

Development of an investigational epigenetic silencer therapy to transcriptionally inactivate viral DNA in chronic hepatitis B

Received: 11 January 2026

Accepted: 27 August 2026

Published online: 21 September 2026

 Check for updates

Yesseinia Anglero-Rodriguez^{1,6}, Qiang Xiong^{1,6},
Martino Alfredo Cappelluti², Sarah B. Voytek¹, Glen Acosta¹,
Rayman Choo-Wing¹, Lenka Hoffman¹, Arogya Khadka¹, Jhon A. Medina¹,
Caroline Mugambwa¹, Chloe Pantano¹, Sivan Harel¹, Amber DiPiazza¹,
Sameer Abraham¹, Erica M. Hildebrand¹, Ricardo N. Ramirez¹, Ava Zhai¹,
Xiaoyun Guo¹, Sahar Abubucker¹, Chih-Wei Ko¹, Cristiana Giancarlo¹,
Bradley Niesner¹, Matteo Conti^{2,3}, Lorena Donnici³, Pietro Spinelli¹,
Taesun Eom¹, Mary S. Morrison¹, Ari E. Friedland¹, Raffaele De Francesco^{3,4},
Angelo Lombardo^{2,5}, Vic E. Myer¹, Aron B. Jaffe¹, Melissa Bonner¹ &
Jennifer L. Marlowe¹✉

Approved chronic hepatitis B therapies rarely result in functional cure, as they fail to permanently silence all transcriptionally active hepatitis B virus (HBV) DNA. CRMA-1001 employs an optimized epigenetic silencer that represses viral transcription through targeted deposition of DNA methylation on episomal and integrated HBV DNAs. Here we showed that in HBV mouse models, a single dose of CRMA-1001 produced $>3\text{-log}_{10}$ reductions in viral biomarkers, which correlated with de novo methylation of HBV DNA. Three doses resulted in up to 90% of animals with undetectable hepatitis B surface antigen and HBV DNA by 6 months post treatment. In non-human primates, CRMA-1001 induced transient liver transaminase elevations only at the highest dose tested. Expression and DNA methylation profiling revealed no detectable unintended targets in the human genome. These findings support the initiation of clinical development of CRMA-1001 as a finite treatment course with the potential for functional cure in individuals living with chronic hepatitis B.

Chronic hepatitis B (CHB) is a serious liver disease, affecting ~250 million people globally, and associated with over 1 million deaths annually from cirrhosis and hepatocellular carcinoma¹. CHB results from unresolved infections of the hepatitis B virus (HBV), an enveloped and partially double-stranded DNA virus. HBV has multiple DNA forms and persists owing to maintenance of covalently closed circular DNA (cccDNA) in nuclei and integrated HBV DNA (intDNA) in the genome of infected hepatocytes². Relaxed circular DNA (rcDNA) is the primary

form of DNA found within viral particles and is the precursor to cccDNA. cccDNA acts as a template for viral RNA transcription and is the driver of HBV persistence. The presence of cccDNA is essential for the synthesis of all viral proteins and production of infective HBV virions. HBV integration does not lead to replication-competent viral particles but is a persistent source of viral messenger RNAs (mRNAs) and proteins, including hepatitis B surface antigen (HBsAg), a component of the viral envelope. High HBsAg serum levels lead to suppression of the host

¹nChroma Bio, Boston, MA, USA. ²San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy. ³Istituto Nazionale Genetica Molecolare ‘Romeo ed Enrica Invernizzi’, Milan, Italy. ⁴Department of Pharmacological and Biomolecular Sciences (DiFeB), University of Milan, Milan, Italy. ⁵Vita-Salute San Raffaele University, Milan, Italy. ⁶These authors contributed equally: Yesseinia Anglero-Rodriguez, Qiang Xiong.

✉e-mail: jmarlowe@nchromabio.com

immune response, resulting in an inability to produce a robust antiviral response required for viral clearance³. Thus, effective therapies for CHB must ultimately address both forms of viral DNA. Elimination or inactivation of cccDNA and intDNA reduces production of viral proteins, precludes the production of infective virions and prevents the production of additional viral proteins, including HBsAg.

HBV gene expression is also regulated by epigenetic modifications, particularly DNA methylation at CpG dinucleotide-rich regions known as CpG islands (CGIs)^{4–6}. The HBV genome contains three CGIs (I, II and III) whose methylation status influences viral replication and serum antigen levels in patients. Specifically, methylation of CGI II and III inversely correlates with cccDNA transcription, lower HBeAg and HBsAg levels, and decreased viral titres^{5,7}.

Nucleos(t)ide analogues (NUCs) remain first-line therapy for CHB, effectively suppressing HBV DNA replication and reducing progression to cirrhosis and hepatocellular carcinoma⁸. However, they rarely achieve HBsAg seroclearance, and most patients do not reach functional cure, defined as sustained loss of HBsAg and undetectable HBV DNA for at least 6 months after stopping therapy^{9,10}. Persistent HBsAg expression from both cccDNA and intDNA contributes to immune tolerance³, underscoring the need for curative strategies that target both viral reservoirs to halt replication, block antigen production and restore antiviral immunity. Long-term adherence to NUCs is challenging¹¹, and discontinuation can trigger relapse or severe flares. Consequently, finite-course therapies capable of achieving functional cure at rates notably above standard of care would offer substantial clinical benefit.

Current investigational approaches, including direct-acting antivirals, immune modulators and RNA silencers, have shown limited ability to sustain HBsAg loss with or without continued use of NUCs¹². More recently, therapeutic modalities with the potential for a durable effect, such as gene editing and epigenetic silencing (ES), have demonstrated promise in preclinical models and have advanced into early clinical development^{13,14}. CRMA-1001 is an ES therapeutic candidate comprising a guide RNA (gRNA) and an mRNA co-encapsulated in a lipid nanoparticle (LNP) for delivery to hepatocytes. The mRNA encodes a single epigenetic effector (EE) fusion protein containing a catalytically inactive *Streptococcus pyogenes* Cas9 (dSpCas9 or dCas9) DNA-binding domain, a Krüppel-associated box (KRAB) transcriptional repression domain and a DNA methylation effector domain consisting of a fusion of the catalytic domain of DNMT3A and the catalytic-like domain of DNMT3L¹⁵. The gRNA directs the EE protein to a specific locus in HBV DNA, where it harnesses the cell's innate epigenetic regulatory machinery to methylate CpGs up to ± 1.5 kb from the gRNA binding site^{16,17}. This targeted modification silences transcription without cutting or nicking the underlying DNA sequence^{17–19}. Although the EE protein and gRNA are short-lived in hepatocytes, these epigenetic changes persist, thus resulting in durable viral gene suppression. Long-term hepatic gene silencing (>1 year) has been reported in mice and in non-human primates (NHPs) for ES platforms with both zinc finger-based and dCas9-based DNA-binding domains^{15,20}. Remarkably, hepatocytes maintain ES-induced methylation and gene suppression following liver regeneration, confirming sustained pharmacology following in vivo cell proliferation¹⁵.

The present study reports on the discovery and preclinical development of CRMA-1001, an ES candidate for CHB, including pharmacology in multiple in vitro and in vivo models, safety and biodistribution in NHPs, and specificity in primary human cells. Through its ability to durably silence HBV transcription from both cccDNA and intDNA reservoirs, CRMA-1001 may offer a finite-course therapeutic strategy with the potential for functional cure for individuals living with CHB.

Screening and identification of HBV-targeted gRNAs and optimization of epigenetic silencer potency

A panel of 121 HBV-targeted gRNAs (HBV-gRNAs) spanning all viral open reading frames (ORFs) across the HBV genome was rationally

designed to achieve broad genotype coverage and maximal target site conservation. The design process leveraged ~7,000 full-length HBV genome sequences spanning genotypes A–E from the HBV database (HBVdb)²¹ (Fig. 1a and Extended Data Fig. 1a). HBV-gRNAs were prioritized on the basis of complete nucleotide identity of the 20-mer spacer sequence across multiple genotypes, presence of a downstream NGG protospacer adjacent motif (PAM) required for dSpCas9 binding activity and localization proximal to a CGI.

All 121 HBV-gRNAs were screened in vitro using HepG2-NTCP cells infected with HBV genotype D and transfected with LNPs containing an EE mRNA and individual gRNAs. HBsAg and HBeAg were quantified in supernatants at 6 days post transfection. Overall, 51 HBV-gRNAs demonstrated >50% silencing of either HBsAg or HBeAg, with 25 showing concurrent silencing of both biomarkers (Extended Data Fig. 1b,c). These active HBV-gRNAs were distributed throughout the HBV genome, suggesting no preferential hotspot for targeted ES of the HBV.

Four HBV-gRNAs (HBV-gRNA1–4) were selected for additional investigation on the basis of antiviral activity, genomic target location and conservation across HBV genotypes. Silencing efficiency was assessed in HepG2.2.15 cells, which stably express integrated full-length HBV genomes²². Cells were treated with the early generation EE4000 construct along with the individual HBV-gRNAs. All candidates demonstrated dose-dependent suppression of viral antigens, with HBV-gRNA2 achieving the most pronounced and durable silencing, sustained over a 4-week observation period including continued cell proliferation (Fig. 1b,c). Importantly, in silico analysis of the HBV-gRNA2 binding site across more than 8,000 genome sequences available in the HBVdb²¹ (Extended Data Fig. 1d,e) revealed 94% conservation across genotypes A–H. Bisulfite sequencing of CpG dinucleotides within the HBV genome revealed a correlation between average methylation (Extended Data Fig. 2) and HBsAg silencing efficiency (Fig. 1c) of the four candidate HBV-gRNAs, with HBV-gRNA2 showing the highest levels of de novo methylation.

Next, an AAV-HBV mouse model harbouring episomal HBV DNA (genotype D) was used to assess relative antigen suppression by the lead gRNAs (Fig. 1d). Each HBV-gRNA was co-encapsulated with EE4000 in an LNP formulation and intravenously administered to AAV-HBV mice. Consistent with in vitro experiments, HBV-gRNA2 demonstrated the largest HBsAg reduction in AAV-HBV mice. Collectively, these results supported selection of HBV-gRNA2 for inclusion in an ES therapeutic candidate.

Then, an endogenous surrogate target was utilized to optimize the EE protein for induction of target methylation and subsequent silencing. The *PCSK9* gene is expressed in hepatocytes and therefore provides a relevant surrogate target in animal models that cannot be infected with HBV, such as NHPs. Using gRNAs cross-reactive to the human and cynomolgus *PCSK9* loci, three EEs (EE4000, EE6000 and EE7000), each employing a different methylation effector domain amino acid sequence (Fig. 1e), were evaluated in both a transgenic mouse model harbouring two copies of the human *PCSK9* locus (Tg-hPCSK9) per diploid host genome²³ and in NHPs. The EE6000 and EE7000 constructs demonstrated potency gains of approximately twofold and fourfold, respectively, over EE4000 in Tg-hPCSK9 mice (Fig. 1f). Similar results were observed in NHPs (Fig. 1g). As previously reported by Tremblay et al.¹⁵, EE7000 achieved saturating reductions in *PCSK9* in NHPs at a single dose of ≥ 1 mg kg⁻¹, with durability of at least 2 years demonstrated herein (Fig. 1h,i and Extended Data Fig. 3). Therefore, EE7000 was selected, along with HBV-gRNA2, for incorporation into the final development candidate CRMA-1001.

CRMA-1001 durably suppresses transcription from intDNA in vivo and in vitro

A transgenic HBV mouse model (Tg-HBV) containing two copies of 1.28×-length HBV genotype A per diploid host genome was used to evaluate the ability of ES to durably silence intDNA²⁴. Proof-of-concept data

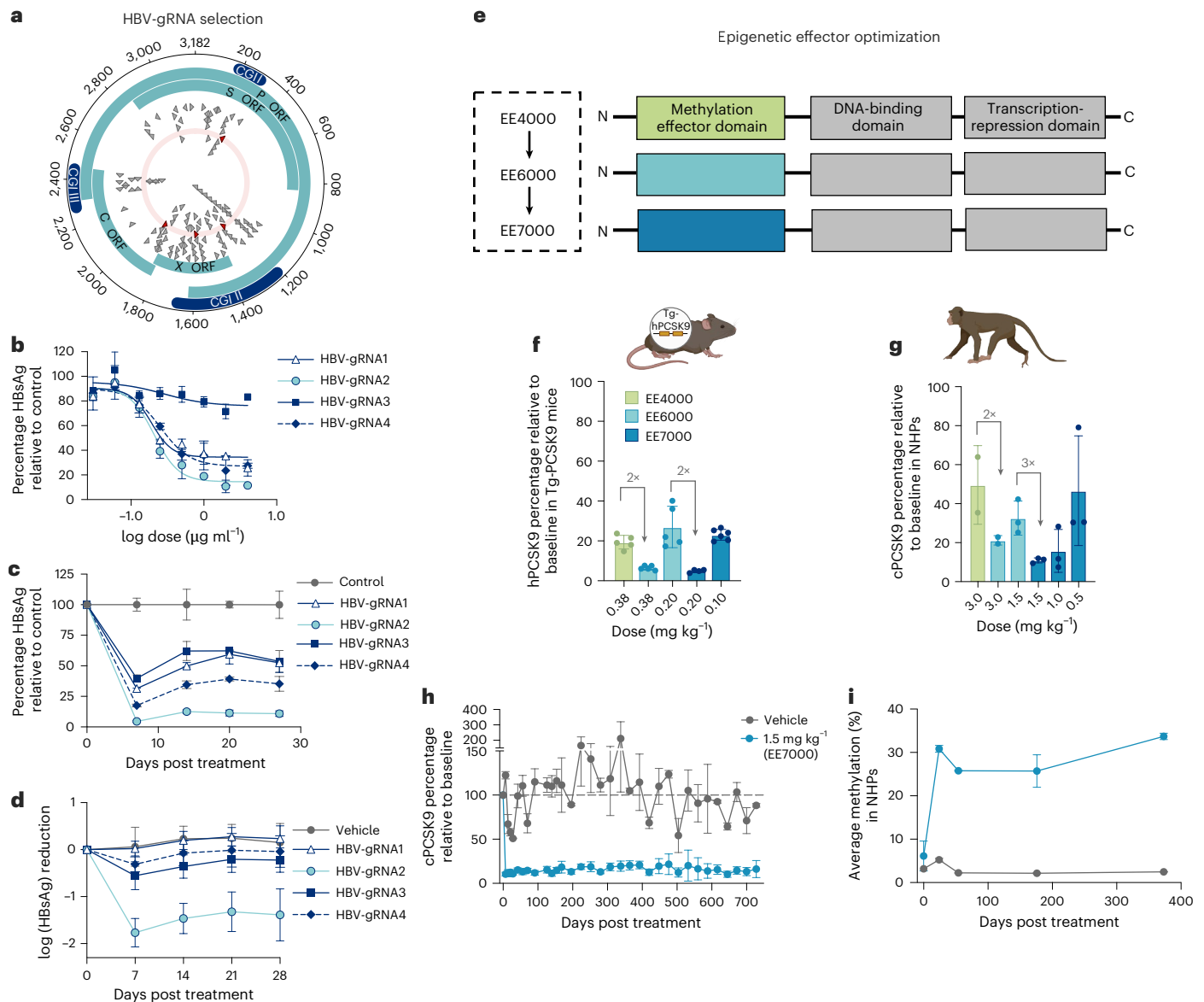


Fig. 1 | Design and functional evaluation of HBV-targeting gRNAs and optimization of effector mRNA potency. **a**, A schematic representation of 121 HBV-targeting gRNAs designed for in vitro screening. Grey triangles indicate the position and orientation of a gRNA target site across the HBV genome, red triangles indicate the lead HBV-gRNAs. Viral ORFs are shown in teal, and CGIs are highlighted in dark blue. **b**, Dose–response curves at 13 days post treatment (relative to control (rhApoE3, mock treatment) mean $\pm$ s.d.; $n = 2$ biological replicates per group). **c**, Longitudinal assessment through 27 days (percentage relative to control (rhApoE3, mock treatment) mean $\pm$ s.d.; $n = 3$ biological replicates per group). In **b** and **c**, HBsAg levels in HepG2.2.1B5 cells following treatment with lead HBV-gRNAs are shown. **d**, Plasma HBsAg levels in AAV-HBV mice for 28 days post treatment with a single 1 mg kg^{-1} dose of LNP-formulated EE4000 and lead HBV-gRNAs. HBsAg levels before and post treatment were measured by ELISA and are shown as mean log₁₀ fold change from baseline $\pm$ s.d.; $n = 5$ animals per group. **e**, Optimization to EEs 4000, 6000 and 7000 focused

on the amino acid sequence of the methylation effector domain, which consists of a fusion of the catalytic domain of DNMT3A and the catalytic-like domain of DNMT3L. N, amino terminus; C, carboxyl terminus. **f**, Plasma human PCSK9 (hPCSK9) reduction measured on day 14 post treatment of various EEs in a transgenic mouse model (Tg-PCSK9, $n = 4$ –6 animals per group). **g**, Plasma cynomolgus PCSK9 (cPCSK9) reduction measured on day 28 post treatment of various EEs in NHPs ($n = 2$ animals in 3.0 mg kg^{-1} dose groups and 3 animals in all other dose groups). **h**, Longitudinal evaluation of pharmacological effects in NHPs following a single dose of 1.5 mg kg^{-1} of PCSK9-targeting EE7000: plasma cPCSK9 (**h**) up to 2 years post treatment and cPCSK9 locus methylation (**i**) up to ~1 year in liver biopsies shown as mean methylation $\pm$ s.d.; $n = 2$ for vehicle and $n = 3$ for EE7000. In vivo potency comparison of different EEs against the surrogate target PCSK9 is shown in **f**–**i**. Data are presented as mean $\pm$ s.d. Illustrations in **f** and **g** created in BioRender; Anglerio, Y. <https://biorender.com/fo9dfim> (2026).

using HBV-gRNA2 and EE4000 co-encapsulated in an LNP (HBV-EE4000) demonstrated a durable, dose-dependent reduction in plasma HBV biomarkers in Tg-HBV mice following a single dose. Maximal reductions of 2.7 log₁₀ HBsAg and 2.9 log₁₀ HBV DNA were observed (Fig. 2a and Extended Data Fig. 4a,b), with suppression maintained throughout the 7-month observation period. All animals receiving $\geq 0.5 \text{ mg kg}^{-1}$ HBV-EE4000 exhibited a progressive decline in both HBsAg and HBV DNA beginning approximately 1 month post treatment. By the end of

the study, comparable levels of suppression were observed across all doses $\geq 0.5 \text{ mg kg}^{-1}$, with 11 of 15 mice achieving undetectable HBsAg levels (Extended Data Fig. 4a). The sustained suppression of HBsAg in HBV-EE4000-treated mice coincided with the emergence of measurable hepatitis B surface antibodies (anti-HBs) (Fig. 2b). Circulating HBeAg levels exhibited a moderate but durable decline ($<1 \text{ log}_{10}$) (Extended Data Fig. 4c), probably due to the known extrahepatic expression of HBeAg in this model²⁵. At study end (day 207), there was

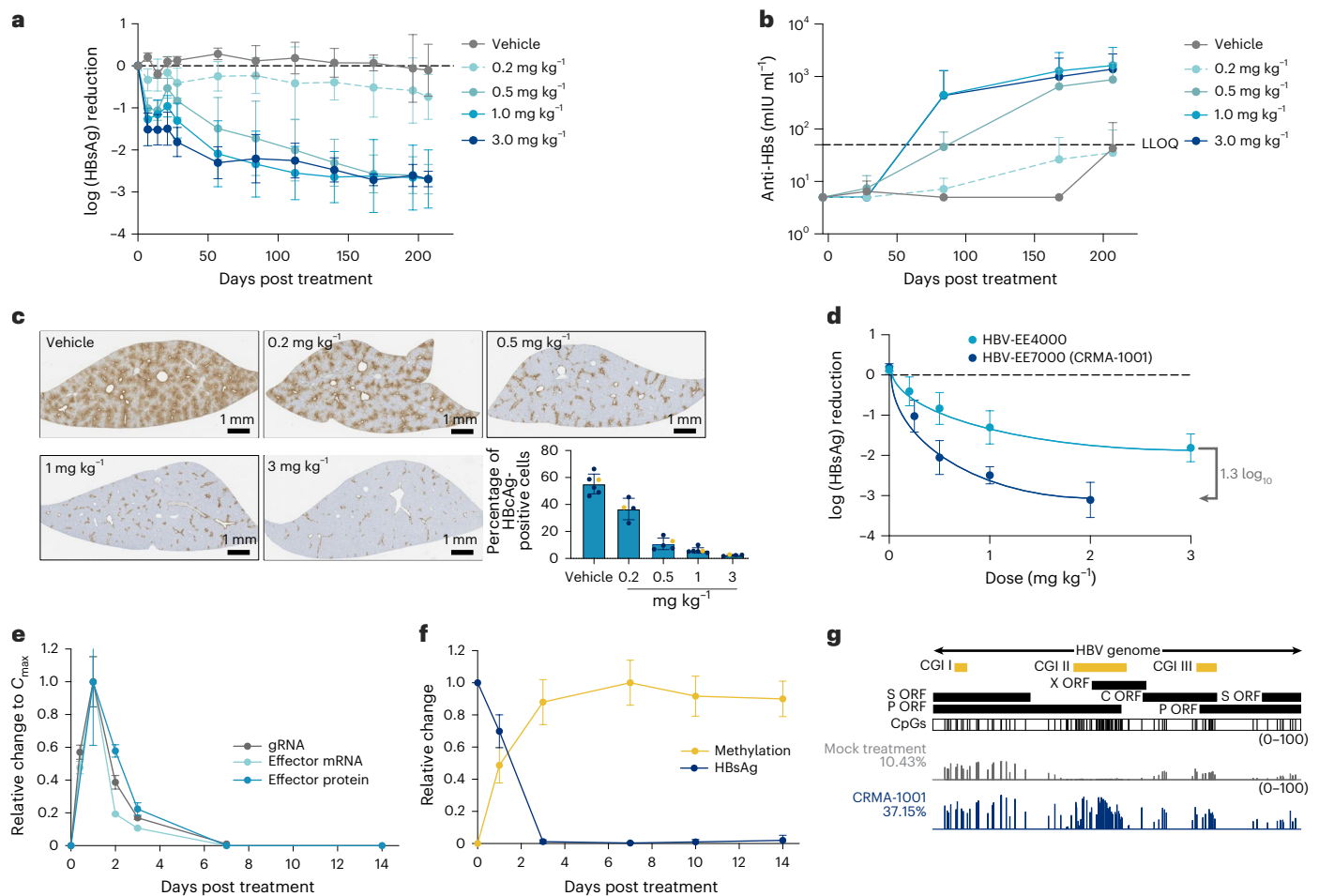


Fig. 2 | Optimized EEs used in CRMA-1001 increased activity by 1.3 log₁₀ in vivo and demonstrated durable methylation of intDNA. a–d, Pharmacological effects in Tg-HBV mice following a single i.v. administration of HBV-EE4000 ($n = 4–6$ per group) or CRMA-1001 ($n = 9–10$ per group; data were presented as mean values $\pm$ s.d. for each group), with HBsAg (a), anti-HBs (b), representative images of HBcAg IHC staining (c) from Tg-HBV livers at study termination (day 207) (brown staining indicates HBcAg-positive cells in the liver; bottom right graph shows quantification of HBcAg-positive cells in liver sections; black dots represent individual animal values; and yellow dots represent the animals from which the IHC images were derived), and HBsAg dose–response (d) at 1 month post dose in animals treated with EE4000 or EE7000. e–g, Longitudinal PKs

and pharmacodynamics of CRMA-1001 in HepG2.2.15 cells showing relative change to C_{max} of gRNA, effector mRNA and effector protein (e), relative change in DNA methylation and HBsAg secretion up to 14 days following treatment (f), and methylation profile of HBV-integrated DNA at day 14 in mock-treatment or CRMA-1001-treated (1.0 μ g ml⁻¹) samples ($n = 3$) (g). Methylation is depicted as a bar plot, with the average percentage methylation across the entire locus for each treatment group. The bar position along the x axis represents the position of a CpG within the HBV genome, and the bar height is the individual CpG methylation percentage. The ORF annotations of HBV genes (black), CGIs (yellow) and positions of individual CpGs (vertical black lines) for HBV genotype D are shown at the top.

a dose-dependent reduction in liver HBcAg staining, with 98% of hepatocytes negative for HBcAg in the highest-dose group despite the presence of intDNA in all cells (Fig. 2c). Methylation sequencing confirmed dose-dependent CpG methylation of the integrated HBV genome, correlating with reduced hepatic HBV transcripts (Extended Data Fig. 4d,e). As expected, methylation extended beyond the immediate target site, consistent with the ± 1.5 -kb region of establishment of epigenetic marks previously described for programmable epigenetic silencers^{16,17}, enabling genome-wide silencing of HBV across viral promoters.

CRMA-1001, which has the same mechanism of action (MoA), gRNA target site and LNP formulation as HBV-EE4000, but includes the optimized EE7000, was subsequently evaluated in Tg-HBV mice (Fig. 2d). Compared with HBV-EE4000 at 3.0 mg kg⁻¹, CRMA-1001 demonstrated -1.3 -log₁₀ greater HBsAg reduction at 2.0 mg kg⁻¹, confirming that optimization of the EE construct using PCSK9 as a surrogate translates to the HBV target. To further characterize the pharmacologic activity of CRMA-1001 against HBV intDNA, pharmacokinetic (PK), pharmacodynamic and mechanistic endpoints were evaluated in a longitudinal study conducted in HepG2.2.15 cells treated with 1 μ g ml⁻¹

CRMA-1001. Peak levels of gRNA, effector mRNA and effector protein were detected 24 h post treatment, with all components undetectable by 7 days post treatment (Fig. 2e). These PK observations correlated with functional outcomes, including an increase in HBV intDNA methylation and HBsAg silencing that peaked 3 days post treatment and persisted through the 14-day experiment (Fig. 2f). CpG methylation analysis confirmed a robust increase in methylation across the integrated HBV genome (Fig. 2g). Consistent with results in HepG2.2.15 cells, silencing of HBsAg was also observed in CRMA-1001-treated PLC/PRF/5 cells, a patient-derived hepatoma cell line with integrated HBV genotype A fragments, thus confirming robust pharmacology in a model of naturally occurring HBV integration (Extended Data Fig. 4f). In addition, CpG methylation in human genome regions junctional to seven HBV intDNA sites was characterized in CRMA-1001-treated PLC/PRF/5 cells (further described in the Supplementary Information and Supplementary Table 4). This study demonstrated that methylation induced by CRMA-1001 is detected up to -1.5 kb nucleotides from the CRMA-1001 gRNA target site, consistent with the extension of epigenetic marks in Tg-HBV mice described above.

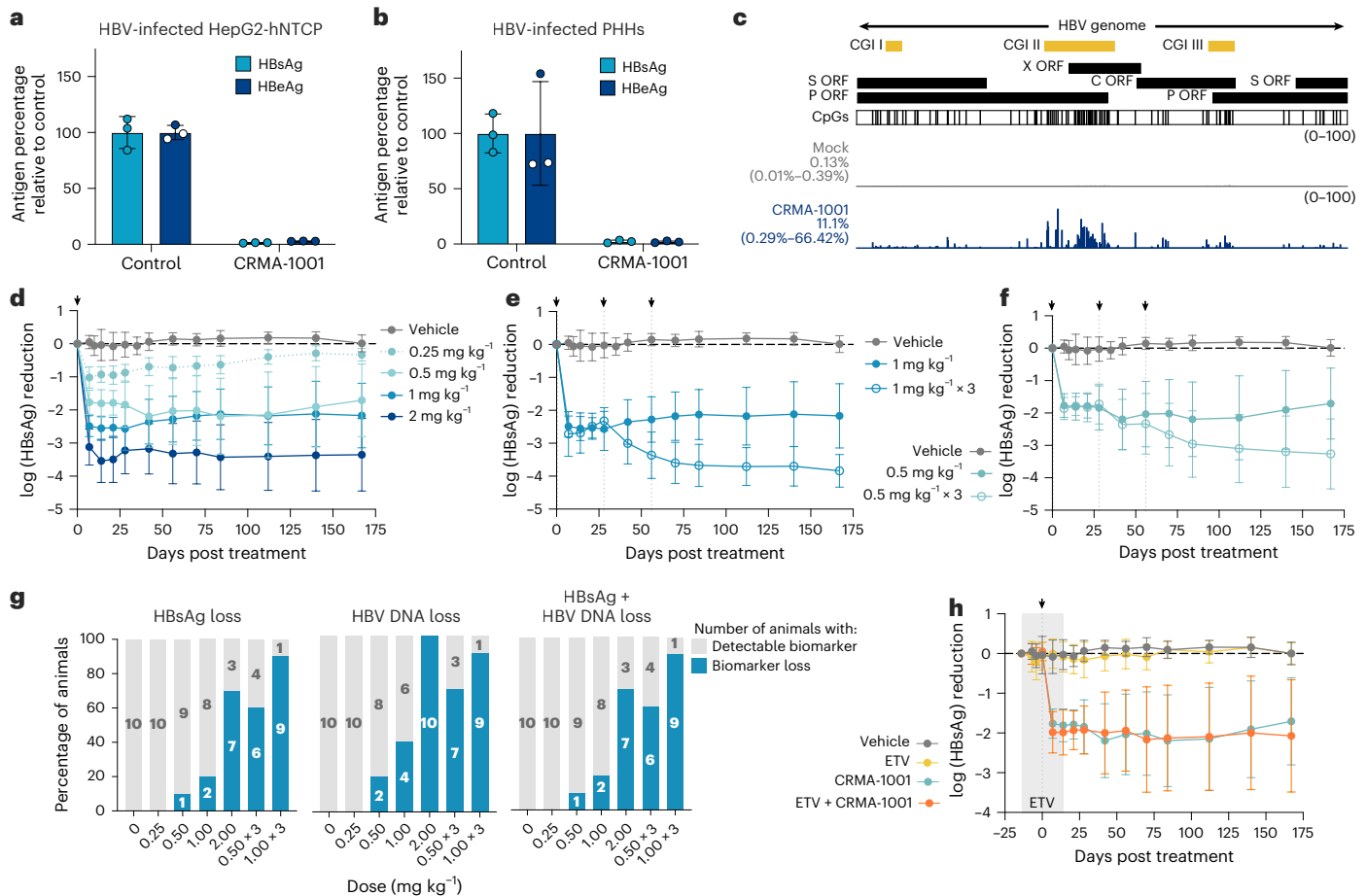


Fig. 3 | CRMA-1001 methylates HBV cccDNA in HBV-infected cells and induces HBsAg and HBV DNA loss in the AAV-HBV mouse model. a, HBsAg and HBeAg in HepG2-hNTCP cells 7 days post treatment (mean $\pm$ s.d.; $n = 3$ biological replicates per group). **b**, HBsAg and HBeAg in PHHs at 14 days post treatment (mean $\pm$ s.d.; $n = 3$ biological replicates per group). **c**, Methylation profile of isolated cccDNA in HBV-infected PHHs at 14 days post treatment. Methylation is depicted as a bar plot of methylation percentage of individual CpGs, with the average (top) and range (bottom) of percentage methylation across the entire locus for each treatment group. Black vertical bars represent the position of a CpG within the HBV genome. ORF annotations (black), CGI locations (yellow) and positions of individual CpGs (vertical black lines) for HBV genotype D are

shown at the top. Graphs in **a–c** show HBV-infected cells either mock-treated (control) or treated with $1 \mu\text{g ml}^{-1}$ CRMA-1001. **d–f**, HBsAg reduction in plasma ($\log_{10}$ fold change to baseline) in AAV-HBV mice was measured following a single dose of CRMA-1001 at the indicated doses (**d**) or Q4W $\times 3$ administrations at 1 mg kg^{-1} (**e**) or 0.5 mg kg^{-1} (**f**). **g**, The percentage of animals with HBV biomarker levels at or below the LLOQ at the terminal time point following CRMA-1001 administration. **h**, HBsAg reduction in plasma following treatment with CRMA-1001, ETV or in combination. The vertical shaded area represents the timeframe of ETV treatment. Graphs in **d–h** show pharmacological effects in AAV-HBV mice following treatment of CRMA-1001. Data are presented as mean $\pm$ s.d.; $n = 10$ animals per group.

Together, these data highlight the enhanced potency of the EE7000 component of CRMA-1001 and demonstrate that CRMA-1001 achieves dose-dependent, durable silencing of HBV intDNA in both in vivo and in vitro models via targeted DNA methylation.

CRMA-1001 durably suppresses HBV cccDNA with a finite course of treatment

HepG2-hNTCP cells and primary human hepatocytes (PHHs) are highly relevant in vitro models of HBV infection, as they support full viral replication and harbour both authentic cccDNA and intDNA²⁶. HepG2-hNTCP cells and PHHs infected with HBV genotype D were treated with $1.0 \mu\text{g ml}^{-1}$ CRMA-1001. HBsAg and HBeAg levels in cell supernatants were reduced by 98% compared with controls in HepG2-hNTCP cells at day 7 post treatment (Fig. 3a) and in PHHs at day 14 post treatment (Fig. 3b). The cccDNA from PHHs was extracted and specifically enriched for evaluation of de novo methylation at CpGs across the HBV genome. Treated cells showed methylation deposition ranging from 0.29% to 66.42%, including the highest levels within CGI II, compared with cccDNA from mock-treated cells, which is relatively devoid of methylation (ranging from 0.01% to 0.39%) (Fig. 3c).

The AAV-HBV mouse model is a well-established model for the assessment of pharmacologic activity against episomal forms of HBV DNA, including cccDNA. Unlike the Tg-HBV model, which contains only two HBV genome copies per diploid host genome, the AAV-HBV model employed in this study delivers a higher HBV DNA load (~ 4.5 HBV genome copies per diploid host genome), providing a potentially more demanding pharmacology model. AAV-HBV mice received either a single intravenous (i.v.) dose or three monthly (Q4W $\times 3$) doses of CRMA-1001 and were monitored for 6 months. A single dose resulted in dose-dependent, durable reductions of viral biomarkers, with maximal mean decreases of $3.4 \log_{10}$ HBsAg, $2.3 \log_{10}$ HBV DNA and $1.7 \log_{10}$ HBeAg over the 6-month study period (Fig. 3d and Extended Data Fig. 5a,b). Q4W $\times 3$ doses markedly enhanced the pharmacological effect of CRMA-1001, leading to a $-1.6 \log_{10}$ further HBsAg reduction compared with a single dose at the same dose level (Fig. 3e,f and Extended Data Fig. 5c–f).

Remarkably, a dose-dependent increase in the proportion of animals with HBsAg (10%, 20% and 70% in the 0.5, 1.0 and 2.0 mg kg^{-1} groups, respectively) and HBV DNA (20%, 40% and 100% in the 0.5, 1.0 and 2.0 mg kg^{-1} groups, respectively) below the assay lower limit

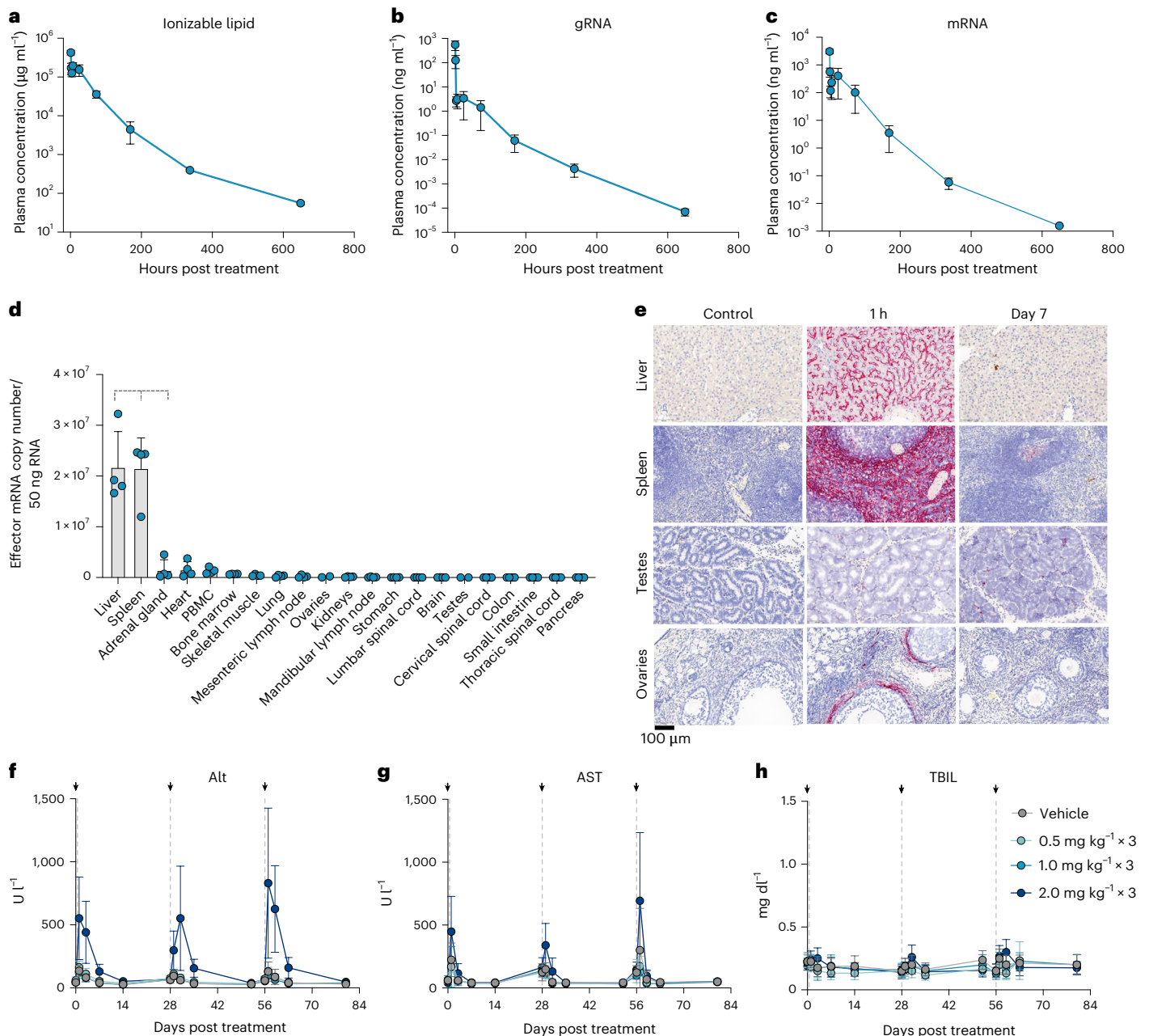


Fig. 4 | PKs, biodistribution and safety of CRMA-1001 in NHPs. **a–c**, Plasma concentrations of ionizable lipid (**a**) (LNP component), gRNA (**b**) and effector mRNA (**c**) after a single i.v. infusion of CRMA-1001 at 2 mg kg^{-1} (mean $\pm$ s.d.; n varies from a maximum of 12 animals/time point at the beginning of the time course to a minimum of 2 animals/time point at the end of the time course owing to the nature of the study design, which included serial terminations). **d**, EE mRNA biodistribution in selected tissues at 1 h post infusion (mean $\pm$ s.d.; $n = 4$ animals; for gonads, $n = 2$ animals). Dotted lines show representative tissues selected for specificity evaluations. **e**, Representative images ($40\times$ magnification) of ISH for EE mRNA (in pink) of selected tissues from control and CRMA-1001-treated NHPs.

ISH analysis was performed on $n = 4$ animals; for gonads, $n = 2$ animals. The left column includes images of tissues collected from control-treated animals at 1 h post treatment. The middle and right columns include images of tissues collected from CRMA-1001-treated animals at 1 h post treatment and 7 days post treatment, respectively. **f–h**, CRMA-1001 toxicity was evaluated in NHPs using Q4W $\times 3$ dosing up to 2.0 mg kg^{-1} , and serial measurements of blood ALT (**f**), AST (**g**) and TBIL (**h**) were performed in NHPs following dosing with either vehicle control or CRMA-1001 (mean $\pm$ s.d., $n = 4$ –10 animals/time point due to serial terminations). The vertical lines with arrows indicate individual dose administrations (three in total). PBMC, peripheral blood mononuclear cells.

of quantification (LLOQ) was observed following a single dose of CRMA-1001 (Fig. 3g). Across all dose groups, 31% (22/70) of the animals achieved HBsAg and HBV DNA below LLOQ at 6 months post treatment. Notably, Q4W $\times 3$ doses further increased the proportion of mice with HBsAg (60% and 90% in the 0.5 and 1.0 mg kg^{-1} groups, respectively) and HBV DNA (70% and 90% in the 0.5 and 1.0 mg kg^{-1} groups, respectively) below LLOQ.

To evaluate whether CRMA-1001 treatment can be effectively combined with NUC therapy, AAV-HBV mice were pretreated with

entecavir (ETV) for 2 weeks to suppress viral replication, followed by CRMA-1001 administration and an additional 2 weeks of ETV treatment. Consistent with the MoA of NUCs, ETV alone reduced HBV DNA by approximately $2 \log_{10}$, but had no effect on HBsAg or HBeAg (Fig. 3h and Extended Data Fig. 5g,h). As expected, ETV withdrawal resulted in a rebound of HBV DNA to baseline levels. By contrast, concomitant CRMA-1001 and ETV administration showed reductions of HBsAg and HBeAg similar to CRMA-1001 alone but led to additive HBV DNA suppression. Finally, when ETV effects had waned, approximately

4 months post treatment, HBV DNA levels in the combination group were comparable to those measured in CRMA-1001-treated animals.

Finally, CRMA-1001 treatment also resulted in dose-dependent reductions in HBV biomarkers in the liver, including HBV mRNA and total HBV DNA (episomal DNA and rcDNA) (Extended Data Fig. 5i,j), whereas no significant changes were observed in episomal HBV DNA levels (Extended Data Fig. 5k). The selective reduction of rcDNA is consistent with CRMA-1001's MoA, namely transcriptional silencing without affecting the underlying DNA sequence.

In summary, CRMA-1001 effectively targets HBV episomal DNA, is compatible with NUC therapy and shows additive antiviral activity when combined with ETV. Repeated dosing enhances the pharmacologic activity of CRMA-1001, resulting in a higher proportion of animals with HBsAg and HBV DNA levels below LLOQ as compared with a single dose.

PKs, biodistribution and safety of CRMA-1001 in NHPs

To evaluate the PK and biodistribution of CRMA-1001 in NHPs, male and female cynomolgus monkeys received a single i.v. infusion of 2 mg kg⁻¹ CRMA-1001 or vehicle. Plasma samples were analysed for ionizable lipid (LNP component), gRNA and EE mRNA, and multiple tissues were analysed for EE mRNA biodistribution. For all analytes, maximum plasma concentrations (C_{max}) were observed at the end of infusion, with mean values of $4.29 \times 10^5 \mu\text{g ml}^{-1}$ for ionizable lipid (Fig. 4a), 495 ng ml⁻¹ for gRNA (Fig. 4b) and 3,090 ng ml⁻¹ for EE mRNA (Fig. 4c). Concentrations declined by more than 60% within the next hour and then entered a terminal clearance phase approximately 24 h after dosing, with levels <0.01% of C_{max} by day 28.

EE mRNA levels and distribution were further assessed by droplet digital PCR (ddPCR) and in situ hybridization (ISH) in selected tissues (Fig. 4d,e). The highest tissue concentrations were observed 1 h after the end of the infusion, and the highest levels of EE mRNA were measured in the liver and spleen (Fig. 4d). Tissue mRNA levels decreased over time, and by day 28 post treatment, mRNA levels in the liver and spleen were <0.1% of the 1-h liver concentration and below the limit of detection in most other tissues.

ISH in tissues collected at 1 h post treatment revealed the highest EE mRNA staining in the sinusoidal endothelium and lower levels in hepatocytes, portal fibrocytes, biliary epithelium and Kupffer cells of the liver. In the spleen, high EE mRNA was noted in the mantle zone cells, red pulp macrophages and germinal centre lymphocytes of the spleen (Fig. 4e), with lighter staining of smooth muscle cells. Testis demonstrated low levels of EE mRNA staining in endothelial and interstitial cells and no staining in the germinal epithelium of seminiferous

tubules. EE mRNA staining was also observed in the theca externa cells of secondary follicles in the ovary and in interstitial cells, but no staining of oocyte germ cells was detected. By day 8 post treatment, the EE mRNA signal in all tissues examined was substantially reduced. Taken together, this analysis supports primary biodistribution of CRMA-1001 to the liver and the spleen and lack of biodistribution to germline cells.

The safety of CRMA-1001 was evaluated in a repeat-dose (Q4W $\times$ 3) Good Laboratory Practice toxicity study in cynomolgus monkeys. Animals were monitored for clinical signs, and samples were collected to evaluate serum chemistry, haematology and coagulation parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL), for up to 80 days post treatment (Fig. 4f–h). CRMA-1001-related effects were limited to serum chemistry findings at the highest dose evaluated (2 mg kg⁻¹). Liver enzymes were transiently elevated following each dose, and recovery was observed by 4 weeks after the last dose. In the absence of associated clinical observations and anatomic pathology correlates, all findings were considered non-adverse.

In summary, CRMA-1001 predominantly distributed to the liver and spleen, with no detectable distribution to germline cells. Findings were limited to transient, reversible liver-related serum chemistry elevations following repeated monthly dosing up to 2.0 mg kg⁻¹.

CRMA-1001 displays a highly specific genome-wide profile

A comprehensive specificity evaluation was performed to identify potential gRNA-mediated unintended target activity of CRMA-1001. The evaluation included a discovery phase that employed whole-genome transcriptomic and DNA methylation analyses, along with in silico prediction of gRNA binding sites, to nominate potential unintended targets. A subsequent confirmatory phase utilized targeted methylation profiling to confirm activity at the nominated unintended target sites. The confirmatory methylation assay and transcriptional profiling were then used to designate verified unintended targets (VUT), defined as genes that exhibit DNA hypermethylation and an associated change in gene expression following treatment with CRMA-1001.

All transcription and methylation profiles were evaluated in primary human cells from three different tissues, including liver (PHHs), spleen (human spleen endothelial cells (HSECs)) and adrenal gland (human adrenal cortical cells (HAdCCs)). These cell types were selected on the basis of NHP biodistribution studies of CRMA-1001 and its components.

Gene expression changes resulting from CRMA-1001 treatment were evaluated using RNA sequencing (RNA-seq; three donors per cell type and nine total cell donors, representing male and female

Fig. 5 | Assessment of the unintended target activity of CRMA-1001 using transcription and methylation assays in three primary cell types.

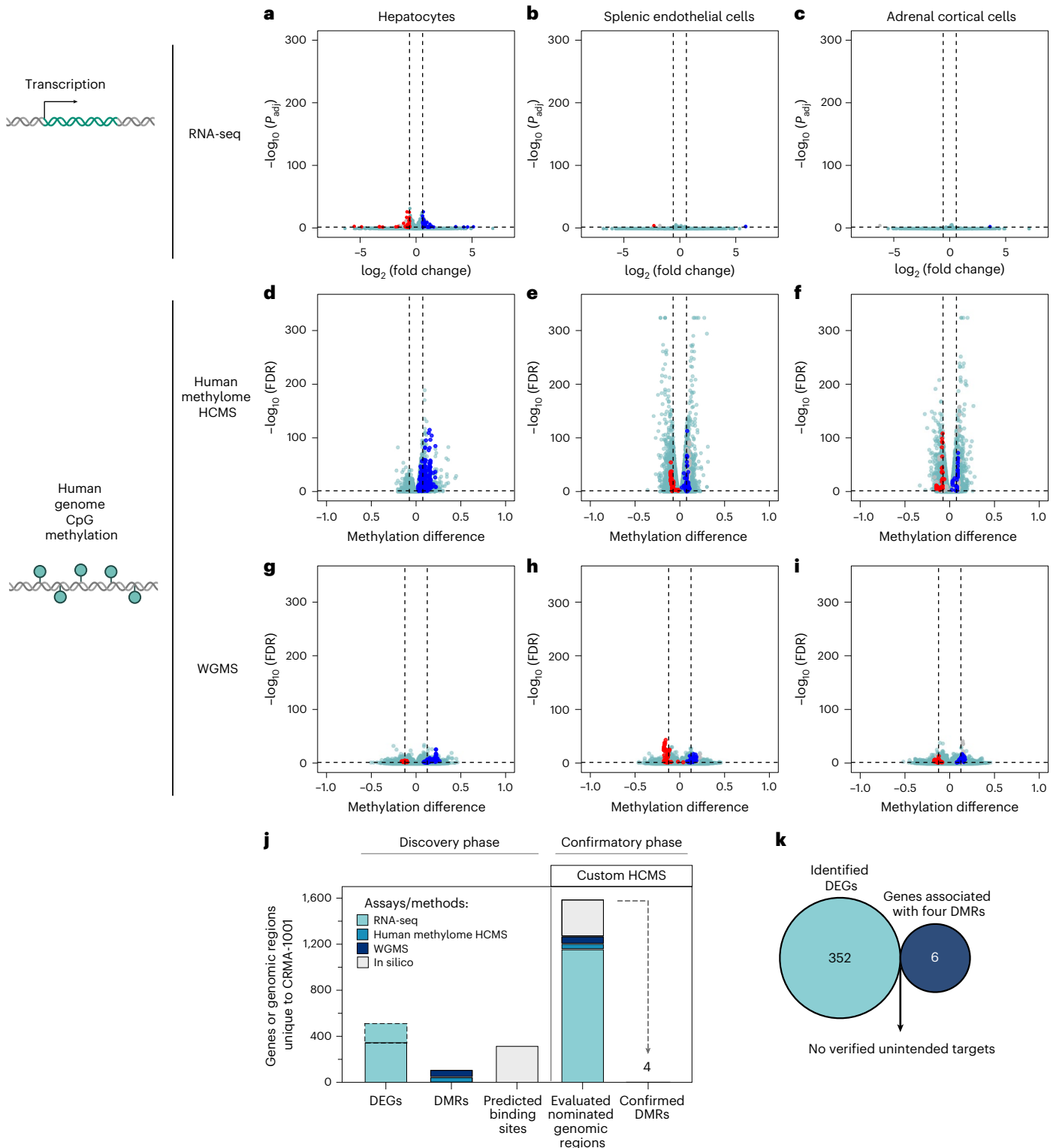
a–i, Differential gene expression (**a–c**) or differential methylation changes (**d–i**) compared with control are shown as volcano plots (one donor for each cell type) for hepatocytes (**a,d,g**), splenic endothelial cells (**b,e,h**) and adrenal cortical cells (**c,f,i**). Grey lines represent thresholds for significant changes; $n \geq 3$ replicates. Dots represent individual genes (blue, upregulated; red, downregulated; teal, not differentially expressed; grey, also observed between control conditions); x axes show change in expression, log₂ (fold change), and y axes represent the statistical significance (DEseq2 Wald test, two-sided, Benjamini–Hochburg multiple comparisons adjustment), $-\log_{10}$ (FDR) (thresholds: adjusted P value (false discovery rate (FDR)) ≤ 0.05 and absolute fold change of >1.5). **d–f**, A Human Methyome HCMS panel, with thresholds including statistical significance based on the adjusted P value (FDR) ≤ 0.05 (DSS Wald test, two-sided, Benjamini–Hochburg multiple comparisons adjustment) and absolute methylation differences >0.075 . **g–i**, A WGMS assay, with thresholds including statistical significance based on the adjusted P value (FDR) ≤ 0.05 (DSS Wald test, two-sided, Benjamini–Hochburg multiple comparisons adjustment) and absolute methylation differences >0.125 . Graphs **d–i** show differential methylation; the x axis shows the methylation difference

(difference in fraction of methylated reads (range of 0–1)) for each CpG, and the y axis represents statistical significance, $-\log_{10}$ (FDR). Dots represent individual CpGs. CpGs are coloured on the basis of inclusion in DMRs (consecutive CpGs with significant changes in methylation) (blue, hypermethylated DMR; red, hypomethylated DMR; teal, not a DMR; grey, also observed between control conditions). **j**, Discovery and confirmatory specificity phases using orthogonal assays to nominate genes or genomic regions. Discovery phase shows numbers of: DEGs by RNA-seq (solid line, ≥ 2 TPM; dotted line, <2 TPM), DMRs by Human Methyome HCMS and WGMS, and in silico-predicted gRNA binding sites. All nominated genomic regions were profiled for differential methylation during the confirmatory phase using custom HCMS. Thresholds included statistical significance based on the adjusted P value (FDR) ≤ 0.01 (DSS Wald test, two-sided, Benjamini–Hochburg multiple comparisons adjustment) and absolute methylation differences >0.11 . **k**, The Venn diagram shows no overlap between identified DEGs and genes associated with confirmed hypermethylated DMRs across all three cell types (three donors per cell type and nine donors in total) and therefore no VUT effects of CRMA-1001. P_{adj} , adjusted P value. Transcription and human genome CpG methylation diagrams created in BioRender; Anglerio, Y. <https://biorender.com/0b91f5q> (2026).

individuals and three ethnicities). There were 514 differentially expressed genes (DEGs) nominated across all cell types and donors, of which 352 were considered expressed above experimental noise (≥ 2 transcripts per million TPM)²⁷ (Fig. 5a–c,j and Extended Data Fig. 6). Two complementary DNA methylation assays were performed on a single donor per cell type to nominate differentially methylated regions (DMRs). Human methylome hybrid capture methylation sequencing (HCMS) and whole-genome methylation sequencing (WGMS) identified 49 and 65 DMRs, respectively, with 2 DMRs in common (Fig. 5d–i). Lastly, an *in silico* analysis was performed to predict CRMA-1001 gRNA

binding sites. This analysis included up to four nucleotide mismatches, up to a two-nucleotide bulge and up to four nucleotides of combined mismatch plus bulge nucleotides, and resulted in 320 unique DNA regions nominated for the confirmatory phase (Fig. 5j).

The specificity discovery phase resulted in a total nomination of 1,591 genomic regions from primary cells and *in silico* analyses, comprising DMRs, predicted binding sites and genomic regions in proximity to all annotated transcription start sites (TSS) of DEGs, as these encompass transcriptional control regions at which methylation effects are most likely to have a functional impact²⁸. A custom HCMS



assay was designed using the 1,591 nominated genomic regions to confirm CRMA-1001-induced hypermethylated DMRs using treated samples from all donors and all cell types. Four hypermethylated DMRs were confirmed in PHHs and none in HSECs or HAdCCs. Two DMRs were shared across at least two PHH donors, and two DMRs were unique to a single PHH donor each. The four DMRs were in genomic proximity (≤ 2 kb) to six genes.

The six genes within genomic proximity of the four confirmed DMRs were compared with the 352 DEGs from RNA-seq to designate VUTs of CRMA-1001; there was no overlap between the gene sets, and therefore, no VUTs of CRMA-1001 were detected in primary human cells representative of tissues with CRMA-1001 biodistribution in NHPs (Fig. 5k).

Discussion

The studies presented here demonstrate the pharmacodynamic, PK, specificity and preclinical safety profiles of CRMA-1001, providing justification for its clinical development as an ES therapeutic with the potential to achieve functional cure in patients living with CHB.

CRMA-1001 was optimized through a comprehensive screen of HBV-gRNAs across multiple viral ORFs and CGIs, combined with iterative engineering of the EE. Using *PCSK9* as an endogenous liver target, each round of EE optimization yielded a ~ 2 – 3 -fold increase in potency in both mice and NHPs. The optimized EE achieved maximal pharmacologic activity at a single 1-mg kg^{-1} dose in NHPs, with suppression of target expression sustained for 2 years so far.

In HBV mouse models, these advances translated into marked improvements in antiviral activity. In Tg-HBV mice, EE optimizations enhanced HBsAg reductions by $\sim 1.3 \log_{10}$ at lower doses compared with the early generation EE4000. A single dose of HBV-EE4000 silenced viral transcripts in the mouse liver for ≥ 7 months and led to undetectable HBcAg in most hepatocytes ($\sim 98\%$), demonstrating durable transcriptional inactivation of HBV DNA by ES. In AAV-HBV mice, CRMA-1001 produced dose-dependent reductions in plasma HBsAg and HBV DNA, with maximal reductions of $>3 \log_{10}$ and $>2 \log_{10}$, respectively. Three doses administered monthly further improved responses, with $\sim 90\%$ of animals achieving undetectable HBsAg and HBV DNA 6 months after the first dose. CRMA-1001 was also compatible with NUC therapy and exhibited additive antiviral effects.

Notably, none of the available HBV in vivo models fully recapitulate human infection and pathobiology; however, these models remain valuable preclinical tools. The models used within were selected to assess the pharmacological activity and therapeutic durability of CRMA-1001, as they possess hepatocytes harbouring HBV genomes (episomal or integrated), exhibit stable and prolonged expression of viral biomarkers, produce viral particles and maintain normal liver physiology, supporting clinically relevant LNP delivery.

Nevertheless, these models have inherent limitations. The AAV-HBV model supports viral expression from episomal HBV DNA that can form cccDNA-like transcriptional templates but does not fully recapitulate authentic infection-derived cccDNA. Tg-HBV mice express HBV transcripts from host-integrated viral DNA; however, this does not reflect the heterogeneous integration patterns observed in patients with CHB. Additional models of HBV infection, specifically liver-humanized mice, were considered but ultimately not pursued owing to the inherent limitations for LNP transduction (further described in the Supplementary Information and Supplementary Fig. 1). The in vitro studies presented herein using HepG2-NTCP cells and PHH recapitulate key aspects of HBV infection, including viral entry, replication and formation of authentic cccDNA. In addition, PLC/PRF/5 cells are a model of natural HBV integration. Together, these systems provide complementary biological context to support the translational relevance of the in vivo findings with CRMA-1001.

A key objective in the preclinical development of CRMA-1001 was to ensure that pharmacological activity translated across species.

Using the endogenous target *PCSK9*, EE7000, the optimized EE mRNA employed in CRMA-1001, led to saturating pharmacology in NHPs at 1.0 mg kg^{-1} . CRMA-1001 also demonstrated a favourable safety profile in NHPs following up to $Q4W \times 3$ doses at 2.0 mg kg^{-1} . The combination of potency, durability and safety supports a relatively wide therapeutic index, a critical feature for LNP-delivered RNA therapeutics. Nonetheless, the absence of an HBV-susceptible NHP model remains a limitation for the field, and ultimately, clinical experience will determine the degree of translation from mouse models of pharmacology to people living with CHB.

Importantly, CRMA-1001 achieved sustained suppression of HBsAg and HBV DNA for ≥ 6 months after treatment in multiple in vivo studies, an outcome consistent with criteria for clinical functional cure and only rarely demonstrated preclinically^{29,30}. Other therapeutic modalities aiming to silence HBV, including small interfering RNA (siRNA) and antisense oligonucleotides (ASOs), only achieved transient biomarker reductions followed by a rebound, both in preclinical models³¹ and in clinical trials³². This is mainly because siRNA and ASOs silence HBV post-transcriptionally, and therefore are effective only as long as the product is present, resulting in relatively low rates of functional cure. By contrast, CRMA-1001 is designed to directly inactivate the transcriptional sources of HBsAg and viral replication intermediates by inducing long-lasting, and potentially permanent, methylation of HBV DNAs. These sustained effects may offer advantages over siRNA or ASOs in promoting reconstitution of host immune function and a higher rate of functional cure³³.

CRMA-1001 reduced expression of HBV biomarkers by up to $>3 \log_{10}$ in vivo and 98% in vitro; however, average DNA methylation across the HBV genome appeared relatively modest, with a maximum average methylation of 37.15% measured in the HepG2.2.15 in vitro model. This apparent discrepancy highlights a key mechanistic difference between ES and genome-editing approaches, which affects how on-target activity is measured. While percent editing at the target site induced by nuclease technologies is closely correlated with the level of silencing³⁴, ES can have effects through both regional and individual CpG methylation^{35–37}. Partial average methylation across a promoter region can result in near-complete silencing, as the level of methylation of individual CpGs can be much higher¹⁵. Many methylated CpGs can promote a heterochromatin state, while individual CpGs may have different effects on gene expression due to impacts on nucleosome positioning and stability, transcription factor binding and other chromatin factors^{35–37}. Therefore, both the average level of promoter methylation across all CpGs and the level of methylation of individual CpGs can impact gene expression at a given locus.

Unlike genome-editing approaches, CRMA-1001 does not rely on the induction of DNA breaks, which carry risks of chromosomal rearrangements and, in HBV, may generate new integration events or smaller, transcriptionally active cccDNA variants as unintended consequences^{14,38,39}. ES therefore provides a precise mechanism for functional inactivation of HBV DNA while preserving genomic integrity, representing a potential safety advantage. Importantly, CRMA-1001 demonstrated robust specificity for the HBV genome. Across four orthogonal assays, three primary cell types and nine human donors, no human genes showed concomitant DNA hypermethylation and associated transcriptional changes following treatment. Together with the lack of germline distribution observed in NHPs, these data indicate the selectivity and preclinical safety of this therapeutic approach for initial clinical development.

In summary, CRMA-1001 achieves up to 3.7-log_{10} sustained reductions of HBV biomarkers and suppression of viral replication by directly silencing transcriptionally active HBV DNA via targeted and specific DNA methylation. In addition, the same EE protein used in CRMA-1001 induces silencing of an endogenous gene (*PCSK9*) in NHPs for at least 2 years following a single administration. This differentiated mechanism has the potential to enable durable antiviral control following

a finite course of treatment, positioning CRMA-1001 as a promising, potentially curative approach for individuals living with CHB.

Methods

Ethics statement

All studies involving the use of animals were conducted in compliance with animal care and use protocols approved by institutional animal care and use committees (IACUC) of corresponding testing facilities: Wuxi Apptec, nChroma Bio and AltaSciences. The health assessment of animals was performed in accordance with IACUC standard operating procedures and guidelines of each testing facility.

In silico HBV conservation and gRNA designs

Guide target conservation analysis was performed by assessing exact matches between the target sequence including presence of the dSp-Cas9 NGG PAM and the reference genomes with A–E genotypes found in HBVdb²¹ using NCBI BLAST⁴⁰. The percentage of exact matches per genotype was used to assess conservation.

RNA production

Using standard methods by commercial suppliers, 100-mer gRNAs were chemically synthesized. The gRNAs used for in vitro screens contain 2′-O-methylation and phosphorothioate linkages at the three terminal nucleotides of both the 5′ and 3′ ends, as previously described¹⁵. Subsequent studies in vitro and in vivo employed gRNAs containing both end-modifications as well as a proprietary pattern of 2′-O-methylation throughout the sequence. All gRNA target sequence used in this study are presented in Supplementary Table 1.

mRNA was produced via in vitro transcription and purification. Plasmids containing an epigenetic silencer or a control construct were linearized by restriction digest and used as a template with T7 RNA polymerase, NTPs (including *N*¹-methylpseudouridine) and cap analogue. mRNAs either underwent enzymatic A-tailing or contained an encoded polyA in the template, as previously described¹⁵. The amino acid sequences of all constructs used in these studies are presented in Supplementary Table 2.

LNP formulations

Small-scale LNP formulations of epigenetic silencer mRNA and gRNA for screens and initial HepG2.2.15 studies were prepared using GenVoy-ILM kits. Formulations were optimized with an aim to attain LNPs of uniform size and high encapsulation efficiency. The ratio of lipids to the total RNA cargo, comprising mRNA and gRNA, was calculated on the basis of the molar weights of lipids and RNAs. Medium-scale LNP formulations used in in vitro, rodent and NHP studies contained an ionizable cationic lipid proprietary to Acuitas Therapeutics, as well as phosphatidylcholine/cholesterol/PEG-lipid. The proprietary lipid and LNP compositions are described in patent application WO2020146805A1. The RNAs were mixed and encapsulated in LNP using a self-assembly process as described previously^{15,41}. The resulting LNPs had a diameter ≤ 70 nm as measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments) instrument and encapsulation efficiencies of more than 97% as measured by Quant-iT RiboGreen Assay (Life Technologies).

In vitro studies

HepG2.2.15 and PLC/PRF/5 cell culture and treatment. HepG2.2.15 cells were cultured in DMEM (Gibco, 11965092) with 10% fetal bovine serum (FBS) (Gibco, A5670501) and 1% penicillin–streptomycin (Gibco, 15140122). A day before treatment, 20,000 cells per well were seeded in collagen type I-coated 96-well plates. On the following day, the cells were treated with 100 μ l of fresh medium supplemented with LNP-formulated epigenetic silencers at a single dose of 0.5 μ g ml^{−1} or an 8-point serial dilution (4–0.031 μ g ml^{−1}) and/or 1 μ g ml^{−1} of recombinant human apolipoprotein E3 (rhApoE3) (a supplement to facilitate LNP transfection in

cell culture), also referred to as mock treatment. Plates were maintained and subcultured (cell trypsinization every 3–4 days), and supernatants or cell samples were collected at various time points from 6 h to 27 days after treatment and stored at -80°C until analysis. Studies to evaluate EE protein were performed in 24-well plate format, and 118,750 cells per well were seeded and treatment with 500 μ l of fresh medium supplemented with 1 μ g ml^{−1} of rhApoE3 and or 1 μ g ml^{−1} of CRMA-1001. PLC/PRF/5 cells (ATCC, CRL-8024) were cultured in Eagle's minimum essential medium (Corning, 10-009-CV) with 10% FBS and 1% penicillin–streptomycin and maintained as described above for 96-well plates. Cells were treated with 1 μ g ml^{−1} of rhApoE3 (mock-treatment control) and/or 1 μ g ml^{−1} of CRMA-1001, then supernatants were collected at 7 and 14 days after treatment and stored at -80°C until analysis.

PHH cell culture, infection and treatment. PHHs isolated from humanized mouse liver (PXB-cells) (PhoenixBio)⁴² were received pre-plated in 24-well plates and maintained in culture medium (dHCGM medium (PhoenixBio) and 10% FBS) and incubated at 37°C and 5% CO₂. To establish HBV infection, 4 days before treatment, HBV was diluted in infection medium (dHCGM, 10% FBS, 4% PEG8000 and 2.5% DMSO) at a multiplicity of genome equivalents (MGE) of 2,000 per cell of HBV genotype D (Imquest BioSciences). The supernatant was collected from all wells and replaced with infection medium. The following day, 16 h after infection, the medium was removed, cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and 500 μ l of fresh maintenance medium (dHCGM, 10% FBS and 2.5% DMSO) was added to each well. For studies with uninfected PHHs, cells were maintained in culture medium upon treatment. On the day of treatment (day 0), PHHs were treated with 500 μ l culture medium supplemented with 1 μ g ml^{−1} of rhApoE3 and 0.5 or 1 μ g ml^{−1} CRMA-1001 or rhApoE3 only (mock-treatment control). At 14 days post treatment, the supernatants were collected, cells washed and both stored at -80°C until analysis.

Primary HSECs and primary HAdCC treatment. Primary HSECs (Innoprot and Accegen) from three different human donors were cultured in medium (endothelial cell medium (ScienCell) with 5% FBS, 1% penicillin–streptomycin and 1% endothelial cell growth supplement (ScienCell)) and incubated at 37°C and 5% CO₂. A day before test or control article treatment, 10,000 cells per well were seeded into a 96-well tissue culture plate precoated with 150 μ g ml^{−1} of human fibronectin and containing 200 μ l culture medium. On the day of treatment (day 0), culture medium was replaced with 100 μ l of fresh medium containing 1 μ g ml^{−1} of rhApoE3 and CRMA-1001 or control articles. On day 14, the cells were washed with 200 μ l DPBS per well, lysed with 63 μ l Beckman Coulter Buffer LBE with Proteinase K, incubated for 30 min at room temperature and frozen at -80°C in plate format.

Primary HAdCCs (Accegen) were cultured in medium (Mesenchymal Stem Cell Medium (Innoprot) with 5% FBS, 1% penicillin–streptomycin and 1% Mesenchymal Stem Cell Growth Serum (Innoprot)) and incubated at 37°C and 5% CO₂. A day before test or control article treatment, 10,000 cells per well were seeded into a 96-well tissue culture plate precoated with 150 μ g ml^{−1} of poly-L-lysine (Sigma-Aldrich) and containing 200 μ l culture medium. On the day of treatment (day 0), the culture medium was replaced with 100 μ l of medium containing 1 μ g ml^{−1} of rhApoE3 and CRMA-1001 or control articles. The plates were maintained and cells subcultured (cell trypsinization every 3–4 days, followed by transfer of $\sim 10,000$ cells to a new 96-well culture plate with fresh medium). On day 14, cells were washed with 200 μ l DPBS per well, lysed with 63 μ l Beckman Coulter Buffer LBE with Proteinase K, incubated for 30 min at room temperature and frozen at -80°C in plate format.

Cell viability assays. Viability was assessed using Cell Titer-Glo (Promega, G924B) or Cell Titer-Blue Assays (Promega, G8080) according to the manufacturer's recommendations. The fluorescent signal obtained was measured using the Infinite 200 PRO Microplate Reader with a

560Ex/590Em filter set (TECAN). To account for technical variability in cell number during subculture, the secreted antigen concentrations were normalized to cell viability in each individual well and expressed as a percentage relative to control-treated cells.

Primary screening in vitro in HepG2-NTCP cells. For the initial screening experiments, EEs were delivered to HepG2-NTCP cells as three individual mRNAs, each encoding an engineered transcriptional repressor as previously described⁴³ (dCas9-KRAB mRNA, DNMT3A mRNA and DNMT3L mRNA) as well as the candidate single guide RNA mixed at a 1:1:1:4 ratio. The RNA mix was formulated into LNPs using the Genvoy-ILM Lipid Mix kit (Precision Nanosystems).

The HepG2-NTCP cell line was generated using HepG2 cells acquired from ATCC and transduced with a lentiviral vector to stably express the NTCP receptor. Cells were maintained in DMEM (Gibco, 21969035) supplemented with 10% FBS, 1% of glutamine and 1% penicillin–streptomycin. HepG2-NTCP cells were plated at 20,000 cells per well in collagen-coated 96-well plates and infected 24 h later with HBV genotype D at 5,000 MGE using DMEM medium supplemented with 4% PEG8000 and 2.5% DMSO. At 16 h post infection, the medium was removed, and the cells were washed with PBS and maintained in DMEM medium supplemented with 2.5% DMSO. Subsequently, 4 days after infection, the cells were treated with LNPs, and the medium was changed 1 and 3 days after LNP treatment. Finally, the supernatant was collected 6 days after transfection, and HBeAg and HBsAg levels were quantified by ELISA (Dia.Pro) and cell viability by Cell Titer-Blue Assay. HBV infection and the antiviral effect of treatments was analysed by HBeAg/HBsAg quantification. Guide potency was assessed by normalizing HBsAg and HBeAg measurement for each guide by the cell viability value for that guide.

In vivo studies

Tg-HBV mice. The study was conducted at WuXi AppTec in accordance with IACUC guidelines. C57BL/6-HBV transgenic mice (Tg-HBV), 5–6 weeks old, carrying two copies per diploid host genome of the 1.28× genotype A HBV genome²⁴ were obtained from Beijing Vitalstar Biotechnology. All animals were acclimated for 10 days during which the baseline levels of circulating HBV biomarkers (HBsAg, HBV DNA, and HBeAg) were measured. On day 0, all animals received a single i.v. bolus administration of vehicle, HBV-EE4000 (0.5, 1 and 3 mg kg⁻¹) or CRMA-1001 (0.25, 0.5, 1 and 2 mg kg⁻¹). Peripheral blood samples were collected for biomarker analysis. At the end of study, all animals were euthanized, and liver samples were collected for further molecular assessment.

AAV-HBV mice. All AAV-HBV mouse experiments were conducted at the nChroma Bio facility in compliance with IACUC guidelines. Male C57BL/6J mice (6–8 weeks old) were obtained from The Jackson Laboratory. Following acclimation, mice were transduced with AAV8-HBV1.2 (genotype D; Signagen, SL100862) via i.v. injection at a dose of 3×10^{11} viral genomes. Circulating HBsAg levels were assessed at 2, 3 and 4 weeks post transduction to establish baseline levels and determine eligibility for study enrollment. Mice with mean baseline HBsAg levels between 5,000 IU ml⁻¹ and 50,000 IU ml⁻¹ were included. Animals meeting this criterion were randomized into treatment groups, with the goal of achieving an average baseline HBsAg level of approximately 10,000 IU ml⁻¹ per group. Eligible animals were further treated with either vehicle or CRMA-1001 (0.25, 0.5, 1 or 2 mg kg⁻¹). In the combination treatment group, CRMA-1001 (0.5 mg kg⁻¹) was administered in conjunction with ETV (Sigma, SML110), which was delivered via daily oral gavage at 0.01 mg kg⁻¹ for 28 days. Fresh ETV dosing solutions (diluted in saline) were prepared weekly. Peripheral blood samples were collected for biomarker analysis throughout the study. At the end of the study, all animals were euthanized, and liver tissues were collected for molecular analyses.

Cynomolgus monkeys. The cynomolgus monkey studies were conducted at Altasciences in accordance with IACUC guidelines. The PK and biodistribution studies consisted of male and female animal monkeys randomized into two groups of one to six animals per sex per group. Groups received a single i.v. infusion over 1 h of vehicle ($n = 3$) (0.9% sodium chloride) or CRMA-1001 at 2 mg kg⁻¹ ($n = 16$). Animals were euthanized at 1 h, 24 h, 7 days and 28 days post infusion to assess PKs and biodistribution of CRMA-1001.

The Good Laboratory Practice toxicity study consisted of male and female naive monkeys randomized into four groups of three to five animals per sex per group. Groups received three i.v. infusions for 1 h of vehicle ($n = 8$) (0.9% sodium chloride), or CRMA-1001 at dose levels of 0.5 mg kg⁻¹ ($n = 6$), 1 mg kg⁻¹ ($n = 10$) or 2 mg kg⁻¹ ($n = 10$), every 4 weeks.

Pretreatment medications of diphenhydramine (5 mg kg⁻¹), dexamethasone (1 mg kg⁻¹) and famotidine (0.5 mg kg⁻¹) were administered to all monkeys via intramuscular injection 1 day and 30–60 min before the start of each infusion across studies. Animals were fasted for at least 4 h before serum chemistry collections with access to water. Blood was collected from a peripheral vein, and serum was obtained for clinical chemistry analyses using a clinical analyzer. Serum chemistry, including ALT, AST and TBIL, was monitored at baseline and through the duration of the study, up to 4 weeks following the last CRMA-1001 dose administration.

PK and biodistribution assessments from cynomolgus monkeys.

Assessments of CRMA-1001 PK in plasma included measurements of EE mRNA, HBV gRNA and ionizable lipid. Assessments of the biodistribution of CRMA-1001 included measurements of the effector mRNA in tissues. In brief, for the effector mRNA analyses, RNA was extracted from monkey plasma using QIAGEN QIAcube HT kits, and a reverse transcription (RT)–ddPCR assay was performed using the following primers: effector mRNA primer 1 (CTGGGACCTAAGAAGTACG), primer 2 (GCAGCTCTTACACTCTTC) and probe (ACAGCCCCACCGTGGCCTAT). For the gRNA analyses, RNA was extracted from monkey plasma samples using the MagMAX mirVana total RNA isolation kit, and an RT–ddPCR assay was performed using the following: primer 1 (GAGTTC-CGCAGTATGGATGTT), primer 2 (CGACTCGGTGCCACTTT) and probe: (AAGGCTAGTCCGTTATCAACTTGAA). For the ionizable lipid analysis, monkey plasma samples were processed using a protein precipitation procedure. The extracts were analysed by HPLC with detection by tandem mass spectrometry. The mobile phase was nebulized using heated nitrogen set to TurboSpray positive ionization mode and the compounds were detected using tandem mass spectrometry. For biodistribution studies, tissue RNA was extracted using QIAGEN QIAcube HT kits, and an RT–ddPCR assay for the effector mRNA was performed using the primers described above. In addition, biodistribution of CRMA-1001 was evaluated using an RNAscope ISH assay for detection of the Cas9 portion of the EE mRNA in tissues. Selected monkey tissues were formalin-fixed according to a protocol provided by Advanced Cell Diagnostics (Biotechnne). Tissues mounted on slides were pretreated for epitope retrieval and then hybridized with oligonucleotide probes targeting the Cas9 RNA in the samples. Detection was performed with a chromogenic substrate, and digital imaging was performed using a bright-field microscope. All images were taken at a magnification of 40×. An independent board-certified pathologist reviewed the slides prepared by Advanced Cell Diagnostics for interpretation and to provide details on specific cell types in which Cas9 mRNA was detected across analysed tissues (Aclairo Pharmaceutical Development Group).

Biomarker assessments

Quantification of secreted HBsAg and HBeAg. HBsAg and HBeAg levels in Tg-HBV mouse plasma were quantified using an HBsAg ELISA kit (Autobio, CLO310) and HBeAg ELISA kit (Autobio, CLO312). The LLOQs for the HBsAg and HBeAg assays were determined to be 5 IU ml⁻¹ and 6 IU ml⁻¹, respectively. HBsAg and HBeAg levels in cell supernatants

or AAV-HBV mouse plasma were analysed using an HBsAg CLIA ELISA kit (Ig Biotechnology, CL18002) and HBeAg CLIA ELISA kit (Ig Biotechnology, CL18004). The LLOQs for the HBsAg and HBeAg assays were determined to be 1.25 IU ml⁻¹ and 6 IU ml⁻¹, respectively. Any plasma samples with HBsAg or HBeAg values below the LLOQ were assigned the LLOQ value to calculate log₁₀ reduction relative to baseline values.

Circulating HBV DNA. To quantify total HBV DNA in mouse plasma, total DNA was extracted from mouse plasma supplemented with Poly(dA:dT) carrier DNA (Cell Signaling, 47945S) using the QIAamp DNA Blood Mini Kit (Qiagen, 51104). HBV DNA was quantified via ddPCR on the QX200 ddPCR system (Bio-Rad) using ddPCR Multiplex Supermix (Bio-Rad, 12005910) and HBV DNA-specific primer/probe set for total HBV DNA detection: primer 1 (GTGTCTGCGGCGTTTATC), primer 2 (GACAAACGGGCAACATACCT) and probe (CCTCATCTTCTGTGTGTTCTTCTGGAC). The LLOQ for the circulating HBV DNA assay was determined as 120 copies per microlitre of mouse plasma. Any samples below the LLOQ were assigned to the LLOQ value to calculate the log₁₀ reduction relative to baseline values.

DNA and RNA isolation. *Genomic DNA and RNA.* Nucleic acids were extracted from Tg-HBV mouse livers or HepG2.215 cells using simultaneous RNA and DNA extraction protocols (Beckman Coulter) for methylation assessments. RNA extraction for Quantification of gRNA and effector mRNA was performed using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, 217684) according to the manufacturer's protocol.

cccDNA isolation. PHH cells were infected with HBV at 2,000 MGE and treated with 1 µg ml⁻¹ of CRMA-1001 or mock treatment, following the infection and treatment protocol as described above. On day 7, the cells were washed with PBS and snap-frozen. DNA was extracted using a modified Hirt protocol, using the MasterPure Complete DNA and RNA Purification Kit (LucigenEpicentre) without the proteinase K digestion. Total DNA was digested using 10 U of T5 exonuclease per 1 µg of DNA for 30 min at 37 °C, followed by heat inactivation at 80 °C for 20 min. One-third of the extracted total DNA was not digested and used as a control. After digestion, all samples were concentrated using a standard sodium acetate DNA precipitation protocol. Around 25 copies of cccDNA per nanogram of total DNA were yielded for both CRMA-1001 and mock treatment, measured by ddPCR, described below.

Quantification of EE protein expression. Quantification was performed using the FastScan Cas9 (*S. pyogenes*) ELISA kit following the manufacturer's instructions. Cell pellets were lysed in 1× cell extraction buffer supplemented with cell extraction enhancer solution and 1× Halt protease inhibitor cocktail (Thermo Fisher). Total protein levels in cell lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher) following the manufacturer's instructions.

Quantification of gRNA and effector mRNA. RNA was quantified via RT-ddPCR on QX200 ddPCR system (Bio-Rad) using Bio-Rad One-Step RT-ddPCR Advanced Kit for Probes and HBV gRNA primer/probe set: primer 1 (GAGTTCGCGAGTATGGATGTT), primer 2 (CGACTCGGTGCCACTTT) and probe (AAGGCTAGTCCGTTATCAACTTGAA; effector mRNA primer/probe set: primer 1 (CTGGGACCCTAAGAAGTACG), primer 2 (CGAGCTCTTTCACACTCTTC) and probe (ACAGCC-CCACCGTGGCCTAT) and the control POLR2J assay (Hs01558819_m1, Thermo Fisher).

Evaluation of HBV transcripts and DNA in liver tissues. Animals were perfused with saline and liver biopsies taken using a 4-mm biopsy punch (Integra, 33-34) and stored in AllProtect Tissue Reagent (Qiagen, 76405). Total RNA was extracted using the RNeasy mini kit (Qiagen). RNA concentration was determined using the Qubit RNA broad range

assay kit (Thermo Fisher). HBV transcripts were quantified via RT-ddPCR on the QX200 ddPCR system (Bio-Rad) using a one-step RT-ddPCR advanced kit for probes (Bio-Rad), and the primers and probe specific for HBV transcripts, primer 1 (CTTCTCCGTCTGCCGTTC), primer 2 (GTGCAGAGGTGAAGCGAA) and probe (CACCTCTCTT-TACGCGGACTCCC). HBV transcript copies were normalized to the amount of total RNA. Total DNA was extracted from the liver samples using the QIAamp DNA Mini Kit (Qiagen). Total HBV DNA copies were quantified using ddPCR using the QX200 AutoDG Droplet Digital PCR System, supermix for probes (no dUTP) (Bio-Rad) and the DNA primer/probe set for total HBV DNA: primer 1 (GTGTCTGCGGCGTTTATC), primer 2 (GACAAACGGGCAACATACCT) and probe (CCTCATCTTCTGTGTGTTCTTCTGGAC; and DNA primer/probe set for episomal HBV DNA: primer 1 (CTCCCCGTCTGTGCCTTC), primer 2 (CCCCAAGCCAC-CCAAGG) and probe (TCGCATGGAGACCACCGTGAACGC). The mouse Tfrc locus was used for quantification of genomic copies Tfrc primer/probe set (TaqMan Copy Number Reference Assay, mouse, Tfrc, 20×; Thermo Fisher, 4458366). The DNA primer/probe set for episomal HBV DNA was also used for quantification of extracted cccDNA from HBV-infected PHH.

IHC staining of HBcAg. HBcAg immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded mouse livers using Ventana BenchMark Ultra by NovoVita Histopath Laboratory. The samples were dewaxed at 79 °C for 24 min, followed by heated EDTA-based antigen retrieval for 64 min. Inhibitor (Roche) was added and heated for 12 min. The samples were incubated with primary rabbit monoclonal anti-HBcAg antibody (Abcam, ab316283-1001) for 60 min, followed by secondary anti-rabbit horseradish peroxidase-conjugated antibody (Roche, 760-4315) for 32 min. DAB chromogen was added, and hematoxylin counterstaining and bluing was performed. Slides were scanned into digital slides using the AperioAT2 scanner. Image quantification was performed using HALO AI (Indica Labs). These quantification data are presented in Supplementary Table 3.

HBV genome on-target methylation sequencing. Genomic or cccDNA was sheared to a target size of 350 base pairs (bp) using a Covaris 96 AFA-Tube TPX plate. Sequencing libraries were then constructed from the sheared genomic DNA using the NEBNext Enzymatic Methyl-seq (EM-seq) kit plus NEBNext Multiplex Oligos for EM-seq. After methylation conversion, a custom panel targeting the appropriate HBV genome (genotype A for Tg-HBV mouse and genotype D for HepG2.2.15 cells and cccDNA) was used to enrich for regions of interest, following the recommended Twist protocol for 8-plex pools. Libraries were then amplified and sequenced using an Illumina NextSeq2000 running NextSeq1000/2000 Control software version 1.7.0.45751 with 2 × 150-bp reads to a sequencing depth of >5 million read pairs per sample.

The nf-core Methyl-Seq pipeline (version 1.6.1) was used to map sequencing reads to an HBV reference genome (GenBank accession number [U95551.1](#) for HepG2.2.15 cells and episomal cccDNA and GenBank accession number [AF305422.1](#) for Tg-Mice) using the Bismark⁴⁴ methylation extraction workflow version 0.24.0, with default parameters, except for the following: clip_r1 = 10, clip_r2 = 10, three_prime_clip_r1 = 10, three_prime_clip_r2 = 10, cytosine_report = true. Visualizations of on-target methylation were implemented in Python using the matplotlib and seaborn packages and show the position of each CpG in HBV coordinates along the x axis and level of methylation along the y axis. Methylation data were loaded from cytosine reports (generated by the Bismark pipeline), and replicate methylation values were averaged and visualized as bar plots using the matplotlib.pyplot.stairs functions. Regions of the HBV sequence in the [AF305422.1](#) reference that were duplications contained no aligned reads and were therefore excluded from the visualized region. Average methylation of the entire HBV genome was calculated for each treatment.

PCSK9 promoter on-target methylation sequencing. Genomic DNA was extracted from NHP liver biopsies using the DNAdvance Kit (Beckman Coulter). Time points for methylation were days −35, 24, 54, 176 and 373. Genomic DNA was further processed and analysed as described in Tremblay et al.¹⁵.

CRMA-1001 specificity assessments

Cell treatment. Uninfected PHHs, HSECs or HAdCCs (three donors per cell type and nine donors in total) were treated with CRMA-1001 at 0.5 $\mu\text{g ml}^{-1}$ (PHHs only) or 1 $\mu\text{g ml}^{-1}$ (all cell types). The selected dose of 1 $\mu\text{g ml}^{-1}$ was empirically determined to be approximately 2.8-fold above the 90% inhibitory concentration (IC_{90}) for viral antigen suppression in infected PHHs. At day 14 post treatment, uninfected cells were collected and processed for RNA-seq and DNA methylation sequencing. Controls included in this experiment for all cell types were EE7000 mRNA only, PCSK9-EE7000 (non-HBV targeting control), mock treatment (rhApoE3 only), and untreated. An HBV CRISPRi control was also included only for PHHs. CRISPRi transiently silences genes using a transcriptional repressor domain (KRAB) but does not contain a DNA methylation domain⁴⁵.

RNA-seq. Total RNA was extracted from PHHs, HSECs and HAdCCs (three donors per cell type and nine donors in total) using a split lysate protocol to extract both RNA and DNA from the same sample lysate (Beckman Coulter). Total RNA-seq libraries were constructed using the Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit. Libraries were sequenced on a NovaSeq X with a 2 × 151-bp configuration to a read depth of >50 million read pairs per sample. For one HSEC donor, the RNA-seq experiment was performed twice, with all results from both experiments included in the specificity assessment. A representative plot of one experiment from this donor is shown. Sequencing reads were aligned to the human reference genome (GRCh38), and gene and transcript counts and normalized counts (TPM) were calculated using the nf-core RNA-seq pipeline (version 3.6) using the STAR⁴⁶ aligner (version 2.7.6d) and RSEM⁴⁷ (version 1.3.3) workflow for transcript quantification. Differential gene expression was detected using DESeq2⁴⁸ (version 1.42.0). The thresholds were experimentally established (manuscript in preparation), resulting in two parameter sets: (1) a threshold of 1.75-fold change and an adjusted P value $\leq 1 \times 10^{-5}$ and (2) a threshold of 1.5-fold change and an adjusted P value ≤ 0.05 . To identify direct unintended targets mediated by the gRNA of CRMA-1001, DEGs detected in comparisons between CRMA-1001-treated cells and controls (EE7000 mRNA only, PCSK9-EE7000) and not detected in comparisons between controls (mock-treated versus untreated, EE7000 mRNA only versus mock treatment, and EE7000 mRNA only versus PCSK9-EE7000) were identified as CRMA-1001 specific and were nominated as potential unintended target sites for confirmatory assessment. Volcano plots of only the CRMA-1001 versus EE7000 mRNA only comparison are shown; however, both comparisons were used in the analysis. Filtering to remove control comparison DEGs was performed using control comparisons from all cell types for the 1.5-fold change and an adjusted P value ≤ 0.05 parameter set, while for the 1.75-fold change and an adjusted P value $\leq 1 \times 10^{-5}$ parameter set, the filtering was performed within each cell type. Filtering to remove genes that were not expressed above the level of noise in the assay (2 TPM) was performed at the level of the comparison where the DEG was detected, with a requirement that the gene was expressed ≥ 2 TPM in either of the conditions within the comparison.

Twist Human Nethylome HCMS. The Twist Human Methylome HCMS panel targets a subset of approximately 4 million CpGs in the human genome near known regulatory regions such as TSSs and CGIs. Genomic DNA was extracted from PHHs, HSECs and HAdCCs using a split lysate protocol to extract both RNA and DNA from the same sample lysate (Beckman Coulter). Genomic DNA was mechanically sheared on a

Covaris R230 Focused Ultrasonicator to a size range of 350–450 bp. Sequencing libraries were then constructed from the sheared genomic DNA using the NEBNext EM-seq kit plus NEBNext Multiplex Oligos for EM-seq. After methylation conversion, the Human Methylome Hybrid Capture panel (Twist Biosciences) was used to enrich for regions of interest, following the recommended Twist protocol for 8-plex pools, and libraries were then amplified and sequenced using an Illumina NovaSeq X instrument with 2 × 151-bp reads to a read depth of >100 million read pairs per sample.

Sequencing reads were mapped to the reference genome (GRCh38) as described above ('HBV genome on-target methylation sequencing'). The R programming language (version 4.3.1) packages Methrix⁴⁹ (version 1.16.0) and DSS^{50–53} (version 2.48.0) were used to filter the results from the Methyl-seq pipeline, and to call DMRs, respectively. The Bismark coverage files were loaded into Methrix objects using `methrix::read_bedgraphs` with the following parameters: `pipeline = 'bismark_cov'`, `stranded = TRUE`, `zero_based = FALSE` and `collapse_strands = TRUE`, and filtered to keep only CpGs that (1) fall within the regions associated with the Twist Human Methylome panel and (2) that meet a minimum 20× coverage threshold, across all libraries in the dataset. CpGs that are in the top 0.01% of coverage were also filtered out of the dataset, as these may represent mapping artifacts or repetitive regions. Next, DSS::DMLTest was used to call significantly differentially methylated CpGs between the experimental and reference conditions, with the following parameters: `smoothing = true`, `smoothing_span = 500` bp. Then, DSS::callDMR was run to aggregate nearby differentially methylated CpGs into DMRs, using a minimum CpG count per DMR of 10. DMRs were detected using DSS, using the experimentally established thresholds (manuscript in preparation), which resulted in two parameter sets: (1) a threshold of absolute methylation difference >0.1 and a P value $\leq 1 \times 10^{-10}$ and (2) a threshold of absolute methylation difference >0.075 and an adjusted P value ≤ 0.05 . To identify direct unintended targets mediated by the gRNA of CRMA-1001, DMRs detected in comparisons between CRMA-1001-treated cells and controls (EE7000 mRNA only, PCSK9-EE7000) and not detected in comparisons between controls (mock-treated versus untreated, EE7000 mRNA only versus mock treatment, EE7000 mRNA only versus PCSK9-EE7000, and HBV CRISPRi versus mock treatment) were identified as CRMA-1001 specific and were nominated as potential unintended target sites for confirmatory assessment. Volcano plots of only the CRMA-1001 versus EE7000 mRNA only comparison are shown; however, both comparisons were used in the analysis.

WGMS. WGMS covers all CpGs genome-wide (approximately 30 million CpGs). Genomic DNA was extracted from PHHs, HSECs and HAdCCs using a split lysate protocol to extract both RNA and DNA from the same sample lysate (Beckman Coulter). Genomic DNA was mechanically sheared on a Covaris R230 Focused Ultrasonicator to a size range of 350–450 bp. Sequencing libraries were then constructed from the sheared genomic DNA using the NEBNext EM-seq kit plus NEBNext Multiplex Oligos for EM-seq. Libraries were then sequenced using an Illumina NovaSeq X instrument with 2 × 151-bp reads to a read depth of >1 billion read pairs per sample.

Sequencing reads were analysed as described above ('Twist Human Methylome HCMS'), with the following differences. Panel region filtering was not applicable for these genome-wide datasets. Data were filtered to retain CpGs that meet a minimum 5× coverage threshold, across all libraries in the dataset. DMRs were detected using DSS, with two parameter sets: (1) a threshold of absolute methylation difference >0.2 and a P value $\leq 1 \times 10^{-10}$ and (2) a threshold of absolute methylation difference >0.125 and an adjusted P value ≤ 0.05 .

Custom specificity panel HCMS. Custom HCMS panels were designed using Twist Biosciences' proprietary methylation probe design technology, with the following input. Genomic sites included on the custom hybrid capture panels represented all unique potential unintended

targets of CRMA-1001 nominated from PHHs, HSECs and HAdCCs using all three nomination assays (RNA-seq, Human Methylome HCMS and WGMS), as well as nominations based on in silico analysis of predicted gRNA binding sites in the human genome. For nominated DEGs, the probes were designed to span 2 kb of the promoter region (± 1 kb from the TSS) for every annotated TSS associated with a nominated DEG with an HGNC identifier, thereby encompassing transcriptional control regions at which methylation by CRMA-1001 is most likely to have a functional impact. For genes without an HGNC identifier, probes were designed to span ± 1 kb from the gene start if on the positive strand and ± 1 kb from the gene end if on the negative strand. Probe designs based on nominated DMRs utilized ± 500 bp from the start and end of the DMR (1,000 bp + DMR length combined). Last, probes were also designed on the basis of the predicted CRMA-1001 binding sites in the human genome from the in silico analysis. Probes were designed to span ± 500 bp of predicted gRNA binding sites (1,000 bp total). Overlapping genomic regions were combined. Three separate panels were used for this analysis. One panel included the in silico sites, and sites from PHHs nominated from DEGs using the 1.75-fold change and an adjusted P value $\leq 1 \times 10^{-5}$ parameter set, and from DMRs using the parameter sets of P value $\leq 1 \times 10^{-10}$ and the absolute methylation difference of 0.1 (Human Methylome HCMS) or 0.2 (WGMS). A second panel included the in silico sites, and sites from the extrahepatic cell types (HSECs and HAdCCs) nominated from DEGs using the 1.75-fold change and an adjusted P value $\leq 1 \times 10^{-5}$ parameter set, and from DMRs using the parameter sets of P value $\leq 1 \times 10^{-10}$ and the absolute methylation difference of 0.1 (Human Methylome HCMS) or 0.2 (WGMS). A third panel included sites from all three cell types nominated from DEGs using the 1.5-fold change and an adjusted P value ≤ 0.05 parameter set and from DMRs using the parameter sets of an adjusted P value ≤ 0.05 and the absolute methylation difference of 0.075 (Human Methylome HCMS) or 0.125 (WGMS).

Genomic DNA was extracted from PHHs, HSECs and HAdCCs using a split lysate protocol to extract both RNA and DNA from the same sample lysate (Beckman Coulter). Genomic DNA was mechanically sheared on a Covaris R230 Focused Ultrasonicator to a size range of 350–450 bp. Sequencing libraries were then constructed from the sheared genomic DNA using the NEBNext EM-seq kit plus NEBNext Multiplex Oligos for EM-seq. After methylation conversion, the custom Hybrid Capture panel (Twist Biosciences) was used to enrich for regions of interest, following the recommended Twist protocol for 8-plex pools, and libraries were then amplified and sequenced using either an Illumina NovaSeq X or an Illumina NextSeq 2000 instrument with 2×151 -bp reads to a read depth of >100 million read pairs per sample.

Sequencing reads were analysed as described above ('Twist Human Methylome HCMS'), with the following differences. Data were filtered to retain CpGs that (1) fall within the regions associated with the custom HCMS panel and (2) meet a minimum $30\times$ coverage threshold, across all libraries in the dataset. DMRs were detected using DSS with a threshold of absolute methylation difference >0.11 and an adjusted P value ≤ 0.01 . Additional criteria were used to determine confirmed DMRs: (1) confirmed DMRs were detected in comparisons between CRMA-1001-treated cells and controls (EE7000 mRNA only and PCSK9-EE7000) and not detected in comparisons between controls (mock-treated versus untreated, EE7000 mRNA only versus mock treatment, EE7000 mRNA only versus PCSK9-EE7000, and HBV CRISPRi versus mock treatment). (2) Confirmed DMRs were reproducibly identified within a donor, that is, compared with both the control conditions (EE7000 mRNA only and PCSK9-EE7000), and if identified at the $0.5 \mu\text{g ml}^{-1}$ dose, the DMR must also be identified at the higher $1 \mu\text{g ml}^{-1}$ dose to be considered confirmed. (3) On the basis of the MoA of CRMA-1001 to methylate DNA, only hypermethylated DMRs were included in the list of confirmed DMRs. Genes were associated with confirmed DMRs on the basis of genomic proximity, with all genes within 2 kb of each DMR included.

Data visualization. Visualization of primary guide screen data, guide conservation analysis, RNA-seq data and methylation sequencing data (both on target and off target) was carried out using either the Python programming language (version 3.12) with the visualization libraries matplotlib⁵⁴ (version 3.10) and seaborn⁵⁵ (version 0.13) libraries or using the R programming language (version 4.3.1) with the visualization library ggplot (version 3.4.1). All other data were graphed and analysed using GraphPad Prism (version 10.2.2).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA-seq and methylation sequencing data have been deposited in NCBI's Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number [GSE318266](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE318266). Publicly available datasets [GSE282432](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE282432), Ensembl reference genomes GRCh38.p13 and Macaca_fascicularis_6.0, HBV reference genomes with GenBank accession numbers [U95551.1](https://www.ncbi.nlm.nih.gov/nuclot/U95551.1) and [AF305422.1](https://www.ncbi.nlm.nih.gov/nuclot/AF305422.1), and HBVdb version 54.0 and version 58.0 were used in this study. Additional data are also provided in a Supplementary Information File. Source data are provided with this paper.

References

1. *Global Hepatitis Report 2024: Action for Access in Low- and Middle-Income Countries* <https://www.who.int/publications/i/item/9789240091672> (WHO, 2024).
2. Wong, G. L. H., Gane, E. & Lok, A. S. F. How to achieve functional cure of HBV: stopping NUCs, adding interferon or new drug development? *J. Hepatol.* **76**, 1249–1262 (2022).
3. Wu, S. et al. Immune mechanisms underlying hepatitis B surface antigen seroclearance in chronic hepatitis B patients with viral coinfection. *Front. Immunol.* **13**, 893512 (2022).
4. Dandri, M. Epigenetic modulation in chronic hepatitis B virus infection. *Semin. Immunopathol.* **42**, 173–185 (2020).
5. Guo, Y., Li, Y., Mu, S., Zhang, J. & Yan, Z. Evidence that methylation of hepatitis B virus covalently closed circular DNA in liver tissues of patients with chronic hepatitis B modulates HBV replication. *J. Med. Virol.* **81**, 1177–1183 (2009).
6. Kim, J.-W. et al. Replicative activity of hepatitis B virus is negatively associated with methylation of covalently closed circular DNA in advanced hepatitis B virus infection. *Intervirology* **54**, 316–325 (2011).
7. Zhang, Y. et al. Transcription of hepatitis B virus covalently closed circular DNA is regulated by CpG methylation during chronic infection. *PLoS ONE* **9**, e110442 (2014).
8. Lampertico, P. et al. EASL 2017 clinical practice guidelines on the management of hepatitis B virus infection. *J. Hepatol.* **67**, 370–398 (2017).
9. Moini, M. & Fung, S. HBsAg loss as a treatment endpoint for chronic HBV infection: HBV cure. *Viruses* **14**, 657 (2022).
10. Lok, A. S. F. Toward a functional cure for hepatitis B. *Gut Liver* **18**, 593–601 (2024).
11. Liaw, Y.-F. Stopping nucleos(t)ide analogue therapy in HBeAg-negative chronic hepatitis B. *Gastroenterology* **159**, 1186 (2020).
12. Lim, S. G. et al. The scientific basis of combination therapy for chronic hepatitis B functional cure. *Nat. Rev. Gastroenterol. Hepatol.* **20**, 238–253 (2023).
13. Smekalova, E. M. et al. Cytosine base editing inhibits hepatitis B virus replication and reduces HBsAg expression in vitro and in vivo. *Mol. Ther. Nucleic Acids* **35**, 102112 (2024).
14. Gorsuch, C. L. et al. Targeting the hepatitis B cccDNA with a sequence-specific ARCUS nuclease to eliminate hepatitis B virus in vivo. *Mol. Ther.* **30**, 2909–2922 (2022).

15. Tremblay, F. et al. A potent epigenetic editor targeting human PCSK9 for durable reduction of low-density lipoprotein cholesterol levels. *Nat. Med.* **31**, 1329–1338 (2025).
16. Stepper, P. et al. Efficient targeted DNA methylation with chimeric dCas9-Dnmt3a-Dnmt3L methyltransferase. *Nucleic Acids Res.* **45**, 1703–1713 (2017).
17. Nuñez, J. K. et al. Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. *Cell* **184**, 2503–2519 (2021).
18. Amabile, A. et al. Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. *Cell* **167**, 219–232 (2016).
19. Qi, L. S. et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 (2013).
20. Cappelluti, M. A. et al. Durable and efficient gene silencing in vivo by hit-and-run epigenome editing. *Nature* **627**, 416–423 (2024).
21. Hayer, J. et al. HBVdb: a knowledge database for hepatitis B Virus. *Nucleic Acids Res.* **41**, D566–D570 (2012).
22. Sells, M. A., Chen, M. L. & Acs, G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl Acad. Sci. USA* **84**, 1005–1009 (1987).
23. Essalmani, R. et al. A single domain antibody against the Cys- and His-rich domain of PCSK9 and evolocumab exhibit different inhibition mechanisms in humanized PCSK9 mice. *Biol. Chem.* **399**, 1363–1374 (2018).
24. Li, H. et al. Inhibition of HBV expression in HBV transgenic mice using AAV-delivered CRISPR–SaCas9. *Front. Immunol.* **9**, 2080 (2018).
25. Guidotti, L. G., Matzke, B., Schaller, H. & Chisari, F. V. High-level hepatitis B virus replication in transgenic mice. *J. Virol.* **69**, 6158–6169 (1995).
26. Guo, H., Urban, S. & Wang, W. In vitro cell culture models to study hepatitis B and D virus infection. *Front. Microbiol.* **14**, 1169770 (2023).
27. Wagner, G. P., Kin, K. & Lynch, V. J. A model based criterion for gene expression calls using RNA-seq data. *Theory Biosci.* **132**, 159–164 (2013).
28. Maston, G. A., Evans, S. K. & Green, M. R. Transcriptional regulatory elements in the human genome. *Genom. Hum. Genet.* **7**, 29–59 (2006).
29. Michler, T. et al. Knockdown of virus antigen expression increases therapeutic vaccine efficacy in high-titer hepatitis B virus carrier mice. *Gastroenterology* **158**, 1762–1775 (2020).
30. Gulck, E. V. et al. Retreatment with HBV siRNA results in additional reduction in HBV antigenemia and immune stimulation in the AAV-HBV mouse model. *Viruses* **16**, 347 (2024).
31. Hua, C., Aftab, S., Rahaman, A., Abbas, Z. & Baloch, Z. siRNA approaches in HBV: from antigen reduction to seroconversion. *Mol. Ther. Nucleic Acids* **36**, 102718 (2025).
32. Yuen, M.-F. et al. Efficacy and safety of bepirovirsen in chronic hepatitis B infection. *N. Engl. J. Med.* **387**, 1957–1968 (2022).
33. Ghany, M. G. & Lok, A. S. Functional cure of hepatitis B requires silencing covalently closed circular and integrated hepatitis B virus DNA. *J. Clin. Investig.* **132**, e163175 (2022).
34. Musunuru, K. Moving toward genome-editing therapies for cardiovascular diseases. *J. Clin. Investig.* **132**, e148555 (2022).
35. Miodownik, I. et al. DNA methylation shapes transcription factor binding beyond canonical CpG contexts. *Proc. Natl Acad. Sci. USA* **122**, e2520814122 (2025).
36. Li, S., Peng, Y., Landsman, D. & Panchenko, A. R. DNA methylation cues in nucleosome geometry, stability and unwrapping. *Nucleic Acids Res.* **50**, 1864–1874 (2022).
37. Pei, Y. & Li, G. Unraveling the interplay of DNA methylation and chromosome organization. *Biochem. Soc. Trans.* **53**, 1433–1444 (2025).
38. Martinez, M. G. et al. CRISPR–Cas9 targeting of hepatitis B virus covalently closed circular DNA generates transcriptionally active episomal variants. *Mbio* **13**, e02888-21 (2022).
39. Aussel, C., Cathomen, T. & Fuster-García, C. The hidden risks of CRISPR/Cas: structural variations and genome integrity. *Nat. Commun.* **16**, 7208 (2025).
40. Camacho, C. et al. BLAST+: architecture and applications. *BMC Bioinform.* **10**, 421 (2009).
41. Maier, M. A. et al. Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* **21**, 1570–1578 (2013).
42. Tatenò, C. & Kojima, Y. Characterization and applications of chimeric mice with humanized livers for preclinical drug development. *Lab. Anim. Res.* **36**, 2 (2020).
43. Migliara, A. et al. In vitro selection of engineered transcriptional repressors for targeted epigenetic silencing. *J. Vis. Exp.* <https://doi.org/10.3791/64403> (2023).
44. Krueger, F. & Andrews, S. R. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27**, 1571–1572 (2011).
45. Larson, M. H. et al. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* **8**, 2180–2196 (2013).
46. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2012).
47. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. *BMC Bioinform.* **12**, 323 (2011).
48. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
49. Mayakonda, A. et al. Methrix: an R/Bioconductor package for systematic aggregation and analysis of bisulfite sequencing data. *Bioinformatics* **36**, 5524–5525 (2020).
50. Feng, H., Conneely, K. N. & Wu, H. A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. *Nucleic Acids Res.* **42**, e69 (2014).
51. Wu, H., Wang, C. & Wu, Z. A new shrinkage estimator for dispersion improves differential expression detection in RNA-seq data. *Biostatistics* **14**, 232–243 (2013).
52. Wu, H. et al. Detection of differentially methylated regions from whole-genome bisulfite sequencing data without replicates. *Nucleic Acids Res.* **43**, e141 (2015).
53. Park, Y. & Wu, H. Differential methylation analysis for BS-seq data under general experimental design. *Bioinformatics* **32**, 1446–1453 (2016).
54. Hunter, J. D. Matplotlib: a 2D graphics environment. *Comput. Sci. Eng.* **9**, 90–95 (2007).
55. Waskom, M. seaborn: statistical data visualization. *J. Open Source Softw.* **6**, 3021 (2021).

Acknowledgements

We thank T. Brambrink, C. Conant, J. Dienstag, A. Espejo, L. Ferreira, D. Ganem, L. Guidotti, M. Hasan, P. Koppa, K. Lewis, D. Litvak, A. Suk-Fong Lok, P. Malyala, S. Martinez, M. May, T. McGregor, J. Merson, S. Parikh, Z. Stoltzner and Y. Weber for their contributions in support of this work. We are grateful to the entire nChroma Bio team for their support, input and discussions throughout the execution of these studies. We also thank the team at Acuitas Therapeutics who supported the work by manufacturing LNP drug product for mouse and NHP studies.

Author contributions

Y.A.-R., M.A.C., J.A.M., C.M., C.P., M.C., L.D., R.D.F., A.L. and A.B.J. designed, performed and/or analysed in vitro studies (inclusive of

unpublished, preceding in vitro studies, performed and analysed by M.A.C. and supervised and coordinated by A.L.). G.A., R.C.-W., L.H., A.K., C.-W.K., C.G., S.H., B.N., S.B.V., Q.X. and J.L.M. designed, performed and/or analysed in vivo studies. A.D., S. Abraham, A.Z., X.G., S. Abubucker, E.M.H., R.N.R. and S.H. designed, performed and analysed the in silico, DNA methylation and/or specificity assessments. V.E.M., A.E.F., M.S.M. and T.E. conceived and directed EE design and testing. Y.A.-R., Q.X. and S.B.V. supervised the work, with planning and scientific input from P.S., oversight from V.E.M., A.B.J., M.B. and J.L.M., and advisory input from A.L. and R.D.F. Y.A.-R. wrote the manuscript in collaboration with Q.X., S.B.V., S.H., B.N., E.M.H. and R.N.R. J.L.M. and M.B. edited the manuscript. All authors reviewed and approved the final version of the manuscript.

Funding

This work was supported by nChroma Bio Inc. The funders of nChroma Bio had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests

Q.X., S.B.V., L.H., A.K., C.M., S.H., S. Abraham., E.M.H., X.G., C.-W.K., T.E., M.B. and J.L.M. are employees of and hold an equity interest in nChroma Bio. Y.A.-R., R.C.-W., R.N.R., S. Abubucker, C.G., P.S., M.S.M., A.E.F. and V.E.M. hold an equity interest in nChroma Bio. A.L. is a cofounder of and holds an equity interest in nChroma Bio. The other authors declare no competing interests. nChroma Bio has filed for patent protection related to various aspects of ES of the HBV.

Additional information

Extended data is available for this paper at <https://doi.org/10.1038/s41551-026-01802-8>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41551-026-01802-8>.

Correspondence and requests for materials should be addressed to Jennifer L. Marlowe.

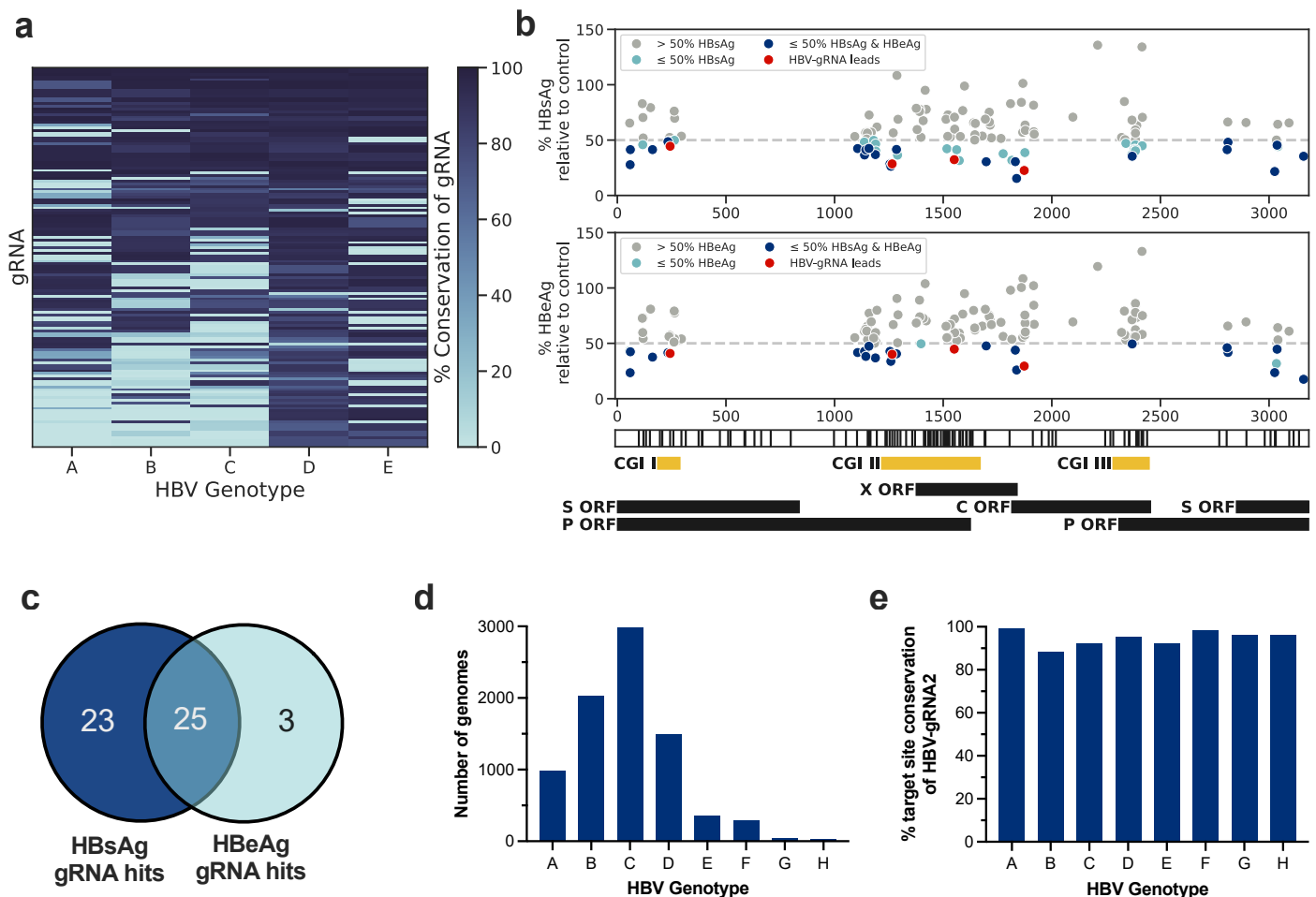
Peer review information *Nature Biomedical Engineering* thanks Luis Schang and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

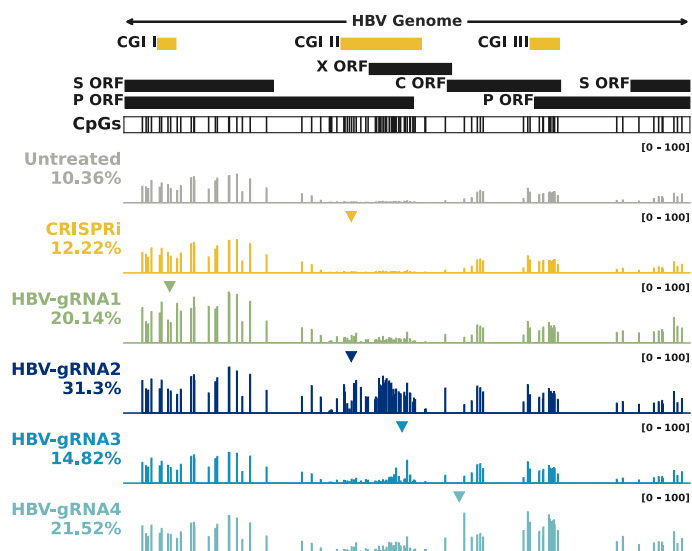
Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2026



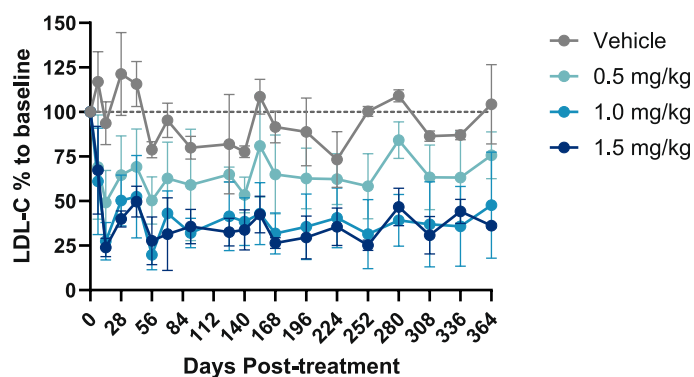
Extended Data Fig. 1 | In silico and in vitro assessments of a panel of 121 HBV-gRNAs spanning all viral ORFs across the HBV genome. (a) In silico conservation analysis of HBV-gRNA target sites using HBV genome sequences (genotypes A–E). **(b)** Primary screen evaluating a total of 121 HBV-gRNAs in HBV-infected HepG2-hNTCP cells using LNP formulated with an early EE generation called engineered transcriptional repressors (ETR)¹⁸. Vertical black bars represent the location of CpGs along the HBV viral genome. The coordinates of viral genes are displayed at the bottom. The secreted viral antigens (*top panel*) HBsAg and (*bottom panel*) HBeAg were measured and normalized to cells treated with a non-targeting control gRNA. Each dot represents a single HBV-gRNA. Grey

dots represent HBV-gRNAs with >50% antigen expression compared to untreated cells, teal dots represent HBV-gRNAs with ≤50% either HBsAg or HBeAg antigen expression, dark blue dots represent HBV-gRNA with ≤50% antigen expression in both HBsAg and HBeAg, and red dots indicate the 4 HBV-gRNA leads. **(c)** Venn diagram of HBV-gRNAs with >50% silencing of HBsAg and/or HBeAg. **(d–e)** Evaluation of HBV-gRNA2 target sequence. **(d)** Number of genomes per HBV genotype evaluated for target conservation. **(e)** Percentage of genomes within each genotype with a perfect nucleotide match to the target sequence of the HBV-gRNA2.



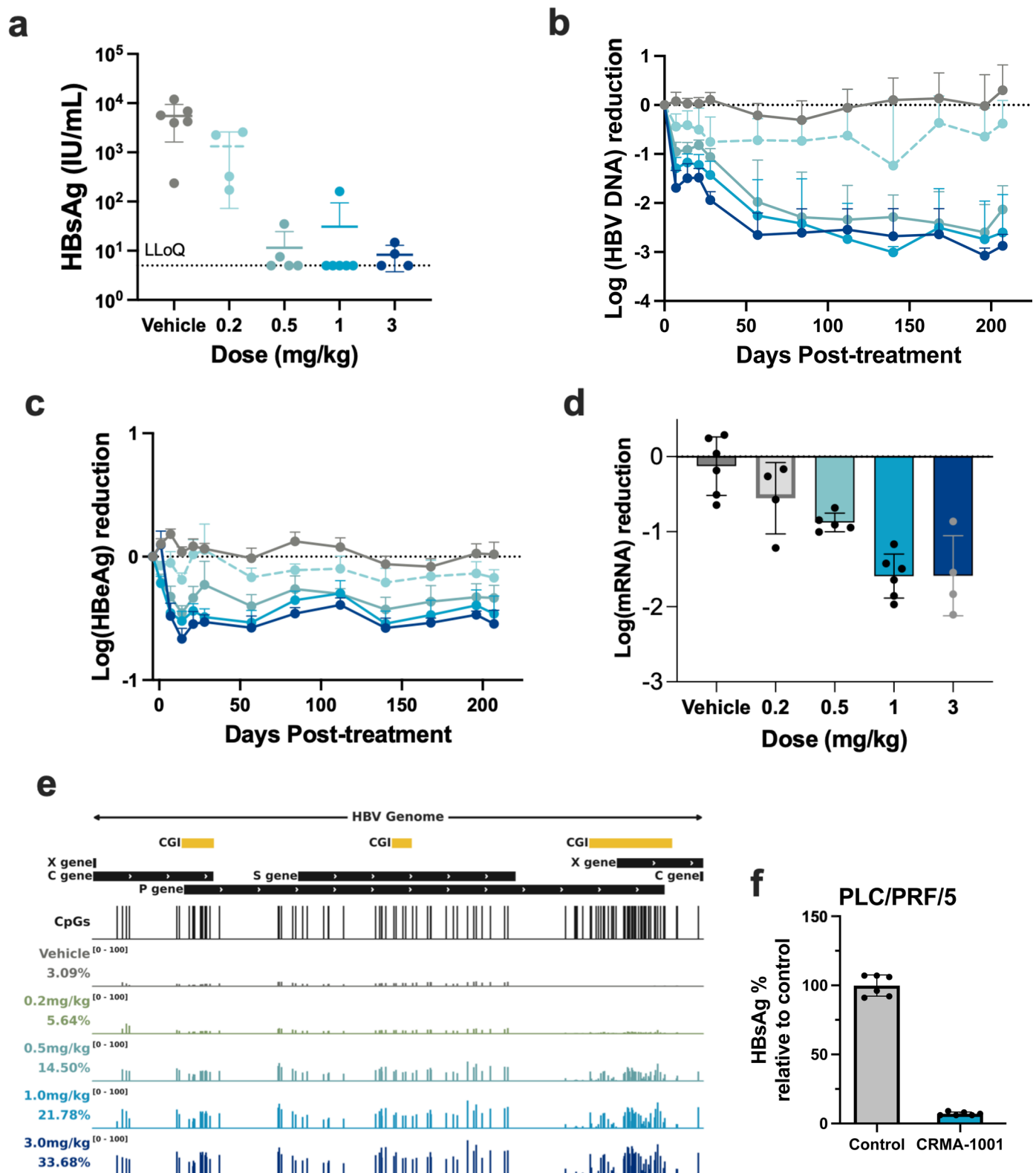
Extended Data Fig. 2 | On-target methylation profiles of lead HBV-gRNAs in HepG2.2.15 cells at 27 days post-treatment. Methylation at CpGs in the HBV genome is depicted as a bar plot. The bar position along the x-axis represents the position of a CpG within the HBV genome, and the bar height is the individual

CpG methylation percentage. The ORF annotations of HBV genes (black), CGIs (yellow), and positions of individual CpGs (black vertical lines) for HBV genotype D are shown at the top. Triangles show each gRNA target site. Average CpG methylation across the HBV sequence is shown on the left.



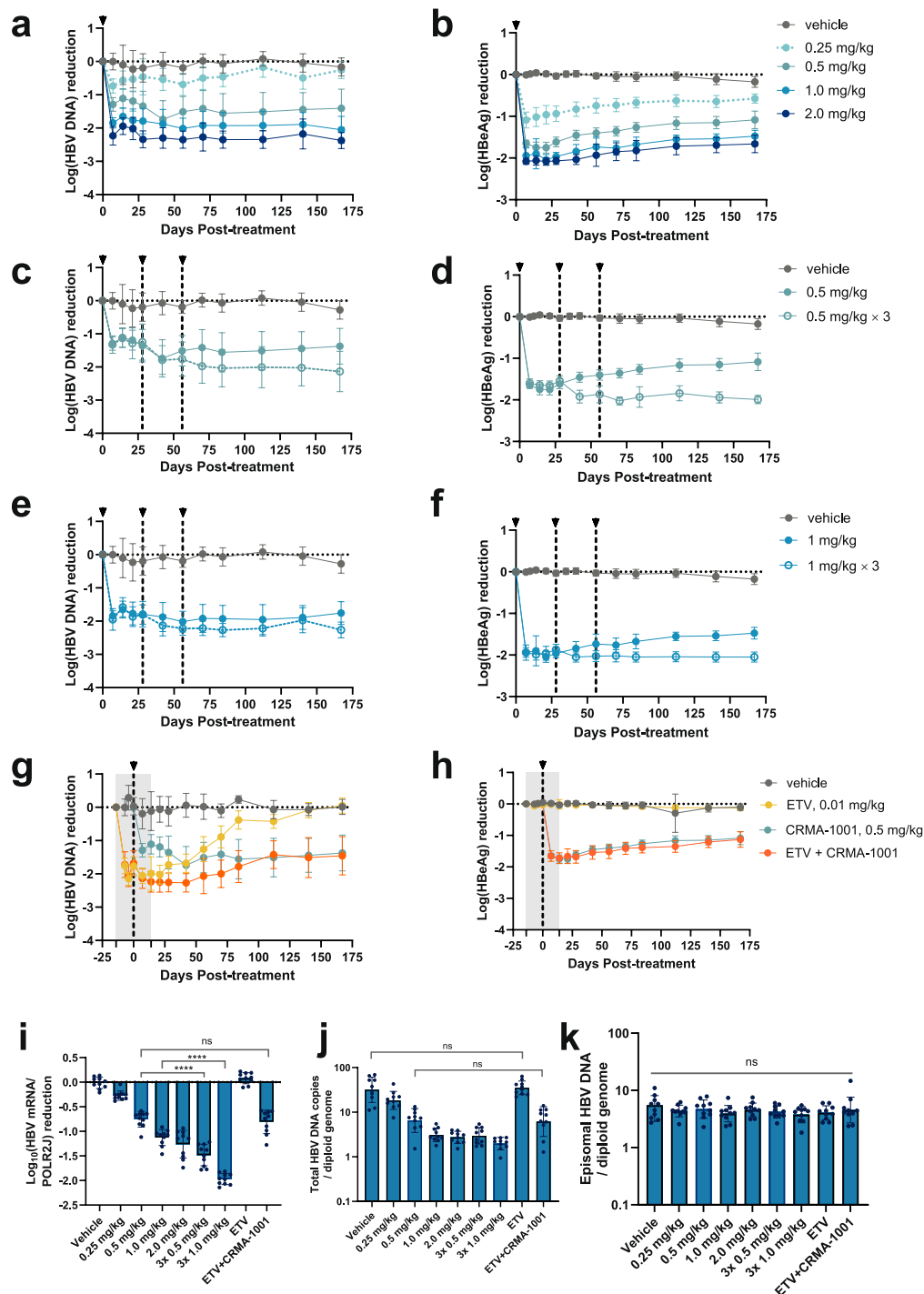
Extended Data Fig. 3 | Reduction of low-density lipoprotein cholesterol associated with silencing of the surrogate target *PCSK9* in NHPs. Low-density lipoprotein cholesterol (LDL-c) levels after single IV administration of PCSK9-EE7000 at 0.5, 1, and 1.5 mg/kg (mean $\pm$ SD; n = 2 in vehicle group and

n = 3 in EE-treated groups) for approximately one-year post-treatment, the observation period for this biomarker, in NHPs. No statistical difference was observed between LDL-c levels of 1.0 and 1.5 mg/kg groups (two-tailed t-test).



Extended Data Fig. 4 | Epigenetic silencers HBV-EE4000 and CRMA-1001 robustly silence viral gene expression from integrated HBV DNA. (a–e) Tg-HBV mice were treated with vehicle (n = 6), or HBV-EE4000 at 0.2 (n = 4), 0.5 (n = 5), 1.0 (n = 6) or 3.0 (n = 4) mg/kg. Data are presented as mean values $\pm$ SD for each group. (a) HBsAg in plasma of individual animals at 207 days post-treatment, (b) HBV DNA in plasma, (c) HBeAg in plasma, and (d) total HBV mRNA copy number in liver. All biomarkers were plotted as $\log_{10}$ -fold reduction compared to baseline levels (HBV DNA and HBeAg) or to the vehicle-treated group (total HBV

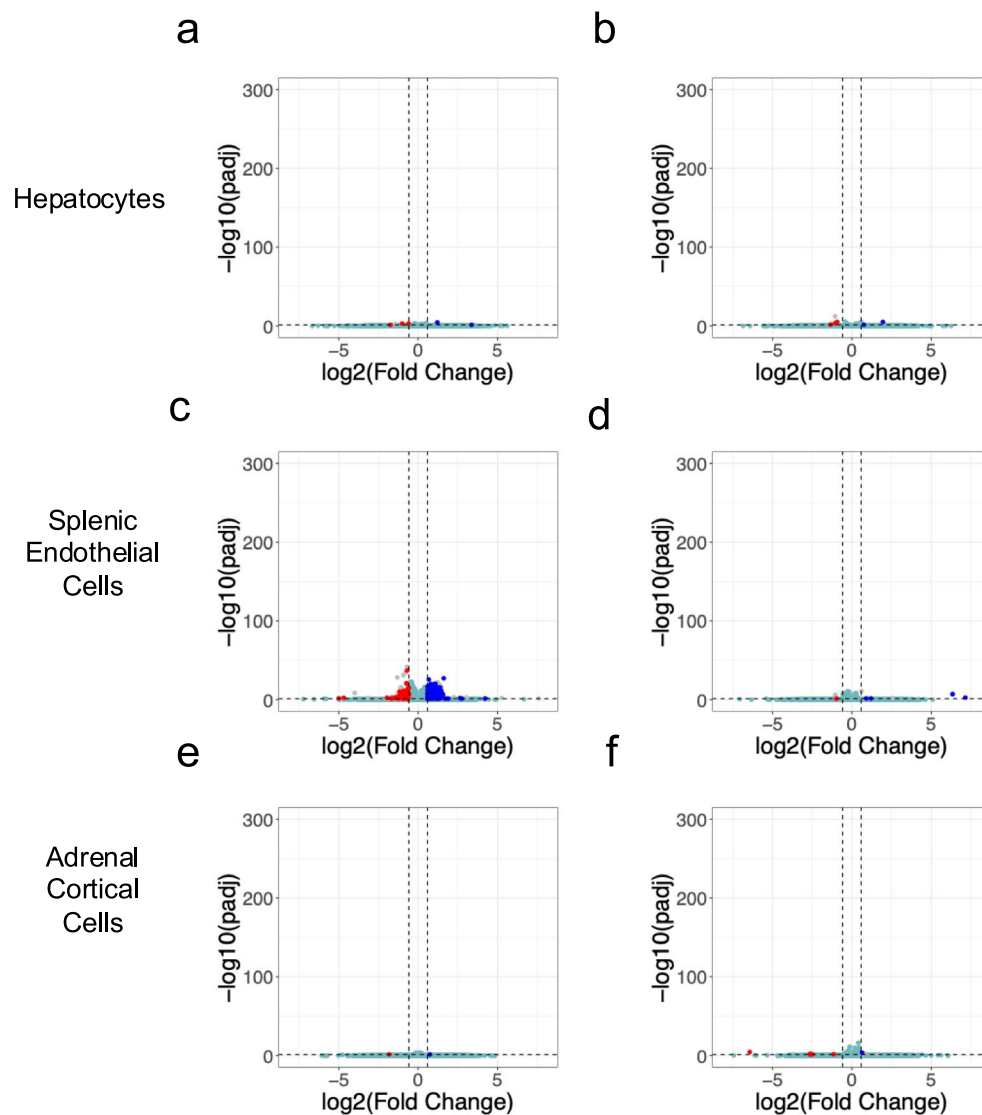
mRNA). (e) DNA methylation profile in liver of HBV-EE4000-treated Tg-HBV mice. Percentage values under each dose group label represent the mean methylation level calculated across all samples from the respective dose group. Annotation of the HBV genome is visualized at the top, with yellow bars representing CGIs, black bars with arrows representing HBV genes and vertical black lines representing positions of individual CpGs. (f) Silencing of HBsAg in PLC/PRF/5 cells on Day 14 following treatment with control (rhApoE3, mock-treatment) or 1 μ g/mL CRMA-1001. Data are presented as mean $\pm$ SD; n = 6 biological replicates per group.



Extended Data Fig. 5 | CRMA-1001 reduces HBV DNA, HBeAg, and HBV viral transcription in liver for 6 months in AAV-HBV mice, with single or multiple dosing, or in combination with ETV treatment. Log₁₀ fold-change in circulating HBV biomarkers following single or three monthly doses of CRMA-1001.

(a) HBV DNA after single dose, (b) HBeAg after single dose, (c) HBV DNA after three doses at 0.5 mg/kg, (d) HBeAg after three doses at 0.5 mg/kg, (e) HBV DNA after three doses at 1 mg/kg, (f) HBeAg after three doses at 1.0 mg/kg. Vertical dashed lines and arrows indicate CRMA-1001 administration and x-axis shows days post-treatment after the first dose. (g) Log₁₀ fold-change in circulating HBV

DNA and (h) HBeAg following treatment with CRMA-1001, ETV or in combination. Vertical shaded area represents the timeframe of ETV treatment. Data are presented as mean ± SD. (i) Normalized HBV mRNA transcript levels in terminal liver samples. Two-tailed t-test statistical analysis, **** $p < 0.0001$. (j-k) HBV DNA levels in liver samples at study termination. (j) Total HBV DNA copies/diploid genome. (k) Episomal HBV DNA per diploid genome. Bars represent mean ± SD with symbols representing individual animals ($n = 10$) One-way ANOVA statistical analysis.



Extended Data Fig. 6 | RNA-seq from two additional donors in three primary cell types. Differential gene expression changes in six individual donors (two per cell type) (a–b) liver (primary human hepatocytes), (c–d) spleen (human spleen endothelial cells), and (e–f) adrenal gland (human adrenal cortical cells) shown as volcano plots, grey lines represent thresholds for significant changes; $n \geq 3$ replicates. Dots represent individual genes

(blue: upregulated; red: downregulated; teal: not differentially expressed; grey: also observed in control conditions); change in expression (x-axis); statistical significance (DESeq2 Wald test, two-sided, Benjamini-Hochburg multiple comparisons adjustment) (y-axis); thresholds: adjusted p-value (FDR) ≤ 0.05 and absolute fold-change of >1.5 .

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

Biotek Gen 5 version 3.12.08
NextSeq 2000: NextSeq1000/2000 Control software version 1.7.0.45751
NovaSeq X Control software

Data analysis

QX Manager Software 2.0 Standard Edition (Bio-Rad)
GraphPad Prism 10
Microsoft Excel
AWS
bcl-convert v4.2.4
bedtools v2.31.1
BiocParallel v1.36.0
Bioframe v0.7.2, v0.8.0
Biopython v1.85
Biostrings v2.70.1
Bismark v0.24.0, v0.24.2
BLAST v2.16.0
bowtie2 v2.5.4
BSgenome v1.70.1
BSgenome.Hsapiens.UCSC.hg38 v1.4.5
Bsseq v1.38.0
CasOffFinder v2.4

CMPlot v4.5.1
 Conda v24.11.0, v25.3.1
 DESeq2 v1.42.0
 DRAGEN BCL convert v4.2.7
 DSS v2.48.0
 FastQC v0.11.9
 GenomeInfoDb v1.38.1
 GenomicRanges v1.54.1
 IRanges v2.36.0
 Latch v2.56.4
 Matplotlib v3.8.0, v3.10.1, v3.10.5
 methImpute tool v1.28.0
 Methprimer (<https://www.methprimer.com/>)
 Methrix v1.16.0
 MultiQC v1.11
 nf-core/methylseq pipeline v1.6.1, v2.3.0dev
 nf-core/rnaseq pipeline v3.6
 Numpy v1.26.4, v2.2.4, v2.3.2
 Pandas v2.2.3, v2.3.2
 Pyranges v0.1.4
 Python v3.12.0, v3.12.8, v3.13.2
 R v4.3.1, v4.3.3, v4.4.3
 Rtracklayer v1.62.0
 Samtools v1.21, v1.22
 Scipy v1.11.3, 1.16.1
 Seaborn v0.13.2
 Seqera Cloud
 Statsmodels v0.14.2
 SummarizedExperiment v1.32.0

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

RNA-seq and methylation sequencing data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE318266. Publicly available datasets GSE282432, Ensembl reference genomes GRCh38.p13 and Macaca_fascicularis_6.0, HBV reference genomes GenBank Accessions: U95551.1 and AF305422.1, and HBVdb V54.0 and V58.0 were used in this study. All other data used to generate Figures and Extended Data Figures within this manuscript are provided as a Source Data File. Additional data is also provided in a Supplementary Information File.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	n/a
Reporting on race, ethnicity, or other socially relevant groupings	n/a
Population characteristics	n/a
Recruitment	n/a
Ethics oversight	n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No specific sample-size calculations were performed. Sample size for in vitro studies and specificity assessments included at least 3 independent samples. The group size for in vivo studies is 5 to 10 animals per group for mouse studies and 2 (controls) to 4 (treatment) animals per group for non-human primate studies. The sample sizes used in this study are consistent with previously published work (Nature Medicine; volume 31, pages1329–1338 (2025); Nature volume 627, pages 416–423 (2024)). The number of replicates for in vitro studies was found to be sufficient to achieve reproducibility of our positive/negative controls.
Data exclusions	In the specificity study, predetermined QC criteria were set for each sequencing data type, and sequencing datasets that did not meet these criteria were excluded and repeated to ensure only high quality data was used for the analysis. No other data was excluded from the analysis. No data were excluded from animal studies.
Replication	Hits from the primary in vitro screen were replicated and confirmed in subsequent experiments. All attempts to replicate the in vitro and in vivo activity of CRMA-1001 were successful.
Randomization	In vitro experiments were not randomized. Animals were randomized into treatment groups based on baseline HBV biomarker levels.
Blinding	In vitro and in vivo pharmacology experiments did not employ any blinding strategy, but all measurements presented in this study were quantitative, and thus less prone to operator bias. Histopathology assessments of mouse and NHP tissues were performed in a blinded fashion.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Anti-HBV core antigen antibody (EPR28251-34). Abcam; Cat# ab316283; Lot# 1081107-06
Validation	Antibody validated in IHC on an automated Leica Biosystems BOND RX™ stainer for easy and quick results by Abcam. https://www.abcam.com/en-us/products/primary-antibodies/hepatitis-b-virus-core-antigen-antibody-epr28251-34-ab316283

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	HepG2.2.15 were obtained from Millipore Sigma (Cat. No. SCC49). HepG2 cells were obtained from the ATCC (Cat. No. HB-8065) and the transduced HepG2-hNTCP cells were kindly donated by Raffaele De Francesco. For specificity studies, primary human hepatocytes were obtained from PhoenixBio (2 female, 1 male), primary human splenic endothelial cells were obtained from Innoprot (Cat. No. P10691, 1 Male) and Accegen (Cat. No. ABC-TC3817, 2 Female), and primary human adrenal cortical cells were obtained from Accegen (Cat. No. ABC-TC3477, 2 Male & 1 Female).
---------------------	---

Authentication	Cells were obtained from commercial vendors and no additional authentication was performed. HepG2-NTCP transduction was experimentally confirmed.
Mycoplasma contamination	All cell lines maintained in nChroma laboratories were routinely tested for Mycoplasma contamination and confirmed to be negative in all experiments.
Commonly misidentified lines (See ICLAC register)	https://www.abcam.com/en-us/products/primary-antibodies/hepatitis-b-virus-core-antigen-antibody-epr28251-34-ab316283

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	C57BL/6-HBV (Tg-HBV mice); C57BL/6J (AAV-HBV mice); Macaca fascicularis (non-human primate)
Wild animals	N/A
Reporting on sex	No sex-specific findings were observed in Tg-HBV mice or in non-human primates. For the AAV-HBV mouse model, only male mice were employed as AAV transduction in male mice is more robust as compared to female mice.
Field-collected samples	All lab animals employed in the studies were housed according to institutional-specific policies or SOPs.
Ethics oversight	All animal studies were performed according to study protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a