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Specific and Nonhepatotoxic Degradation of Nuclear Hepatitis B Virus cccDNA

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Abstract

Current antivirals can control but not eliminate hepatitis-B-virus (HBV), because HBV establishes a stable nuclear cccDNA. Interferon-α treatment can clear HBV but is limited by systemic side effects. Here we describe how interferon-α can induce specific degradation of the nuclear viral DNA without hepatotoxicity and propose lymphotoxin-β-receptor activation as a therapeutic alternative. Interferon-α and lymphotoxin-β-receptor activation up-regulated APOBEC3A and 3B cytidinedeaminases, respectively, in HBV-infected cells, primary hepatocytes and human liver-needle biopsies. HBV-core protein mediated the interaction with nuclear cccDNA resulting in cytidine-deamination, apurinic/apyrimidinic site formation and finally cccDNA degradation that prevented HBV-reactivation. Genomic DNA was not affected. Thus, inducing nuclear deaminases

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- e.g., by lymphotoxin-β-receptor activation - allows development of new therapeutics that combined with existing antivirals may cure hepatitis B.

Hepatitis B virus (HBV) infection remains a major public health threat with more than 350 million humans chronically infected worldwide at risk of developing end-stage liver disease and hepatocellular carcinoma. Each year, more than 600,000 humans die from consequences of chronic HBV infection. A prophylactic vaccine has been available for hepatitis B for almost thirty years, but the overall number of chronic infections remains high.

HBV is a small, enveloped DNA virus replicating via an RNA intermediate. The encapsidated viral genome consists of a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA) molecule. The virus has optimized its life-cycle for long-term persistence in the liver (1). Upon translocation to the nucleus, the rcDNA genome is converted into a covalently closed circular DNA (cccDNA), which serves as the template for viral transcription and secures HBV persistence. Nucleos(t)ide analogs are efficient antivirals but only control and do not cure HBV infection owing to the persistence of HBV cccDNA. Therefore, long-term treatment is required, which is expensive and may lead to concomitant resistance (2). Interferon (IFN)-α is licensed for hepatitis B therapy and treatment with this cytokine can result in virus clearance in a proportion of patients; however, its efficacy is limited and high doses are not tolerated (3). Thus, efficient and nontoxic elimination of cccDNA in hepatocytes is a major goal of HBV research.

Using animal models, it has been shown that HBV replication, and in particular the cccDNA content of the liver, can be affected by noncytopathic mechanisms involving cytokines such as interferons and tumor necrosis factor (TNF), which influence RNA and capsid stability (4–7). Here, we describe an antiviral mechanism that interferes with cccDNA stability and is distinct from influences of antiviral cytokines on cccDNA activity (8).

High-Dose IFN-α Leads to cccDNA Degradation in HBV-Infected Hepatocytes

IFN-α is known to exert transcriptional, post-transcriptional and epigenetic antiviral effects on HBV (8–12). To study the effect of IFN-α on HBV cccDNA, we used HBV-infected, differentiated HepaRG (dHepaRG) cells and primary human hepatocytes (PHH). These are human cell types susceptible to HBV infection (13, 14) and responsive to IFN-α treatment in vitro (fig. S1A). IFN-α treatment did not lead to detectable hepatotoxicity, even at very high doses (fig. S1B). Treating dHepaRG cells with 500 or 1000 IU/ml IFN-α controlled HBV-DNA synthesis as efficiently as 0.5 μM (5-fold EC50) of the nucleoside analog lamivudine (LAM). IFN-α, however, unlike LAM also significantly reduced expression of HBV-RNA and hepatitis B surface (HBsAg) and e (HBeAg) antigens (Fig. 1A and fig. S1C).

In patients, interruption of LAM treatment results in a rebound of HBV replication (2). Using IFN-α, we observed only a partial or no rebound in HBV-infected dHepaRG cells after treatment cessation (Fig. 1A). Because dHepaRG don't allow virus spread, reduction of HBeAg and lacking rebound indicated an effect of IFN-α on the established HBV cccDNA transcription template besides the known antiviral effects on viral replication (14). By

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cccDNA-specific qPCR, we determined an 80% reduction of cccDNA after 10 days of treatment (Fig. 1B). Reduction of cccDNA was confirmed by Southern blot analysis (fig. S1D) and was dose dependent (fig. S1E). cccDNA reduction could be induced at any time point (Fig. 1C) and persisted over time (Fig. 1, A and C). The effect was corroborated in HBV-infected primary human hepatocytes (PHH) (Fig. 1D). In contrast to IFN-α, LAM and even more potent nucleoside analog entecavir (ETV) at very high doses (0.5 μM, 1000-fold IC-50) only inhibited reverse transcription and thus HBV replication, but not viral persistence (Fig. 1E). Pretreatment with ETV did not enhance the effect of IFN-α (Fig. 1F) indicating that IFN-α induces the decay of established HBV cccDNA. Since the doses of IFN-α used to achieve this effect were high, we screened for other cytokines showing similar antiviral effects at moderate doses.

LTβR Activation Controls HBV and Leads to cccDNA Degradation in HBV-Infected Cells

IFN-γ and TNF-α are known to control HBV in a noncytopathic fashion (4, 7), but cannot be used as therapeutics because they cause severe side effects. We tested the effect of lymphotoxin (LT) β receptor (LTβR) activation as an alternative therapeutic option. TNF superfamily members LTα, LTβ and CD258 are the physiological ligands for LTβR and activate several inflammatory, anti-inflammatory, pro- and anti-survival pathways (15). Like hepatocytes (16), dHepaRG (14) and HepG2-H1.3 cells permit HBV replication (17) and express the LTβR (fig. S2, A and B). To activate LTβR, we used a super-agonistic tetravalent bispecific antibody (BS1) and a bivalent anti-LTβR monoclonal antibody (CBE11) (18, 19). As expected, LTβR agonists activated canonical (20) and noncanonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways to trigger p100 cleavage (fig. S2C), RelA phosphorylation (fig. S2D), nuclear RelB and RelA translocation (fig. S2, E and F), and up-regulation of known target genes (fig. S2G) without causing any detectable hepatocytotoxicity (fig. S2H).

To test the effect of LTβR-activation on HBV infection, dHepaRG cells were treated with BS1 for 12 days starting 24 hours prior to HBV infection. LTβR-activation decreased levels of all HBV-markers, including cccDNA by approximately 90% without toxicity (Fig. 2A). The antiviral effect was highly potent with an EC_{5O} of approximately 0.01 μg/mL (fig. S3A). Inhibition of apoptosis did not alter antiviral activity (fig. S4). Neither IFN-β nor classic IFN-stimulated genes were upregulated upon BS1-treatment (fig. S2G) and antiviral activity was independent of IFN-induction (fig. S5).

In vivo, activation of the murine LTβR by systemic application of an agonistic antibody (ACH6) induced RelA and RelB nuclear translocation in hepatocytes of HBV-transgenic mice (fig. S6A), reduced HBV viremia (fig. S6B), HBV RNA (fig. S6C) and HBV core (HBc) protein expression in the liver (fig. S6, D and E). Neither signs of hepatocyte apoptosis (fig. S6F) nor elevation of aminotransferases (ALT) (fig. S6G, right panel) were observed indicating good in vivo tolerability of LTβRactivation. Since HBV-transgenic mice do not establish HBV cccDNA, this indicated additional antiviral effects of LTβR-activation

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on HBV RNA transcription or stability. Accordingly, discontinuation of LTβRactivation induced an immediate, strong rebound of HBV replication (fig. S6G).

To investigate whether LTβR-activation would affect established HBV cccDNA in the context of a persistent infection and prevent HBV reactivation, dHepaRG cells were treated with LTβR agonists BS1 or CBE11 when a stable, nuclear cccDNA pool had established. All HBV markers, including HBV cccDNA, were reduced upon LTβR-activation in HBV-infected dHepaRG cells (Fig. 2, B and C, and fig. S3) as well as in stably transfected HepG2H1.3 cells containing high levels of cccDNA (Fig. 2C). In HBV-infected primary human hepatocytes (PHH), LTβR agonisation reduced HBV cccDNA, HBeAg secretion and even more pronounced HBV-DNA replication (Fig. 2D). cccDNA degradation was more effective (up to 95%) when treatment was prolonged (fig. S3, C and D). Treatment interruption for 10 days was almost as efficient as continuous treatment (fig. S3C) indicating that LTβR agonists induce a persistent antiviral effect. In contrast to LAM treatment, no rebound of HBV-replication was observed when BS1 treatment stopped (Fig. 2E). Hence, LTβR activation not only suppressed HBV replication but also caused nuclear cccDNA degradation, needed to achieve virus elimination.

LTβR Activation and IFN-α Treatment Induce Deamination and Apurinic/Apyrimidinic (AP) Site Formation in cccDNA

To investigate if cccDNA degradation upon LTβR-activation or IFN-α treatment was a result of DNA damage, we examined cccDNA deamination by differential DNA denaturation PCR (3D-PCR) (21). Low denaturing temperatures were sufficient for cccDNA amplification from HBV-infected dHepaRG cells and for PHH treated with IFN-α or BS1, compared with untreated, LAM- or ETV-treated cells (Fig. 3A and fig. S7, C and D). Using a cocktail of recombinant proteins containing all enzymes necessary for DNA repair (preCR mix), we could reverse the denaturation of cccDNA (Fig. 3A, lower panels). The fact, that denaturation temperatures of mock, LAM and ETV treated cells also shifted, indicated that this modification of HBV cccDNA existed even without exposure to exogenous drugs. Deamination of cccDNA (Fig. 3A, right panel) and a drop in cccDNA levels after treatment with CBE11 (table S1) was confirmed in vivo in human liver chimeric uPA-SCID mice infected with HBV. Sequencing analyses showed G/A transitions occurred under treatment (Fig. 3B and fig. S7, A and B) indicating deamination of cytidines to uridine in the HBV cccDNA minus strand. At lower denaturation temperatures G/A transitions became more obvious (Fig. 3C and fig. S7A). These data showed that both LTβR-activation and IFN-α treatment led to cccDNA deamination in vitro and in vivo, and help to explain the G/A hypermutation observed in patient samples (21).

Importantly, neither deamination nor mutations of genomic DNA were observed by 3D-PCR (fig. S8A) or by deep sequencing of selected housekeeping or IFN- and LTβR-target genes (fig. S8B). This indicated that DNA modifications were specifically targeted to viral cccDNA.

After cytidine deamination, DNA-glycosylases recognize the damaged DNA and cleave N-glycosidic bonds to release the base and create an accessible AP site that can then be cleaved

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by endonucleases (22). These AP sites can either be repaired, can lead to mutations upon DNA replication or can induce DNA degradation (23). We quantified AP sites created by LTβR-activation or IFN-α treatment. However, no increase of AP sites in total DNA extracts from dHepaRG cells or PHH treated with IFN-α or LTβR-agonists (fig. S8C) was found, reassuring that our treatments did not lead to detectable damage in genomic DNA. Because AP sites in the small (3.2 kb) cccDNA are very likely to be missed by this analysis, we digested total DNA extracts with an AP-endonuclease (APE1) and then amplified cccDNA by qPCR. APE digestion further decreased cccDNA extracted from dHepaRG cells and PHH treated with IFN-α or LTβR-agonists but not with LAM (Fig. 3D). Taken together, our data indicate that both, LTβR-activation or IFN-α treatment induced deamination and AP-site formation in HBV cccDNA leading to its degradation, but did not affect genomic DNA.

LTβR Activation and IFN-α Treatment Up-Regulate Expression of Nuclear APOBEC3 Deaminases

IFN-α is known to induce several cytidine deaminases (23, 24). We performed genome-wide expression profiling of HBV-infected dHepaRG cells after LTβR-activation (fig. S9A) and classified regulated genes according to their activity and properties (fig. S9B). Hereby, APOBEC3B (A3B) was identified to be the most up-regulated gene with nucleic acid binding properties (fig. S9C).

Analysis of all APOBEC3 family members showed that LTβRactivation leads to strong up-regulation of A3B and to minor extent A3G in HBV-infected dHepaRG and PHH, and after systemic application in human liver chimeric uPA-SCID mice (fig. S10A). A3B expression was induces by LTβR-activation in a dose-dependent manner and expression levels steadily increased during continuous treatment (fig. S11) correlating with a concomitant increase in treatment efficacy over time (fig. S3C). Treatment of PHH isolated from different donors with LTβRagonist BS1 resulted in cccDNA degradation at different levels (Fig. 3E and fig. S10B), which could neither be explained by the level of A3B upregulation (Fig. 3E) nor by detection of a previously described (25) genomic deletion of the A3B allele, which seems to correlate with HBV persistence in infected patients (fig. S10, B and C).

In contrast to LTβR-activation, IFN-α treatment induced mainly A3A, but also A3F and A3G expression in HBV-infected dHepaRG cells and PHH (fig. S12A), and A3D expression in isolated PHH. By systemic IFN treatment of chimpanzees (26), A3A was strongly upregulated in liver needle biopsies (fig. S12B). Activation of A3A, A3F and A3G after IFN-α treatment was dose- and time-dependent, and decreased after an initial peak despite continuous treatment indicating that cells become refractory to IFN-α (fig. S13). In patients treated with subcutaneous pegylated IFN-α, needle biopsies obtained at different time points confirmed a rapid, strong upregulation of A3A and to a lower extend of A3G in the liver peaking at 16 hours post treatment (fig. S12C). Expression levels declined after this time point and remained low until day 6 post treatment confirming a fast but only transient induction of A3A by IFN-α treatment. Interestingly, the level of A3B or A3A induction in BS-1 and IFN-α treated PHH, respectively, did not directly correlate with the level of cccDNA degradation (Fig. 3E). The fact that IFN-α only induces a transient A3A induction

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and cells rapidly become refractory to IFN-α may account for the limited effect of IFN-α treatment in HBV-infected patients (3).

APOBEC3A or APOBEC3B Activity Is Essential to Induce cccDNA

Degradation

Among the APOBEC3 family members up-regulated in our experiments, only A3A and A3B located to the nucleus (fig. S14) where they can gain access to cccDNA. To verify that they are indeed responsible for the induction of cccDNA degradation, we overexpressed the HIV-Vif protein (known to promote the degradation of all APOBEC3 proteins except A3B (27, 28)) in dHepaRG cells in a tetracycline-regulated fashion. Expression of HIV-Vif reduced A3A, A3F and A3G expression (fig. S15A), reverted IFN-α-induced cccDNA deamination and prevented cccDNA degradation induced by IFN-α treatment (Fig. 4A). However, expression of HIV-Vif did not alter A3B levels (fig. S15B) and had no impact on cccDNA degradation by LTβR-activation (fig. S15C). To specifically address the role of A3A or A3B in cccDNA degradation we further knocked down A3A and A3B in dHepaRG cells under IFN-α or LTβR-agonist treatment, respectively, and observed reduced cccDNA deamination (Fig. 4, B and C, left panels). A3A as well as A3B knock-down completely reverted cccDNA degradation, but could not rescue the additional effect of IFN-α or LTβR-activation on HBV replication (Fig. 4, B and C, right panels).

To confirm the impact of A3A and A3B on cccDNA deamination, we overexpressed A3A and A3B, respectively, in HBV-replicating HepG2-H1.3 (Fig. 4, D and E). Cytidine-deamination of nuclear cccDNA by A3A and A3B is in accordance with other studies showing that both localize to the nucleus (29) and may be involved in the elimination of foreign DNA (23).

APOBEC3A Interacts with the HBV Core Protein and Binds to cccDNA

APOBECs have evolved to restrict retroviral replication (30) as well as DNA transfer into cells. They are able to clear foreign nuclear DNA (23, 31), but it remains unclear how HBV cccDNA DNA was recognized and whether it was specifically targeted in our experiments. To assess specificity, we generated cell lines replicating a mammalian replicon plasmid pEpi containing a linear HBV 1.3-fold overlength sequence. From the linear HBV-genome, HBV replication was initiated and in addition to the pEpi-H1.3 replicon HBV cccDNA was established in the nucleus. Treatment with either IFN-α or LTβR-agonist BS1 inhibited HBV replication and resulted in deamination and degradation of HBV cccDNA, but not of the HBV-sequence containing replicon (fig. S16). This indicated that deamination and subsequent degradation induced by both treatments is HBV cccDNA specific.

HBV core protein associates with A3G (32) and HBV cccDNA (33) and thus was a candidate to mediate the targeting of A3 deaminases to HBV cccDNA. Confocal microscopy indicated a co-localization of A3A and A3B with HBV core in different cell lines and PHH (Fig. 5 and fig. S17). Chromatin immunoprecipitation (ChIP) experiments using stably (fig. S18A) or transiently transfected HepG2H1.3 cells or HBV-infected and IFN-α treated dHepaRG cells, showed that HBV core protein and A3A both bind to the cccDNA

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minichromosome (Fig. 6A). Supporting the possibility that a guardian protein prevents A3A direct binding to DNA (34), we could not detect A3A binding to genomic DNA (fig. S18B) even in the presence of HBV core, which has been reported to also bind to cellular DNA (35).

HBV core protein co-immunoprecipitated A3A in HepG2H1.3 cells and transfected HuH7 cells indicating physical interaction with A3A (fig. S19). Direct interaction of HBV core expressed after HBV infection and A3A induced by IFN-α was confirmed by proximity ligation assay (PLA) (Fig. 6B and fig. S20) and fluorescence resonance energy transfer (FRET) analysis (Fig. 6C). By deletion analysis, we determined that the central region of HBc (aa 77 to 149) is involved in the interaction with A3A (Fig. 6C and fig. S21).

These data suggest that A3A is targeted to cccDNA by interaction with HBV core. No such targeting to genomic DNA has been described so far. Since APOBEC3 deaminases are thought to act on single stranded DNA (36), one possibility is that A3A and A3B act on cccDNA when it is transiently rendered single-stranded by RNA polymerase II before transcription initiation.

We suggest, therefore, the following mechanism of APOBECdependent degradation of HBV cccDNA (Fig. 6D). High dose IFN-α treatment or LTβR-activation up-regulate the expression of A3A and A3B, respectively, which subsequently co-localize or directly interact with HBV core in infected hepatocytes, translocate to the nucleus, where they are brought into close contact with cccDNA by HBV core. Now, APOBECs can deaminate cccDNA that is transiently rendered singlestranded during transcription. Uracils in HBV cccDNA are recognized and excised by cellular DNA glycosylases leading to formation of AP sites, which are then recognized by cellular AP endonculeases (23) leading to cccDNA digestion. Why cccDNA is degraded instead of being repaired by the cellular DNA repair machinery remains elusive so far. Using a mixture of various enzymes, we were able to repair deaminated cccDNA *in tubo* (Fig. 3A) suggesting induction of an additional factor promoting DNA degradation or an impaired function of the repair machinery rather than a lack of recognition by the repair machinery. Thus, we can only speculate that either the number of AP sites introduced after treatment is too high and exceeds the capacity of the cellular repair machinery or that IFN-α treatment or LTβR-activation or even HBV itself (37) modulate the repair machinery. This may shift the equilibrium from cccDNA repair (38) to degradation.

Ideally, a cure for HBV infection needs to eliminate cccDNA. Therefore, cytokines or cytokine-receptor agonists that can trigger HBV cccDNA deamination and its degradation are interesting antiviral candidates. Antivirals that induce A3A/B activity should be combined with nucleos(t)ide analogs to avoid the replenishment of nuclear cccDNA after degradation. LTβR-agonists were active at low doses and we did not observe any toxicity in vitro or in vivo nor did we detect any modification of genomic DNA. Constitutive overexpression of LTα/β for more than one year has been associated with inflammatory liver disease and hepatocellular carcinoma (16). As antivirals, however, LTβR-agonists would only be used for a limited period of time minimizing the risk of side effects. Moreover, LTβR-activation was already explored as a cancer treatment (18).

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A recent study has shown a significantly higher frequency of an A3B deletion allele in persistent HBV carriers and hepatocellular carcinoma patients compared with healthy controls (25). This finding was further supported by the moderate deamination of cccDNA even in absence of treatment, and by the observation that knockdown of A3B in the absence of any treatment increased cccDNA levels. Although deregulated expression of A3A and A3B has been shown to correlate with genomic DNA mutations (39, 40), we did not detect any alterations of genomic DNA using analyses of AP sites, 3D-PCR analysis and deep sequencing of a set of human genes.

Our data indicate that cccDNA degradation is possible and can be induced without side-effects on the infected host cell. An important task will be testing of combinations of nucleos(t)ide analogs with novel antiviral strategies (e.g., LTβR agonists or adoptive T-cell therapy (41)) to activate A3A or A3B to cure hepatitis B.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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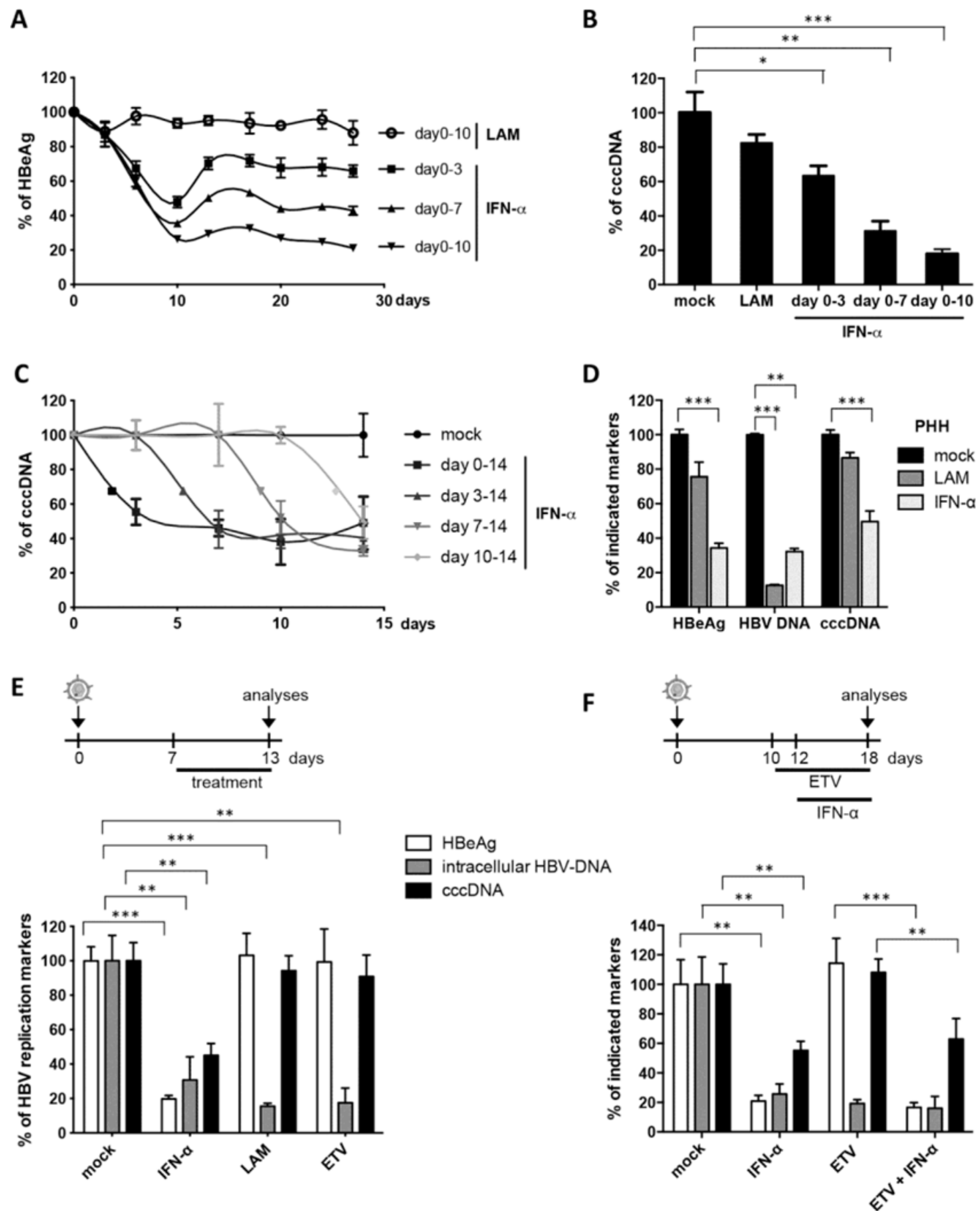

Fig. 1. Degradation of cccDNA in IFN-α treated HepaRG cells and primary human hepatocytes.
(**A**, **B**, **C**, **E**, and **F**) HBV-infected dHepaRG were treated with IFN-α at day 10 post-infection (dpi). Different regimens of treatment were applied as indicated. (**D**) HBVinfected primary human hepatocyte (PHH) were treated with IFN-α at dpi 3 for 13 days. Levels of HBeAg, total intracellular DNA and cccDNA are given relative to mock treated cells. LAM: lamivudine; ETV: entecavir. Mean values +/– standard deviation of replicates from independent experiments are given; data were analyzed by *t* test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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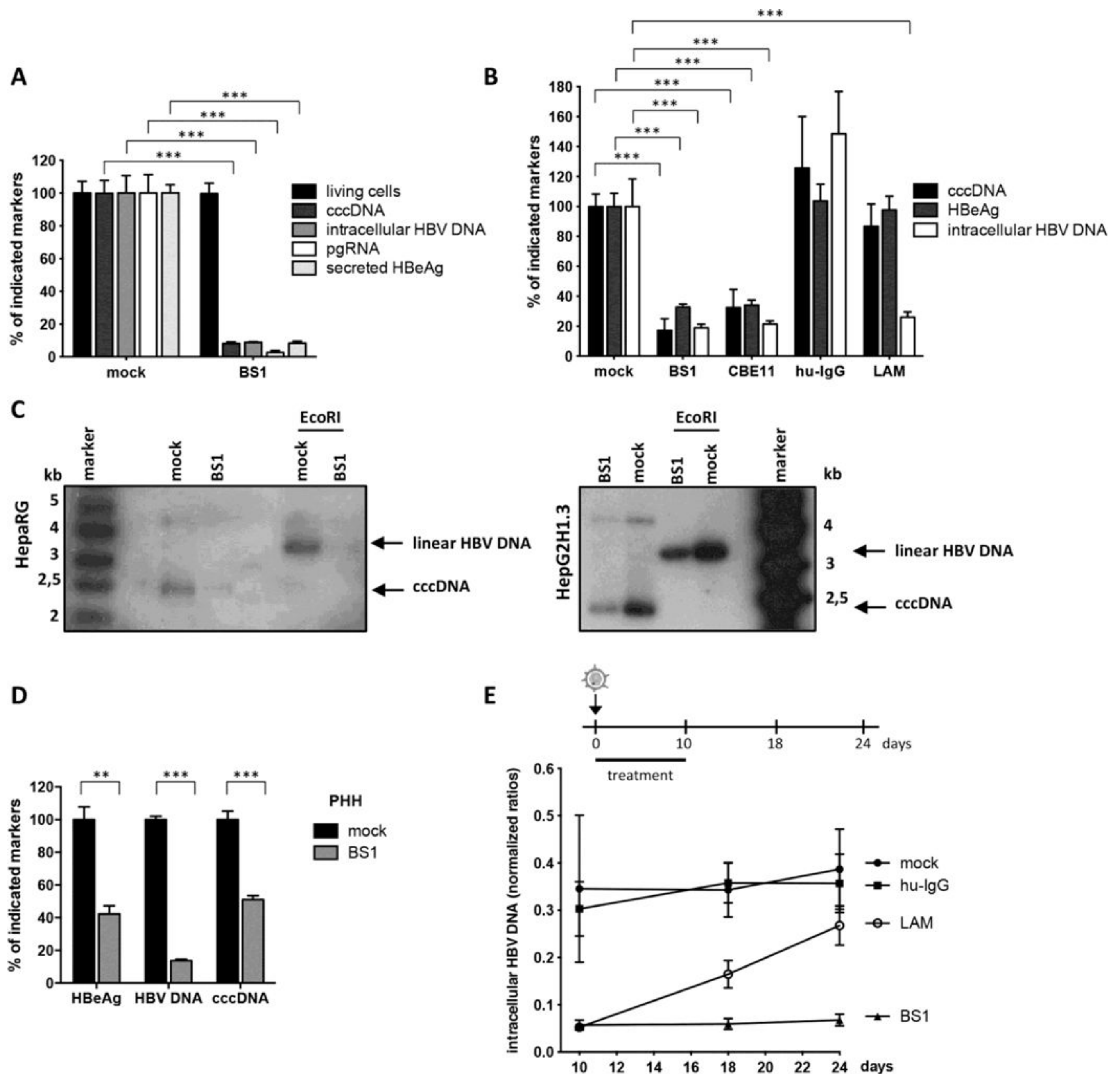

Fig. 2. LTβR-activation inhibits HBV infection and leads to cccDNA degradation in HepaRG cells and PHH.

(**A** and **B**) HBV-infected dHepaRG were treated with BS1, CBE11, hu-IgG control or lamivudine (LAM). (A) Treatment started 24h before infection for 12 days or (B) at 18 dpi for 10 days. Levels of the indicated HBV markers as well as cell viability are given relative to untreated controls (mock). (**C**) cccDNA levels were analyzed after 14 days of BS1 treatment by Southern blot in HBV-infected dHepaRG and HBV-replicating HepG2H1.3 cells. Supercoiled cccDNA bands were identified by their expected size and linearization upon *EcoRI* digestion (3,2 kb). (**D**) PHH were infected with HBV and treated with BS1 at 7 dpi for 10 days. Levels of the indicated HBV markers were compared to untreated PHH of

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the same donor (donor 3) (mock). (**E**) HBV-infected dHepaRG were treated with BS1, hu-IgG control or LAM. Intracellular HBV-DNA was analyzed 8 and 14 days after treatment cessation. Mean values +/− standard deviation of replicates from independent experiments are given; data were analyzed by *t* test. * $p < 0.05$, *** $p < 0.001$.

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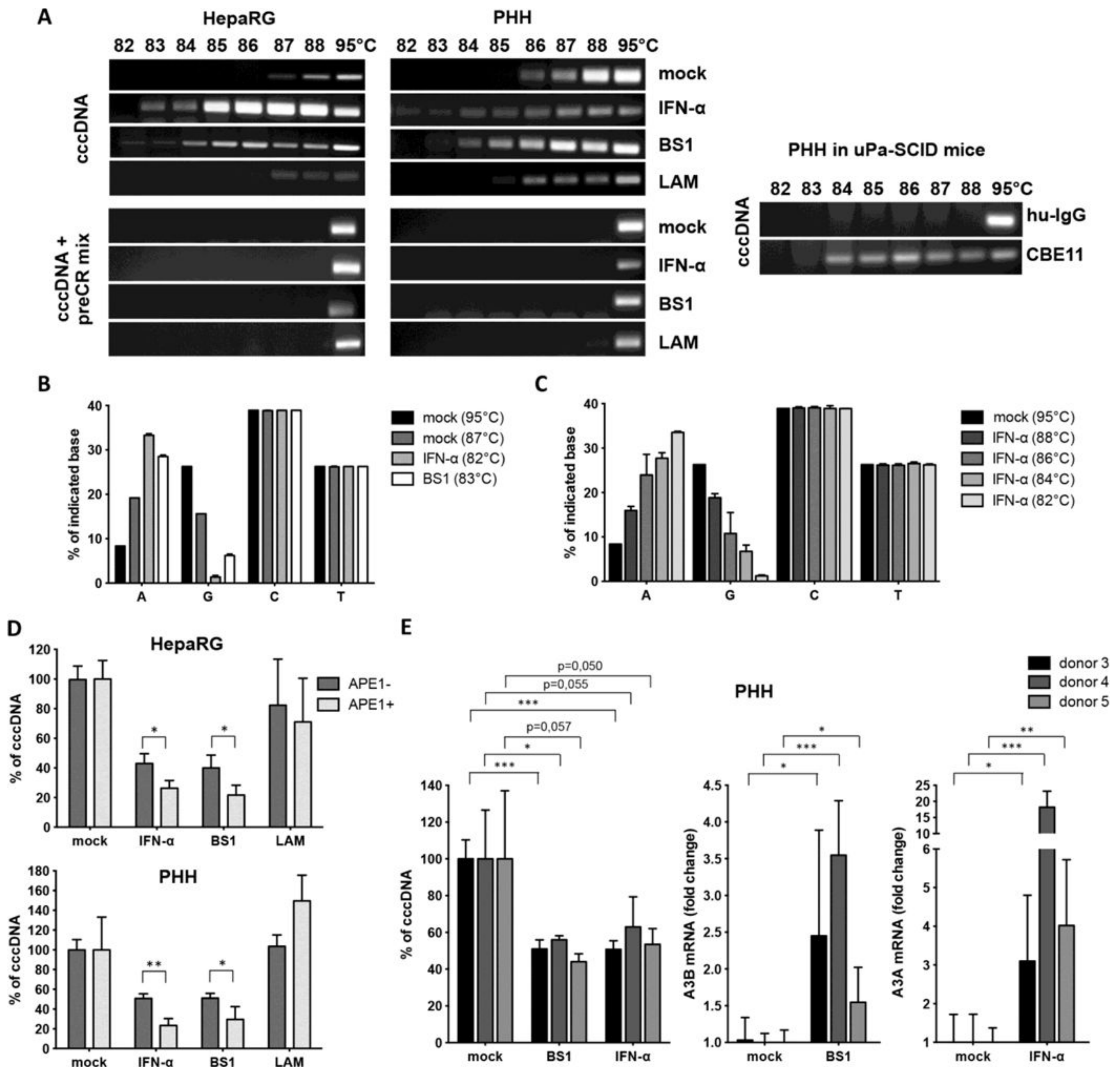

Fig. 3. Deamination and AP-site formation in cccDNA upon IFN-α treatment and LTβR-activation.

(**A**) dHepaRG (left) and PHH (middle panel) were infected with HBV and treated with IFN-α, BS1 or LAM. Human chimeric uPA/SCID mice were treated with CBE11 or hu-IgG control (right panel). 3D-PCR analyses were performed on cccDNA left either untreated (upper panels) or treated with a PreCR mix (lower panels). (B and C) 3D-PCR products from HBV-infected dHepaRG cells treated as indicated (IFN-α, BS1 or mock) were cloned and sequenced and mutations were analyzed. (D) Total DNA extracts from HBV-infected cells treated as indicated were digested with APE1, and cccDNA content was compared to mock-treated cells. In (B), (C), and (D), mean values +/− standard deviation of biological

triplicates from two independent experiments are given; data were analyzed by *t* test. * $p < 0.05$, ** $p < 0.01$. (E) PHH were infected with HBV and treated with BS1 or IFN-α at 7 dpi for 10 days. Levels of the indicated cccDNA as well as A3A and A3B mRNA expression were compared to untreated PHH (mock) of the same donor.

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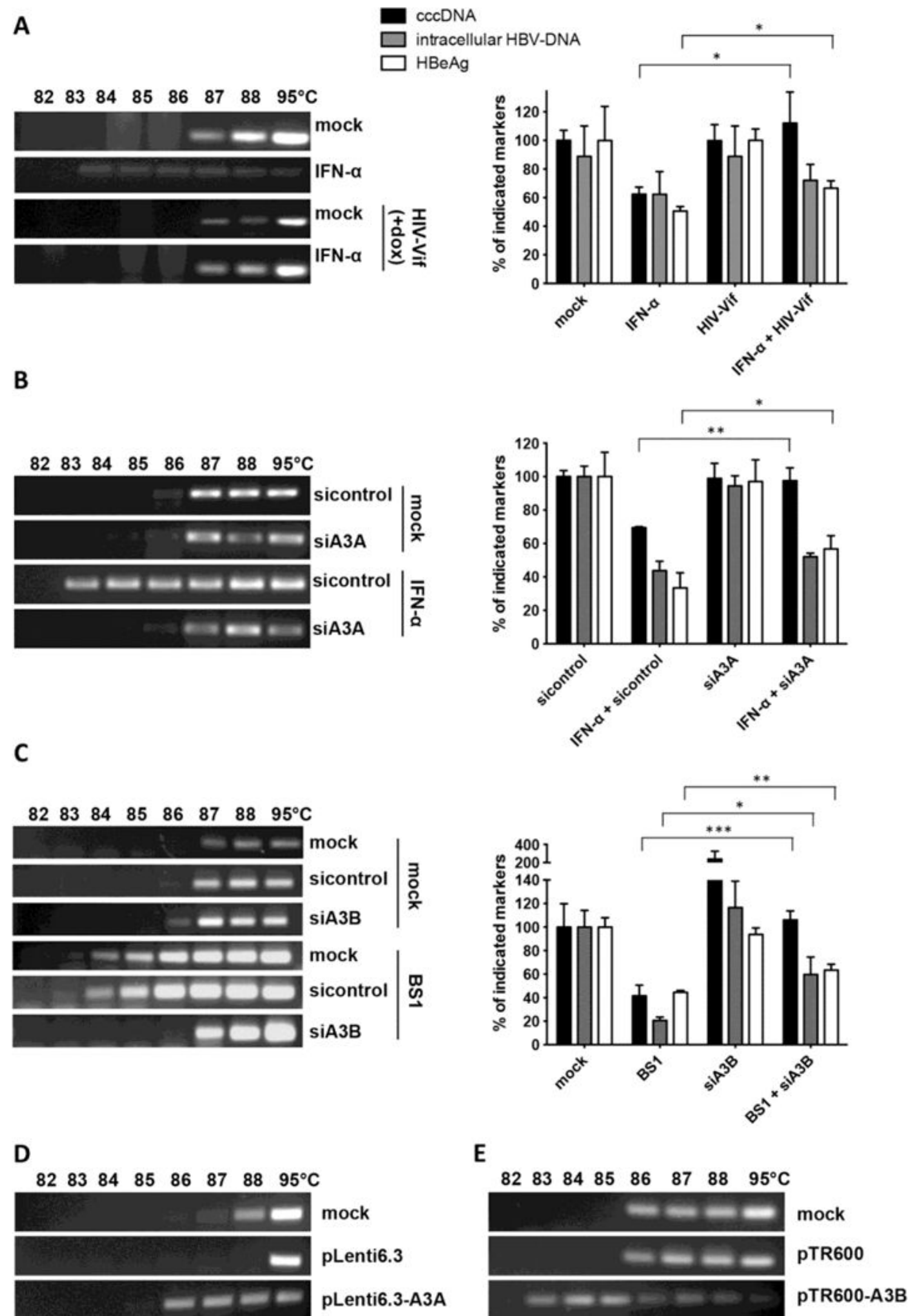

Fig. 4. Analysis of cccDNA deamination and degradation.

(**A** to **C**) cccDNA denaturation was analyzed by 3D-PCR (left panels); levels of HBeAg, total intracellular DNA and cccDNA are given relative to mock treated cells (right panels). (A) dHepaRG-tA-Vif cells treated with IFN-α for 10 days with and without doxycycline (dox)-induced HIV-Vif expression. HBV-infected dHepaRG cells treated with (B) IFN-α or (C) BS1 transfected with siRNA against A3A or A3B, respectively, or sequence nonspecific siRNA (sicontrol). Mean values +/− standard deviation of independent replicates and experiments are given; data were analyzed by *t* test. * $p < 0.05$, ** $p < 0.01$ and *** p <

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0.001. (**D**) cccDNA denaturation analysis by 3D-PCR in HepG2-H1.3 cells overexpressing A3A or (**E**) A3B from lentiviral vector plasmid pLenti6.3 or pTR600, respectively, for 5 days.

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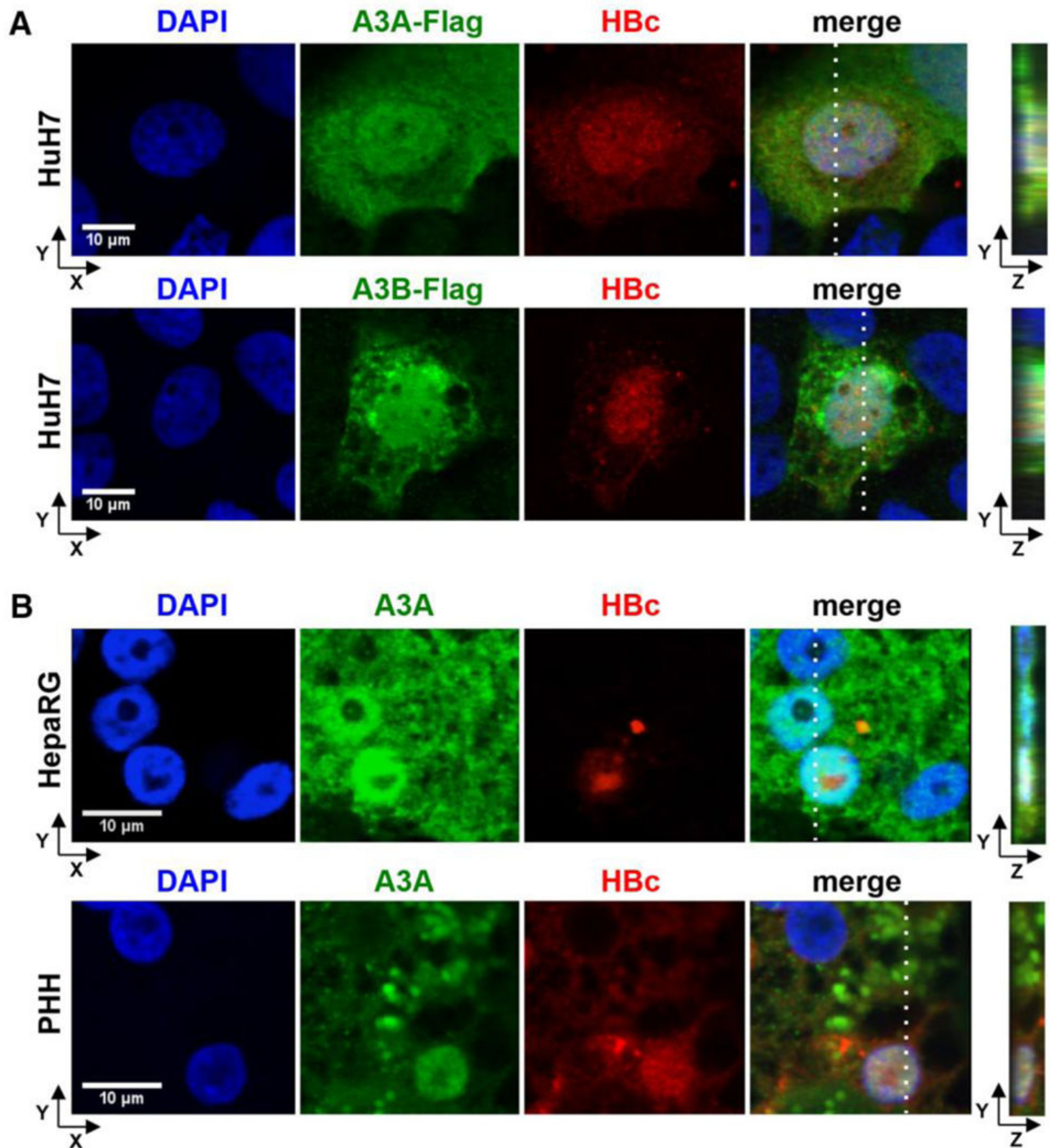

Fig. 5. Co-localization of A3A and A3B with HBV core protein (HBc).

(**A**) HuH7 cells were co-transfected with an HBV1.1-fold genome and A3A-Flag or A3B-Flag expressing plasmids and stained using DAPI, anti-HBc and anti-FLAG antibodies. (**B**) HBV-infected dHepaRG and PHH were treated with IFN-α at day 7 post infection for 3 days. A3A and HBc were analyzed by immunofluorescence staining. Right panels indicate z stacks taken at the dotted lines.

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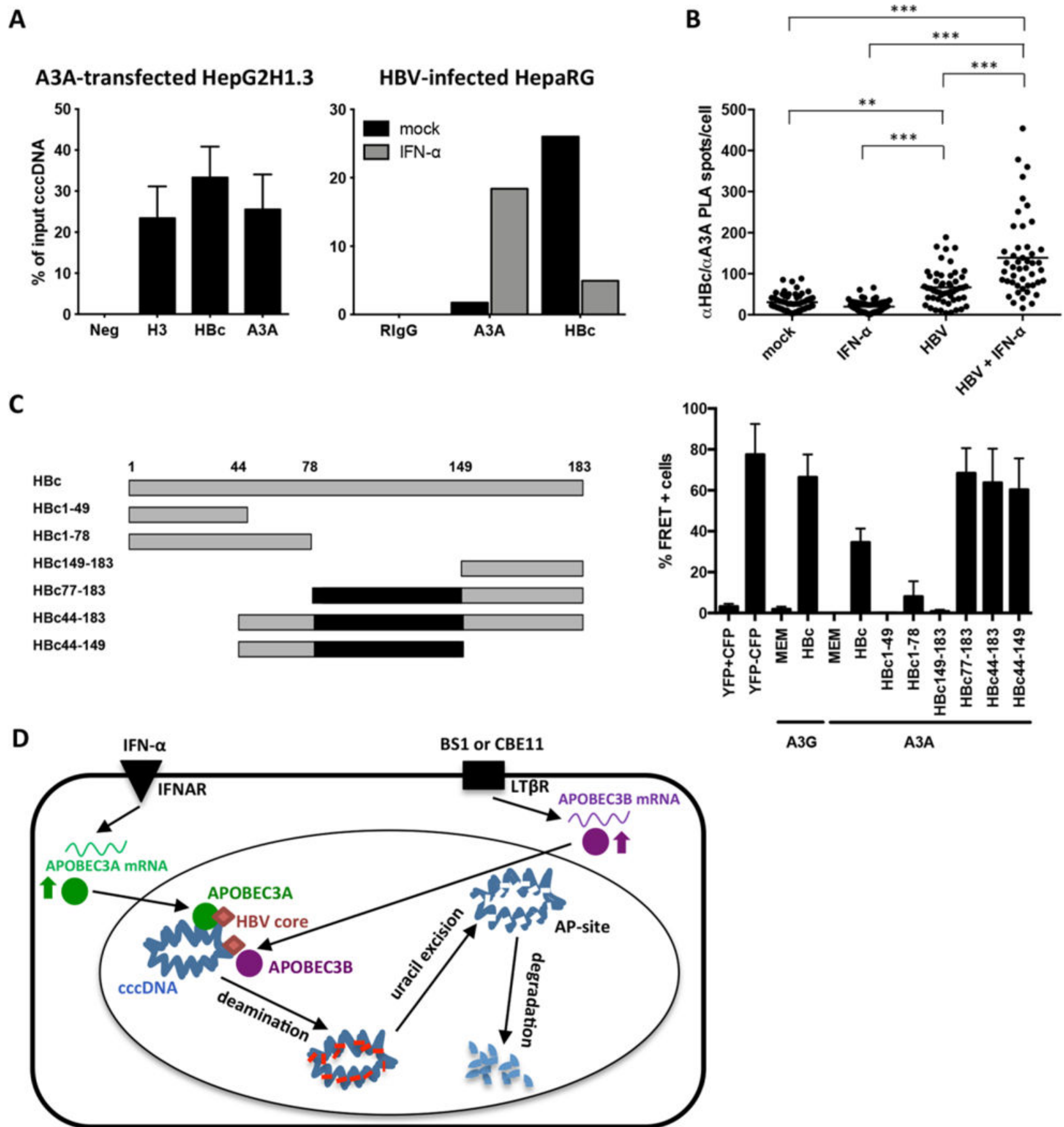

Fig. 6. Interaction of A3A, HBV core protein (HBc) and cccDNA.

(**A**) Chromatin immunoprecipitation (ChIP) was performed using lysates of HepG2H1.3 cells transfected with A3Aexpressing plasmid, or HBV-infected dHepaRG cells treated with IFN-α for 3 days. IPs using antibodies against histone H3, A3A, HBc and control rabbit IgG (RIgG) were analyzed by qPCR for cccDNA. (**B**) Interaction between HBc and A3A was assessed by proximity ligation assay (PLA) in HBV-infected, IFN-α treated dHepaRG. PLA-spots were quantified in single cells by software-based spot-counting. Data were analyzed by one-way ANOVA. ** $p < 0.01$ and *** $p < 0.001$. (**C**) Serial HBV core-deletion

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mutants (left panel) were fused to CFP and interaction with A3A-YFP was assessed by FACS-FRET in HuH7.5 hepatoma cells (right panel). Cells cotransfected with CFP and YFP served as controls to exclude false positive FRET and subtract background signals. A CFP-YFP fusion construct was used as positive control. Mean values ± standard deviation of FRET-positive cells from 3–4 independent experiments are given. Black boxes indicate shared regions of HBc mutants giving a FRET signal. (**D**) Model of cccDNA degradation induced by IFN-α treatment or LTβR-activation.