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

Bawono, Rheza Gandi ; Abe, Takayuki ; Qu, Mengting ; Kuroki, Daisuke ; Deng, Lin ; Matsui, Chieko ; Ryo, Akihide ; Suzuki, Tetsuro ; Matsuura…

(Citation)
Journal of General Virology,102(10):1668

(Issue Date)
2021-10-18

(Resource Type)
journal article

(Version)
Accepted Manuscript

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

Rheza Gandi Bawono[1,2], Takayuki Abe[1], Qu Mengting[1], Daisuke Kuroki[1], Lin Deng[1], Chieko Matsui[1], Akihide Ryo[3], Tetsuro Suzuki[4], Yoshiharu Matsuura[5], Masaya Sugiyama[6], Masashi Mizokami[6], Kunitada Shimotohno[6], and Ikuo Shoji[1,#]

[1] Division of Infectious Disease Control, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

[2] Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

[3] Department of Microbiology, Yokohama City University School of Medicine, Yokohama, Japan

[4] Department of Virology and Parasitology, Hamamatsu University School of Medicine, Shizuoka, Japan

[5] Center for Infectious Diseases Education and Research（CiDER）

Research Institute for Microbial Diseases (RIMD)

Osaka University, Osaka, Japan

[6] Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan

[#]**Correspondence:** Ikuo Shoji, MD, PhD

Professor, Division of Infectious Disease Control, Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Japan.

Tel.: +81-78-382-5500; Fax: +81-78-382-5519.

Email: ishoji@med.kobe-u.ac.jp

Short Title: HBx-ISGylation promotes viral replication

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

ABSTRACT [213 words]

Ubiquitin and ubiquitin-like protein modification play important roles in modulating the 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-like protein; this modification is called ISGylation. Immunoblot analyses revealed that HBx proteins derived from four different HBV genotypes accepted ISGylation in cultured cells. Site-directed mutagenesis revealed that three lysine residues (K91, K95, and K140) on the HBx protein, which are well conserved among all the HBV genotypes, are involved in acceptance of ISGylation. Using expression plasmids encoding three known E3 ligases involved in the ISGylation to different substrates, we found that HERC5 functions as an E3 ligase for HBx-ISGylation. Treatment with type I and type III IFNs resulted in the limited suppression of HBV replication in Hep38.7-Tet cells. When cells were treated with IFN-α, silencing of ISG15 resulted in a marked reduction of HBV replication in Hep38.7-Tet cells, suggesting a role of ISG15 in the resistance to IFN-α. In contrast, the silencing of USP18 (an ISG15 de-conjugating enzyme) increased the HBV replication in Hep38.7-Tet cells. Taken together, these results suggest that the HERC5-mediated ISGylation of HBx protein confers pro-viral functions on HBV replication and participates in the resistance to IFN-α-mediated antiviral activity.

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).

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

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

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

ISG15/ISGylation has been reported to play an antiviral role, but in the case of several infections—including infections with HBV and hepatitis C virus (HCV)—ISG15/ISGylation has been reported to have a pro-viral function (20, 25, 26). In the case of HBV infection, Li and colleagues (25) reported that the overexpression of ISG15 and subsequent ISGylation could promote HBV production in HBV-persistent replicating cells, although the putative ISGylated viral protein(s) remain to be determined. There is also a report suggesting that high 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 ISG15/ISGylation has a pro-viral function, but the exact mechanisms underlying this 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.

RESULTS

HBx protein is a substrate of ISGylation

At first, to investigate the effects of ISGylation on several HBV proteins, including HBc, HBx, HBV polymerase (HBV pol), and large HBV surface (LHBs) from genotype (GT)-C (C-AT_JPN), we co-transfected HEK293T cells with C-terminal Myc-His_6-tagged HBV proteins and N-terminal FLAG-tagged-ISG15 together with E1 activating enzyme (UBE1L), E2 conjugating enzyme (UbcH8) and HA-tagged E3 ligase (HA-HERC5), followed by immunoprecipitation with anti-FLAG and detection with each of the anti-HBV specific antibodies. The immunoprecipitation analysis coupled with immunoblotting revealed that HBx-Myc-His_6, but not HBc, HBV pol, and LHBs proteins, was precipitated with FLAG-ISG15 (Fig. 1A, lane 3 in the first panel and Supplementary Fig. 1). These results suggest 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_6 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 HBx protein 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.

To further seek for the evidence suggesting that HBx proteins are indeed ISGylated, HEK293T cells were co-transfected with HBx-Myc-His_6 together with plasmids expressing ISGylation components and cell lysates were pulled down with Ni-Nitrilotriacetic (NTA) resin under denaturing condition (6M guanidine-HCl). The Ni-NTA pull-down analysis coupled with immunoblotting revealed that C-terminal His-tagged HBx and ISGylated HBx proteins were detected in the same eluted fractions (Supplementary Fig. 3, upper, right panel, lanes 7 to 9). These results suggest that the slowly migrating forms of HBx protein are indeed 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

RIPA buffer containing 300 mM NaCl and 150 mM NaCl. These results indicate that the slowly migrating forms of HBx protein are indeed covalently ISGylated HBx protein.

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

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

To determine the an E3 ligase involved in the ISGylation of HBx, we co-transfected HEK293T cells with HBx-Myc-His_6 and FLAG-ISG15 together with E1 (UBE1L), E2 (UbcH8), and each of the HA-tagged E3 ligases, including HA-HERC5, HA-TRIM25, or HA-HHARI, followed by immunoprecipitation with anti-FLAG and detection with anti-HBx rabbit pAb. The immunoprecipitation analysis coupled with immunoblotting revealed that the transfection of HERC5, but not TRIM25 and HHARI, strongly induced the ISGylation of HBx (Fig. 2A, first and second panels, lane 2; asterisks indicate ISGylated HBx). These 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),

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_6
209 (WT), but not HBx-Myc-His_6 (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_6 (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_6 (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_6 (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_6 (K91/K95/K140) mutant was used instead of the HBx-Myc-His_6 (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 10^3 units/ml of type I (IFN-α and IFN-β) 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-α. 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-α 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-α (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-α (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-α-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 pro-viral 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 IFN-mediated antiviral activity (Fig. 7).

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.

Ubiquitin-like modifiers, such as NEDDylation, SUMOylation, and ISGylation, play important roles in modulating the function of viral proteins to mediate pathogenesis (18). For example, HBx protein was reported to accept NEDDylation mediated by the HDM2 E3 ligase, which enhances the stability of HBx to prevent its proteasomal degradation (19). Similarly, Dengue virus (DENV) NS5 protein and HCV NS5A protein were shown to accept SUMOylation mediated by several SUMO-conjugating enzymes to enhance protein stability and positively regulate the viral replication (29, 30). These studies suggest that the inhibition 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_6

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 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.

MATERIALS & METHODS

Cell culture and reagents. Huh7.5 cells were kindly provided by Dr. C.M. Rice (The Rockefeller University, NY). Huh7.5 cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (High Glucose) with L-glutamine (Wako, Osaka, Japan) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France) at 37°C in a 5% CO_2 incubator. A doxycycline (Dox)-inducible HBV expression cells, Hep38.7-Tet cells (kindly provided by Dr. K. Watashi, NIID, Japan), were maintained in DMEM/F-12 (Gibco) supplemented with 10 mM HEPES (Gibco), 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco), 10% FBS (Biowest), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 400 μg/ml G418 (Nacalai Tesque, Kyoto, Japan), and 400 ng/ml Dox (Sigma-Aldrich). Cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Promega, Madison, WI). 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).

Plasmids. The plasmids pEF1A-HBx-Myc-His_6 and pEF1A-HBc-Myc-His_6 have been previously described (17). The cDNA fragments of LHBs from genotype (GT)-C (C-AT_JPN) was inserted into the EcoRI site of pEF1A-Myc-His_6 using the In-Fusion HD-Cloning 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_6 as a template. The specific primers used for the PCR were as follows: sense primer (K91R), 5'-CAGGTCTTGCCCAGGGTCTTATAT-3'; antisense primer (K91R), 5'-ATATAAGACCCTGGGCAAGACCTG-3', sense primer (K95R), 5'-AAGGTCTTATATAGGAGGACTCTT-3'; antisense primer (K95R), 5'-AAGAGTCCTCCTATATAAGACCTT-3', sense primer (K113R), 5'-GAGGCATACTTCAGAGACTGTTTG-3'; antisense primer (K113R), 5'-CAAACAGTCTCTGAAGTATGCCTC-3', sense primer (K118R), 5'-GACTGTTTGTTTAGGGACTGGGAG-3'; antisense primer (K118R), 5'-CTCCCAGTCCCTAAACAAACAGTC-3', sense primer (K130R), 5'-GAGATTAGGTTAAGGATTTTTGTA-3'; antisense primer (K130R), 5'-TACAAAAATCCTTAACCTAATCTC-3', sense primer (K140R), 5'-GGCTGTAGGCATAGATTGGTCTGT-3'; antisense primer (K140R), 5'-ACAGACCAATCTATGCCTACAGCC-3'. We designated the resulting plasmid as pEF1A-HBx(K-Null)-Myc-His_6. 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

using pEF1A-HBx(K-Null)-Myc-His_6 as a template. The specific primers used for PCR were as follows: sense primer (K91), 5'-CAGGTCTTGCCCAAGGTCTTATAT-3'; antisense primer (K91), 5'-ATATAAGACCTTGGGCAAGACCTG-3', sense primer (K95), 5'-AGGTCTTATATAAGAGGACTCTT-3'; antisense primer (K95), 5'-AAGAGTCCTCTTATATAAGACCCT-3', sense primer (K113), 5'-GAGGCATACTTCAAAGACTGTTTG-3'; antisense primer (K113), 5'-CAAACAGTCTTTGAAGTATGCCTC-3', sense primer (K118), 5'-GACTGTTTGTTTAAGGACTGGGAG-3'; antisense primer (K118), 5'-CTCCCAGTCCTTAAACAAACAGTC-3', sense primer (K130), 5'-GAGATTAGGTTAAAGATTTTTGTA-3'; antisense primer (K130), 5'-TACAAAAATCTTTAACCTAATCTC-3', sense primer (K140), 5'-GGCTGTAGGCATAAATTGGTCTGT-3'; antisense primer (K140), 5'-ACAGACCAATTTATGCCTACAGCC-3'. The construction of HBx Lys (K) mutants, containing some combination mutants of Lys to Arg at the indicated Lys residues (referred to as K91R/K95R and K91R/K95R/K140R) were generated by PCR mutagenesis using pEF1A-HBx-Myc-His_6 as a template and specific primers described above. The HBV expression plasmids pUC19-HBV-Ae_US, pUC19-HBV- Bj_JPN56, and pUC19-HBV-C-AT_JPN were kindly provided by Dr. M. Mizokami (NCGM, Japan). The cDNA fragments of HBx from HBV genotypes (GT): GT-A (Ae_US), GT-B (Bj_JPN56), and GT-C (C-AT_JPN) were inserted into the EcoRI site of pEF1A-Myc-His_6 using the In-Fusion HD-Cloning kit (Clontech). The cDNA fragments encoding HBx from GT-D (Ayw) were amplified by reverse transcription-PCR from the total RNA of Hep38.7-Tet cells and were cloned into pEF1A-Myc-His_6 using the In-Fusion HD-Cloning kit (Clontech). The HBx-deficient HBV

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

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

Antibodies. The mouse monoclonal antibodies (mAbs) used in this study were anti-FLAG (M2) mAb (F-3165, Sigma-Aldrich), anti-NS5A mAb (MAB8694, Millipore, Billerica, MA), anti-NS3 mAb (MAB8691, Millipore), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAb (MAB374, Millipore), anti-c-Myc mAb (9E10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Hep B preS1 mAb (AP1, Santa Cruz Biotechnology), anti-Hep B pol mAb (2C8, Santa Cruz Biotechnology), anti-UbcH8 mAb (ab56502-100), and anti-ISG15 mAb (F-9, Santa Cruz Biotechnology). The rabbit polyclonal (pAbs) or monoclonal antibodies (mAbs) used in this study were anti-HA pAb (H-6908, Sigma-Aldrich), anti-HBx pAb (39716, Abcam, Cambridge, UK), anti-STAT1 pAb (#9172, Cell Signaling Technology, Beverly, MA), anti-USP18 mAb (D4E7, Cell Signaling Technology), anti-Ube1L mAb

(ab108929, Abcam), and anti-HBc pAb (kindly provided by Prof. T. Suzuki, Hamamatsu University School of Medicine, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibody (Cell Signaling Technology) were used as secondary antibodies.

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

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

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

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

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

Real-time PCR. Total RNA was prepared from each of the cells using a RNeasy mini kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using the GoScript™ reverse transcription system (Promega). The real-time PCR was performed using SYBR *Premix Ex Taq*™ II (Tli RNaseH plus) (TaKaRa Bio) according to the manufacturer's protocol. Fluorescent signals were analyzed by a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). The HBV RNA, ISG15, and GAPDH genes were amplified using the specific primer pairs 5'-GACCACCAAATGCCCCTATC-3' and 5'-GATTGAGATCTTCTGCGACGC-3'; 5'-AGCGAACTCATCTTTGCCAGTACA-3' and 5'-CAGCTCTGACACCGACATGGA-3'; and 5'-GCCATCAATGACCCCTTCATT-3' and 5'-TCTCGCTCCTGGAAGATGG-3', respectively. The expression level of each gene was determined by the $\mathbf{\Delta\Delta}C_T$ method using GAPDH as an internal control. To quantitate intracellular and extracellular HBV rcDNA, cell pellets and culture supernatants were 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 primer pairs 5'-GGAGGGATACATAGAGGTTCCTTGA-3' and 5'-GTTGCCCGTTTGTCCTCTAATTC-3'.

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

Funding information

This work was supported by the Program for Basic and Clinical Research on Hepatitis from the Japan Agency for Medical Research and Development (AMED) under Grant No. 20fk0310104h9904 and 20fk0310104s1204. R.G.B was supported by the Program for Nurture of Next Generation Leaders Guiding Medical Innovation in Asia of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan. This research was supported in parts by The Daiichi Sankyo Foundation of Life Science (T.A.) and The Mochida Memorial Foundation for Medical and Pharmaceutical Research (T.A.).

Acknowledgments

We are grateful to Dr. C.M. Rice (The Rockefeller University, New York, NY) for providing the Huh7.5 cells. We thank Y. Kozaki for the secretarial work.

Author Contributions

R.G.B., T.A., and I.S. conceived and designed the experiments. R.G.B. and T.A. carried out most of the experiments. Q.X., C.M., D.K., and L.D. assisted the construction of plasmids and the data analysis. A.R., K.S., Y.M, T.S., S.M. and M.M. contributed to the materials providing. T.A. and I.S. wrote the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interest.

REFERENCES

1. **Trépo C, Chan HLY, Lok A.** Hepatitis B virus infection. *The Lancet* 2014;384:2053-2063.
2. **Revill PA, Chisari FV, Block JM, Dandri M, Gehring AJ, *et al*.** A global scientific strategy to cure hepatitis B. *Lancet Gastroenterol Hepatol* 2019;4:545-558.
3. **Ng SA, Lee C.** Hepatitis B virus X gene and hepatocarcinogenesis. *J Gastroenterol* 2011; 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.
6. **Lee TH, Elledge SJ, Butel JS.** Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. *J Virol* 1995;69:1107-1114.
7. **Sitterlin D, Lee TH, Prigent S, Tiollais P, Butel JS, *et al*.** Interaction of the UV-damaged DNA-binding protein with hepatitis B virus X protein is conserved among mammalian hepadnaviruses and restricted to transactivation-proficient X-insertion mutants. *J Virol* 1997;71:6194-6199.
8. **Benhenda S, Ducroux A, Riviere L, Sobhian B, Ward MD, *et al*.** Methyltransferase PRMT1 is a binding partner of HBx and a negative regulator of hepatitis B virus transcription. *J Virol* 2013;87:4360-4371.

9. **Kouwaki T, Okamoto T, Ito A, Sugiyama Y, Yamashita K, *et al*.** Hepatocyte Factor JMJD5 Regulates Hepatitis B Virus Replication through Interaction with HBx. *J Virol* 2016;90:3530-3542.
10. **Tanaka Y, Kanai F, Ichimura T, Tateishi K, Asaoka Y, *et al*.** The hepatitis B virus X protein enhances AP-1 activation through interaction with Jab1. *Oncogene* 2006;25:633-642.
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.
14. **Pang R, Lee TK, Poon RT, Fan ST, Wong KB, *et al*.** Pin1 interacts with a specific serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis. *Gastroenterology* 2007;132:1088-1103.
15. **Kumar M, Jung SY, Hodgson AJ, Madden CR, Qin J, *et al*.** Hepatitis B virus regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation of beta interferon. *J Virol* 2011;85:987-995.
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.

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

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.
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.
28. **Dos Santos PF, Mansur DS.** Beyond ISGlylation: Functions of Free Intracellular and Extracellular ISG15. *J Interferon Cytokine Res* 2017;37:246-253.
29. **Lee HS, Lim YS, Park EM, Baek SH, Hwang SB.** SUMOylation of nonstructural 5A 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.
31. **Sekiba K, Otsuka M, Ohno M, Yamagami M, Kishikawa T, *et al*.** Inhibition of HBV Transcription From cccDNA With Nitazoxanide by Targeting the HBx-DDB1 Interaction. *Cell Mol Gastroenterol Hepatol* 2019;7:297-312.
32. **Wong JJ, Pung YF, Sze NS, Chin KC.** HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. *Proc Natl Acad Sci U S A* 2006;103:10735-10740.
33. **Wang Y, Ding Q, Xu T, Li CY, Zhou DD, *et al*.** HZ-6d targeted HERC5 to regulate p53 ISGylation in human hepatocellular carcinoma. *Toxicol Appl Pharmacol* 2017;334:180-191.
34. **Henkler F, Hoare J, Waseem N, Goldin RD, McGarvey MJ, *et al*.** Intracellular localization of the hepatitis B virus HBx protein. *J Gen Virol* 2001;82:871-882.

35. **Keasler VV, Hodgson AJ, Madden CR, Slagle BL.** Hepatitis B virus HBx protein localized to the nucleus restores HBx-deficient virus replication in HepG2 cells and in vivo in hydrodynamically-injected mice. *Virology* 2009;390:122-129.
36. **Kornyeyev D, Ramakrishnan D, Voitenleitner C, Livingston CM, Xing W, *et al*.** Spatiotemporal Analysis of Hepatitis B Virus X Protein in Primary Human Hepatocytes. *J Virol* 2019;93: e00248-19.
37. **Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM.** Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc Natl Acad Sci U S A* 2005;102:10200-10205.
38. **Giannakopoulos NV, Luo JK, Papov V, Zou W, Lenschow DJ, *et al*.** Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. *Biochem Biophys Res Commun* 2005;336:496-506.
39. **Minami N, Abe T, Deng L, Matsui C, Fukuhara T, *et al*.** Unconjugated interferon-stimulated gene 15 specifically interacts with the hepatitis C virus NS5A protein via domain I. *Microbiol Immunol* 2017;61:287-292.

FIGURE LEGENDS

Fig. 1. HBx is a substrate for ISGylation. A: The expression plasmid encoding HBx-Myc-His_6 from genotype (GT)-C (C-AT_JPN) (pEF1A-HBx-Myc-His_6) was co-expressed 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) in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb. The membrane was stripped by stripping buffer and was reprobed with anti-FLAG mouse mAb. IP: immunoprecipitation. IB: immunoblotting. LC: immunoglobulin light chain. **B:** 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, pCAG-UbcH8, and pCAG-HA-HERC5 in Huh7.5 cells, followed by immunoprecipitation with anti-FLAG mouse mAb 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 membrane was stripped by stripping buffer and was reprobed with anti-FLAG mouse mAb. The asterisks (*) indicates the ISG15-conjugated HBx proteins (ISGylated-HBx). The western blots are representative for three independent experiments.

Fig. 2. HERC5 is a specific E3 ligase for HBx-ISGylation. A: The expression plasmid encoding HBx-Myc-His_6 from genotype (GT)-C (C-AT_JPN) (pEF1A-HBx-Myc-His_6) was co-expressed with pCAG-FLAG-ISG15 and an HA-tagged E3 ligase, i.e., (*a*) pCAG-HA-HERC5, (*b*) pCAG-HA-TRIM25, or (*c*) pCAG-HA-HHARI together with pCAG-UBE1L

(E1) and pCAG-UbcH8 (E2) in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb. **B:** The expression plasmid encoding HBx-Myc-His_6 from GT-C (C-AT_JPN) (pEF1A-HBx-Myc-His_6) was co-expressed with pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and pCAG-HA-HERC5 (WT) or an inactive mutant HERC5 (C994A) (pCAG-HA-HERC5 C994A) in 293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb and detection with anti-HBx rabbit pAb. The membrane was stripped by stripping buffer and was reprobed with anti-FLAG mouse mAb. The faint band in lane 2 was the remaining of previously blotted HBx. **C:** The expression plasmid encoding HBx-Myc-His_6 from GT-C (C-AT_JPN) (pEF1A-HBx-Myc-His_6) was co-expressed with pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and pCAG-HA-HERC5 or with the depletion of E3 or both E2 and E3 plasmids in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb 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, anti-ISG15 mouse mAb, or anti-FLAG mouse mAb. The asterisks indicate the ISG15-conjugated HBx proteins (ISGylated-HBx). The membrane was stripped by stripping buffer and was reprobed with anti-FLAG mouse mAb. The faint band in lane 4 was the remaining of previously blotted HBx. The western blots are representative for three independent experiments.

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 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_6 (pEF1A-HBx-Myc-His_6) 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_6 from genotype (GT)-C (C-AT_JPN) (pEF1A-
693 HBx-Myc-His_6) 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_6 from GT-C (C-
699 AT_JPN) (pEF1A-HBx-Myc-His_6) 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

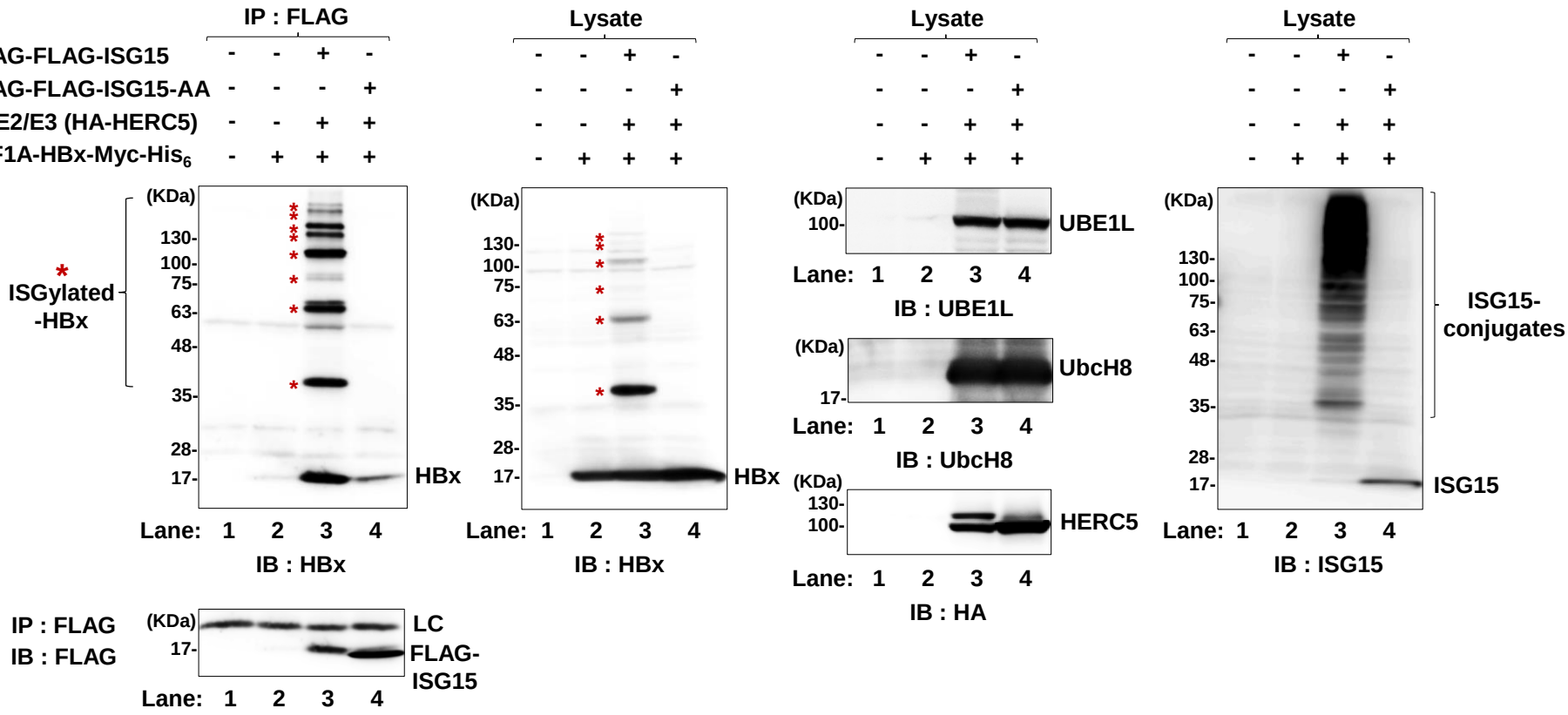
mutants containing a point mutation of Lys to Arg at a corresponding Lys residue (indicated as K91R/K95R and K91R/K95R/K140R) was co-expressed with pCAG-FLAG-ISG15 together with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and HA-tagged E3 ligase (pCAG-HA-HERC5) in HEK293T cells, followed by immunoprecipitation with anti-FLAG mouse mAb 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. The asterisks indicate the ISG15-conjugated HBx proteins (ISGylated-HBx). The western blots are representative for three independent experiments.

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

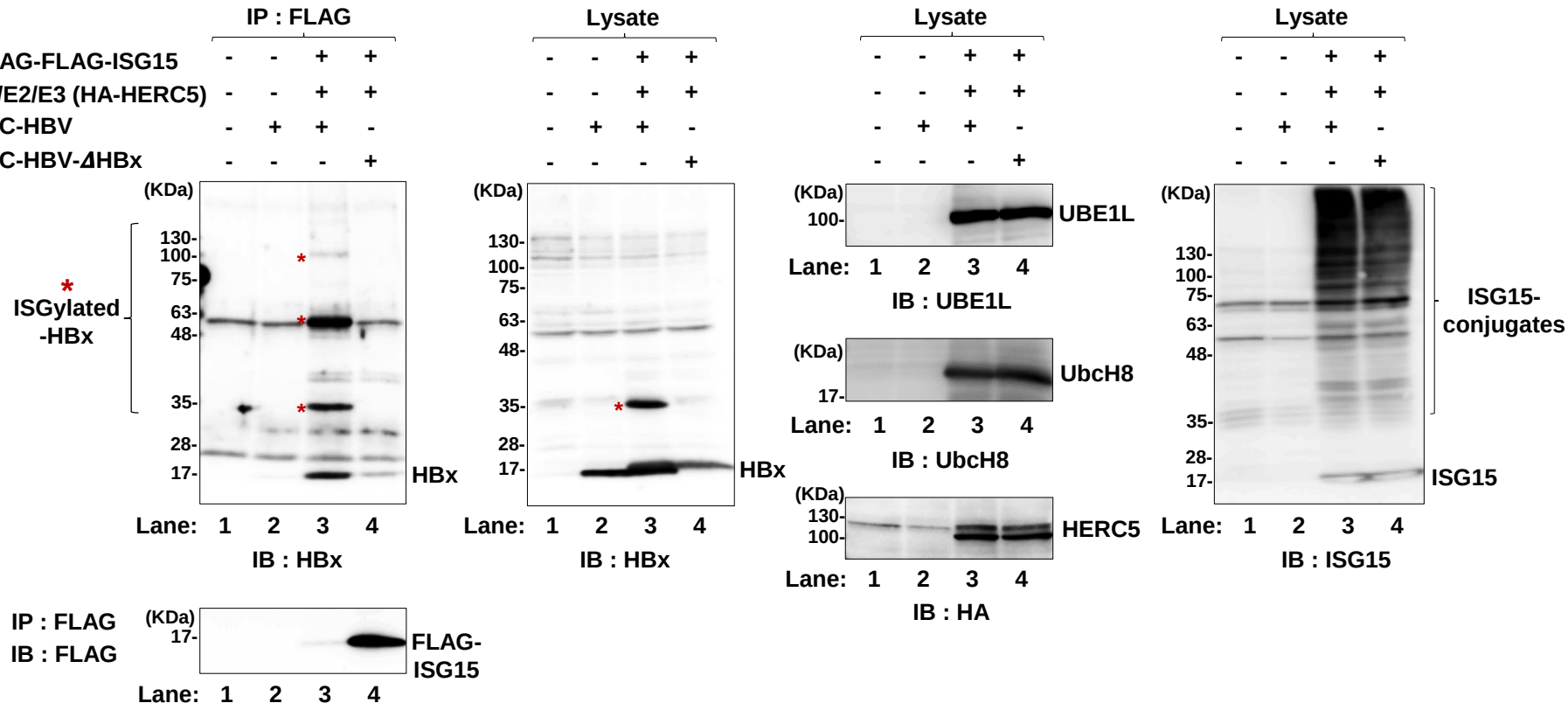
Fig. 6. ISG15 functions as an IFN-resistance factor in HBV-replicating cells. Hep38.7-Tet cells were cultured in the medium without 400 ng/ml doxycycline. After 5 days of culture, cells were harvested and then treated with 50 nM siRNA targeted to ISG15 (siISG15) (**A)**, USP18 (siUSP18) (**B)**, or negative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

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.

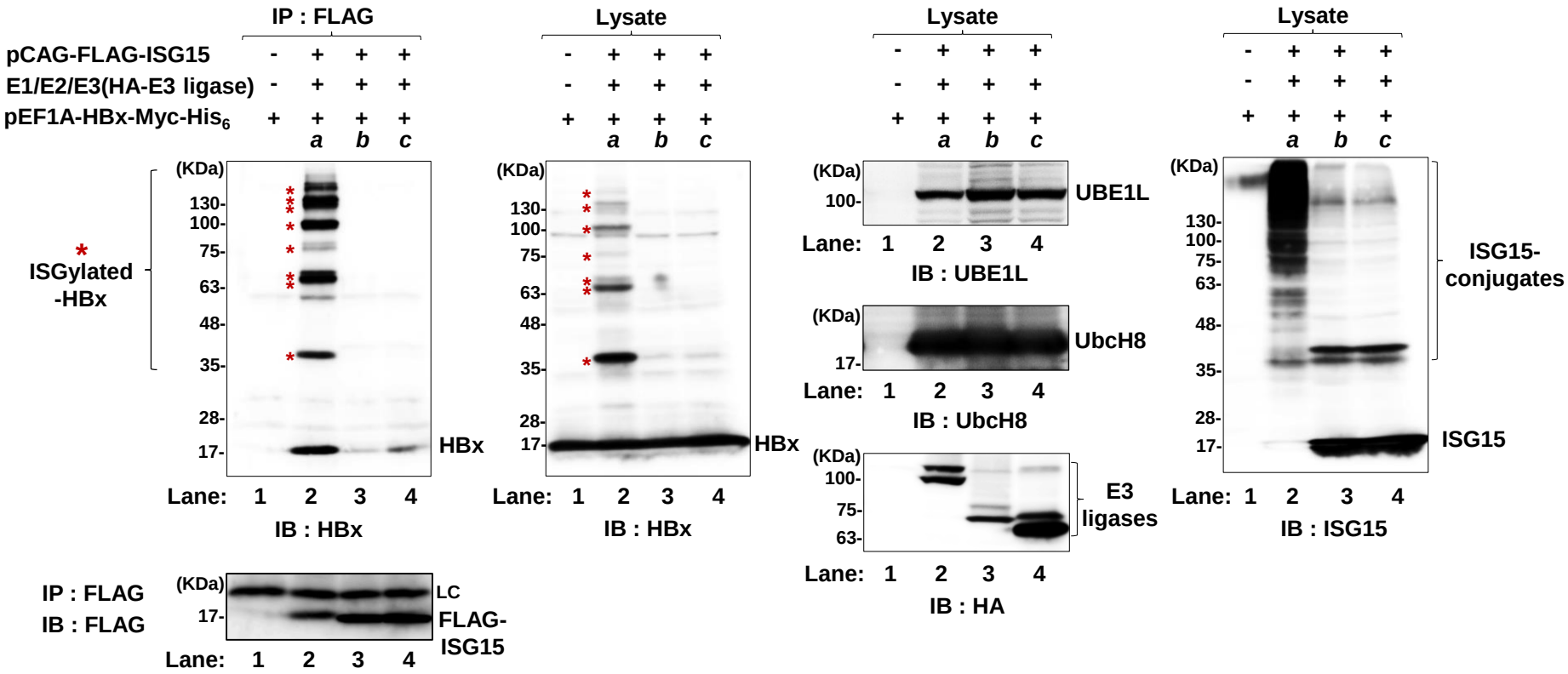
A.


IP : FLAG
Lysate
Lysate
Lysate
pCAG-FLAG-ISG15 - - + -
pCAG-FLAG-ISG15-AA - - - +
E1/E2/E3 (HA-HERC5) - - + +
pEF1A-HBx-Myc-His6 - + + +
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
* ISGylated -HBx
HBx
Lane: 1 2 3 4
IB : HBx
IB : HBx
(KDa)
100-
UBE1L
Lane: 1 2 3 4
IB : UBE1L
(KDa)
17-
UbcH8
Lane: 1 2 3 4
IB : UbcH8
(KDa)
130-
100-
HERC5
Lane: 1 2 3 4
IB : HA
ISG15-conjugates
ISG15
Lane: 1 2 3 4
IB : ISG15
IP : FLAG
IB : FLAG
(KDa)
17-
LC
FLAG-ISG15
Lane: 1 2 3 4

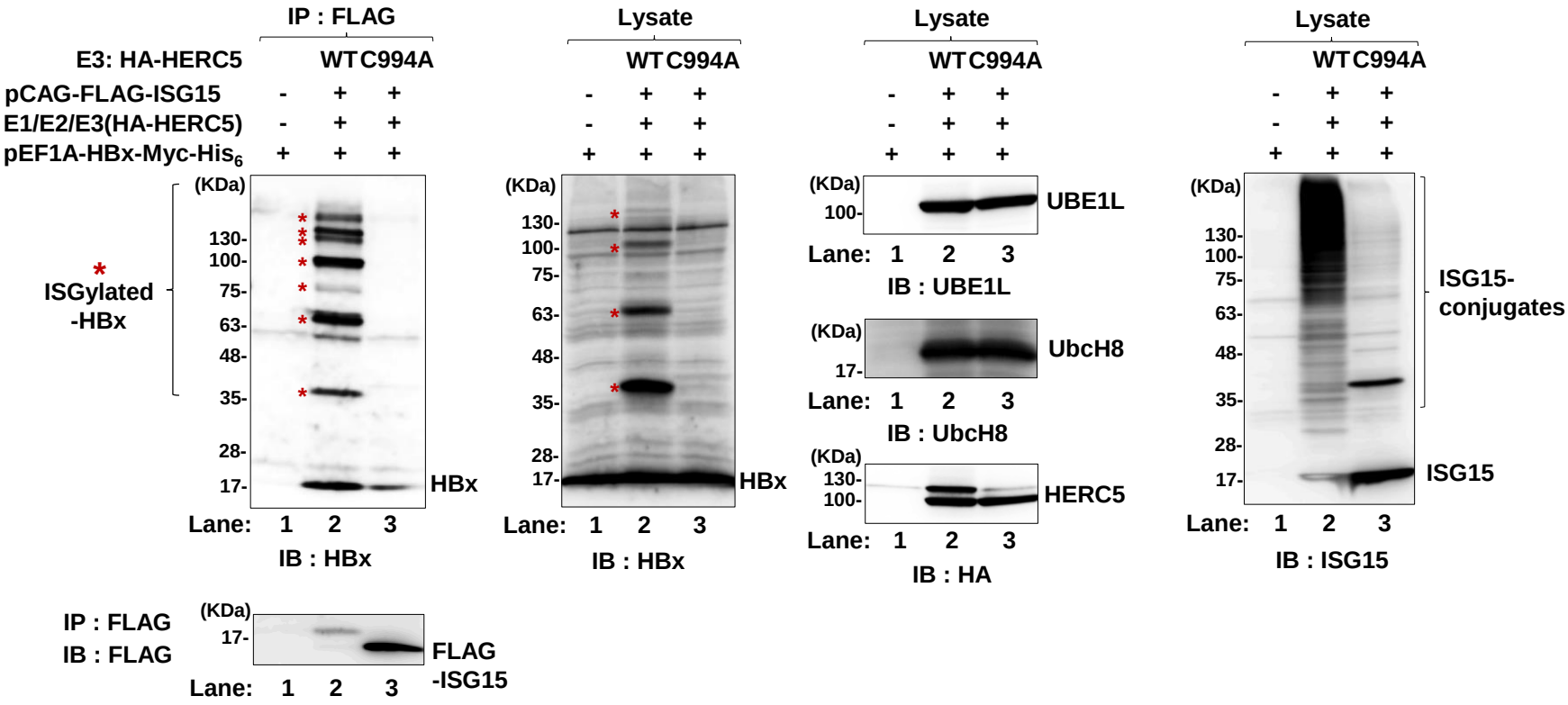
B.


IP : FLAG
Lysate
Lysate
Lysate
pCAG-FLAG-ISG15 - - + +
E1/E2/E3 (HA-HERC5) - - + +
pUC-HBV - + + -
pUC-HBV-ΔHBx - - - +
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
*
ISGylated -HBx
HBx
Lane: 1 2 3 4
IB : HBx
IB : HBx
IP : FLAG
IB : FLAG
FLAG-ISG15
100-
UBE1L
IB : UBE1L
UbcH8
IB : UbcH8
130-
HERC5
IB : HA
ISG15-conjugates
ISG15
IB : ISG15

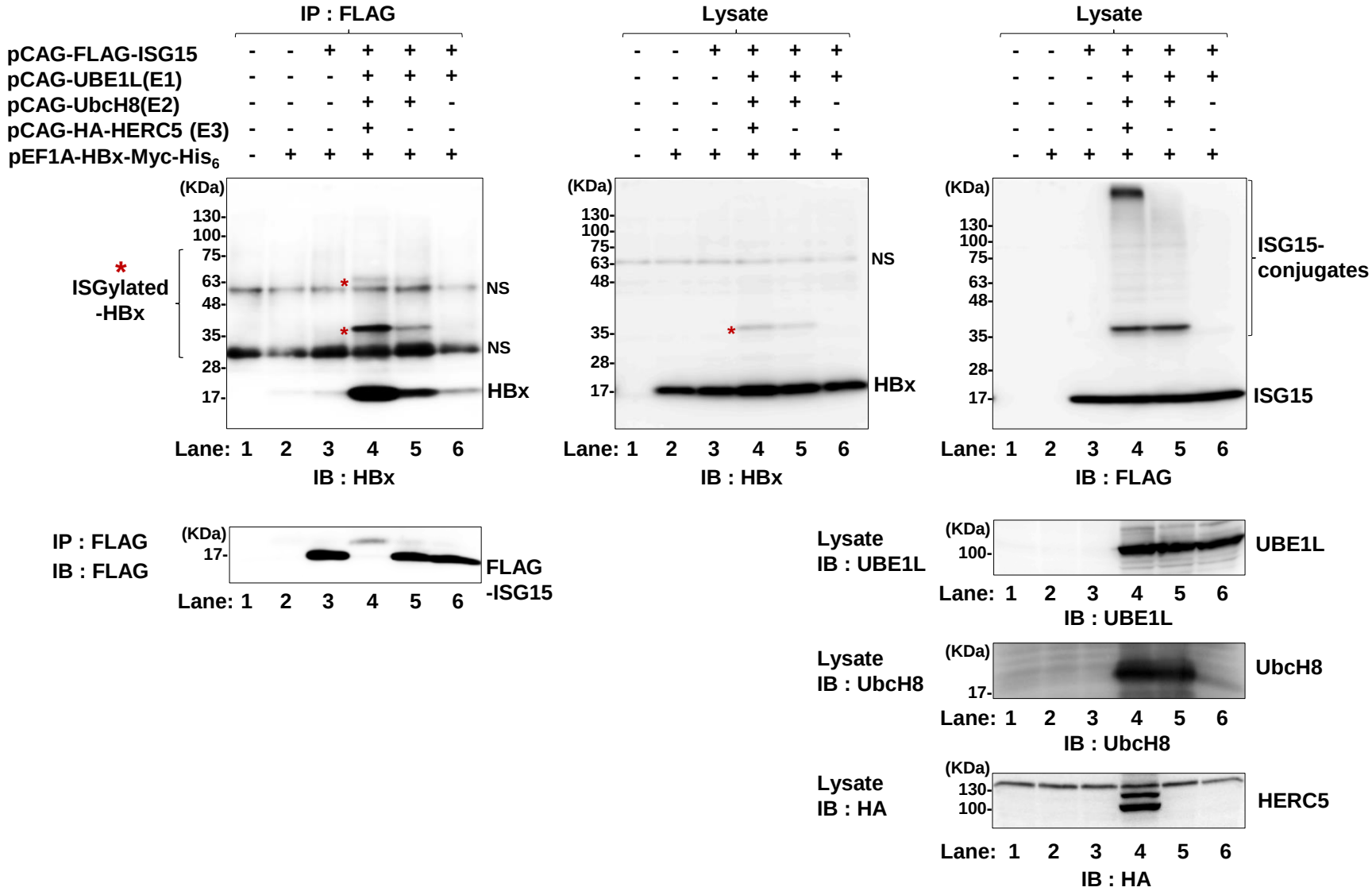
A.


IP : FLAG
Lysate
Lysate
Lysate
pCAG-FLAG-ISG15 - + + +
E1/E2/E3(HA-E3 ligase) - + + +
pEF1A-HBx-Myc-His6 + + + +
a b c
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
* ISGylated -HBx
HBx
Lane: 1 2 3 4
IB : HBx
IB : HBx
IP : FLAG
IB : FLAG
LC
FLAG-ISG15
UBE1L
IB : UBE1L
UbcH8
IB : UbcH8
E3 ligases
IB : HA
ISG15-conjugates
ISG15
IB : ISG15

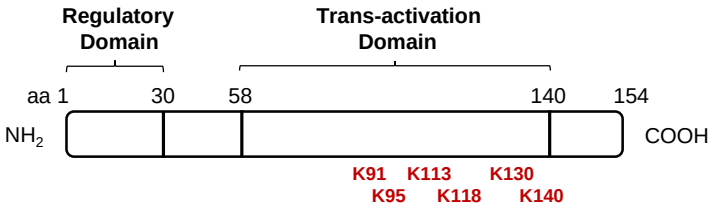
B.


IP : FLAG
Lysate
Lysate
Lysate
E3: HA-HERC5
WT C994A
pCAG-FLAG-ISG15
E1/E2/E3(HA-HERC5)
pEF1A-HBx-Myc-His6
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
ISGylated -HBx
HBx
Lane: 1 2 3
IB : HBx
UBE1L
IB : UBE1L
UbcH8
IB : UbcH8
HERC5
IB : HA
ISG15-conjugates
ISG15
IB : ISG15
IP : FLAG
IB : FLAG
FLAG -ISG15

C.


IP : FLAG
Lysate
Lysate
pCAG-FLAG-ISG15
pCAG-UBE1L(E1)
pCAG-UbcH8(E2)
pCAG-HA-HERC5 (E3)
pEF1A-HBx-Myc-His6
(KDa)
130
100
75
63
48
35
28
17
ISGylated -HBx
NS
NS
HBx
ISG15-conjugates
ISG15
Lane: 1 2 3 4 5 6
IB : HBx
IB : HBx
IB : FLAG
IP : FLAG
IB : FLAG
FLAG -ISG15
Lysate IB : UBE1L
UBE1L
IB : UBE1L
Lysate IB : UbcH8
UbcH8
IB : UbcH8
Lysate IB : HA
HERC5
IB : HA

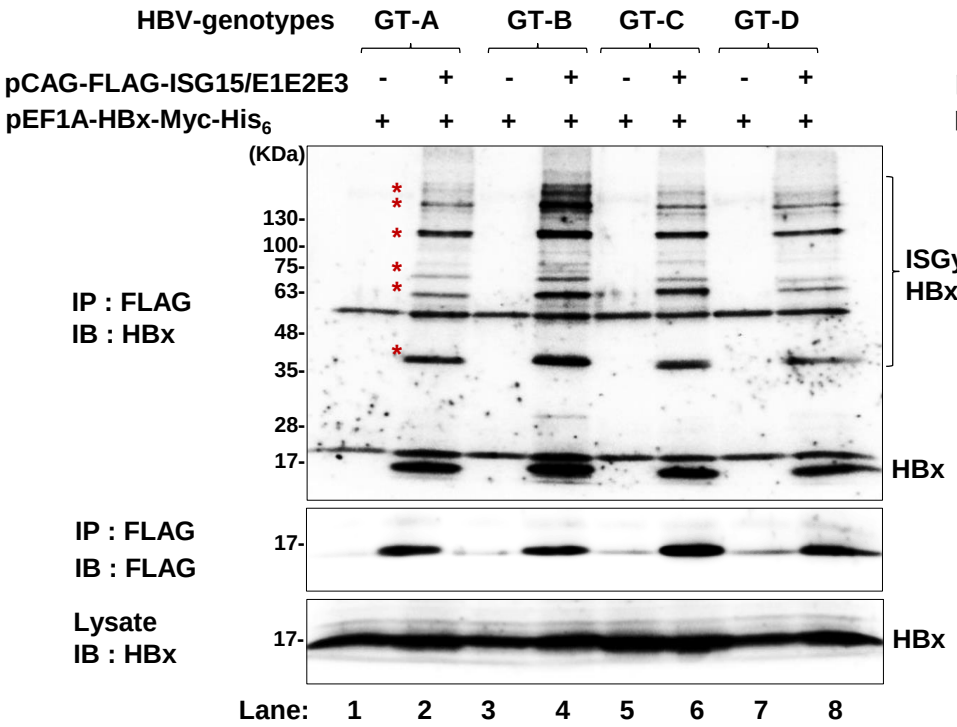
A.

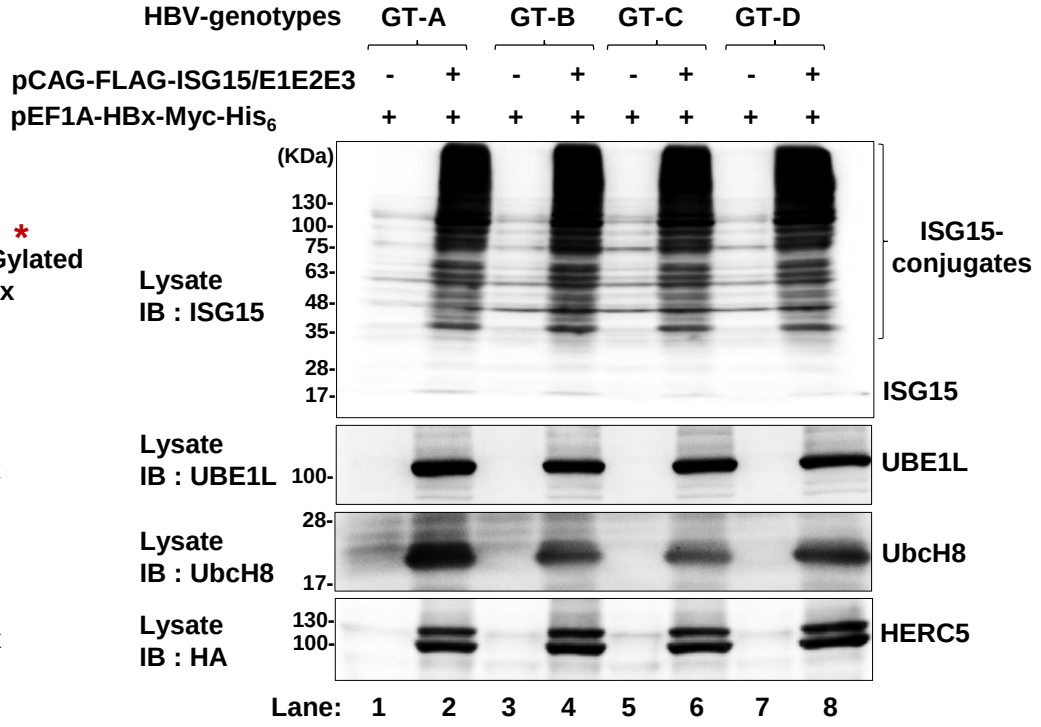


B.

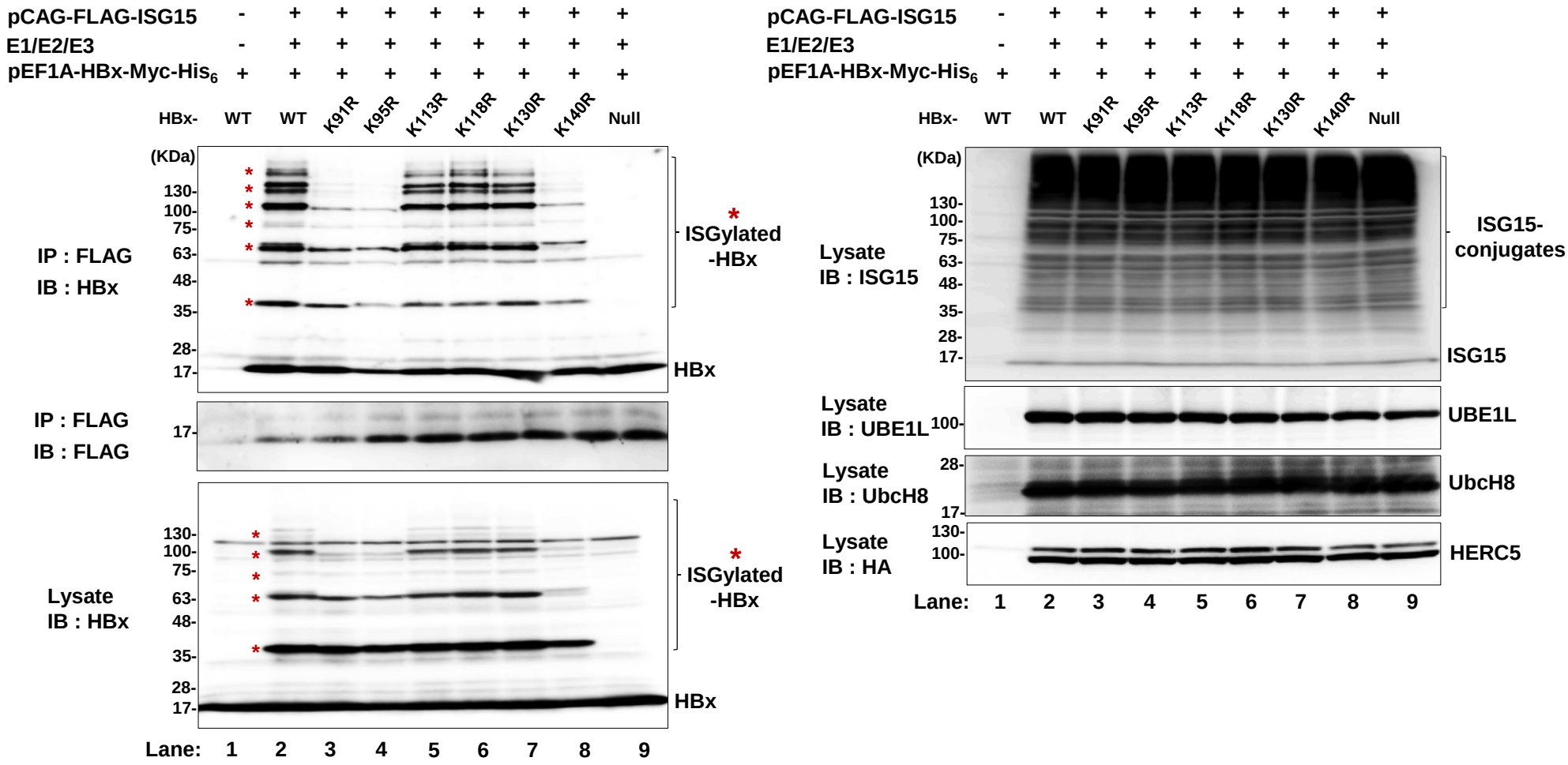
```
GT-A    1 MAARLYCQLDPSRDVLCLRPVGAESRGRPLSGPLGTFSSPSPSAVPADHGAHLSLRGLPV   aa 60
GT-B    1 MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPSAVSTDHGAHLSLRGLPV     60
GT-C    1 MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLSSPSSSAVPADHGAHLSLRGLPV     60
GT-D    1 MAARLCCQLDPARDVLCLRPVGAESCGRPFSGSLGTLSSPSPSAVPTDHGAHLSLRGLPV     60
          ****..*****.************.*** .*..*.....*.***. **************
                                      91 95              113  118
GT-A   61 CAFSSAGPCALRFTSARCMETTVNAHQILPKVLHKRTLGLPAMSTTDLEAYFKDCVFKDW   aa 120
GT-B   61 CAFSSAGPCALRFTSARRMETTVNAHRNLPKVLHKRTLGLSPMSTTDLEAYFKDCVFNEW     120
GT-C   61 CAFSSAGPCALRFTSARRMETTVNAHQVLPKVLYKRTLGLSAMSTTDLEAYFKDCLFKDW     120
GT-D   61 CAFSSAGPCALRFTSARRMETTVNAHRMLPKVLHKRTLGLSAMSTTDLEAYFKDCLFKDW     120
          ****************.******** *****.******..************ *.*.*
                    130        140
GT-A  121 EELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA                          aa 154
GT-B  121 EELGEEIRLKVFVLGGCRHKLVCSPAPCNFFTSA                            154
GT-C  121 EELGEEIRLKIFVLGGCRHKLVCSPAPCNFFTSA                            154
GT-D  121 EELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA                            154
          **********.************ **********
```

C.

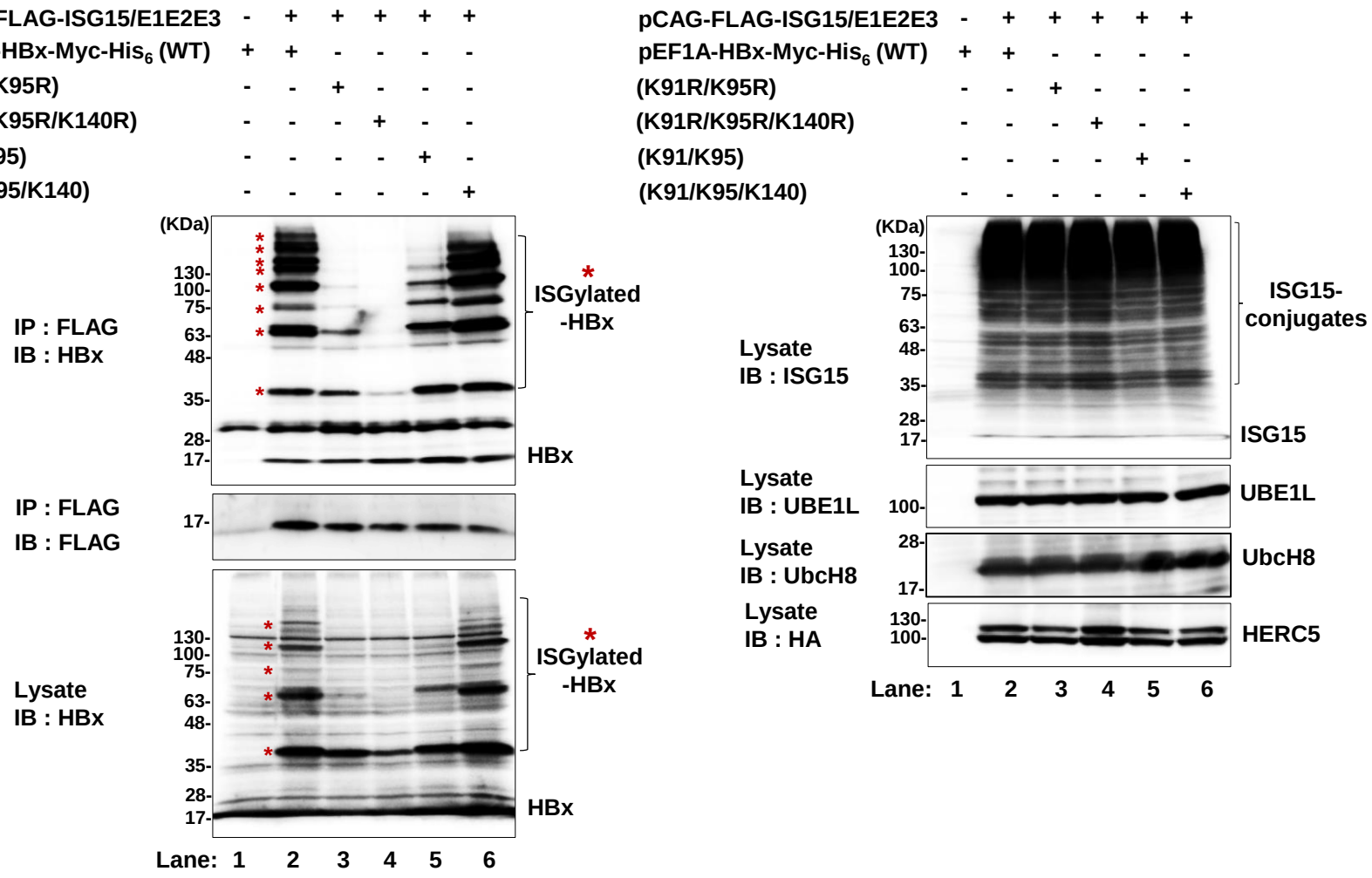


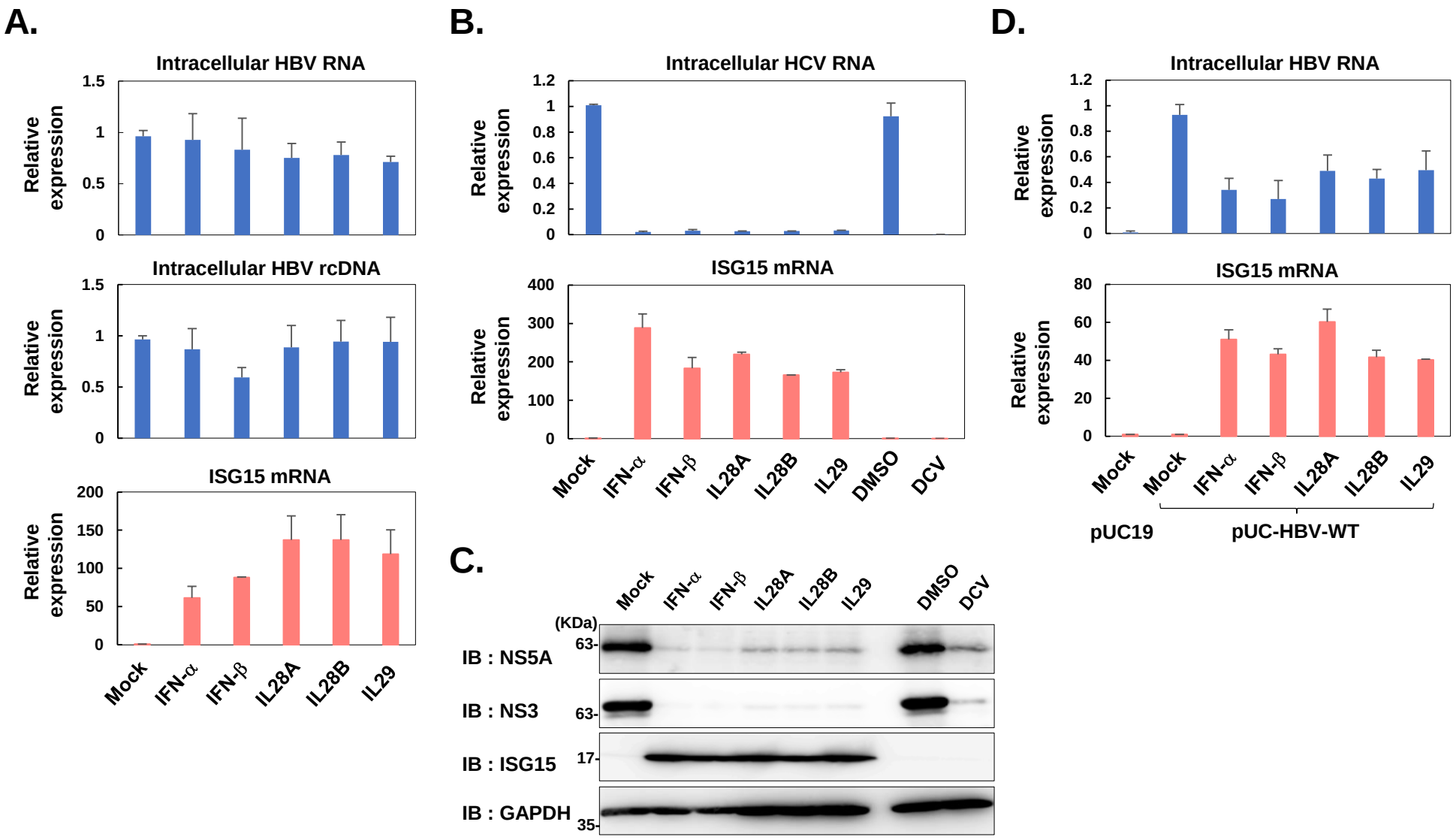


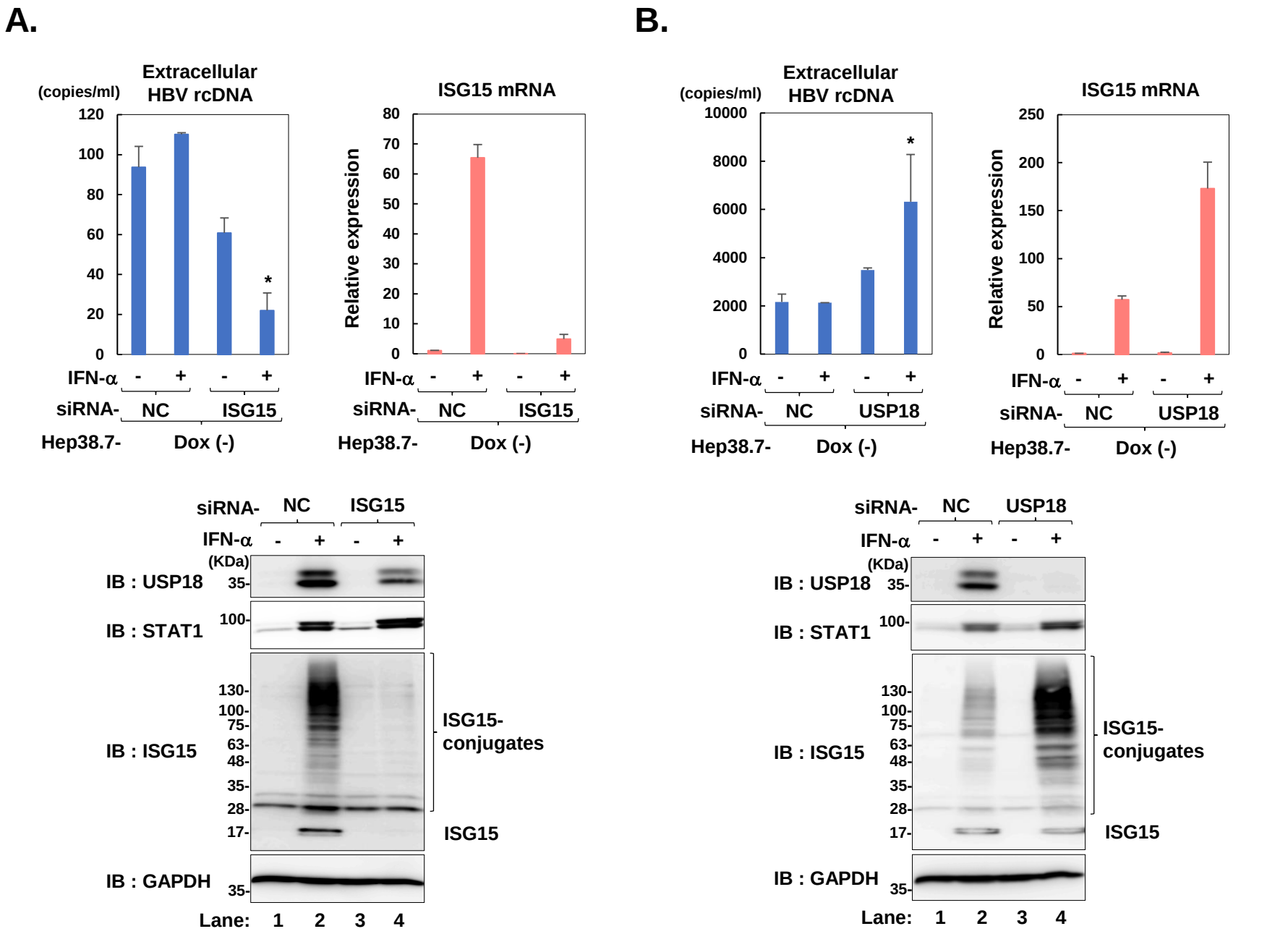
A.

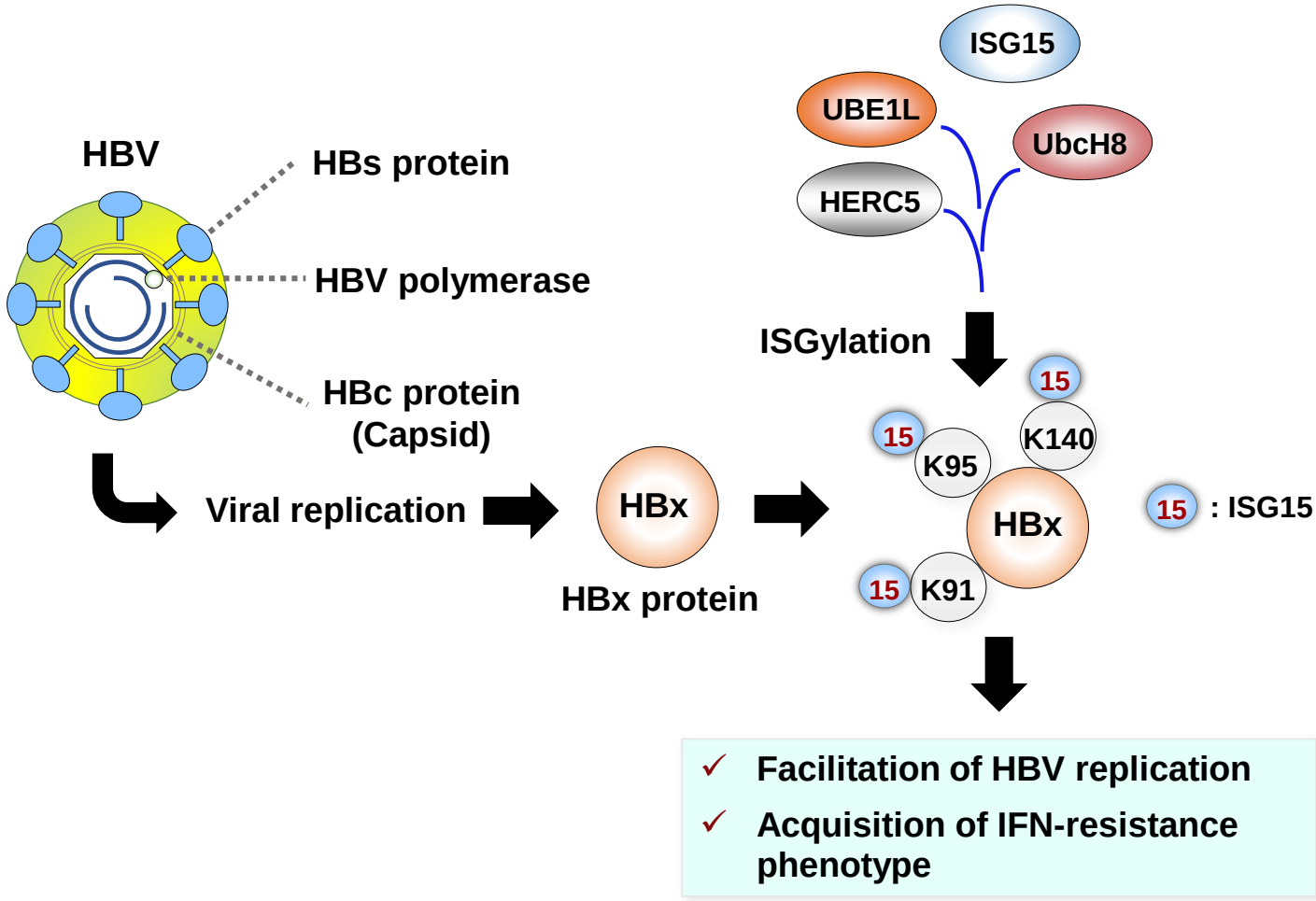

pCAG-FLAG-ISG15 - + + + + + + + +
E1/E2/E3 - + + + + + + + +
pEF1A-HBx-Myc-His6 + + + + + + + + +
HBx- WT WT K91R K95R K113R K118R K130R K140R Null
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
IP : FLAG
IB : HBx
ISGylated -HBx
HBx
IP : FLAG
IB : FLAG
Lysate
IB : HBx
Lane: 1 2 3 4 5 6 7 8 9
Lysate
IB : ISG15
ISG15-conjugates
ISG15
Lysate
IB : UBE1L
UBE1L
Lysate
IB : UbcH8
UbcH8
Lysate
IB : HA
HERC5

B.


pCAG-FLAG-ISG15/E1E2E3 - + + + + +
pEF1A-HBx-Myc-His6 (WT) + + - - - -
(K91R/K95R) - - + - - -
(K91R/K95R/K140R) - - - + - -
(K91/K95) - - - - + -
(K91/K95/K140) - - - - - +
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
IP : FLAG
IB : HBx
ISGylated
-HBx
HBx
IP : FLAG
IB : FLAG
17-
Lysate
IB : HBx
ISGylated
-HBx
HBx
Lane: 1 2 3 4 5 6
pCAG-FLAG-ISG15/E1E2E3 - + + + + +
pEF1A-HBx-Myc-His6 (WT) + + - - - -
(K91R/K95R) - - + - - -
(K91R/K95R/K140R) - - - + - -
(K91/K95) - - - - + -
(K91/K95/K140) - - - - - +
(KDa)
130-
100-
75-
63-
48-
35-
28-
17-
Lysate
IB : ISG15
ISG15-
conjugates
ISG15
Lysate
IB : UBE1L
100-
UBE1L
Lysate
IB : UbcH8
28-
17-
UbcH8
Lysate
IB : HA
130-
100-
HERC5
Lane: 1 2 3 4 5 6


A.
Intracellular HBV RNA
Relative expression
1.5
1
0.5
0
Intracellular HBV rcDNA
Relative expression
1.5
1
0.5
0
ISG15 mRNA
Relative expression
200
150
100
50
0
Mock
IFN-α
IFN-β
IL28A
IL28B
IL29
B.
Intracellular HCV RNA
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
ISG15 mRNA
Relative expression
400
300
200
100
0
Mock
IFN-α
IFN-β
IL28A
IL28B
IL29
DMSO
DCV
C.
Mock
IFN-α
IFN-β
IL28A
IL28B
IL29
DMSO
DCV
(KDa)
IB : NS5A
63-
IB : NS3
63-
IB : ISG15
17-
IB : GAPDH
35-
D.
Intracellular HBV RNA
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
ISG15 mRNA
Relative expression
80
60
40
20
0
Mock
Mock
IFN-α
IFN-β
IL28A
IL28B
IL29
pUC19
pUC-HBV-WT


A.
Extracellular HBV rcDNA
(copies/ml)
120
100
80
60
40
20
0
*
IFN-α - + - +
siRNA- NC ISG15
Hep38.7- Dox (-)
ISG15 mRNA
Relative expression
80
70
60
50
40
30
20
10
0
IFN-α - + - +
siRNA- NC ISG15
Hep38.7- Dox (-)
B.
Extracellular HBV rcDNA
(copies/ml)
10000
8000
6000
4000
2000
0
*
IFN-α - + - +
siRNA- NC USP18
Hep38.7- Dox (-)
ISG15 mRNA
Relative expression
250
200
150
100
50
0
IFN-α - + - +
siRNA- NC USP18
Hep38.7- Dox (-)
siRNA- NC ISG15
IFN-α - + - +
(KDa)
IB : USP18
35-
IB : STAT1
100-
IB : ISG15
130-
100-
75-
63-
48-
35-
28-
17-
ISG15-conjugates
ISG15
IB : GAPDH
35-
Lane: 1 2 3 4
siRNA- NC USP18
IFN-α - + - +
(KDa)
IB : USP18
35-
IB : STAT1
100-
IB : ISG15
130-
100-
75-
63-
48-
35-
28-
17-
ISG15-conjugates
ISG15
IB : GAPDH
35-
Lane: 1 2 3 4


ISG15
UBE1L
UbcH8
HERC5
HBV
HBs protein
HBV polymerase
HBc protein
(Capsid)
ISGylation
15
15
K140
15
K95
HBx
15 : ISG15
15
K91
Viral replication
HBx
HBx protein
✓ Facilitation of HBV replication
✓ Acquisition of IFN-resistance phenotype

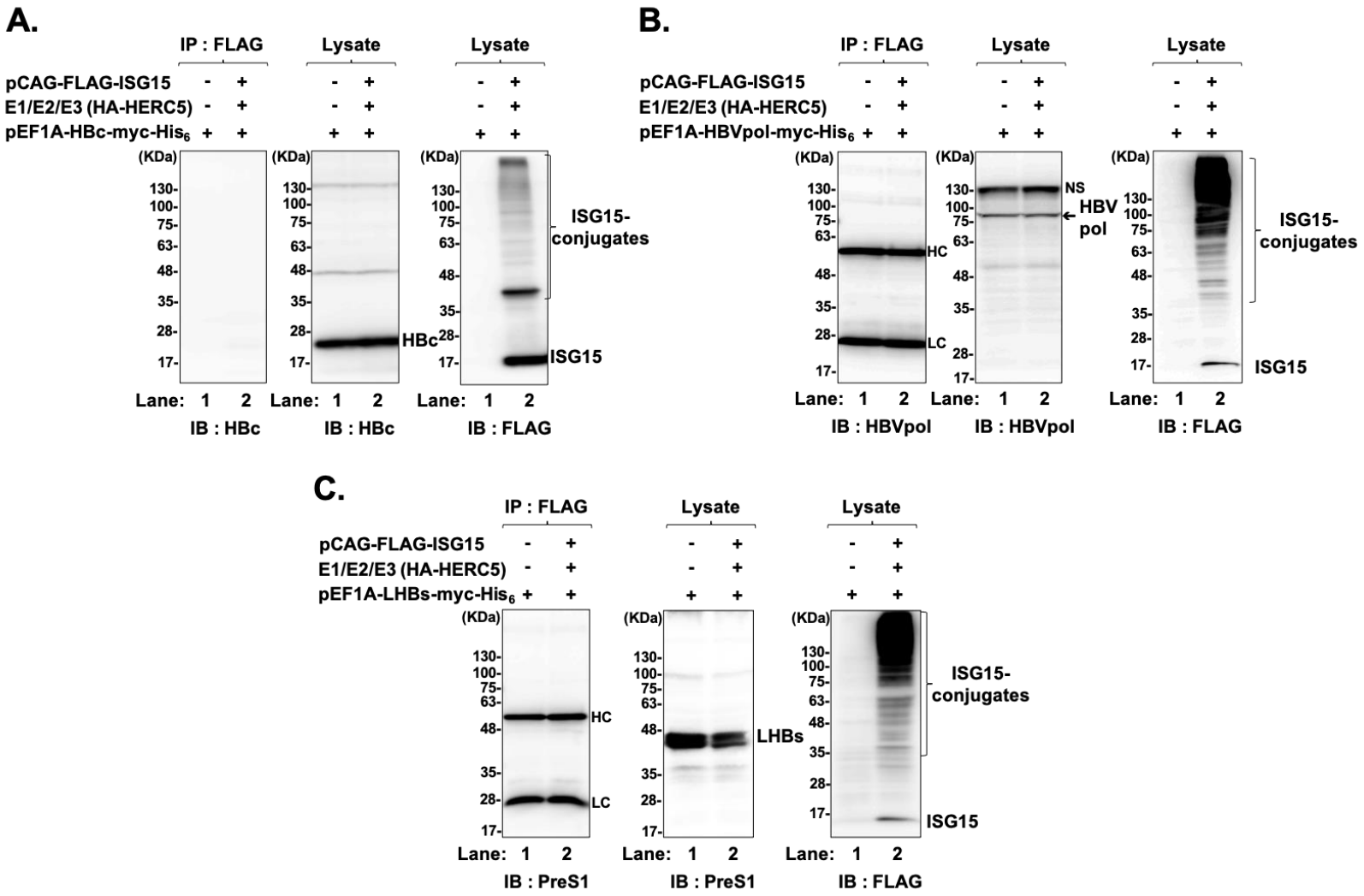


Fig. S1. Other HBV proteins, including HBc, HBV polymerase, and HBs proteins, do not accept ISGylation.
HEK293T cells were co-transfected with the expression plasmid encoding HBc (A), HBV polymerase (HBV pol) (B), or large HBV surface (LHBs) (C) genes from genotype (GT)-C (C-AT_JPN) together with pCAG-FLAG-ISG15, 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.

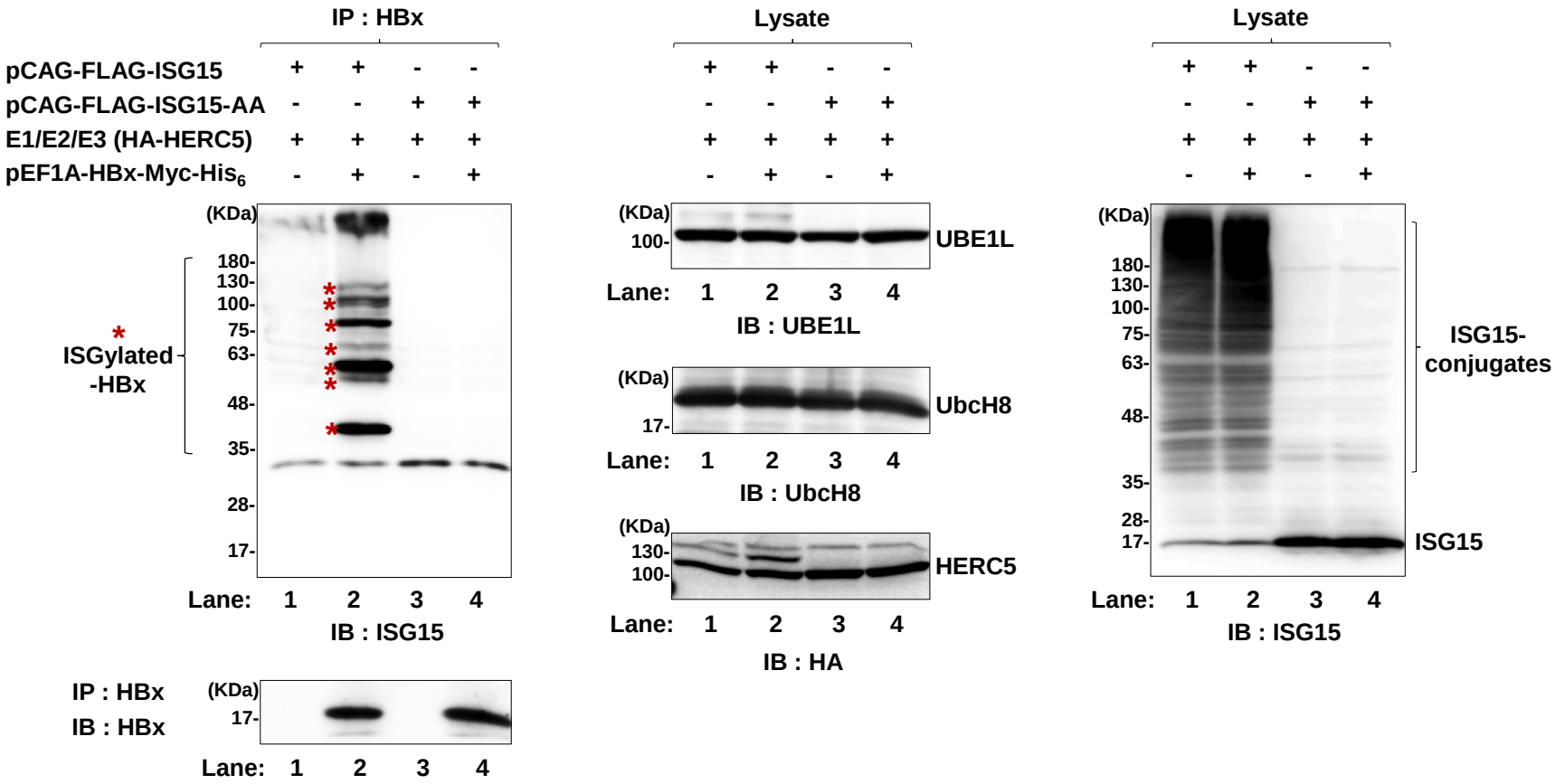


Fig. S2. HBx protein is a substrate of ISGylation.
HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His$_6$ from genotype (GT)-C (C-AT_JPN) (pEF1A-HBx-Myc-His$_6$) with either pCAG-FLAG-ISG15 or FLAG-ISG15 mutant (pCAG-FLAG-ISG15-AA) together with pCAG-UBE1L (E1), pCAG-UbcH8 (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.

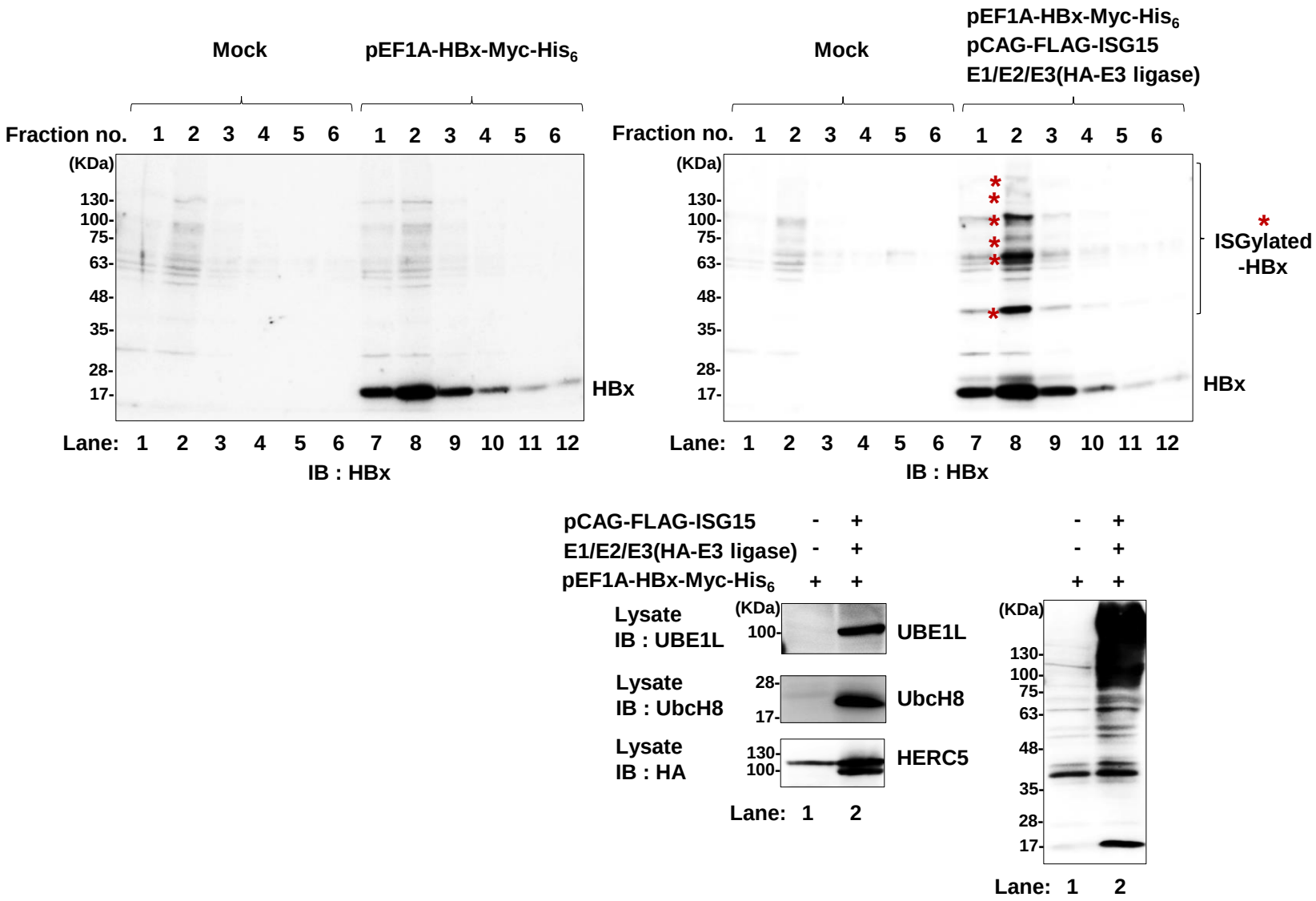


Fig. S3. Ni-NTA pull-down analysis for ISGylated HBx proteins.

HEK293T cells were co-transfected with the expression plasmid encoding HBx-Myc-His$_6$ from genotype (GT)-C (C-AT_JPN) (pEF1A-HBx-Myc-His$_6$) 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.

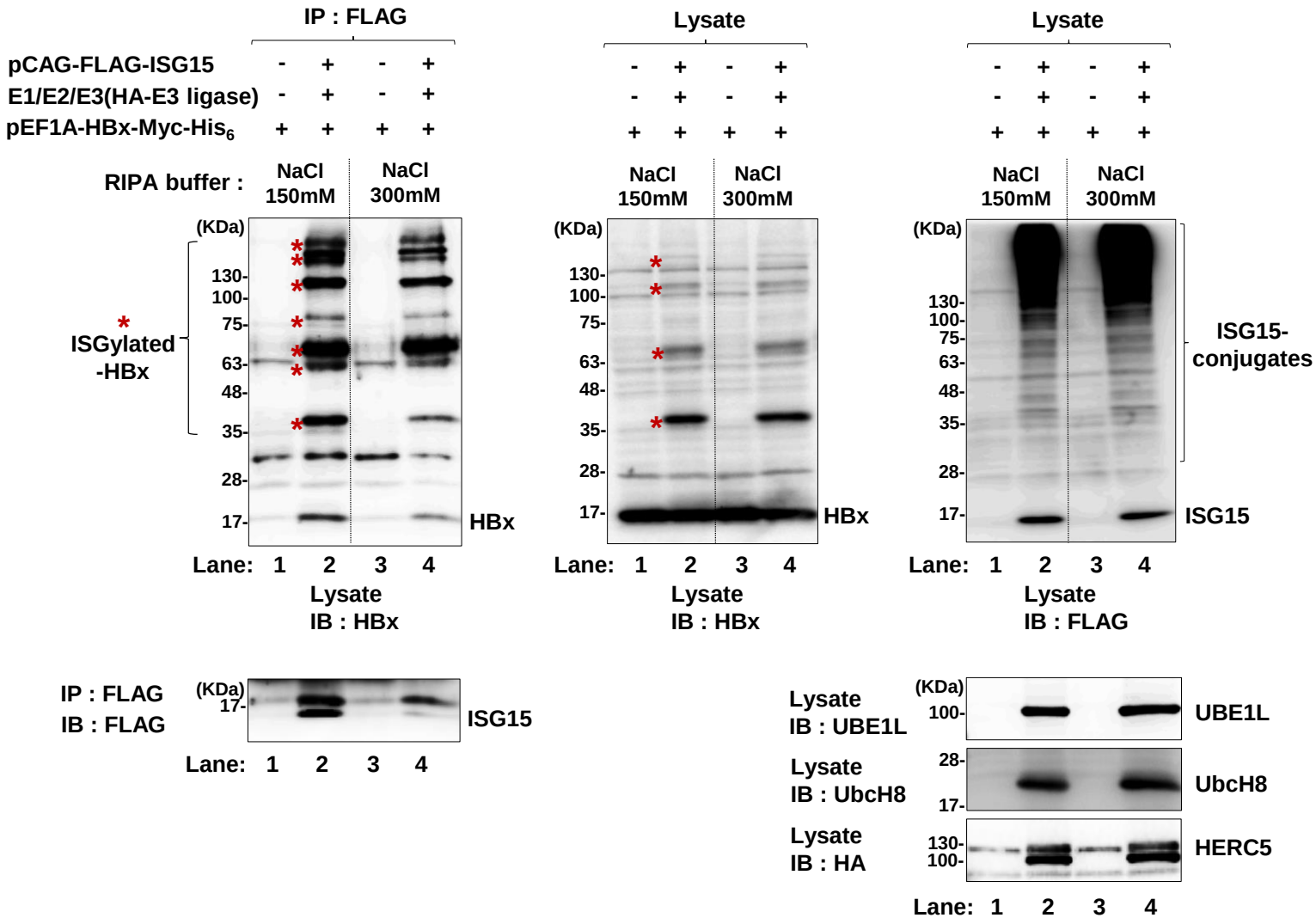


Fig. S4. HBx protein interacts with ISG15 via protein-protein interaction.
HEK293T cells were co-transfected with the expression plasmid pEF1A-HBx-Myc-His$_6$ and pCAG-FLAG-ISG15 together with pCAG-UBE1L, pCAG-UbcH8, and pCAG-HA-HERC5. At 2days after transfection, cells were lysed with RIPA buffer containing 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.

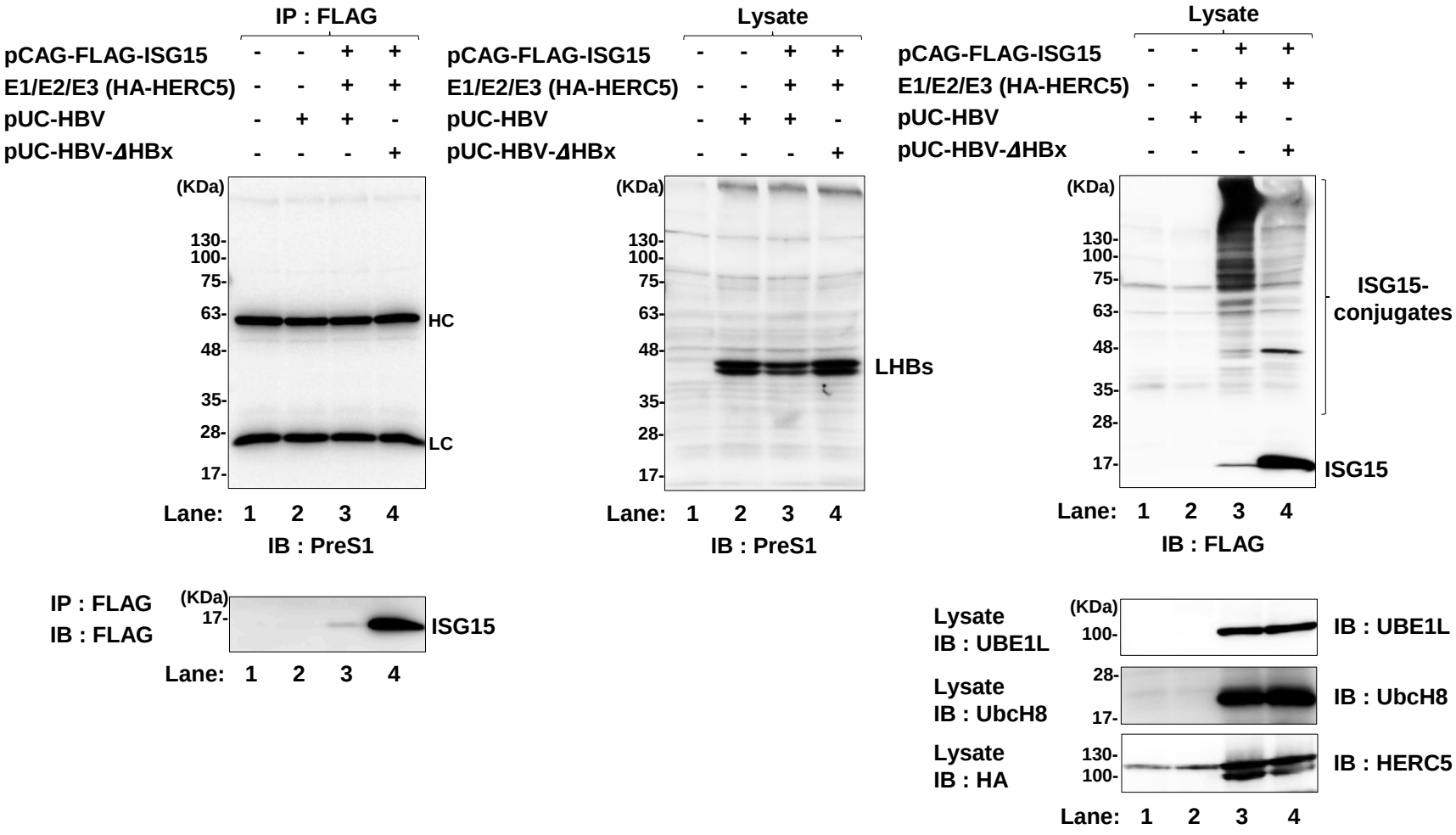


Fig. S5. HBs protein does not accept ISGylation in the cells transfected with pUC-HBV.
The expression plasmid encoding either the full-length HBV gene (pUC-HBV) from GT-C (C-AT_JPN) or an HBV gene lacking the HBx region (pUC-HBV-ΔHBx) was co-expressed with pCAG-UBE1L (E1), pCAG-UbcH8 (E2), and pCAG-HA-HERC5 (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.