SUPPLEMENTARY MATERIALS AND METHODS

Human samples

Human samples (FFPE embedded tissue blocks and their donor characteristics) were collected from deceased multi organ donors by the Beta-cell bank UZ Brussels, as part of their clinical islet transplantation program. Pancreata of Whipple resections, chronic pancreatitis and autopsy samples plus patient characteristics were obtained from the department of Anatomopathology of UZ Brussels. The study included FFPE pancreatic cancer tissue blocks selected from the Anatomopathology department of Erasme Hospital (ULB-Brussels). Ethical consent was given by the Committee of Medical Ethics - UZ Brussels and samples were obtained through the Central Biobank UZ Brus sel (17-183) and partner biobank at Erasme Hospital (B2020/001).

Mouse samples

Mice were sacrificed in accordance with institutional ethical guidelines and regulations and were approved by VUB Animal Ethics Committee (ethical approval 19-595-3). Mouse experiments in the Cell Differentiation lab received ethical approval (16-277-1 (LA1230277)). Ethical approval for the mouse experiments at de Duve institute received ID 2019/UCL/MD/005. Mouse experiments in the University of Pittsburgh Medical Centre received ethical approval under ID 18022411.

Haematoxylin-eosin Saffron staining

After baking, deparaffinization and rehydration, sections were stained with haematoxylin (Sigma-Aldrich, St-Louis, MO, USA) for 5 minutes and were then differentiated with alcoholic acid and were blued in lithium carbonate. The slides were then stained with Erythrosine B (Sigma-Aldrich) for 2 minutes and 30 seconds. Slides were dehydrated in propanol and were then stained with saffron for 30 seconds and mounted with Pertex mounting medium.

Immunohistochemistry

For singleplex ΔNp63 stainings on human samples, the automated stainer Ventana Benchmark ULTRA was used with the anti-p40 antibody, clone BC28 (790-4950, Roche, Switzerland). For brightfield duplex stainings, the Benchmark ULTRA was used with the anti-p40 antibody in combination with the anti-CA19.9 antibody (760-2609, Roche) and the anti-MUC6 antibody (760-4390, Roche).

On mouse pancreas, we performed manual DAB stainings as the automated stainer detects all mouse IgGs. One to three random sections were assessed per block. Sections were baked, deparaffinised and rehydrated. Endogenous peroxidase activity was blocked using 3% Hydrogen Peroxidase in methanol for 30 minutes. Antigen retrieval was performed using citrate buffer (Sigma-Aldrich) in a pressure cooker for 40 minutes, and protein block was done using casein block (Thermo Fisher Scientific, Waltham, Massachusetts, USA) concentrated 25%. The primary antibody was incubated overnight at 4°C. The antibodies anti-p40 (ab167612, Abcam, Cambridge, UK; diluted 1/200), anti-p40 (ABS552, Sigma-Aldrich; diluted 1/50.000), anti-KRT14 (1/2000, HPA023040, Atlas antibodies, Bromma, Sweden) or anti-KRT5 (1/200, ab52635, Abcam) were used, which gave identical results. The next day, slides were incubated for 30 minutes with biotinylated goat anti-rabbit IgG antibody (Vector, Burlingame, California, USA, BA-1000, 1/200). Afterwards, slides were incubated for 30 minutes with a Streptavidin-biotin-HRP complex (VECTASTAIN(R) ELITE(R) ABC HRP Detection Kit, Vector). DAB incubation was done by diluting DAB concentrate 10 times in peroxide buffer (11718096001, Roche), after which slides were counterstained with haematoxylin for 30 seconds, differentiated in alcoholic acid and blued in
lithium carbonate. Slides were dehydrated and mounted with Pertex mounting medium. For these stainings, a positive control of either a mouse mammary gland or mouse skin were included.

For multiplex IHC staining the same protocol as above was followed however with minor modifications. On the second day Ultrabrite IHC Red Chromogen (BioVision, Milpitas, California, USA) was used instead of DAB. Afterwards, casein blocking was performed again and slides were incubated overnight at 4°C with the second primary antibody. On day three, UltraBrite IHC green chromogen (BioVision) was used. Slides were rinsed, air-dried and mounted with Pertex mounting medium.

For immunofluorescence (IF), we resorted to a P63 antibody (ab735, Abcam) as the ΔNp63 antibodies mentioned before, and additionally anti-p40 (ab172731, Abcam) were not suitable for multiplex IF. Slides were processed as above and incubated overnight at 4°C with a cocktail of antibodies, always including the P63 antibody (1/50). Other primary antibodies used were E-cadherin (1/100, AB5733, Sigma-Aldrich), KRT19 (1/100, TROMA-III, obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242 ), SOX9 (1/1000, ABE2868, Sigma-Aldrich), HNF1β (1/100, sc-7411, Santa Cruz, Dallas, Texas, USA), PDX1 (1/100, AF2419, RandD), KRT5 (1/100, ab52635, Abcam), KRT14 (1/1000, HPA023040, Atlas antibodies), S100A2 (1/200, HPA062451, Atlas antibodies), KRT7 (1/1000, ab181598, Abcam), DCLK1 (1/500, ab109029, Abcam), CD142 (1/100, AF2339, RandD), OLFM4 (1/5000, HPA077718, Atlas antibodies), MUC6 (1/4000, ab223846, Abcam), Ki67 (1/1000, 14-5698-82, Ebioscience, Thermo Fisher Scientific), NANOG (1/2000, 49035, Cell Signalling Technology, Danvers, Massachusetts, USA) and OCT3/4 (1/1000, 561556, BD Biosciences, Franklin Lakes, New Jersey, USA). The next day, slides were incubated with a cocktail of secondary antibodies for 1 hour. Antibodies used were donkey anti-mouse Cy3 (1/500), donkey anti-mouse AF647 (1/500), donkey anti-rabbit AF647 (1/500), donkey anti-chicken Cy3 (1/100), donkey anti-rabbit AF647 (1/500), donkey anti-rat AF647 (1/500), donkey anti-goat AF647 (1/500) and donkey anti-rabbit AF488 (1/500), all from Jackson ImmunoResearch (Ely, UK). After rinsing, slides were mounted with fluorescent mounting medium with DAPI added at 10 µg/ml. A section of human skin was included as positive control for ΔNp63.

RNA in situ hybridization (BaseScope)

For the BaseScope analysis, the standard protocol for BaseScope on FFPE tissue from ACDBio (Newark, California, USA) was used with the Basescope RED v2 kit. In short, 5µm slides were baked and deparaffinized, slides were incubated with hydrogen peroxide and next target retrieval was performed for 15 minutes. Protease III was applied for 15 minutes, and then the probe was incubated for 2 hours. A custom probe was designed to bind specifically to the promoter region of the delta isoform of P63, therefore not detecting the TA isoform. Standard hybridization followed, completed by signal detection and subsequent haematoxylin counterstaining. Slides were mounted with Pertex mounting medium.

FLIP-IT

FFPE processing

Samples were verified for P63 presence in 2D sections. 5mm punches (up to 37 mm³ of tissue) were acquired with the Tissue-Tek Quick-Ray Tissue Microarray System (Sakura, Torrance, USA). The paraffin was visually eliminated from the punches using a heater at 65°C and then incubated in Hemo-De (Laborimpex 23412-01) overnight at room temperature. Afterwards samples were incubated in ethanol and rehydrated with 1xPBS-Triton 0,5%. Then washed for 3 incubations in 1xPBS-Triton 0,5%. All incubation steps were performed on an orbital shaker unless stated otherwise.
Delipidation

Samples were incubated with clearing solution consisting of 10% sodium dodecyl sulfate and 200mM boric acid pH7 at 45°C (human) or 54°C (mouse) for 3 days. The clearing solution was refreshed every day. Samples were washed 3x1h at room temperature (RT) with PBS-Triton 0,5% to rinse out remaining micelles.

Blocking and immunostaining

Samples were blocked overnight in blocking solution with 1x PBS-Triton 0,5% containing 25% casein block (Thermo Fisher Scientific, 37528) pH 7,5.

Immunostaining FLIP-IT

Samples were incubated with primary antibody solution containing 1xPBS-Triton 0,5%, 25% casein block for 3 days. Primary antibodies used: KRT5 (1/200, ab52635, Abcam), KRT7 (1/100, ab181598, Abcam), TROMAIII (1/50, DSHB), P63 (1/50, ab735, Abcam) and Laminin (1/25, L9393, Merck, Kenilworth, New Jersey, USA). After primary antibody incubation, samples were washed three times with wash buffer 1xPBS-Tween 0,2% containing 0,1% Heparin (LEO Pharma, Lier, Belgium) pH7,5 for 1 hour each and then incubated with 1/100 diluted Alexa Fluor donkey anti-mouse 647-, anti-rat Cy3-, and anti-rabbit 488-conjugated secondary antibody in wash buffer for 3 days. Next, samples were washed 3 times with washing buffer for 1 hour. All steps were performed at room temperature while samples were gently shaken in amber 5mL tubes (Eppendorf, Aarschot, Belgium) to protect the samples from light.

Refractive index matching and agarose gel embedding of the cleared tissue

FFPE samples were incubated in 50% and 100% CUBIC-R for at least 6h each and 2% CUBIC-R agarose was made by dissolving low melting point agarose powder (Sigma-Aldrich, A4018) in Fresh CUBIC-R in the microwave. To form the bottom gel layer (2mm height), 0.304mL of the solution was transferred with a P1000 pipette (Eppendorf) in a custom-made glass chamber, covered with a cover glass (Leica, Wetzlar, Germany) and incubated at 4°C for 15 minutes. To form the middle gel layer, 1.5mL of the mixture was poured into a 5mL tube to which the sample was added and carefully poured in the chamber and incubated at 4°C for 30 minutes. To form the top gel layer, the remaining mixture was poured in the chamber until the surface protruded slightly, covered with a cover glass, and incubated at 4°C for at least 30 minutes. The sample was then removed from the chamber and CR-ECi was started by immersion of the agarose block in 50% MilliQ-diluted CUBIC-R for at least 6h followed by 100% at least 12h. Next, the sample was dehydrated in ascending ethanol solutions (25,50,75,2x100%; each 30min with last incubation overnight) and subsequently incubated in ascending ECI solutions (25,50,75,2x100%; each 30min). The next day the sample was glued to the mount with superglue. All steps were performed while being protected from light as much as possible. The sample was immersed in fresh ECI for LSFM image acquisition.

LSFM Imaging

Images were acquired using a Zeiss Lighsheet Z.1 (Zeiss, Oberkochen, Germany) fitted with 405,488,561,638nm lasers. Samples were optically sectioned using a z-plane optimally adjusted. Overview images were acquired using 20x objective, NA=1 with zoom 0.36 – 8bit. High magnification
images were acquired using 20x objective with zoom between 1 and 2,5 (magnification 20x and 50x)-16bit.

Additional information FLIP-IT

Human samples

For FFPE samples, the complementary effect of CUBIC-R and FDA-approved optical clearing agent ECI allowed safe handling, versatile imaging, storage, transport and retention of fluorescence intensity over longer periods of time. Hong et al. were able to clear thick (0.5cm) fresh/FFPE human pancreatic samples using a modified iDISCO method. They used liquid dibenzyl ether (DBE) which we refrained from using since dipping high magnifying objectives in DBE could dissolve the glues within the objectives. Moreover, the iDISCO protocol induces severe sample shrinkage which to some extent would distort our assessments. Hence, we devised a novel methodology, FLIP-IT, that yielded consistently transparent pancreas samples ready for LSFM within two weeks, whether applied to fresh or FFPE stored specimens (some over 25 years old) and without sample shrinkage. The duration does not take into account required imaging time which is considerably longer for confocal microscopy.

Furthermore FLIP-IT allows the complete undisturbed imaging in agarose as it is compatible with CUBIC-agarose embedding. Regular water-agarose embedding results in opacity when using FFPE tissues. The opacity creates more refraction in the sample and subsequently imaging artefacts. This compatibility allows to image the complete sample without compromise (due to the use of glue directly on the sample).

We provide the adjacent figure showing FLIP-IT delipidation of fresh-fixed normal human pancreas. FLIP-IT is superior to FLASH (CLARITY-derived) and CUBIC-HL as the delipidation buffer retains sample integrity and size whereas FLASH and CUBIC HL induce shrinkage and take longer for homogenous delipidation.

Mouse samples

Messal et al. used methyl salicylate for RI-matching again dissolving glues when using dipping objectives (1). Dipping objectives are required for high resolution light sheet fluorescence microscopy. The duration in S Fig 7 does not take into account required imaging time which is considerably longer for confocal microscopy. Additionally should the microscope be resistant to methyl salicylate the room would require additional hoods to eliminate the hazardous odor which is not require for ECI.
Within the FLASH protocol there is no indication whether it is suited for FFPE samples. The effect of methyl salicylate on FFPE processed tissue for LSF is not yet been clarified.

For further in depth comparison as well as the comparison with other methods, we like to draw the attention to the publication of Messal et al. Nature protocols 2020 (1)-extended data figures 3,5-8 showing the comparison between the different protocols in fresh-fixed mouse pancreas, brain, mammary gland, lung and liver. The scope of our paper is centered around the use of formalin-fixed paraffin-embedded human pancreas hence we investigated the additional benefit of in particular FLASH (CLARITY), iDISCO, CUBIC clearing principles in this approach. Please note that FLASH and CLARITY are related in terms of clearing buffer (SDS-based) but due to the principle going back to CLARITY we tend to see FLASH as a major modification on the original CLARITY protocol. Where we place the FLIP-IT as a major enhancement of the FLASH (especially in regards to FFPE samples).

We provide the adjacent figure showing FLIP-IT delipidation of fresh-fixed mouse pancreas still attached to spleen and duodenum to maintain correct anatomical orientation. Although FLIP-IT and FLASH use the same delipidation buffer for mouse samples, the FLASH is not permissable for LSF machines due to the manner of aggressive solvent for optical sample clearing.

<table>
<thead>
<tr>
<th>After excision</th>
<th>After Clearing (Fresh-fixed over night; O/N)</th>
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<tr>
<td>Cubic 2-2days</td>
<td>FLASH-O/N</td>
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### Dissociation and FACS analysis of pancreatic cells

Adult mouse pancreas from Sox9: eGFP transgenic mice (2) were harvested and digested in 1.4 mg/mL collagenase-P (Roche) at 37 °C for 20-30 min. Peripheral acinar-ductal units, depleted of endocrine islets, were prepared as described in (3). Following multiple washes with HBSS supplemented with 5% FBS, collagenase-digested pancreatic tissue was filtered through 600µm and 100µm polypropylene mesh (BD). Peripheral acinar-ductal units containing intercalated ducts and centroacinar cells (hereinafter called small ducts of less than 100µm), intercalated (inter- and intra-lobular ducts, hereinafter called medium ducts of 100-µm) and main ducts and its ramifications (hereinafter called big ducts of more than 500µm) were further dissociated for FACS analysis in TrypLE (Invitrogen, Thermo Fisher Scientific) and incubated at 37 °C for 5 min. Dispersed cells were filtered through 40µm polypropylene mesh (BD). Dissociated cells were then resuspended at 1:106 cells per ml in HBSS supplemented with 0.5% FBS. Cell sorting was performed using a FACS-Aria II (BD). The sorting gate for Sox9:eGFP positive ductal cells was established by using a WT mouse pancreas sample as negative control. Cells were directly sorted in RNasy lysis buffer (Qiagen, Hilden, Germany) for RNA extraction or sorted in complete organoid media for culture.

### Organoid culture

Entire ducts were embedded in GFR Matrigel, and cultured in organoid expansion medium (4) (AdDMEM/F12 medium supplemented with HEPES (1x, Invitrogen), Glutamax (1x, Invitrogen),...
penicillin/streptomycin (1x, Invitrogen), B27 (1x, Invitrogen), N-acetyl-L-cysteine (1 mM, Sigma-Aldrich), RSPO1-conditioned medium (10% v/v), Noggin-conditioned medium (10% v/v), epidermal growth factor (EGF, 50 ng/ml, Peprotech, London, UK), Gastrin (10 nM, Sigma-Aldrich) and fibroblast growth factor 10 (FGF10, 100 ng/ml, Preprotech). After 3 passages we used the organoids for RNA or immunolocalization analysis. For genetic manipulation TP63 siRNA (NM_011641, Sigma-Aldrich) and the Thermo Fisher’s Lipofectamine 3000 Transfection Kit (L3000-008) were used. In short, organoids were enzymatically disintegrated into single cells. Single cells were incubated with the lipofectamine 3000-siRNA mix for 4 hours at 37°C. After incubation, single cells were re-embedded in GFR Matrigel. Organoids were harvested 48 hours after genetic manipulation for RNA.

Cell lines and PDAC primary culture

HPDE cell line was purchased from Kerafast (Boston, USA). Cells were cultured in Keratinocyte Serum Free Medium, supplemented with Bovine Pituitary Extract and EGF (all from Thermo Fisher Scientific) in a humidified incubator at 37°C and 5% CO₂, according to previously published protocol (5). Cells were plated in a 6-well plate at a confluency of 70-80%, 24 hours prior to transfection. For the silencing of ΔNp63, a customized siRNA was designed at IDT (Iowa, USA) and three other validated siRNA’s against full TP63 (TP63 TriFECTa DsiRNA kit, IDT) were tested. In short, 10nM of the siRNA diluted in opti-MEM was added to lipofectamine RNAiMAX(1:10 diluted in Opti-MEM). siRNA-lipofectamine RNAiMAX complexes were added to the well and incubated for a minimum of 4 hours. After 4 hours medium containing the complex was removed and replaced with complete medium. RNA and protein were collected 48 hours after transfection.

PDAC primary cell cultures were derived from 44 PDTX samples as described in (6). Briefly, PDX samples were split into several small pieces (1 mm³) and processed in a biosafety chamber. After a fine mincing, they were treated with collagenase type V (C9263; Sigma-Aldrich) and trypsin/EDTA (25200-056; Gibco, Sigma-Aldrich) and suspended in DMEM supplemented with 1% w/w penicillin/streptomycin (Gibco, Life Technologies) and 10% of fetal bovine serum (Lonza, Basel, Switzerland). After centrifugation, cells were re-suspended in Serum Free Ductal Media (SFD) and conserved at 37°C in a 5% CO₂ incubator.

RNA analysis

Total RNA from organoids was isolated using the RNeasy Minikit (Qiagen) or TRizol followed by DNase I treatment (Invitrogen) RNA was reverse transcribed with SuperScript III Reverse Transcriptase and random hexamers according to the manufacturer’s instructions. qPCR of reverse-transcribed RNA samples was performed on a 7900 Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, Verenigde Staten) using the Power SYBR Green reagent (Applied Biosystems).

Total RNA from HPDE’s was extracted with the NucleoSpin RNA isolation kit (Machery-Nagel, Düren, Germany), according to manufacturer’s protocol. RNA concentration was measured using the NanoDrop 2000 (Thermofisher). Total RNA was reversed transcribed into cDNA using the GoScript Reverse Transcription System (Invitrogen). qPCR was performed using FastSYBRGreen 5x MasterMix on a QuantStudio 6 (Invitrogen). Primers were obtained from IDT. Analysis was done by determination of the comparative threshold cycle. For normalization GAPDH and HPRT were used.

PDAC Primary cultures total RNA was extracted using RNeasy mini kit (Qiagen), mRNA profiles were obtained using Illumina’s TrueSeq Stranded mRNA LT protocol. Sequencing followed oligo-dT capture and was done on a paired-end 100 pair flow cell. mRNA libraries were prepared and sequenced by AROS Applied Biotechnology A/S (Aarhus, Denmark). RNAseq reads were mapped using STAR 18 with the proposed ENCODE parameters) and SMAP on the human hg19 and mouse mmu38 genomes and
transcript annotation (Ensembl 75). Gene expression profiles were obtained using FeatureCount. Only genes with at least three read counts in at least 3 samples were kept for further analysis. Gene counts were normalized using the upper-quartile approach.

RNA sequencing and data analysis

Differential gene expression between HPDE knockdown and control was performed on the Illumnia NovaSeq 6000 instrument at the VIB, Nucleomics Core, Leuven, Belgium. Library prep was performed with the Truseq. Low quality ends (<Q20) were trimmed using FastX 0.0.14. Reads shorter than 35bp after trimming were removed. FastX 0.0.14 and ShortRead 1.40.0 were used to remove polyA-reads (more than 90% of the bases equal A), ambiguous reads (containing N), low quality reads (more than 50% of the bases < Q25) and artifact reads (all but 3 bases in the read equal one base type). Pre-processed reads were aligned to the reference genome of Homo sapiens (GRCh38). Expression levels were computed by counting the number of reads in the alignment that overlapped with gene features using featureCounts 1.5.3. Within and between sample normalization was conducted with the EDASeq package from Bioconductor. FMPK values were determined by dividing normalized counts by the total number of counts (in millions) for each sample. For each gene, the scaled counts were divided by the gene length (in kbp), resulting in the number of Fragments Per Kilobase of gene sequence and per Million fragments of library size. Differential gene expression between ΔNp63 KD and control was determined with the edgeR 3.24.3 package of Bioconductor.

Analysis of ΔNp63 isoform and correlation with molecular subtypes

To establish the link between Δn63 specific isoform and the PDAC subtypes, transcriptome data from 44 primary cell cultures and 6 HPDE cells were used to determine their PAMG (7). Raw count quantification was performed using Kallisto quant tool (8) and Homo sapiens genome (GRCh38) from ENSEMBL database. Raw counts were normalized using the upper-quartile approach (9) and log2 transformed. The PAMG scores were obtained from transcriptomes by projection on the previously published molecular signature (7). Pearson’s correlation test was used to evaluate the relationship between the expression level of Δn63 isoform and PAMG. The classification into basal-like and classical subtypes was performed using the PurIST classifier (10).

Whole mount organoid staining

For whole mount organoid staining the protocol described in Dekkers et al. (11) was followed with minor modifications. Antibodies used were anti-SOX9 (Sigma-Aldrich AB5535) and anti-P63 (ab735, Abcam).

Immunostainings on HPDE cell pellet

KD and control samples were shortly centrifuged and fixed for 24 hours at room temperature in 4% paraformaldehyde. Cells were resuspended in pre-warmed agarose, centrifuged and embedded into paraffin. 4µm tissue sections were cut. For staining the abovementioned protocol for immunofluorescence was followed. Primary antibodies used were: anti-KRT5 (1/100, ab52635, Abcam), anti-P63 (1/50, ab735, Abcam) and anti-KRT19 (1/100, GA61561-2, Dako). Secondary antibodies used were: anti-rabbit AF647 (1/500) and anti-mouse Cy3 (1/500), both from Jackson.

Immunoblotting

Total protein samples were extracted using the RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris pH 8.0), supplemented with Protease and phosphatase inhibitors (both from Sigma-Aldrich). Protein concentration was determined using the Bradford Assay.
Samples were mixed with loading buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue, and 0.125M Tris-HCl pH 6.8), boiled for 5 minutes at 95°C, followed by short centrifugation at maximum speed for 1 minute at 4°C. Equal amounts of proteins were loaded on a 10% polyacrylamide gel and transferred overnight on a Nitrocellulose membrane. Membranes were blocked with Tris-Buffered Saline and Tween 20 (TBST) with 5% non-fat milk. After 1 hour of blocking, membranes were incubated overnight at 4°C with the primary antibodies in 3%BSA in TBS-T. Primary antibodies used were: anti-P40 (ABS552, Sigma-Aldrich, 1/500) and anti-β-actin (A1978, Sigma-Aldrich, 1/100). After washing, membranes were incubated with the secondary antibodies for 1 hour at room temperature. Secondary antibodies used were: anti-rabbit 800CW or anti-mouse 680RD (Li-Cor Biosciences, Lincoln, Nebraska, USA). Protein signal was visualized with the Li-Cor Odyssey Fc Imaging System.

Image acquisition and processing

DAB slides were visualized and scanned with the Aperio CS2, the 3DHistech Pannoramic SCAN slide scanner and Zeiss AXIOSCAN Z.1. Slides were viewed with the Pathomation PMA.view software.

Fluorescent multiplex stainings were visualized with EVOS FL Auto Cell Imaging System or Zeiss AXIOSCAN Z.1. Confocal imaging was done using the ZEISS LSM 800 system. A merged Z-stack was created and saved as a PNG using the ZEISS Zen Lite program. BaseScope slides were also imaged with the ZEISS LSM 800 system, using the Cy3 channel to detect the BaseScope signal, and the brightfield channel to detect the haematoxylin staining.

Acquisitioned 3D data was processed using Zen black software using online dualside fusion algorithm. If necessary DualsideFusion files underwent background subtraction or were deconvolved using deconvolution module set to medium strength constrained iterative deconvolution. Afterwards tiled images were imported in Arivis 3.0 for stitching. 3D renderings, movies and images were acquired using Arivis software.

Data analysis

The HALO image analysis platform was used for all quantifications of 2D slides. Prior to analysis, scans were cleaned for possible processing artifacts. To quantify the total ΔNp63⁺ cells over all cells located in ducts, ducts were first annotated on one annotation layer and were then calculated using the multiplex IHC v3.0 quantification algorithm. To calculate the optical density of haematoxylin and eosin in ΔNp63⁺ cells and duct cells, two annotation layers were created, and a selection of ΔNp63⁺ cells and duct cells were annotated in a separate layer each. The optical density was quantified using the Area Quantification v2.1.3 algorithm. Finally, ΔNp63 expression in tumours was analysed on tissue microarrays (TMAs), which were first segmented using the TMA module and whole slides. ΔNp63 signal was quantified using the multiplex IHC v3.0. For the ΔNp63 quantification in murine samples, HALO AI was trained for nuclei and ductal morphology segmentation on 25% of used sections. HALO AI – nuclei phenotyper plugin was specifically applied on nuclei and ductal phenotype segmentation. Mouse skin tissue served as a positive training control. ΔNp63-Ki67 co-expression was quantified using HALO AI - nuclei phenotyper plugin. All returned data underwent visual quality control.

Arivis v3.2 was used to analyze 3D high resolution images. Voxel operations, membrane segmentation and 3D object building pipelines were created in Arivis for identification of KRT7⁺ and KRT5⁺ cells while excluding cells touching the edges. Membrane segmenter was set to plane-wise segmentation allowing holes and full connectivity in X/Y/Z. The segments were interrogated for Sphericity (3D roundness/shape) and volume. Manual quality control was performed for omission of false-positive and -negative results.
H-scoring

125 cases of PDAC were analyzed using the ΔNp63 immunostaining to assess the extent of nuclear immunoreactivity of ΔNp63 in the cancer cells, the H-score was applied. Only cancer cells with a clear invasive growth were analyzed (N=122). All non-cancerous areas and areas with carcinoma in situ were excluded from analysis. All images were scanned and digitally reviewed by a trained pathologist using the HALO 3.2 software.

Intensity of positive nuclear staining of the cancer cells was determined according the following scheme: 0, no nuclear staining of cancers cells; 1+, weak nuclear staining; 2+, moderate nuclear staining; 3+, strong nuclear staining. The H-score was obtained by the formula: 3 x percentage of strongly staining nuclei + 2 x percentage of weak/moderately staining nuclei + percentage of faint staining nuclei, giving a range of 0 to 300.

Statistical analysis

Experimental data were analysed by two-tailed unpaired Student t test, unpaired t test with Welch’s correction, paired t-test, Mann–Whitney or one-way Anova with Turkey’s multiple comparisons test using GraphPad Prism8.0 and statistical significance was accepted at P < 0.05. The results are shown as mean ± standard error of mean (SEM). The number of independent experiments (n) is indicated in the figure legends. We tested positive rate and prevalence using meta-analysis and Clopper-Pearson.

List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Species</th>
<th>Sequences</th>
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<tbody>
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<td>Human</td>
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<tr>
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<td></td>
<td>Reverse: GCC ATG GAC TGT GGT CAT GAG</td>
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References


