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Supplementary Methods

Cloning of *CRISPR-SB*. To generate the *CRISPR-SB* vector, *pX330* (Addgene #42230 (1)) was sequentially opened with *AflIII* and *NotI* single cutters. *Sleeping Beauty (SB)* terminal repeats were amplified from *pTnori* (2) with *AflIII* and *NotI* overhangs, respectively and cloned into *pX330*.

Design and cloning of single guide RNA (sgRNA) sequences. The 20-bp sgRNA sequences were designed using the CRISPR design tool (http://crispr.mit.edu) (3) and are depicted in Table S6. For *Tet2* we used the sgRNA sequence as described in (4). Cloning of sgRNA sequences into *CRISPR-SB* was performed following protocols provided by the depositor of *pX330*.

T7 Endonuclease 1 (T7E1) assay for identification of suitable sgRNAs for somatic mutagenesis. The mouse pancreatic cancer cell line *PPT-53631* was cultured in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (Biochrom). Eighty thousand cells per well were sown in a 24-well-plate and transfected the next day with 450ng *CRISPR-SB* plasmid and 50ng *pcDNA™6.2/EmGFP-Bsd/V5-DEST* (Life Technologies) using Lipofectamine® 2000 (Life Technologies). Twenty four hours after transfection, cells were selected with 5µg/mL Blasticidin (Life Technologies). After two days of selection, cells were lysed with DirectPCR lysis reagent (Viagen). PCR amplification of the target region was performed with Q5® High-Fidelity DNA Polymerase (New England Biolabs) using primers listed in Table S8. We purified PCR products by gel extraction (QIAquick Gel Extraction Kit, Qiagen) and denatured and reannealed 200ng of the purified PCR product in NEBuffer 2 (New England Biolabs) using a thermocycler. Hybridized PCR products were treated with 10U of T7E1 (New England Biolabs) at 37°C for 15min in a reaction volume of 20µL. Reactions were stopped by the addition of 2µL 0.5M EDTA and analyzed by electrophoresis using a 10% polyacrylamide gel. Indel frequency was calculated according to (5).

Surveyor nuclease assay for determining indel frequency in targeted genes. For Surveyor assays we used the mouse pancreatic cancer cell lines *PPT-53631* and *PPT-4072*, which were cultured in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (Biochrom). Eighty thousand cells per well were sown in a 24-well plate and transfected the next day with 450ng *CRISPR-SB* plasmid and 50ng *pcDNA™6.2/EmGFP-Bsd/V5-DEST* (Life Technologies) using Lipofectamine® 2000 (Life Technologies). Twenty four hours after transfection, cells were selected with 5µg/mL Blasticidin (Life Technologies). After two days of selection, cells were lysed with DirectPCR lysis reagent (Viagen). Amplifications of the target regions were performed with TaKaRa Ex Taq DNA Polymerase (Clontech) using primers listed in Table S8. PCR products were denatured and reannealed in NEBuffer 2 (New England Biolabs) using a thermocycler. Surveyor nuclease reaction was performed according to manufacturer's instructions (Transgenomic) and indel frequency was calculated according to (5).

Animal Experiments. For hydrodynamic tail vein injections (HTVI), 10µg/mL *hSB5 transposase* (2) and ten (eighteen) *CRISPR-SB* sgRNA vectors (10µg/mL in total) were dissolved in 2mL 0.9% saline and injected into the tail vein of eight weeks old mice over six to ten seconds (2). In order to accelerate liver tumorigenesis, we used *Alb-Cre;Kras*$^{LSL\text{-}G12D/+}$ mice (6, 7). For chemical acceleration of tumorigenesis, wild type mice were treated nine times with a weekly intraperitoneal injection of 1µL/g body weight 10% carbon tetrachloride (CCl_4, Sigma-Aldrich) in Corn Oil (Sigma-Aldrich) beginning two weeks after HTVI. Mice were monitored for tumor development by regular magnetic resonance imaging (MRI) screening, starting at 20 weeks post HTVI. Animals were sacrificed as soon as hepatic tumors were diagnosed or when signs of sickness were apparent (in one case of early onset metastasized cancer at 20 weeks prior to MRI screening; see data in Figure 4).

For subcutaneous implantation of cell lines derived from mouse primary hepatic tumors, trypsinized cells were washed twice with DBPS (Life Technologies) and counted using a hemocytometer. Concentration was adjusted to $3.3x10^6$ cells per mL DPBS and 150µL cell suspension ($5x10^5$ cells) was subcutaneously injected into the right and left flank of NOD scid gamma (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice using a 1mL syringe with a 27 gauge needle. Mice were monitored regularly for general health and tumor growth and were sacrificed once tumors reached a size of 1cm in diameter (about two weeks post implantation).

All animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees

(IACUC) of Technische Universität München, Regierung von Oberbayern and the UK Home Office.

Immunofluorescence test for HTVI-based co-delivery of multiple plasmids to hepatocytes. To test whether hepatocytes can be transfected simultaneously with multiple plasmids by HTVI, *Rosa26*^{mTmG} reporter mice (8) were co-injected with two transposon constructs containing expression cassettes of Flag-YAP and Cre^{ERT2} and with *hSB5 transposase* vector (9). Cre^{ERT2} activation by Tamoxifen ten days post HTVI leads to conversion of the *Rosa26*^{mTmG} allele and subsequent expression of cell-membrane localized GFP. Six month after injection, livers were embedded and sections were immunostained for Flag-YAP (M2-Flag antibody, Sigma-Aldrich; green) and GFP (GFP Tag antibody, Life Technologies; red).

Magnetic resonance imaging (MRI) screening. MRI was performed using a 3 Tesla clinical MRI system (Ingenia 3T, Philips Healthcare) with a human 8-channel wrist coil (SENSE Wrist coil 8 elements) following a previously described protocol that was adapted to the 3 Tesla scanner (10). Starting at 20 weeks after HTVI, mice were screened on a regularly basis. To this end, longitudinal T2-weighted (T2w) turbo spin-echo imaging (slice thickness=0.7mm, in-plane resolution=0.3x0.38mm^2, TR/TE=TR/TE=4352ms/101ms, TF=21, NSA=9, total scan duration 5.22min) was performed for tumor detection and volumetric analysis. Mice were sacrificed once tumors reached a size greater or equal 3mm in diameter.

Histology and immunohistochemistry. Histological analysis was performed for all tumors >1mm. Mouse tissues were fixed in 4% formalin solution, embedded in paraffin and cut into 2µm sections. Hematoxylin and eosin (H&E) staining was performed according to standard protocols. Immunohistochemistry was conducted using primary antibodies listed in Table S7. As secondary antibodies we used a rabbit-anti-rat antibody (1:1000, Jackson Immuno Research) and a rabbit-anti-goat antibody (1:300, DAKO) and detection was performed with the Bond Polymer Refine Detection Kit (Leica). Detailed protocols of individual staining procedures are available upon request.

DNA isolation and microdissection. DNA was isolated from tissue samples stored in RNAlater (Sigma-Aldrich) with DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. For heterogeneity analysis, we microdissected sections of Tu1 under a microscope using 20 gauche needles. DNA was isolated from the microdissected regions in the same manner as from the freshly frozen tissues mentioned above with an extended tissue lysis time of 60h.

Sequencing of sgRNA target regions. Genomic sgRNA target regions (5ng DNA per 30µL reaction) were amplified with Q5® High-Fidelity DNA Polymerase (New England Biolabs) using primers displayed in Table S8. For Sanger capillary sequencing, PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and each PCR product was sequenced individually. For amplicon-based next generation sequencing, the ten (eighteen) PCR products of each sample were pooled and purified (QIAquick PCR Purification Kit, Qiagen). Library preparation was carried out as described previously (11). Briefly, after end repair and A-tailing, an Illumina paired end adapter was ligated (NEBNext® Ultra DNA Library Prep Kit for Illumina®, New England Biolabs; sequences depicted in Table S8) and the individual sample pools were barcoded with eight cycles of PCR (2x KAPA HiFi HotStart ReadyMix, Kapa Biosystems; sequences listed in Table S8). Barcoded samples were pooled and quantified with qPCR (KAPA SYBR® Fast qPCR ABI Prism Mix, Kapa Biosystems) and the single pool was sequenced (300bp, paired end) on the Illumina MiSeq Desktop Sequencer (Illumina). To verify the next generation sequencing results, we cloned the PCR products for a subset of target regions into the *pCR® 2.1-TOPO® TA* vector (TOPO® TA Cloning® Kit, Life Technologies). For each sample, we picked 30 colonies and sequenced them individually using Sanger capillary sequencing.

Sequencing of sgRNA off-target sites. Coordinates of potential off-target sites for the ten sgRNAs were downloaded from the CRISPR design tool (http://crispr.mit.edu; Table S13) (3). For the top five off-targets (exonic, intronic, intergenic) and (if not already included in the top five list) top three exonic off-targets of each sgRNA flanking PCR primers (Table S14) were designed. PCRs and amplicon-based next generation sequencing were performed in the same manner as described above for the sgRNA target regions.

Bioinformatic analyses. MiSeq Illumina paired 300 nucleotide reads were mapped onto *mm10* assembly with *BBMAP short read aligner* (http://bbmap.sourceforge.net) using default settings. Among a number of other tested aligners, only this particular aligner was able to map correctly large deletions, such as 178bp in Tu2 (Figure S7). BAM files were sorted and indexed with *samtools (v0.1.19)* (12). After mapping, only paired reads (about 3% were unpaired) were extracted based on *bitwise flag 0x2*. This resulted in BAM files containing only correctly paired reads. In order to obtain data in pileup format with the number of reads covering sites we employed *samtools (v0.1.6) pileup command with option (-i)* which only displays lines containing indels. Pileup files were processed with *VarScan (v2.3.6) pileup2indel command* (13).

Establishment of cancer cell lines. To derive cell lines from primary mouse cancers and metastases, tumor tissues were first washed with sterile DPBS (Life Technologies) and cut into small pieces, followed by digestion in RPMI 1640 (Life Technologies) with 10% FBS (Biochrom) and 1x PenStrep (Life Technologies), supplemented with 200 U/mL collagenase (Collagenase Type II, Worthington) at 37°C until tissue pieces were disintegrated completely. Cells were then centrifuged, resuspended in RPMI 1640 containing 10% FBS and 1x PenStrep and sown in six-well plates coated with 0.1% gelatin (Sigma-Aldrich).

Quantitative Cas9 analysis. To detect *hSpCas9* presence in the liver samples of mice two weeks post HTVI, 7.5ng genomic DNA was used for real time quantitative PCR (SYBR® Select Master Mix, Life Technologies). *HSpCas9* copy numbers were normalized to mouse *Apolipoprotein B (ApoB)* copy numbers. Primer sequences are listed in Table S9.

Quantitative guide distribution analysis. To analyze the distribution of sgRNAs in liver samples of mice two weeks after HTVI, 10ng DNA per 20µL sample was amplified with Taq Polymerase (New England Biolabs) using *CRISPR-SB-fwd* und *CRISPR-SB-rev* primers (Table S10). PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and 10pg purified PCR product was used for guide specific real time quantitative PCR (SYBR® Select Master Mix, Life Technologies). The universal forward primer (*CRISPR-SB-quant-fwd*) and the guide specific reverse primers are displayed in Table S10.

***CRISPR-SB* integration analysis**. To test for integration of the *CRISPR-SB* vector into the genome of mouse liver tumors, 10ng genomic DNA per 50µL reaction was amplified (Q5® High-Fidelity DNA Polymerase, New England Biolabs) using *CRISPR-SB* specific primers as depicted in Table S12. Liver samples of mice two weeks post HTVI (containing episomal *CRISPR-SB* vectors) functioned as positive controls. 25µL of each PCR was loaded on a 1.5% agarose gel.

Fusion analysis for detection of large chromosomal deletions. To test for possible intrachromosomal fusion products caused by combinatorial sgRNA targeting, we performed PCRs spanning the potential location of the fusions as predicted by the sgRNA target sites. To this end, we used 10ng genomic DNA in 30µL PCR reactions (TaKaRa Ex Taq DNA Polymerase, Clontech) using the respective forward and reverse primers of the target sites (Table S8). Resulting PCR products were purified for Sanger capillary sequencing (QIAquick PCR Purification Kit, Qiagen). To quantify the *Cdkn2a* fusion product in regions 1, 2 and 3 of Tu1, we used 10ng DNA of the respective samples for real time quantitative PCR (SYBR® Select Master Mix, Life Technologies) with primers displayed in Table S11. Primer *Cdkn2a-ex1β-quant-fwd* with *Cdkn2a-ex2-quant-rev* was used for quantification of the fusion product and *Cdkn2a-ex2-quant-fwd* and *Cdkn2a-ex2-quant-rev* were used for quantification of other alleles at that position (wild type and with small indels).

Multicolor fluorescence in situ hybridization (M-FISH). To analyze interchromosomal fusions/rearrangements in liver tumor cell lines derived from mice injected with *hSpcas9* and sgRNAs, M-FISH was carried as described before (14).

Array comparative genomic hybridization (aCGH). aCGH was carried out using Agilent 60k mouse CGH arrays with custom design (AMADID 041078) as described previously (15). CGH data was preprocessed with the Agilent Genomic Workbench software. Raw log ratios were recentered by adding or subtracting a constant value to insure that the zero point reflects the most common ploidy state (legacy centralization option). Segmentation and aberration calling were done with the implemented ADM-2 algorithm. Normalized data was imported in R version 3.1.3 (http://www.r-project.org). For each detected aberration the closest off-targets surrounding the aberration borders down- and upstream were investigated. The distance and the number of probes between the aberration border and the predicted off-target were calculated. An

aberration was called potentially induced by an off-target if 20 probes or less are located between the aberration and the off target and the distance between them is lower than 500,000 nucleotides.

Statistics. To test if CRISPR/Cas9-induced target site mutations across tumors show a random or non-random distribution, we performed a χ^2 test. To examine if some of the targeted tumor suppressor genes undergo positive selection (in comparison to *Brca1/Brca2* which serve as negative controls) we performed Fisher's Exact tests and corrected p values for multiple testing with the Benjamini Hochberg procedure. Results were considered as significant for p values <0.05.

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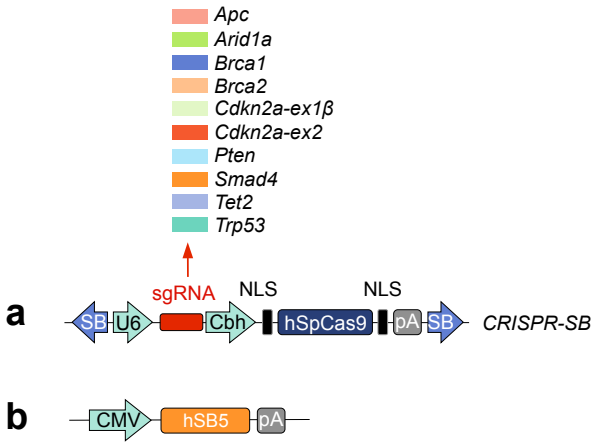
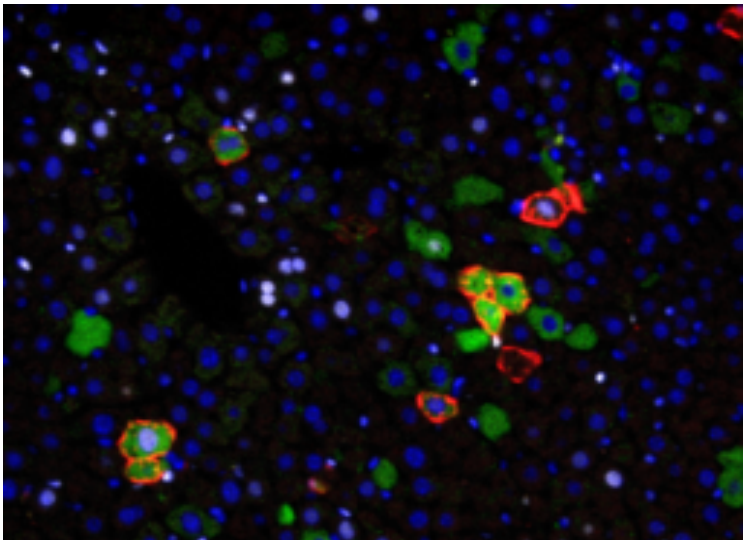
Supplementary Figures

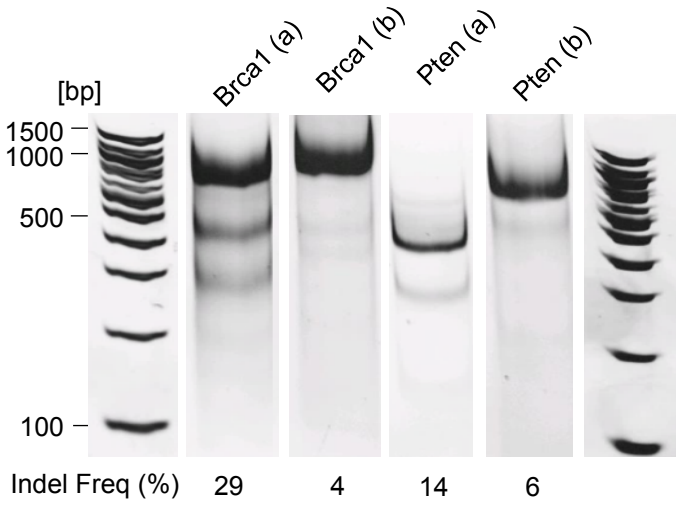
Supplementary Figure S1. Vectors used for hepatic delivery of multiplexed CRISPR/Cas9 for somatic mutagenesis in mice. (A) *CRISPR-SB* bicistronic expression vector consisting of a U6 promoter-driven single guide RNA (sgRNA) and a *CBA (chicken β-actin) hybrid intron* (CBh) promoter-driven human codon-optimized *Streptococcus pyogenes* Cas9 (*hSpCas9*) flanked by *Sleeping Beauty* (SB) inverted terminal repeats. (B) A cytomegalovirus (CMV) promoter-driven *Sleeping Beauty* transposase (*hSB5*) can mobilize/integrate the *CRISPR-SB* vector into the liver cell genome. NLS, nuclear localization signal; pA, polyadenylation signal.



Supplementary Figure S2. Simultaneous delivery of multiple vectors into hepatocytes upon hydrodynamic tail vein injection (HTVI). *Rosa26*^{mTmG} reporter mice were co-injected with *hSB5 transposase* vector and with two transposon constructs containing expression cassettes of *Flag-YAP* and *Cre*^{ERT2}. *Cre*^{ERT2} activation by Tamoxifen 10 days post HTVI leads to conversion of the *Rosa26*^{mTmG} allele and subsequent expression of cell-membrane localized GFP. Six month after injection, livers were embedded and sections were immunostained for *Flag-YAP* (M2-Flag antibody, Sigma-Aldrich; green) and GFP (GFP Tag antibody, Life Technologies; red).



Supplementary Figure S3. Identification of suitable sgRNAs for somatic mutagenesis. Prior to *in vivo* application, multiple sgRNAs per gene were tested for their efficiency to induce frameshift-causing mutations in combination with transiently expressed Cas9. Indel frequencies were determined upon T7E1 assays in a mouse pancreatic cancer cell line. Results were used to choose the most efficient sgRNAs for *in vivo* application (in these instances: *Brca1(a)* and *Pten(a)*).

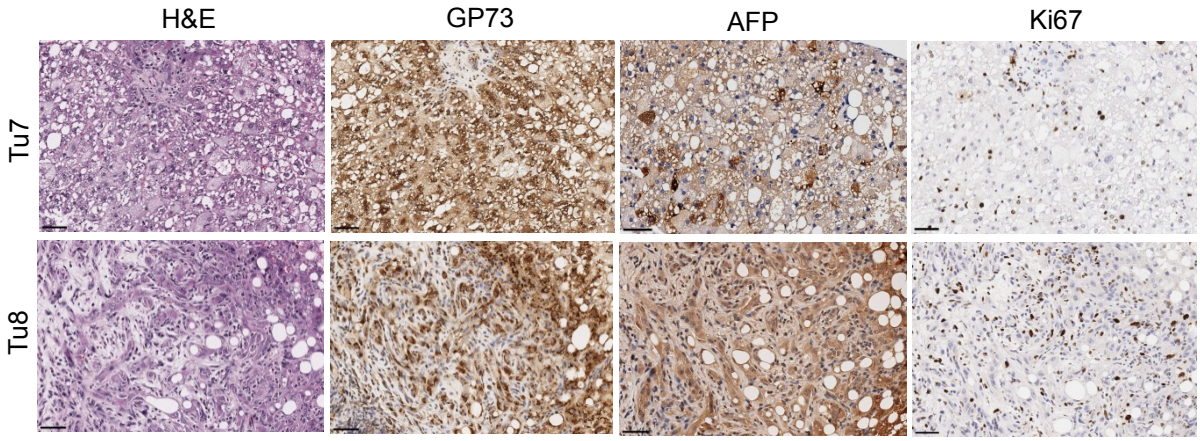


Supplementary Figure S4. Microscopic images of hepatocellular carcinomas. Representative microscopic images of hepatocellular carcinomas (HCCs) derived from mice injected with *hSpCas9* and ten sgRNAs.

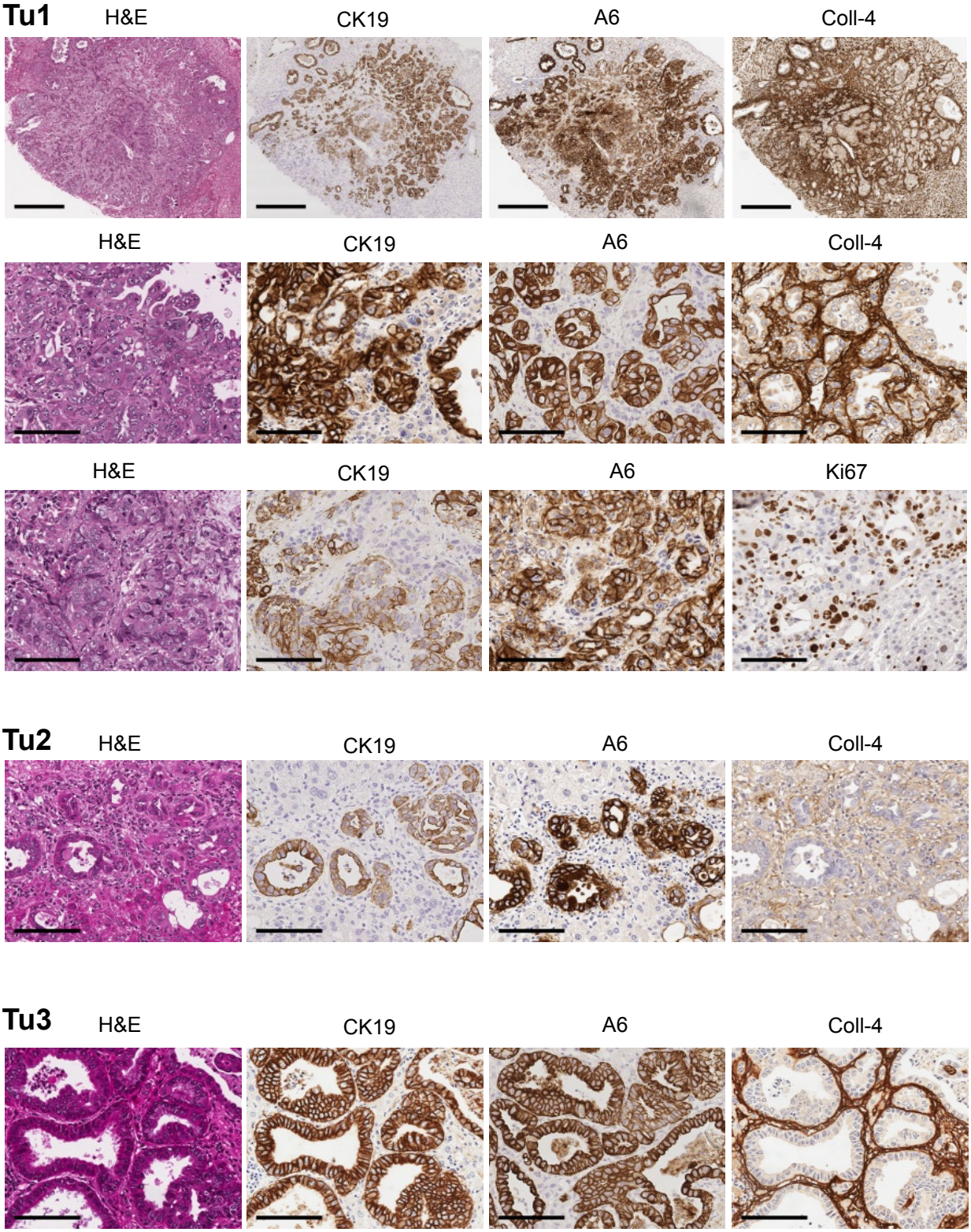
Tu7 (upper panels), a moderately differentiated HCC with trabecular to solid growth pattern (H&E staining; first image), shows strong *Golgi phosphoprotein 2/Golgi membrane protein GP73* (GOLM1/ GP73) expression (second image) and high proliferation activity (Ki67 staining; forth image). *α-fetoprotein* (AFP) is expressed slightly by the majority of tumor cells, scattered neoplastic cells show a strong expression of AFP (third image).

Tu8 (lower panels), a poorly differentiated HCC, shows fatty changes and a slight to moderate fibroplasia (H&E staining; first image). Tumor cells strongly express GP73 (second image) and show a very high proliferative activity (Ki67 staining; forth image). AFP is expressed with slight to moderate intensity by the majority of neoplastic cells (third image).

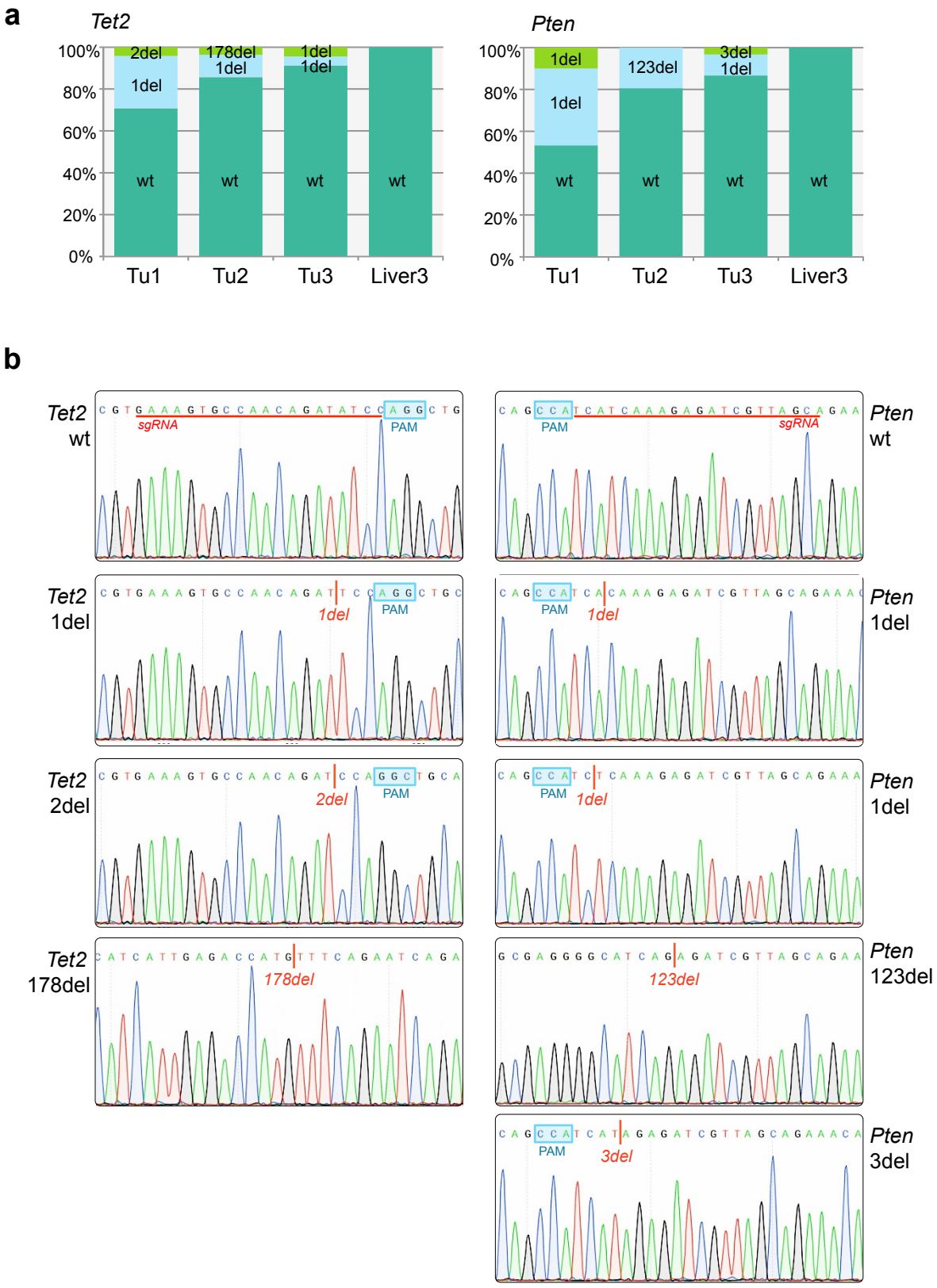
Bars, 50µm.



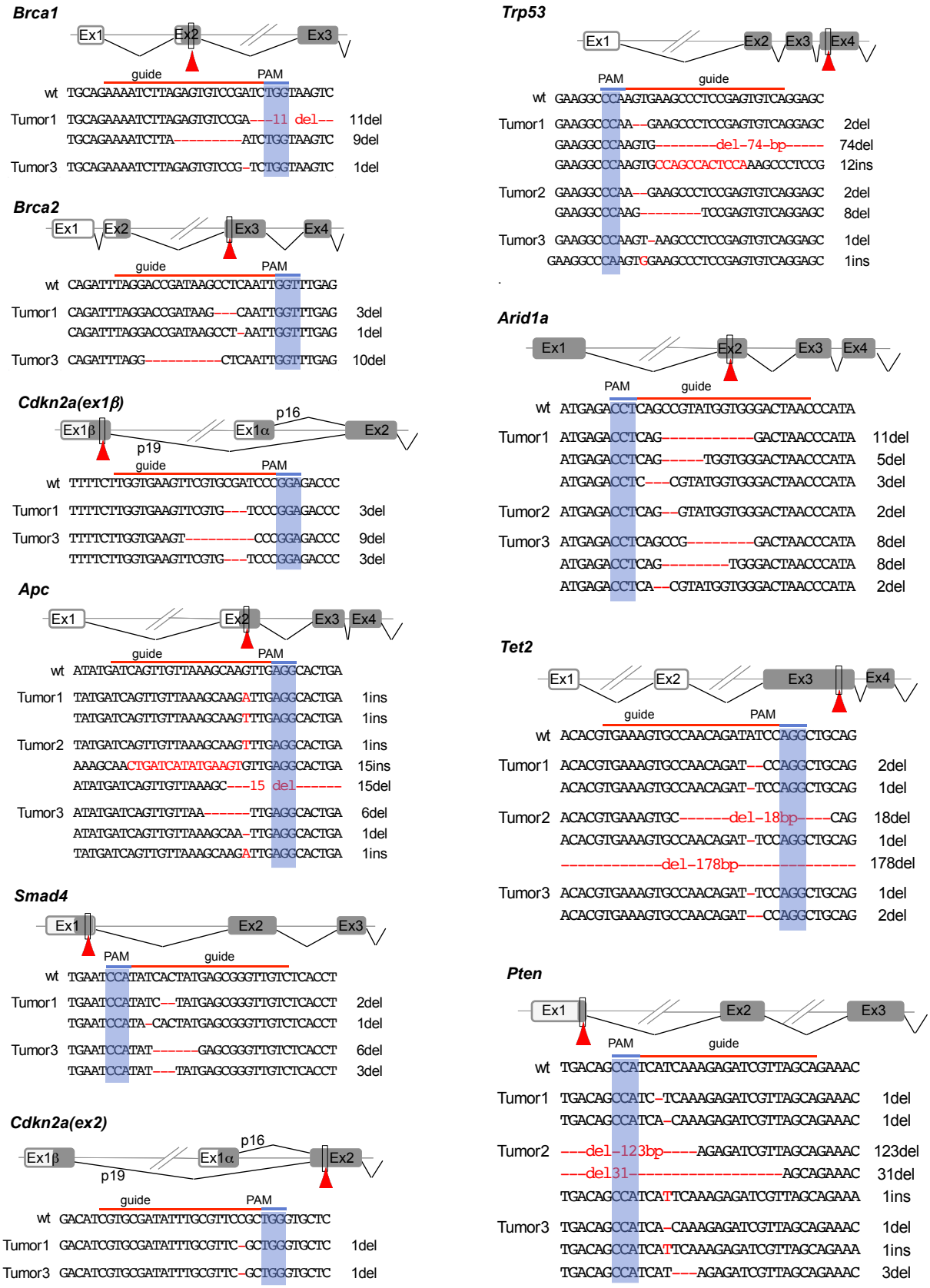
Supplementary Figure S5. Histology and IHC stainings of three intrahepatic cholangiocarcinomas (ICC). Tu1: Moderately to poorly differentiated ICC with a tubular growth pattern in the tumor periphery (middle panels) and cord-like to solid growth pattern in the central part (lower panels). Tumor cells intensely express cytokeratin 19 (CK19) and A6 (oval cell surface antigen). Collagen-4 (Coll-4) is strongly expressed in tumor-associated stroma. Tumor cells show a high proliferation rate (Ki67 in bottom right image). Bars, 400µm (upper row), 100µm (middle and lower row). **Tu2:** Well to moderately differentiated ICC with strong expression of CK19, A6. Coll-4 is expressed in the tumor-associated stroma. Bars, 100µm. **Tu3:** Well differentiated ICC. Bars, 100µm.



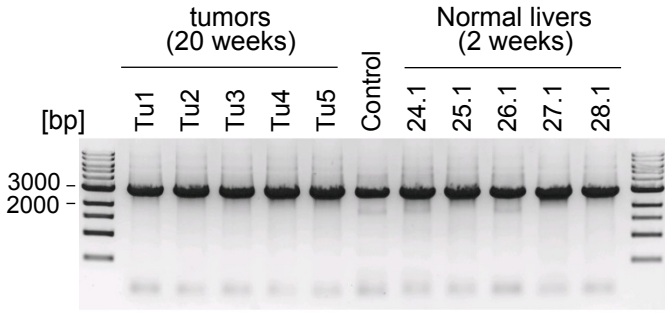
Supplementary Figure S6. Validation of NGS data by Sanger capillary sequencing of PCR-amplified sgRNA target regions. Data are shown for *Tet2* and *Pten* in Tu1, Tu2 and Tu3 as well as in healthy liver sample of a tumor-bearing mouse. PCR products of sgRNA target sites have been cloned into *E. coli* and 30 clones per target site and tumor were subjected to Sanger capillary sequencing. (A) Bar charts display percentages of wild type (wt) clones and clones with respective indels. (B) Graphical display shows alignment of the sequence traces with wt sequence using SnapGene® 2.4.3. Sanger sequencing confirmed the results from NGS, which also identified in some cases additional lower-frequency indels (see Figure S8), as expected.



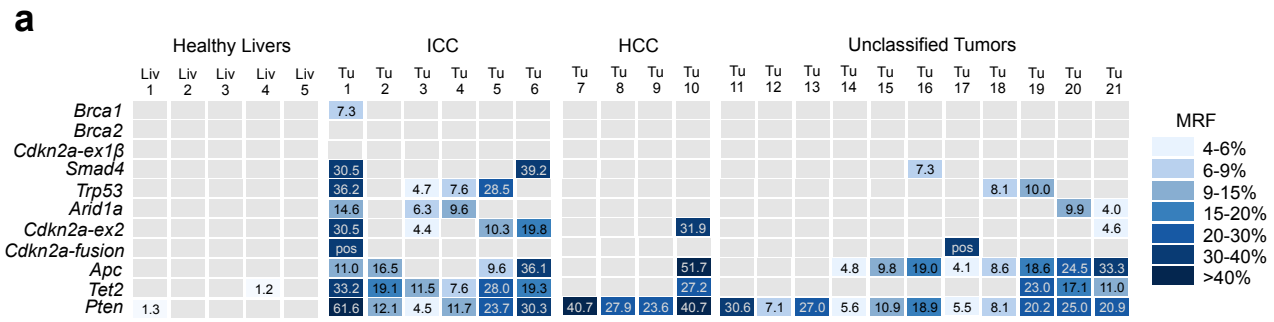
Supplementary Figure S7. Schematic view of indels induced by multiplexed CRISPR/Cas9 mutagenesis in mice. The schemes display all mutant target site sequences with frequencies above 1% in Tu1-Tu3 (the data relate to experiments described in figures 1 and 2; cancers induced by ten sgRNA multiplexing). For each mutation the altered sequence is marked in red. Note that mutations in *Brca1* and *Brca2* are either in-frame or have very low MRFs that do not exceed background mutation frequencies in healthy livers, except the 11del mutation in *Brca1* (see also Fig. S8 and Figure 2).

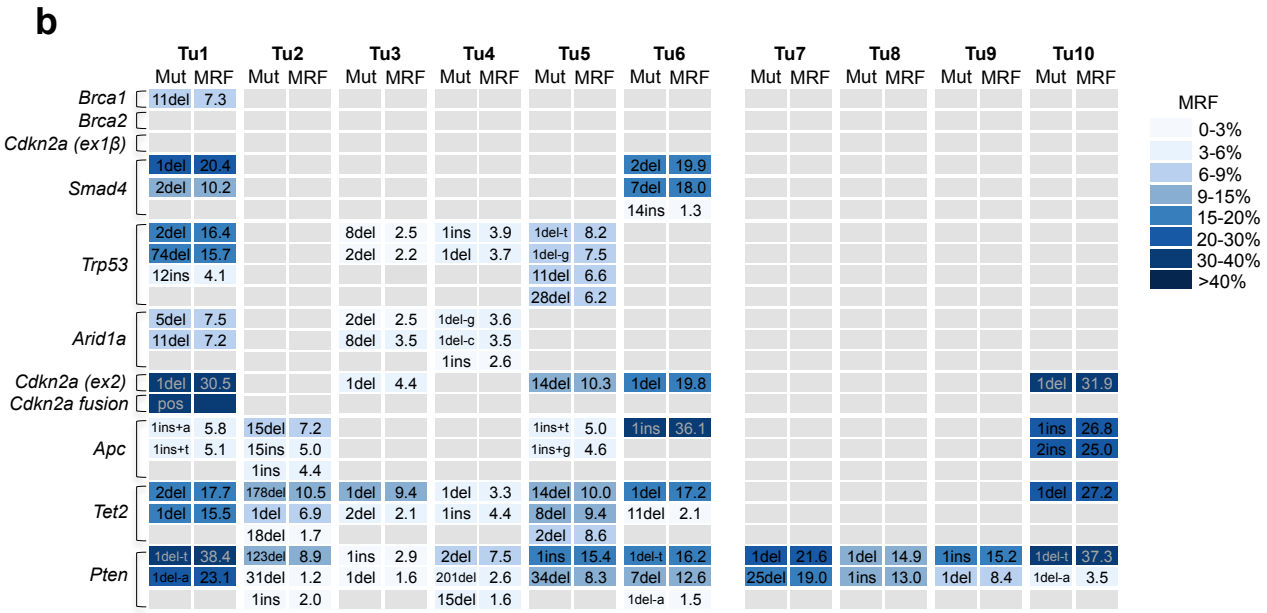


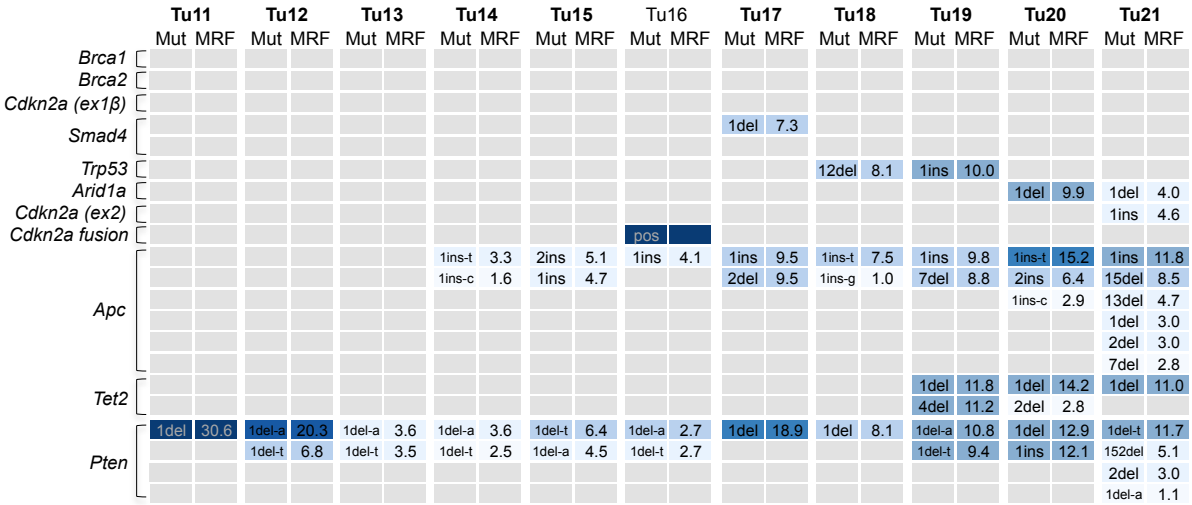
Supplementary Figure S8. Screening for large deletions at the *Cdkn2a* locus in tumors and healthy liver samples. Analysis of the *Cdkn2a-ex2* target site using long-range PCR (2900bp) was performed to screen for large deletions that could span a region beyond the standard PCR-amplified and sequenced 400-500 base pairs around target sites. No large deletion at this site could be found in any of the analysed CRISPR/Cas9-induced tumor samples or normal livers.



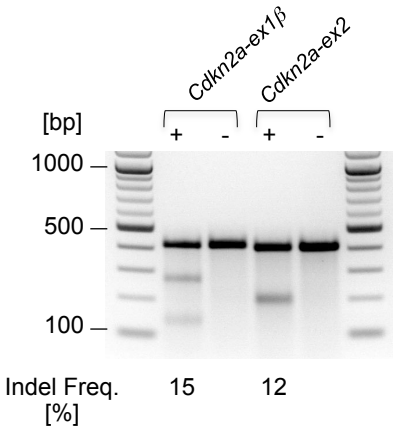
Supplementary Figure S9. Description of all frame shift causing indels in liver tumors of mice injected with *hSpCas9* and ten sgRNAs. (A) Cumulative indel frequencies above 4% are displayed for individual target sites (as also shown in figure 2). In (B) all different mutations at individual target sites are displayed separately and mutant read frequencies (MRF) are shown individually.



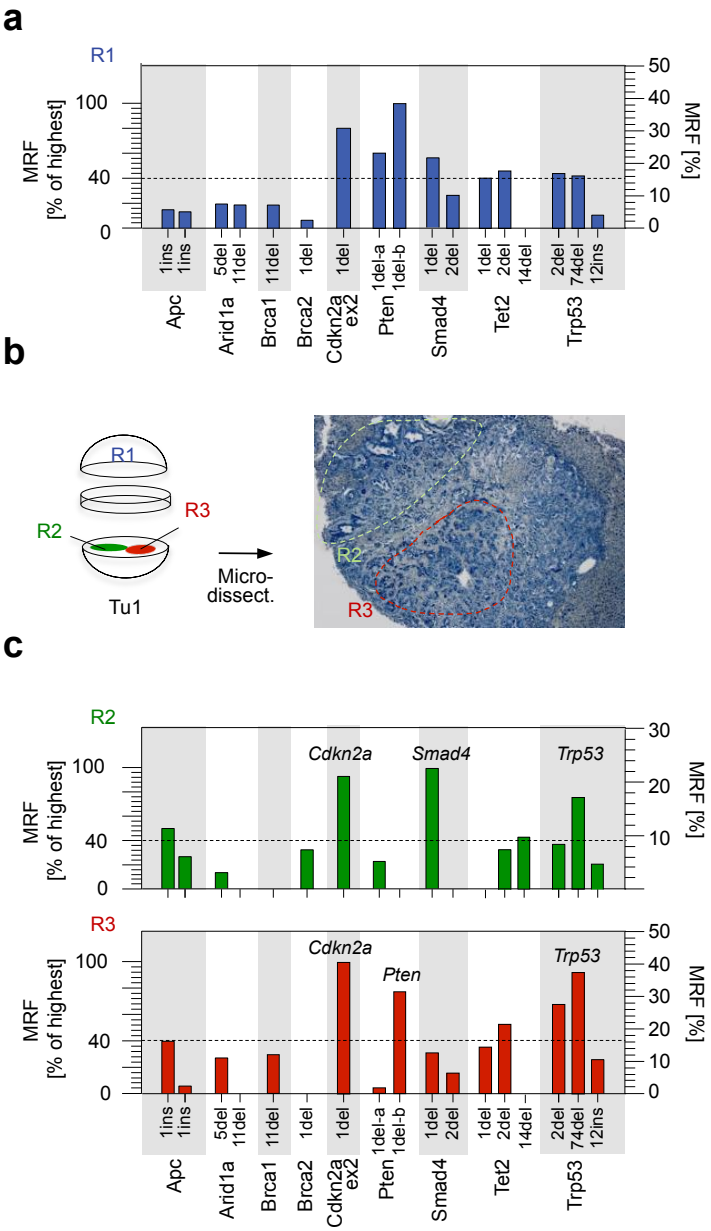




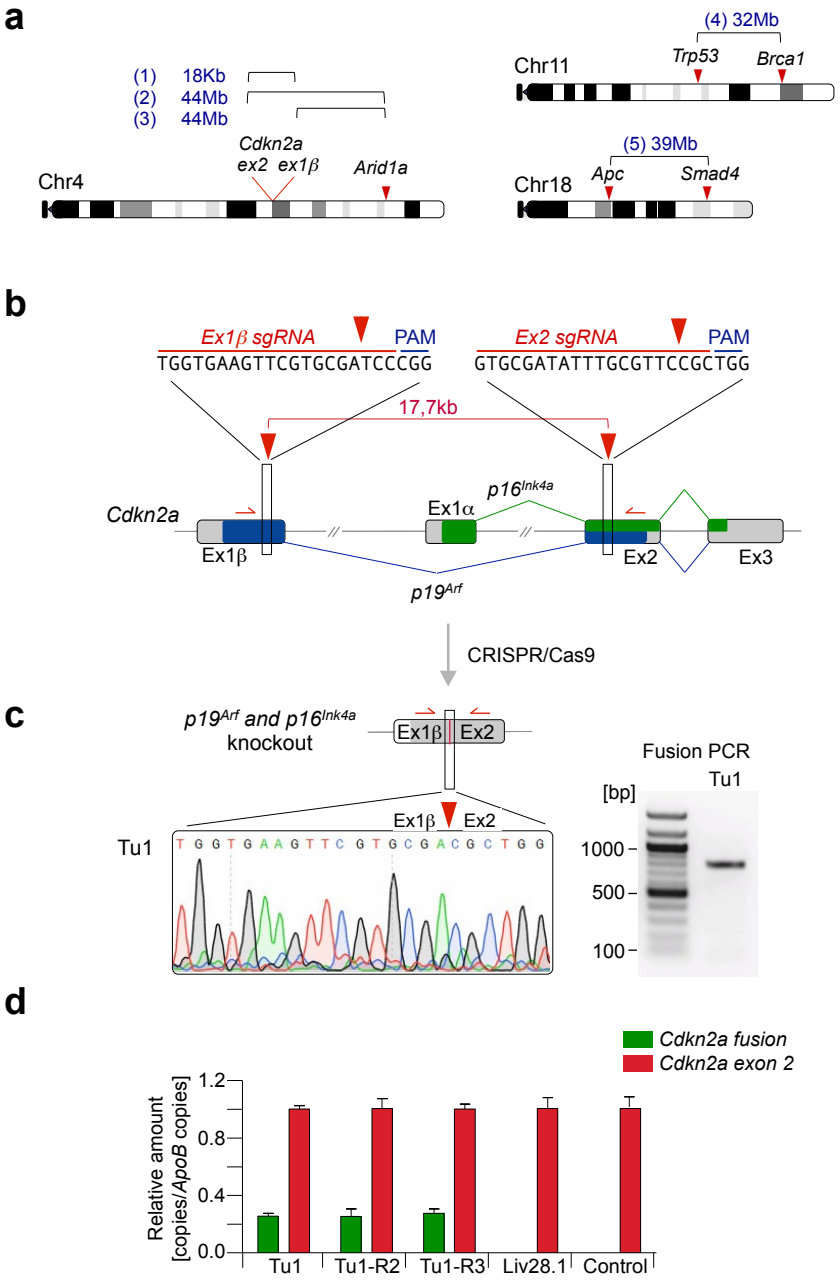
Supplementary Figure S10. Comparison of sgRNA targeting efficiencies at *Cdkn2a-ex1β* and *Cdkn2a-ex2*. Efficiencies of the two sgRNAs targeting the *Cdkn2a* locus were assessed using Surveyor assays in murine pancreatic cancer cell lines upon transient transfection of *CRISPR-SB* plasmid as described in the Methods section. The mouse pancreatic cancer cell line *4072-PPT* was chosen because it had - in contrast to most other available mouse cancer cell lines - an intact *Cdkn2a* locus. (+) Cell line transfected with the sgRNA as indicated above; (-) cell line transfected with the non-targeting sgRNA served as a negative control. The results show that mutation of $p19^{Arf}$ (using *Cdkn2a-ex1β* sgRNA) or induction of the $p19^{Arf}/p16^{Ink4a}$ double-mutant (using *Cdkn2a-ex2* sgRNA) are equally efficient.



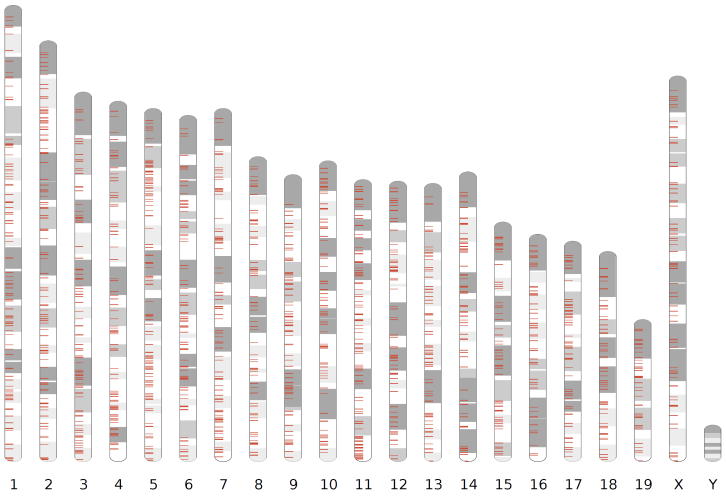
Supplementary Figure S11. Analysis of intratumor heterogeneity in a CRISPR/Cas9-induced cancer. (A) Mutant read frequencies (MRF) of frame shift causing indels detected at target sites in region-1 (R1, blue) of Tu1. (B) Regions of Tu1 used for heterogeneity analysis. R1 contains a large proportion of the tumor, R2 and R3 were microdissected. (C) MRF of frame shift causing indels for R2/ R3.



Supplementary Figure S12. Intrachromosomal fusions induced by combinatorial sgRNA targeting in mice. (A) Scheme of all chromosomes with two or more CRISPR/Cas9 target sites in the ten sgRNA multiplexing approach. All targeted genes are listed in Figure 1. Out of the 105 possible deletions in 21 tumors we found evidence for fusion products between the *Cdkn2a-ex1β* and *Cdkn2a-ex2* sgRNA target sites in three cancers (see also Figure 2). In all three cases the resulting deletion of approximately 18kb led to inactivation of both *p16*^{Ink4a} and *p19*^{Arf}. (B) Scheme of *Cdkn2a* dual targeting using 2 sgRNAs. (C) Example of the PCR-amplified fusion product and its sequence trace resulting from deletion of the 17.7kb fragment in Tu1. Red arrows indicate primers. (D) Relative copy numbers of the fusion allele in comparison to other *Cdkn2a-ex2* alleles (including wild type and alleles with small indels) in Tu1, as determined by qPCR. Error bars, SEM from triplicate determinations.

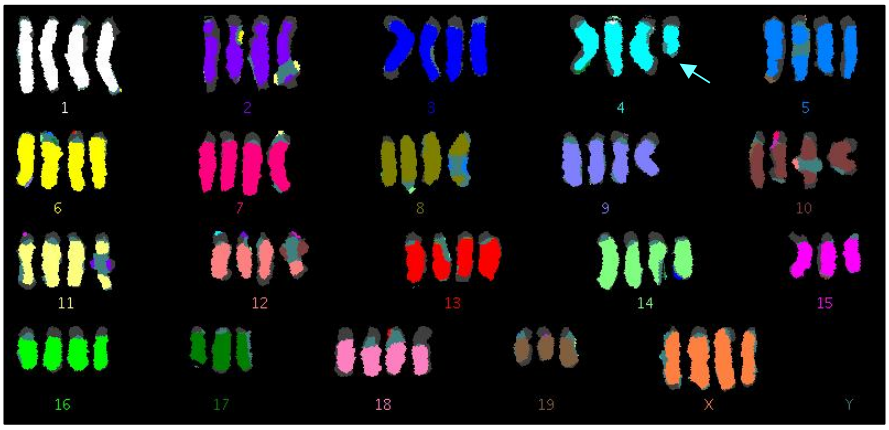


Supplementary Figure S13. Analysis of off-target effects resulting in intrachromosomal deletions. Array CGH was performed on six different tumors and analysed for aberrations (see Methods section). Intrachromosomal deletions were screened for 18 on-target and 1550 off-target sites which are distributed throughout all chromosomes.

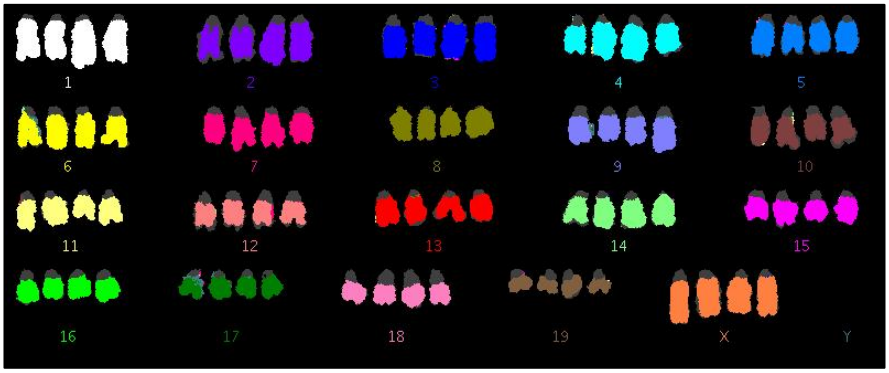


Supplementary Figure S14. M-FISH analysis of tumor cell lines. To analyze potential interchromosomal rearrangements in liver tumor cell lines derived from mice injected with *hSpCas9* and sgRNAs, multicolor in situ hybridization (M-FISH) was performed.
M-FISH analysis revealed a tetraploid stable chromosome set for both analyzed cell lines (a, cell line Tu23; b, cell line Tu24). The composite karyotype for cell line Tu23 is 77,XXXX,Del(4),-15,17,-19 (a) and for cell line Tu24 80,XXXX (b). For Tu23, the CRISPR/Cas9 induced large deletion on chromosome 4 is clearly visible in one out of four chromosomes (a, arrow). Further analysis of additional metaphases of cell line Tu23 confirms three different states of chromosome 4 as already identified by PCR: 1. without any visible alterations (wt), 2. with the CRISPR/Cas9 induced 17Mb deletion (small del; *Arid1a-Errfi1* fusion) and 3. with the CRISPR/Cas9 induced 62Mb deletion (large del; *Cdkn2b-Errfi1* fusion) (c).

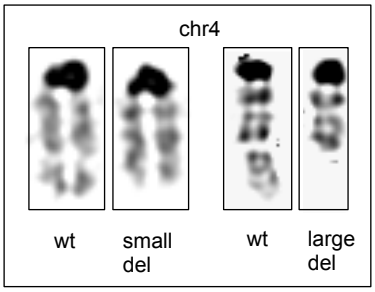
a



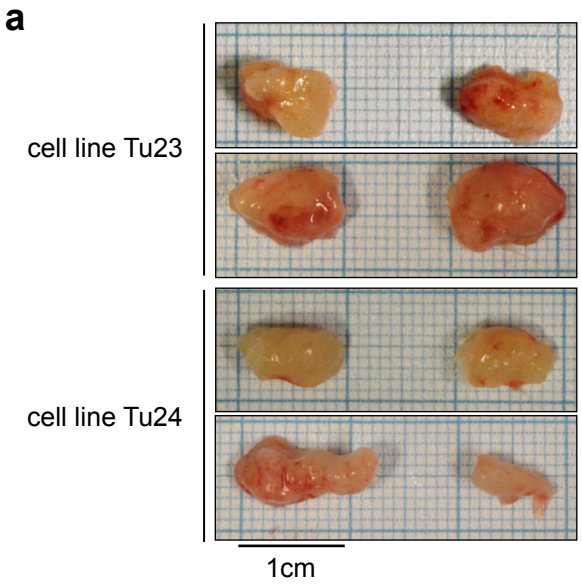
b

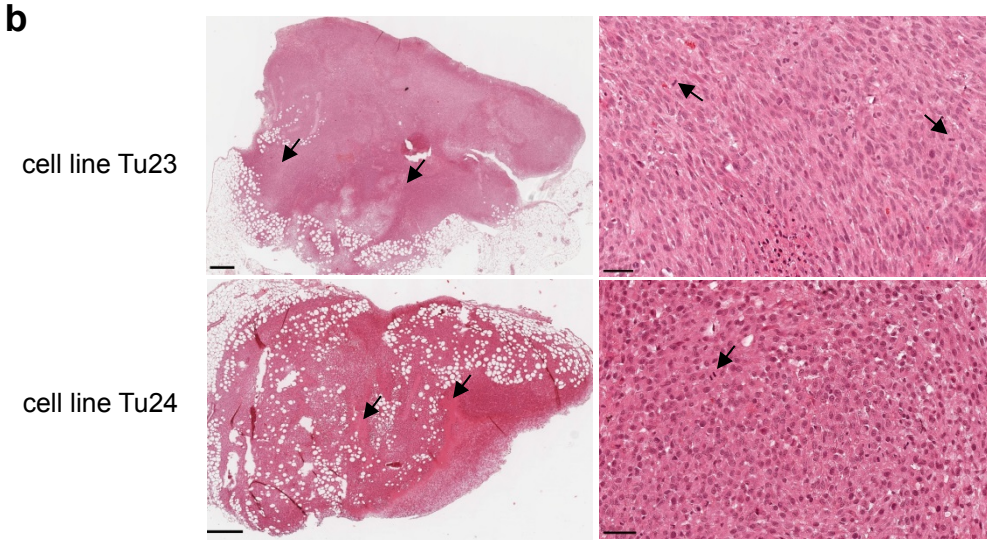


c

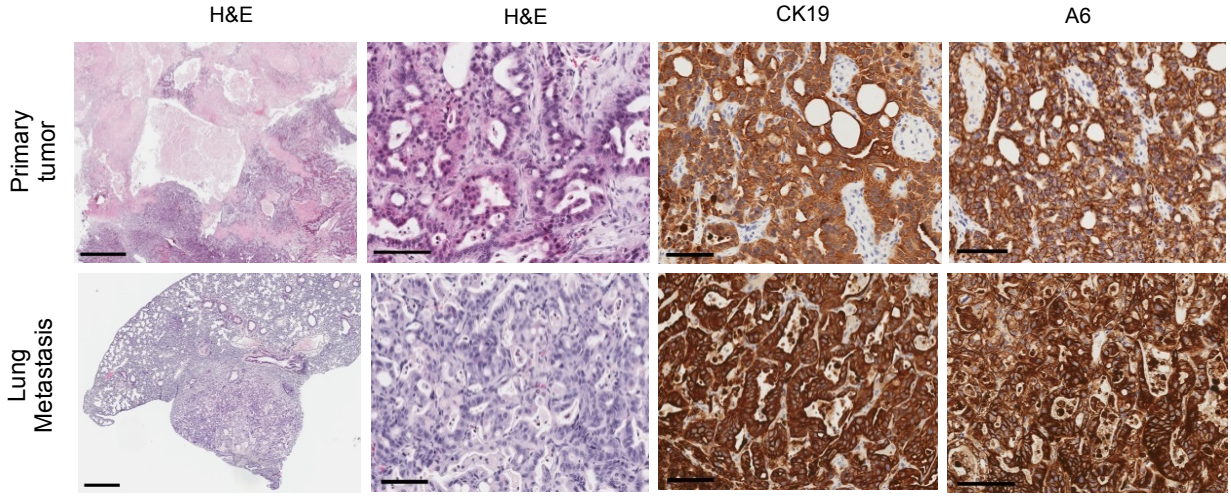


Supplementary Figure S15. Subcutaneous implantation of tumor cell lines. Cell lines derived from two primary intrahepatic cholangiocarcinomas (Tu23 and Tu24; $5x10^5$ cells/implantation) were implanted subcutaneously into the right and left flanks of NOD scid gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice. (A) All mice developed tumors (n=4 per cell line) up to 1cm in diameter within two weeks after implantation. (B) Representative microscopic H&E images of the allograft tumors. The neoplasias show a solid growth pattern with infiltration of the adjacent adipose tissue (left panel) and multifocal necroses (arrows, left panel). The tumor cells are elongated (cell line Tu23, upper left image) or polygonal (cell line 24, lower left image) with a high number of mitoses (arrows, right panel). Bars, 500µm left panels, 50µm right panels.





Supplementary Figure S16. Microscopic images of Tu24 and a corresponding lung metastasis. Moderately to poorly differentiated intrahepatic cholangiocarcinoma (ICC) with extensive central necrosis (upper panels). Tumor cells intensely express cytokeratin 19 (CK19) and A6 (oval cell surface antigen). Subpleural metastasis of the moderately to poorly differentiated cholangiocarcinoma (lower panels). The metastasis shows a tubular growth pattern with CK19 and A6 positivity, as seen in the primary tumor. Bars, 100µm except upper left: 500µm and lower left: 1mm.



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Supplementary Tables

Supplementary Table 1. Literature-based analysis of tumor suppressor gene alterations in human liver cancers. A systematic literature-based analysis of tumor suppressor gene alterations in human intrahepatic cholangiocarcinoma (ICC) (A) and hepatocellular carcinoma (HCC) (B) was performed. The tables show alterations found in the ten tumor suppressor genes targeted with CRISPR/Cas9 in the mouse liver using hydrodynamic tail vein injection (HTVI). We used information about mutated genes (MUT) found in recent whole genome/whole exome sequencing studies and about genes being described to be located in commonly deleted regions (DEL) or to be silenced by promoter methylation (PM). In addition, studies analyzing expression of the respective proteins in ICC/HCC (LOSS; loss of expression) were taken into account. For APC, WNT pathway activation (WNT) in ICC was studied.

A

Gene	Type	Range [%]	References
APC	PM	26.6 - 47.2	(*1, 2*)
	WNT		
	- reduced membranous expression of β-catenin	82	(*3*)
	- aberrant nuclear expression of β-catenin	15	(*3*)
ARID1A	MUT	9 - 35.5	(*4-7*)
BRCA1	MUT	0 - 3.6*	(*4-7*)
BRCA2			
CDKN2A	MUT	0 - 5.6	(*4-7*)
	PM	15.7 - 83.0	(*1, 2, 8, 9*)
	LOSS	35.7	(*10*)
	DEL	18.0	(*11*)
PTEN	MUT	0 - 10.7	(*4-7*)
	PM	35.3	(*8*)
SMAD4	MUT	0 - 16.7	(*4-7*)
	LOSS	45.2	(*10*)
TET2			
TP53	MUT	6 - 44.4	(*4-7*)
	PM	61.1	(*12*)

* Only 1 B*rca1* mutation in 1 patient

Legend

LOSS	loss of expression
DEL	large deletions
MUT	mutations found in exome sequencing studies
PM	promoter methylation
WNT	Wnt pathway activation

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B

Gene	Type	Range [%]	References
APC	MUT DEL LOSS	0 – 3.0 0 – 0.5 53.0	(*13-20*) (*13-15, 18*) (*21*)
ARID1A	MUT DEL	2.0 – 16.0 0 – 1.4	(*13-20*) (*13-15, 18*)
BRCA1	MUT DEL	0 – 2.0 0 – 0.3	(*13-20*) (*13-15, 18*)
BRCA2	MUT DEL	0 - 5.7 0 – 0.8	(*13-20*) (*13-15, 18*)
CDKN2A	MUT DEL PM LOSS	0 - 2.9 4.0 - 6.4 17.6 72.2	(*13-20*) (*13-15, 18*) (*22*) (*22*)
PTEN	MUT DEL PM LOSS	0 – 4.0 4.0 16.1 40.9 – 57.1	(*13-20*) (*13-15, 18*) (*23*) (*24, 25*)
SMAD4	MUT DEL	0 - 0.9 0 - 0.8	(*13-20*) (*13-15, 18*)
TET2	MUT DEL	0 – 2.0 0 - 0.8	(*13-20*) (*13-15, 18*)
TP53	MUT DEL	18 - 51.8 0 - 3.0	(*13-20*) (*13-15, 18*)

Legend

LOSS	loss of expression
DEL	large deletions
MUT	mutations found in exome sequencing studies
PM	promoter methylation

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Supplementary Table 2. Indel analysis in healthy livers and liver tumors derived from mice injected with *hSpCas9* and ten sgRNAs. *CRISPR-SB* vectors expressing *hSpCas9* and ten sgRNAs (targeting ten different tumor suppressor genes) were delivered into livers of *Alb-Cre;Kras*$^{LSL-G12D/+}$ mice using hydrodynamic tail vein injection (HTVI). 20 to 30 weeks post HTVI, mice developed liver tumors (ICCs and HCCs). DNA was isolated from healthy livers of tumor-bearing mice (n=5; Liver1-Liver5) and liver tumors (n=21; Tu1-Tu21) (see Figure 2). CRISPR/Cas9 target regions were amplified and sequenced using amplicon-based next-generation sequencing (see Methods section). The table below shows all indels derived from healthy livers of tumor-bearing mice and liver tumors detected with a mutant read frequency (MRF) of 0.2% or higher. This cut-off was set to account for technical sequencing errors.

Liver 1				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	0.23%	68del	11: 101549019	-CGG...ATA
Apc	0.20%	27del	18: 34261044	-AGT...TGA
Apc	0.21%	1del	18: 34261059	-T
Apc	0.25%	1ins	18: 34261059	+A
Apc	0.55%	1ins	18: 34261059	+T
Trp53	0.22%	11del	11: 69587432	-GCC...GAA
Trp53	0.22%	3del	11: 69587440	-GAA
Arid1a	0.36%	1del	4: 133723009	-C
Tet2	0.37%	11del	3: 133485654	-TGG...TGT
Tet2	0.20%	2del	3: 133485657	-AT
Tet2	0.73%	1del	3: 133485658	-T
Pten	0.50%	14del	19: 32758455	-CAT...TCG
Pten	1.30%	1del	19: 32758456	-A
Pten	0.27%	1del	19: 32758457	-T
Liver 2				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Cdkn2a(Ex2)	0.21%	7del	4: 89276737	-GCGGAAC
Pten	0.67%	23del	19: 32758440	-AGA...AAG
Pten	0.83%	1ins	19: 32758457	+T
Liver 3				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Pten	0.48%	11del	19: 32758457	-TCA...ATC
Pten	0.31%	2del	19: 32758458	-CA
Liver 4				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Smad4	0,95%	3del	18: 73675798	-GTG
Apc	0,29%	6del	18: 34261053	-AGCAAG
Apc	0,27%	1ins	18: 34261058	+G
Apc	0,37%	1ins	18: 34261059	+T
Trp53	0,68%	1del	11: 69587439	-T
Trp53	0,52%	28del	11: 69587440	-GAA...CTG
Trp53	0,66%	1del	11: 69587440	-G
Trp53	0,66%	11del	11: 69587440	-GAA...CCG
Arid1a	0,54%	3del	4: 133723006	-CGG
Tet2	0,94%	8del	3: 133485653	-CTGGATAT
Tet2	0,40%	2del	3: 133485657	-AT
Tet2	1,17%	14del	3: 133485658	-TAT...ACT
Pten	0,85%	1ins	19: 32758457	+T
Liver 5				
Gene	**MRF**	**Mut**	**Position**	**Indel**
-	0.0%	-	-	-

Tu1				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	7.29%	11del	11: 101549007	-GAC...AGA
Brca1	7.28%	9del	11: 101549019	-CGGACACTC
Brca2	5.31%	3del	5: 150529485	-CCT
Brca2	2.52%	1del	5: 150529488	-C
Cdkn2a(Ex1β)	0.51%	3del	4: 89294435	-ATC
Cdkn2a(Ex1β)	0.58%	15del	4: 89294436	-TCG...CAC
Cdkn2a(Ex1β)	9.88%	3del	4: 89294436	-TCG
Apc	5.13%	1ins	18: 34261059	+T
Apc	5.84%	1ins	18: 34261059	+A
Smad4	10.17%	2del	18: 73675798	-GT
Smad4	20.36%	1del	18: 73675801	-A
Cdkn2a(Ex2)	30.53%	1del	4: 89276739	-G
Trp53	0.46%	2del	11: 69587437	-AG
Trp53	16.38%	2del	11: 69587438	-GT
Trp53	0.37%	2del	11: 69587439	-TG
Trp53	0.20%	2del	11: 69587440	-GA
Trp53	0.34%	1del	11: 69587440	-G
Trp53	0.21%	2ins	11: 69587441	+CC
Trp53	0.34%	1ins	11: 69587441	+C
Trp53	4.09%	12ins	11: 69587441	+CCA...CCA
Trp53	15.75%	74del	11: 69587441	-AAG...GCC
Trp53	0.26%	1ins	11: 69587446	+A
Arid1a	7.16%	11del	4: 133722998	-CCA...CGG
Arid1a	7.48%	5del	4: 133723004	-TACGG
Arid1a	8.35%	3del	4: 133723008	-GCT
Tet2	17.69%	2del	3: 133485657	-AT
Tet2	15.47%	1del	3: 133485658	-T
Pten	23.13%	1del	19: 32758456	-A
Pten	38.43%	1del	19: 32758457	-T
Tu1 - Region2				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca2	12.50%	3del	5: 150529485	-CCT
Brca2	7.57%	1del	5: 150529488	-C
Apc	5.88%	1ins	18: 34261059	+T
Apc	11.11%	1ins	18: 34261059	+A
Smad4	21.56%	1del	18: 73675801	-A
Cdkn2a(Ex2)	20.46%	1del	4: 89276739	-G
Trp53	0.20%	2del	11: 69587437	-AG
Trp53	8.23%	2del	11: 69587438	-GT
Trp53	0.21%	11ins	11: 69587441	+CCA...TCC
Trp53	0.24%	12ins	11: 69587441	+CCA...CCC
Trp53	0.25%	2ins	11: 69587441	+CC
Trp53	0.38%	1ins	11: 69587441	+C
Trp53	5.10%	12ins	11: 69587441	+CCA...CCA
Trp53	16.65%	74del	11: 69587441	-AAG...GCC
Trp53	0.21%	1ins	11: 69587446	+A
Arid1a	2.97%	5del	4: 133723004	-TACGG
Tet2	7.43%	2del	3: 133485657	-AT
Tet2	9.48%	14del	3: 133485658	-TAT...ACT
Pten	4.86%	1del	19: 32758456	-A

Tu1 - Region3				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	11.93%	11del	11: 101549007	-GAC...AGA
Brca1	6.40%	9del	11: 101549019	-CGGACACTC
Cdkn2a(Ex1β)	1.00%	3del	4: 89294435	-ATC
Cdkn2a(Ex1β)	19.48%	3del	4: 89294436	-TCG
Apc	2.34%	1ins	18: 34261059	+T
Apc	15.99%	1ins	18: 34261059	+A
Smad4	6.21%	2del	18: 73675798	-GT
Smad4	12.30%	1del	18: 73675801	-A
Cdkn2a(Ex2)	40.58%	1del	4: 89276739	-G
Trp53	0.75%	2del	11: 69587437	-AG
Trp53	27.32%	2del	11: 69587438	-GT
Trp53	0.72%	2del	11: 69587439	-TG
Trp53	0.24%	74del	11: 69587440	-GAA...TGC
Trp53	0.33%	12ins	11: 69587440	+TCC...TCC
Trp53	0.32%	2ins	11: 69587441	+CC
Trp53	0.34%	11ins	11: 69587441	+CCA...TCC
Trp53	0.37%	12ins	11: 69587441	+CCA...CCC
Trp53	0.66%	1ins	11: 69587441	+C
Trp53	10.09%	12ins	11: 69587441	+CCA...CCA
Trp53	37.12%	74del	11: 69587441	-AAG...GCC
Trp53	0.45%	1ins	11: 69587446	+A
Arid1a	10.95%	5del	4: 133723004	-TACGG
Arid1a	16.93%	3del	4: 133723008	-GCT
Tet2	21.18%	2del	3: 133485657	-AT
Tet2	14.29%	1del	3: 133485658	-T
Pten	1.72%	1del	19: 32758456	-A
Pten	30.86%	1del	19: 32758457	-T

Tu2				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Apc	7.18%	15del	18: 34261056	-AAG...TGA
Apc	4.99%	15ins	18: 34261057	+CTG...AGT
Apc	4.35%	1ins	18: 34261059	+T
Cdkn2a(Ex2)	0.29%	7del	4: 89276737	-GCGGAAC
Cdkn2a(Ex2)	0.28%	1del	4: 89276739	-G
Arid1a	0.41%	1del	4: 133723007	-G
Arid1a	1.29%	2del	4: 133723007	-GG
Tet2	10.53%	178del	3: 133485574	-CAT...TTC
Tet2	1.74%	18del	3: 133485647	-TGC...TGT
Tet2	6.87%	1del	3: 133485658	-T
Pten	8.87%	123del	19: 32758338	-AGC...CAA
Pten	1.19%	31del	19: 32758440	-AGA...GTT
Pten	2.00%	1ins	19: 32758457	+T

Tu3				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	1.43%	1del	11: 101549018	-T
Brca2	1.64%	10del	5: 150529476	-ACC...AGC
Cdkn2a(Ex1β)	2.99%	9del	4: 89294435	-ATCGCACGA
Cdkn2a(Ex1β)	1.19%	3del	4: 89294436	-TCG
Apc	1.65%	6del	18: 34261051	-AAAGCA
Apc	1.52%	1del	18: 34261058	-G
Apc	0.23%	1ins	18: 34261059	+T
Apc	1.56%	1ins	18: 34261059	+A
Smad4	5.59%	6del	18: 73675795	-ATAGTG
Smad4	3.33%	3del	18: 73675798	-GTG
Cdkn2a(Ex2)	4.39%	1del	4: 89276739	-G
Trp53	2.23%	2del	11: 69587438	-GT
Trp53	2.46%	8del	11: 69587439	-TGAAGCCC
Trp53	0.85%	5ins	11: 69587440	+TAGAA
Trp53	0.64%	15ins	11: 69587443	+CTT...AAT
Arid1a	1.89%	8del	4: 133722998	-CCACCATA
Arid1a	1.91%	8del	4: 133723001	-CCATACGG
Arid1a	2.51%	2del	4: 133723009	-CT
Tet2	9.35%	2del	3: 133485657	-AT
Tet2	2.15%	1del	3: 133485658	-T
Pten	0.38%	2del	19: 32758457	-TC
Pten	1.59%	1del	19: 32758457	-T
Pten	2.94%	1ins	19: 32758457	+T
Pten	3.15%	3del	19: 32758458	-CAA
Tu4				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Cdkn2a(Ex1β)	3.04%	1ins	4: 89294436	+T
Apc	1.84%	3del	18: 34261058	-GTT
Trp53	3.73%	1del	11: 69587440	-G
Trp53	3.87%	1ins	11: 69587440	+G
Arid1a	3.56%	1del	4: 133723007	-G
Arid1a	2.61%	1ins	4: 133723009	+A
Arid1a	3.45%	1del	4: 133723009	-C
Tet2	4.37%	1ins	3: 133485657	+A
Tet2	3.27%	1del	3: 133485658	-T
Pten	2.62%	201del	19: 32758270	-AGC...GTT
Pten	1.62%	15del	19: 32758447	-ACA...AAA
Pten	0.25%	2del	19: 32758456	-AT
Pten	7.49%	2del	19: 32758457	-TC
Tu5				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Apc	4.08%	6del	18: 34261053	-AGCAAG
Apc	4.59%	1ins	18: 34261058	+G
Apc	5.01%	1ins	18: 34261059	+T
Smad4	5.67%	3del	18: 73675798	-GTG
Cdkn2a(Ex2)	10.28%	14del	4: 89276738	-CGG...TAT
Trp53	8.23%	1del	11: 69587439	-T
Trp53	6.16%	28del	11: 69587440	-GAA...CTG
Trp53	6.62%	11del	11: 69587440	-GAA...CCG
Trp53	7.53%	1del	11: 69587440	-G
Arid1a	7.78%	3del	4: 133723006	-CGG
Tet2	9.42%	8del	3: 133485653	-CTGGATAT
Tet2	8.63%	2del	3: 133485657	-AT
Tet2	9.96%	14del	3: 133485658	-TAT...ACT
Pten	8.28%	34del	19: 32758439	-CAG...TAG
Pten	0.43%	1ins	19: 32758456	+C
Pten	15.40%	1ins	19: 32758457	+T

Tu6				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Smad4	1.33	14del	18: 73675797	-AGT...TTC
Smad4	17.96	7del	18: 73675800	-GATATGG
Smad4	19.93	2del	18: 73675798	-GT
Arid1a	19.89	6del	4: 133723007	-GGCTGA
Arid1a	20.98	3del	4: 133723006	-CGG
Cdkn2a(Ex2)	19.79	1del	4: 89276739	-G
Apc	36.13	1ins	18: 34261059	+T
Tet2	0.22	2del	3:133485657	-AT
Tet2	2.10	11del	3:133485654	-TGG...TGT
Tet2	17.22	1del	3:133485658	-T
Pten	0.71	1ins	19: 32758457	+C
Pten	1.45	1del	19: 32758456	-A
Pten	12.61	7del	19: 32758456	-ATCAAAG
Pten	16.21	1del	19: 32758457	-T
Tu7				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Pten	19.04	25del	19: 32758453	-ATC...GAA
Pten	21.61	1del	19: 32758456	-A
Tu8				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	0.74	59ins	11: 101549027	+TTG...TAG
Pten	12.98	1ins	19: 32758457	+T
Pten	14.93	1del	19: 32758456	-A
Tu9				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Pten	0.40	1ins	19: 32758457	+A
Pten	0.51	1ins	19: 32758457	+C
Pten	8.37	1del	19: 32758457	-T
Pten	15.19	1ins	19: 32758456	+A
Tu10				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Cdkn2a(Ex2)	31.86	1del	4: 89276739	-G
Apc	24.96	2ins	18: 34261059	+TT
Apc	26.78	1ins	18: 34261058	+G
Tet2	27.18	1del	3:133485658	-T
Pten	3.45	1del	19: 32758456	-A
Pten	37.25	1del	19: 32758457	-T
Tu11				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Apc	0.24	1ins	18: 34261059	+T
Apc	0.24	1ins	18: 34261059	+A
Pten	30.55	1del	19: 32758456	-A
Tu12				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Cdkn2a(Ex1β)	1.73	1ins	4: 89294435	+A
Pten	3.47	1del	19: 32758457	-T
Pten	3.59	1del	19: 32758456	-A
Tu13				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Pten	6.75	1del	19: 32758457	-T
Pten	20.25	1del	19: 32758456	-A
Tu14				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca1	1.52	3del	11: 101549016	-GAT
Cdkn2a(Ex1β)	1.93	6del	4: 89294430	-CCGGGA
Arid1a	2.19	3del	4: 133723006	-CGG
Cdkn2a(Ex2)	1.59	1del	4: 89276739	-G
Apc	1.56	1ins	18: 34261058	+G
Apc	1.60	3del	18: 34261058	-GTT
Apc	3.25	1ins	18: 34261059	+T
Tet2	0.21	1del	3: 133485658	-T
Pten	2.47	1del	19: 32758456	-A
Pten	3.13	1del	19: 32758457	-T

Tu15				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Apc	4.70	1ins	18: 34261059	+T
Apc	5.10	2ins	18: 34261059	+TT
Pten	4.46	1del	19: 32758456	-A
Pten	6.44	1del	19: 32758457	-T
Tu16				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Smad4	7.33	1del	18: 73675801	-A
Apc	9.47	1ins	18: 34261056	+A
Apc	9.54	2del	18: 34261059	-TT
Pten	18.87	1del	19: 32758456	-A
Tu17				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Smad4	0.32	2del	18: 73675798	-GT
Smad4	0.36	7del	18: 73675800	-GATATGG
Cdkn2a(Ex2)	0.20	1del	4: 89276739	-G
Apc	4.14	1ins	18: 34261059	+T
Tet2	1.77	1ins	3:133485659	+A
Tet2	2.04	1del	3:133485658	-T
Pten	2.72	1del	19: 32758456	-A
Pten	2.74	1del	19: 32758457	-T
Tu18				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Brca2	0.27	1del	5: 150529488	-C
p53	8.13	12del	11: 69587427	-TGA...AAG
Apc	1.04	1ins	18: 34261061	+G
Apc	7.50	1ins	18: 34261059	+T
Pten	0.27	22del	19: 32758448	-CAG...CGT
Pten	0.28	1del	19: 32758457	-T
Pten	8.12	1del	19: 32758456	-A
Tu19				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Trp53	9.97	1ins	11: 69587440	+A
Apc	8.83	4del	18: 34261052	-AAGC
Apc	9.82	1ins	18: 34261059	+T
Tet2	11.20	4del	3: 133485654	-TGGA
Tet2	11.78	1del	3: 133485658	-T
Pten	0.40	1ins	19: 32758456	+A
Pten	9.40	1del	19: 32758457	-T
Pten	10.84	1del	19: 32758456	-A
Tu20				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Cdkn2a(Ex1β)	3.55	1ins	4: 89294436	+T
Arid1a	9.85	1del	4: 133723007	-G
Apc	2.92	1ins	18: 34261059	+C
Apc	6.38	2ins	18: 34261059	+TT
Apc	15.18	1ins	18: 34261059	+T
Tet2	2.89	2del	3:133485657	-AT
Tet2	14.21	1del	3:133485658	-T
Pten	12.06	1ins	19: 32758457	+T
Pten	12.93	1del	19: 32758456	-A

Tu21				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Smad4	3.22	1del	18: 73675801	-A
Arid1a	3.94	1del	4: 133723007	-G
Cdkn2a(Ex2)	4.59	1ins	4: 89276740	+A
Apc	2.75	7del	18: 34261052	-AAGCAAG
Apc	2.96	2del	18: 34261055	-CA
Apc	3.02	1del	18: 34261058	-G
Apc	3.54	1ins	18: 34261059	+T
Apc	3.86	1ins	18: 34261059	+A
Apc	4.05	1ins	18: 34261058	+G
Apc	4.66	13del	18: 34261045	-GTT...CAA
Apc	5.06	3del	18: 34261058	-GTT
Apc	8.49	15del	18: 34261056	-AAG...TGA
Tet2	10.96	1del	3:133485658	-T
Pten	0.94	35ins	19: 32758590	+AGA...GAC
Pten	1.14	1del	19: 32758456	-A
Pten	2.98	2del	19: 32758455	-CA
Pten	4.40	6del	19: 32758457	-TCAAAG
Pten	5.14	152del	19: 32758305	-CCA...TCA
Pten	11.65	1del	19: 32758457	-T

Supplementary Table 3. Overview of cohorts and tumor prevalence in CCl_4 treated wild type mice and Alb-Cre;$Kras^{LSL-G12D/+}$ upon 18sgRNA CRISPR/Cas9 mutagenesis. In the carbon tetrachloride (CCl_4) treated cohorts, wild type mice received *hSpCas9* only (control cohort) or *hSpCas9* and 18 sgRNAs (experimental cohort) by hydrodynamic tail vein injection (HTVI). Beginning two weeks after HTVI mice were treated nine times with a weekly intraperitoneal injection of 1µL/g body weight 10% CCl_4. Whereas no tumors were observed in the control cohort by magnetic resonance imaging (MRI) screening or at necropsy, 7 of 16 mice in the experimental cohort developed signs of illness between 30 to 60 weeks post HTVI. All of these mice had HCCs at necropsy. 35 tumors were collected and analyzed so far. In the *Alb-Cre;*$Kras^{LSL-G12D/+}$ cohorts, three out of three mice in the experimental cohorts developed signs of illness between 21 and 32 weeks post HTVI. Six tumors were collected and analyzed. None of the control animals developed ICCs or HCCs within this time span.

Cohort	Alive	Dead		
wild type & CCl_4	number of mice	number of mice	weeks post HTVI	necropsy
hSpCas9 only (control cohort)	4 (> 50 weeks)	4	20-30	no HCC/ ICC
hSpCas9 & 18 sgRNA (experimental cohort)	9 (> 60 weeks)	7	30-60	all 7 mice had HCCs (Tu28-Tu62)
Alb-Cre; $Kras^{LSL-G12D/+}$	number of mice	number of mice	weeks post HTVI	necropsy
hSpCas9 only (control cohort)	-	8	20-30	no HCC/ ICC
hSpCas9 & 18 sgRNA (experimental cohort)	-	3	21-32	all 3 mice had ICCs/ HCCs (Tu22-Tu27)

Supplementary Table 4. Indel analysis in primary tumors and metastases derived from one mouse injected with *hSpCas9* and 18 sgRNAs. *CRISPR-SB* vectors expressing *hSpCas9* and 18 sgRNAs (targeting 18 different tumor suppressor genes) were delivered into livers of *Alb-Cre;Kras*$^{LSL-G12D/+}$ mice using hydrodynamic tail vein injection (HTVI). While most liver tumors were detected 20 to 30 weeks post HTVI due to regular MRI screening and were thus small (1-3mm), one animal developed a large early onset ICC 20 weeks post HTVI (before start of MRI screening) with multiple metastases to lymph nodes, peritoneum and lungs (see Figure 4). DNA was isolated from different areas of the primary tumor (n=10) and from numerous metastases (n=9). Cell lines were generated from primary tumor (n=9) and metastases (n=9). CRISPR/Cas9 target sites in all samples (n=37) were amplified and sequenced using amplicon-based next-generation sequencing. Indel analysis revealed three independent primary tumors (Tu22, Tu23, Tu24), with the largest part of the tumor mass being formed by Tu24 (eight out of ten samples). The table below shows indels detected in the Tu22, Tu23 and Tu24 (for Tu24 two representative samples are shown) and corresponding cell lines (for Tu23 and Tu24). Indels with a mutant read frequency (MRF) of 0.2% or higher are shown. This cut-off takes technical sequencing errors into account.

Tu22				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Apc	6.8%	1ins	18:34261059	+T
Apc	3.9%	3del	18:34261058	-GTT
Apc	3.8%	4del	18:34261052	-AAGC
Arid1b	0.9%	16del	17:5040686	-CGG...GCA
Arid1b	24.0%	1del	17:5040687	-G
Cdkn2a(Ex2)	8.2%	1del	4:89276739	-G
Errfi1	0.6%	2del	4:150866429	-GT
Errfi1	5.1%	1ins	4:150866431	+G
Errfi1	6.9%	4del	4:150866427	-GCGT
Errfi1	10.0%	1del	4:150866431	-G
Igsf10	11.9%	1del	3:59336469	-C
Pten	19.4%	1del	19:32758457	-T
Pten	5.9%	1del	19:32758456	-A
Tet2	9.0%	1del	3:133485658	-T
Tet2	8.4%	1ins	3:133485658	+T
Tet2	7.8%	2del	3:133485657	-AT
Tet2	9.2%	2del	3:133485656	-GA
Trp53	8.2 %	1del	11: 69587440	-G
Tu23				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	31.4%	2del	17:5040686	-CG
Arid2	33.4%	1del	15: 9628729	-G
Igsf10	15.9%	1del	3:59336469	-C
Igsf10	17.4%	5del	3:59336465	-AACGC
Pten	15.7%	1ins	19:32758457	+T
Pten	27.8%	1del	19:32758456	-A
Pten	20.1%	1del	19:32758457	-T
Tu23 cell line				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	52.4%	2del	17:5040686	-CG
Arid2	99.8%	1del	15: 9628729	-G
Igsf10	51.2%	1del	3:59336469	-C
Igsf10	48.7%	5del	3:59336465	-AACGC
Pten	50.5%	1ins	19:32758457	+T
Pten	30.1%	1del	19:32758456	-A
Pten	19.3%	1del	19:32758457	-T

Tu24.1				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	39.2%	1del	17:5040686	-C
Arid1b	46.6%	16del	17:5040686	-CGG...GCA
Arid2	76.2%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	73.1%	37del	4:89294408	-CGC...GAA
Errfi1	73.6%	2del	4:150866429	-GT
Irf2	75.3%	1ins	8:46806498	+T
Pten	41.2%	4del	19:32758457	-TCAA
Pten	43.6%	2ins	19:32758457	+TG
Trp53	43.1%	1del	11:69587440	-G
Tu24.1 cell line				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	47.2%	1del	17:5040686	-C
Arid1b	52.8%	16del	17:5040686	-CGG...GCA
Arid2	99.4%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	99.6%	37del	4:89294408	-CGC...GAA
Errfi1	99.8%	2del	4:150866429	-GT
Irf2	99.8%	1ins	8:46806498	+T
Pten	50.5%	4del	19:32758457	-TCAA
Pten	49.2%	2ins	19:32758457	+TG
Trp53	50.6%	1del	11:69587440	-G
Tu24.2				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	37.8%	1del	17:5040686	-C
Arid1b	43.7%	16del	17:5040686	-CGG...GCA
Arid2	75.1%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	77.4%	37del	4:89294408	-CGC...GAA
Errfi1	68.4%	2del	4:150866429	-GT
Irf2	69.4%	1ins	8:46806498	+T
Pten	37.6%	4del	19:32758457	-TCAA
Pten	46.0%	2ins	19:32758457	+TG
Trp53	41.2%	1del	11:69587440	-G
Tu24.2 cell line				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	46.0%	1del	17:5040686	-C
Arid1b	54.0%	16del	17:5040686	-CGG...GCA
Arid2	99.6%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	99.7%	37del	4:89294408	-CGC...GAA
Errfi1	99.7%	2del	4:150866429	-GT
Irf2	99.8%	1ins	8:46806498	+T
Pten	48.7%	4del	19:32758457	-TCAA
Pten	51.1%	2ins	19:32758457	+TG
Trp53	53.3%	1del	11:69587440	-G
Met24.1				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	35.8%	1del	17:5040686	-C
Arid1b	43.8%	16del	17:5040686	-CGG...GCA
Arid2	68.5%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	72.3%	37del	4:89294408	-CGC...GAA
Errfi1	64.3%	2del	4:150866429	-GT
Irf2	64.0%	1ins	8:46806498	+T
Pten	39.2%	4del	19:32758457	-TCAA
Pten	43.4%	2ins	19:32758457	+TG
Trp53	37.6%	1del	11:69587440	-G
Met24.1 cell line				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	44.9%	1del	17:5040686	-C
Arid1b	54.9%	16del	17:5040686	-CGG...GCA
Arid2	99.2%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	99.4%	37del	4:89294408	-CGC...GAA
Errfi1	99.0%	2del	4:150866429	-GT
Irf2	99.2%	1ins	8:46806498	+T
Pten	47.0%	4del	19:32758457	-TCAA
Pten	52.8%	2ins	19:32758457	+TG
Trp53	49.3%	1del	11:69587440	-G

Met24.2				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	34.5%	1del	17:5040686	-C
Arid1b	43.1%	16del	17:5040686	-CGG...GCA
Arid2	75.8%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	80.0%	37del	4:89294408	-CGC...GAA
Errfi1	66.1%	2del	4:150866429	-GT
Irf2	59.3%	1ins	8:46806498	+T
Pten	39.1%	4del	19:32758457	-TCAA
Pten	42.7%	2ins	19:32758457	+TG
Trp53	41.8%	1del	11:69587440	-G
Met24.2 cell line				
Gene	**MRF**	**Mut**	**Position**	**Indel**
Arid1b	45.7%	1del	17:5040686	-C
Arid1b	54.1%	16del	17:5040686	-CGG...GCA
Arid2	99.4%	3del	15: 9628729	-GCG
Cdkn2a(Ex1β)	99.9%	37del	4:89294408	-CGC...GAA
Errfi1	98.7%	2del	4:150866429	-GT
Irf2	99.3%	1ins	8:46806498	+T
Pten	45.6%	4del	19:32758457	-TCAA
Pten	54.3%	2ins	19:32758457	+TG
Trp53	51.6%	1del	11:69587440	-G

Supplementary Table 5. Mono- versus biallelic mutations at CRISPR/Cas9 target sites. Analysis of the allelic status of CRISPR/Cas9 target sites in cell lines derived from Tu23 and Tu24. Target sites were regarded as being altered 'homozygously' if no wild type (wt) reads for the respective amplicon emerged from amplicon-based next generation sequencing. In this case, both alleles harbored indels and/or large deletions (fusions). The same indel or large deletion at both alleles indicates either independent identical CRISPR/Cas9-induced mutations on each allele or loss of heterozygosity. Target sites were considered as being altered 'heterozygously' if 50% of reads for the respective amplicon harbored an indel or large deletion and 50% of reads were wt.

Target site	NGS and fusion PCR results	Heterozygous	Homozygous
Tu23			
Arid1b	50% indel - 50% wt	1	
Arid2	100% indel - no wt		1
Igsf10	50% indel - 50% indel		1
Pten	28% indel - 20% indel - 16% indel		1
Arid1a	fusion - wt	1	
Cdkn2b	fusion - no wt		1
Errfi1	fusion - fusion		1
Tu24			
Trp53	50% indel - 50% wt	1	
Irf2	100% indel - no wt		1
Errfi1	100% indel - no wt		1
Pten	50% indel - 50% indel		1
Arid1b	50% indel - 50% indel		1
Cdkn2a-Ex1β	fusion - indel		1
Cdkn2a-Ex2	fusion - no wt		1
	sum	**3**	**11**
	percent	**21%**	**79%**

Supplementary Table 6. Sequences of the 18 sgRNAs used to target tumor suppressor genes in the mouse liver by hydrodynamic tail vein injection (HTVI).

Gene	Transcript (Ensembl transcript ID)	CCDS	sgRNA sequence (PAM)	Exon
Apc	Apc-001 (ENSMUST00000079362)	CCDS29125	TCAGTTGTTAAAGCAAGTTG (AGG)	2
Arid1a	Arid1a-201 (ENSMUST00000105897)	CCDS38908	TTAGTCCCACCATACGGCTG (AGG)	2
Brca1	Brca1-001 (ENSMUST00000017290)	CCDS25474	AAATCTTAGAGTGTCCGATC (TGG)	2
Brca2	Brca2-201 (ENSMUST00000044620)	CCDS39411	TAGGACCGATAAGCCTCAAT (TGG)	3
Cdkn2a (ex1β)	Cdkn2a-201 (ENSMUST00000107131)	CCDS18350	TGGTGAAGTTCGTGCGATCC (CGG)	1
Cdkn2a (ex2)	Cdkn2a-001 (ENSMUST00000060501) Cdkn2a-201 (ENSMUST00000107131)	CCDS38812 CCDS18350	GTGCGATATTTGCGTTCCGC (TGG)	2
Pten	Pten-001 (ENSMUST00000013807)	CCDS29753	GCTAACGATCTCTTTGATGA (TGG)	1
Smad4	Smad4-001 (ENSMUST00000025393)	CCDS29337	GACAACCCGCTCATAGTGATA (TGG)	2
Tet2	Tet2-201 (ENSMUST00000098603)	CCDS51071	GAAAGTGCCAACAGATATCC (AGG)	3
Trp53	Trp53-202 (ENSMUST00000171247)	CCDS48826	GACACTCGGAGGGCTTCACT (TGG)	4
Arid1b	Arid1b-201 (ENSMUST00000115797)	CCDS49929	CTGTGCACCTGGGGGACCGT (AGG)	2
Arid2	Arid2-001 (ENSMUST00000096250)	CCDS37185	AGGCGCCTCCGGACGAGCGG (AGG)	1
Arid5b	Arid5b-201 (ENSMUST00000020106)	CCDS35929	GCTATGCAAATCGGATCCTT (TGG)	2
Atm	Atm-001 (ENSMUST00000118282)	CCDS40636	GGCTGTCAACTTCCGAAAAC (GGG)	7
Cdkn2b	Cdkn2b-201 (ENSMUST00000097981)	CCDS18351	GGCGCCTCCCGAAGCGGTTC (AGG)	1
Errfl1	Errfi1-001 (ENSMUST00000030811)	CCDS18974	AAGCTCGGGACAGCGTGAAG (AGG)	4
Igsf10	Igsf10-201 (ENSMUST00000039419)	CCDS50915	TGAGTCCGTAAAACGCCTCG (GGG)	4
Irf2	Irf2-201 (ENSMUST00000034041)	CCDS22295	GTGCCGAGCCGCATGCATCC (AGG)	3

Supplementary Table 7. Primary antibodies used for immunohistochemistry.

Antibody	Company/Source	Host	Pretreatment	Dilution
A6	Engelhardt et al.. Differentiation; research in biological diversity, 1990	rat	Proteinase; 37°C; 10min	1:100
AFP	R&D (AF5369)	goat	Citrate; 100°C; 30min	1:100
Collagen-4	Cedarlane (CL50451AP)	rabbit	Proteinase; 37°C; 10min	1:50
Cytokeratin 19	Hybridoma bank (TROMAIIIc)	rat	EDTA; 100°C; 20min	1:500
Ki67	Neo Markers (Clone SP6)	rabbit	EDTA; 95°C; 30min	1:200

Supplementary Table 8. Primer sequences for target site amplicons. Primers used for amplifying CRISPR/Cas9 target sites (length of PCR products is between 400bp and 600bp) and oligonucleotides used for amplicon-based next-generation sequencing.

Guide	Forward primer	Reverse primer
Apc	GCGAATAAGCACCACTCCTC	AAGAATGAACCAACACCAAGG
Arid1a	GTTCTGATTCCTGTGCTCGC	TCCATCACCTACCTGCTGTG
Brca1	AGCGTGAGAACTCCTCCAAA	CTGCCATGAGGAAGAACACA
Brca2	TCACGAGTTTCTCCGTGTCA	GCTCTGGCTGTCTCGAACTT
Cdkn2a (ex1β)	TCTCACCTCGCTTGTCACAG	AAGTACTCCATCTCCCGGGA
Cdkn2a (ex2)	TCAACTACGGTGCAGATTCG	CGGGTGGGTAAAATGGGAAC
Pten	TGCGAGGATTATCCGTCTTC	CATCCGTCTACTCCCACGTT
Smad4	TGCAGTGTCACAGATGCTCA	CTCAGGAACTGGAGGAAGCA
Tet2	CAGATGCTTAGGCCAATCAAG	AGAAGCAACACACATGAAGATG
Trp53	ACATAGCAAGTTGGAGGCCA	CCACTCACCGTGCACATAAC
Arid1b	AGTTCTGGGGTACTTGGAATCA	GGTACTGCAAGCCTCCCA
Arid2	ATGACTGAGCCCCGCCA	GAGCAGACTTTTCCGAGCAG
Arid5b	TGGCTTGCACGGACCTTATA	ATCAGCAGTTGGACGGTCTT
Atm	TCCTTTTCAACTGTTCCTGTTACA	GACAATGGAAAGGCGAGTCA
Cdkn2b	CCGAAGCTACTGGGTCTCC	CACTTGCCCAGCTTGTACG
Errfl1	GTGTTCCCCTACTCTGGCTC	TCTTCAGAGATGGGCAGTGG
Igsf10	CTGTCCACCTGAGTCCACTT	TGTCAGCCGGTTTCCTTCTA
Irf2	TGTCTGACAGTCGACTTCCC	ACTGGGAACTTCTGGGATGG
Oligonucleotides for library preparation		
PE adapter top strand	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	
PE adapter bottom strand	GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	
PE 1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	
iPCRtag	CAAGCAGAAGACGGCATACGAGATXXXXXXXXCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	

Supplementary Table 9. qPCR primers for *hSpCas9* quantification by real time quantitative PCR.

Target	Forward primer	Reverse primer
hSpCas9	GCCTATTCTGTGCTGGTGGT	ATCCCCAGCAGCTCTTTCAC
Apob	CACGTGGGCTCCAGCATT	TCACCAGTCATTTCTGCCTTTG

Supplementary Table 10. qPCR primers for sgRNA distribution analysis. Primers used for sgRNA distribution analysis (real time quantitative PCR). *CRISPR-SB-fwd* and *CRISPR-SB-rev* amplify a 763bp product containing the cloned 20bp sgRNA sequence. Nested real time quantitative PCR was performed to analyze distribution of specific sgRNAs using *CRISPR-SB-quant-fwd* and the specific reverse oligonucleotides of each sgRNA.

Primer	Sequence
CRISPR-SB-fwd	GAGGGCCTATTTCCCATGAT
CRISPR-SB-rev	CGACTCGGTGCCACTTTT
CRISPR-SB-quant-fwd	ACTATCATATGCTTACCGTAAC
Apc_rev	AAACCAACTTGCTTTAACAACTGAC
Arid1a_rev	AAACCAGCCGTATGGTGGGACTAAC
Brca1_rev	AAACGATCGGACACTCTAAGATTTC
Brca2_rev	AAACATTGAGGCTTATCGGTCCTAC
Cdkn2a_(ex1β)_rev	AAACGGATCGCACGAACTTCACCAC
Cdkn2a_(ex2)_rev	AAACGCGGAACGCAAATATCGCAC
Pten_rev	AAACTCATCAAAGAGATCGTTAGC
Smad4_rev	AAACTATCACTATGAGCGGGTTGTC
Tet2_rev	AAACGGATATCTGTTGGCACTTTC
Trp53_rev	AAACAGTGAAGCCCTCCGAGTGTC

Supplementary Table 11. qPCR primers used for quantification of *Cdkn2a exon-1β/exon-2* fusion products by real time quantitative PCR.

Target	Forward primer	Reverse primer
Cdkn2a-ex1β-quant	CAAGAGAGGGTTTTCTTGGTGA	
Cdkn2a-ex2-quant	ACAACATGTTCACGAAAGCCA	GGGACATCAAGACATCGTGC

Supplementary Table 12. Primers used for *CRISPR-SB* integrations analysis.

Target	Forward primer	Reverse primer
CRISPR-SB-int	GAGGGCCTATTTCCCATGAT	CGACTCGGTGCCACTTTT

Supplementary Table 13. Information about off-target sites. Location, sequence and number of mismatches (in regard to the on-target) for each potential off-target site analyzed by amplicon-based next-generation sequencing.

Name	Chr	Strand	Location (mm9)	Sequence	Mismatches	Gene
Apc						
OT_Apc_1	9	-1	26741476	TCAGTTATTAAAGCAAATTGGGG	2	None
OT_Apc_2	14	1	78004236	GCAGTTGAGAAAGCAAGTTGGAG	3	None
OT_Apc_3	3	-1	114079728	TCAGATTATAAAGCAAGTTGTGG	3	None
OT_Apc_4	5	1	76213674	TTAGCTGTTAAAGCAAGTTACAG	3	None
OT_Apc_5	10	1	93842567	TCAGATGGGAAAGCAAGTTGCAG	3	None
OT_Apc_6	13	1	107647475	TAAGTTGCTATAGCAACTTGAAG	4	NM_029665
OT_Apc_7	7	1	13570187	TCAGTTCCTACAGCAAGTTCCAG	4	NM_001168561
OT_Apc_8	10	-1	51914804	AGAGTTCTTAAAGCAAGGTGGAG	4	NM_011282
Arid1a						
OT_Arid1a_1	17	1	27589623	CCAGGCCCACCATATGGCTGAGG	4	None
OT_Arid1a_2	5	-1	123157730	TGAGCCCCACTTTACGGCTGCGG	4	NM_011026
OT_Arid1a_3	8	1	92089504	TGGGACCCACCATACCGCTGTGG	4	None
OT_Arid1a_4	16	-1	13033016	TTAAACCCACCATACGCCTAAAG	4	None
OT_Arid1a_5	8	-1	128820603	ATAGTCCATCCATAGGGCTGAAG	4	None
OT_Arid1a_6	15	1	79748335	TGTGTCCCACCACAAGGCTGGAG	4	NM_144811
OT_Arid1a_7	1	1	166141978	ATAGACCCACCCTTCGGCTGGAG	4	NM_007976
Brca1						
OT_Brca1_1	18	1	11328992	AAATCTTGGAGTGTCCGGTCAAG	2	None
OT_Brca1_2	10	-1	124791436	AAATTTTAGTGTGTCCCATCAAG	3	None
OT_Brca1_3	11	1	111352929	AATTCTTAGAATGTCCCATCCAG	3	None
OT_Brca1_4	5	-1	34545145	ACATCTGTGAGTGTCCCATCCAG	4	None
OT_Brca1_5	3	-1	7612605	AAGTCTGGGAGTTTCCGATCCAG	4	None
OT_Brca1_6	17	1	35264433	GAACCTTGGAGTGTCCGCTCAAG	4	NM_033477
OT_Brca1_7	12	1	112094143	ACATCTTACAGTGTCAGATGGGG	4	NM_001199785
OT_Brca1_8	1	1	60543179	AAATCTTAAATTGTCTTATCTGG	4	NM_001045513
Brca2						
OT_Brca2_1	16	16	7627877	TATGACCAATGAGCCTCAATAAG	3	None
OT_Brca2_2	5	5	37739749	GATGACCCATAAGCCTCAAAGAG	4	NM_145920
OT_Brca2_3	7	7	90824129	TGGAACAGCTAAGCCTCAATCAG	4	None
OT_Brca2_4	6	6	101189127	TTAGACCTATAAACCTCAATGAG	4	None
OT_Brca2_5	X	X	10413660	GAGAACTGATTAGCCTCAATAGG	4	None
OT_Brca2_6	12	12	37203260	AAGGACAGATAAACCTCATTAGG	4	NM_178629
OT_Brca2_7	5	5	124269242	TAGGAACGCTACGGCTCAATAGG	4	NM_001042421
Cdkn2a-ex1β						
OT_Cdkn2a-ex1β_1	8	1	45813437	TGATTAAGTTCGTGAGATCCTGG	3	None
OT_Cdkn2a-ex1β_2	2	-1	30064240	CAGTGAAGTGCCTGCGATCCCAG	4	None
OT_Cdkn2a-ex1β_3	1	1	39634432	TGGGGAAGTTTGTGCGCTCCGGG	3	None
OT_Cdkn2a-ex1β_4	6	-1	87783496	AGGTGTGGTGCGTGCGATCCCAG	4	None
OT_Cdkn2a-ex1β_5	16	1	39487138	AAGTGAAGTTTGTGCGTTCCCAG	4	None
OT_Cdkn2a-ex1β_6	13	-1	50513278	TGCTGCAGTTCGTGCGGGCCAAG	4	NM_175401
OT_Cdkn2a-ex1β_7	11	-1	120670835	TGTGGAAGTTCGTCAGATCCTGG	4	NM_007988
OT_Cdkn2a-ex1β_8	X	1	56172330	TGGTGAAGTTTCTGAGCTCCAAG	4	NM_023774
Cdkn2a-ex2						
OT_Cdkn2a-ex2_1	4	1	148248166	GTGGGAGATCTGCGTTCCGTAAG	4	None
OT_Cdkn2a-ex2_2	7	1	134068454	GTGCGTTCTTTGCGTTGCGTGGG	4	None
OT_Cdkn2a-ex2_3	4	1	45268111	GTGCCATATTCCAGTTCCGCAAG	4	None
OT_Cdkn2a-ex2_4	2	1	180660612	GTGGGACATTTGGGTTCCTCTGG	4	None
OT_Cdkn2a-ex2_5	8	-1	74680985	GTTCAATATTTGTGTTCTGCCAG	4	None
OT_Cdkn2a-ex2_6	11	-1	95536456	GTGTGATATTGACGTTCTGCAAG	4	NM_008831
OT_Cdkn2a-ex2_8	3	-1	53278662	GTGCGATAGTTGCATGCGGCCGG	4	NM_173382

Pten						
OT_Pten_1	1	-1	98296790	CCTATCGATTTCTTTGATGATGG	3	None
OT_Pten_2	10	1	11506620	AATACCGGTCTCTTTGATGATGG	4	None
OT_Pten_3	6	1	110090641	TGTCACGATGTCTTTGATGAAGG	4	None
OT_Pten_4	1	-1	148546230	GCTTACGATGTATTTGATGATGG	3	None
OT_Pten_5	12	-1	8417202	AGTAGCTATCTCTTTGATGAGAG	4	None
OT_Pten_6	2	1	37283155	TGTAACAATGTCTTTGATGAAAG	4	NM_146253
OT_Pten_7	3	1	138114184	GCTGACACTGTCTTTGATGATAG	4	NM_007410
OT_Pten_8	10	-1	62480288	GGAAACGATGGCTTTGATGACAG	4	NM_001079824
Smad4						
OT_Smad4_1	18	1	39860049	AATAGCAGCTCATAGTGATAGAG	4	None
OT_Smad4_2	6	-1	127173197	ATAACCCGCTTATAGTGATGTGG	3	None
OT_Smad4_3	8	-1	88373851	ACAGCCCTTACATAGTGATAGGG	4	None
OT_Smad4_4	13	1	31227591	ACAAACATCTCCTAGTGATATGG	4	None
OT_Smad4_5	1	1	170162378	GAAACCAGCTCAAAGTGATAGAG	4	None
OT_Smad4_6	10	1	20868341	ACTATCTGCTCAAAGTGATACGG	4	NM_001198914
Tet2						
OT_Tet2_1	9	1	45417670	TAGTGTGACAACAGATATCCTGG	4	None
OT_Tet2_2	2	1	30832627	GATTATGACAACAGATATCCTGG	4	None
OT_Tet2_3	1	1	89767330	GGAATTGCCAACAGATCTCCTGG	3	None
OT_Tet2_4	17	1	30344787	GCCAGTGCCAACAGATTTCCCAG	3	None
OT_Tet2_5	13	-1	51963540	GAAAGACCCTACAGATATCCCAG	3	None
OT_Tet2_6	7	-1	115756426	GACAGTGCCAACAGATATAGTGG	3	NM_001011871
OT_Tet2_7	10	1	5219200	GAACGTGCTTACAGATATCAAAG	4	NM_001079686
OT_Tet2_8	4	1	132622017	GACACTGCCTACAGGTATCCAGG	4	NM_146155
Trp53						
OT_Trp53_1	17	-1	54559163	AACACTTGGAGGGCTTCACTTGG	2	None
OT_Trp53_2	5	-1	107143107	ATCACTTGGAGGGCTTCACTCAG	3	None
OT_Trp53_3	10	-1	109084970	GGCTGTCAGAGGGCTTCACTCAG	4	None
OT_Trp53_4	9	-1	49608135	GTCTGTCAGAGGGCTTCACTGAG	4	None
OT_Trp53_5	5	-1	117474920	CACACTGGGAAGGCTTCACTTAG	3	None
OT_Trp53_6	2	-1	35586131	GGCAGTCAGAGGTCTTCACTCAG	4	NM_001114125
OT_Trp53_7	2	-1	62339193	GACAGTCTGAAGGCTTCACATGG	4	NM_007986
OT_Trp53_8	2	-1	158087116	AACACTCGGAGGCCATCACTGGG	3	NM_177850

Supplementary Table 14. Primer sequences for off-target site amplicons. Primers used for amplifying CRISPR/Cas9 off-target sites (length of PCR products is between 400bp and 450bp).

Name	Forward primer	Reverse primer
Apc		
OT_Apc_1	CTGAGTGTGGTGCTATACTCAAG	ACTAGGATTAGGACCTAGGAAACA
OT_Apc_2	AGATCTGCAGTTCACCCCAA	GGGAGTCCAGGAAGCAGAAT
OT_Apc_3	AGTTACTGGTGGCTGTAAGACA	AGAGTGGCAGTTCAAGGTAGT
OT_Apc_4	ATCCAACGCTGATTCCTTGC	GGGAGGTGATTGAGAGGGAC
OT_Apc_5	CCTGGTTTTACGTTGCTGCT	CTATTTGCCTGCACCTCCAG
OT_Apc_6	CAATGCAAAAGGTGTTCTGACA	TCACCACCCTTGCTGTAACT
OT_Apc_7	CACTTGCTTCAGTCTGAGCC	CCTGCAGTCAACCTTGGTTC
OT_Apc_8	CGAACCTGTCAGTTGCAAGT	TGCGATGTTCTGGGCTATCT
Arid1a		
OT_Arid1a_1	TCCAGATGCCAACCCCTATC	GCCACAGACCCTATTCCTCA
OT_Arid1a_2	TGAGAGGGTCACGAGTTGG	CTATTGCCCCAGACCCAGAG
OT_Arid1a_3	TGTCTACGATCACAGTGCAGT	ACACAGGCTGTAACTCTGAAGA
OT_Arid1a_4	CAGAGGAAGTTGGGTGAGGA	TCATGCTCATCAGGGCTTCT
OT_Arid1a_5	GCCAACAGGTGAGTCTTCTAAC	CAGGCCCATGTTGTCTGAAG
OT_Arid1a_6	CGGCAAGTTCTGTTTGTGCT	GTCTGGGTCTCATCTCCTGG
OT_Arid1a_7	TCCTCGAAGTAGACATATCCACA	TGCAAAGGTTCTTCTGGAGC
Brca1		
OT_Brca1_1	GACTTCGTGGACAGAATGGC	TCCAGCCCTGTTTGATTCCT
OT_Brca1_2	GAGAACTGCAGAGCCCATTG	ACCGACATTTTCCCCTCCTT
OT_Brca1_3	TCCAAAGGCTGCTAGTGGAA	CCTCGACCCCTCCCAATTTT
OT_Brca1_4	CCCAACACAGCCCACTACA	ACCTGCAGAGTAAAGGGCTC
OT_Brca1_5	TGGATTCCAGCCTCTGTCAA	TGTCCCTAGCCAGTACCTCT
OT_Brca1_6	TAGCAGGGACCTCAAAGTGG	ATAGCAGCCCATGAAGCCAG
OT_Brca1_7	GCACTGTAAGCTCAACCCAG	CCTCTGCCACATGAGTACCA
OT_Brca1_8	ACATGACTGGAGTTAGAAAAGGA	TGTGCTTGCTATTCCTATGATGA
Brca2		
OT_Brca2_1	CACAGTAGGTTGGGTCTTCC	GACAGGGTTGGAGAGTGCC
OT_Brca2_2	GCGCTGTTATTTCCTCCGTT	AGCAAGGCCAGTGATCTCAT
OT_Brca2_3	TGAGCAAGTCACTTTGGAAAACA	AAGTGGGAACTTCAGGAGGG
OT_Brca2_4	CACTGAGTGTCATGCTTGGC	ACTAGTGAGCCCTGCCTTTC
OT_Brca2_5	GACACAGGAAGAGGGAGACA	ATCAAGCCACCAGAATCCCT
OT_Brca2_6	TGCATTTCCTTTGACACCAGT	ATCAGAGATCTCCGTGGCTG
OT_Brca2_7	AGAAGGAATTTGGGATTTTGGCA	TGGAGAGTGAGCTAGCCAAG
Cdkn2a-ex1β		
OT_Cdkn2a-ex1β_1	GCTTCCCTGAAACCTGCATC	CATCAAGGACTAGGAGCAATGA
OT_Cdkn2a-ex1β_2	GTTGCCCTCATCTCAGACCT	TTCCAAGTGCAGCAAAGGTC
OT_Cdkn2a-ex1β_3	GCGACTCACTCCAGGCTG	ACAAAAGGCATCTGGACAACT
OT_Cdkn2a-ex1β_4	GGGGAGAGGGTCTAGAAGGA	TCCACAGATCATTGGCGAGA
OT_Cdkn2a-ex1β_5	GGCATCTTTTCATTTGTCAGCC	ACACAGACACACAGATCCAAT
OT_Cdkn2a-ex1β_6	ACTTCAGTGATCGCTAGGCC	CACACAGTGGGGCATAGAGA
OT_Cdkn2a-ex1β_7	TGAGGACATGCACACAGACT	AATGCTTGGCTGGGTGATTG
OT_Cdkn2a-ex1β_8	CTGCAGAGAGTTCCCAGGAA	CTCTTCATTGCTGATCCGCC
Cdkn2a-ex2		
OT_Cdkn2a-ex2_1	TGGGCTTGTTTTAAAGGGGC	CAATGTCTGCTGCTCACCTG
OT_Cdkn2a-ex2_2	GTCTGTTTGGATGCCCTTGG	AGGCTACTCTTGCTGTCTCC
OT_Cdkn2a-ex2_3	AAACTGAACTTGCTCGGCTC	TTGAGCATGAGAGGGAAGCA
OT_Cdkn2a-ex2_4	TACCACTTCCTTCCCTGCAG	ATTGACTGTCCTACCCTGGG
OT_Cdkn2a-ex2_5	TTACCTAACTCCTGGGGCAG	CAGGAAGCTAGACTGTGCCT
OT_Cdkn2a-ex2_6	CCATCCTGTCAGTGGTTCCT	GCTACCTACCCACCACTACTC
OT_Cdkn2a-ex2_7	ACTGGGGCATCTTCAGTCTC	AGTGAAAAGCCCCAATGATAAGT
Pten		
OT_Pten_1	CAAGAGAAAGACAAGGCATGGT	AGAAGGGAGGAGGGAAGGAA
OT_Pten_2	GGAGCAGCTTGGAGTCTGAT	CATTGCCAGCACAGTTCTCA
OT_Pten_3	GGAACATTAAGAGTGAAACAGCT	AAATAGGTGGCAGAACGGGT
OT_Pten_4	CATGCAACAACAGAGGACACA	TCCTTCTTCTGACCAAATGTGA
OT_Pten_5	AACAATGCTCAGAGGGTCCC	GATGGAATGTTGGGCCTCAA
OT_Pten_6	AAGGGTGGACTACAAAAGAGC	ACAGAAAGGTTTGTCTTGGCC
OT_Pten_7	GCTGTGGTATTTCAACTGGCT	TGACCTTCACGTTGCCAATG
OT_Pten_8	CCATAGCCATGTCCTCCCAT	GCTGCAAACATTAATGAAGAAGC

Smad4		
OT_Smad4_1	CATCATCTCCAAGGCCCTCA	GCCATTCCAGGGATCAAACC
OT_Smad4_2	CAGATATGGTGGTGCATGCC	TTGGAAAGCAGAGCAACAGG
OT_Smad4_3	GGGGTTCCTGGGAGTCTTTT	TACTGTGGCCTTGAGAAGCA
OT_Smad4_4	TAAGCAGCACTCACCACCAA	GCTCAGTCACCTAAGCTTGT
OT_Smad4_5	AAAGTGGGACTCATAGGGCC	TCCCGTCTCAGGTCACAAAA
OT_Smad4_6	TAATGCCTGCTGTCCCTTCA	TGAGATCATCTGACGGGCAA
Tet2		
OT_Tet2_1	AATTCAAGTGCAGAGCCAGG	GCCAGTCTGCAAATGAAATCT
OT_Tet2_2	CAACACACCTGCCTCCAAC	CTGAGTTCACTGTGCAAGCA
OT_Tet2_3	TCTAGGGAATGTGGCCTGAG	CCCTGCAGATCCCCTAAATGA
OT_Tet2_4	CCGCACCCATTTTCTGATAGG	CTTTCCGGTCCAGTTTCACC
OT_Tet2_5	GCTGTCCTGGAACTCACTCT	ACTGAGCCTAAGATTGTCCCA
OT_Tet2_6	TAATGCATCCTCCTTCACCCT	GGGGTTCAACATGGGGATCA
OT_Tet2_7	ACATGACCCAAGATTTCCCAA	GGCCTGAGAAGCGAAATGAG
OT_Tet2_8	CTATGAAGGCAAGGTGGGC	CATCCCCAGACTTACCCAGG
Trp53		
OT_Tp53_1	CCTAGCATTCAGGCCCTCAT	TGAGGGGAGGAGAGTACAGT
OT_Tp53_2	GGATTGTCCCTTGTACCACTTC	AACAAATGTGCGGGCAACTT
OT_Tp53_3	GCATGCACTGAACAGAAATTGG	TCAGAGGAGATTTGCTTGGGA
OT_Tp53_4	CCCTGGCTCTTCTGTGTGTA	GAACCCGCAGCATGTGATAG
OT_Tp53_5	CATGATGCCTGTTCACGAGG	CTGGTAAAAGGTGCTGGCTT
OT_Tp53_6	CATGCTGTTTGGGTGGAAGG	AGAAAAGAGGGGCTGGTTCC
OT_Tp53_7	CTACCCGGCAATGAACAGGT	CCAAGTGGCCAAGAAGCAAA
OT_Tp53_8	GGCTTGCCGTCTTTGTTGAT	AAGTGGACAGTTCTCCCAGC