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Restriction of exogenous DNA expression by SAMHD1

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ABSTRACT

SAMHD1 (**S**terile **A**lpha **M**otif and **H**istidine-aspartate **D**omain containing protein 1) has been documented as a host factor that restricts HIV-1 and some DNA viruses. In this work, we attempted to explore possible effects of SAMHD1 on exogenous DNA and show that SAMHD1 exerts a general inhibition on the expression of exogenous DNA *in vitro* and in mice. This inhibition is achieved through repressing transcription of exogenous DNA. Intriguingly, unlike SAMHD1's restriction of HIV-1, such restriction does not require the dNTPase or RNase activities, or T592 phosphorylation of SAMHD1. Mechanistically, SAMHD1 enhances the expression of interferon regulatory factor-1 (IRF1), while IRF1 upregulation was demonstrated to inhibit exogenous DNA expression in a similar fashion as SAMHD1. IFNλ1, whose induction has been associated with IRF1 activation, is dispensable for SAMHD1/IRF1-mediated restriction of exogenous DNA, and neither type I nor II interferons appear to be involved. We also demonstrate that SAMHD1/IRF1-mediated restriction can effectively inhibit hepatitis B virus (HBV) antigen expression and progeny virus production in mouse models. In conclusion, these data support restriction of exogenous DNA as a novel function of SAMHD1.

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1. Introduction

SAMHD1 (**S**terile **A**lpha **M**otif and **H**istidine-aspartate **D**omain containing protein 1) is expressed in both cycling and noncycling cells, and has been documented as a host restriction factor in the intrinsic antiviral immune system [1]. It possesses a triphosphohydrolase activity that catalyzes the conversion of deoxyribonucleoside triphosphates (dNTP) to deoxyribonucleosides (dN) and triphosphates [2–4]. Depletion of intracellular pool of dNTP by such a dNTPase activity has been linked to SAMHD1-mediated restriction of various retroviruses including HIV-1 and a few DNA viruses [5,6]. A potential ribonuclease (RNase) activity and phosphorylation at T592 of SAMHD1 have also been reported to regulate its restriction of HIV-1 [7,8]. SAMHD1 has also been shown to play a dual role in affecting HBV infection as well as progeny virus production, although the detailed mechanisms are not yet clear [9].

On the other hand, SAMHD1 appears to suppress innate immune responses to viral infections and inflammatory stimuli by inhibiting the NF-kappaB activation and type I interferon (IFN-I) induction [10]. It has also been observed that SAMHD1 can prevent interferon induction in response to DNA damage by stimulating the exonuclease activity of MRE11 to degrade nascent DNA at stalled replication forks [11].

Interferon regulatory factor-1 (IRF1) is a transcription factor expressed constitutively in all cell types except early embryonic cells. IRF1 can be activated by viruses, lipopolysaccharide (LPS), interleukin-1 (IL-1), and various immune effectors including type I and type II interferons (IFN) [12]. IRF1 regulates the transcription of various target genes like 2′,5′-oligo (A) synthetase (OAS) [13], cyclin-dependent kinase (Cdk) inhibitor $p21^{WAF1/CIP1}$ (p21) [14] and double-stranded RNA-dependent protein kinase R (PKR) [15], which are involved in diverse physiological and pathological processes, such as antiviral response, tumor suppression, immunomodulation, and pro-inflammatory responses [16]. The mechanisms underlying IRF1's functions are not fully understood. Earlier studies have addressed IRF1 as a regulator for type I inter-

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feron production [17]. A more recent work, however, has associated activation of IRF1 with the induction of IFNλ1 (a type III interferon), which was involved in the recognition of cytosolic exogenous DNA mediated by Ku70 [18].

Given SAMHD1's restriction of HIV-1 and some DNA viruses, we wonder whether SAMHD1 might have a general restrictive effect on exogenous DNA. In the present study, we show that SAMHD1 exerts a general inhibition of the expression of exogenous DNA *in vitro* and in mice. The inhibition is achieved through repression of the transcription of exogenous DNA and does not require the dNTPase or RNase activities, or phosphorylation at T592 of SAMHD1. SAMHD1 upregulates the expression of IRF1, while increased IRF1 expression was shown to inhibit exogenous DNA expression similarly to SAMHD1. We provide evidence that interferon-independent pathways are involved in SAMHD1/IRF1-mediated restriction of exogenous DNA. Finally, we demonstrate that SAMHD1/IRF1-mediated restriction can efficiently inhibit hepatitis B virus (HBV) antigen expression and replication in mouse models.

2. Materials and methods

2.1. Cell culture and establishment of stable cell lines

The cell lines used in this study include human embryonic kidney HEK293 and HEK293FT, human cervical carcinoma HeLa, human hepatocellular carcinoma (HCC) Huh7 and MHCC97H, human hepatoma HepG2 and HepG2-NTCP, and mouse HCC Hepa1-6. These cell lines were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, USA) containing 2 mmol/L L-glutamine, 50 U/mL penicillin, 100 mg/mL streptomycin, and 10% (vol/vol) fetal bovine serum (Gibco). Primary mouse hepatocytes (PMH) were prepared following a two-step perfusion protocol as previously described [19] and cultured in Williams E medium (Gibco) supplemented with 10% fetal bovine serum, 3 mg/mL insulin, 2 mmol/L L-glutamine, 18 mg/mL hydrocortisone, 50 U/mL penicillin and 100 mg/mL streptomycin.

Gene knockdown and knockout cell lines were established by transductions of Huh7 or HEK293 cells with the lentiviruses that encode gene-specific shRNA and gRNA-Cas9 respectively. The transducted cells were selected and cultured in medium supplemented with 4 μg/mL puromycin (Sigma, St Louis, USA). Control cell lines were similarly established except with the lentiviruses that encode nonspecific shRNA and gRNA-Cas9 respectively.

2.2. Plasmids and lentiviruses

pCMV-FLAG-SAMHD1 that encodes the N-terminally FLAG-tagged SAMHD1 was constructed based on pcDNA3.0 (Invitrogen, Carlsbad, USA) and was a generous gift from Dr. Yongxiang Wang (Fudan University, China). Human *IRF1* cDNA were obtained using PCR from HEK293 cDNA pool. Murine *IRF1* and *SAMHD1* (isoform 1 and 2) cDNA were from Hepa1-6 cDNA pool. Point mutations (D137N, D207N, D311A, Q548A, T592A, T592E, ΔATG), truncations of human SAMHD1 and point mutation (D169N) of mSAMHD1 were generated through site-directed mutagenesis using KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) and were verified by DNA sequencing (GENEWIZ, Suzhou, China). cDNAs of FLAG-tagged truncates and mutants of SAMHD1 and HA-tagged IRF1 were inserted in pcDNA3.0 respectively. pSV40-Luc (firefly luciferase), pCMV-RLuc (*Renilla* luciferace), pCMV-NanoLuc and pCMV-SecNanoLuc were from Promega (Madison, USA). Plasmid pCMV-SecNanoLuc encodes NanoLuc, a small-size secretory luciferase. The linear pSV40-Luc was generated by digesting pSV40-Luc with *Bam*H I (NEB, Ipswich, USA). The SV40-Luc fragment without plasmid backbone was generated by PCR. Both were recovered by gel purification using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, USA). pCMV-Luc, pHBCP-Luc, and pHRE-Luc were constructed by insertion of the respecitve promoter (CMV, human cytomegalovirus promoter; HBCP, human β-catenin promoter; HRE, human hypoxia-response element) into the luciferase reporter vector pGL3-Basic (Promega). The Tet-Off and Tet-On gene expression systems were from Clontech (Mountain View, USA). prcccDNA and pCMV-Cre plasmids were constructed previously [20,21]. prcccDNA contains a precursor of recombinant HBV cccDNA which can be formed by Cre-mediated recombination in cells and mice. Plasmid pHBV1.3 encoded a 1.3-mer overlength HBV genome (GenBank accession No. V01460.1).

Lentiviruses were produced by co-transfection of HEK293FT cells with the packaging vector psPAX2 (Addgene, Watertown, USA), the envelope expression plasmid pMD2.G (Addgene) and the pLKO.1- or plentiCRISPRv2-based vector (at a ratio of 3:1:4) using polyethylenimine (PEI). The medium was changed 8 h post-transfection. The virus-containing supernatant was harvested and passed through a 0.45-μm filter 72 h post-transfection. The lentivirus stock was used for transductions.

Gene-specific and non-specific shRNA coding sequences were inserted into pLKO.1 (Addgene) between the sites of *Age* I and *Eco*R I. The following target sequences were used: sh-SAMHD1#1, GCCATCATCTTGGAATCCAAA; sh-SAMHD1#2, GCCAGTGCTAAACCCAAAGTA; sh-mSAMHD1#1, CCCTCTCCTTATCAGAATCAT; sh-mSAMHD1#2, GCCGCAATCTATACAAGTATT; sh-IRF1#1, GCGTGTCTTCACAGATCTGAA; sh-IRF1#2, GCTACACAGTTCCAGGCTACA; sh-IFNλ1#1, GCCACATTGGCAGGTTCAAAT; sh-IFNλ1#2, GGAGCTAGCGAGCTTCAAGAA; sh-IFNα1/2#1, GGAGGTTGTCAGAGCAGAAAT; sh-IFNα1/2#2, GACAGACATGACTTTGGATTT; sh-IFNβ#1, GCTCCTGTGGCAATTGAATGG; sh-IFNβ#2, GCTACAACTTGCTTGGATTCC; sh-IFNγ#1, CTCATCCAAGTGATGGCTGAA; sh-IFNγ#2, GGTTCTCTTGGCTGTTACTGC. Non-specific, CCTAAGGTTAAGTCGCCCTCG.

Gene-specific disruption/knockout was induced by lentivirus transducted CRISPR-Cas9 system. *SAMHD1* guide RNAs (gRNAs) were CTCGGGCTGTCATCGCAACG and GGCAGACTGGTCCCCGGGCC. Non-targeting control gRNA was GCCCCGCCGCCCTCCCCTCC. These oligonucleotides were annealed and inserted into plentiCRISPRv2 (Addgene).

2.3. Transfection and transduction

Transfections of HeLa and HCC cell lines (Huh7, HepG2, MHCC97H and Hep1-6) were performed using TurboFect Transfection Reagent (Thermo Fisher Scientific) following the manufacturer's instructions while transfections of HEK293 and HEK293FT cells were carried out using polyethylenimine (PEI). Empty vectors were added whenever necessary to ensure equal amounts of DNA in parallel transfections.

Cells were transducted by lentiviruses in the presence of 4 μg/mL polybrene (Sigma). Medium was changed 24 h post-transduction and cells were selected with 4 μg/mL puromycin (Sigma) 3 days after transduction. Western blot or reverse transcription PCR (RT-PCR) was performed to confirm the overexpression, knockdown or knockout of the target genes.

2.4. Luciferase activity assay

Cells were transfected with appropriate luciferase reporter plasmids for 48 h before cell harvest. Firefly and *Renilla* luciferase activities in cell lysates were assayed using Luciferase Assay System (Promega) following the manufacturer's instructions. NanoLuc luciferase activities in culture supernatants of transfected cells and mouse sera were determined using Nano-Glo Luciferase Assay System (Promega) following the manufacturer's instructions. The

results were derived from at least three independent experiments, each performed in triplicate.

2.5. Cell viability and cytotoxicity assays

The Cell Couting Kit-8 (Dojindo, Kumamoto, Japan) and Cytotoxicity LDH Assay Kit-WST (Dojindo) were used to assess cell viability and cytotoxicity LDH according to the manufacturer's instructions. Briefly, Huh7 and HEK293 cells transfected with pCMV-FLAG-SAMHD1 were dispensed into the 96-well plate (5000 cells/well) and incubated at 37 ℃ respectively. 48 h after transfection, assay solutions of the two kits were added, and then the absorbances were measured using a microplate reader (Bio-Rad, Hercules, USA).

2.6. RNA extraction and qRT-PCR analysis

Total cellular RNA was extracted from Huh7, HEK293 cells or mouse liver tissues using TRIzol reagent (Invitrogen) following the manufacturer's instructions. After phenol/chloroform extraction and isopropanol precipitation, 1 μg of RNA was reverse transcribed using cDNA Synthesis Kit (TaKaRa, Kusatsu, Japan).

Quantative real-time PCR (qRT-PCR) was performed in a 20 μL reaction containing SYBR Green (TaKaRa) on MXP3000 cycler (Stratagene, La Jolla, USA) with the following program: initial denaturation at 95 ℃ for 300 s; 40 cycles of 95 ℃ for 15 s, 55 ℃ for 20 s, and 72 ℃ for 20 s; followed by a melt curve step. The values were normalized against those of the *GAPDH* gene. Data were expressed as fold change over control treatment, and derived from at least three independent experiments, each performed in triplicate. PCR primers (Genewiz) were as following (forward/reverse): GAPDH, 5′-GGAGCGAGATCCCTCCAAAAT-3′/5′-GGCTGTTGTCATACTTCTCATGG-3′; Luciferase, 5′-CTCGCATGCCAGAGATCCTA-3′/5′-AAGGCTCCTCAGAAACAGCT-3′; IRF1, 5′-ATGCCCATCACTCGGATGC-3′/5′-CCCTGCTTTGTATCGGCCTG-3′; IFNλ1, 5′-CACATTGGCAGGTTCAAATCTCT-3′/5′-CCAGCGGACTCCTTTTTGG-3′; IFNβ, 5′-GATTCATCTAGCACTGGCTGG-3′/5′-CTTCAGGTAATGCAGAATCC-3′; rcccDNA, 5′-CAAGACAGGTTTAAGGAGAC-3′/5′-GAGAGAAAGGCAAAGTGGAT-3; HBV, 5′-AATGCCCCTATCTTATCAACAC-3′/5′-GAGATTGAGATCTTCTGCGACG-3′; OAS1, 5′-TGTCCAAGGTGGTAAAGGGTG-3′/5′-CCGGCGATTTAACTGATCCTG-3′; OAS2, 5′-GAAGGAGTTCGTAGAGAAGGCG-3′/5′-CCCTTGACAGTTTTCAGCACC-3′; OAS3, 5′-AGGTGGCTCCTATGGACGG-3′/5′-TTTATCGAGGATGTCACGTTGG-3′; p21, 5′-TGTCCGTCAGAACCCATGC-3′/5′-AAAGTCGAAGTTCCATCGCTC-3′; PKR, 5′-GCCGCTAAACTTGCATATCTTCA-3′/5′-TCACACGTAGTAGCAAAAGAACC-3′.

2.7. Isolation of DNA from transfected or mouse liver cells

Total cellular DNA was extracted and purified from Huh7, HEK293 or mouse liver cells using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Briefly, cells were collected by centrifugation and lysed with proteinase K. Buffering conditions were adjusted and the lysates were loaded onto the spin column. The remaining contaminants and enzyme inhibitors were removed by washing and cellular DNA was eluted. DNA was analyzed with southern blot using a digoxigenin-labeled specific probe.

2.8. Immunoprecipitation and western blot analysis

Co-immunoprecipitation (Co-IP) was performed using cell lysates pre-cleared by centrifuge. All lysates were lysed in cell lysis buffer (Thermo Fisher Scientific). Proteins were precipitated using indicated antibodies and conjugated to Protein A/G PLUS-Agarose (Santa Cruz, Dallas, USA). The beads were washed with RIPA buffer (50 mmol/L Tris-HCl, 0.5% NP-40, 1 mmol/L EDTA, 150 mmol/L NaCl, pH 8.0), boiled in SDS buffer (50 mmol/L Tris-HCl, 1% SDS, 1 mmol/L EDTA, 150 mmol/L NaCl, pH 8.0), and analyzed with western blot.

Western blot analysis was performed following the standard method. Briefly, cells were lysed in SDS buffer and the protein concentration was estimated using BCA Protein Assay (Sangon Biotech, Shanghai, China). Cell lysates were denatured at 100 ℃ for 5–10 min before loading onto 10% gel. The membranes (Millpore, Billerica, USA) were probed overnight at 4 ℃ with the following antibodies respectively in 5% skim-milk solution to detect the target protein: 1:10000 anti-FLAG antibody (Sigma), 1:10000 anti-GFP antibody (Abmart, Berkeley Heights, USA); 1:50000 anti-β-Actin antibody (Sigma), 1:5000 anti-GAPDH antibody (Proteintech, Wuhan, China), 1:1000 anti-SAMHD1 antibody (Abcam, Cambridge, UK), 1:1000 anti-p65 (Santa Cruze), 1:1000 anti-IRF1 antibody (Cell Signaling Technology, Beverly, USA), 1: 1000 anti-Histone H3 antibody (Cell Signaling Technology). Horseradish-peroxidase-conjugated sheep anti-mouse and anti-rabbit (Bio-Rad) were used as the secondary antibodies. Signal detection was performed using CLARITY ECL Blotting Substrate (Millpore). Each analysis was carried out at least three times.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described with minor modifications [22]. Briefly, transfected cells were fixed in 1% formaldehyde for 20 min at room temperature, re-suspended in lysis buffer (50 mmol/L Tris-HCl, 0.5% NP40, 1 mmol/L EDTA, and 100 mmol/L NaCl, pH 8.0) and incubated for 10 min at 4 °C. Lysates were centrifuged at 13,000 r/min for 2 min and supernatants were removed. Nuclei were extracted with SDS buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris, pH 8.0). Chromatin solutions were sonicated to generate 300–400 bp DNA fragments. DNA fragments were diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl, 167 mmol/L NaCl, pH 8.0). ChIP was performed overnight at 4 °C using indicated antibodies and Protein A/G PLUS-Agarose (Santa Cruz). Antibodies were as following: anti-SAMHD1 antibody (1:1000, Abcam), acetyl-histone H3 antibodies (anti-H3K9ac, H3K14ac, H3H18ac, H3K27ac and H3K56ac, 1:1000, Cell Signaling Technology), trimethyl-histone H3 antibodies (anti-H3K4me3, H3K9me3, H3K27me3, H3K36me3 and H3K79me3, 1:1000, Cell Signaling Technology). Immunoprecipitated chromatins were analyzed using qRT-PCR with the following primers (forward/reverse): Luciferase, 5′-CTCGCATGCCAGAGATCCTA-3′/5′-AAGGCTCCTCAGAAACAGCT-3′; IRF1 promoter, 5′-ATGTGTCAGGTTCTCAGGGG-3′/5′-AAGCAAAGTGGAGGGCAATG-3′; HBV, 5′-AATGCCCCTATCTTATCAACAC-3′/5′-GAGATTGAGATCTTCTGCGACG-3′.

2.10. RNA-seq cDNA library preparation and analysis

Total RNA of Huh7 cells transfected with pCMV-FLAG-SAMHD1 or empty vector from two independent experiments was extracted using TRIzol Reagent (Invitrogen), and the RNA of each sample was quantified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA), NanoDrop (Thermo Fisher Scientific) and 1% agrose gel. 1 μg total RNA with RIN value above 7 was used for following library construction according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®).

The poly(A) mRNA isolation was performed using Ribo-Zero™ rRNA removal Kit (Illumina, San Diego, USA). The mRNA fragmentation and priming were performed using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. First

strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen, Union City, USA), and fragments of ~360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies), and quantified by Qubit 2.0 Fluorometer (Invitrogen). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina). Sequencing was carried out using a 2 × 150 bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ.

Reads that are unmapped, low quality, with duplicates or multiple mappings, or not primary were excluded from the analysis by Trimmomatic (v0.30). Hisat2 (v2.0.1) was used to index reference genome sequence. Clean data were aligned to reference genome via software Hisat2 (v2.0.1). HTSeq (v0.6.1) estimated gene and isoform expression levels from the pair-end clean data. Differential expression analysis used the DESeq Bioconductor package. After adjusted by Benjamini and Hochberg's approach for controlling the false discovery rate, *P*-value of genes were setted <0.05 to detect differential expressed ones. Eventually, the gained data indicated the accurate quantification of expression of all transcripts and the transcripts were processed through interactive comparative analysis of most active networks and functional processes.

2.11. Confocal microscopy

Cells grown on coverslip-placed dishes were fixed in 4% paraformaldehyde, blocked with blocking buffer (Thermo Fisher Scientific), stained overnight with the indicated primary antibodies (1:200, Thermo Fisher Scientific) at 4 ℃, and finally incubated with Alexa Fluor 488- or 546-labeled secondary antibodies (1:1000, Thermo Fisher Scientific) for 1 h at 37 ℃. Cell-coated coverslips were mounted using ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were captured with the SP8 confocal microscope (Leica, Wetzlar, Germany).

2.12. HBV infection

HepG2-NTCP cells were used for HBV infection as described [23]. Cells were cultured in PMM medium (DMEM medium supplemented with 2.5% DMSO) for 24 h and then inoculated with HBV at a multiplicity of infection (M.O.I) of 1000 (GE, genome equivalent) in the presence of PEG8000 (Sigma) for 12 h. Cells were washed 5 times with PBS and maintained in PMM for 5 days with medium change every other day.

2.13. Serum analyses

Extracellular and serum hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) were determined using commercial ELISA kits (KHB, Shanghai, China) following the manufacturer's instructions. Serum HBV DNA was analysed using commercial quantitative assays (Adicon, Shanghai, China).

2.14. Animals

Male 6-week-old to 8-week-old specific pathogen-free BALB/c mice were purchased from Experimental Animal Centre (Slaccas, Shanghai, China). Mice were randomly separated into several groups (6 each). pCMV-SecNanoLuc (5 μg) and pCMV-GFP (5 μg) were hydrodynamically injected into the tail vein together with pCMV-FLAG-mSAMHD1 (20 μg) or pcDNA3.0 (20 μg) or psh-mSAMHD1 (20 μg) or pLKO.1 (20 μg) within 5–6 s in a volume of saline equivalent to 8% of the mouse body weight. To evaluate SAMHD1-mediated restriction on HBV, prcccDNA (4 μg) and pCMV-Cre (4 μg) were hydrodynamically delivered into the mouse liver. Two days later, the mouse was infected with the lentivirus (5×10^7 Transducing Unit (TU)) encoding mSAMHD1 or sh-mSAMHD1 or the empty vector. The *in vivo* experiments on *mIRF1* were similarly executed.

All mice were housed in pathogen-free conditions. Mice were sacrificed at indicated time points, sera and liver tissues were used for experiments. The animal experimental protocols were approved by the Animal Ethic Committee of School of Basic Medical Sciences, Fudan University.

2.15. Immunohistochemical staining

Staining methodology was performed as described previously [22]. Briefly, formalin-fixed, paraffin-embedded slides were dewaxed by Xylene and rehydrated in ethyl alcohol gradient. Mouse liver tissues were retrieved with microwave heating in antigen retrieval buffer. Incubation was carried out at 4 ℃ overnight with the indicated primary antibodies mentioned above. Secondary antibody was incubated at room temperature. Color development was performed using HRP/DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, USA) according to the manufacturer's instruction.

2.16. Statistical analysis

Data were analyzed using Prism 7 (GraphPad) and are presented as mean ± SEM. Statistical significance was determined using the unpaired two-tailed Student's *t* test for single variables and two-way ANOVA followed by Bonferroni posttests for multiple variables. A *P* value of <0.05 was considered statistically significant and is presented as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

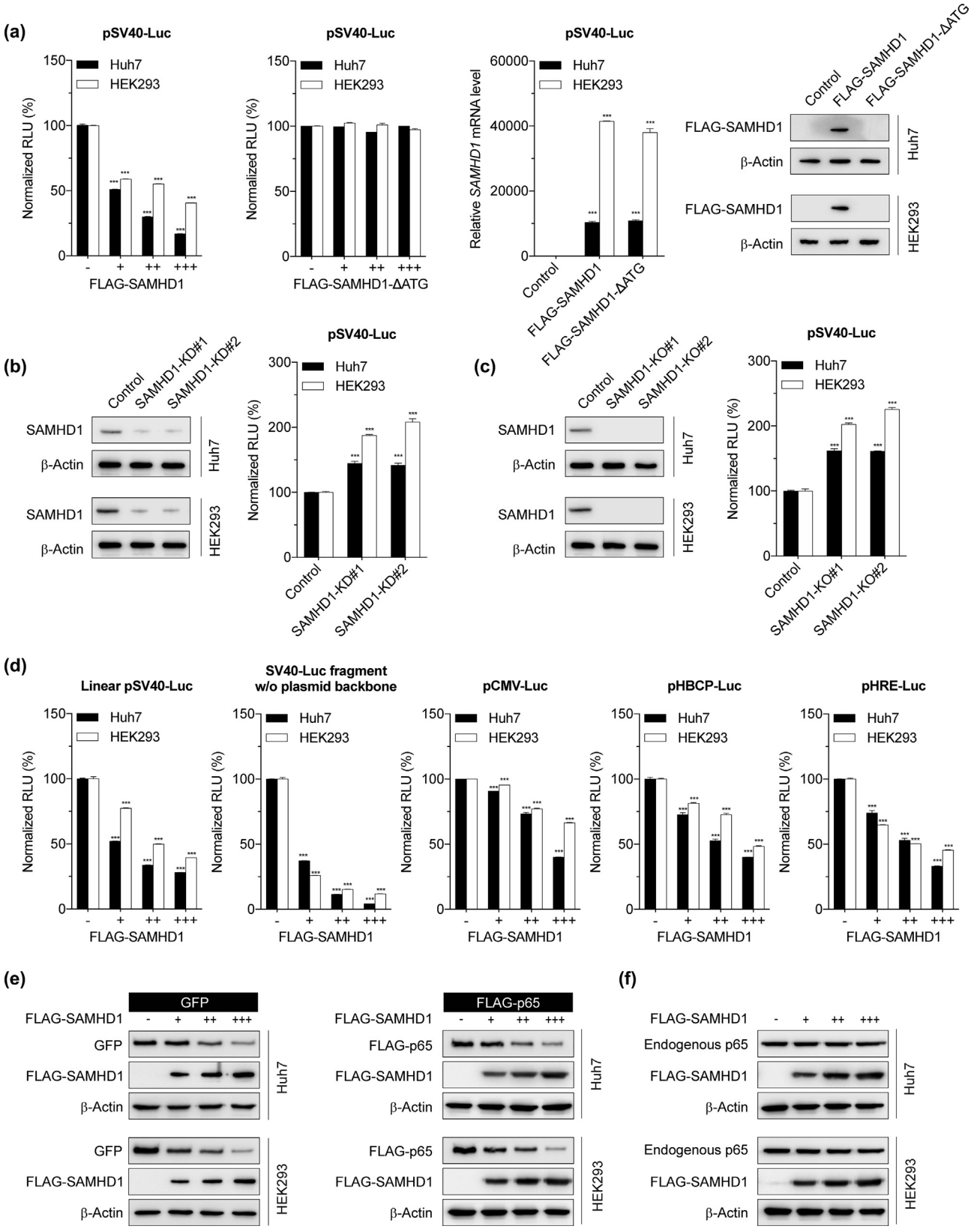
3. Results

3.1. SAMHD1 inhibits the expression of exogenous DNA

To assess the effects of SAMHD1 on exogenous DNA, we transfected Huh7 or HEK293 cells with luciferase reporter plasmid pSV40-Luc and SAMHD1 expression plasmid pCMV-FLAG-SAMHD1, or its derivative pCMV-FLAG-SAMHD1-ΔATG with the start codon mutated. A SAMHD1-dose-dependent decrease in the luciferase activity was observed in *SAMHD1*-co-transfected but not *SAMDH1-ΔATG*-co-transfected cells (Fig. 1a). SAMHD1 overexpression was not cytotoxic for Huh7 or HEK293 cells during the observation period (up to 48 h post transfection, Fig. S1a, b online), despite the fact that prolonged SAMDH1 overexpression (>5 d) had been reported to inhibit proliferation of THP-1 cells and induce apoptosis [24]. Neither *Renilla* luciferase nor NanoLuc showed similar effect on exogenous DNA expression in co-transfected cells (Fig. S2a, b online). These data indicate that such SAMHD1-

mediated suppression is not the result of saturated cellular transcription or translation machinery, reduced cell proliferation, or induced apoptosis, and therefore is attributable to SAMHD1 protein itself.

Similar inhibitory effects on exogenous DNA expression by SAMHD1 overexpression were also observed in human HeLa, HepG2 and MHCC97H cell lines (Fig. S3a online). Murine SAMHD1 (mSAMHD1) has two isoforms that differ at the carboxyl terminus



(Fig. S3b online). Both isoforms suppressed the exogenous DNA expression in co-transfected mouse Hep1-6 HCC cell line (Fig. S3c online).

SAMHD1 knockdown and knockout cell lines were then established by lentivirus transduction with *SAMHD1*-specific shRNA and gRNA-Cas9 respectively. When transfected with pSV40-Luc, *SAMHD1* knockdown or knockout cells displayed significantly higher luciferase expression compared to control cells (Fig. 1b, c). SAMHD1-mediated inhibition of exogenous DNA expression did not depend on DNA circularity or linearity, bacterial sequences in plasmid backbone, promoter (CMV promoter, HBCP and HRE) driving the expression of the reporter gene, or reporter gene used (*GFP* and human *p65*) (Fig. 1d, e). In contrast, SAMHD1 overexpression did not affect the expression of endogenous chromosomal gene *p65* (Fig. 1f).

To rule out any effects of variable transfection efficiency, Huh7 or HEK293 cells were co-transfected with pTRE-SAMHD1, pCMV-rtTA (Tet-On) and pSV40-Luc. In this setting, SAMHD1 expression was driven by the *TRE* promoter that was only activated by co-transfected rtTA in the presence of doxycycline (Fig. S4a online). The same number of transfected cells were treated with doxycycline or vehicle, and doxycycline-treated cells expressed much lower luciferase compared to vehicle-treated cells (Fig. S4b, c online). These results collectively suggest that SAMHD1 has a novel function that restricts gene expression from exogenous DNA.

3.2. SAMHD1 inhibits the transcription of exogenous DNA

In order to identify which step of gene expression from exogenous DNA is targeted by SAMHD1, DNA and RNA were extracted from Huh7 and HEK293 cells co-transfected with pSV40-Luc and pCMV-FLAG-SAMHD1 or pCMV-FLAG. Southern blot showed that pSV40-Luc plasmid DNA level was not affected by SAMHD1 overexpression (Fig. 2a). In contrast, qRT-PCR results showed that SAMHD1 overexpression dose-dependently decreased luciferase mRNA levels in co-transfected cells (Fig. 2b). Furthermore, time course analysis showed that luciferase protein and mRNA levels built up more slowly in both SAMHD1-overexpressing Huh7 and HEK293 cells than in control cells (Fig. 2c). To determine the kinetics of luciferase mRNA decay, Huh7 or HEK293 cells were co-transfected with pCMV-tTA (Tet-Off), pTRE-Luc and pCMV-FLAG-SAMHD1. When these cells were exposed to doxycycline, luciferase transcription would be turned off by tTA and the decay of existing luciferase mRNA could be measured. As shown in Fig. 2d, the decrease of luciferase mRNA level in SAMHD1-overexpressing cells was comparable to control cells. Taken together, these results indicate that SAMHD1 inhibits the expression of exogenous DNA at the transcription level.

SAMHD1 has been reported to bind ssDNA [25,26]. Although we used exclusively dsDNA in the preceding experiments and ssDNA formation from such transfected dsDNA has not been reported, there remains a possibility that SAMHD1 might directly or indirectly bind to exogenous DNA to restrict expression. We thus performed chromatin immunoprecipitation (ChIP) to probe whether SAMHD1 could bind transfected DNA. The results showed that SAMHD1 was not associated with transfected DNA (Fig. 2e). For further verification, HEK293 cells were co-transfected with biotin-tagged linear TRE-SV40-luciferase PCR amplicon, pCMV-FLAG-SAMHD1 and pCMV-tTA (Tet-Off). Avidin pull-down analysis following formaldehyde cross-linking clearly showed the binding of tTA to the amplicon DNA, but no binding of SAMHD1 was detected (Fig. 2f). These results suggest that SAMHD1-mediated restriction does not involve or require its binding to exogenous DNA.

3.3. IRF1 is involved in SAMHD1-mediated restriction of exogenous DNA

SAMHD1 possesses dNTPase and potential RNase activities [27]. To determine whether these enzymatic activities are required for SAMHD1-mediated inhibition of exogenous DNA expression, a series of mutants were constructed with defective dNTPase (D137N), RNase (Q548A) or both (D207N, D311A) activities [28,29]. Given that T592 phosphorylation is important for the restriction of HIV-1 [7], two mutants with T592 replaced by a phosphor-mimetic (T592E) and a non-phosphorylatable residue (T592A) were also constructed respectively (Fig. S5a online). Co-transfection results showed that all of these mutants inhibited luciferase expression as efficiently as the wild type (Fig. S5b, c online), indicating that dNTPase and RNase activities as well as T592 phosphorylation are not involved in SAMHD1-mediated restriction of exogenous DNA.

Lack of binding to target DNA and dispensability of its known enzymatic activities suggest that SAMHD1-mediated restriction of exogenous DNA is possibly achieved through other factors. RNA sequencing (RNA-seq) analysis was therefore used to detect differential gene expression between SAMHD1-overexpressing and control Huh7 cells (Fig. 3a). GO analysis of the differentially expressed genes revealed that these genes were involved in several biological processes and pathways (Fig. 3b). We verified the RNA-seq results with qRT-PCR and selected 11 genes (7 downregulated, <0.7 fold and 4 upregulated, >2 fold) related to transcriptional regulation for further verification in co-transfection assay (Fig. 3c), of which only IRF1 significantly inhibited luciferase expression from co-transfected pSV40-Luc (Figs. 3d and S6 (online)).

Compared with control cells, both mRNA and protein levels of IRF1 increased in SAMHD1-overexpressing Huh7 and HEK293 cells (Fig. 4a), and decreased in *SAMHD1* knockdown and knockout cells (Fig. 4b, c). Similar effects were observed in mouse primary hepatocytes transduced by recombinant lentiviruses causing overexpression or knockdown of *mSAMHD1* (Fig. 4d, e). The expression of IRF1 also increased in the Huh7 and HEK293 cells that overexpressed the *SAMHD1* mutants used above (Fig. 4f). In line with these observations, transcription of known IRF1 target genes was activated in SAMHD1-overexpressing cells (Fig. S7a online).

In Huh7 and HEK293 cells co-transfected with pSV40-Luc and pCMV-HA-IRF1, luciferase protein and mRNA levels decreased in an IRF1-dose-dependent manner (Fig. 4g). Similarly, knockdown

Fig. 1. SAMHD1 inhibits the expression of exogenous DNA. (a) Relative luciferase activities, *SAMHD1* mRNA and protein levels in Huh7 and HEK293 cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-FLAG-SAMHD1 or pCMV-FLAG-SAMHD1-ΔATG (0.2, 0.4, 0.8 μg) in 24-well plates. The luciferase activities and mRNA values from the corresponding cells transfected with pSV40-Luc and pCMV-FLAG were taken as 100 and 1.0 respectively (the same below). (b) *Left*, western blot verification of SAMHD1 expression in *SAMHD1* knockdown (SAMHD1-KD) cells. *Right*, relative luciferase activities in SAMHD1-KD cells. (c) *Left*, western blot verification of SAMHD1 expression in *SAMHD1* knockout (SAMHD1-KO) cells. *Right*, relative luciferase activities in SAMHD1-KO cells. (d) Relative luciferase activities in the cells co-transfected with pSV40-Luc derivative (0.2 μg) and pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg) in 24-well plates. The plasmid form and the promoter controlling the reporter gene were indicated. (e) Western blot analysis of the reporter proteins (GFP and FLAG-p65) in Huh7 and HEK293 cells co-transfected with pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg) and pCMV-GFP (0.2 μg) or pCMV-FLAG-p65 (0.2 μg) in 24-well plates. GFP and FLAG-p65 were detected by anti-GFP mAb and anti-FLAG mAb respectively. (f) Western blot analysis of endogenous p65 in Huh7 and HEK293 cells transfected with pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg) in 24-well plates. p65 was detected by anti-p65 mAb. Data from three independent experiments were analyzed and presented as mean ± SEM. Data are compared to that of the control group and statistical significance calculated using unpaired two-tailed *t*-test. $^{***}P < 0.001$.

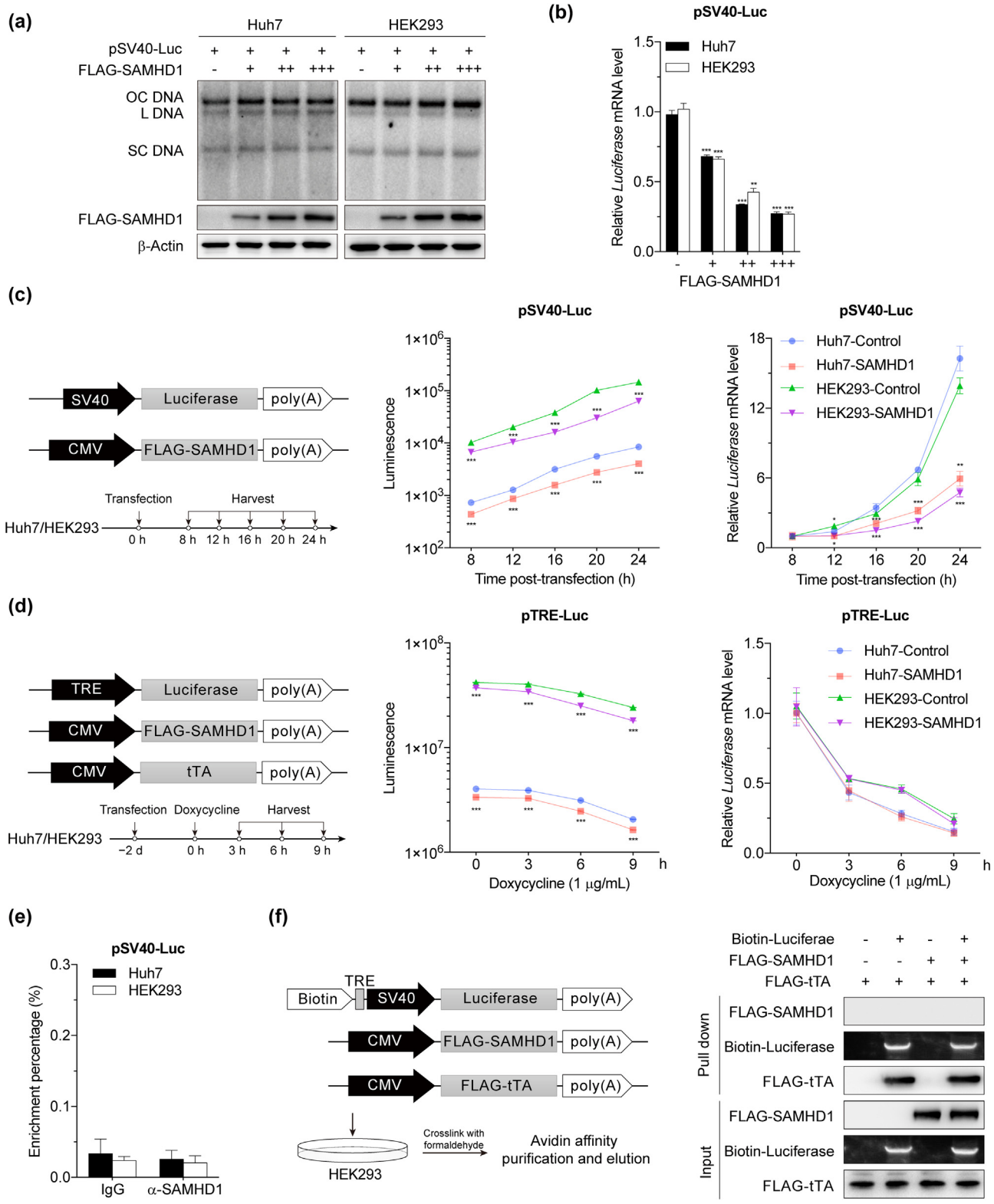


Fig. 2. SAMHD1 inhibits the transcription of exogenous DNA. (a) Southern blot analysis of pSV40-Luc DNA in the cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg) in 24-well plates. OC DNA: open circular DNA; L DNA: linear DNA; SC DNA, super-coiled DNA. The expression of FLAG-SAMHD1 was verified with western blot. (b) Quantitative analysis of the expression of luciferase in the cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg) in 24-well plates. (c) The kinetics of luciferase activity (*Left*) and expression (*Right*) in Huh7 and HEK293 cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-FLAG-SAMHD1 (0.4 μg) or pCMV-FLAG (0.4 μg) in 24-well plates. (d) The kinetics of luciferase activity (*Left*) and mRNA decay (*Right*) in Huh7 and HEK293 cells co-transfected with pTRE-Luc (0.2 μg), pCMV-tTA (0.8 μg) and pCMV-FLAG-SAMHD1 (0.4 μg) in 24-well plates. (e) Chromatin immunoprecipitation (ChIP) analysis of pSV40-Luc bound SAMHD1. Huh7 and HEK293 cells were plated in 6-well plates and transfected with pSV40-Luc (0.2 μg). (f) *Left*, schematic representation of transfected constructs. *Right*, Co-immunoprecipitation (Co-IP) analysis of Biotin-tagged SV40-Luc DNA and FLAG-SAMHD1 or tTA. HEK293 cells were plated in 6-well plates and co-transfected with pCMV-FLAG-tTA (2.0 μg), Biotin-tagged SV40-Luc (1.0 μg) and pCMV-FLAG-SAMHD1 (1.0 μg). tTA: tetracycline transcriptional activator. Data from three independent experiments were analyzed and presented as mean ± SEM. Data are compared to that of the control group and statistical significance calculated using unpaired two-tailed *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

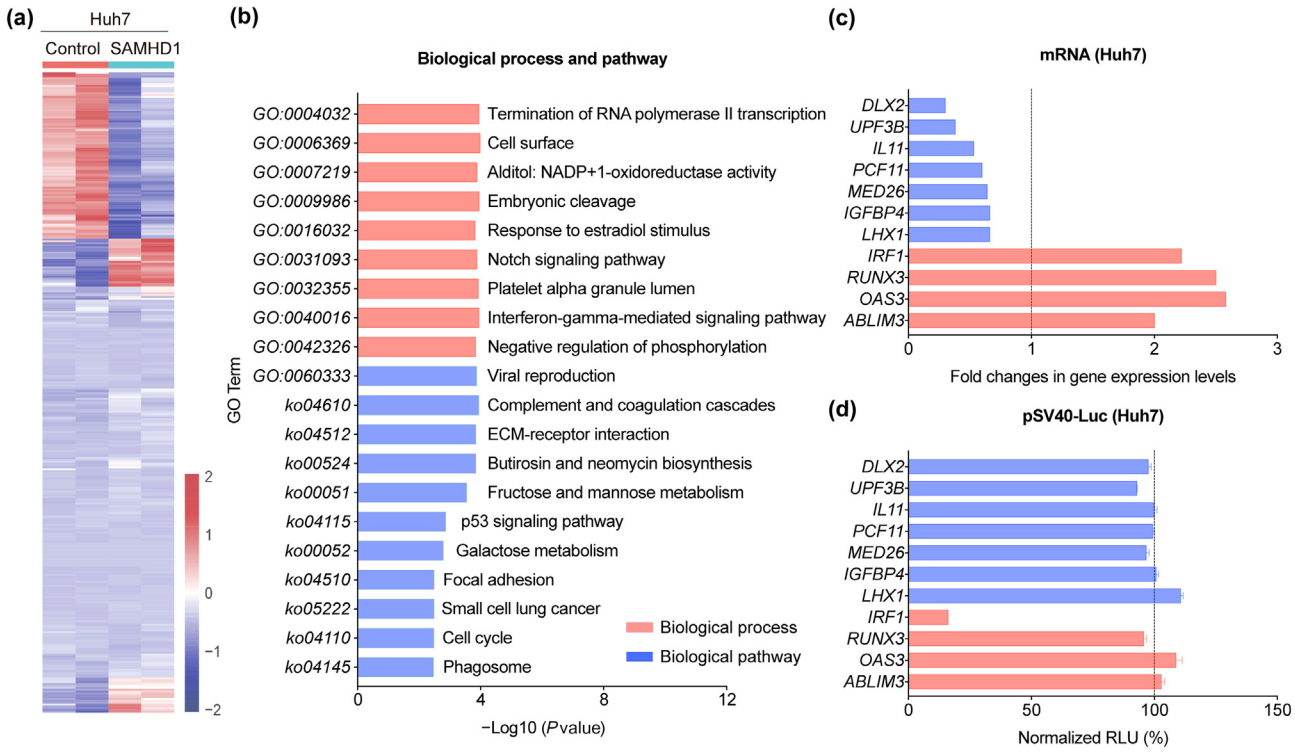


Fig. 3. Differential gene expression in Huh7 cells with and without SAMHD1 overexpression. (a) Heatmap of genes whose expression was upregulated (red) or downregulated (blue) in SAMHD1 overexpressed Huh7 cells against that in control cells. (b) GO enrichment analysis of differentially expressed genes in (a), with biological processes and biological pathways represented by red and blue bars respectively. (c) Fold changes in gene expression level verified using qRT-PCR between SAMHD1 overexpressed Huh7 cells and control cells, with upregulated and downregulated genes represented by red and blue bars respectively. (d) Normalized luciferase activities from Huh7 cells co-transfected with pSV40-Luc (0.2 μg) and the expression plasmid of the indicated gene (0.8 μg) in 24-well plates. The value was calculated against that of Huh7 cells co-transfected with pSV40-Luc and pCMV-FLAG-SAMHD1. Data from three independent experiments were analyzed and presented as mean ± SEM.

of endogenous *IRF1* expression increased the luciferase activity in pSV40-Luc-transfected Huh7 or HEK293 cells (Fig. 4h). Nevertheless, knockdown of IRF1 in both Huh7 and HEK293 did not completely relieve SAMHD1-mediated inhibition of exogenous DNA expression (Fig. 4i, compared to Fig. 1a), suggesting that IRF1 is not the only effector involved in the SAMHD1-mediated restriction of exogenous DNA. Furthermore, SAMHD1 was found to be associated with *IRF1* promoter using ChIP analysis (Fig. 4j).

Consistent with the observed transcriptional repression, overexpression of SAMHD1 or IRF1 resulted in decreased H3K4 trimethylation, H3K9 acetylation and H3K14 acetylation on co-transfected pSV40-Luc (Fig. 4k). Trimethylations of H3K9, H3K27, H3K36 and H3K79, and acetylations of H3K18, H3K27 and H3K56 were not markedly affected (Figs. 4k and S7b (online)).

Same as SAMHD1, IRF1-mediated inhibition of exogenous DNA expression did not depend on DNA circularity or linearity, bacterial sequences in plasmid backbone, promoter or reporter gene, did not affect endogenous chromosomal gene, and did not involve changes to the level of exogenous DNA (Fig. S8a–d online). Also like SAMHD1, binding of IRF1 to exogenous DNA was not detected (Fig. S8e online).

3.4. SAMHD1 interacts with IRF1

According to the functional protein association network prediction (https://string-db.org/, Fig. S9 online), there might be an interaction between IRF1 and SAMHD1. Indeed, endogenous SAMHD1 and IRF1 could be co-immunoprecipitated from both Huh7 and HEK293 cells (Fig. 5a). Moreover, marked overlap of intranuclear localization between endogenous SAMHD1 and IRF1 could be observed in Huh7 and HEK293 cells (Fig. 5b).

SAMHD1 is composed of three regions [30]: the N-terminus (residues 1–109), the HD domain (dNTPase catalytic core domain, residues 110–599) and the C-terminus (residues 600–626) (Fig. 5c). The sterile α-motif (SAM) domain (residues 40–109) and HD domain perform diverse functions [30]. To determine the IRF1-interacting region in SAMHD1, a series of FLAG-tagged truncated SAMHD1 mutants were constructed and transfected into Huh7 and HEK293 cells. Co-immunoprecipitation results then mapped IRF1-binding to the central HD domain of SAMHD1 (Fig. 5d). When cells were co-transfected with pSV40-Luc and FLAG-tagged truncated SAMDH1 mutants, the SAMHD1 mutant without the HD domain lost the suppressive capacity (Fig. 5e).

IRF1 has been reported to induce the production of IFNλ1 to suppress exogenous DNA expression in HEK293 cells [18]. Overexpression of SAMHD1 or IRF1 did increase the expression of *IFNλ1* in both Huh7 and HEK293 cells (Fig. S10a online). However, blocking of secreted IFNλ1 by anti-IFNλ1 or knockdown of *IFNλ1* did not relieve the inhibition of exogenous DNA expression by SAMHD1 or IRF1 overexpression (Fig. S10b online). In addition, baseline expression of *IFNλ1* was not affected by knockdown or knockout of *SAMHD1*, or knockdown of *IRF1* (Fig. S10c online). Furthermore, knockdown of *IFNα1/2*, *IFNβ* or *IFNγ* did not relieve the inhibition by SAMHD1 or IRF1 overexpression (Fig. S10d online). These results suggest that interferon-independent pathways are involved in SAMHD1/IRF1-mediated inhibition of exogenous DNA expression.

3.5. SAMHD1 and IRF1 restrict the expression of exogenous DNA and HBV in mice

We went on to test the inhibitory effect of SAMHD1 on exogenous DNA expression in mice (Fig. 6a). pCMV-GFP and

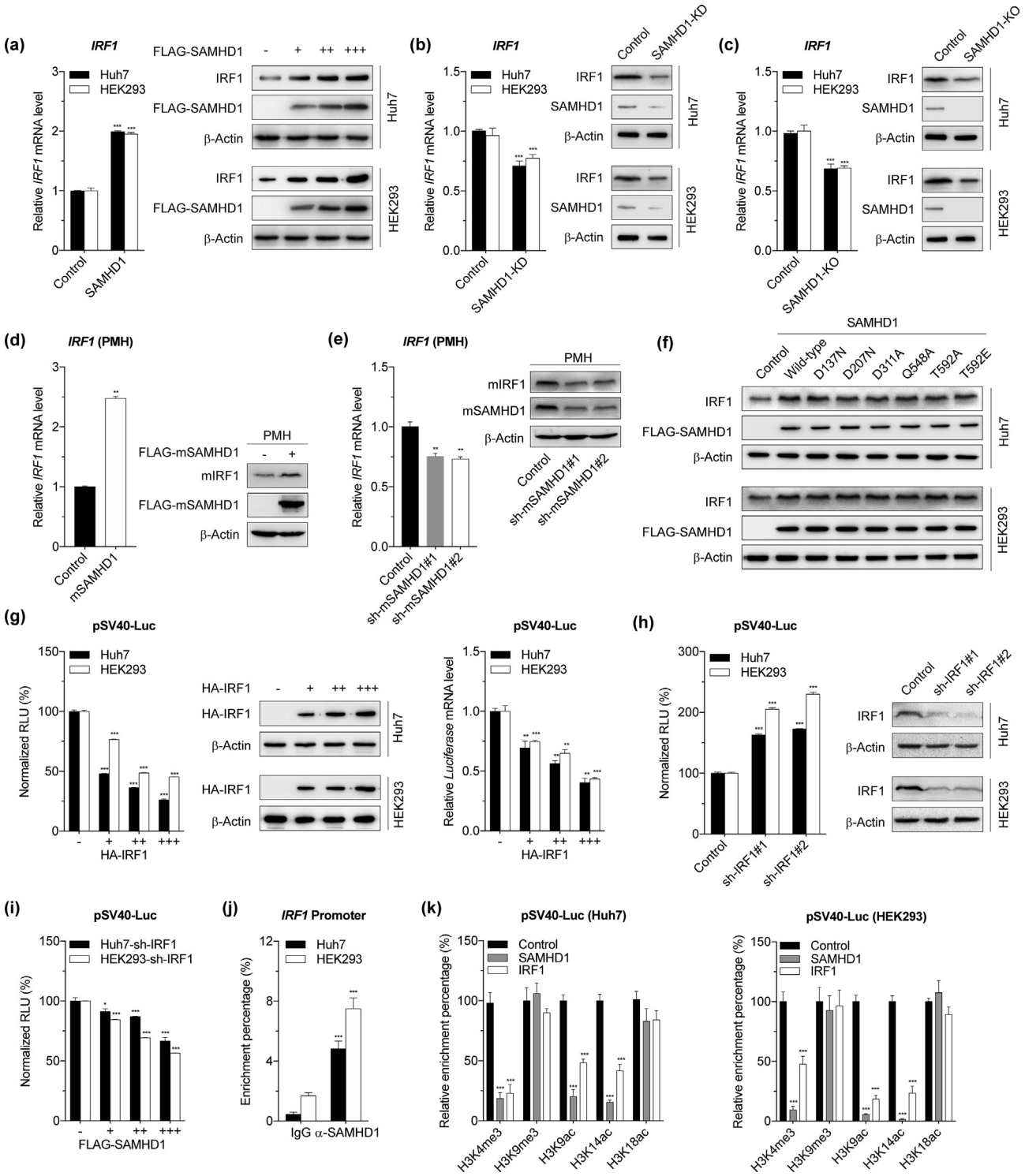


Fig. 4. IRF1 is involved in SAMHD1-mediated restriction of exogenous DNA expression. *IRF1* expression and protein levels in (a) SAMHD1 overexpressed Huh7 and HEK293 cells, (b) *SAMHD1* knockdown (SAMHD1-KD), (c) knockout (SAMHD1-KO) Huh7 and HEK293 cells, (d) lentivirus-transducted SAMHD1 overexpressed primary mouse hepatocytes (PMH), and (e) lentivirus-transducted *SAMHD1* knockdown PMH. Cells were plated in 6-well plates and transfected with pCMV-HA-IRF1 (0.5, 1.0, 2.0 μg) or transducted with indicated lentivirus. The mRNA values from the corresponding cells transfected with pCMV-HA were taken as 1.0. (f) Western blot analysis of IRF1 in Huh7 and HEK293 cells with the overexpression of each *SAMHD1* mutant (2.0 μg) in 6-well plates. (g) Relative luciferase activities (*Left*), HA-IRF levels (*Middle*) and *luciferase* mRNA (*Right*) in Huh7 and HEK293 cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-HA-IRF (0.2, 0.4, 0.8 μg). The luciferase activities and mRNA values from the corresponding cells transfected with pSV40-Luc and pCMV-HA were taken as 100 and 1.0 respectively (the same below). (h) *Left*, relative luciferase activities from Huh7 and HEK293 cells with *IRF1* knockdown. *Right*, western blot verification of IRF1 in IRF1-KD cells. Cells were plated in 24-well plates and transfected with the same amount of pSV40-Luc (0.2 μg). (i) Relative luciferase activities in Huh7 and HEK293 IRF1-KD cells co-transfected with pSV40-Luc (0.2 μg) and pCMV-FLAG-SAMHD1 (0.2, 0.4, 0.8 μg). (j) Chromatin immunoprecipitation (ChIP) analysis of *IRF1* promoter bound SAMHD1. (k) ChIP analysis of selected histone modifications on pSV40-Luc. Precipitated pSV40-Luc DNA was measured using qPCR. me, methylated; ac, acetylated. Data from three independent experiments were analyzed and presented as mean ± SEM. Data are compared to that of the control group and statistical significance calculated using unpaired two-tailed *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

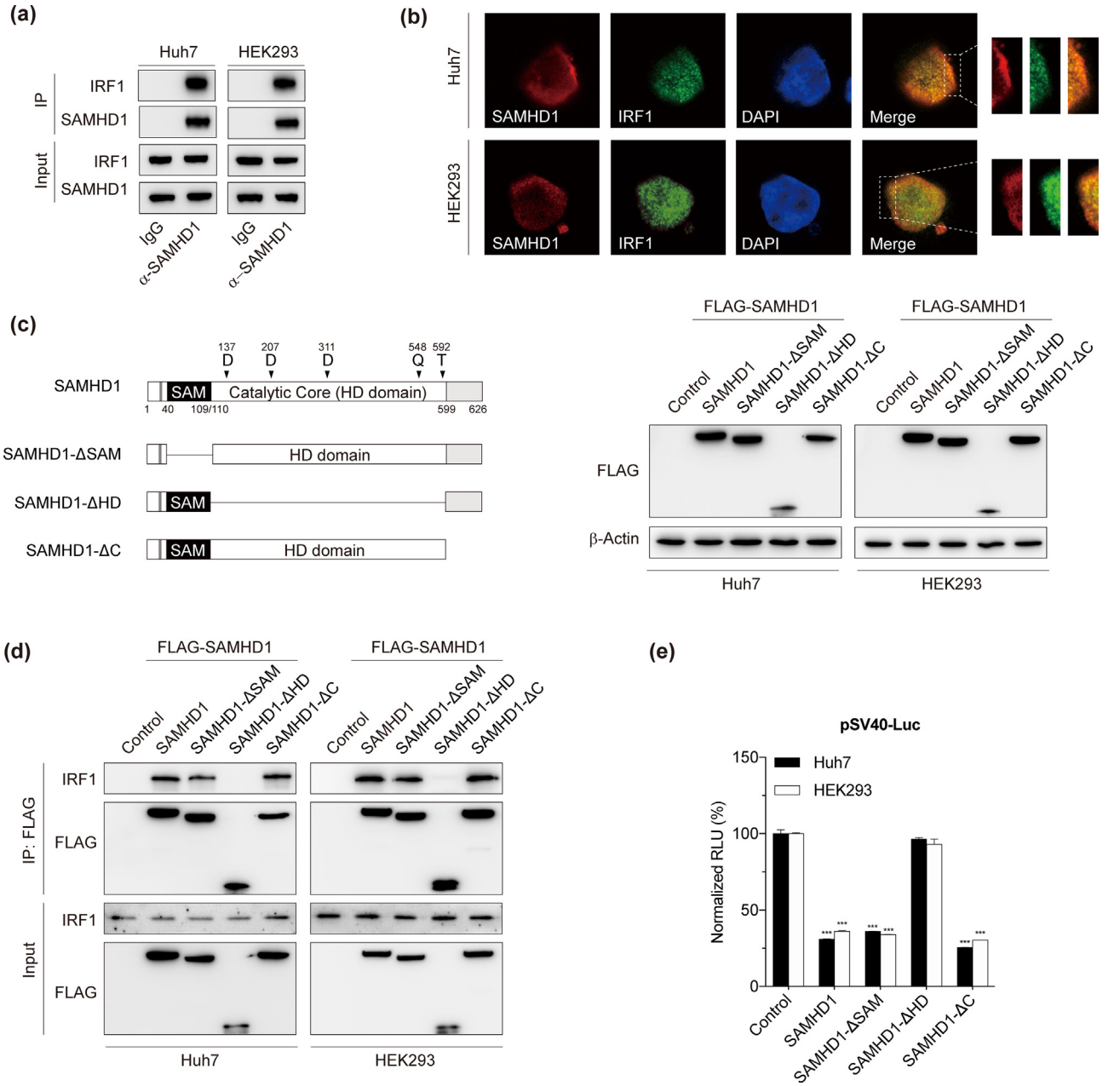


Fig. 5. SAMHD1 interacts with IRF1. (a) Co-immunoprecipitation verification of the interaction of endogenous SAMHD1 and IRF1 in Huh7 and HEK293 cells. (b) Visualization of co-localization (merge) of endogenous SAMHD1 (red) and IRF1 (green) in the nucleus (blue) of Huh7 and HEK293 cells using immunofluorescent confocal microscopy. (c) *Left*, schematic representation of truncated SAMHD1 proteins used in this study. *Right*, western blot verification of FLAG-SAMHD1 mutants in Huh7 and HEK293 cells. (d) Mapping of the IRF1-interacting region in SAMHD1. Co-immunoprecipitation was performed using anti-FLAG mAb and the captured IRF1 was detected by anti-IRF1 mAb. Representative blots and graphs were presented from three independent experiments. (e) Relative luciferase activities in Huh7 and HEK293 cells co-transfected with pSV40-Luc (0.2 μg) and each *SAMHD1* mutant (0.8 μg). Data from three independent experiments were analyzed and presented as mean ± SEM. Data are compared to that of the control group and statistical significance calculated using unpaired two-tailed *t*-test. ***$P < 0.001$.

pCMV-SecNanoLuc that encodes a secreted luciferase (NanoLuc) were used as target DNA. Levels of secreted NanoLuc were reduced by SAMHD1 overexpression in pCMV-SecNanoLuc transfected cells (Fig. S11a online).

Since both mSAMHD1 isoforms were capable of inhibiting exogenous DNA in Hep1-6 cells (Fig. S3 online), we used isoform 1 for *in vivo* experiments. Mice were hydrodynamically injected with pCMV-SecNanoLuc, pCMV-GFP and pCMV-FLAG-mSAMHD1 or pshmSAMHD1 that encodes a *mSAMHD1*-specific shRNA with proven knockdown competence (Fig. S11b online). Compared to mice co-injected with vector control, mice co-injected with pCMV-FLAG-mSAMHD1 showed much lower serum NanoLuc activity (Fig. 6b) and reduced expression of GFP in the liver (Fig. 6c). In contrast, knockdown of *mSAMHD1* by co-transfected pshmSAMHD1 increased both serum NanoLuc activity (Fig. 6b) and hepatic GFP expression (Fig. 6c). The endogenous SAMHD1 signal on the blot was not altered because only a very small percentage of liver cells received HDIed constructs (Fig. 6c). Immunohistochemical staining further proved that exogenous mSAMHD1 inhibited hepatic expression of GFP (Fig. 6d). Similarly, human IRF1 and murine IRF1 (mIRF1) inhibited the expression of NanoLuc *in vitro* (Fig. S11c online) and *in vivo* (Fig. S11d online) and the expression of exogenous GFP in hydrodynamically injected mice (Fig. S11e, f online). These results indicate that SAMHD1/

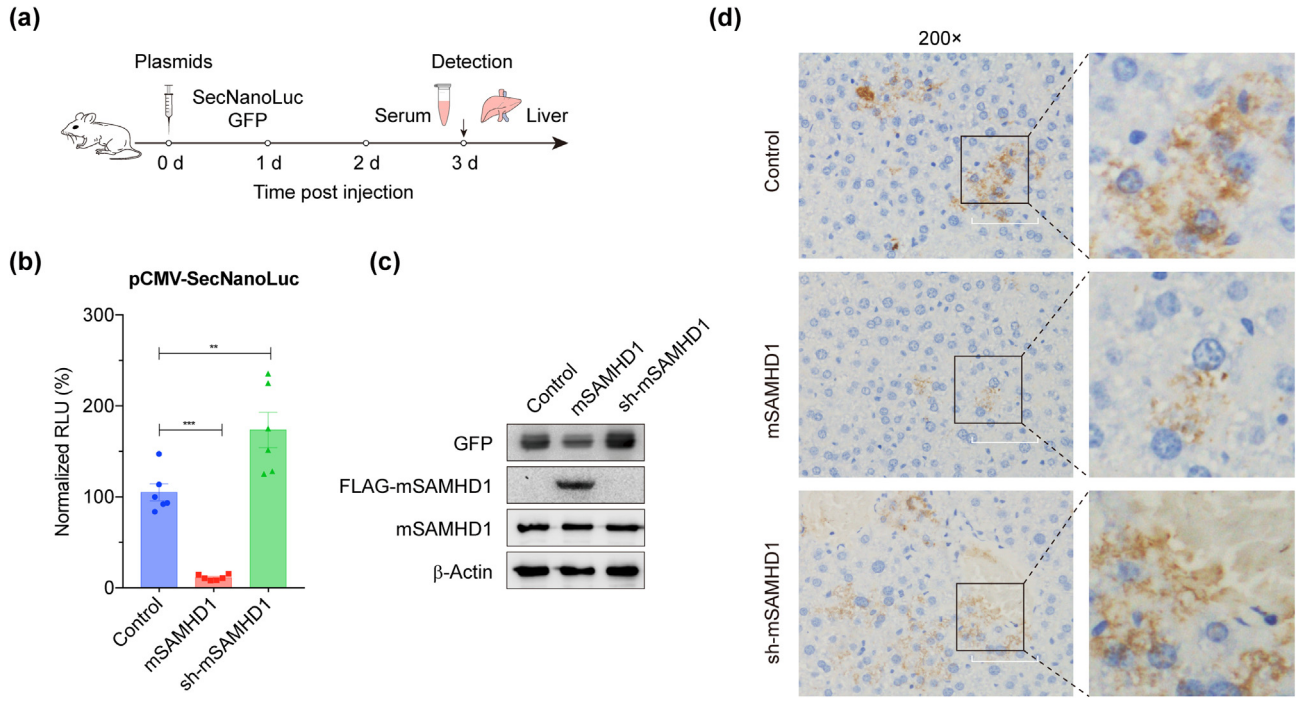


Fig. 6. SAMHD1 restricts the expression of exogenous DNA in mice. (a) Schematic representation of the hydrodynamic injection (HDI) of the indicated plasmids and ensuing assays. (b) Relative serum NanoLuc activities post HDI. Data are compared to that of the control group and statistical significance calculated using unpaired two-tailed *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$. (c, d) Western blot analysis and immunohistochemical staining of GFP in tissue samples from the liver post HDI. Scale bars: 200 μm. Each group contains 6 mice. Representative blots and graphs were presented.

IRF1-mediated inhibition of exogenous DNA expression also occurs *in vivo*, at least in mice.

Next, we tested the inhibitory effect of SAMHD1 on HBV in mice. Overexpression of SAMHD1 or IRF1 inhibited HBV promoter-driven reporter expression in Huh7 cells (Fig. S12a, b online) and HBeAg expression in HBV infected HepG2-NTCP cells (Fig. S12c online). A ChIP experiment was performed in Huh7 cells to examine the binding between SAMHD1 and nuclear HBV DNA that normally includes HBV rcDNA, double-stranded linear DNA and cccDNA. As shown in Fig. S12i (online), no obvious binding of SAMHD1 to HBV DNA was detected. For *in vivo* study, a mouse model of HBV cccDNA was employed [20,21]. In such a system, a recombinant cccDNA (rcccDNA) precursor plasmid (prcccDNA) and pCMV-Cre were hydrodynamically injected through tail vein to transfect mouse liver. prcccDNA was then processed by Cre to form rcccDNA that mimicked wild type HBV cccDNA. Three days after prcccDNA/pCMV-Cre injection, mice were further injected with recombinant lentivirus expressing mSAMHD1 or *mSAMHD1*-specific shRNA. At day 6 post prcccDNA injection, mice were sacrificed and serum and liver samples were taken for analysis (Fig. 7a). Serum HBV antigen (HBsAg/HBeAg) levels (Fig. 7b), intrahepatic HBV DNA (Fig. 7c) and RNA levels (Fig. 7d) were significantly lower in mSAMHD1-overexpressing mice, but higher in *mSAMHD1*-knockdown mice. However, intrahepatic HBV rcccDNA levels were not affected (Fig. S12d online). The mSAMHD1 mutant (D169N) with defective dNTPase (analogous to D137N in human SAMHD1) also suppressed serum HBV antigen, DNA and RNA levels, although to a slightly lesser extent compared to wild type mSAMHD1 (Fig. 7e–g). Similar results were obtained with mIRF1 overexpression in mice (Fig. S12e–h online).

4. Discussion

In this study, we demonstrate for the first time that SAMHD1 has a general inhibitory effect on the expression of exogenous DNA expression. SAMHD1 is able to inhibit gene expression from both cyclic and linear DNA forms and does not discriminate different promoters or genes (Fig. 1d, e). In addition, its activity of inhibiting exogenous gene expression is effective in various cell lines and in mice.

The mechanism of SAMHD1-mediated general inhibition of exogenous DNA expression is different from SAMHD1-mediated restriction of HIV-1 and certain DNA viruses reported previously. For HIV-1, SAMHD1 represses the reverse transcription of HIV-1 by depleting dNTP pool and degrading RNA via its intrinsic dNTPase and RNase activities respectively [2,4,28]. Besides, the phosphorylation level of T592 of SAMHD1 has also been linked to the restriction of HIV-1 [7,31]. For SAMHD1-mediated inhibition of exogenous DNA expression, however, the enzymatic activities as well as the T592 phosphorylation are not required (Fig. S5 online). In addition, SAMHD1 inhibits transcription of mRNA from exogenous DNA, manifesting as reduced mRNA production and decrease in the levels of histone activation markers such as H3K4me3, H3K9ac and H3K14ac associated with target DNA (Fig. 2b, 4k). How SAMHD1 carries out this function has been partially explained by this study. SAMHD1 does not bind to exogenous DNA (Fig. 2e, f), although it resides primarily in the nucleus. IRF1 may play a key role in SAMHD1-mediated inhibition of exogenous DNA expression. Several lines of evidence support this view. First, the effects on exogenous DNA expression by overexpression and knockdown of *IRF1* are similar to those of *SAMHD1*. Second, SAMHD1 associates with the promoter of *IRF1* and stimulates its expression. Third, SAMHD1 also directly interacts with IRF1 and such interaction correlates with inhibition of exogenous DNA transcription (Fig. 5d, e). Nevertheless, how IRF1 eventually suppresses exogenous gene expression remains to be addressed.

IRF1 has been reported to induce the production of IFNλ1 to suppress exogenous DNA expression in HEK293 cells [18]. Indeed, overexpression of either SAMHD1 or IRF1 could stimulate *IFNλ1* expression. However, our results in HEK293 and Huh7 cells

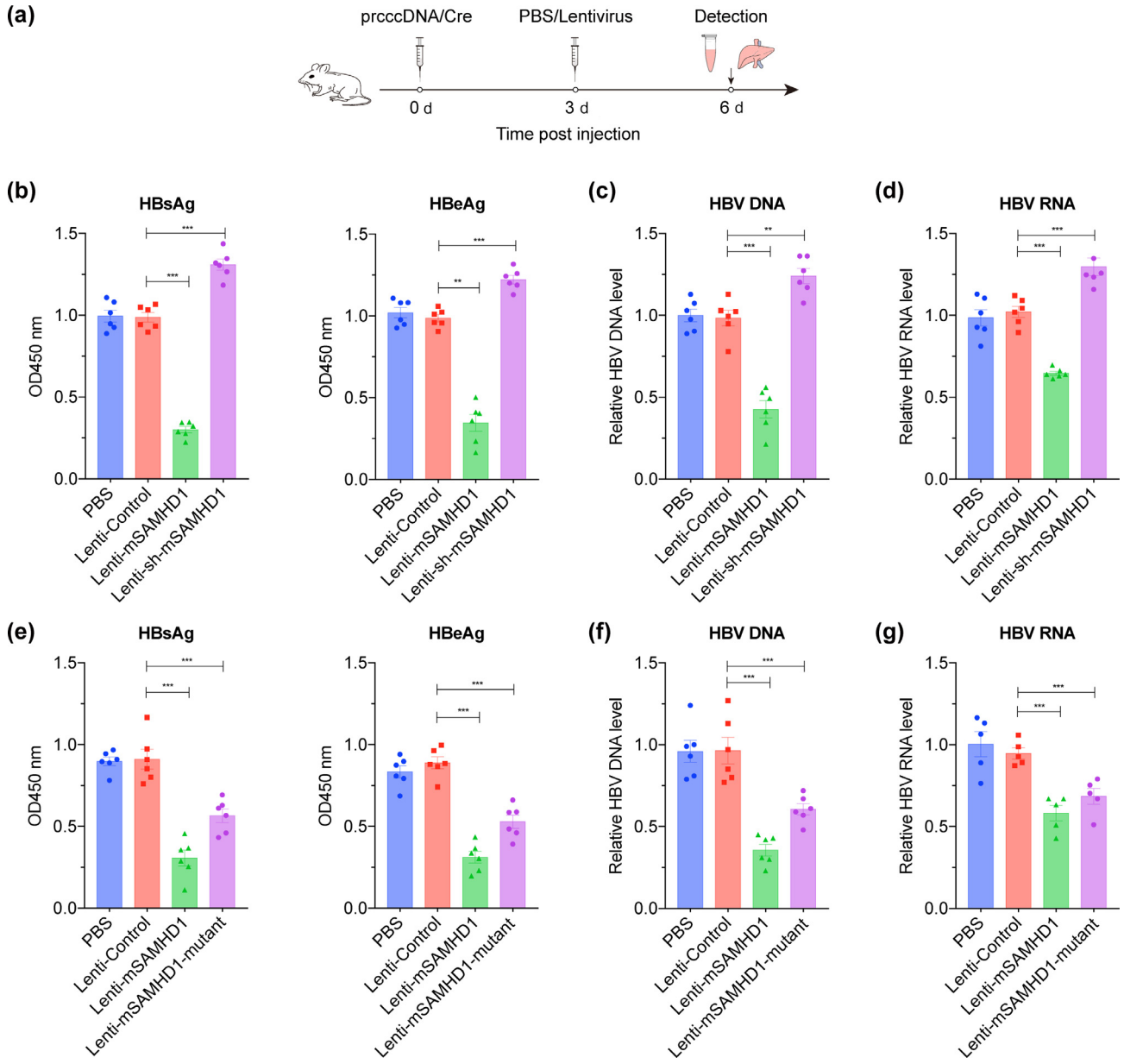


Fig. 7. SAMHD1 restricts HBV *in vitro* and *in vivo*. Schematic representation of the hydrodynamic injection (HDI) of the indicated treatments and ensuing assays. (b, e) Evaluation of HBV antigens (HBsAg/HBsAg) post lentivirus transduction. Serum HBV DNA (c, f) and RNA levels in liver tissues (d, g) determined by qPCR post the transduction by the indicated lentivirus. Each group contains 6 mice. Data were analyzed and mean ± SEM was presented. Data are compared to that of the control group or PBS treated group and statistical significance calculated using unpaired two-tailed *t*-test, $^{**}P < 0.01$ or $^{***}P < 0.001$.

showed that knockdown of endogenous *IFNλ1* expression did not significantly relieve SAMHD1- or IRF1-mediated inhibition of exogenous DNA expression, nor did knockdown of endogenous *IFNα1/2*, *IFNβ* or *IFNγ* (Fig. S10d online). Therefore, we speculate that SAMHD1/IRF1-mediated inhibition of exogenous DNA expression involves IFN-independent pathways, which remains to be elucidated. Moreover, SAMHD1 and IRF1 do not affect the expression of endogenous genes (Figs. 1f and S8c (online)). How SAMHD1/IRF1 discriminates the transcription of exogenous DNA and chromosomal DNA awaits exploration. The associated transcriptional factors and DNA modifications might be different between transfected DNA and genome-integrated DNA.

We verified SAMHD1-mediated restriction of exogenous DNA and HBV cccDNA mimic in mice. HBV is a liver-tropic DNA virus that can persistently infect human hepatocytes, which may lead to chronic hepatitis, liver fibrosis, cirrhosis and HCC [32]. The relaxed circular DNA genome of HBV is converted to cccDNA in the nucleus of the infected hepatocyte. cccDNA is in effect a small plasmid. It serves as the template for viral gene transcription and is the primary reason for HBV persistence [33]. Mice are not susceptible to HBV infection but mouse hepatocytes efficiently support viral replication from introduced HBV replicons. However, cccDNA formation is deficient in mouse hepatocytes [34,35]. To circumvent this obstacle, we previously established a recombinant cccDNA (rcccDNA) mouse model [20,21]. With the rcccDNA model, we demonstrate a strong SAMHD1-mediated inhibition of gene expression from rcccDNA and progeny virus production (Fig. 7). Similar to SAMHD1-mediated restriction of exogenous DNA in cells, SAMHD1-mediated restriction of rcccDNA does not reduce the quantity of rcccDNA, which is consistent with previous studies using HBV infection cell models [36]. However, it is noteworthy that the dNTPase activity of SAMHD1, which has been implicated

in SAMHD1-mediated restriction of HBV replication in cell models [5,36], is dispensable for SAMHD1-mediated restriction of rcccDNA (Fig. 7). Recently, SAMHD1 has been shown to promote HBV cccDNA formation in HepG2-NTCP cells [9]. Since data presented here established transcriptional repression of cccDNA by SAMHD1, how such apparently conflicting functions of SAMHD1 were reconciled in natural infections remain to be addressed. Nevertheless, many double-stranded DNA viruses, such as polyomaviruses, herpesviruses and papillomaviruses, also rely on nuclear episomal DNA for viral gene expression and SAMHD1-mediated restriction of these DNA viruses is a possibility that warrants investigation.

When cells encounter bacterial, viral and ingested exogenous DNA, pattern-recognition receptors (PRRs) recognize the invading DNA and activate the innate immune response [37]. A number of PRRs for exogenous DNA have been documented, such as IFI16, Ku70, and cGAS-STING. Among them, cGAS-STING is the most well-characterized. cGAS serves as a cytosolic DNA sensor, which is activated by double-stranded DNA (dsDNA). The activated cGAS produces second messenger cGAMP, which activates the adaptor STING, leading to type I interferon expression [38,39]. A recent work has observed the deficiency STING expression in mouse and human hepatocytes [40]. In human HCC cell lines, STING expression is undetectable in Huh7 cells but is present in HepG2 hepatoma cells [41]. In STING-deficient Huh7 cells (Fig. S13a online), no induction of SAMHD1, IRF1 or interferons was detected upon DNA transfection (Fig. S13b, c online). IRF1 and IFNλ1 expression was reported to be induced by transfected plasmids in HEK293 cells but not in HEK293T cells [42]. However, we were unable to detect the induction of SAMHD1, IRF1 or IFNλ1 by transfected plasmids in HEK293 cells (Fig. S13d, e online). Also, the distributions of SAMHD1 and IRF1 in the nucleus and cytoplasm were not changed upon DNA transfection in both HEK293 and Huh7 cells (Fig. S13f online). Based on these observations, it can be concluded that the expression of endogenous SAMHD1 is not induced by exogenous DNA, whereas the fact that SAMHD1 does not bind target DNA (Fig. 2e, f) also largely rules out it as a DNA sensor. However, there remains the possibility that SAMHD1 protein modification, rather than its expression level, was affected by exogenous DNA, for instance via signaling through as yet unidentified DNA sensor(s), which modulates the activity of SAMHD1.

SAMHD1's restriction of exogenous DNA expression may act as a constitutive defense against exogenous DNA. Given that STING expression is absent in hepatocytes, SAMHD1-mediated restriction of exogenous DNA expression may be especially important in liver, an organ that is exposed to a large amount of pathogens and exogenous DNA on a routine basis [43]. In this context, the fact that SAMHD1-mediated restriction of exogenous DNA expression does not involve interferon may be additionally beneficial and protects the host from frequent stimulation by interferons. In fact, SAMHD1 has been reported to prevent type I interferon induction [1,10,11]. On the other hand, SAMHD1 expression can be stimulated by interferons [5,44], which might reflect a complexed crosstalk between interferon-dependent and -independent pathways that restrict exogenous DNA.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Author contributions

Youhua Xie and Jing Liu drafted the manuscript, designed the project involving the experiments. The majority of the experiments and data analysis were performed by Chenjian Gu and Lijun Ming. The other authors participated in the data analyses and manuscript revision. All authors discussed the results, commented on the manuscript, and approved the manuscript finally.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://doi.org/10.1016/j.scib.2019.12.028.

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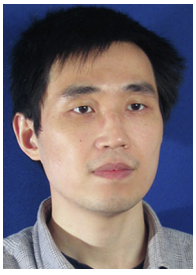

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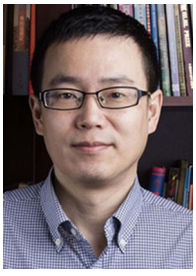

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