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Supplementary Materials for

An AAV gene therapy computes over multiple cellular inputs to enable precise targeting of multifocal hepatocellular carcinoma in mice

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Materials and Methods

Cell lines

HuH-7 cells were purchased from the Health Science Research Resources bank of the Japan Health Sciences Foundation (Cat#JCRB0403). HepG2 cells were purchased from American Type Culture Collection (ATCC), (Cat#HB-8065). HeLa cells were purchased from ATCC (Cat#CCL-2). Hep3B cells were purchased from ATCC (Cat#HB-8064). HCT-116 cells were purchased from Deutsche Sammlung Von Microorganismen and Zellkulturen (DMZ), Cat#ACC-581). SW-620 cells were purchased from ATCC (Cat#CCL-227). LoVo cells were purchased from ATCC (Cat#CCL-229). A549 cells were purchased from ATCC (Cat#CCL-185). SH4 cells were purchased from ATCC (Cat#CRL-7724). IGROV1 cells are part of the NCI-60 panel and were obtained from the National Cancer Institute (NIH).

All cell lines were cultured at 37 ºC, 5% CO_2. HuH-7, HeLa, Hep3B, HCT-116, SW-620, LoVo, A549 and SH4 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cat#41966-029, Gibco), supplemented with 10% FBS (Sigma-Aldrich, Cat#F9665 or Life Technologies, Cat#10270106) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Cat#P4333). HepG2 and IGROV1 were cultured in RPMI1640 (Cat#A10491-01, Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Cat#F9665 or Life Technologies, Cat#10270106) and 1% penicillin/streptomycin solution (Corning, Cat#30-002CI). Cell splitting was performed every 3-4 days, cells were detached using 0.25% Trypsin-EDTA (Cat#25200-072).

HepG2-LC: HepG2 cells were stably modified with the vector pIK014 (39) encoding constitutively-expressed mCitrine fluorescent protein and firefly luciferase gene, integrated using AAVS1-specific TALENs pIK006 and pIK007 (39) and sorted for homogenous mCitrine expression, to create an HepG2-LC cell line (fig. S3A). The linear relationship between cell number and bioluminescence signal was confirmed in vitro (fig. S3B).

Mycoplasma tests

Cells were tested for mycoplasma using a PCR-based method as described in (63). The thermocycler program used for detection was as follows: 1 cycle of 7 minutes at 95 ºC, 3 minutes at 72 ºC and 2 minutes at 65 ºC; 32 cycles of 4 seconds at 95 ºC, 8 seconds at 50 ºC and 45 seconds at 68 ºC. Primers PR1843, PR1844, PR1845, PR1846, PR1847 and PR1848 were used for detection. For positive control, an extra set of primers (PR0673 and PR0674) apart from the ones mentioned above were used. Cell cultures for in vitro experiments were propagated to a maximum of twenty passages before being replaced with a fresh stock.

Recombinant DNA methods

All plasmids were generated using standard molecular cloning techniques (see table S1 for the overview of the cloning procedures). All restriction enzymes used in this work were purchased from New England Biolabs (NEB) and digestion was carried out at the temperature suggested by the supplier. DNA amplification was performed using Phusion High Fidelity DNA Polymerase (NEB, Cat#M0491L) or Q5 High-Fidelity DNA Polymerase (NEB, Cat#M0491L). De-salted oligonucleotides to be used as primers or to be annealed and subcloned were ordered from Sigma Aldrich. De-salted gene fragments/synthetic DNA sequences were ordered from IDT (gBlocks). Digestion fragments and PCR products were either PCR purified using MinElute PCR purification (Qiagen, Cat#28004), Qiaquick PCR purification kit (Qiagen, Cat#28104), GenElute PCR Clean-up Kit (Sigma-Aldrich, Cat#NA1020-1KT) or separated by electrophoresis and extracted from gel using MinElute Gel extraction kit (Qiagen, Cat#28604) or Qiaquick Gel Extraction kit (Qiagen, Cat#28706), depending on the requirements of the specific experiment. Oligonucleotide annealing was performed by mixing 25 µL each of the phosphorylated oligonucleotide and then incubating in a thermocycler at 95 ºC for 3 minutes followed by a decrease of 0.5 ºC every minute for the next 170 minutes. 1 µL of 1:20 diluted (with ddH_2O) annealed oligonucleotides was used for ligation reaction. Ligation reactions were performed using T4 DNA ligase (NEB, Cat#M0202L), optimizing temperature, ligation time and molar ratio on a case by case basis. Gibson assembly (64) was performed at 50 °C for 1 hour in 20 µL final volume by mixing vector (50 ng) and

inserts (5 molar equivalent) in 1x Gibson assembly buffer (0.1 M Tris-HCl, pH 7.5, 0.01M $MgCl_2$, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.01 M DTT, 5% (w/v) PEG-8000, 1 mM NAD), 0.04 units of T5 exonuclease, 0.25 units of Phusion DNA polymerase and 40 units of Taq DNA ligase. Phosphorylation of oligonucleotides was performed by combining together 3 µL oligonucleotide (100 µM), 5 µL 10xPNK buffer, 5 µL ATP (10 mM), 1.5 µL of T4 PNK (10 U/µL) and 34 µL ddH_2O followed by incubation at 37 ºC for 30 minutes.

The assembly products were transformed in chemically competent E. coli, either TOP10 cells prepared in-house or in low recombination strains Stbl3 cells (Thermo Fisher Scientific, Cat#C737303) or NEB Stable (NEB, Cat#C3040H) when containing ITR or LTR sequences, known to be recombination prone. The transformed cells were plated on LB Agar with appropriate antibiotics selection (ampicillin, 100 µg/ml, chloramphenicol, 25 µg/ml, kanamycin, 50 µg/ml). The resulting colonies were screened by restriction digestion or via colony PCR using Quick-Load *Taq* 2x Master Mix (NEB, Cat#M0271L). Plasmid isolation was performed using GenElute Plasmid Mini-prep kit (Sigma-Aldrich, Cat#PLN350-1KT). All plasmids were verified using Sanger sequencing service provided by Microsynth. The DNA used in cell transfection was isolated from 100 ml bacterial cultures using HiPure Plasmid Filter Midi-prep kit (Invitrogen, Cat#K2100-14) followed by endotoxin removal (Norgen, Cat#52200). The DNA used for large scale viral productions was isolated from large scale cultures (2 L) and purified using Qiagen Endo Free Plasmid Mega Kit (Qiagen, Cat#12362). For all kits, supplier instructions were followed unless otherwise indicated.

Small-scale viral vector production

All small-scale viral productions were performed in 6-well plates (Thermo Fisher Scientific, Cat#140675). HEK 293T clone 17 (ATCC, Cat#CRL-11268) cells were seeded at a density of $8*10^5$ cells/well 24h before transfection. The cell transfection was performed using polyethylenimine (PEI) reagent (Chemie Brunschwig AG, Cat#24765-1). For each reaction, 15 µl of PEI were diluted in 500 µl of Opti-MEM (Gibco, Cat#31985-062) and incubated for 5 minutes. During incubation, 1.5 µg of helper plasmid (pHelper from Cell Biolabs Inc.,

kit#VPK-400-DJ) was mixed with 750 ng of the AAV-DJ capsid plasmid (pAAV-DJ from Cell Biolabs Inc., kit#VPK-400-DJ) and 750 ng of plasmid encoding the payload of interest and dissolved in 500 µl of Opti-MEM for each reaction. Following the incubation time, the PEI mix was added to the DNA and incubated for 20 min before being added to the cells. After 72 h the grow medium containing viral capsids was collected and used directly for transduction or frozen and stored at -80 C. For viral transduction we add the viral containing medium directly to the cells to be transduced in a 1:1 ratio with their growing medium.

Large-scale viral vector production

HEK 293T cells clone 17 (ATCC, Cat#CRL-11268) were seeded in 150-cm^2 plate (Thermo Fisher Scientific, Cat#1638381) at a density of $2*10^7$ cells/plate the day before transfection. Cell transfection was performed using PEI (Chemie Brunschwig AG) with a 5:1 PEI:DNA ratio. For each plate 180 µl of PEI were diluted in 1.5 ml of Opti-MEM (Gibco, Cat#31985-062) and incubated for 5 min. During incubation, 17 µg of helper plasmid (pHelper from Cell Biolabs Inc., kit#VPK-400-DJ) was mixed with 10 µg of the AAV capsid plasmid (pAAV-DJ from Cell Biolabs Inc., kit#VPK-400-DJ or pGG-B1, Addgene #78504 (48)) and 10 µg of genome plasmid encoding the payload of interest and diluted into 1.5 ml of Opti-MEM for each reaction. Following the incubation time, the Opti-MEM PEI solution was added to the DNA, the resulting mix was gently vortexed and incubated for 20 min before being added to the cells. Cells were harvested 72 hours post-transfection. Briefly, culture medium was collected and the virus in the medium was precipitated using polyethylene glycol (PEG) (Promega, Cat#V3011) by incubation on ice and successive centrifugation (4000 g, 30 min). Cells were detached using PBS and repeated pipetting to avoid exposing the virus to proteases. Harvested cells were lysed by 3 freeze-thaw cycles, the resulting lysate was combined with the PEG precipitated viral pellet and incubated for 1 hour at 37°C with 1500 units per plate of Benzonase (Sigma-Aldrich, Cat#E1014-25KU). The Benzonase digestion product was centrifuged (2x4000g, 20 min), the pellet containing cellular debris was discarded and the clarified supernatant was further purified via density gradient centrifugation. The Iodixanol gradient was prepared according to previous reports (59) using Opti-Prep (Sigma-Aldrich, Cat#D1556-250ML) in Quick-

Seal centrifuge tubes (Beckman Coulter, Cat#344326). After ultracentrifugation (2 hours, 63000 rpm in a Beckman TI 70 rotor) the 40% Iodixanol layer was extracted. The extracted iodaxinol layer was run through an Amicon Ultra-15 column (Merck Millipore, Cat#UFC910096) to concentrate the virus and exchange the buffer with AAV storage buffer (PBS with 2mM KCl and 1mM $MgCl_2$). Before further use the purity of the virus was checked by SDS-PAGE and the concentration in viral genomes/ml was determined by qPCR. In brief, the purified virus was diluted 1:100 and mixed with 2xSYBR Green I Master Mix (Roche, Cat#04707516001) and PR4304-4305 primers (table S2). The amplification was run on a Roche Lightcycler 96 with the following program: 10 min denaturation at 95°C followed by 40x: 10 sec at 95°C, 10 sec at 60°C, 15 sec at 72°C and the presence of a single melting peak was confirmed. For absolute quantification, a viral vector (YB6-6) with a titer of $5x10^{13}$ vg/ml (produced and quantified using a certified procedure at the Zurich viral vector facility) served as a reference. The reported concentration of the YB6-6 standard sample was additionally confirmed by qPCR using a calibration with the quantified plasmid DNA of its genome (pMD26). Serial dilutions of YB6-6 were generated to create the respective regression curves. Threshold and Ct values were determined automatically by Roche's analysis software for the Roche Lightcycler 96 (version 1.1).

Transfections

Transfections were performed using Lipofectamine 2000 as transfection reagent, according to the guidelines suggested by the producer. The transfections were carried out in 24-well plates (Thermo Fisher Scientific, Cat#142475). Cells were seeded 24 hours prior to transfection at a density of $5.5*10^4$ cells/well for HeLa and $6.5*10^4$ cells/well for HepG2, Huh-7 and Hep3B, to have around 80% confluency at the time of transfection. Appropriate plasmid amounts for each transfection were mixed and diluted in Opti-MEM (Gibco, Cat#31985-062) to a final volume of 50 µL (Opti-MEM DNA mix). The Lipofectamine 2000 was diluted in 50 µL Opti-MEM in a DNA (µg) to lipofectamine 2000 (µL) ratio of 1:2.5 and incubated 5 min (Opti-MEM Lipofectamine mix). After incubation this mix was added to the Opti-MEM DNA mix, gently vortexed and incubated for 15 min before adding it to the cell culture. When required by the experimental design, 5 pmol of miRIDIAN

mimics were added to the Opti-MEM DNA mix. The mimics were not counted when calculating the DNA to Lipofectamine ratio.

Primary hepatocyte isolation and culture

Hepatocytes were isolated as described by Zhang *et al.* (62) with slight modification. Briefly, a retrograde liver perfusion was conducted by cannulating the inferior vena cava with a 25G Venofix butterfly needle (B. Braun, Cat#4056502-01) while the portal vein was cut for drainage. The liver was perfused initially with 60ml Hanks' balanced salt solution (HBSS, Gibco, Cat#14175-053) at a flow rate of 6-8 ml/min and the perfusion is continued with 60-80 ml digestion solution (DMEM-low glucose; 1% penicillin/streptomycin; 15mM HEPES) containing 100 CDU/ml collagenase IV (Sigma-Aldrich, Cat#C5138) for 8-10 min. Following the digestion, the liver was removed and dissociated gently in cold solution (DMEM-high glucose; 1% penicillin/streptomycin; 15 mM HEPES; 10% FBS). The cell suspension was filtered through a 100 μm strainer (Falcon, Cat#352360) and centrifuged for 3 min at 50 g and 4°C. After two washing steps, fresh hepatocytes were resuspended in hepatocyte plating medium (Lonza, Cat#MP100) and seeded on collagen-coated plates. During the first hour plates were kept in a 37°C/5% CO_2 incubator and shaken frequently to facilitate even cell distribution. After 60 min medium was aspirated and replaced with fresh hepatocyte plating medium (300 μl/well). After 8 h the plating medium was replaced with hepatocyte maintenance medium. To determine the best culturing medium and protocol combination for efficient AAV transduction of hepatocyte in vitro we conducted an optimization experiment (fig. S2). The plated hepatocytes were maintained in two different media, either hepatocyte culture medium (Lonza, Cat#CC3198) supplemented with HCM Single-Quots (Lonza, Cat#CC4182) or William Medium (Gibco, Cat#A12176) supplemented with primary hepatocyte maintenance supplement (Gibco, Cat#CM4000) and HepExtend (Gibco, Cat#A2737501) and exposed to different viral titer for either 24 h or 48 h. The morphology of non-transduced hepatocytes was monitored over time for a qualitative evaluation of the uniformity and duration of the cellular monolayer (fig. S2B). The hepatocytes cultured in Lonza HBM seemed to maintain a healthy morphology for a longer time (evident at

day 3 and day 5). The hepatocyte transduction efficiency was evaluated via microscopy and flow cytometry (fig. S2, C and D). Based on these observations we performed all successive hepatocyte culture and transduction using Lonza medium. In brief, the hepatocytes were seeded in 24-well plate (Gibco, Cat#A1142802) at a density of $1.5x10^5$ cells/well. The hepatocytes used for in vitro efficacy experiments were seeded in 96-well plate (Gibco, Cat#A1142803). During the first hour plates were kept in a 37°C/5% CO_2 incubator and shaken frequently to facilitate even cell distribution. After 60 min medium was aspirated and replaced with fresh hepatocyte plating medium (300 µl for 24-well plates, 100 µl for 96-well plates). The cells were allowed a minimum total of 4-6 h post-seeding to facilitate cell attachment before hepatocyte plating medium was replaced with hepatocyte culture medium (Lonza, Cat#CC3198) supplemented with HCM Single-Quots (Lonza, Cat#CC4182), 500 µl for 24-well plates or 100 µl for 96-well plates. The culture medium was replaced every 24 h.

Cell preparation for in vivo tumor establishment

HepG2-LC cells were cultured and passaged until 70–80% confluence in T-75 or T-150 flasks. For in vivo injection we used cells with low passage number (passage 12 or less). Cells were detached by removing the growth medium, washing with PBS (10 ml for T-75 or 20 ml for T-150), and dissociating the cells with trypsin (Gibco, Cat#25200056) (2 ml for T-75 or 6 ml for T-150 Flask) for 5 min at 37 °C. The cell suspension was diluted with 8 mL (T-75) or 24 ml (T-150) of PBS, gently resuspended by pipetting, and subsequently filtered in a 50 ml Falcon tube through a 100 µm cell strainer (Falcon, Cat#352360) to obtain a single cell suspension. Additional 10 ml of PBS was used to wash the filter (T-75) or 20 ml (T-150), further diluting the cells to a total volume of 20 ml (T-75) or 50 ml (T-150). Cell suspension was centrifuged at 498 rpm at 4 °C for 9 min. The resulting pellet was washed with 20 ml of PBS and centrifuged at 498 rpm at 4 °C for 6 min two more times to remove any trace of trypsin. Each cell pellet was resuspended in 250-300 µl of PBS volume and an aliquot was diluted 1:50 and 1:100 for manual counting of live cells using Neubauer chamber and trypan blue. At least four independent counts were taken per cell suspension and the average value was used to determine the

number of cells to be injected. Cell suspension was further inspected visually under the microscope to verify the absence of large clumps. The final volume was adjusted with PBS to 2×10^7 cells/ml. To minimize manipulation and improve viability, the suspension was divided in multiple stocks (2 or 3 tubes) and kept on ice for the duration of the surgical procedures. Given the high cell concentration, the cells required resuspension before each injection to avoid clumps in the bolus.

Microscopy

For in vitro experiments, fluorescent protein expression was imaged using fluorescence microscopy at 48 h post transfection or 72 h post transduction. Images were acquired utilizing Nikon Eclipse Ti microscope equipped with a mechanized stage and temperature control chamber held at 37 °C. The excitation light was generated by a Nikon IntensiLight C-HGFI mercury lamp or LED source and filtered through a set of optimized Semrock filter cubes. The resulting images were collected by a Hamamatsu, ORCA R2 or Flash4. Cell cultures were imaged using a 10X objective. Organ imaging was performed using a 4x magnification objective. The following optimal excitation (Ex), emission (Em) and dichroic (Dc) filter sets were used to minimize the crosstalk between different fluorescent channels: mCitrine (Ex 500/24nm, Em 542/27nm, Dc 520nm), mCherry (Ex 562/40nm, Em 624/40nm, Dc 593nm), CFP/mCerulean (Ex 438/24 or Em 483/32nm, Dc 458nm). Image processing for presentation in figures was performed using Fiji software and Illustrator. The microscopy images were acquired at multiple exposures of 10 ms, 30 ms, 100 ms, 300 ms, 600 ms, 1000 ms, and 2000 ms for mCherry; 10 ms, 30 ms, 100 ms, 300 ms, 600 ms, and 2000 ms for Cerulean; 200 ms and 600 ms for Citrine. All channels were used for signal quantification. In the figures, we show the micrographs using the exposure that best conveys the information (balancing sensitivity and signal over-saturation); the exposures are indicated in the figures.

Flow cytometry

All cell lines were detached by replacing the culture medium with 120 µL of Trypsin-EDTA (Gibco, Cat#15400-054) diluted 1:2 with PBS (Gibco, Cat#10010-015) and incubating for 4-5 minutes at 37 ºC. Cells

were re-suspended, transferred to micro-dilution tubes (Life Systems Design, Cat#02-1412-0000) and kept on ice. The cells were measured using a BD LSR Fortessa II Cell Analyzer. The excitation lasers (Ex) and emission filters (Em) used for respective fluorescent protein measurements are as follows: mCherry (Ex: 561 nm, Em: 610/20 nm, longpass filter 600 nm), mCerulean (Ex:445 nm, Em: 473/10 nm), mCitrine (Ex: 488 nm, Em: 530/11 nm, longpass filter 505 nm), and iRFP (Ex: 633 nm, Em:780/60 nm, longpass filter 750 nm).

Data processing and quantification

Flow cytometry: Data analysis for bar charts was performed using FlowJo software. Live cells were gated based on forward scatter area vs side-scatter area. Negative gating for each fluorescent channel was established using an untreated control sample (non-transfected or non-transduced) such that 99.9% of the control population falls in the negative gate. In all experiments, each channel was used to quantify one fluorescent reporter (see the paragraph above for channel definition). In the experimental samples for each channel, we calculated the percentage of cells that were positive in a given channel among all live cells (defined as frequency of positive cells) and the mean value of their fluorescence intensity. The absolute expression units in a given channel, (abs. u.) were therefore calculated as follows:

Absolute expression units (abs. u.) in a given channel = (frequency of cells that are positive in the channel of interest) x (mean fluorescence of cells that are positive in the channel of interest)

The normalized units (norm. u.) were obtained by dividing absolute expression units in a given channel by the frequency of cells that are positive for the expression of the transfection control. As the transfection control, we used a separate plasmid harboring a CMV-driven iRFP. This normalization allowed for correction for sample-to-sample variability in the transfection efficiency.

Normalized units (norm. u.) in a given channel = (absolute expression units in a given channel) / (frequency of cells positive for the expression of the transfection control)

To more precisely evaluate the repression induced by different miRNAs and better evaluate their functional activity we used bidirectional reporters in which two different fluorescent proteins were driven by

identical promoter sequences with the gene encoding for one of the proteins harboring targets for a given miRNAs in the 3'-UTR (reporter protein), while the other gene harbored an inert 3'-UTR (control protein). For these measurements we defined relative units (rel. u.) as the ratio between the absolute expression of the miRNA-regulated reporter protein and the absolute expression of the control protein.

Relative units (rel. u.) = (absolute expression units of reporter protein) / (absolute expression units of control protein)

Microscopy: Image quantification of organ sections to produce bar charts was performed using ImageJ software. A schematic representation of the image analysis pipeline is depicted in fig. S5A. In brief, for each section the region of interest (ROI) was defined based on the phase image picture of the section. The mean signal level in the ROI was measured at different exposure times of 10 ms, 30 ms, 100 ms, 300 ms, 600 ms, 1000 ms, and 2000 ms for mCherry; and 10 ms, 30 ms, 100 ms, 300 ms, 600 ms, and 2000 ms for Cerulean. Linear regressions were performed aggregating all replicates for a given viral vector and organ combination measured at different exposure times to determine the linear range of the signal (fig. S5B). The signal was considered linear in the range that guaranteed a goodness-of-fit (R^2) ≥ 0.95. For organs and vector combinations that did not reach saturation (linear across the whole range) the mean signal level measured at 2000 ms exposure was used directly. For organs and vector combinations showing signal saturation, the linear fit to the linear signal range was used to extrapolate a corresponding calculated mean signal level at 2000 ms exposure in the absence of saturation according to:

Calculated Signal= mX+b

where X=2000 ms. This approach was used to extend the dynamic range of microscopy imaging and enable the quantification of signals that span several orders of magnitude. The same procedure was applied to the organs from control animals (injected only with PBS) and the value obtained was used as a measure of the autofluorescence background for each organ and depicted in the bar charts.

For bar charts in Fig. 3C (activity of bidirectional microRNA reporters), for each reporter, the mCherry and mCerulean values were calculated separately using the procedure above. Then, the mCherry/mCerulean ratio was calculated to represent the relative knock-down of the reporter arm (mCherry) vs internal control output (mCerulean). Whereas a control vector bearing TFF5 target should theoretically represent the upper bound on normalized mCherry/mCerulean ratio, it was apparent that TFF5 target responded to cryptic inputs in vivo and many reporters resulted in normalized readouts above the ratio obtained with TFF5 target; therefore, comparisons in the bar charts of Fig. 3C should be made to the highest normalized readout in each plot (values between 5-10).

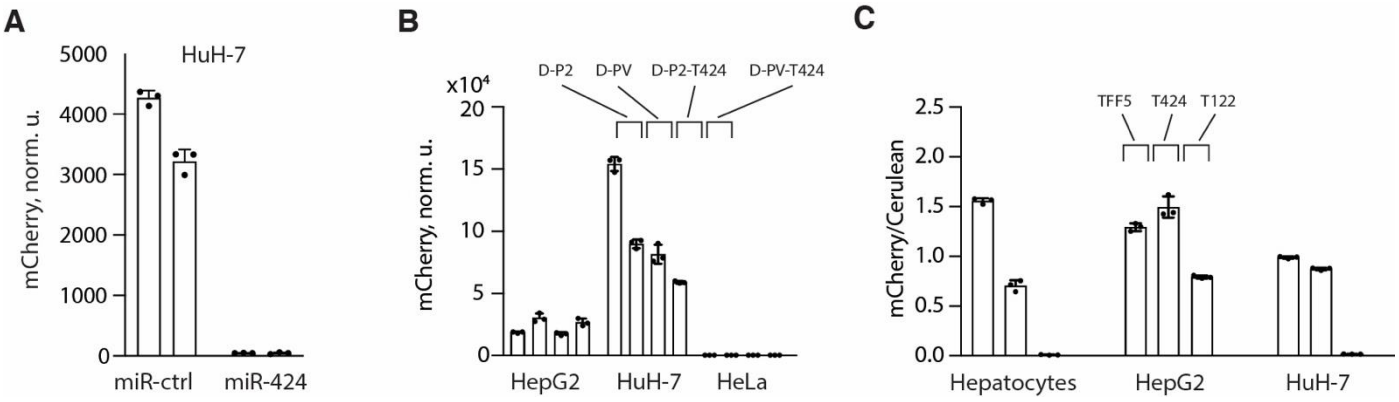

Figure S1. Additional characterization of various constructs. (**A**) Functionality of the miRNA NOT gate in the presence of endogenous TF inputs. mCherry output of the constructs D-P2-T424 and D-PV-T424 (Y axis) was measured in HuH-7 cells with and without transfection of the miR-424 mimic (indicated under X axis). (**B**) Evaluation of the miRNA target inclusion effect on mCherry output expression obtained with the plasmids encoding the indicated constructs, in two HCC cells lines (HepG2 and HuH-7), and non-HCC (HeLa) cell line as a control. The correspondence between the bars and the constructs is indicated. (**C**) In vitro evaluation of miRNA-424 and miR-122 for their activity in healthy primary hepatocytes and HCC cell lines (X axis labels), with the help of AAV-DJ-encoded bidirectional miRNA reporters. The correspondence between the bars and the constructs is indicated, with the notation TFF5, T424 and T122 representing, respectively, the control, miR-424, and miR-122 reporter. In all panels, shown are individual data points, mean±SD for biological triplicate, ***n***=3.

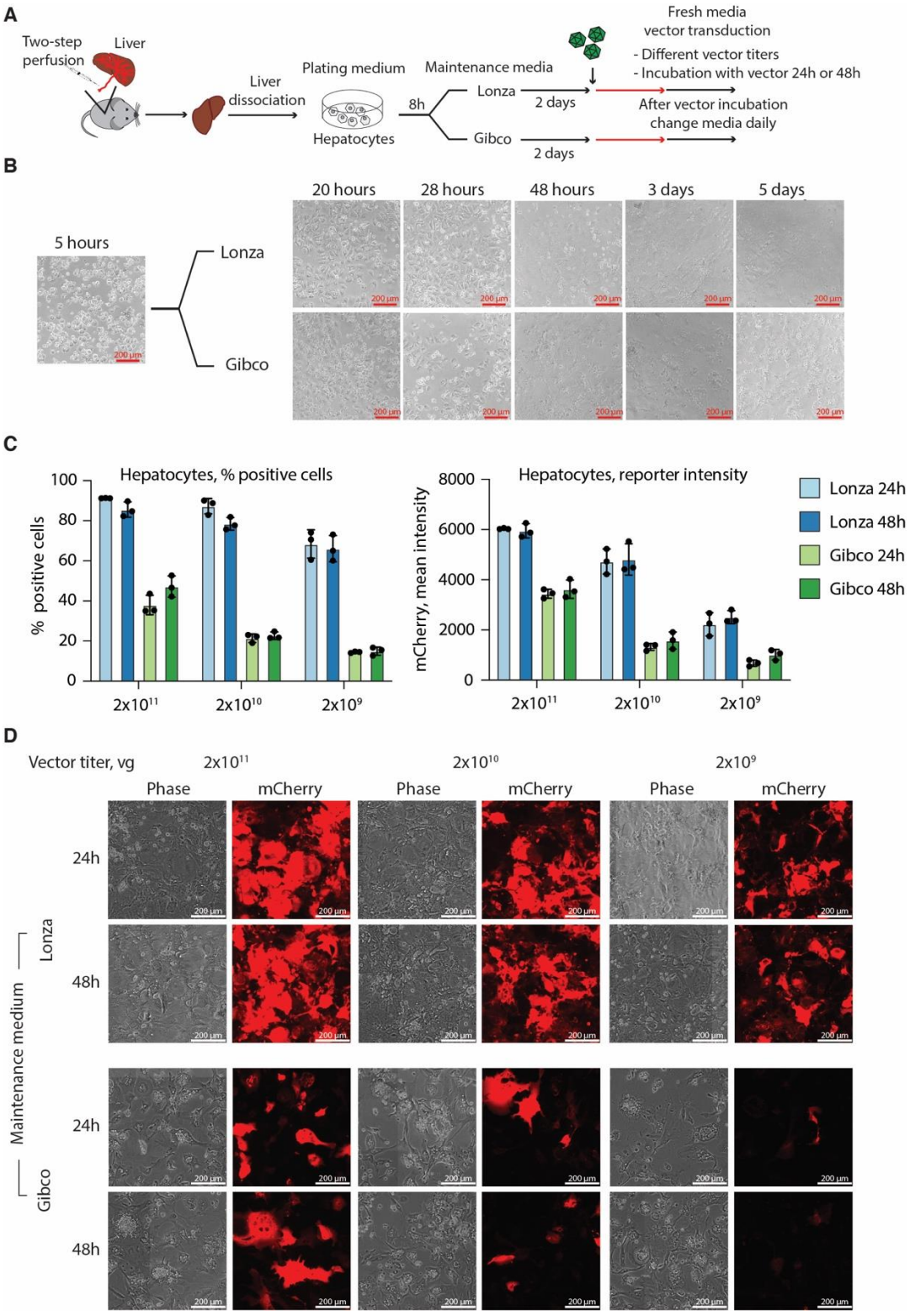

Figure S2. Optimization of primary hepatocyte culture conditions and viral vector transduction. (**A**) Schematic representation of the workflow used to optimize primary hepatocyte culture conditions and viral transductions. After extraction and plating, primary hepatocyte culture was carried out using two different complete maintenance media (Lonza or Gibco). The cells were transduced with viral vector at different titers spanning two orders of magnitude and the vector-containing medium was incubated with the cells for either 24 h or 48 h. (**B**) The morphology of primary

hepatocytes at different time points in culture evaluated by bright field microscopy. The time elapsed after extraction and media type is indicated. (**C**) Flow cytometry quantification of primary hepatocyte transduction using a DJ-pseudotyped constitutive reporter (pMD26) at different titers ($2x10^9$ – $2x10^{11}$ vg) with different maintenance media (Gibco or Lonza), keeping the cells in contact with the vector-containing medium for different duration (24 h or 48 h). The charts show the percentage of mCherry-positive cells (left) and the mean fluorescence intensity of the mCherry-positive population (right). Shown are individual data points, mean±SD for biological triplicate, ***n***=3. (**D**) Micrographs showing hepatocyte morphology and mCherry expression right before the flow cytometry measurement (day 5) for different viral titers, maintenance media, and transduction time. Scale bars in all images correspond to 200 μm.

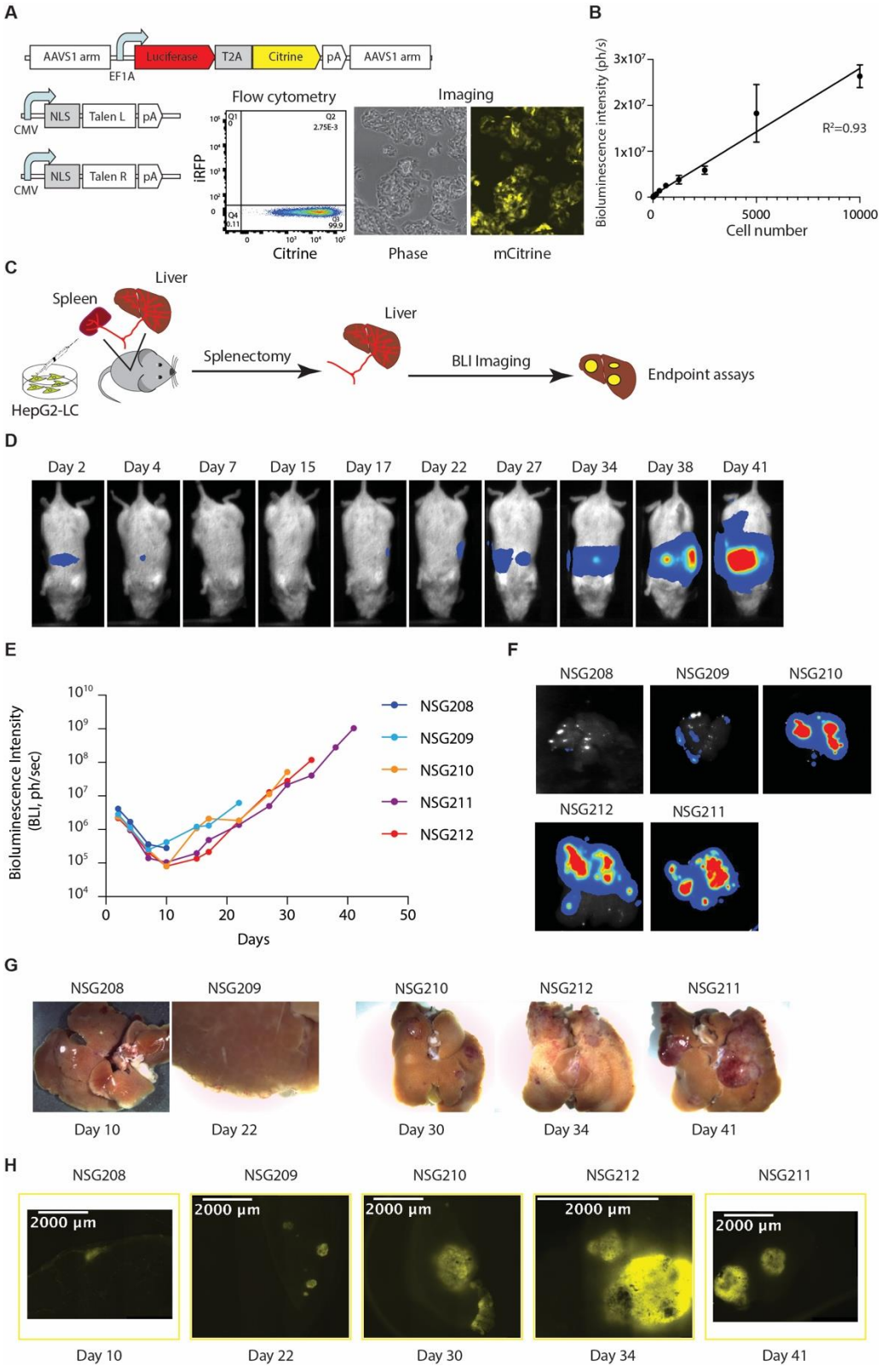

Figure S3. Calibrating the orthotopic mouse model of HCC. (**A**) Schematic representation of the components used to generate the human HCC HepG2-LC stable cell line via transcription activator-like effector nuclease (TALEN)-mediated genome editing (left). The two TALEN (L and R) were designed to target the AAVS1 locus in the human chromosome 19. The reporter cassette is flanked by AAVS1 homology arm to guide its genomic insertion and is composed by an EF1A promoter driving the bicistronic expression of luciferase and mCitrine linked via a 2A peptide.

NLS, nuclear localization signal. T2A, 2A peptide derived from *Thosea asigna*. The flow cytometry measurements and microscopy imaging of the clone selected to establish the animal model are shown (right). (**B**) In vitro measurements confirm the linear relationship between HepG2-LC cell number and measured BLI signal. Shown are mean±SD for biological triplicate, ***n***=3. (**C**) Schematic illustration of the surgical procedure used to establish the hepatocellular carcinoma (HCC) mouse model and tumor follow-up. The HepG2-LC cells were delivered to the liver for colonization via splenic injection. After the surgery the spleen was removed and the tumor was followed over time via BLI imaging. At a selected endpoint the animals were terminated to obtain the gross morphology of liver tumors *in situ* and to harvest the liver for further analysis. (**D**) Whole-body bioluminescence imaging of a representative animal over time after intrasplenic tumor cell inoculation. (**E**) The progression of tumor burden after the inoculation of 10^6 HepG2 LC cells in NSG mice. Each curve represents data obtained from a single animal. Each datapoint represents one BLI measurement of a single animal, as indicated. The animals were terminated at different time points to establish a qualitative relationship between measured signal level and physical tumor size. (**F**) Liver tumor burden at different termination points, quantified by bioluminescence. The images are superpositions of the livers (grayscale) and the tumor-derived bioluminescent signal. (**G**) Gross morphology of the liver and tumors at termination. The day post cell inoculation at which the termination took place is shown under each image. (**H**) Fluorescence micrographs of liver sections. The day post cell inoculation at which the termination took place is shown under each image. The scale bar corresponds to 2000 μm.

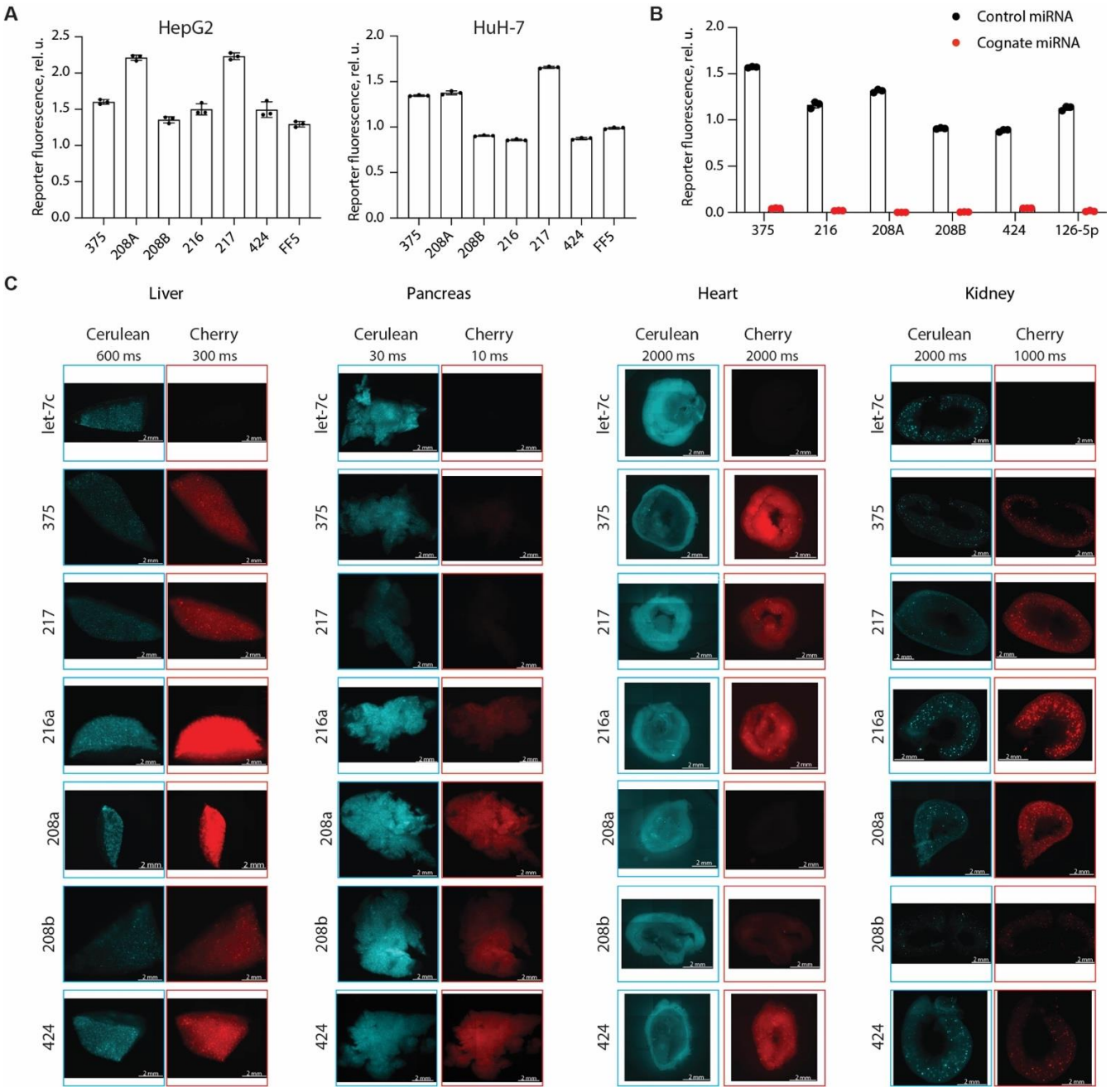

Figure S4. In vitro and in vivo functional testing of miRNA inputs to the tumor-specific computation. (**A**) Reporter fluorescence obtained with a panel of miRNA reporters packaged in DJ-pseudotyped vectors and transduced in HepG2 (left) and HuH-7 cells (right). FF5 is a synthetic control target. (**B**) Evaluation of miRNA reporter functionality and repressibility. Each reporter plasmid was used to transfect HuH-7 cells (previously confirmed as negative for the candidate miRNA) together with its cognate miRNA (red dots) or with a scrambled miRNA control (black dots). In (A) and (B), shown are individual data points, mean±SD for biological triplicate, ***n***=3. (**C**) Representative micrographs of reporter expression in mouse liver, pancreas, heart and kidney. The name of the miRNA sensed by a reporter is indicated on the left. Cerulean pseudocolor shows the expression of the constitutive mCerulean internal control. Red pseudocolor shows the expression of the mCherry reporter, furnished with the indicated miRNA target. The exposures used to image the tissues are shown. The scale bars correspond to 2 mm.

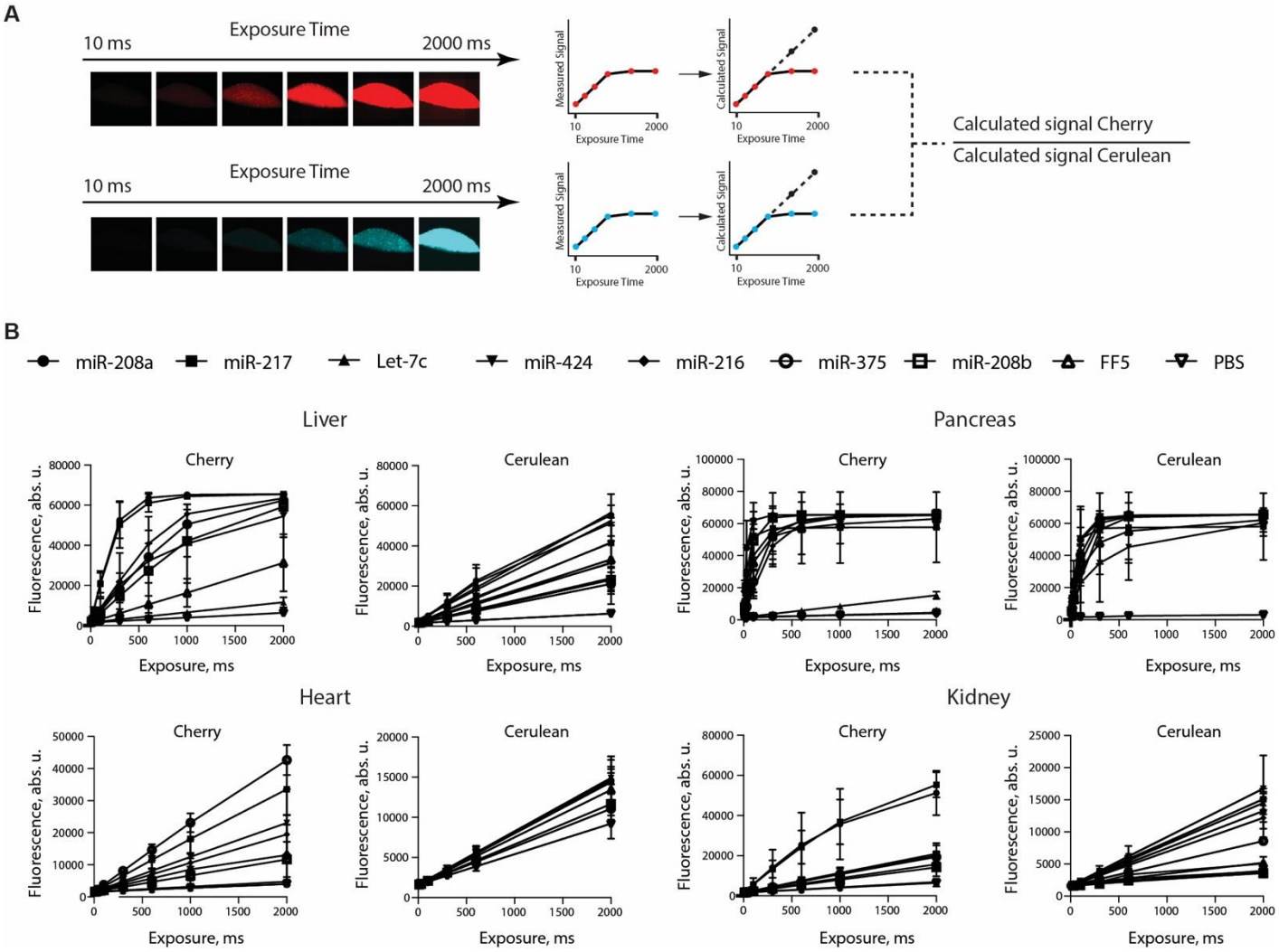

Figure S5. Image quantification pipeline. (**A**) The schematics of image quantification pipeline. Each sample was imaged at different exposures ranging between 10 ms and 2000 ms in both the mCherry and Cerulean channels. The measured signal was plotted against the exposure time to uncover signal linearity range. If the signal measured at 2000 ms for a given sample was in the linear range, the measured value was used directly as "Calculated signal". For samples showing signal saturation, we used the linear portion of the curve to extrapolate the value of the corresponding "Calculated signal" at 2000 ms via linear regression. The "Reporter fluorescence" in relative units (rel. u.) shown in Fig. 3C was the ratio of "Calculated signals" for mCherry and Cerulean. (**B**) Measured signal as a function of the exposure time for each miRNA reporter in different mouse organs. The mCherry and Cerulean channels are shown separately. All samples from each miRNA reporter were aggregated and the error was calculated to show the general signal saturation profile for each reporter in each organ. Shown are individual data points, mean±SD, obtained during image processing from at least two organ sections per organ per animal, from ***n***=3 animals.

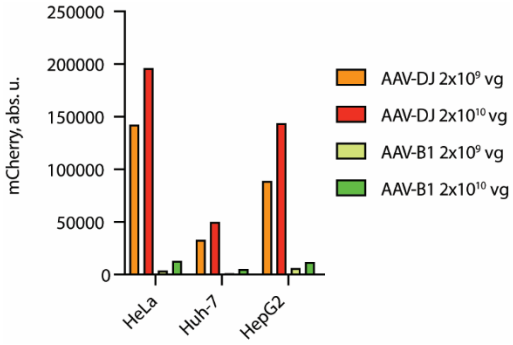

Figure S6. Comparison of AAV-DJ and AAV-B1 transduction efficiency in cell lines in vitro. Cultured HeLa, Huh-7 and HepG2 cells were transduced with the indicated titer of either DJ- or B1-typed AAV vector encoding the constitutive bidirectional expression vector. The intensity of the mCherry readout is shown. The transductions were performed once for each cell line and titer. This evidence of much stronger AAV-DJ transduction of cultured cells in vitro compared to AAV-B1, was reproduced in additional observations.

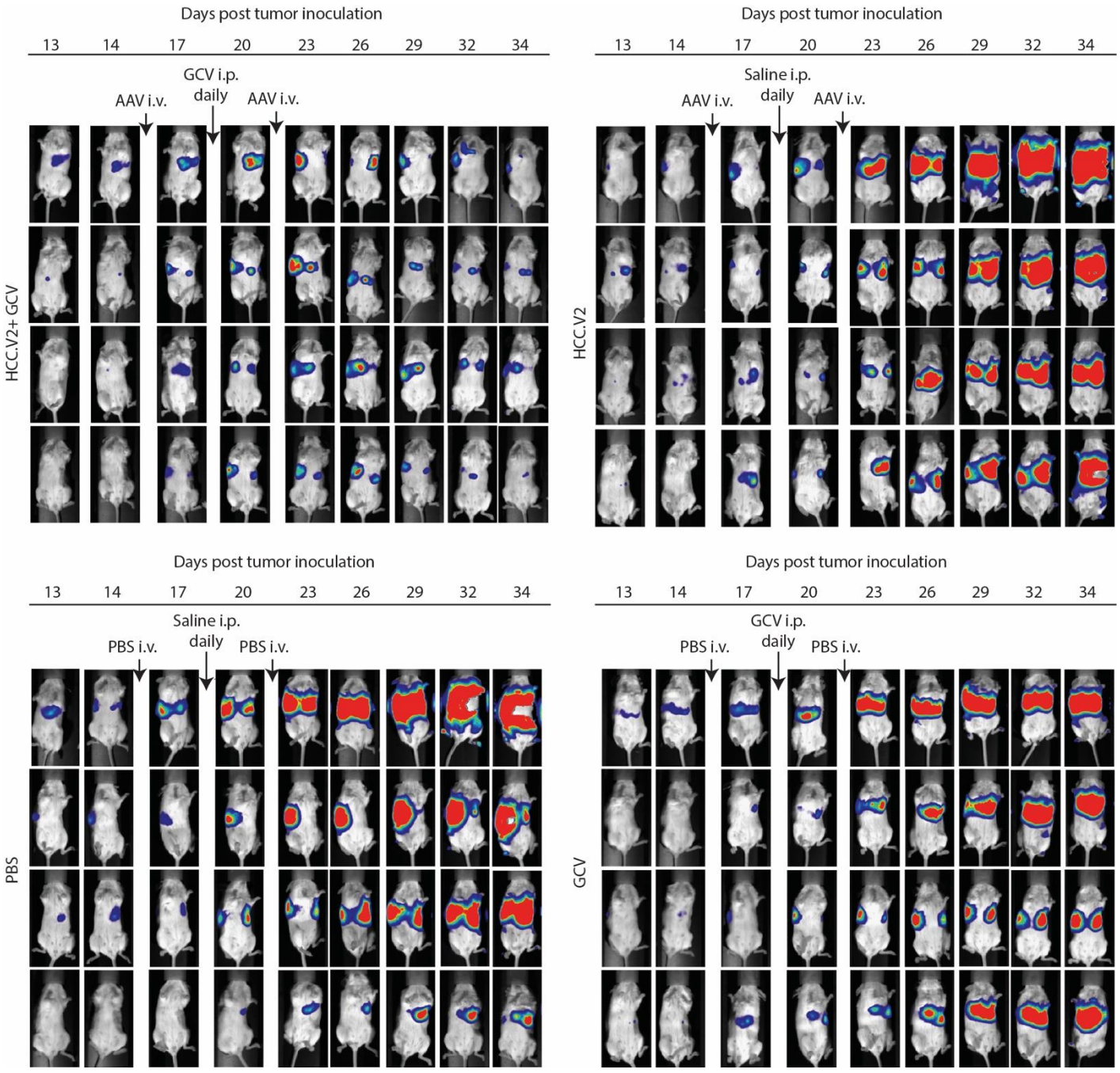

Figure S7. Tumor burden dynamics. Tumor burden progression over time for all the animals used in the four indicated groups in the efficacy experiment in Fig. 8. Tumor burden was measured via in vivo whole-body bioluminescence imaging. Time series related to individual animals are arranged in rows. The treatment description is shown on the left. The timing of interventions is indicated at the top of every image group.

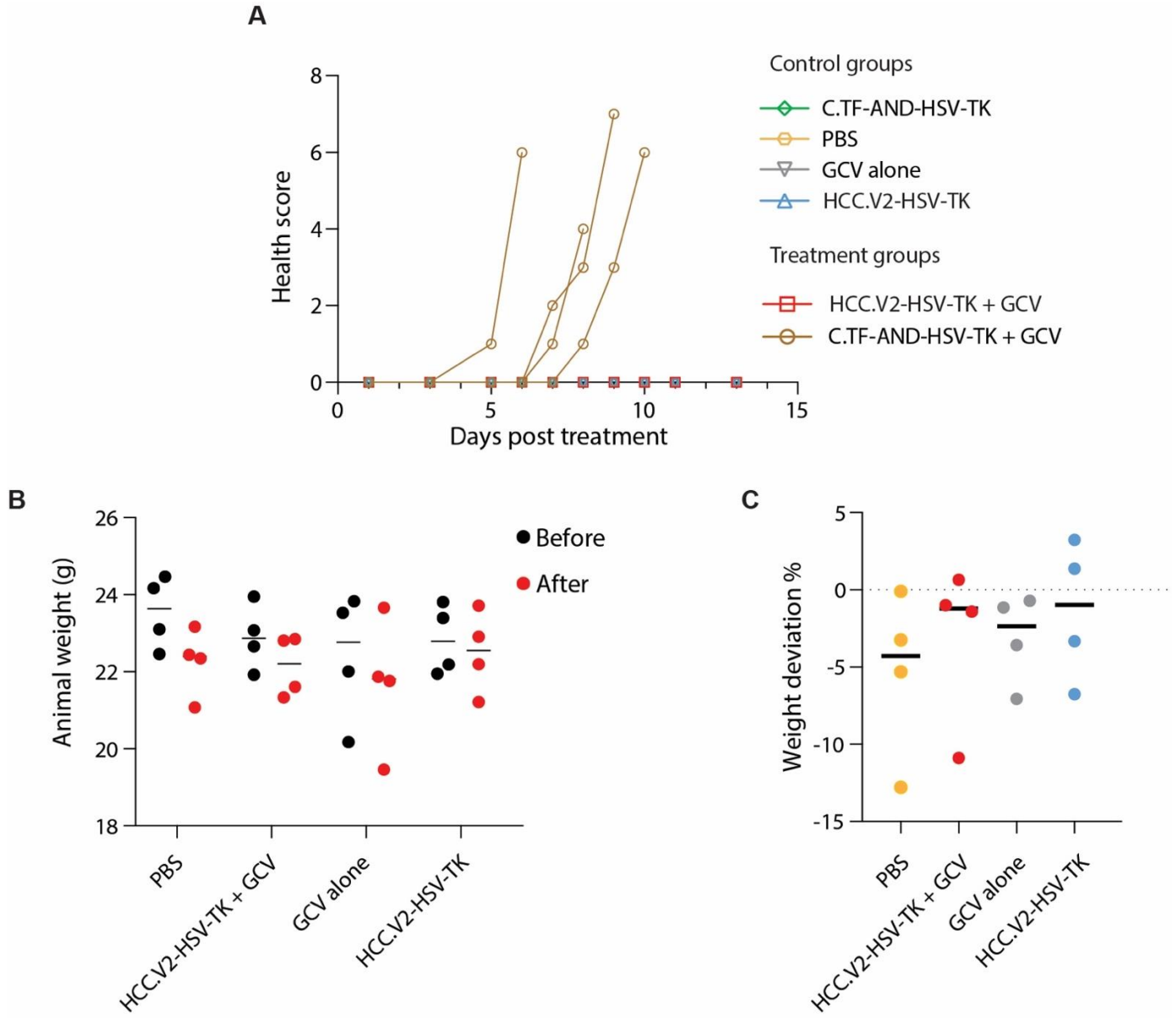

Figure S8. Assessment of adverse effects. (**A**) Health status monitoring of animals undergoing treatment, using a score sheet. Each animal was monitored and scored for multiple parameters: body condition (critical score), behavior (critical score), abdominal distension, and piloerection and grooming. Higher score corresponded to poorer wellbeing (see Methods). The scores for all animals are shown. (**B**) Animal weight in different groups before the treatment (black dots) and at termination (red dots). The average weight change was not statistically significant or any of the groups (P>0.05). (**C**) Relative change in animal weight between the start and the end of the treatment. In all panels, individual data points obtained with individual animals are shown, with horizontal bars in (B) and (C) representing the mean values.

Table S1. Plasmid cloning procedures.	
Plasmid number, acronym used in manuscript	Cloning procedure
pMD29 (precursor)	pMD26 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR2902-PR2903 were phosphorylated, annealed and ligated with the digested backbone
pBA497 (precursor)	pBA489 (28) was digested with AgeI and HindIII. PIT2 was PCR-amplified from pBA427 (28) using primers GAGAGTAAGCTTTCCTTAGGAGCTGATCTGACTCA and GATATTGCCACCACCGGTATGAGTCGAGGAGAGGTGCGC, digested with AgeI and HindIII and cloned into the digested pBA489.
pBA928 (precursor)	pMD13 (39) was digested with XbaI and BamHI and the backbone (4006 bp) was gel purified. The region encoding 3x SOX9/10 YB TATA PIT2 was amplified from pBA497 using the primers CGCCTTGCAGGCCAGGGTTTTCCCAGTCAC and CGACGGTACCGCGGGCCCGGGAATTCGAAGCTTTCCTTAGGAGCTGATCT and gel purified (1633 bp). The region encoding for PIR 3x HNF1A/B YB TATA Cyan F4 was amplified from pBA066 (28) using the primers AAACCCTGGCCTGCAAGGCGATTAAGTTGGGTAACGC - GTGGTATGGCTGATTATGATCCTCCTAGGCTTCGAATCGATTTAGAAGGGCACCACGGAGG and gel purified (1383 bp). The three fragments were combined by Gibson assembly.
pBA929 (precursor)	pMD13 (39) was digested with XbaI and BamHI and the backbone (4006 bp) was gel purified. The region encoding 3x SOX9/10 YB TATA PIT2 was amplified from pBA497 using the primers PR3723-PR3724 and gel purified (1654 bp). The region encoding for PIR 3x HNF1A/B YB TATA Cyan F4 was amplified from pBA066 (28) using the primers PR3725-PR3726 and gel purified (1362 bp). The three fragments were combined by Gibson assembly.
pBA930 (precursor)	pMD13 (39) was digested with EagI and the backbone (2887 bp) was gel purified. The region encoding 3x SOX9/10 YB TATA PIT2 was amplified from pBA497 using the primers PR3648-PR3652 and gel purified (1719 bp). The region encoding for PIR 3x HNF1A/B YB TATA Cyan F4 was amplified from pBA066 (28) using the primers PR3648-PR3649 and gel purified (1449 bp). A SV40 bidirectional PolyA was amplified from pJS88 with PR3654 and PR3655 and gel purified. The four fragments were combined by Gibson assembly.
pBA977 (precursor)	pBA929 was digested with AgeI-HF, dephosphorylated using CIP and the backbone was extracted from gel (6489 bp) and purified. pBA928 was PCR amplified with PR3803 and PR3884 the resulting product was gel purified (552 bp) digested with AgeI-HF, PCR purified and ligated with the digested backbone.
pBA979 (precursor)	pBA930 was digested with BsrGi and HpaI, dephosphorylated with CIP and the backbone was gel purified (5703 bp). pBA977 was digested using BsrGI and HpaI the resulting fragment (403 bp) was gel purified and ligated to pBA930 digested backbone.
pBA981 **C-P2**	pBA979 was digested with BamHI-HF and AvrII-HF, dephosphorylated with CIP and the backbone was gel purified (5021 bp). mCherry was amplified from pMD43 (39) using PR4078 and PR4083 digested with BamHI-HF and AvrII-HF, purified on gel and ligated with the digested pBA979 backbone.
pBA982 (precursor)	pBA977 was digested with BamHI-HF and KpnI-HF, dephosphorylated with CIP and the backbone was gel purified (5862 bp). HSV-TK was amplified from pMD43 (39) using as primers the oligos PR4076 and PR4077, the resulting product was extracted from gel, digested with BamHI-HF and KpnI-HF, PCR purified and ligated with pBA977 digested backbone.
pBA983 **D-P2**	pBA977 was digested with BamHI-HF and KpnI-HF, dephosphorylated with CIP and the backbone was gel purified (5862 bp). mCherry was amplified from pMD43 (39) using PR4078 and PR4079 digested with BamHI-HF and KpnI-HF, purified on gel and ligated with pBA977 backbone.
pBA985 **C-PV**	pBA981 was digested with AgeI-HF and HindIII-HF, dephosphorylated with CIP and the backbone was gel purified (4362 bp). The PIT-VP16 transactivator was amplified from pBA481 (28) with PR1308 and PR336, digested with AgeI-HF and HindIII-HF, gel purified and ligated with pBA981 digested backbone.

pBA986 **C.TF-HSV-TK**	pBA982 was digested with AgeI-HF and HindIII-HF, dephosphorylated with CIP and the backbone was gel purified (5627 bp). PIT-VP16 transactivator was amplified from pBA481 using as primers the oligos PR3106 and PR4272, the resulting product was extracted from gel, digested with AgeI-HF and HindIII-HF, PCR purified and ligated with the digested backbone.
pBA987 **D-PV (C.TF-AND)**	pBA983 was digested with AgeI-HF and HindIII-HF, dephosphorylated with CIP and the backbone was gel purified (5206 bp). The PIT-VP16 transactivator was amplified from pBA481 (28) with PR1308 and PR336, digested with AgeI-HF and HindIII-HF, gel purified and ligated with pBA983 digested backbone.
pBA996 (precursor)	pBA983 was digested with AvrII-HF and ClaI dephosphorylated with CIP and the backbone was gel purified (6576 bp). PR4099-PR4100 were phosphorylated, annealed and ligated with pBA983 digested backbone
pBA997 (precursor)	pBA987 was digested with AvrII-HF and ClaI dephosphorylated with CIP and the backbone was gel purified (6366 bp). PR4099-PR4100 were phosphorylated, annealed and ligated with pBA987 digested backbone
pBA998 (precursor)	pBA981 was digested with AvrII-HF and ClaI dephosphorylated with CIP and the backbone was gel purified (5731 bp). PR4095-PR4096 were phosphorylated, annealed and ligated with pBA981 digested backbone
pBA999 (precursor)	pBA985 was digested with AvrII-HF and ClaI dephosphorylated with CIP and the backbone was gel purified (5522 bp). PR4095-PR4096 were phosphorylated, annealed and ligated with pBA985 digested backbone
pBA1003 **D-P2-T424**	pBA996 was digested with Kpn-HF, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6658 bp). PR4097-PR4098 were phosphorylated, annealed and ligated with pBA996 digested backbone
pBA1004 **D-PV-T424**	pBA997 was digested with Kpn-HF, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6448 bp). PR4097-PR4098 were phosphorylated, annealed and ligated with pBA997 digested backbone
pBA1006 **C-P2-T424**	pBA998 was digested with HindIII-HF dephosphorylated with CIP and the backbone was gel purified (5827 bp). PR4101-PR4102 were phosphorylated, annealed and ligated with pBA998 digested backbone. Positive clones were screened to select the correct target orientation.
pBA1007 **C-PV-T424**	pBA999 was digested with HindIII-HF dephosphorylated with CIP and the backbone was gel purified (5618 bp). PR4101-PR4102 were phosphorylated, annealed and ligated with pBA999 digested backbone. Positive clones were screened to select the correct target orientation.
pBA1033 **miR-424-5p reporter**	pMD26 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR3792-PR4157, encoding 4 repeats of miR-424-5p targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1071 **HCC.V1-mCherry**	pBA987 was digested with Kpn-HF, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6365 bp). PR4446-PR4447 were phosphorylated, annealed and ligated with pBA987 digested backbone
pBA1072 (precursor)	pBA987 was digested with KpnI, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6365 bp). Primers PR4448 and PR4449 were phosphorylated, annealed and ligated with the digested backbone.
pBA1074 **HCC.V1-HSV-TK**	pBA986 was digested with Kpn-HF, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6365 bp). PR4446-PR4447 were phosphorylated, annealed and ligated with pBA986 digested backbone
pBA1134 **miR-124 reporter**	*pMD30* (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5125b-PR5126b, encoding 4 repeats of miR-124-3p targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1135 **miR-208a reporter**	*pMD30* (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR4536-PR4537, encoding 4 repeats of miR-208a targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1137 **miR-217 reporter**	*pMD30* (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5131b-PR5132b, encoding 4 repeats of miR-217 targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1138 **miR-375 reporter**	*pMD30* (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR3434-PR3435, encoding 4 repeats of miR-375 targets, were phosphorylated, annealed and ligated with the digested backbone

pBA1136 **miR-216a reporter**	pMD30 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5129b-PR5130b, encoding 4 repeats of miR-216a targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1145 **let-7c reporter, C.Let-7c**	pMD26 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5388-PR5389, encoding 4 repeats of miR-Let7c targets were phosphorylated, annealed and ligated with the digested backbone
pBA1146 **miR-26b reporter**	pMD26 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5390-PR5391, encoding 4 repeats of miR-26 targets were phosphorylated, annealed and ligated with the digested backbone
pBA1147 **miR-22 reporter**	pMD26 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5392-PR5393, encoding 4 repeats of miR-22 targets were phosphorylated, annealed and ligated with the digested backbone
pBA1150 **miR-208b reporter**	pMD30 (39) was digested with BamHI-HF and SalI-HF, dephosphorylated with CIP and the backbone was gel purified (6296 bp). PR5440-PR5441, encoding 4 repeats of miR-208b targets, were phosphorylated, annealed and ligated with the digested backbone
pBA1151 (precursor)	pBA1072 was digested with Kpn-HF, PCR purified, the resulting linearized product was digested with BglII, dephosphorylated with CIP and the backbone was gel purified (6365 bp). PR5501-PR5502, encoding 4 repeats of let-7c were phosphorylated, annealed and ligated with pBA1072 digested backbone.
pBA1152 **HCC.V2-mCherry**	pBA1151 was digested with AvrII-HF and ClaI, dephosphorylated with CIP and the backbone was gel purified (6442 bp). PR5503-PR5504, encoding 4 repeats of let-7c were phosphorylated, annealed and ligated with the digested backbone.
pBA1170 **HCC.V2-HSV-TK**	pBA1152 was digested with BamHI-HF and KpnI-HF, dephosphorylated with CIP and the backbone was gel purified (5810 bp). pBA986 was digested with BamHI-HF and KpnI-HF to extract the HSV-TK gene, the relevant fragment was gel purified (1148 bp) and ligated to PBA1152 digested backbone.
pAA001 (precursor)	pBA983 was digested with KpnI-HF and HindIII-HF and the backbone (4013 bp) was gel purified. The region encoding 3x HNF1A/B YB TATA mCherry was amplified from pBA983 using the primers PR4300B-PR4301B and gel purified (980 bp). The region encoding for PIT2 transactivator was amplified from pBA066 (28) using the primers PR4302B-PR4303B and gel purified (1300 bp). The three fragments were combined by Gibson assembly.
pAA002 (precursor)	pAA001 was digested with AsI and NdeI, dephosphorylated with CIP and the backbone (6341 bp) was gel purified. The oligos PR2068-PR2069, encoding 3X SOX9/10 response elements were phosphorylated, annealed and ligated with the digested backbone
pAA003 **C.HNF-FB**	pAA001 was digested with AvrII-HF and EcoRI-HF, dephosphorylated with CIP and the backbone (6403 bp) was gel purified. A synthesized spacer sequence (gBlock237) was amplified using as primers the oligos PR4306-PR4307, purified on gel, digested with AvrII and EcoRI-HF, PCR purified and ligated with the digested backbone.
pAA004 **C.SOX-FB**	pAA002 was digested with AvrII-HF and EcoRI-HF, dephosphorylated with CIP and the backbone (6394 bp) was gel purified. A synthesized spacer sequence (gBlock237) was amplified using as primers the oligos PR4306-PR4307, purified on gel, digested with AvrII and EcoRI-HF, PCR purified and ligated with the digested backbone.

Table S2 (Excel). List of primers and other genetic building blocks used in the study.

Data File S1 (Excel). Individual data points for main figures.

Data File S2 (Excel). Individual data points for supplementary figures.