Supplementary Information for

Hepatitis B Virus X protein Recruits Methyltransferases to Affect Co-transcriptional N6-Methyladenosine Modification of Viral/Host RNAs

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Supplementary Material and Methods.

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Material and Methods

RNA-protein UV-crosslinking assay

Before harvesting, platted cells were washed with ice-cold PBS and then, exposed with 245 nm UV for 250 mJ/cm. Ice-cold PBS was treated into a culture plate and cells were harvested. Cell pellets were lysed with SDS-lysis buffer (0.5% SDS, 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1mM DTT, 150 mM NaCl) supplemented with a protease inhibitor and RNase inhibitor (Thermo Fisher Scientific). Purified lysates were immunoprecipitated with Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) or YTHDC2 antibodies-protein G bead complex for 2 h on rotator at 4ºC. The bead complex was washed with NP-40 lysis buffer 5 times. After the wash step, beads were resuspended with NP-40 lysis buffer. The immunoprecipitated lysates were extracted from half of the bead. The other half of the bead was treated with proteinase K for 90 min at 37ºC. Immunoprecipitated RNA was extracted using Trizol (Invitrogen).

S1 Table. Primers of this article

Primers	Sequence
HBV pgRNA F	5'-CCTGCACCACCAACTGCTTA-3'
HBV pgRNA R	5'-CATGAGTCCTTCCACGATACCA-3'
HBV transcripts F	5'-GTCAACGACCGACCTTG-3'
HBV transcripts R	5'-TGATTAGGCAGAGGTGAAAAAG-3'
GAPDH F	5'-CCTGCACCACCAACTGCTTA-3'
GAPDH R	5'-CATGAGTCCTTCCACGATACCA-3'
PTEN F	5'- CCGTTACCTGTGTGTGGTGATATC-3'
PTEN R	5'-GAATGTATTTACCCAAAAGTGAAACATT-3'
ChIP cccDNA F	5'-CTCCCCGTCTGTGCCTTCT-3'
ChIP cccDNA R	5'- GCCCCAAAGCCACCCAAG-3'
ChIP PTEN 3'-UTR F	5'-ACATCCACAGGGTTTTGACAC-3'
ChIP PTEN 3'-UTR R	5'-GCCACAAGTGCAAAGGGGTA-3'
CREBBP F	5'-ACAAGCGAAACCAACAAACC-3'
CREBBP R	5'-CCTGTGCCAACAGAACCAA-3'
HPRT1 F	5'-TGACACTGGCAAAACAATGCA-3'
HPRT1 R	5'-GGTCCTTTTCACCAGCAAGCT-3'

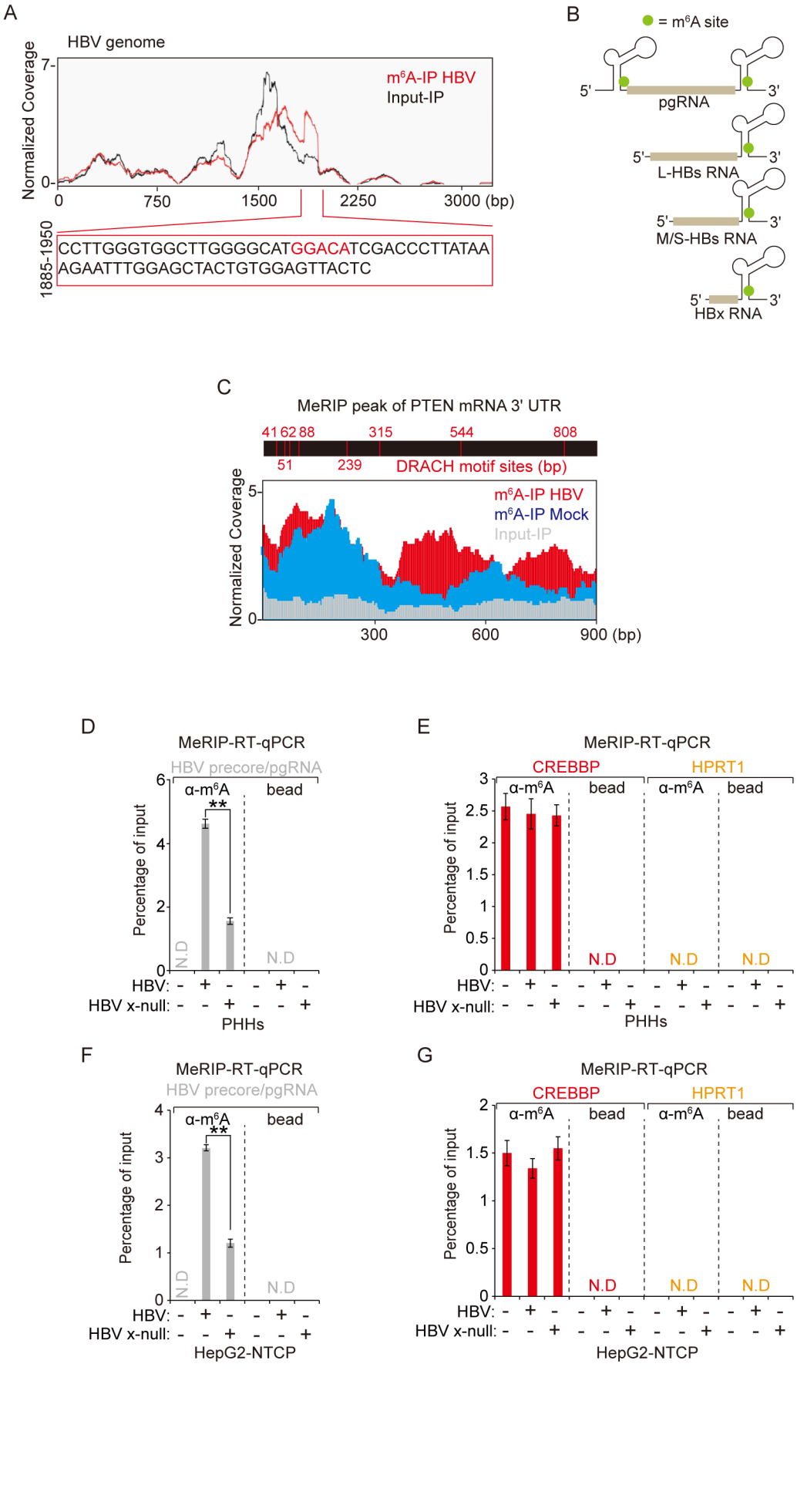
A
HBV genome
Normalized Coverage
m6A-IP HBV
Input-IP
0
750
1500
2250
3000 (bp)
1885-1950
CCTTGGGTGGCTTGGGGCATGGACATCGACCCTTATAA
AGAATTTGGAGCTACTGTGGAGTTACTC
B
= m6A site
5'
3'
pgRNA
L-HBs RNA
M/S-HBs RNA
HBx RNA
C
MeRIP peak of PTEN mRNA 3' UTR
41 62 88
315
544
808
51
239
DRACH motif sites (bp)
m6A-IP HBV
m6A-IP Mock
Input-IP
Normalized Coverage
300
600
900 (bp)
D
MeRIP-RT-qPCR
HBV precore/pgRNA
α-m6A
bead
Percentage of input
N.D
HBV:
HBV x-null:
PHHs
E
MeRIP-RT-qPCR
CREBBP
HPRT1
α-m6A
bead
Percentage of input
N.D
HBV:
HBV x-null:
PHHs
F
MeRIP-RT-qPCR
HBV precore/pgRNA
α-m6A
bead
Percentage of input
N.D
HBV:
HBV x-null:
HepG2-NTCP
G
MeRIP-RT-qPCR
CREBBP
HPRT1
α-m6A
bead
Percentage of input
N.D
HBV:
HBV x-null:
HepG2-NTCP

Fig. S1. Related to Figure 1. (A) Analysis of m^{6}A sites of HBV transcripts by MeRIP-seq of polyA-RNA isolated from HBV transfected cells. The red read coverage is for MeRIP-seq and black read coverage is for input RNA-seq. One m^{6}A site was identified after normalizing for coverage. The Inset presents nt 1885–1950 of the HBV genome, with the m^{6}A site highlighted by red text. (B) Schematic showing the position of the m^{6}A site (A1907), indicated by the green filled circle in all of the HBV transcripts. Note that it is present at both the 5′ and 3′ ends of pgRNA and only at the 3' ends of the other HBV transcripts. (C) m^{6}A peaks of PTEN mRNA 3'-UTR were analyzed. Read coverage, aligned to PTEN mRNA 3′-UTR, is in blue for MeRIP-seq from control cells, is in red for MeRIP-seq from HBV transfected cells, or is in gray for RNA-seq. PTEN mRNA 3′-UTR contains eight DRACH motif sites, indicated by the red bar within the black bar that depicts a linear representation of PTEN mRNA 3'-UTR. (D and E) PHHs were infected with HBV or HBV x-null particles. After 7 days, cells were harvested and m^{6}A methylated HBV pgRNA level was analyzed by MeRIP-qRT-PCR (D). *CREBBP* and *HPRT1* were analyzed as positive and negative controls, respectively (E). (F and G) HepG2-NTCP cells were infected with HBV or HBV x-null particles. m^{6}A methylated HBV pgRNA (F), *CREBBP*, or *HPRT1* (G) levels were quantified by MeRIP-qRT-PCR. In (D-G), data are mean ± s.d. $^{**}P < 0.01$; by unpaired one-tailed Student's *t*-test.

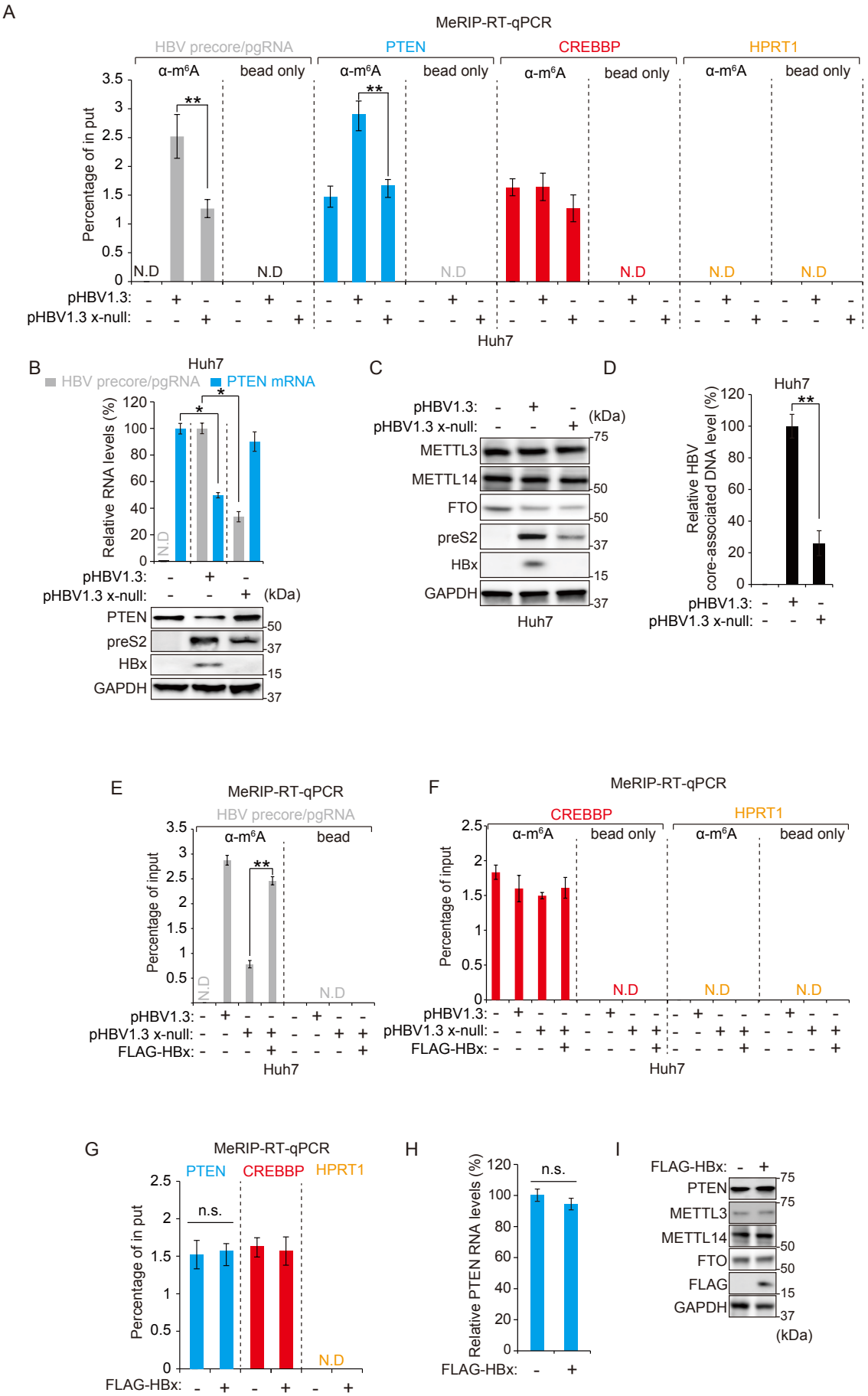
A
MeRIP-RT-qPCR
HBV precore/pgRNA
PTEN
CREBBP
HPRT1
α-m⁶A
bead only
Percentage of in put
N.D
pHBV1.3:
pHBV1.3 x-null:
Huh7
B
Huh7
HBV precore/pgRNA
PTEN mRNA
Relative RNA levels (%)
pHBV1.3:
pHBV1.3 x-null:
(kDa)
PTEN
preS2
HBx
GAPDH
C
pHBV1.3:
pHBV1.3 x-null:
(kDa)
METTL3
METTL14
FTO
preS2
HBx
GAPDH
Huh7
D
Huh7
Relative HBV core-associated DNA level (%)
pHBV1.3:
pHBV1.3 x-null:
E
MeRIP-RT-qPCR
HBV precore/pgRNA
α-m⁶A
bead
Percentage of input
pHBV1.3:
pHBV1.3 x-null:
FLAG-HBx:
Huh7
F
MeRIP-RT-qPCR
CREBBP
HPRT1
α-m⁶A
bead only
Percentage of input
pHBV1.3:
pHBV1.3 x-null:
FLAG-HBx:
Huh7
G
MeRIP-RT-qPCR
PTEN
CREBBP
HPRT1
Percentage of in put
n.s.
N.D
FLAG-HBx:
H
Relative PTEN RNA levels (%)
n.s.
FLAG-HBx:
I
FLAG-HBx:
PTEN
METTL3
METTL14
FTO
FLAG
GAPDH
(kDa)

Fig. S2. Related to Figure 1. (A-D) Huh7 cells were transfected with pHBV 1.3 or pHBV 1.3 x-null. After 72 h, RNA and lysates were extracted to assess m^6A methylated RNAs by MeRIP-qRT-PCR (A). HBV pgRNA and PTEN mRNA levels were analyzed by qRT-PCR (B) and the indicated proteins were assess by immunoblotting (B and C). HBV core-associated DNA was analyzed from pHBV 1.3 or pHBV 1.3 x-null transfected Huh7 cells by qPCR (D). In all panels, data are mean ± s.d. * $P < 0.05$; ** $P < 0.01$; by unpaired one-tailed Student's *t*-test. (E and F) Huh7 cells were transfected with pHBV 1.3, pHBV 1.3 x-null, or co-transfected with pHBV 1.3 x-null and pcDNA3.1 FLAG-HBx. After 72 h, cells were harvested to assess m^6A methylated pgRNA (E), *CREBBP*, or *HPRT1* (F) by MeRIP-qRT-PCR. (G-I) Huh7 cells were transfected with pcDNA 3.1 FLAG-HBx. After 72 h, cells were harvested to assess m^6A-methylated PTEN mRNA (G), PTEN mRNA levels (H), or immunoblotting for the indicated proteins (I). In all panels, data are mean ± s.d. * $P < 0.05$; ** $P < 0.01$; n.s., non-significant; by unpaired one-tailed Student's *t*-test.

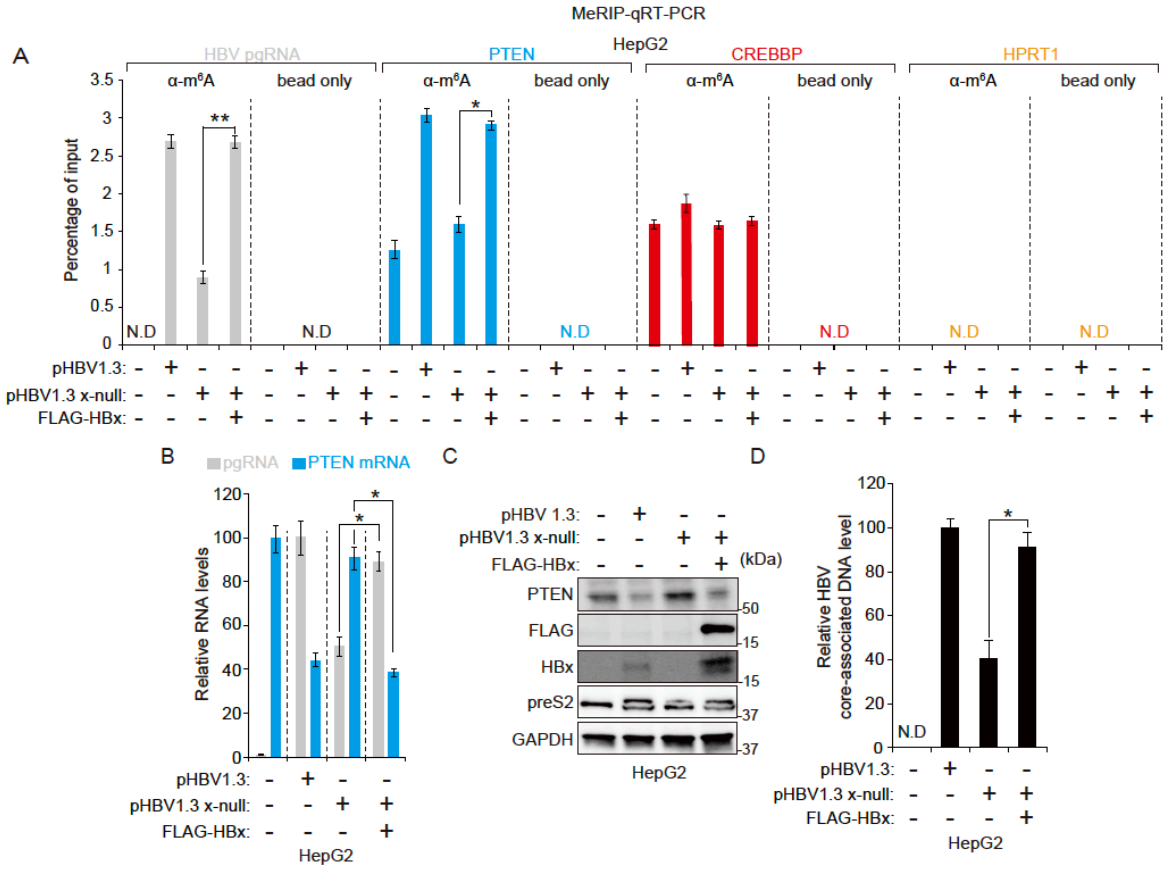

Fig. S3. Related to Figure 1. (A-C) HepG2 cells were transfected with pHBV 1.3, pHBV 1.3 x-null, or co-transfected with pHBV 1.3 x-null and pcDNA3.1 FLAG-HBx. After 72 h, cells were harvested to assess m^{6}A methylation of the indicated RNAs by MeRIP-qRT-PCR (A). HBV pgRNA and PTEN mRNA levels were analyzed by qRT-PCR (B). The indicated protein expressions were assessed by immunoblotting (C). (D) HBV core-associated DNA was extracted from HepG2 cells transfected with pHBV 1.3, pHBV 1.3 x-null, or co-transfected with pHBV 1.3 x-null and pcDNA3.1 FLAG-HBx and analyzed by qPCR. In all panels, data are mean ± s.d. * $P < 0.05$; ** $P < 0.01$; by unpaired one-tailed Student's *t*-test.

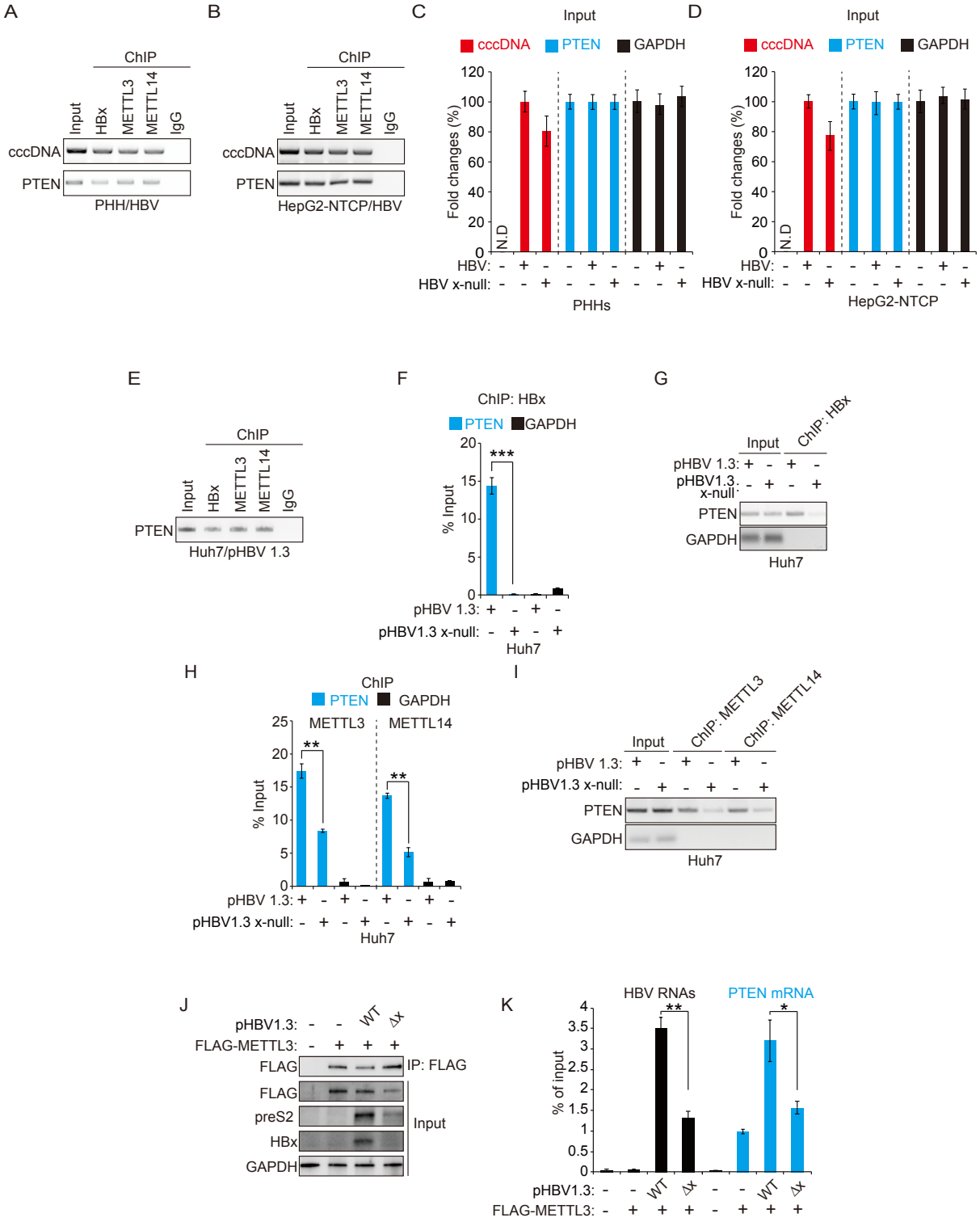

Fig. S4. Related to Figure 2. (A and B) ChIP assay was performed as described in Fig. 2, with anti-HBx, anti-METTL3, anti-METTL14, or IgG antibody. IgG was used as a negative control. (C and D) HBV cccDNA, PTEN, and GAPDH input levels from Fig. 2 were analyzed by qPCR. (E-I), Huh7 cells were transfected with pHBV 1.3 or x-null and incubated for 72 h. Before

harvesting, cells were treated with formaldehyde for crosslinking the protein and the DNA. ChIP assay was performed with anti-HBx, anti-METTL3, anti-METTL14, or IgG antibody. IgG was used as a negative control (E). The HBx associated PTEN 3′-UTR DNA or GAPDH DNA levels were analyzed by ChIP assay (F and G). The levels of PTEN 3'-UTR DNA or GAPDH DNA associated with m^6A methyltransferases were assessed by ChIP assay (H and I). (J and K) The pHBV1.3 WT or x-null plasmid was treated into FLAG-METTL3 expressed Huh7 cells for 48 h. Before harvesting, cells were exposed with 245 nm ultraviolet for cross-linking of the protein-RNA complex. Lysates were immunoprecipitated with anti-FLAG (METTL3), and the immunoprecipitated lysates were used to assess immunoblotting for the indicated proteins (J) and also used to extract RNA followed by RT-PCR to show enriched PTEN mRNA and HBV RNA (K). In all panels, data are mean ± s.d. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; by unpaired one-tailed Student's *t*-test.

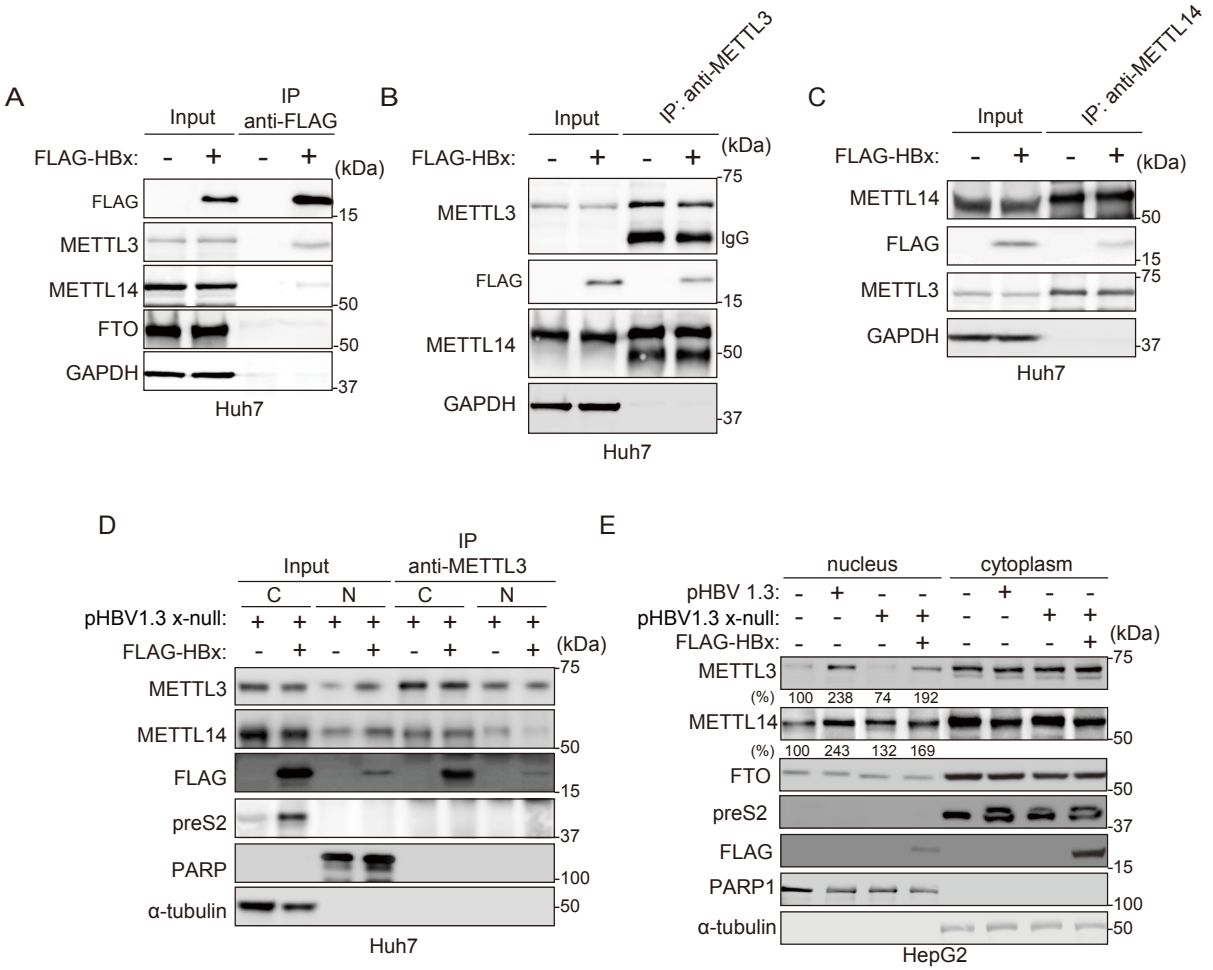

Fig. S5. Related to Figure 4. (A-C) Huh7 cells were transfected with pcDNA 3.1 FLAG-HBx. Lysates were immunoprecipitated using anti-FLAG (A), anti-METTL3 (B), or anti-METTL14 (C) antibodies, followed by immunoblotting for the indicated proteins. (D) HepG2 cells were transfected with pHBV 1.3, pHBV 1.3 x-null, or co-transfected with pHBV 1.3 x-null and pcDNA3.1 FLAG-HBx. After 72 h, cytoplasm and nuclear lysates were isolated to assess the indicated proteins by immunoblotting. e, Huh7 cells, which were transfected with pHBV 1.3 x-null, were treated with control or pcDNA 3.1 FLAG-HBx. Cytoplasmic and nuclear lysates were immunoprecipitated with anti-METTL3, followed by immunoblotting for the indicated proteins.

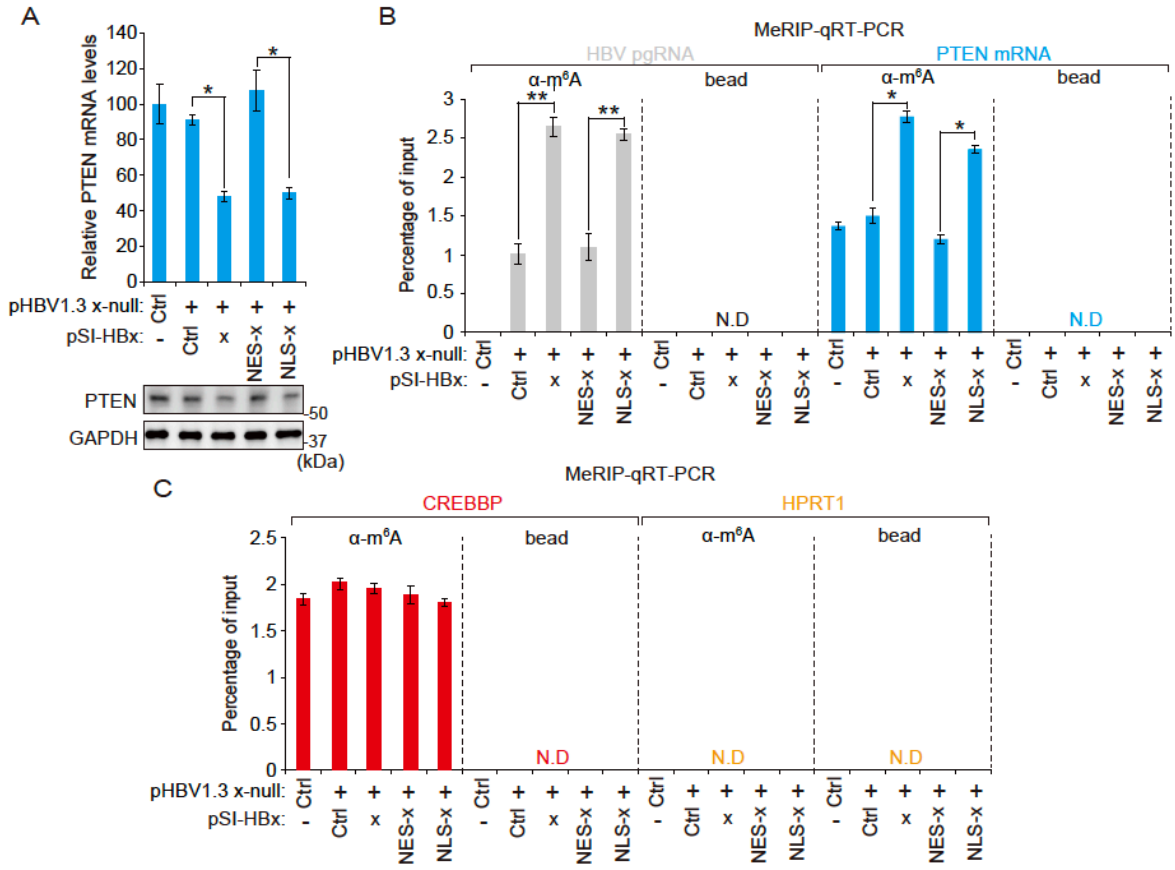

Fig. S6. Related to Figure 5. (A-C) Huh7 cells were transfected with HBV 1.3 x-null together with pSI-x, pSI-NES-x, or pSI-NLS-x for 48 h, before quantification of PTEN mRNA and protein (A) or MeRIP-qRT-PCR (B and C). In all panels, data are mean ± s.d. * $P < 0.05$; ** $P < 0.01$; by unpaired one-tailed Student's *t*-test.

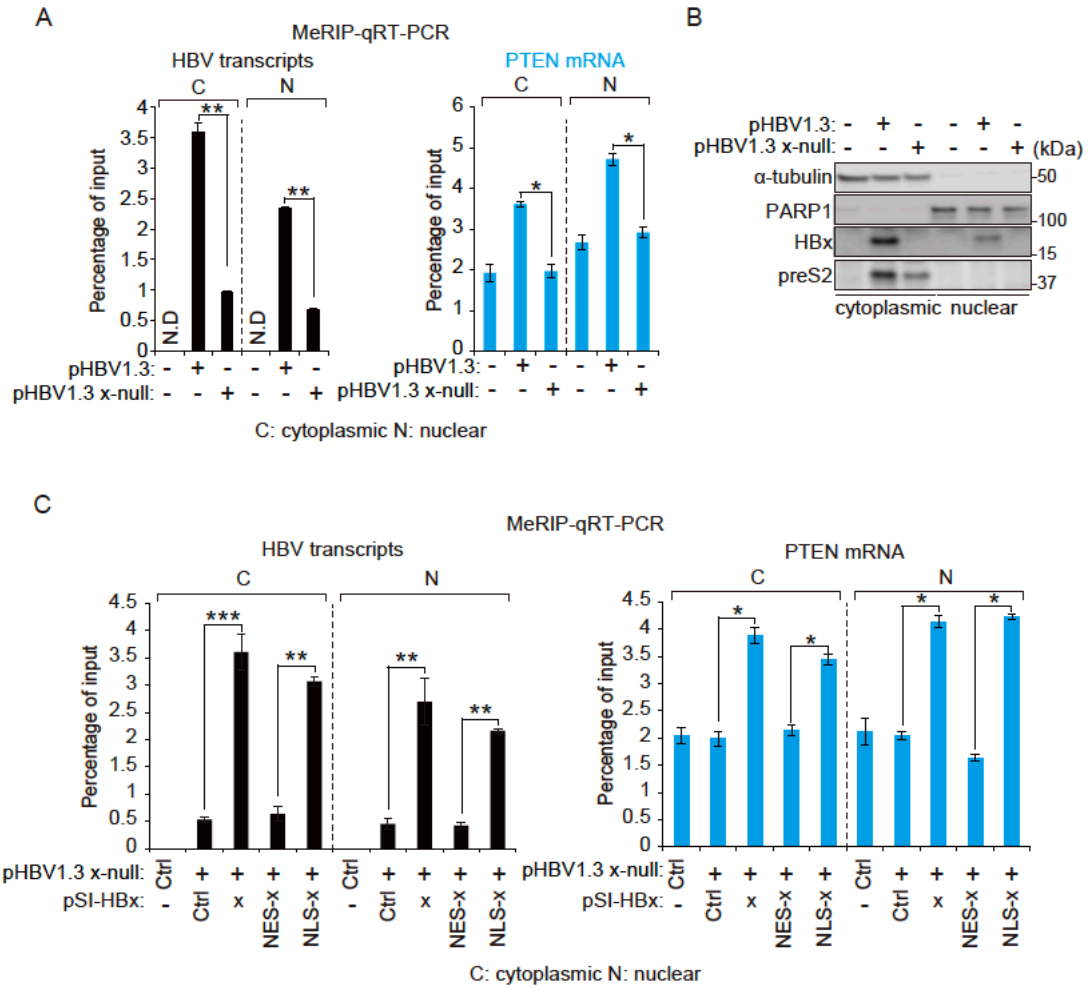

Fig. S7. Related to Figure 5. (A and B) Huh7 cells were transfected with pHBV 1.3 or pHBV 1.3 x-null. RNA was extracted from each cytoplasmic and nuclear fractions to assess m^{6}A modified HBV pgRNA and PTEN (A) mRNA by MeRIP-qRT-PCR. The indicated proteins were analyzed from cytoplasmic and nuclear lysates by immunoblotting (B). (C) Analysis of m^{6}A methylated viral transcripts and PTEN mRNA levels in RNA sample from Fig. 5f. In all panels, data are mean ± s.d. *P < 0.05; **P < 0.01; ***P < 0.001; by unpaired one-tailed Student's t-test.

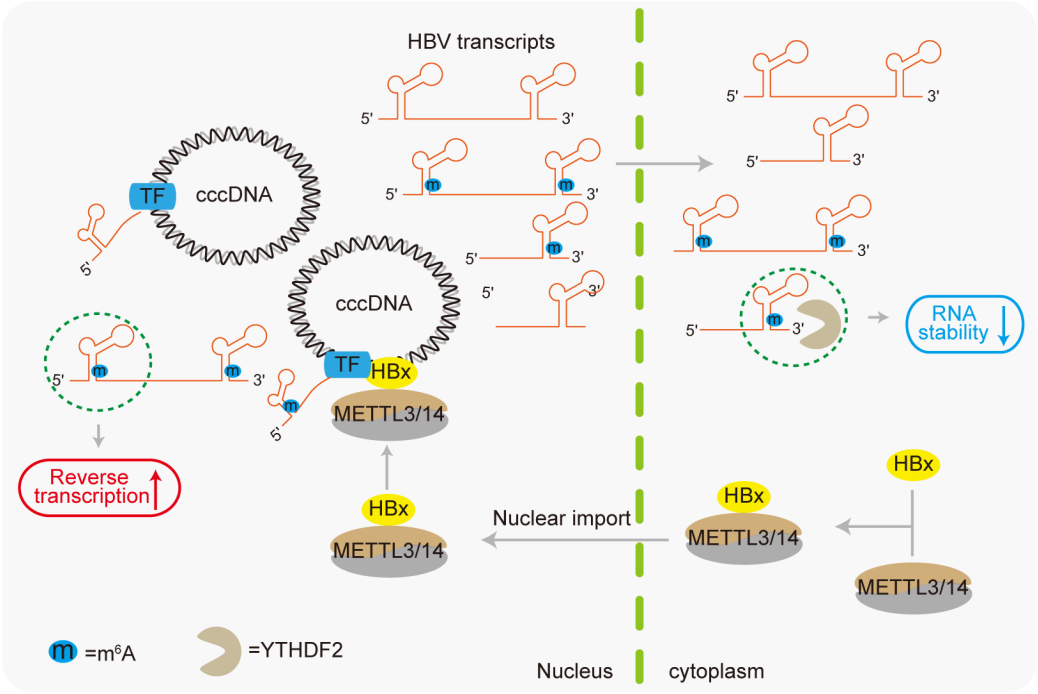

Fig. S8. Model of HBx-mediated regulation of m^{6}A modification. HBx protein binds and enhances METLL3/14 complex nuclear import, and recruits m^{6}A machinery onto HBV cccDNA and PTEN chromosomal locus to promote m^{6}A modification co-transcriptionally. m^{6}A modification of 5'-epsilon loop of HBV pgRNA increases reverse transcription which occurs in the encapsidated core particles, while m^{6}A modification of 3'-epsilon loop of HBV transcripts reduces RNA stability in the cytoplasm via interaction with YTHDF2.