Title

Full title: NEDD4-binding protein 1 suppresses HBV replication by degrading pgRNA

Short title: Effect of NEDD4-binding protein 1 on the replication of HBV

Authors

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34 **Abstract**

35 Chronic infection with hepatitis B virus (HBV) places patients at increased risk for liver cirrhosis
36 and hepatocellular carcinoma. Although nucleos(t)ide analogs are mainly used for the treatment of
37 HBV, they require long-term administration and may lead to the emergence of drug resistant mutants.

Therefore, to identify targets for the development of novel anti-HBV drugs, we screened for HBV-suppressive host factors using a plasmid expression library of RNA-binding proteins (RBPs). We screened 132 RBPs using an expression plasmid library by measuring HBV relaxed circular DNA (rcDNA) levels in hepatocellular carcinoma. After we identified one RBP which suppressed rcDNA, the domain-deficient mutants were generated to determine the region contributing to the anti-HBV effect. Also, we measured pregenomic RNA (pgRNA) and covalently closed circular DNA (cccDNA) level in the RBP transfected cells and confirmed the degradation of pgRNA by Northern blotting. Our screen identified NEDD4-binding protein 1 (N4BP1) having an anti-HBV effect. In hepatocellular carcinoma cell lines transfected or infected with HBV, overexpression of N4BP1 decreased rcDNA levels while knockdown or knockout of N4BP1 expression rescued rcDNA levels. We found that both the KH-like and RNase domains of N4BP1 were required for the protein's anti-HBV effect. N4BP1 suppressed HBV replication by promoting pgRNA, PreS1 and PreS2/S degradation. In summary, we found that N4BP1 is a newly identified host factor able to counteract HBV production by reducing HBV RNA levels.

Author summary

There is still a large number of HBV-infected people in the world today because of no curative treatment for HBV infection. In this study, we focused on and screened RNA-binding proteins to

identify new host factors which inhibit HBV replication. As a result, we found that NEDD4-binding protein 1 (N4BP1) expression suppresses rcDNA production by promoting the degradation of pregenomic RNA, 2.4kb and 2.1kb HBV RNA. Furthermore, KH-like domain or RNase domain of N4BP1 were involved in this anti-HBV effect. In addition, the N4BP1 levels were lower in HCC resection samples of exacerbated patients, suggesting that individual N4BP1 levels might be related to HCC progression. This novel factor can potentially become a key to new HBV treatments.

Introduction

Hepatitis B virus (HBV) infection causes either acute or chronic hepatitis, with approximately 296 million people worldwide suffering from chronic hepatitis B and an estimated 1.5 million new cases arising each year. Chronically infected individuals are at increased risk for liver disease, and annually ~820,000 die from cirrhosis and hepatocellular carcinoma (HCC) caused by HBV infection[1]. Although there is a prophylactic vaccine for HBV, there are still many new cases of HBV infection due to the large number of chronic HBV infected patients still in Africa, Asia, and Eastern Europe and the low vaccination rates in these highly endemic areas[2].

HBV is an enveloped DNA virus with a small, partially double-stranded genome of 3.2 kbp. Entry into hepatocytes is mediated by binding to the bile acid transporter sodium-taurocholate cotransporting polypeptide (NTCP). After being released into the nucleus, the HBV genome is

converted from its relaxed circular DNA (rcDNA) form to covalently closed circular DNA (cccDNA) using the host DNA repair system[3-6]. The cccDNA serves as the transcription template for viral mRNAs, including the HBV pregenomic RNA (pgRNA). pgRNA is used to synthesize the negative-strand DNA first and then the positive-strand is synthesized to generate rcDNA.

Interferon (IFN) and nucleos(t)ide analogs (NAs) are currently used as treatments for HBV infection, and both have shown efficacy in controlling viremia[7, 8]. IFNs are involved in the inhibition of HBV replication, transcription, and other important processes by inducing the expression of various IFN-stimulated genes (ISGs) in host cells[9]. However, IFN treatment can cause considerable side effects, including liver damage, and is thus unsuitable for patients with severe liver dysfunction[10].

In contrast, NAs impair further HBV intrahepatic spreading by blocking production of new virions. But the HBV antigens HBs or HBe are not eliminated with treatment as NAs inhibit the reverse transcription step that occurs after these antigens have already been translated. Furthermore, NAs do not directly target cccDNA, allowing the virus to persist in hepatocytes[11, 12]. Consequently, to prevent relapse due to reactivation of HBV after discontinuing NAs, long-term treatment is recommended[13].

In efforts to overcome the limitations of these current therapeutic options, research has looked to identify host factors with an antiviral role. Understanding the mechanism of action of such factors

92 could aid in the development of new antiviral treatments. Several host factors are known to inhibit
93 HBV replication, including RNA-binding proteins (RBPs). For example, the CCCH zinc finger
94 antiviral protein ZAP (ZC3HAV1) binds to HBV RNA with its four zinc finger motifs at the N-
95 terminus, promoting pgRNA degradation[14]. Similarly, MCPIP1 (ZC3H12A, regnase-1) has also
96 been reported to inhibit HBV replication by binding to HBV pgRNA through its zinc finger motif,
97 directly degrading pgRNA via its RNase activity[15]. Furthermore, PUF60 is a host factor that not
98 only positively regulates HBV 3.5 kb precore plus pregenomic RNA expression by binding to the
99 HBV enhancer region II with the transcription factor TCF7L2 but can also promote degradation of the
100 HBV 3.5 kb RNA by binding via its N-terminal RNA recognition motif[16]. Despite these descriptions,
101 HBV produces high levels of HBV RNAs in infected primary human hepatocytes (PHHs) and i*n vivo*,
102 so the antiviral effects of these host factors appears limited.

103 To determine if other RBPs may have an antiviral role, we performed a comprehensive screen
104 using an expression library of RBPs. We identified NEDD4-binding protein 1 (N4BP1) as a host factor
105 with anti-HBV activity. We subsequently elucidated the mechanism of how N4BP1 suppresses HBV
106 replication by promoting pgRNA degradation.

107

108 **Results**

109 **Screen for RNA-binding proteins that inhibit HBV propagation**

110 To identify novel RBPs involved in HBV propagation, we screened 132 RBPs using an
111 expression plasmid library. Huh7 cells were co-transfected with an RBP expression plasmid and a 1.3-
112 fold-overlength HBV genome (genotype C). At 3 days post-transfection (dpt), cells were collected to
113 measure intracellular rcDNA levels. As shown in Fig 1A, 23 out of 132 RBPs suppressed rcDNA
114 levels by at least 50% compared to the control.

115 As a secondary screen, we then examined the effect of these 23 RBPs on HBV entry and
116 replication. HepG2-hNTCP C4 cells expressing each of the 23 RBPs were infected with HBV
117 (genotype D), and intracellular rcDNA measured by quantitative PCR (qPCR) at 14 days post-
118 infection (dpi). Seven of the 23 RBPs (N4BP1, TIA-1, ZC3H12B, PJA1, HNRNPC, ZCCHC10, and
119 KHNYN) reduced rcDNA levels in comparison with the control (Fig 1B). These results were
120 consistent with previous reports that found TIA-1 and PJA1 inhibit expression of HBs antigen and
121 HBV transcription and replication, respectively[17, 18].

122 Of the five remaining factors, we found by western blot that three (N4BP1, HNRNPC, and
123 ZCCHC10) are endogenously expressed in Huh7 and HepG2-hNTCP C4 cells (Fig 1C). To test
124 whether these proteins affect HBV replication, we knocked down each one and assessed rcDNA levels
125 following transfection with the 1.3-fold-overlength genome of HBV genotype C. The antiviral drug
126 entecavir, a nucleoside analog, was used as a positive control. Knockdown of each factor was
127 confirmed by western blotting (WB), but unlike knockdown of N4BP1, knockdown of HNRNPC or

128 ZCCHC10 did not consistently increase the levels of rcDNA depending on the siRNA used (Fig 2A-
129 2C). Thus, N4BP1 became the focus of our study.
130
131 **N4BP1 is a suppressive host factor of HBV replication**
132 Next, to determine whether N4BP1 also had an antiviral effect against other HBV genotypes, we
133 co-transfected Huh7 cells with a plasmid expressing N4BP1 and a plasmid containing 1.3-fold-
134 overlength genomes of HBV genotypes A, B, and C. At 3 dpt, rcDNA levels were decreased in cells
135 transfected with each genotype (Fig 3A).
136 To further elucidate the role of N4BP1 in HBV replication, we constructed Huh7 N4BP1
137 knockout (KO) cells using CRISPR/Cas9. We generated two clones of N4BP1 KO Huh7 cells (Fig
138 3B) and once more assessed whether intracellular rcDNA levels changed at 3 dpt with a plasmid
139 containing the 1.3-fold-overlength genome of HBV genotype C. In both KO lines, rcDNA levels
140 increased approximately 3-fold compared to control cells (Fig 3B). After rescuing N4BP1 expression
141 in our KO line, rcDNA levels became significantly decreased again (Fig 3C, 3D), suggesting that
142 N4BP1 is a suppressive host factor of HBV replication. In addition, we performed a transcriptomic
143 analysis to determine whether host factors are involved in the anti-HBV activity of N4BP1. As
144 visualized by heat maps, host factors differentially expressed between N4BP1-overexpressing PHHs
145 and control PHHs (S1 Fig A). We found 25 genes that were upregulated by 2-fold or more in N4BP1-

146 vs control PHHs and 17 genes that were downregulated by 0.5-fold or more (S1 Fig B, S1 Table).
147 None of these factors were related to immune pathways nor had been previously reported to be
148 involved in HBV replication.
149

150 **N4BP1 expression is not induced by HBV**

151 We then evaluated whether N4BP1 expression level was increased by HBV infection. A plasmid
152 containing the 1.3-fold-overlength genome of HBV genotype C was transfected into Huh7 cells, and
153 N4BP1 expression assessed by WB and qPCR at 3 dpt. Transfection of the HBV genome did not
154 significantly affect N4BP1 expression at either the transcriptional or protein level (Fig 4A, 4B).
155 N4BP1 expression also did not significantly change in HepG2-hNTCP C4 cells after infection with
156 HBV (Fig 4A, 4C). Finally, we measured N4BP1 expression in HepAD38.7 cells that stably secrete
157 HBV particles unless cultured in the presence of tetracycline. In this system, removal of tetracycline
158 and induction of HBV particle production also did not result in any change in N4BP1 expression (Fig
159 4A, 4D). Together, these results indicate that N4BP1 expression is not affected by HBV infection.
160

161 **The KH-like and RNase domains of N4BP1 are critical for the anti-HBV effect of N4BP1**

162 As shown in Fig 5A, N4BP1 is composed of 896 amino acids and contains a KH-like domain at
163 its N terminus side and an RNase domain at its C terminus[19]. To examine which N4BP1 domains

164 are needed to suppress HBV replication, we constructed deletion mutants lacking either the KH-like
165 (ΔKH; deletion of amino acids 59-143) or the RNase domain (ΔRNase; deletion of amino acids 617-
166 769).

167 The plasmid encoding wild-type or mutant N4BP1 was respectively co-transfected into naïve
168 Huh7 cells with the plasmid containing the 1.3-fold-overlength genome of HBV genotype C and the
169 rcDNA levels measured. The expression of the deletion mutants was confirmed by WB using an HA-
170 tagged antibody (Fig 5B, upper). The rcDNA level in N4BP1 overexpressing cells was significantly
171 reduced, while the rcDNA levels in the ΔKH- or ΔRNase-expressing cells were comparable to those
172 in the control cells (Fig 5B, lower).

173 Residue D623 in the RNase domain of N4BP1 (Fig 5A) has been shown to be important for the
174 protein's RNase activity[20]. A D623N substitution causes loss of RNase activity without changing
175 the structure of N4BP1. Co-transfecting the Huh7 cells with a plasmid expressing this N4BP1 D623
176 mutant and the HBV overlength genome also did not lead to a decrease in rcDNA levels (Fig 5C).
177 These results suggest that both the KH-like and RNase domains are critical for the anti-HBV effect of
178 N4BP1.

179

180 **N4BP1 promotes pgRNA degradation**

181 Having shown the effect of N4BP1 on rcDNA, we then sought to determine more specifically

182 which step of HBV replication N4BP1 suppresses. To that end, we assessed the impact of N4BP1
183 overexpression on cccDNA and pgRNA levels. HepG2-hNTCP C4 cells were infected with HBV and
184 cells were harvested at 14 dpi. While cccDNA levels were not affected by overexpression of N4BP1,
185 pgRNA and rcDNA levels were both significantly reduced (Fig 6A-6C).

186 For further verification, we performed the same experiments using PHHs. Adenovirus vectors
187 were used to overexpress N4BP1 in PHHs (Fig 6D), and the cells were then infected with HBV
188 genotype C. As shown in Fig 6E-6G, even in PHHs, cccDNA levels were comparable to control cells,
189 but pgRNA and rcDNA levels were both significantly decreased. Collectively, these results indicate
190 that N4BP1 augmented pgRNA degradation.

191 To elucidate the mechanism of pgRNA suppression by N4BP1, we turned to HepAD38.7 cells.
192 First, the cells were cultured for 2 days in media without tetracycline to induce HBV replication. Then
193 N4BP1 or the domain deletion mutant plasmids were transfected, and tetracycline added at 2 dpt to
194 stop viral replication. Cells were collected starting 2 days after adding tetracycline to measure the
195 pgRNA levels (Fig 6H). As shown in Fig 6I, the expression of wild-type N4BP1 augmented the rate
196 of pgRNA degradation. In contrast, both domain deficient mutants resulted in pgRNA degradation at
197 a rate similar to the control, suggesting that N4BP1 affects the degradation rate of pgRNA. Next, we
198 examined whether N4BP1 expression also suppresses HBV RNAs other than pgRNA. We co-
199 transfected Huh7 cells with a plasmid expressing N4BP1 and a plasmid containing 1.3-fold-overlength

200 genomes of HBV genotype C. At 3 dpt, cells were collected and HBV RNA expression was measured
201 by Northern blotting. Results showed that control Huh7 cells showed intense expression of 3.5kb,
202 2.4kb, and 2.1kb HBV RNA, whereas N4BP1 overexpression Huh7 cells showed weak expression of
203 all these RNAs (Fig 6J).
204
205 **N4BP1 expression is induced by IFN-α/λ in PHHs**
206 It has been reported that N4BP1 expression is increased 3- to 5-fold with IFN-α treatment in
207 Jurkat cells and THP-1 cells[20]. To see if this was the case in PHHs and hepatoma cell lines, we
208 treated Huh7, HepG2-hNTCP C4, and PHHs with 1000 U/ml of IFN-α and 100 ng/ml of IFN-λ, which
209 are known to also inhibit HBV replication. Cells were collected at 24 and 48 hours post-drug treatment
210 and N4BP1 was examined by qPCR and WB (Fig 7A). The ISG15 was included as a positive control.
211 As shown in Fig 7B and 7C, levels of N4BP1 mRNA as measured by qPCR were slightly increased
212 in both Huh7 and HepG2-hNTCP C4 cells by IFN-α or IFN-λ. WB also showed that IFN-α or IFN-λ
213 treatments had no effect on N4BP1 protein levels in Huh7 cells and HepG2-hNTCP C4 cells. In
214 contrast, IFN-α and IFN-λ treatment in PHHs resulted in an approximately 2-fold increase in N4BP1
215 mRNA expression and protein level was also increased. Collectively, these results indicate that IFN-
216 α and IFN-λ treatment slightly induce N4BP1 expression in Huh7 and HepG2-hNTCP C4 cells while
217 significantly inducing N4BP1 expression in PHHs.

218 Since N4BP1 expression was induced by IFN treatment in the primary cells and not the hepatoma
219 cell lines, we thought that N4BP1 expression may differ depending on the immune status of the human
220 liver. Therefore, we assessed ISG15 and N4BP1 mRNA expression in HBV-HCC resection human
221 liver tissue samples. ISG15 (Fig 8A) and N4BP1 (Fig 8B) mRNA expression in the HBV DNA
222 negative group tended to be higher than in the HBV DNA positive group.
223

224 **Discussion**

225 NAs are generally well tolerated, very effective in inhibiting HBV replication, and are currently
226 the most commonly used treatment for chronic hepatitis B. However, since NAs inhibit reverse
227 transcription, HBV cccDNA remains in the nucleus of hepatocytes so the patient is never completely
228 cured. Indeed, patients who discontinue NA treatment can experience virologic relapse and thus must
229 continue NA therapy for extended periods. Long-term treatment with NA is associated with the
230 development of antiviral drug resistance; for example, mutations in the HBV polymerase gene, which
231 is a target of NAs, have been reported[21-23]. Resistance to NA therapy can lead to hepatitis relapse
232 and even death. Thus, we sought to identify host factors involved in HBV proliferation that would be
233 distinct from the viral sites targeted by NAs. In a library of 132 RBPs containing proteins with known
234 RNA-binding domains such as RNA recognition motif domains or zinc finger domains, we found that
235 N4BP1 inhibited HBV replication, was negatively correlated with HBV rcDNA levels in hepatoma

236 cell lines and suppressed HBV replication by promoting pgRNA degradation.

237 We found that both the KH-like domain and the RNase domain of N4BP1 were essential for the
238 protein's anti-HBV effect (Fig 5B). It has been reported that the KH-like domain binds to RNA or
239 single-strand DNA and is involved in splicing, transcriptional regulation, and translational
240 regulation[19, 24, 25]. On the other hand, the RNase domain of N4BP1 is also involved in regulating
241 genome stability and repressing the expression of target genes through mRNA strand breaks[26]. Thus,
242 it is possible that N4BP1 binds to HBV RNA through its KH-like domain and degrades HBV RNA by
243 the RNase activity of its RNase domain. In fact, in this study, N4BP1 suppressed HBV replication at
244 the step of pgRNA production in the HBV life cycle (Fig 6B, 6F, 6I). We also found that N4BP1
245 suppresses not only the 3.5 kb RNA of HBV, but also the 2.4 kb and 2.1 kb RNAs by NB (Fig 6J),
246 which means N4BP1 suppresses pgRNA, PreS1 and PreS2/S.

247 N4BP1 is known to be an inhibitor of innate immunity and inflammation-mediated cytokine
248 production. NF-κB is an important transcription factor for the elicitation of cytokine responses, and
249 its activation is necessary for many immune responses. N4BP1 interacts with the NF-κB signal
250 essential modulator (NEMO, also known as IκB kinase γ) to suppress Toll-like receptor (TLR)–
251 dependent NF-κB activation [27]. Furthermore, even in vivo, it has been reported that N4BP1-/- mice
252 develop mild inflammation and have exacerbated TLR-dependent inflammatory responses[28].
253 Recently, N4BP1 was found as a new hotspot gene for HBV integration[29]. Although the significance

254 of HBV integration to N4BP1 remains unknown, it is interesting that the virus integration occurred to
255 N4BP1 which has anti-HBV activity. Regarding virus replication, N4BP1 is known to directly
256 recognize and degrade retroviruses such as HIV-1[20] and to be involved in replication of the Porcine
257 Reproductive and Respiratory Syndrome Virus[30]. In both cases, the RNase activity of N4BP1 was
258 found to inhibit viral replication by directly degrading viral RNA, consistent with our data. In addition,
259 the RNase domain of N4BP1 has a similar structure to that of MCPIP1[31], which has been reported
260 to recognize and degrade HBV pgRNA directly[15]. However, MCPIP1 is a cytoplasmic RNase,
261 whereas N4BP1 is mainly localized on the nucleus. Furthermore, MCPIP1 has not been reported to
262 suppress 2.4 kb and 2.1 kb HBV RNAs, whereas we have demonstrated that N4BP1 suppresses these
263 RNAs. These results suggest that MCPIP1 and N4BP1 may regulate HBV degrading mRNAs through
264 different processes. The mechanism behind our finding that the KH-like domain of N4BP1 was also
265 important for the anti-HBV effect of this protein is presumably due to its binding to HBV RNA but
266 further work is needed to elucidate the mechanism more precisely.

267 Although N4BP1 expression has been reported to be induced by type I IFN[20], stimulation of
268 N4BP1 expression by IFN-α (type I IFN) and IFN-λ (type III IFN) treatment varied between the PHHs
269 and hepatoma cell lines we tested. This could be due to the disrupted IFN signaling reported in the cell
270 lines Huh7 and HepG2 that result in them being less sensitive to IFN[32, 33]. Also, ISG15 is an
271 inducible factor by IFN-α and IFN-λ[34, 35]. In characterizing N4BP1 expression, we found that

272 N4BP1 expression was not induced by HBV infection or transfection in hepatoma cell lines (Fig 4A-
273 4D). Furthermore, in clinical liver tissue samples, N4BP1 expression was significantly lower in HBV-
274 DNA positive vs HBV-DNA negative samples (Fig 8B). These results suggest that N4BP1 expression
275 may differ according to each individual and that the inherent expression level of N4BP1 is related to
276 HBV DNA negative conversion. Otherwise, it is thought that factors upstream of N4BP1 may cause
277 differences in N4BP1 expression and affect HBV DNA negative conversion. Regardless, a greater of
278 samples needs to be examined in order to further understand the relationship between the expression
279 of N4BP1 and HBV.

280 In conclusion, to investigate different treatments against HBV infection from currently approved
281 HBV therapeutics, we focused on RBPs. We found that N4BP1 is a RBP with novel anti-HBV activity,
282 inhibiting HBV replication via degradation of HBV pgRNA. Although the detailed molecular
283 mechanism behind the anti-HBV effect of N4BP1 remains to be uncovered, understanding the
284 association between N4BP1 and HBV could contribute to the development of new HBV treatments.

285

286 **Materials and methods**

287 **Cell culture**

288 All cells were grown at 37℃ in a 5% (v/v) CO2 environment. Huh7 cells and HEK293T cells
289 were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan,

290 JCRB0403 and JCRB9068). HepG2-hNTCP C4 cells and HepAD38.7 cells were kindly provided by
291 Takaji Wakita (NIID, Tokyo, Japan). PHHs were obtained from Phoenix Bio (Hiroshima, Japan).
292 Huh7 cells and HEK293T cells were maintained in DMEM (Nacalai Tesque, Kyoto, Japan)
293 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin (Nacalai Tesque) and 10% fetal
294 bovine serum (FBS, Sigma, MO, USA). HepG2-hNTCP C4 cells were maintained in the
295 aforementioned media containing 400 μg/ml G418 (Nacalai Tesque). HepAD38.7 cells were
296 maintained in DMEM/Ham's F-12 media (Nacalai Tesque) supplemented with 10% FBS, 100 U/ml
297 of penicillin, 100 μg/ml of streptomycin, 400 μg/ml of G418, 5 μg/ml of insulin (Nacalai Tesque), and
298 400 ng/ml of tetracycline (Nacalai Tesque). PHHs were maintained in PHH-specific media provided
299 by the manufacture (Phoenix Bio).
300
301 **Generation of RBP overexpressing cells**
302 RBP-overexpressing Huh7 cells were generated by transfection of RBP expression plasmids
303 using TransIT LT-1 transfection reagent (Mirus, WI, USA). Briefly, Huh7 cells were seeded in 24-
304 well plates (Greiner, Bad Haller, Austria) 1 day prior to the transfection. The following day, 1 μl of
305 TransIT LT-1 transfection reagent, 0.3 μg of RBP expression plasmid, and 0.1 μg of a plasmid
306 containing a 1.3-fold-overlength genome of HBV (genotype C) were mixed in 50 μl of Opti-MEM
307 (Thermo Fisher Scientific, MA, USA). After 15 minutes incubation at room temperature, the mixture

308 was added into the supernatant of the cultured Huh7 cells. Entecavir (Fujifilm, Tokyo, Japan) was
309 added at 5 μM the day after transfection as a positive control.

310 RBP-overexpressing HepG2-hNTCP C4 cells were made by lentiviral transduction. Lentiviruses
311 were made by transfecting HIV gag/pol, VSV-G, REV, and pcs-II EF RBP expression plasmids into
312 HEK293T cells using TransIT LT-1 transfection reagent. At 2 dpt, the supernatant of HEK293T was
313 collected and spun at 4,200 rpm for 2 minutes at room temperature and the supernatant added to
314 HepG2-hNTCP C4 cells. Entecavir or heparin was used as a positive control. Entecavir was added at
315 5 μM the day after infection. Heparin (Mochida Pharmaceutical Co., Tokyo, Japan) was added at 5
316 U/ml 30 min before infection.

317 To express N4BP1 in PHHs, we used an adenovirus vector (AdV). The N4BP1expressing
318 adenovirus vector (N4BP1 AdV) was constructed using the cosmid cassette pAxEFwit2, which has a
319 cloning site *SwaI* flanked by the EF1α promoter and rabbit β-globin polyA signal in the E1-deleted
320 region of the adenovirus genome. The N4BP1 cDNA was prepared from the pCS2-EF-N4BP1-HA
321 plasmid and inserted into the *SwaI* cloning site. The AdV was prepared as previously described[36,
322 37]. The AdV was titrated using the methods described by Pei et al[38]. Briefly, the copy numbers of
323 viral genome successfully transduced into target cells were measured by qPCR. For generating
324 N4BP1-overexpressing PHHs, cells were infected with N4BP1 AdV at a multiplicity of infection
325 (MOI) of 5 two days before HBV infection.

326

327 **Generation of RBP knockdown cells**

328 RBP knockdown cell lines were generated using Silencer Select siRNA (Thermo Fisher
329 Scientific) and Lipofectamine RNAiMAX (Thermo Fisher Scientific) following the manufacturer's
330 protocol. Briefly, 1.5 µl of Lipofectamine RNAiMAX and 5 pg of siRNA were mixed in 50 µl of Opti-
331 MEM. After incubating for 5 minutes at room temperature, the mixture was gently added to the cell
332 supernatant. The following siRNA reagents were used in this study: control siRNA (Silencer Select
333 Negative Control No.1 siRNA, 4390843), ZCCHC10 siRNA#1 (s29507), ZCCHC10 siRNA#2
334 (s29508), ZCCHC10 siRNA#3 (s29509), HNRNPC siRNA#1 (s6719), HNRNPC siRNA#2 (s6720),
335 HNRNPC siRNA#3 (s6721), N4BP1 siRNA#1 (s18638), N4BP1 siRNA#2 (s18639).

336

337 **Generation of N4BP1 knockout cells**

338 N4BP1 KO Huh7 cells were generated by CRISPR/Cas9 using pX330-U6-Chimeric_BB-CBh-
339 hSpCas9 (pX330, Addgene, MA, USA) and HR110PA-1 (System Biosciences, CA, USA). The
340 pX330-puro vector was constructed by integrating the puromycin resistance gene sequence from the
341 HR110PA-1 vector into the pX330 vector. The pX330-puro vector was digested using *BbsI* (Biolabs,
342 MA, USA) and the target sequence was subcloned. The plasmid was transfected into Huh7 cells using
343 TransIT LT-1 transfection reagent and after selection with puromycin dihydrochloride (Invivogen, CA,

344 USA), a single colony was isolated using cloning cylinders (Merck, Darmstadt, Germany) and
345 propagated. KO of the target gene was confirmed by WB and DNA sequencing.
346
347 **Plasmid Constructions**
348 We used the following plasmids containing 1.3-fold-overlength genomes of different HBV
349 genotypes: pUC19-Ae_us (genotype A, accession no. AB246337), pGEM-Bj-JPN56 (genotype B,
350 accession no. AB246342), and pUC19-C_JPNAT (genotype C, accession no. AB246345).
351 Each RBP expression plasmid had a C-terminus HA tag and was inserted between the *XhoI* and
352 *XbaI* sites of the pcs-II EF vector. The KH-like domain deletion mutant (ΔKH), RNase domain
353 deletion mutant (ΔRNase), and D623N mutant were generated from the N4BP1 expression plasmid.
354 ΔKH plasmid was generated by deleting amino acids 59-143 from N4BP1; ΔRNase was generated by
355 deleting amino acids 617-769 N4BP1; and D623N was generated by changing the 623rd amino acid
356 from asparagine acid to asparagine. To insert these mutated N4BP1 genes into the pcs-II EF vector
357 between the *XhoI* and *XbaI* sites, we used the following primer sets and In-Fusion Snap Assembly
358 Master Mix (Takara Bio, Shiga, Japan) per the manufacturer's directions. Each N4BP1 mutant plasmid
359 had a C-terminus HA tag.
360 ΔKH: 5'- CGCTACCGGTCTCGAGATGGCGGCCCGGGCGGTGCT -3' (forward)
361 5'- GGGTAGGTTCTCTTTCGCCCCGCAGAGCTGCAGCC -3' (reverse) , and

362 5’- CAGCTCTGCGGGGCGAAAGAGAACCTACCCAGTAG -3’ (forward)

363 5’- GGTACATGGTCTCGAGATCCAACACCATGGCAGAAA -3’ (reverse)

364 ΔRNase: 5’- CGCTACCGGTCTCGAGATGGCGGCCCGGGCGGTGCT -3’ (forward)

365 5’- TTCCTTCTGAAGAAAATCCGTTCTCCCTGGTTCAT -3’ (reverse) , and

366 5’- CCAGGGAGAACGGATTTTCTTCAGAAGGAAGTCTG -3’ (forward)

367 5’- GGGTAGGTTCTCTTTCGCCCCGCAGAGCTGCAGCC -3’ (reverse)

368 D623N: 5’- CGCTACCGGTCTCGAGATGGCGGCCCGGGCGGTGCT -3’ (forward)

369 5’- AACATTGCTCCCATTTATAACAATGTGTTTCAAAT -3’ (reverse) , and

370 5’- AATGGGAGCAATGTTGCAATTACCCATGGTCTGAA -3’ (forward)

371 5’- GGTACATGGTCTCGAGATCCAACACCATGGCAGAAA -3’ (reverse)

372

373 **Viruses**

374 The production of HBV particles (genotype D) was adapted from a previous report[39]. Briefly,

375 HepAD38.7 cells were cultured under tetracycline (400 ng/mL) for maintenance. To produce HBV

376 particles, tetracycline was removed and the supernatant collected after 6 and 9 days. The collected

377 supernatant was mixed with 30% polyethylene glycol (PEG) 8000 (Sigma) and incubated overnight

378 at 4℃. Then it was spun at 3,000 rpm for 20 minutes at 4℃ for concentration. Before HBV infection,

379 HepG2-hNTCP C4 cells were seeded in collagen type I-coated 24-well plates (Iwaki, Tokyo, Japan)

380 and transduced with lentivirus to express RBP (see “Generation of RBP-overexpressing cells” above).
381 At 2 days post-transduction, the cells were infected with 5,000 genome equivalents (GEq)/cell of HBV
382 in DMEM containing 3% DMSO (Nacalai Tesque) and 4% PEG 8000, and the media was changed
383 every 2-3 days until 14 dpi.

384 HBV genotype C for PHHs infection was purchased from Phoenix Bio. The infection into PHHs
385 was performed at 5 Geq/cell in PHH-specific media containing 40% PEG 8000 following the
386 manufacturer’s protocol. The culture media was replaced every 4 days. Cells were harvested at 14 dpi
387 for WB and reverse transcription quantitative PCR (RT-qPCR).

388

389 **Western blotting**

390 Cells were lysed with 2 × Laemmli sample buffer (Bio Rad Laboratories, CA, USA) containing
391 10% 2-mercaptoethanol (Sigma) and boiled for 10 minutes at 100℃. Then lysates were loaded on a
392 10-20% gradient SDS-PAGE gel (Atto, Tokyo, Japan) and transferred onto a polyvinylidene fluoride
393 transfer membrane (Millipore, MA, USA). Membranes were immersed in Block Ace (Kac, Kyoto,
394 Japan) containing 1% bovine serum albumin (Sigma) and incubated for 1 hour at room temperature.
395 The blocking solution was then decanted, and replaced with Can Get Signal Solution 1 (Toyobo, Osaka,
396 Japan) containing the appropriate antibodies described below and incubated for 1 hour. The
397 membranes were washed with 1 × PBS (Fujifilm) containing 0.1% polyoxyethylene (20) sorbitan

398 monolaurate (Fujifilm) and incubated with Can Get Signal Solution 2 (Toyobo) containing horseradish
399 peroxidase-conjugated antibody against rabbit or mouse immunoglobulins for 1 hour. After addition
400 of Amersham ECL Prime Western Blotting Detection Reagents (Cytiva, Tokyo, Japan), the
401 membranes were subsequently imaged via LuminoGraph I (Atto).

402 The following antibodies were used in this study: anti-N4BP1 (Bethyl Laboratories, TX, USA),
403 anti-ZC3H12B (Gene Tex, CA, USA), anti-HNRNPC (abcam, Cambridgeshire, UK), anti-ZCCHC10
404 (Sigma), anti-KHNYN (Fujifilm), anti-HA tag (Biolegend, CA, USA), anti-rabbit IgG (H+L) HRP
405 (Jackson Immuno Research Laboratories, MD, USA), anti-mouse IgG (H+L) HRP (Thermo Fisher
406 Scientific), and anti-GAPDH (Fujifilm). The antibodies used in this study are listed in S2 Table.

407

408 **Purification of intracellular HBV rcDNA**

409 To extract intracellular HBV DNA, cells were washed twice with PBS, and cells were lysed in
410 lysis buffer (100 mM Tris-HCl [pH 8.0], 0.2% NP-40) for 15 minutes at 4℃. After spinning at 13,000
411 rpm for 1 minute at room temperature, the supernatant was collected and incubated with 1 M MgCl2,
412 0.2 mg/ml of DNase I (Sigma), and 10 mg/ml of RNase A (Nacalai Tesque) for at least 3 hours at
413 37℃. After adding 500 mM EDTA and 5 M NaCl, the lysates were digested with 20 mg/ml of
414 proteinase K (Kanto Chemical, Tokyo, Japan) and 10% SDS for 5 hours at 55℃. The extracted HBV
415 DNA was purified using phenol-chloroform-isoamyl alcohol (Sigma), then precipitated with

416 isopropanol, 0.5 mg/ml of glycogen, and 3M sodium acetate. After washing with 70% ethanol, the
417 purified HBV DNA was resolved in 50 μl pure water. HBV rcDNA was detected by qPCR using
418 Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). As a standard for rcDNA,
419 pUC19-C_JPNAT was used. qPCR procedures were following the manufacturer's protocol. Briefly,
420 the samples were incubated at 50℃ for 2 minutes and at 95℃ for 10 minutes to initial denaturation.
421 Then, the samples were incubated at 95℃ for 15 seconds and at 60℃ for 1 minute for 40 cycles to
422 polymerase reaction. The following primer pair was used in this study.
423 rcDNA: 5'- GGAGGGATACATAGAGGTTCCTTGA -3' (forward)
424 5'- GTTGCCCGTTTGTCCTCTAATTC -3' (reverse)
425
426 **Quantification of intracellular HBV cccDNA level**
427 To extract intracellular HBV cccDNA, the cells were lysed by lysis buffer (10mM Tris-HCl [pH
428 7.4], 10mM EDTA, 0.5% SDS) and 5M NaCl was added. After incubation overnight at 4℃, the
429 samples were spun at 15,000 rpm for 30 minutes at 4℃ and the supernatant was collected to isolate
430 protein-free DNA. The DNA was purified a total of three times by phenol (Nacalai Tesque), phenol-
431 chloroform-isoamyl, and chloroform (Fujifilm). After precipitation with isopropanol and ethanol,
432 Plasmid-Safe ATP-Dependent DNase (Lucigen, WI, USA) was added to the purified DNA to remove
433 double-stranded and circular DNA. The Purified DNA was extracted in 30 μl of 10mM Tris-Cl (pH

434 8.5) using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan). HBV cccDNA
435 was measured by qPCR using TaqMan Fast Advanced Master Mix (Applied Biosystems, CA, USA).
436 As a standard for cccDNA, pUC19-C_JPNAT was used. qPCR procedures were done per the
437 manufacturer's protocol. In brief, the samples were incubated at 50℃ for 2 minutes and at 95℃ for 10
438 minutes to initiate denaturation. Then, the samples were incubated at 95℃ for 15 seconds and at 60℃
439 for 1 minute for 50 cycles for the polymerase reaction. The following primer pair and probe were used
440 in this study.
441 cccDNA: 5'- CGTCTGTGCCTTCTCATCTGC -3' (forward)
442 5'- GCACAGCTTGGAGGCTTGAA -3' (reverse)
443 5'- CTGTAGGCATAAATTGGT -3' (Probe)
444
445 **Measurement of intracellular HBV pgRNA**
446 Total RNA including pgRNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden,
447 Germany) following the manufacturer's protocol. The extracted RNA was resolved in 50 µl pure water
448 and measured by RT-qPCR using Power SYBR Green RNA-to-Ct 1-step Kit (Applied Biosystems).
449 The pgRNA expression level was normalized against the expression level of GAPDH as a
450 housekeeping gene and calculated by the ΔΔCT method. RT-qPCR procedures were done following
451 manufacturer's protocol. First, the samples were incubated at 50℃ for 2 minutes and at 95℃ for 10

452 minutes to intiate denaturation. Then, the samples were incubated at 95℃ for 15 seconds and at 60℃
453 for 1 minute for 40 cycles for the polymerase reaction. Samples were then incubated at 95℃ for 15
454 seconds, 60℃ for 1 minute, and 95℃ for 15 seconds for the melting curve measurement. The following
455 primer pairs were used in this study.
456 pgRNA: 5'- TCCCTCGCCTCGCAGACG -3' (forward)
457 5'- GTTTCCCACCTTATGAGTC -3' (reverse)
458 GAPDH: 5'- AGAAGGCTGGGGCTCATTTG -3' (forward)
459 5'- AGGGGCCATCCACAGTCTTC -3' (reverse)
460
461 **pgRNA degradation assay**
462 HepAD38.7 cells were seeded in collagen type I-coated 12-well plates (Iwaki) and cultured
463 overnight in media containing tetracycline hydrochloride. After washing twice with PBS, cells were
464 cultured without tetracycline for 48 hours. Then, the cells were transfected with N4BP1 wild-type
465 expression plasmid, ΔKH expression plasmid, ΔRNase expression plasmid, or control vector. These
466 plasmids were transfected as follows: 100 μl of Opti-MEM, 6 μl of TransIT LT-1, and 3μg of
467 expression plasmid. After transfection of plasmids, cells were cultured for 48 hours and changed to a
468 tetracycline-containing media in order to stop HBV production. Cells were collected at 2, 3, and 4
469 days after changing to tetracycline-containing media. Total RNA, including pgRNA, was extracted as

470 described above for RT-qPCR.

471

472 **Drug treatment assay**

473 Huh7 cells, HepG2-hNTCP C4 cells, and PHHs were stimulated with 1000 U/ml of IFN-α (PBL
474 Assay Science, NJ, USA) or 100 ng/ml of IFN-λ (Pepro Tech, NJ, USA). At 24 and 48 hours post-
475 drug stimulation, total RNA in cells was extracted for qPCR using the RNeasy Mini Kit, and cDNA
476 was synthesized using SuperScript IV VILO Master Mix (Invitrogen). For WB, the cells were lysed
477 with 2 × Laemmli sample buffer containing 10% 2-mercaptoethanol and processed as described above.
478 qPCR was performed using Power SYBR Green PCR Master Mix. The mRNA expression levels of
479 N4BP1 and ISG15 were normalized against the expression level of GAPDH as a housekeeping gene
480 and calculated by the ΔΔCT method. The following primer pairs were used in this study.

481 N4BP1: 5'- CCCGATGATCCTCTGGGAAG -3' (forward)

482 5'- TTTGGCAGGGCACTGAGTAG -3' (reverse)

483 ISG15: 5'- ACTCATCTTTGCCAGTACAGGAG -3' (forward)

484 5'- CAGCATCTTCACCGTCAGGTC -3' (reverse)

485 GAPDH: see above

486

487 **Analysis of N4BP1 expression in clinical liver samples**

488 To assess the N4BP1 expression of HBV-HCC resection samples, we confirmed that all
489 specimens were HBs-Ag (+), HBe-Ag (-), and HBe-Ab (+) as documented in the patient medical
490 records. The samples were divided into an HBV DNA positive group (n=9) and an HBV DNA negative
491 group (n=8). In all cases, we used tissue from non-cancerous areas. RNA was extracted from the
492 resected tissues using the RNeasy Mini Kit and N4BP1 expression was measured by qPCR as
493 described above. This study was approved by the Institutional Review Board of the Graduate School
494 of Medicine, Hokkaido University (No. 14-015, 022-0195). All patients participated in this study
495 provided written informed consent for the use of their samples and clinical data and they were
496 informed of the opportunity to opt out.
497
498 **Northern blotting**
499 Huh7 cells were co-transfected with a plasmid containing the 1.3-fold-overlength genome of
500 HBV genotype C and N4BP1 using TransIT LT-1 transfection reagent, and cells were collected at 3
501 dpt. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) and subjected to
502 electrophoresis in 1% agarose-formaldehyde gel and morpholinepropanesulfonic acid buffer (Nacalai
503 Tesque). Ribosomal RNA was visualized by ethidium bromide staining, and electrophoresed RNA
504 was transferred onto a positively charged nylon membrane (Roche, Basel-Stadt, Switzerland). An
505 HBV RNA probe and GAPDH RNA probe were linearized and synthesized by digoxigenin RNA

506 labeling kit (Roche). Hybridization and detection were performed using the DIG Northern Starter kit
507 (Roche) following the manufacturer's protocol. The signals were detected by LuminoGraph I (Atto).
508

509 **RNA sequencing**

510 PHHs were analyzed for RNA sequencing. N4BP1-overexpressing PHHs were infected with HBV
511 genotype C (5 Geq/cell) as described above. At 3 dpi, total RNA in PHHs was extracted using RNeasy
512 Mini Kit following the manufacturer's protocol. RNA sequencing was performed by Osaka University
513 (Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University,
514 Osaka, Japan). We ran 2 well (N4BP1-overexpressing) vs 2 well (control) samples. Whole
515 transcriptome sequencing was applied to RNA samples using the Illumina NovaSeq 6000 platform in
516 a 101 bp single-end mode. Sequenced reads were mapped to the human reference genome sequence
517 (hg19) using TopHat version 2.2.1 in combination with Bowtie 2 version 2.2.3 and SAMtools version
518 1.0. The number of fragments per kilobase of exon per million mapped fragments was calculated using
519 Cufflinks version 2.2.1. The obtained data were analyzed by iDEP.951[40]. The cutoff value of gene
520 expression was set at fold change >2 or < 0.5 and FDR < 0.05 compared to control cells. To review
521 GEO data, access https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225322. The accession
522 No. is GSE225322.
523

524 **Statistical analysis**

525 Data are averages of three or more independent experiments. All data in figures are presented as
526 the mean ± standard deviation (SD). Evaluation of data was performed by Mann-Whitney *U* test. *P*-
527 value of < 0.05 was considered statistically significant. Statistical analysis was performed using JMP
528 Pro version 16.

529

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533

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560 for any part of the work.

561

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702

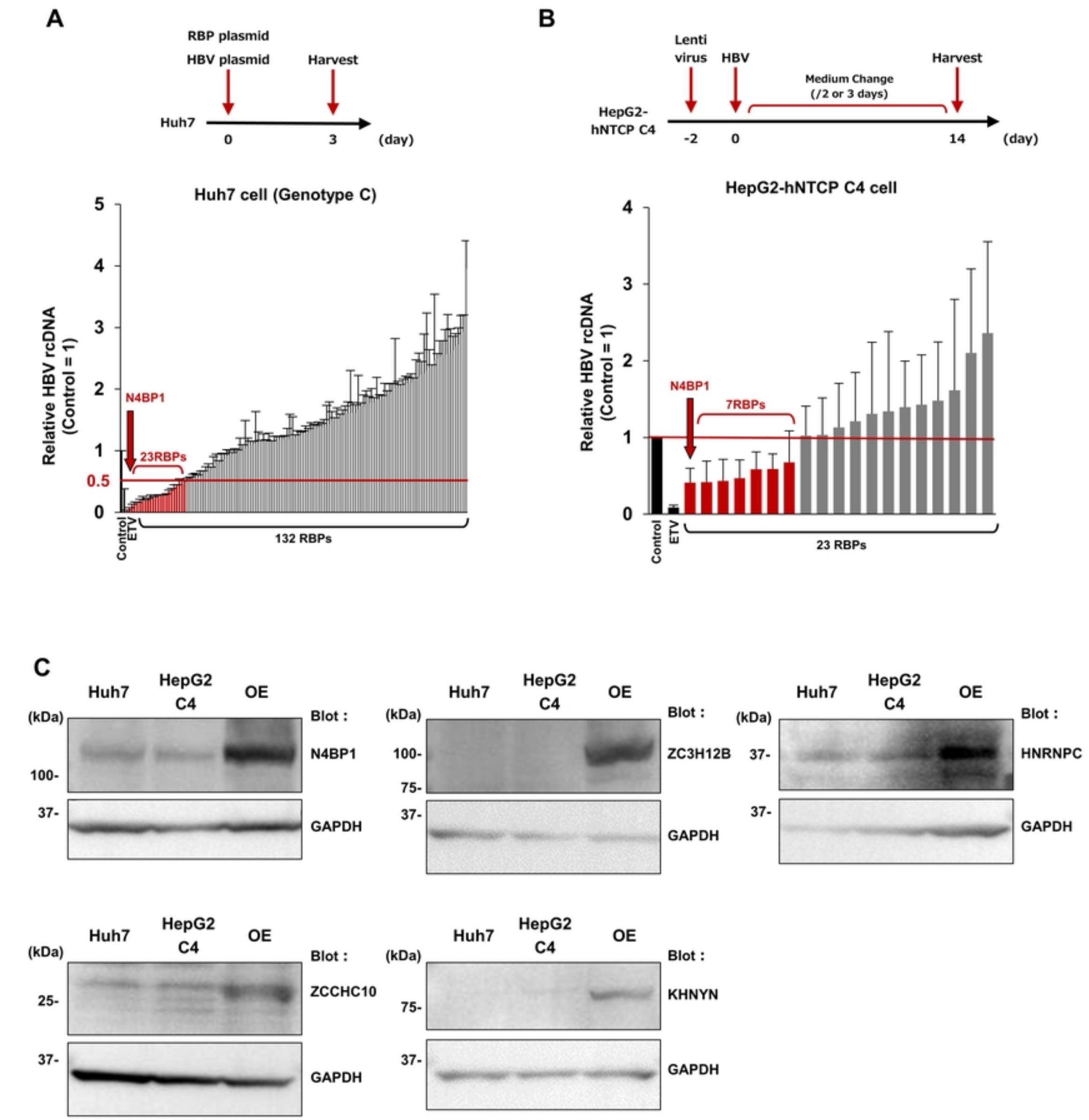
A
RBP plasmid
HBV plasmid
Harvest
Huh7
0
3
(day)
Huh7 cell (Genotype C)
Relative HBV rcDNA (Control = 1)
N4BP1
23RBPs
0.5
Control
ETV
132 RBPs
B
Lenti virus
HBV
Harvest
Medium Change (/2 or 3 days)
HepG2-hNTCP C4
-2
0
14
(day)
HepG2-hNTCP C4 cell
Relative HBV rcDNA (Control = 1)
N4BP1
7RBPs
Control
ETV
23 RBPs
C
Huh7
HepG2 C4
OE
(kDa)
Blot :
N4BP1
100-
37-
GAPDH
ZC3H12B
75-
HNRNPC
ZCCHC10
25-
KHNYN

Figure1

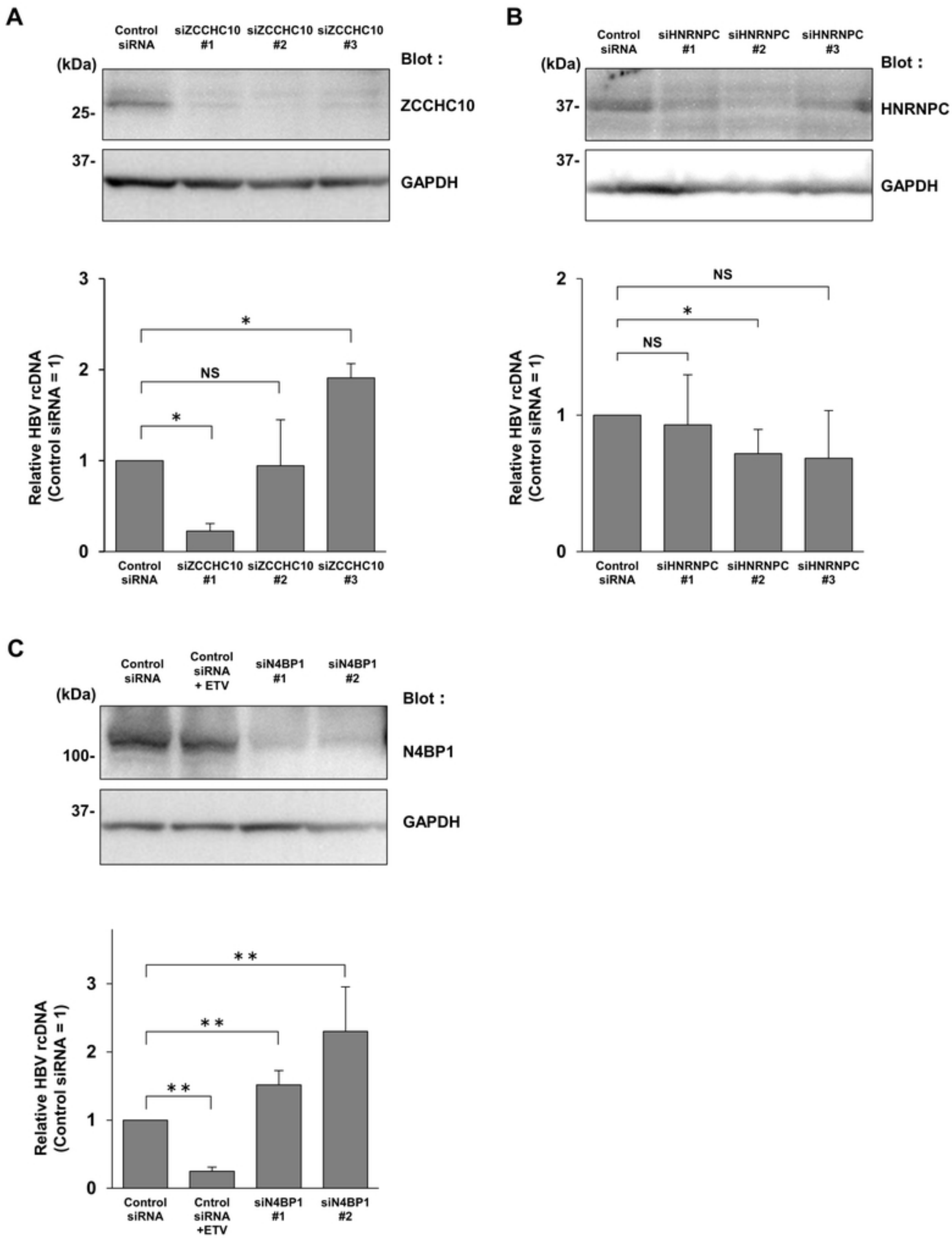
A
Control siRNA
siZCCHC10 #1
siZCCHC10 #2
siZCCHC10 #3
(kDa)
Blot :
25-
ZCCHC10
37-
GAPDH
Relative HBV rcDNA (Control siRNA = 1)
*
NS
*
B
Control siRNA
siHNRNPC #1
siHNRNPC #2
siHNRNPC #3
HNRNPC
NS
C
Control siRNA
Control siRNA + ETV
siN4BP1 #1
siN4BP1 #2
100-
N4BP1
Cntrol siRNA +ETV
**

Figure2

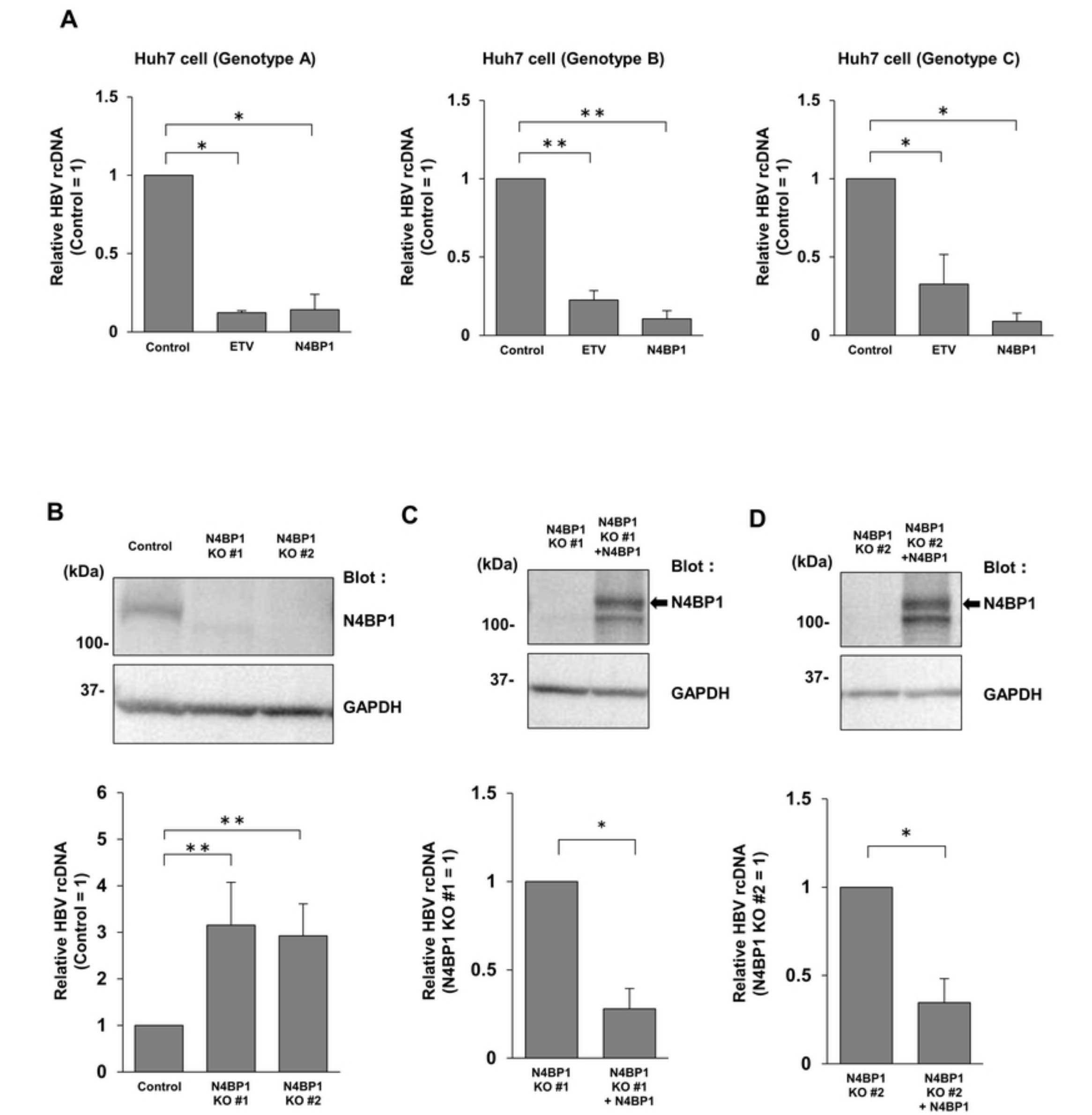
A
Huh7 cell (Genotype A)
Huh7 cell (Genotype B)
Huh7 cell (Genotype C)
Relative HBV rcDNA (Control = 1)
1.5
1
0.5
0
*
**
Control
ETV
N4BP1
B
Control
N4BP1 KO #1
N4BP1 KO #2
(kDa)
Blot :
N4BP1
100-
37-
GAPDH
C
N4BP1 KO #1
N4BP1 KO #1 +N4BP1
D
N4BP1 KO #2
N4BP1 KO #2 +N4BP1
Relative HBV rcDNA (Control = 1)
6
5
4
3
2
1
0
Relative HBV rcDNA (N4BP1 KO #1 = 1)
Relative HBV rcDNA (N4BP1 KO #2 = 1)
N4BP1 KO #1 + N4BP1
N4BP1 KO #2 + N4BP1

Figure3

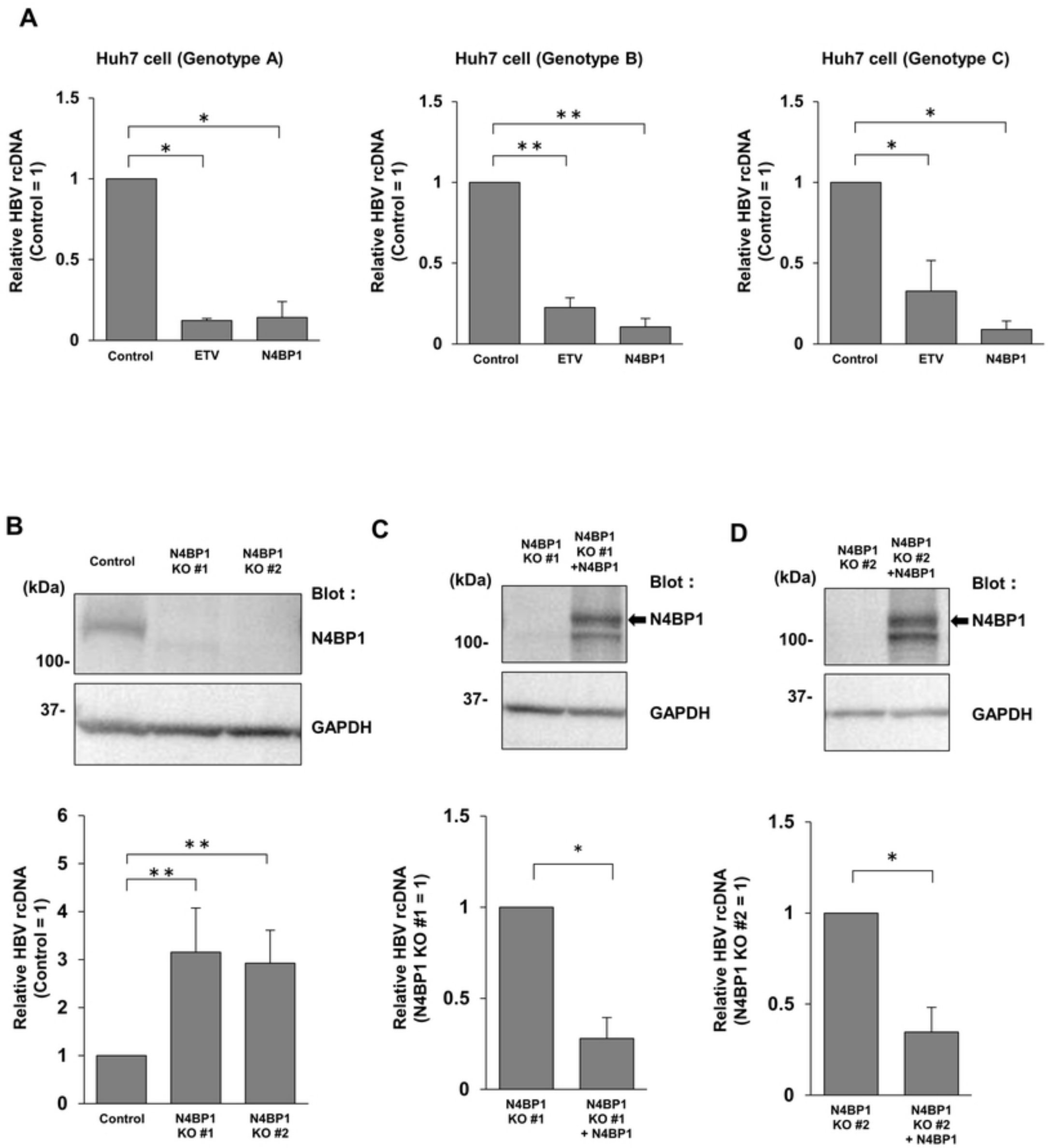
A
Huh7 cell (Genotype A)
Huh7 cell (Genotype B)
Huh7 cell (Genotype C)
Relative HBV rcDNA (Control = 1)
1.5
1
0.5
0
*
**
Control
ETV
N4BP1
B
Control
N4BP1 KO #1
N4BP1 KO #2
(kDa)
Blot :
N4BP1
100-
37-
GAPDH
Relative HBV rcDNA (Control = 1)
6
5
4
3
2
1
0
C
N4BP1 KO #1
N4BP1 KO #1 +N4BP1
Relative HBV rcDNA (N4BP1 KO #1 = 1)
D
N4BP1 KO #2
N4BP1 KO #2 +N4BP1
Relative HBV rcDNA (N4BP1 KO #2 = 1)
N4BP1 KO #1 + N4BP1
N4BP1 KO #2 + N4BP1

Figure4

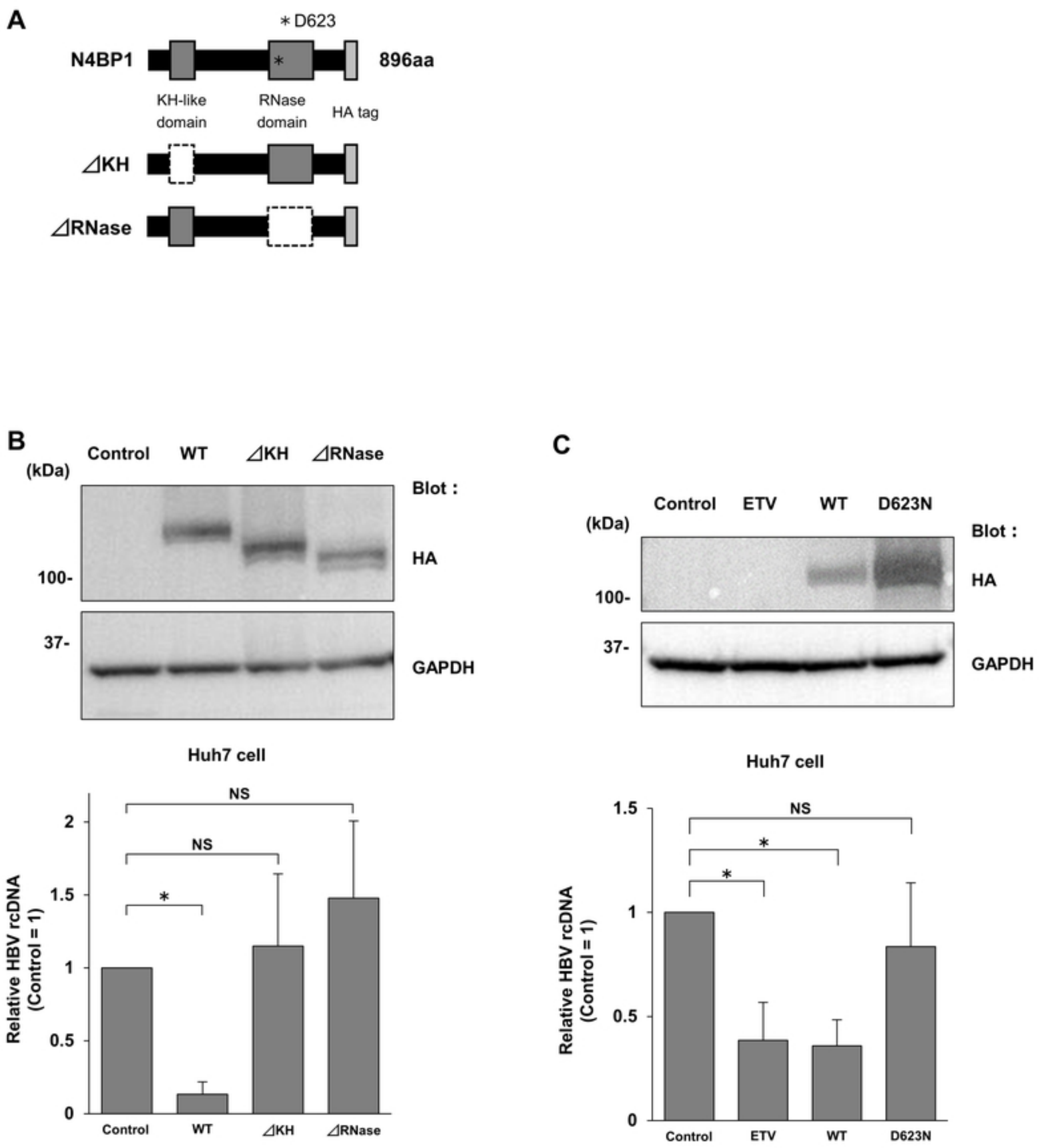
A
∗ D623
N4BP1
896aa
KH-like domain
RNase domain
HA tag
⊿KH
⊿RNase
B
Control
WT
⊿KH
⊿RNase
(kDa)
Blot :
HA
100-
37-
GAPDH
Huh7 cell
NS
NS
∗
Relative HBV rcDNA (Control = 1)
2
1.5
1
0.5
0
Control
WT
⊿KH
⊿RNase
C
Control
ETV
WT
D623N
(kDa)
Blot :
HA
100-
37-
GAPDH
Huh7 cell
NS
∗
∗
Relative HBV rcDNA (Control = 1)
1.5
1
0.5
0
Control
ETV
WT
D623N

Figure5

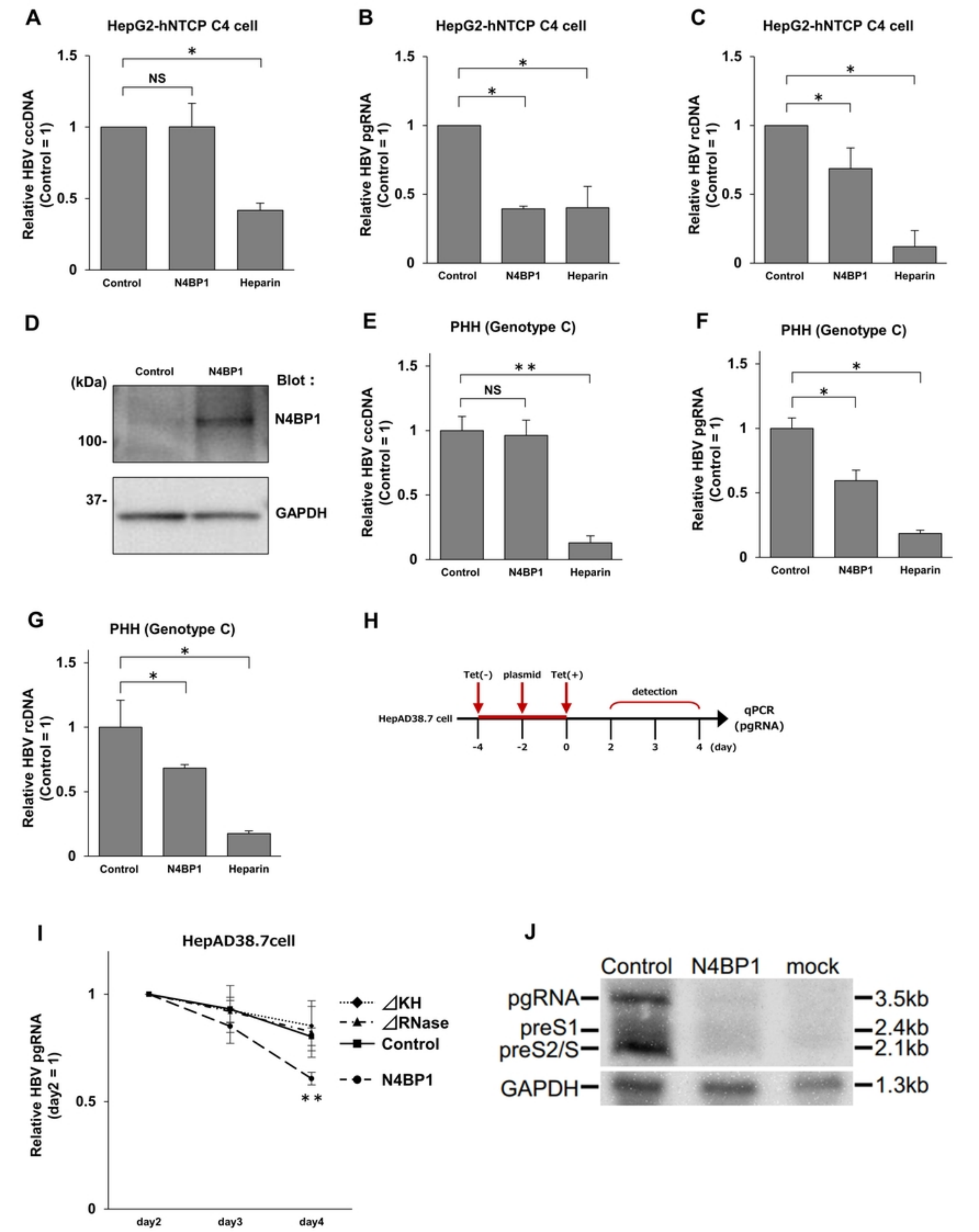
A
HepG2-hNTCP C4 cell
Relative HBV cccDNA (Control = 1)
NS
*
Control
N4BP1
Heparin
B
HepG2-hNTCP C4 cell
Relative HBV pgRNA (Control = 1)
C
HepG2-hNTCP C4 cell
Relative HBV rcDNA (Control = 1)
D
(kDa)
Control
N4BP1
Blot :
100-
37-
GAPDH
E
PHH (Genotype C)
Relative HBV cccDNA (Control = 1)
**
F
PHH (Genotype C)
Relative HBV pgRNA (Control = 1)
G
PHH (Genotype C)
Relative HBV rcDNA (Control = 1)
H
Tet(-)
plasmid
Tet(+)
detection
HepAD38.7 cell
qPCR (pgRNA)
-4
-2
0
2
3
4
(day)
I
HepAD38.7cell
Relative HBV pgRNA (day2 = 1)
⊿KH
⊿RNase
Control
N4BP1
day2
day3
day4
J
Control
N4BP1
mock
pgRNA
preS1
preS2/S
GAPDH
3.5kb
2.4kb
2.1kb
1.3kb

Figure6

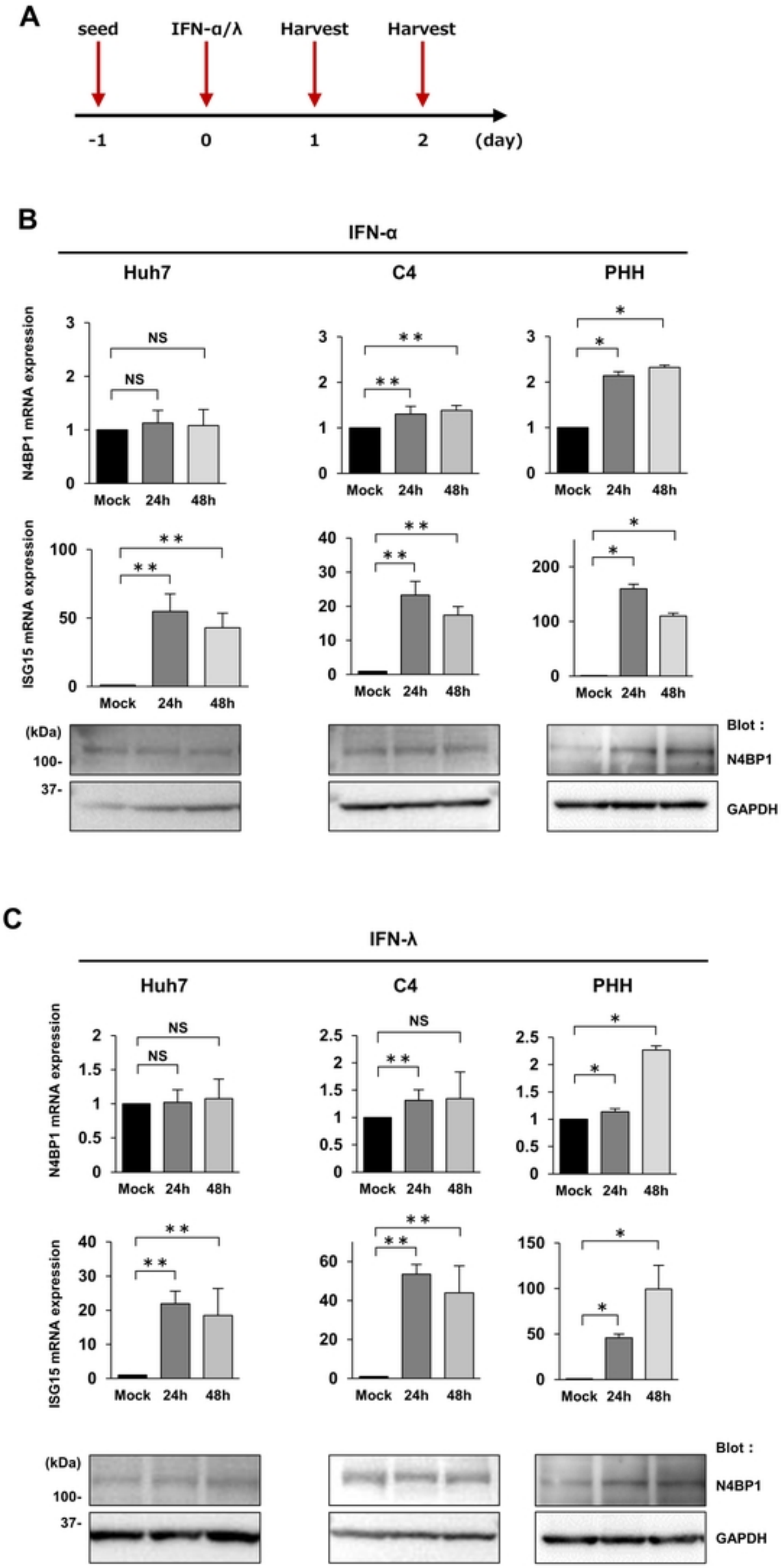
A
seed
IFN-α/λ
Harvest
Harvest
-1
0
1
2
(day)
B
IFN-α
Huh7
C4
PHH
N4BP1 mRNA expression
ISG15 mRNA expression
NS
Mock
24h
48h
(kDa)
100-
37-
Blot :
N4BP1
GAPDH
C
IFN-λ
Huh7
C4
PHH
N4BP1 mRNA expression
ISG15 mRNA expression
NS
Mock
24h
48h
(kDa)
100-
37-
Blot :
N4BP1
GAPDH

Figure7

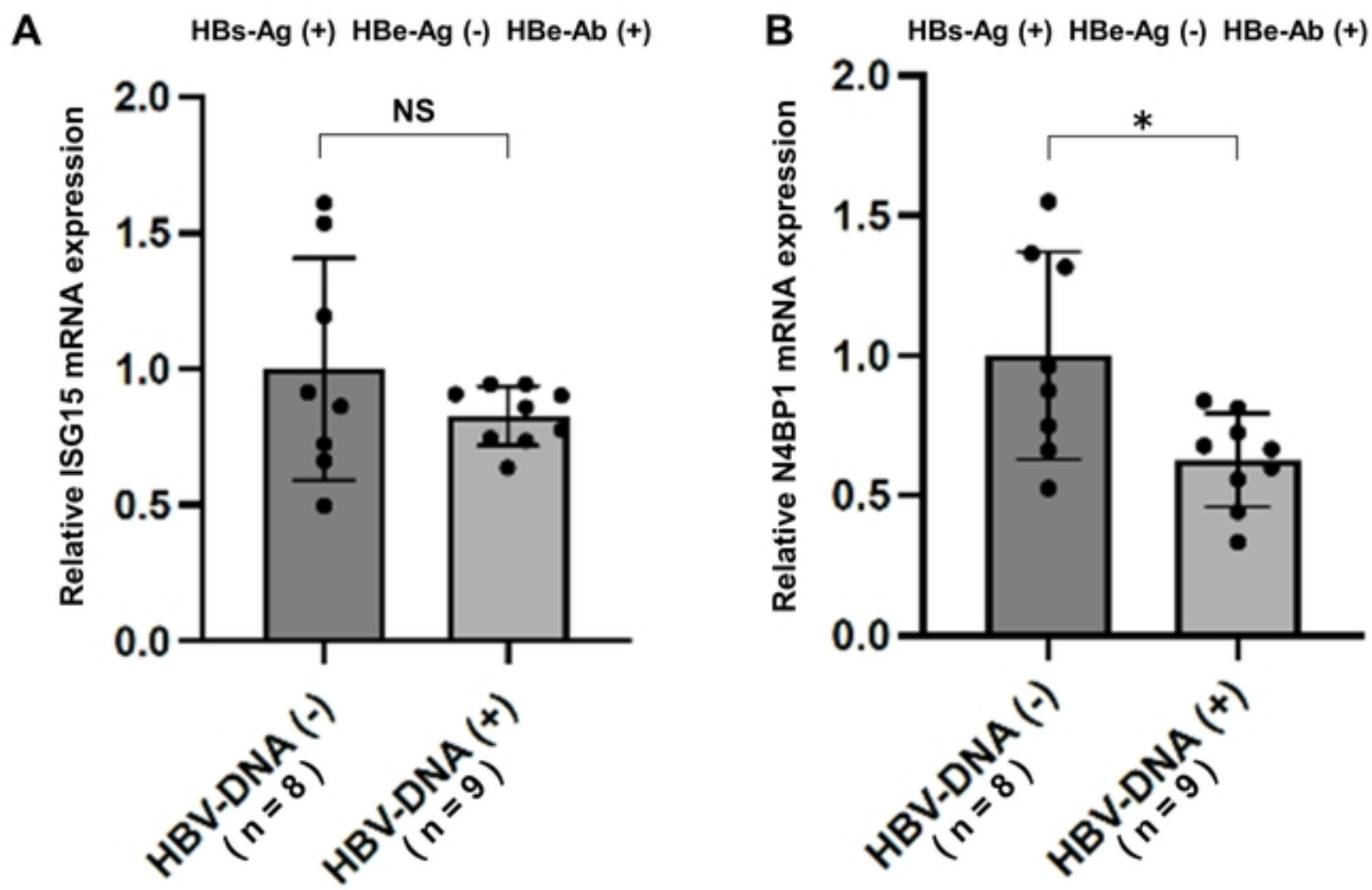
A
HBs-Ag (+) HBe-Ag (-) HBe-Ab (+)
Relative ISG15 mRNA expression
2.0
1.5
1.0
0.5
0.0
NS
HBV-DNA (-) (n = 8)
HBV-DNA (+) (n = 9)
B
HBs-Ag (+) HBe-Ag (-) HBe-Ab (+)
Relative N4BP1 mRNA expression
2.0
1.5
1.0
0.5
0.0
*
HBV-DNA (-) (n = 8)
HBV-DNA (+) (n = 9)

Figure8

1 **Figure legends**

2 **Fig 1. Screening for RNA binding proteins (RBPs) that inhibit HBV propagation.**

3 (A) A plasmid containing the 1.3-fold-overlength genome of HBV genotype C and 132 RBP
4 expression plasmids were co-transfected into Huh7 cells. Intracellular rcDNA levels were assessed at
5 3 dpt. Using the 50% of rcDNA level of the control as a cutoff, 23 out of the 132 RBPs suppressed
6 HBV rcDNA levels. (B) HepG2-hNTCP C4 cells overexpressing the 23 RBPs were infected with
7 HBV genotype D. At 14 dpi, cells were harvested and intracellular rcDNA levels measured by qPCR.
8 Seven out of the 23 RBPs (N4BP1, TIA-1, ZC3H12B, PJA1, HNRNPC, ZCCHC10, and KHNYN)
9 reduced rcDNA levels in comparison to the control. (C) WB analyses in naïve Huh7 cells, HepG2-
10 hNTCP C4 cells of the five factors (N4BP1, ZC3H12B, HNRNPC, ZCCHC10, and KHNYN)
11 identified in the secondary screening. As a positive control, WB were performed on Huh7 cells
12 transfected to overexpress each factor. Error bars represent the SD. Abbreviations: RBP (RNA binding
13 protein), ETV (entecavir), HepG2 C4 (HepG2-hNTCP C4), OE (overexpression)

14

15 **Fig 2. Measurement of rcDNA levels after RBP knockdown.**

16 (A) Huh7 cells were transfected with siRNA, then co-transfected with the 1.3-fold-overlength genome
17 of HBV genotype C and an RBP expression plasmid. WB analyses and qPCR of rcDNA were
18 performed at 3 dpt. ZCCHC10 (A), HNRNPC (B), and N4BP1 (C). GAPDH was used as a loading

19 control and ETV was used as a positive control. Error bars represent the SD. *P < 0.05, **P < 0.01,

20 as determined by Mann-Whitney U test. All qPCR experiments were independently performed at least

21 three times. Abbreviations: NS (not significant)

22

23 **Fig 3. N4BP1 is a suppressive host factor of HBV replication.**

24 (A) Plasmids containing 1.3-fold-overlength genomes of HBV genotypes A, B, or C were co-

25 transfected with N4BP1 expression plasmids into Huh7 cells. At 3 dpt, the rcDNA levels were

26 measured by qPCR. Genotype A (left), genotype B (middle), and genotype C (right). (B) WB analysis

27 and rcDNA levels of N4BP1 KO Huh7 cells. N4BP1 KO Huh7 cells were transfected with the plasmid

28 containing 1.3-fold-overlength genomes C and WB and qPCR were performed at 3 dpt. N4BP1

29 expression by WB (upper, anti-N4BP1). GAPDH was used as a loading control (lower, anti-GAPDH).

30 rcDNA levels were increased relative to control in both N4BP1-KO lines that were generated. N4BP1

31 KO Huh7 cell lines #1 (C) and #2 (D) were transfected with the N4BP1 plasmid to restore N4BP1

32 expression (upper, anti-N4BP1). GAPDH was used as a loading control (lower, anti-GAPDH). In the

33 N4BP1-rescued KO Huh7 cells, the level of rcDNA was significantly lower than in the non-rescued

34 N4BP1 KO Huh7 cells. Error bars represent the SD. *P < 0.05, **P < 0.01, as determined by Mann-

35 Whitney U test. All qPCR experiments were independently performed at least three times.

36

37 **Fig 4. N4BP1 expression is not induced by HBV.**

38 (A) We evaluated whether N4BP1 expression is induced by HBV infection or transfection in
39 hepatocyte cell lines. In each system, we confirmed by qPCR whether pgRNA was induced. (B) Huh7
40 cells were transfected with a plasmid containing the 1.3-fold-overlength genome of HBV genotype C
41 and cells were collected at 3 dpt (upper). N4BP1 mRNA expression was assessed by qPCR (middle)
42 and protein expression by WB (lower). (C) HepG2-hNTCP C4 cells were infected with HBV genotype
43 D and cells were collected at 3 dpi (upper). N4BP1 expression level by qPCR (middle). N4BP1
44 expression levels by WB (lower). (D) Tetracycline was removed from the medium of HepAD38.7
45 cells and the cells were collected 3 days later (upper). N4BP1 expression levels by qPCR (middle).
46 N4BP1 expression levels by WB (lower). Error bars represent the SD. *$P < 0.05$, **$P < 0.01$, as
47 determined by Mann-Whitney U test. All qPCR experiments were independently performed at least
48 three times.

49

50 **Fig 5. KH-like domain and RNase domain are involved in the anti-HBV effect of N4BP1.**

51 (A) Schematic of N4BP1. N4BP1 is composed of 896 amino acids and has both a KH-like domain
52 and an RNase domain. We constructed a KH-like domain deficient mutant (deletion of amino acids
53 59-143) and an RNase domain deficient mutant (deletion of amino acids 617-769). Each plasmid had
54 a HA tag at its C-terminus. (B) Huh7 cells were transfected with the plasmid containing the 1.3-fold-

55 overlength genome of HBV genotype C and each expression plasmid (control vector, N4BP1 WT,
56 ΔKH, and ΔRNase) and cells were collected at 3 dpt. WB was used to confirm the expression of each
57 N4BP1 mutant (upper, anti-HA tag). GAPDH was used as a loading control (lower, anti-GAPDH).
58 HBV levels were assessed by measuring intracellular rcDNA levels at 3 dpt. (C) Huh7 cells were
59 transfected with the plasmid containing the 1.3-fold-overlength genome of HBV genotype C and each
60 expression plasmid (control vector, WT, and D623N) and cells were collected at 3 dpt. WB was used
61 to confirm the expression of each N4BP1 mutant (upper, anti-HA tag). GAPDH was used as a loading
62 control (lower, anti-GAPDH). HBV levels were assessed by measuring intracellular rcDNA levels at
63 3 dpt. ETV was used as a positive control. Error bars represent the SD. *$P < 0.05$ as determined by
64 Mann-Whitney U test. All qPCR experiments were independently performed at least three times.
65
66 **Fig 6. N4BP1 suppresses pgRNA levels by promoting pgRNA degradation.**
67 HepG2-hNTCP C4 cells were infected with HBV genotype D and cells were collected at 14 dpi.
68 Comparison of cccDNA (A), pgRNA (B), and rcDNA (C) levels in N4BP1-overexpressing HepG2-
69 hNTCP C4 cells and control at 14 dpi. Heparin was used as a positive control. PHHs were infected
70 with HBV genotype C and cells were collected at 14 dpi. (D) WB analysis of PHHs expressing N4BP1
71 using adenovirus vectors (upper, anti-N4BP1). GAPDH was used as a loading control (lower).
72 Comparison of cccDNA (E), pgRNA (F), and rcDNA (G) levels in N4BP1-overexpressing PHHs and

73 control at 14 dpi. Heparin was used as a positive control. (H) Time course of pgRNA degradation

74 assay in HepAD38.7 cells. (I) pgRNA levels over time in HepAD38.7 cells overexpressing N4BP1

75 WT, ΔKH, ΔRNase, or control. Error bars represent the SD. *P < 0.05, **P < 0.01, as determined by

76 Mann-Whitney U test. All experiments were independently performed at least three times. (J) Northern

77 blotting analyses in control, N4BP1-overexpressing and naïve Huh7 cells.

78

79 **Fig 7. N4BP1 expression level is induced by IFN-alpha and IFN-lambda in PHHs.**

80 (A) Time course of the drug treatment assay. Cells were harvested at 24 or 48 hours post-IFN-α/λ

81 treatment. (B) N4BP1 expression levels by qPCR in Huh7 cells, HepG2-hNTCP C4 cells, and PHHs

82 treated with IFN-α (upper). ISG15 expression levels by qPCR (middle). N4BP1 protein expression

83 levels by WB. GAPDH was used as a loading control (lower). (C) N4BP1 expression levels by qPCR

84 in Huh7 cells, HepG2-hNTCP C4 cells, and PHHs treated with IFN-λ (upper). ISG15 expression levels

85 by qPCR (middle). N4BP1 protein expression levels by WB. GAPDH was measured as loading control

86 (lower).

87

88 **Fig 8. ISG15 and N4BP1 expression levels in HBV-infected human liver tissues.**

89 The mRNA levels of ISG15 (A) and N4BP1 (B) from non-cancerous tissues of HBV-HCC patients

90 were examined by qPCR. The samples were divided into HBV DNA positive group (n=9) and HBV

91 DNA negative group (n=8). Error bars represent the SD. *P < 0.05, **P< 0.01, as determined by

92 Mann-Whitney U test.