Supplementary Information for

N6-methyladenosine modification of a parvovirus-encoded small noncoding RNA facilitates viral DNA replication through recruiting Y-family DNA polymerases

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SI Materials and Methods

Cell lines.

(i) Cell lines: Human embryonic kidney (HEK) 293 cells (#CRL-1573, American Type Culture Collection [ATCC], Manassas, VA) and HEK293T cells (#CRL-11268, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (#SH30022; Cytiva Life Science, Marlborough, MA) with addition of 10% fetal bovine serum (FBS, #F0926, MilliporeSigma, St. Louis, MO) at 37°C under 5% CO_2 atmosphere. CuFi-8 cells were immortalized human primary airway epithelial cells isolated from an anonymous donor by expressing hTERT and HPV E6/E7 genes (1), and were cultured in collagen-coated 100-mm dishes in PneumaCult™Ex Plus medium (#05040; StemCell, Vancouver, BC).

(ii) Human airway epithelium (HAE)-air liquid interface (ALI) cultures: Polarized primary HAE-ALI cultures were prepared by the Tissue and Cell Culture Core of the Center for Gene Therapy, University of Iowa as previously described (2). Briefly, the human airway (tracheobronchial) epithelial were isolated from the lungs of different donors (#B37-22, #B12-23, #B13-23) and cultured on collagen-coated transwell permeable supports (#3470; Corning, Corning, NY). The cells were differentiated at an ALI for 3–4 weeks in USG medium (DMEM/F12 medium [50%/50%] supplied with 2% Ultroser G (#15950-017, Sartorius, Goettingen, Germany). HAE-ALI cultures were also generated from the immortalized airway epithelial cell line CuFi-8 as described (3,4). Briefly, cells growing in PneumaCult™-Ex Plus Medium (#05040, StemCell) were polarized for 3–4 weeks in PneumaCult™-ALI medium (#05001, StemCell) before use. After polarization, an epithelial Volt-Ohm Meter (#MERS00002, MilliporeSigma) was used to measure the transepithelial electrical resistance (TEER) of the HAE-ALI cultures, and those that possessed a TEER value of > 1,500 $\Omega \cdot cm^2$ were selected for further experiments. Primary human airway (tracheobronchial) epithelial cells freshly isolated from lung donors were de-identified.

Plasmid constructs.

(i) pIHBoV1 plasmids: An HBoV1 infectious clone plasmid, pIHBoV1, and the BocaSR knockout mutant, pIHBoV1$^{\Delta BocaSR}$, have been described previously (5,6).

(ii) pLKO plasmids: Lentiviral vector pLKO.1 (#10879, Addgene) with a mCherry expression cassette (pLKO.1-mCherry) (7,8) was used to clone shRNA hairpin sequences using the AgeI and EcoRI sites. pLKO.1-mCherry containing a scramble shRNA (shScram) sequence was used as a shRNA control (9). shRNAs (listed in **Table S1**) were obtained from MilliporeSigma or references for the knockdown of genes: *METTL3*, *METTL14*, *METTL4*, *METTL16*, *YTHDF1* (10), *YTHDF2* (11), *YTHDF3*, and *YTHDC1*.

(iii) pLentiCRISPRv2 plasmids: Two sgRNAs targeting *METTL3* (12) (**Table S2**) were cloned into plentiCRISPRv2 vectors to construct plentiCRISPRv2-METTL3KO. The lentiCRISPRv2-SsgRNA that expresses a scramble sgRNA (SsgRNA, **Table S2**) was chosen as a control.

(iv) m6A modification-depleted plasmids: The potential m6A modification sites on BocaSR were chosen based on the m6A-conserved purine (G>A) m6AC(A/C/U) motif (13) or predicted with Sequence-based RNA Adenosine Methylation site Predictor (SRAMP) (14). The BocaSR m6A-modification depleted plasmids and control plasmids were constructed based on pIHBoV1 through synthesized DNA and assembly. The following mutants were

constructed by mutating A sites to guanine (G) in the indicated BocaSR sites: pIHBoV1^{m6A}M1 [site: 18 (in BocaSR)], pIHBoV1^{m6A}M2 (sites: 18, 67, and 102), pIHBoV1^{m6A}M3 (sites: 18, 39, 67, 70, 102, 111, and 122), pIHBoV1^{m6A}M4 (sites: 18, 39, 67, 70, 92, 102, 111, and 122), pIHBoV1^{m6A}M5 (site: 92), pIHBoV1^{m6A}Ctrl1 (sites: 1, 2, 13, 20, and 83), and pIHBoV1^{m6A}Ctrl2 (sites: 1, 2, 13, and 135).

(v) pLenti-based plasmids. The METTL3 ORF was codon-optimized(opt) and synthesized by Twist bioscience (South San Francisco, CA). The pLenti-optMETTL3-Blast and pLenti-mCherry-Blast plasmids were constructed by cloning optMETTL3 and mCherry ORFs into pLenti-Cas9-Blast (#52962, Addgene), respectively, via AgeI and BamHI sites.

(vi) p3Z-based plasmids. WT BocaSR-, BocaSR^{Ctrl1}-, and BocaSR^{M4}-coding DNA fragments were ligated into pGEM-3Z (p3Z) vector (#P2151, Promega, Madison, WI) using the AgeI and BamHI sites as described (6).

Plasmid DNA transfection.

HEK293 cells were transfected using Lipofectamine 3000 (#L3000015; ThermoFisher, Waltham, MA) following the manufacturer's instructions. For lentivirus production, HEK293T cells were transfected using PEI MAX (#24765-2; Polyscience, Warrington, PA) as previously described (15). Briefly, 2 μg of plasmid was used for each well of the 6-well plate and a total of 30 μg plasmid was used for each 150-mm plate.

Lentivirus production and transduction.

Lentiviruses were prepared by transfection of HEK293T cells with the sh/sgRNA-expressing plasmids (shRNA-expressing pLKO plasmids and pLentiCRISPRv2-METTL3KO) together with packaging plasmids (psPAX2 and pMD2.G) by using PEI MAX reagent. The collected lentiviruses were concentrated through a 20% sucrose gradient by ultracentrifugation in a SureSpin 630 rotor (ThermoFisher) at 19,400 rpm for 3 h. The transduction unit (TU) of the produced lentiviruses were titrated as previously described (16).

For lentivirus transduction, HEK293 cells and proliferating CuFi-8 cells were transduced at an MOI of ~5 TUs/cell. At 2 days post-transduction, the cells were treated with puromycin (2 μg/ml) or blasticidin (5 μg/ml) to select the transduced cells or carry out single cell cloning.

Establishment of gene knockout and METTL3-complemented cell lines.

Proliferating CuFi-8 cells were transduced with LentiCRISPRv2 expressing single guide (sg)RNAs targeting *METTL3* or a scramble (S)sgRNA control before differentiation. At 48 hpt, transduced cells were selected by the addition of puromycin (2 μg/ml) for 1 week, followed by limiting dilutions on collagen-coated 96-well plates for colony expansion from a single cell. When single-cell clones became confluent, the cells were expanded and screened for gene KO using Western blotting. For polarized HAE-ALI cultures, the CuFi^{METTL3KO} and CuFi^{SsgRNA} cells were differentiated on permeable supports of transwells for 3–4 weeks, using PneumaCult™-ALI medium. For the establishment of METTL3-complemented (CuFi$^{METTL3KO+optMETTL3}$) and the control (CuFi$^{METTL3KO+mCherry}$) cell lines, CuFi^{METTL3KO} cells were transduced by lentiviruses expressing optMETTL3 or mCherry, respectively. Transduced cells were selected with blasticidin (5 μg/ml) for 1 week.

Subsequentially, the blasticidin-resistant cell pools were differentiated in transwells at an ALI for 3–4 weeks. The matured HAE-ALI cultures for experiments were chosen based on the measurement of TEER (>1,500 $\Omega.cm^2$).

Virus production, infection, and quantification.

(i) Virus production. Thirty micrograms of pIHBoV1 or its mutants were transfected into HEK293 cells on 150-mm plate following a previously described method (5). A 5-plate scale was used for each virus production, respectively. At 3 days post-transfection (dpt), the viruses were harvested and purified as previously described (5).

(ii) Virus infection. HAE-ALI cultures were infected with HBoV1 from the apical side at a multiplicity of infection (MOI) of 100 DNase I digestion-resistant particles (DRP) per cell. After infection, the released virions were collected by incubating 100 µl of phosphate-buffered saline (PBS, pH7.4) on the apical side of the insert (5,17).

Virus and BocaSR quantifications.

(i) Virus quantification. The purified virus preparations and progeny viruses collected in apical washes were quantified for virus particles as DRP using quantitative PCR (qPCR) as previously described (3).

(ii) BocaSR quantifications. Total RNA was extracted in the transfected cells by TRIzol reagent (#15596026, ThermoFisher) according to the manufacturer's protocol. BocaSR was quantified using TaqMan probe-based qPCR with random primers (#48190011, ThermoFisher), BocaSR-specific probe (BocaSR-p: 5' 6-FAM/AGC TCA TGG GTT CAA GAC CAC AGC-3'/IABkFQ) and primers (BocaSR-F: 5'-GGA AAG TGA AGG GTG ACT GTA G-3' and BocaSR-R: 5'-TCG AGA CGG TAA CAC CAC TA-3').

Antisense oligonucleotides (ASO) and transfection.

(i) ASOs. Posphorothioate (PS) and 2′-O-methoxy-ethyl (2′-MOE) modifications were used in ASOs. ASOs targeting BocaSR "loop" regions were: ASO-1 (nt, in BocaSR), 23–39, 5'-TCA CCC TTC ACT TTC CG-3'), and ASO-2 (nt: 98–114, 5'-CTG TCG GCT AGG TTC GA-3'). Control (Ctrl) ASOs targeting the BocaSR "stem" regions were: ASO^{Ctrl1} (nt: 2–22, 5'-CTT GTC CAT TGA GGA GGA ACT-3'), ASO^{Ctrl2} (nt: 40–56, 5'-TGA GCT CAG GAC TAC AG-3'), and ASO^{Ctrl3} (nt: 74–89, 5'-CAC CAC TAC CAT CGG G-3').

(ii) Transfection. HEK293 cells were seeded onto 60-mm dishes (#628160, Greiner Bio-One, Monroe, NC). When cells reach 80% confluent, ASOs were transfected at the indicated concentrations by using DharmaFECT 4 transfection reagent (#T-2004-02, Horizon Discovery, Waterbeach, United Kingdom). At 6 hpt, subsequent transfection of pIHBoV1 was performed using Lipofectamine 3000 reagent.

Determination of *in vivo* secondary structures of BocaSR.

(i) In-cell DMS treatment. The cells of HBoV1-infected HAE-ALI cells were detached from the supportive membrane by Accutase (#AT104, Innovative Cell Technologies, San Diego, CA) and resuspended in DMEM in 2% FBS at 6.7×10^6 cells/mL. 2 µL of DMS (#D186309, MilliporeSigma) was added into pre-warmed 200 µL of cell suspension and mixed by repetitively pipetting. After incubation for 5 min at room temperature (RT), the DMS

reaction was quenched with 100 µL 50% β-mercaptoethanol (#444203, MilliporeSigma) in PBS. The cells were immediately pelleted and the DMS-modified total RNA was extracted by TRIzol reagent according to the manufacturer's protocol with RNase-free DNase I (Qiagen) treatment. For the DMS-untreated sample, total RNA was prepared following the same protocol in parallel.

(ii) Library preparation and sequencing. All samples were prepared in duplicates. The reverse transcription of DMS-treated and untreated RNA samples was conducted according to the literature (18) but at 55°C. The PCR cycle numbers were chosen at the end of the exponential stage and judged by electrophoresis to avoid over-amplification. The amplicons were purified by agarose gel electrophoresis. The primers used for RT-PCR were: BocaSR-Fw, 5'-AAG TTC CTC CTC AAT GGA CA-3' and BocaSR-Rv, 5'-AC AGC TCC CCC CAC A-3'. The DNA amplicon samples were quantified with the High Sensitivity (HS) Qubit dsDNA quantification assay kit (#Q32851, ThermoFisher), and 4200 TapeStation system (#G2991BA, Agilent, Santa Clara, CA) to determine the quality and base-pair size. The sequencing libraries were constructed using 100–500 ng of amplicon DNA with the Illumina DNA Prep sequencing library kit (#20060060, Illumina, San Diego, CA) following the manufacturer's protocol. The sequencing library construction includes tagmentation of the amplicon DNA using a bead-based transposome complex to simultaneously fragment and tag the DNA with adapter sequences. Following tagmentation, unique index adapters are added in a PCR amplification step to the ends of the DNA fragments. The libraries were quantified and validated by Qubit and TapeStation assays. The sequencing library preps were pooled equally by mass, and the molarity of the pool was verified with a KAPA library quantification kit (#KK4824, Roche, Basel, Switzerland). An Illumina NextSeq 550 system was used to generate paired-end, 150-base sequence reads from the multiplexed libraries at a depth of 15×10^6 reads per amplicon library. Base-calling was carried out by the instrument's Real-Time analysis (RTA) software. The base call (bcl) files were demultiplexed and converted to compressed FASTQ files by bcl2fastq2 Conversion Software (v2.20, Illumina).

Methylated RNA immunoprecipitation (MeRIP).

MeRIP was performed as previously described (19). Briefly, 1-3 µg total RNA were extracted from pIHBoV1-transfected HEK293 cells and supplemented with 300 µL of 1 × IP buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 0.1% NP40, 40U/µL RNase Inhibitor [#Y9240L, Enzymatics, Beverly, MA]) containing 2 µg anti-m6A/m5C/m7G/o8G/ Ψ antibodies, respectively. After incubation with head-over-tail rotation at 4°C for 2 h, the antibodies were immunoprecipitated by addition of 20 µL M-280 anti-rabbit/mouse IgG (0.5% BSA blocked) superparamagnetic beads. The RNA binding to the m6A-/m5C-/m7G-/o8G-/Ψ- beads was carried out with head-over-tail rotation at 4°C for 2 h, and pelleted by 4000 g at 4°C for 2 min. The supernatant was kept, and the beads were then washed three times with 500 µL of 1 × IP buffer and twice with 500 µL of Wash buffer (50 mM Tris-HCl, pH7.4, 50 mM NaCl, 0.1% NP40, 40 U/µL RNase Inhibitor). The enriched RNA was eluted with 200 µL of Elution buffer (10 mM Tris-HCl, pH7.4, 1 mM EDTA, 0.05% SDS, 40U proteinase K) at 50°C for 1 h and extracted by acid phenol-chloroform and ethanol precipitated. IP RNA was marked as "MeRIP", and the supernatant RNA marked as "supernatant".

MeRIP-qPCR.

MeRIP-qPCR was performed as previously described (19). Briefly, the first strand cDNA of the immunoprecipitated BocaSR RNAs were synthesized by using SuperScript III reverse transcriptase (#18080093, ThermoFisher), and the percentage of indicated modification (% Input) for each MeRIP fraction was quantified by using qPCR with primers specifically targeting BocaSR (BocaSRmF [forward]: 5'-GTT CCT CCT CAA TGG ACA AGC-3'; BocaSRmR [Reverse]: 5'-GTA ACA CCA CTA CCA TCG GGC-3' and SYBR Green Real-time qPCR master mix (#AS-MR-006-5, Arraystar, Rockville, MD). Relative enrichment of each methylated RNA was normalized to the input (% Input) using the following formula: $\%Input = 2^{-Ct\ MeRIP}/(2^{-Ct\ MeRIP}+2^{-Ct\ Supernatant})$.

Immunofluorescence assay.

Immunofluorescence staining was performed as previously described (17,20). Briefly, cells were digested and washed twice with PBS. After being cytospun onto slides and fixed with 3.7% paraformaldehyde (PFA) at RT for 15 min, the cells were permeabilized with 0.5% Triton X-100 for 5 min, blocked and incubated with the primary antibodies in PBS-2%FBS for 1 h and subsequently with the correspondent secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Confocal images were visualized under a Leica TCS SP8 STED 3x super resolution microscope.

RNA fluorescence in situ hybridization (FISH) assay

FISH assay was performed as previously described (6). Briefly, HBoV1-infected HAE-ALI cultures or plasmid transfected-HEK293 cells were digested, cytospun onto slides and fixed with 3.7% paraformaldehyde, followed by permeabilization with 70% ethanol for at least 1 h. The cells were then blocked with 3% bovine serum albumin (BSA, #A7030, Sigma) in 4 x SSC buffer (0.6 M NaCl plus 0.06 M sodium citrate, 0.2% Tween 20) for 1 h, washed once with Wash buffer A (#SMF-WA1-60, Biosearch Technologies, Novato, CA), and incubated with a set of four biotin-labeled antisense oligonucleotide probes synthesized from IDT (125 nM each): Probe 1, 5'-/5BiosG/TAC AGT CAC CCT TCA CTT T-3'; Probe 2, 5'-/5BiosG/TAA CAC CAC TAC CAT CGG G-3'; Probe 3, 5'-/5BiosG/TGT CGG CTA GGT TCG AGA C-3'; and Probe 4, 5'-/5BiosG/TCC CCC CAC AAT GTA CAA G-3') in Hybridization buffer (#ENZ-33808, Enzo Life Sciences, Farmingdale, NY) at 37°C overnight. The secondary antibodies (Jackson ImmunoResearch Inc., West Grove, PA) were applied, and the slides were sequentially washed with Wash buffers A and B (#SMF-WB1-20, Biosearch Technologies). The nuclei were stained with DAPI. Confocal images were taken under a Leica TCS SP8 microscope.

Immunoprecipitation of ribonucleoprotein (RNP) complexes.

The RNA-immunoprecipitation (RIP) assay was modified based on a previous publication (21). Briefly, HEK293 cells seeded on 150-mm plate were transfected with 30 μg of desired plasmids by using Lipofectamine 3000 (ThermoFisher). At 3 dpt, the cells were treated with RNase-free DNase I (Qiagen), washed once with PBS, and pelleted by centrifuge at 400 × g for 3 min. Ten percent of the cells were prepared for the extraction of

total RNA by using TRIzol for the detection of total BocaSR. Other cells were cross-linked by resuspension of the pellets in 25 ml of 1.0% formaldehyde diluted in PBS. After slow rotation for 10 min at RT, the cells were pelleted and washed once with PBS, and supplemented with 0.25 M glycine (#G8898, MilliporeSigma) to quench any residual formaldehyde for 5 min at RT. After being washed twice with ice-cold PBS, the pellets are resuspended in 500 µL of Lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM DTT, 20 U RNase Inhibitor [#EO0382, ThermoFisher] and 1 tablet of Protease Inhibitors [#S8830, Sigma-Aldrich] in 10ml) for 10 min on ice and sonicated. Cell debris was removed by centrifugation for 10 min at 17, 000 g (4°C). 10 µg of anti-m6A antibody were added into the supernatant and rotated at 4°C for 2 h followed by the addition of 20 µL of blocked Dynabeads per sample. The mixtures were rotated at 4°C for 2 h, and the pelleted beads were washed three times with 500 µL of Lysis buffer and twice with 500 µL of Wash buffer (50 mM Tris-HCl, pH7.4, 50 mM NaCl, 0.1% NP40, 40 U RNase Inhibitor). The m6A-enriched samples were then split into two halves for qPCR to quantify the BosaSR following RNA extraction using TRIzol and for Western blotting analyses to probe BocaSR-binding proteins with specific antibodies.

***In vitro* transcription of BocaSR and transfection.**

(i) *In vitro* transcription. BocaSR was *in vitro* transcribed by using MEGAscript SP6 Transcription Kit (#AM1330, ThermoFisher) following the manufacturer's instructions with complementation of Biotin-dUTP (#11093070910, MilliporeSigma). The template was amplified from p3Z-BocaSR (6) or p3Z-$BocaSR^{M4}$ using a primer set targeting BocaSR (F-BocaSRT: 5'- CGG ATG CAT TTA GGT GAC ACT ATA GAG CTT ACA AGT TCC TCC TCA ATG G-3'; R-BocaSRT: 5'- AC AGC TCC CCC CAC AAT GTA CAA GGG-3').

(ii) Transfection. HEK293 cells were seeded onto 60-mm dishes. When the cells reached 80% confluent, 2 µg of *in vitro* transcribed BocaSR were complemented by transfection, using DharmaFECT 4 transfection reagent in the indicated wells. At 2 hpt, 4 µg of pIHBoV1 or pIHBoV1$^{\Delta BocaSR}$ were transfected, as indicated, by using Lipofectamine 3000 transfection.

Biolayer Interferometry (BLI) assay.

A series of oligoes based on the sequences of HBoV1 LEH and REH were synthesized by IDT (**Table S3**) for interaction with BocaSR in the BLI assay. The BLI kinetics analysis was performed using Octet Red96e (Sartorius, Bohemia, NY). In brief, after hydration in Kinetic buffer (20 mM tris-HCl pH 7.4 and 100 mM NaCl) for 10 min, the Octet streptavidin biosensor (#18-5019, Sartorius) was loaded. An amount of 80 pM of biotin-labeled BocaSR or $BocaSR^{M4}$ in Kinetic buffer was then mounted on the streptavidin biosensors to generate the baseline. The association (Ka) and disassociation (Kd) kinetics were determined by the addition of synthesized oligoes. BLI was carried out on Octet Red96e. Data (Ka and Kd) were acquired using Fortebio Data Acquisition 11.1.0.11 and analyzed using Fortebio Data Analysis 11.1.0.4 for K_D (mean ± SD). The binding assays were repeated at least three times.

Proximity ligation assay (PLA).

The PLA assay was performed with a Duolink PLA kit (#DUO92014, # DUO92002, and # DUO92004, MilliporeSigma) according to the manufacturer's instructions as previously described (17). Briefly, the cells on the slides were fixed with 3.7% PFA for 15 min and permeabilized with 0.5% Triton X-100 for 5 min. After being blocked with a blocking buffer for 30 min, the cells were incubated with the indicated primary antibodies for 1 h and two diluted PLA probes were applied to the cells. After incubation for 1 h at 37°C, hybridized oligonucleotides were ligated and amplified for 100 min. The cells were then stained with DAPI, washed with PBS, mounted and observed under a Leica TCS SP8 microscope.

Western blotting.

The cell lysates were separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (22). 2.5 μL of a protein ladder (#P008; GoldBio, St. Louis, MO) was loaded as size marker. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (#IPVH00010; MilliporeSigma), blocked with 5% non-fat milk, and probed with primary and secondary antibodies sequentially. Signals were visualized with an Odyssey imaging system (LI-COR Corporate, Lincoln, NE) (4).

Southern blotting.

Southern blotting was performed according to our previously reported method (23,24). Briefly, the Hirt DNA samples were prepared from cells, digested with DpnI, resolved on a 1% agarose gel, blotted onto a nitrocellulose membrane, and probed with a [α-32P]dCTP-labeled HBoV1 dsDNA genome excised from pIHBoV1 (5). Hybridization signals were captured with a storage phosphor screen and visualized on the Amersham Typhoon Biomolecular Imager (Cytiva, Marlborough, MA), and quantified using ImageQuant Tl (IQTL) 8.2 (Cytiva).

Antibodies used in the study.

(i) Primary antibodies: The following first antibodies were purchased: anti-m6A antibody (#202003) from Synaptic Systems (Goettingen, Germany); anti-m5C (#C15200006-500) and anti-Pseudouridine (#C15200247) from Diagenode (Denville, NJ); anti-m7G (#RN017M) from MBL international corporation (Woburn, MA); anti-Biotin (#ab201341) from Abcam (Waltham, MA); anti-METTL3 (#15073-1-AP and #67733-1-Ig), anti-METTL14 (#26158-1-AP), anti-METTL16 (#19924-1-AP), anti-YTHDF1 (#17479-1-AP), anti-YTHDF2 (#24744-1-AP), anti-YTHDF3 (#25537-1-AP), anti-YTHDC1 (#14392-1-AP), and anti-FTO (# 27226-1-AP) from Proteintech (Rosemont, IL); anti-METTL4 (#A9294), anti-m6A (#A19841), anti-Pol η (#A1833), and anti-Pol κ (#A6122) from Abclonal (Woburn, MA); anti-Pol κ (#AT3371a) from Abcepta (San Diego, CA); anti-o8G (#MAB3560), anti-β-actin (#A5441) and anti-β-tubulin IV (#T7941) from MilliporeSigma; anti-ZO-1(#610966) from BD Bioscience (San Jose, CA). Dynabeads™ M-280 anti-rabbit IgG (#11203D) and anti-mouse (#11202D) superparamagnetic beads were purchased from ThermoFisher.

(ii) Secondary antibodies: The following secondary antibodies were used: DyLight 800 conjugated anti-rabbit IgG (#5151S) and DyLight 800-conjugated anti-mouse IgG (#5257S) from Cell Signaling (Danvers, MA); Alexa Fluor 488-conjugated streptavidin

(#S11223) from ThermoFisher; Alexa Fluor 594-conjugated anti-mouse IgG (#115-585-146) from Jackson ImmunoResearch (West Grove, PA).

Statistics analysis.

Statistical analysis was performed with GraphPad Prism (version 9.5, GraphPad software, Boston, MA). Data are representative of triplet experiments and shown as mean ± standard deviation (SD). Statistical significance P values were determined by using Student's *t* test. ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 were regarded as statistically significant, and n.s. was regarded as statistically insignificant.

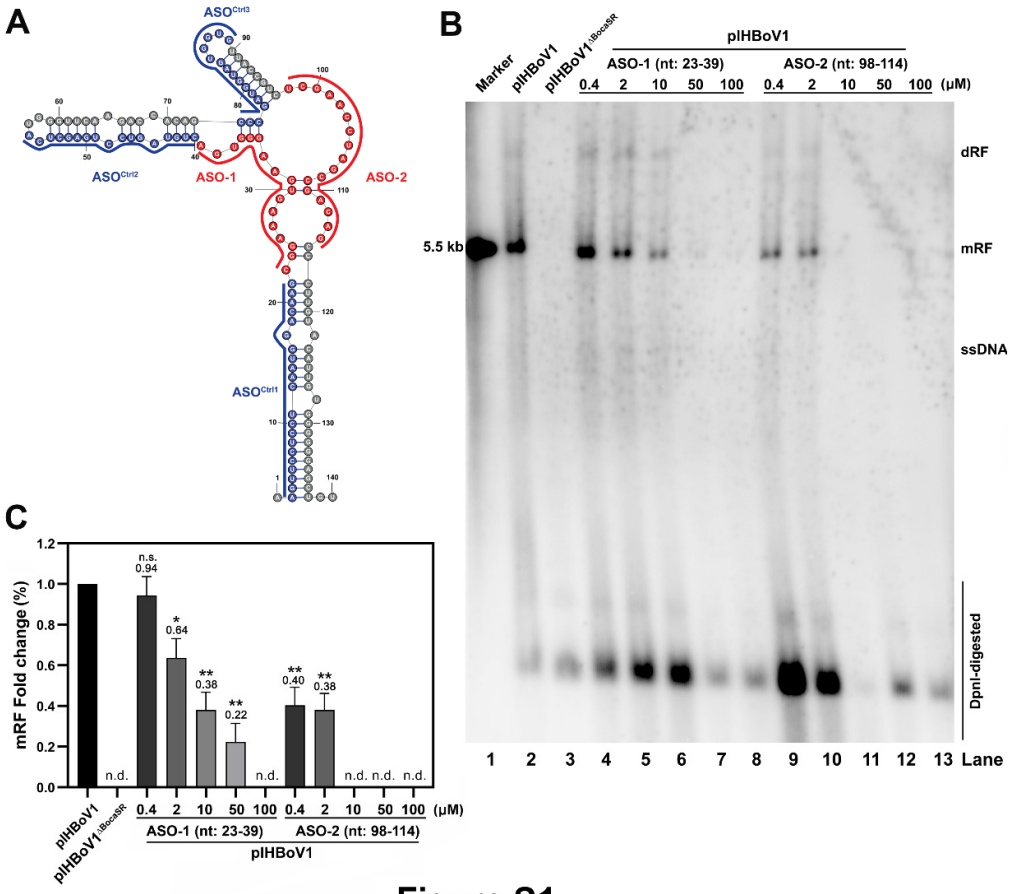


Figure S1

Figure S1. Antisense oligonucleotides (ASOs) targeting BocaSR loops inhibits HBoV1 DNA replication.

(A) Positions of ASOs. ASOs targeting the stem regions (in blue, ASO^{Ctrl1}, nt: 2–22, in BocaSR; ASO^{Ctrl2}, nt: 40–56; ASO^{Ctrl3}, nt: 74–89), and loop regions (in red, ASO-1, nt: 23–39; ASO-2, nt: 98–114) of BocaSR are depicted. **(B) Southern blotting.** ASO-1 and ASO-2 were transfected into HEK293 cells 6 h prior to pIHBoV1 transfection. At 2 dpt of pIHBoV1, the cells were collected for the extraction of Hirt DNA, followed by Southern blotting. HBoV1 dsDNA genome excised from pIHBoV1 was used as a size marker (Lane 1, Marker, ~5.5 kb). dRF, mRF, and ssDNA represent dimer, monomer replicative form DNA and single-stranded DNA, respectively. Signals in the lower gel are the residues of the DpnI-digested plasmid DNA. **(C) Quantification of mRF DNA.** The intensity of mRF DNA bands on the blot was quantified using ImageQuant TI software, and DpnI-digested bands served as a loading control. The values (mean ± SD) obtained from three blots were normalized to the replication of pIHBoV1, which is arbitrarily set up to 1. The shown data were analyzed by Student's *t* test (n.d., not detectable; n.s., not significant; *, $p<0.05$; **, $p<0.01$).

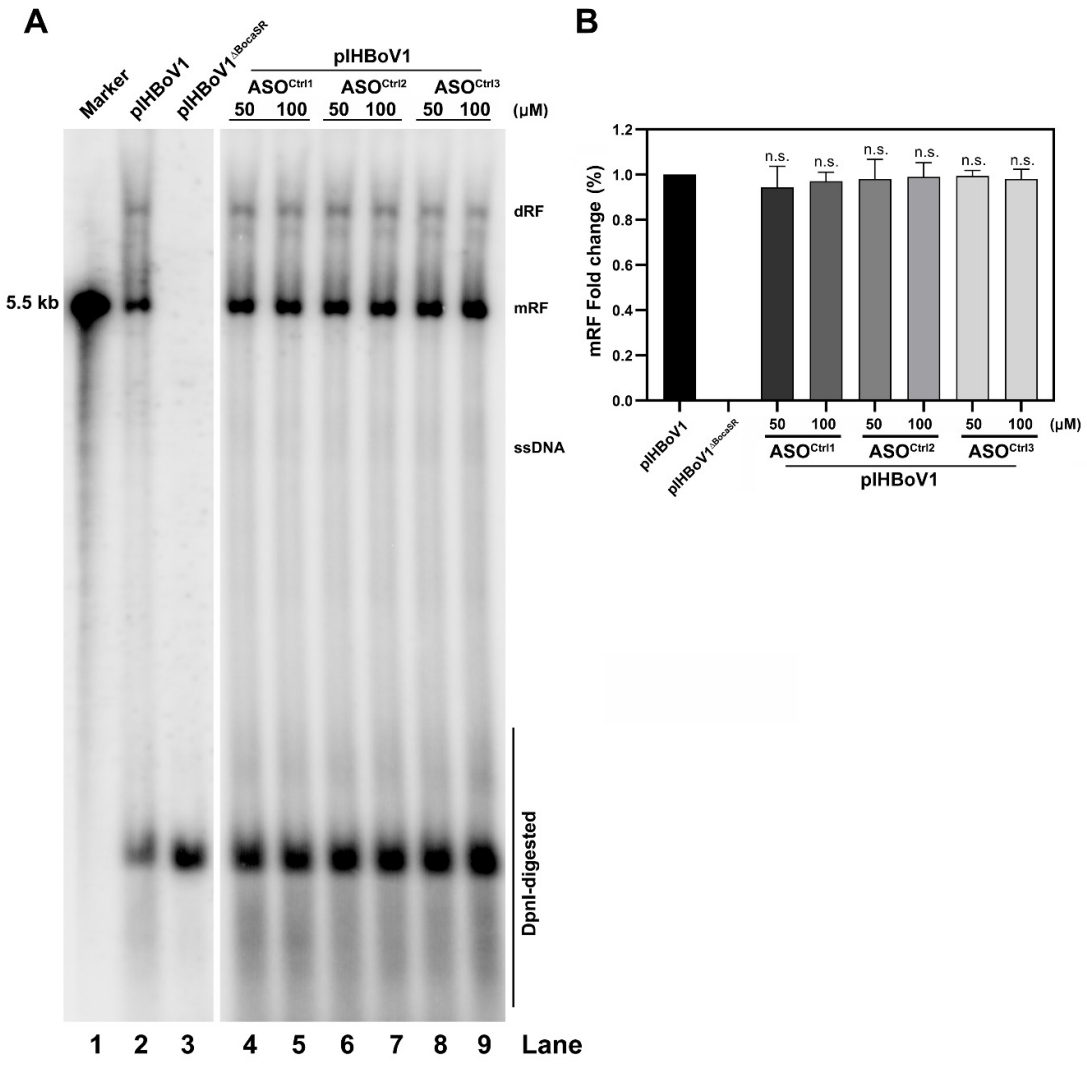


Figure S2

Figure S2. Antisense oligonucleotides (ASO) controls targeting BocaSR stems did not inhibit HBoV1 DNA replication.

(A) Southern blotting. ASO^{Ctrl1} (nt: 2–22), ASO^{Ctrl2} (nt: 40–56), and ASO^{Ctrl3} (nt: 74–89), targeting to BocaSR stems, respectively, were transfected into HEK293 cells at 6 h prior to the transfection of pIHBoV1. At 2 dpt, Hirt DNA was extracted, DpnI-digested, and subjected to Southern blotting. A ~5.5 kb size marker (Marker) was loaded on Lane 1. dRF, mRF, and ssDNA represent the dimer, monomer replicative form DNA and single-stranded DNA, respectively. **(B) Quantification of mRF DNA.** The intensity of mRF DNA bands on the blot was quantified using ImageQuant TI software, and DpnI-digested bands served as an internal (transfection) control. The values obtained from three blots were normalized to the replication of pIHBoV1, which is arbitrarily set up to 1. Data shown are mean ± SD and were analyzed by Student's *t* test (n.s., not significant).

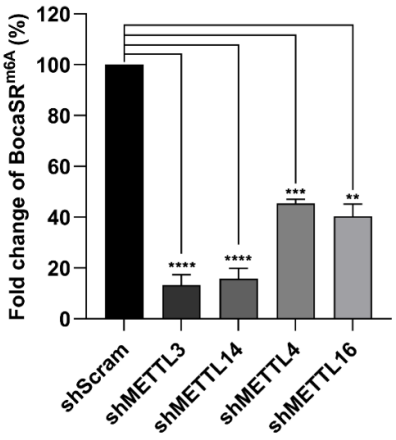


Figure S3

Figure S3. m6A writer gene silencing decreases m6A modification of BocaSR.
HEK293^{shScram}, HEK293^{shMETTL3}, HEK293^{shMETTL14}, HEK293^{shMETTL4} and HEK293^{shMETTL16} cells were transfected with p3Z-BocaSR plasmid. At 2 dpt, total RNA was extracted using TRIzol reagent. m6A-modified BocaSR was pulled down by an m6A-specific antibody and quantified by MeRIP-qPCR. The percentage of m6A modified BocaSR (BocaSR^{m6A}) in HEK293^{shScram} cells were arbitrarily set up to 1 and the relative percentages of BocaSR^{m6A} in HEK293^{shMETTL3}, HEK293^{shMETTL14}, HEK293^{shMETTL4}, and HEK293^{shMETTL16} cells are shown. The data were analyzed by Student's t test (**, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

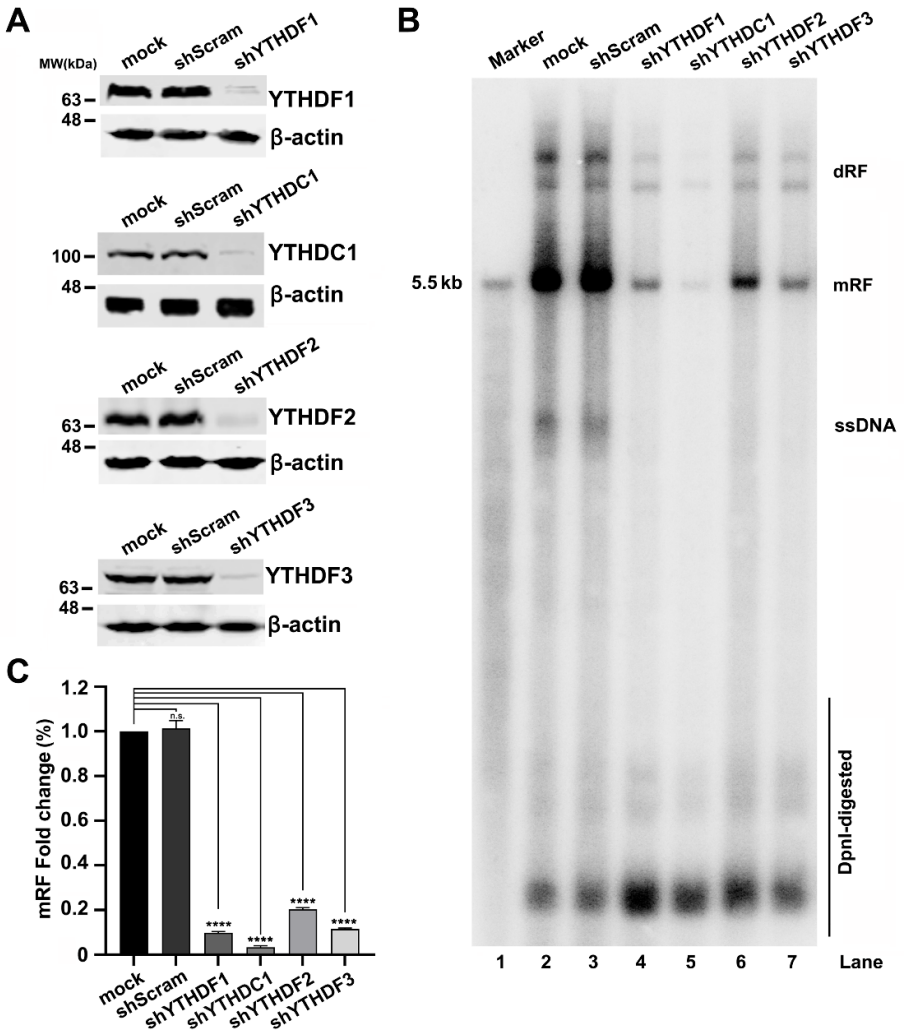


Figure S4

Figure S4. Silencing of endogenous m6A Reader genes inhibits HBoV1 DNA replication.

(A) Western blotting. HEK293 cells were chosen to silence m6A Reader genes (*YTHDF1*, *YTHDC1*, *YTHDF2*, and *YTHDF3*) using specific shRNA-expressing lentiviruses and further selected for the establishment of cell line. The control cell line was established using lentiviral vector expressing shScram. The cell lines were tested for m6A "Reader" gene expression using Western blotting. β-actin was probed as a loading control. **(B) Southern blotting.** Each cell line was transfected with pIHBoV1. At 2 dpt, Hirt DNA was extracted, DpnI-digested, and subjected to Southern blotting with a full-length HBoV1 genome probe. dRF, mRF, and ssDNA represent dimer, monomer replicative form DNA, and single-stranded DNA, respectively. HBoV1 duplex DNA was used as a size marker (lane 1, Marker, ~5.5 kb). **(C) Quantification of mRF DNA.** The intensity of mRF DNA bands was quantified by ImageQuant TI software, and DpnI-digested bands served as an internal control. The values shown (mean ± SD) obtained from three blots were normalized to the replication of pIHBoV1 in mock HEK293 cells, which was arbitrarily set to 1. The shown data were analyzed by Student's *t* test (n.s., not significant; ****, p<0.0001).

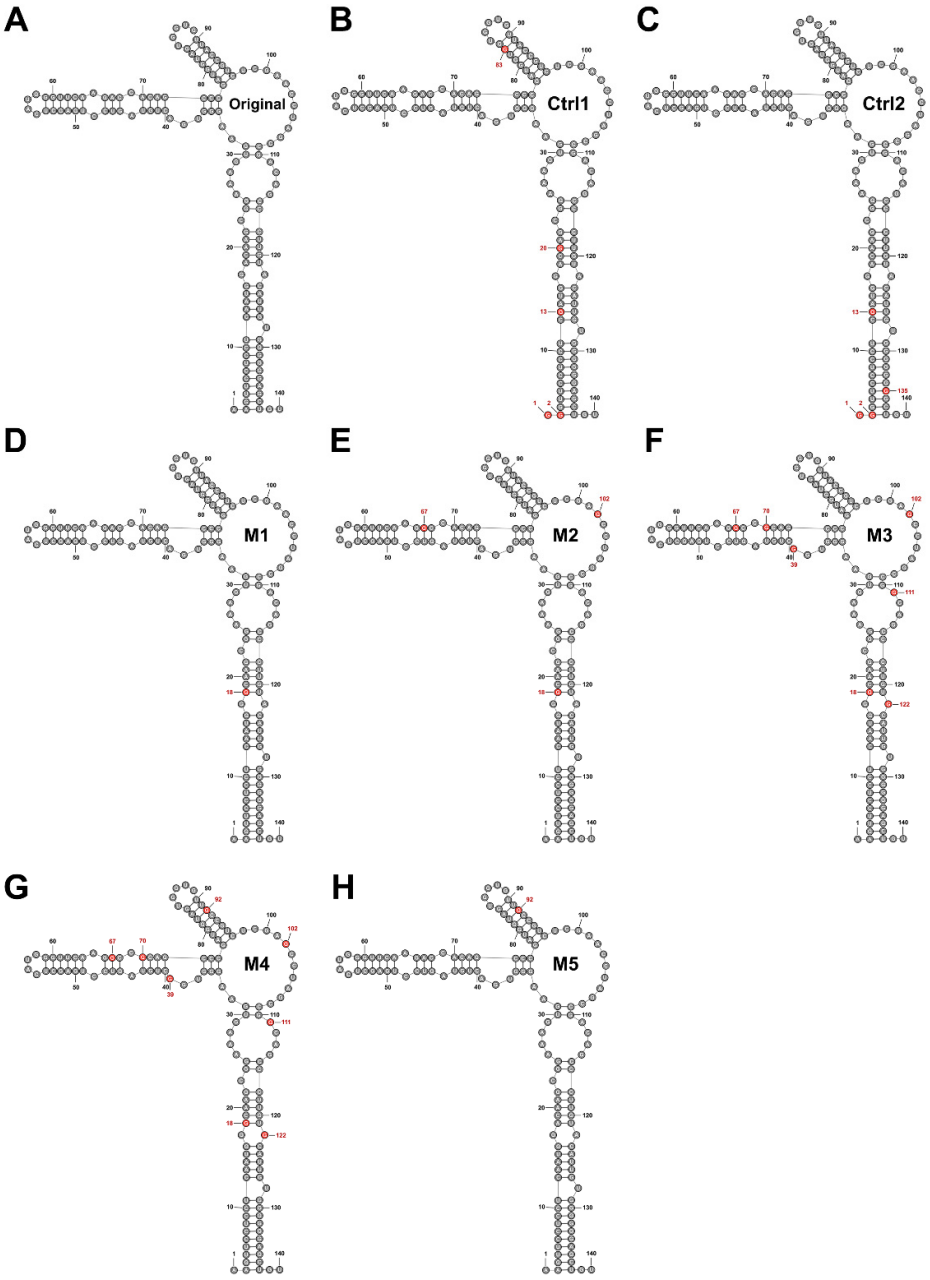


Figure S5

Figure S5. Predicted secondary structure of BocaSR mutants.

The secondary structure of BocaSR mutants were predicted by M-fold (25) and Kinefold (26). To maintain the secondary structure unchanged, we mutated the predicated modified A sites to guanine (G) in BocaSR. The secondary structure of the original BocaSR was shown as control (**A**, Original). Two BocaSR random control mutants were constructed by introducing A to G mutations in the stems, respectively (**B-C**). The potential m6A sites, which are consistent with m6A-conserved purine (G>A) m6AC(A/C/U) motif or predicted by Sequence-based RNA Adenosine Methylation site Predictor (SRAMP), were mutated (A to G) and marked in red as indicated (**D-H**).

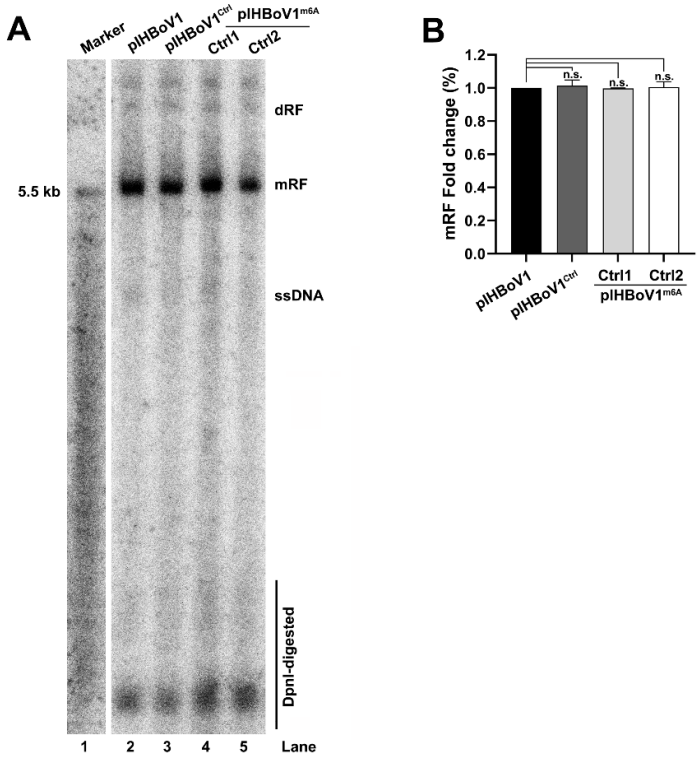


Figure S6

Figure S6. Random mutations (controls) of BocaSR do not alter HBoV1 DNA replication.

Two BocaSR mutated pIHBoV1 control plasmids were constructed with random mutations on adenines that were not predicted m6A-modified. WT pIHBoV1, pIHBoV1^{Ctrl}, pIHBoV1^{m6A}Ctrl1, and pIHBoV1^{m6A}Ctrl2 were transfected into HEK293 cells. **(A) Southern blotting.** At 2 dpt, Hirt DNA of the transfected cells were extracted, DpnI-digested, and subjected to Southern blotting. (**B) Quantification of mRF DNA.** The intensity of mRF DNA bands was quantified by ImageQuant TI. The shown values (mean ± SD) obtained from three blots were normalized to the replication of pIHBoV1, which is arbitrarily set to 1. All of the experiments were performed in triplicate, and the statistical analysis was carried out by Student's *t* test (n.s., not significant).

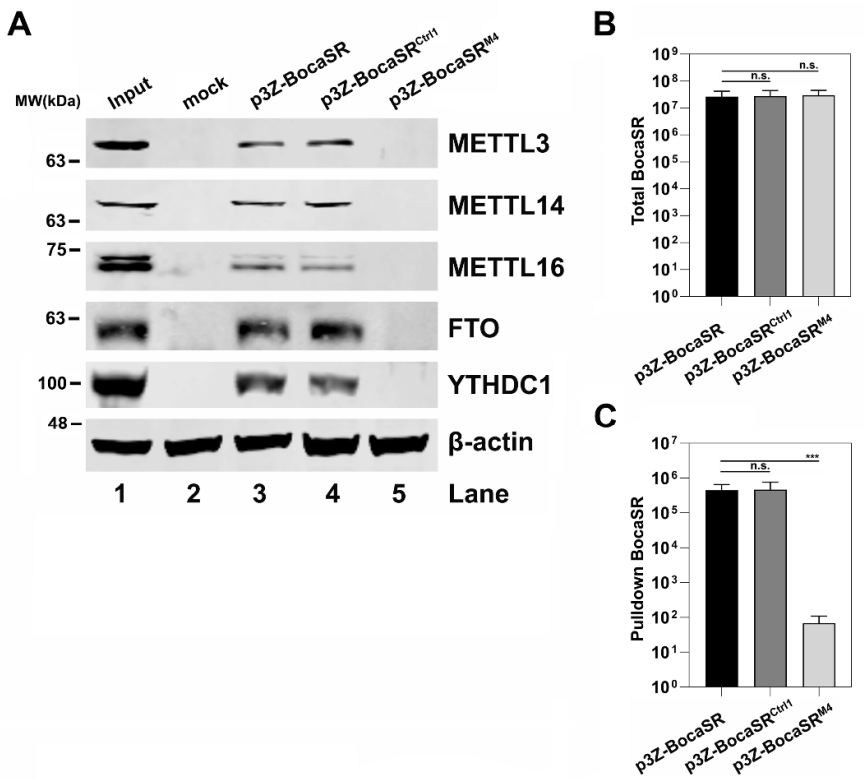


Figure S7

Figure S7. BocaSR interacts with m6A-processing proteins.

p3Z-BocaSR, p3Z-BocaSR^{Ctrl1}, and p3Z-BocaSR^{M4} were transfected into HEK293 cells, respectively. At 3 dpt, the cells were collected for the RNA pulldown experiment followed by Western blotting, and the extraction of total RNA. Untransfected cells served as a control. **(A) RNA pulldown of m6A-processing proteins.** The transfected and mock-transfected cells were collected and cross-linked by formaldehyde before lysis, followed by addition of a rabbit anti-m6A antibody. Dynabeads (M-280) of anti-rabbit IgG were added into the cell lysate to precipitate m6A-biogeneisis proteins (Writers METTL3, METTL14, and METTL16; Eraser FTO; Reader YTHDC1). The precipitated proteins were probed with specific antibodies as indicated. β-actin was used as a loading control. Ten percent of the cell lysis were loaded as inputs. **(B&C) Quantification of BocaSR.** The input (Total) (B) and precipitated (Pulldown) (C) BocaSR were quantified with a BocaSR-specific RT-qPCR. All the experiments were performed in triplicate, and data shown were analyzed by Student's *t* test (n.s., not significant; ***, $P < 0.001$).

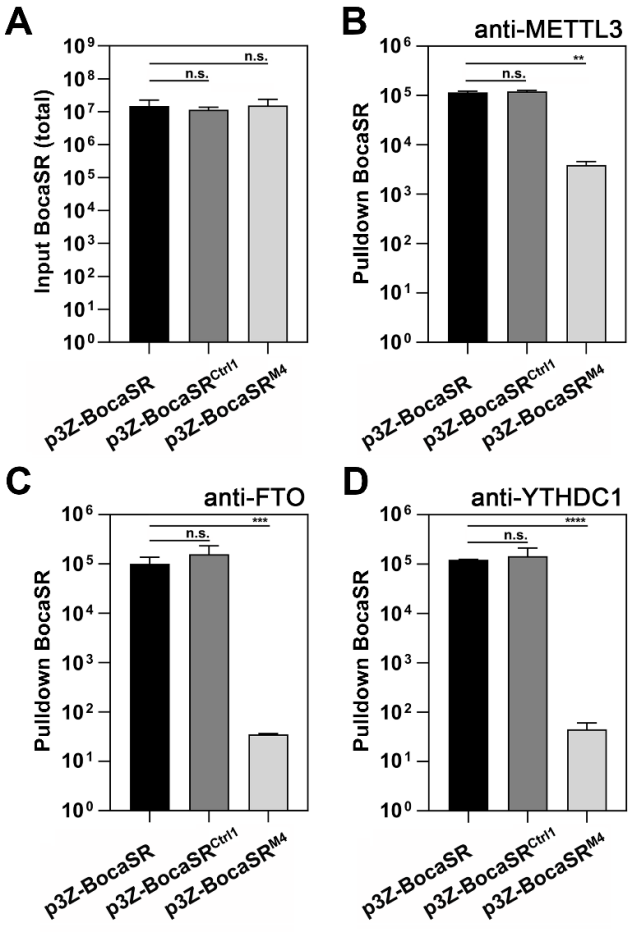


Figure S8

Figure S8. BocaSR pulldown assays using specific antibodies against m6A Writer, Eraser and Reader.

HEK293 cells were transfected by p3Z-BocaSR, p3Z-BocaSR^{Ctrl1}, and p3Z-BocaSR^{M4}. At 2 dpt, the cells were collected and cross-linked by formaldehyde before lysis, followed by pulldown using antibodies target METTL3 (**B**), FTO (**C**), and YTHDC1 (**D**), and addition of protein A/G beads. The total RNA on the beads were extracted by using TRIzol reagent. The input (Total) (**A**) and precipitated (Pulldown) (**B-D**) BocaSR were quantified with a BocaSR-specific RT-qPCR. All the experiments were performed in triplicate, and data shown were analyzed by Student's t test (n.s., not significant; **, P < 0.01; ***, P < 0.001); ****, P < 0.0001.

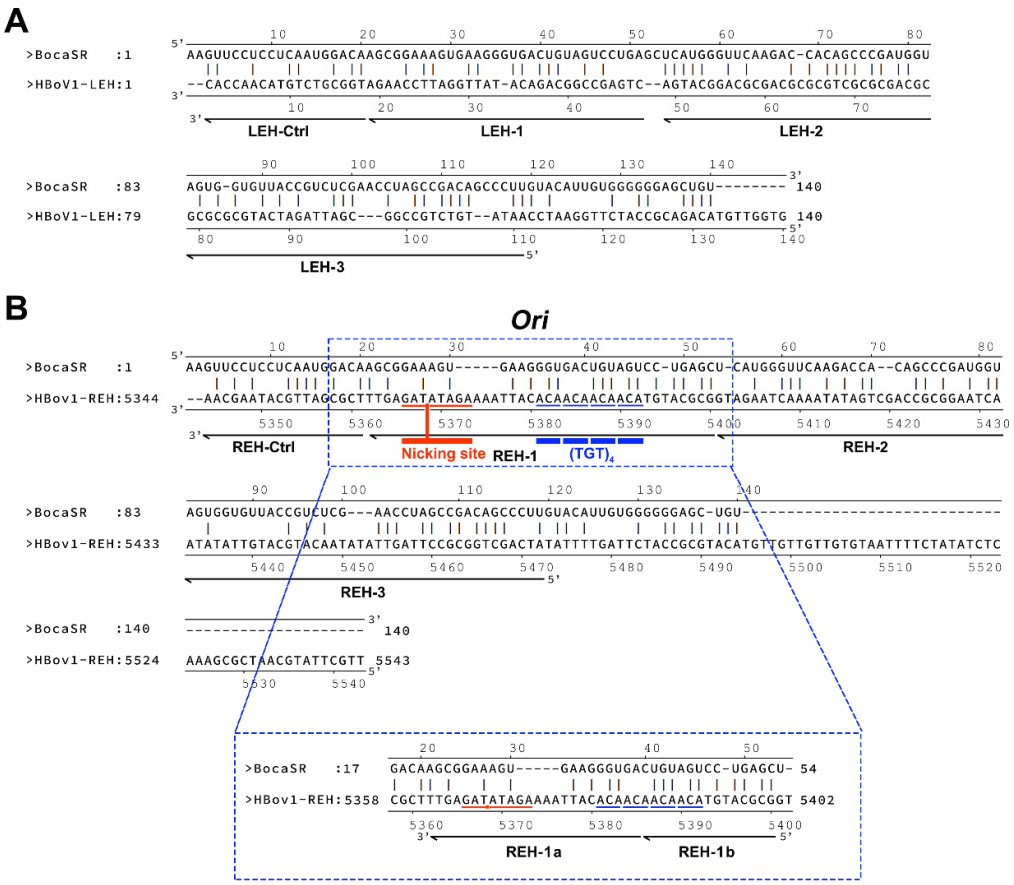


Figure S9

Figure S9. Alignment of the *BocaSR* gene with the left- and right-end hairpin (LEH and REH) of HBoV1.

The *BocaSR* gene was aligned with HBoV1 LEH (**A**) and REH (**B**), respectively, by using ClustalW (27). The paired nucleotides are matched with lines. Dashed frame indicates the HBoV1 minimal DNA replication origin (*Ori*) at the REH diagramed with the nicking site in red and NS1-binding sites $(TGT)_4$ in blue. Synthesized oligos are underlined. The nucleotide numbers of HBoV1 and BocaSR sequences refers to GenBank accession number: JQ923422.

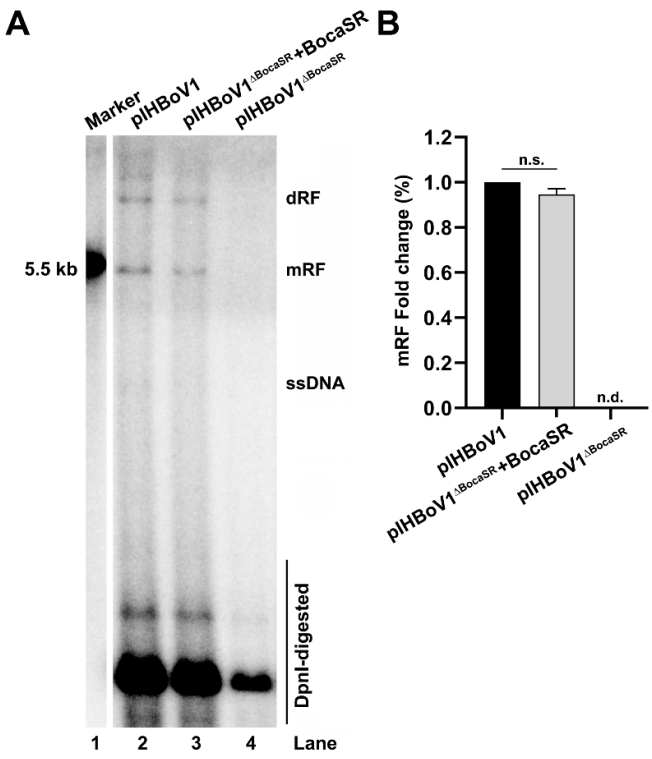
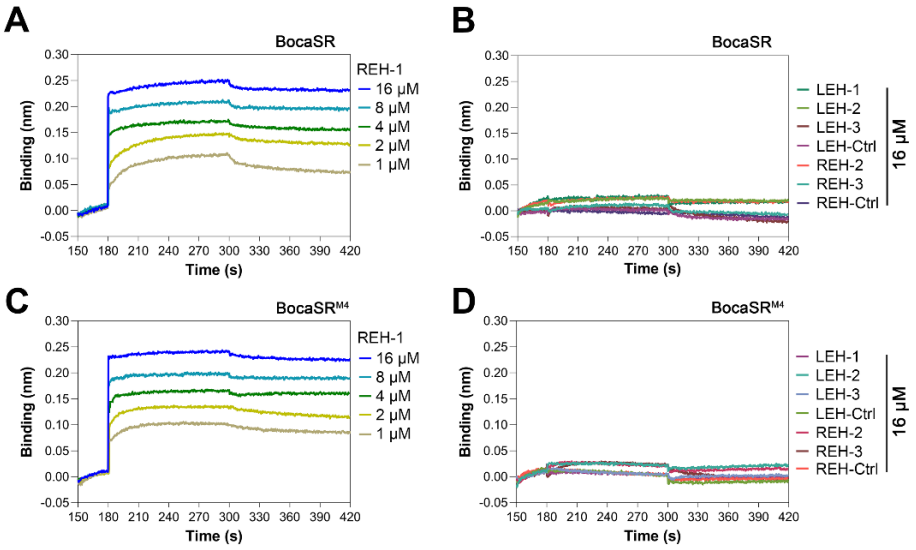


Figure S10

Figure S10. *In vitro* transcribed BocaSR facilitates HBoV1 replication.

(A) Southern blotting. HEK293 cells was transfected with pIHBoV1 (lane 2), pIHBoV1$^{\Delta BocaSR}$ complemented with *in vitro* transcribed BocaSR (lane 3), and pIHBoV1$^{\Delta BocaSR}$ (lane 4), respectively. At 2 dpt, Hirt DNA was prepared for Southern blotting. A size marker was indicated (~5.5 kb, lane 1). DpnI-digested bands served as inputs. (**B) Quantification of mRF DNA.** The intensity of mRF DNA bands was quantified by ImageQuant TI. The shown values (mean ± SD) obtained from three blots were normalized to the replication of pIHBoV1, which was arbitrarily set to 1. All the experiments were performed in triplicate and analyzed by Student's *t* test (n.s., not significant; n.d., not detectable).



E

Binding parameters of BocaSR/BocaSR^{M4} with REH-1

	K_D(nM)	K_a(1/Ms)	K_d(1/s)
BocaSR	1.2	7.2E5 ± 9.4E4	8.9E-4 ± 2.3E-4
BocaSR^{M4}	1.3	7.0E5 ± 1.2E5	8.9E-4 ± 1.1E-4

Figure S11

Figure S11. BocaSR interacts with the unwound DNA replication origin (*Ori*) of the REH.

(A-D) Kinetics of the *in vitro* interaction of oligos spanning HBoV1 LEH/REH with BocaSR or BocaSR^{M4}. Biotin-dUTP labeled BocaSR (A&B) or BocaSR^{M4} (C&D) was mounted on streptavidin biosensors. The binding kinetics depicted the associations and dissociations of 80 pM BocaSR/BocaSR^{M4} with the indicated oligos at the concentrations shown**. (E) The binding parameters of BocaSR and BocaSR^{M4} with REH-1 oligo.** Equilibrium dissociation constant K_D value represents the ratio of dissociation [K_d (1/s)] and association [K_a (1/Ms)] computed from the real-time binding curves of BocaSR/BocaSR^{M4} with the REH-1 oligo. Data shown were generated from at least three repeated experiments.

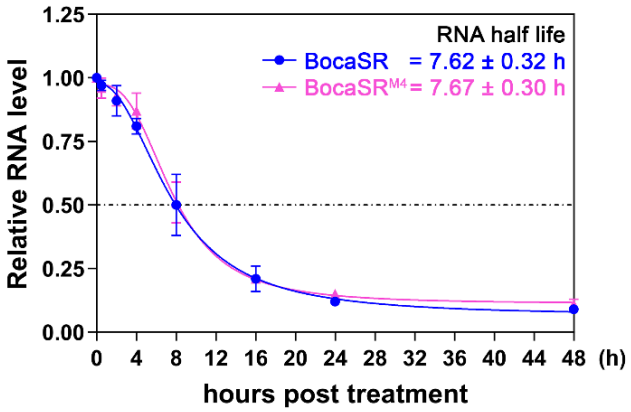


Figure S12

Figure S12. Stability assays of BocaSR and BocaSR^{M4}.

HEK293 cells were transfected with p3Z-BocaSR or p3Z-BocaSR^{M4}. After 48 hpt, 20 μM of the RNA Pol III inhibitor ML-60218 (#HY-122122, MedChemExpress, NJ) were supplemented in the media, and this time point was regarded as 0 h post treatment (28,29). Total RNA of the treatment cells was prepared by using TRIzol at different time points as indicated. The levels of BocaSR and BocaSR^{M4} were quantified by using BocaSR-specific qRT-PCR and normalized to the RNA level at 0 h.

Table S1. List of shRNAs used in the study.

Gene	**shRNA sequence (5' → 3')**
Scramble shRNA control (Scram)	CCG GCC TAA GGT TAA GTC GCC CTC GCT CGA GCG AGG GCG ACT TAA CCT GTT TTT G
METTL3	CCG GGC CAA GGA ACA ATC CAT TGT TCT CGA GAA CAA TGG ATT GTT CCT TGG CTT TTT G
METTL14	CCG GCC ATG TAC TTA CAA GCC GAT ACT CGA GTA TCG GCT TGT AAG TAC ATG GTT TTT G
METTL4	CCG GGC CTG CAG TTT GTC AGA ATT ACT CGA GTA ATT CTG ACA AAC TGC AGG CTT TTT G
METTL16	CCG GCC TGT ACT TAC CTA CTC ACA TCT CGA GAT GTG AGT AGG TAA GTA CAG GTT TTT G
YTHDF1	CCG GGG CGT GTG TTC ATC ATC AAG ACT CGA GTC TTG ATG ATG AAC ACA CGC CTT TTT G
YTHDC1	CCG GTG CCT CCA GAG AAC CTT ATA ACT CGA GTT ATA AGG TTC TCT GGA GGC ATT TTT G
YTHDF2	CCG GGC TAC TCT GAG GAC GAT ATT CCT CGA GGA ATA TCG TCC TCA GAG TAG CTT TTT G
YTHDF3	CCG GGA TAA GTG GAA GGG CAA ATT TCT CGA GAA ATT TGC CCT TCC ACT TAT CTT TTT G

Table S2. gRNA sequences

Gene	**gRNA sequence (5' → 3')**
Scramble sgRNA control (SsgRNA)	GTA TTA CTG ATA TTG GTG GG
METTL3	AGA GTC CAG CTG CTT CTT GT
METTL3	GAA GCA GGA CTC GGG GCA CT

Table S3. Oligos used in *in vitro* binding (BLI) assay.

Oligos	**Sequence (5' → 3')**
LEH-Ctrl	TGG CGT CTG TAC AAC CAC
LEH-1	CTG AGC CGG CAG ACA TAT TGG ATT CCA AGA
LEH-2	CGC AGC GCG CTG CGC GCA GCG CAG GCA TGA
LEH-3	AAT ATG TCT GCC GGC GAT TAG ATC ATG CGC GCG
REH-Ctrl	TCG CGA TTG CAT AAG CAA
REH-1	GCG CAT GTA CAA CAA CAA CAC ATT AAA AGA TAT AGA GTT
REH-1a	CAA CAC ATT AAA AGA TAT AGA GTT
REH-1b	GCG CAT GTA CAA CAA
REH-2	ACT AAG GCG CCA GCT GAT ATA AAA CTA AGA TG
REH-3	ATC AGC TGG CGC CTT AGT TAT ATA ACA TGC ATG TTA TAT A

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