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PDF issue: 2025-02-25

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(Citation)

Journal of General Virology, 102(10):1668

(Issue Date) 2021-10-18

(Resource Type) journal article

(Version) Accepted Manuscript

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(URL)

https://hdl.handle.net/20.500.14094/90009262



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28	Short Title: HBx-ISGylation promotes viral replication
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30	Keywords: hepatitis B virus, HBx, ISG15, ISGylation, interferon, viral replication
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#### 32 ABSTRACT [213 words]

33 Ubiquitin and ubiquitin-like protein modification play important roles in modulating the 34 functions of viral proteins in many viruses. Here we demonstrate that hepatitis B virus (HBV) X protein (HBx) is modified by ISG15, which is a type I interferon (IFN)-inducible, ubiquitin-35 36 like protein; this modification is called ISGylation. Immunoblot analyses revealed that HBx 37 proteins derived from four different HBV genotypes accepted ISGylation in cultured cells. 38 Site-directed mutagenesis revealed that three lysine residues (K91, K95, and K140) on the 39 HBx protein, which are well conserved among all the HBV genotypes, are involved in 40 acceptance of ISGylation. Using expression plasmids encoding three known E3 ligases 41 involved in the ISGylation to different substrates, we found that HERC5 functions as an E3 42 ligase for HBx-ISGylation. Treatment with type I and type III IFNs resulted in the limited 43 suppression of HBV replication in Hep38.7-Tet cells. When cells were treated with IFN-a, 44 silencing of ISG15 resulted in a marked reduction of HBV replication in Hep38.7-Tet cells, 45 suggesting a role of ISG15 in the resistance to IFN- $\alpha$ . In contrast, the silencing of USP18 (an 46 ISG15 de-conjugating enzyme) increased the HBV replication in Hep38.7-Tet cells. Taken 47 together, these results suggest that the HERC5-mediated ISGylation of HBx protein confers 48 pro-viral functions on HBV replication and participates in the resistance to IFN-α-mediated 49 antiviral activity.

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#### 52 INTRODUCTION

Hepatitis B virus (HBV) infection is a leading cause of chronic hepatitis, liver cirrhosis and
hepatocellular carcinoma (HCC), and remains a major public health burden worldwide (1).
Current approved therapies against chronic HBV infection are conducted using several
nucleos(t)ide analogs and pegylated-interferon (IFN); these drugs reduce the viral load, but
they rarely achieve complete cure in HBV-infected patients (2).

58 HBV is a member of the *Hepadnaviridae* family and has a circular, partially double-59 stranded 3.2-kb DNA genome encoding a viral envelope, capsid protein, viral polymerase, 60 and regulatory X protein (HBx). HBx, a 154-amino acid (aa) polypeptide with a molecular 61 weight of 17 kDa, is a multi-functional regulatory protein involved in the modulation of cell 62 proliferation, apoptosis, and transcriptional gene expression, whose processes are associated 63 with the development of HBV-related chronic liver diseases (3). Cumulative evidence 64 suggests that HBx is essential for efficient HBV replication and pathogenesis via its 65 association with a large number of host factors, including HBx-interacting protein (4), p53 66 (5), damaged DNA binding protein 1 (DDB1) (6, 7) and other proteins (8-16). In addition, we 67 reported that peroxiredoxin 1 (Prdx1), a novel HBx-interacting protein, plays a role in the 68 degradation of HBV RNA (17). However, the precise functions of HBx during the HBV life 69 cycle remain to be elucidated.

Ubiquitylation and ubiquitin-like protein modifications, such as NEDDylation, SUMOylation, and ISGylation, play important roles in modulation of the functions of many viral proteins (18), but little is known about the exact mechanism how these protein modifications affect HBV infection. Liu and colleagues (19) recently reported that the E3 ligase HDM2 promotes the NEDDylation of HBx protein to maintain HBx stability and 75 chromatin localization, which promotes HBx-dependent transcriptional regulation, cell76 proliferation, and tumor development.

77 IFN-stimulated gene 15 (ISG15) is the ubiquitin-like protein that is induced by stimulation with type I IFN or by viral or bacterial infection (20). ISG15 is covalently 78 79 conjugated to a substrate protein via specific lysine (K) residues by three enzymes: E1 80 activating enzyme (UBE1L), E2 conjugating enzyme (UbcH8), and E3 ligase. HERC5, 81 TRIM25 (also referred to as EFP) and human homolog of Ariadne (HHARI) have been 82 reported to function as E3 ligases for ISGylation of different substrate proteins (21-23). This 83 process, known as ISGylation, is a post-translational protein modification that is similar to 84 ubiquitin and other ubiquitin-like proteins. Covalently conjugated ISG15 can be removed 85 from substrates by USP18 (also referred to as UBP43), an ISG15 de-conjugating enzyme, 86 which cleaves isopeptide bonds between ISG15 and the substrate protein (24). In addition to 87 the regulation of signal transduction and tumorigenesis, ISG15 has anti-viral effects on 88 Sindbis virus, influenza A and B viruses, and herpes simplex virus 1 infections (18, 20).

89 ISG15/ISGylation has been reported to play an antiviral role, but in the case of several 90 infections-including infections with HBV and hepatitis C virus (HCV)-ISG15/ISGylation 91 has been reported to have a pro-viral function (20, 25, 26). In the case of HBV infection, Li 92 and colleagues (25) reported that the overexpression of ISG15 and subsequent ISGylation 93 could promote HBV production in HBV-persistent replicating cells, although the putative 94 ISGylated viral protein(s) remain to be determined. There is also a report suggesting that high 95 ISG15 expression levels were correlated with both the HBV-DNA loads in patients' serum and the progression of HBV-related HCC (27). These observations suggest that intracellular 96 97 ISG15/ISGylation has a pro-viral function, but the exact mechanisms underlying this 98 correlation remain to be elucidated.

In the present study we aimed to elucidate the role of HBx-ISGylation in HBV infection.
We analyzed the ISGylation sites on HBx protein via specific Lys (K) residues, which are
well conserved among several HBV genotypes. We demonstrate evidence suggesting that the
E3 ligase HERC5 promotes HBx-ISGylation to confer pro-viral functions on HBV replication
and participates in the resistance to IFN-α-mediated antiviral activity.

104

105 RESULTS

#### 106 HBx protein is a substrate of ISGylation

107 At first, to investigate the effects of ISGylation on several HBV proteins, including HBc, 108 HBx, HBV polymerase (HBV pol), and large HBV surface (LHBs) from genotype (GT)-C 109 (C-AT\_JPN), we co-transfected HEK293T cells with C-terminal Myc-His<sub>6</sub>-tagged HBV 110 proteins and N-terminal FLAG-tagged-ISG15 together with E1 activating enzyme (UBE1L), 111 E2 conjugating enzyme (UbcH8) and HA-tagged E3 ligase (HA-HERC5), followed by 112 immunoprecipitation with anti-FLAG and detection with each of the anti-HBV specific 113 antibodies. The immunoprecipitation analysis coupled with immunoblotting revealed that 114 HBx-Myc-His<sub>6</sub>, but not HBc, HBV pol, and LHBs proteins, was precipitated with FLAG-115 ISG15 (Fig. 1A, lane 3 in the first panel and Supplementary Fig. 1). These results suggest 116 that only HBx protein can accepts ISGylation.

Using anti-HBx rabbit polyclonal antibody (pAb), we observed that the slowly migrating forms of HBx protein were clearly detected in the cells co-transfected with FLAG-ISG15, E1, E2, and E3 plasmids compared to the cells transfected with HBx-Myc-His<sub>6</sub> alone (Fig. 1A, first and second panels, lanes 2 and 3; asterisks indicate ISGylated HBx). These slowly migrating forms of HBx protein were also detected when anti-HBx antibody was used for immunoprecipitation, followed by detection with anti-ISG15 antibody (Supplementary Fig. 2, lane 2, left panel; asterisks indicate ISGylated HBx). These results suggest that HBxprotein actually accepts ISGylation.

Next, to verify that the slowly migrating forms of HBx are ISGylated HBx proteins,
we performed an immunoprecipitation analysis using a conjugation-defective ISG15 mutant
that possesses a Gly-Gly to Ala-Ala substitution within the C-terminal LRLRGG motif,
termed FLAG-ISG15-AA. The expression of FLAG-ISG15, but not FLAG-ISG15-AA,
resulted in the induction of HBx-ISGylation (Fig. 1A, first and second panels, lanes 3 and 4).
These results indicate that the slowly migrating forms of HBx protein are indeed ISGylated
HBx proteins.

132 To further seek for the evidence suggesting that HBx proteins are indeed ISGylated, 133 HEK293T cells were co-transfected with HBx-Myc-His<sub>6</sub> together with plasmids expressing 134 ISGylation components and cell lysates were pulled down with Ni-Nitrilotriacetic (NTA) 135 resin under denaturing condition (6M guanidine-HCl). The Ni-NTA pull-down analysis 136 coupled with immunoblotting revealed that C-terminal His-tagged HBx and ISGylated HBx 137 proteins were detected in the same eluted fractions (Supplementary Fig. 3, upper, right panel, 138 lanes 7 to 9). These results suggest that the slowly migrating forms of HBx protein are indeed 139 covalently ISGylated HBx protein.

To ask a question why unmodified HBx was co-precipitated with FLGA-ISG15 (Fig.1A, lane 3; Fig. 1B, lane 3), we examined HBx-ISGylation under more stringent condition using RIPA buffer containing 300mM NaCl. The IP-western blot analysis revealed that the level of unmodified HBx (~17 kDa) was markedly reduced compared to the regular RIPA buffer containing 150mM NaCl (Supplementary Fig. 4, left panel, lane 4). These results suggest that the unmodified HBx can interact with ISG15 via protein-protein interaction. The IP-western blot analysis also revealed that ISGylated HBx proteins were detected using the 147 RIPA buffer containing 300 mM NaCl and 150 mM NaCl. These results indicate that the148 slowly migrating forms of HBx protein are indeed covalently ISGylated HBx protein.

149 To seek for evidence suggesting that HBx protein produced from replicating HBV is 150 ISGylated, we co-transfected Huh7.5 cells with HBV genotype C plasmid, pUC-HBV-C-151 AT JPN, which carries 1.3-mer overlength HBV genome, together with ISGylation 152 components. Transfection of pUC-HBV, but not HBx-deficient HBV plasmid (pUC-HBV-153  $\Delta$ HBx), exhibited the ISGylated HBx proteins (Fig. 1B, lanes 3 and 4, first and second panels; 154 asterisks indicate ISGylated HBx). No LHBs-ISGylation was detected in the cells transfected 155 with pUC-HBV when anti-PreS1 antibody was used for immunoblotting (Supplementary Fig. 156 5, lane 3 and lane 4, 2nd panel). These results suggest that the HBx protein produced from 157 replicating HBV is indeed ISGylated.

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#### 159 HERC5 E3 ligase functions as an E3 ligase for ISGylation of HBx protein

160 To determine the an E3 ligase involved in the ISGylation of HBx, we co-transfected 161 HEK293T cells with HBx-Myc-His<sub>6</sub> and FLAG-ISG15 together with E1 (UBE1L), E2 162 (UbcH8), and each of the HA-tagged E3 ligases, including HA-HERC5, HA-TRIM25, or HA-163 HHARI, followed by immunoprecipitation with anti-FLAG and detection with anti-HBx 164 rabbit pAb. The immunoprecipitation analysis coupled with immunoblotting revealed that the 165 transfection of HERC5, but not TRIM25 and HHARI, strongly induced the ISGylation of 166 HBx (Fig. 2A, first and second panels, lane 2; asterisks indicate ISGylated HBx). These 167 results suggest that HERC5 E3 ligase is involved in ISGylation of HBx protein.

Next, to determine whether HERC5 E3 ligase activity mediates ISGylation of HBx
protein, we performed a cell-based ISGylation assay using the catalytically inactive mutant
HERC5 (C994A). The transfection of wild-type (WT) HERC5, but not HERC5 (C994A),

strongly induced the HBx-ISGylation (Fig. 2B, first and second panels, lanes 2 and 3;
asterisks indicate ISGylated HBx), indicating the requirement of HERC5 ligase activity for
ISGylation of HBx protein.

174 To further confirm the involvement of HERC5 E3 ligase for ISGylation of HBx protein, 175 we depleted each of the ISGylation components in the cells co-transfected with FLAG-ISG15 176 plasmid. The cell-based ISGylation assay coupled with the immunoprecipitation and 177 immunoblotting clearly revealed ISGylation of HBx protein when all components were 178 expressed (Fig. 2C, left and middle panels, lane 4; asterisks indicate ISGylated HBx proteins). 179 In contrast, ISGylation of HBx protein was markedly reduced when plasmid expressing E3 180 (HERC5) or both E3 (HERC5) and E2 (UbcH8) was depleted (Fig. 2C, left and middle panels, 181 lanes 5 and 6). These results indicate that HERC5 functions as an E3 ligase for ISGylation of 182 HBx protein. HBx ISGylation in the absence of transfected HERC5 (Fig. 2C, lane 5) was 183 presumably due to the presence of endogenous HERC5 in the cells.

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#### 185 Lys residues for ISGylation of HBx protein from four HBV genotypes

186 We then investigated Lys residues for ISGylation of HBx protein from four different HBV 187 genotypes (GTs): GT-A (Ae-US), GT-B (Bj\_JPN56), GT-C (C-AT\_JPN), and GT-D (Ayw). 188 As shown in Fig. 3A and 3B, there are six conserved Lys residues on HBx among these HBV 189 genotypes, except GT-B (Bj\_JPN56). GT-A (Ae-US), GT-C (C-AT\_JPN), and GT-D (Ayw) 190 have Lys residue at aa 118, whereas GT-B (Bj\_JPN56) has Asparagine (Asp; N) residue at aa 191 118 (Fig. 3B). To test whether the HBx proteins from all the HBV genotypes accept 192 ISGylation in cultured cells, each of the plasmids encoding HBx derived from the four 193 different HBV genotypes was co-transfected with ISGylation components similarly. The cell-194 based ISGylation assay coupled with the immunoprecipitation and immunoblotting revealed that the HBx proteins derived from all four different HBV genotypes were ISGylated in their
co-transfected cells (Fig. 3C, left panel, lanes 2, 4, 6, and 8; asterisks indicate ISGylated HBx).
In the immunoblot analysis with anti-HBx, anti-HA, or anti-ISG15, the equivalent expression
levels of HBx, HERC5, or ISG15 were detected in the cells co-transfected with plasmids
expressing HBx and E1/E2/E3 ligases (Fig. 3C, indicated panels). These results suggest that
the HBx proteins of all four different HBV genotypes investigated in this study accept
ISGylation.

202

#### 203 The residues K91, K95, and K140 on HBx protein are acceptor lysines for ISGylation

204 HBx from GT-C (C-AT JPN) has six Lys residues (K91, K95, K113, K118, K130, and K140) 205 within the C-terminal trans-activation domain on the coding sequence (Fig. 3A). To identify 206 the ISGylation sites on HBx protein, we constructed a series of HBx mutants, containing a 207 point mutation of Arg (R) at a corresponding Lys (K) (K/R-mutant series). Consistently, the 208 immunoprecipitation analysis coupled with immunoblotting revealed that HBx-Myc-His<sub>6</sub> 209 (WT), but not HBx-Myc-His<sub>6</sub> (K-Null) with all Lys (K) residues mutated to Arg (R), exhibited 210 HBx-ISGylation (Fig. 4A, upper and middle panels, lanes 2 and 9; asterisks indicate 211 ISGylated HBx), indicating that these slowly migrating bands are dependent on the Lys 212 residues of HBx protein.

A further immunoblot analysis using the HBx K/R-mutants revealed that the ISGylation of HBx protein was markedly reduced in the HBx-Myc-His<sub>6</sub> (K91R, K95R, and K140R) mutants compared to HBx (WT) (Fig. 4A, upper and middle panels, lanes 3, 4, and 8; asterisks indicate ISGylated HBx). Although slight HBx-ISGylation was still present in the cells transfected with the HBx-Myc-His<sub>6</sub> (K91R/K95R) double mutant, in which residues K91 and K95 on HBx were replaced with R (Fig. 4B, upper and middle panels, lane 3), the

219 expression of the HBx-Myc-His<sub>6</sub> (K91R/K95R/K140R) mutant (which contains an additional 220 mutation at the residue K140) exhibited the complete loss of HBx-ISGylation (Fig. 4B, upper 221 and middle panels, lane 4). We also constructed a series of HBx mutants in which all lysines 222 except the indicated lysines are mutated to Arg (R) residues. Conversely, when the HBx-Myc-223 His<sub>6</sub> (K91/K95/K140) mutant was used instead of the HBx-Myc-His<sub>6</sub> (K91/K95) mutant, the 224 level of ISGylation of HBx protein was comparable to that of HBx (WT) (Fig. 4B, upper and 225 middle panels, lanes 5 and 6). Collectively, these results suggest that the residues K91, K95, 226 and K140 are acceptor lysines for ISGylation on HBx protein.

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# The persistent HBV replication is resistant to the treatment of various IFNs in theHep38.7-Tet cells

Next, to determine the effects of various IFNs on HBV replication, we analyzed intracellular HBV RNA and HBV rcDNA in Hep38.7-Tet cells, which are a doxycycline (Dox)-inducible HBV cell culture model. At 2 days after doxycycline removal, Hep38.7-Tet cells were treated with  $10^3$  units/ml of type I (IFN- $\alpha$  and IFN- $\beta$ ) or type III (IL28A, IL28B, and IL29) IFNs for 5 days, and the expression levels of intracellular HBV RNA and HBV rcDNA were measured by real-time polymerase chain reaction (PCR).

As shown in Fig. 5A, the treatment with type I and type III IFNs did not result in reduction of the levels of HBV RNA or HBV rcDNA in Hep38.7-Tet cells, although high levels of ISG15 mRNA expression were observed in the IFN-treated cells. After the treatment with type I and type III IFNs, a limited suppression of HBV RNA was observed in the cells transfected with the full-length HBV expression plasmid (pUC-HBV-C-AT\_JPN) (Fig. 5D). In contrast, the treatment of the cells with type I and type III IFNs resulted in marked reductions of both HCV RNA and the levels of viral proteins in the HCV subgenomic replicon cells (1b, Con1 strain) (Fig. 5B and Fig. 5C). These results indicate that the treatment withIFNs had a limited ability to suppress the HBV replication in the Hep38.7-Tet cells.

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#### 246 ISG15 functions as an IFN-resistance factor in HBV-replicating cells

247 We hypothesized that ISG15 is involved in the IFN-resistance of HBV in HBV-replicating 248 cells. To determine a role(s) of ISG15 in the IFN-resistance of HBV in HBV-replicating cells, 249 Hep38.7-Tet cells were transfected with siRNA targeting ISG15 or USP18, which is an ISG15 250 de-conjugating enzyme, followed by treatment with  $10^3$  units/ml of IFN- $\alpha$ . The expressions 251 of extracellular HBV rcDNA and ISG15 mRNA were measured by real-time PCR. Consistent 252 with the results in Fig. 5A, the treatment with IFN- $\alpha$  did not result in the suppression of HBV 253 replication in Hep38.7-Tet cells treated with negative control (NC) siRNA (Fig. 6A, upper 254 bar graph, second bar). Interestingly, the silencing of ISG15 resulted in a partial reduction (to 255 30%) of HBV rcDNA expression in Hep38.7-Tet cells (Fig. 6A, upper bar graph, third bar), 256 and this inhibitory effect was enhanced by treatment with IFN- $\alpha$  (Fig. 6A, upper bar graph, 257 fourth bar). In contrast, the silencing of USP18 resulted in increase of HBV rcDNA in the 258 USP18-silenced cells treated with or without IFN- $\alpha$  (Fig. 6B, upper bar graph, third and fourth 259 bars). Immunoblot analysis revealed that IFN-α induced STAT1 expression in both ISG15 260 and USP18 silencing cells treated with IFN-α (Fig. 6A and 6B, second panels, lanes 2 and 4). 261 In addition, the marked reduction of IFN-α-induced ISGylation was observed in the ISG15-262 silencing cells, whereas the enhancement of IFN-a-induced ISGylation was observed in 263 USP18-silencing cells (Fig. 6A and 6B, third panels, lanes 2 and 4). These results suggest that 264 ISG15 functions as an IFN-resistance factor for HBV replication.

Collectively, these results suggest that ISGylation of HBx protein functions as a proviral factor in HBV replication and that ISGylation is also involved in the IFN-resistance of
HBV in HBV-replicating cells.

Taken together, we propose a model in which HBV HBx protein is ISGylated at the
residues K91, K95, and K140 via the action of three enzymes: E1 activating enzyme (UBE1L),
E2 conjugating enzyme (UbcH8) and E3 ligase (HERC5) in HBV-replicating cells, thereby
promoting the viral replication and conferring the machinery of immune evasion from IFNmediated antiviral activity (Fig. 7).

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#### 274 DISCUSSION

The IFN-inducible protein ISG15 and ISGylation are believed to play important roles in antiviral activity against many types of RNA and DNA viruses (20, 28). Our present results demonstrated that ISG15 functions as a pro-viral factor in HBV replication through HBx-ISGylation at multiple specific Lys residues. We demonstrated that the HERC5 E3 ligase mediates ISGylation of HBx protein. We further demonstrated that ISG15 is involved in the IFN-resistance of HBV, although the detailed mechanism remains to be elucidated.

281 Ubiquitin-like modifiers, such as NEDDylation, SUMOylation, and ISGylation, play 282 important roles in modulating the function of viral proteins to mediate pathogenesis (18). For 283 example, HBx protein was reported to accept NEDDylation mediated by the HDM2 E3 ligase, 284 which enhances the stability of HBx to prevent its proteasomal degradation (19). Similarly, 285 Dengue virus (DENV) NS5 protein and HCV NS5A protein were shown to accept 286 SUMOylation mediated by several SUMO-conjugating enzymes to enhance protein stability 287 and positively regulate the viral replication (29, 30). These studies suggest that the inhibition 288 of the specific conjugating enzymes involved in the regulation of ubiquitin-like modifiers

289 could be a novel approach for the treatment of viral infection. In fact, there was a report 290 suggesting that MLN4924, a specific inhibitor of NEDD8-activating enzyme (NAE), has the 291 ability to suppress HBV replication through HBx destabilization (31). We demonstrated that 292 HERC5 mediates the ISGylation of HBx protein to promote HBV replication. HERC5 is 293 induced by type I IFN and is not essential for basic host functions (22, 32). Therefore, HERC5 294 could be a potential therapeutic target against HBV infection without side effects. 295 Interestingly, the quinazoline derivative compound known as HZ-6d was reported to inhibit 296 the HERC5-mediated p53-ISGylation to prevent its proteasomal degradation, leading to the 297 suppression of tumor growth (33). Since we demonstrated that HERC5 is involved in HBx-298 ISGylation, it is interesting to investigate whether HZ-6d or other compounds may inhibit 299 HBV replication.

300 It was reported that the induction of ISGylation promoted HBV particle production in 301 HBV-persistent replicating cells (HepG2.2.15 cells), although it remained to be determined 302 which viral proteins are target substrates for ISGylation (25). In the present study, we 303 discovered that 1) HBx protein is a target substrate for ISGylation, 2) the residues K91, K95, 304 and K140 on HBx protein are ISGylation sites, 3) HERC5 E3 ligase functions as an E3 ligase 305 for ISGylation of HBx protein, and 4) ISGylation may function as an IFN-resistance factor 306 for HBV replication. There are three acceptor lysine residues for ISGylation on HBx protein. 307 However, immunoblot analyses indicated ISGylated-HBx protein showed more than 130 kDa, 308 suggesting that poly-ISGylation or ISG15-ubiquitin mixed chains might be involved in HBx 309 ISGylation. We reported similar findings in HCV NS5A ISGylation (26).

We unexpectedly detected approximately 18 kDa-band, which is slightly higher than
HBx-band, in lane 3 and lane 4 (the second panel, Fig.1B), whereas the 18 kDa-band was not
detected in lane 2. When HEK293T cells were transfected with pEF1A-HBx-Myc-His<sub>6</sub>

together with pCAG-FLAG-ISG15 and E1/E2/E3, we couldn't detect the 18 kDa-band
(Fig.1A, lanes 3 and 4). When Huh7.5 cells were transfected with either pUC-HBV or pUCHBV-deltaHBx together with pCAG-FLAG-ISG15 and E1/E2/E3, we could detect the 18
kDa-band (Fig. 1B, lanes 3 and 4). Therefore, we speculate that host proteins in Huh7.5 cells
might be ISGylated and cross-reacted with anti-HBx rabbit polyclonal antibody.

We previously demonstrated that HCV NS5A protein interacts with the unmodified ISG15, leading to acceptance of ISGylation via covalent bond (26, 39). Thus, we considered that HBx protein may interact with ISG15 via protein-protein interactions, leading to acceptance of ISG15 conjugation via covalent bond. We demonstrated that ISG15 proteins were co-precipitated with HBx, suggesting the interaction between HBx and ISG15 via protein-protein interaction.

324 HBx has been shown to localize in both the cytoplasm and the nucleus (34, 35), 325 although both studies employed physiologically irrelevant conditions (e.g., abundant 326 expression of HBx protein by plasmid transfection or viral vector transduction). Most recently, 327 Kornyeyev and colleagues (36) described a novel monoclonal antibody that enables the 328 detection of HBx protein in HBV-infected primary human hepatocytes (PHHs), which reflect 329 more physiologically relevant condition. They demonstrated that HBx is predominantly 330 localized in the nucleus in HBV-infected PHHs. It was also reported that >100 host proteins 331 may accept ISGylation (37, 38). The roles of HBx-ISGylation as well as host protein 332 ISGylation in the subcellular distribution of HBx and in HBV replication remain to be 333 clarified. We are currently investigating the effect of ISGylation on nuclear localization of 334 HBx protein. Further study will be needed to clarify the role(s) of ISGylated HBx protein in 335 viral replication.

Interestingly, the ISG15 mRNA was increased upon USP18 knockdown (Fig. 6B). We
speculate that a host factor, which is involved in the negative regulation of ISG15 mRNA
expression, (e.g. regulator for mRNA decay), might be affected by the USP18 knockdown.
However, to clarify the detailed mechanism, further study will be needed.

In summary, we demonstrated evidence suggesting that HERC5 E3 ligase specifically mediates ISGylation of HBx protein to promote HBV replication. We identified the ISGylation sites on HBx protein, which are well conserved among HBV genotypes. Our results suggest that HBx-ISGylation participates in the positive regulation of viral replication and IFN-resistance of HBV. Targeting the ISGylation machinery on the HBx protein could lead to the development of novel therapeutics for the treatment of chronic HBV infection.

346

#### 347 MATERIALS & METHODS

348 Cell culture and reagents. Huh7.5 cells were kindly provided by Dr. C.M. Rice (The 349 Rockefeller University, NY). Huh7.5 cells and 293T cells were maintained in Dulbecco's 350 modified Eagle's medium (DMEM) (High Glucose) with L-glutamine (Wako, Osaka, Japan) 351 supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Gibco, Grand Island, NY), 352 and 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France) at 37°C in a 353 5% CO<sub>2</sub> incubator. A doxycycline (Dox)-inducible HBV expression cells, Hep38.7-Tet cells 354 (kindly provided by Dr. K. Watashi, NIID, Japan), were maintained in DMEM/F-12 (Gibco) 355 supplemented with 10 mM HEPES (Gibco), 50 IU/ml penicillin, 50 µg/ml streptomycin 356 (Gibco), 10% FBS (Biowest), 5 µg/ml insulin (Sigma-Aldrich, St. Louis, MO), 400 µg/ml 357 G418 (Nacalai Tesque, Kyoto, Japan), and 400 ng/ml Dox (Sigma-Aldrich). Cells were 358 transfected with plasmid DNA using FuGene 6 transfection reagents (Promega, Madison, WI). 359 Daclatasvir (DCV) was purchased from Sigma-Aldrich. Recombinant type-I (IFN-α and IFN-

β) and type-III IFNs (IL-28A, IL-28B, and IL-29) were purchased from PBL assay science
(Piscataway, NJ).

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Plasmids. The plasmids pEF1A-HBx-Myc-His<sub>6</sub> and pEF1A-HBc-Myc-His<sub>6</sub> have been
previously described (17). The cDNA fragments of LHBs from genotype (GT)-C (CAT\_JPN) was inserted into the EcoRI site of pEF1A-Myc-His<sub>6</sub> using the In-Fusion HDCloning kit (Clontech, Mountain View, CA). The cDNA fragments of HBx (C-AT\_JPN) with
all of the Lys (K) residues mutated to Arg (R) were generated by PCR mutagenesis using
pEF1A-HBx-Myc-His<sub>6</sub> as a template. The specific primers used for the PCR were as follows:
sense primer (K91R), 5'-CAGGTCTTGCCCAGGGTCTTATAT-3'; antisense primer (K91R),

370	5'-ATATAAGACCCTGGGCAAGACCTG-3',	sense	primer	(K95R),	5'-
371	AAGGTCTTATATAGGAGGACTCTT-3';	antisense	primer	(K95R),	5'-
372	AAGAGTCCTCCTATATAAGACCTT-3',	sense	primer	(K113R),	5'-
373	GAGGCATACTTCAGAGACTGTTTG-3';	antisense	primer	(K113R),	5'-
374	CAAACAGTCTCTGAAGTATGCCTC-3',	sense	primer	(K118R),	5'-
375	GACTGTTTGTTTAGGGACTGGGAG-3';	antisense	primer	(K118R),	5'-
376	CTCCCAGTCCCTAAACAAACAGTC-3',	sense	primer	(K130R),	5'-
377	GAGATTAGGTTAAGGATTTTTGTA-3';	antisense	primer	(K130R),	5'-
378	TACAAAAATCCTTAACCTAATCTC-3',	sense	primer	(K140R),	5'-
379	GGCTGTAGGCATAGATTGGTCTGT-3';	antisense	primer	(K140R),	5'-
380	ACAGACCAATCTATGCCTACAGCC-3'. We	designated	the resulting	plasmid as pE	F1A-

HBx(K-Null)-Myc-His<sub>6</sub>. The construction of HBx Lys (K) mutants, including those with all
of the Lys (K) residues mutated to Arg (R) except for some combinations of the indicated Lys
residue (referred to as K91/K95 and K91/K95/K140), were generated by PCR mutagenesis

384	using pEF1A-HBx(K-Null)-Myc-His <sub>6</sub> as a terr	plate. The spe	cific primer	s used for PCR w	vere
385	as follows: sense primer (K91), 5'-CAGG	ICTTGCCCA	AGGTCTT	ATAT-3'; antise	ense
386	primer (K91), 5'-ATATAAGACCTTGGGG	CAAGACCTG	-3', sense	primer (K95),	5'-
387	AGGTCTTATATAAGAGGACTCTT-3';	antisense	primer	(K95),	5'-
388	AAGAGTCCTCTTATATAAGACCCT-3',	sense	primer	(K113),	5'-
389	GAGGCATACTTCAAAGACTGTTTG-3';	antisense	primer	(K113),	5'-
390	CAAACAGTCTTTGAAGTATGCCTC-3',	sense	primer	(K118),	5'-
391	GACTGTTTGTTTAAGGACTGGGAG-3';	antisense	primer	(K118) <b>,</b>	5'-
392	CTCCCAGTCCTTAAACAAACAGTC-3',	sense	primer	(K130),	5'-
393	GAGATTAGGTTAAAGATTTTTGTA-3';	antisense	primer	(K130) <b>,</b>	5'-
394	ТАСАААААТСТТТААССТААТСТС-3',	sense	primer	(K140),	5'-
395	GGCTGTAGGCATAAATTGGTCTGT-3';	antisense	primer	(K140) <b>,</b>	5'-
396	ACAGACCAATTTATGCCTACAGCC-3'. 7	The constructi	on of HBx	K Lys (K) muta	nts,
397	containing some combination mutants of Lys t	o Arg at the in	dicated Lys	residues (referre	d to
398	as K91R/K95R and K91R/K95R/K140R) were	e generated by	PCR mutage	enesis using pEF	1A-
399	HBx-Myc-His <sub>6</sub> as a template and specific pr	imers describe	ed above. T	he HBV express	sion
400	plasmids pUC19-HBV-Ae_US, pUC19-HBV-	Bj_JPN56, and	d pUC19-H	BV-C-AT_JPN w	vere
401	kindly provided by Dr. M. Mizokami (NCGN	A, Japan). The	cDNA frag	ments of HBx f	rom
402	HBV genotypes (GT): GT-A (Ae_US), GT-	B (Bj_JPN56)	, and GT-C	C (C-AT_JPN) w	vere
403	inserted into the EcoRI site of pEF1A-My	c-His <sub>6</sub> using	the In-Fusi	on HD-Cloning	kit
404	(Clontech). The cDNA fragments encoding	HBx from G	T-D (Ayw)	were amplified	by
405	reverse transcription-PCR from the total RN.	A of Hep38.7	-Tet cells a	nd were cloned	into
406	pEF1A-Myc-His <sub>6</sub> using the In-Fusion HD-Cle	oning kit (Clor	ntech). The	HBx-deficient H	IBV

407 plasmid (referred to as pUC-HBV-ΔHBx) was kindly provided by Dr. Y. Matsuura (RIMD,
408 Osaka University, Japan).

409 The expression plasmids for pCAG-FLAG-ISG15 and its conjugation defective mutant 410 (pCAG-FLAG-ISG15-AA) were previously described (39). The cDNA fragments encoding 411 UBE1L, UbcH8, and HERC5 were cloned into the NotI/BglII and SmaI/KpnI site of pCAG-412 MCS2 using the In-Fusion HD-Cloning kit. The cDNA fragments encoding HERC5, TRIM25, 413 and HHARI were also cloned into the NotI/BgIII site of pCAG-HA using the In-Fusion HD-414 Cloning kit. The point mutant HERC5 (C994A) was generated by PCR mutagenesis using 415 pCAG-HA-HERC5 as a template. The specific primers used for PCR were as follows: sense 416 primer (C994A), 5'-AGAGCACTGACAGCTTTCAGTGTC-3'; antisense primer (C994A), 417 5'-GACACTGAAAGCTGTCAGTGCTCT-3'. All the PCR applications were employed by 418 use of Tks DNA polymerase (TaKaRa Bio, Shiga, Japan). The sequences of the inserts were 419 extensively confirmed by sequencing (Eurofins Genomics, Tokyo, Japan).

420

421 Antibodies. The mouse monoclonal antibodies (mAbs) used in this study were anti-FLAG 422 (M2) mAb (F-3165, Sigma-Aldrich), anti-NS5A mAb (MAB8694, Millipore, Billerica, MA), 423 anti-NS3 mAb (MAB8691, Millipore), anti-glyceraldehyde-3-phosphate dehydrogenase 424 (GAPDH) mAb (MAB374, Millipore), anti-c-Myc mAb (9E10, Santa Cruz Biotechnology, 425 Santa Cruz, CA), anti-Hep B preS1 mAb (AP1, Santa Cruz Biotechnology), anti-Hep B pol 426 mAb (2C8, Santa Cruz Biotechnology), anti-UbcH8 mAb (ab56502-100), and anti-ISG15 427 mAb (F-9, Santa Cruz Biotechnology). The rabbit polyclonal (pAbs) or monoclonal 428 antibodies (mAbs) used in this study were anti-HA pAb (H-6908, Sigma-Aldrich), anti-HBx 429 pAb (39716, Abcam, Cambridge, UK), anti-STAT1 pAb (#9172, Cell Signaling Technology, 430 Beverly, MA), anti-USP18 mAb (D4E7, Cell Signaling Technology), anti-Ube1L mAb 431 (ab108929, Abcam), and anti-HBc pAb (kindly provided by Prof. T. Suzuki, Hamamatsu
432 University School of Medicine, Japan). Horseradish peroxidase (HRP)-conjugated goat anti433 mouse IgG and goat anti-rabbit IgG antibody (Cell Signaling Technology) were used as
434 secondary antibodies.

435

Immunoprecipitation and immunoblot analysis. Cells were transfected with the plasmids 436 437 using FuGene 6 (Promega), harvested at 48 h post-transfection, and suspended in 500 µl of 438 RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% SDS, 1% NP-40, 439 0.5% deoxycholate (DOC), and protease inhibitor cocktail tablets (Roche Molecular 440 Biochemicals, Mannheim, Germany). Cell lysates were incubated at 4°C for 2h and 441 centrifuged at 20,400 × g (TOMY centrifuge MX-307, Rotor Rack AR015-SC24) at 4°C for 442 30 min. The supernatant was immunoprecipitated with protein G Sepharose 4 fast flow (GE 443 Healthcare, Buckinghamshire, UK) and incubated with appropriate antibodies at 4°C 444 overnight.

445 After being washed with the RIPA buffer five times, the samples were boiled in 15  $\mu$ l of 446 sodium dodecyl sulfate (SDS) sample buffer and then subjected to SDS-10% polyacrylamide 447 gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes 448 (PVDF) (Millipore). The membranes were blocked with Tris-buffered saline containing 20 449 mM Tris-HCl (pH 7.6), 135 mM NaCl, and 0.05% Tween 20 (TBST) supplemented with 5% 450 bovine serum albumin (BSA) at room temperature for 2 h and incubated with corresponding 451 antibodies. The membranes were then incubated with HRP-conjugated secondary antibody at 452 room temperature for 2 h. The immune complexes and cell lysates were visualized with ECL 453 western blotting detection reagents (GE Healthcare) and detected by a LAS-4000 image 454 analyzer system (GE Healthcare).

455

456 Nickel-Nitrilotriacetic Acid (Ni-NTA) pull-down analysis. Cells were transfected with the 457 plasmids using FuGene 6 (Promega), harvested at 48 h post-transfection, and extracted sample 458 using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% SDS, 1% 459 NP-40, 0.5% deoxycholate (DOC), and protease inhibitor cocktail tablets. The extracted 460 sample was then added into His60 Ni Superflow Resin & Gravity Columns (TaKaRa Bio) 461 and rotated at 4°C for 2h. Precipitates were washed three times with His60 Ni equilibration 462 buffer containing 50 mM sodium phosphate, 6 M guanidine-HCl, 300 mM NaCl, and 20 mM 463 imidazole (pH 7.4) and then eluted by His60 Ni Elution buffer containing 50 mM sodium 464 phosphate, 6 M guanidine-HCl, 300 mM NaCl, and 300 mM imidazole (pH 7.4). The 465 collected fractions were boiled in SDS sample buffer and then subjected to immunoblotting. 466

467 RNA interference and stable ISG15 knockdown cell clones. Small interfering (si)RNAs 468 targeted to the human ISG15 (5'-UGAGCACCGUGUUCAUGAAdTdT-3') was purchased 469 from Sigma Genosys (Hokkaido, Japan). The siRNA targeted to the human USP18 (ID: 470 s22261) and negative control (NC) siRNA (the silencer Select Negative Control No. 1) were 471 purchased from Ambion (Austin, TX). Hep38.7-Tet cells were transfected with 50 nM siRNA 472 using RNAiMax transfection reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. At 72 h post-transfection, the cells were treated with 10<sup>3</sup> units/ml 473 474 of type-I IFN-α for 24 h. The short hairpin RNA (shRNA) fragment targeted to ISG15 (5'-475 UGAGCACCGUGUUCAUGAA-3') was inserted into the pSilencer 2.1 U6 puro vector 476 following the manufacturer's instruction (Ambion). To establish the stable ISG15knockdown cell clones, Hep38.7-Tet cells were transfected with the shRNA plasmids, and 477

drug-resistant clones were selected by the treatment of puromycin (Sigma-Aldrich) at a finalconcentration of 1 μg/ml.

480

481 Real-time PCR. Total RNA was prepared from each of the cells using a RNeasy mini kit 482 (Qiagen, Valencia, CA). First-strand cDNA was synthesized using the GoScript<sup>TM</sup> reverse 483 transcription system (Promega). The real-time PCR was performed using SYBR Premix Ex 484 Taq<sup>TM</sup> II (Tli RNaseH plus) (TaKaRa Bio) according to the manufacturer's protocol. 485 Fluorescent signals were analyzed by a StepOnePlus real-time PCR system (Applied 486 Biosystems, Foster City, CA). The HBV RNA, ISG15, and GAPDH genes were amplified 487 specific primer pairs 5'-GACCACCAAATGCCCCTATC-3' and using the 5'-488 GATTGAGATCTTCTGCGACGC-3'; 5'-AGCGAACTCATCTTTGCCAGTACA-3' and 5'-489 CAGCTCTGACACCGACATGGA-3'; and 5'-GCCATCAATGACCCCTTCATT-3' and 5'-490 TCTCGCTCCTGGAAGATGG-3', respectively. The expression level of each gene was 491 determined by the  $\Delta\Delta C_T$  method using GAPDH as an internal control. To quantitate intracellular and extracellular HBV rcDNA, cell pellets and culture supernatants were 492 493 collected from Hep38.7-Tet cells, and viral DNA was extracted using a QIAamp DNA mini kit (Qiagen). Extracted DNA was purified, followed by the real-time PCR analysis using the 494 495 primer pairs 5'-GGAGGGATACATAGAGGTTCCTTGA-3' and 5'-496 GTTGCCCGTTTGTCCTCTAATTC-3'.

497

498 Statistics. Results are expressed as the mean ± standard error. Student's t-test was used to
499 analyze the data. P-values <0.05 was considered significant.</li>

- 500
- 501 Funding information

502	This work was supported by the Program for Basic and Clinical Research on Hepatitis from
503	the Japan Agency for Medical Research and Development (AMED) under Grant No.
504	20fk0310104h9904 and 20fk0310104s1204. R.G.B was supported by the Program for
505	Nurture of Next Generation Leaders Guiding Medical Innovation in Asia of the Ministry of
506	Education, Culture, Sports, Science, and Technology (MEXT) of Japan. This research was
507	supported in parts by The Daiichi Sankyo Foundation of Life Science (T.A.) and The Mochida
508	Memorial Foundation for Medical and Pharmaceutical Research (T.A.).
509	
510	Acknowledgments
511	We are grateful to Dr. C.M. Rice (The Rockefeller University, New York, NY) for providing
512	the Huh7.5 cells. We thank Y. Kozaki for the secretarial work.
513	
514	Author Contributions
515	R.G.B., T.A., and I.S. conceived and designed the experiments. R.G.B. and T.A. carried out
516	most of the experiments. Q.X., C.M., D.K., and L.D. assisted the construction of plasmids
517	and the data analysis. A.R., K.S., Y.M, T.S., S.M. and M.M. contributed to the materials
518	providing. T.A. and I.S. wrote the manuscript.
519	
520	Conflict of Interest
521	The authors declare that there are no conflicts of interest.
522	

523	REFERENCES
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- Trépo C, Chan HLY, Lok A. Hepatitis B virus infection. *The Lancet* 2014;384:2053 2063.
- 527 2. Revill PA, Chisari FV, Block JM, Dandri M, Gehring AJ, *et al.* A global scientific
  528 strategy to cure hepatitis B. *Lancet Gastroenterol Hepatol* 2019;4:545-558.
- 529 3. Ng SA, Lee C. Hepatitis B virus X gene and hepatocarcinogenesis. *J Gastroenterol* 2011;
  530 46:974-990.
- 4. Melegari M, Scaglioni PP, Wands JR. Cloning and characterization of a novel hepatitis
  B virus x binding protein that inhibits viral replication. *J Virol* 1998;72:1737-1743.
- 5. Truant R, Antunovic J, Greenblatt J, Prives C, Cromlish JA. Direct interaction of the
  hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response
  element-directed transactivation. *J Virol* 1995;69:1851-1859.
- 536 6. Lee TH, Elledge SJ, Butel JS. Hepatitis B virus X protein interacts with a probable
  537 cellular DNA repair protein. *J Virol* 1995;69:1107-1114.
- 538 7. Sitterlin D, Lee TH, Prigent S, Tiollais P, Butel JS, *et al.* Interaction of the UV539 damaged DNA-binding protein with hepatitis B virus X protein is conserved among
  540 mammalian hepadnaviruses and restricted to transactivation-proficient X-insertion
  541 mutants. *J Virol* 1997;71:6194-6199.
- 542 8. Benhenda S, Ducroux A, Riviere L, Sobhian B, Ward MD, *et al.* Methyltransferase
  543 PRMT1 is a binding partner of HBx and a negative regulator of hepatitis B virus
  544 transcription. *J Virol* 2013;87:4360-4371.

- 545 9. Kouwaki T, Okamoto T, Ito A, Sugiyama Y, Yamashita K, *et al.* Hepatocyte Factor
  546 JMJD5 Regulates Hepatitis B Virus Replication through Interaction with HBx. *J Virol*547 2016:90:3530-3542.
- 548 10. Tanaka Y, Kanai F, Ichimura T, Tateishi K, Asaoka Y, et al. The hepatitis B virus X
- 549 protein enhances AP-1 activation through interaction with Jab1. *Oncogene* 2006;25:633-550 642.
- 551 11. Zhang T, Xie N, He W, Liu R, Lei Y, *et al.* An integrated proteomics and bioinformatics
- analyses of hepatitis B virus X interacting proteins and identification of a novel interactor
  apoA-I. *J Proteomics* 2013;84:92-105.
- 12. Geng X, Huang C, Qin Y, McCombs JE, Yuan Q, *et al.* Hepatitis B virus X protein
  targets Bcl-2 proteins to increase intracellular calcium, required for virus replication and
  cell death induction. *Proc Natl Acad Sci U S A* 2012;109:18471-18476.
- 13. Hong A, Han DD, Wright CJ, Burch T, Piper J, *et al.* The interaction between hepatitis
  B virus X protein and AIB1 oncogene is required for the activation of NFkappaB signal
  transduction. *Biochem Biophys Res Commun* 2012;423:6-12.
- 560 14. Pang R, Lee TK, Poon RT, Fan ST, Wong KB, *et al.* Pin1 interacts with a specific
  561 serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis.
  562 *Gastroenterology* 2007;132:1088-1103.
- 563 15. Kumar M, Jung SY, Hodgson AJ, Madden CR, Qin J, *et al.* Hepatitis B virus
  564 regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation of beta
- 565 interferon. *J Virol* 2011;85:987-995.
- 566 16. Kalra N, Kumar V. The X protein of hepatitis B virus binds to the F box protein Skp2
- and inhibits the ubiquitination and proteasomal degradation of c-Myc. *FEBS Lett*2006;580:431-436.

- 569 17. Deng L, Gan X, Ito M, Chen M, Aly HH, *et al.* Peroxiredoxin 1, a Novel HBx570 Interacting Protein, Interacts with Exosome Component 5 and Negatively Regulates
  571 Hepatitis B Virus (HBV) Propagation through Degradation of HBV RNA. *J Virol*572 2019;93: e02203-18.
- 573 18. Isaacson MK, Ploegh HL. Ubiquitination, ubiquitin-like modifiers, and deubiquitination
  574 in viral infection. *Cell Host Microbe* 2009;5:559-570.
- 575 19. Liu N, Zhang J, Yang X, Jiao T, Zhao X, *et al.* HDM2 Promotes NEDDylation of
  576 Hepatitis B Virus HBx To Enhance Its Stability and Function. *J Virol* 2017;91: e00340-
- **577** 17.
- 578 20. Zhang D, Zhang DE. Interferon-stimulated gene 15 and the protein ISGylation system.
  579 *J Interferon Cytokine Res* 2011;31:119-130.
- 580 21. Zou W, Zhang DE. The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP
  581 also functions as an ISG15 E3 ligase. *J Biol Chem* 2006;281:3989-3994.
- 582 22. Dastur A, Beaudenon S, Kelley M, Krug RM, Huibregtse JM. Herc5, an interferon-
- induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J Biol Chem* 2006;281:4334-4338.
- 585 23. Okumura F, Zou W, Zhang DE. ISG15 modification of the eIF4E cognate 4EHP
  586 enhances cap structure-binding activity of 4EHP. *Genes Dev* 2007;21:255-260.
- 587 24. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18)
  588 specifically removes ISG15 from conjugated proteins. *J Biol Chem* 2002;277:9976-9981.
- 589 25. Li Y, Li S, Duan X, Chen Y, Jiao B, et al. Interferon-Stimulated Gene 15 Conjugation
- 590 Stimulates Hepatitis B Virus Production Independent of Type I Interferon Signaling
- 591 Pathway In Vitro. *Mediators Inflamm* 2016;7417648.

- 592 26. Abe T, Minami N, Rheza Gandi Bawono, Chieko M, Lin D, et al. ISGylation of
- hepatitis C virus NS5A protein promotes viral RNA replication via recruitment of
  cyclophilin A. *J Virol* 2020;94:e00532-20.
- 595 27. Hoan NX, Van Tong H, Giang DP, Toan NL, Meyer CG, et al. Interferon-stimulated
- gene 15 in hepatitis B-related liver diseases. *Oncotarget* 2016;7:67777-67787.
- 597 28. Dos Santos PF, Mansur DS. Beyond ISGlylation: Functions of Free Intracellular and
  598 Extracellular ISG15. *J Interferon Cytokine Res* 2017;37:246-253.
- 599 29. Lee HS, Lim YS, Park EM, Baek SH, Hwang SB. SUMOylation of nonstructural 5A
  600 protein regulates hepatitis C virus replication. *J Viral Hepat* 2014;21:e108-117.
- 30. Su CI, Tseng CH, Yu CY, Lai MMC. SUMO Modification Stabilizes Dengue Virus
  Nonstructural Protein 5 To Support Virus Replication. *J Virol* 2016;90:4308-4319.
- 603 31. Sekiba K, Otsuka M, Ohno M, Yamagami M, Kishikawa T, et al. Inhibition of HBV
- 604 Transcription From cccDNA With Nitazoxanide by Targeting the HBx-DDB1 Interaction.
  605 *Cell Mol Gastroenterol Hepatol* 2019;7:297-312.
- 606 32. Wong JJ, Pung YF, Sze NS, Chin KC. HERC5 is an IFN-induced HECT-type E3 protein
- 607 ligase that mediates type I IFN-induced ISGylation of protein targets. *Proc Natl Acad Sci*608 USA 2006;103:10735-10740.
- 609 33. Wang Y, Ding Q, Xu T, Li CY, Zhou DD, et al. HZ-6d targeted HERC5 to regulate p53
- 610 ISGylation in human hepatocellular carcinoma. *Toxicol Appl Pharmacol* 2017;334:180-
- **611** 191.
- 612 34. Henkler F, Hoare J, Waseem N, Goldin RD, McGarvey MJ, et al. Intracellular
- 613 localization of the hepatitis B virus HBx protein. *J Gen Virol* 2001;82:871-882.

614	35. Keasler VV, Hodgson AJ, Madden CR, Slagle BL. Hepatitis B virus HBx protein
615	localized to the nucleus restores HBx-deficient virus replication in HepG2 cells and in
616	vivo in hydrodynamically-injected mice. Virology 2009;390:122-129.

- 617 36. Kornyeyev D, Ramakrishnan D, Voitenleitner C, Livingston CM, Xing W, et al.
- 618 Spatiotemporal Analysis of Hepatitis B Virus X Protein in Primary Human Hepatocytes.
- 619 *J Virol* 2019;93: e00248-19.
- 620 37. Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM. Human ISG15 conjugation
- 621 targets both IFN-induced and constitutively expressed proteins functioning in diverse
  622 cellular pathways. *Proc Natl Acad Sci U S A* 2005;102:10200-10205.
- 623 38. Giannakopoulos NV, Luo JK, Papov V, Zou W, Lenschow DJ, *et al.* Proteomic
  624 identification of proteins conjugated to ISG15 in mouse and human cells. *Biochem*625 *Biophys Res Commun* 2005;336:496-506.
- 626 39. Minami N, Abe T, Deng L, Matsui C, Fukuhara T, et al. Unconjugated interferon-
- 627 stimulated gene 15 specifically interacts with the hepatitis C virus NS5A protein via
- 628 domain I. *Microbiol Immunol* 2017;61:287-292.

#### 629 FIGURE LEGENDS

630

631 Fig. 1. HBx is a substrate for ISG vlation. A: The expression plasmid encoding HBx-Myc-632 His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) was co-expressed with 633 either pCAG-FLAG-ISG15 or FLAG-ISG15 mutant (pCAG-FLAG-ISG15-AA) together 634 with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-635 HERC5) in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb 636 and detection with anti-HBx rabbit pAb. The membrane was stripped by stripping buffer and 637 was reprobed with anti-FLAG mouse mAb. IP: immunoprecipitation. IB: immunoblotting. 638 LC: immunoglobulin light chain. **B**: The expression plasmid encoding either the full-length 639 HBV gene (pUC-HBV) from GT-C (C-AT\_JPN) or an HBV gene lacking the HBx region 640 (pUC-HBV-ΔHBx) was co-expressed with pCAG-UBE1L, pCAG-UbcH8, and pCAG-HA-641 HERC5 in Huh7.5 cells, followed by immunoprecipitation with anti-FLAG mouse mAb and 642 detection with anti-HBx rabbit pAb. Input samples (indicated as Lysate) were detected with 643 anti-HBx rabbit pAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, anti-HA rabbit pAb, 644 or anti-ISG15 mouse mAb as indicated. The membrane was stripped by stripping buffer and 645 was reprobed with anti-FLAG mouse mAb. The asterisks (\*) indicates the ISG15-conjugated 646 HBx proteins (ISGylated-HBx). The western blots are representative for three independent 647 experiments.

648

Fig. 2. HERC5 is a specific E3 ligase for HBx-ISGylation. A: The expression plasmid
encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) was
co-expressed with pCAG-FLAG-ISG15 and an HA-tagged E3 ligase, i.e., (*a*) pCAG-HAHERC5, (*b*) pCAG-HA-TRIM25, or (*c*) pCAG-HA-HHARI together with pCAG-UBE1L

653 (E1) and pCAG-UbcH8 (E2) in HEK293T cells, followed by immunoprecipitation with anti-654 FLAG mouse mAb and detection with anti-HBx rabbit pAb. B: The expression plasmid 655 encoding HBx-Myc-His<sub>6</sub> from GT-C (C-AT JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) was co-656 expressed with pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), 657 and pCAG-HA-HERC5 (WT) or an inactive mutant HERC5 (C994A) (pCAG-HA-HERC5 658 C994A) in 293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb and 659 detection with anti-HBx rabbit pAb. The membrane was stripped by stripping buffer and was 660 reprobed with anti-FLAG mouse mAb. The faint band in lane 2 was the remaining of 661 previously blotted HBx. C: The expression plasmid encoding HBx-Myc-His<sub>6</sub> from GT-C (C-662 AT JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) was co-expressed with pCAG-FLAG-ISG15 together 663 with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and pCAG-HA-HERC5 or with the depletion 664 of E3 or both E2 and E3 plasmids in HEK293T cells, followed by immunoprecipitation with 665 anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb. Input samples (indicated as 666 Lysate) were detected with anti-HBx rabbit pAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse 667 mAb, anti-HA rabbit pAb, anti-ISG15 mouse mAb, or anti-FLAG mouse mAb. The asterisks 668 indicate the ISG15-conjugated HBx proteins (ISGylated-HBx). The membrane was stripped 669 by stripping buffer and was reprobed with anti-FLAG mouse mAb. The faint band in lane 4 670 was the remaining of previously blotted HBx. The western blots are representative for three 671 independent experiments.

672

Fig. 3. The Lys residues responsible for HBx-ISGylation in four different HBV
genotypes. A: Schematic diagram of HBx from genotype (GT)-C (C-AT\_JPN). The amino
acid sequence of 1 to 30 and 58 to 140 indicate regulatory-domain and transactivation-domain,

677 respectively. **B**: The sequence alignment of HBx from four HBV genotypes (GTs): GT-A 678 (Ae-US), GT-B (Bj\_JPN56), GT-C (C-AT\_JPN), and GT-D (Ayw). These HBx GTs except 679 for GT-B, have six conserved Lvs (K) residues (K91, K95, K113, K118, K130, and K140) 680 (highlighted in pink). HBx GT-B has Asn (N) residue at amino acid 118. C: The expression 681 plasmid encoding HBx-Myc-His<sub>6</sub> (pEF1A-HBx-Myc-His<sub>6</sub>) derived from four different HBV 682 GTs was co-expressed with pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-683 UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5) in HEK293T cells, followed by 684 immunoprecipitation with anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb. 685 Input samples (indicated as Lysate) were detected with anti-HBx rabbit pAb, anti-UBE1L 686 rabbit mAb, anti-UbcH8 mouse mAb, anti-HA rabbit pAb, or anti-ISG15 mouse mAb. The 687 asterisks indicate the ISG15-conjugated HBx proteins (ISGylated-HBx). The western blots 688 are representative for three independent experiments.

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691 Fig. 4. K91, K95, and K140 on HBx protein are responsible for ISGylation. A: The 692 expression plasmid encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT JPN) (pEF1A-693 HBx-Myc-His<sub>6</sub>) or its mutant, in which all Lys (K) residues were mutated to Arg (R) 694 (indicated as Null) and mutants containing a point mutation of Lys to Arg at a corresponding 695 Lys residue (K/R mutant series), was co-expressed with pCAG-FLAG-ISG15 together with 696 pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5) in 697 HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb and detection 698 with anti-HBx rabbit pAb. B: The expression vector encoding HBx-Myc-His<sub>6</sub> from GT-C (C-699 AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) or its mutant consisting of the Lys residues mutated to 700 Arg except for the indicated Lys residues (indicated as K91/K95 and K91/K95/K140) and 701 mutants containing a point mutation of Lys to Arg at a corresponding Lys residue (indicated 702 as K91R/K95R and K91R/K95R/K140R) was co-expressed with pCAG-FLAG-ISG15 703 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-704 HA-HERC5) in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse 705 mAb and detection with anti-HBx rabbit pAb. Input samples (indicated as Lysate) were 706 detected with anti-HBx rabbit pAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, anti-707 HA rabbit pAb, or anti-ISG15 mouse mAb. The asterisks indicate the ISG15-conjugated HBx 708 proteins (ISGylated-HBx). The western blots are representative for three independent 709 experiments.

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711 Fig. 5. HBV replication is resistant to the treatment of various IFNs in the Hep38.7-Tet 712 cells. Hep38.7-Tet cells (A) and HCV subgenomic replicon cells (SGR; genotype 1b, Con1) 713 (**B**) were treated with  $10^3$  units/ml of type I (IFN- $\alpha$  and IFN- $\beta$ ) or type III IFNs (IL-28A, IL-714 28B, and IL-29) for 5 days, and the levels of intracellular HBV RNA and HBV relaxed 715 circular DNA (rcDNA) or HCV RNA, and ISG15 mRNA were measured by real-time PCR. 716 The level of mRNA expression was normalized by GAPDH expression. C: The cell lysates 717 prepared from HCV-SGR cells treated with indicated IFNs or 1 µM daclatasvir (DCV) for 5 718 days were subjected to immunoblotting with the indicated antibodies. DMSO was treated as 719 the control. D: An expression vector encoding the full-length HBV gene from GT-C (C-720 AT\_JPN) (pUC-HBV-WT) was transfected in HepG2-NTCP cells and then treated with 10<sup>3</sup> 721 units/ml of type I (IFN- $\alpha$  and IFN- $\beta$ ) or type III IFNs (IL-28A, IL-28B, and IL-29) for 5 days. 722 The levels of intracellular HBV RNA and ISG15 mRNA were measured by real-time PCR. 723 The level of mRNA expression was normalized by GAPDH expression. Results are the mean 724 values from triplicates  $\pm$  SD (n=3 biological replicates).

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726 Fig. 6. ISG15 functions as an IFN-resistance factor in HBV-replicating cells. Hep38.7-727 Tet cells were cultured in the medium without 400 ng/ml doxycycline. After 5 days of culture, 728 cells were harvested and then treated with 50 nM siRNA targeted to ISG15 (siISG15) (A), 729 USP18 (siUSP18) (B), or negative control (NC) siRNA (siRNA-NC). After 72 h of incubation, cells were treated with or without  $10^3$  units/ml of type I IFN- $\alpha$  for 24 h. The levels of 730 731 extracellular HBV rcDNA and ISG15 mRNA were measured by real-time PCR. Data from 732 the real-time PCR were normalized to the amount of GAPDH mRNA expression. The cell 733 lysates were subjected to immunoblotting with the indicated antibodies (A, B: lower blotting 734 panels). \*p<0.05 vs. the results for the cells treated with NC siRNA without IFN-α. Results 735 are the mean values from triplicates  $\pm$  SD (n=3 biological replicates).

736

Fig. 7. A proposed model of the functional role of HBx-ISGylation. HBx protein is
expressed as a nonstructural protein during viral life cycle and is involved in the viral
replication and pathogenesis. HBx protein is ISGyalyed at the residues K91, K95, and K140
via the action of three enzymes: E1 activating enzyme (UBE1L), E2 conjugating enzyme
(UbcH8) and E3 ligase (HERC5) in HBV-replicating cells, thereby promoting the viral
replication and acquisition of IFN-resistant phonotype.

## Α.



## Β.



















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C.



Fig.3

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#### Fig. S1. Other HBV proteins, including HBc, HBV polymerase, and HBs proteins, do not accept ISGylation.

HEK293T cells were co-transfected with the expression plasmid encoding HBc (A), HBV polymerase (HBV pol) (B), or large HBV surface (LHBs) (C) genes from genotype (GT)-C (C-AT\_JPN) together with pCAG-FLAG-ISG15, pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5), followed by immunoprecipitation with anti-FLAG mouse mAb and detection with the anti-HBV specific antibodies. Input samples (indicated as Lysate) were detected with anti-HBc rabbit pAb, anti-Hep B preS1 mouse mAb, anti-Hep B pol mouse mAb, or anti-FLAG mouse mAb as indicated. IP: immunoprecipitation. IB: immunoblotting. HC: immunoglobulin heavy chain. LC: immunoglobulin light chain.



#### Fig. S2. HBx protein is a substrate of ISGylation.

HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) with either pCAG-FLAG-ISG15 or FLAG-ISG15 mutant (pCAG-FLAG-ISG15-AA) together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5), followed by immunoprecipitation with anti-HBx rabbit pAb and detection with anti-ISG15 mouse mAb. Input samples (indicated as Lysate) were detected with anti-HBx rabbit pAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, anti-HA rabbit pAb, or anti-ISG15 mouse mAb as indicated. The asterisks (\*) indicates the ISG15-conjugated HBx proteins (ISGylated-HBx). IP: immunoprecipitation. IB: immunoblotting.



#### Fig. S3. Ni-NTA pull-down analysis for ISGylated HBx proteins.

HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) and pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5), followed by pull-down with Ni-NTA resin under denaturing condition (6M guanidine-HCI) and detection with anti-HBx rabbit pAb. Input samples (indicated as Lysate) were detected with anti-HBx rabbit pAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, anti-HA rabbit pAb, or anti-ISG15 mouse mAb as indicated. The asterisks (\*) indicates the ISG15-conjugated HBx proteins (ISGylated-HBx). IB: immunoblotting.



#### Fig. S4. HBx protein interacts with ISG15 via protein-protein interaction.

HEK293T cells were co-transfected with the expression plasmid pEF1A-HBx-Myc-His<sub>6</sub> and pCAG-FLAG-ISG15 together with pCAG-UBE1L, pCAG-UbcH8, and pCAG-HA-HERC5. At 2days after transfection, cells were lysed with RIPA buffer containing 150 mM NaCl or 300 mM NaCl, followed by immunoprecipitation with anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb or anti-FLAG mouse mAb. Input samples (indicated as Lysate) were detected with anti-HBx rabbit pAb, anti-FLAG mouse mAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, and anti-HA rabbit pAb.



#### Fig. S5. HBs protein does not accept ISGylation in the cells transfected with pUC-HBV.

The expression plasmid encoding either the full-length HBV gene (pUC-HBV) from GT-C (C-AT\_JPN) or an HBV gene lacking the HBx region (pUC-HBV-∆HBx) was co-expressed with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and pCAG-HA-HERC5 (E3) in Huh7.5 cells, followed by immunoprecipitation with anti-FLAG mouse mAb and detection with anti-Hep B preS1 mouse mAb. Input samples (indicated as Lysate) were detected with anti-Hep B preS1 mouse mAb, anti-UBE1L rabbit mAb, anti-UbcH8 mouse mAb, anti-HA rabbit pAb, or anti-ISG15 mouse mAb as indicated. IP: immunoprecipitation. IB: immunoblotting. HC: immunoglobulin heavy chain. LC: immunoglobulin light chain.