



# HERC5 E3 ligase mediates ISGylation of hepatitis B virus X protein to promote viral replication

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1 **HERC5 E3 ligase mediates ISGylation of hepatitis B virus X protein to promote viral**  
2 **replication**

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28 **Short Title:** HBx-ISGylation promotes viral replication

29

30 **Keywords:** hepatitis B virus, HBx, ISG15, ISGylation, interferon, viral replication

31

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)  
35 X protein (HBx) is modified by ISG15, which is a type I interferon (IFN)-inducible, ubiquitin-  
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- $\alpha$ ,  
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- $\alpha$ -mediated  
49 antiviral activity.

50

51

52 **INTRODUCTION**

53 Hepatitis B virus (HBV) infection is a leading cause of chronic hepatitis, liver cirrhosis and  
54 hepatocellular carcinoma (HCC), and remains a major public health burden worldwide (1).  
55 Current approved therapies against chronic HBV infection are conducted using several  
56 nucleos(t)ide analogs and pegylated-interferon (IFN); these drugs reduce the viral load, but  
57 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.

70 Ubiquitylation and ubiquitin-like protein modifications, such as NEDDylation,  
71 SUMOylation, and ISGylation, play important roles in modulation of the functions of many  
72 viral proteins (18), but little is known about the exact mechanism how these protein  
73 modifications affect HBV infection. Liu and colleagues (19) recently reported that the E3  
74 ligase HDM2 promotes the NEDDylation of HBx protein to maintain HBx stability and

75 chromatin localization, which promotes HBx-dependent transcriptional regulation, cell  
76 proliferation, and tumor development.

77 IFN-stimulated gene 15 (ISG15) is the ubiquitin-like protein that is induced by  
78 stimulation with type I IFN or by viral or bacterial infection (20). ISG15 is covalently  
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  
96 and the progression of HBV-related HCC (27). These observations suggest that intracellular  
97 ISG15/ISGylation has a pro-viral function, but the exact mechanisms underlying this  
98 correlation remain to be elucidated.

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

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

123 Fig. 2, lane 2, left panel; asterisks indicate ISGylated HBx). These results suggest that HBx  
124 protein actually accepts ISGylation.

125 Next, to verify that the slowly migrating forms of HBx are ISGylated HBx proteins,  
126 we performed an immunoprecipitation analysis using a conjugation-defective ISG15 mutant  
127 that possesses a Gly-Gly to Ala-Ala substitution within the C-terminal LRLRGG motif,  
128 termed FLAG-ISG15-AA. The expression of FLAG-ISG15, but not FLAG-ISG15-AA,  
129 resulted in the induction of HBx-ISGylation (Fig. 1A, first and second panels, lanes 3 and 4).  
130 These results indicate that the slowly migrating forms of HBx protein are indeed ISGylated  
131 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.

140 To ask a question why unmodified HBx was co-precipitated with FLGA-ISG15  
141 (Fig.1A, lane 3; Fig. 1B, lane 3), we examined HBx-ISGylation under more stringent  
142 condition using RIPA buffer containing 300mM NaCl. The IP-western blot analysis revealed  
143 that the level of unmodified HBx (~17 kDa) was markedly reduced compared to the regular  
144 RIPA buffer containing 150mM NaCl (Supplementary Fig. 4, left panel, lane 4). These results  
145 suggest that the unmodified HBx can interact with ISG15 via protein-protein interaction. The  
146 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 the  
148 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.

158

### 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.

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

171 strongly induced the HBx-ISGylation (Fig. 2B, first and second panels, lanes 2 and 3;  
172 asterisks indicate ISGylated HBx), indicating the requirement of HERC5 ligase activity for  
173 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.

184

### 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

195 that the HBx proteins derived from all four different HBV genotypes were ISGylated in their  
196 co-transfected cells (Fig. 3C, left panel, lanes 2, 4, 6, and 8; asterisks indicate ISGylated HBx).  
197 In the immunoblot analysis with anti-HBx, anti-HA, or anti-ISG15, the equivalent expression  
198 levels of HBx, HERC5, or ISG15 were detected in the cells co-transfected with plasmids  
199 expressing HBx and E1/E2/E3 ligases (Fig. 3C, indicated panels). These results suggest that  
200 the HBx proteins of all four different HBV genotypes investigated in this study accept  
201 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.

213 A further immunoblot analysis using the HBx K/R-mutants revealed that the  
214 ISGylation of HBx protein was markedly reduced in the HBx-Myc-His<sub>6</sub> (K91R, K95R, and  
215 K140R) mutants compared to HBx (WT) (Fig. 4A, upper and middle panels, lanes 3, 4, and  
216 8; asterisks indicate ISGylated HBx). Although slight HBx-ISGylation was still present in the  
217 cells transfected with the HBx-Myc-His<sub>6</sub> (K91R/K95R) double mutant, in which residues K91  
218 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.

227  
228 **The persistent HBV replication is resistant to the treatment of various IFNs in the**  
229 **Hep38.7-Tet cells**

230 Next, to determine the effects of various IFNs on HBV replication, we analyzed intracellular  
231 HBV RNA and HBV rcDNA in Hep38.7-Tet cells, which are a doxycycline (Dox)-inducible  
232 HBV cell culture model. At 2 days after doxycycline removal, Hep38.7-Tet cells were treated  
233 with 10<sup>3</sup> units/ml of type I (IFN- $\alpha$  and IFN- $\beta$ ) or type III (IL28A, IL28B, and IL29) IFNs for  
234 5 days, and the expression levels of intracellular HBV RNA and HBV rcDNA were measured  
235 by real-time polymerase chain reaction (PCR).

236 As shown in Fig. 5A, the treatment with type I and type III IFNs did not result in  
237 reduction of the levels of HBV RNA or HBV rcDNA in Hep38.7-Tet cells, although high  
238 levels of ISG15 mRNA expression were observed in the IFN-treated cells. After the treatment  
239 with type I and type III IFNs, a limited suppression of HBV RNA was observed in the cells  
240 transfected with the full-length HBV expression plasmid (pUC-HBV-C-AT\_JPN) (Fig. 5D).  
241 In contrast, the treatment of the cells with type I and type III IFNs resulted in marked  
242 reductions of both HCV RNA and the levels of viral proteins in the HCV subgenomic replicon

243 cells (1b, Con1 strain) (Fig. 5B and Fig. 5C). These results indicate that the treatment with  
244 IFNs had a limited ability to suppress the HBV replication in the Hep38.7-Tet cells.

245

### 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- $\alpha$  induced STAT1 expression in both ISG15  
260 and USP18 silencing cells treated with IFN- $\alpha$  (Fig. 6A and 6B, second panels, lanes 2 and 4).  
261 In addition, the marked reduction of IFN- $\alpha$ -induced ISGylation was observed in the ISG15-  
262 silencing cells, whereas the enhancement of IFN- $\alpha$ -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.

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

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

273

## 274 **DISCUSSION**

275 The IFN-inducible protein ISG15 and ISGylation are believed to play important roles  
276 in antiviral activity against many types of RNA and DNA viruses (20, 28). Our present results  
277 demonstrated that ISG15 functions as a pro-viral factor in HBV replication through HBx-  
278 ISGylation at multiple specific Lys residues. We demonstrated that the HERC5 E3 ligase  
279 mediates ISGylation of HBx protein. We further demonstrated that ISG15 is involved in the  
280 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).

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

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

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



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

340 In summary, we demonstrated evidence suggesting that HERC5 E3 ligase specifically  
341 mediates ISGylation of HBx protein to promote HBV replication. We identified the  
342 ISGylation sites on HBx protein, which are well conserved among HBV genotypes. Our  
343 results suggest that HBx-ISGylation participates in the positive regulation of viral replication  
344 and IFN-resistance of HBV. Targeting the ISGylation machinery on the HBx protein could  
345 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, Nuaille, 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-

360  $\beta$ ) and type-III IFNs (IL-28A, IL-28B, and IL-29) were purchased from PBL assay science  
361 (Piscataway, NJ).

362

363 **Plasmids.** The plasmids pEF1A-HBx-Myc-His<sub>6</sub> and pEF1A-HBc-Myc-His<sub>6</sub> have been  
364 previously described (17). The cDNA fragments of LHBs from genotype (GT)-C (C-  
365 AT\_JPN) was inserted into the EcoRI site of pEF1A-Myc-His<sub>6</sub> using the In-Fusion HD-  
366 Cloning kit (Clontech, Mountain View, CA). The cDNA fragments of HBx (C-AT\_JPN) with  
367 all of the Lys (K) residues mutated to Arg (R) were generated by PCR mutagenesis using  
368 pEF1A-HBx-Myc-His<sub>6</sub> as a template. The specific primers used for the PCR were as follows:

369 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 GACTGTTTGTGTTAGGGACTGGGAG-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 pEF1A-

381 HBx(K-Null)-Myc-His<sub>6</sub>. The construction of HBx Lys (K) mutants, including those with all

382 of the Lys (K) residues mutated to Arg (R) except for some combinations of the indicated Lys

383 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 template. The specific primers used for PCR were  
 385 as follows: sense primer (K91), 5'-CAGGTCTTGCCCAAGGTCTTATAT-3'; antisense  
 386 primer (K91), 5'-ATATAAGACCTTGGGCAAGACCTG-3', sense primer (K95), 5'-  
 387 AGGTCTTATATAAGAGGACTCTT-3'; antisense primer (K95), 5'-  
 388 AAGAGTCCTTATATAAGACCCT-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), 5'-  
 392 CTCCCAGTCCTTAAACAAACAGTC-3', sense primer (K130), 5'-  
 393 GAGATTAGGTTAAAGATTTTTGTA-3'; antisense primer (K130), 5'-  
 394 TACAAAAATCTTTAACCTAATCTC-3', sense primer (K140), 5'-  
 395 GGCTGTAGGCATAAATTGGTCTGT-3'; antisense primer (K140), 5'-  
 396 ACAGACCAATTTATGCCTACAGCC-3'. The construction of HBx Lys (K) mutants,  
 397 containing some combination mutants of Lys to Arg at the indicated Lys residues (referred to  
 398 as K91R/K95R and K91R/K95R/K140R) were generated by PCR mutagenesis using pEF1A-  
 399 HBx-Myc-His<sub>6</sub> as a template and specific primers described above. The HBV expression  
 400 plasmids pUC19-HBV-Ae\_US, pUC19-HBV- B<sub>j</sub>\_JPN56, and pUC19-HBV-C-AT\_JPN were  
 401 kindly provided by Dr. M. Mizokami (NCGM, Japan). The cDNA fragments of HBx from  
 402 HBV genotypes (GT): GT-A (Ae\_US), GT-B (B<sub>j</sub>\_JPN56), and GT-C (C-AT\_JPN) were  
 403 inserted into the EcoRI site of pEF1A-Myc-His<sub>6</sub> using the In-Fusion HD-Cloning kit  
 404 (Clontech). The cDNA fragments encoding HBx from GT-D (Ayw) were amplified by  
 405 reverse transcription-PCR from the total RNA of Hep38.7-Tet cells and were cloned into  
 406 pEF1A-Myc-His<sub>6</sub> using the In-Fusion HD-Cloning kit (Clontech). The HBx-deficient HBV

407 plasmid (referred to as pUC-HBV- $\Delta$ HBx) 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/BglII 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 anti-  
433 mouse IgG and goat anti-rabbit IgG antibody (Cell Signaling Technology) were used as  
434 secondary antibodies.

435  
436 **Immunoprecipitation and immunoblot analysis.** Cells were transfected with the plasmids  
437 using FuGene 6 (Promega), harvested at 48 h post-transfection, and suspended in 500  $\mu$ l 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  $\times$  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'-UGAGCACCGUGUUCAUGAAAdTdT-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  
473 manufacturer's instructions. At 72 h post-transfection, the cells were treated with 10<sup>3</sup> units/ml  
474 of type-I IFN- $\alpha$  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 ISG15-  
477 knockdown cell clones, Hep38.7-Tet cells were transfected with the shRNA plasmids, and

478 drug-resistant clones were selected by the treatment of puromycin (Sigma-Aldrich) at a final  
479 concentration 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™ reverse  
483 transcription system (Promega). The real-time PCR was performed using SYBR *Premix Ex*  
484 *Taq*™ 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 using the specific primer pairs 5'-GACCACCAAATGCCCTATC-3' and 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  
492 intracellular and extracellular HBV rcDNA, cell pellets and culture supernatants were  
493 collected from Hep38.7-Tet cells, and viral DNA was extracted using a QIAamp DNA mini  
494 kit (Qiagen). Extracted DNA was purified, followed by the real-time PCR analysis using the  
495 primer pairs 5'-GGAGGGATACATAGAGGTTCCCTTGA-3' and 5'-  
496 GTTGCCCGTTTGTCCTCTAATTC-3'.

497  
498 **Statistics.** Results are expressed as the mean  $\pm$  standard error. Student's t-test was used to  
499 analyze the data. P-values <0.05 was considered significant.

500

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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**

524

- 525 1. **Trépo C, Chan HLY, Lok A.** Hepatitis B virus infection. *The Lancet* 2014;384:2053-  
526 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.
- 531 4. **Melegari M, Scaglioni PP, Wands JR.** Cloning and characterization of a novel hepatitis  
532 B virus x binding protein that inhibits viral replication. *J Virol* 1998;72:1737-1743.
- 533 5. **Truant R, Antunovic J, Greenblatt J, Prives C, Cromlish JA.** Direct interaction of the  
534 hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response  
535 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 UV-  
539 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  
552 analyses of hepatitis B virus X interacting proteins and identification of a novel interactor  
553 apoA-I. *J Proteomics* 2013;84:92-105.
- 554 12. **Geng X, Huang C, Qin Y, McCombs JE, Yuan Q, et al.** Hepatitis B virus X protein  
555 targets Bcl-2 proteins to increase intracellular calcium, required for virus replication and  
556 cell death induction. *Proc Natl Acad Sci U S A* 2012;109:18471-18476.
- 557 13. **Hong A, Han DD, Wright CJ, Burch T, Piper J, et al.** The interaction between hepatitis  
558 B virus X protein and AIB1 oncogene is required for the activation of NFkappaB signal  
559 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  
567 and inhibits the ubiquitination and proteasomal degradation of c-Myc. *FEBS Lett*  
568 2006;580:431-436.

- 569 17. **Deng L, Gan X, Ito M, Chen M, Aly HH, et al.** Peroxiredoxin 1, a Novel HBx-  
570 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-  
583 induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J Biol*  
584 *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  
593 hepatitis C virus NS5A protein promotes viral RNA replication via recruitment of  
594 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  
596 gene 15 in hepatitis B-related liver diseases. *Oncotarget* 2016;7:67777-67787.
- 597 28. **Dos Santos PF, Mansur DS.** Beyond ISGylation: 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.
- 601 30. **Su CI, Tseng CH, Yu CY, Lai MMC.** SUMO Modification Stabilizes Dengue Virus  
602 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 *U S A* 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 ISGylation. 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 reprobbed 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 reprobbed 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

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

673

674 **Fig. 3. The Lys residues responsible for HBx-ISGylation in four different HBV**  
675 **genotypes. A:** Schematic diagram of HBx from genotype (GT)-C (C-AT\_JPN). The amino  
676 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 Lys (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.

689  
690

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.

710

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  $\mu$ M 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  $10^3$   
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).

725

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,

730 cells were treated with or without  $10^3$  units/ml of type I IFN- $\alpha$  for 24 h. The levels of

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- $\alpha$ . Results

735 are the mean values from triplicates  $\pm$  SD (n=3 biological replicates).

736

737 **Fig. 7. A proposed model of the functional role of HBx-ISGylation.** HBx protein is

738 expressed as a nonstructural protein during viral life cycle and is involved in the viral

739 replication and pathogenesis. HBx protein is ISGylated at the residues K91, K95, and K140

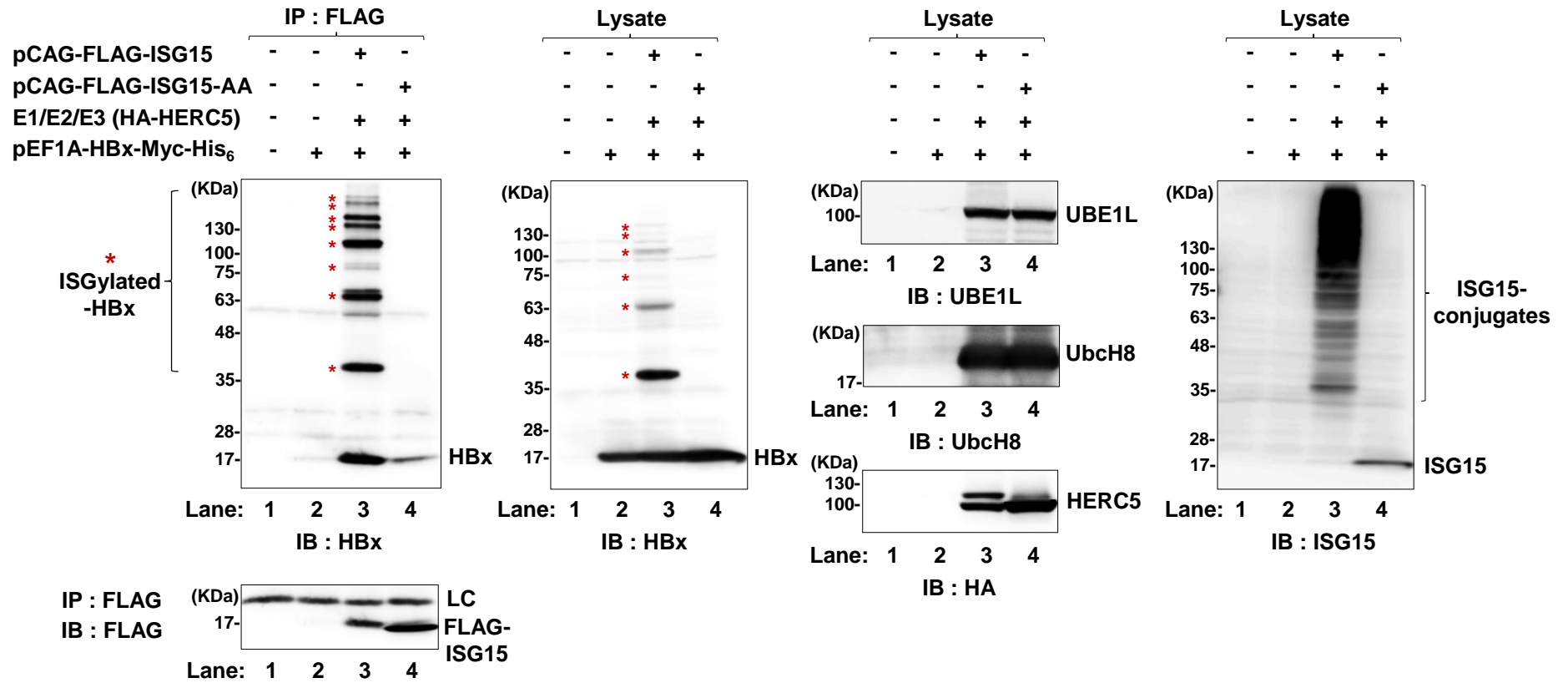
740 via the action of three enzymes: E1 activating enzyme (UBE1L), E2 conjugating enzyme

741 (UbcH8) and E3 ligase (HERC5) in HBV-replicating cells, thereby promoting the viral

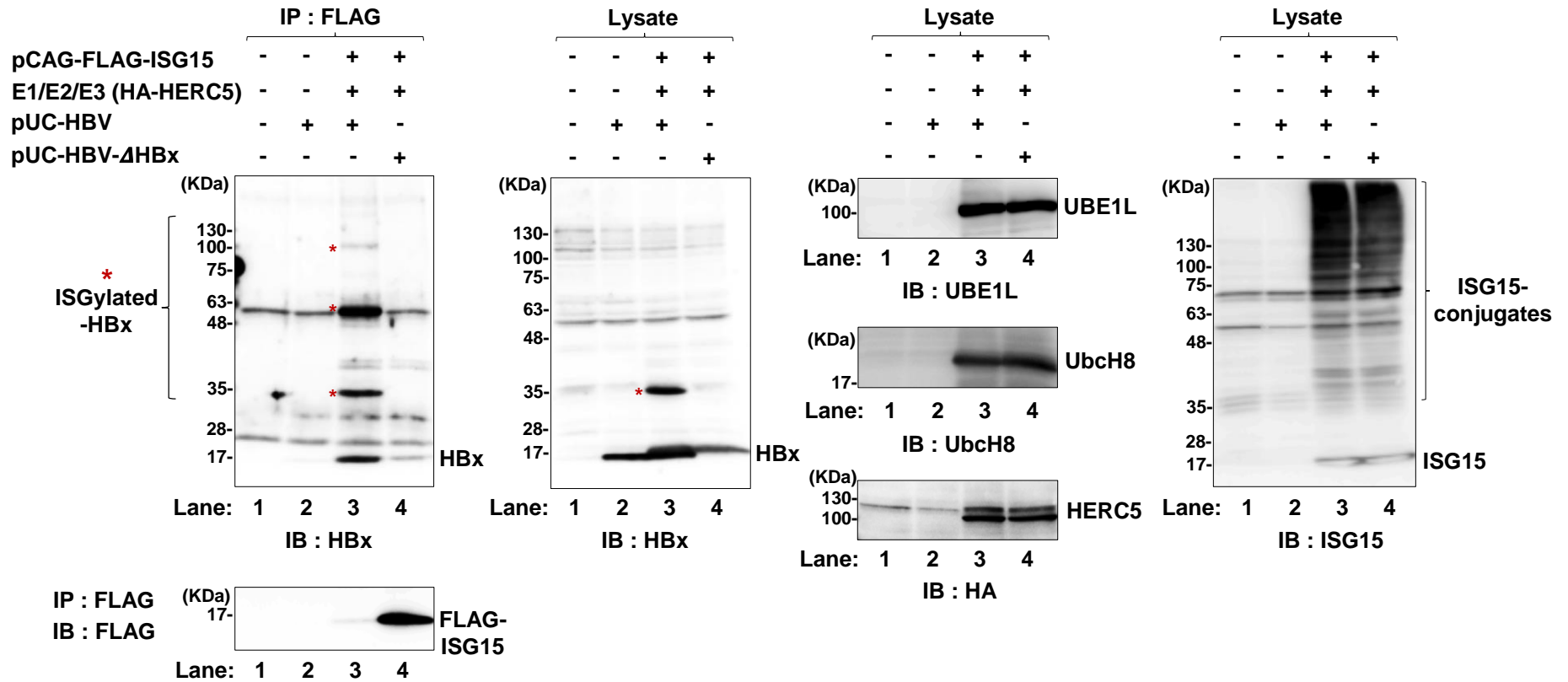
742 replication and acquisition of IFN-resistant phenotype.

743

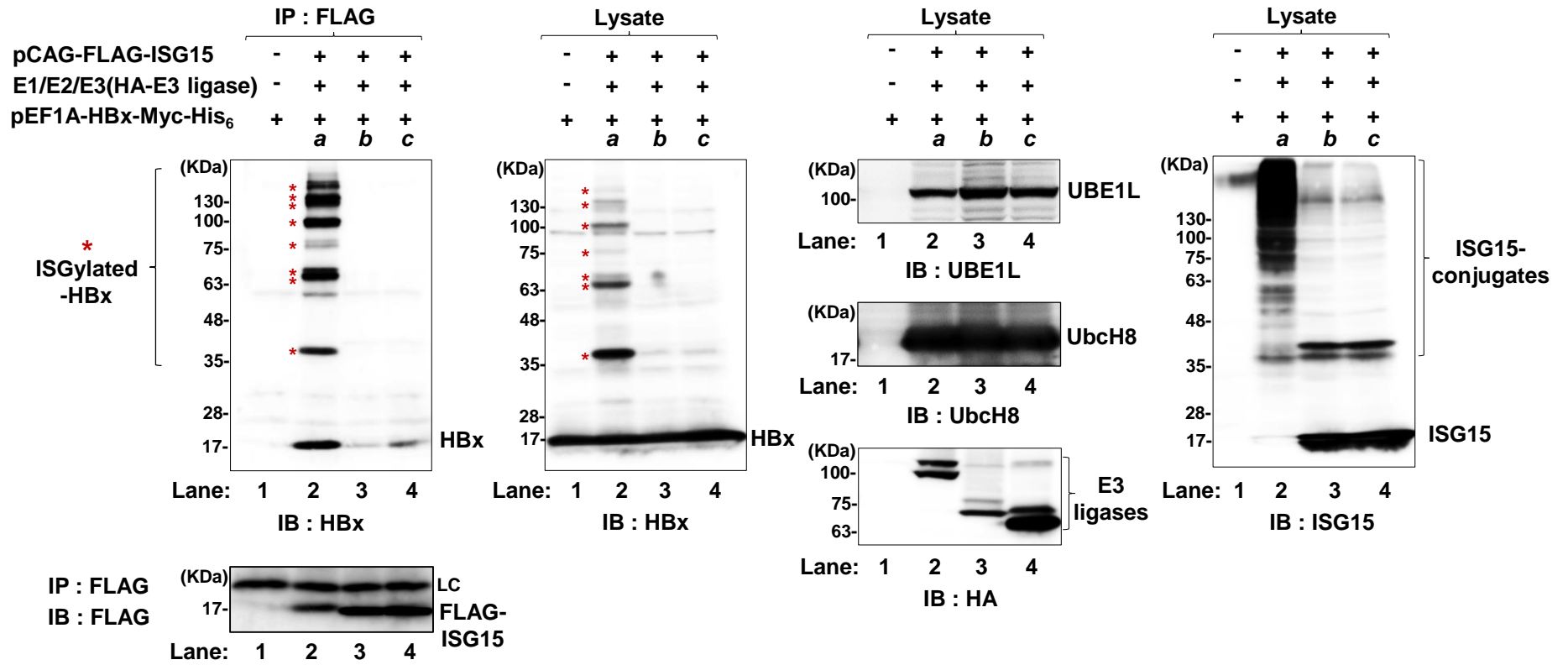
A.



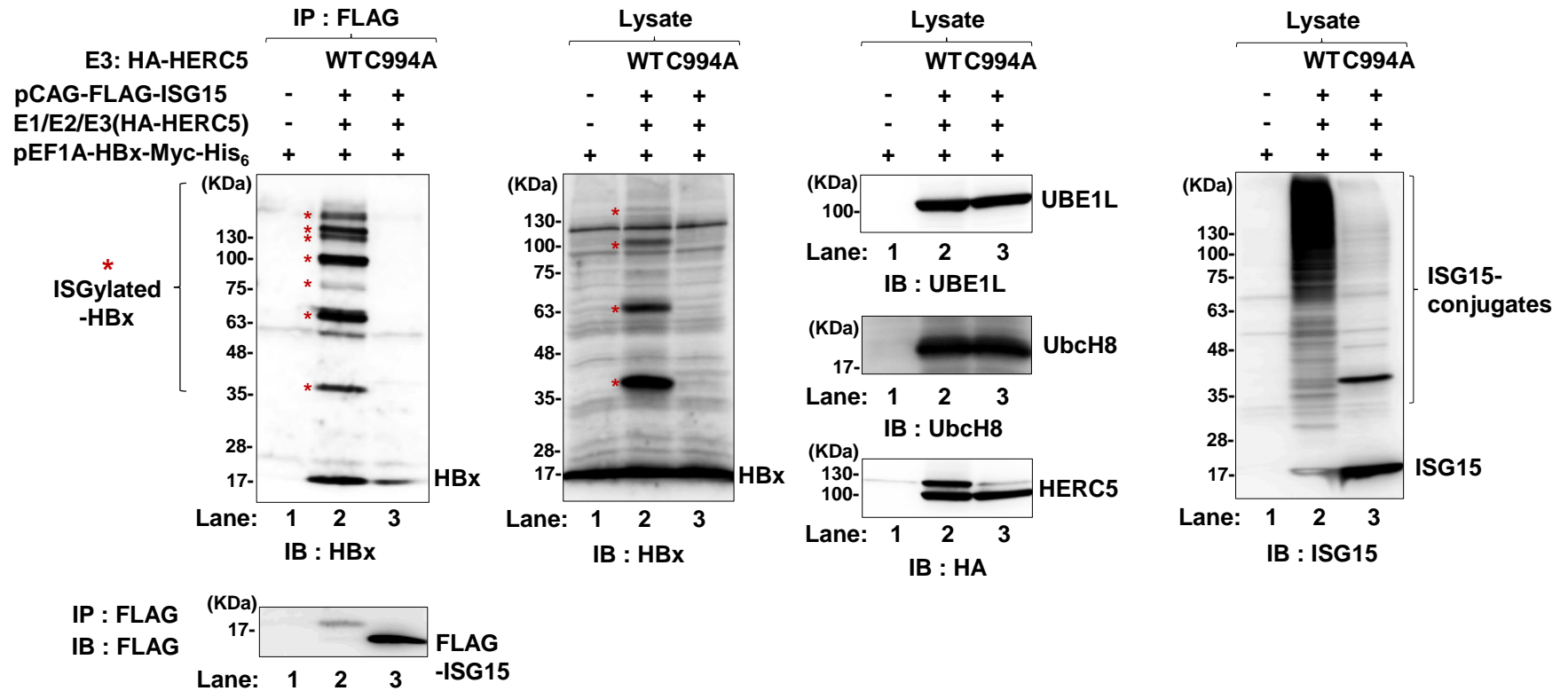
**B.**



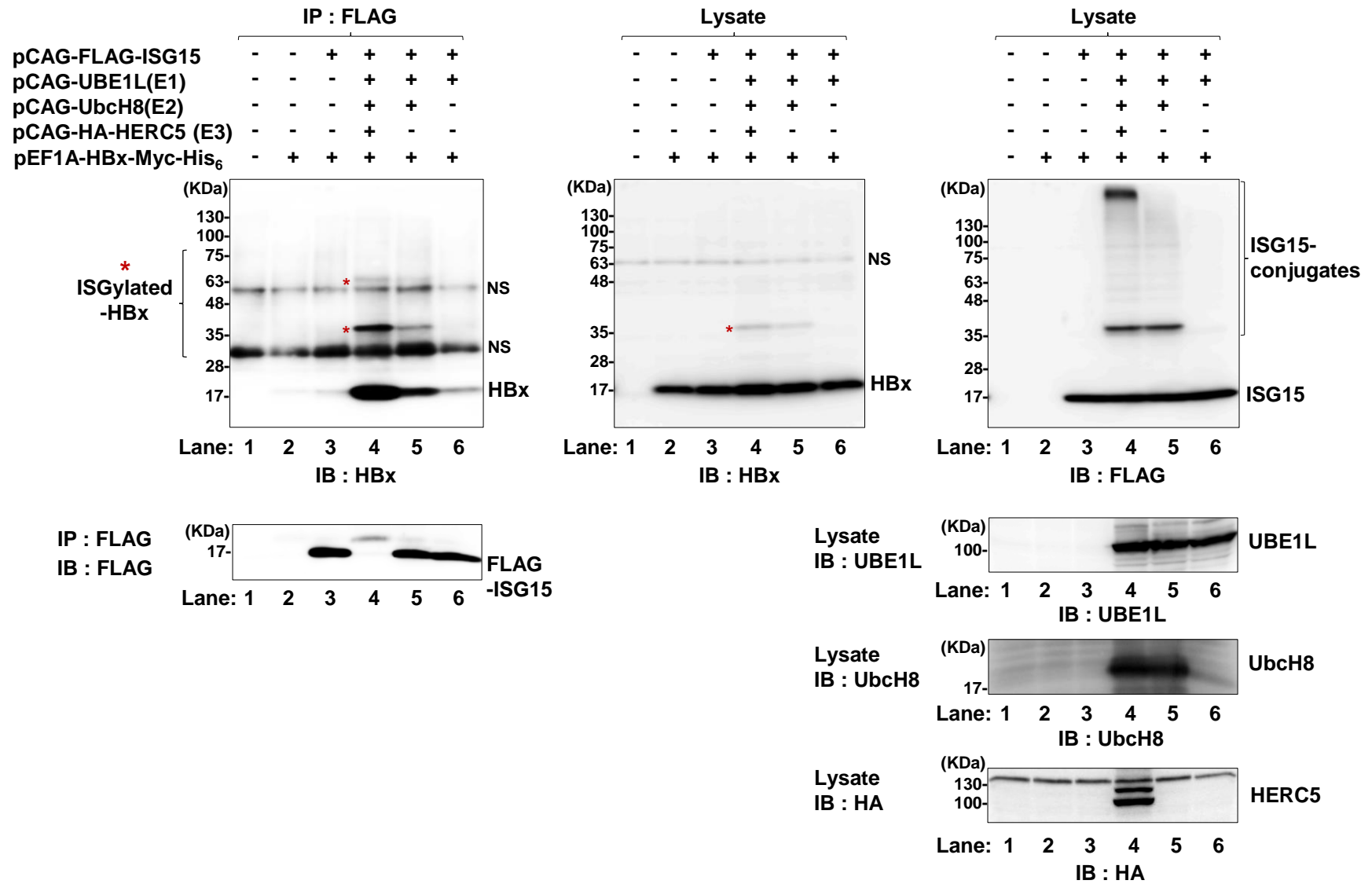
A.



B.



C.

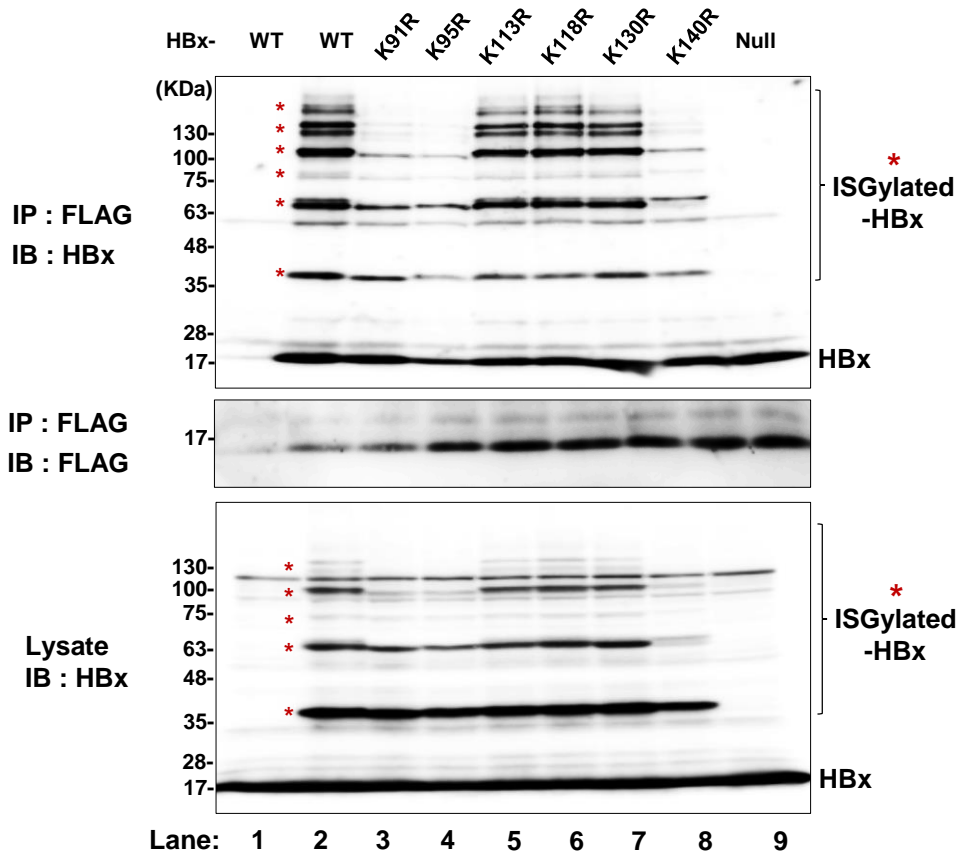






A.

|                                |   |   |   |   |   |   |   |   |   |
|--------------------------------|---|---|---|---|---|---|---|---|---|
| pCAG-FLAG-ISG15                | - | + | + | + | + | + | + | + | + |
| E1/E2/E3                       | - | + | + | + | + | + | + | + | + |
| pEF1A-HBx-Myc-His <sub>6</sub> | + | + | + | + | + | + | + | + | + |



IP : FLAG  
IB : FLAG

17-

Lysate  
IB : HBx

(KDa)

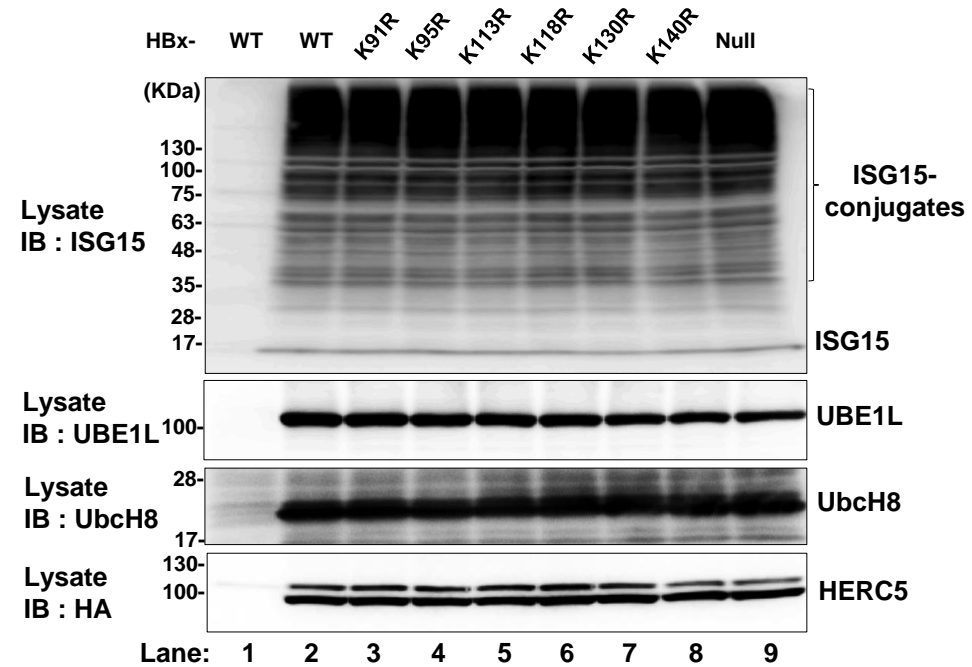
130-  
100-  
75-  
63-  
48-  
35-  
28-  
17-

ISGylated  
-HBx

HBx

Lane: 1 2 3 4 5 6 7 8 9

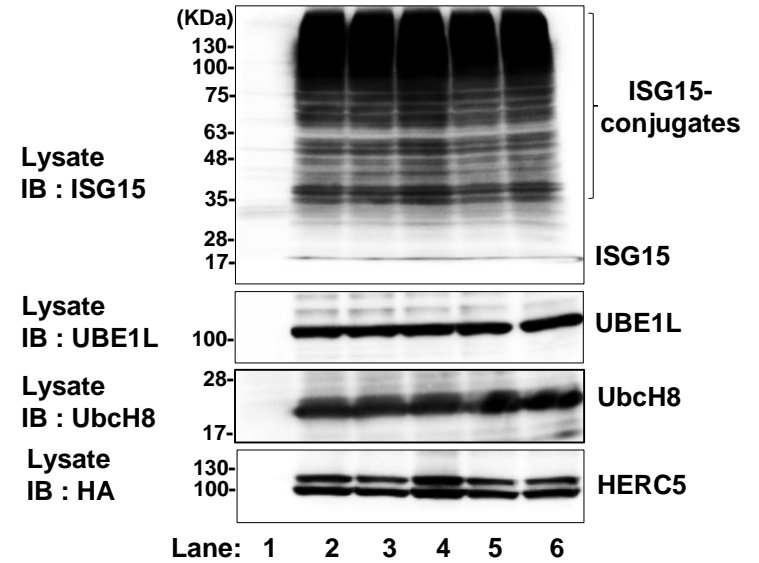
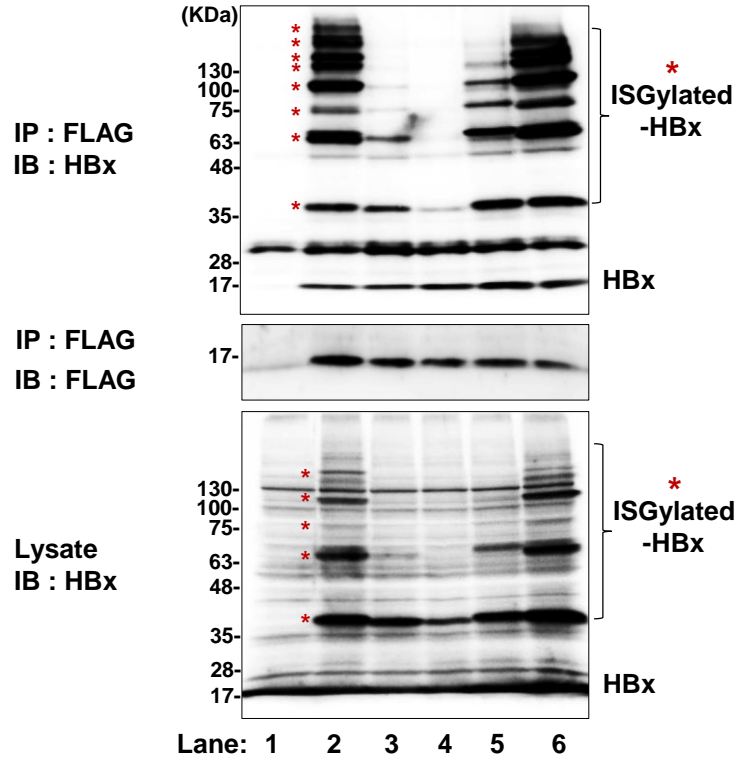
|                                |   |   |   |   |   |   |   |   |   |
|--------------------------------|---|---|---|---|---|---|---|---|---|
| pCAG-FLAG-ISG15                | - | + | + | + | + | + | + | + | + |
| E1/E2/E3                       | - | + | + | + | + | + | + | + | + |
| pEF1A-HBx-Myc-His <sub>6</sub> | + | + | + | + | + | + | + | + | + |



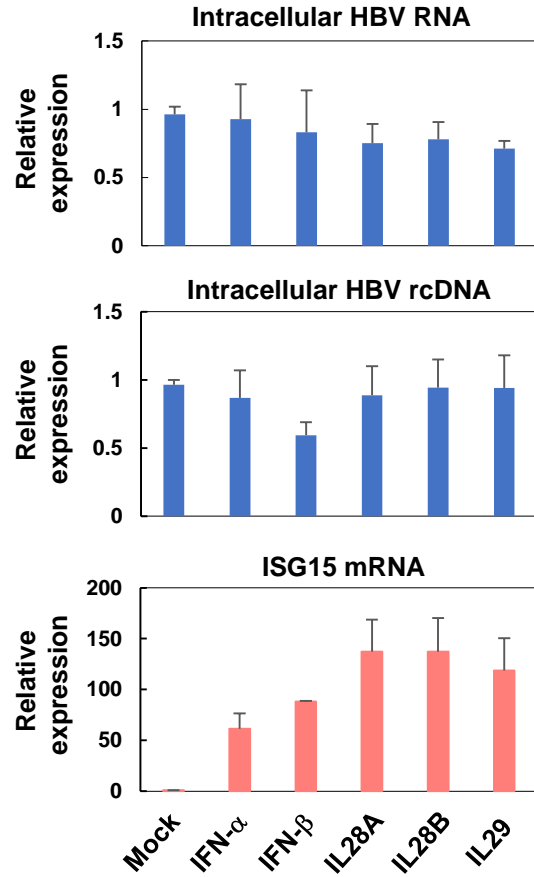
**B.**

|                                     |   |   |   |   |   |   |
|-------------------------------------|---|---|---|---|---|---|
| pCAG-FLAG-ISG15/E1E2E3              | - | + | + | + | + | + |
| pEF1A-HBx-Myc-His <sub>6</sub> (WT) | + | + | - | - | - | - |
| (K91R/K95R)                         | - | - | + | - | - | - |
| (K91R/K95R/K140R)                   | - | - | - | + | - | - |
| (K91/K95)                           | - | - | - | - | + | - |
| (K91/K95/K140)                      | - | - | - | - | - | + |

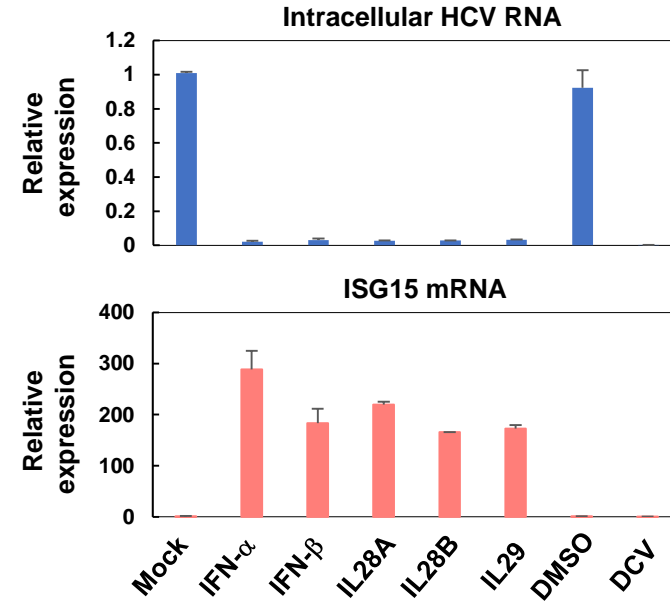
|                                     |   |   |   |   |   |   |
|-------------------------------------|---|---|---|---|---|---|
| pCAG-FLAG-ISG15/E1E2E3              | - | + | + | + | + | + |
| pEF1A-HBx-Myc-His <sub>6</sub> (WT) | + | + | - | - | - | - |
| (K91R/K95R)                         | - | - | + | - | - | - |
| (K91R/K95R/K140R)                   | - | - | - | + | - | - |
| (K91/K95)                           | - | - | - | - | + | - |
| (K91/K95/K140)                      | - | - | - | - | - | + |



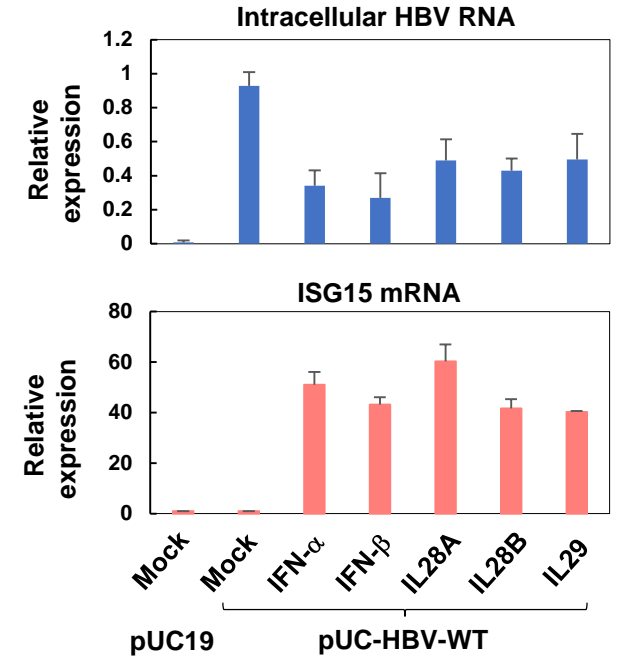
A.



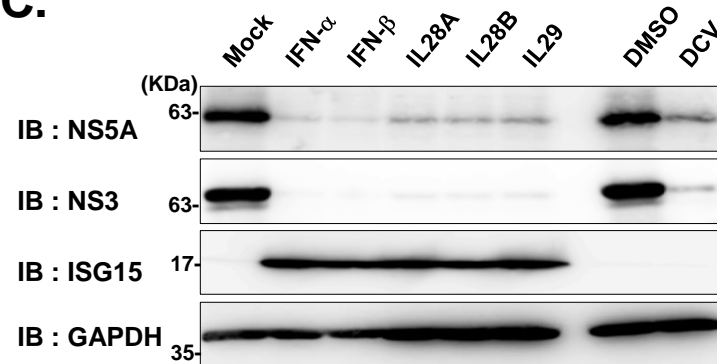
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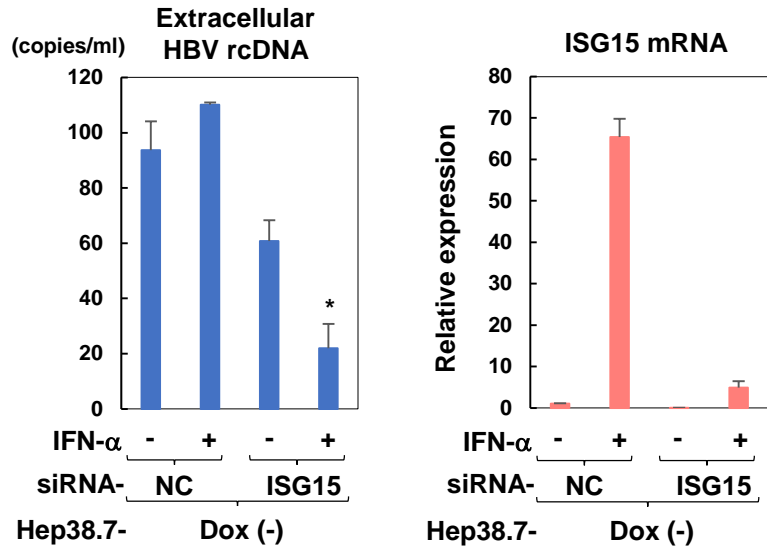
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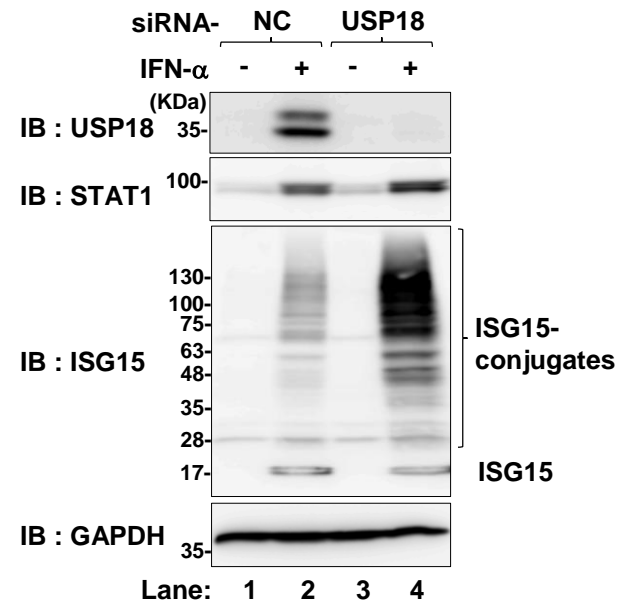
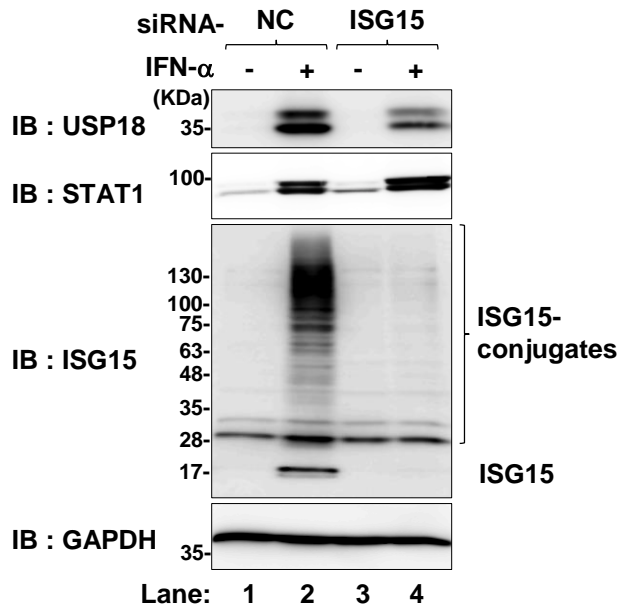
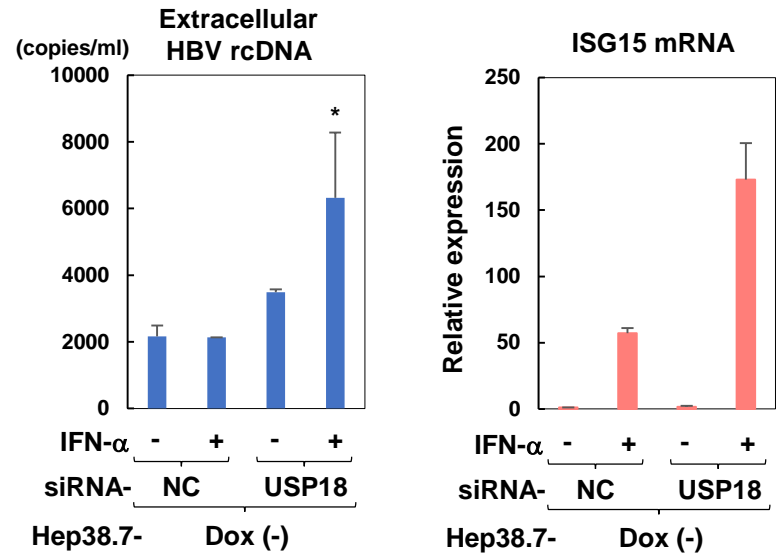
C.

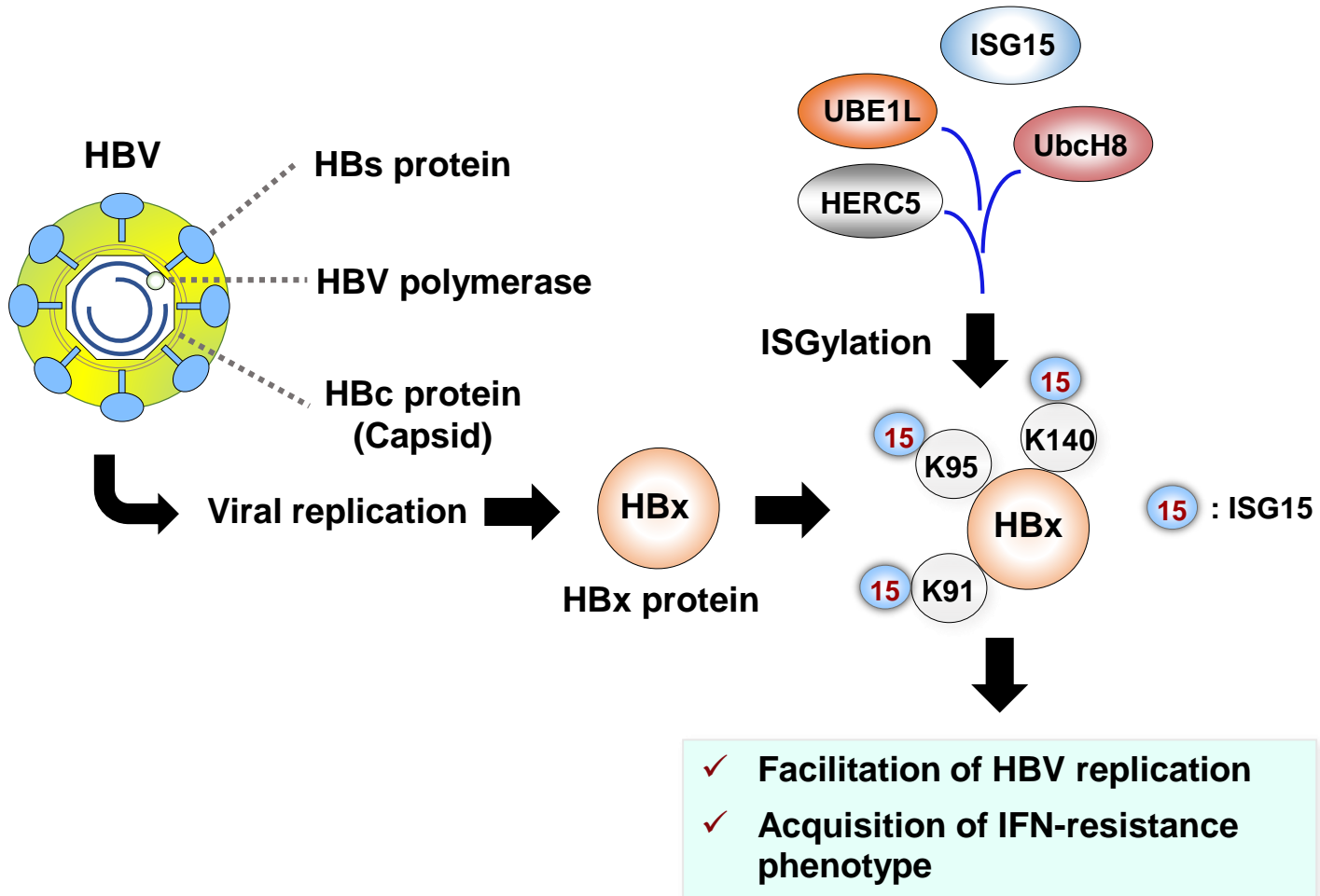


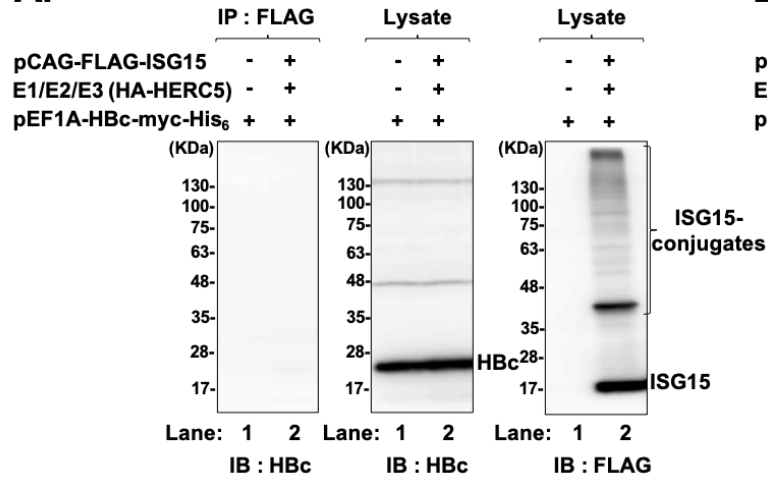
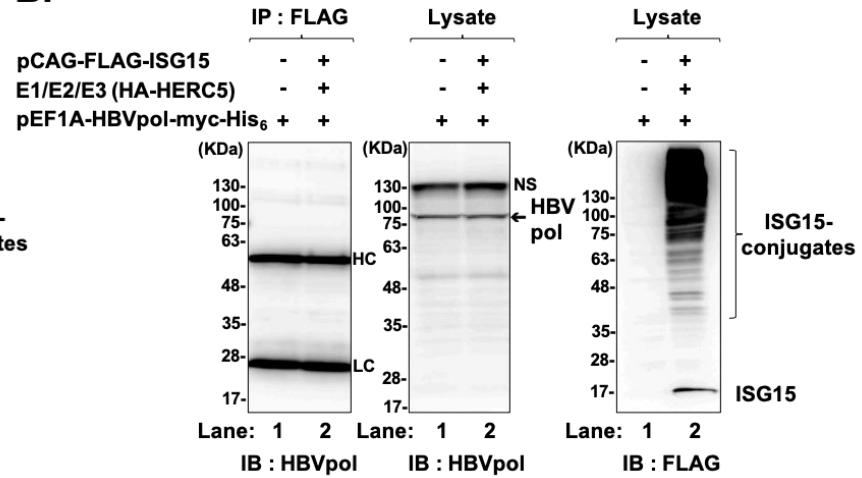
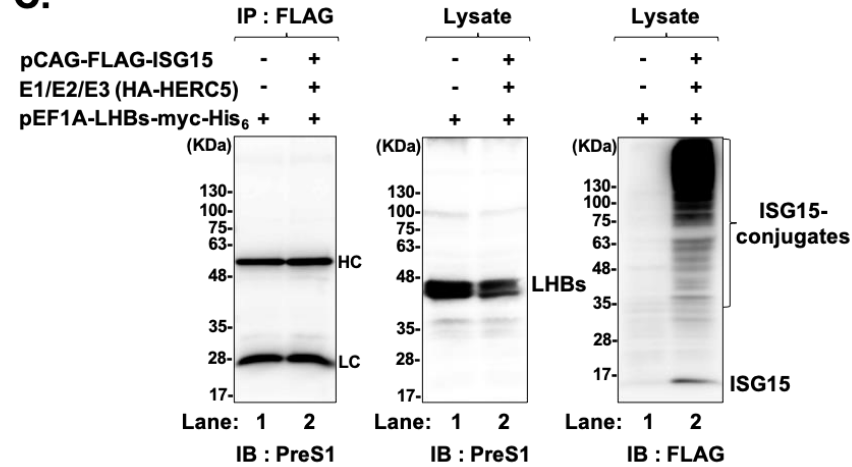
**A.**



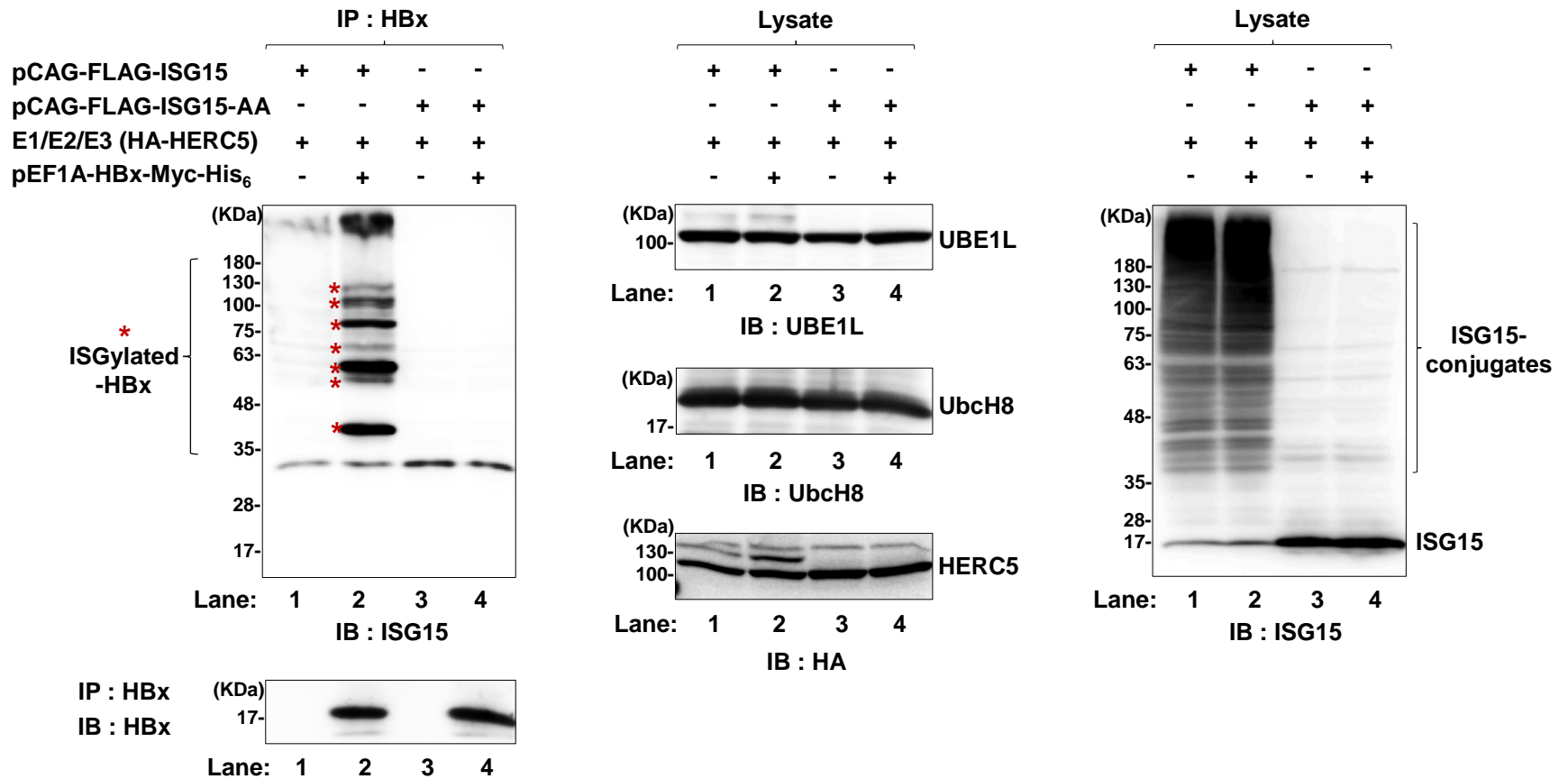
**B.**





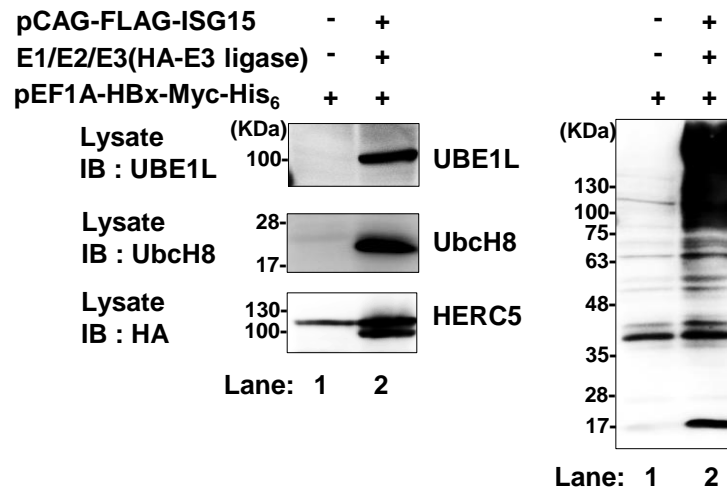
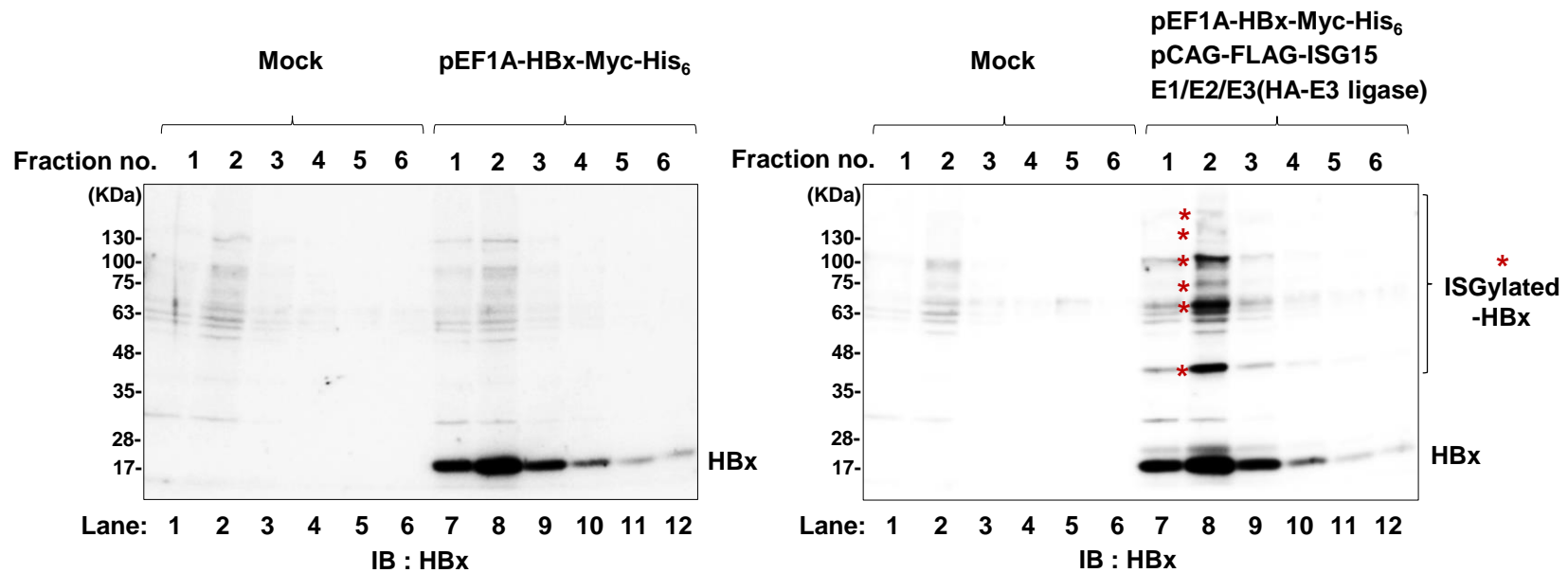
**A.****B.****C.****Fig. S1. Other HBV proteins, including HBc, HBV polymerase, and HBs proteins, do not accept ISGylation.**

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.**

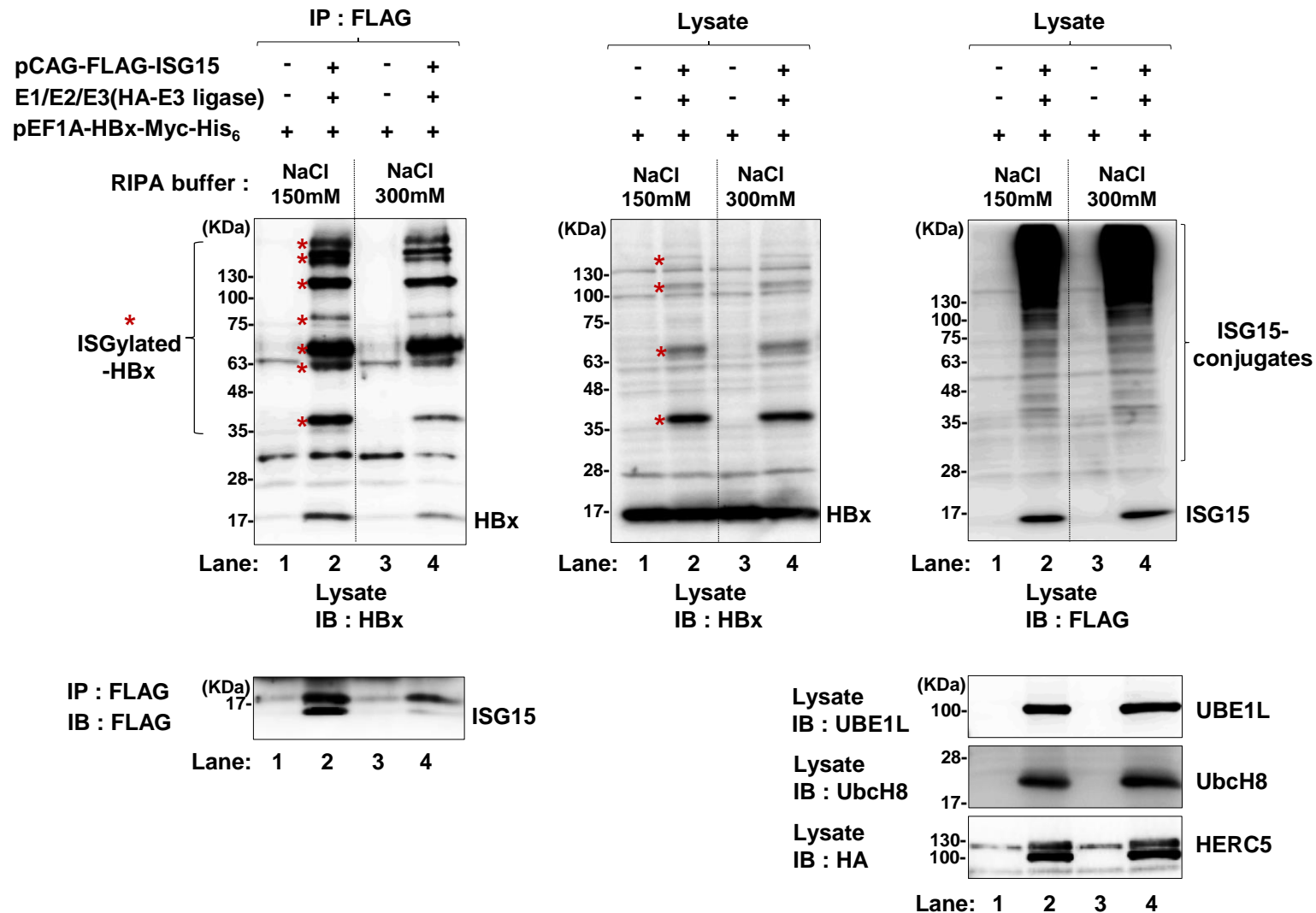
HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) with either pCAG-FLAG-ISG15 or FLAG-ISG15 mutant (pCAG-FLAG-ISG15-AA) together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (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.**

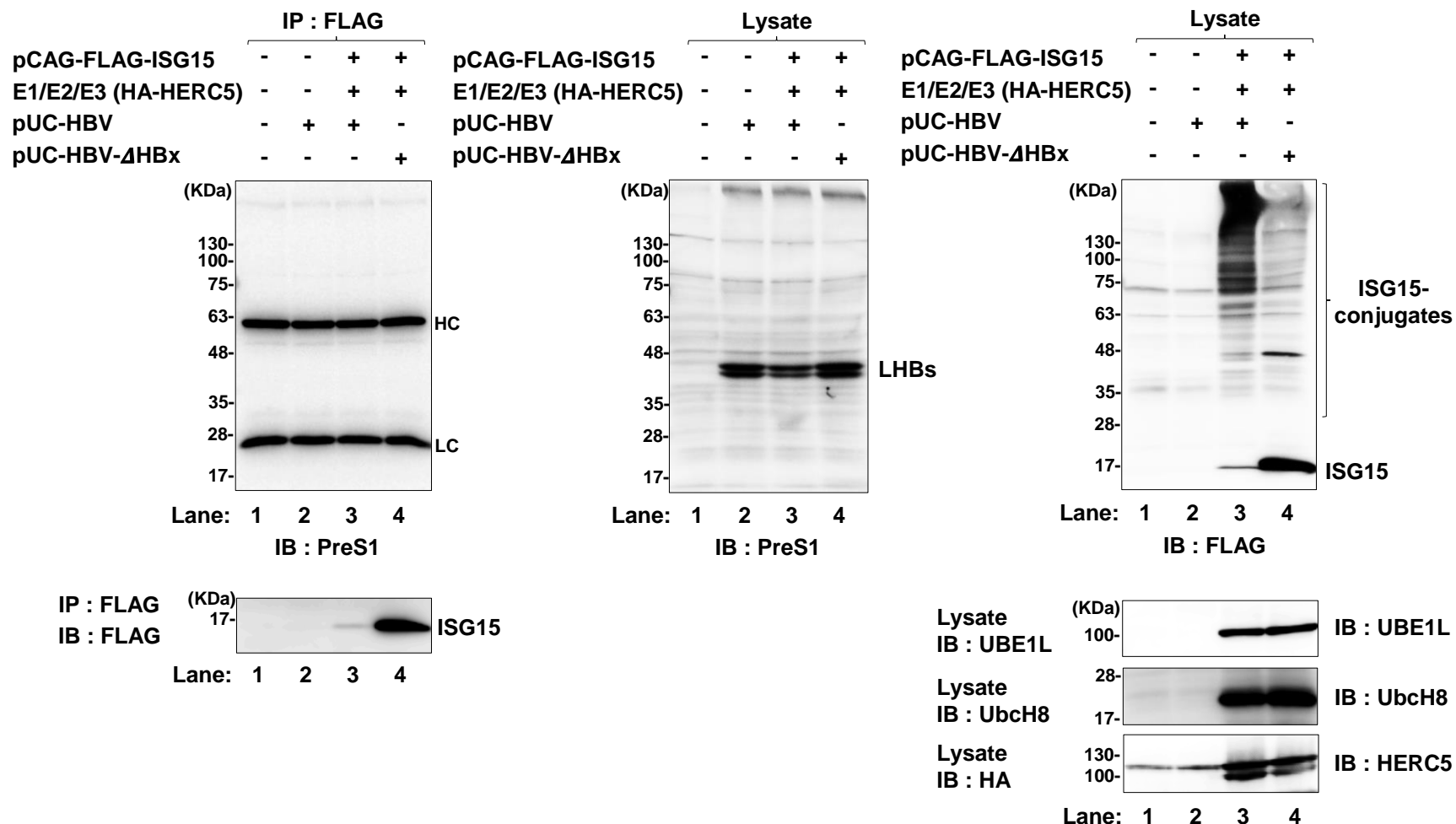
HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His<sub>6</sub> from genotype (GT)-C (C-AT\_JPN) (pEF1A-HBx-Myc-His<sub>6</sub>) and pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5), followed by pull-down with Ni-NTA resin under denaturing condition (6M guanidine-HCl) 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 2 days 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-ΔHBx) 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.