Supplementary Legends

Table S1. Primers used in the T7EI assay for each target region.

Table S2. Predicated off-target sites for gRNA-S4 in the human genome.

Table S3. Primers used in the off-target assay for each possible off-target region.

Fig S1. Homologous sequence analysis of gRNA target region in different HBV serotypes.

Fig S2. Efficiency of transfection in HepG2.A64 72 h after puromycin screening was routinely ~90%. Scale bars represent 200 μm.

Fig S3. Representative target S4 region insertion in gRNA-S4-treated cells (A) and HBV-Tg mice (B), generated using the Integrative Genomics Viewer.

Fig S4. Suppression of HBV expression and replication in T11. (A, B) Titers of HBsAg and HBV DNA in cell culture supernatants during 9 consecutive days' cultivation. The HBsAg test results were always negative (<0.05 IU/ml), and the amount of HBV DNA was near or below the negative critical value in T11 (n = 3). (E) The amount of cccDNA in T11 and N1on the 9th day after consecutive cultivation. (F) The amount of HBV DNA inside viral nucleocapsid in T11 was 10,000-fold lower than that in N1 on the 9th day after culture consecutive.

Fig S5. Primers that amplify the A1AT gene and HBS-gene were used to detect genomic DNA contamination. DNA was amplified from genomic DNA by A1AT gene specific primers, but not from HBV cccDNA.

Primers	Sequence (5'-3')							
S4-F	ACCCTGCGCTGAACATGGAG							
S4-R	AAGCCCTACGAACCACTGAACAA							
S5-F	TCAACTACCAGCACGGGACC							
S5-R	GCCCAAAAGACCCACGATTC							
XP-F	GCAGGCTTTCACTTTCTCGC							
XP-R	GGAACGGCAGATGAAGAA							
URR-F	CGCTCCGAAAGTTTCCTT							
URR-R	GAGTGCGAATCCACACTCC							
BCP-F	CCTTCTTCATCTGCCGTTCC							
BCP-R	ATGTCCATGCCCCAAAGCC							

Table S1. Primers used in the T7EI assay for each target region.

Table S2. Predicated off-target sites for gRNA-S4 in the human genome.

No.	Sequence	Score	Mismatches	UCSC gene	Locus			
1	GCCCTCTCTGGATGTGTCTGAGG	1.6	3MMs [3:4:7]		chr11:+75047287			
2	GCTGTTGCTAGATGTGTCTGAAG	1.4	3MMs [4:6:10]		chr8:+47864962			
3	TGTTTCGTTGGATGTGTCTGAGG	1.4	4MMs [1:2:4:8]		chr17:-74488431			
4	AATTTCGTTGGATGTGTCTGTGG	1.4	4MMs [1:2:4:8]		chr10:-36941610			
5	CCTAGCGCTGGATGTGACTGTGG	1.3	3MMs [1:5:17]		chr22:+49133545			
6	CCTGTCGCTGGAGGTGTCTGTGG	1.2	3MMs [1:4:13]	NR_024527	chr11:-118015895			
7	TCTAGCATTGGATGTGTCTGTGG	0.9	4MMs [1:5:7:8]		chr7:-20747255			
8	ACTTTCCCTTGATGTGTCTGAAG	0.9	4MMs [1:4:7:10]		chr5:-125646588			
9	CTAATGGCTGGATGTGTCTGGGG	0.8	4MMs [1:2:3:6]		chr5:+178213889			
10	TCCGTGGCTGGATGTGTCTGAGG	0.8	4MMs [1:3:4:6]		chr18:-10706739			
11	GCAGGTGCTGGATGTGTCTGGGG	0.8	4MMs [3:4:5:6]	NM_020676	chr3:+58279310			
12	GCATTTGCTTGATGTGTCTGGGG	0.8	4MMs [3:4:6:10]		chr1:+194264939			
13	GGTGTCGCTCGCTGTGTCTGTAG	0.7	4MMs [2:4:10:12]		chr2:+69959464			
14	GCGGACGCTGGATGTGGCTGCAG	0.6	4MMs [3:4:5:17]		chr2:+129349323			
15	CCTCTCCCCGGATGTGTCTGGGG	0.6	4MMs [1:4:7:9]		chr8:+99952106			
16	CCTAAAACTGGATGTGTCTGTGG	0.6	4MMs [1:5:6:7]		chr7:-57842027			
17	GAAATAACTGGATGTGTCTGCAG	0.5	4MMs [2:3:6:7]		chr3:+2365959			
18	GATAACCCTGCATGTGTCTGAGG	0.5	4MMs [2:5:7:11]		chr8:-115773972			
19	GCTGCACCTGGATGTGTCTGGGG	0.5	4MMs [4:5:6:7]		chr10:+50257134			
20	CCTGTTGCAGGATGTGTCTGCAG	0.5	4MMs [1:4:6:9]		chr14:+99788041			

Primers	Sequence (5'-3')						
off-target1F	GGAAACCCCTCAGCTCACTC						
off-target1R	CTGGCATGGACAGCTCAGAA						
off-target2F	CTAGGGCAGATTAGGGGGCCA						
off-target2R	CATGACAAGTCCACCCGTCC						
off-target3F	CCTGCCCCATAAAGCCCATA						
off-target3R	GATGCCAAAGAGACCTGGGG						

Table S3. Primers used in the off-target assay for each possible off-target region.

Figure S1

gRNA-S3						gRNA-SP-II										
Sequence Name	< Pos = 24	D						Sequence Name	< Pos = 3071							
+								+								
Consensus	TACCACAGAGT	CTAGACTCGTGGT	GGACTTCTCTC	AATTTTCTA	AGGGGGAGC	ACCCACGTO	FICCIGG	Consensus	GAGGICTITIGGGG	IGGAGCCCTC	AGGCTCAGGG	CATATTGACAA	CAGTGCCAGC	AGCGCCTCCT	CCTGCCTCCAC	CAATCGG
5 Sequences	40 25	0 260	270	280	290	30	10	5 Sequences	'0 3080	3090	3100	3110	3120	3130	3140	3150
HQ638218.1	TACCACAGAGT	CTAGACTCGTGGT	GGACTTCTCTC	AATTTTCTA	GGGGGGAGC	ACCCACGTO	TCTTGG	HQ638218.1	GCGGTCTTTTGGGG	IGGAGCCCTC	AGGCTCAGGG	CATATTGACAC	CAGTGCCAGC	AGCGCCTCCT	CCTGCCTCCAC	CAATCGG
EF103278.1	TACCGCAGAGT	CTAGACTCGTGGT	GGACTTCTCTC	AATTTTCTA	GGGGGACC	ACCCGTGTG	TCTTGG	EF103278.1	GAGGTGTCTTGGGG	IGGAGCCCIC	AGGCTCAGGG	CATATIGGCCA	CAGTGCCAGC	AGTGCCTCCT	CCTGCCTCCAC	CANTOGA
NC_003977.1	TACCACAGAGT	CTAGACTCGTGGI	GGACTTCTCTC	AATTTTCTA	GGGGGGAGC	ACCCACGTO	FICCIGG	NC_003977.1	GAGGCCTTTTGGGG	IGGAGCCCTC	AGGCTCAGGG	CATATTGACAA	CACTGCCAGC	AGCACCTCCT	CCTGCCTCCAC	CAATCGG
AB299858.1	TACCACAGAGT	CTAGACICGIGGI	GGACITCICIC	AATTTICIA	GGGGGGAGC	ACCCACGIG	FICCIGG	AB299858.1	GCGGTCTTTTGGGG	IGGAGCCCTC	AGGCTCA	CATATIGICAA	CAGTGCCAGT	AGCCCCTCCT	CCTGCCTCCAC	CANTOGG
gRNA-54	4							gRNA-2	(P							
Sequence Name	< Pos = 346							Sequence Name	<pre> < Pos = 1232</pre>							
-	CTOTTGTCCTCCAN	TTTGTCCTCCCT	COTOSTOTOTOTO	TACAGOST	******		COTOCTO	-								
Consensus 5 Sequences	350 3	60 370	380	390	400	410	420	Consensus	GGCGCATGCGTGG	AACCTITIGTG	SCTCCTCTG	CCGATCCATAC	TGCGGAACTO	CTAGCAGCTI	IGTITIGCTCO	SCAGCCG
80638218.1	CTCTTGTCCTCCAA	TTTGTCCTGCTAT	GOTGGATGTGT	CGGCGTT	TTATCATATT	CTCTTCAT	CTGCTG	5 Sequences	1 1240	1250	1260	1270	1280	1290	1300	131
U95551.1	CTCCTGTCCTCCAA	CTTGTCCTGGTTAT	CGCTGGATGTGT	TGCGGCGTT	TTATCATCTT	CCTCTTCAT	CCTGCTG	HQ638218.1 U95551.1	AGCGCGTGCGTGG	AACCITITGIG	GCTECTCIG	CCGATCCATAC	RECGGAACTO	CTAGCAGCT	GITTIGCTCG	GCAGCCG(GCAGCAG)
EF103278.1	CTCCTGTCCTCCAA	ITTGICCIG GITAT	CGCTGGATGTGTC	TECGGCGITI	ΤΤΑΤCΑΤΑΤΤ	CCTCTTCAT	CCTGCAG	EF103278.1	AGCGCATGCGTGG	ACCTITIGTG	TCTCCTCTG	CCGATCCATAC	тессбаласто	CAAGCCGCTT	GGTTTGCTCG	GCAGCCG
AB299858.1	CTCTTGTCCTCCAX	TTTGTCCTGGCTAT	CGCTGGATGTGTG	TECGGCGIT	TTATCATATT	CCTCTTCAT	CCIGCIG	NC_003977.1	GGCGCATGCGTGG	AACCITIGIG	GCT CCTCTG	CCGATCCATAC	TGCGGAACTC	CTAGCAGCTI	IGTITIGCTCO	SCAGCCG4
gRNA-S	5							gRNA-0	CP-BCP							
Sequence Name	< Pos = 666							Sequence Name	e < Pos = 1844							
								-								
Consensus	TCTCCTGGCTCAGT	TTACTAGTGCCAT	TGTTCAGTGGTT	CGTAGGGCT	TTCCCCCACT	GTTTGGCTT	TCAGTTA	🔀 Consensus	ATCTCATGTTCAT	GTCCTACTG	TTCAAGCCT	CCANGCTGTG	CTTEGETEG	CTTTGGGGGCA	TGGACATTGA	ACCCGTA
5 Sequences	670 6	690 690	700	710	720	730	74	5 Sequences	1850	1860	1870	1880	1890	1900	1910	192
HQ638218.1	TCTCATGGCTCAGT	TICTTAGIGCCAT	TGITCAGIGGI	CGTAGGGCT	TTCCCCCACT	GITIGGCTT	TCAGITA	HQ638218.1	ATCTCATGTTCAT	GTCCTACTG	TTCAAGCCT	CCAAGCTGTGG	CONT GGGTGG	CTITGGGGGCA	TGGACATTGA	ACCCGTA
EF103278.1	TCTCTTGGCTCAG	TTACTAGTGCCAT	TGTTCAGIGGT	CGTAGGGCT	TTCCCCCACT	GTTTGGCTT	TCAGCTA	EF103278.1	ATCTCTTGTACAT	GTCCCACTT	TTCAAGCCT	CCAAGCTGTG	GGATGG	CTTTGGGGGCA	TGGACATTGA	ACCCTTA
NC_003977.1	TCTCCTGGCTCAGT	TTACTAGTGCCAT	TGTTCAGTGGTT	CGTAGGGCT	TTCCCCCACT	GTTTGGCTT	TCAGCTA	NC_003977.1	ATCTCATGTTCAT	GICCIACIG	TTCAAGCCT	CCAAGCTGTGG	CTT GGGTGG	CTTTGGGGGCA	TGGACATTGA	ACCCGTA
AB299858.1	TCTCCTGGCTCAG	TTACTAGIGCCAT	TGITCAGIGGI	CGTAGGGCT	TTCCCCCACT	GIIIGGCII	TCAGTTA	AB299858.1	ATCTCATGTTCAT	GTCCTACTG	TTCAAGCCT	CCAAGCTGTG	GGGIGG	CTTTGGGGGCA	төөлслттөл	ACCCGTA
gRNA-SI	P-I							gRNA-0	CP-URR							
Sequence Name	< Pos = 2405							Sequence Name	< Pos - 1622							
+								Sequence ivanie	< P05 = 1052							
Consensus	CGAAGGTCTCAATC	GCCGCGTCGCAGAA	GATCTCAATCTCO	GGAATCTCA	ATGTTAGTAT	CCCTTGGAC	TCATAAG	Consensus	CACCAGGICTIGCCCAA	GICTIACATA	AGAGGACTCTT	GGACTCTCAGC	ANTGTCANCON	CGACCTTGAG	SCATACTTCAAA	GACTGTT:
5 Sequences	2410 2	420 2430	2440	2450	2460	2470	248	5 Sequences	1640 1	650 16	160 16	70 1680	1690	1700	1710	1720
HQ638218.1	CGAAGGTCTCAATC	GCCGCGTCGCAGAA	GATCTCAATCTC	GGAATCTCA	ATGTTAGTAT	CCCTTGGAC	SCATAAC	HQ638218.1	CACCAGGTCTTGCCCAA	GICTIACATA	AGAGGACTCTT	GGACTCTCAGC	ATGTCAACGA	CGACCTTGAG	CATACTTCAAA	GACTGIT:
EF103278.1	CGAAGATCTCAATC	GCCGCGTCGCAGAA	GATCTCAATCTC	GGAATCTCA	ATGTTAGTAT	CCTTGGAC	TCATAAG	095551.1 EF103278.1	CACCGAATGTTGCCCAAG	GICTTACATA	AGAGGACTCTT	GGACTCCCAGC	ATGTCAACGAO AATGTCAACGAO	CGACCTTCAG	CATACITCAAA SCCTACITCAAA	GACTGTT.
NC_003977.1	CGAAGGTCTCAATC	GCCGCGTCGCAGAA	GATCTCAATCTC	GGAATCTCA	ATGTTAGTAT	CCCTTGGAC	TCATAAG	NC_003977.1	CACCAGGTCTTGCCCAA	GICTIACACA	AGAGGACTCTT	GGACTCTCAGC	ATGTCAACGA	CGACCTTGAG	CATACTTCAAA	GACTGTT
AB299858.1	CGAAGGTCTCAATC	GCCGCGTCGCAGAA	GATCTCAATCTC	GGAATCTCA	ATGTTAGTAT	CCCTTGGAC	ICATAAG	AB299858.1	CACCAGGTCTTGCCCAA	GICTIACATA	AGAGGACTCTT	GGACTCTCAGC	MATGTCAACGAO	CGACCTTCAG	CGTACTTCAAA	GACTGTT:

Fig S1.Homologous sequence analysis of gRNA target region in different HBV serotypes.

Figure S2



Fig S2. Efficiency of transfection in HepG2.A64 72 h after puromycin screening was routinely \sim 90%. Scale bars represent 200 μ m.



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Figure S4



Fig S4. Representative target S4 region insertion in gRNA-S4-treated cells (A) and HBV-Tg mice (B), generated using the Integrative Genomics Viewer.



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