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Supplemental Information

CRISPR-Cas9 gene editing of hepatitis B virus in chronically infected humanized mice

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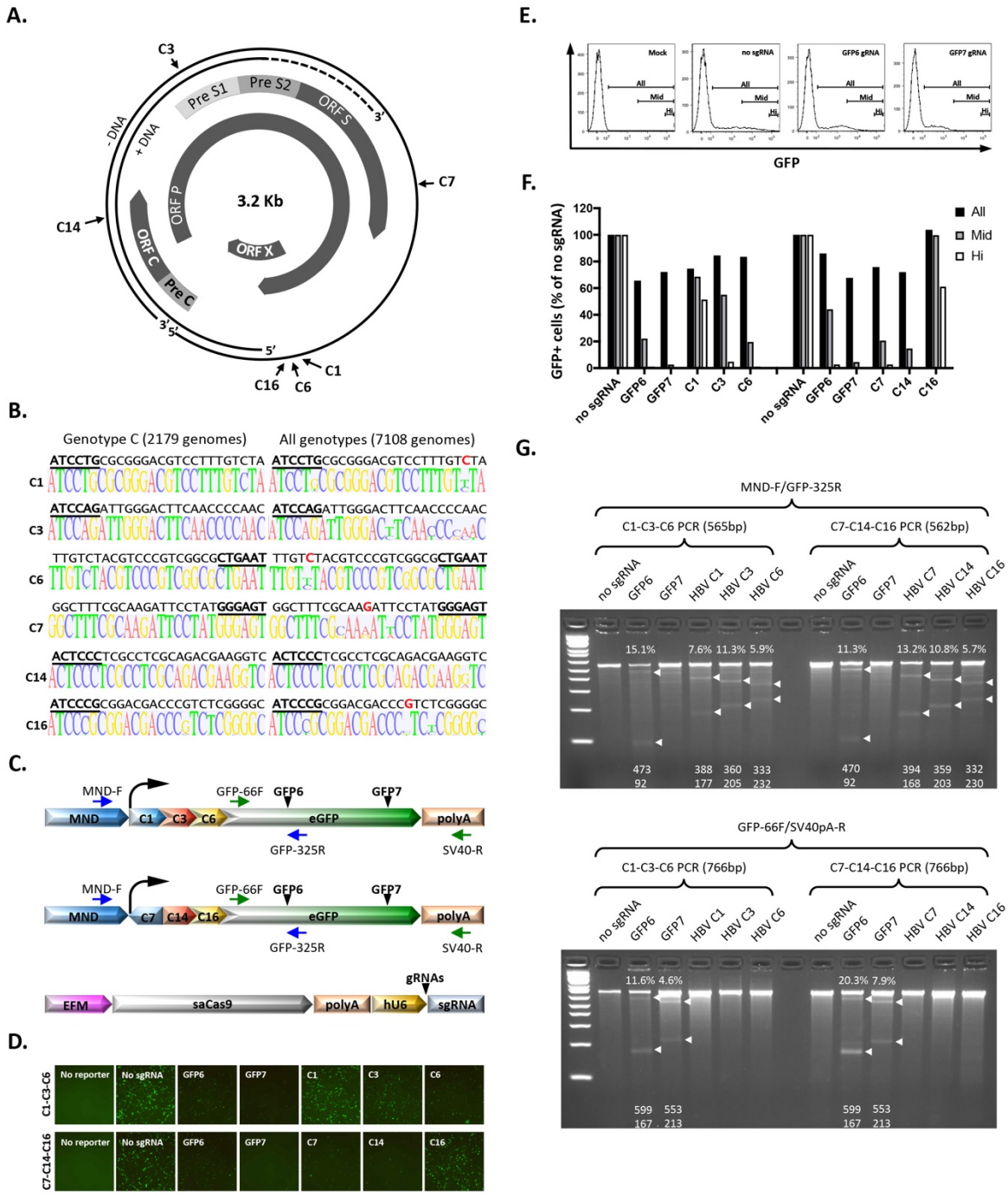
Supplemental Table 1. sgRNA target site sequences. DNA sequences targeted by each indicated sgRNA are shown along with the ORF targeted. PAM sequences are underlined in bold. Black = forward orientation, red = reverse orientation.

Name	sgRNA target sequence	ORFs targeted
C1	TAGACAAAGGACGTCCCGCG**CAGGAT**	HBV P/X
C3	GTTGGGGTTGAAGTCCCAAT**CTGGAT**	HBV P/S
C6	TTGTCTACGTCCCGTCGGCG**CTGAAT**	HBV P/X
C7	GGCTTTCGCAAGATTCCTAT**GGGAGT**	HBV P/S
C14	GACCTTCGTCTGCGAGGCGA**GGGAGT**	HBV P/C
C16	GCCCCGAGACGGGTCGTCCG**CGGGAT**	HBV P/X
GFP6	GTCGTGCTGCTTCATGTGGT**CGGGGT**	eGFP
GFP7	GGGTCTTTGCTCAGGGCGGA**CTGGGT**	eGFP

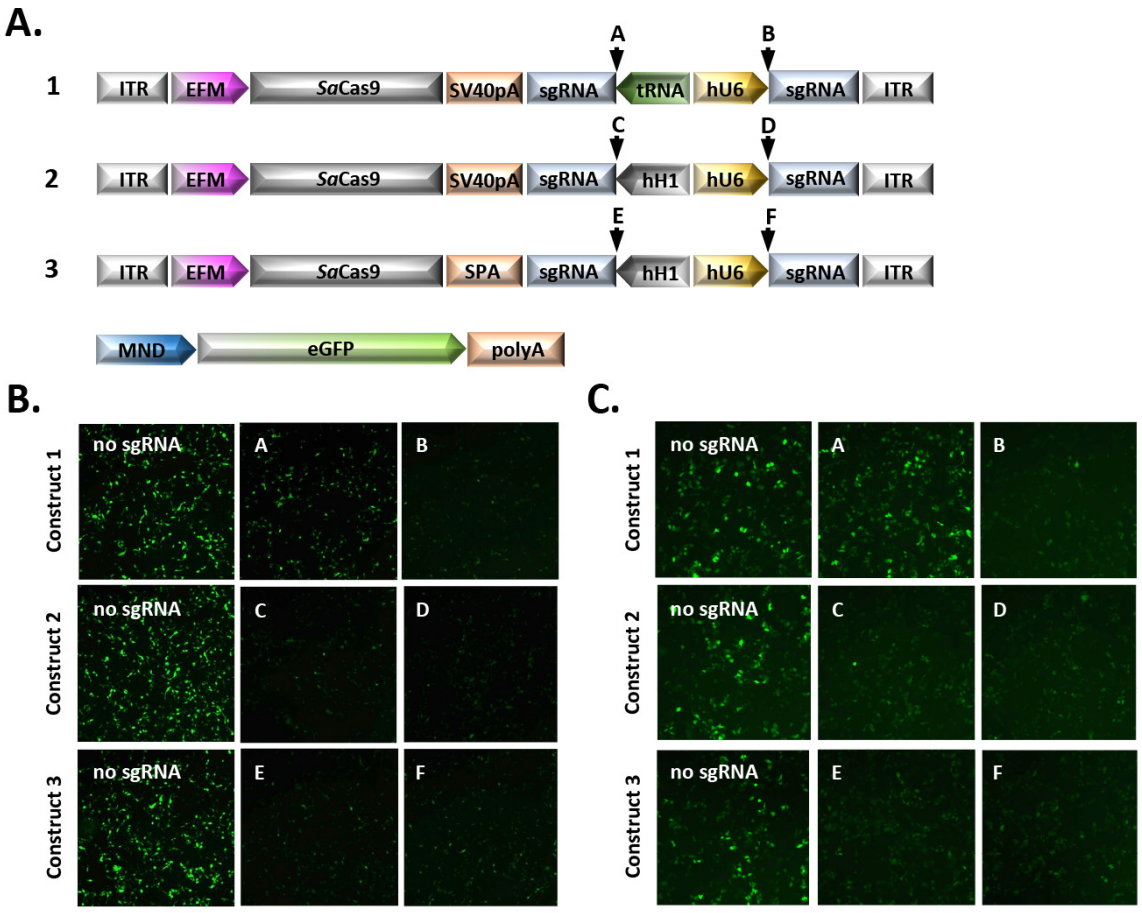
Supplemental Table 2. DNA sequences for human and mouse off-target sites. The chromosomal locations and DNA sequences of C7 (left) or C14 (right), and human (upper 5) or mouse (lower 5) off-target sites. Forward (red) or reverse (green) orientations of each chromosomal target site are indicated.

Locus	Target sequence	Locus	Target sequence
CHR1 246105087	GtCTTTgGCAAGAaTCCTgTTAGGAT	CHR2 219477308	GAgCTTCGTgTaCGAGGaGAATGAGT
CHR3 157310788	GGCTTTtaaAAGATTCCTcTTGGAGT	CHR5 98654280	GACCTTCaTCTGCaAGGCaaCTGAGT
CHR3 197878238	GGCTTTCGgAAGcaTCCTcTGAGGGT	CHR5 145229209	GACCTTCaTCTGgGgGGCcAGCGAGT
CHR13 77156174	GGCTTcaGCcAGATTCaTATGCGGAT	CHR7 13308669	GACCTTgGTCTGCaAGttGAAAGAAT
CHR16 66851217	GGCTTggGCcAGATTCCTcTTTGGGT	CHR7 158773226	GACCTgCGTCTGCcAGGtGAATGGGT
CHR1 37584565	GGCTTTgGgAAGAaTCCTATAGGGAT	CHR2 53456299	GACCTTgGTCTaCaAGGtGAATGAAT
CHR3 9624137	tGCTTTCtCAAGATTCtTcTATGGGT	CHR5 16637046	GcCCTTCaTCTGCctGGCGACAGGGT
CHR5 7182552	aGCTTTCtCAAGATTCtTAaCAGAGT	CHR7 16889254	GACCTcCGTCTcCcAGGtGATGGGAT
CHR7 142846242	GGCaTTCcCAAGATTCtTAcTGGAGT	CHR8 13415181	GACtgTCtTCTGaGgGGCGAGGGAGT
CHR8 104545290	tGCTTTCcCAAGATTCtTaTTAGAAT	CHR8 108709798	GACCTTCcTCTGCtAGGatgATGGGT

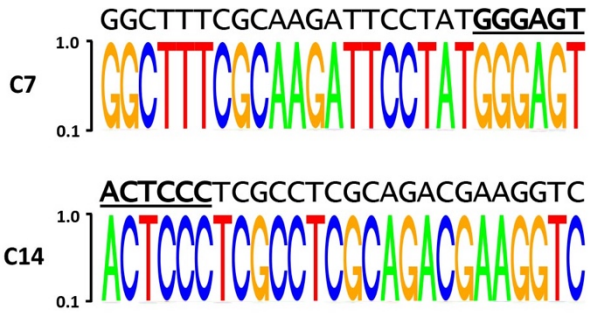


Supplemental Figure 1. Location and validation of HBV-specific sgRNAs. A, Six highly conserved sites within regions of the HBV genome containing overlapping ORFs were identified from a consensus sequence for genotype C. **B**, Triple sgRNA target site GFP reporter and *Sa*Cas9/sgRNA expression constructs used to validate HBV-specific sgRNA activity. **C**, Control

GFP-specific or HBV-specific *Sa*Cas9/sgRNA-mediated knockdown of GFP reporter activity was assayed in 293 cells 24h after plasmid co-transfection by fluorescent microscopy (**C**), or flow cytometry (**D**/**E**). Flow cytometry analysis of GFP knockdown relative to the no sgRNA control cells in all GFP+ (All), mid plus high MFI GFP+ (Mid) and high MFI GFP+ (Hi) cell populations by control GFP-specific sgRNAs is shown for the C7-C14-C16 reporter (**D**). **E**, Knockdown of GFP expression from the C1-C3-C6 and C7-C14-C16 reporters by GFP- or HBV-specific sgRNAs. Data from one of duplicate experiments is shown. Relative transfection efficiencies of 20.1% and 18.6% GFP+ cells were seen in the no sgRNA controls for C1-C3-C6 and C7-C14-C16 reporters respectively. **F**, T7 endonuclease I assays show cleavage bands (arrow heads) indicative of gene editing at all HBV (upper panel) and control GFP (lower panel) target sites. 1Kb Plus DNA ladder is shown along with percent mutation (above) and expected band sizes (below). **G**, Logo plots for each sgRNA target site generated from 2179 genotype C sequences (left panel), and 7108 sequences from genotypes A-H (right panel) from the Hepatitis B Virus database [37]. sgRNA target site PAM sequences are underlined and bold, while differences from the consensus sequence are shown in red. MND - murine leukemia virus and myeloproliferative sarcoma virus hybrid LTR promoter; EFM – EF1a short/minute virus of mouse intron hybrid promoter; *Sa*Cas9 – *S. aureus* Cas9; polyA – SV40 polyA; sgRNA – single guide RNA; hU6 - human U6 promoter.



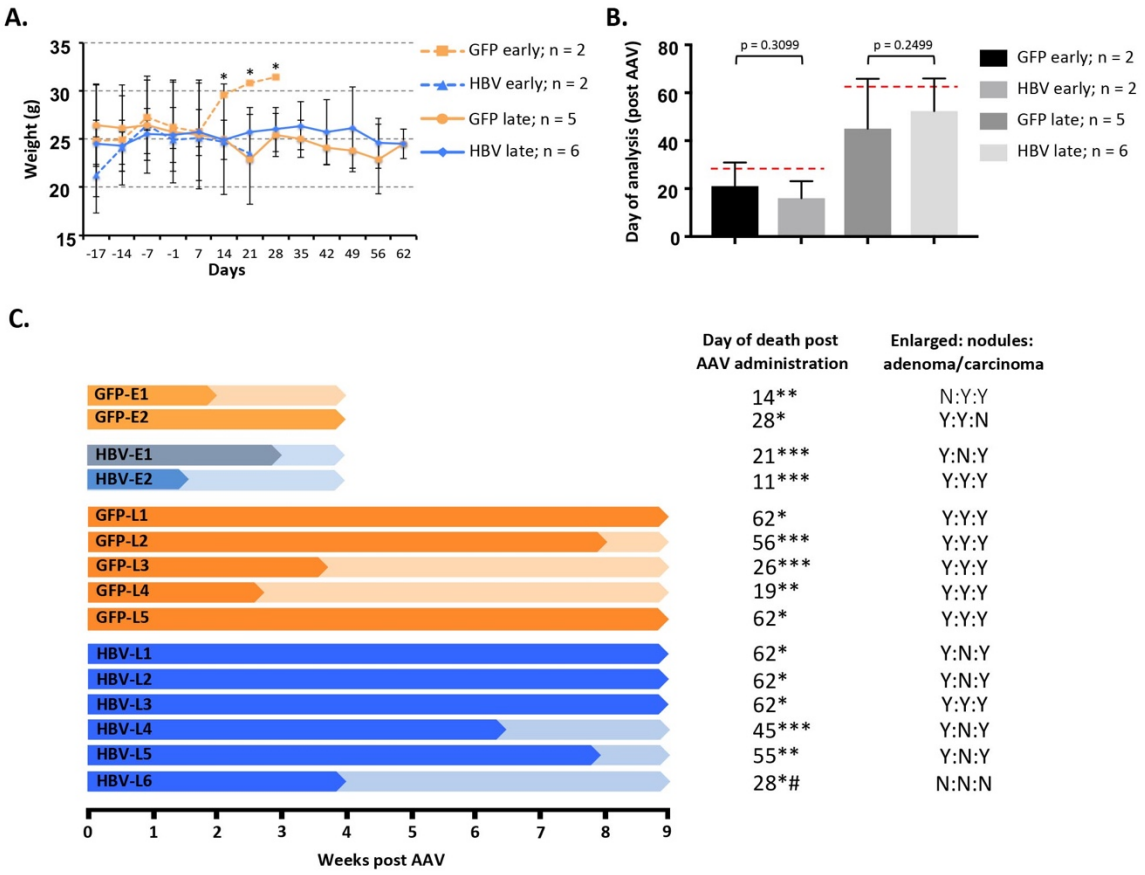
Supplemental Figure 2. AAV-*Sa*Cas9 vector optimization. A, *Sa*Cas9 and dual sgRNA-expressing AAV vector plasmids 1-3 with sgRNA insertion sites A through F and GFP reporter plasmid. Knockdown of GFP reporter expression in 293 (**B**) or Huh7 (**C**) cells 24 hours after co-transfection of GFP reporter along with each indicated AAV vector construct expressing *Sa*Cas9 and the GFP7 sgRNA from the indicated sgRNA insertion site. ITR – inverted terminal repeat; EFM – EF1α short/minute virus of mouse intron hybrid promoter; *Sa*Cas9 – *S. aureus* Cas9; SPA – synthetic polyA; sgRNA – single guide RNA; MND - fusion of murine leukemia virus and myeloproliferative sarcoma virus LTRs.



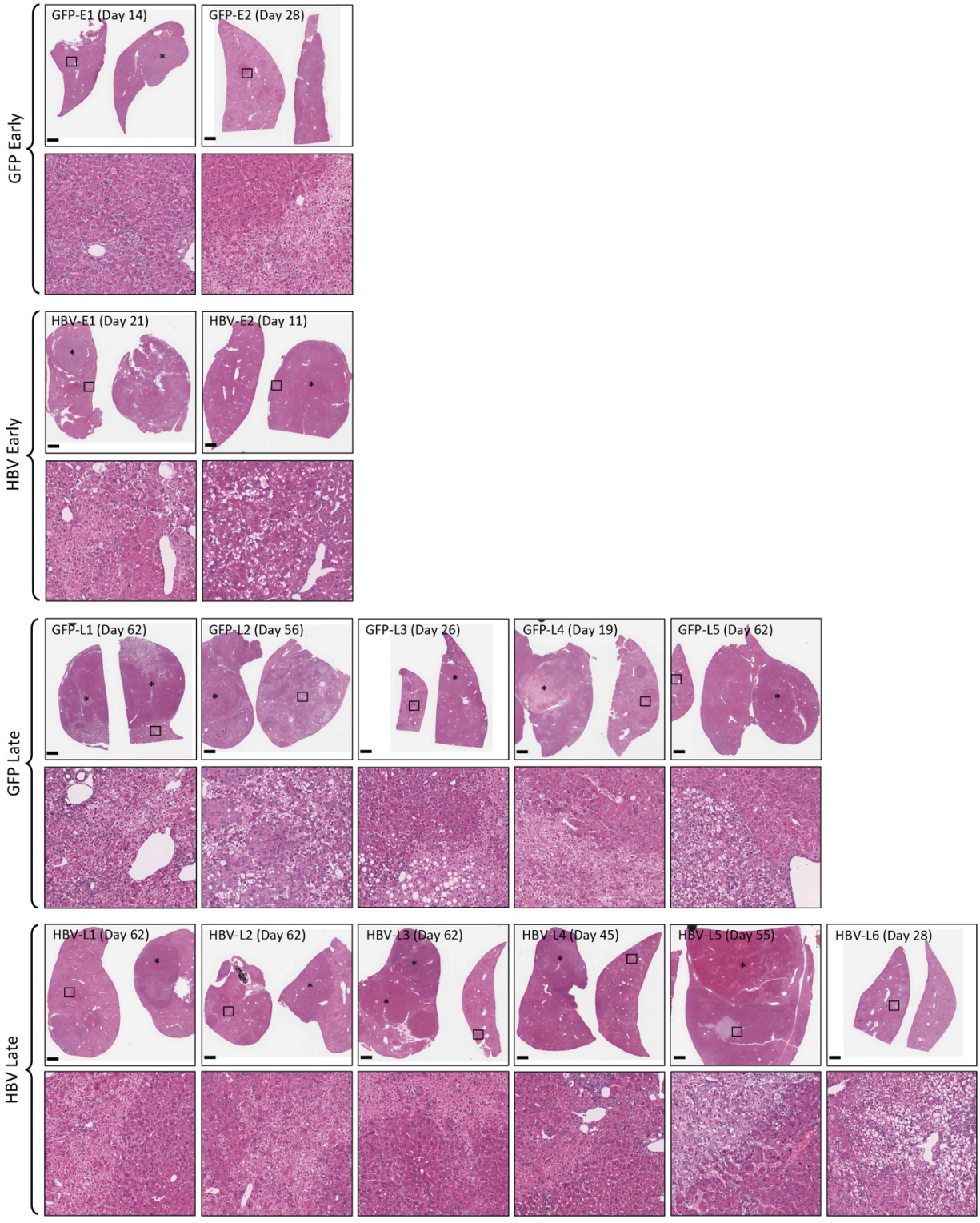
Supplemental Figure 3. HBV inoculum target site sequencing. PCR amplicons spanning each target site were amplified from a pre-inoculation sample of the HBV genotype C isolate used to challenge the FRG mice. HBV target sites for sgRNAs C7 and C14 were analyzed by Illumina sequencing, and Logo plot consensus sequences are shown. The Logo plot scale represents probability occurrence within the inoculum. sgRNA PAM sequences are underlined/bold.

Mouse ID	Indel (bp)	Sequence	Read count
	wtC7	**GGCTTTCGCAAGATTCCTAT▼GGGAGT**	
HBV-E2	−1	GGCTTTCGCAAGATTCCTAT−GGAGT	x2
HBV-L2	−1	GGCTTTCGC−AGATTCCTATGGGAGT	x3
HBV-L3	−1	GGCTTTCGCAAGATTCCTAT−GGAGT	x2
HBV-L4	−2	GGCTTT−−CAAGATTCCTATGGGAGT	x2
	wtC14	**ACTCCC▼TCGCCTCGCAGACGAAGGTC**tcaatc	
HBV-L1	−3	ACTCCCTCG−−−CGCAGACGAAGGTC	x3
	−1	ACT−CCTCGCCTCGCAGACGAAGGTC	x2
HBV-L2	−6	ACTCCCTCG−−−−−−AGACGAAGGTC	x2
HBV-L3	−7	ACTCCCTCGCCTCGCAGACGA−−−−−−−aatc	x6

Supplemental Figure 4. HBV target site mutations. Indels identified within the C7 or C14 *Sa*Cas9 sgRNA target sequences in livers of anti-HBV AAV-*Sa*Cas9 treated humanized FRG mice. Bold = sgRNA target site; underlined = PAM sequence; red dash = deletion; lower case = target site adjacent HBV sequence; arrow = *Sa*Cas9 cut site; C7 = HBV + strand; C14 = HBV – strand.

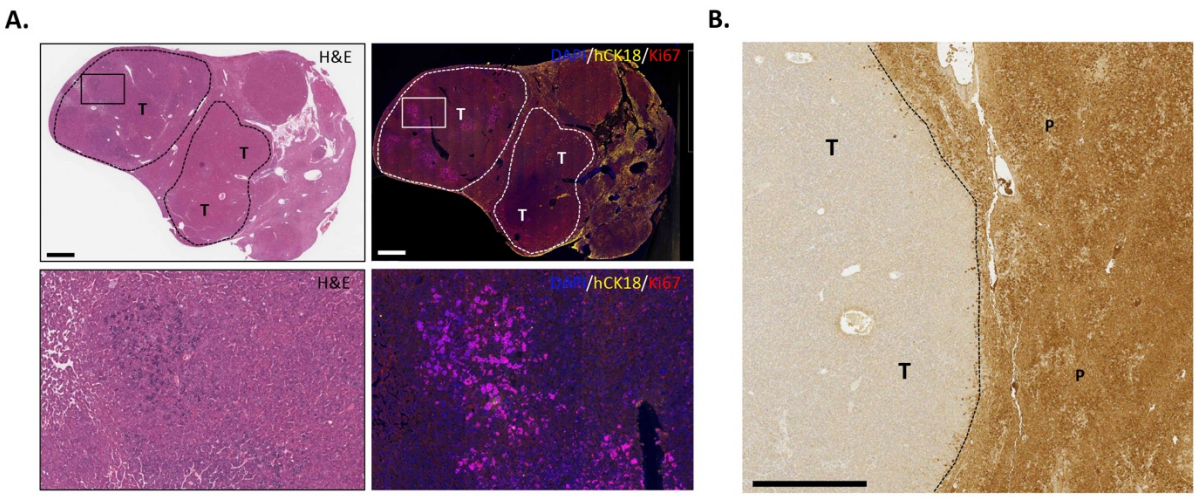


Supplemental Figure 5. Weight and experimental timeline for HBV infected humanized FRG mice. Longitudinal body weights for study animals by treatment group (**A**). * n=1 for group GFP early from days 14-28. Scheduled experimental timeline and actual end point for grouped (**B**) and individual (**C**) HBV infected humanized FRG mice. For each mouse the scheduled (longest or lightest color arrow) and actual (darkest color arrow) experimental duration is indicated (**B**). * scheduled sacrifice; ** found dead; *** sacrificed due to health; # sacrificed early as a histology replacement for the prematurely dead group anti-HBV early mice. The presence or absence of enlarged livers, liver nodules and tumors at death is indicated.



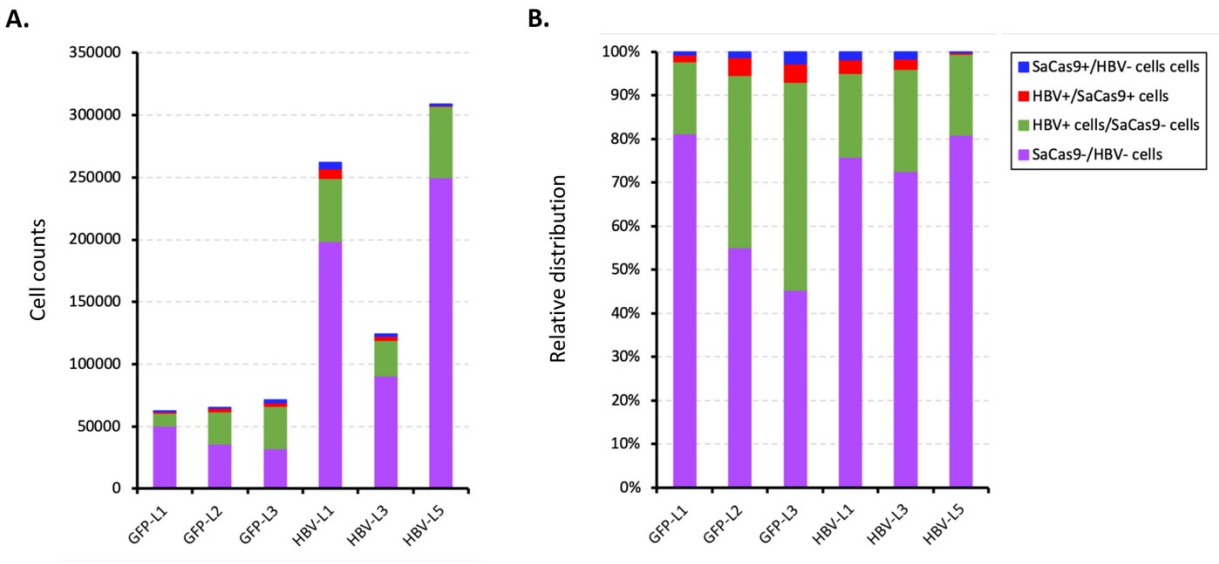
Supplemental Figure 6. H&E staining of humanized FRG mouse livers. Liver lobes are shown for all study mice at the indicated time point post AAV administration. Low power (scale

bar = 1mm) and high power (black inset boxes) images are shown, and liver tumors are indicated (asterisk, adenoma/carcinoma).

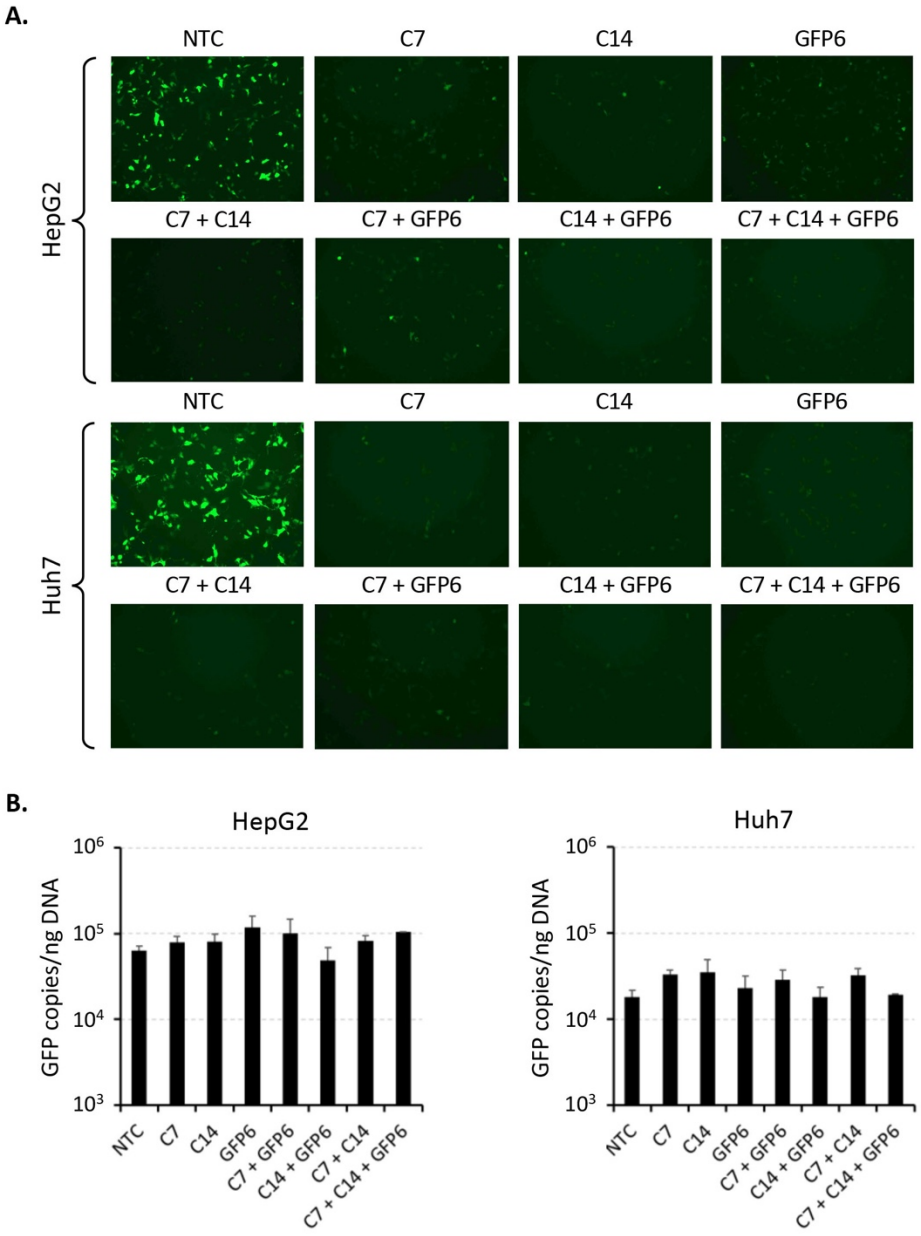


Supplemental Figure 7. Characterization of liver tumors in humanized FRG mouse livers.

A, Serial liver sections from humanized liver FRG mice were stained with hematoxylin and eosin (H&E), or co-labelled via immunohistochemistry with human cytokeratin 18 (hCK18) in combination with Ki67. Sections are shown for animal HBV-L3, which was sacrificed at 62 days post AAV delivery. Asterisks show areas of mouse derived tumor. Lower panels show a Ki67 positive area of a proliferating mouse tumor. **B,** Liver sections were also stained for expression of human fumarylacetoacetase (FAH) to determine the origin of liver tumors. T – tumor; P – liver parenchyma. Dotted lines indicate approximate tumor margins. Scale bars = 1mm.



Supplemental Figure 8. Quantification of HBV+ and *Sa*Cas9+ cells. Liver sections from three AAV-*Sa*Cas9 control and three AAV-*Sa*Cas9 treated humanized liver FRG mice were subjected to RNAscope using custom HBV and *Sa*Cas9-specific probes. Cells in each image were assigned based on DAPI+ staining and those containing foci indicative of HBV and/or *Sa*Cas9 RNA probe hybridization were quantified using a RRScell image analysis algorithm which was used to assign individual cell types as HBV+/*Sa*Cas9+, HBV+/*Sa*Cas9-, HBV-/*Sa*Cas9+ and HBV-/*Sa*Cas9-. Cell counts are from taken non-tumor regions of a single section per animal as shown in **Fig 8**, except for HBV-L3 which uses an average from 2 sections. Total cell counts (**A**) and relative distribution (**B**) are shown for each animal.



Supplemental Figure 9. Template gene editing and degradation. The C7-C14-C16 reporter plasmid (200ng) was transfected into HepG2 or Huh7 cells along with a total of 300ng of *Sa*Cas9/sgRNA expressing plasmids expressing no sgRNA, C7 sgRNA, C14 sgRNA or GFP6 sgRNA as indicated using Lipofectamine 3000. Fluorescent images were taken at 48 hours post

transfection (**A**), and intracellular reporter levels were quantified by GFP-specific qPCR at 72 hours post transfection (**B**).