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Hepatitis D virus interferes with hepatitis B virus RNA production via interferon-dependent and -independent mechanisms

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1 **Interferon-dependent and independent mechanisms drive**
2 **Hepatitis Delta Virus interference on Hepatitis B Virus RNA production**
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46 **Key Words**

47 Hepatitis Delta virus; Hepatitis B virus; interference, interferon
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49

50 **List of abbreviations**

51 AAV, adeno-associated virus;

52 cccDNA, covalently closed circular DNA;

53 CHIP, chromatin immunoprecipitation;

54 dpi, days post-infection;

55 HDag: HDV antigens;

56 HBV, Hepatitis B virus;

57 HCV, Hepatitis C virus;
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1 HDV, hepatitis Delta virus;
2 HDAg, HDV antigens;
3 HuHep mice, liver humanized mice i.e mice with human hepatocytes;
4 IFN, interferon;
5 ISG, interferon-stimulated gene;
6 LHD, large hepatitis delta antigen;
7 moi, multiplicity of infection;
8 rcDNA relaxed circular DNA;
9 pgRNA, pregenomic RNA;
10 PHH, primary human hepatocyte;
11 RIP, RNA immunoprecipitation;
12 SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2;
13 SHD, small hepatitis delta antigen;
14 wpi, weeks post-infection.

19 **Author contributions**

- 20 - study concept and design: JL, DD, AS
- 21 - acquisition of data: JL, DA, CP, MM, FF, FA, AR, AFL, EC, SV, RF, CB
- 22 - analyses and interpretation of data: JL, RR, ERV, AS, DD
- 23 - drafting of the manuscript: JL
- 24 - funding acquisition: JL, ERV, TFB, AS, DD
- 25 - material support: MR, EV, AH, RB, FZ, AB, HB, FLC, SF, GP, NG

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3

4 **Competing financial interest**

5 JL and DD received a research grant from Gilead Sciences to perform experiments. RR, AH,
6 RKB and SPF are employees of Gilead Sciences, Inc.
7

8 **Data Availability Statement**

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10 The data presented in this manuscript are available through the corresponding author (Julie
11 Lucifora) upon reasonable request.
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Abstract

Background & Aims. Chronic co-infection with hepatitis B and D viruses (HBV and HDV) leads to the most aggressive form of chronic viral hepatitis. Here, we aimed at elucidating the molecular mechanisms leading to the interference of HDV on HBV observed in most co-infected patients.

Methods. Patient liver tissues, primary human hepatocytes, HepaRG cells and human liver chimeric mice were used to analyze the effect of HDV on HBV using virological and RNA-seq analyses, as well as RNA synthesis, stability and association assays.

Results. Transcriptomic analyses in cell culture and mouse models of co-infection allowed to define the HDV-induced signature mainly composed of interferon (IFN)-stimulated genes (ISGs). We also provide evidence that ISGs are upregulated in HDV-HBV chronically infected patients but not in cells only expressing the HDV antigens (HDAg). Inhibition of the hepatocyte IFN response partially rescued the levels of HBV parameters. We observed less HBV RNAs synthesis upon HDV infection or HDV protein expression. Additionally, HDV infection or expression of HDAg alone specifically accelerated the decay of HBV RNAs and HDAg are associated with HBV RNAs. On the contrary, HDAg expression did not affect other viruses such as hepatitis C virus (HCV) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Conclusions. Our data indicate that HDV interferes with HBV through both IFN-dependent and IFN-independent mechanisms. Specifically, we uncover a new viral interference mechanism in which proteins of a satellite virus affect RNA production of its helper virus. Exploiting these finding could pave the way to the development of new therapeutic strategies against HBV.

Impact and implications

Although, the molecular mechanisms remained unexplored, it was known for long that despite its dependency, HDV decreased HBV viremia in patients. Here, using *in vitro* and *in vivo* models, we showed that HDV interferes with HBV through both IFN-dependent and IFN-independent mechanisms affecting HBV RNAs metabolism and we defined the HDV-induced modulation signature. The mechanisms we uncovered could pave the way to the development of new therapeutic strategies against HBV by mimicking and/or increasing the effect of HDAg on HBV RNAs and the HDV-induced modulation signature may allow to the draw correlation

withthe responsiveness to IFN alpha treatment and thereby ultimately help in the management of HBV/HDV co-infected patients.

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Introduction

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4 Hepatitis B Virus (HBV) functionally persists in the nucleus of hepatocytes as a non-integrated,
5 episomal DNA, the so-called covalently closed circular DNA (cccDNA) that serves as template
6 for transcription of all HBV RNAs by the cellular RNA polymerase II (RNA Pol-II). It thereby
7 drives production of viral RNAs, antigens (HBeAg and HBsAg), and progeny virions (containing
8 a partially double-stranded relaxed circular DNA (rcDNA) that results from reverse
9 transcription of a pregenomic RNA (pgRNA) within assembled nucleocapsid). CccDNA is
10 organized into a chromatin-like structure that displays the typical beads-on-a-string
11 arrangement in electron microscopy [1, 2]. Viral proteins (HBx, HBc), host histones but also
12 non-histone cellular proteins are directly or indirectly bound to cccDNA [3-5] and its
13 transcriptional activity is subjected to epigenetic regulations [4, 6, 7]. As an example, the
14 recruitment of H3 and H4 histones, as well as of several cellular transcription factors and
15 histone modifying enzymes has been shown to modulate cccDNA transcriptional activity [4, 6,
16 8]. Hepatitis Delta Virus (HDV) contains a ribonucleoprotein composed of a circular single-
17 stranded negative RNA genome, presenting a “quasi” double-stranded conformation, coated
18 with both small and large isoforms of the HDV antigen (HDAg). HDV is a defective satellite
19 virus that uses the envelope of HBV [9] to egress from and to re-enter into hepatocytes. Apart
20 from those steps, HDV intracellular RNA replication is thought to be independent of HBV. In
21 the nucleus of HDV-infected cells, incoming HDV genomes serve as templates for the synthesis
22 of replicative intermediates called anti-genomes, as well as for HDV mRNAs transcription by
23 the RNA Pol II [10, 11]. Two isoforms of HDAg are produced upon the HDV life cycle: S-HDAg
24 and L-HDAg that are essential for replication and secretion, respectively (see [12] for review).

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47 HDV co-infection has been shown to reduce HBV replication in the majority of HBV/HDV co-
48 infected patients as well as in different animal models [9, 13-22]. A cross-sectional study in a
49 small cohort of HBV/HDV infected patients reported HDV dominance in 75% of the cases [23].
50 There is limited understanding of how HDV interferes with HBV. Interference is thought to be
51 independent of adaptive immunity since it was also observed in isolated hepatocytes in the
52 absence of immune cells [24, 25]. Overexpression models suggested that both forms of HDAg
53 might inhibit HBV replication by repressing the HBV enhancer activity [26]. Alternatively, we
54 found that the levels of HBV RNAs, but not of cccDNA, were decreased in the presence of HDV

1 in non-transformed and immune-competent hepatocytes infected *in vitro*, suggesting an HDV-
2 induced inhibition of HBV RNAs transcription or stability [24]. Here, we aimed to better
3 characterize the mechanism(s) underlying interference of HDV on HBV using highly relevant
4 infection models including primary human hepatocytes (PHH), differentiated HepaRG cells
5 (dHepaRG), liver chimeric humanized mouse model (HuHep) as well as patient liver tissues. In
6 addition to advance our current knowledge on the interplay between a satellite virus and its
7 helper virus, these data may help identifying new antiviral targets for treatment of both
8 viruses.
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Results

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4 **HDV infection impairs HBV replication *in vivo* and *in vitro*.** We previously reported that super-
5 infection of HBV-infected differentiated HepaRG cells with HDV results in lower levels of HBV
6 replication [24] similarly to what was previously described in HDV-infected patients [16-21].
7 We confirmed an interference of HDV on HBV in a co-infection setting *in vitro* using dHepaRG
8 cells and PHH, in which a decrease in intracellular HBV RNAs as well as secreted HBeAg and
9 HBsAg, but not cccDNA, in co-infected vs. HBV mono-infected cells was observed (**Figure 1A,**
10 **1B and S1**). Northern blot analyses showed that the levels of all detectable HBV RNA species
11 are affected by HDV co-infection (**Figure 1A, 1B**). On the contrary, the presence of HBV had
12 no impact on the level and kinetics of intracellular HDV replication (**Figure S1**, left panels) and
13 no cell toxicity (measured by LDH release in cells supernatants) was observed in the presence
14 of HDV (**Figure S2**). To further confirm the interference of HDV on HBV *in vivo* and extend the
15 data published in uPa-SCID mice reporting a decreased HBV viremia in the presence of HDV
16 [15], FRG liver humanized mice (HuHep) were either infected with HBV alone (HBV), co-
17 infected with HBV and HDV (HBV + HDV) or infected with HBV first and then super-infected
18 with HDV, four weeks later (HBV->HDV). Except for the levels of cccDNA, all the other HBV
19 replication parameters, including intrahepatic HBV DNA, viremia and the two secreted viral
20 antigens (HBeAg and HBsAg) decreased in the presence of HDV (**Figure 1C**). The interference
21 of HDV on HBV was more pronounced in the co-infection setting compared to the super-
22 infection setting (**Figure 1C**). Interestingly, in each individual super-infected mouse, the peaks
23 of HDV RNA correlated with a decrease in HBV parameters (**Figure S3**). Using dHepaRG cells
24 with an integrated HBV genome or HBV-infected HuHep mice, we observed that HDV also
25 interfered with other HBV genotypes (C and E) than HBV genotype D (mainly used in our study)
26 (**Figure S4**).

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50 **Identification of the HDV-induced signature.** We and others did show that, in contrast to HBV
51 genotype D, HDV genotype 1 induced a strong increase in mRNA levels of classical interferon
52 (IFN) stimulated genes (ISGs), such as MX1, OAS1 or RSAD2 [24, 26, 27]. We further confirmed
53 these observations in HDV-infected PHH, from three different donors, in which induction of
54 the RSAD2 (coding for Viperin) ISG occurred concomitantly to HDV replication (**Figure S5A,**
55 **S1**). To identify host factors consistently modulated by HDV in an unbiased approach, we
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1 performed RNA sequencing analyses of samples from dHepaRG or PHH infected with HDV
2 and/or HBV (co-infections or mono-infection), as well as liver samples from infected HuHep
3 mice. First, we confirmed that HBV did not induce strong transcriptional modulations *in vitro*
4 [28] since no significant changes in the levels of any cellular RNA were observed in HBV
5 infected cells at day six post-infection (**Figure 2**). Conversely, HDV induced strong changes in
6 the levels of cellular mRNAs (**Figure 2**) in dHepaRH and PHH that were similar whether HBV
7 was present or not (**Figure 2**). Pathway analyses revealed that innate-immune related
8 pathways, and notably the IFN pathway, are consistently up-regulated upon HDV infection
9 (**Figure 2A**) and that most of the significant HDV-induced genes in dHepaRG and PHH were
10 also induced following a 24h treatment with IFN- α (non-infected cells, **Figure S5B**). We
11 identified a subset of 73 ISGs that were significantly up-regulated across the three models that
12 likely represent a “HDV signature” of virus-induced perturbations (**Figure 2B**). Since RSAD2
13 was among the most up-regulated genes in HDV-infected hepatocytes, it was used as a
14 biomarker of the HDV-induced IFN pathway throughout the rest of the study. Interestingly, a
15 similar modulation of IFN-related genes was observed in patients. Despite a generally more
16 advanced liver disease in HBV mono-infected patients in our cohort (**Table1**), possibly
17 suggesting a higher level of inflammation, IFN-related genes were more strongly induced in
18 HBV-HDV co-infected patients (**Figure 2A**). Notably, a set of ISGs that was the most strongly
19 induced by HDV in cells and mice was found significantly and specifically associated to HDV
20 co-infection in patients, such as MX1, RSAD2, IFITM1, OASL, IFIT3, and IFI27 (**Figure 2B**).

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41 **The HDV-induced IFN response is partially responsible for the interference on HBV.** Since
42 some genes up-regulated by HDV have been previously described to be capable of inhibiting
43 HBV (**Figure 2**), we hypothesized that the interference of HDV on HBV could be due to its
44 capacity to induce the IFN pathway. We therefore performed a series of experiments to
45 analyze HBV parameters in the absence of the HDV-induced IFN pathway. First, we used TPCA-
46 1, a chemical inhibitor of the IKKb and IFN pathways [29]. In a co-infection setting and in the
47 presence of TPCA-1, the induction of the IFN pathway by HDV (measured here by RSAD2 and
48 MxA expression) was markedly reduced (**Figure 3A, S6A**) and we observed a partial rescue of
49 the levels of secreted HBV antigens and particles as well as intracellular HBV RNAs (**Figure 3B,**
50 **S6A**). We confirmed these data using JAK1 inhibitors (**Figure S6B, S6C**). Second, as MDA5 has
51 been described to be the cellular receptor responsible for HDV recognition and induction of
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1 the IFN pathway in hepatocytes [30], we generated an MDA5 knock-out (KO) HepaRG cell line
2 using a CRISPR/Cas9 approach and IRF3_{KO} cell line to block intracellular induction of ISGs. We
3 also generated a Cas9 control (CTL) cell line as well as a RIG-I_{KO} control cell line (RIG-I not being
4 involved in the HDV-induced IFN pathway) [30]. As RIG-I and MDA5 are almost not detectable
5 at basal levels, treatment with poly(I:C) or IFN- α (**Figure S7A**) as well as infection with HDV
6 (**Figure S7B**) were performed to confirm the knock-out phenotype of the different cell lines.
7 Moreover, even though differences in viral replications were probably due to their
8 differentiation status, all cell lines were still susceptible to both HBV and HDV infections
9 (**Figure 4A**) and exogenous IFN- α was also still efficient to inhibit both viruses in all the cell
10 lines (**Figure S7C**). As expected, HDV did not induce any response in dHepaRG-Cas9-MDA5_{KO}
11 [30], but also not in dHepaRG-Cas9-IRF3_{KO} cells, whereas it did induce ISGs in both dHepaRG-
12 Cas9-CTL and dHepaRG-Cas9-RIG-I_{KO} cells (**Figures 4B, 4C**). Again, we observed a partial rescue
13 of the levels of HBV parameters in all the cells that displayed a lack of HDV-induced IFN
14 response (**Figure 4D, 4E, 4F**). Of note, levels of HDV RNAs and proteins are only slightly
15 increased in dHepaRG-Cas9-MDA5_{KO} compared to dHepaRG-Cas9-RIG-I_{KO} cells and kinetics of
16 appearance and decrease are similar (**Figure S7D**). Altogether, our results show that the
17 induction of the IFN pathway by HDV is only partially responsible for the inhibition of HBV and
18 suggest that HDV itself may play a direct role, independent of the IFN response, likely through
19 its proteins as previously documented [26].
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39 **HDV proteins expression does not induce an IFN response but specifically reduces HBV in**
40 **cell-based models.** We next addressed the direct role of HDV proteins in the interference of
41 HDV on HBV. In dHepaRG cells either stably producing S-HDAg and L-HDAg in a Tet-inducible
42 manner or transduced with recombinant AAV vectors expressing these genes (AAV-SHD and
43 AAV-LD), the two viral proteins were mainly expressed in hepatocytes, exclusively located in
44 the nucleus (**Figure S8A, S9A**) and did not induce an IFN response as shown by the absence of
45 RSAD-2 and RIG-I expression modulation (**Figure S8B, S8C, S9B, S9C**). Of note, probably due
46 to a leak in the inducible system, the HepaRG-TR-LHD cells that expressed low but detectable
47 levels of L-HDAg in the absence of Tet (**Figure S8B**), could not be productively infected by HDV
48 as evidenced by a lack of detection of S-HDAg (**Figure S8B, HBV+HDV condition**) and RSAD2
49 induction (**Figure S8C, HBV+HDV condition**), confirming data from previous studies suggesting
50 that even low levels of L-HDAg expression have a negative trans-dominant effect on HDV
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1 replication [31, 32]. We transduced HBV-infected dHepaRG cells or HuH7.5-NTCP cells with
2 AAV-SHD and AAV-LHD vectors (**Figure 5A, S10A**) and observed a decrease of the levels of HBV
3 RNAs and secreted HBV antigens (**Figure 5B, S10B**) without induction of ISGs (**Figure 5B, S8B,**
4 **S8C, S9B, S9C**) or profound modulations in the levels of HNF4a, a transcription factor crucial
5 for the synthesis of HBV RNAs (**Figure 5B**). Importantly, expression of S-HDAg or L-HDAg had
6 no effect on the levels of intracellular RNAs for other viruses such as hepatitis C virus (HCV) or
7 SARS-Cov2 (**Figure S10C, S11**) strongly suggesting a specific phenotype on HBV. Of note, we
8 already reported rather low rates of HBV/HDV co-positive cells [24] and confirmed that with
9 liver slices from co-infected HuHep mice (**Figure S12A**) as also already reported by others [27].
10 To exclude the involvement of secreted cellular factors (unrelated to the IFN response)
11 following HDV infection/HDAg expression that could act in cis or trans, HBV-infected dHepaRG
12 cells were treated with fresh undiluted media from cells transduced with AAV-SHD or AAV-
13 LHD (that do not induce an IFN response) and we did not observe any reduction in HBV
14 parameters (**Figure S12B**) strongly suggesting that HBV/HDV co-infection of the same cells is
15 needed for the interference of HDAg on HBV.
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31 **HDV decreases the level of nascent HBV RNAs and their stability.** The decrease in HBV RNAs
32 observed with no effect on cccDNA amounts suggests that HDV may affect either HBV RNAs
33 transcription and/or stability. We thus performed run-on and RNA stability assays to test these
34 hypotheses. We first confirmed the specificity of both assays using RG7834, a molecule known
35 to decrease the levels of HBV RNAs (**Figure 6A**) [33] without affecting their synthesis (**Figure**
36 **6B, 6C**) but by reducing their stability (**Figure 6D, 6E**) [33], and IFN- α , known to affect both
37 HBV RNAs synthesis (**Figure 6B, 6C**) and stability (**Figure 6D**) [34]. We observed a decrease in
38 the levels of nascent HBV RNAs in dHepaRG cells co-infected with HDV or transduced with
39 AAV-SHD and AAV-LHD (**Figure 6B, 6C**) without any significant effect on the levels of nascent
40 cellular *PRNP* mRNAs (**Figure S13A**). In addition, with RNA stability assays performed in PHH
41 (because transcription inhibitors were toxic in dHepaRG cells), we observed a faster decrease
42 in the level of HBV RNAs when cells were co-infected with HDV or transduced with AAV-SHD
43 and AAV-LHD (**Figure 6D, 6E**). Of note, the stability of the cellular *PRNP* mRNAs was not
44 affected by any of the infection/transduction/treatment (**Figure S13B, S13C**). Altogether,
45 these data strongly suggest that HDV/HDAg expression interfere with RNAs production. It has
46 been proposed that m6A modifications induced within the 3' epsilon stem loop of HBV
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1 transcripts controls their stability [35]. However, we observed a similar decrease in the levels
2 of HBV RNAs upon transduction with the AAV-SHD and AAV-LHD vectors in cells transfected
3 with plasmids encoding a wild type HBV genome or a mutated HBV genome that abrogate the
4 m6A site of the epsilon loop of HBV RNAs (**Figure S14**).
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9 **HDV proteins interact with HBV RNAs.** We hypothesized that HDAg might be associated to
10 the HBV mini-chromosome and/or RNAs derived from its transcription and may thereby
11 influence RNA production. To test this hypothesis, we set up a protocol to be able to
12 determine in one sample 1) the efficiency of the immunoprecipitation by western blot
13 analyses, 2) the association of a given protein (i.e., RNA Pol-II or HDAg for instance) to cccDNA
14 by qPCR analyses, as well as 3) the association of a given protein (i.e., RNA Pol-II or HDAg for
15 instance) to HDV RNAs (as controls since these proteins are known to associate to HDV RNAs)
16 or HBV RNAs by RT-qPCR analyses. Western blot analyses after immunoprecipitations (IPs) of
17 cross-linked samples from HBV/HDV co-infected PHH, dHepaRG and HuHep mice showed
18 efficient IPs of both HDAg and RNA Pol-II (**Figure 7A, 7B, 7C, blots on the left**). RNA extractions
19 followed by RT-qPCR to detect HDV RNAs after IPs, validated our methods since we found an
20 association of HDAg and RNA Pol-II to HDV RNAs (**Figure 7A, 7B, 7C, HDV RNAs after IP**). DNA
21 extractions followed by specific qPCR for cccDNA detection after IPs showed no association of
22 HDAg to cccDNA in the presence of HDV in PHH and dHepaRG cells (**Figure 7A, 7B, cccDNA**
23 **after IP**) and we observed a positive signal in only one of the HuHep mouse tested (**Figure 7C,**
24 **cccDNA after IP**). Of note, we did not observe modifications of the amount of HBc, acetylated
25 histone H3 (H3K27) or RNA Pol-II associated to cccDNA in the presence of HDV (**Figure S15A**).
26 As phosphorylation of serine 2 and 5 of RNA Pol-II is a key feature for its transcriptional
27 activity, we performed additional immunoprecipitation assays and did not observe
28 modifications of the phosphorylation of serine 2 and 5 of the cccDNA-bound RNA Pol-II (**Figure**
29 **S15B**). Interestingly, RT-qPCR to detect HBV RNAs after IPs revealed an association of HDAg to
30 HBV RNAs in *in vitro* as well as *in vivo* samples (**Figure 7A, 7B, 7C, HBV RNAs after IP**). Using
31 AAV-SHD and AAV-LHD vectors or dHepaRG-TR-SHD and dHepaRG-TR-LHD cells, we further
32 showed that both S-HDAg and L-HDAg are associated to HBV RNAs in HBV-infected dHepaRG
33 cells (**Figure 7D, S16**). We also tested the association of HDAg to the cellular *PRNP* mRNAs and
34 did not observe any positive signal by RT-qPCR after IPs with anti-HDAg antibodies (**Figure**
35 **S17**), suggesting that HDAg does not associate to any random RNA in the cells. Even though
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1 the amount of RNAs recovered after IPs was lower with cells that were cross-linked with UV
2 (compared to formaldehyde cross-linking) (**Figure S18A**), we still observed an association of
3 HDAg with HBV RNAs (**Figure S18B**) suggesting a direct interaction of HDAg with HBV RNAs.
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9 **Discussion**

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12 Although the inhibitory phenotype is observed in a majority of co-infected patients [23], the
13 mechanism underlying the interference of HDV on HBV replication remained unclear. We
14 previously showed that it is independent of adaptive immunity since it was observed *in vitro*
15 in HBV-infected hepatocytes super-infected by HDV [24]. Using various cell culture models as
16 well as liver-humanized mice, we extend here our initial observations by showing that this
17 phenomenon can also occur in co-infection settings. Additionally, our data suggest that this is
18 specific for HBV and HDV since neither HCV [36] nor SARS-Cov2 [37] RNAs levels were found
19 to be affected by HDV expression. Cell culture assays as well as experiments with mice [24,
20 26, 27, 30, 38-41], already showed that HDV induces a strong innate response with an increase
21 of mRNA levels of classical interferon ISGs, such as MX1, OAS1 or RSAD2. Here, we confirm
22 this result and define an HDV-induced modulation signature by performing RNA sequencing
23 analyses with samples from the most relevant *in vitro* and *in vivo* models. Notably, we confirm
24 the relevance of this signature in an exploratory cohort of patients. Although the number of
25 patients is low and the levels of inflammation complicates the analysis of innate immune-
26 related gene expression, we believe that the HDV signature we characterized in infection
27 models may reflect the specific response to HDV infection in patients. The data should be
28 confirmed by single-cell RNAseq (to exclude the contribution of immune cells and high
29 inflammation levels) in a larger cohort of patients (with a careful matching of potential
30 confounders as fibrosis and inflammatory activity, as well as HBV and HDV replicative activity)
31 to address if this “pan-model” signature would be relevant for the clinical management of
32 chronic HDV infection. Indeed, a poor response of HCV-infected patients to IFN- α -based
33 treatments was related to basal up-regulation of ISGs expression, as well as a particular
34 polymorphism of the IL28b gene [42, 43]. Therefore, the analysis of the baseline (i.e. before
35 an IFN-based treatment is started) expression profiles of human HDV-infected samples might
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1 provide information to investigate underlying mechanisms and find biomarkers of IFN- α
2 treatment failure/success.
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5 In contrast to what previously reported in transformed cells (HuH7) [26], the over-expression
6 of S-HDAg or L-HDAg alone in either dHepaRG or PHH did not induce an IFN response,
7 suggesting that HDV genomic/antigenomic RNA amplification was the sole driver of the IFN
8 response in relevant cell types. Whether these discrepancies are due to HDAg expression
9 levels or the use of non-transformed vs. transformed character of hepatocytes remains to be
10 determined. Using stable cell lines or AAV vectors, we also observed that L-HDAg and S-HDAg
11 are exclusively located in the nucleus in the absence of HDV RNAs consistent with previous
12 reports [44]. This reinforces the hypothesis that the transport of HDV proteins to the
13 cytoplasm requires their association to HDV RNAs.
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25 Since type I IFNs decrease both synthesis and stability of HBV RNAs [8, 45], it was suggested
26 that some HDV-induced ISGs might be responsible for the interference of HDV on HBV
27 replication [46]. Hence, TRIM22, which is up-regulated upon HDV-infection, exhibited an
28 antiviral activity against HBV by acting as a transcriptional repressor [47]. DDX60, a
29 cytoplasmic RNA helicase, which functions as a cell-type specific sentinel for recognition and
30 degradation of viral RNAs, also up-regulated upon HDV infection, promotes the degradation
31 of HBV RNAs [48]. Similarly, ISG20, an exo-ribonuclease, induces the degradation of HBV RNAs
32 [45] through recognition of m6A modifications on the epsilon stem loop [49]. Here, using
33 chemical and genetic approaches we show that the HDV-induced ISGs are only partially
34 responsible for the interference of HDV on HBV since abrogation of the HDV-induced IFN
35 response did not fully revert this phenomenon. Along with these experiments, we confirm
36 that MDA5, but not RIG-I, through IRF3, is involved in HDV recognition [30]. We also show that
37 the HDV-induced IFN response has a minor effect on HDV replication itself since the kinetics
38 of HDV RNAs and proteins appearance/decrease are very similar in dHepaRG-Cas9-RIG_{KO} and
39 dHepaRG-Cas9-MDA5_{KO} cells. These data suggest that the ISGs involved in the antiviral effect
40 observed against HDV in the context of the clinical use of Peg-IFN alpha as well as *in vitro* [50],
41 might not be among the 73 genes of the HDV signature we have identified in this work. The
42 data also strongly suggest that the synthesis of L-HDAg, rather than an innate response of
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1 hepatocyte, is more likely responsible for the natural decline of HDV replication observed *in*
2 *vitro*.
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5 The comparable levels of cccDNA in the different groups of mice (co-infection versus super-
6 infection) ruled out that the interference in the co-infection setting was due to cell entry
7 competition. In contrast, we found that the levels of HBV RNAs (and all subsequent HBV
8 replication markers) were markedly decreased in the presence of HDV/HDAg suggesting an
9 inhibition of HBV RNA transcription and/or an acceleration of HBV RNA decay. Previous
10 studies using an overexpression system suggested that both forms of HDAg might inhibit HBV
11 replication by repressing the HBV enhancer activity [26]. Here, we extended the finding in an
12 infection setting with a nascent HBV RNA assay approach in the context of HDV co-infection
13 or HDAg expression. The mechanism by which HDV/HDAg affects HBV RNA production is still
14 unclear. Indeed, in the presence of HDV, we found no major modification of the amounts of
15 the RNA Pol-II, HBc or acetylated histone H3 (H3K27) associated to cccDNA. We also did not
16 observe significant differences in the in the pool of total and phosphorylated (S2 and S5) RNA
17 Pol-II associated to cccDNA. Because analysis of epigenetic modifications at targeted positions
18 in cccDNA is currently not possible, these results do not exclude that HDAg may directly or
19 indirectly repress HBV RNA synthesis. Alternatively, it is possible that rather than directly
20 inhibiting HBV RNAs transcription per se, HDV/HDAg might act by rapidly affecting the stability
21 of nascent HBV RNAs. Following RNA stability assays, we indeed observed a faster decay of
22 HBV RNAs upon HDV infection/HDAg expression. By performing RNA immunoprecipitation
23 assays, we showed that both S-HDAg and L-HDAg proteins are associated to the HBV RNAs.
24 Preliminary results using UV cross-linking suggest that the interaction might be direct. This
25 should be confirmed by biochemical approaches to identify and narrow down the binding site
26 of HDAg on HBV RNAs as well as determined whether HDAg binds as multimers as it does with
27 HDV RNAs [51-53]. Association of HDAg to HBV RNAs should be also tested in liver biopsies
28 from chronically HBV/HDV infected patients for which no viral interference is observed [23].
29 As HDAg is a typical RNA binding protein [54] one could hypothesize that it might non-
30 specifically bind to any RNAs or even DNAs [53]. However, it was already suggested that
31 interactions of HDAg with cellular RNAs was more specific than expected because only the
32 GSTP1 mRNA was recovered as potential RNA binder to HDAg after a SELEX [41]. Here we
33 could not see any association of HDAg to the *PRNP* transcript but further genome wide
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1 analyses (RIP-seq) should be performed to determine if HDAG may also associate with cellular
2 RNAs and thereby possibly induce pathogenesis.

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4 It is tempting to speculate that the association of HDAG to HBV RNAs could contribute to/favor
5 their degradation. The mechanism underlying how HDAG association to HBV RNAs may induce
6 their degradation will be investigated. Several mechanisms controlling HBV RNA stability were
7 recently uncovered. For instance, m6A modifications induced within the 3' epsilon stem loop
8 of all HBV transcripts controls their stability [35] and ISG20 was shown to be the exonuclease
9 responsible for HBV RNAs degradation [45]. We excluded such a mechanism by evidencing
10 interference of HDV on HBV even when the m6A site on the epsilon loop of HBV RNAs was
11 mutated. Extensive mixed tailing of viral RNAs generated via a mechanism exploiting the
12 TENT4–ZCCHC14 complex was discovered (TENT4 enzymes generate 'mixed tails' of diverse
13 nucleotides at 3' ends of RNAs via non-templated nucleotide addition to protect messenger
14 RNAs from de-adenylation) [55, 56] and RG7834 was identified as a molecule inhibiting
15 TENT4A/B and thereby inducing HBV RNAs degradation [33]. Even though, we did not observe
16 any shift in the migration of HBV RNAs in the presence of HDV (that would be a reflection of
17 the shorter polyA tails) contrary to what is observed in cells treated with RG7834, this
18 mechanism of HBV RNAs destabilization should be further investigated in the context of HDV
19 interference on HBV.
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36 While IFN-dependent interference of one virus with another is commonly observed with
37 respiratory viruses [57], very few IFN-independent mechanisms of viral interference have
38 been described especially when considering a satellite virus and its helper virus. Among them,
39 the AAV2 Rep68 protein can specifically bind to the double-stranded herpes simplex virus 1
40 (HSV-1) genome and inhibit its replication [58, 59]. To our knowledge, we describe here a new
41 mechanism of viral interference, in which the proteins of a satellite virus can influence viral
42 RNA production of its helper virus.
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50 Considering that we found no impact of HBV on the level and kinetics of intracellular HDV
51 replication, it remains to be determined why the mechanisms behind the interference of HDV
52 on HBV have been selected. Indeed, a decrease in HBsAg, which is required for HDV secretion,
53 might be considered counter-productive for HDV. However, it should be noticed that the HDV-
54 induced decrease in HBV is limited (rarely more than 50-60% reduction of HBV RNAs after 13
55 days of co-infection). This might be because HDAG may bind to HBV RNAs with low affinity
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1 compared to binding to HDV RNAs and HBsAg is produced in large excess upon HBV infection.
2 Finally, infectious HDV particles can be also produced in HBV non-replicating cells producing
3 HBsAg from integrated HBV DNA [60] suggesting that chronic HDV infection could persist even
4 when HBV replication is suppressed.
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7 To conclude, our data indicate that HDV interferes with HBV through both IFN-dependent and
8 IFN-independent mechanisms affecting HBV RNAs metabolism. We therefore provide here
9 important observations that could pave the way to the development of new therapeutic
10 strategies against HBV by mimicking and/or increasing the effect of HDV on HBV RNAs for
11 instance.
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18 **Methods**

19 See supplementary file.
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25 **References**

26 Author names in bold designate shared co-first authorship.
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Figure legends

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4 **Figure 1. HDV reduces the levels of HBV RNAs, secreted HBV antigens and DNA but not**
5 **cccDNA *in vivo* and *in vitro*.** (A, B) Differentiated HepaRG cells (dHepaRG, at least 3 batches
6 of differentiation) or primary human hepatocytes (PHH, 3 donors) were infected with HBV
7 alone or with HBV and HDV at multiplicity of infection of 500 and 50 vge/cell respectively.
8 Thirteen days post-infection, the levels of intracellular HBV RNAs were analyzed by RT-qPCR
9 or Northern blot, the levels of cccDNA by qPCR whereas the levels of secreted HBeAg and
10 HBsAg were assessed by ELISA. Data were normalized to the “HBV” condition after being
11 normalized to a housekeeping gene (*PRNP*) in the case of the RT-qPCRs. Results are the mean
12 +/- SD of three independent experiments each performed with three biological replicates. (C)
13 Chimeric liver humanized mice (FRG NOD) were infected with HBV alone, with HBV and HDV
14 or with HBV first and with HDV 4 weeks later. At the indicated weeks post (HBV) infection
15 (wpi), sera were collected and HBV and HDV viremia were analyzed by qPCR and qRT-PCR
16 whereas the levels of secreted HBeAg and HBsAg were assessed by ELISA. Twelve wpi, mice
17 were sacrificed and the levels of intrahepatic total HBV DNAs as well as cccDNA were analyzed
18 by qPCR respectively and normalized to B-Globin. Results are the mean +/- SEM of 6 to 5 mice
19 per group. Statistical analyses were performed using Mann-Whitney tests and p values are
20 indicated (significance when $p < 0.05$).

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39 **Figure 2. HDV-induced transcriptomic modulations.** Differentiated HepaRG cells (dHepaRG)
40 or primary human hepatocytes (PHH, 3 donors) were infected with HBV alone, with HDV alone
41 or with HBV and HDV at multiplicity of infection of 500 and 50 vge/cell respectively or treated
42 with IFN- α (1000 UI/mL). RNA samples collected at day 6 post-infection or day 1 post
43 treatment with IFN- α (for PHH and dHepaRG) or at weeks 12 post-HBV infection (8 weeks
44 post-HDV infection) for mice or from human liver biopsies were analyzed by RNA sequencing.
45 (A) Pathways activities relative to non-infected conditions are presented for each model. The
46 color corresponds to the difference in GSEA scores for each model compared to uninfected
47 control samples. *nominal p-value < 0.05 (B) ISGs that are significantly different ($\log_2(\text{Fold}$
48 Change) > 1 , nominal $p < 0.05$) in the HDV models. In this case the color corresponds to the
49 $\log_2(\text{Fold Change})$. ISGs were defined either being included in IFN- α by Molecular Signatures Database
50 Hallmark gene sets (the same set used for or if they significantly upregulated in the PHHs or
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HepaRGs by IFN- α). The genes selected had at least nominal p-values below 0.05 and log₂(Fold Change) of at least 1 in all three models.

Figure 3. Inhibition of the HDV-induced IFN response by TPCA-1 partially restores the levels of HBV RNAs and secreted HBV antigens and particles. Differentiated HepaRG cells were infected with HBV, HDV or with HBV and HDV at multiplicity of infection of 500 (HBV) and 50 (HDV) vge/cell and treated or not with TPCA-1 (1 μ M). (A) Six days post-infection, the levels of RSAD2 mRNAs, MxA mRNAs, HDV RNAs and HBV RNAs were analyzed by RT-qPCR. (B) Thirteen days post infection, the levels of secreted HBeAg and HBsAg were assessed by ELISA, the levels of secreted HBV DNA by qPCR and the levels of intracellular HBV and HDV RNAs were analyzed by RT-qPCR. In the case of the RT-qPCRs, all the data were normalized to a housekeeping gene (*PRNP*) and in addition to the “non treated HBV” condition for (B). Results are the mean +/- SD of two independent experiments each performed with three biological replicates. Statistical analyses were performed using Mann-Whitney tests and p values are indicated (significance when $p < 0.05$).

Figure 4. Abrogating the HDV-induced IFN response partially restores levels of HBV RNAs and secreted HBV antigens. Differentiated HepaRG, HepaRG-Cas9-CTL, HepaRG-Cas9-RIG-I_{KO}, HepaRG-Cas9-MDA5_{KO} or HepaRG-Cas9-IRF3_{KO} cells were infected with HBV and HDV at multiplicity of infection of 500 and 50 vge/cell respectively. (A, B, C) RNA samples collected at day 6 post-infection were analyzed for the levels of (A) HBV and HDV RNAs and (B) RSAD2 mRNAs by RT-qPCR or (C) RNA sequencing. The heat map represents log₂ fold changes without transformation. (D, E, F) Thirteen days post infection, the levels of secreted (D) HBsAg and (E) HBeAg were assessed by ELISA and the levels of (F) intracellular HBV RNAs were analyzed by RT-qPCR. Data were normalized to the “HBV” condition after being normalized to a housekeeping gene (*PRNP*) in the case of the RT-qPCRs except for RSAD2 mRNAs for which data were normalized to *PRNP* mRNA only. (B, D, E, F) Results are the mean +/- SD of three to five independent experiments each performed with three biological replicates. Statistical analyses were performed using Mann-Whitney tests and p values are indicated (significance when $p < 0.05$).

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Figure 5. Expression of HDAg alone decreases the levels of HBV RNAs and secreted antigens without inducing an IFN response. dHepaRG were infected with HBV at a multiplicity of infection of 500 vge/cell. Three days later, cells were infected with HDV at a multiplicity of infection of 50 vge/cell or transduced with AAV-GFP, AAV-SHD or AAV-LHD at the indicated multiplicity of infection (vge/cells). (A) At days 14 post-HBV infection, cells were lysed and the presence of HDAg was assessed by western blot analyses. (B) The levels of intracellular HBV RNAs, HNF4a, RSAD2 and MxA mRNAs were analyzed by RT-qPCR whereas the levels of secreted HBeAg and HBsAg were assessed by ELISA. Data were normalized to the “AAV-GFP 10³” condition after being normalized to a housekeeping gene (*PRNP*) in the case of the RT-qPCRs except for RSAD2 and HNF4a mRNAs for which data were normalized to *PRNP* mRNAs only. Results are the mean +/- SD of two independent experiments each performed with three biological replicates. Statistical analyses were performed using Mann-Whitney tests and p values are indicated (significance when p<0.05).

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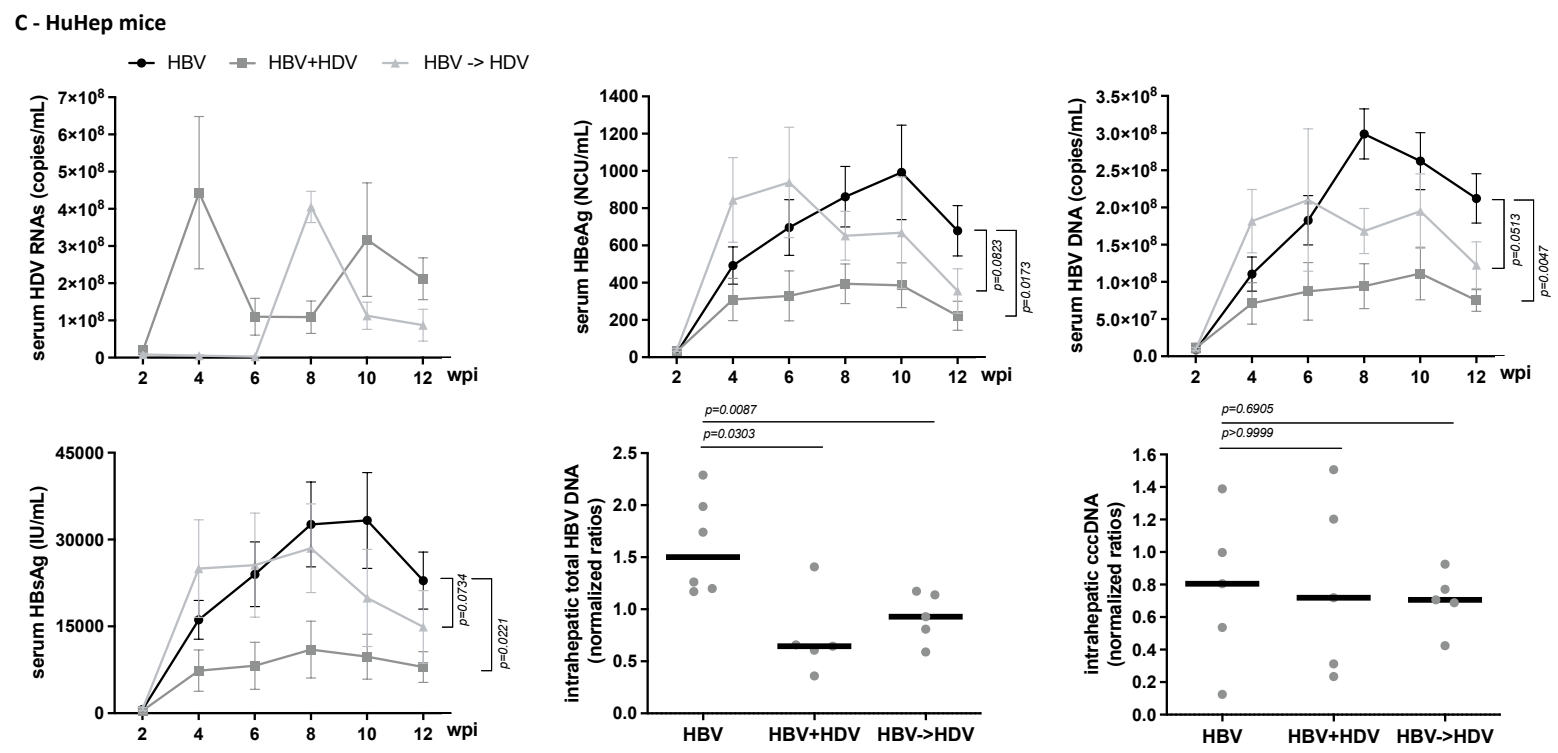
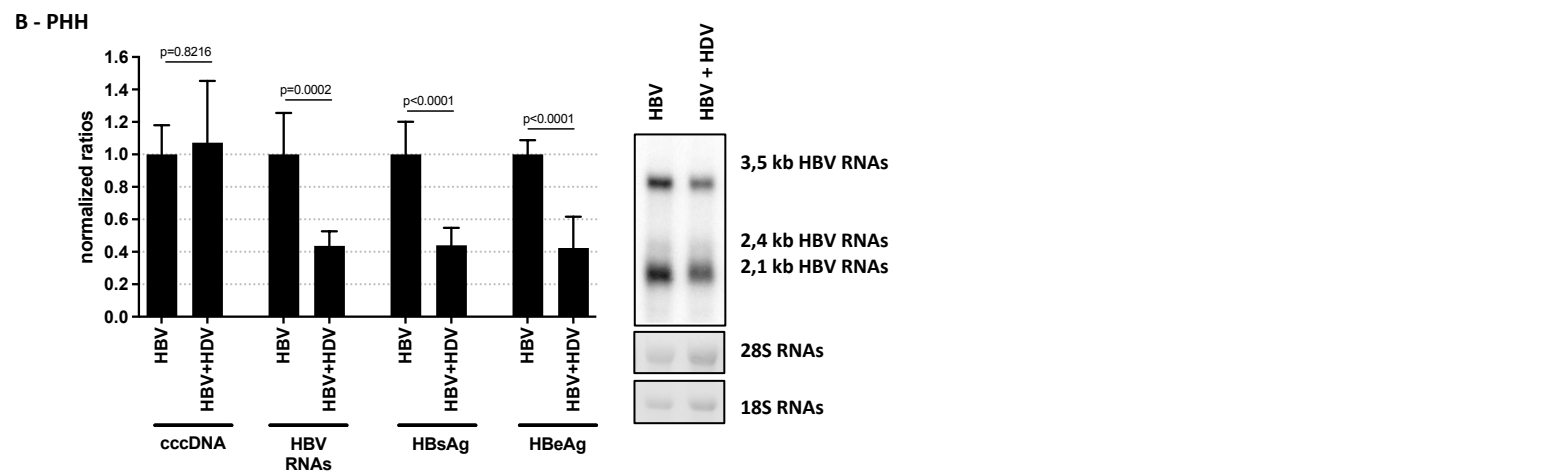
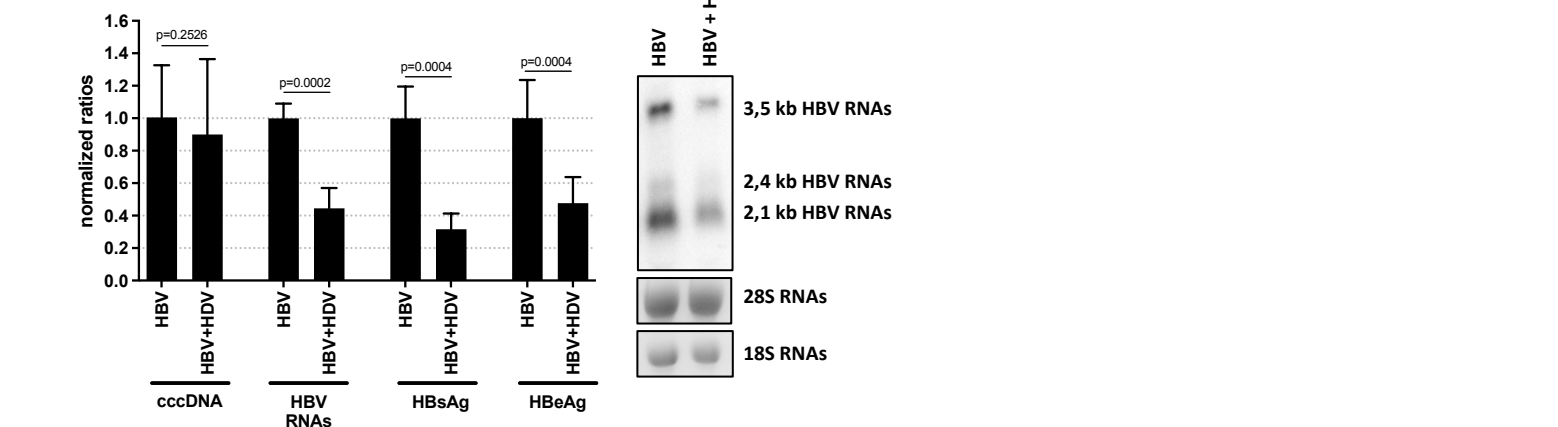
Figure 6. HDV decreases the synthesis and stability of HBV RNAs. (A) dHepaRG were infected with HBV at multiplicity of infection of 500 vge/cell with or without HDV at multiplicity of infection of 50 vge/cell. At day 10 post infection with HBV, some cells were treated with RG7834 (1 uM). At day 13 post infection with HBV, cells were collected, RNA extracted and analyzed by Northern Blot. (B) dHepaRG were infected with HBV at multiplicity of infection of 500 vge/cell with or without HDV at multiplicity of infection of 50 vge/cell. At day 3 post infection, some cells were treated with RG7834 (1 uM) or IFN- α (500 IU/mL) for 10 days. At day 13 post infection with HBV, cells were incubated or not with bromo-uridine (BrU, 2 mM) for 2h. As a control, some cells were treated with actinomycin D (Act D, 1 ug/mL) for 1h before incubation with BrU. Cells were then collected, RNAs extracted and the levels of total intracellular HBV RNAs (normalized to *PRNP*) as well as BrU-labeled HBV RNAs (nascent HBV RNAs normalized to the mock) were analyzed by RT-qPCR. Results are the mean +/- SD of two independent experiments performed with 4 biological replicates. (C) dHepaRG were infected with HBV at multiplicity of infection of 500 vge/cell. Three days later cells were either infected by HDV at multiplicity of infection of 50 vge/cell, transduced with the indicated AAV vectors at multiplicity of infection of 5000 vge/cell or treated with RG7834 (1 uM) or IFN- α (500 IU/mL) for 10 days. At day 13 post infection with HBV, cells were incubated or not with bromo-uridine (BrU, 2 mM) for 2h. Cells were then collected, RNAs extracted and the levels of total

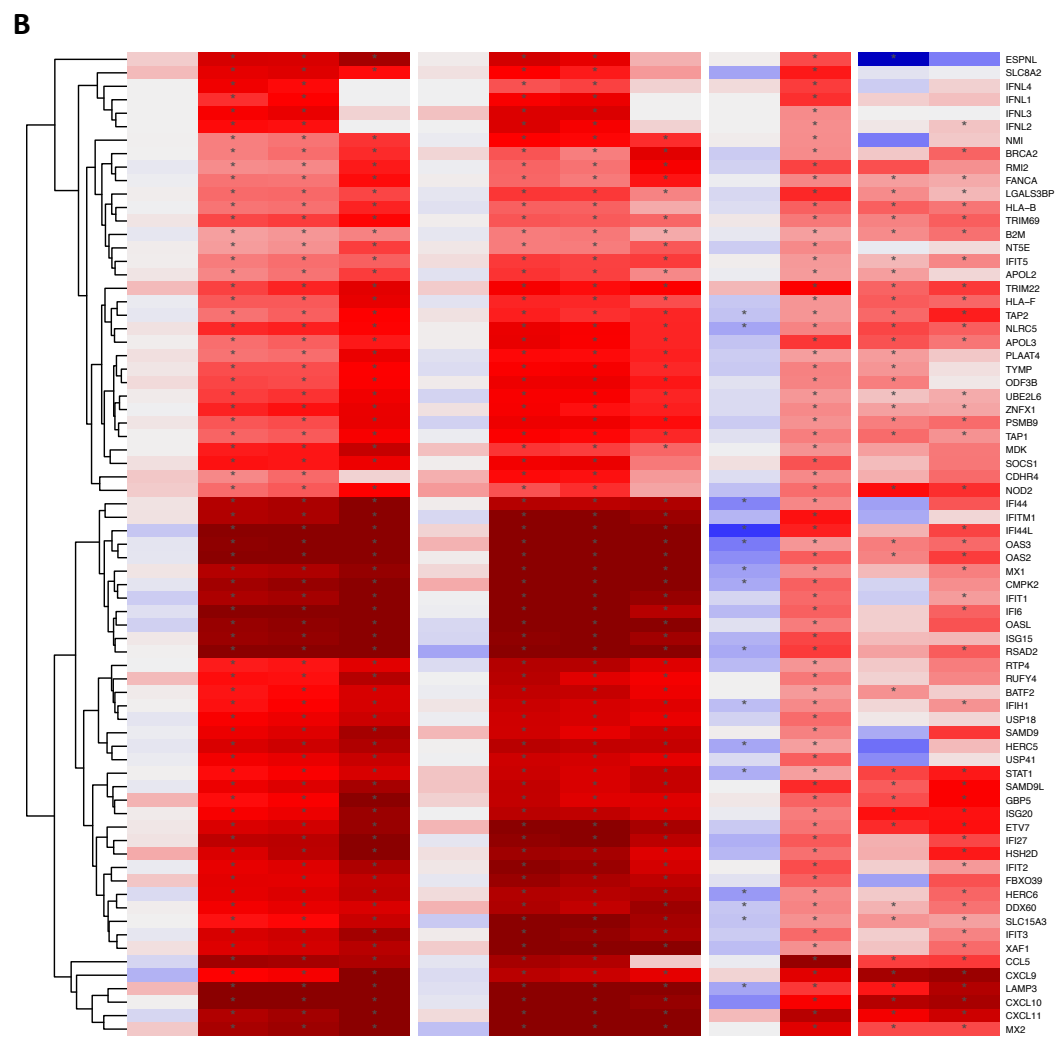
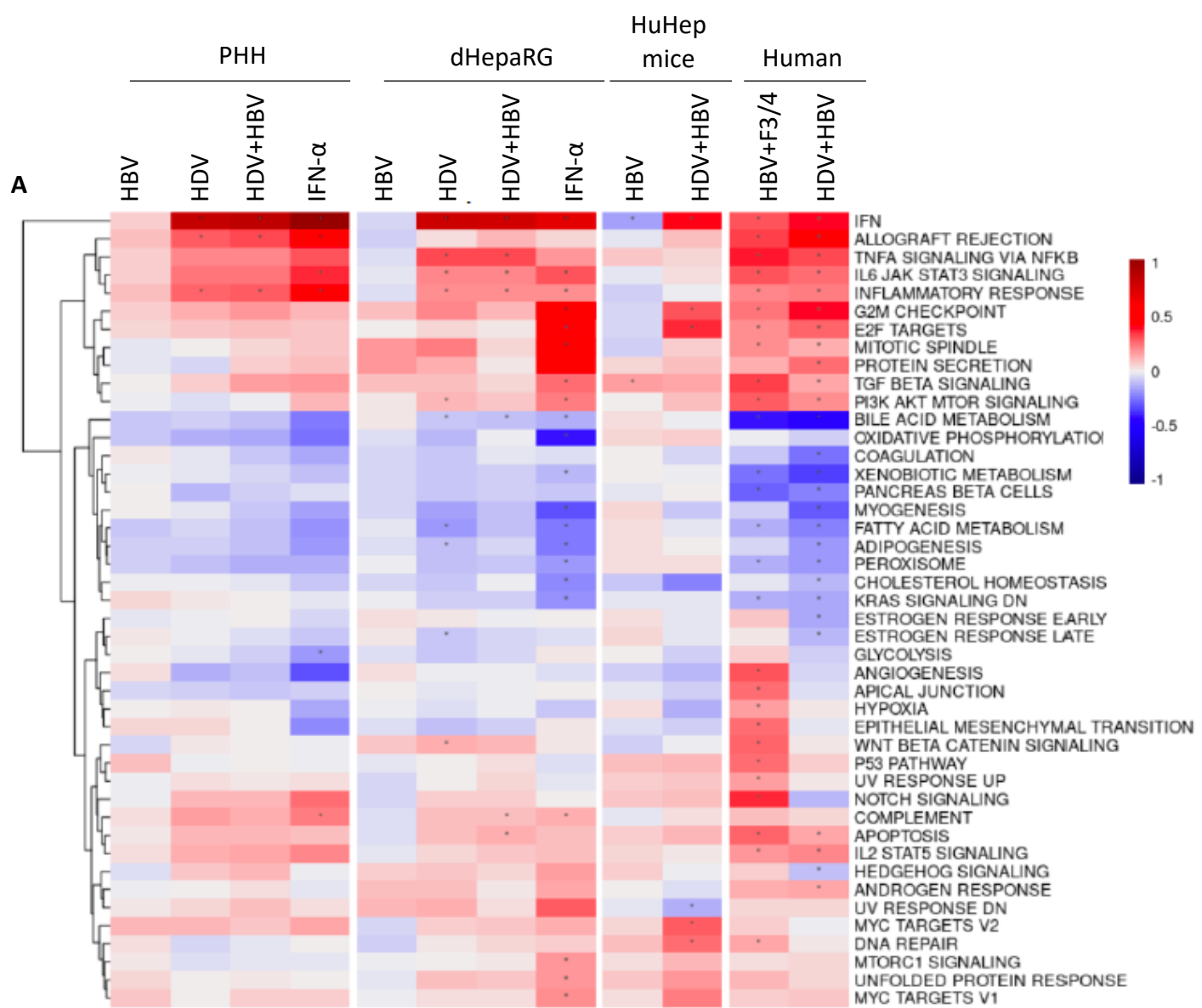
1 intracellular HBV RNAs (normalized to *PRNP*) as well as BrU-labeled HBV RNAs (nascent HBV
2 RNAs normalized to *PRNP*). Results are the mean +/- SD of three independent experiments
3 performed with 3 biological replicates. (D) Freshly isolated PHH were infected with HBV at
4 multiplicity of infection of 500 vge/cell with or without HDV at multiplicity of infection of 50
5 vge/cell. At day 5 post infection with HBV, some cells were treated with RG7834 (1 uM) or IFN-
6 α (500 IU/mL). At day 7 post infection with HBV, all cells were treated with triptolide (1 uM)
7 to block RNA synthesis. At the indicated time post-treatment with Triptolide, cells were
8 collected, RNA extracted and the levels of HBV RNAs were assessed by RT-qPCR analyses.
9 Results are the mean +/- SD of two independent experiments each performed with 3 biological
10 replicates. (E) Freshly isolated PHH were infected with HBV at multiplicity of infection of 500
11 vge/cell. Three days later cells were either infected by HDV at multiplicity of infection of 50
12 vge/cell, transduced with the indicated AAV vectors at multiplicity of infection of 5000 vg/cell.
13 At day 7 post infection with HBV, some cells were treated with RG7834 (1 uM). At day 10 post
14 infection with HBV, all cells were treated with Triptolide (1 uM) to block RNA synthesis. At the
15 indicated time post-treatment with Triptolide, cells were collected, RNA extracted and the
16 levels of HBV RNAs were assessed by RT-qPCR analyses. Results are the mean +/- SD of one
17 experiment performed with 3 biological replicates. Statistical analyses were performed using
18 Mann-Whitney tests and p values are indicated (significance when $p < 0.05$).

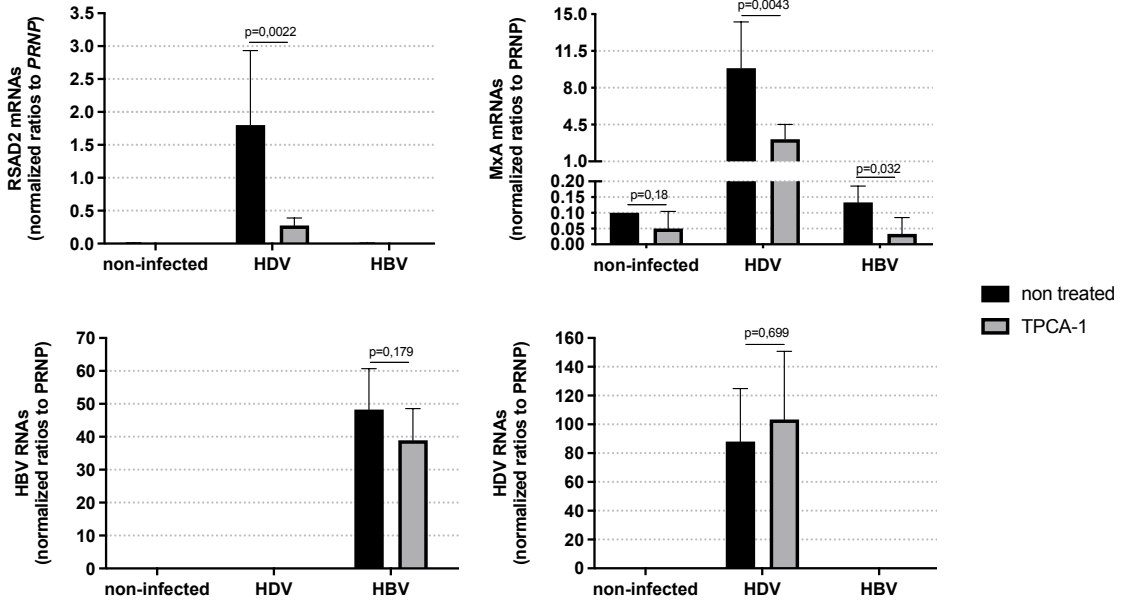
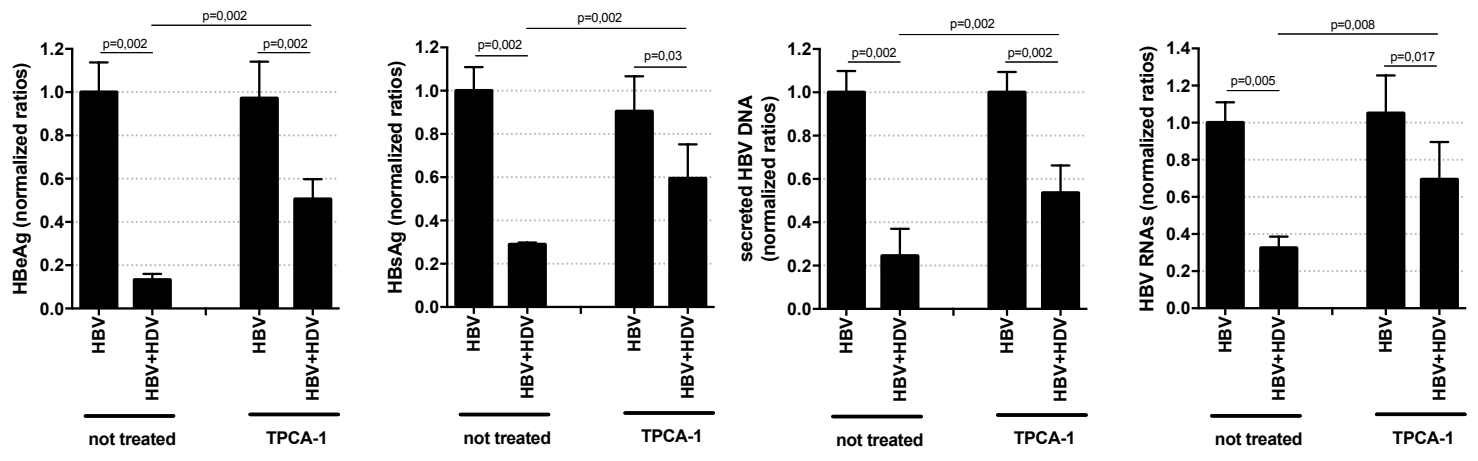
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37 **Figure 7. HDAg are associated to HBV RNAs in HBV/HDV infected PHH, dHepaRG and HuHep**
38 **mice.** (A, B, C) Cells pellets from HBV/HDV infected (A) PHH or (B) dHepaRG (9 days post-
39 infection) or (C) frozen liver from HBV/HDV infected HuHep mice (12 weeks post-infection)
40 were cross-linked with 1% formaldehyde before immunoprecipitation with the indicated
41 antibodies (IgG mouse, anti-HBc, anti-RNA Pol-II, pre-immune sera or anti-HDAg). 0.16^e of the
42 immunoprecipitated materials (IP) as well as 0.04^e of the the flowthrough (FT) were analyzed
43 were by western blot. RNAs were extracted from the 0,28^e of the immunoprecipitated
44 materials and the levels of HDV or HBV RNAs associated to the indicated proteins were
45 analyzed by RT-qPCR. DNAs were extracted from the 0.56^e of the immunoprecipitated
46 materials and the levels of cccDNA associated to the indicated proteins were analyzed by
47 qPCR. Results are the mean +/- SEM of three independent experiments. (D) dHepaRG were
48 infected with HBV at multiplicity of infection of 500 vge/cell. Four days later, cells were
49 infected with HDV at multiplicity of infection of 50 vge/cell or transduced with the indicated

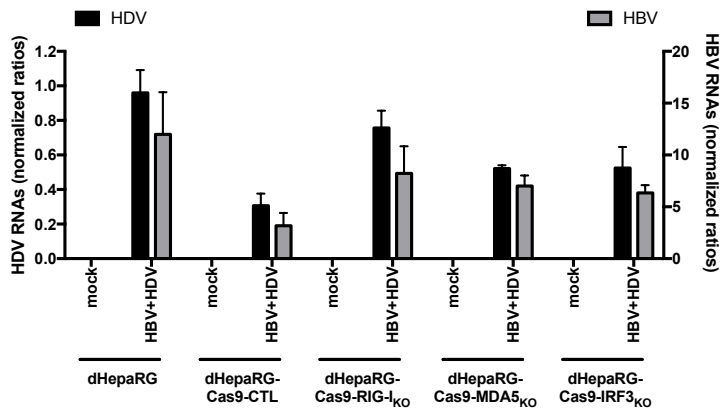
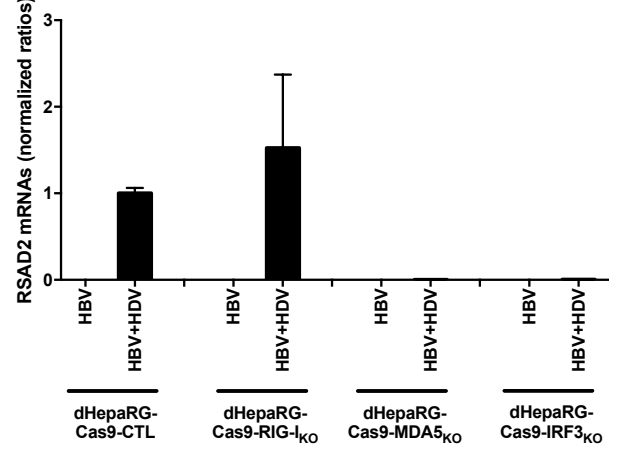
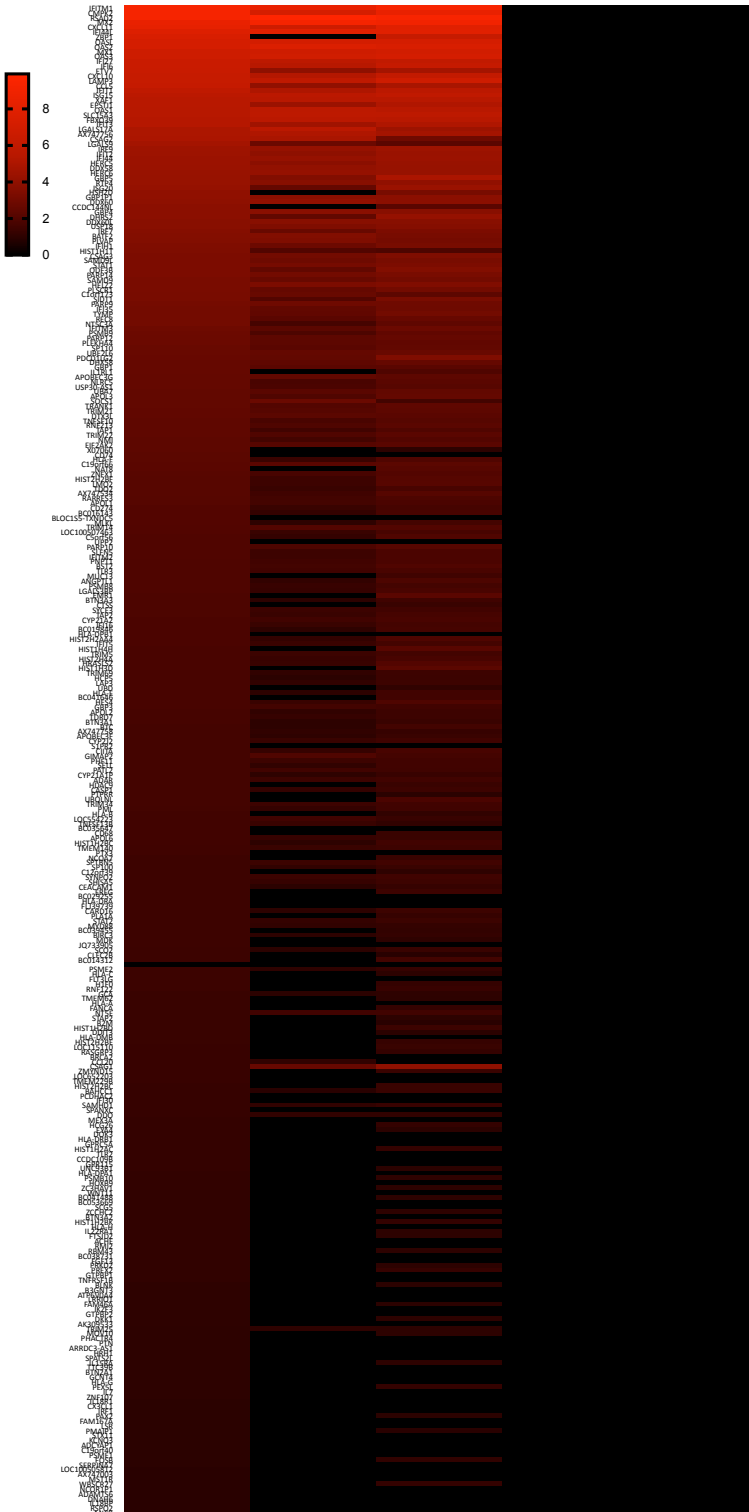
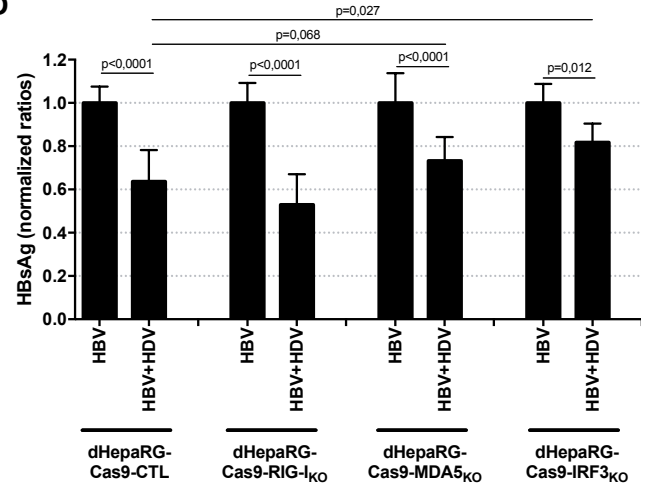
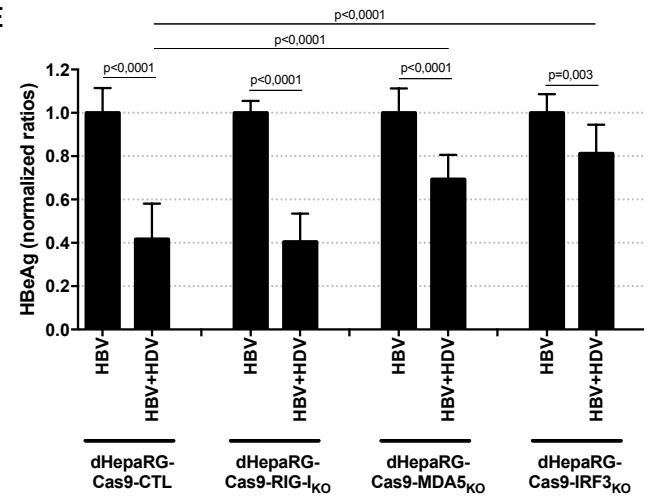
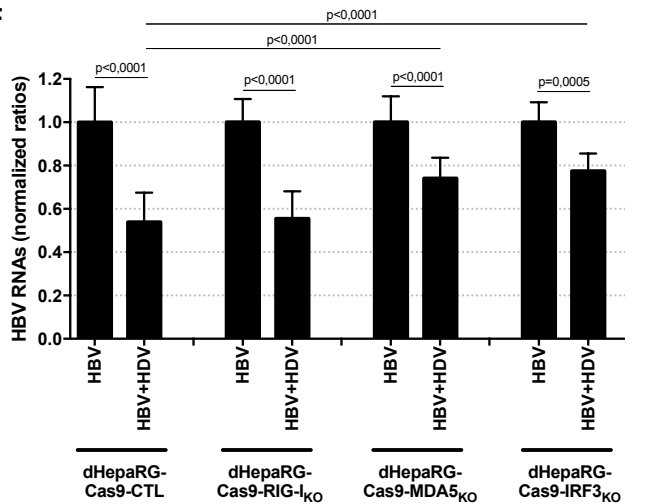
AAV vectors at multiplicity of infection of 5000 vg/cell. Eleven days post infection with HBV, cells were cross-linked with formaldehyde before immunoprecipitation with the indicated antibodies (IgG mouse or anti-HDAg). RNAs were extracted from the immunoprecipitated materials and the levels of HBV RNAs were analyzed by RTqPCR. Results are the mean +/- SEM of two independent experiments each performed with two biological replicates.

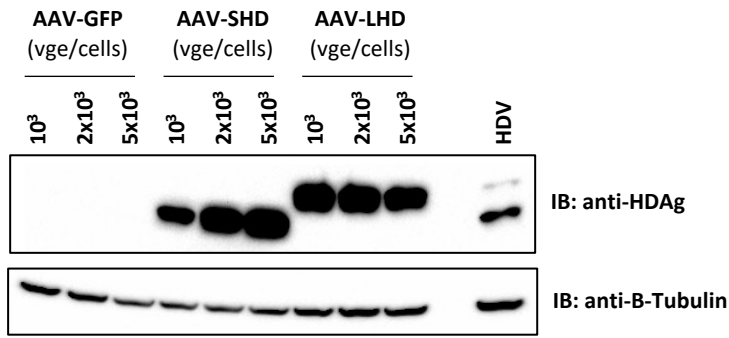
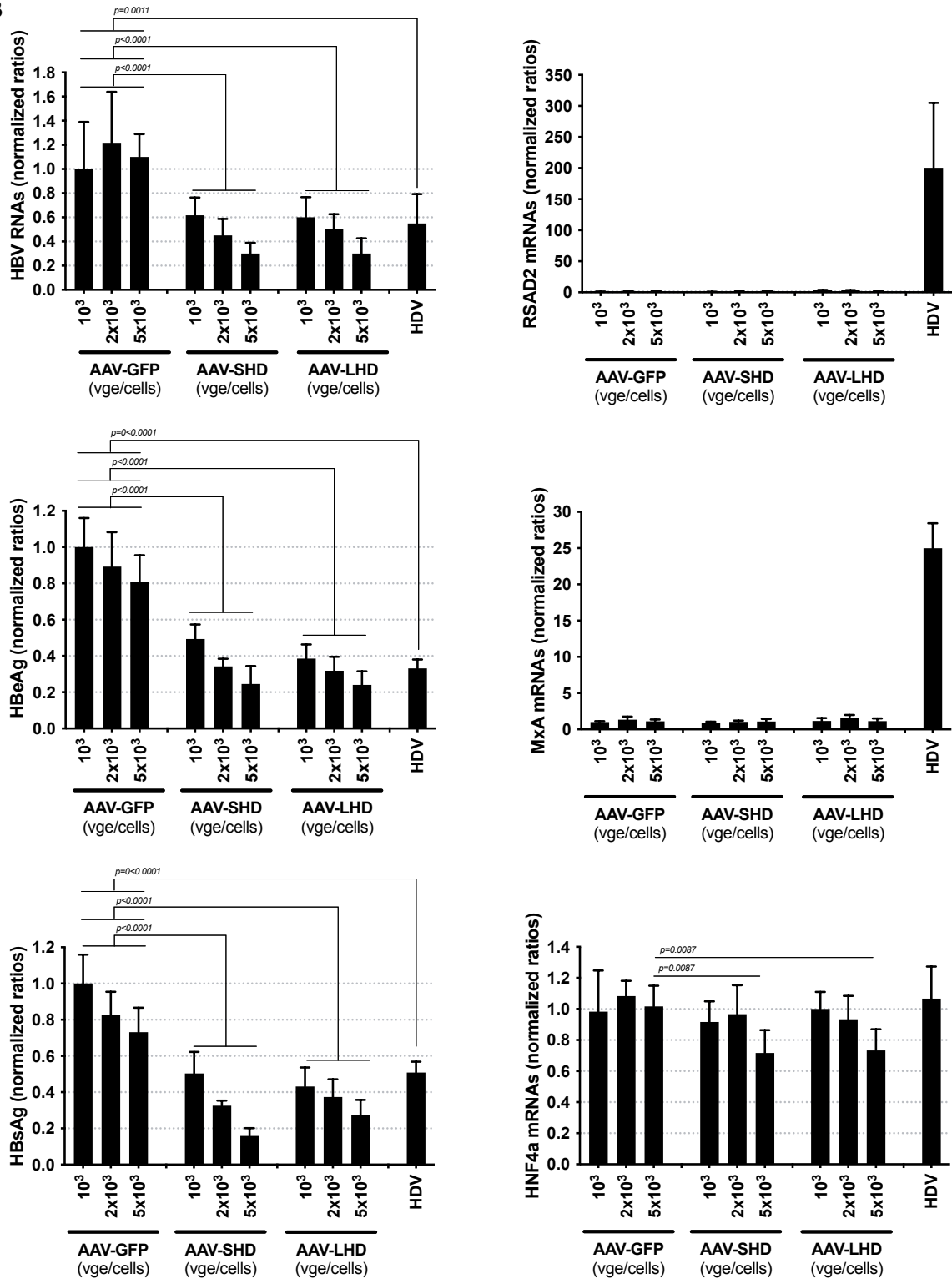
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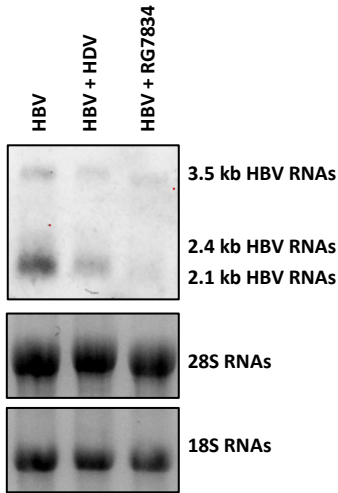


A**B**

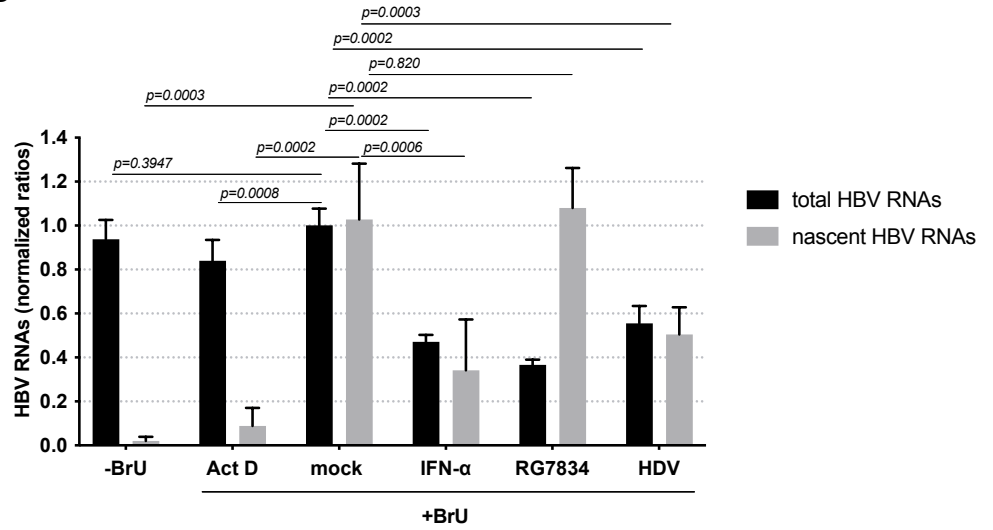
A**B****C****D****E****F**

A**B**

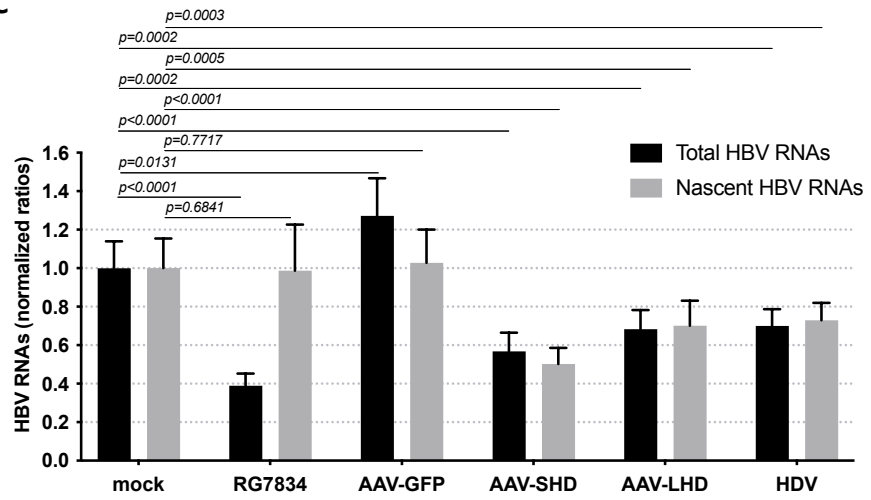
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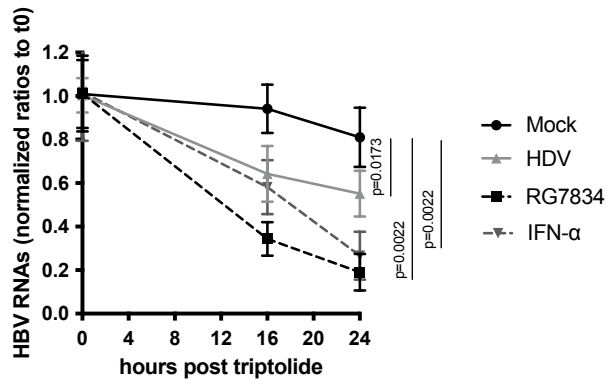
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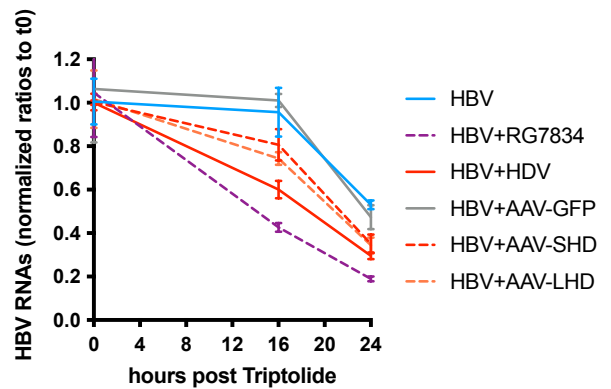
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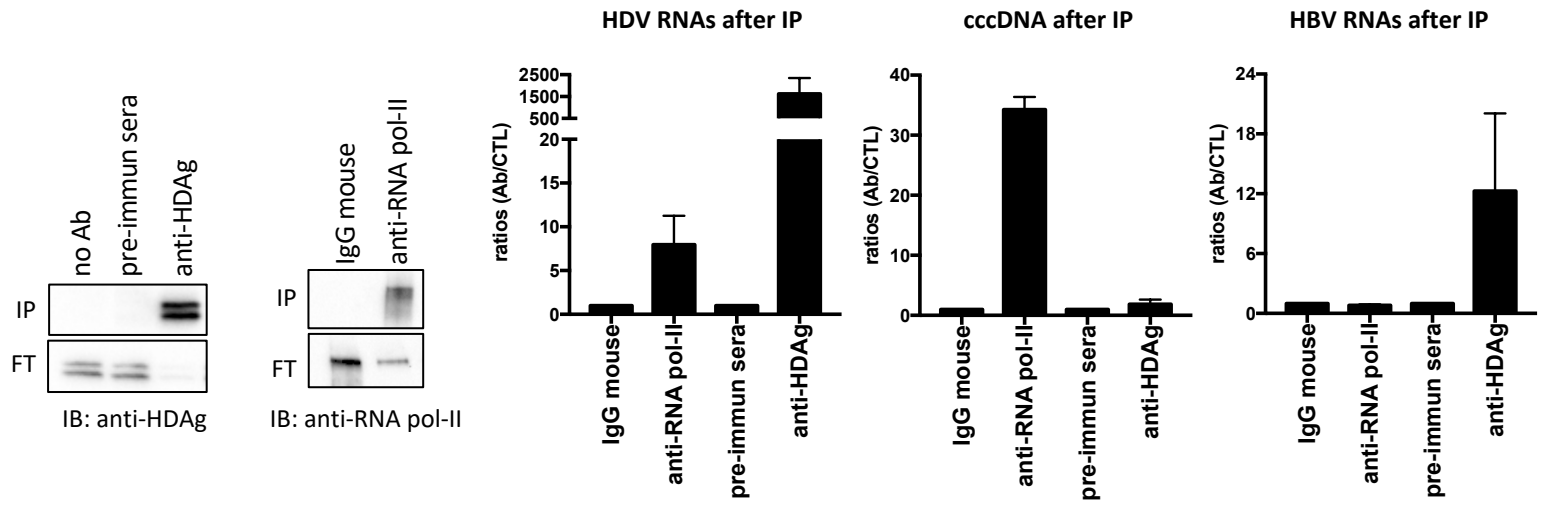
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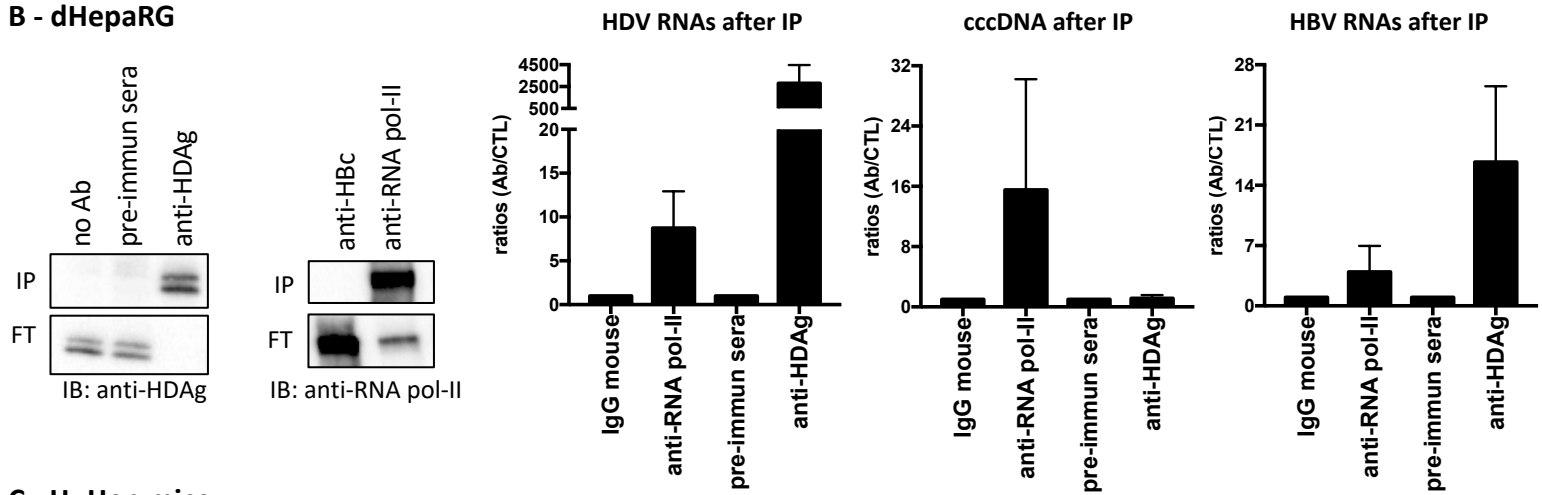
E



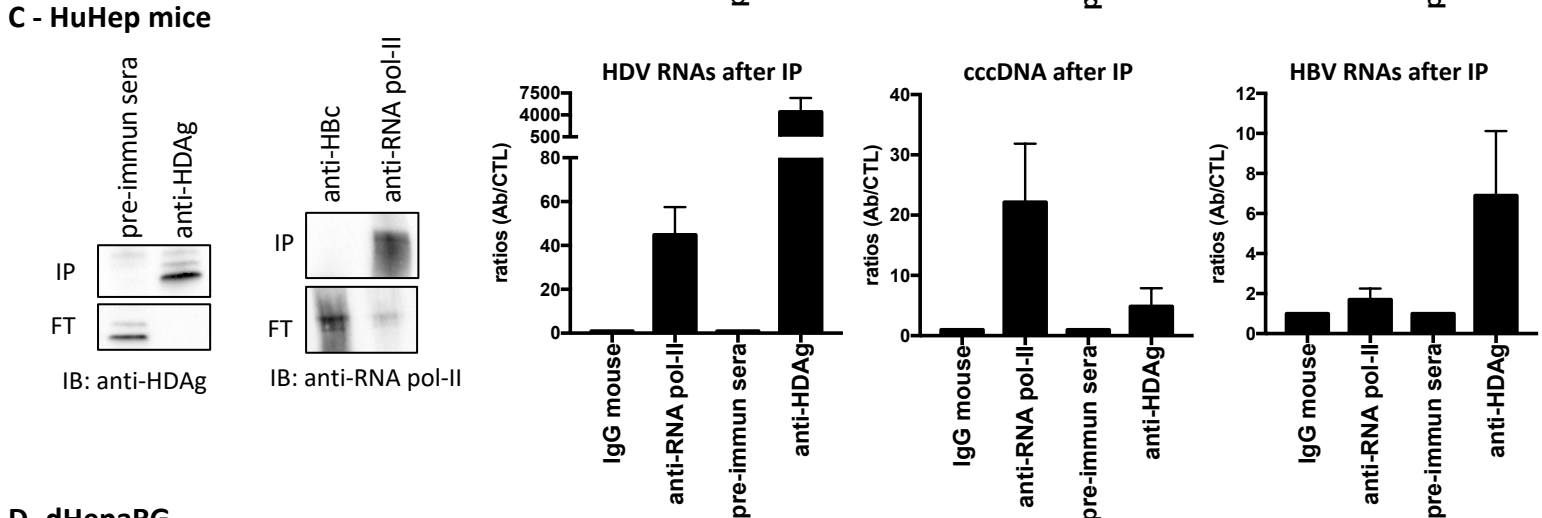
A - PHH



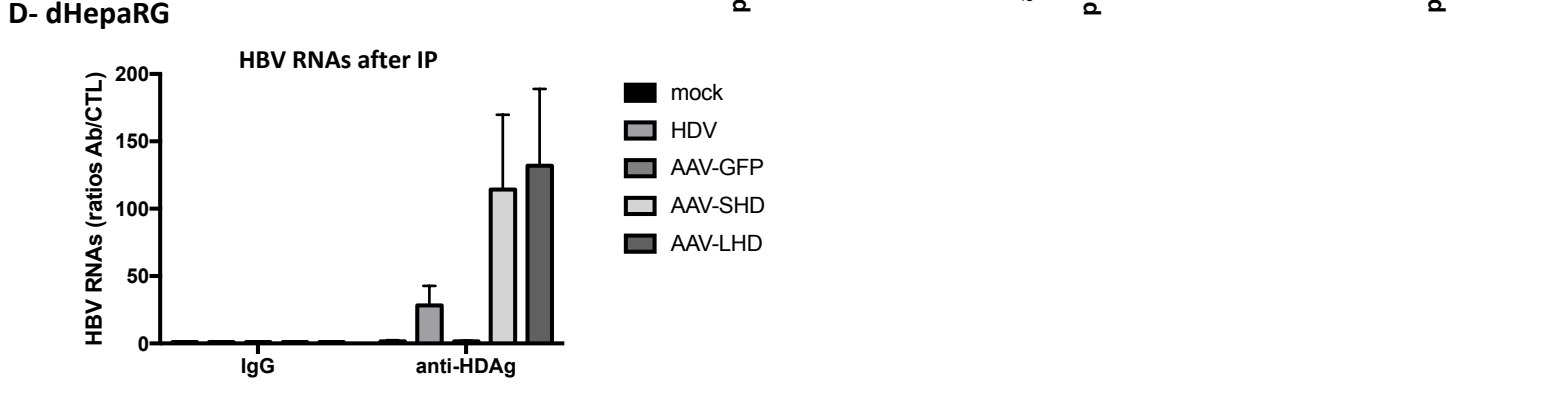
B - dHepaRG



C - HuHep mice



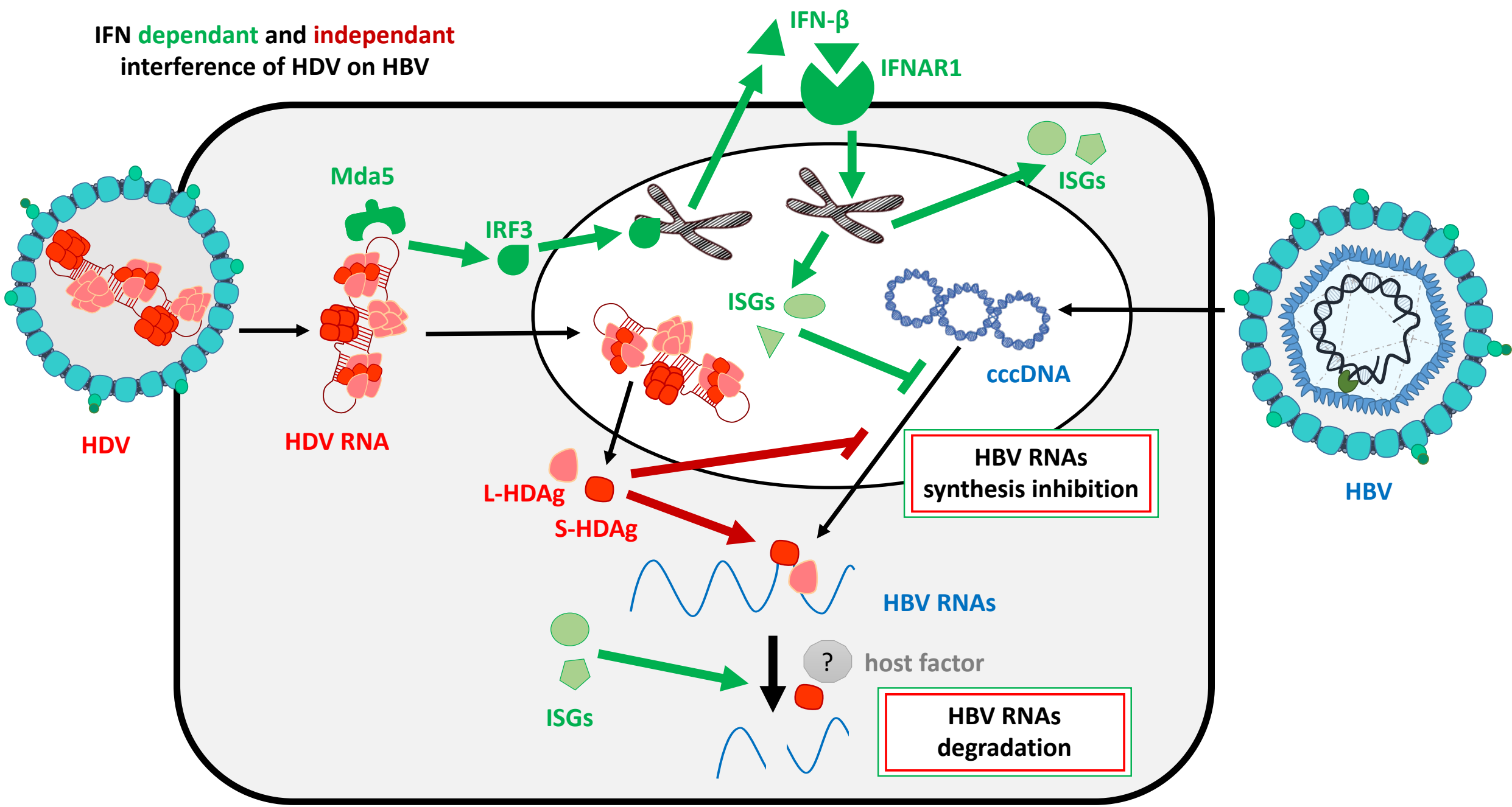
D - dHepaRG



Biopsy ID	H-number	Ethnicity	Age	Phase	Gender	Log HBV DNA	HbeAg	Anti-HBeAg	ALT	AST	HBsAg	Genotype	Interface_inflam	Lobulaire_inflam	Portale_inflam	confluent_crosis	NASH	METAVIR	Steatosis	Comments
AB.02	Healthies	Caucasian	52	HC	F	NA	NA	NA	24	27	Negative	NA	No	No	No	#N/A	No	F0	No	
AB.04	Healthies	Caucasian	51	HC	M	NA	NA	NA	21	23	Negative	NA	No	Moderate	No	#N/A	No	F0	No	
AB.03	Healthies	Caucasian	55	HC	F	NA	NA	NA	28	28	Negative	NA	No	No	No	#N/A	No	F0	No	
AB.07	Healthies	Caucasian	57	HC	F	NA	NA	NA	24	28	Negative	NA	No	No	No	#N/A	No	F0	No	
AB.01	Healthies	Caucasian	27	HC	M	NA	NA	NA	61	36	Negative	NA	#N/A	Moderate	#N/A	#N/A	No	NA	No	
AB.145	Healthies	Other	28	HC	M	NA	Negative	Negative	33	23	Negative	NA	No	No	No	#N/A	No	NA	No	
AB.146	Healthies	Caucasian	63	HC	M	NA	NA	NA	28	23	Negative	NA	No	No	No	#N/A	No	NA	No	
AB.05	Healthies	Caucasian	49	HC	M	NA	NA	NA	NA	NA	Negative	NA	#N/A	#N/A	#N/A	#N/A	No	NA	No	
AB.06	Healthies	Other	43	HC	M	NA	NA	NA	NA	NA	Negative	NA	#N/A	#N/A	#N/A	#N/A	No	NA	No	
AB.23	F3/F4	Asian	19	F34	M	4,245	Negative	Positive	56	45		C	Mild/Moderate	<2 foci	Moderate/Marked	No	No	4	No	
AB.18	F3/F5	Asian	48	F34	M	8,052	Positive	Negative	80	128		ND	Moderate	2-4 foci	Moderate/Marked	No	No	4	No	
AB.45	F3/F6	Asian	32	F34	F	3,374	Negative	Positive	122	80		C	Moderate	2-4 foci	Moderate/Marked	No	No	4	No	
AB.59	F3/F7	Caucasian	24	F34	M	5,23	Positive	Negative	142	84		ND	Moderate	5-10 foci	Moderate/Marked	No	No	4	No	
AB.51	F3/F8	Caucasian	37	F34	M	9,055	Negative	Positive	204	99		D	Mild/Moderate	<2 foci	Moderate	No	No	4	Yes	
AB.138	HBV/HDV	African	43	HBV/HDV	F	4,08	Negative	Positive	36	38		E	Mild/Moderate	<2 foci	Moderate/Marked	No	No	3	No	severe fibrosis or cirrhosis, no treatment
AB.140	HBV/HDV	Caucasian	44	HBV/HDV	M	<3	Negative	Positive	112	88		ND	Yes	Yes	#N/A	#N/A	No	F4	No	history of iv drug use, had cleared HCV, has liver cirrhosis, no treatment
AB.142	HBV/HDV	African	28	HBV/HDV	M	1,39	Negative	Positive	53	57		ND	#N/A	#N/A	#N/A	#N/A	No	#N/A	No	severe fibrosis, macronocular cirrhosis
AB.143	HBV/HDV	Other	49	HBV/HDV	F	1,65	Negative	Positive	93	48	>150	ND	#N/A	present	moderate	#N/A	No	F2	yes, mild	no therapy
AB.144	HBV/HDV	Other	50	HBV/HDV	M	ND	Negative	Positive	ND	ND	>150	ND	#N/A	present	#N/A	#N/A	No	severe fibrosis	yes, mild	severe fibrosis to cirrhosis, on treatment with tenofivir

Table 1. Patient characteristics linked to the human liver biopsies used for RNA sequencing.

IFN dependant and independant interference of HDV on HBV



Highlights

- we define the HDV-induced signature transcriptomic modulations mainly composed of ISGs
- ISGs are upregulated upon HDV infection but not in cells only expressing HDAg alone
- HDV interferes with HBV through both IFN-dependent and IFN-independent mechanisms
- HDV infection and HDAg expression decrease the levels of nascent HBV RNAs and accelerated the decay of HBV RNA
- HDAg are associated with HBV RNAs



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Supplementary material

JHEPAT-D-22-00233 - revised 221212 - sup.docx

