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TGF-β suppression of HBV RNA through AID-dependent recruitment of an RNA exosome complex

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Abstract:	Transforming growth factor (TGF)-β inhibits hepatitis B virus (HBV) replication although the intracellular effectors involved are not determined. Here, we report that reduction of HBV transcripts by TGF-β is dependent on AID expression which significantly decreases both HBV transcripts and viral DNA, resulting in inhibition of viral replication. Immunoprecipitation reveals that AID physically associates with viral P protein that binds to specific virus RNA sequence called epsilon. AID also binds to an RNA degradation complex (RNA exosome proteins), indicating that AID, RNA exosome, and P protein form a RNP complex. Suppression of HBV transcripts by TGF-β was abrogated by depletion of either AID or RNA exosome components, suggesting that AID and the RNA exosome involve in TGF-β mediated suppression of HBV RNA. Moreover, AID-mediated HBV reduction does not occur when P protein is disrupted or when viral transcription is inhibited. These results suggest that induced expression of AID by TGF-β causes recruitment of the RNA exosome to viral RNP complex and the

	RNA exosome degrades HBV RNA in a transcription-coupled manner.
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We appreciate all reviewers' comments and questions, which greatly improved our manuscript. The reviewers' comments are in *italics* below.

Reviewer #1: In the revised submission Liang et al. have addressed many of the earlier concerns however some of the most important concerns still remain unaddressed.

> *HBV replicon cells do not represent a good model system to study host cell response to HBV infection. The observation made with replicon cells should be further substantiated using cell culture models which resemble natural HBV infection or in HBV stable cell lines harboring integrated HBV transgene. This is essential to rule out any AID-mediated effect on transfected HBV plasmid and to ascertain that the observed inhibition of HBV replication is a post-transcriptional event.*

Response

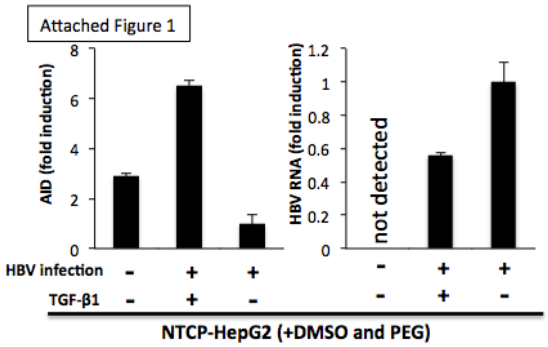
Thanks for reminding us this important question. As shown in our results, experiments using HBV stable cell lines harboring an integrated HBV transgene were performed. First, a B cell line containing a chromosomally integrated HBV transgene (Fig. 3I–K) demonstrated that endogenous AID expression induced by cytokine stimulation (CIT) downregulates HBV RNA. Second, experiments using 7T7-8 cells, a stable Huh7 cell line that has the chromosomally integrated HBV transgene, demonstrated that TGF-β1 downregulates HBV RNA and TGF-β1-mediated downregulation of HBV RNA is dependent on AID and Exosc3 (Fig. 7). Thus, we think that the experiments requested by reviewer 1 have been done.

In addition, we would like to add other evidences to strengthen our conclusion. In this study, we excluded the possibility of an AID-mediated effect on transfected HBV plasmid based on three pieces of experimental evidence. (1) The two HBV stable cell lines mentioned above rule out any artifacts due to transient transfection. (2) AID- and TGF-β1-mediated HBV reductions were rescued by knocking down of RNA exosome proteins. (3) AID-mediated HBV

reduction was no longer observed in the absence of intact HBV P protein, which cannot be explained by an AID-mediated effect on the HBV plasmid. If AID affects plasmids, AID should also affect the HBV P protein mutant replicon. However, we did not observe AID-mediate HBV RNA downregulation in the mutant replicon.

As for an in vitro model mimicking natural HBV infection, our collaborator previously demonstrated that AID expression is induced by IL-1β stimulation in HBV-infected HepaRG cells and IL-1β restricts HBV replication in infected HepaRG cells. Moreover, Dr. Watashi showed that AID is essential for the antiviral activity of IL-1β (JBC 2013, Watashi et al.). Therefore, involvement of AID in an antiviral pathway against HBV was suggested using a HepaRG model of natural HBV infection in our previous collaborative study; however, the molecular mechanism by which AID suppresses HBV replication was not determined at that time.

To further confirm the involvement of AID in TGF-β1-mediated restriction of HBV replication in an HBV infection model, we asked our previous collaborators, Drs. Wakita and Watashi, to send an HBV-producing cell line and NTCP-expressing HepG2 cells. Wakita's group has demonstrated that they can infect their NTCP-expressing HepG2 cells with HBV (BBRC 2013, 440:515). Those cell lines were received by us very recently (in Japan, material transfer of infectious research tool is time-consuming), and we performed a preliminary experiment of HBV infection by using their protocol, the results of which are shown below.



NTCP-expressing HepG2 cells were seeded with medium containing 4% DMSO. HBV was concentrated in PEG. NTCP-expressing HepG2 cells were infected with HBV (8000 genome equivalent / cell). One day after infection, one group was treated with

TGF-β1 and the other was not treated. After 3 days of TGF-β1 treatment, cells were harvested for RT-qPCR to determine AID, GAPDH, and HBV mRNA levels. Non-infected-NTCP HepG2 cells (treated with only PEG) were also used as a control. After normalization to GAPDH levels, the fold induction of AID and HBV RNA were determined. Cells infected with HBV but without TGF-β1 treatment were defined as one-fold induction.

The results above indicate that TGF-β1 upregulates AID mRNA, and TGF-β1 reduces HBV RNA levels in HBV-infected NTCP-HepG2 cells, which is consistent with our major claim in the manuscript; that is, AID downregulates HBV transcripts.

In summary, experimental evidence from two HBV stable cell lines (Figs. 3I–K and 7) and two natural infection models (attached Fig. 1, and our previous paper JBC 2013 Watashi et al.), ruled out an AID effect on transfected plasmid, and those results are consistent with AID-mediated HBV RNA reduction.

Reviewer #1
It is also important to consider viral escape strategies involving TGFb signaling which may have been developed in cells chronically infected, like HBV stable cell lines.

Response
Thank you for intriguing comment.
AID-mediated HBV RNA reduction depends on HBV P protein (Fig. 4C). Logically, the more efficiently AID reduces HBV RNA, the lower the level of P protein. Under the condition where P protein is limiting, AID-mediated HBV RNA downregulation is relatively inefficient. We think that reducing the copy number of HBV genome per cell is a plausible escape mechanism in HBV infection.
It would be also possible for HBV to develop other escape mechanisms. Therefore, we want to leave this question open for future study.

Reviewer #1

2) The microscopic analysis done is very weak. Proper confocal microscopy should be performed and images need to be captured at higher magnification to be able to properly discern various subcellular sites and precisely determine the colocalization between HBV P protein and AID.

Response

We do not have access to a confocal microscope; thus, we tried very hard to detect AID and HBV P proteins by immunostaining using conventional fluorescence microscopy (together with the approach using GFP and DsRed fusion proteins, which was shown in the first revision).
However, high background fluorescence and/or low specific signals of AID and P proteins prevented us from conclusively interpreting the results.
Meanwhile, we demonstrated a complex formation between AID and P proteins by immunoprecipitation following subcellular fractionation (Supplementary Fig. 3).
Those results indicate that AID and P proteins form complexes in both the nucleus and cytoplasm. Moreover, we also determined the subcellular fraction containing the AID/exsoc3/HBV-RNA complex (Fig. 5 and Supplementary Fig. 4). Since AID-mediated HBV RNA reduction was observed in the nuclear fraction (Supplementary Fig. 6), we think that AID, P protein, RNA exosomes, and HBV RNA form RNP complexes in both the nucleus and cytoplasm, and that RNA degradation occurs at least in the nucleus.

Reviewer #1

3) According to the authors AID and HBV P interact both in the cytoplasm and nucleus and all the HBV transcripts are likely affected. Which subcellular site is predominantly responsible for AID mediated degradation of HBV pgRNA.

Response

We appreciate this important question. Because AID, P protein, RNA exosome as well as HBV RNA molecules distribute to both nucleus and cytoplasm, it is not easy to conclude which subcellular site is predominantly responsible for AID mediated HBV RNA reduction.

To this end, we biochemically fractionated nuclear RNA and cytoplasmic RNA and determined the subcellular fractions in which AID reduces HBV RNA. The results show that AID-dependent HBV RNA reduction is observed in both fractions. Since nuclear RNA is an upstream of cytoplasmic RNA, we think that nuclear HBV RNA may be a primary target for AID-mediated RNA reduction. In the revised manuscript, these results are shown as Supplementary Fig. 6, and the main text was modified accordingly (lines 244–248, in red).
However, we do not exclude that AID also triggers cytoplasmic viral RNA decay. To conclude this, we need to find a condition that AID does not induce viral nuclear RNA but cytoplasmic RNA decay. At present, we have not found such a condition (like use mutant HBV, AID mutant or inhibition of AID nuclear export), and once this system is established, we can make a conclusion by experimental results.

Reviewer #1
Is HBV P protein required for the effect of AID on all other HBV transcripts?

Response
Yes.
Northern blotting in Fig. 4 demonstrates that AID expression reduces all types of HBV RNA in the presence of P protein while AID does not change the pattern of HBV RNA, as detected by northern blot, in the absence of P protein. In the 1st revised manuscript, we showed that all of HBV transcripts contain the eplison RNA structure that HBV P protein binds to. Therefore, HBV P protein is required for the effect of AID on all other HBV RNA.

Reviewer #1
4) Analysis of clinical samples from HBV patients would give more comprehensive understanding.

Response

This is an important analysis that we are also very interested. However, to add any relevant information from clinical samples, we would need to obtain RNA samples from liver biopsies within a very short period, which is not feasible. Again, Japan has strict relevant laws not letting us to obtain patients' samples in a short time.
Moreover, to add supportive evidence of AID-mediated HBV RNA reduction, we would need two types of liver samples (high and low AID expression). Unfortunately, useful SNP markers associated with differential expression of AID or AID-deficient patients are not available in the public data base.

Reviewer #1

5) Recently a similar mechanism involving ZAP protein mediated posttranscriptional degradation of HBV RNA has been reported (Mao et al, PLos Pathogens, 2013, e1003494). Is ZAP involved in AID mediated degradation of HBV RNA, the authors should silence ZAP and determine if AID activity is affected or not.

Response

Thank you for the excellent suggestion.
According to the study by Mao et al. (Plos Pathogenes 2013), transcriptional upregulation of ZAP expression by either IFNα or IPS-1 is important for ZAP-mediated HBV RNA reduction, especially for the ZAP short form. To explore the potential involvement of ZAP in AID-mediated HBV RNA reduction, we determined ZAP mRNA expression levels, and RT-qPCR shows no change in ZAP expression by AID expression. These results are included in Supplementary Fig. 2.

Next, as recommended by reviewer #1, we knocked down ZAP expression using siRNAs. The results demonstrated that knocking down of ZAP increases basal HBV RNA levels; however, it did not affect AID-mediated HBV RNA reduction. We think that ZAP is dispensable for AID-mediated HBV RNA reduction. These results will help readers to understand AID-mediated HBV RNA reduction; therefore, we mention knocking down of ZAP in the Discussion and the results

are shown as Supplementary Fig. 8 in the revised manuscript. (See lines 342–351 in red)

Reviewer #2:
The authors answered all my questions. Most of the new data provided are satisfied, except for the following two points.

> *In Figure 2A, loading of the first lane has problem because the loading control GAPDH in this lane is much weaker than other lanes. The new figure is needed to replace this one.*

Response
Thanks for this reminding.
We repeated the western blot and reconfirmed expression of FLAG-A3 proteins as well as GAPDH. Revised Fig. 2 was updated by replacing with new blots.

Reviewer #2
2) In Figure 5A, the labeling of the third lane is wrong, GFP-Exosec3 should be positive in this lane.

Response
Thank you very much. We corrected it.

TGF-β Suppression of HBV RNA through AID-dependent Recruitment of an RNA Exosome Complex

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The authors declare no competing financial interests.

Abstract

Transforming growth factor (TGF)-β inhibits hepatitis B virus (HBV) replication although the intracellular effectors involved are not determined. Here, we report that reduction of HBV transcripts by TGF-β is dependent on AID expression, which significantly decreases both HBV transcripts and viral DNA, resulting in inhibition of viral replication. Immunoprecipitation reveals that AID physically associates with viral P protein that binds to specific virus RNA sequence called epsilon. AID also binds to an RNA degradation complex (RNA exosome proteins), indicating that AID, RNA exosome, and P protein form an RNP complex. Suppression of HBV transcripts by TGF-β was abrogated by depletion of either AID or RNA exosome components, suggesting that AID and the RNA exosome involve in TGF-β mediated suppression of HBV RNA. Moreover, AID-mediated HBV reduction does not occur when P protein is disrupted or when viral transcription is inhibited. These results suggest that induced expression of AID by TGF-β causes recruitment of the RNA exosome to viral RNP complex and the RNA exosome degrades HBV RNA in a transcription-coupled manner.

Introduction

Hepatitis B virus (HBV) is recognized as the major causative factor of severe liver diseases such as cirrhosis and hepatocellular carcinoma. The clinical outcomes and development of hepatocellular carcinoma and cirrhosis are modulated by viral replication and antiviral immunity against HBV [1]. After entry into the host hepatocyte, HBV forms covalently closed circular DNA (cccDNA) in the nucleus and it initiates the transcription of viral RNAs, including a replicative intermediate known as pregenomic (pg) RNA. Two viral proteins (core and P protein) encapsidate pgRNA to form nucleocapsids, where P protein reverse-transcribes pgRNA to produce relaxed circular (RC)-DNA. These nucleocapsids associate with three types of viral surface proteins for secretion as infectious virions [1,2]. Although the mechanism of HBV replication has been well studied, the mechanisms of antiviral immunity against HBV remain unclear.

Several members of the apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC) family were recently identified as new types of antiviral factors [3-5]. In humans, the APOBEC family comprises at least 11 members, including activation-induced cytidine deaminase (AID), APOBEC 1, 2, 3A, 3B, 3C, 3D, 3F, 3G, 3H, and 4. Most family members deaminate cytidine bases on DNA and/or RNA to generate uridine [3-5]. Accumulating evidence from *in vitro* experiments has further revealed that A3 proteins can inhibit the replication of various types of viruses, including human immunodeficiency virus type 1 (HIV-1) and HBV [4,5]. Among APOBEC deaminases, the molecular mechanism of A3G antiviral activity has been well characterized. In cases of HBV, A3G restricts viral replication through hypermutation and inhibition of reverse-transcription [4,5]. AID is another member

of the APOBEC family [4,5] and was originally isolated as a cytidine deaminase that triggered class switch recombination (CSR) and somatic hypermutation (SHM) of transcribed immunoglobulin genes in B cells [6-9]. AID expression was recently shown to be upregulated in human hepatocytes *in vitro* after stimulation with cytokines, including TGF-β1, TNFα, and IL-1β and in the liver in chronic hepatitis patients, and AID involvement in viral infection was suggested [10-17]. Higher serum TGF-β1 levels were reported in some HBV infections *in vivo* [18,19], and TGF-β1 reduces HBV replication *in vitro* [18,20]. However, the precise mechanisms remain elusive. In the present study, we examined the involvement of AID in TGF-β1-mediated restriction of HBV replication. We have demonstrated that TGF-β1 induces AID expression in hepatocytes, which leads to the downregulation of HBV transcripts and inhibition of nucleocapsid formation. AID-dependent downregulation of HBV transcripts requires a viral RNA binding protein (P protein) and RNA exosome components. These data suggest a novel antiviral pathway in which AID recruits the RNA exosome to downregulate viral RNA in HBV infected hepatocytes.

Results

TGF-β1-mediated anti-HBV activity

To investigate the involvement of APOBEC deaminases in TGF-β1-mediated antiviral activity against HBV, human hepatocytes (Huh7) were transfected with a HBV replicon plasmid (pPB) [21] and the cells were then treated with TGF-β1. Concentrations of 5−20 ng/mL TGF-β1 were used to match the range reported in chronic HBV and hepatocellular carcinoma patients [19]. HBV replication was evaluated by measuring HBV transcript levels using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 1A) and Northern blotting (Fig. 1D). Viral DNA in secreted virions was determined using qPCR (Fig.

1B), and nucleocapsid formation was estimated using native agarose gel electrophoresis (NAGE). Subsequently, cytoplasmic nucleocapsid core protein and nucleocapsid associated DNA (NC-DNA) levels were determined using western blotting and Southern blotting, respectively (Fig. 1C). Collectively, TGF-β1 dose-dependently inhibited the production of HBV transcripts, nucleocapsid core protein, and nucleocapsid NC-DNA in both cytoplasmic and secreted samples.

In further experiments, qRT-PCR was used to determine the expression of APOBEC deaminases in the presence and absence of TGF-β1. Initially, relative expression levels of APOBEC deaminases in non-stimulated Huh7 cells were determined. Huh7 cells expressed all APOBEC3 deaminases. A3G and A3C were highly expressed among A3 deaminases (Fig. 1E), whereas APOBEC1 expression was not detected in Huh7 cells. In TGF-β1-treated Huh7 cells, expression of most APOBEC deaminases, including A3A, A3B, A3C, A3F, and AID (Fig. 1F, upper and lower) was upregulated. Western blotting also detected AID protein in TGF-β1-stimulated Huh7 cells (Fig. 1G).

TGF-β1-mediated reduction of HBV transcripts depends on AID expression

It has been demonstrated that APOBEC3 proteins suppress HBV replication *in vitro* [1,4,5]. HBV plasmids and APOBEC deaminase expression vectors were transfected into Huh7 cells, and nucleocaspid formation was estimated using NAGE followed by Southern and western blotting (NAGE assay). The expression of A3G and A3F, but not A3A, reduced NC-DNA levels in cytoplasmic nucleocapsids but did not reduce nucleocapsid core protein levels (Fig. 2A). HBV virion DNA was also reduced by A3C, A3G and A3F expression, whereas total HBV transcript levels were not affected by A3C, A3G or A3F (Fig. 2B and C). It was proposed that minus-strand DNA synthesis was the primary target of A3G-mediated anti-

114 HBV activity in hepatocytes that were transiently transfected with HBV plasmids [1,4,5]. Our
115 results support this proposed mechanism of A3G antiviral activity. In contrast with A3
116 deaminases, the overexpression of AID reduced HBV transcript levels, nucleocapsid
117 formation, and virion secretion (Fig. 2A-C and Supplementary Fig. 1A and B). Nucleocapsid
118 NC-DNA levels were also reduced in AID-expressing cells, as indicated by Southern blotting
119 using purified nucleocapsid NC-DNA (Fig. 2D). Importantly, AID expression did not
120 suppress host cell gene transcripts (Supplementary Fig. 2), suggesting that AID expression
121 may specifically suppress viral RNA. In accordance with the HBV life cycle, these data
122 suggest that AID-mediated reduction of HBV transcripts leads to the downregulation of
123 nucleocapsid core protein and NC-DNA.
124
125 To investigate the contributions of APOBEC deaminases to TGF-β1-mediated anti-HBV
126 activity, small interfering (si) RNAs targeting specific deaminases were transfected with the
127 HBV plasmid into Huh7 cells. Cells were further treated with TGF-β1 to assess the effects
128 on TGF-β1-mediated reduction of HBV transcripts. TGF-β1 stimulation in siGFP-
129 transfected control cells reduced HBV transcript levels by 76% compared with non-
130 stimulated cells (Fig. 2E, top, lane 4 vs. 8). Transfection of siAID, siA3A, or siA3G
131 suppressed the corresponding endogenous genes by up to 51%, 40%, and 56%, respectively.
132 However, the knockdown of A3A and A3G did not affect TGF-β1-mediated reduction of
133 HBV RNA in comparison with the siGFP control. In contrast, TGF-β1-mediated
134 downregulation of HBV RNA was significantly attenuated by the knockdown of AID (Fig.
135 2E, top, lane 1 vs. 4). These data suggest that TGF-β1-mediated downregulation of HBV
136 transcripts is dependent on endogenous AID expression. Partial rescue of HBV transcript
137 levels in siAID-transfected cells also suggests the involvement of either residual AID or other

unidentified effectors in TGF-β1-mediated reduction of HBV transcripts.

AID expression levels required for initiating class switching are sufficient for AID-mediated reduction of HBV transcripts

We previously demonstrated that the induction of AID in B cells triggers class switch recombination (CSR) in immunoglobulin genes [7-9], which validates B cells as a model to study AID functions. In addition, it is anticipated that peripheral blood mononuclear cells and B cells can be extrahepatic reservoirs for HBV infection [22,23]. Thus, we investigated whether endogenous AID expression that could trigger CSR is also sufficient to trigger a reduction in HBV transcripts. AID expression and IgA class switching can be induced in CH12F3-2 mouse B cells following co-stimulation with CD40 ligand, IL-4, and TGF-β1 (designated CIT) [6,24]. CH12F3-2 cells transiently transfected with the HBV plasmid were divided into two groups, and were treated with (or without) CIT to induce IgA switching, a GFP expression vector was co-transfected to verify transfection efficiency. At three days post-transfection, HBV replication and CSR were determined (Fig. 3A-D), and showed that CIT induced AID protein expression and initiated IgA class switching, as previously reported [6,24]. Moreover, NAGE assays and qRT-PCR revealed that HBV transcripts, nucleocapsid NC-DNA, and core protein were downregulated in CIT-stimulated cells, whereas the expression of GFP remained intact after CIT stimulation (Fig. 3B, C). These data indicate that CIT stimulation specifically inhibits HBV replication in mouse B cells. We further used siRNAs against mouse AID (simAID-1 and -2) to assess the contribution of AID to the suppression of HBV products in CIT-stimulated cells. Although simAIDs knocked down endogenous AID transcripts to only 39% determined by qRT-PCR (Fig. 3E), western blotting revealed clear suppression of endogenous AID protein levels (Fig. 3F). Furthermore, flow cytometric analyses revealed that IgA class switching is attenuated by the knockdown of AID

(Fig. 3G), and qRT-PCR revealed that HBV transcript levels are inversely correlated with AID expression and IgA switching efficiency (Fig. 3G, H). To avoid artifacts due to the transfection process, a tetracycline-dependent stable line of the HBV replicon plasmid was established in CH12F3-2 cells (CH12-HBV; Fig. 3I). CH12-HBV cells were treated with CIT to induce IgA switching, and HBV transcript levels were determined. Subsequent qRT-PCR analyses demonstrated significant reductions of HBV transcript levels upon IgA switching (Fig. 3J–K). These data clearly demonstrate that endogenous AID expression sufficient to trigger CSR is also sufficient to downregulate HBV transcripts.

Another putative activity of AID involves the initiation of somatic hypermutation (SHM) in immunoglobulin variable genes [8,9],[25] previously demonstrated that human BL2 B cells autonomously induce SHM, which is absent following AID gene disruption by gene targeting. Thus, we transiently transfected the HBV replicon plasmid into BL2 cells and compared HBV replication in *Aicda*+/+ and *Aicda*−/− BL2 cells. We previously demonstrated that nucleocapsid NC-DNA and core protein are suppressed in *Aicda*+/+ in comparison with *Aicda*−/− BL2 cells, although co-transfected GFP expression levels were similar in both cell types [26]. Using identical samples, we here showed that HBV transcript levels in *Aicda*+/+ BL2 cells were almost 50% of those in *Aicda*−/− BL2 cells (Fig. 3L).

Both mouse and human B cell lines collectively demonstrated that endogenous AID activity that can initiate either CSR or SHM of immunoglobulin genes is sufficient to trigger downregulation of HBV transcripts.

AID-mediated downregulation of HBV transcripts requires intact P protein structure

To investigate the mechanism of AID-mediated downregulation of HBV transcripts, we initially focus on the viral P protein, because AID, P protein and HBV transcripts form RNP

complex [26]. In these experiments, we applied a mutant HBV replicon plasmid (pPB-ΔP, Fig. 4A) that expresses a mutant P protein lacking the C-terminal half including catalytic DNA polymerase and RNase H domains [26]. Transfection with pPB-ΔP did not support nucleocapsid DNA synthesis due to inhibition of reverse-transcription, although HBV transcription and core protein synthesis remained intact in Huh7 cells (Fig. 4C, lanes 1 and 4). AID-mediated downregulation of HBV transcripts was compared between pPB- and pPB-ΔP-transfected Huh7 cells. As shown in Fig. 4C, AID-mediated downregulation of HBV transcripts was not observed in pPB-ΔP-transfected Huh7 cells, indicating that AID-mediated downregulation of HBV transcripts requires intact viral P protein.

The requirement of cytidine deaminase activity for AID was also investigated. AID mutant P19 was isolated from a class switch deficient patient and the deaminase activity was negligible owing to a missense mutation in catalytic cytidine deaminase domain [27]. P19 was then co-transfected with the wild-type HBV plasmid, and HBV transcript levels were compared with that in wild-type AID controls. These experiments showed that the P19 mutant significantly reduced HBV transcript level, although less effectively than wild-type AID (Fig. 4C). Therefore, under experimental conditions of AID over-expression, cytidine deaminase activity is not exclusively required for AID-mediated downregulation of HBV transcripts.

In subsequent experiments, we generated an expression vector (pFLAG-PΔC) for the mutant P protein which was a corresponding mutant P protein produced from pPB-ΔP-transfected cells (Fig. 4B). Then the physical association between AID and the mutant P protein was examined. Immunoprecipitation analyses showed that wild type P protein co-precipitated AID in an RNase A-sensitive manner (Fig. 4D, lane 5, 8, 9), whereas the mutant P protein

(FLAG-PΔC) precipitated only trace levels of AID protein, suggesting that AID may not efficiently form RNP complex with the mutant P protein in pPB-ΔP-transfected cells. To explore which subcellular sites are responsible for AID and P protein interaction, cells were biochemically fractionated into three fractions (cytoplasmic, soluble nuclear, and insoluble nuclear) (Supplementary Fig. 3). Immunoprecipitation analyses using cytoplasmic and soluble nuclear proteins revealed that AID can associate with P protein in both nucleus and cytoplasm. It is of note that robust signals of AID and P proteins were found in the insoluble fraction that contains chromatin and other nuclear proteins.

AID-mediated downregulation of HBV transcripts requires the RNA exosome complex

AID was recently shown to physically interact with RNA exosome proteins and promote CSR in transcribed immunoglobulin genes [28,29]. The RNA exosome comprises a ring-like structure and two catalytic components, and plays a major role in various RNA processing and degradation pathways [30,31]. Exosome component 3 (Exosc3, also known as Rrp40) is non-catalytic but is essential for the degradation and processing of target RNA, and the knockdown of Exosc3 severely diminished the RNA exosome function [32]. Thus, we investigated whether Exosc3 is involved in TGF-β1-mediated downregulation of HBV transcripts in Huh7 cells. As shown in Fig. 5A, immunoprecipitation of AID co-purified Exosc3, but did not precipitate GAPDH or GFP. Exosc3 immunoprecipitation also co-purified AID but not GAPDH or GFP (Fig. 5B), indicating a physical association between AID and Exosc3 proteins. This study found a physical association between AID and the RNA exosome proteins (Exosc 2, 3, 7) in Huh7 cells in the absence of HBV replication (Fig. 5D). As expected, Exosc3 immunoprecipition also copurified with other RNA exosome proteins (Exosc2 and 7) in Huh7 cells (Fig. 5D). Furthermore, we found that AID can also associate with RNA exosome in both nucleus and cytoplasm (Supplementary Fig. 4A). Consistent with

AID-RNA exosome interaction, RNA exosome proteins localized to both cytoplasm and nucleus (Supplementary Fig. 5A and B). We previously demonstrated a physical association between HBV transcripts and AID in HBV-replicating Huh7 cells [26]. In current study, we examined whether Exosc3 associates with HBV transcripts. As shown in Fig. 5C, qRT-PCR analysis demonstrated enrichment of HBV but not HPRT transcripts in Exosc3 immunoprecipitates, which was observed only when AID was present (Fig. 5C, lane 1). This is also true when nuclear or cytoplasmic Exosc3 was separately precipitated (Supplementary Fig. 4B). AID-mediated downregulation of HBV transcripts was observed in both nucleus and cytoplasm, and efficiency of downregulation was comparable between nucleus, cytoplasm, and whole cell samples (Supplementary Fig. 6A and B). These results suggest that AID recruits the RNA exosome proteins to HBV transcripts and AID downregulates HBV RNA in nucleus.

To further confirm that the RNA exosome is involved in AID-mediated downregulation of HBV transcripts, we used the siRNA knockdown of Exosc3, which is essential for the RNA exosome function [32]. In these experiments, siRNAs against Exosc3 were co-transfected with the HBV plasmid and AID (or GFP) expression vectors, and HBV replication was determined. Northern blotting, NAGE assays, and qRT-PCR analyses showed the attenuation of AID-mediated downregulation of HBV transcripts and nucleocapsid formation in siExosc3 transfectants (Fig. 5E-G). In contrast, AID, GFP, and GAPDH expression were not affected by Exosc3 depletion (Fig. 5E, bottom). Importantly, knock down of Exosc3 did not increase HBV RNA levels in GFP transfected samples. Moreover, siExosc3 transfection attenuated TGF-β1-mediated downregulation of HBV transcripts and nucleocapsid formation in a similar manner to that observed after transfection with siAID (Fig. 6A-F). In further experiments, knockdown of another RNA exosome component Exosc6 also attenuated TGF-

262 β 1-mediated downregulation of HBV transcripts and nucleocapsid formation, albeit less
263 effectively than the knockdown of siExosc3 and AID (Fig. 6A-F). Similarly, the contributions
264 of AID and Exosc3 to TGF- β 1-mediated downregulation of HBV transcripts were examined
265 in stably HBV-transfected Huh7 cells (7T7-8) [26]. The short hairpin (sh) RNA expressing
266 lentivirus was transduced into 7T7-8 cells, and two stable transfectants (shAID and
267 shExosc3) and a control transfectant (shLuc) were established after puromycin selection.
268 These cells were then cultured in the presence or absence of TGF- β 1 (Fig. 7A). Subsequent
269 qRT-PCR and western blotting showed reduced endogenous AID and Exosc3 expression (Fig.
270 7B-E). Comparison of HBV transcript levels between TGF- β 1-treated and non-treated 7T7-
271 8 cells revealed that TGF- β 1-mediated reduction of HBV transcripts is restored by the
272 knockdown of AID and Exosc3 (Fig. 7F). Taken together, these data indicate that RNA
273 exosome proteins (Exosc3 and Exosc6) and AID are required for TGF- β 1-mediated
274 downregulutation of HBV transcripts.
275
276 **AID-mediated downregulation of HBV transcripts depends on transcription**
277 Immunoglobulin gene diversification triggered by AID is coupled with the transcription of
278 immunoglobulin locus [8,9]. Here we examined whether AID-mediated HBV RNA
279 downregulation is also coupled with transcription using a transcription inhibitor actinomycin D
280 (ActD). Using a stable HBV transfectant (7T7-8), we generated experimental conditions in
281 which endogenous or ectopic AID is expressed in HBV-replicating cells. ActD was then added
282 to evaluate whether it could downregulate HBV RNA even in ActD-treated cells. As shown in
283 Fig. 8A and B, no significant synergistic reduction in HBV RNA levels by ActD and AID was
284 observed in TGF- β 1-treated and AID-overexpressing cells, indicating that AID was unable to
285 reduce HBV RNA levels in ActD-treated cells. These results suggest that AID-mediated HBV

RNA downregulation depends on transcription, similar to the immunoglobulin gene diversification triggered by AID.

Discussion

AID is a key molecule involved in the diversification of immunoglobulin genes [8,9], and thus its role in B cells is well understood. AID expression has been also found in non-B cells [11-13], however, its role in non-B cells remains elusive. In the present study, we assessed AID involvement in TGF-β1-dependent anti-HBV activity and demonstrated the following: (1) AID expression is upregulated in TGF-β1-stimulated hepatocytes and reduces HBV RNA levels (Fig. 1 and 2); (2) TGF-β1-mediated downregulation of HBV transcripts is inhibited by AID knockdown (Fig. 2); and (3) endogenous AID protein levels in B cells capable of inducing immunoglobulin diversification also downregulate HBV transcript levels in a transcription-coupled manner (Fig. 3 and 8). These data indicate that AID is involved in a TGF-β1-mediated anti-HBV pathway.

Which part of the virus life cycle that is targeted by AID-mediated downregulation of HBV transcripts? Another APOBEC protein, A3A, which was previously proposed to hypermutate transfected plasmids in human peripheral monocytes [33]. However, AID did not change HBV transcript levels in hepatocytes transfected with the mutant HBV replicon (pPB-ΔP) (Fig. 4C). In contrast, HBV transcripts in hepatocytes transfected with the wild-type replicon (pPB) were specifically downregulated by following the expression of AID expression (Fig. 2 and 4). Intact HBV transcript levels in AID-expressing pPB-ΔP transfectants suggest that AID-mediated reduction of HBV transcripts is not due to plasmid targeting or promoter interference by AID activity. Otherwise, targeting of HBV plasmid or promoter activity would result in reduction of HBV transcripts in both pPB- and pPB-ΔP-transfectants

311 because those HBV plasmids share the exactly same DNA sequences except 4 base insertion
312 within *P* gene in pPB- Δ P. Previous our study demonstrated that chicken AID can
313 downregulate cccDNA of duck hepatitis virus in a uracil-DNA glycosylase (UNG)-dependent
314 manner [34], therefore, the next obvious candidate for AID target is cccDNA of HBV. We
315 determined cccDNA levels of transfectants using the rolling circle amplification (RCA) assay,
316 which specifically amplifies circular DNA, including cccDNA. As per our results, cccDNA
317 was clearly detected in a cccDNA-producing control cell line (HepG2.2.5) [10-15,35];
318 however, the HBV-replicating transfectants used in this study rarely produced cccDNA
319 (Supplementary Fig. 7A and B). Therefore, the majority of the HBV transcripts produced
320 from HBV transfectants in the present experimental systems are derived from HBV replicon
321 plasmids and not from cccDNA. That means that targeting of cccDNA does not explain the
322 observed downregulation of HBV transcripts in the present experimental systems. AID over-
323 expression was previously shown to deaminate nucleocapsid NC-DNA and encapsidated
324 pgRNA [10,13,26]. However, because NC-DNA is reverse transcribed from HBV pgRNA,
325 AID activity against NC-DNA fails to explain the downregulation of HBV transcripts.
326 Reduction of HBV RNA by the catalytically dead mutant AID (p19) indicates that
327 encapsidated pgRNA editing is distinct from AID-mediated reduction of HBV RNA. Thus,
328 we concluded that AID directly targets HBV transcripts.
329
330 The viral P protein is a reverse transcriptase that binds 5′ -epsilon RNA structure in pgRNA
331 and encapsidates pgRNA to the nucleocapsid [1,2] (see also Supplementary Fig. 1). It is
332 demonstrated that P protein can also bind 3′ -epsilon RNA structure present in 2.4-, 2.1-, and
333 0.7-kb viral mRNAs [36], indicating that P protein binds all types of HBV transcripts. AID
334 and TGF-β downregulate HBV transcripts containing 3′ -epsilon but not cellular transcripts
335 (Figs. 1D, 4C, 5E, 6A, and Supplementary Figs. 1, 2). AID-mediated HBV RNA reduction

did not occur in hepatocytes expressing a mutant P protein (Fig. 4C). We demonstrated that AID can physically associate with viral RNP complexes comprising P protein [26] (Fig. 4). Therefore, AID-mediated HBV RNA reduction is dependent on the presence of intact P protein and P protein may determine the target specificity for AID-mediated HBV RNA reduction.

Mao *et al.* recently reported that zinc finger antiviral protein (ZAP) inhibits the replication of HBV by binding the 5′ -epsilon RNA structure of HBV and degrading viral RNA [37]. To explore the molecular mechanism of AID-mediated downregulation of HBV transcripts, we first investigated the possible involvement of ZAP. RT-qPCR revealed that AID expression did not affect the level of ZAP mRNA (Supplementary Fig. 2). Knocking down of ZAP by transfection of siRNAs against ZAP increased HBV RNA levels, which indicates that ZAP reduces the basal level of HBV RNA; however, AID-mediated downregulation of HBV transcripts was not affected by knocking down of ZAP expression (Supplementary Fig. 8). These results imply that the ZAP antiviral pathway is dispensable for AID-mediated downregulation of HBV transcripts.

Next, we explored the possible involvement of the RNA exosome. Basu *et al.* [29] demonstrated that AID binds and recruits the RNA exosome complex to R-loop structures in immunoglobulin genes. Here, we investigated whether AID forms a complex with RNA exosome proteins in hepatocytes. The immunoprecipitation of AID and Exosc3 revealed the formation of a RNP complex comprising AID and RNA exosome proteins in both nucleus and cytoplasm of hepatocytes, and that HBV transcripts formed a specific complex with the RNA exosome in an AID-dependent manner (Fig. 5, Supplementary Fig. 4). Furthermore, AID-dependent downregulation of HBV transcripts was inhibited in the absence of the

361 essential RNA exosome component Exosc3 (Fig. 5). We also demonstrated that AID-
362 mediated downregulation of HBV transcripts does not occur when P protein loses the C-
363 terminus domain, which is essential for AID binding (Fig. 4C). Inhibition of transcription
364 resulted in blocking of AID-mediated downregulation of HBV transcripts (Fig. 8). Taken
365 together, we suggest that AID recruits the RNA exosome to transcribing HBV RNA through
366 an association with the P protein, and thereby downregulates HBV transcripts (Fig. 8C).
367
368 AID has been shown to reduce the transpositioning of the reverse transcriptase-dependent
369 retroelement L1 [14,15]. Moreover, MacDuff et al. demonstrated that a catalytically dead
370 mutant and wild-type AID suppress L1 transpositioning. Here, we showed that the AID-
371 mediated HBV RNA reduction depends on HBV reverse transcriptase (P protein), and
372 catalytically dead mutant AID (p19) reduces HBV transcript levels (Fig. 4). It would be
373 interesting to examine whether suppression of transpositioning by AID is also dependent on
374 the RNA exosome.
375 To our knowledge, this is the first study to show that AID mediates the downregulation of
376 viral RNA through the RNA exosome complex. However, further studies are required to
377 elucidate the mechanisms of AID-mediated HBV RNA downregulation, and to investigate the
378 involvement of AID in anti-HBV activity *in vivo*.
379
380 **Materials and Methods**
381 **NAGE assays**
382 NAGE assays were performed as previously described [20,26,38,39]. In brief, intact
383 nucleocapsid particles were separated from crude extracts of HBV-replicating cells using
384 agarose gel electrophoresis. Nucleocapsid particles within the gel were then denatured under
385 alkaline conditions, and were transferred onto nitrocellulose membranes (Roche).

386 Nucleocapsid DNA and core proteins were detected using Southern and western blotting with
387 a double-stranded HBV DNA probe spanning the whole viral genome and an anti-core
388 antibody, respectively.

389 **Cell culture and transfection**

390 Plasmids were transfected into Huh7 cells using CalPhos (Clontech) or Fugene 6 (Roche).
391 The total transfected plasmid per sample was normalized by supplementation with empty or
392 GFP expression plasmids. Co-transfection of plasmid and siRNA was performed using
393 lipofectamine 2000 according to the manufacturer's instructions. Stealth-grade siRNA for
394 mouse and human AID, A3A, A3G, Exosc3, Exosc6, and control were purchased from
395 Invitrogen. In all transfection experiments, control siRNA was designed to differ from all
396 mammal transcripts. BL2 [25] and CH12F3-2 cell culture, CIT stimulation, and transfection
397 by electroporation were performed as previously described [24-26,40]. The HBV-replicating
398 Huh7 cell line (7T7-8) was established and described previously [26]. The pTre-HBV [41]
399 vector was transfected into tetracycline activator expressing CH12F3-2 cells (FTZ14 [42]) to
400 establish the CH12-HBV cell line. Subsequently, shLuc, shAID, and shExosc3 expressing
401 7T7-8 cells were established by infection with recombinant lentivirus followed by puromycin
402 selection. Recombinant lentiviruses were generated by transient transfection of shLuc-,
403 shAID-, and shExosc3-pLKO1-puro and packaging plasmids (pMD2.D and psPAX2,
404 Addgene plasmid 12259 and 12260, respectively, kind gifts of Dr. Trono) in 293T cells
405 according to the manufacturer's instructions.

406

407 **Expression vectors and reagents**

408 Human TGF-β1 and IL-4 were purchased from R&D systems. Actinomycin D was
409 purchased from Sigma-Aldrich. The HBV replicon plasmid (pPB) contains 1.04 copies of
410 HBV genomic DNA and expresses pgRNA under the control of the CMV promoter [21]. The

411 pPB-ΔP plasmid contains a frame-shift mutation in codon 306 of the *P* gene, leading to loss
412 of the C-terminal 539 amino acids, which comprise catalytic and RNase H domains [26].
413 Probe labeling and northern and Southern blots were developed using the AlkPhos direct
414 labeling system (Amersham). Signals for northern, Southern, and western blots were
415 analyzed using a LAS1000 Imager System (FujiFilm). Other expression vectors are listed in
416 Supplementary Table S1.
417
418 **Immunoprecipitation and western blotting**
419 Cells were lysed in buffer containing 50-mM Tris-HCl (pH 7.1), 20-mM NaCl, 1% NP-40, 1-
420 mM EDTA, 2% glycerol, and protease inhibitor cocktail (Roche). After centrifugation,
421 supernatants were incubated with the indicated antibodies and protein G sepharose (GE
422 Healthcare) or anti-FLAG M2 agarose beads (Sigma, A2220). For IP-qRT-PCR experiments,
423 cells were lysed with PBS containing 0.1% Tween 20, 1% triton-X, 1-mM EDTA, protease
424 inhibitor cocktail (Roche), and 2% glycerol. After centrifugation, crude lysates were
425 subjected to anti-FLAG M2 beads for 4 h. Immune complexes were washed in lysis buffer 10
426 times and were then washed in lysis buffer containing an additional 100-mM NaCl. FLAG-
427 Exosc3 and RNA complexes were eluted using free 3 × FLAG peptides (Sigma, F4799).
428 Western blotting was performed using standard methods with rabbit anti-GAPDH (Sigma,
429 G9545), mouse anti-FLAG (Sigma, F3165), rabbit anti-GFP (Clontech, 632376), anti-rabbit
430 Igs HRP (Biosource, ALI3404), anti-rat Igs HRP (Jackson ImmunoResearch, 712-035-153),
431 rabbit and mouse IgG TrueBlot (eBioscience, 18-8816, 18-8877), rat monoclonal anti-AID
432 (MAID2, eBioscience, 14-5959), rabbit anti-A3G[38], anti-core (Dako, B0586), anti-human
433 Exosc3 (GenWay Biotech, GNB-FF795C, F8130F), and isotype control (eBioscience 14-
434 4321) antibodies. To generate a polyclonal antibody against AID, the C-terminal AID peptide
435 (EVDDLRDAFRMLGF) was conjugated with cysteine and rabbits were immunized using

keyhole limpet hemocyanin (KLH). Subsequently, anti-AID rabbit serum and rat monoclonal anti-AID were isolated and used in IP experiments. IgA class switching was determined by detecting surface IgA expression using flow cytometry as previously described[7,24,40].

Quantitative PCR and RT-PCR

Total RNA was extracted using TRIsure (Bioline), was treated with DNase I (Takara) to eliminate transfected plasmids, and was then re-purified using TRIsure. For qRT-PCR analyses, 1 μg of total RNA was treated with amplification grade DNase I (Invitrogen) and was then reverse-transcribed using oligo-dT or random primers with SuperScript III (Invitrogen). Subsequently, cDNA was amplified using SYBR green ROX (Toyobo) with MX3000 (Stratagene) according to the PCR protocol. A1, AID, A3A, A3B, A3C, A3D, A3F, A3G, A3H, Exosc3, Exosc6, 18S ribosomal RNA, HPRT, and β-actin expression and HBV transcription were determined using PCR conditions of 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 70°C for 30 s, and one cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. For A3A amplification, an annealing temperature of 60°C was used. Copy numbers of APOBECs were determined using plasmid standard curves for each APOBEC (Fig. 2A). Fold induction of APOBEC expression following treatment of cells with TGF-β1 was determined using the $\Delta\Delta$CT method [43]. To eliminate transfected plasmids, purified NC-DNA from secreted virions and cytoplasmic lysates was obtained after serial DNase I digestion, proteinase K and SDS digestion, phenol–chloroform extraction, and isopropanol precipitation. NC-DNA copy numbers were determined using a HBV plasmid standard curve. Transcript expression levels in this study (except Fig. 2A) are presented as fold induction relative to unstimulated cells. In transfection experiments, expression levels of mock-, GFP-, siGFP-, and siLuc-transfected cells were defined as one. Expression levels in qRT-PCR analyses were normalized to the amplification of internal controls (HPRT, β-actin,

461 or 18S ribosomal RNA). Primers are listed in Supplementary Table S2.

462

463 **Statistical analysis**

464 Differences were identified using the two-tailed unpaired Student's *t*-tests and were
465 considered significant when $P < 0.05$.

466

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469 cells, respectively. We also thank Ms. Imayasu for their technical support, and Dr. T. Honjo
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471

472 **Author Contributions**

473 G.L., G.L., and K.K. performed the experiments. K.W, S.C., M.K., A.M., M. S., and W.Z.
474 assisted with the experiments. G.L., K.K. edited the manuscript, and M.M. directed the study
475 and wrote the manuscript.

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595

596

597 **FIGURE. LEGENDS**

598 **Figure 1. TGF-β1 upregulates APOBEC3 expression and suppresses HBV replication**

599 **in Huh7 cells**

600 Six hours after transfection of pPB, Huh7 cells were treated with TGF-β1 for 3 days, and

601 HBV replication was evaluated. (A) qRT-PCR shows dose-dependent reduction of HBV

602 transcripts by TGF-β1. (B) NC-DNA levels in secreted virions were also measured using

603 qPCR. (C) Nucleocapsid NC-DNA and core protein levels in crude cytoplasmic extracts were

604 assessed using NAGE assays. GAPDH protein levels in the same crude extracts were

605 determined using western blotting. (D) Huh7 cells were treated with 150 ng/mL IL-4 or 10

606 ng/ml TGF-β1 for 3 days. Levels of HBV RNA and GAPDH mRNA were determined by

607 Northern blot. Control: non-stimulated Huh7 cells. (E) Relative expression levels of

608 APOBEC deaminases in non-stimulated Huh7 cells; Relative expression levels were

609 determined using qPCR with cDNA from non-stimulated Huh7 cells and standard curves of

610 control APOBEC deaminase DNA. Relative copy numbers of A3B were defined as one. (F)

611 Induction of APOBEC deaminase expression in TGF-β1-treated Huh7 cells was estimated

612 using qRT-PCR. Fold induction of APOBEC deaminases is shown in the top (10 ng/mL TGF-

613 β1 for 24 or 48 h) and bottom (10 or 20 ng/mL TGF-β1 for 24 h) panels. (G) Huh7 cells

614 were treated with indicated concentrations of TGF-β1 for 3 days. AID protein was

615 immunoprecipitated using an anti-AID antibody (or an isotype control IgG, most right) and

616 immunoprecipitated AID protein was determined by western blot. One lane contains

617 immunoprecipitated protein harvested from 60% of 15 cm dish. All data are representative of

618 two to four independent experiments. Error bars represent standard errors of the mean.

Figure 2. AID is responsible for TGF-β1-mediated reduction of HBV transcripts

To evaluate antiviral activity of indicated APOBEC proteins, Huh7 cells were co-transfected with FLAG-tagged A3A, A3C, A3F, A3G, GFP or GFP-tagged AID expression vectors and pPB. Cells were cultured for 3 days, and then HBV replication was estimated using NAGE assays (A). Protein expression is shown (A, bottom). qRT-PCR analyses of HBV transcripts (B), and qPCR analyses of NC-DNA in secreted virions (C). (D), Secreted virions in the culture medium and cytoplasmic extracts were treated with proteinase K and SDS to digest nucleocapsids, and levels of HBV DNA were determined using Southern blotting. (E), To evaluate contribution of indicated APOBEC proteins, Huh7 cells were co-transfected with pPB and the indicated siRNAs. Six hours later, cells were incubated in the presence or absence of 10 ng/mL TGF-β1. Three days later, total RNA was extracted, and qRT-PCR performed to determine expression levels of HBV transcripts, AID, A3A, and A3G. Although siAID significantly reduced AID expression and prevented the downregulation of HBV transcripts in TGF-β1-stimulated Huh7 cells (lane 1), siA3A and siA3G had no effects against the downregulation of HBV transcripts (lanes 2–4). siGFP was used as a control. Expression levels in lane 8 are defined as one fold induction. $^{**}P < 0.01$ (*t*-test); Data are representative of two to three independent experiments and error bars represent standard errors of the mean.

Figure 3. IgA switching activity correlates with reduction of HBV transcripts in B cells

(A, B, C, D) pPB and GFP expression vectors were transiently co-transfected into a mouse B cell line (CH12F3-2). Six hours after transfection, cells were divided into two groups and stimulated with (or without) CD40 ligand, IL-4, and TGF-β1 (CIT) for 3 days to induce IgA switching; (A) Schematic diagram of experimental design; (B) Nucleocapsid formation was

644 measured using NAGE assays and GFP expression was used to confirm transfection. (C)
645 HBV transcripts and AID expression levels were determined using qRT-PCR. (D) AID
646 dependent IgA switching was determined using flow cytometry. (E, F, G, H) CH12F3-2 cells
647 were co-transfected with pPB and the indicated siRNA against mouse AID (simAID-1 and -2)
648 or controls (siCtrl and siGFP), and after 6 hours incubation, cells were further stimulated with
649 CIT for 3 days. HBV transcript levels, knock down efficiency of AID, and IgA switching
650 were determined using qRT-PCR, western blotting, and flow cytometry, respectively. (I, J, K)
651 A tetracycline promoter-regulating HBV plasmid (pTre-HBV) was stably transfected into
652 CH12F3-2 transfectants expressing tetracycline-responsible transactivator (Tet-off).
653 Established CH12F3-2 transfectants were designated CH12-HBV; (I) Schematic diagram of
654 CH12-HBV; (J) CH12-HBV cells were incubated in the presence or absence of CD40 ligand,
655 IL-4, or TGF-β1 (CIT) and tetracycline as indicated for 2 days to induce endogenous AID
656 expression and IgA switching. HBV transcription and AID expression levels were determined
657 using qRT-PCR. (K) IgA switching was detected according to surface expression of IgA using
658 flow cytometry. (L) AICDA-deficient and -wild type BL2 cells were transfected with HBV
659 plasmid (pPB), and qRT-PCR was performed at 3 day post-transfection. *$P < 0.05$, **$P <$
660 0.01 (*t*-test). Data are representative of two to three independent experiments and error bars
661 represent standard errors of the mean.
662
663 **Figure 4. Intact P protein is required for AID-mediated downregulation of HBV**
664 **transcripts and AID associates with HBV P protein.**
665 (A) Schematic diagram of wild-type and mutant HBV replicon plasmids. Partially redundant
666 HBV genomic DNA is shown as black boxes and the positions of 5' - ε and 3' - ε are
667 shown. Open reading frames corresponding to C, P, S, and X genes are shown as open boxes.
668 The position of the frame-shift mutation in the mutant replicon plasmid (pPB- Δ P) is

669 indicated as an open triangle. This frame-shift mutation results in loss of the C-terminal
670 portion (polymerase and RNase H domains) from the P protein; pCMV, CMV promoter. (B)
671 Schematic diagram of P protein domain structure; (C) Replicon plasmid (pPB or pPB-ΔP)
672 and GFP fusion expression vectors (mock, AID, and p19-mutant AID) were transfected into
673 Huh7 cells, and after four days, AID-mediated downregulation of HBV transcripts was
674 compared between two replicon plasmids or between wild-type and p19 mutant AID using
675 northern blotting. Expression of HBV core and GFP fusion proteins (mock, AID, and p19-
676 mutant AID) was confirmed using SDS-PAGE and western blotting. (D) Wild-type replicon
677 plasmid (pPB) and indicated protein expression vectors (FLAG-P, FLAG-PΔC, or AID)
678 were transfected into Huh7 cells. Three days later, physical associations between AID and
679 FLAG-P (or FLAG-PΔC) proteins were determined using immunoprecipitation (IP). In lane
680 9, crude extract was incubated with RNase A before immunoprecipitation. Positions of
681 FLAG-P and FLAG-PΔC proteins are indicated by arrows and diamonds, respectively. The
682 structure of FLAG-PΔC protein is shown in B. Input; crude extract. Data are representative
683 of two to three independent experiments.
684
685 **Figure 5. AID inducing HBV RNA reduction depends on Exosc3**
686 (A, B) Huh7 cells were co-transfected with pPB and the indicated protein expression vectors,
687 and were cultured for 3 days. Crude extracts (input) were then subjected to IP using an anti-
688 FLAG antibody, and crude extracts and IP fractions were analyzed using western blotting. (C)
689 Fold enrichment of HBV or HPRT transcripts upon anti-FLAG-Exosc3 IP; To determine
690 RNA coprecipitation with the RNA exosome component Exosc3, Huh7 cells were transfected
691 with pPB, pFLAG-Exosc3, and pCMV-AID (or pEGFP-C2), and were cultivated for 3 days.
692 IP using anti-FLAG antibody was then performed, complexes of FLAG-Exosc3 were then
693 eluted using free FLAG peptides, and the eluted RNA was analyzed using qRT-PCR.

694 Combination of expression vectors used for transfection is the same with B (see numbers
695 below the graph), and values in lane 3 were defined as 1. Error bars represent standard errors
696 of the mean. (D) Associations of AID with RNA exosome proteins; Huh7 cells were co-
697 transfected with indicated expression vectors, and were cultured for 3 days. Crude extracts
698 (input) were subjected to IP with FLAG antibody, and crude extracts and IP fractions were
699 analyzed using western blotting. Expression levels of GFP-Exosc7 were too low to be
700 visualized in the crude extract (lanes 4 and 9, input), but GFP-Exosc7 was clearly detectable
701 after FLAG-AID and FLAG-Exosc3 immunoprecipitation (lanes 4 and 9, IP). (E) Huh7 cells
702 were co-transfected with pPB and either AID or GFP expression vectors and each of the
703 siRNAs indicated in E and F, and cells were cultured for 3 days. HBV transcript levels,
704 nucleocapsid formation, and Exosc3 expression were estimated using northern and western
705 blotting, NAGE assays (E), and qRT-PCR analyses (F and G); siGFP and siCtrl were used as
706 controls; $^{**}P < 0.01$ (*t*-test); Data are representative of two to three independent experiments
707 and error bars represent standard errors of the mean.
708
709 **Figure 6. TGF-β1-mediated downregulation of HBV transcripts requires RNA exosome**
710 **proteins**
711 Huh7 cells were transfected with pPB and indicated siRNAs. Six hours after transfection, the
712 cells were incubated in the presence or absence of 10-ng/mL TGF-β1 for 3 days. Total RNA
713 was analyzed using northern blotting (A) and qRT-PCR to determine HBV transcription of
714 AID, Exosc3, and Exosc6 (B, D, E, F). In C, NC-DNA from secreted virions was also
715 measured by qPCR. Transfection of siAID and siExosc3 partially restored TGF-β1-mediated
716 downregulation of HBV transcripts and viral production; $^{*}P < 0.05$, $^{**}P < 0.01$ (*t*-test); error
717 bars represent standard errors of the mean. Data are representative of two to three
718 independent experiments.

Figure 7. TGF-β1-mediated reduction of HBV transcripts depends on AID and Exosc3

Stable HBV transfectant Huh7 cells (7T7-8) were infected with recombinant lentiviruses to express indicated short hairpin (sh) RNA, and then cells were incubated in the presence or absence of 15 ng/ml TGF-β1 for 3 days. (A) Schematic diagram of experimental design; (B) AID expression levels in qRT-PCR and (C) IP western blotting. Crude extract before IP was also blotted (input). Crude extracts from TGF-β1-treated 7T7-8 transfectants were immunoprecipitated by anti-AID antibody. Loading control: anti-(adenosine deaminase acting on RNA) ADAR. (D) Exosc3 expression level in qRT-PCR or western blotting (E); shLuc was used as a control; (F) Reductions of HBV transcript levels following TGF-β1 treatment are compared between shAID-, shExosc3-, and shLuc-expressing 7T7-8 cells. HBV transcript levels of each non-stimulated transfectant are defined as 1; shLuc was used as a non-targeted control. $*P < 0.05$, $**P < 0.01$ (*t*-test), error bars represent standard errors of the mean. Data are representative of two to three independent experiments.

Figure 8. Transcription dependency for TGF-β1-mediated reduction of HBV transcripts and a proposed model

HBV-expressing 7T7-8 cells were treated with 10 ng/ml TGF-β1 (A) or transfected with AID (or GFP) expression plasmid (B) and cultivated for 3 days. At 18 h before harvest, 100 ng/ml actinomycin D (ActD) was added to block transcription. Total RNA was extracted to measure HBV RNA levels (normalized by HPRT) by qRT-PCR. HBV RNA levels in non-treated (A) and GFP transfected cells (B) were taken as one. $**P < 0.01$ (*t*-test); Data are representative of two independent experiments and error bars represent standard errors of the mean. (C) Hypothetical model: Left panel, the canonical HBV life cycle; (a) After the entry of HBV into a hepatocyte, nucleocapsid NC-DNA is converted into cccDNA. (b)

745 Subsequently, cccDNA expresses viral transcripts, including pgRNA, pre-S1, pre-S2/S, X,
746 and pre-C mRNAs. In this study, most viral RNAs were transcribed from the HBV plasmid
747 instead of cccDNA. All transcripts possess the 3' - ε RNA stem-loop structure; only pgRNA
748 is shown. (c) P protein binds to the ε structure and stabilizes it, and the core protein
749 (indicated by hexagons) is then recruited to form the nucleocapsid. (d) Inside the
750 nucleocapsid, P protein reverse transcribes pgRNA to produce NC-DNA. A mature
751 nucleocapsid gains S proteins and is secreted as an infectious virion. The minor fraction of
752 nucleocapsid may enter a second intracellular viral cycle. (e) Right panel, TGF- β 1
753 stimulation of hepatocytes induces AID expression. (f) AID associates with the RNA
754 exosome proteins. The RNA exosome comprises ring-like core and exonuclease catalytic
755 components. AID associates with HBV transcripts and P proteins. (g) Consequently, AID
756 bridges the RNA exosome with the RNP complex of HBV transcripts and P protein, which
757 may trigger the degradation of HBV transcripts.

758
759
760 **Supplementary Results**

761 **Supplementary Figure 1. AID suppresses all HBV transcripts.**

762 (A) Schematic diagram of putative HBV transcripts. Structure of the HBV replicon plasmid
763 (pPB) is shown on the top. Red arrows indicate the position of the X gene primers. The
764 putative HBV transcripts are depicted on the bottom. (B, C) qRT-PCR analysis of HBV RNA.
765 The RNA samples used in Figure 2B and 2E (lanes 4 and 8) were subjected to qRT-PCR
766 analysis using the X gene primers. $^{**}P < 0.01$ (*t*-test). Data are representative of two to three
767 independent experiments and error bars represent standard errors of the mean.

768 **Supplementary Figure 2. AID does not downregulate host cell gene expression.**

769 qRT-PCR analysis of cellular gene expression. The RNA samples used in Figure 2B were
770 subjected to qRT-PCR analysis using the indicated gene primers. Expression levels of control

771 GFP-expressing cells are defined as 1-fold. Error bars represent standard errors of the mean.

772 **Supplementary Figure 3. AID binds to P protein in both the cytoplasm and nucleus in**
773 **HBV replicating hepatocytes**

774 Huh7 cells were transfected with the indicated expression vectors and pPB. Two days after
775 transfection, cells were harvested and biochemically separated into three fractions
776 (cytoplasmic, soluble nuclear, and insoluble nuclear fractions) using the Subcellular Protein
777 Fractionation Kit (Thermo Scientific) as recommended by the manufacturer.
778 Immunoprecipitation with FLAG agarose M2 beads was performed. Expected positions for
779 AID-GFP and GFP proteins are indicated at the left side of the anti-GFP blot. PCNA is a
780 putative soluble nuclear protein and was used as a control. Interaction of AID with P protein
781 was determined by Western blot analysis.

782 **Supplementary Figure 4. AID binds to Exosc3 in both the cytoplasm and nucleus in**
783 **HBV replicating hepatocytes.**

784 Huh7 cells were transfected with the indicated expression vectors and pPB. Two days after
785 transfection, cells were harvested and biochemically separated into three fractions
786 (cytoplasmic, soluble nuclear, and insoluble nuclear fractions) using the Subcellular Protein
787 Fractionation Kit (Thermo Scientific) as recommended by the manufacturer.
788 Immunoprecipitation with FLAG agarose M2 beads was performed. Expected positions for
789 AID-GFP and GFP proteins are indicated at the left side of the anti-GFP blot. Interaction of
790 AID with Exosc3 was determined by Western blot analysis (A). Transcripts in the indicated
791 fractions in A were subjected to RT-PCR analysis to determine coprecipitation of HBV and
792 HPRT transcripts (B).

793 **Supplementary Figure 5. Subcellular localization of the RNA exosome proteins.**

794 GFP expression of Huh7 transfectants used in Figure 5D was observed by fluorescence
795 microscopy. GFP expression of the transfectants in Figure 5D (lanes 1–4 and 6–9) are shown

in (A) and (B), respectively. GFP-tagged Exosc3, 2, and 7 are localized in both the cytoplasm and nucleus. A nuclear pattern of a GFP fusion protein is observed in some cells, especially in GFP-Exosc2- and -Exosc7-expressing cells.

Supplementary Figure 6. AID downregulates HBV RNA in the nucleus.

Huh7 cells were transfected with an AID-GFP (or GFP) expression vector and pPB. Two days after transfection, cells were harvested and biochemically separated into three fractions (soluble cytoplasmic, soluble nuclear, and whole cell extract) using the Subcellular Protein Fractionation Kit (Thermo Scientific) as recommended by the manufacturer. Expression of AID-GFP, GFP, and PCNA were detected by western blot (A) and HBV RNA levels were determined by qRT-PCR analysis (B). $*P < 0.05$, $**P < 0.01$ (*t*-test), error bars represent standard errors of the mean. Levels of HBV RNA from GFP transfectants were defined as one.

Supplementary Figure 7. cccDNA level in Huh7 cells transfected with HBV replicon plasmids.

Rolling circle amplification (RCA) is capable of amplifying circular DNA such as HBV plasmid and cccDNA. cccDNA production in pPB-transfected Huh7 cells and 7T7-8 cells was compared with that in cccDNA-producing cells (HepG2.2.15). (**A**) Schematic diagram of RCA and analysis of cccDNA is shown. (**B**) Huh7 cells were transfected with pPB (or pPB-dP) and cultivated for 3 days. Huh7T7-8 cells were cultivated in the absence of tetracycline for 3 days. HepG2.2.15 cells were used as a cccDNA producing control cells. Nuclear fraction of each transfectant was subjected to Hirt extraction to extract cccDNA. cccDNA was amplified by RCA. As a standard reaction, 10^7, 10^8, and 10^9 copies of HBV plasmids were amplified side by side as a standard reaction. Amplified RCA products were digested by EcoRI (for plasmid standard reactions, EcoRV) and agarose electrophoresis image visualized by ethidium bromide is shown (top). EcoRI digestion converts concatemeric cccDNA into 3.2

kb monomer, while EcoRV digestion converts concatemeric HBV plasmids into 3.2-kb and 4.2-kb DNA. Nucleocapsid production of each transfectants was also determined by extraction of nucleocapsid RC-DNA following PCR detection of HBV DNA (bottom).

Supplementary Figure 8. Knocking down of ZAP expression did not affect AID-mediated downregulation of HBV transcripts.

Huh7 cells were transfected with an AID (or GFP) expression vector with indicated siRNA together with pPB. Cells were harvested after 3 days of incubation. Levels of ZAP expression and HBV RNA were determined by RT-qPCR. siZAP-1 and -2 were obtained from Invitrogen. siZAP-3 [37] was obtained from Santa Cruz Biotechnology. (A) Fluorescence microscopic image of GFP transfectants on day 3 after transfection. Knocking down of GFP expression is obvious in siGFP transfectants. (B) ZAP mRNA expression levels. The expression level of ZAP in the GFP-transfected, GFP siRNA (siGFP)-transfected (far right) was defined as one. (C) HBV RNA levels: HBV RNA level in the GFP-transfected, GFP siRNA (siGFP)-treated (far right) was defined as one. AID expression reduces HBV RNA levels in both siZAP and siGFP transfectants. (D) HBV RNA levels: The same data set in (C) was plotted with HBV RNA levels in siGFP-AID and siGFP-GFP transfectants defined as one. Knocking down of ZAP increases HBV RNA levels in both AID and GFP transfectants. $**P < 0.01$ (*t*-test), error bars represent standard errors of the mean.

Table S1. List of plasmids used in this study.

Table S2. List of primers used in this study.

Figure 1
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Figure 1

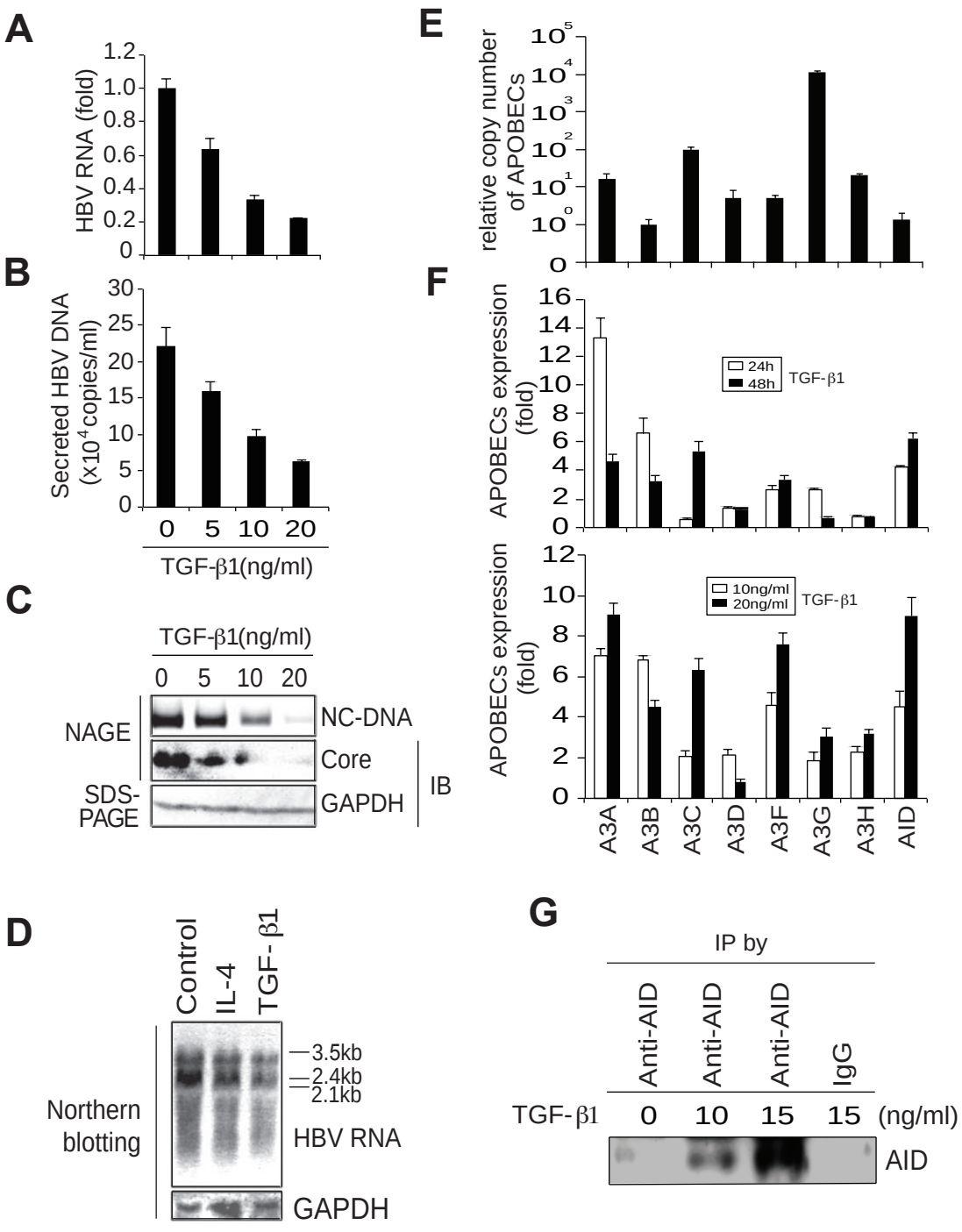

Figure 2
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Figure 2

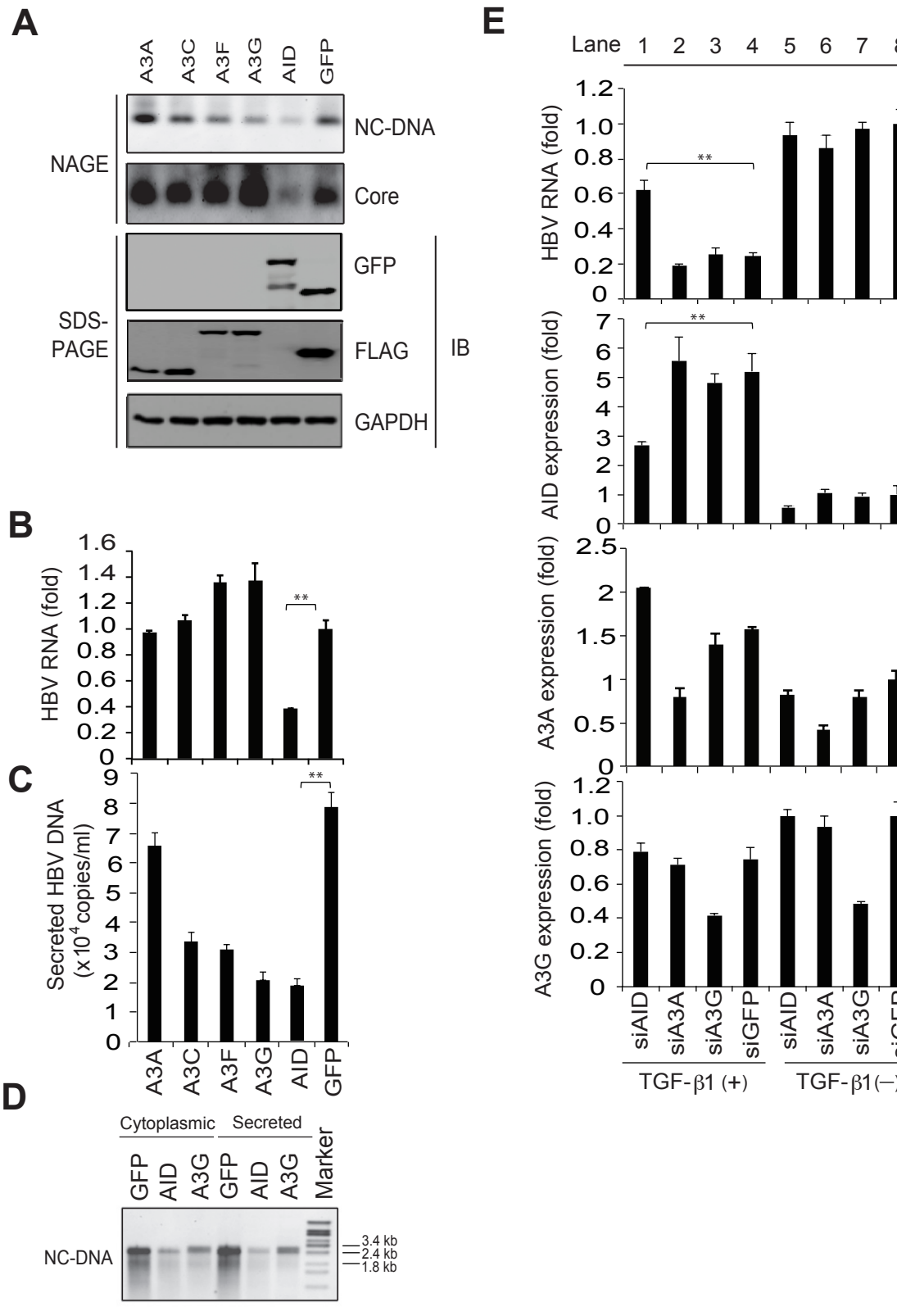

Figure 3
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Figure 3

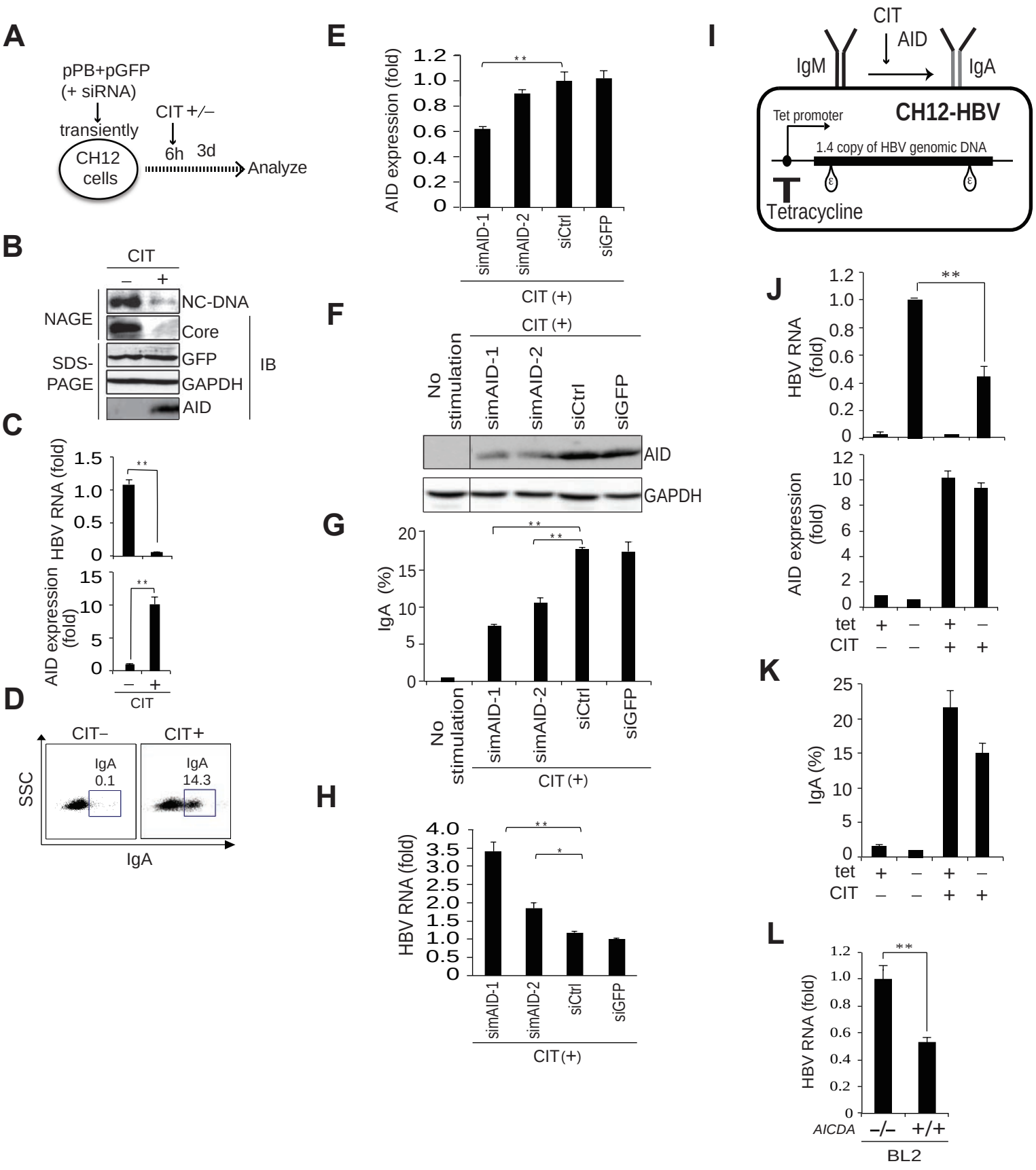

Figure 4

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Figure 4

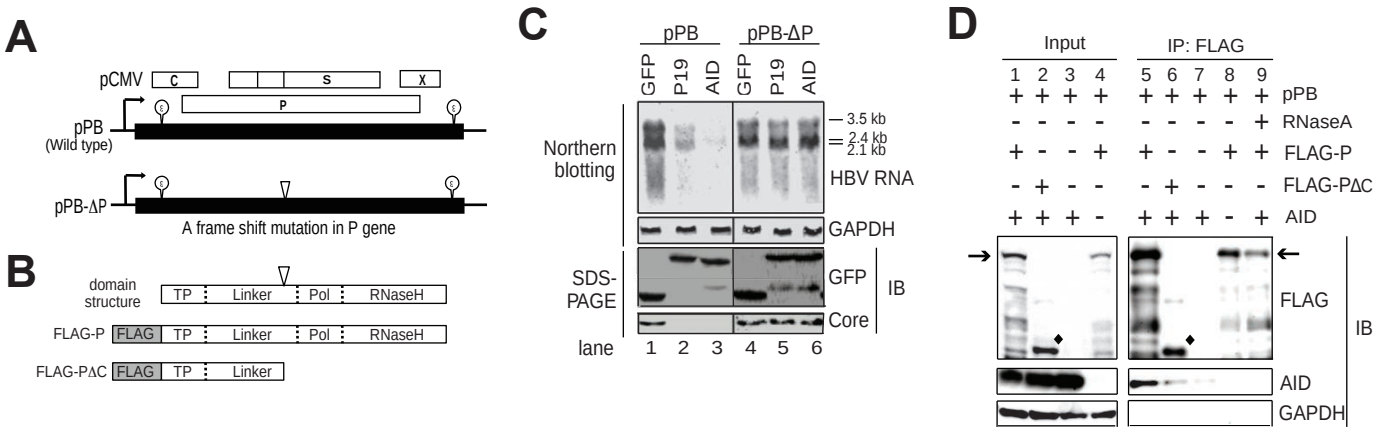

Figure 5

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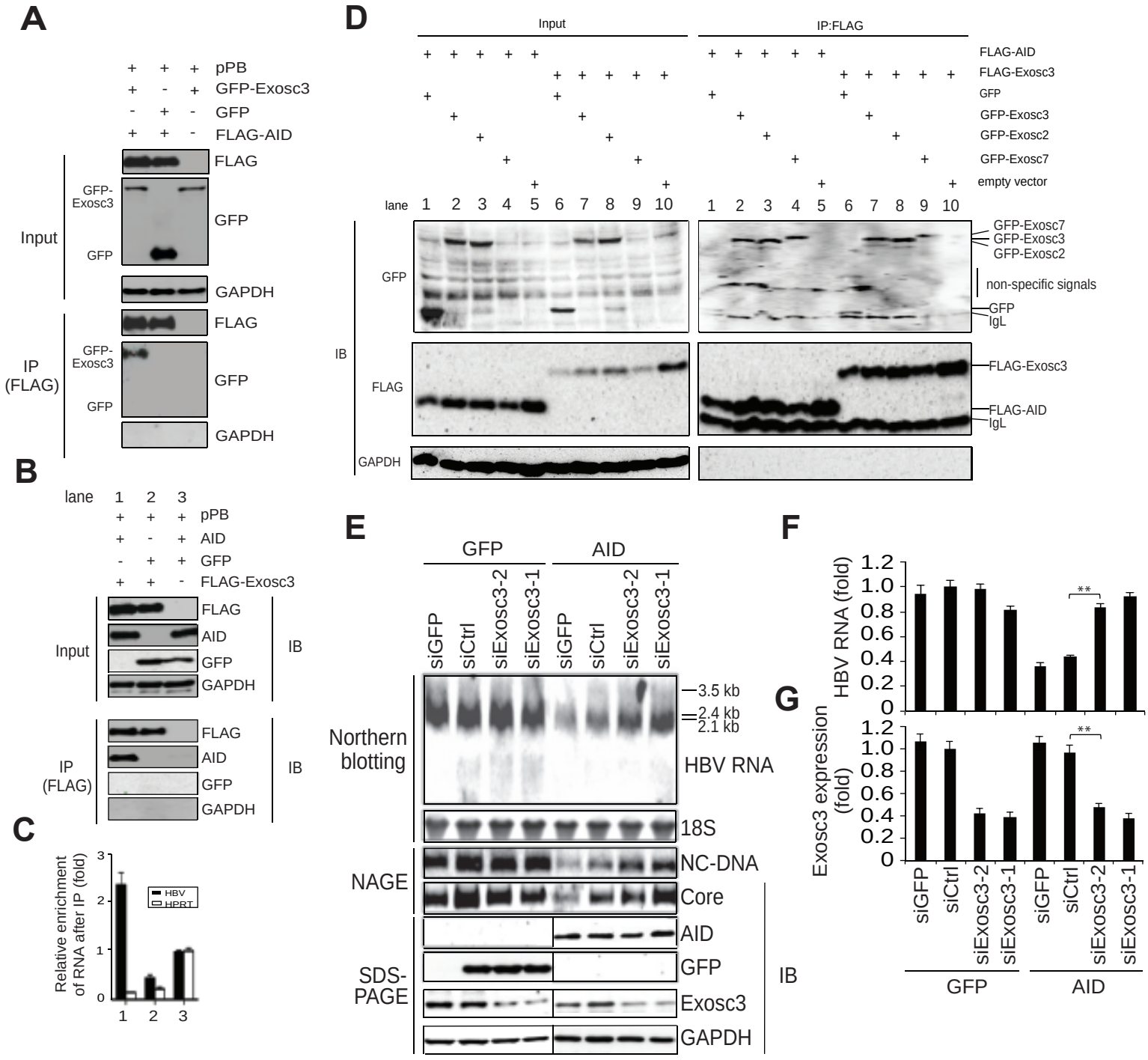

Figure 6
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Figure 6

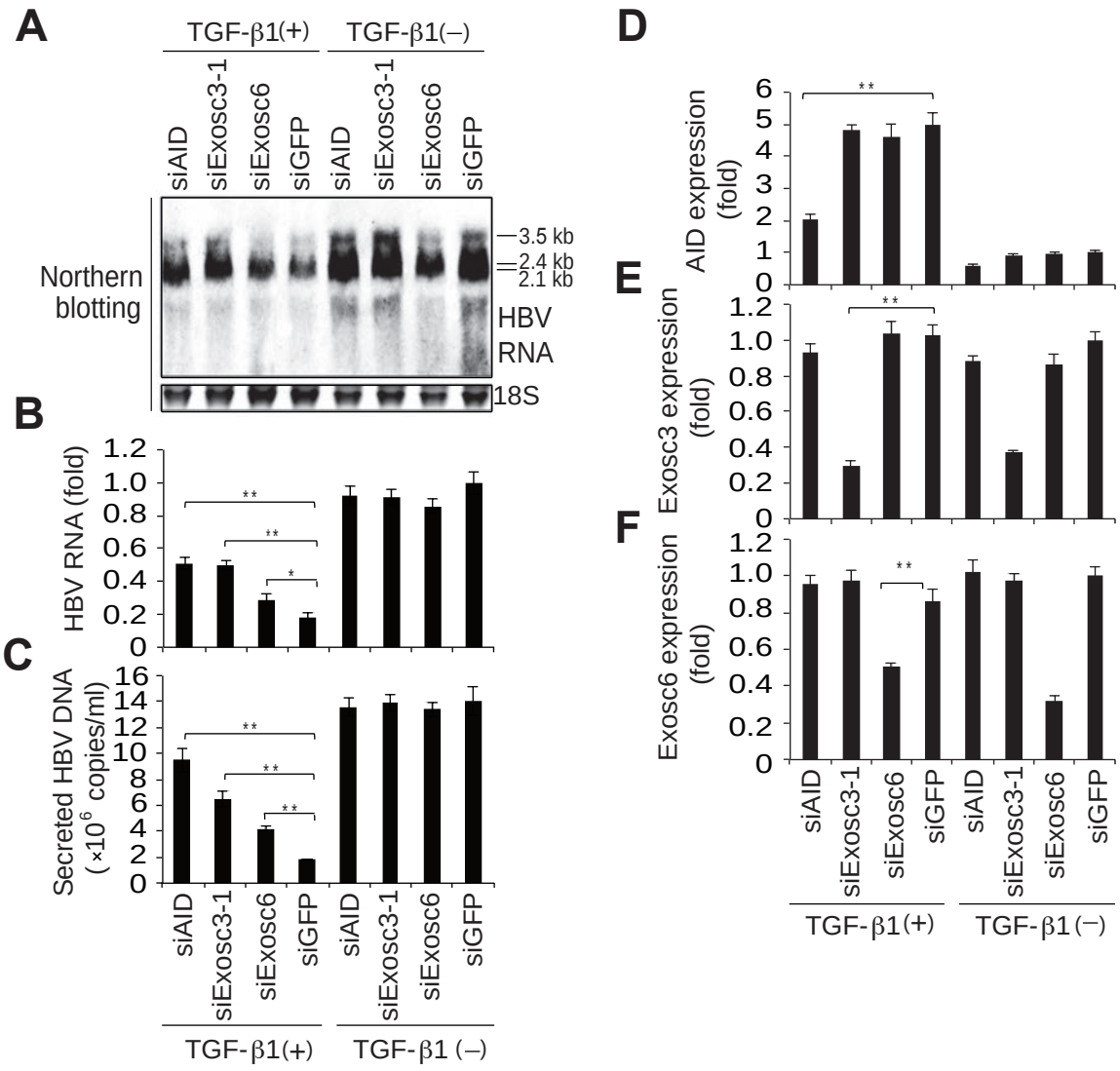

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Figure 7

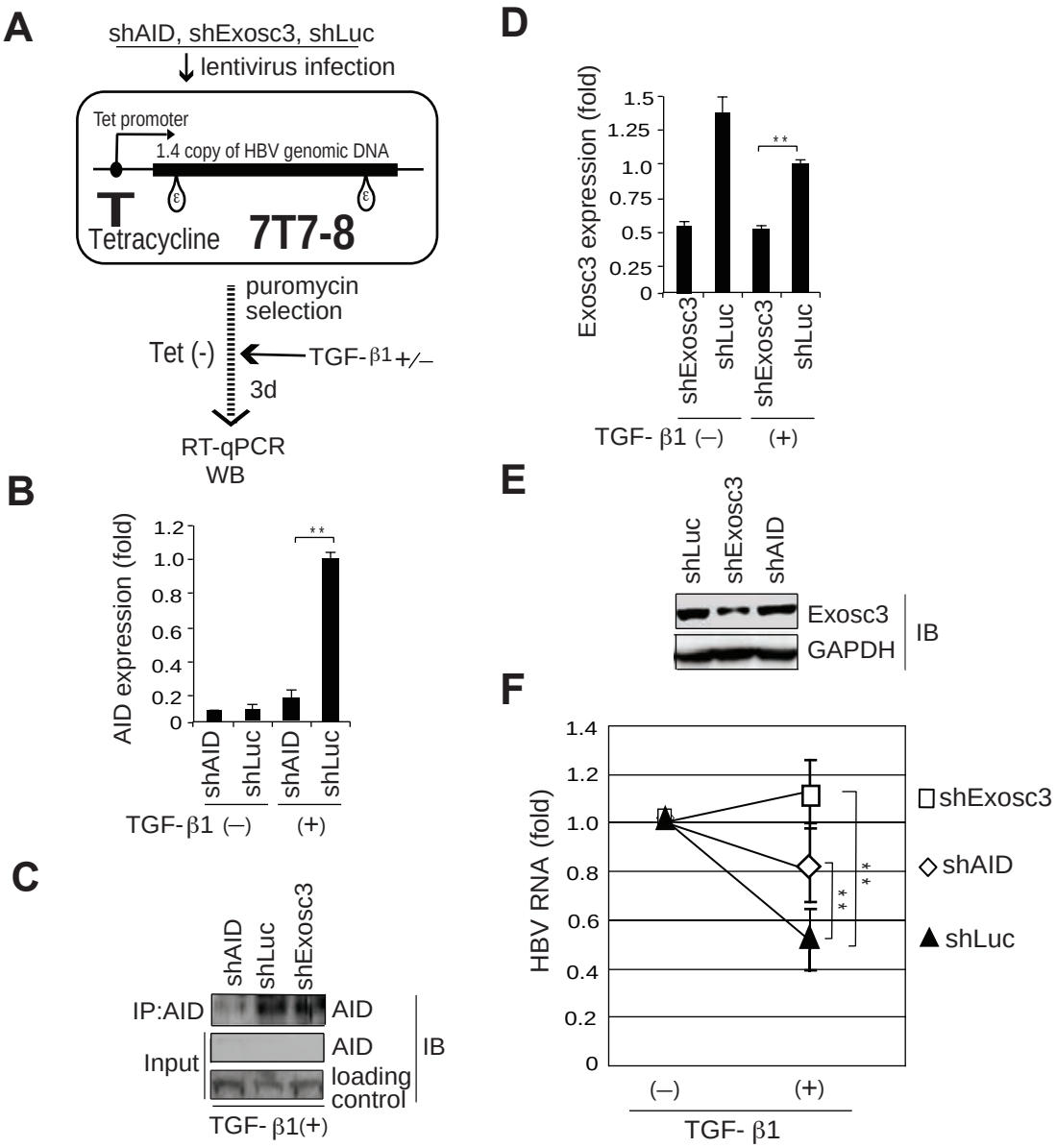

Figure 8

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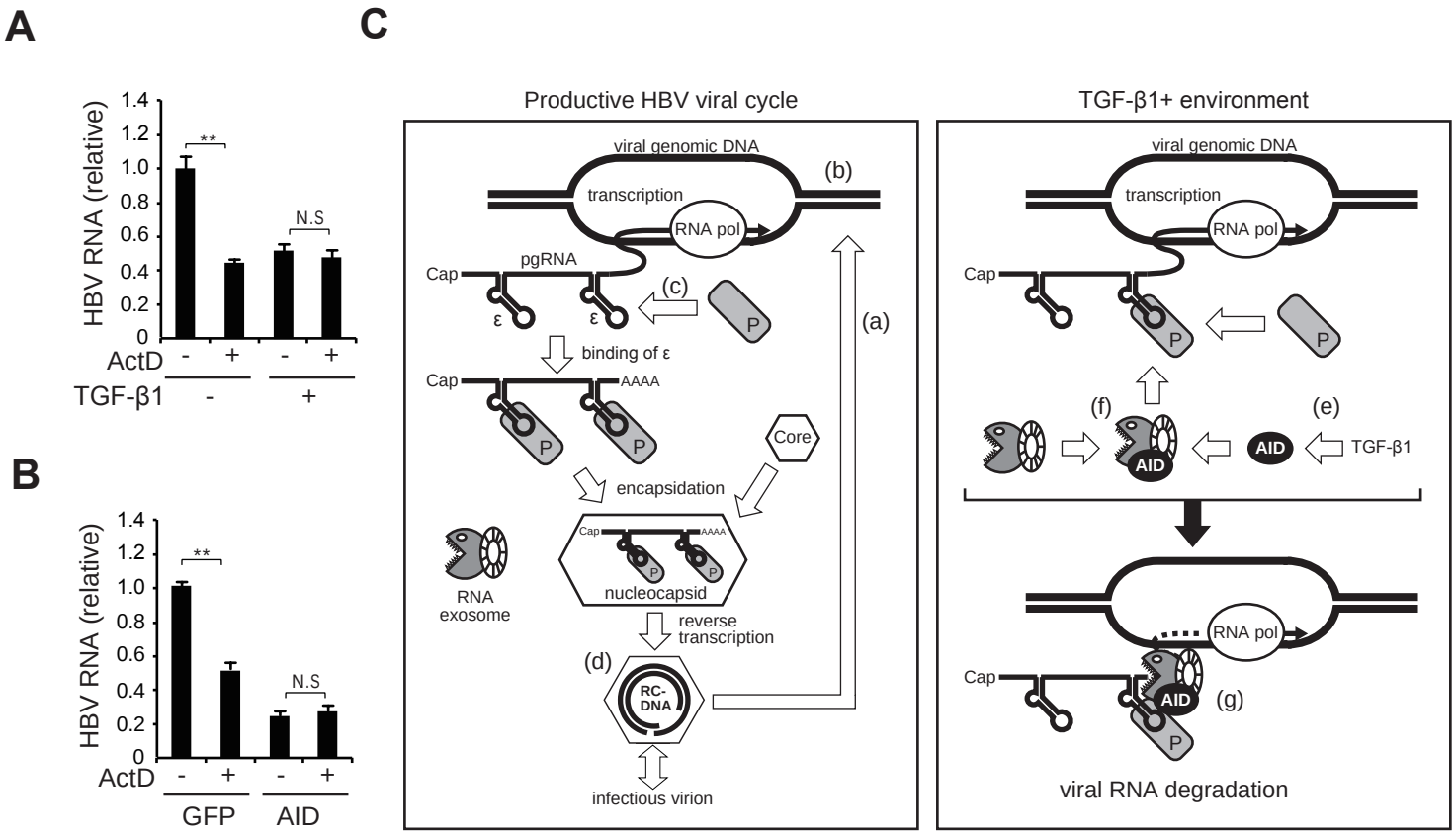
Figure 8



Supplementary Figures

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