relative to ductal or sarcomatoid, respectively ($p(\text{exp}|\text{ductal})/p(\text{exp})$, $p(\text{exp}|\text{sarcomatoid})/p(\text{exp})$), at increasing distance across the tissue. Scatter plots of representative tissues with each dot representing a single cell using original cell coordinates, colored by cell type annotation.

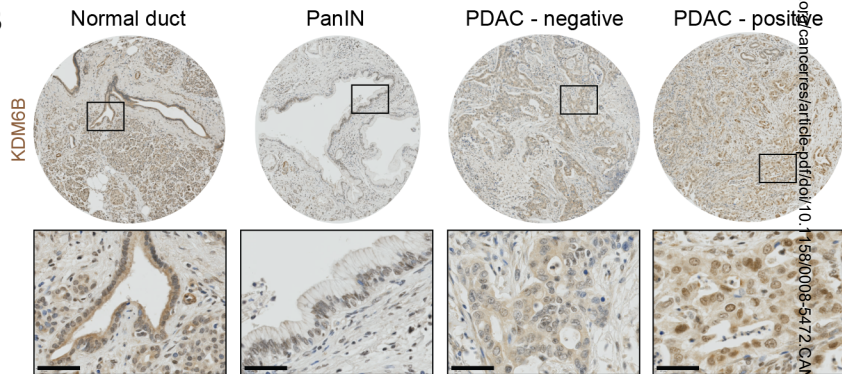
Figure 7: Kdm6b tunes human PDAC phenotypes via Jagged1. (A) Average Immunoreactive Scores of sarcomatoid carcinoma of the pancreas (SCP) cases and a TUM PDAC tissue microarray stained for KDM6B, NOTCH1 or JAG1. (B) Representative images of corresponding hematoxylin and eosin (H&E) and immunostaining quantified in A. Scale bars denote 50 μm . (C) Scatterplots of Immunoreactive Scores of JAG1 vs. KDM6B for the indicated human PDAC tissues with Spearman correlation and lines depicting linear regression. (D) CellTiter-Glo assay with tumor-derived cell lines treated with different concentrations of GSK-J4 for 72 hours. Values are normalized to respective DMSO controls and curves obtained by nonlinear regression. Data points are the average of 3 wells per cell line. Smaller graph is showing IC50 values for each cell line. (E) Western blot analysis of cell lines treated with 1.5 μM GSK-J4 or DMSO for 72 hours. (F) Quantification of Western blot shown in E, with intensities of Jagged1 bands normalized to corresponding β -actin bands. Unpaired t-test. (G)-(J) 3D CellTiter-Glo assays of two different metastatic patient-derived PDAC organoid lines with indicated treatments for 4 or 6 days, respectively, normalized to DMSO control. 10 μM GSK-J4, Gem: Gemcitabine, used at 25 nM (G, H) or 5 nM (I, J). Mean of 3-4 independent experiments $\pm$ SD shown with unpaired t-test. Representative brightfield images on day 4 or 6, respectively, are shown in G and I. Scale bars denote 100 μm . * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 1

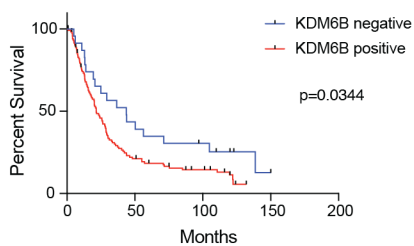
A



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C



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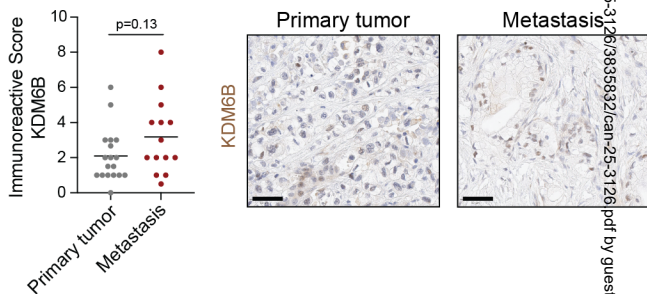


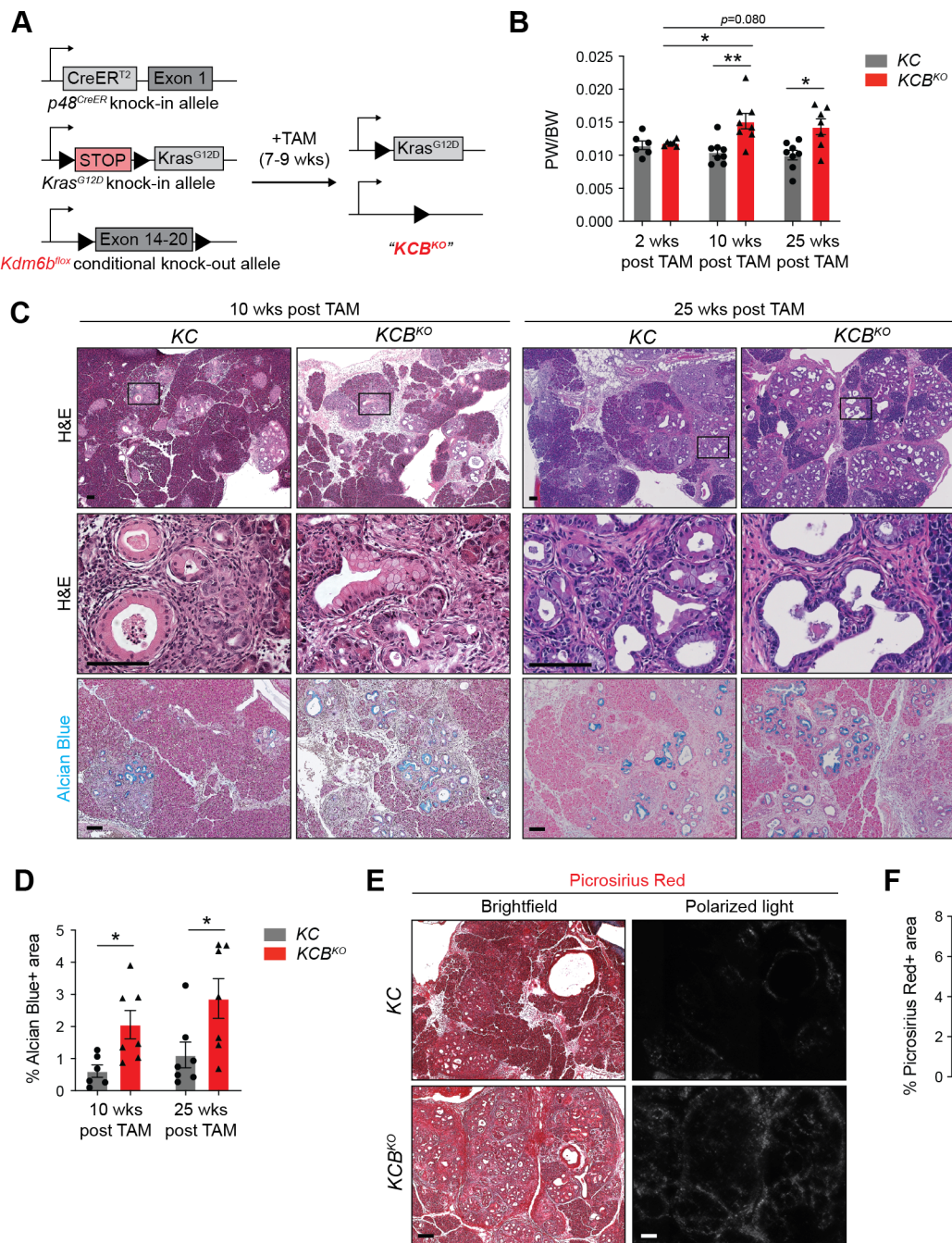
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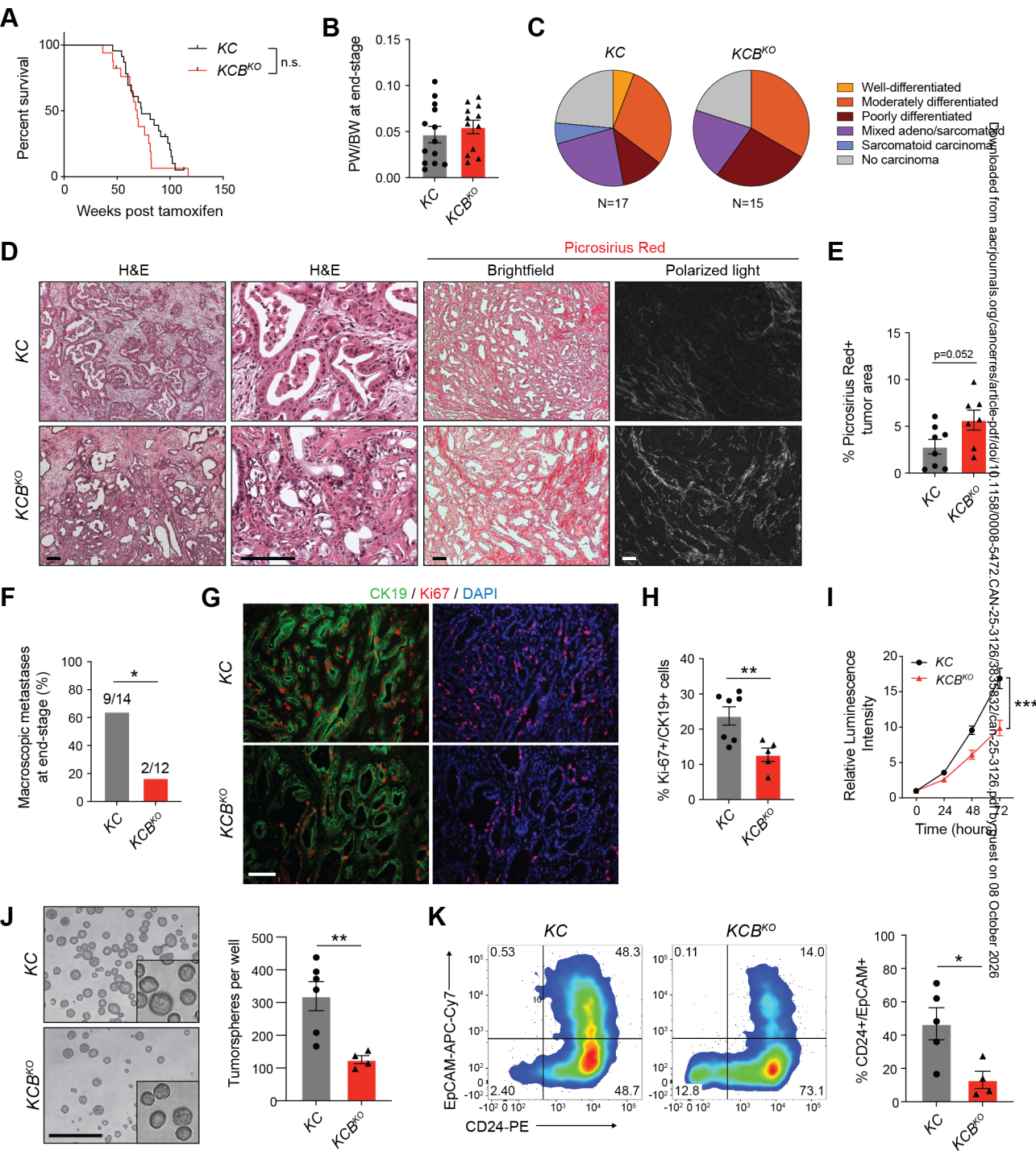
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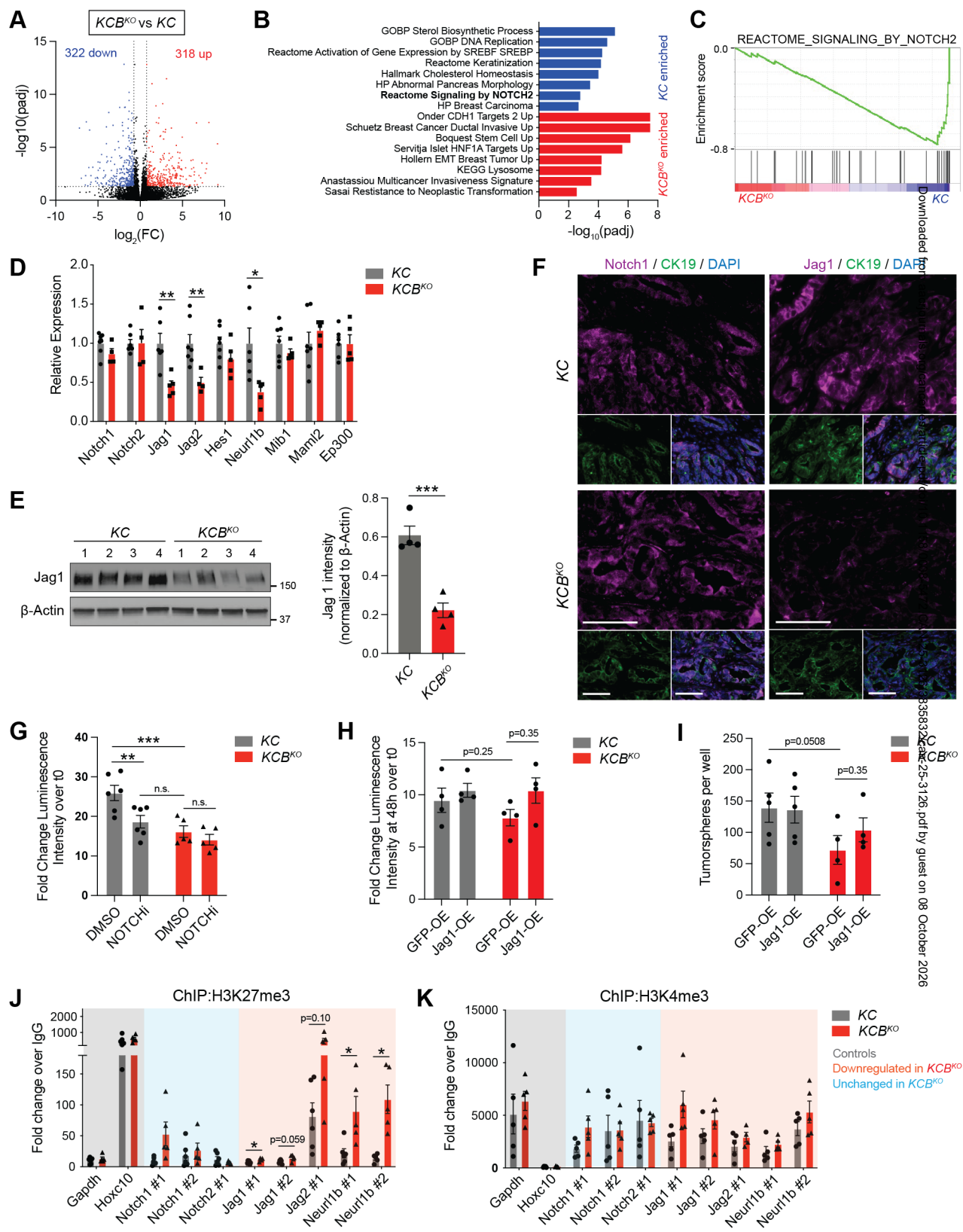
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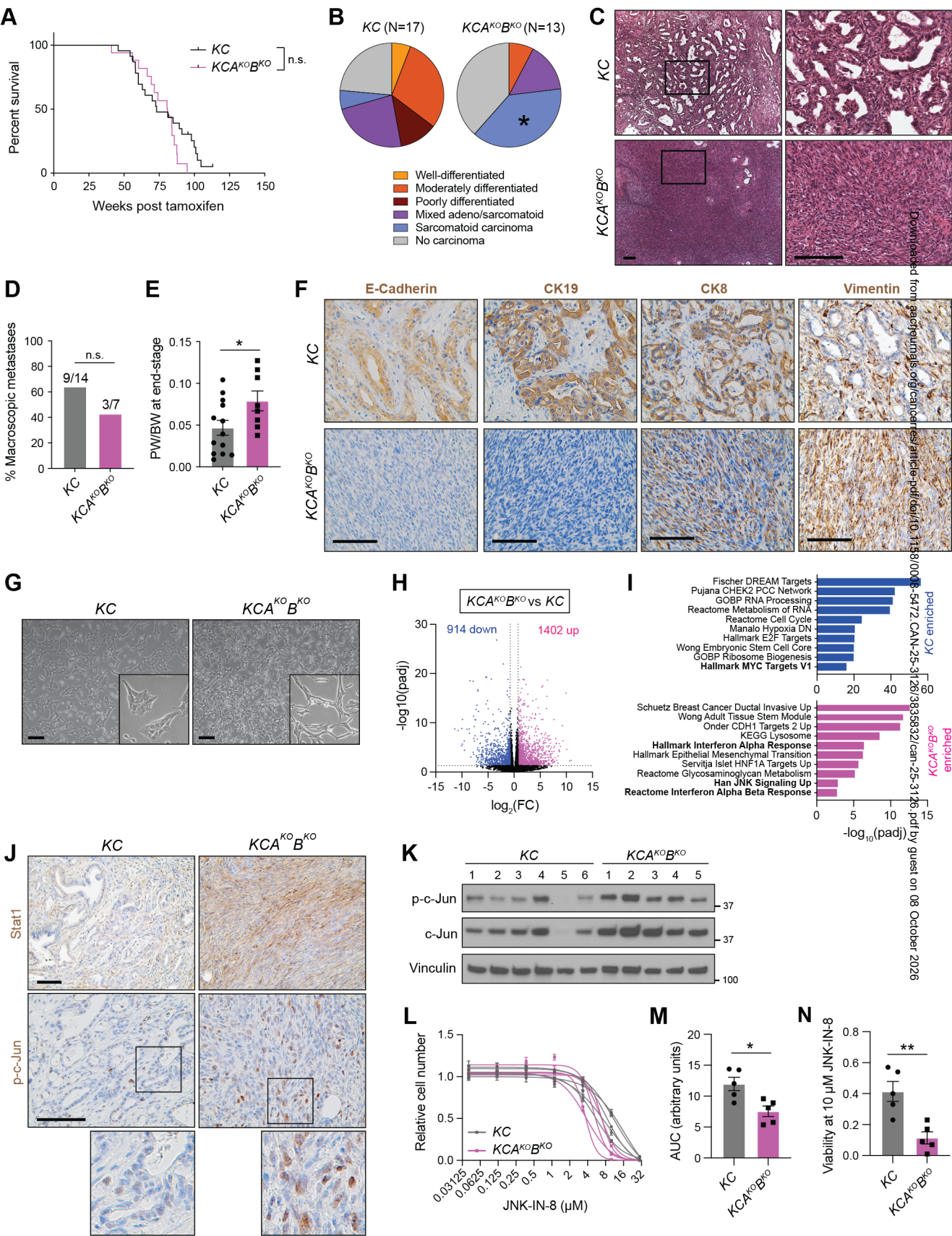
Figure 5

Figure 6

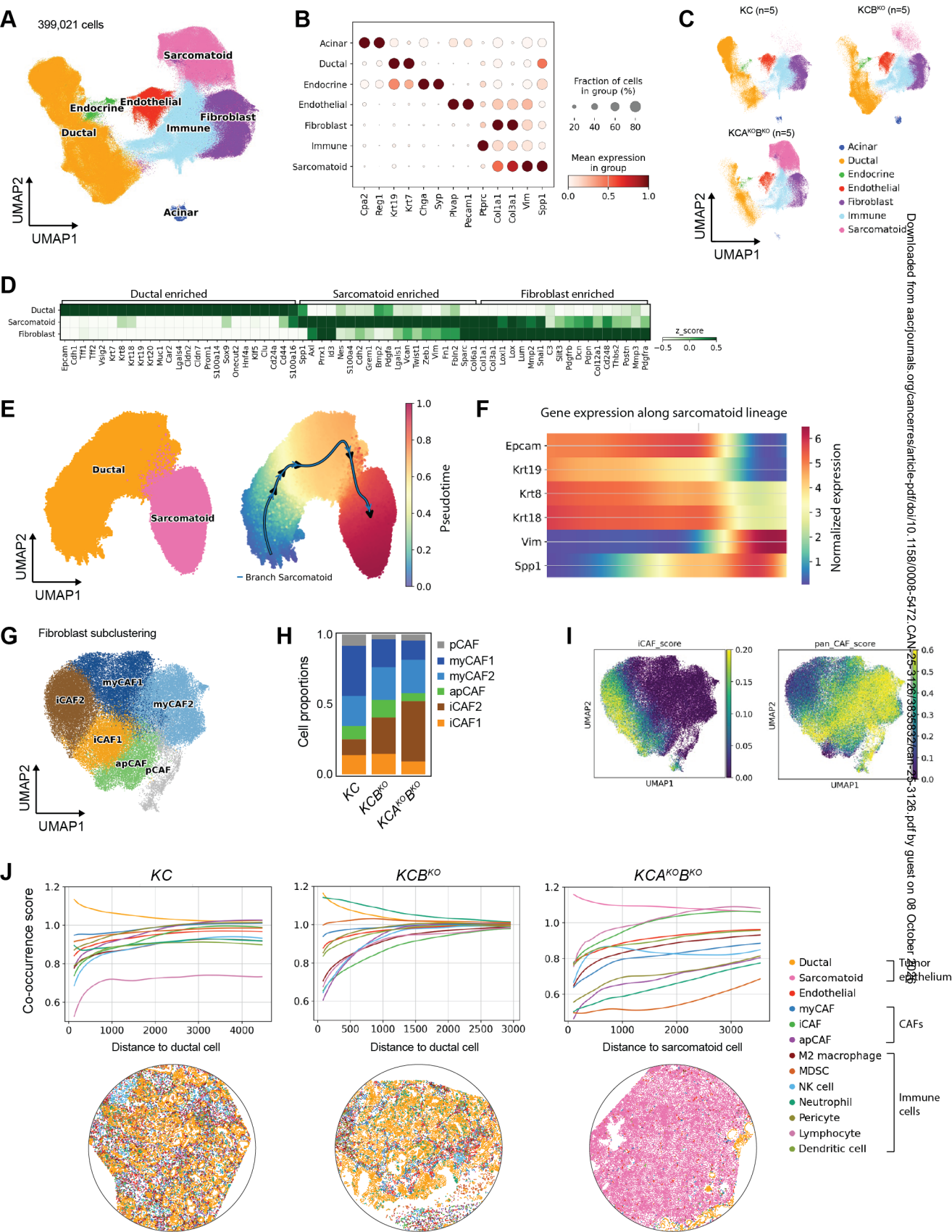


Figure 7