

1 **High-level expression of STING restricts susceptibility to HBV by mediating type**

2 **III IFN induction**

4 Running title: STING-mediated restriction of HBV

6 Hiromichi Dansako^{*,1}, Hirotaka Imai¹, Youki Ueda¹, Shinya Satoh¹, Kunitada
7 Shimotohno², Nobuyuki Kato¹

9 ¹Department of Tumor Virology, Okayama University Graduate School of Medicine,
10 Dentistry and Pharmaceutical Sciences, Okayama, Japan

11 ²Research Center for Hepatitis and Immunology, National Center for Global Health and
12 Medicine, Chiba, Japan.

13 ^{*}Corresponding author. Tel: +81 86 235 7386; E-mail: dansako@md.okayama-u.ac.jp

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16 STING, type III interferon

17

18 **Abstract**

19 Hepatitis B virus (HBV) is a hepatotropic DNA virus causing hepatic diseases such as
 20 chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. To study HBV, human
 21 hepatoma HepG2 cells are currently used as an HBV infectious cell culture model
 22 worldwide. HepG2 cells exhibit susceptibility to HBV by exogenously expressing
 23 sodium taurocholate cotransporting polypeptide (NTCP). We herein demonstrated that
 24 human immortalized hepatocyte NKNT-3 cells exhibited susceptibility to HBV by
 25 exogenously expressing NTCP (NKNT-3/NTCP cells). By comparing the cGAS-STING
 26 signaling pathway in several NKNT-3/NTCP cell-derived cell clones, we found that
 27 STING was highly expressed in cell clones exhibiting resistance but not susceptibility
 28 to HBV. High-level expression of STING was implicated in HBV-triggered induction of
 29 type III IFN and a pro-inflammatory cytokine, IL-6. In contrast, RNAi-mediated
 30 knockdown of STING inhibited type III IFN induction and restored the levels of HBV
 31 total transcript in an HBV-infected cell clone exhibiting resistance to HBV. These
 32 results suggest that STING regulates susceptibility to HBV by its expression levels.

33 STING may thus be a novel target for anti-HBV strategies.

34

35 **Introduction**

36 Hepatitis B virus (HBV) is a hepatotropic virus classified into the *Hepadnaviridae*
 37 family. HBV infection causes chronic hepatitis, liver cirrhosis, and finally
 38 hepatocellular carcinoma (HCC) [1, 2]. The progression of hepatic diseases is tightly
 39 associated with the HBV-triggered host innate immune response and inflammatory
 40 response. To prevent the progression of hepatic diseases, it is important to suppress the
 41 HBV-triggered host innate immune response and inflammatory response.

42 The cytoplasmic DNA sensor, cyclic GMP-AMP synthetase (cGAS), is known to
 43 recognize viral DNA and other non-self exogenous DNAs as pathogen-associated
 44 molecular patterns (PAMPs) [3, 4]. After the recognition of non-self exogenous DNA,
 45 cGAS produces cyclic GMP-AMP (cGAMP) and then uses cGAMP to activate a
 46 stimulator of interferon genes (STING). STING mediates activation of the transcription
 47 factor interferon regulatory factor 3 (IRF-3) and subsequently the induction of interferon
 48 (IFN)- β (type I IFN) [5], IFN- λ 1, λ 2, and λ 3 (type III IFN) [6]. Both type I and type III

IFNs stimulate the induction of numerous IFN-stimulated genes (ISGs) such as ISG15 and ISG56 through the JAK-STAT signaling pathway [7]. On the other hand, STING also mediates the induction of pro-inflammatory cytokines such as IL-6 and IL-8 through the NF- κ B signaling pathway [8, 9]. As described here, both cGAS and STING are required for the innate immune response and inflammatory response. We previously reported that cGAS recognized HBV DNA and subsequently triggered an innate immune response in human hepatoma Li23 cells [10]. However, in that study, we could not examine the HBV-triggered inflammatory response, since Li23 cells were a human hepatoma cell line. To study HBV-triggered inflammatory responses, it will be necessary to establish an HBV infectious cell culture model from normal human hepatic cells rather than human hepatoma cells.

Sodium taurocholate cotransporting polypeptide (NTCP) is a functional receptor for HBV [11]. Human hepatoma HepG2 cells exhibit susceptibility to HBV by exogenously expressing NTCP [11]. HepG2/NTCP cells (HepG2 cells stably expressing exogenous NTCP) are currently used as an HBV infectious cell culture model for the study of HBV worldwide. However, we previously reported that HepG2 cells exhibited defective

expression of endogenous cGAS [10]. This result suggests that HepG2/NTCP cells cannot be used for the study of endogenous cGAS-triggered innate immune response and inflammatory response. Our previous study also showed that cGAS was expressed in immortalized human hepatocyte NKNT-3 cells [10]. In the present study, we established NKNT-3 cells exhibiting susceptibility to HBV by the exogenous expression of NTCP. In addition, we obtained several NKNT-3/NTCP-derived cell clones exhibiting susceptibility or resistance to HBV. Interestingly, STING was highly expressed in a cell clone exhibiting resistance to HBV. Here, we show that STING is an important host factor that regulates susceptibility to HBV by its expression levels. We also show that NKNT-3/NTCP cells are a novel HBV infectious cell culture model for the study of HBV-triggered innate immune responses and inflammatory responses.

Results

The immortalized human hepatocyte NKNT-3 cells exhibited susceptibility to HBV via their expression of exogenous NTCP.

Since HepG2 cells were a human hepatoma cell line and exhibited defective

81 expression of endogenous cGAS [10], we tried to establish HBV infectious cell culture
82 model from immortalized human hepatocyte NKNT-3 cells, which has been exhibited a
83 non-neoplastic phenotype [12] and the endogenous expression of cGAS [10]. HepG2
84 cells have been reported to exhibit susceptibility to HBV through their expression of
85 exogenous NTCP [11]. Therefore, to establish NKNT-3 cells exhibiting susceptibility to
86 HBV, we first prepared NKNT-3 cells stably expressing exogenous NTCP-myc
87 (designated NKNT-3/NTCP cells; Fig. 1A). The cell surface expression of NTCP was
88 detected in both NKNT-3/NTCP cells and HepG2/NTCP cells (HepG2 cells stably
89 expressing exogenous NTCP-myc), but not in NKNT-3/Control cells (NKNT-3 cells
90 stably expressing the control vector) (Fig. 1B). By using two kinds of inoculum,
91 HBV/NLuc (genotype C) [13] and HBV (the supernatant of HBV-replicating
92 HepG2.2.15 cells, genotype D) [14], we compared the levels of susceptibility to HBV in
93 NKNT-3/NTCP cells with that in NKNT-3/Control cells. After the infection with
94 HBV/NLuc or HBV, both level of NLuc activity and HBV total transcript were
95 increased in NKNT-3/NTCP cells in a time-dependent manner, but not in
96 NKNT-3/Control cells (Figs. 1C and 1D). We next compared the level of susceptibility

to HBV in NKNT-3/NTCP cells with that in HepG2/NTCP cells. The levels of NLuc activity, HBV total transcript, and pgRNA in HBV/NLuc- or HBV-infected NKNT-3/NTCP cells were almost ten times lower than those in HBV/NLuc- or HBV-infected HepG2/NTCP cells (Figs. 1E and 1F). We further examined whether or not the exogenous NTCP was functional in NKNT-3/NTCP cells. Cyclosporin A (CsA) was previously reported to inhibit HBV entry by targeting NTCP [15]. When administered before and during HBV inoculation, CsA inhibited the levels of HBV total transcript in HBV-infected NKNT-3/NTCP cells as well as in HBV-infected HepG2/NTCP cells (Fig. 1G). These results suggest that NKNT-3 cells exhibit susceptibility to HBV by exogenously expressing functional NTCP.

The level of susceptibility to HBV in NKNT-3/NTCP #28.3.8 cells approximated that in HepG2/NTCP cells.

Since susceptibility to HBV in NKNT-3/NTCP cells was lower than that in HepG2/NTCP cells (Figs. 1E and 1F), we next tried to select a subcloned cell line exhibiting higher susceptibility to HBV than NKNT-3/NTCP cells (Fig. 2A). During

three-round serial limited dilution, we obtained three distinct cell clones (#28, #28.3, and #28.3.8 cells, respectively; Fig. 2A) that met this criterion (Fig. 2B). Exogenous NTCP was expressed on the cell surface in all three clones (Fig. 2C). Among them, the NKNT-3/NTCP #28.3.8 cells exhibited the highest levels of HBV total transcript after HBV infection (Fig. 2D). Therefore, we next compared the levels of susceptibility to HBV in NKNT-3/NTCP #28.3.8 cells with those in HepG2/NTCP cells. Upon the infection with HBV/NLuc or HBV, both levels of NLuc activity (Fig. 2E) and HBV total transcript (Fig. 2F) in NKNT-3/NTCP #28.3.8 cells approximated those in HepG2/NTCP cells. Consistent with these results, Northern blot analysis also showed that the levels of HBV pregenomic RNA (pgRNA) and 2.1/2.3 kb RNA in NKNT-3/NTCP #28.3.8 cells were roughly the same as those in HepG2/NTCP cells after HBV infection (Fig. 2G). These results suggest that NKNT-3/NTCP #28.3.8 cells are useful as an HBV infectious cell culture model in the manner of HepG2/NTCP cells.

HBV triggered the induction of type III IFNs in NKNT-3/NTCP #28.3.25.13 cells exhibiting resistance to HBV.

129 During the three-round limited dilution, we obtained NKNT-3/NTCP #28.3.8 cells
130 that exhibited higher susceptibility to HBV than the parent NKNT-3/NTCP cells (Figs.
131 2B and 2D). On the other hand, during the additional limited dilution (Fig. 3A), we
132 unexpectedly obtained a cell clone (#28.3.25.13) exhibiting greater resistance to HBV
133 compared with NKNT-3/NTCP #28.3.8 cells (Fig. 3B). We conjectured that the innate
134 immune response might be induced in cell clones exhibiting resistance to HBV. To
135 examine this possibility, we first compared the HBV-triggered innate immune responses
136 among cell clones exhibiting susceptibility or resistance to HBV. At 5 days after HBV
137 infection, ISG56 was strongly induced in NKNT-3/NTCP #28.3.25.13 cells, but not in
138 NKNT-3/NTCP #28.3.8 cells (Fig. 3C). Since HBV-triggered ISG56 induction in
139 NKNT-3/NTCP #28.3.25.13 cells was higher than that in #28.3. 30.20.3 cells (another
140 cell clone exhibiting resistance to HBV, Fig. 3B), we mainly focused the innate immune
141 response to HBV in NKNT-3/NTCP #28.3.25.13 cells. We first compared the time
142 course of *ISG56* mRNA induction after HBV infection between NKNT-3/NTCP #28.3.8
143 and #28.3.25.13 cells (Fig. 3D). At 5 or 9 days after HBV infection, *ISG56* mRNA was
144 strongly induced in NKNT-3/NTCP #28.3.25.13 cells, but not in #28.3.8 cells (Fig. 3D).

145 These results suggest that HBV infection induces the innate immune response in cell
146 clone exhibiting resistance but not susceptibility to HBV. We next examined whether
147 type I and/or type III IFN was required for *ISG56* mRNA induction after HBV infection
148 in NKNT-3/NTCP #28.3.25.13 cells. Interestingly, at 9 days after HBV infection,
149 *IFN- λ 1* and *IFN- λ 2/3* (type III IFN) mRNA, but not *IFN- β* (type I IFN) mRNA, were
150 induced in NKNT-3/NTCP #28.3.25.13 cells (Figs. 3E and 3F). In addition, *IFN- λ 1*
151 mRNA (Fig. 3G), *ISG15* (Fig. 3H), and *ISG56* (Fig. 3H) were induced at 9 days after
152 HBV infection, but not mock or ultraviolet-inactivated HBV (UV-HBV) infection, in
153 NKNT-3/NTCP #28.3.25.13 cells. Consistent with these results, HBV induced *IFN- λ 1*
154 and *IFN- λ 2/3*, but not *IFN- β* mRNA, in HBV-replicating HepG2.2.15 cGAS/STING
155 cells stably expressing both exogenous cGAS and STING [10] (Fig. 3I). In addition, the
156 induction levels of *IFN- λ 1* and *IFN- λ 2/3* mRNA in HepG2.2.15 cGAS/STING cells
157 were higher than those in HepG2.2.15 cGAS GSAA/STING cells stably expressing
158 both exogenous cGAS GSAA (the inactive mutant of cGAS) and STING [10]. These
159 results suggest that HBV induces type III IFN through the cGAS/STING signaling
160 pathway in NKNT-3/NTCP #28.3.25.13 cells, but not in #28.3.8 cells. These results also

suggest that the expression levels of cGAS/STING signaling pathway-associated host factor(s) are different between NKNT-3/NTCP #28.3.8 cells and #28.3.25.13 cells.

High-level expression of STING was implicated in HBV-triggered type III IFN induction in NKNT-3/NTCP #28.3.25.13 cells exhibiting resistance to HBV.

Since our results suggested that the expression levels of cGAS/STING signaling pathway-associated host factor(s) were different between NKNT-3/NTCP #28.3.8 cells and #28.3.25.13 cells, we next compared the levels of p-dGdC (the synthetic ligand for the cGAS/STING signaling pathway)-triggered type III IFN induction. We found that the p-dGdC-triggered *ISG56* and *IFN-lambda1* mRNA induction in NKNT-3/NTCP #28.3.25.13 cells was several times higher than that in NKNT-3/NTCP #28.3.8 cells (Fig. 4A). We next tried to identify the host factor(s) responsible for the higher responsiveness to p-dGdC in NKNT-3/NTCP #28.3.25.13 cells. Among cGAS/STING signaling pathway-associated host factor(s), we found that *STING* mRNA (Fig. 4B) and STING protein (Fig. 4C) were highly expressed in NKNT-3/NTCP #28.3.25.13 cells. These results suggest that the high-level expression of STING enhances

p-dGdC-triggered type III IFN induction in NKNT-3/NTCP #28.3.25.13 cells compared with #28.3.8 cells. We further compared the phosphorylation levels of STING among several NKNT-3/NTCP cell-derived cell clones. STING was highly phosphorylated in p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 cells, but not in #28.3.8 cells (Fig. 4D, lower-left panel). In addition, STING was also highly phosphorylated in p-dGdC-treated NKNT-3/NTCP #28.3.25 cells (the parent cells of #28.3.25.13) but not in the parent cells, or in #28 and #28.3 cells (the common parent cells of #28.3.8, #28.3.25 and #28.3.25.13, respectively). *IFN-λ1* mRNA was strongly induced in NKNT-3/NTCP cells highly phosphorylating STING such as NKNT-3/NTCP #28.3.25 and #28.3.25.13 cells (Fig. 4D, upper-left panel). Consistent with these results, the knockdown of STING reduced *IFN-λ1* mRNA induction in p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 cells (Fig. 4D, upper-right panel). These results suggest that STING regulate p-dGdC-triggered type III IFN induction by its expression level in NKNT-3/NTCP cells.

We next examined whether high-level expression of STING was required for HBV-triggered type III IFN induction in NKNT-3/NTCP #28.3.25.13 cells. We found

that knockdown of STING decreased the induction of *IFN-λ1* mRNA (Fig. 4E, upper panel) and subsequently ISG56 (Fig. 4E, lower panel) in HBV-infected NKNT-3/NTCP #28.3.25.13 cells. The knockdown of STING also increased the amounts of HBV total transcript in HBV-infected NKNT-3/NTCP #28.3.25.13 cells (Fig. 4F). On the other hand, the stable expression of exogenous STING, but not STING I200N which causes the conformational disruption [16], increased the induction of *IFN-λ1* mRNA (Fig. 4G, upper panel) and subsequently ISG56 (Fig. 4G, lower panel) in HBV-infected NKNT-3/NTCP #28.3.8 cells. The stable expression of exogenous STING also decreased the amounts of HBV total transcript in HBV-infected NKNT-3/NTCP #28.3.8 cells (Fig. 4H). These results suggest that high-level expression of STING is implicated in HBV-triggered type III IFN induction in NKNT-3/NTCP #28.3.25.13 cells.

High-level expression of STING was required for the HBV-triggered inflammatory response in NKNT-3/NTCP #28.3.25.13 cells.

Since high-level expression of STING mediated HBV-triggered type III IFN induction in NKNT-3/NTCP #28.3.25.13 cells (Figs. 4D and 4E), we next examined

209 whether high-level expression of STING was implicated in the induction of not only
210 type III IFN but also pro-inflammatory cytokine including IL-6 through the NF- κ B
211 signaling pathway. *IL-6* mRNA induction in p-dGdC-transfected NKNT-3/NTCP
212 #28.3.25.13 cells was higher than that in p-dGdC-transfected NKNT-3/NTCP #28.3.8
213 cells (Fig. 5A). In addition, the knockdown of STING reduced *IL-6* mRNA induction in
214 p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 cells (Fig. 5B). Since the
215 phosphorylation of NF- κ B p65 at Ser536 was required for the activation of
216 noncanonical NF- κ B signaling pathway [17], we next compared the phosphorylation of
217 NF- κ B p65 at Ser536 between p-dGdC-transfected NKNT-3/NTCP #28.3.8 cells and
218 #28.3.25.13 cells. Our results indicated that NF- κ B p65 was phosphorylated at Ser536
219 in p-dGdC-treated NKNT-3/NTCP #28.3.25.13 cells, but not #28.3.8 cells (Fig. 5C).
220 These results suggest that high-level expression of STING enhances p-dGdC-triggered
221 *IL-6* mRNA induction through the noncanonical NF- κ B signaling pathway in
222 NKNT-3/NTCP #28.3.25.13 cells. We next examined whether HBV infection also
223 triggered *IL-6* mRNA induction through the noncanonical NF- κ B signaling pathway in
224 NKNT-3/NTCP #28.3.25.13 cells. Interestingly, HBV infection, but not mock or

UV-HBV infection, triggered the phosphorylation of NF- κ B p65 at Ser536 (Fig. 5D) and subsequently induced *IL-6* mRNA (Fig. 5E) in NKNT-3/NTCP #28.3.25.13 cells. These results suggest that high-level expression of STING is implicated in HBV-triggered pro-inflammatory cytokine induction through the noncanonical NF- κ B signaling pathway in NKNT-3/NTCP #28.3.25.13 cells. NKNT-3/NTCP #28.3.25.13 cells are a useful tool for studying hepatic carcinogenesis caused by the HBV-triggered inflammatory response through the NF- κ B signaling pathway.

Discussion

Cytoplasmic DNA or RNA sensors trigger the innate immune responses and the inflammatory responses by recognizing viral PAMPs. We previously reported that one of the cytoplasmic DNA sensors, cGAS, recognized HBV DNA as viral PAMPs and subsequently induced the innate immune response through its adaptor protein, STING [10]. In the present study, we found that the immortalized human hepatocyte NKNT-3 cells exhibited HBV susceptibility by stably expressing the exogenous NTCP (Figs. 1C and 1D). Cells of one of the NKNT-3/NTCP cell-derived clones, NKNT-3/NTCP

#28.3.25.13, highly expressed STING and exhibited resistance to HBV through STING-mediated type III IFN induction (Figs. 4C, 4E, and 4F). Interestingly, STING was highly phosphorylated in p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 cells, but not in the parent, #28, #28.3, or #28.3.8 cells (Fig. 4D). However, it is uncertain why the expression and phosphorylation levels of STING differed among the NKNT-3/NTCP cell-derived cell clones. In humans, several single nucleotide polymorphisms (SNP) of STING have been discovered [18]. SNPs of STING have been shown to cause autoinflammatory diseases such as STING-associated vasculopathy with onset in infancy [19] and familial chilblain lupus [20]. These SNPs are implicated in the dysregulation of host innate immune responses and inflammatory responses through a loss-of-function mutation or a gain-of-function mutation of STING. Further analysis is needed to identify the gain-of-function mutation(s) in STING in NKNT-3/NTCP #28.3.25.13 cells.

In the present study, we showed that HBV infection induced type III IFN, but not IFN- β (type I IFN), through a STING-mediating signaling pathway in NKNT-3/NTCP #28.3.25.13 cells (Fig. 5F). Sato et al. previously reported that a cytoplasmic RNA

257 sensor, RIG-I, recognized HBV pgRNA and subsequently induced type III but not type I
258 IFN through its adaptor protein, IPS-1, in human primary hepatocytes [21]. These
259 results suggest that HBV suppresses the induction of type I IFN but not type III IFN.
260 One of the HBV proteins, HBV polymerase, suppressed STING-mediated IFN- β
261 induction by disrupting K63-linked ubiquitination of STING [22]. Another study also
262 reported that HBx bound IPS-1 and suppressed the activation of IFN- β [23]. However,
263 in these studies, it was unclear whether HBV suppressed the induction of type III IFN
264 through these HBV proteins. Our results showed that HBV transiently induced *ISG56*
265 mRNA induction at 5 and 9 days, but not at 13 days, after HBV infection in
266 NKNT-3/NTCP #28.3.25.13 cells (Fig. 3D). This result suggests that HBV possesses
267 two opposite functions to simultaneously trigger or suppress the induction of type III
268 IFN. Further analysis is needed to examine whether or not HBV suppresses the
269 induction of type III IFN.

270 We also showed that HBV infection induced a pro-inflammatory cytokine, IL-6,
271 through the noncanonical NF- κ B signaling pathway in NKNT-3/NTCP #28.3.25.13
272 cells (Fig. 5F). STING also mediates host inflammatory responses by triggering its

downstream NF- κ B signaling pathway [8, 9]. A STING-triggered host inflammatory response has been reported to be associated with hepatic diseases [24, 25]. In nonalcoholic fatty liver disease, STING promotes hepatocyte injury by inducing inflammation [24]. In addition, STING mediates liver injury and fibrosis in mice administered CCl₄ (a chemical inducer of hepatocyte death) [25]. Moreover, based on the results of several previous studies, STING is also thought to play an important role in tumor development [26]. Interestingly, STING may exert two opposite effects (tumor-suppressing and tumor-promoting effects) on tumor development under different situations. For example, in breast cancer, STING and its downstream signaling may suppress the tumor or the cancer metastasis [27, 28]. In contrast, STING is also required for cell survival and regrowth in breast cancer [29, 30]. However, the results of the present study do not clarify whether the HBV-triggered NF- κ B signaling pathway causes liver diseases and tumor development. Further analysis will also be needed to examine how HBV causes liver diseases and finally HCC through a STING-mediated NF- κ B signaling pathway.

In the present study, we established a novel HBV infectious cell culture model by

using NKNT-3 cells. Since NKNT-3 cells exhibit a non-neoplastic phenotype [12], our HBV infectious cell culture model is expected to be a useful tool for the study of hepatic carcinogenesis caused by HBV-triggered innate immune responses and inflammatory responses.

Materials and Methods

Cell culture

Human immortalized hepatocyte NKNT-3 cells, which were kindly provided by N. Kobayashi and M. Namba (Okayama University). Human hepatoma HepG2/NTCP cells were cultured as previously described [10]. HepG2.2.15 Cont, HepG2.2.15 cGAS/STING, and HepG2.2.15 cGAS GSAA/STING cells were maintained in medium including blasticidin and puromycin as previously described [10].

Establishment of an NKNT-3 cell line stably expressing exogenous NTCP and the derivation of its cell clones

NKNT-3 cells stably expressing exogenous NTCP (designated NKNT-3/NTCP cells)

were established as previously described [10]. NKNT-3/NTCP-derived cell clones were isolated from their parental cells by the limited dilution method. We evaluated HBV susceptibility by HBV/NLuc assay [13] and, from the several tens of cell clones obtained, selected a cell clone exhibiting susceptibility or resistance to HBV. By repeating the cell cloning and selection process, we obtained cell clones exhibiting the different levels of susceptibility to HBV.

HBV/NLuc assay

HBV/NLuc was prepared as previously reported [13]. Intracellular NLuc activity was measured at 5, 9, and 13 days after the inoculation of HBV/NLuc. For the measurement of NLuc activity, we used a Nano-Glo luciferase assay system (Promega, Madison, WI, USA). Data are the means \pm SD from three independent experiments.

Western blot analysis

Western blot analysis was performed as previously described [31]. Anti-Myc (PL14; Medical & Biological Laboratories, Nagoya, Japan), anti-ISG15 (H-150; Santa Cruz

321 Biotechnology, Dallas, TX, USA), anti-ISG56, anti-cGAS, anti-phospho-STING
 322 (Ser366), anti-STING, anti-phospho-NF- κ B p65 (Ser536), anti-NF- κ B p65 (Cell
 323 Signaling Technology, Beverly, MA, USA), and anti- β -actin (AC-15; Sigma-Aldrich, St.
 324 Louis, MO, USA) were used as primary antibodies.

325

326 **Flow cytometric analysis**

327 Cell surface expression of exogenous NTCP was detected by a flow cytometer as
 328 previously reported [32]. Anti-Myc (PL14; Medical & Biological Laboratories), and
 329 FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories,
 330 West Grove, PA, USA) were used as primary and secondary antibody, respectively.

331

332 **Analysis of HBV RNA**

333 HBV was prepared from the supernatant of HepG2.2.15 cells as previously reported
 334 [10]. Cells were infected with HBV at 10^3 HBV genome equivalents per cell, unless
 335 otherwise described. For the analysis of intracellular HBV RNA after the infection of
 336 HBV, we performed quantitative RT-PCR analysis and Northern blot analysis as

previously reported [10].

Quantitative RT-PCR analysis

At 5, 9, and 13 days after HBV inoculation or at 6 hours after the transfection of an *in vitro*-synthesized ligand, p-dGdC (Invivogen, San Diego, CA, USA), we performed quantitative RT-PCR analysis as previously described [33]. For quantitative RT-PCR analysis, we used primer sets previously described for ISG56 [34], IFN- β [34], cGAS [10], STING [10], IL-6 [33], and GAPDH [33]. We also prepared forward and reverse primer sets for IFN- λ 1 (5'- CTGGGAAGGGCTGCCACATT-3' (forward) and 5'- TTGAGTGACTCTTCCAAGGCG-3' (reverse)) and IFN- λ 2/3 (5'- CAGCTGCAGGTGAGGGAG-3' (forward) and 5'-CTGGGTCAGTGTCAGCGG-3' (reverse)).

RNA interference

The day after mock or HBV infection, we introduced small interfering RNAs (siRNAs) targeting STING or nontargeting siRNAs into NKNT-3/NTCP #28.3.25.13 cells as

previously described [35]. At 4 days after the introduction of siRNAs, we isolated the total RNA or cell lysate, and subjected it to quantitative RT-PCR analysis or western blot analysis, respectively.

Generation of cells stably expressing exogenous STING

To construct pCX4bleo/HA-STING retroviral vector, we introduced STING (accession no. NM_198282) cDNA containing a full-length ORF into the pCX4bleo/HA retroviral vector as previously reported [36]. pCX4bleo/HA-STING I200N [16] was also constructed using PCR mutagenesis with primers containing base alterations. These vectors were introduced into NKNT-3/NTCP #28.3.8 cells by retroviral transfer and then the cells stably expressing exogenous STING or STING I200N were selected by Zeocin (Thermo Fisher Scientific, Carlsbad, CA, USA).

Statistical analysis

Statistical analysis was performed to determine the significance of differences among groups by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

369

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382 **Author Contributions**

383 HD and NK designed the research. HD performed most of the experiments. HI

384 contributed pCX4bleo HA-STING I200N. NK performed the cell cloning by the limited

dilution method. HD, HI, YU, SS and NK analyzed the data. HD wrote the paper. All

authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Chen DS (1993) From hepatitis to hepatoma: lessons from type B viral hepatitis.

Science **262**: 369-370

2. Kao JH, Chen DS (2002) Global control of hepatitis B virus infection. *Lancet Infect*

Dis **2**: 395-403

3. Sun L, Wu J, Du F, Chen X, Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA

sensor that activates the type I interferon pathway. *Science* **339**: 786-791

4. Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ (2013) Cyclic GMP-AMP is an

endogenous second messenger in innate immune signaling by cytosolic DNA. *Science*

339: 826-830

- 401 5. Ishikawa H, Ma Z, Barber GN (2009) STING regulates intracellular DNA-mediated,
402 type I interferon-dependent innate immunity. *Nature* **461**: 788-792
- 403 6. Sui H, Zhou M, Imamichi H, Jiao X, Sherman BT, Lane HC, Imamichi T (2017)
404 STING is an essential mediator of the Ku70-mediated production of IFN- λ 1 in response
405 to exogenous DNA. *Sci Signal* **10**: eaah5054
- 406 7. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA,
407 Sheikh F, Dickensheets H, Donnelly RP (2003) IFN- λ s mediate antiviral protection
408 through a distinct class II cytokine receptor complex. *Nat Immunol* **4**: 69-77
- 409 8. Abe T, Barber GN (2014) Cytosolic-DNA-mediated, STING-dependent
410 proinflammatory gene induction necessitates canonical NF- κ B activation through TBK1.
411 *J Virol* **88**: 5328-5341
- 412 9. Blaauboer SM, Gabrielle VD, Jin L (2014) MPYS/STING-mediated TNF- α , not type
413 I IFN, is essential for the mucosal adjuvant activity of
414 (3'-5')-cyclic-di-guanosine-monophosphate in vivo. *J Immunol* **192**: 492-502
- 415 10. Dansako H, Ueda Y, Okumura N, Satoh S, Sugiyama M, Mizokami M, Ikeda M, Kato
416 N (2016) The cyclic GMP-AMP synthetase-STING signaling pathway is required for

- 417 both the innate immune response against HBV and the suppression of HBV assembly.
- 418 *FEBS J* **283**: 144-156
- 419 11. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, *et al*
- 420 (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for
- 421 human hepatitis B and D virus. *eLife* **1**:, e00049
- 422 12. Kobayashi N, Fujiwara T, Westerman KA, Inoue Y, Sakaguchi M, Noguchi H,
- 423 Miyazaki M, Cai J, Tanaka N, Fox IJ, *et al* (2000) Prevention of acute liver failure in
- 424 rats with reversibly immortalized human hepatocytes. *Science* **287**: 1258-1262
- 425 13. Nishitsuji H, Ujino S, Shimizu Y, Harada K, Zhang J, Sugiyama M, Mizokami M,
- 426 Shimotohno K (2015) Novel reporter system to monitor early stages of the hepatitis B
- 427 virus life cycle. *Cancer Sci* **106**: 1616-1624
- 428 14. Sells MA, Chen ML, Acs G (1987) Production of hepatitis B virus particles in Hep
- 429 G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* **84**:
- 430 1005-1009
- 431 15. Watashi K, Sluder A, Daito T, Matsunaga S, Ryo A, Nagamori S, Iwamoto M,
- 432 Nakajima S, Tsukuda S, Borroto-Esoda K, *et al* (2014) Cyclosporin A and its analogs

- 433 inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane
- 434 transporter, sodium taurocholate cotransporting polypeptide (NTCP). *Hepatology* **59**:
- 435 1726-1737
- 436 16. Yin Q, Tian Y, Kabaleeswaran V, Jiang X, Tu D, Eck MJ, Chen ZJ, Wu H (2012)
- 437 Cyclic di-GMP sensing via the innate immune signaling protein STING. *Mol Cell* **46**:
- 438 735-745
- 439 17. Sasaki CY, Barberi TJ, Ghosh P, Longo DL (2005) Phosphorylation of RelA/p65 on
- 440 Serine 536 Defines an I κ B α -independent NF- κ B Pathway. *J Biol Chem* **280**:
- 441 34538-34547
- 442 18. Li Y, Wilson HL, Kiss-Toth E (2017) Regulating STING in health and disease. *J*
- 443 *Inflammation* **59**: 14:11
- 444 19. Liu Y, Jesus AA, Marrero B, Yang D, Ramsey SE, Sanchez GAM, Tenbrock K,
- 445 Wittkowski H, Jones OY, Kuehn HS, *et al* (2014) Activated STING in a vascular and
- 446 pulmonary syndrome. *N Engl J Med* **371**: 507-518
- 447 20. König N, Fiehn C, Wolf C, Schuster M, Cura Costa E, Tüngler V, Alvarez HA,
- 448 Chara O, Engel K, Goldbach-Mansky R, *et al* (2017) Familial chilblain lupus due to a

- 449 gain-of-function mutation in STING. *Ann Rheum Dis* **76**: 468-472
- 450 21. Sato S, Li K, Kameyama T, Hayashi T, Ishida Y, Murakami S, Watanabe T, Iijima S,
451 Sakurai Y, Watashi K, *et al* (2015) The RNA sensor dually functions as an innate sensor
452 and direct antiviral factor for hepatitis B virus. *Immunity* **42**: 123-132
- 453 22. Liu Y, Li J, Chen J, Li Y, Wang W, Du X, Song W, Zhang W, Lin L, Yuan Z (2015)
454 Hepatitis B virus polymerase disrupts K63-linked ubiquitination of STING to block
455 innate cytosolic DNA-sensing pathways. *J Virol* **89**: 2287-2300
- 456 23. Kumar M, Jung SY, Hodgson AJ, Madden CR, Qin J, Slagle BL (2011) Hepatitis B
457 virus regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation
458 of beta interferon. *J Virol* **85**: 987-995
- 459 24. Qiao JT, Cui C, Qing L, Wang LS, He TY, Yan F, Liu FQ, Shen YH, Hou XG, Chen
460 L (2018) Activation of the STING-IRF3 pathway promotes hepatocyte inflammation,
461 apoptosis and induces metabolic disorders in nonalcoholic fatty liver disease.
462 *Metabolism* **81**: 13-24
- 463 25. Iracheta-Vellve A, Petrasek J, Gyongyosi B, Satishchandran A, Lowe P, Kodys K,
464 Catalano D, Calenda CD, Kurt-Jones EA, Fitzgerald KA, *et al* (2016) Endoplasmic

- 465 Reticulum Stress-induced Hepatocellular Death Pathways Mediate Liver Injury and
- 466 Fibrosis via Stimulator of Interferon Genes. *J Biol Chem* **291**: 26794-26805
- 467 26. He L, Xiao X, Yang X, Zhang Z, Wu L, Liu Z (2017) STING signaling in
- 468 tumorigenesis and cancer therapy: A friend or foe? *Cancer Lett* **402**: 203-212
- 469 27. Bhatelia K, Singh A, Tomar D, Singh K, Sripada L, Chagtoo M, Prajapati P, Singh R,
- 470 Godbole MM, Singh R (2014) Antiviral signaling protein MITA acts as a tumor
- 471 suppressor in breast cancer by regulating NF-kappaB induced cell death. *Biochim*
- 472 *Biophys Acta* **1842**: 144-153
- 473 28. Chandra D, Quispe-Tintaya W, Jahangir A, Asafu-Adjei D, Ramos I, Sintim HO,
- 474 Zhou J, Hayakawa Y, Karaolis DK, Gravekamp C (2014) STING ligand c-di-GMP
- 475 improves cancer vaccination against metastatic breast cancer. *Cancer Immunol Res* **2**:
- 476 901-910
- 477 29. Lemos H, Mohamed E, Huang L, Ou R, Pacholczyk G, Arbab AS, Munn D, Mellor
- 478 AL (2016) STING promotes the growth of tumors characterized by low antigenicity via
- 479 IDO activation. *Cancer Res* **76**: 2076-2081
- 480 30. Gaston J, Cheradame L, Yvonnet V, Deas O, Poupon MF, Judde JG, Cairo S, Goffin

- 481 V (2016) Intracellular STING inactivation sensitizes breast cancer cells to genotoxic
482 agents. *Oncotarget* **7**: 77205-77224
- 483 31. Dansako H, Ikeda M, Kato N (2007) Limited suppression of the interferon-beta
484 production by hepatitis C virus serine protease in cultured human hepatocytes. *FEBS J*
485 **274**: 4161-4176
- 486 32. Dansako H, Imai H, Ueda Y, Satoh S, Wakita T, Kato N (2018) ULBP1 is induced
487 by hepatitis C virus infection and is the target of the NK cell-mediated innate immune
488 response in human hepatocytes. *FEBS Open Bio* **8**: 361-371
- 489 33. Dansako H, Ikeda M, Ariumi Y, Wakita T, Kato N (2009) Double-stranded
490 RNA-induced interferon-beta and inflammatory cytokine production modulated by
491 hepatitis C virus serine proteases derived from patients with hepatic diseases. *Arch Virol*
492 **154**: 801-810
- 493 34. Dansako H, Yamane D, Welsch C, McGivern DR, Hu F, Kato N, Lemon SM (2013) Class A
494 scavenger receptor 1 (MSR1) restricts hepatitis C virus replication by mediating Toll-like
495 receptor 3 recognition of viral RNAs produced in neighboring cells. *PLoS Pathog* **9**: e1003345

35. Dansako H, Hiramoto H, Ikeda M, Wakita T, Kato N (2014) Rab18 is required for viral assembly of hepatitis C virus through trafficking of the core protein to lipid droplets. *Virology* **462-463C**: 166-174

36. Dansako H, Naganuma A, Nakamura T, Ikeda F, Nozaki A, Kato N (2003) Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element. *Virus Res* **97**: 17-30

Figure legends

Figure 1 - The immortalized human hepatocyte cell line NKNT-3 exhibited susceptibility to HBV by expressing exogenous NTCP.

A Western blot analysis of exogenous NTCP in NKNT-3/NTCP cells. Anti-Myc antibody was used for the detection of NTCP-Myc in NKNT-3/NTCP cells. β -actin was included as a loading control.

B Flow cytometric analysis of the cell surface NTCP in NKNT-3/Control cells, NKNT-3/NTCP cells, or HepG2/NTCP cells. Signals of the cell surface NTCP are shown in green. An isotype control was used as a negative control (violet area).

512 C Comparison of NLuc activity after HBV/NL inoculation between NKNT-3/Control
513 cells and NKNT-3/NTCP cells. Intracellular NLuc activity was measured at 5, 9, and 13
514 days after HBV/NL inoculation. $**P < 0.01$, $***P < 0.001$ versus HBV/NL-infected
515 NKNT-3/Cont cells.

516 D Quantitative RT-PCR analysis of the amount of HBV total transcript in
517 HBV-infected NKNT-3/Control cell or NKNT-3/NTCP cells. The supernatant of
518 HepG2.2.15 cells was used as an HBV inoculum. The amounts of HBV total transcript
519 were measured at 5, 9, and 13 days after HBV inoculation. $**P < 0.01$, $***P < 0.001$
520 versus HBV-infected NKNT-3/Cont cells.

521 E, F Comparison of the susceptibility to HBV between HepG2/NTCP cells and
522 NKNT-3/NTCP cells. Intracellular NLuc activity was measured after HBV/NL
523 inoculation. The amounts of HBV total transcript and the pgRNA were measured after
524 HBV inoculation by quantitative RT-PCR analysis. $**P < 0.01$, $***P < 0.001$ versus
525 HBV/NL- or HBV-infected HepG2/NTCP cells, respectively.

526 G Functional analysis of NTCP in NKNT-3/NTCP cells using CsA as an HBV-entry
527 inhibitor. CsA was administered before and during HBV inoculation. $*P < 0.05$, $***P <$

528 0.001 versus 0 μ M of CsA-administered HBV-infected cells.

529

530 **Figure 2 - The level of susceptibility to HBV in NKNT-3/NTCP #28.3.8 cells**
 531 **approximated that in HepG2/NTCP cells.**

532 A Outline of cell cloning by the limited dilution method. NKNT-3/NTCP #28.3.8
 533 cells were selected by three-round limited dilution. Red arrows with solid lines show the
 534 selection of a cell clone exhibiting higher susceptibility to HBV.

535 B Comparison of susceptibility to HBV among parent NKNT-3/NTCP cells and their
 536 derived cell clones by using HBV/NL assay. $**P < 0.01$, $***P < 0.001$ versus
 537 HBV/NL-infected parent NKNT-3/NTCP cells.

538 C Flow cytometric analysis of the cell surface NTCP in their derived cell clones.
 539 Signals of the cell surface NTCP are shown in green. An isotype control was used as a
 540 negative control (violet area).

541 D Comparison of the amounts of HBV total transcript after HBV infection among
 542 parent NKNT-3/NTCP cells and their derived cell clones. The amount of HBV total
 543 transcript was measured after HBV infection by quantitative RT-PCR analysis. $*P <$

544 0.05 versus HBV-infected parent NKNT-3/NTCP cells.

545 E, F Comparison of susceptibility to HBV between HepG2/NTCP cells and
546 NKNT-3/NTCP #28.3.8 cells. Intracellular NLuc activity or the amounts of HBV total
547 transcript were measured as described in Figs. 1E and 1F. NS; not significant, $**P <$
548 0.01, $***P < 0.001$ versus HBV/NL- or HBV-infected HepG2/NTCP cells, respectively.

549 G Comparison of susceptibility to HBV between HepG2/NTCP cells and
550 NKNT-3/NTCP #28.3.8 cells by Northern blot analysis. Total RNA was isolated from
551 HBV-infected cells at 13 days after HBV inoculation. 28S rRNA and 18S rRNA were
552 included as a loading control. NKNT-3/NTCP #28.8.4 is another clone, which has been
553 estimated to exhibit susceptibility to HBV by HBV/NL assay (data not shown).

554

555 **Figure 3 – HBV induced type III IFN in NKNT-3/NTCP #28.3.25.13 cells exhibiting**
556 **resistance to HBV.**

557 A Outline of cell cloning by the limited dilution method. NKNT-3/NTCP
558 #28.3.25.13 and #28.3.30.20.3 cells were selected by their distinct serial limited dilution,
559 respectively. Blue arrows with dashed lines show the selection of a cell clone exhibiting

560 resistance to HBV.

561 B Quantitative RT-PCR analysis of the amounts of HBV total transcript in
562 HBV-infected NKNT-3/NTCP #28.3.8, #28.3.25.13, or #28.3.30.20.3 cells. * $P < 0.05$,
563 ** $P < 0.01$ versus HBV-infected NKNT-3N #28.3.8 cells.

564 C Western blot analysis of ISG56 in HBV-infected NKNT-3/NTCP #28.3.8,
565 #28.3.25.13, or #28.3.30.20.3 cells. Cell lysates were prepared from mock- or
566 HBV-infected cells at 5 days after HBV inoculation.

567 D Quantitative RT-PCR analysis of *ISG56* mRNA in HBV-infected NKNT-3/NTCP
568 #28.3.8 or #28.3.25.13 cells. Cells were infected with HBV at 10^3 or 10^4 HBV genome
569 equivalents per cell, respectively. Each mRNA level was calculated relative to the level
570 in mock-infected NKNT-3/NTCP #28.3.25.13 cells, which was set at 1. * $P < 0.05$, ** P
571 < 0.01 , *** $P < 0.001$ versus mock-infected NKNT-3N #28.3.25.13 cells.

572 E Quantitative RT-PCR analysis of *IFN- $\lambda 1$* and *IFN- $\lambda 2/3$* mRNA in HBV-infected
573 NKNT-3/NTCP #28.3.8 or #28.3.25.13 cells. Cells were infected with HBV at 10^3 or
574 10^4 HBV genome equivalents per cell, respectively. Each mRNA level was calculated as
575 described in Fig. 3D. ND, not detected. NS; not significant, * $P < 0.05$, ** $P < 0.01$

576 versus mock-infected NKNT-3/NTCP #28.3.25.13 cells.

577 F Quantitative RT-PCR analysis of *IFN-β* mRNA in HBV-infected NKNT-3/NTCP
578 #28.3.8 or #28.3.25.13 cells. Cells were infected with HBV at 10^3 or 10^4 HBV genome
579 equivalents per cell, respectively. Each mRNA level was calculated as described in Fig.
580 3D. NS; not significant versus mock-infected NKNT-3/NTCP #28.3.25.13 cells.

581 G (left panel) Quantitative RT-PCR analysis of the amounts of HBV total transcript
582 in mock-, HBV-, or UV-HBV-infected NKNT-3/NTCP #28.3.25.13 cells. (right panels)
583 Quantitative RT-PCR analysis of *IFN-λ1* mRNA in mock-, HBV-, or UV-HBV-infected
584 NKNT-3/NTCP #28.3.25.13 cells. Each mRNA level was calculated as described in Fig.
585 3D. $**P < 0.01$ versus mock- or UV-HBV-infected NKNT-3/NTCP #28.3.25.13 cells,
586 respectively.

587 H Western blot analysis of ISG15 and ISG56 in mock-, HBV-, or UV-HBV-infected
588 NKNT-3/NTCP #28.3.25.13 cells. The cell lysate was prepared as described in Fig. 3C.

589 I Quantitative RT-PCR analysis of *IFN-λ1*, *IFN-λ2/3*, and *IFN-β* mRNA in
590 HepG2.2.15 cGAS/STING cells. Each mRNA level was calculated relative to the level
591 in HepG2.2.15 Cont cells, which was set at 1. $*P < 0.05$, $**P < 0.01$ versus HepG2.2.15

Cont cells or HepG2.2.15 cGAS GSAA/STING cells, respectively.

Figure 4 – High-level expression of STING was implicated in HBV-triggered type

III IFN induction in NKNT-3/NTCP #28.3.25.13 cells.

A (upper panel) Quantitative RT-PCR analysis of *ISG56* and *IFN- λ 1* mRNA in p-dGdC-transfected NKNT-3/NTCP #28.3.8 or #28.3.25.13 cells. Each mRNA level was calculated relative to the level in vehicle-transfected NKNT-3/NTCP #28.3.25.13 cells, which was set at 1. *** $P < 0.001$ versus p-dGdC-transfected NKNT-3/NTCP #28.3.8 cells. (lower panel) Western blot analysis of ISG56 in p-dGdC-transfected NKNT-3/NTCP #28.3.8 or #28.3.25.13 cells. The cell lysate was prepared as described in Fig. 3C.

B Quantitative RT-PCR analysis of *cGAS* and *STING* mRNA in NKNT-3/NTCP #28.3.8 or #28.3.25.13 cells. Each mRNA level was calculated relative to the level in NKNT-3/NTCP #28.3.25.13 cells, which was set at 1. NS; not significant, *** $P < 0.001$ versus NKNT-3/NTCP #28.3.8 cells.

C Western blot analysis of cGAS and STING in NKNT-3/NTCP #28.3.8 or

608 #28.3.25.13 cells.

609 D (upper-left panel) Quantitative RT-PCR analysis of *IFN- λ 1* mRNA in the parent

610 NKNT-3/NTCP cells and in the several cell clones derived from them after transfection

611 with p-dGdC. Each mRNA level was calculated relative to the level in NKNT-3/NTCP

612 #28.3.25.13 cells transfected with vehicle, which was set at 1. NS; not significant, ***P*

613 < 0.01 versus p-dGdC-transfected parent NKNT-3/NTCP cells. (lower-left panel)

614 Western blot analysis of phosphorylated STING at Ser366 in the original

615 NKNT-3/NTCP cells and in the several cell clones derived from them after transfection

616 with p-dGdC. The cell lysate was prepared as described in Fig. 3C. (upper-right panel)

617 Quantitative RT-PCR analysis of *IFN- λ 1* mRNA in NKNT-3/NTCP #28.3.25.13 cells

618 transfected with STING-specific (designated NKNT-3/NTCP #28.3.25.13 siSTING) or

619 control (designated NKNT-3/NTCP #28.3.25.13 siCont) siRNA followed by p-dGdC.

620 Each mRNA level was calculated relative to the level in vehicle-transfected

621 NKNT-3/NTCP #28.3.25.13 siCont cells, which was set at 1. ****P* < 0.001 versus

622 p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 siCont cells. (lower-right panel)

623 Western blot analysis of phosphorylated STING at Ser366 in NKNT-3/NTCP

624 #28.3.25.13 siSTING cells after transfection with p-dGdC. The cell lysate was prepared
625 as described in Fig. 3C.

626 E (upper panel) Quantitative RT-PCR analysis of *IFN- λ 1* mRNA in mock- or
627 HBV-infected NKNT-3/NTCP #28.3.25.13 siSTING cells or NKNT-3/NTCP
628 #28.3.25.13 siCont cells. Each mRNA level was calculated relative to the level in
629 mock-infected NKNT-3/NTCP #28.3.25.13 siCont cells, which was set at 1. (lower
630 panel) Western blot analysis of ISG56 in HBV-infected NKNT-3/NTCP #28.3.25.13
631 siCont cells or NKNT-3/NTCP #28.3.25.13 siSTING cells. The cell lysate was prepared
632 as described in Fig. 3C. $**P < 0.01$ versus HBV-infected NKNT-3/NTCP #28.3.25.13
633 siCont cells.

634 F Quantitative RT-PCR analysis of the amount of HBV total transcript in
635 HBV-infected NKNT-3/NTCP #28.3.25.13 siCont cells or NKNT-3/NTCP #28.3.25.13
636 siSTING cells. $**P < 0.01$ versus HBV-infected NKNT-3/NTCP #28.3.25.13 siCont
637 cells.

638 G (upper panel) Quantitative RT-PCR analysis of *IFN- λ 1* mRNA in mock- or
639 HBV-infected NKNT-3/NTCP #28.3.8 cells stably expressing exogenous STING wild

640 type (designated NKNT-3/NTCP #28.3.8 STING wt) or STING I200N (designated
641 NKNT-3/NTCP #28.3.8 STING I200N). Each mRNA level was calculated relative to
642 the level in HBV-infected NKNT-3/NTCP #28.3.8 STING I200N cells, which was set at
643 1. ND, not detected. *** $P < 0.001$ versus HBV-infected NKNT-3/NTCP #28.3.8 STING
644 I200N cells. (lower panel) Western blot analysis of ISG56 in HBV-infected
645 NKNT-3/NTCP #28.3.8 STING wt cells or NKNT-3/NTCP #28.3.8 STING I200N cells.
646 The cell lysate was prepared as described in Fig. 3C.

647 H Quantitative RT-PCR analysis of the amount of HBV total transcript in
648 HBV-infected NKNT-3/NTCP #28.3.8 STING wt cells or NKNT-3/NTCP #28.3.8
649 STING I200N cells. *** $P < 0.001$ versus HBV-infected NKNT-3/NTCP #28.3.8 STING
650 I200N cells.

651

652 **Figure 5 – High-level expression of STING was required for HBV-triggered**
653 **inflammatory response in NKNT-3/NTCP #28.3.25.13 cells.**

654 A (upper panel) Quantitative RT-PCR analysis of *IL-6* mRNA in p-dGdC-transfected
655 NKNT-3/NTCP #28.3.8 or #28.3.25.13 cells. Each mRNA level was calculated relative

656 to the level in vehicle-transfected NKNT-3/NTCP #28.3.25.13 cells, which was set at 1.

657 $^{**}P < 0.01$ versus p-dGdC-transfected NKNT-3/NTCP #28.3.8 cells. (lower panel)

658 Western blot analysis of IL-6 in p-dGdC-transfected NKNT-3/NTCP #28.3.8 or

659 #28.3.25.13 cells. The cell lysate was prepared as described in Fig. 3C.

660 B (upper panel) Quantitative RT-PCR analysis of *IL-6* mRNA in p-dGdC-transfected

661 NKNT-3/NTCP #28.3.25.13 siCont cells or NKNT-3/NTCP #28.3.25.13 siSTING cells.

662 Each mRNA level was calculated relative to the level in vehicle-transfected

663 NKNT-3/NTCP #28.3.25.13 siCont cells, which was set at 1. $^{**}P < 0.01$ versus

664 p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 siCont cells. (lower panel) Western

665 blot analysis of IL-6 in p-dGdC-transfected NKNT-3/NTCP #28.3.25.13 siCont cells or

666 NKNT-3/NTCP #28.3.25.13 siSTING cells. The cell lysate was prepared as described in

667 Fig. 3C.

668 C Western blot analysis of phosphorylated NF- κ B p65 at Ser536 in

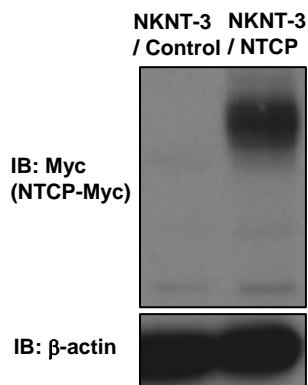
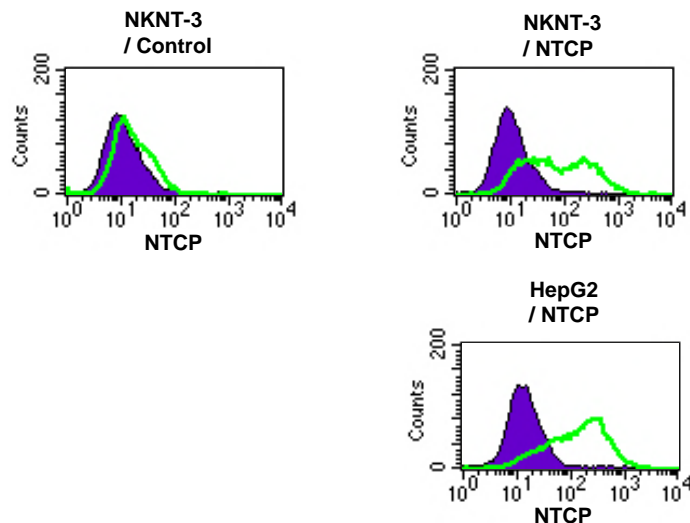
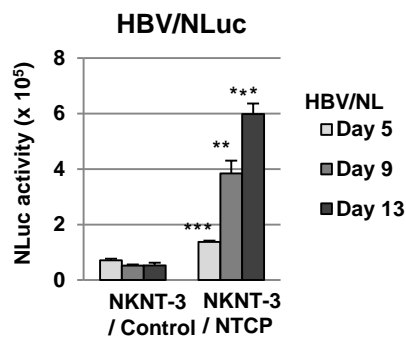
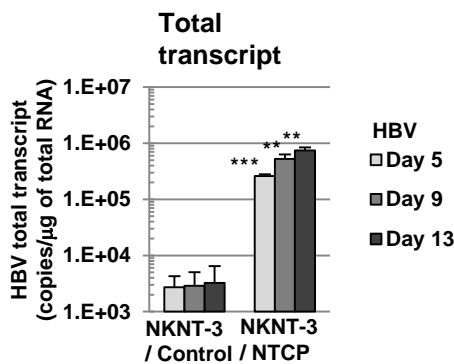
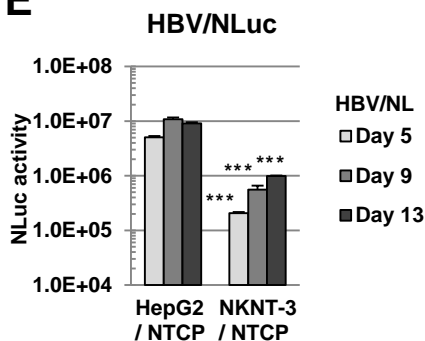
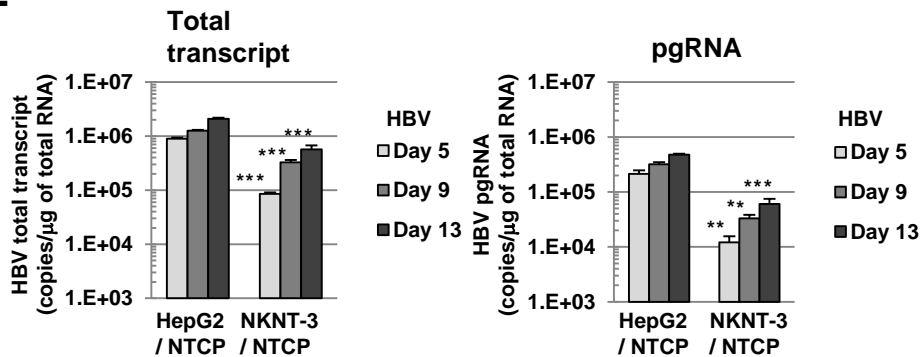
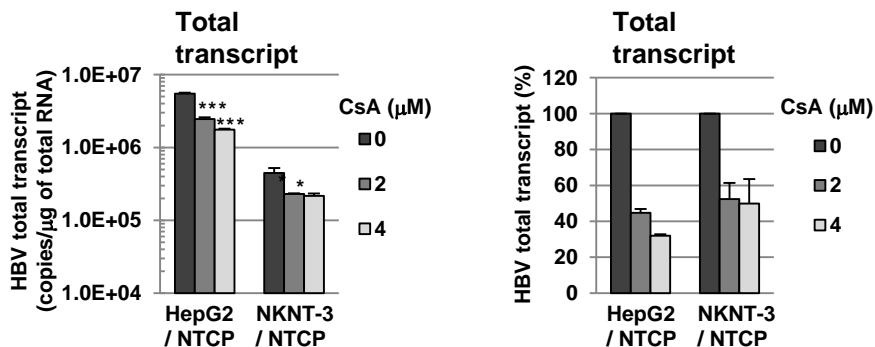
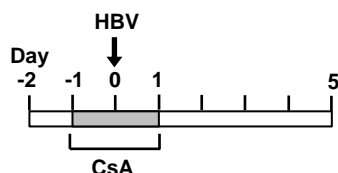
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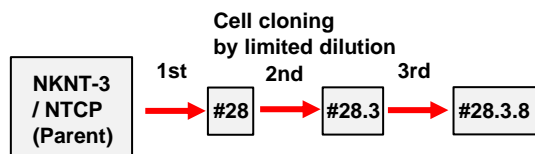
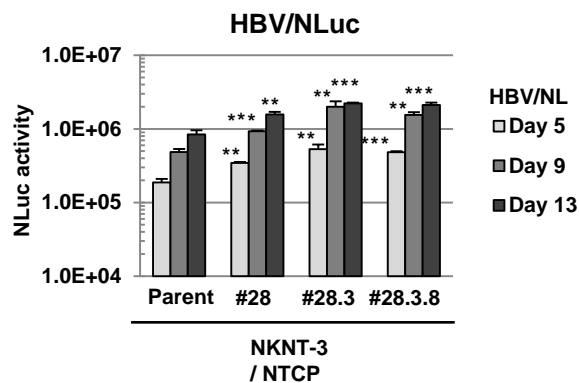
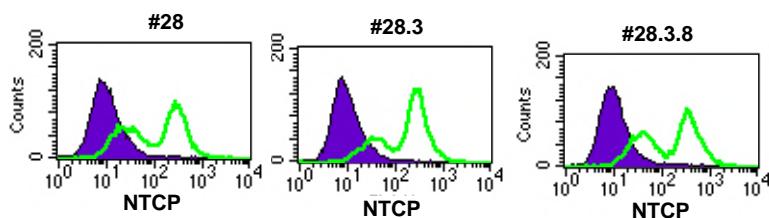
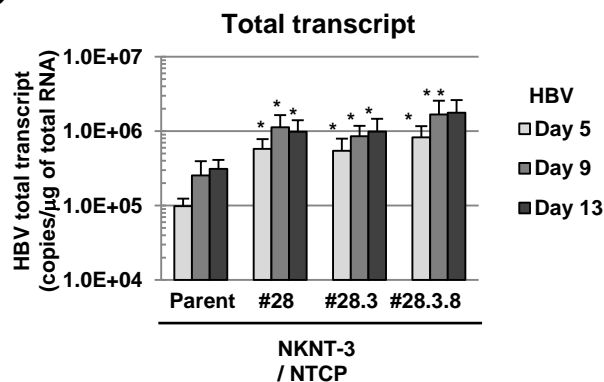
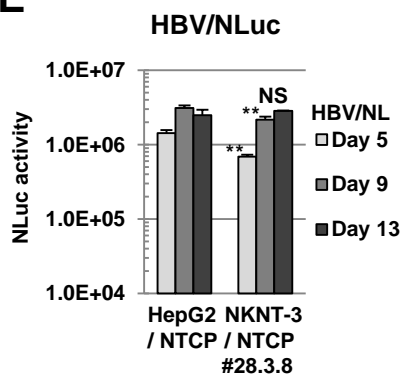
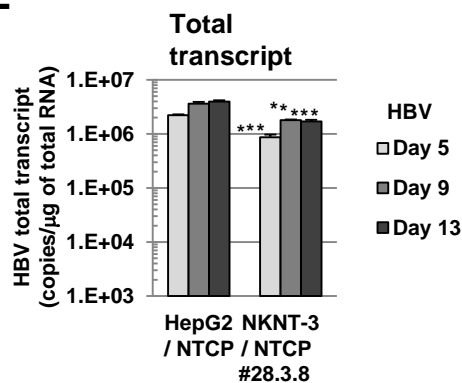
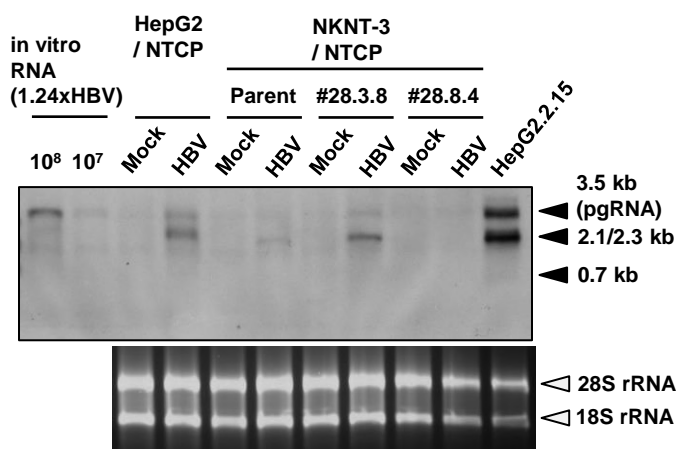
670 D Western blot analysis of phosphorylated NF- κ B p65 at Ser536 in mock-, HBV-, or

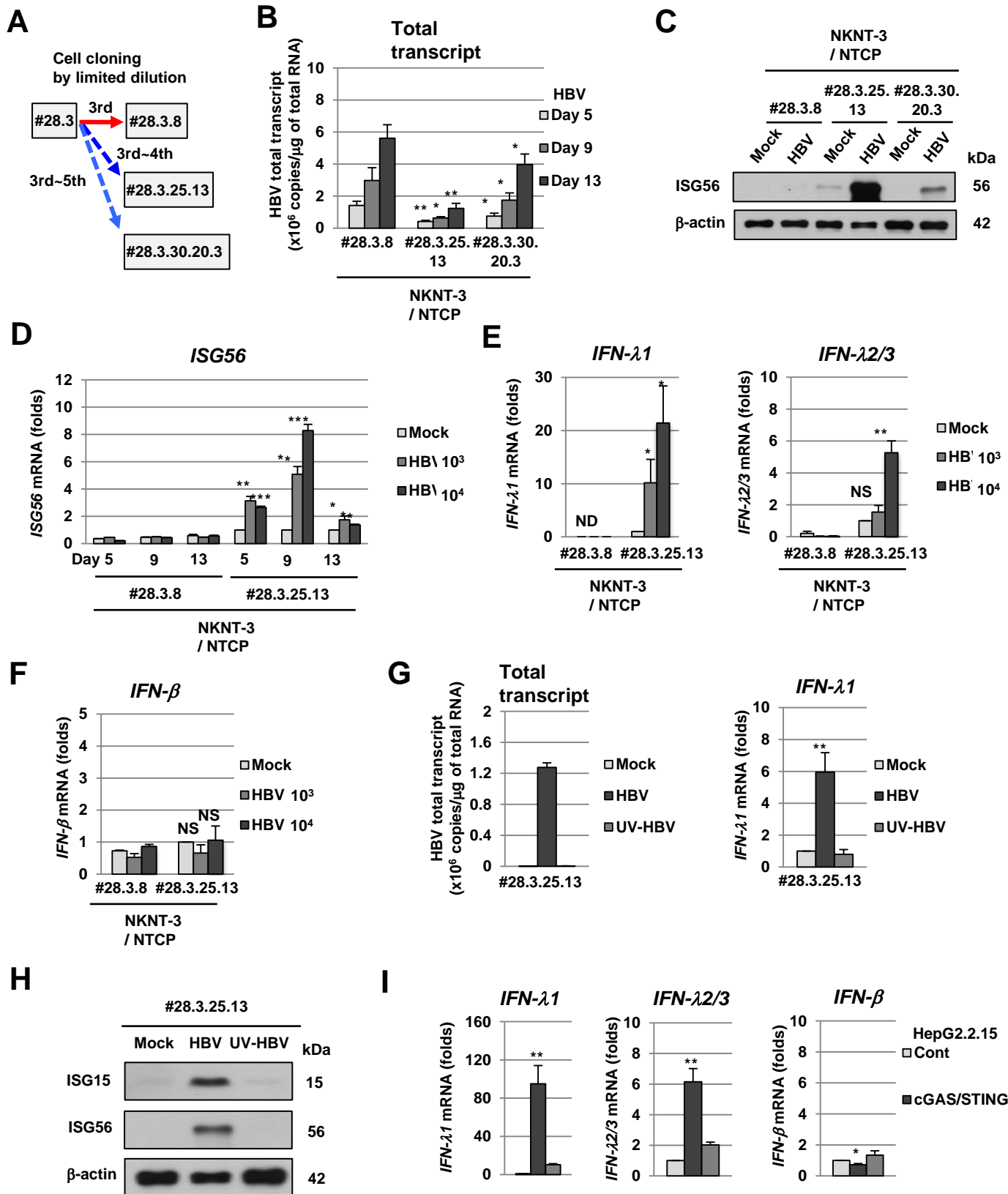
671 UV-HBV-infected NKNT-3/NTCP #28.3.25.13 cells.

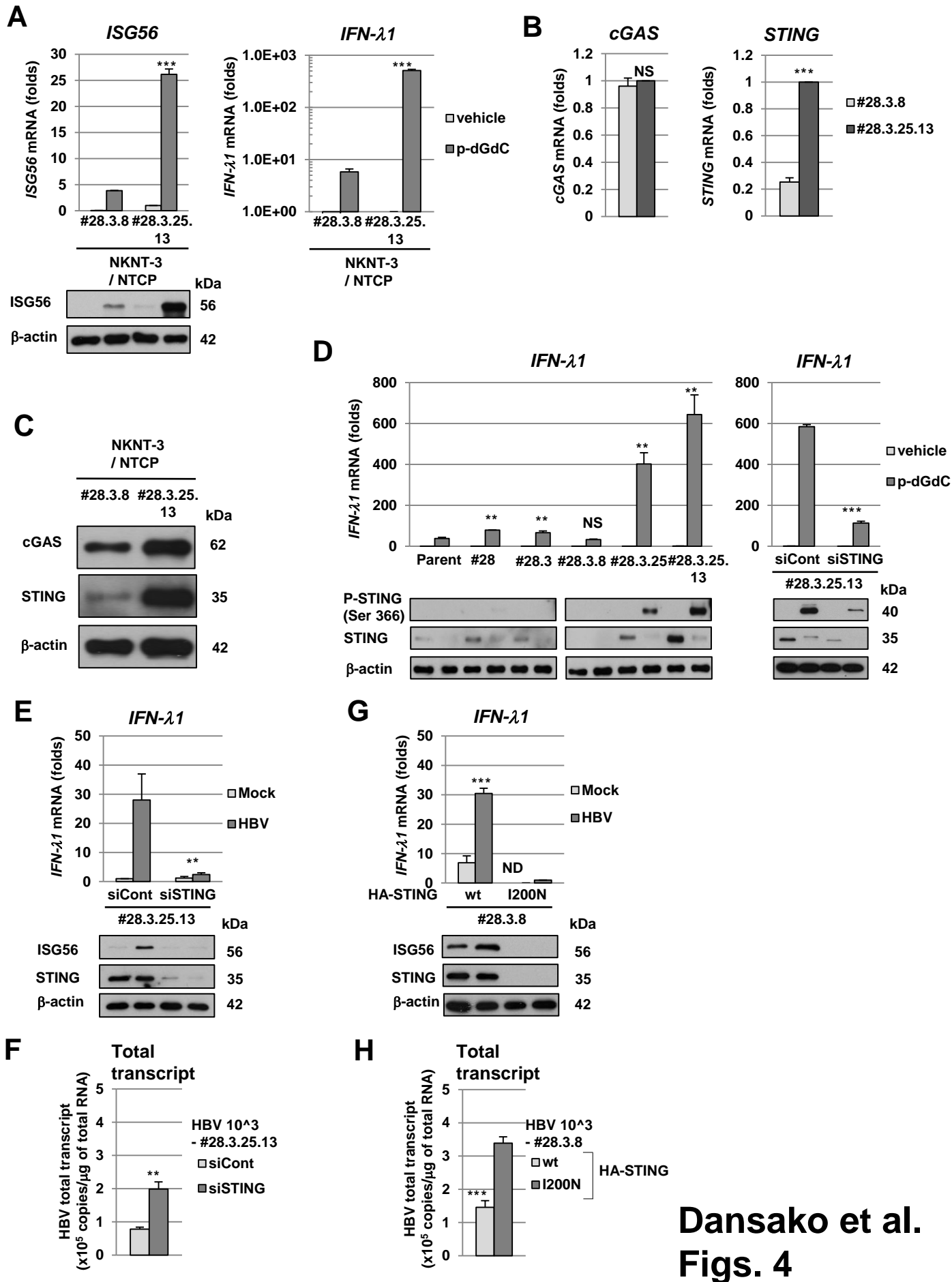
672 E (upper panel) Quantitative RT-PCR analysis of *IL-6* mRNA in mock-, HBV-, or
 673 UV-HBV-infected NKNT-3/NTCP #28.3.25.13 cells. Each mRNA level was calculated
 674 as described in Fig. 3D. *** $P < 0.001$ versus mock- or UV-HBV-infected
 675 NKNT-3/NTCP #28.3.25.13 cells, respectively. (lower panel) Western blot analysis of
 676 *IL-6* mRNA in mock-, HBV-, or UV-HBV-infected NKNT-3/NTCP #28.3.25.13 cells.

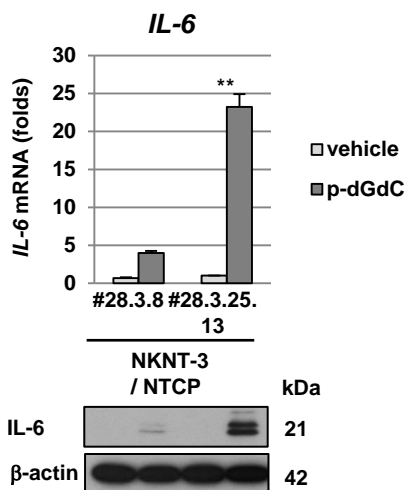
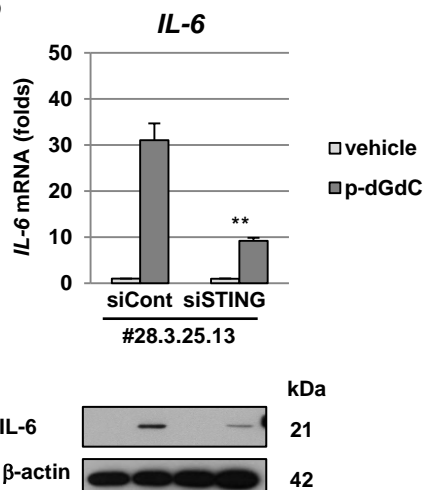
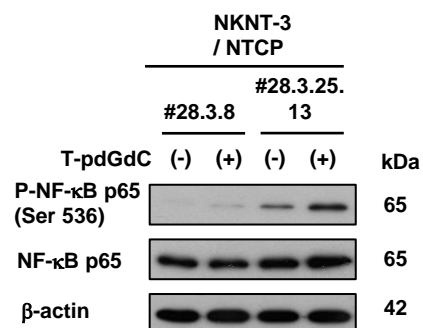
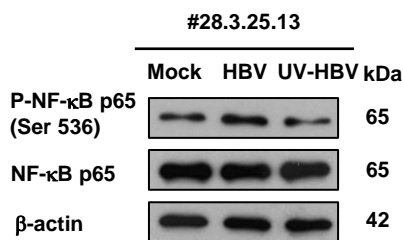
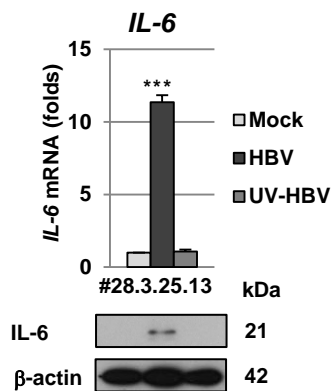
677 F Proposed model of the HBV-triggered host innate immune response and
 678 inflammatory response through STING.

A**B****C****D****E****F****G**

A**B****C****D****E****F****G**





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