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Hepatitis B virus evasion from cGAS sensing in human hepatocytes

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27 **Key words** antiviral, capsid, innate immunity, liver, recognition.

28

29 **List of Abbreviations**

30 HBV: hepatitis B virus; cGAS: cyclic GMP-AMP synthase; HSPG: heparan sulfate proteoglycan;
31 NTCP: Na⁺/taurocholate cotransporting polypeptide; rcDNA: relaxed circular DNA; cccDNA:
32 covalently closed circular DNA; pgRNA: pregenomic RNA; PRR: pattern recognition receptors;
33 cGAMP: cyclic GMP-AMP; IFN: interferon; STING: stimulator of IFN genes; ISG: IFN-stimulated
34 gene; PHH: primary human hepatocyte; PEG: polyethylene glycol; siRNA: small interfering RNA;
35 HIV: human immunodeficiency virus; VSV-G: vesicular stomatitis virus glycoprotein; sgRNA:
36 single-guide RNA; PMM: primary hepatocyte maintenance medium; dsIDNA: double stranded
37 linear DNA; dsDNA: double stranded DNA; GSEA: gene set enrichment analysis; FDR: false
38 discovery rate; FISH: fluorescence in situ hybridization; KO: knock-out. SeV: Sendai virus. IAR:
39 innate antiviral response gene set. Dpi: days post infection.

40

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50 **Abstract**

51 Chronic hepatitis B virus (HBV) infection is a major cause of chronic liver disease and cancer
52 worldwide. The mechanisms of viral genome sensing and the evasion of innate immune
53 responses by HBV infection are still poorly understood. Recently, the cyclic GMP-AMP synthase
54 (cGAS) was identified as a DNA sensor. In this study, we aimed to investigate the functional role
55 of cGAS in sensing of HBV infection and elucidate the mechanisms of viral evasion. We performed
56 functional studies including loss- and gain-of-function experiments combined with cGAS effector
57 gene expression profiling in an infectious cell culture model, primary human hepatocytes and
58 HBV-infected human liver chimeric mice. Here we show that cGAS is expressed in the human
59 liver, primary human hepatocytes and human liver chimeric mice. While naked relaxed-circular
60 HBV DNA is sensed in a cGAS-dependent manner in hepatoma cell lines and primary human
61 hepatocytes, host cell recognition of viral nucleic acids is abolished during HBV infection,
62 suggesting escape from sensing, likely during packaging of the genome into the viral capsid.
63 While the hepatocyte cGAS pathway is functionally active, as shown by reduction of viral cccDNA
64 levels in gain-of-function studies, HBV infection suppressed cGAS expression and function in cell
65 culture models and humanized mice. **Conclusion:** HBV exploits multiple strategies to evade
66 sensing and antiviral activity of cGAS and its effector pathways.

67 With more than 250 million chronically infected patients, hepatitis B virus (HBV) infection is a
68 leading cause of liver disease and hepatocellular carcinoma (1, 2). Current antiviral therapies
69 effectively control viral load, but largely fail to cure (3). HBV is a partially double stranded DNA
70 (dsDNA) virus infecting human hepatocytes after initial attachment to heparan sulfate
71 proteoglycans (HSPG) and its receptor Na⁺/taurocholate cotransporting polypeptide (NTCP;
72 reviewed in (4)). Following uncoating, the viral nucleocapsid is released into the cytoplasm. The
73 viral genome is imported into the nucleus through mechanisms which are still poorly understood.
74 The viral genome it is converted in the nucleus into a covalently closed circular DNA (cccDNA)
75 (5). This minichromosome serves as a template for both pregenomic RNA (pgRNA) and viral
76 mRNA transcription. While recent studies suggested sensing of the pgRNA or other HBV RNAs
77 by either MDA5 (6) or RIG-I (7), the recognition of the viral nucleic acids by the regular pattern
78 recognition receptors (PRRs) still remains elusive. In general, HBV does not or only marginally
79 activate innate immune responses in cell culture models and *in vivo* (8-14), leading to the concept
80 that HBV behaves like a “stealth” virus avoiding viral DNA and RNA sensing (15). Other studies
81 have suggested an active inhibition of the innate immune responses by HBV proteins (16).
82 Consequently, the interaction of HBV and the innate immune system of hepatocytes, and in
83 particular the sensing of HBV DNA, is only poorly understood.

84 Foreign DNA recognition by cytosolic DNA sensors triggers an early antiviral innate
85 immune response, including type I and type III IFN production (17). Recently, the cyclic GMP-
86 AMP (cGAMP) synthase (cGAS) was identified as a DNA sensor exhibiting an antiviral activity
87 against a broad range of DNA and RNA viruses (18-20). cGAS is encoded by *MB21D1* gene and
88 directly binds to dsDNAs inducing the production of cGAMP which is recognized by the stimulator
89 of IFN genes (STING, encoded by *TMEM173*) triggering the expression of IFN-stimulated genes
90 (ISGs) through TBK1 activation (21-23). While two studies have investigated cGAS-HBV

91 interactions in viral replication and assembly (24, 25), the functional role of cGAS in sensing of
92 the viral genome during natural infection of human hepatocytes remains unknown.

93 The understanding of HBV-host interactions, including innate immune response after
94 infection, has been hampered for long time by the absence of robust cell culture model system
95 for the study of viral infection (26). The development of HBV-susceptible NTCP-overexpressing
96 hepatoma cells, such as HepG2-NTCP cells, allows the study of the full life cycle in a robust and
97 easy-to-use cell culture model (26). HepG2 cells are capable of mounting an efficient innate
98 immune response after infection by hepatitis C virus (27). Moreover, another study took
99 advantage of HBV-infected HepG2-NTCP for studying the interaction between RIG-I and HBV
100 RNA (7), suggesting that this cell line is suitable for the study of innate immune response after
101 HBV infection. Here, we aimed to understand the functional role of cGAS for the HBV life cycle in
102 human hepatocytes and unravel the mechanisms of viral evasion using loss- and gain-of-function
103 experiments combined with cGAS effector gene expression profiling in human liver chimeric mice.

104

105 **EXPERIMENTAL PROCEDURES**

106 **Human subjects.** Human material including liver tissue from patients undergoing surgical
107 resection or HBV-positive serum was obtained with informed consent from all patients. Protocols
108 were approved by the Ethics Committee of the University of Strasbourg Hospitals, France (CPP
109 10-17 and DC-2016-2616).

110

111 **Animal Experimentation.** All mice were kept in a specific pathogen-free animal housing facility
112 at Inserm U1110. The respective protocols were approved by the Ethics Committee of the
113 University of Strasbourg Hospitals and authorized by the French Ministry of Research (number
114 02014120416254981AL/02/19/08/12, AL/01/18/08/1202014120416254981, 0201412051105

115 4408). Mice were kept in individual ventilated cages, with bedding composed of irradiated sawdust
116 and chips from spruce and pine and enriched with cotton cocoon and aspen bricks. The animal
117 diet consists of 25kGy irradiated RM3(E) (SDS) and mice were not fasted. Primary human
118 hepatocytes (PHH) were transplanted into 3 week-old uPA/SCID-bg mice (male and female) by
119 intrasplenic injection as described (28). Engraftment and viability of PHH were assessed by
120 quantification of human serum albumin by ELISA (E80-129, Bethyl Laboratories; (28)). uPA/SCID-
121 bg mice were then infected with serum-derived HBV and sacrificed 16 weeks after virus
122 inoculation. Serum HBV load was determined by qPCR (Realtime HBV viral load kit, Abbott)
123 before sacrifice. Interventions were all performed during light cycle.

124

125 **Cell lines and human hepatocytes.** HEK 293T (29) and HepG2-NTCP (30) cells and isolation
126 of PHH have been described (29).

127

128 **Reagents and plasmids.** DMSO, PEG 8000 (polyethylene glycol), Poly (I:C) and calf thymus
129 DNA (control dsDNA) were obtained from Sigma-Aldrich, pReceiver-Lv151 plasmid from
130 GeneCopoeia™ and lentiCas9-Blast and lentiGuide-Puro plasmids were gifts from Feng Zhang
131 (Addgene #52962 and #52963).

132

133 **Small interfering RNAs for functional studies.** Pools of ON-TARGET plus (Dharmacon) small
134 interfering RNA (siRNA) targeting *MB21D1* (cGAS), *TMEM173* (STING), *TBK1*, and *IFI16*
135 expression were reverse-transfected into HepG2-NTCP using Lipofectamine RNAi-MAX
136 (Invitrogen) as described (29). RNA was purified from cells harvested two days after transfection
137 and gene expression was analyzed using qRT-PCR.

138

139 **HepG2-NTCP-cGAS overexpressing and *MB21D* knock-out cells.** Lentivirus particles were
140 generated in HEK 293T cells by cotransfection of plasmids expressing the human
141 immunodeficiency virus (HIV) gap-pol, the vesicular stomatitis virus glycoprotein (VSV-G) and
142 either the human *MB21D1* full open reading frame (ORF) encoding plasmid, or the *MB21D1*-
143 targeting single-guide RNA (sgRNA) encoding plasmids, or the Cas9 expressing plasmid in the
144 ratio of 10:3:10. HepG2-NTCP cells were then plated and transduced with lentivirus encoding
145 either the human *MB21D1* ORF or the *eGFP* ORF in pReceiver-Lv151 vector (GeneCopoeia™).
146 After 3 days, transduced cells were selected with 200 µg/ml of neomycin (G418). The cGAS-over-
147 expressing and control HepG2-NTCP cells were then further cultured in presence of G418 at 200
148 µg/ml. For the generation of *MB21D1* knock-out cell lines, one *MB21D1*-targeting sgRNA was
149 designed using CRISPR Design Tool (Broad Institute: [http://www.genome-](http://www.genome-engineering.org/crispr/?page_id=41)
150 [engineering.org/crispr/?page_id=41](http://www.genome-engineering.org/crispr/?page_id=41)). The sgRNA sequence targeting the exon 1 of *MB21D1*
151 (sgcGAS 5'-CACCGCGCCCCCATTCTCGTACGG-3') was inserted into lentiGuide-Puro
152 plasmid (31). We first generated Cas9 expressing HepG2-NTCP cells after transduction of cells
153 with the lentiCas9-Blast plasmid (31). Cells were then selected with 6 µg/ml blasticidin for 10
154 days. HepG2-NTCP-Cas9 cells were seeded in six-well plates at 50% confluency 24 h prior to
155 transduction with the sgcGAS-encoding plasmid. Subpopulations of cells were selected from the
156 whole population and cultured independently. cGAS expression was controlled by Western blot.
157 Finally, two cGAS-deficient cell lines (cGAS_KO#1 and cGAS_KO#2) were selected.

158

159 **Analysis of gene expression using qRT-PCR.** Total RNA was extracted using ReliaPrep™ RNA
160 Miniprep Systems (Promega) and reverse transcribed into cDNA using Maxima First Strand cDNA
161 Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Gene expression
162 was then quantified by qPCR using a CFX96 thermocycler (Bio-Rad). Primers and TaqMan®

163 probes for *MB21D1* (cGAS), *TMEM173* (STING), *TBK1*, *IFI16*, *IFNB1*, *IFNL1*, and *GAPDH* mRNA
164 detection were obtained from ThermoFisher (TaqMan® Gene expression Assay, Applied
165 Biosystems). All values were normalized to *GAPDH* expression.

166

167 **Protein expression.** The expression of cGAS, STING, and β -actin proteins was assessed by
168 Western blot as described (30) using two polyclonal rabbit anti-cGAS antibodies (HPA031700,
169 Sigma & NBP1-86761, Novus Biologicals, see Supporting Information), a polyclonal rabbit anti-
170 STING antibody (19851-1-AP, Proteintech), and monoclonal anti- β -actin antibody (mAbcam8226,
171 Abcam). Protein expression was quantified using ImageJ software.

172

173 **Infection of HepG2-NTCP cells and PHH.** The purification of infectious recombinant HBV
174 particles from HepAD38 cells and infection of HepG2-NTCP cells has been described (30). Briefly,
175 HepG2-NTCP and derived cells were plated one day prior to incubation with HBV in presence of
176 4% PEG at multiplicity of infection (MOI) ~ 500 genome equivalent/cell (GEq/cell) except
177 otherwise stated. Sixteen hours after HBV inoculation, cells were washed with PBS and then
178 cultured in 3.5% DMSO primary hepatocyte maintenance medium (PMM) for ten days. HBV
179 infection was assessed by quantification of HBV pgRNA using qRT-PCR or HBV total DNA using
180 qPCR as described (30), or by immunofluorescence (IF) using anti-HBsAg antibody (1044/329,
181 Bio-Techne) and AF647-labelled goat antibody targeting mouse IgGs (115-605-003, Jackson
182 Research) as described (30). Southern blot detection of HBV cccDNA was performed using DIG-
183 labelled (Roche) specific probes as described (32). Total DNA from HBV-infected cells was
184 extracted using the previously described HIRT method (33). Specific DIG-labelled probes for the
185 detection of HBV and mitochondrial DNAs were synthesized using the PCR DIG Probe Synthesis
186 Kit (Roche) and the primers indicated in Table S1. PHH were plated one day prior to incubation

187 with a HBV preS1- or a control peptide for one hour at 37°C as described (30). PHH were then
188 infected with recombinant HBV particles for ten days. HBV infection was assessed by
189 quantification of HBV pgRNA using qRT-PCR and immunofluorescence as described above.

190

191 **Sendai virus (SeV) infection.** HepG2-NTCP cells and PHH were infected with SeV DI-H4 at an
192 MOI of 10 as described (13).

193

194 **Extraction of HBV rcDNA from HBV infectious particles.** HBV rcDNA was extracted from HBV
195 preparations using QiaAMP DNA MiniKit protocol (Qiagen). PEG-precipitated cell supernatants
196 from naive HepG2-NTCP cells were used as non-virion controls. The presence of HBV DNA was
197 confirmed by PCR and quantified by qPCR as described (30) (see Supporting Information). One
198 µg of rcDNA or dsDNA (calf thymus DNA) was transfected in cells using Lipofectamine 2000
199 (Invitrogen) and CalPhos Mammalian Transfection Kit (Clontech) according to manufacturer's
200 instructions. Cells transfected with HepG2-NTCP control supernatants were used as a control.
201 Three days after transfection, total RNA was extracted and purified as described above.

202

203 **Transcriptomic analysis by digital multiplexed gene profiling using nCounter NanoString.**

204 Transcriptomic analyses using nCounter NanoString were performed according to manufacturer's
205 instructions. Specific probes for a set of 36 innate antiviral response-related (IAR) genes
206 (according to (20) and additional genes listed in Table S2) were obtained from the manufacturer.
207 HepG2-NTCP cells, HepG2-NTCP derived cell lines and PHH were either infected with HBV or
208 SeV, or were transfected with Poly (I:C) (100ng) for two days. Alternatively, HepG2-NTCP-Cas9
209 and HepG2-NTCP-KO_cGAS#2 cells were transfected with rcDNA (1µg) or dsDNA (calf thymus
210 DNA, 1µg) for three days. Total RNA was then extracted and subjected to nCounter Digital

211 Analyzer system (NanoString). Alternatively, total liver RNA was extracted from HBV-infected mice
212 and gene expression was assessed by either qRT-PCR (*MD21D1* expression) or nCounter Digital
213 Analyzer system. The 36 genes were considered as an artificial gene set termed innate antiviral
214 response (IAR) gene set. Its perturbation by infection, transfection or gain-/loss-of -function
215 studies was assessed through Gene Set Enrichment Analysis (GSEA (34)). GSEA determines
216 whether an a priori defined set of genes shows statistically significant differences between two
217 biological states. False discovery rate (FDR) < 0.05 was considered statistically significant.
218 Heatmaps illustrating the induction (red) or repression (blue) of the genes of the IAR compared
219 to control were illustrated using Morpheus software (Broad Institute of MIT and Harvard,
220 Cambridge, MA, USA). The heatmap illustrating the induction (red) or repression (blue) of
221 individual genes in chimeric mouse livers were designed using GenePattern (Broad Institute of
222 MIT and Harvard, Cambridge, MA, USA).

223

224 **FISH analyses.** Fluorescence in situ hybridization (FISH) analyses were performed as described
225 (28, 35). Briefly, liver samples were collected from mice and then immediately embedded into
226 optimal cutting temperature compound (OCT). OCT-embedded liver sections were cryosectioned
227 (10 μ m) using a cryostat (Leica). Upon fixation with 4% formaldehyde at 4°C, washing, and
228 dehydration in ethanol, tissue sections were boiled at 90–95°C for 1 min in a pretreatment solution
229 (Affymetrix-Panomics), followed by a 10 min digestion in protease QF (Affymetrix-Panomics) at
230 40°C. Sections were then hybridized using specific probe sets targeting HBV (target region
231 nucleotides 483-1473 of HBV [Genotype D, GenBank V01460]) and human *MD21D1* (VA1-
232 3013492-VC, Affymetrix-Panomics). Pre-amplification, amplification and detection of bound
233 probes were performed according to the manufacturer's instructions. Finally, pictures were
234 acquired by LSCM (LSM710, Carl Zeiss Microscopy) and Zen2 software.

235

236 **Statistical Analysis.** Except otherwise stated, cell culture experiments were performed at least
237 three times in an independent manner. Statistical comparisons of the samples were performed
238 using a two-tailed Mann-Whitney U test. For *in vivo* experiments, a two-tailed unpaired Student's
239 t-test was performed for comparing gene expression from non-infected and HBV-infected mice. p
240 < 0.05 (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. Significant p values are
241 indicated by asterisks in the figures. Each digital multiplexed gene profiling experiment was
242 performed using three biological replicates per condition and the induction or repression of the
243 gene set was analyzed using GSEA. FDR < 0.05 was considered statistically significant.

244

245 RESULTS

246 **Expression of cGAS in primary human hepatocytes and an infectious HBV cell culture**
247 **model.** Prior to its functional characterization, we studied cGAS/*MB21D1* expression in primary
248 hepatocytes and HBV permissive cell lines. As shown in Figure 1A, cGAS protein expression was
249 easily detectable in PHH from three independent donors. Since HBV infection of primary cells is
250 highly variable and does not allow robust perturbation studies, we used an HBV infectious cell
251 culture model based on differentiated hepatocyte-derived HepG2 cells overexpressing NTCP (30)
252 – a key HBV entry factor. As shown in Figure 1A, cGAS protein is expressed in HepG2-NTCP
253 cells. We validated the specificity of cGAS detection using a siRNA specifically targeting the
254 *MD21B1* expression (sicGAS) and Western blots applying two antibodies (Figure 1A, Figure S1).
255 Moreover, we generated CRISPR-mediated *MB21D1* knock-out (KO) cells using a specific sgRNA
256 (Figure 1B). Two cell lines, KO_cGAS#1 and KO_cGAS#2 were selected for further studies
257 (Figure 1B). Interestingly, the adaptor STING was also detected in HepG2-NTCP cells, suggesting
258 a fully functional cGAS-STING pathway (Figure 1C). To test the suitability of these cells as a

259 model to analyze cGAS-mediated innate immune response after virus infection, we stimulated
260 cells with different analogs of viral nucleic acids. Stimulation by Poly (I:C) or dsDNA transfection
261 elicited a dose-dependent *IFNB1* expression in HepG2-NTCP cells (Figure 1D). These results
262 suggest that the cGAS sensing machinery is present, functional, even if it is less efficient than the
263 RNA sensing complex. Moreover, cGAS protein expression was induced by both Poly (I:C) and
264 dsDNA stimulation confirming an efficient IFN response through the upregulation of ISGs such as
265 *MB21D1* (36) after RNA or DNA stimulation (Figure 1E). Collectively, these data show that the
266 HepG2-NTCP model is suitable to study innate immune responses.

267

268 **HBV evades cGAS sensing.** Next, we investigated whether HBV was sensed in HBV permissive
269 cells. To address this question, we infected HepG2-NTCP cells with recombinant HBV (MOI: 500
270 GEq/cell) and studied the expression of *IFNB1* at early time points after HBV infection. As it has
271 been described that HBV infection may induce the expression of type III IFN (7), *IFNL1* expression
272 was also quantified. As shown in Figure 2A-B, the lack of increase in *IFNB1* and *IFNL1* expression
273 in spite of efficient infection (Figure S2) indicates poor or absent detection of HBV by cellular
274 sensors. In contrast, SeV, known to induce a strong IFN response in hepatocytes (13), strongly
275 induced *IFNB1* and *IFNL1* expression (Figure 2 AB). Since cGAS has been shown to induce the
276 expression of a large set of innate effector genes (such as *OAS2* or *IFI44*, see (20)), the analysis
277 of expression of a single effector gene such *IFNB1* may not be sufficient to evaluate cGAS
278 sensing. Therefore, we designed a 36 innate antiviral response gene set (named IAR), comprising
279 29 ISGs whose expression is modulated by cGAS activity described by Schoggins and Rice in
280 (20) as well as 7 established innate immune response effector genes (Table S2). We then infected
281 HepG2-NTCP cells with HBV or SeV, and measured the innate antiviral immune response at day
282 2 post infection by analysis of IAR gene expression using digital multiplexed gene profiling

283 (nCounter NanoString) and GSEA-based analysis. Whereas Poly (I:C) transfection and SeV
284 infection induced a marked modulation of cGAS effector/IAR gene expression (FDR = 0.004 and
285 < 0.001, respectively), no significant modulation of IAR gene expression was observed in HBV-
286 infected cells (Figure 2C), as further illustrated by the expression of *IFNB1* and *IFI44* (Figure 2E).
287 To measure the impact of cGAS expression on cellular response to HBV infection, we then
288 infected cGAS-depleted (KO_cGAS#2) and -overexpressing (cGAS_OE) HepG2-NTCP cells with
289 HBV and analyzed the expression of the cGAS-related genes after infection. As shown in Figure
290 2D, no significant modulation of the IAR signature was observed in HBV-infected samples, as
291 further illustrated by absent modulation of *IFNB1* and *IFI44* expression (Figure 2F).

292 To exclude the possibility that low HBV infection could be the reason for the absence of
293 IFN induction, we performed additional experiments using increasing MOIs. As shown in Figure
294 3A-C, no induction of *IFNB1* (Figure 3A) was observed even at a MOI 10000, despite very high
295 infection efficiency as shown by quantitation of pre-genomic RNA and immunofluorescence of
296 HBsAg (Figure 3B-C). To investigate whether IFN induction occurs potentially at a very early step
297 of viral infection and was missed in the experimental design shown above, we performed a time
298 course studying IFN response to HBV within the first 24 hours post infection. As shown in Figure
299 3D, HBV infection did not induce a measurable IFN response during early steps of HBV infection.
300 In contrast, SeV, an established inducer of IFN showed robust induction of IFN responses in
301 HepG2-NTCP cells (Figure 3D). Taken together, these data suggest an absence of sensing of
302 HBV infection by the cGAS-STING pathway in HepG2-NTCP cells.

303 As the HBV genome is packaged into the nucleocapsid (37), we investigated whether
304 packaging shields virion DNA from cGAS recognition. We purified HBV genomic rcDNA from HBV
305 infectious particles (Figure S3) and transfected the naked viral genome into HepG2-NTCP cells
306 (1 μ g, corresponding to approximately 10^6 - 10^7 HBV DNA copies/ μ L). As shown in Figure 4A, a

307 significant (FDR = 0.02) induction of the IAR signature (illustrated by *IFNB1* and *IFI44* expression,
308 Figure 4B) was observed after both rcDNA and dsDNA transfection, suggesting sensing of the
309 naked HBV genome. Interestingly, the amount of cellular HBV DNA copies was higher in HBV-
310 infected cells compared to rcDNA transfected cells (Figure 4C), confirming that the levels of HBV
311 DNA in HBV infected cells are sufficient to trigger IFN signaling and the absence of HBV sensing
312 in infected cells was not due to low MOIs. Moreover, the induction of the IAR gene expression
313 was absent in HepG2-NTCP-KO_cGAS#2 cells, suggesting a cGAS-specific activation of innate
314 immunity by both dsDNA and rcDNA transfection in our model.

315 To validate these observations in a more physiological model, we infected PHH with HBV
316 and control cGAS gene expression after two days of infection. Interestingly, while SeV strongly
317 induced IAR gene expression (Figure 5B), a highly efficient HBV infection (Figure 5A) did not
318 induce the induction of the expression of innate antiviral response genes (Figure 5B). In contrast,
319 the transfection of rcDNA and dsRNA into PHH from four different donors robustly triggered the
320 expression of *IFNB1* and *IFNL1* (Figure 5C), suggesting a robust sensing of viral DNA in human
321 hepatocytes. Collectively, these data suggest that non-encapsidated HBV DNA is sensed by
322 cGAS, but this sensing is impaired during HBV infection.

323

324 **The cGAS-STING pathway exhibits robust antiviral activity against HBV infection with**
325 **reduction of cccDNA levels.** As cGAS exhibits an antiviral activity against a broad range of DNA
326 and RNA viruses (meaning even in absence of direct viral sensing) (20), we then investigated the
327 antiviral effect of the cGAS-STING signaling pathway in HBV infection. We silenced the
328 expression *MB21D1*, *TMEM173* (encoding the STING protein), *TBK1* and *IFI16* (encoding the
329 gamma-interferon-inducible protein 16, another cytoplasmic DNA sensor able to directly activate
330 STING (17)) in HepG2-NTCP cells prior to infection with HBV. As shown in Figure 6A-B, silencing

331 of *MB21D1*, *TMEM173* and *TBK1* expression induced a marked increase in HBV infection. In
332 contrast, the silencing of *IFI16* had no effect on HBV infection. CRISPR/Cas9-mediated KO or
333 overexpression of cGAS protein resulted in a marked increase or decrease in HBV infection and
334 HBV cccDNA levels – the key viral nucleic acid responsible for viral persistence (Figure 6C-E).
335 Notably, the overexpression of cGAS did not affect NTCP expression at the cell surface,
336 suggesting that the susceptibilities of the different cell lines to HBV infection are equivalent (Figure
337 S4). Taken together, our results suggest that cGAS is functional and exerts antiviral activity in
338 HBV permissive cells.

339

340 **HBV infection induces repression of cGAS and its effector gene expression in cell culture**
341 **and in liver chimeric mice.** As several reports have suggested that HBV proteins can inhibit IFN-
342 signaling pathways (16), we next investigated whether HBV infection interferes with the
343 expression of cGAS-related gene by quantifying *MB21D1*/cGAS mRNA and protein expression
344 (Figure 7A-B). Interestingly, cGAS protein expression (Figure 7B) as well as the expression of
345 *MB21D1*, *TMEM173*, and *TBK1* mRNA (Figure 7C) were significantly inhibited in HBV-infected
346 cells. To confirm this observation *in vivo*, we then investigated the expression of human *MB21D1*
347 expression in HBV-infected human liver chimeric mice. *MB21D1* was expressed at low but
348 detectable levels (Figure 7D). As shown in Figure 7E, *MB21D1* expression was significantly ($p =$
349 0.013) downregulated in HBV-infected mice compared to non-infected control mice, confirming
350 our results in the cell culture model. Importantly, *MB21D1* expression levels did not correlate with
351 HBV genotype (Table 1). An absent correlation of human serum albumin with either *MB21D1*
352 expression (Table 1, Figure 7E) or status of HBV infection (Table 1, t-test HBV versus Ctrl: $p =$
353 0.26) largely excludes that the observed differences in cGAS expression are due to different
354 human hepatocyte repopulation levels or due to a decrease of human hepatocyte cell viability in

355 individual animals. To investigate whether HBV modulates cGAS effector function, we analyzed
356 virus-induced changes on cGAS effector gene expression using gene expression profiling in three
357 control mice and the three HBV-infected mice exhibiting the lowest levels of *MB21D1* expression
358 (Table 1). As shown in Figure 7F, HBV infection resulted in a significant (FDR = 0.047) down-
359 regulation of the expression of cGAS effector genes in human hepatocytes in chimeric mice. The
360 data showed that HBV represses expression of cGAS and its effector genes *in vivo*.

361

362 **DISCUSSION**

363 The interaction between HBV and the innate immune system is a complex process still remaining
364 elusive and controversial (15). Collectively, our data demonstrate that in human hepatocytes (i)
365 naked HBV genomic rcDNA is sensed in a cGAS-dependent manner whereas the packaged HBV
366 genome appears not to be recognized during viral infection; (ii) cGAS-STING pathway exhibits
367 antiviral activity against HBV infection including reduction of viral cccDNA levels; (iii) HBV infection
368 suppresses both cGAS expression and function in cell culture and humanized liver chimeric mice.

369 The detection of HBV DNA by the cellular sensors within infected cells is still poorly
370 understood and remains controversial. *In vitro* and *in vivo* data strongly suggest that HBV behaves
371 like a stealth virus unable to trigger any innate immune response (8, 9, 11). Other studies have
372 suggested that HBV-derived dsDNA fragments (25) and viral nucleocapsid destabilization and
373 disassembly (38, 39) could induce innate immune responses. Our results demonstrate, that in
374 human hepatocytes - the natural target cell of HBV infection - the exposure of the naked HBV
375 genome leads to the activation of innate antiviral immune responses. In contrast, sensing is
376 largely absent during HBV infection, most likely due to packaging into the viral capsid. These
377 results extend a previous observation in hepatoma cell lines transfected with replication-
378 competent HBV DNA that the HBV genome itself can be recognized by the classical sensors (25).

379 Interestingly, the capsid of HIV-1 also prevents the sensing of HIV cDNA by cGAS following
380 reverse-transcription up to integration, whereas HIV-2 capsid may unmask the cDNA leading to a
381 stronger sensing by cGAS and a lower pathogenicity of the strain (40).

382 Another explanation of this absence of sensing would be the lack a functional STING
383 protein in hepatocyte, as it has been recently reported (41). In our study, rcDNA and dsDNA were
384 sensed in a cGAS-dependent manner and were able to activate the cGAS-mediated antiviral
385 response in HepG2-NTCP cells (Figure 2). Moreover, we detected STING at the protein level in
386 accordance with a recent study (42) and specific silencing of *TMEM173* (STING) expression was
387 associated with a significant increase in HBV infection (Figure 6). Consequently, it is likely that
388 STING is functionally active in HepG2 cells. The observed HBV DNA sensing in PHH (Figure 5)
389 suggests that the foreign DNA detection pathways are active in PHH as well. This observed innate
390 immune response in spite of a weak STING expression may suggest a STING-independent
391 activity of cGAS as it has been recently reported (43), including in hepatocytes (44). To understand
392 the impact of the cGAS and STING expression on innate immune response to HBV infection, it
393 would be of further interest to analyze the HBV-induced modulation of gene expression in Kupffer
394 cells following phagocytosis, as they exhibit higher STING- and cGAS levels compared to
395 hepatocytes (41, 45) and respond to HBV infection (11). In the same vein, cGAMP has been
396 shown to be packaged in viral particles (46). It would be of interest to determine whether HBV
397 particles can incorporate cGAMP during viral assembly and to test their ability to stimulate other
398 cell types through this indirect pathway.

399 Moreover, our results show conclusive evidence that cGAS basal expression has antiviral
400 activity against HBV infection including reduction of viral cccDNA. This finding extends a previous
401 studies showing that cGAS exhibited an antiviral activity against a broad range of RNA and DNA
402 viruses (20) and that the cGAS/STING pathway can impair HBV replication and assembly in

403 transfection studies (24, 25). Schoggins and colleagues have proposed that the expression of
404 cGAS may be responsible for the establishment of a basal antiviral level in the cells through its
405 activation by an unknown ligand. cGAS-depleted cells may then be more susceptible to viral
406 infections through the downregulation of the basal level of innate antiviral genes (20).

407 Given its antiviral function, cGAS is a target of choice for viruses in order to evade immune
408 responses. It has been reported that the Kaposi's sarcoma-associated herpesvirus negatively
409 regulated cGAS-dependent signaling pathway (47, 48). In the same vein, HBV viral proteins have
410 been shown to interfere with the JAK-STAT signaling pathway (16). Our data suggest that HBV
411 can repress the expression of the cGAS and its related genes, such as *MB21D1*, *TMEM17* and
412 *TBK1*. More interestingly, *MB21D1* expression was downregulated in the liver of HBV-infected
413 mice, validating the relevance of these findings *in vivo*. It still needs to be determined whether
414 HBV can directly target cGAS and cGAS-related factors for an active inhibition of this signaling
415 pathway. A recent study elegantly demonstrated an active inhibition of cGAS pathway by Dengue
416 virus through NS2B protein (49). On the other hand, *MB21D1* (as a classical member of the ISGs
417 (20, 36)) downregulation may be the consequence of the global inhibition of the canonical IFN
418 pathways by HBV as suggested by some investigators (16, 50), but not by others (11, 13, 14).
419 Given the antiviral activity of the cGAS-signaling pathway against HBV including reduction of HBV
420 cccDNA (Figure 5, (24, 25)) the virus-mediated restriction of *MB21D1* expression may play an
421 additional role in HBV immune evasion.

422

423

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554

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561

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563

564 **Author contribution.** TFB initiated the study. TFB, ERV and CS designed and supervised
565 research. ERV, SAY, LH, CB, VTL, SD, LM, JL, TC and DD performed the experiments. ERV, SAY,
566 LH, CB, VTL, JL, DD, MBZ, NP, CS and TFB analyzed the data. PP provided liver resections for
567 PHH isolation. NM, IH and NP made substantive intellectual contributions. ERV, SAY, CS and TFB
568 wrote the paper.

569 **FIGURE LEGENDS**

570

571 **Figure 1. cGAS expression and function in human hepatocytes and in a cell culture model**572 **for HBV infection.** (A) Detection of endogenous cGAS protein expression in different cellular

573 models by Western blot. Cell lysates from Huh7.5.1, HepG2, HepG2-NTCP cells, and PHH from

574 three independent donors were used. HepG2-NTCP cells were reverse transfected with a siRNA

575 targeting *MB21D1* (siCGAS) or a non-targeting siRNA control (siCtrl) two days before cGAS576 detection. β -actin was used as a Western blot control. Individual representative experiments are577 shown. (B) Generation of *MB21D1* knock out (KO) cells. *MB21D1* KO HepG2-NTCP cell lines

578 were generated via CRISPR/Cas9 technology. The absence or presence of cGAS protein was

579 controlled by Western blot using the HPA031700 anti-cGAS antibody in Cas9-expressing HepG2-

580 NTCP cells (Cas9) and in different cell lines after transduction with the sgRNA targeting *MB21D1*

581 (line-A, line-B, line-C, cGAS_KO#1 and cGAS_KO#2). One experiment is shown. (C) Detection

582 of endogenous STING protein in HepG2-NTCP cells. siRNA targeting *TMEM173* (siSTING) or a

583 non-targeting siRNA (siCtrl) were reverse-transfected into HepG2-NTCP cells. Silencing efficacy

584 was assessed by Western blot. One experiment is shown. (D-E) Poly (I:C) and dsDNA transfection

585 induce *IFNB1* and *MB21D1* expression in HepG2-NTCP cells. HepG2-NTCP cells were

586 transfected with increasing doses of Poly (I:C) or calf thymus DNA at the indicated concentrations.

587 *IFNB1* mRNA expression was quantified by qRT-PCR 24 h after transfection and cGAS protein

588 expression was assessed by Western blot 24 h and 48 h after transfection. qRT-PCR data (D)

589 are expressed as means \pm SD relative *IFNB1* expression (log10) compared to non-transfected

590 control (0, set at 100) from four independent experiments performed in triplicate (dsDNA) or from

591 three independent experiments performed in triplicate (Poly I:C). One representative Western blot

592 experiment is shown (E).

593

594 **Figure 2. Impaired cGAS-mediated sensing of HBV infection in HepG2-NTCP cells.** (A-B)

595 HBV infection does not induce *IFNB1* or *IFNL1* expression. HepG2-NTCP cells were infected with

596 HBV (MOI: 500) or SeV (MOI: 10) and total RNA was extracted every day for 3 days. RNA

597 extracted from naive cells before infection was used as a control (D0). *IFNB1* (A) and *IFNL1* (B)

598 expression was then assessed by qRT-PCR. Results are expressed as means \pm SD *IFNB1/IFNL1*

599 relative expression (log10) compared to controls (D0, all set at 1) from three independent

600 experiments performed at least in duplicate (SeV) or four independent experiments performed in

601 duplicate (HBV). No robust *IFNL1* expression was detected in HBV-infected samples

602 (representative dots are presented under the “detection limit” dotted line). (C, E) cGAS-related

603 ISGs are not affected by HBV infection. HepG2-NTCP cells were infected with HBV or SeV.

604 Alternatively, HepG2-NTCP cells were transfected with Poly (I:C) (100ng). Two days after infection

605 or transfection, total RNA was extracted. Gene expression of IAR signature was then analyzed

606 using multiplexed gene profiling. Results were analyzed by GSEA enrichment compared to non-

607 transfected or non-infected controls (C) or by *IFNB1* and *IFI44* gene expression (log10) compared

608 to non-transfected or non-infected controls (set at 1) (E). One experiment performed in triplicate

609 is shown. (D, F) cGAS expression level does not affect the cellular response to HBV infection.

610 HepG2-NTCP-Cas9 (Cas9), HepG2-NTCP-KO_cGAS#2 (Ko_cGAS#2), HepG2-NTCP-Ctrl_ORF

611 (Ctrl_ORF), and HepG2-NTCP-cGAS_OE (cGAS_OE) were infected with HBV. Two days after

612 infection, total RNA was extracted. Gene expression of IAR signature gene set was then analyzed

613 using multiplexed gene profiling. Results were analyzed by GSEA enrichment compared to non-

614 transfected or non-infected controls (D) or by *IFNB1* and *IFI44* gene expression (log10) compared

615 to non-transfected or non-infected controls (set at 1) (F). One experiment performed in triplicate

616 is shown. IAR: innate antiviral response gene set.

617

618 Figure 3. Impaired sensing of HBV infection at high MOIs or early time points of infection.

619 (A-C) HepG2-NTCP cells were infected with HBV at increasing MOIs (0, 50, 500, and 10000
620 GEq/cell) or transfected with Poly (I:C) (100 ng). Two days after infection or transfection, cells
621 were lysed, total RNA was extracted, and *IFNB1* expression (A) as well as HBV pgRNA levels (B)
622 were quantified by qRT-PCR. (A) Results are expressed as means \pm SD relative *IFNB1*
623 expression (log10) compared to mock infected cells (MOI 0, set at 1) from three independent
624 experiments performed in triplicate. (B) Results are expressed means \pm SD relative HBV pgRNA
625 levels compared to mock infected cells (MOI 0, set at 100%) from three independent experiments
626 performed in triplicate. Alternatively, HBV infection was assessed at 10 days post infection by IF
627 of HBsAg (C). One representative experiment is shown. (D) HBV infection does not induce *IFNB1*
628 expression at early time points. HepG2-NTCP cells were either infected by HBV or SeV, or
629 transfected with Poly (I:C). Total RNA was extracted at 2 h, 6 h, 18 h, and 24 h post
630 infection/transfection and *IFNB1* expression was assessed by qPCR. Results are expressed as
631 means \pm SD relative *IFNB1* expression (log10) compared to naive cells (0, set at 1) from three
632 independent experiments performed in triplicate.

633

634 Figure 4. Sensing of HBV rcDNA in HepG2-NTCP cells. (A-B) Transfection of purified HBV

635 rcDNA genome induces a cGAS-mediated innate immune response. HBV rcDNA was extracted
636 from recombinant HBV virions as described in Experimental Procedures and quantified by qPCR
637 (Figure S3). HBV rcDNA (1 μ g) and positive control dsDNA (1 μ g) were transfected into HepG2-
638 NTCP-Cas9 and HepG2-NTCP-KO_cGAS#2 cells. Three days after transfection, total RNA was
639 extracted. Gene expression of IAR set was then analyzed using multiplexed gene profiling. The
640 transcripts were analyzed by GSEA enrichment compared to non-transfected control (A) or by

641 *IFNB1* and *IFI44* gene expression (log10) compared to non-transfected control (set at 1) (B). One
642 experiment in triplicate is shown. (C) HBV DNA in HepG2-NTCP cells after rcDNA transfection
643 and HBV infection are similar. HepG2-NTCP were infected with HBV or transfected with HBV
644 rcDNA (1µg). At day 2 after transfection/infection, DNA was extracted and total HBV DNA was
645 quantified by qPCR. Results are expressed as means ± SD total DNA copies per µg of total DNA
646 (log10) from three independent experiments performed in triplicate (HBV infection) and two
647 independent experiments performed in triplicate (HBV rcDNA transfection). IAR: innate antiviral
648 response gene set.

649

650 **Figure 5. Sensing of naked HBV rcDNA but not infectious HBV virions in primary human**
651 **hepatocytes.** (A-B) HBV infection does not induce ISG expression in PHH. PHH were treated
652 with a preS1 peptide (PreS1) or a scrambled peptide (Ctrl) for one hour before infection with HBV.
653 As positive controls, PHH were transfected with Poly (I:C) (100ng) or infected with SeV (MOI ~
654 10). HBV infection of PHH was assessed 10 days post infection by qRT-PCR (A, left panel) or
655 immunofluorescence (A, right panel). qRT-PCR results are expressed as means ± SD ratio HBV
656 pgRNA RNA/ GAPDH mRNA from one experiment corresponding to the gene profiling experiment
657 and performed in duplicate. Two days after infection, total RNA was extracted and gene
658 expression of IAR was then analyzed using multiplexed gene profiling. Results were analyzed by
659 GSEA enrichment compared to non-transfected control (B). One experiment in triplicate is shown.
660 (C) Induction of *IFNB1* and *IFNL1* expression in PHH. PHH were transfected with dsDNA (4 µg)
661 or HBV rcDNA (4 µg). *IFNB1*- and *IFNL1* expression was assessed 24 h after transfection by qRT-
662 PCR. Results are expressed as means ± SD log10 *IFNB1* or *IFNL1* expression compared to non-
663 transfected control cells (Ctrl, set at 1) from five independent experiments performed at least in
664 duplicate. IAR: innate antiviral response gene set.

665
666
667 **Figure 6. Antiviral activity of cGAS results in reduction of HBV cccDNA.** (A-B) Silencing of
668 cGAS-related gene expression increases HBV infection. siRNA targeting *MB21D1* (siGAS),
669 *TMEM173* (siSTING), *TBK1* (siTBK1), *IFI16* (siIFI16) or a non-targeting siRNA (siCtrl) were
670 reverse-transfected into HepG2-NTCP cells 2 days prior to HBV infection. Silencing efficacy was
671 assessed by qRT-PCR 2 days after transfection (A). Results are expressed as means \pm SD %
672 gene expression relative to siCtrl (set at 100%) from four independent experiments performed in
673 technical duplicate. HBV infection was assessed by quantification of HBV pgRNA by qRT-PCR
674 10 days after infection (B). Results are expressed as means \pm SD % HBV pgRNA expression
675 relative to siCtrl (set at 100%) from four independent experiments performed in technical duplicate.
676 (C) KO of *MB21D1* gene increases HBV infection. cGAS_KO#1, cGAS_KO#2, and the control
677 Cas9 cells were then infected with HBV and viral infection was assessed 10 days after infection
678 as described above. Results are expressed as means \pm SD % HBV pgRNA expression relative
679 to control cell line (Cas9, set at 100%) from three independent experiments performed in triplicate.
680 (D) cGAS overexpression reduces HBV infection. HepG2-NTCP cells were transduced with
681 lentivirus encoding either a control plasmid (Ctrl_ORF) or a plasmid encoding the full length
682 *MB21D1* ORF (cGAS_OE). *MB21D1* expression was assessed by qRT-PCR (left panel). Results
683 are expressed as means \pm SD % relative *MB21D1* expression (log10) relative to control cell line
684 (Ctrl_ORF, set at 1) from three independent experiments performed in duplicate. HepG2-NTCP,
685 Ctrl_ORF, and cGAS_OE cells were then infected with HBV for ten days and HBV infection was
686 assessed as described above. Results are expressed as means \pm SD % HBV pgRNA expression
687 relative to control cell line (Ctrl_ORF, set at 100%) from three independent experiments performed
688 in triplicate. (E) Detection of HBV cccDNA by Southern blot. HepG2-NTCP-derived cGAS_KO- or

689 cGAS_overexpressing cell lines were infected for 10 days with HBV. Total DNA from indicated
690 HBV infected cells was extracted and HBV DNA were detected by Southern blot. Two different
691 DNA ladders (L1 & L2) were used. *XhoI* digestion of DNA extracted from HBV-infected HepG2-
692 NTCP-Cas9 cells was used as a control and resulted in a single 3.2 kb band (dsI HBV DNA).
693 Mitochondrial DNA (mt DNA) was detected as a loading control. One experiment is shown.

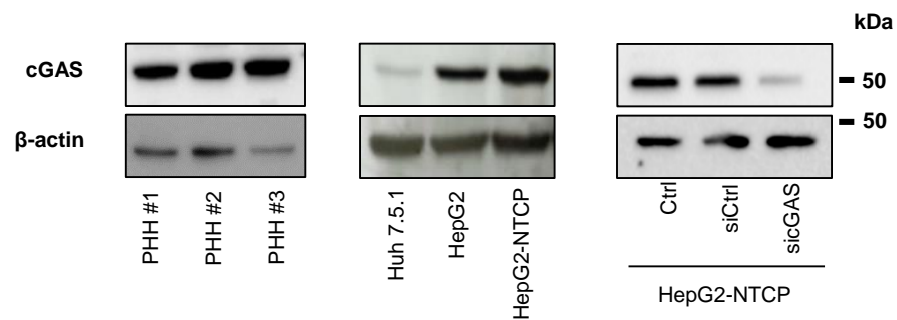
694

695 **Figure 7. HBV infection suppresses the expression of the cGAS-related genes cell culture**
696 **and humanized liver chimeric mice *in vivo*.** (A-C) HepG2-NTCP cells were infected with HBV
697 for 10 days. HBV infection was assessed by quantification of HBV pgRNA by qRT-PCR. Results
698 are expressed as means \pm SD from three experiments performed in triplicate. cGAS protein
699 expression was assessed 10 days after infection (B, one experiment is shown). Gene expression
700 relative to non-infected control cells of *MB21D1*, *TMEM173* and *TBK1* were assessed by qRT-
701 PCR at day 10 after infection (C). Results are expressed as means \pm SEM from three independent
702 experiments performed in triplicate. (D-E) *MB21D1*- and IAR gene set expression is impaired in
703 HBV-infected mice. uPA-SCID mice were infected with HBV for 16 weeks. Mice were then
704 sacrificed and HBV infection was assessed by HBV RNA specific in situ hybridization (D) and
705 quantification of HBV viral load in the serum (Table 1). Human *MB21D1* expression was detected
706 in human hepatocytes by FISH from one HBV-infected mouse (D) and by qRT-PCR from 7 HBV-
707 infected mice and 4 control mice (E, left panel). Results are expressed as the ratio *MB21D1* mRNA
708 / *GAPDH* mRNA. All individual mice are presented as well as means \pm SD for each group (Mock-
709 and HBV-infected mice). The level of *MB21D1* expression was independent of the viability of
710 engrafted human hepatocytes as indicated by an absent correlation between *MB21D1* expression
711 and the human serum albumin expression in humanized mice ($R^2 = 0.231$, E, right panel). (F) The
712 IAR gene set was analyzed using the nCounter NanoString in mice 6472, 6251, and 6254 (Mock-

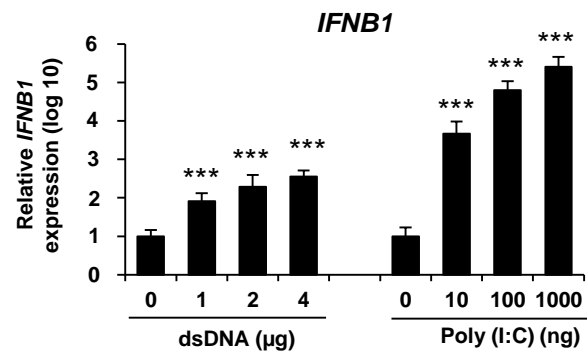
713 infected mice, Table 1) and 4766, 4771, and 4847 (HBV-infected mice, Table 1). A significant
714 downregulation (FDR = 0.047) of the gene set was observed in HBV-infected mice compared to
715 control mice. Individual Z-scores for the genes significantly modulated between the two groups
716 according to GSEA analysis are presented. Negative Z-score (blue) and positive Z-score (red)
717 correspond to repression and induction of the indicated genes, respectively. Dpi: days post
718 infection.

Figure 1.

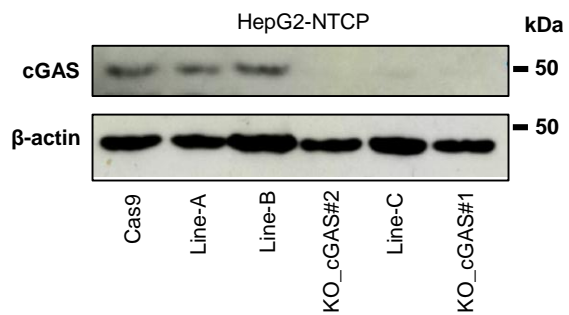
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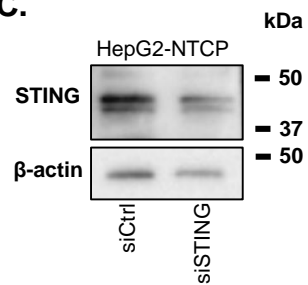
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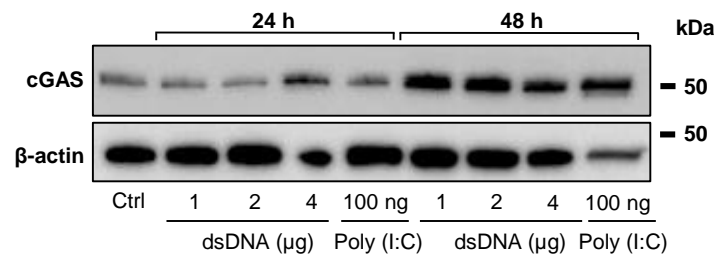
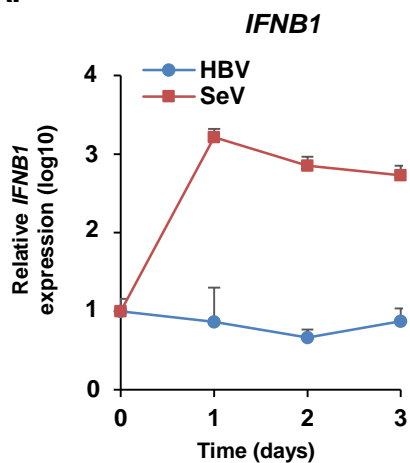
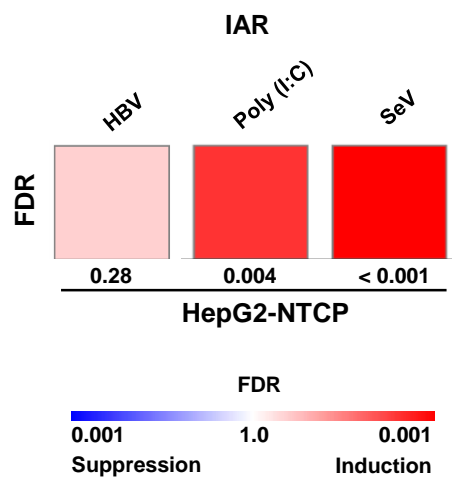


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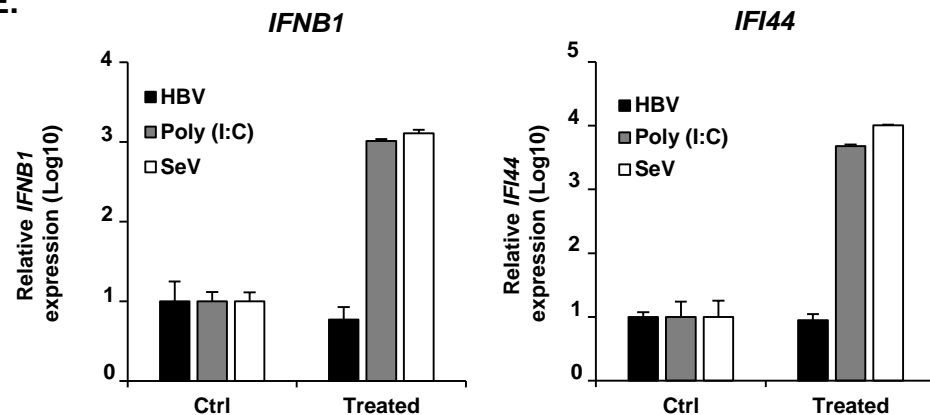
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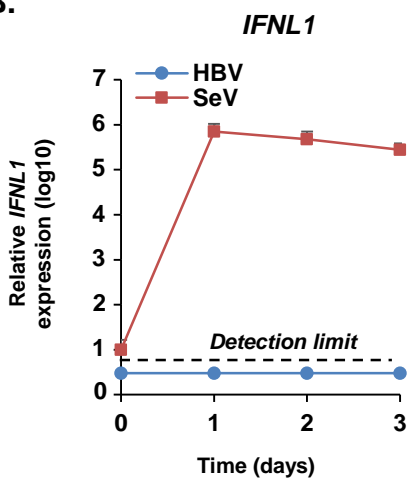
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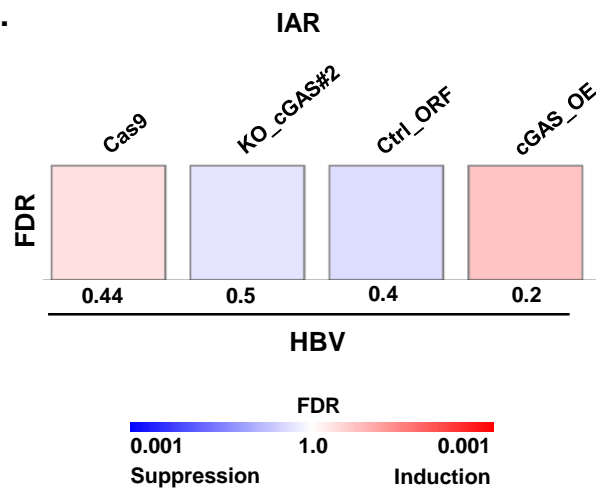
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F.

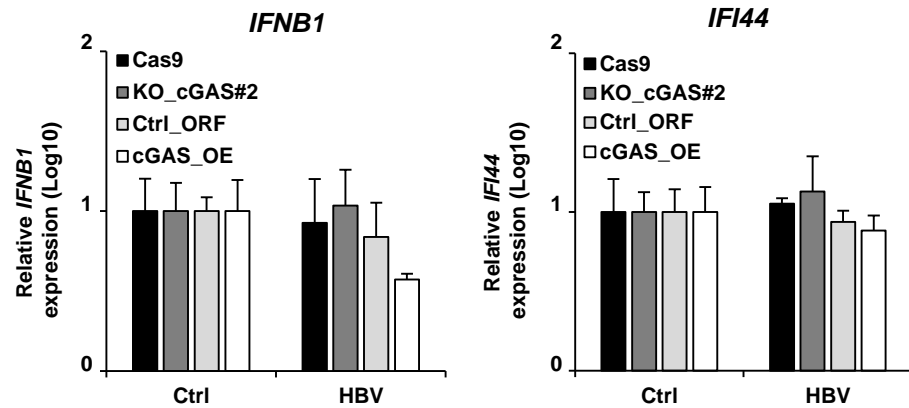
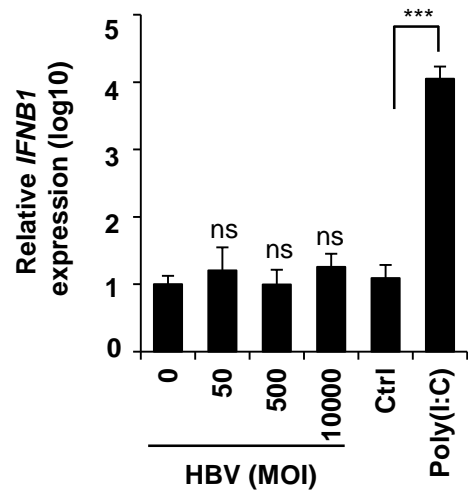
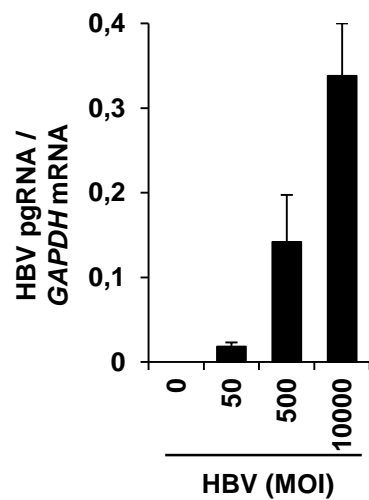


Figure 3.

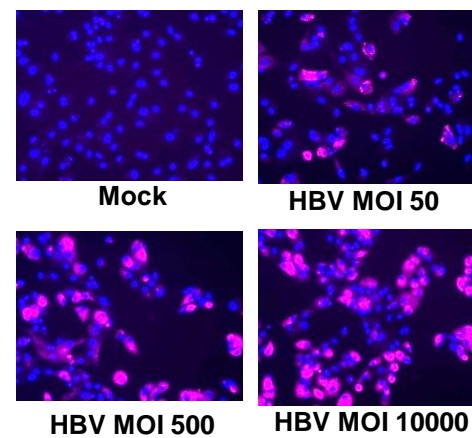
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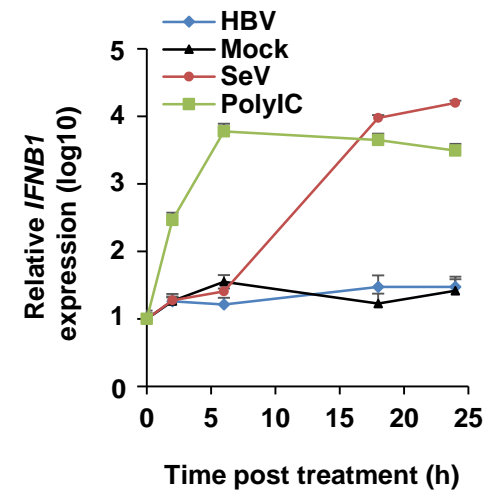
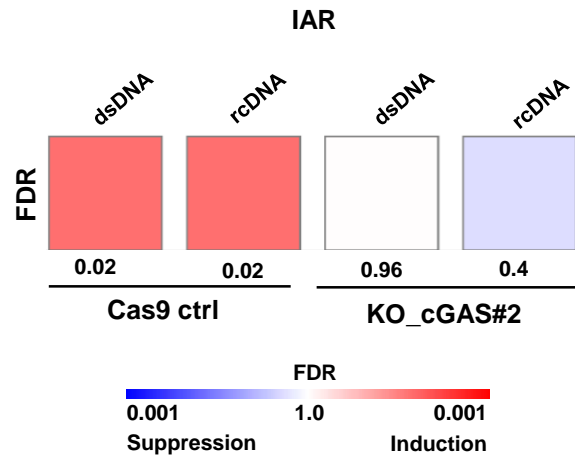
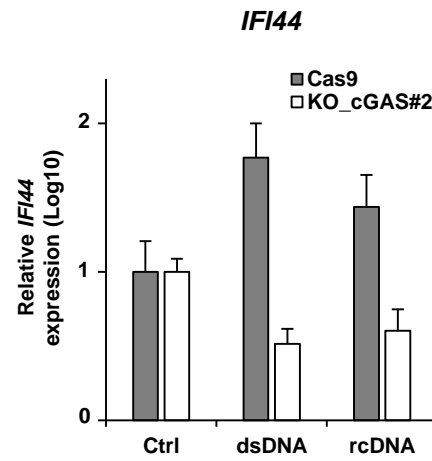
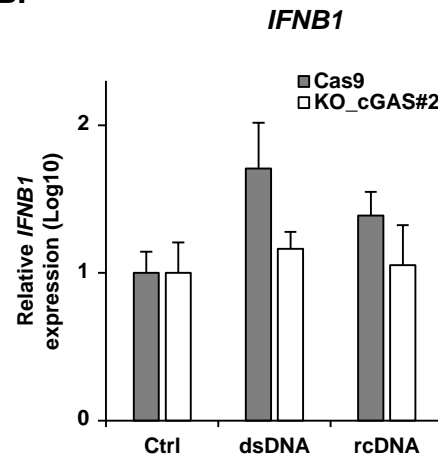


Figure 4.

A.



B.



C.

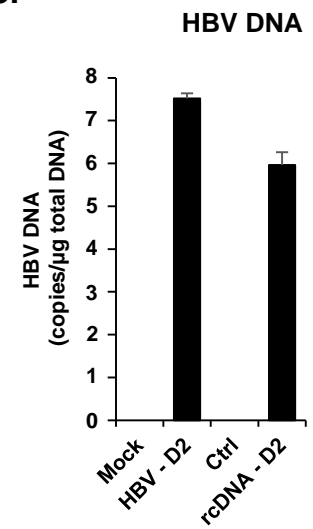


Figure 5.

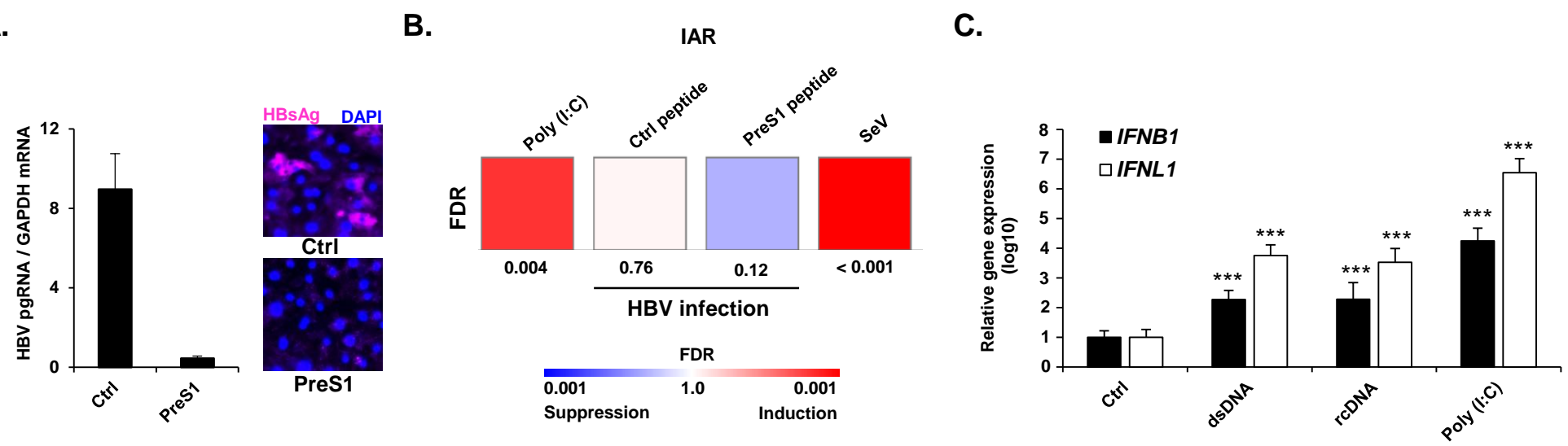


Figure 6.

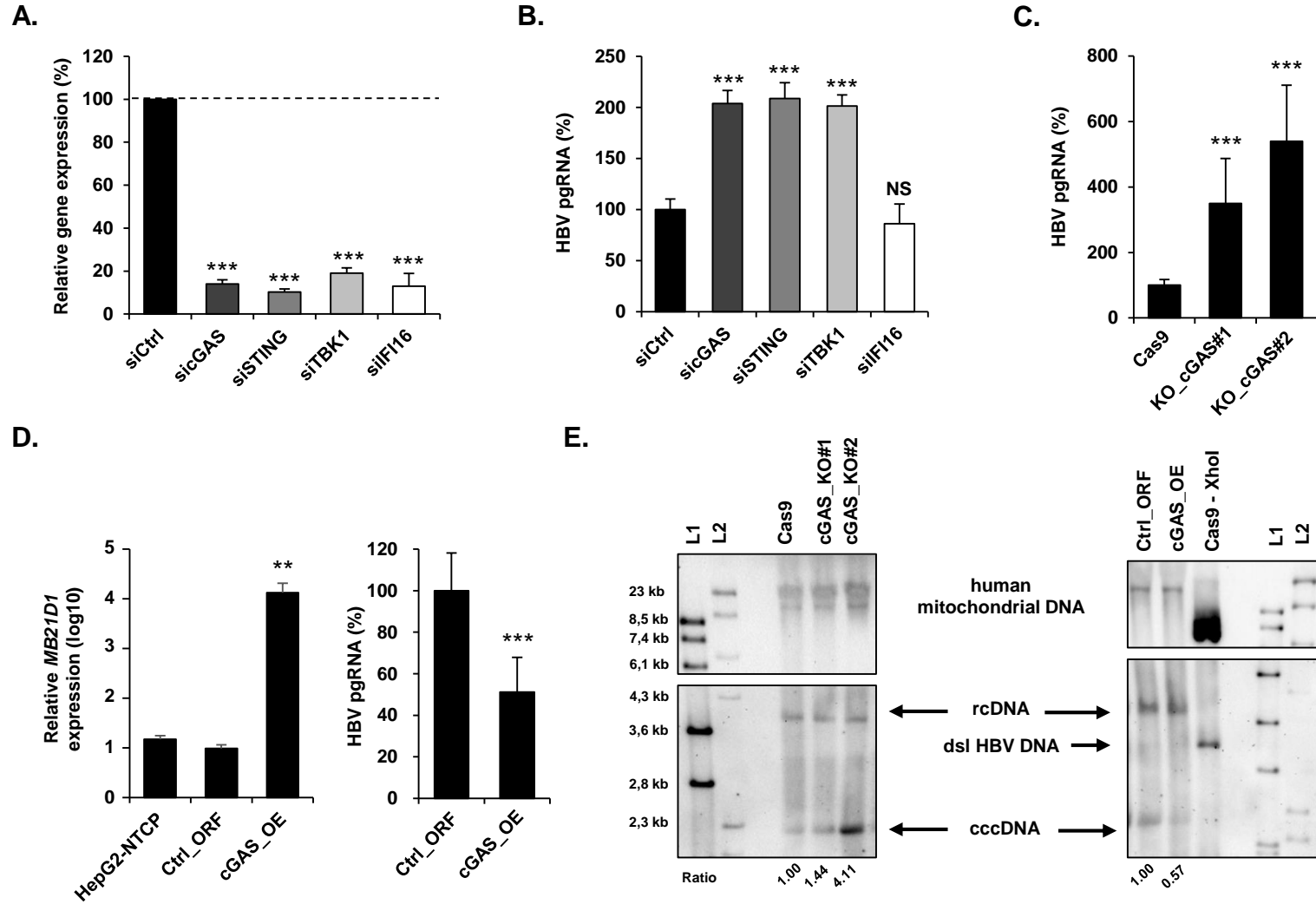
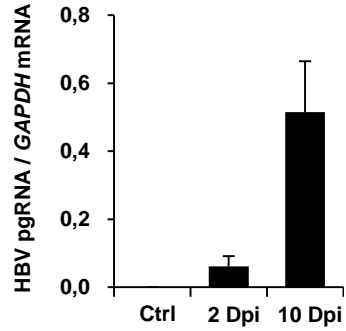
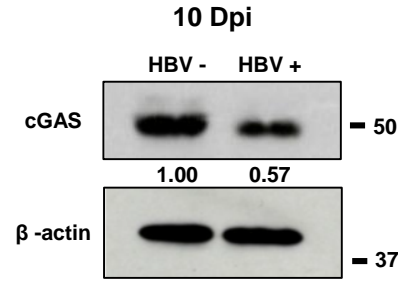


Figure 7.

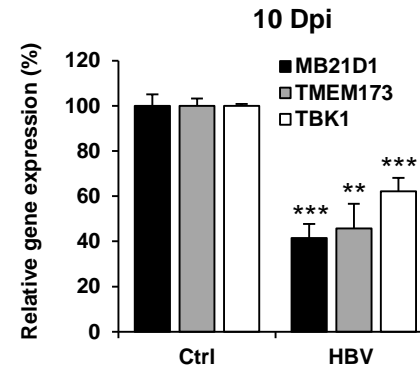
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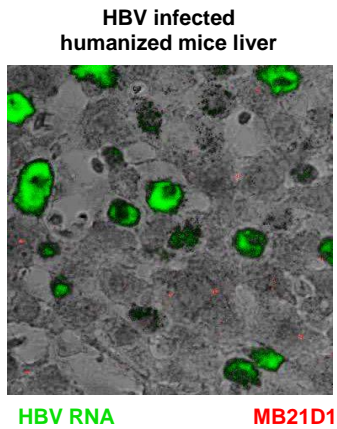
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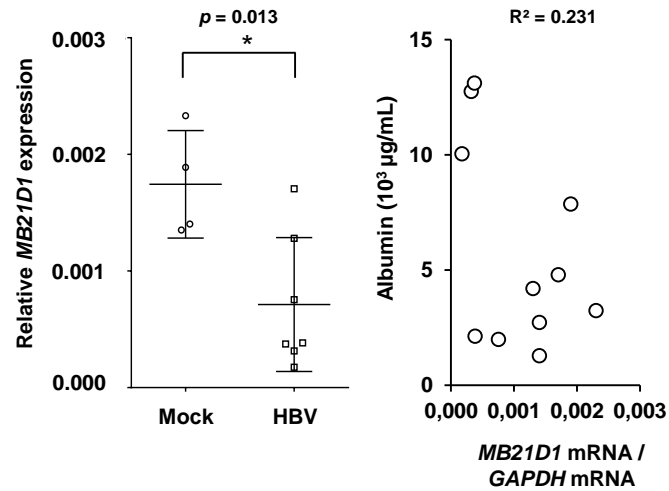
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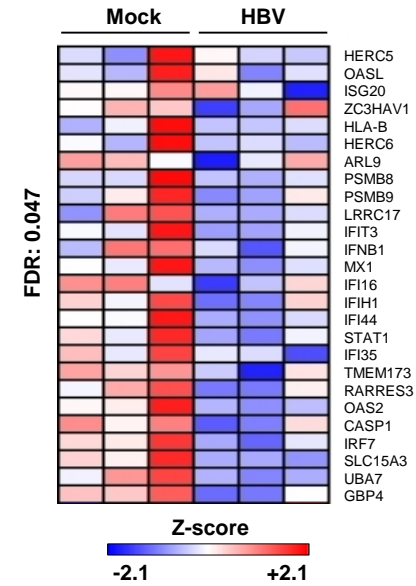
D.



E.



F.



Hepatitis B virus evasion from cGAS sensing in human hepatocytes

Eloi R. Verrier, Seung-Ae Yim, Laura Heydmann, Houssein El Saghire, Laurent Mailly, Charlotte Bach, Vincent Turon-Lagot, Sarah C. Durand, Julie Lucifora, David Durantel, Patrick Pessaux, Nicolas Manel, Ivan Hirsch, Mirjam B. Zeisel, Nathalie Pochet, Catherine Schuster and Thomas F. Baumert

SUPPORTING INFORMATION

Supplementary Experimental Procedures

Detection of cGAS protein expression using two independent antibodies. HepG2-NTCP cells were transfected with a siRNA targeting *MB21D1* expression (siC_{GAS}) or with a non-targeting siRNA control (siCtrl). Three days after transfection, cells were lysed and cGAS protein expression was assessed as described in the Experimental Procedures section using two rabbit polyclonal anti-cGAS antibodies (HPA031700, Sigma & NBP1-86761, Novus Biologicals). Given the proximity of the bands, another western blot was run in parallel for the detection of β -actin as a control. Molecular weights were assessed using the Precision Plus Protein™ Standards molecular weight marker (Bio-Rad).

Detection of HBV DNA by PCR and qPCR. DNA was extracted using QiaAMP DNA MiniKit (Qiagen) following manufacturer's instructions. The presence of HBV DNA was confirmed by PCR using the following primers (expected band size: 148 bp) (1) : forward primer 5'-CACCTCGCCTAATCATC-3', reverse primer 5'-GGAAAGAAGTCAGAAGGCA-3'. For qPCR quantification of HBV DNA, The presence of HBV DNA was confirmed by PCR and quantified by qPCR using the following primers and probe (1) : forward primer 5'-CACCTCGCCTAATCATC-3', reverse primer 5'-GGAAAGAAGTCAGAAGGCA-3'; TaqMan probe 5'-[6FAM]-TGGAGGCTTCAACAGTAGGACATGAAC-[BHQ1]-3'. Copy number of HBV was determined using a standard curve.

Detection of NTCP expression by flow cytometry. HepG2 cells, HepG2-NTCP-Ctrl_ORF cells, and HepG2-NTCP-cGAS_OE cells were treated with the AF647-labelled pres1 peptide for one hour at 37°C as described (1). Cells were then fixed with 2% paraformaldehyde for 20 minutes at room temperature. NTCP expression was then quantified by flow cytometry using MacsQuant instrument (Miltenyi).

Supplementary Figures

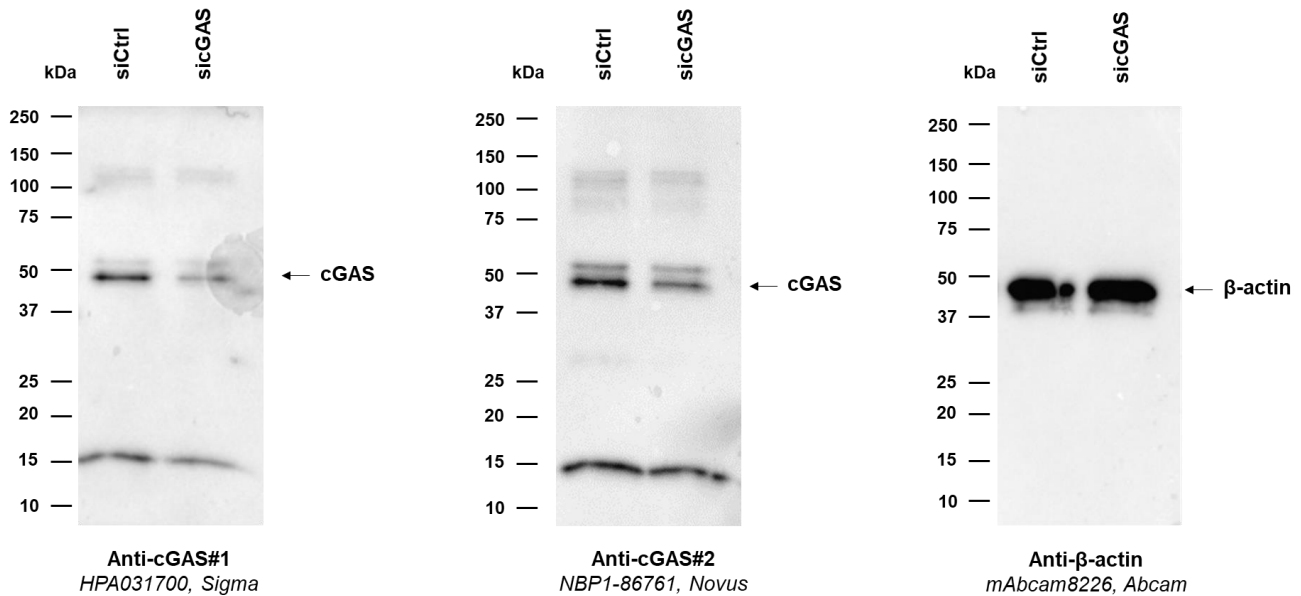


Figure S1. Detection of cGAS protein in HepG2-NTCP cells (related to Figure. 1). HepG2-NTCP cells were transfected for three days with a siRNA targeting *MB21D1* expression (sicGAS) or with a non-targeting siRNA control (siCtrl). Cells were then lysed and cGAS protein expression was assessed using two rabbit polyclonal anti-cGAS antibodies (HPA031700, Sigma, used in the main manuscript & NBP1-86761, Novus Biologicals). β-actin expression was assessed as a control. One representative experiment is shown.

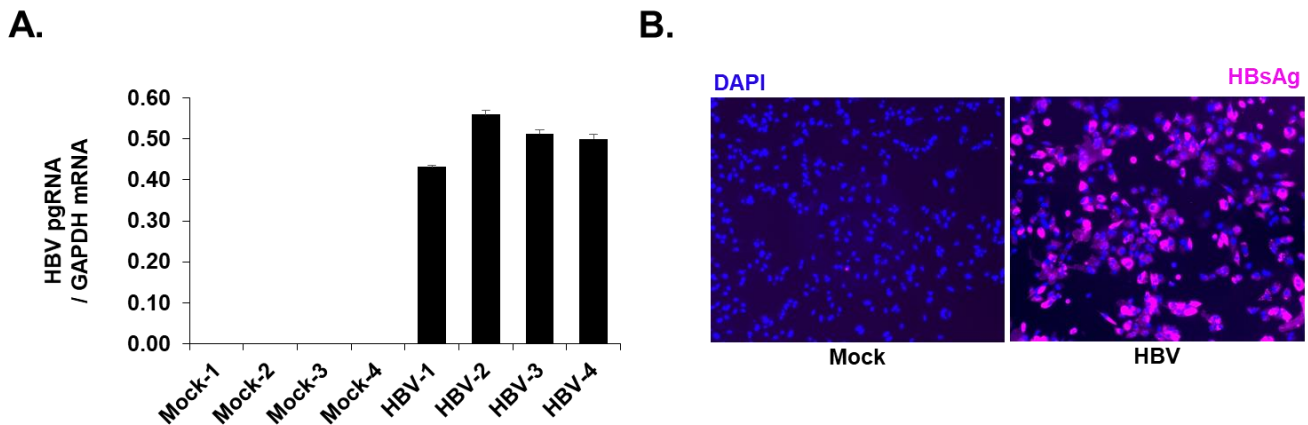


Figure S2. Analysis of HBV infection in HBV time course samples with quantification of HBV pregenomic RNA and HBsAg expression (related to Figure 2). HepG2-NTCP cells were infected with HBV as described in Experimental Procedures. 10 days after infection, total RNA was extracted and HBV infection was assessed by quantification of HBV pgRNA as described in Methods. Results are expressed as means \pm SD HBV pgRNA / GAPDH mRNA from four independent experiments performed in duplicate (corresponding to the four experiments shown in Figure 2A). Alternatively, cells were infected for 10 days in HBV, and HBV infection was assessed by immunofluorescence using an anti-HBsAg antibody. One representative experiment is shown.

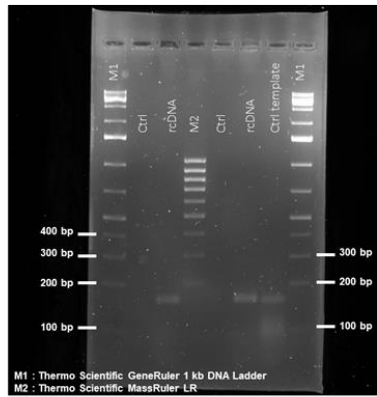
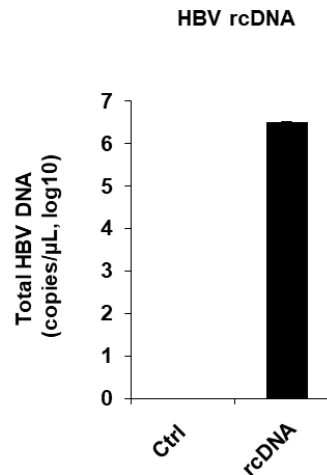
A.**B.**

Figure S3. Analysis and quantification of HBV DNA extracted from HBV infectious particles by PCR (related to Figure 4). A-B. HBV genomic DNA (rcDNA) was extracted from cell culture-derived HBV virions. Extraction from naive HepG2-NTCP control supernatants without virus was used as a control (Ctrl). HBV DNA standard preparation used as a template for the calculation of HBV DNA concentration was used as a positive control (Ctrl template). The presence of HBV DNA was controlled by PCR (expected band size: 148 base pairs [bp]) (A) and quantified by qPCR (B). Two independent experiments (A) and one experiment performed in triplicate (B) are shown. C. Quantification of total HBV DNA in transfected or infected cells. HepG2-NTCP were infected with HBV or transfected with rcDNA. Three days after transfection/infection, DNA was extracted and total HBV DNA was quantified by qPCR.

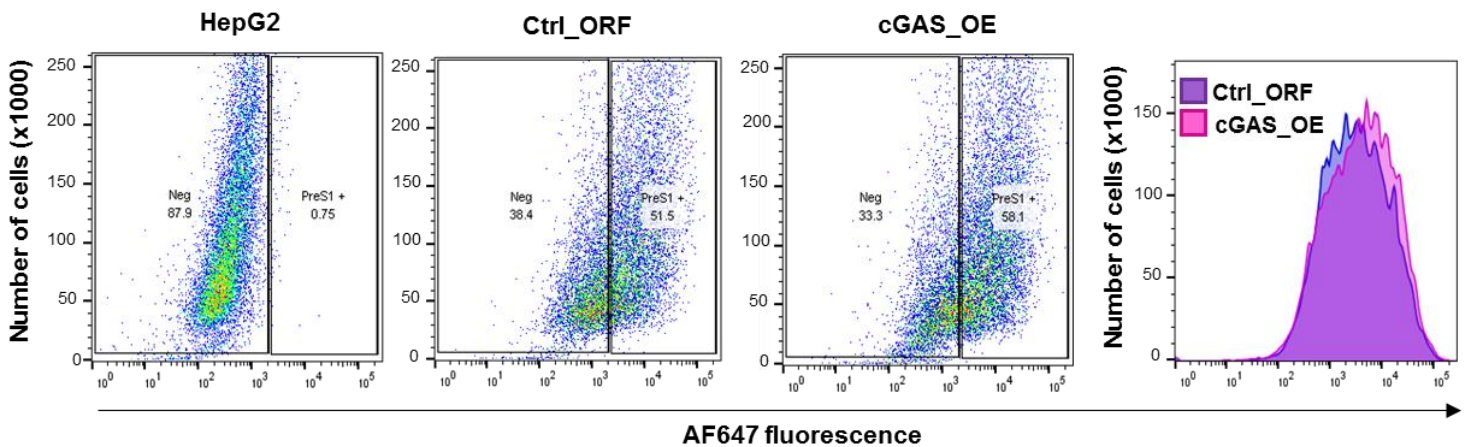


Figure S4. PreS1-binding/NTCP cell surface expression is independent on cGAS expression. HepG2 cells, HepG2-NTCP-Ctrl_ORF cells, and HepG2-NTCP-cGAS_OE cells were treated with the AF647-labelled preS1 peptide for one hour at 37°C. PreS1 binding corresponding to NTCP expression was quantified by flow cytometry.

Supplementary Tables

Tables S1: Specific probes used for the detection of HBV and Mitochondrial DNA (2).

Target	Name	Sequence
HBV	HBV-F1	TAGCGCCTCATTGTTGTGGT
	HBV-R1	CTTCCTGTCTGGCGATTGGT
	HBV-F2	TAGGACCCCTGCTCGTGTTA
	HBV-R2	CCGTCCGAAGGTTTGGTACA
	HBV-F3	ATGTGGTATTGGGGGCCAAG
	HBV-R3	GGTTGCGTCAGCAAACACTT
	HBV-F4	TGGAACCTTTTCGGCTCCTC
	HBV-R4	GGGAGTCCGCGTAAAGAGAG
	HBV-F6	TACTGCACTCAGGCAAGCAA
	HBV-R6	TGCGAATCCACACTCCGAAA
	HBV-F8	AGACGAAGGTCTCAATCGCC
	HBV-R8	ACCCACAAAATGAGGCGCTA
Mitochondrial DNA	Fw_huND1	CCCTACTTCTAACCTCCCTGTTCTTAT
	Rw_huND1	CATAGGAGGTGTATGAGTTGGTCGTA
	Fw_huND5	ATTTTATTTCTCCAACATACTCGGATT
	Rw_huND5	GGGCAGGTTTTGGCTCGTA
	Fw_huATP6	CATTTACACCAACCACCAACTATC
	Rw-huATP6	CGAAAGCCTATAATCACTGTGCC

Tables S2: Specific probes of the IAR gene set for multiplexed gene profiling analysis.

Gene	Accession Number	Target Sequence	Note
ATP5B	NM_001686.3	GAAATTCTGGTGACTGGTATCAAGGTTGTCGATCTGCTA GCTCCCTATGCCAAGGGTGGCAAATTGGGCTTTTTGGT GGTGCTGGAGTTGGCAAGACTG	HG
BAG6	NM_001199698.1	CATTGATCACGGGGCTAGAAGAGTATGTGCGGGAGAGTT TTTCCTTGGTGCAGGTTTCAGCCAGGTGTGGACATCATCC GGACAAACCTGGAATTTCTCCA	HG
NDUFA2	NM_001185012.1	ATGGGCTAGGCTTTAGGGTCCGCGTTGGTCAGACCGG AGCACTTGGCCTGAAGACCTGGAATTGGCGACTTCGATA TTAACAAGGATGGCGGCGGCCGC	HG
ARL9	NM_206919.1	CAGATATCCATGAAGCTTTGGCATTATCTGAAGTGGAAA TGACAGGAAGATGTTCTTGTGGAACTACCTGACTAAG AATGGCTCAGAGATACCCTC	Schoggins
CASP1	NM_001223.3	TGGAGACATCCACAATGGGCTCTGTTTTATTGGAAGA CTCATTGAACATATGCAAGAATATGCCTGTTCTGTGATG TGGAGGAAATTTCCGCAAGG	Schoggins
CXCL16	NM_001100812.1	CCATGGGTTCCAGGAATTGATGAGCTGTCTTGATCTCAA GAATGTGGACATGCTTACTCGGGGATTGTGGCCCACCAG AAGCATTACTTCTACCAGCC	Schoggins
CXCL8	NM_000584.2	ACAGCAGAGCACACAAGCTTCTAGGACAAGAGCCAGGAA GAAACCACCGGAAGGAACCATCTCACTGTGTGTAACAT GACTTCCAAGCTGGCCGTGGCT	Schoggins
HERC5	NM_016323.2	TGGGCTGCTGTTTACTTTCCGGTGGTGGAAAACATGGGCA ACTTGGTCATAATTCAACACAGAATGAGCTAAGACCCTGT TTGGTGGCTGAGCTTGTGGG	Schoggins

HERC6	NM_001165136.1	TCCATCACCCAGATTTATACTTAGAGTCAGACGAAGTCGC CTGGTTAAAGATGCTCTGCGTCAATTAAGTCAAGCTGAA GCTACTGACTTCTGCAAAGTA	Schoggins
HLA-B	NM_005514.6	CCCTGAGATGGGAGCCGCTTCCCAGTCCACCGTCCCC ATCGTGGGCATTGTTGCTGGCCTGGCTGTCTAGCAGTT GTGGTCATCGGAGCTGTGGTCGC	Schoggins
HLA-H	NR_001434.3	GAGCGGGAGGGGCCGAGTATTGGGACCGGAACACACA GATCTGCAAGGCCAAGCACGGACTGAACGAGAGAACC TGCGGATCGCGCTCCGCTACTACA	Schoggins
IFI35	NM_005533.3	TGCCCTCTGCTTGCAGGGCTCTGCTCTGATCACCTTTGAT GACCCCAAAGTGGCTGAGCAGGTGCTGCAACAAAAGGA GCACACGATCAACATGGAGGAGT	Schoggins
IFI44	NM_006417.4	GATGAAAGAAAGATAAAAGGGGTCAATTGAGCTCAGGAAG AGCTTACTGTCTGCCCTTGAGAACTTATGAACCATATGGAT CCCTGGTTCAACAAATACGAA	Schoggins
IFIH1	NM_022168.2	GCTTGGGAGAACCCTCTCCCTTCTCTGAGAAAGAAAGAT GTGGAATGGGTATTCCACAGACGAGAATTTCCGCTATCT CATCTCGTGCTTCAGGGCCAGG	Schoggins
IFIT3	NM_001031683.2	CGCTGCTAAGGGATGCCCTTCAGGCATAGGCAGTATT TTCCTGTCAGCATCTGAGCTTGAGGATGGTAGTGAGGAA ATGGGCCAGGGCCGAGTCAGCT	Schoggins
ISG20	NM_002201.5	AGCCCGCCGAGGGCTGCCCGCCTGGCTGTGTCAGACT GAAGCCCCATCCAGCCCGTCCGCAGGGACTAGAGGCT TTCGGCTTTTTGGGACAGCAACTA	Schoggins
LRRC17	NM_001031692.1	CAGCACAACCAGATCAAAGTCTTGACGGAGGAAGTGTT ATTTACACACCTCTCTTGAGCTACCTGCGTCTTTATGACA ACCCCTGGCACTGTACTTTG	Schoggins
MX1	NM_002462.2	GCCTTTAATCAGGACATCACTGCTCTCATGCAAGGAGAG GAAACTGTAGGGGAGGAAGACATTCGGCTGTTTACCAGA CTCCGACACGAGTTCCACAAAT	Schoggins
OAS2	NM_016817.2	TGAAAAACAATTTTCGAGATCCAGAAGTCCCTTGATGGGTT CACCATCCAGGTGTTCAAAAAAATCAGAGAATCTCTTTC GAGGTGCTGGCCGCCTCAA	Schoggins
OASL	NM_198213.1	GGCGTTTCTGAGCTGTTTCCACAGCTTCCAGGAGGCAGC CAAGCATCACAAAGATGTTCTGAGGCTGATATGAAAAAC CATGTGGCAAAGCCAGGACCTG	Schoggins
PLCG2	NM_002661.2	GCTTGAAAATCTTACACCAGGAAGCGATGAATGCGTCCA CGCCCACCATTATCGAGAGTTGGCTGAGAAAGCAGATAT ATTCTGTGGATCAAACCAGAAG	Schoggins
PSMB8	NM_004159.4	ACTCACAGAGACAGCTATTCTGGAGGCGTTGTCAATATG TACCACATGAAGGAAGATGGTTGGGTGAAAGTAGAAAGT ACAGATGTCAGTGACCTGCTGC	Schoggins
PSMB9	NM_002800.4	TCAGGTATATGGAACCCTGGGAGGAATGCTGACTCGACA GCCTTTTGCCATTGGTGGCTCCGGCAGCACCTTTATCTA TGTTATGTGGATGCAGCATAT	Schoggins
RARRES3	NM_004585.3	CTGACCCCTCGTCCCTGTCTCAGGCGTTCTCTAGATCCT TTCCTCTGTTTCCCTCTCTCGCTGGCAAAGTATGATCTA ATTGAAACAAGACTGAAGGAT	Schoggins
SLC15A3	NM_016582.1	GCCGCTTCTTCAACTGGTTTTACTGGAGCATCAACCTGG GTGCTGTGCTGTGCTGCTGGTGGTGGCGTTTATTACAGC AGAACATCAGCTTCTGCTGGG	Schoggins
TNFRSF1B	NM_001066.2	CCCAGCTGAAGGGAGCACTGGCGACTTCGCTTCCAGT TGGACTGATTGTGGGTGTGACAGCCTTGGGTCTACTAAT AATAGGAGTGGTGAACGTGTGC	Schoggins
UBA7	NM_003335.2	GCGGGAGGATGGTCCCTGGAGATTGGAGACACAACAA CTTTCTCTCGGTACTTGCCTGGTGGGGCTATCACTGAAG TCAAGAGACCCAAGACTGTGAGA	Schoggins
UBE2L6	NM_004223.3	TGTTTCAAACCCTTGCATCCTGTTAGATTGCCAGTTC CTGGGACCAGGCCTCAGACTGTGAAGTATATATCCTCCA GCATTACAGTCCAGGGGGAGCC	Schoggins
ZC3HAV1	NM_020119.3	CTCCTTCTTACATCGTAGAAACATGGCATATAGGGCTAG AAGCAAGAGTAGAGATCGGTTCTTTAGGGCAGCCAAGA ATTTCTTGGCTGTGCTCAGC	Schoggins
ZMYND15	NM_032265.1	CCTCAGAGCGGCCGACAACACTGCATGTCCTGGTACTGCAA TGCCCTTCACTTCCACCTGGTTTACAAGCCTGCTCAAGG GAGCGGGGCCCGCCCGGCC	Schoggins

PMAIP1	NM_021127.2	CTAGTGTTTTTGCCGAAGATTACCGCTGGCCTACTGTGA AGGGAGATGACCTGTGATTAGACTGGGCGGCTGGGGAG AAACAGTTCAGTGCATTGTTGTT	Schoggins
GBP4	NM_052941.4	TTCTACAAGATATGCCATGGGCCTTTTCACAGGGGACAC AGGCTTCTTAAACAACCCGGCTTCCTCACCCCTATGTCCT TTATTTACAAAGCTGTGCTCC	Schoggins
TMEM173	NM_198282.1	CTGGCATGGTCATATTACATCGGATATCTGCGGCTGATC CTGCCAGAGCTCCAGGCCCGGATTCTGAACCTTACAATCAG CATTACAACAACCTGCTACGGG	STING
IFI16	NM_005531.1	ACGACTGAACACAATCAACTGTGAGGAAGGAGATAAACT GAAACTCACCAGCTTTGAATTGGCACCGAAAAGTGGGAA TACCGGGGAGTTGAGATCTGTA	
IFNB1	NM_002176.2	ACAGACTTACAGGTTACCTCCGAAACTGAAGATCTCCTA GCCTGTGCCTCTGGGACTGGACAATTGCTTCAAGCATTC TTCAACCAGCAGATGCTGTTTA	
IRF3	NM_001571.5	TCATGGCCCCAGGACCAGCCGTGGACCAAGAGGCTCGT GATGGTCAAGGTTGTGCCACGTGCCTCAGGGCCTTGG TAGAAATGGCCCGGTAGGGGGTG	
IRF7	NM_001572.3	CGCAGCGTGAGGGTGTGTCTTCCCTGGATAGCAGCAGC CTCAGCCTCTGCCTGTCCAGCGCCAACAGCCTCTATGAC GACATCGAGTGCTTCCCTTATGGA	
STAT1	NM_139266.1	ACAGTGTTAGAAAAGCAAGACTGGGAGCACGCTGCCAA TGATGTTTTCATTTGCCACCATCCGTTTTTCATGACCTCCTG TCACAGCTGGATGATCAATAT	
TBK1	NM_013254.2	ACCAGTCTTCAGGATATCGACAGCAGATTATCTCCAGGT GGATCACTGGCAGACGCATGGGCACATCAAGAAGGCAC TCATCCGAAAGACAGAAATGTAG	

HG: Housekeeping genes

Schoggins: cGAS-related genes described by Schoggins et al., (3)

Supplementary References

1. Verrier ER, Colpitts CC, Bach C, Heydmann L, Weiss A, Renaud M, Durand SC, et al. A targeted functional RNAi screen uncovers Glypican 5 as an entry factor for hepatitis B and D viruses. *Hepatology* 2016;63:35–48.
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3. Schoggins JW, MacDuff DA, Imanaka N, Gainey MD, Shrestha B, Eitson JL, Mar KB, et al. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* 2014;505:691-695.