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Supplemental information

Cytosine base editing inhibits hepatitis B virus replication and reduces HBsAg expression *in vitro* and *in vivo*

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Table S1.
gRNAs introducing Stop-codons (NGG-PAM).

Name	Guide	% Stop Edit (1)	Guide Strand	% Conservation All Genotypes (2)	Stop in HBV Gene
MSPbeam52	TCAATCCCAACAAGGACACC	58,84	1	15,3	Pol
MSPbeam50	GGGAACAAGATCTACAGCAT	51,93	1	22,3	Pol
MSPbeam46	TCCAAGGAATACTAACATTG	50,79	-1	3,0	Pol
MSPbeam47	TTCCAATGAGGATTAAAGAC	45,95	-1	3,3	Pol
MSPbeam54	TGCTCCAGCTCCTACCTTGT	45,57	-1	16,2	Pol
EMSbeam95	CGCCCACCGAATGTTGCCCA	45,29	1	0,2	X
MSPbeam58	CGATAACCAGGACAAGTTGG	44,43	-1	18,3	Pol
MSPbeam191	CTGCCAACTGGATCCTGCGC	41,43	1	72,5	X
MSPbeam56	AGCCACCAGCAGGGAAATAC	41,17	-1	16,7	Pol
MSPbeam51	GGAACAAGATCTACAGCATG	40,48	1	3,3	Pol
MSPbeam190	GCTGCCAACTGGATCCTGCG	36,53	1	76,3	X
MSPbeam53	GACGCCAACAAGGTAGGAGC	36,32	1	15,9	Pol
MSPbeam37	GAAAGCCCAGGATGATGGGA	31,87	-1	40,6	S
MSPbeam49	TGGGAACAAGATCTACAGCA	30,73	1	22,3	Pol
MSPbeam40	CCATGCCCCAAAGCCACCCA	30,21	-1	64,6	Core
MSPbeam55	CCACCAATCGCCAGACAGGA	27,87	1	0,7	Pol
MSPbeam63	GGTCTCCATGCGACGTGCAG	27,5	-1	68,9	Pol
MSPbeam39	AAGCCACCCAAGGCACAGCT	27,31	-1	94,6	Core
MSPbeam57	ACCAGGACAAGTTGGAGGAC	18,71	-1	16,2	Pol
MSPbeam34	TACCGCAGAGTCTAGACTCG	6,7	1	37,1	S
MSPbeam42	CAGGCAAGCAATTCTTTGCT	6,22	1	9,6	Core
MSPbeam61	TCAACGAATTGTGGGTCTTT	5,2	1	23,5	Pol
MSPbeam41	TCAGGCAAGCAATTCTTTGC	4,01	1	9,6	Core
MSPbeam36	CACCACGAGTCTAGACTCTG	3,14	-1	94,3	S
MSPbeam60	CCCCATCTCTTTGTTTTGTT	2,03	-1	5,2	Pol
MSPbeam62	CAACGAATTGTGGGTCTTTT	1,6	1	24,8	Pol
MSPbeam48	TGCAATTGATTATGCCTGCT	1,16	1	8,9	Pol
MSPbeam43	AGGCAAGCAATTCTTTGCTG	0,91	1	9,6	Core
MSPbeam35	CGCAGAGTCTAGACTCGTGG	0,53	1	37,1	S
MSPbeam59	CCCATCTCTTTGTTTTGTTA	0,45	-1	4,9	Pol
MSPbeam44	GGCAAGCAATTCTTTGCTGG	0,32	1	8,2	Core
MSPbeam38	CCACCCAAGGCACAGCTTGG	0,31	-1	94,5	Core
MSPbeam45	GCAAGCAATTCTTTGCTGGG	0,02	1	8,2	Core

(1) Color intensity indicates high-to-low percentage of editing efficiency in HEK293T cells (represented as % Stop Edit)
(2) Color intensity indicates high-to-low percentage of overall conservation across all HBV genotypes.

Table S2.
gRNAs targeting highly conserved sequences across all HBV genotypes and predicted to introduce missense mutations (NGG-PAM).

Name	Guide	Highest % C>T Edit (1)	Guide Strand	% Conservation All Genotypes (2)	Predicted missense in HBV Gene
EMSbeam4	AGGAGTTCCGCAGTATGGAT	54,23	-1	93,2	Pol
EMSbeam20	TCCTCTGCCGATCCATACTG	50,74	1	89,6	Pol
EMSbeam19	TCCGCAGTATGGATCGGCAG	44,63	-1	90,9	Pol
EMSbeam12	GACTTCTCTCAATTTTCTAG	43,91	1	94,4	Pol,S
EMSbeam21	TGGACTTCTCTCAATTTTCT	15,40	1	94,2	Pol,S
EMSbeam23	TTTGCTGACGCAACCCCCAC	6,35	1	90,3	Pol
EMSbeam15	GGACTTCTCTCAATTTTCTA	5,00	1	94,1	Pol,S

(1) Color intensity indicates high-to-low percentage of efficiency to introduce C-to-T editing in HEK293T cells
(2) Color intensity indicates high-to-low percentage of overall conservation across all HBV genotypes.

Table S3.
On-target editing and number of off-target sites detected by rhAmpSeq analysis for each combination of editor and guide RNA.

Base Editor	gRNA37		gRNA40	
	On-target editing, %	Off-target, number of sites	On-target editing, %	Off-target, number of sites
BE4	**59%**	**19**	**79%**	**7**
BE4-PpAPOBEC1	**45%**	**8**	**62%**	**1**
CBE-T	**35%**	**2**	**28%**	**0**

Table S4.
Off-target edit annotation and frequency for each particular base editor with g37 or g40. Blank indicates no detectable off-target editing.

gRNA37			Editing frequency (%)		
Name	Off-target gene annotation	Genome position/ Consequence	BE4	ppApobec1	TadC
chr3:52779133-52779156(+)	ITIH1	Intronic	47.52	3.87	9.30
chr4:105642670-105642693(+)	ARHGEF38	Intronic	44.58	34.14	
chr4:102211240-102211263(-)		Intergenic	20.34	20.00	1.32
chr1:155769412-155769435(+)	GON4L	Intronic	8.21	7.23	
chr19:51365484-51365507(+)	ETFB	Intronic	6.39		
chr9:95900557-95900580(+)	ERCC6L2	Intronic	4.17		
chr17:34837229-34837252(+)	AC022903.1	Intergenic	4.13	2.13	
chr13:113320588-113320611(-)	LAMP1	Intronic	3.02		
chr17:6792613-6792636(-)	TEKT1	Intronic	2.56	1.37	
chr6:99560845-99560868(+)	CCNC	Intronic	2.05	1.12	
chr11:119631394-119631417(-)	NECTIN1	Intronic	1.93	1.65	
chr2:228692395-228692418(-)		Intergenic	0.90		
chr12:98693730-98693753(-)	APAF1	Intronic	0.86		
chr6:75987249-75987272(+)	IMPG1	Intronic	0.76		
chrX:17338831-17338854(+)		Intergenic	0.73		
chr12:88142172-88142195(+)	CEP290	5PRIME_UTR	0.57		
chr12:122044012-122044035(+)	BCL7A	Stop gained/ non-synonymous	0.45		
chr5:146756069-146756092(+)	PPP2R2B	Intronic	0.35		
chr10:126741924-126741947(+)		Intergenic	0.27		
gRNA40			**Editing frequency (%)**		
Name	Off-target gene annotation	Genome position/ Consequence	BE4	ppApobec1	TadC
chr8:29821564-29821587(+)	AC131254.1	noncoding change	4.43	6.61	
chr16:55782506-55782529(+)	CES1P1	Intronic	3.82		
chr14:101522882-101522905(+)		Intergenic	2.25		
chr1:173702424-173702447(+)		Intergenic	1.95		
chr15:72861565-72861588(+)	AC103874.1	Intronic	0.99		
chr1:18923399-18923422(+)	IFFO2	Intronic	0.41		
chr12:83905050-83905073(-)		Intergenic	0.36		

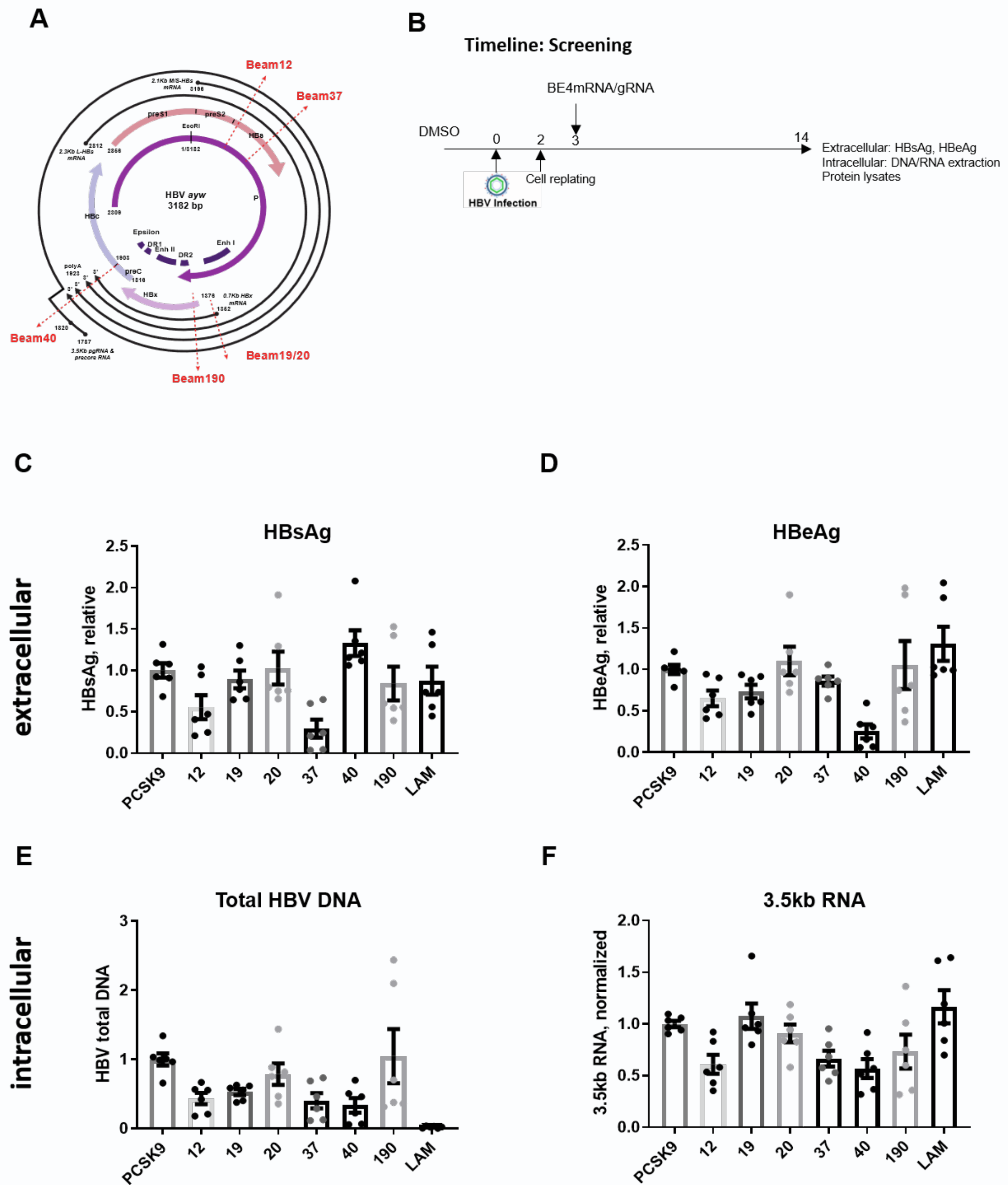


Figure S1. Effect of BE4 with the six selected gRNAs on HBV parameters in HepG2-NTCP cells. (A) cccDNA organization with the location of the selected gRNAs. (B) Schematic representation of the experiments performed in HepG2-NTCP. (C-F) Antiviral parameters assessed 14 days post infection: extracellular HBsAg and HBeAg were measured by ELISA; total HBV DNA was quantified by qPCR from DNA extracted from cell lysates; total cellular RNA was extracted and HBV 3.5kb RNA levels were quantified by qRT-PCR. Data were normalized to the control condition (CBE with *PCSK9* control gRNA). Error bars indicate SEM of 6 replicates. LAM, Lamivudine

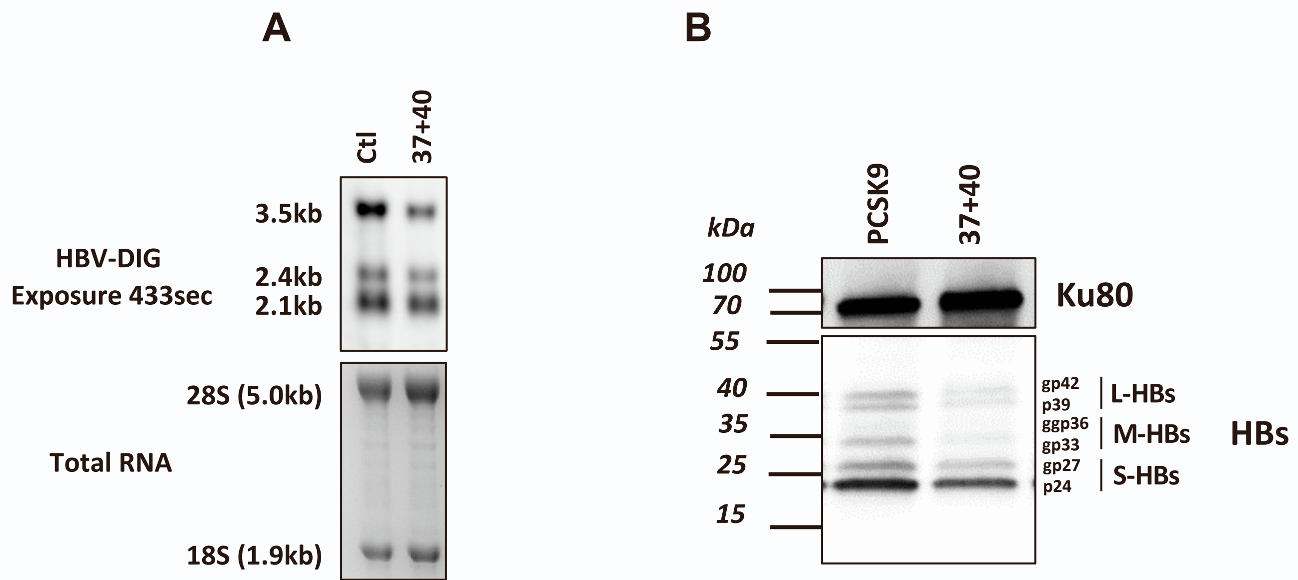


Figure S2. Effect of BE4 with the g37+g40 combination on HBV RNAs and HBs proteins levels in HepG2-NTCP cells. (A) Northern and (B) Western blots showing the effect of the gRNAs (g37+g40) on HBV RNAs and intracellular HBs isoforms, respectively, in 3TC untreated cells. Ctl represents non-transfected condition.

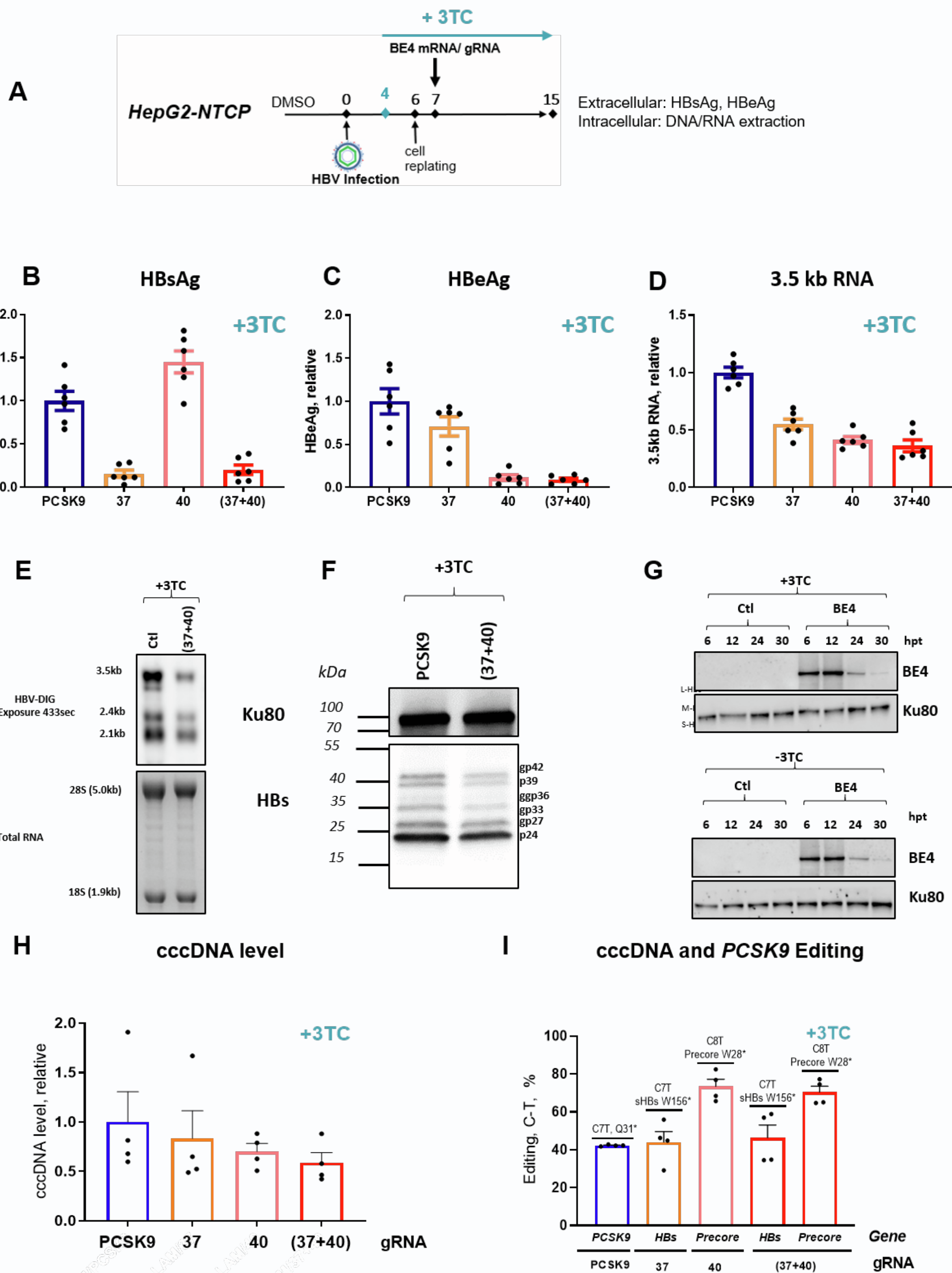
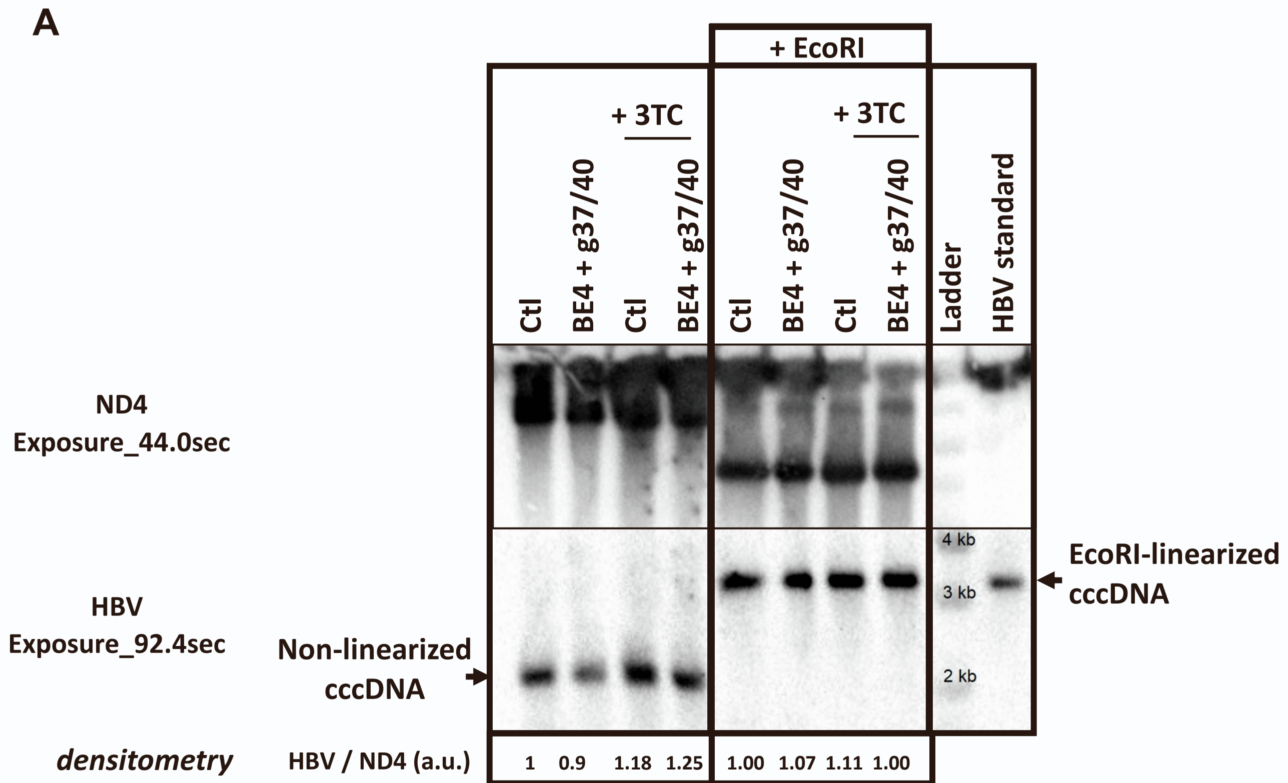


Figure S3: Effect of BE4 with g37, g40 and (g37+g40) on HBV parameters in 3TC-treated HepG2-NTCP cells. (A-D) A protocol similar to Figure 1B was used to test the effect of transfection with BE4 and either gRNA g37, g40, or the combination (g37+g40) on HBsAg, HBeAg, and 3.5kb RNA in 3TC pre-treated cells. (E-F) Northern and Western blots showing the effect of gRNAs (g37+g40) on HBV RNAs and intracellular HBs isoforms, respectively in 3TC pre-treated cells. Ctl represents untransfected condition. (G) BE4 Western blot with Cas9 antibody, showing the delivery and time-dependent expression of BE4 in HepG2-NTCP cells, with or without 3TC pretreatment. Cells were collected at 6 h, 12 h, 24 h and 30 h post transfection. Ku80 was used as endogenous normalizer. (H) cccDNA level was assessed by qPCR on the DNA samples pretreated with ExoI/III in HepG2-NTCP. (I) Level of the C>T functional editing that leads to the introduction of the stop codons in *HBs* and *Precore* genes, assessed by NGS on ExoI/III-treated cccDNA samples from HepG2-NTCP, as well as *PCSK9* (assessed on total DNA). Data are represented as mean ± SEM for n = 4 to 6 replicates



B

Sample	g37site C7T (sHBs W156*)	g40 site C8T (precore W28*)
BE4+g37/40 (-3TC)	47.55 %	60.8 %
BE4+g37/40 (+3TC)	68.12%	66.5 %

Figure S4. Base Editing functions through cccDNA editing, without reducing cccDNA level. (A) Southern blotting was performed on HIRT extracted ExoI/III-digested DNA samples from non-treated or 3TC pre-treated cells. By densitometry analysis, no effect of BE4/(g37+g40) editing on cccDNA was observed. cccDNA band was confirmed by a shift to the expected 3.2kb size upon EcoRI linearization. Ctl represents untransfected condition. (B) Level of the C>T functional editing that leads to the introduction of the Stop codons in *HBs* and *Precore* genes, in HIRT extracted DNA used for Southern blot analysis (HepG2-NTCP).

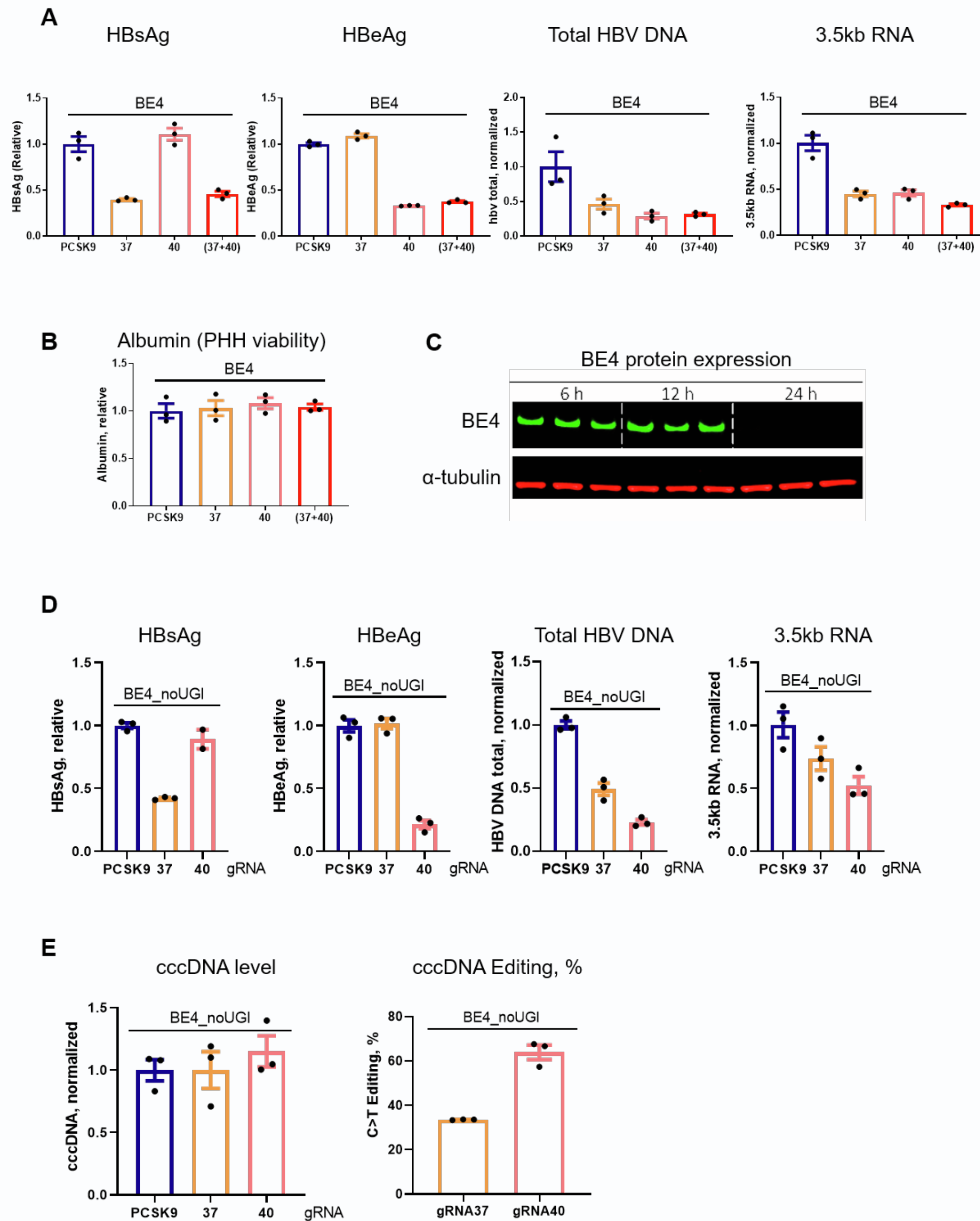


Figure S5. **Antiviral efficacy of the base editing in HBV-PHH.** (A) Transfection with BE4 and selected gRNA g37(Stop-S) and g40 (Stop-Precore) leads to the reduction of the respective viral markers in HBV-PHH. Multiplexing the two lead gRNAs simultaneously reduces HBsAg, HBeAg, total HBV DNA, and 3.5kb RNA. Viral parameters assessed at the end of the experiment, day 25 post infection. (B) Transfection with the base editing reagents does not influence PHH cell functionality – assessed through the measurement of albumin level in the PHH cell supernatant at the end of the experiment (day 25). (C) Expression of the base editor is temporary: BE4 protein is detected within the first 6-12 hours and disappears 24 hours after the mRNA transfection in PHH. (D-E) Base editor lacking uracil glycosylase inhibitor UGI (BE4_noUGI) reduced HBV viral parameters and resulted in robust cccDNA editing but did not affect cccDNA levels in PHH. The data suggests that in our experimental conditions base editing in the absence of uracil glycosylase inhibitor UGI does not promote cccDNA degradation through tethering uracil glycosylase to deaminated cccDNA.

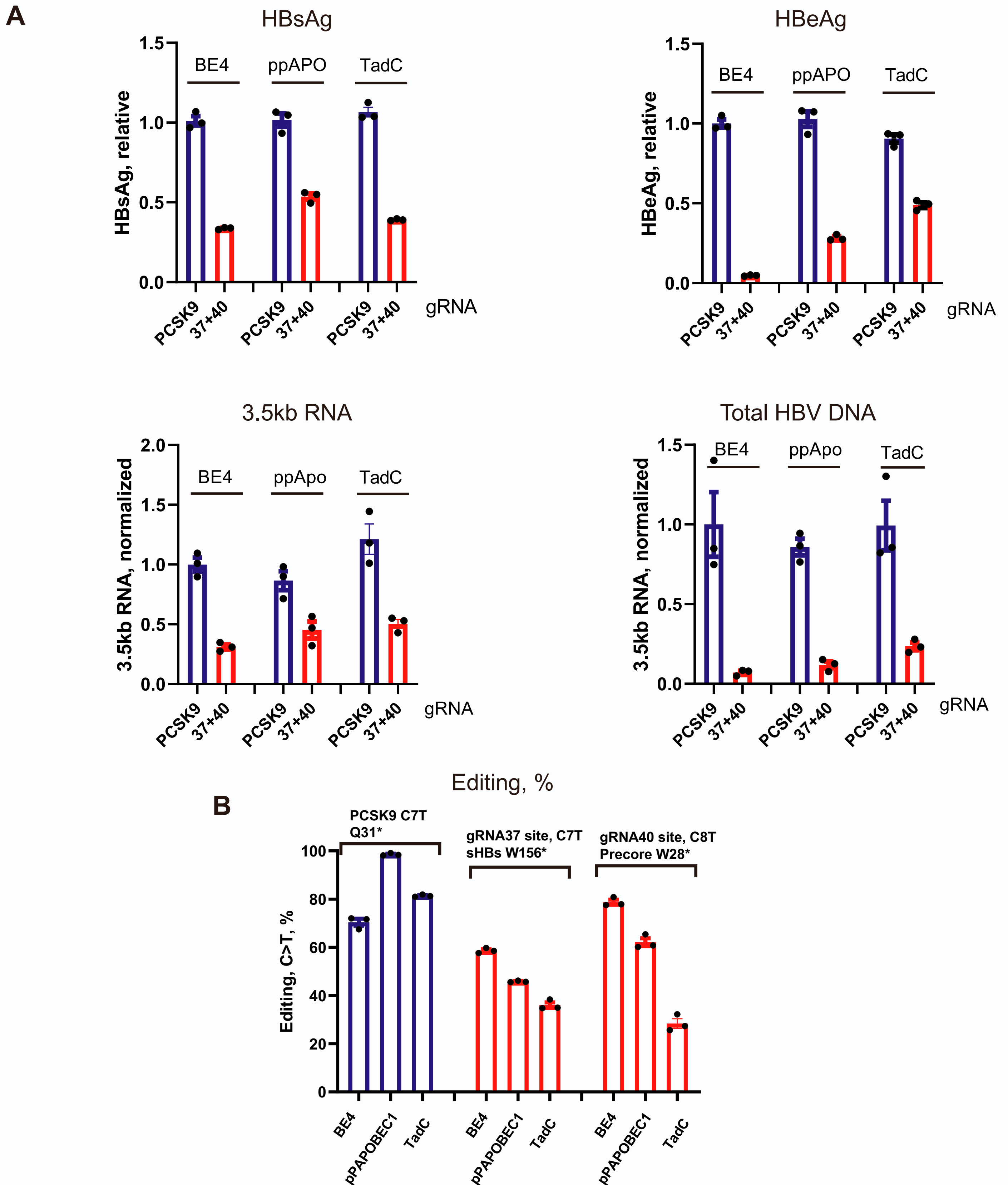


Figure S6. **Anti-viral efficacy of the gRNAs (g37+g40) tested with the next generation cytosine base editor BE4-PpAPOBEC1, CBE-T (TadC) or prototypical BE4.** (A) Viral parameters (HBsAg, HBeAg, 3.5kb RNA and total HBV DNA) were assessed at the end of the experiment, day 25 post infection. (B) C>T functional editing was assessed on ExoI/III pretreated cccDNA samples (g37+g40) and total DNA samples (*PCSK9*).

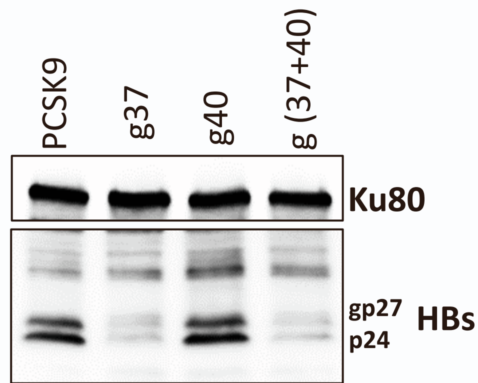


Figure S7. Effect of BE4 with g37, g40 and (g37+g40) on HBs proteins levels in 3TC-treated HepG2.2.15 cells. Intracellular HBs protein levels were assessed by Western blotting in HepG2.2.15 cells after the transfection with the base editing reagents. Ku80 served as an endogenous normalizer.

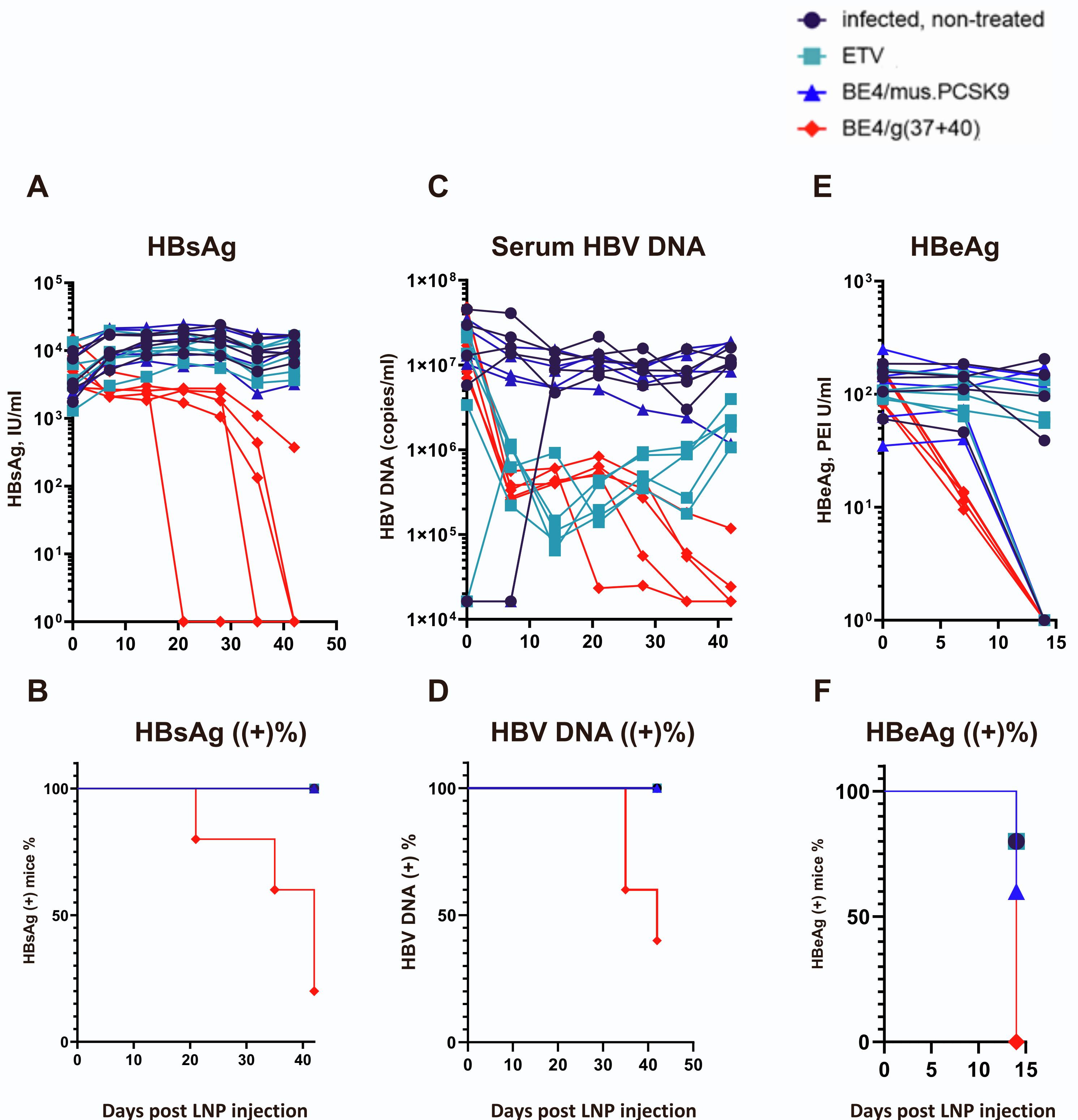


Figure S8. In vivo base editing in HBV minicircle mouse model leads to sustained reduction of viral markers. (A) Serum HBsAg levels were assessed weekly during the study for individual mice. One mouse in HBV-specific LNP treated group died after week 5, for the reasons not related to the treatment. This mouse was HBeAg and HBsAg negative prior to death. (B) Percentages of HBsAg-positive mice in different groups. (C) Serum HBV DNA assessed weekly by qPCR for individual mice. (D) Percentages of HBV DNA-positive mice in different groups. (E) Serum HBeAg levels were assessed weekly during the study for individual mice. (F) Percentages of HBeAg-positive mice in different groups.