Supplementary material and methods

Collagenase liver perfusion

Collagenase liver perfusion was performed as described in the main document. To expose the cannulation site, a midline laparotomy was performed and the intestines were mobilized to the left. After successful cannulation the catheter was connected to the perfusion line. After the first perfusion, the subhepatic vena cava was cut allowing the drainage of the perfusate prior to perfusion of the digestion medium. When portal vein cannulation failed or proved difficult, the subhepatic vena cava was used instead as cannulation site. In this fashion, after successful cannulation the suprahepatic vena cava was clamped and the portal vein was cut to allow a retro-grade perfusion of the liver.

Plasmid designs

The STAR reporter used in this study (unless indicated otherwise) is an optimized version of the original reporter (1), enabling high-throughput imaging with easy quantifications, high sensitivity, and is well suitable for *in vivo* studies. The optimized STAR technology, termed movieSTAR, is based on a transposase-based integration method (movieSTAR: Tol2 insulator-8xSTAR-min.pLGR5-sTomato-NLS-pA-PGK-H2BmNeonGreen-2A-Puro, Figure S1I-J). A tandem cHS4 insulator precedes 8xSTAR repeats followed by a sTomato with a nuclear localisation signal (NLS) and a polyA sequence. Additionally, the murine PGK promoter is driving the expression of a H2B-mNeonGreen fusion protein alongside with a 2A sequence and a puromycin resistance cassette.

Experiments depicted in Figures 4, S3A-B, and S4 were performed with the previously published STAR version suitable for lentiviral integration (1): A blue or red STAR reporter in which 4xSTAR repeats are followed by TagBFP2 or tagRFP, an IRES sequence, and a blasticidin selection cassette (pLV-4xSTAR-min.pLGR5-TagBFP2-IRES-Blast or pLV-4xSTAR-min.pLGR5-TagRFP-IRES-Blast, Figure S1J). These reporters were combined with CMV-driven expression of an H2B-mNeonGreen fusion protein, a 2A sequence, and a puromycin selection cassette (pLV-CMV-H2BmNeonGreen-2A-Puro).

Inducible YAP and YTIP overexpression plasmids were cloned into a modified pInducer backbone which contains a ubiquitously expressed resistance cassette. YAP activation and

inhibition was achieved by overexpression of the constitutively active YAP^{5SA} (pLV-pTREG-YAP^{5SA}-Ubc-rtTA-IRES-Blast) and the YAP mimetic YITP (2) together with the fluorescent protein mMaroon1 (pLV-pTREG-mMaroon-NLS-2A-YTIP-Ubc-rtTA-IRES-Blast), respectively.

Plasmids were stably integrated into the genome of organoids as previously described (3). Plasmids generated in this study can either be found on Addgene or are available upon request.

Intravital imaging on liver metastases

Stage coordinates and visual landmarks inside the liver (such as blood vessels) were used for orientation. Metastases were identified and size-measured through RFP expression. Lesions were included into the analysis if tracked on at least 3 days.

Metastatic size and phenotype calling

IHC for human CEA was used to identify and measure the size of P19Tb-derived liver metastases. The stem cell expression pattern was assessed upon staining for RFP to be either heterogeneous or negative. Lesions smaller than 5,000 μm^2 were classified to be micrometastases. A Fisher's exact test was used to test the contingency between metastatic size and the stem cell expression pattern.

Organoid cultures

PDO lines used in this study are listed on COSMIC and comprise P19Tb (COSMIC: I2L-P19Tb-Tumor-Organoid, ID 2433500), P16T (COSMIC: I2L-P16-Tumor-Organoid, ID 2433496), and P9T (COSMIC: I2L-P9-Tumor-Organoid, ID 2433492). Additionally, the following engineered human CRC and colon WT lines were used: A/K/P/S ($APC^{KO/KO}$; $KRAS^{G12D/-}$; $TP53^{KO/KO}$; $SMAD4^{KO/KO}$), A/K/P ($APC^{KO/KO}$; $KRAS^{G12D/-}$; $TP53^{KO/KO}$), APC/KRAS ($APC^{KO/KO}$; $KRAS^{G12D/-}$), APC ($APC^{KO/KO}$), and WT (4).

Human organoid lines were maintained in Matrigel (Corning, Cat# 356231) as previously described (4,5), with culture medium containing Advanced DMEM/F12 (Invitrogen, Cat# 12634-028) supplemented with 10 mM Hepes (Invitrogen, Cat# 15630-056), 1% GlutaMAX (Invitrogen, Cat# 35050-038), 1% Penicillin-Streptomycin (Lonza, Cat# DE17-602E) as well as 1x B27 supplement (Invitrogen, Cat# 17504001), 1.25 mM N-Acetyl-L-cysteine (Sigma-Aldrich, Cat# A9165), 10 mM Nicotinamide (Sigma-Aldrich, Cat# N0636), 500 nM A83-01 (Tocris, Cat#

2939/10), 10 µM SB202190 (Gentaur, Cat# A1632), 10 % R-Spondin1 conditioned medium, 10 % Noggin conditioned medium, and 50 ng/mL human EGF (PeproTech).

For organoid splitting, the Matrigel was degraded through incubation with 1 mg/mL Dispase II (Life Technologies, Cat# 17105041) for 10 min at 37°C and organoids were enzymatically dissociated using Trypsin-EDTA (Sigma-Aldrich, Cat# 25200056). Trypsin activity was subsequently abrogated through trypsin inhibitor (Sigma-Aldrich, Cat# T9003) in a 1:1 ratio. For maintenance, organoids were cultured in the presence of 10 µM Y-27632 (Gentaur, Cat# A3008) during the first two days.

The murine CRC model of the genetic background *VillinCre-ER^{T2}; Apc$^{FL/FL}$; Kras$^{LSL-G12D/+;}$ Tp53$^{KO/KO}$; R26R-Confetti; Lgr5$^{DTR-eGFP}$* was generated as previously described, while RFP^{+} confetti clones were isolated by FACS in order to generate the corresponding organoid line (6). This line was cultured in BME (AMS Bio), growing in Advanced DMEM/F12 with Hepes, GlutaMAX, B27, N-Acetyl-L-cysteine, and Noggin at concentrations specified above.

Absence of microplasm and the identity of all organoid lines was confirmed in regular intervals by targeted PCRs and sequencing of identifying loci, respectively.

Time-lapse microscopy of organoid outgrowth

Light-sheet recordings were performed using a LS1 light-sheet microscope from Viventis Microscopy equipped with temperature control at 37°C and 5.0% CO_2 overflow. CRC organoids were dissociated to small fragments and plated in Matrigel inside the light-sheet chamber. Organoids were imaged every 10 min for 7-13 days, while re-adjusting the stage position every couple of days (small jump in movies). For each channel, the data was background subtracted and a maximum intensity projection was performed for each time point. Data is represented with constant intensity scale over the entire movie.

Analysis of STAR dynamics around plasticity events

Plasticity events were selected by sudden appearance of STAR signal in time-lapse movies recording the outgrowth of p19Tb STAR organoids. Analysis was performed from the preceding mitosis up until two mitoses later. All 4 cells were analysed backwards in time down to t=0, so that each mother cell has at least 2 overlapping traces describing the same cell. STAR and H2B intensities were measured, the ratio STAR/H2B calculated, and the ratio is plotted as rolling average of 3 time points. Mitoses can be recognized by a sudden dip in the ratio which

is affected by the nuclear envelope breakdown leading to dilution of STAR over the entire cell. The third round of mitoses is not outlined by asterisks but can be recognized by the shape. Cells for which the analysis could not be reliably conducted, are excluded from the plots.

FACS isolation of STAR populations

Two-week-old organoid were dissociated to single cells, incubated with 1.5 µM DRAQ7 (Cell Signaling Technology), and sorted using an Aria III machine (Becton Dickinson). STAR populations were collected in organoid medium with 100 µg/mL primocin and 2 % v/v Matrigel.

Flow analysis of STAR expression

3-day-old organoids grown from single cells were treated with either 2 µg/mL doxycycline (Bio-Connect, Cat# 0219504401) or with DMSO (VWR). 48 h later, organoids were dissociated to single cells and fixed using 1mL of 4% paraformaldehyde (VWR, Cat# 47377.9L) for 15 min at RT. Samples were kept at 4°C in PBS with 0.05% Tween (VWR, Cat# 8221840500) until analysis.

For each line, 20,000-200,000 single, alive cells were analysed (gating on scattering properties and H2B-mNeonGreen expression) on a BD FACSCelesta™ flow cytometer. For YTIP overexpression lines, additional gating on mMaroon1^{+} cells was performed. STAR gates were defined as STAR-high (top 15%), STAR-low (bottom 15%), and STAR-mid (remaining 70%) based on the DMSO sample using FlowJo 10.6.1 (https://www.flowjo.com). The fraction of cells within each STAR gate was plotted as mean + SEM of 3-4 independent experiments for each line.

Relative viability and proliferation was assessed by comparing the number of DAPI-negative and EdU-positive cells, respectively, in drug-treated samples to their respective DMSO control.

Organoid tracking experiment

Organoids were scanned daily with a 40X water objective (HC PL APO CS2; NA 1.1) on a Leica SP8X confocal microscope equipped with a culture chamber held at 37°C with 5.0% CO_2 overflow. The images were analysed with Imaris (v9.3, Oxford Instruments) and organoids with at least 5 time points were included into the analysis.

For the data represented in Figure S3, organoid size is represented as the number of nuclei per organoid cross-section at its widest position, while STAR fate was assessed after investigating the entire organoid in 3D in Fiji (https://imagej.nih.gov/ij/).

EdU incorporation assay

Intact organoids were fixed inside Matrigel using 4% paraformaldehyde with 0.25% glutaraldehyde for 15 min at RT and subsequent washing with PBS. Background signal was quenched with 1 % sodium borohydride (Sigma- Aldrich, Cat# 480886) for 10 min at RT thrice, followed by 3 PBS washing steps. Click-iT™ EdU Cell Proliferation Kit for Imaging (ThermoFisher, Cat# 12043795) was used with a 647 nm dye according to the manufacturer's instructions.

Fluorescent image analysis

Fluorescent images were imported into Fiji (https://imagej.nih.gov/ij/) for analysis (unless indicated otherwise). If necessary, background subtraction, correction for bleed through, smoothening, cropping / rotating, and linear contrast adjustment were applied.

Bulk RNA sequencing analysis

Sequencing reads were aligned with Hisat2 version 2.0.4 (7) to the provided and pre-indexed hg38 transcript assembly from UCSC, with alignment tailoring for transcript assemblers enabled. Reads per gene were counted with the htseq-count script from the Hisat2 software suite using the GTF file corresponding to the transcript assembly, with strandness disabled and identification attribute set to gene_id. The Wald test followed by correction for multiple testing using Benjamini-Hochberg method was used to identify significantly changing genes. The 'rlogTransformation' function in DESeq2 was used to normalise, $\log_2$ transform and noise-stabilize the expression data for visualisation purposes. Heatmaps were created with the ComplexHeatmap package (version 4.2.4) (8).

Gene set enrichment analyses were run with the following parameters: Nominal p-values were determined by 1000000 gene-set permutations. The repair gene signature was defined by upregulated genes with a p-value < 0.01 and fold change of at least 1.5. The foetal gene signature included genes that were significant at p-value < 0.01 and with a fold change of at least 3. Both LCC signatures were defined as genes with a fold change of at least 2 and an adjusted p-value < 0.05.

Micro-organoid gene selection

Bulk RNA-seq data was mined for genes with a high YAP score and/or a high fold change in expression between micro- and macro-organoids using two different strategies. Filtering strategy 1: (1) FDR size comparison < 0.001, (2) normalised mean sample expression > 9, (3) YAP score > 1.5. Filtering strategy 2: (1) FDR size comparison < 0.001, (2) normalized mean sample expression > 9, (3) fold change expression micro-/macro-organoid samples > 3. Gene lists were ranked by decreasing YAP score (strategy 1) or decreasing fold change in expression (strategy 2). From these list, 17 *micro-organoid-related genes* were manually selected (Table S3).

Single-cell RNA-sequencing data analysis

After mapping of the reads, cells with >200 unique transcripts and <10% mitochondrial reads were included into the analysis. The first 16 principle components were used for unsupervised hierarchical clustering (default settings at resolution 0.5). Differential gene expression was performed using a wilcox test (FindMarkers with min.pct = 0.25) and genes with adjusted p-value < 10e-3 and absolute average log2 fold change > 0.58 are displayed.

The co-expression of Lgr5 with other SC markers was tested for cells harvested on Day 27. The expression of both Lgr5 alleles (WT and DTR-eGFP gene fusion) were summed up and cells were grouped by their normalised expression value into three classes: negative (zero), mid (up to 1.5), and high (above 1.5). For each class, the mean expression of indicated SC markers was computed and depicted as mean over row mean in Figure S7B (R package ComplexHeatmap version 2.6.2).

Supplementary references

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