

LUC7L3/CROP inhibits replication of hepatitis B virus via suppressing enhancer II/basal core promoter activity

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2 **enhancer II/basal core promoter activity**

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23

1 **Abstract**

2 The core promoter of hepatitis B virus (HBV) genome is a critical region
3 for transcriptional initiation of 3.5 kb, pregenome and precore RNAs and for the
4 viral replication. Although a number of host-cell factors that potentially regulate
5 the viral promoter activities have been identified, the molecular mechanisms of
6 the viral gene expression, in particular, regulatory mechanisms of the
7 transcriptional repression remain elusive. In this study, we identified LUC7 like 3
8 pre-mRNA splicing factor (LUC7L3, also known as hLuc7A or CROP) as a novel
9 interacting partner of HBV enhancer II and basal core promoter (ENII/BCP), key
10 elements within the core promoter, through the proteomic screening and found
11 that LUC7L3 functions as a negative regulator of ENII/BCP. Gene silencing of
12 LUC7L3 significantly increased expression of the viral genes and antigens as
13 well as the activities of ENII/BCP and core promoter. In contrast, overexpression
14 of LUC7L3 inhibited their activities and HBV replication. In addition, LUC7L3
15 possibly contributes to promotion of the splicing of 3.5 kb RNA, which may also
16 be involved in negative regulation of the pregenome RNA level. This is the first to
17 demonstrate the involvement of LUC7L3 in regulation of gene transcription and in
18 viral replication.

19

20

1 **Introduction**

2 Hepatitis B virus (HBV) infection is a major cause of acute and chronic
3 hepatitis and is closely associated with development of cirrhosis and
4 hepatocellular carcinoma (HCC) worldwide. HBV is a small enveloped virus with
5 a partially double-stranded DNA genome. The 3.2 kb HBV genome transcribes
6 four major species of unspliced RNA transcripts; 3.5, 2.4, 2.1 and 0.7 kb from the
7 core, preS1, preS2/S and X promoters, respectively, as well as a variety of
8 spliced viral RNAs. Core promoter produces 3.5-kb transcripts; the pregenome
9 and precore RNAs (termed as pgRNA in this study). HBV pgRNA not only serves
10 as the mRNA that codes for core and polymerase proteins but can be packaged
11 into nucleocapsid as a pregenomic RNA intermediate. Two enhancers; ENI and
12 ENII are also involved in modulation of the viral transcription basically via
13 up-regulating the activities of the HBV promoters. The transcriptional regulation
14 of HBV genes is prerequisite for the control of the viral replication^{1,2,3}. In contrast,
15 functional roles and significance of spliced RNAs in HBV lifecycle remain largely
16 unclear.

17 The core promoter and ENII are located in the region that overlaps with
18 the X gene. The core promoter can be divided into two elements; basal core
19 promoter (BCP)(nucleotide [nt] 1743-1849) sufficient to initiate transcription and
20 an upper regulatory region (nt 1643-1742). The latter region is composed of the
21 core upstream regulatory sequence (CURS) that stimulates the BCP and of
22 negative regulatory element (NRE). ENII is located at nt **1685-1773**, immediately
23 upstream of the BCP and overlaps with the CURS of the core promoter. ENII
24 stimulates the viral promoter activities in the manner independent (preS1,

1 preS2/S and X) or dependent (BCP) on position/orientation. ENII function is the
2 results of various interactions with liver-enriched or ubiquitous transcription
3 factors including the nuclear receptor superfamily. Nevertheless, further studies
4 to elucidate regulatory mechanisms for accurate HBV gene modulation on the
5 complex interplay between cellular factors and their corresponding sites within
6 these critical elements are required^{2,3,4}.

7 The course of chronic HBV infection frequently leads to alteration of the
8 viral genome. Several nucleotide mutations that naturally occurred in the
9 ENII/BCP region have been shown affecting viral function in cultured cells^{5,6,7,8,9}.
10 It is assumed that ENII/BCP mutations potentially affect binding between the
11 region and cellular factors involved in the transcriptional regulation, leading to
12 less production of the 3.5-kb mRNA and HBe antigen. Although effects of
13 ENII/BCP mutations on the development of HCC has been poorly investigated, it
14 has been reported that mutations within the region, for instance, T1762/A1764 in
15 the BCP are often found in patients with advanced liver diseases such as
16 HCC^{10,11,12,13}.

17 In this study, to further understand the molecular mechanisms
18 underlying pivotal roles of ENII/BCP in HBV replication and possibly in the viral
19 pathogenesis, we employed a proteome-based screening to identify candidate
20 proteins interacting with ENII/BCP of the HBV genome. One of the identified
21 proteins, LUC7 like 3 pre-mRNA splicing factor (LUC7L3, also known as hLuc7A
22 or CROP) was found to be a negative regulator of ENII/BCP activity. The
23 silencing or overexpression of LUC7L3 regulated HBV replication. Ectopic
24 expression of the siRNA-resistant LUC7L3 apparently restored down-regulation

1 of the core promoter activity mediated by the gene silencing. Mutational analyses
2 demonstrated that the N-terminal zinc finger motif within LUC7L3 is involved in
3 the negative regulation of pgRNA expression and that the nt 1666 -1700 region in
4 ENII is important for LUC7L3-mediated negative regulation of the ENII/BCP
5 activity. In addition, it is likely that LUC7L3 plays a role in promotion of the pgRNA
6 splicing. Our data suggested a role of LUC7L3 as a novel negative regulator in
7 HBV replication process.

8

9

10 **Results**

11 **Identification of nuclear proteins that interact with the enhancer II/basal** 12 **core promoter region and control the HBV core promoter activity**

13 To identify novel host factors which are involved in regulating the
14 transcription of 3.5-kb pregenome/precore RNA (pgRNA) of HBV, we first carried
15 out proteomics screening in which nuclear proteins binding to biotinylated
16 capture DNA of HBV sequence covering enhancer II and basal core promoter
17 (ENII/BCP)(nt 1627-1817) were isolated using the magnetic microbeads
18 separation system ([Supplementary Table S1](#)). Among 89 proteins identified by
19 LC-MS/MS analysis, seven proteins; ACIN1, CNBP1, LUC7L3, BCLAF1, CTCF,
20 HDGFRP2, HMGN1, were selected because of their involvement in transcription
21 and RNA metabolism, followed by testing as to whether they play roles in the
22 HBV gene expression. The effect of siRNA-based gene silencing of each
23 candidate on the activity of HBV core promoter was assessed. After 24 h of
24 siRNA transfection, cells were transfected with the reporter luciferase plasmids,

1 followed by measuring luciferase activities after further 48-h culture. We found
2 that LUC7L3 knock-down led to marked increase in the promoter activities
3 derived from both genotypes A and B (Fig. 1A).

4 LUC7L3, a human homolog of yeast U1 snRNP-associated factor, is
5 known as a nuclear protein that is possibly involved in pre-mRNA splicing^{14,15}.
6 Expression of LUC7L3 gene in human organs was determined by RT-quantitative
7 PCR (RT-qPCR). Although LUC7L3 expression was detected in most of organs
8 tested, the highest expression was observed in the small intestine and the
9 relatively high levels were found in the liver, ovary and colon (Fig. 1B).

11 **Negative regulation of enhancer II/basal core promoter activity and HBV** 12 **replication by LUC7L3**

13 The functional relevance of LUC7L3 on transcriptional regulation of
14 pgRNA was further assessed. Knockdown of LUC7L3 resulted in significant
15 increase in the ENII/BCP activity, likewise the activity of entire core/pregenome
16 promoter (Fig. 2A; upper panel). Knockdown efficiencies were confirmed by
17 RT-qPCR (Fig. 2A; lower panel). Effect of over-expression of LUC7L3 on the
18 promoter activities was also determined. After 48 h of co-transfection with the
19 LUC7L3-expression plasmid and the reporter plasmid for entire core promoter or
20 ENII/BCP, luciferase activities in cell lysates were measured. As expected, both
21 activities for the entire core promoter and ENII/BCP were significantly reduced in
22 cells transfected with the LUC7L3-expression plasmid (Fig. 2B). Effect of the
23 gene silencing of LUC7L3 by siRNA was apparently restored by ectopic
24 expression of the siRNA-resistant LUC7L3 but not by that of wild-type LUC7L3

1 (Fig. 2C), confirming that the knockdown-mediated increase in the core promoter
2 activity observed was not due to the off-target effect by siRNA used.

3 Next we investigated loss- and gain-of-functions of LUC7L3 with respect
4 to the replication and production of HBV. After 48 h of siRNA transfection, cells
5 were transfected with a plasmid carrying a 1.24-fold HBV genome derived from
6 genotype A, B or C (pUC-HB-Ae, -HB-Bj or -HB-CAT). Further 48-h culture later,
7 the supernatants and cells were harvested and used to determine the
8 particle-associated HBV DNA by qPCR and HBs and HBc antigens by
9 immunoblotting, respectively. The viral DNA levels in the supernatants were 5- to
10 9-fold higher in the cultures with knockdown of LUC7L3 compared with those with
11 introducing negative control siRNA (Fig. 2D, upper panel). Steady-state levels of
12 HBs and HBc antigens in cells with LUC7L3 knockdown were obviously higher
13 compared to the control cells (Fig. 2D, lower panel). In contrast, over-expression
14 of LUC7L3 in cells replicating the HBV genome resulted in reduced expression of
15 the viral RNA and antigens (Fig. 2E), indicating that increased expression of
16 LUC7L3 potentially leads to the opposite effect on the HBV replication as
17 observed in case of the gene silencing. **It is noted that neither over-expression
18 nor knockdown of LUC7L3 exhibited an influence on the cell viability under the
19 experimental conditions used (Supplementary Fig. S1).**

21 **A key role of the N-terminal zinc finger motif within LUC7L3 in the negative** 22 **regulation of expression of HBV pgRNA**

23 As represented in Fig. 3A (upper), LUC7L3 contains two
24 cysteine/histidine zinc finger motifs in its N terminus and central region (ZF1,

1 ZF2) and two leucine zipper-like repeats in the **N-terminal** half (LZ1, LZ2). Its
2 C-terminal half is extremely hydrophilic and contains the arginine/serine-rich
3 domain (RS) that has been observed in RNA splicing factors. To define the region
4 in LUC7L3 responsible for its effect on the pgRNA expression, we constructed a
5 series of LUC7L3 deletion mutant plasmids; pCAG-LUC7L3-D1~-D6, each
6 expressing LUC7L3 that lacks one of the domains/motifs mentioned above or
7 coiled-coil domains (CC1, CC2), with a FLAG tag (Fig. 3A). Effects of
8 over-expression of each LUC7L3 deletion mutant on the viral RNA level were
9 assessed in cells co-transfected with pUC-HB-Bj (Fig. 3B). Among mutants
10 tested, expression of LUC7L3-D1 exhibited no or only a limited influence on the
11 ENII/BCP activity and the pgRNA level, demonstrating that the N-terminal zinc
12 finger motif (ZF1), which is the C3H-type (three cysteines and one histidine), is
13 important for the negative regulation of the ENII/BCP activity mediated by
14 LUC7L3. To determine a role of LUC7L3 in HBV replication, HuH7 cells were
15 transfected with pUC-HB-Bj together with either expression construct for
16 wild-type LUC7L3 or LUC7L3-D1, subjecting to Southern blotting at 5 days
17 post-transfection (Fig. 3C). As expected, the levels of intracellular HBV DNA, in
18 particular, a possible form of covalently closed circular DNA (cccDNA) was
19 markedly lower in cells expressing wild-type LUC7L3. **A lower level of the**
20 **cccDNA was also seen in cells expressing LUC7L3-D1 but its difference**
21 **compared to the level in control cells was limited.**

22 The subcellular distribution of LUC7L3 and its mutants was also
23 determined (Fig. 3D). As previously reported, LUC7L3 was detected throughout
24 the nucleus with a speckled staining pattern. In contrast to LUC7L3-D1 and -D2

1 being more exclusively granular nuclear patterns, LUC7L3-D6 showed a
2 homogeneous nuclear distribution, found largely in the nucleoplasm. It is likely
3 that several motifs or domains, including ZF1, known to be involved in DNA/RNA
4 binding potentially contribute to subcellular localization of LUC7L3.

5

6 **The nt 1666 -1700 region within enhancer II/basal core promoter is**
7 **important for its negative regulation mediated by LUC7L3**

8 To identify the element **within the ENII/BCP sequence responsible for**
9 **the negative regulation of its transcriptional activity mediated by LUC7L3**, a
10 series of reporter constructs with partial ENII/BCP deletions were generated (Fig.
11 4A, left) and the reporter activity in the transfected cells with or without LUC7L3
12 expression was measured 2 days post-transfection. Although all deletions tested
13 led to a decrease in the reporter activity, the deletion of nt 1666-1700 region
14 (HBenIIcp-del-4) resulted in the complete loss of inhibitory effect by LUC7L3 (Fig.
15 4A, right). Interaction between LUC7L3 and the ENII/BCP region detected by
16 DNA pull-down assay, in which the biotinylated ENII/BCP DNA probe can be
17 complexed with LUC7L3 in the nuclear extract, was cancelled by deleting nt
18 1666-1700 sequence in the probe (ENIIBCP-del4 DNA)(Fig. 4B). However, the
19 electrophoretic mobility shift assay based on in vitro synthesized LUC7L3 and a
20 series of oligonucleotide probes resulted in no specific signal for the direct
21 LUC7L3-DNA binding (**Supplementary Fig. S2**). It is thus likely that LUC7L3
22 recognizes indirectly the ENII/BCP presumably through nt 1666-1700 region.
23 There are potential binding sites for HNF4 α and C/EBP α , which are conserved
24 among HBV genotypes, within nt 1666-1700 region. These transcription factors

1 are known to function as positive regulators for HBV pregenome expression^{2,4}.
2 One may hypothesize that LUC7L3 suppresses ENII/BCP activity via possibly
3 interacting either with HNF4 α or C/EBP α . We addressed this possibility by
4 knocking-down either HNF4 α or C/EBP α , followed by introducing pUC-HB-Bj or
5 the ENII/BCP reporter plasmid together with or without LUC7L3-expression
6 plasmid (Fig. 4C). Knockdown either of HNF4 α or C/EBP α led to a significant
7 decrease in the pgRNA level and the ENII/BCP activity. However, the inhibitory
8 effect of LUC7L3 on the pgRNA level and the ENII/BCP activity was not
9 influenced by the knockdown; the pregenome levels in the knockdown- and
10 control cells, respectively, were reduced to 30% (siHNF4a), 29% (siC/EBPa) and
11 26% (siCont) by LUC7L3 expression. LUC7L3 might regulate the ENII/BCP
12 activity negatively by its interacting with certain transcription activator unidentified
13 to date.

14

15 **Possible involvement of LUC7L3 in the regulation of pgRNA splicing**

16 Since LUC7L3 is known to be a component of the U1 snRNP and is
17 involved in the pre-mRNA splicing, whether LUC7L3 modulates splicing of HBV
18 pregenome RNA was assessed by RT-qPCR (Fig. 5A) and semi-quantitative
19 RT-PCR (Fig. 5B). After 48 h of co-transfection with pUC-HB-Ae or -HB-Bj and
20 the LUC7L3-expression plasmid, total cellular RNAs were isolated and HBV
21 spliced RNAs derived from pregenome, such as most abundant 2.2 kb
22 singly-spliced RNA that lacks intron nt 2447/489, and unspliced, 3.5-kb pgRNA
23 were separately determined. In RT-qPCR assay, the levels of unspliced pgRNA
24 were lower in cells transfected with the LUC7L3-expression plasmid compared to

1 control cells (Fig. 5A, top), analogous to Fig. 2E. By contrast, no or a little change
2 in the spliced RNA levels was found in the LUC7L3-expressing cells (Fig. 5A,
3 middle). As a result, over-expression of LUC7L3 led to increase in ratios of the
4 spliced RNAs/unspliced pgRNA in HBV genotypes A and B (Fig. 5A, bottom).
5 Such changes in the spliced/unspliced RNA ratios were confirmed by agarose gel
6 electrophoresis of the corresponding cDNAs (Fig. 5B).

7 Thus, in addition to the inhibitory effect on the viral ENII/BCP activity,
8 positive effect on the RNA splicing may be involved in negative regulation of the
9 pgRNA level mediated by LUC7L3 during HBV lifecycle.

10

11

12 **Discussion**

13 LUC7L3 is the human homologue of yeast splicing factor Luc7p, a
14 component of the yeast U1 snRNP. This is categorized in the SR protein family
15 and contains the arginine/serine-rich RS domain, which is generally located at
16 the C terminus of pre-mRNA splicing factors and plays a role in splicing activation
17 or alternative splicing regulation by engaging in protein-protein interactions^{16,17}. It
18 has been reported that LUC7L3 is possibly involved in alternative splicing of
19 some cellular genes such as proapoptotic Bcl-x¹⁸ and a sodium channel member
20 SCN5A¹⁹ through its association with another splicing factor RBM25. Besides the
21 splicing regulation, only limited evidence for biological roles of LUC7L3 is
22 available to date. LUC7L3 has also been demonstrated as cisplatin
23 resistance-associated protein, cloned from cisplatin resistant cell lines by
24 differential display^{20,21}. Independently, LUC7L3 or its closely related human

1 protein has been identified as a protein binding to the cAMP response element
2 (CRE) by the yeast one-hybrid system²². However, the functional roles in the drug
3 resistance or as the CRE-binding protein have not been elucidated.

4 In this study, among nuclear proteins capable for interacting with the
5 ENII/BCP region of HBV genome as identified by the proteomics screening (Fig.
6 1A), we found that LUC7L3 functions as a negative regulator of HBV replication
7 (Fig. 2). In addition to involvement in the pgRNA splicing (Fig. 5), LUC7L3
8 inhibited expression of pgRNA through down-regulation of the ENII/BCP activity
9 (Fig. 2B and 4A). Further, LUC7L3 expression decreased the levels of the viral
10 cccDNA in cells (Fig. 3C) and of the particle-associated HBV DNA in the culture
11 supernatants (Fig. 2E), indicating decrease in the viral replication induced by
12 LUC7L3. The lower level of HBV replication potentially led to decrease in
13 expression of whole viral antigens including HBc and HBs antigens (Fig. 2E).
14 Deletion mutation analysis revealed that the C3H-type zinc finger motif at the N
15 terminus of LUC7L3 is important for its negative regulation of the transcriptional
16 activity. However, despite the fact that the motif is commonly found as a DNA
17 binding motif in eukaryotic transcription factors or in RNA-binding proteins^{23,24,25},
18 no direct binding between LUC7L3 and the ENII/BCP sequence was observed in
19 the in vitro gel shift assay (Supplementary Fig. S2). Considering the results
20 obtained in this study, a likely scenario is that the negative regulation of the
21 ENII/BCP activity by LUC7L3 includes its interaction with yet-unidentified,
22 positive regulator(s) bound to the ENII/BCP region, thus modulating the
23 transcriptional activity. Recently, structural maintenance of chromosome complex
24 SMC5/6 has been identified as a restriction factor for HBV gene expression²⁶. We

1 assessed effect of knockdown or over-expression of LUC7L3 on gene expression
2 of SMC5 and SMC6 (Supplementary Figure S3). Although LUC7L3 knockdown
3 resulted in a moderate increase in SMC5 and SMC6 mRNAs, over-expression of
4 LUC7L3 exhibited no impact on expression of SMC5 and SMC6. Besides the
5 cases with DNA/RNA binding, SR proteins are known to interact with RNA
6 polymerase II or to act as cofactor to transcription factors such as CREB or
7 AP-1^{27,28}. Alternatively, LUC7L3 may contribute to down-regulation of expression
8 of such positive regulator(s) through transcriptional or post-transcriptional
9 process. Although further analysis to understand the mechanism underlying
10 regulation of the LUC7L3-mediated ENII/BCP activity is needed, this study is the
11 first to demonstrate the involvement of LUC7L3 in the transcriptional regulation.

12 It has been demonstrated that certain RNA splicing factors are involved
13 in transcriptional regulation via interacting with transcription factors and/or basal
14 transcriptional machinery. For example, PSF and p54nrb, in addition to their roles
15 in facilitating RNA splicing, have been shown to function as transcriptional
16 repressors for several nuclear receptors including progesterone receptor and
17 androgen receptor²⁹. Further study has indicated that PSF plays a role in
18 repression of STAT6-mediated transcription through recruitment of the HDAC
19 complex³⁰. It has been reported that splicing factors such as PGC-1, CoAA, and
20 CAPERs potentially coactivate the transcriptional activity of nuclear
21 receptors^{31,32,33}. Although for many years it has been thought that transcription
22 and splicing are independent events, evidence to support the existence of
23 functional links between these two processes is accumulating. Both transcription
24 and pre-mRNA splicing are extremely complex steps where certain molecular

1 interactions and kinetic constraints might be relevant. To understand functional
2 roles of relatively poorly characterized LUC7L3 in the transcription/RNA
3 processing machineries and their regulatory mechanisms, comprehensive
4 analyses to identify a variety of interactions of LUC7L3 with the DNA/RNA
5 sequences derived from viral and cellular target genes in the presence or
6 absence of nuclear protein fractions should be required.

7 Among a number of host-cell factors that have been identified to
8 regulate the HBV transcriptional activities via interactions with the ENII/BCP
9 region, the majority are activators that stimulate the activities of the *cis*-acting
10 elements. For example, liver-enriched or ubiquitous transcription factors such as
11 C/EBP α , RXR, PPAR, HNF4, HNF3, FTF/LRH-1, TBP, FXR α , PGC-1 α , SIRT1
12 and Sp1^{2,4} can bind to the ENII and contribute to the up-regulation of the core
13 promoter activity. Regarding mechanisms for transcriptional repression of HBV
14 gene expression, a negative regulatory element (NRE) that is located
15 immediately upstream of ENII is known to be involved in down-regulation of the
16 core promoter activity in an orientation-independent manner^{2,4}. In addition, ENII,
17 although less widely reported, is involved in negative regulation of the activity.
18 Prox1, which is known as a corepressor of FTF/LRH-1, inhibits
19 FTF/LRH-1-mediated activation of ENII³⁴. IL-4 suppresses the core promoter
20 activity presumably through down-regulation of C/EBP α expression³⁵. It has also
21 shown that TRIM proteins and COUP-TF1 potentially contribute to inhibition of
22 ENII activity^{36,37}. LUC7L3, a member of SR protein family, is a novel type of
23 ENII-mediated negative regulator of HBV replication.

24 The study provided evidence valuable for understanding the HBV-host

1 cell interaction during the viral replication cycle. Further studies to elucidate the
2 molecular mechanism underlying LUC7L3-mediated suppression of HBV
3 replication may provide new information on antiviral strategies.

4 5 6 **Methods**

7 8 **Plasmids.**

9 Plasmids containing HBV genomes; pUC-HB-Ae, pUC-HB-Bj, and
10 pUC-HB-CAT³⁸, were gifts from Dr. Mizokami (National Center for Global Health
11 and Medicine, Japan). DNA fragments of HBV core promoter derived from
12 genotypes A and B were designed in accordance with the most common
13 nucleotide among genotypes Ae and Bj, respectively, and synthesized (Eurofins
14 Genomics, Ebersberg, Germany). To construct pGL4.10-HBpg-Ae/Bj, the
15 synthesized fragments corresponding to nt 900-1817 region of HBV genome
16 digested by KpnI and HindIII were inserted into pGL4.10 luciferase reporter
17 (Promega, CA, USA). To construct pGL4.10-HBenIcp-Ae/Bj, the synthesized
18 fragments of nt 1627-1817 were amplified by PCR and digested by KpnI and
19 HindIII, followed by being inserted into pGL4.10. A series of deletion mutants,
20 pGL4.10-HBenIcp-Bj-del1/del2/del3/del4/del5/del6/del7/del8/del9/del10 were
21 generated based on pGL4.10- HBenIcp-Bj. To create LUC7L3-expression
22 plasmid pCAG-Flag-LUC7L3, human LUC7L3 sequence (Gene ID: 51747) was
23 amplified by PCR using cDNA derived from HuH7 cells as a template, followed by
24 digesting with BamHI and NotI and inserted into pCAG-Neo (Wako, Osaka,

1 Japan). Parts of LUC7L3 gene were deleted in pCAG-Flag-LUC7L3, resulting in
2 pCAG-Flag-LUC7L3-del1/del2/del3/del4/del5/del6. To create HNF4 α -expression
3 plasmid, the HNF4 α sequence (Gene ID: 3172) was amplified by PCR as
4 above, digested by EcoRI, and inserted into pCAGGS. Expression plasmids for
5 C/EBP α and C/EBP β were generated previously³⁹.

6

7 **Cell culture, transfection, RNA interference and cell viability assay**

8 HuH7 and 293T cells were maintained in Dulbecco modified Eagle
9 medium with 10% fetal bovine serum. Cells (1×10^5 cells/well in a 12-well plate)
10 were transiently transfected with **0.5 μ g of** plasmid DNA mixed with Lipofectamine
11 LTX (Invitrogen, CA, USA). ACIN1-specific siRNA, BCLAF1-specific siRNA,
12 CNBP1-specific siRNA, CTCF-specific siRNA, HDGFRP2-specific siRNA,
13 HMGN1-specific siRNA, LUC7L3-specific siRNA (siLUC7) and the negative
14 control RNA (siNC) was provided by Bonac (Fukuoka, Japan). siRNAs for HNF4 α
15 (siHNF4a), C/EBP α (siC/EBP α) and the negative control RNA (siNC-2) were
16 **purchased from** Ambion (CA, USA). The synthetic siRNAs (**50 pmol**) were
17 transfected into cells using ScreenFect A (Wako). Total RNAs and proteins of
18 cells were prepared at 72 h post-transfection. **Cell viability was measured by**
19 **using a CellTiter-Glo luminescent cell viability assay (Promega).**

20

21 **Immunoblotting and immunocytochemistry.**

22 Immunoblotting and immunocytochemistry were preformed essentially
23 as described⁴⁰. Cell lyses with 1% NP-40, 0.1% SDS, 1% sodium deoxycholate,
24 25mM Tris-HCl, pH 7.6, 150mM NaCl, 1mM EDTA, protease inhibitor cocktail

1 (Roche Diagnostics, Switzerland), were separated by SDS–PAGE and
2 transferred onto PVDF membranes. After blocking for 1 h, the membranes were
3 incubated with an antibody against HBc, which was generated by immunizing
4 rabbits with bacterially-expressed HBc protein, HBs (Institute of Immunology,
5 Tokyo, Japan), LUC7L3 (SIGMA Aldrich), HNF4 α (Santa Cruz Biotechnology,
6 Texas, USA), C/EBP α (Santa Cruz Biotechnology), FLAG M2 (Sigma-Aldrich,
7 Tokyo, Japan) or GAPDH (Santa Cruz Biotechnology) for 1 h. After washing,
8 membranes were incubated with HRP-conjugated secondary antibody (Cell
9 Signaling, MA, USA) for 0.5-1 h. Antigen-antibody complexes were detected
10 using the ECL prime Western blotting Detection Reagent (GE Healthcare, Bucks,
11 UK). For immunocytochemistry, cells grown on a glass bottom plate were fixed
12 with 4% paraformaldehyde (Wako) for 15 min and permeabilized in 0.5% Triton
13 X-100 in PBS, followed by blocking with 1% bovine serum albumin.
14 Immunocytochemistry was performed with the anti-LUC7L3 antibody (Sigma
15 Aldrich) for 2 h, followed by Alexa Fluor 488 anti-rabbit IgG (H+L) antibody
16 (Vector Laboratories, CA, USA) for 2 h. Double-stranded DNA was stained with
17 Hoechst 33342 (Dojin, Tokyo, Japan). Subcellular localization of LUC7L3 was
18 observed under the confocal microscope FV1000-D (Olympus, Tokyo, Japan).

19

20 **Luciferase reporter assay.**

21 Cells were transiently co-transfected with the **firefly** luciferase reporter
22 and pGL4.75 carrying **Renilla** luciferase gene with CMV promoter (Promega),
23 which was used for normalizing transfection efficiency. At 48 h post-transfection,
24 luciferase activities in cell lysates were measured with the dual luciferase reporter

1 assay kit (Promega).

2

3 **Quantification of DNA and RNA of HBV.**

4 To determine HBV pgRNA and spliced RNAs derived from pgRNA, total
5 RNAs were extracted from transfected cells with TRI-reagent (MRC, OH, USA)
6 according to the manufacture's instruction. The RNAs were treated with DNase I
7 (TaKaRa, Shiga, Japan) and RNase inhibitor (TaKaRa). cDNA templates were
8 synthesized using SuperScript VILO cDNA synthesis kit (Invitrogen), and qPCR
9 was carried out using the SYBR qPCR Mix kit (Toyobo, Osaka, Japan) with the
10 following primer sets; 5'-TCCCTCGCCTCGCAGACG-3' and
11 5'-GTTTCCCACCTTATGAGTC -3' for unspliced, 3.5-kb pgRNA, and
12 5'-CCGCGTCGCAGAAGATCT-3' and 5'-CTGAGGCCCACTCCCATAGG-3' for
13 spliced RNAs derived from pgRNA. The GAPDH gene was adopted as an
14 internal standard for the assay and the primers;
15 5'-AACAGCCTCAAGATCATCAGC-3' and 5'-GGATGATGTTCTGGAGAGCC-3'
16 were used. For semi-quantitative RT-PCR, the cDNA templates were amplified
17 with the following primers, 5'-AGCCTCCAAGCTGTGCCTTGGGTG-3' and
18 5'-AACCACTGAACAAATGGCACTAGTAACTGAGC-3'. Agarose gel
19 electrophoresis was used for the separation of PCR products corresponding to
20 unspliced and spliced forms of pgRNA.

21 To quantify the particle-associated HBV DNA, culture supernatants
22 collected were treated with PNE buffer (8.45%PEG, 0.445 M NaCl, 13 mM
23 EDTA) on ice for 1 h. After centrifugation at 12000 rpm for 15 min, the pellets
24 were treated with DNase I and RNase for 1 h at 37°C to remove free nucleic acids.

1 The samples were then lysed with the lysis buffer (13.33 mM Tris-HCl, 6.6 mM
2 EDTA, and 0.67% SDS) containing proteinase K (Wako) at 56°C overnight.
3 DNAs in the lysates were isolated by phenol/chloroform extraction and ethanol
4 precipitation. The viral DNA was then analyzed by qPCR with primers used for
5 the analysis of unspliced pgRNA as indicated above.

6

7 **mRNA quantification of cellular genes.**

8 Expression of LUC7L3 mRNA in various tissue was examined by using
9 the multiple tissue cDNA (MTC) human panels I and II (Clontech laboratories, CA,
10 USA). The panels contained a set of normalized single-strand cDNAs, produced
11 from poly(A)+ RNA from various normal human tissues. qPCRs were performed
12 using the SYBR qPCR Mix kit (Toyobo, Osaka, Japan). Expression of LUC7L3
13 mRNA was standardized by that of RNA polymerase II (RPII) gene. The primers
14 used were 5'-TCAAGCCGAACATCAGACAG-3' and
15 5'-GCTTCTGCTTCTTCGTGAT-3' for LUC7L3 and
16 5'-GCACCACGTCCAATGACAT-3' and 5'-GTGCGGCTGCTTCCATAA-3' for
17 RPII. For RT-qPCR to determine mRNAs of SMC5 and SMC6, total cellular RNAs
18 isolated by TRI reagent were transcribed using SuperScript VILO cDNA
19 Synthesis Kit. Aliquots of cDNAs were subjected to 45 cycles of PCR
20 amplification using THUNDERBIRD SYBR qPCR mix. The primers used were 5'-
21 AGAAGCAAGATGTTATAGAAAGGAAAG-3' and 5'-
22 TCCTCTGTCGGTCAAGCTCT-3' for SMC5 and 5'-
23 TGCATCAATTCTGGACAAAGA-3' and 5'- TGCTTCTTGGTACTGCCTCA-3' for
24 SMC6. The RNA expression data were normalized to levels of reference gene

1 **GAPDH using the comparative threshold cycle method.**

2

3 **Southern blot analysis.**

4 Extraction of protein-free DNAs that contain cccDNA and protein-free
5 relaxed circular (rc)DNA was carried out by using a modified Hirt extraction
6 procedure^{41,42}. Briefly, cells collected from a 10 cm diameter dish were lysed in 3
7 ml of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.7% SDS. After 30 min
8 incubation at room temperature, the lysate was transferred into a 15-ml tube,
9 followed by addition of 0.8 ml of 5 M NaCl and incubation at 4°C overnight. The
10 lysate was then clarified by centrifugation at 12,000 × g for 30 min at 4°C and
11 extracted twice with phenol and once with phenol:chloroform. The resulting DNA
12 was precipitated with two volumes of ethanol overnight at room temperature. 10
13 µg of the protein-free DNA sample was resolved in a 1.2% agarose gel and
14 transferred onto Hybond-XL membrane. Detection of HBV DNA was performed
15 using the DIG RNA Labeling Mix (Roche Diagnostics). Membranes were probed
16 with a DIG-labeled 0.4 kb HBV RNA probe corresponding to nt 1998-2453
17 (GenBank. No. AB246338). Hybridization was carried out in 5 ml of DIG EASY
18 Hyb reagent (Roche Diagnostics) at 68°C overnight, followed by washing with
19 0.1× SSC and 0.1% SDS at 68°C. The membrane was blocked with blocking
20 solution for 30 min, and incubated with anti-DIG antibody (Roche Diagnostics) for
21 1 h. CDP-star (Roche Diagnostics) was used as a substrate and hybridization
22 signals on the membrane were detected by ChemiDoc Touch Gel Imaging
23 System (Bio-Rad Laboratories, Tokyo, Japan).

24

1 **Detection of DNA-protein interactions.**

2 Nuclear proteins that interact with the DNA sequence corresponding to
3 the ENII/BCP (nt 1627-1817) were isolated using FactorFinder Kit (Miltenyi
4 Biotec, Bergisch Gladbach, Germany) according to the manufacturer's
5 instructions. The biotin-labeled capture ENII/BCP DNA probe was generated by
6 PCR with biotinylated primers; 5'- CGTGAACGCCACCGGAACC-3' and
7 5'-GTTGCATGGTGCTGGTGAAC-3'. The streptavidin-conjugated microbeads
8 were used to isolate DNA-protein complexes, followed by mass-spectrometry
9 (MS) analysis to identify proteins using mass spectrometry Q Exactive Hybrid
10 Quadrupole-Orbitrap Mass Spectrometer (Thermo fisher, CA, USA) with Xcalibur
11 (version 2.2). The Proteome Discoverer software (version 1.4; Thermo Fisher
12 Scientific) was used to generate peak lists from the raw MS data files. The
13 resulting peak lists were subsequently submitted to a SEQUEST search engine
14 (Thermo Fisher Scientific) and compared against the human protein sequences
15 in the UniProt protein database (Reviewed-Human+[9606]-20130806; 70236
16 sequences) to identify peptides. The SEQUEST search parameters were as
17 follows: variable modifications including oxidation of methionine and
18 carbamidomethyl of cysteine; peptide mass tolerance of ± 10 ppm; fragment
19 mass tolerance of ± 0.02 Da.

20 For detection of the DNA-LUC7L3 interaction, nuclear extracts obtained
21 from cells transfected with a plasmid expressing FLAG-tagged LUC7L3 or its
22 deletion mutant were used. The DNA-protein complexes were analyzed by
23 immunoblotting with anti-FLAG antibody. To prepare ampicillin DNA probe as an
24 unrelated capture DNA probe, PCR was performed using biotinylated primers;

- 1 5'-TTCGTTTCGTCCATAGTGGCCTG-3' and
- 2 5'-CATAGACTGGATGGAGGCGGAC-3', and pGL4.10 (Promega) as a
- 3 template.
- 4

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2

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22

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7

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13 **Contributions**

14 Y.L., M.I., S.S., and K.N. performed the experiments; Y.L., M.I., and T.S. wrote
15 the manuscript; M.I. and T.S. designed the research; Y.L., M.I., and T.S.
16 prepared the figures; Y.L., M.I., S.S., T.C., and K.N. analyzed the data; and all
17 authors commented on the manuscript, and approved the final version.

18

19 **Competing interests**

20 The authors declare that they have no conflicts of interest.

21

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24

1 **Figure legends**

2

3 Figure 1. Effect of knockdown of ENII/BCP-interacting factors on the viral core
4 promoter and distribution of LUC7L3 mRNA expression in human tissues. (A)
5 The luciferase reporter constructs harboring HBV cis-acting elements for the
6 entire core promoter (pGLHBp900/1817) and for the ENII/BCP region
7 (pGLHBp1627/1817) with numbering nucleotide positions used are depicted
8 (upper). HuH7 cells introducing siRNA for each ENII/BCP-interacting factor
9 identified by the proteomics screening (siRNA for each factor) or its negative
10 control (siNC) were transfected with pGLHBp900/1817, in which the viral
11 sequence is derived from genotype A (GT-A) or B (GT-B) of HBV. Two days later,
12 cells were harvested and used for the reporter luciferase assays. Values (RLU)
13 are normalized with the values of transfection with control siRNAs and GT-A
14 reporter, set at 100%. Values shown represent the means with standard errors of
15 the means (SEM) from three independent transfection of siRNA. (B) Expression
16 of LUC7L3 gene in various human tissues was assessed by quantitative PCR
17 using the tissue-specific human cDNA panel. Values shown were normalized by
18 those of RNA polymerase II gene expression. The relative value for liver was set
19 at 1.

20

21 Figure 2. Negative regulation of ENII/BCP activity and HBV replication by
22 LUC7L3. (A) Effect of LUC7L3 knockdown on HBV ENII/BCP activity was
23 determined. At 48 h after introducing LUC7L3 siRNAs (siLUC7) or its negative
24 control (siNC), HuH7 cells were transfected with pGLHBp1627/1817 whose HBV

1 sequence is derived from genotype A (GT-A) or B (GT-B), followed by further
2 48-h culture. Cells were used for the luciferase assay as indicated in Figure 1A
3 (upper) and for determination of LUC7L3 mRNA expression by RT-qPCR (lower).
4 (B) Effect of over-expression of LUC7L3 on HBV promoter activities was tested.
5 HuH7 cells were transfected with the LUC7L3-expression plasmid (LUC7) or an
6 empty vector (EV) together with pGLHBp900/1817 (900/1817) or
7 pGLHBp1627/1817 (1627/1817). Cells were harvested two days later to
8 determine the luciferase activities. (C) Restoration of the promoter activity by
9 expression of siRNA-resistant LUC7L3 was shown. HuH7 cells were transfected
10 with LUC7L3 siRNAs (siLUC7) or its negative control (siNC) together with
11 LUC7L3-expression plasmids (LUC7) or siRNA-resistant LUC7L3 (LUC7-siR).
12 After 24 h, cells were further transfected with pGLHBp900/1817 for evaluating the
13 luciferase activities. Expression of LUC7L3 proteins and GAPDH was assessed
14 by immunoblotting (lower). (D) Effect of LUC7L3 knockdown on HBV replication
15 and production of the viral proteins were determined. At 48 h after introducing
16 LUC7L3 siRNAs (siLUC7) or its negative control (siNC), cells were transfected
17 with pUC-HB-Ae, -Bj or -CAT, which carries a 1.24-fold HBV genome derived
18 from genotype A, B or C, respectively, followed by 48-h culture. The supernatants
19 and cells were subjected to qPCR of the particle-associated HBV DNA (upper)
20 and immunoblotting of HBs and HBc antigens, LUC7L3 and GAPDH (lower),
21 respectively. (E) Effect of over-expression of LUC7L3 on HBV replication and
22 production of the viral proteins were determined. HuH-7 cells were transfected
23 with the LUC7L3-expression plasmid (LUC7) or an empty vector (EV) together
24 with pUC-HB-Ae, -Bj or -CAT. At 72 h post-transfection, the supernatants and

1 cells were harvested to determine the particle-associated HBV DNA (upper) and
2 the viral antigens (lower) as indicated in (D). Values shown represent the means
3 with SEM obtained from three independent transfection of siRNAs or plasmids.

4

5 Figure 3. Effects of deletions within LUC7L3 on its subcellular distribution and on
6 expression of the viral pgRNA. (A) A series of deletion mutants of LUC7L3 used
7 in this study were indicated. A schematic diagram of predicted domain structures
8 of LUC7L3 is indicated at the top. ZF, zinc-finger domain; LZ, leucine zipper
9 domain; SR, SR-rich domain; CC, coiled-coil domain. (B) HuH7 cells were
10 transfected with FLAG-tagged LUC7L3 (LUC7L3) or its partial deletions (D1-D6)
11 or empty vector (EV) together with pGLHBp1627/1817 (1627/1817). Cells were
12 harvested three days later to determine the luciferase activities (upper) and
13 expression of LUC7L3 proteins as well as of GAPDH was assessed by
14 immunoblotting (lower). (C) HuH7 cells were transfected with FLAG-tagged
15 LUC7L3 (LUC7L3) or its partial deletion (LUC7L3 D1) or empty vector (EV)
16 together with pUC-HBAe, -Bj or pUC19 (-). At 5 days post-transfection, Hirt DNA
17 was prepared from the transfected cells and subjected to Southern blot analysis.
18 RC, rcDNA; CCC, cccDNA; SS, single-strand DNA. cccDNA was resistant to
19 denaturation at 85°C for 5 min (Heat), but sensitive to EcoRI digestion
20 (Heat+EcoRI). D) Subcellular localization of LUC7L3 and its deletion mutants
21 were determined by confocal microscopy images of cells transfected with
22 expression plasmids for FLAG-tagged LUC7L3 (LUC7L3) or its partial deletions
23 (D1-D6) or empty vector (EV). Cells were immunostained with anti-FLAG
24 antibody as a first antibody (LUC7L3), and counterstained with Hoechst 33342 to

1 label nuclei (DNA). Scale bar, 10 μ m. **Statistical significances compared with EV**
2 **were shown. * $p < 0.05$, Student's t test.**

3
4 Figure 4. Effects of deletions within the ENII/BCP sequence on down-regulation
5 of the ENII/BCP activity mediated by LUC7L3. (A) Deletion constructs of the
6 ENII/BCP region of the HBV genome linked to the firefly luciferase gene are
7 shown. Cells were co-transfected with one of these reporter constructs and the
8 LUC7L3-expressing plasmid or an empty vector. At 48 h post-transfection, cells
9 were harvested and subjected to the luciferase assay. Values obtained without
10 LUC7L3 expression were set as 1 for expression of each reporter. Results
11 represent the means with SEM from three independent transfectants. (B)
12 Interaction of LUC7L3 with the ENII/BCP sequence but not with its deletion, del4
13 as indicated in (A), was shown by DNA pull-down assay. In vitro synthesized
14 biotinylated DNA derived from entire ENII/BCP or its deletion of nt 1666-1700
15 region was mixed with the nuclear extract prepared from cells transfected with
16 LUC7L3 construct. Biotinylated DNA-protein complexes were captured with
17 streptavidin-conjugated beads, followed by immunoblotting with anti-FLAG
18 antibody. (C) Effects of knockdown of HNF4 α or C/EBP α on down-regulation of
19 the ENII/BCP activity and the pgRNA expression were evaluated. At 24 h after
20 introducing siRNA against HNF4 α (siHNF4a) or C/EBP α (siC/EBPa) or negative
21 control (siCont), cells were transfected with pUC-HB-Ce or pGLHBp1627/1817
22 together with the LUC7L3-expressing plasmid (LUC7) or an empty vector (EV)
23 and cultured for further 48 h. The reporter activities (upper left) and the pgRNA
24 levels (upper right) were determined by the luciferase assay and RT-qPCR,

1 respectively. Knockdown efficiency of HNF4 α (lower left) and C/EBP α (lower
2 right) was assessed by RT-qPCR. Results represent the means with SEM from
3 three independent transfectants.

4

5 Figure 5. Effect of LUC7L3 expression on splicing of pgRNA. (A) HuH7 cells were
6 transfected with the LUC7L3-expression plasmid (LUC7L3) or an empty vector
7 (EV) together with either pUC-HB-Ae (GT-A), or -Bj (GT-B). At 48 h

8 post-transfection, total RNAs were prepared from cells and subjected to

9 RT-qPCRs for 3.5-kb pgRNA and pgRNA-derived spliced RNAs separately.

10 Results represent the means with SEM from three independent transfectants. (B)

11 Total RNAs obtained from the transfected cells as indicated above were used for

12 semi-quantitative RT-PCR. cDNA bands corresponding to unspliced 3.5 kb

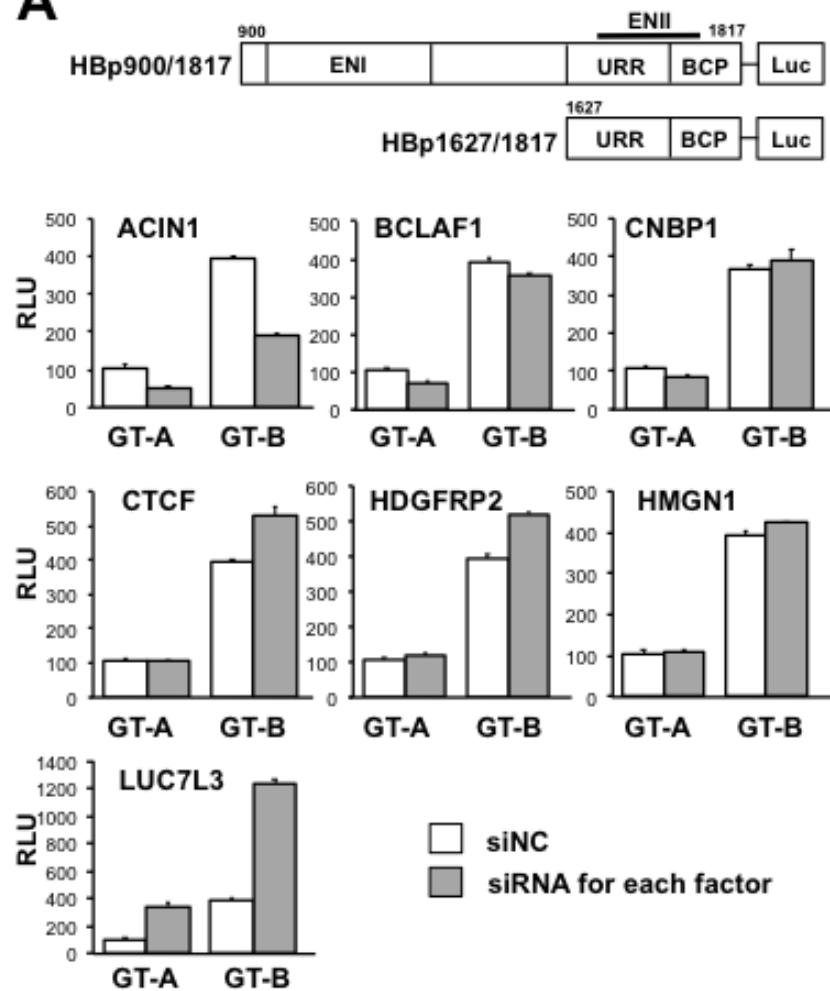
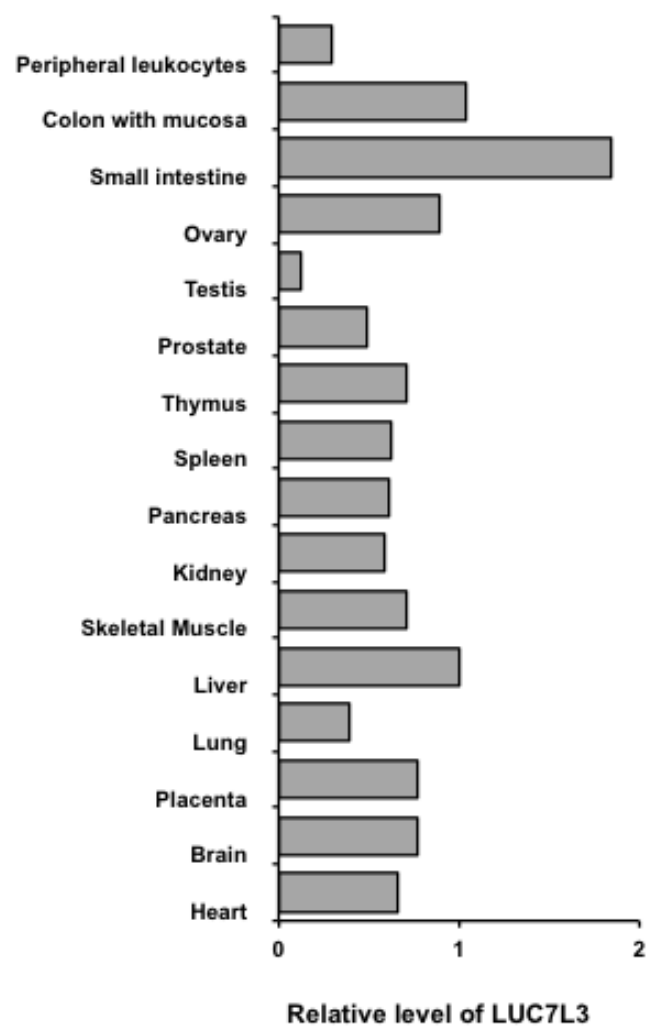
13 pgRNA as well as several spliced forms including major spliced RNA derived from

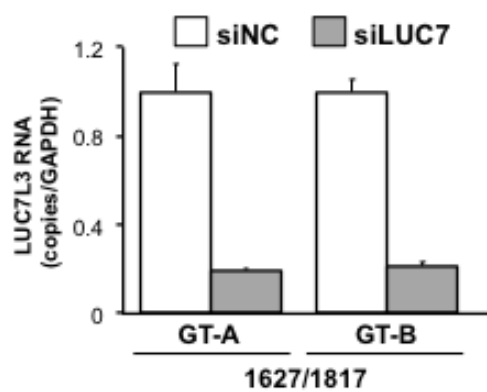
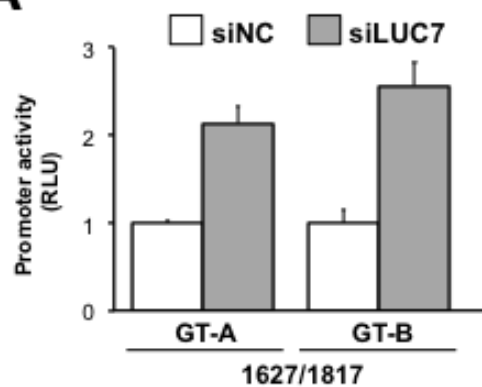
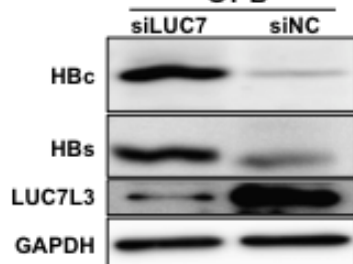
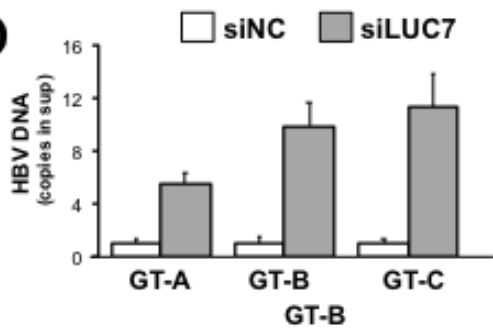
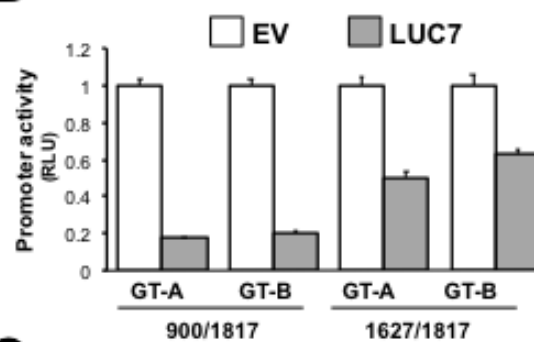
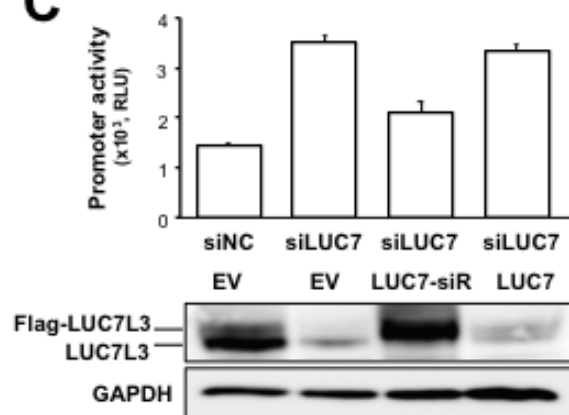
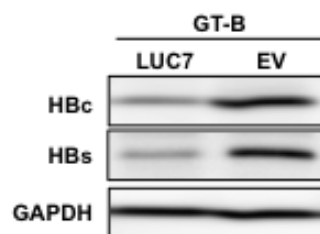
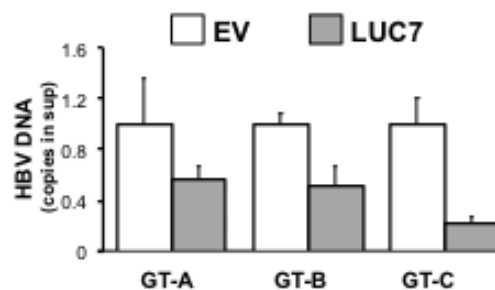
14 pgRNA were detected by agarose gel electrophoresis. The 28S and 18S

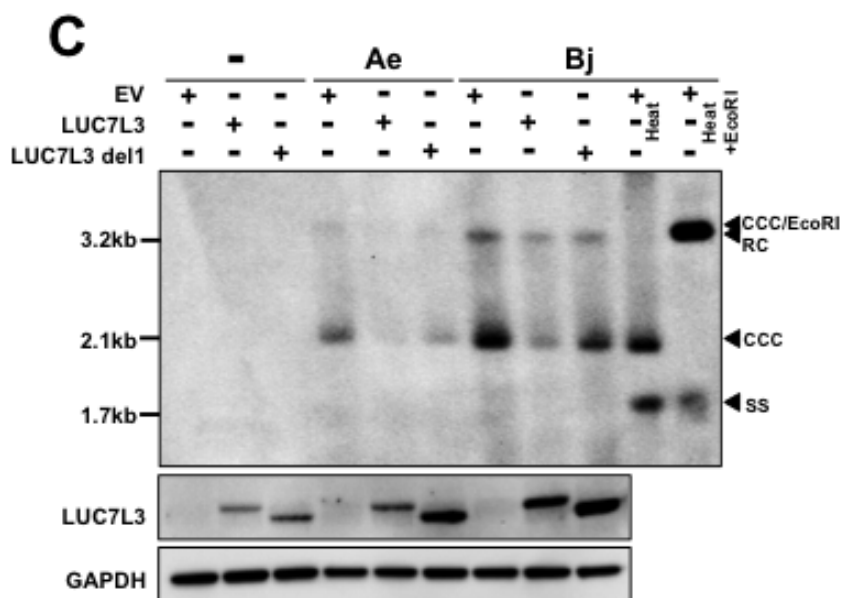
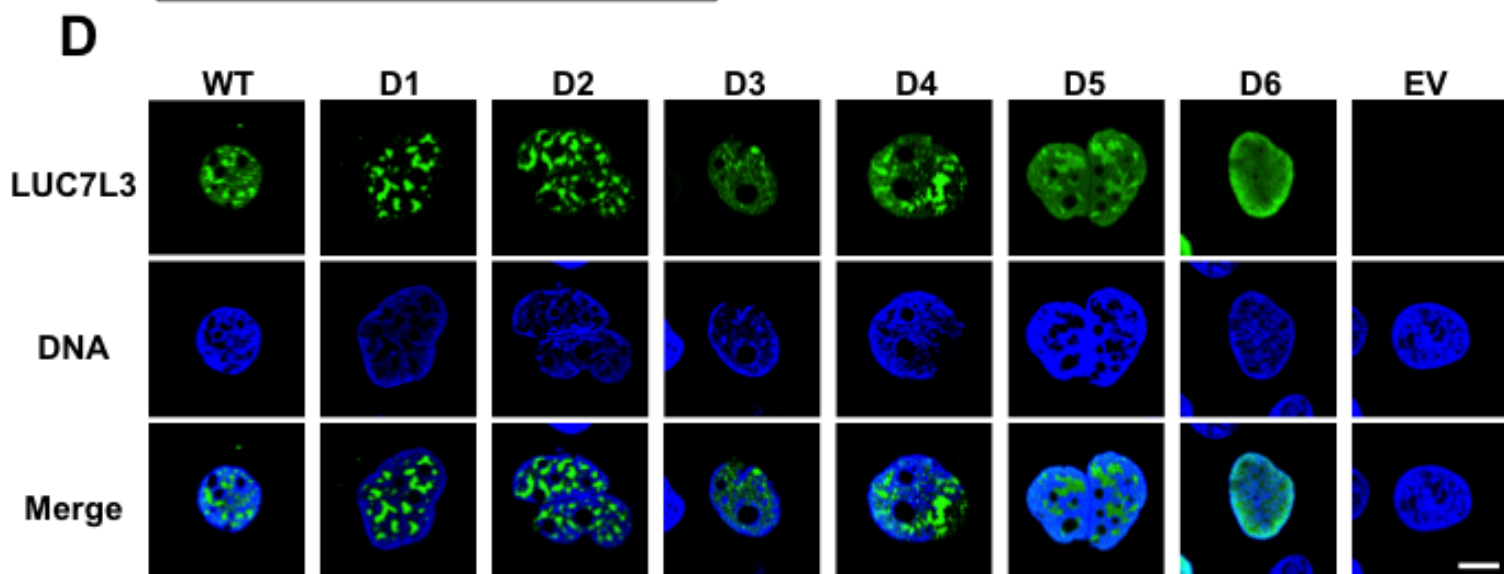
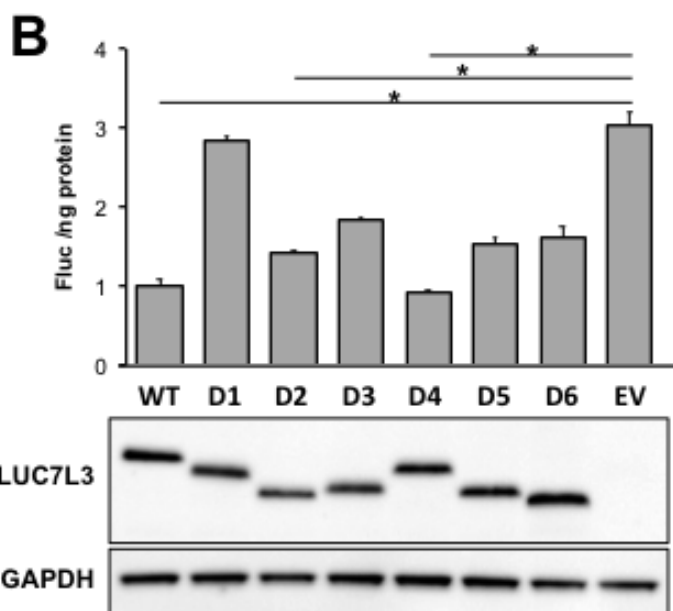
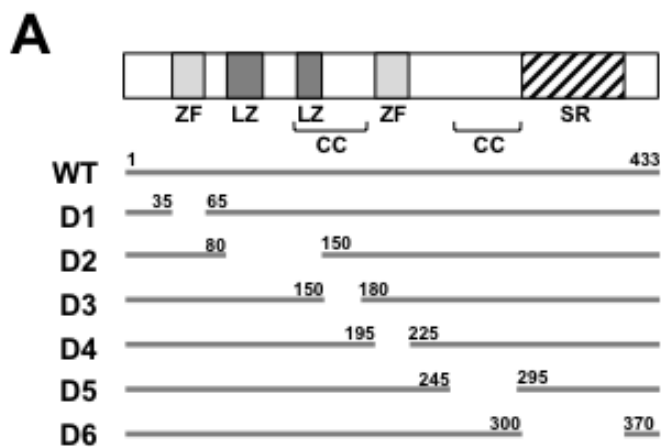
15 ribosomal RNAs were also detected. Statistical significances compared with EV

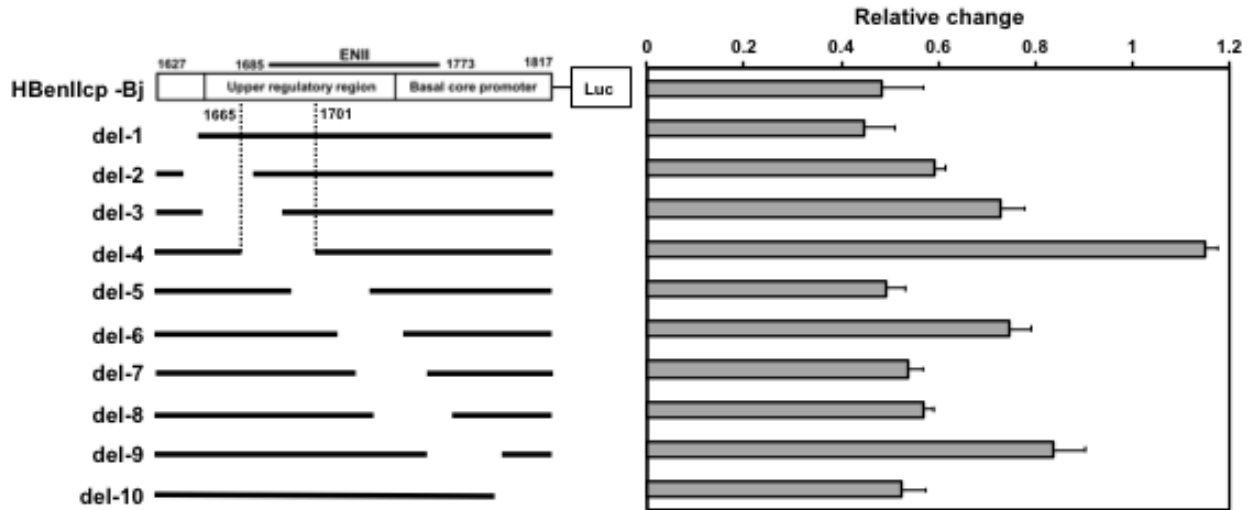
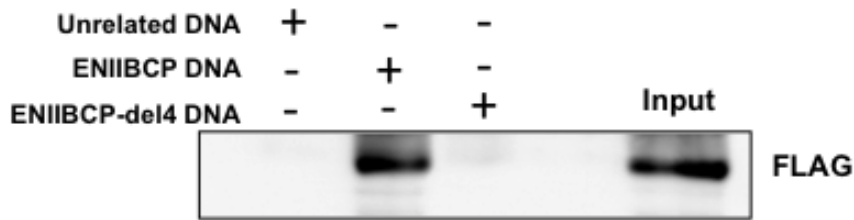
16 were shown. *p<0.05, **p<0.01, Student's t test.

17

A**B**

A**D****B****C****E**



A**B****C**