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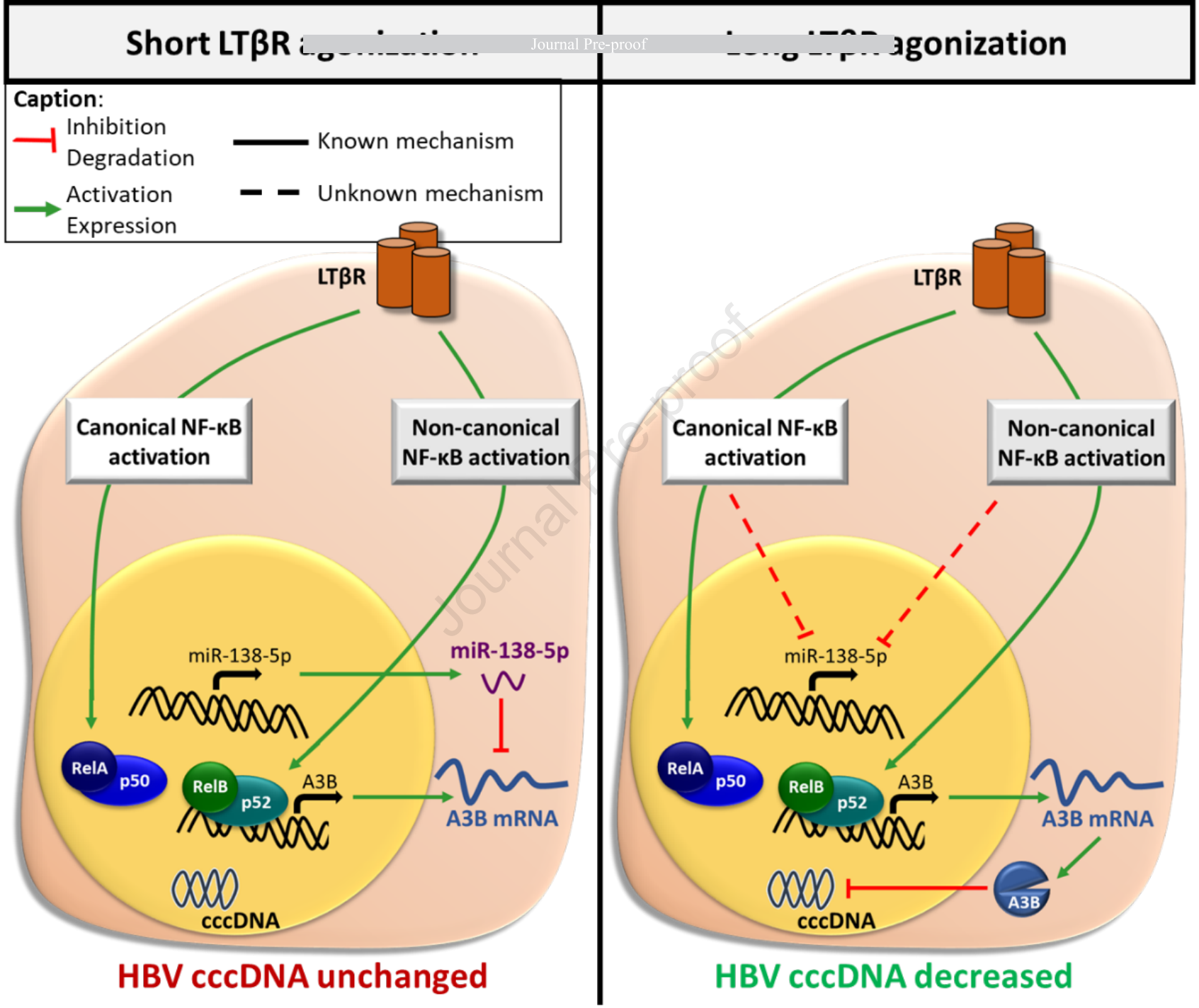

Control of APOBEC3B induction and cccDNA decay by NF-κB and miR-138-5p

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Author contributions

- Conceptualization: SFD, TR, MR, FR, DD, JL, ED, MH
- Methodology: SFD, TR, MR, FR, DD, JL, ED, MH

- Formal analysis: SFD, TR, MR, ZH
- Investigation: SFD, TR, MR, ZH, FR, KN, SS, CR, NG, MS, MSt, JW, RB, RP, LCS, RF, SP, CG, RO, TE, KR, RR, KU, DT, JL, DBL
- Resources: KU, UP, DD, JL, ED, MH
- Data curation: SFD, TR, MR, ZH
- Writing-original draft: SFD, TR, DD, JL, ED, MH
- Visualization: SFD, TR, MR, ED, MH
- Supervision: ED, MH
- Project administration: ED, MH
- Funding acquisition: DD, JL, ED, MH

Data availability statement: The data that support the findings of this study are available from the corresponding author, MH, upon reasonable request.

Abstract

Background & Aims: Immune-mediated induction of cytidine deaminase APOBEC3B (A3B) expression leads to hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) decay. Here, we aimed to decipher the signalling pathway(s) and regulatory mechanism(s) involved in A3B induction and related HBV control.

Methods: Differentiated HepaRG cells (dHepaRG) knocked-down for NF-κB signalling components, transfected with siRNA or micro RNAs (miRNA), and primary human hepatocytes +/- HBV or HBVΔX or HBV-RFP, were treated with lymphotoxin beta receptor (LTβR)-agonist (BS1). The biological outcomes were analysed by RT-qPCR, immunoblotting, luciferase activity, ChIP, EMSA, targeted-bisulfite-, miRNA-, RNA-, genome-sequencing, and mass-spectrometry.

Results: We found that canonical and non-canonical NF-κB signalling pathways are mandatory for A3B induction and anti-HBV effects. The degree of immune-mediated A3B production is independent of A3B promoter demethylation but is controlled post-transcriptionally by the micro RNA 138-5p expression (hsa-miR-138-5p), promoting A3B mRNA decay. Hsa-miR-138-5p over-expression reduced A3B levels and its antiviral effects. Of note, established infection inhibited BS1-induced A3B expression through epigenetic modulation of A3B promoter. Twelve days of treatment with a lymphotoxin beta receptor-specific agonist BS1 is sufficient to reduce the cccDNA pool by 80% without inducing significant damages to a subset of cancer-related host genes. Interestingly, the A3B-mediated effect on HBV is independent of the transcriptional activity of cccDNA as well as on rcDNA synthesis.

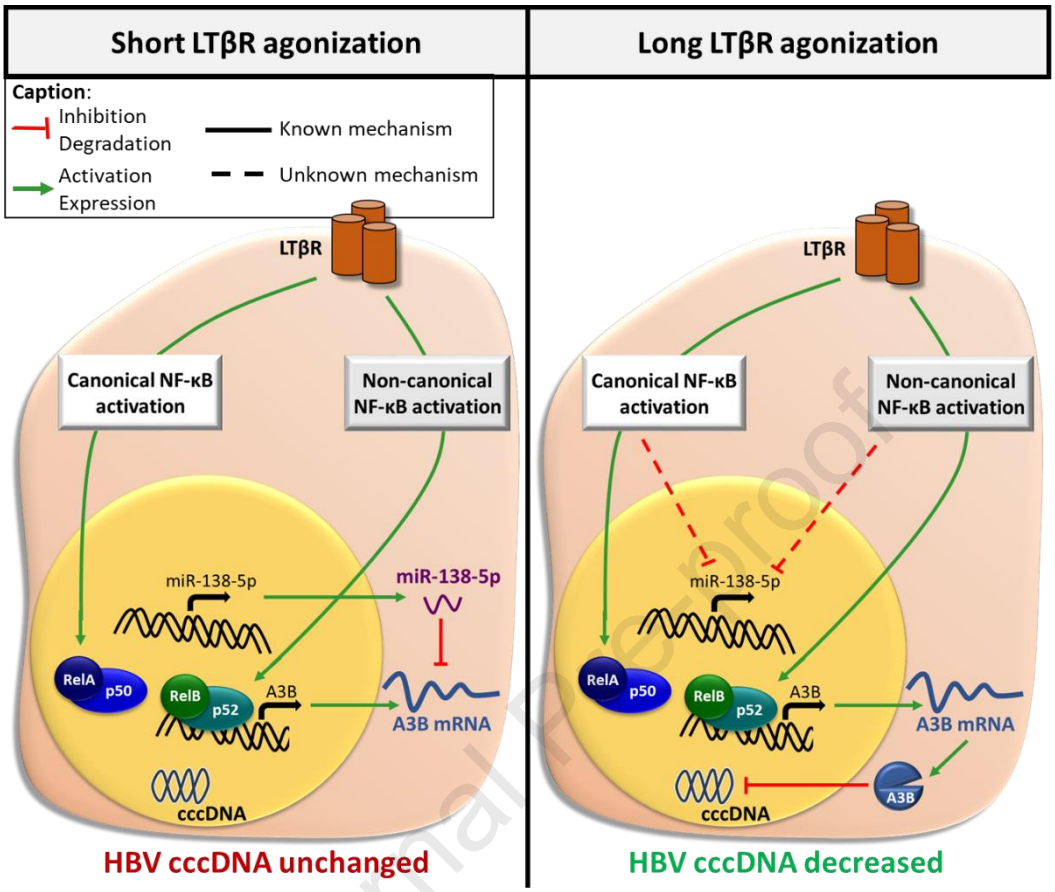
Conclusion: Altogether, A3B represents the only described enzyme to target both transcriptionally active and inactive cccDNA. Thus, inhibiting hsa-miR-138-5p expression should be considered in

the combinatorial design of new therapies against HBV, especially in the context of immune-mediated A3B induction.

Lay Summary

Immune-mediated induction of cytidine deaminase APOBEC3B is transcriptionally regulated by NF-κB signalling and post-transcriptionally down-regulated by the hsa-miR-138-5p expression, leading to cccDNA decay. Timely controlled APOBEC3B-mediated cccDNA decay occurs independently of cccDNA transcriptional activity and without damages to a subset of cancer-related genes. Thus, APOBEC3B-mediated cccDNA decay could offer an efficient therapeutic alternative to target hepatitis B virus chronic infection.

Graphical Abstract



Introduction

Hepatitis B virus (HBV) is a major global health burden with more than 250 million people chronically infected and about 900,000 related death per year (WHO, 2017). Chronic hepatitis B patients (CHB) are at high risk of developing end-stage liver disease and hepatocellular carcinoma (WHO, 2017). Current treatments (e.g. nucleos(t)ides analogues such as Tenofovir or pegylated-interferon alpha) allow the control of the infection but not its complete eradication due to the persistence of the viral minichromosome, called covalently-closed-circular DNA (cccDNA) [1]. Upon treatment arrest, due to side effects or development of resistance, the infection can relapse [1]. Therefore, new treatments are urgently needed to cure chronic HBV infection.

We and others previously showed that the cytidine deaminase apolipoprotein B mRNA editing enzyme catalytic subunit 3B (APOBEC3B, A3B) is upregulated upon immune-mediated Lymphotoxin-β receptor (LTβR) agonization [2,3]. A3B induction subsequently leads to cccDNA hypermutation and viral decrease in a non-hepatotoxic manner *in vitro* [2]. Notably, it is the extent and quality of hepatic inflammation that can contribute to HBV elimination (e.g. in the setting of an acute HBV infection) [3,4]. These results opened the door for new R&D strategies to improve *functional cure* in CHB [2]. LTβR is expressed on different hepatic cells (e.g. hepatocytes, endothelial cells, hepatic stellate cells) [5] and direct, chronic agonization in CHB patients with the current tools available (e.g. BS1 - a LTβR agonist with non-hepatocyte-specific targeting) might affect liver biology. Thus, understanding the mechanisms of A3B regulation in hepatocytes is an important first step towards the targeted development of new therapies aiming at hepatocyte-specific cccDNA decay.

In distinct cancer types, A3B induction has been shown to be mediated by the nuclear factor-kappa B (NF-κB) pathways [6]. However, whether NF-κB signalling is mandatory for A3B induction in non-cancerous hepatocytes or in the context of a chronic HBV infection has remained unknown. The NF-κB-signalling pathway can be divided into two arms: the classical/canonical and the alternative/non-canonical pathways [7]. The canonical pathway, commonly activated by tumour necrosis factor (TNF) family members, signals through the IKK complex (inhibitor of nuclear factor κ-B kinase complex, consisting of NEMO/IKKα/IKKβ), triggering the phosphorylation and ubiquitination of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) and the release of p50/RelA heterodimer [7]. In addition to the canonical pathway, LTβR agonization signals through the non-canonical pathway by activating the NF-κB inducing kinase (NIK). This leads to phosphorylation of IKKα and p100 and its processing into p52 forming p52/RelB heterodimers which translocate to the nucleus to activate target genes such as immune mediators [8].

The liver displays an overall immunosuppressive environment [9]. In order to regulate the immunosuppressive state, as well as to prevent inappropriate and/or chronic inflammation induced by pathogen recognition, a large number of immune factors can additionally be regulated at the post-transcriptional level by micro RNAs (miRNA) [10]. miRNAs are small non-coding RNA involved in mRNA silencing and post-transcriptional regulation through base-pairing with complementary RNA sequences [10]. Protein synthesis of mRNAs targeted by miRNAs is then reduced, either because of the cleavage of the mRNA strand, destabilization of mRNAs by shortening of the poly(A) tail, or reduced translation of the mRNA [10].

Here we describe the regulatory mechanisms of A3B induction upon immune-mediated LTβR agonization at the transcriptional and post-transcriptional level, and identify the regulation of hsa-miR-138-5p as novel antiviral strategy against HBV.

Material and Methods

Cell culture

HepaRG, a non-transformed progenitor cell line that can be differentiated into hepatocytes, were cultured as described previously [11]. HEK293T cells (ATCC® CRL-1573™, for lentivirus production) and HEK293T/17 cells (293T/17; ATCC CRL-11268, for luciferase assays) were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco) and 50 U/mL Penicillin/Streptomycin (Gibco). Primary human hepatocytes (PHH) were isolated were isolated and cultured as previously described [12]. Work with primary cells was approved by the local ethics committee (French ministerial authorizations (AC 2013-1871, DC 2013–1870, AFNOR NF 96 900 sept 2011)). Written consent was obtained from all subjects. HBV, HDV, or HIV chronically infected specimens were excluded.

Transgenic cell line preparation

Knock-out HepaRG cell lines were generated by lentiviral transduction of a double-sgRNA containing construct into HepaRG-iCas9-TR (from David Durantel, unpublished). Briefly, HepaRG cells were transduced with pLenti6-TR to introduce the tetracyclin repressor (TetR), and subsequently with pLenti4/TO/V5 (Invitrogen), in which the coding sequence of an N-terminally 3x FLAG-tagged Cas9 was inserted between the EcoRI and XhoI sites of the vector. The generation of double-sgRNA containing vectors for the knock-out cell line generation was described previously [13]. In short, sgRNAs were chosen based on high scoring and no high scoring off-targets using CHOPCHOP v2 web tool [14]. These sgRNAs were inserted into pUSEPR (generous gift from Dr. Tscharaganeh, unpublished) based on methods as described elsewhere [15].

Preparation of lentiviral particles and transduction of HepaRG cells were performed based on protocols from Addgene. After each transduction step, HepaRG were selected with blasticidin (Invitrogen; 5 µg/mL; TetR), Zeocin (Invitrogen; 300 µg/mL; Cas9) and puromycin (Sigma Aldrich; 10 µg/mL; sgRNAs) until non-transduced cells have died.

Additional material and methods can be found in the supplementary material.

Results

Canonical and non-canonical NF-κB signalling induces APOBEC3B upon LTβR agonization

We and others have shown that agonization of LTβR triggers A3B transcription [8]. However, in non-transformed hepatocytes, the signalling pathways activated remain to be identified. First, we confirmed that of among APOBEC3 family members, A3B displayed the strongest upregulation (2-fold increase by mRNA sequencing and 3-fold increase by mass spectrometry (MS), respectively) at 1 day post-treatment with BS1, an antibody agonising LTβR (**Figures S1A-B**). In addition to the MS results, we also observed an enrichment of A3B mRNA in the polysomes fractions of BS1-treated dHepaRG cells compared to untreated cells (**Figures S1C-D**). Of note, this increase was mostly based on signals located in heavy polysomes, indicative of strong translational activity (**Figure S1E**). EDTA release control [16] confirmed that A3B transcript signal was of polysomal origin (**Figure S1F**).

To decipher the pathway activated by BS1, RNA sequencing was performed and highlighted that constant BS1 treatment up to 40 days downregulated metabolic pathways (e.g. cytochrome P450 mediated detoxification of drugs and xenobiotics) and "complement and coagulation cascades", whereas pathways usually activated during virus-infection were upregulated (**Figure S2A**). In-depth analysis of specific pathways highlighted a strong induction of many of the NF-κB signalling proteins by LTβR agonization (**Figure S2B**), as well as an upregulation of many transcripts of proteins involved in MAPK, NOD-like receptor, IL17, and TNF-signalling pathways (**Figures S2C-F**).

Because LTβR activates the two NF-κB signalling pathways (canonical and non-canonical) [8], we performed *in silico* analyses of the proximal A3B promoter region to find putative NF-κB binding sites (**Figure 1A**). Two κB sites (κB1 and κB2) were identified and tested in mobility shift

assays (EMSA) with nuclear extracts of BS1-treated dHepaRG cells (**Figures 1A and S3A**). Both κB probes displayed NF-κB binding activities, although with different patterns. In addition, the contribution of both κB sites to the A3B transcriptional activity was monitored with a luciferase vector containing the proximal A3B promoter with wild type and/or mutated κB1/2 sites. We observed that all NF-κB heterodimers tested were able to induce a luciferase activity with varying efficacy, but p50/RelA and p50/RelB displayed the poorest activity (**Figure 1B**). Our mutagenesis analysis of each κB sites revealed that κB1 site was the major active site, as its mutation strongly decreased luciferase activity (**Figure 1B**). Chromatin immunoprecipitation of the A3B proximal promoter in HepaRG cells highlighted an increase of binding for RelB, p52, and p50 but not RelA, upon BS1 treatment (**Figures 1C and S3B**). Of note, binding of p52 as well as polymerase II (a marker of active transcription) was constant from day 1 to 6 post treatment (**Figures 1C and 1D**). Furthermore, siRNAs against NIK, or specific IKKβ inhibitors [17,18], or a combination of both, blocked BS1-induced A3B upregulation at the mRNA and protein level (**Figures 1E and S3C**). Results were confirmed using other NF-κB inducing agents (**Figures S3D and S3E**). In addition, HepaRG knock-out lines of genes involved in canonical (i.e. IKKβ, RelA) and non-canonical (i.e. NIK, RelB) NF-κB pathways were generated (**Figures S3F-H**). A significant impairment of BS1-induced A3B upregulation was observed in all tested cell lines (**Figures 1F and S3I**). Knock-down of NIK in combination with IKKβ was most efficient to prevent A3B upregulation.

Taken together, these data highlight that both arms of NF-κB signalling play an important role in the induction of immune-mediated APOBEC3B expression, and confirmed our previous findings on the crucial role of RelB for A3B promoter activation [19].

APOBEC3B is as an atypical NF-κB target

Unlike prototypic C-X-C motif chemokine 10 (CXCL10) or A20 (typical NF-κB target genes), which have an induction peak shortly after treatment start, A3B mRNA was significantly, but only weakly induced at 24h after BS1 exposure (**Figures 2A and S4A**). Whereas continued BS1 treatment led to decreased CXCL10 and A20 expression, A3B mRNA levels remained low during a 4days "lag phase" followed by a constant rise after 4 days of constant or pulse-chase BS1 treatments (**Figures 2A and S4A, respectively**). Similarly, A3B protein level remained low during a 4days "lag phase" and increased after 4 days of BS1 treatment (**Figures 2B)**. RelA phosphorylation, p100 processing to p52, and RelB protein levels were elevated from 18 hours post-treatment (**Figures 2B**). Notably, similar results for A3B expression were obtained with other NF-κB inducing cytokines (**Figure S4B**). The "lag phase" was not linked to delayed A3B transcription because a constant binding of p52 and polymerase II on A3B promoter were detected from 1 day post treatment onwards (**Figures 1C and 1D**). As promoter demethylation is a key factor of gene expression and could play a role in the observed "lag phase", targeted bisulfite sequencing was performed [20]. Over a 12 days period of constant BS1 treatment, no change in A3B promoter methylation was detected (**Figure S4C**).

These data highlighted that A3B is an atypical NF-κB target which displays a "lag phase" profile upon NF-κB activation.

Hsa-miR-138-5p post-transcriptionally regulates APOBEC3B mRNA

Our results suggest that A3B "lag phase" induction might be due to a post-transcriptional regulation by miRNAs. Therefore, we hypothesized that miRNAs might be negative regulators of A3B mRNA to buffer A3B induction upon short-time LTβR stimulation, which potentially leads

to genomic DNA damage [21]. However, a sustained stimulation would lead to a repression of the miRNAs leading to high A3B mRNA levels, needed for an efficient antiviral effect (**Figure 2C)**.

Combined unbiased small RNA sequencing, RT-qPCR, and *in silico* target prediction algorithms[22] revealed 3 clusters of dysregulated miRNAs in untreated compared to BS1-treated HepaRG cells (**Figure 2D**): **(I)** miRNAs highly expressed at day 2 post-treatment (when A3B mRNA is low) but downregulated at day 4 (when A3B mRNA is higher). This group includes the candidates of interest; **(II)** miRNAs lowly expressed at day 2 but with increased expression at day 4; **(III)** miRNAs downregulated under BS1 treatment.

Our *in silico* analysis revealed that among the 30 miRNAs identified in cluster I, only hsa-miR-138-5p was predicted to have a high binding affinity within the 3'UTR of A3B (**Figure 2E**). Next, we confirmed by RT-qPCR the reduced expression of hsa-miR-138-5p between day 2 (i.e. during "lag phase") and day 4 (i.e. after "lag phase") post BS1-treatment (**Figure 2F**).

To assess the functional activity of miR-138-5p on A3B, we fused the luciferase gene upstream of the 3'UTR of A3B and co-transfected the plasmid together with, either a miRNA ctrl or a miRNA-138-5p expression vector. We observed that expression of miRNA-138-5p decreased luciferase activity while expression of miRNA control did not **(Figures 2G-H; 3B-3'-UTR-138)**. Conversely, insertion of point mutations within the miRNA-138-5p binding site of the 3'UTR of A3B abrogated the sensitivity to the miRNA-138-5p (**Figures 2G-H; 3B-3' UTR-138 mut**). We next extended our *in silico* analysis for the presence of miR-138 binding site in the 3'UTR of other APOBEC3 family members. We found that APOBEC3G (A3G) displays one single site while APOBEC3A (A3A) contains a pseudo-miR-138-5p binding site with a single point mutation. Interestingly, an A3A variant with a matching miR-138-5p binding site in its 3'UTR has been identified in a low percentage of the population (SNP rs1367248965). We confirmed that A3G

(3G-3'-UTR-138) and A3A (3A-3'-UTR SNP) variant containing of intact miR-138 binding site were responsive to the expression of miR-138 as opposed to their mutant counterpart (3G-3'-UTR-138 mut and 3A-3'-UTR) **(Figures 2G-H).** The sensitivity of these APOBEC mRNAs to miRNA-138 were similar when the full coding sequences (CDS) of the APOBEC3 genes (**Figures S5A-B**) or the minimal miRNA-138-5p binding site was cloned downstream of the luciferase gene (**Figures S5C-D**).

Noteworthy, in the human genome, two different loci encode hsa-miR-138-5p genes (**Figures S5E-F**). However, dHepaRG cells mainly express the hsa-miR-138-1 located on chromosome 3 (**Figure S5G**). We observed that BS1-mediated hsa-miR-138-5p repression was prevented either by inhibiting IKK kinase activities (i.e. by using TPCA, an IKK inhibitor) or by depleting NIK in HepaRG cells **(Figures S5H-I).** Altogether, these results suggest that activation of NF-κB acts as a positive regulator of A3B transcription while inhibiting hsa-miR-138-5p transcription.

Inhibition of NF-κB signalling or forced expression of hsa-miR-138-5p mimics abolish A3B-mediated anti-HBV activity

We next investigated how NF-κB and miRNA-138-5p modulate HBV viremia. HepaRG control and KO for different NF-κB signalling proteins were treated with BS1or Tenofovir (Teno) for either 6 or 12 days and cccDNA and viremia were monitored **(Figure 3A)**. We observed that BS1-mediated anti-HBV effects (on cccDNA and viremia) was significantly reduced (**Figures 3B and S6A**). Tenofovir (Teno), a nucleoside analogue that reduces secreted DNA but not the cccDNA content, was used as control. These results correlate with the absence of BS1-mediated A3B induction and hsa-miR138-5p repression in NIK or IKKβ-deficient cells (**Figure 3C, S6B and S6C)** and was not the consequence of induced cell death (**Figure S6D**).

These results were also phenocopied using a miRNA mimic approach (see experimental timeline **Figure 3D**). Indeed, transfection of hsa-miR-138-5p mimics reduced A3B levels (**Figure 3E-F**) and prevented antiviral effects on cccDNA (**Figure 3G**). These results further validate our observations in **Figure 2H** showing the effect of the miR-138 binding site in the 3'UTR of A3B and A3G.

Of note, from the 704 hsa-miR-138-5p predicted targets genes (**Table S3**), only 6 genes were related to NF-κB signalling or hepatocyte function (HIF1α, HNF4α, JMJD8, MAPKBP1, RelA, UBE2V1). None of the latter was significantly affected by hsa-miR-138-5p mimics nor BS1 treatment (**Figure S6E;** UBE2V1 could not be detected in dHepaRG), highlighting that the effect of the 138-5p-mimic was most probably limited to A3B.

Interestingly, 7 days post infection (d.p.i.), even though both non-infected and infected cells (HepaRG or PHH) showed a significant upregulation of A3B mRNA upon BS1 treatment, HBV-infected cells displayed a 50% reduction of A3B mRNA expression as compared to the non-infected counterpart, which was (i) independent of an increase of hsa-miR-138-5p expression levels and (ii) not sufficient to prevent the antiviral effect on HBV secreted protein (**Figures 4A-E**). ChIP on the activating epigenetic mark H3K4Me3 highlighted that the increase of H3K4Me3 on the A3B promoter induced by BS1 treatment was lost when the cells were infected with HBV (**Figure S6F)**.

In summary, disrupting A3B induction prevents the immune-mediated effect on HBV cccDNA levels. Moreover, HBV infection itself partially counteracts A3B upregulation during persistent infection.

Transient APOBEC3B induction triggers cccDNA decay without inducing damages to cancer-related genes

One of the major risks in the induction of A3B to eliminate cccDNA might possibly be a DNA-modifying effect. Indeed, A3B expression has been described to be associated with cancer development [21]. As we have previously described that A3B-induced cccDNA decay do not lead to rebounds of HBV infection *in vitro* [2], we hypothesized that short-term A3B induction could be sufficient to ensure viral decay without affecting genomic DNA, in line with the observation that high A3B levels are present in acute, self-limiting HBV infection in patients [4].

Whereas 12 days of BS1-treatment led to an ~80% decrease of cccDNA (**Figures 3A-B and 5A**), no significant mutational load was observed on subset of genes related to cancer development, as analysed by targeted deep-sequencing of 766 genes associated with somatic mutations in tumours (e.g. tumour suppressors; oncogenes) (**Table S4**). Of 2868 detected SNPs (compared to the human reference genome hg19), only 13 were shared by all BS1-treated cells, whereas 12 were also shared in non-treated cells above cut-off level (**Figure 5B**). Closer analyses of SNPs in the tri-nucleotide context revealed no significant differences in SNP frequencies between non-treated and BS1-treated cells (**Figures 5C-E**).

Altogether, transient upregulation of A3B in hepatocytes is sufficient to eliminate cccDNA without inducing a detrimental mutational load to a subset of cancer-related genes *in vitro*.

The antiviral effects of APOBEC3B expression are independent of cccDNA transcriptional activity and can occur on double-stranded DNA

Finally, two of the suggested limitations of A3B-induced cccDNA decay are that: (i) transcriptionally inactive cccDNA (i.e. during occult infection), might escape deamination and

lead to HBV relapses further on; (ii) like other members of the APOBEC3 family (e.g. A3G), A3B might act only on single-stranded DNA during HBV reverse transcription [23,24].

X-protein deficient HBV (HBV ΔX) cccDNA has been shown to be transcriptionally inactive and have a condensed chromatin state [25,26]. To address if A3B can target transcriptionally inactive cccDNA, dHepaRG were infected with HBV wt or HBV ΔX (**Figure 6A**). In both HBV wt- and HBV ΔX-infected cells, a similar reduction of cccDNA levels was observed upon BS1-treatment (**Figure 6B**). The decrease of cccDNA levels in BS1-treated HBV ΔX-infected dHepaRG cells was confirmed by Southern-blot analysis **(Figure 6C)**.

Moreover, we infected dHepaRG with a tRFP-NLS recombinant strain of HBV (tRFP-rHBV), in which the Pol/HBsAg ORF was disrupted by the insertion of a TTR promoter driving a tRFP-NLS reporter (i.e. there is no reverse transcription, and no produced relaxed circular DNA or rcDNA) (**Figure 6D**). Infected cells were positive for RFP (**Figure 6E**). BS1 treatment decreased the number RFP-positive cells (**Figure 6F**), induced A3B (**Figure 6G**), and reduced cccDNA levels (**Figures 6H**), as well as pregenomic RNA (pgRNA) and RFP mRNA (**Figure 6I**). As no rcDNA can be formed in these cells, the reduction of HBV DNA observed was specific of cccDNA.

Thus, A3B acted directly on inactive cccDNA in a reverse transcription-independent manner.

Discussion

APOBEC3B (A3B) has been proposed to be an antiviral enzyme, targeting a multitude of DNA viruses [2,27,28]. We have previously shown that induction of A3B by LTβR agonization leads to non-cytolytic degradation of nuclear HBV cccDNA, enabling long-term inhibition of HBV-replication without rebound, even after treatment arrest [2]. These findings were also independently confirmed *in vivo* by T cell-mediated LTβR activation [3].

Previous studies identified A3B as a NF-κB target gene in cancer cell lines [29]. Here, we describe that both NF-κB pathways (canonical and non-canonical) are involved in LTβR-induced A3B in non-transformed human hepatocytes (dHepaRG). *In silico* analysis identified two putative NF-κB binding sites in the proximal promoter of A3B. These sites were bound by NF-κB complexes in mobility shift and chromatin immunoprecipitation assays, as well as activated in luciferase assays. Chemical based-approaches combined with genetic loss of function of IKKβ and NIK further highlighted the involvement of both NF-κB pathways for A3B induction. A time course analysis of NF-κB (p52 and RelB) and polymerase II recruitment to the A3B promoter and the level of A3B transcript highlighted a post-transcriptional mechanism involving the hsa-miR-138-5p. Amongst the miRNAs previously identified to repress A3B *in silico* [30], only hsa-miR-138-5p was detected in our miRNA analysis. An IKKβ- and NIK-dependent inverse correlation between the expression of hsa-miR-138-5p and A3B was observed in BS1-stimulated cells. These results suggest that NF-κB pathways regulate the expression of a repressor of hsa-miR-138-5p expression. Alternatively, generation of p52/p52 dimers could compete out transcriptionally active NF-κB dimers on the hsa-miR-138-5p promoter region.

The peculiar regulation of A3B might be a conserved evolutionary mechanism to avoid a detrimental A3B-mediated genome editing [21]. Several studies have demonstrated a link between hsa-miR-138-5p and tumour development suggesting a tumour suppressor activity for hsa-miR-138-5p [31]. Thus, it will be interesting to assess whether the hsa-miR-138-5p is downregulated in cancer harbouring an A3B signature or high A3B expression.

We observed that interfering with A3B transcriptionally or post-transcriptionally severely impaired BS1-mediated cccDNA decay. Thus, these findings raise important considerations concerning new therapeutic tools involving LTβR activation for the treatment of CHB patients. As chronic inflammation and tumour development might develop with long-lasting BS1 treatment, a time-restricted administration (e.g. 4 weeks) would be mandatory. Indeed, we confirmed *in vitro* that 12 days BS1-treatment was sufficient to strongly decrease cccDNA levels without inducing mutations within a subset of cancer-related genes.

A repression of A3B was observed in infected cells, upon BS1 treatment and independently of hsa-miR-138-5p. We showed that HBV infection inhibited A3B transcription activation at the epigenetic level, as previously described for interferon β [32]. Thus, understanding the full repertoire of HBV-inhibitory mechanisms on hepatic immune responses might reveal promising targets to enable full A3B induction as well as other immune mediators. Understanding the HBV-mediated A3B expression, the mechanisms of downregulation of the hsa-miR-138-5p, and - as recently published - the inhibition of HIF1α stabilization, could ensure effective immune-mediated control of the viral infection [19].

Although A3B has been proposed to deaminate only ssDNA [23,33], as described for A3A and A3G [34], we have shown that an X-deficient HBV with a transcriptionally inactive cccDNA and a replication-deficient virus (i.e. no reverse transcription) were still susceptible to cccDNA

427 degradation. These results ruled out that the antiviral effects are due to the editing of replicative
428 intermediate of HBV, *i.e.* the relaxed circular DNA, in the cytoplasm, and the nuclear re-import of
429 dysfunctional, mutated HBV genomes. Whether A3B can either induce unwinding of the cccDNA
430 via yet to be described helicase activity, act on ssDNA that naturally occurs in a transcription-
431 independent manner, or act on dsDNA, remains to be determined. Thus, we propose that A3B
432 induction could possibly be used in the treatment of patients with poorly active cccDNA (*i.e.*
433 inactive carrier), in order to eliminate the virus before any reactivation.

434 It will be also important to assess the effect of A3B on integrated HBV genomes, as it is a recurrent
435 event which has been described to be involved in liver pathogenesis [35]. Moreover, as integrations
436 risks increase over time, it is important to diagnose and treat the patients early on. Indeed, if
437 patients are treated before or soon after integrations, we could hypothesize that both the elimination
438 of HBV cccDNA by A3B, as well as the natural renewal of hepatocytes within the liver, might
439 lead to elimination of hepatocytes in which the HBV genome has been integrated.

440 In summary, we have shown that LTβR agonization and activated NF-κB signalling pathways lead
441 to APOBEC3B induction **(Figure 7)**. Moreover, hsa-miR-138-5p negatively regulates
442 APOBEC3B expression and aberrant hsa-miR-138-5p expression inhibits A3B-mediated cccDNA
443 decay, as measured by qPCR and Southern-blot analyses. We believe that blocking hsa-miR-138-
444 5p expression or preventing hsa-miR-138-5p binding to A3B might represent a new therapeutic
445 approach (e.g. in a combinatorial regiment with other treatments) that should be considered to
446 ensure the full functionality of LTβR agonists-based treatments.

447

448 **Abbreviations**

449 A20: tumor necrosis factor alpha-induced protein 3

450 APOBEC3A/A3A: apolipoprotein B mRNA editing catalytic polypeptide-like A

451 APOBEC3B/A3B: apolipoprotein B mRNA editing catalytic polypeptide-like B

452 APOBEC3G/A3G: apolipoprotein B mRNA editing catalytic polypeptide-like G

453 BCA: Bicinchoninic acid assay

454 cccDNA: covalently closed circular DNA

455 CHB: chronic hepatitis B

456 ChIP: chromatin immune precipitation

457 CXCL10: C-X-C motif chemokine ligand 10

458 DNA: desoxyribonucleic acid

459 d.p.i: days post infection

460 EDTA: ethylenediaminetetraacetic acid

461 EMSA: electrophoretic mobility-shift assay

462 H3K4Me3: histone 3 lysine 4 trimethylation

463 HBV: hepatitis B virus

464 IFNα/γ: interferon alpha/gamma

465 IKKα/β: IκB kinase alpha/beta

466 IL-17: interleukin 17

467 JMJD8: jumonji domain containing 8

468 LPS: lipopolysaccharide

469 LTβR: lymphotoxin beta receptor

470 MAPK: mitogen-activated protein kinase

miRNA: micro RNA

NEMO: NF-kB Essential Modulator

NF-κB: nuclear factor kappa B

NIK: NF-κB inducing kinase

NT: non treated

RelA: NF-kappa-B p65 subunit

RNA: ribonucleic acid

RT-qPCR: reverse transcription-quantitative polymerase chain reaction

siCTRL: siRNA control

TNF: tumour necrosis factor

UBE2V1: ubiquitin conjugating enzyme E2 V1

UTR: untranslated region

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599

Figures Legends

Figure 1 - Lymphotoxin beta receptor agonization induces APOBEC3B through NF-κB signalling. (A) dHepaRG were treated for indicated times with 0.5 µg/mL BS1. Upper panel: schematic representation of the experiment. Lower panel: labelled probes containing the NF-κB binding sites were analysed by EMSA. **(B)** HEK293T cells were co-transfected with a luciferase construct containing APOBEC3B promoter (-230, +18, distance to transcription start site) WT or mutated for each NF-κB binding sites, together with NF-κB-transcription-factors-expressing plasmids. Upper panel: schematic representation of the experiment. Lower panel: schematic representation of the downstream promoter region with the inserted mutations of the NF-κB sites. Luciferase activity was assessed 48h post-transfection. Heat map represents the mean of one experiment performed in triplicates. **(C-D)** dHepaRG were treated for the indicated time with 0.5 µg/mL BS1. Upper panel: schematic representation of the experiment. Lower panel: binding of **(C)** p52 and **(D)** polymerase II to APOBEC3B promoter was analysed by ChIP and qPCR**. (E)** dHepaRG were transfected with 10 nM of control or NIK-targeting siRNAs for 24h before being left untreated (NT) or treatment with 0.5 µg/mL of BS,1 +/- 10 µM of TPCA-1 or 5 µM PHA-408. Upper panel: schematic representation of the experiment. Lower panel: mRNAs were isolated and analysed by RT-qPCR. **(F)** Knock-out dHepaRG lines for NIK (sgNIK), IKKβ (sgIKKβ), NIK and IKKβ (sgNIK+sgIKKβ), RelB (sgRelB), or RelA and RelB (sgRelA+sgRelB), as well as control dHepaRG (sgCtrl) were left untreated (NT) or treated with 0.5 µg/mL of BS1 for 3 days. Upper panel: schematic representation of the experiment. Lower panel: mRNAs were isolated and analysed by RT-qPCR. Bars represent the mean +/- SD of **(E)** two, **(F)** three, or **(C-D)** four independent experiments. Data were submitted to **(C-F)** one-way ANOVA. **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.001$; ns: not significant.

Figure 2 – APOBEC3B is post-transcriptionally regulated by the miRNA-138-5p. (A-B) Treatment of dHepaRG with 0.5 µg/mL BS1 was started sequentially and stopped altogether at the indicated time points. **(A)** Upper panel: schematic representation of the experiment. Lower panel: mRNAs of interest were extracted and analysed by RT-qPCR. **(B)** Proteins were analysed by immunoblotting. **(C)** Schematic representation of the working hypothesis. **(D)** dHepaRG were treated for 2 or 4 days with 0.5 µg/mL of BS1. Upper panel: schematic representation of the experiment. Lower panel: RNAs were extracted and small RNA were sequenced. Top 50 significantly dysregulated miRNAs of combined sequencing and RT-qPCR data was unbiased clustered and plotted. Cluster I represents miRNAs highly expressed at day 2 and lowly expressed at day 4 (i.e. miRNAs of interest); Cluster II represents miRNAs lowly expressed at day 2 and highly expressed at day 4; Cluster III represents miRNA lowly expressed at day 2 and day 4. **(E)** Schematic illustration of the miRNA-138-5p binding site on the APOBEC3B 3' UTR. **(F)** dHepaRG were left untreated (NT) or treated with 0.5 µg/mL of BS1 for 2 or 4 days (see schematic representation of the experiment in D). miRNAs were extracted and analysed by RT-qPCR. **(G-H)** HEK293T cells were co-transfected with luciferase-3' UTR fusion constructs and either miR-138-5p-expressing plasmids or control miR-expressing plasmids. **(G)** Schematic representations of luciferase-3' UTR fusions used. **(H)** Upper panel: schematic representation of the experiment. Lower panel: luciferase activity was assayed 48h post transfection. Bars, respectively points, represent the mean +/- SD of **(F, H)** one, or **(A)** three experiment performed in triplicates Data were submitted to **(A, F, H)** unpaired student's t-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.001$; ns: not significant.

Figure 3 – Dysregulation of APOBEC3B expression by disruption of NF-κB signalling and mi-RNA 138-5p prevent the antiviral effect. (A) Schematic representation of the experiment presented in panels **B** and **C**. **(B-C)** Knock-out dHepaRG lines for NIK (sgNIK), IKKβ (sgIKKβ), or NIK and IKKβ (sgNIK+sgIKKβ), as well as control dHepaRG (sgCtrl) were infected with HBV. 7 d.p.i cells were left untreated (NT) or treated with 0.5 µg/mL of BS1 or 0.5 µM of Tenofovir for 12 days. **(B)** DNA and **(C)** RNAs were isolated and analysed by RT-qPCR or qPCR. **((D-G)** dHepaRG were infected with HBV and 10 and 13 d.p.i. transfected with 10 nM microRNA (miR)-138-5p or control mimics. Cells were then left untreated (NT) or treated for 6 days with 0.5 µg/mL of BS1 or 0.5 µM of Tenofovir. **D)** Schematic representation of the experiment presented in panels **E** to **G**. **(E)** RNAs, **(F)** proteins and **(G)** DNA were isolated and analysed by RT-qPCR, immunoblotting, and qPCR, respectively. Bars represent the mean +/- SD of **(B-C)** three or **(E, G)** six independent experiments. Data were submitted to **(B-C, E, G)** one-way ANOVA. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.001$; ns: not significant.

Figure 4 – HBV-mediated inhibition of APOBEC3B expression is not sufficient to prevent the antiviral effect. (A) Schematic representation of the experiment presented in panels **B** to **E**. **(B-C)** dHepaRG or **(D-E)** PHH were infected with HBV and left untreated (NT) or treated with 0.5 µg/mL of BS1 starting 1 d.p.i, for 6 days. **(B, D)** RNAs were isolated and analysed by RT-qPCR or qPCR. **(C, E)** Levels of HBeAg were detected in the cell culture supernatant via ELISA. Bars represent the mean +/- SD of **(D-E)** one, or **(B-C)** three independent experiments. Data were submitted to **(B-E)** unpaired student's t-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.001$; ns: not significant.

Figure 5 – APOBEC3B induction does not induce *de novo* mutations in a subset of genes related to cancer development. (A-E) dHepaRG were infected and 10 d.p.i., were left untreated (NT) or treated with 0.5 µg/mL BS1 for 12 days. DNA was extracted and subjected to panel sequencing of a panel containing 766 genes (CeGaT CancerPrecision panel). 2,868 SNVs (single nucleotide variants) were detected in total. **(A)** Schematic representation of the experiment. **(B)** These SNVs were then filtered to identify SNVs, that occur in all samples with a number of alleles (NAF) > 5% and a coverage > 30 (2,404), only in all treated samples (13) and only in all 'not treated' samples (12). 439 SNVs were not found in all samples but they were not specific to either of the two groups. Inspection of the 13 and 12 genes showed that they have NAFs close to the cutoff of 5% but are detected in the other samples as well. **(C-E)** SNVs in every possible trinucleotide context were analysed for their frequency. **(C)** Comparison of the frequency of SNVs between non-treated and BS1 treated samples. In the table, the median frequency and the interquartile range (IQR) of SNVs are presented. In the box plot, every data point represents a SNV in a trinucleotide context. Data was submitted to Wilcoxon-signed Rank Sum test. **(D)** Frequency for all SNVs in a trinucleotide context of non-treated samples. **(E)** Frequency for all SNVs in a trinucleotide context of non-treated samples.

Figure 6 – APOBEC3B effect on double stranded DNA is independent of transcription. (A-C) dHepaRG were infected with wild type (wt) HBV or HBx deficient (ΔX) HBV. 7 d.p.i, cells were left untreated (NT) or treated with 0.5 µg/mL BS1 for 11 days. **(A)** Schematic representation of the experiment. **(B-C)** DNA were extracted and analysed by (B) qPCR and **(C)** Southern-blotting. **(D-I)** dHepaRG were infected with a recombinant tRFP-rHBV virus. 7 d.p.i., cells were left untreated (NT) or treated with 0.5 µg/mL BS1 or 0.5 µM of Tenofovir for 9 days. **(D)**

693 Schematic representation of the experiments presented in panels **E** to **I**. **(E)** Representative photos
694 of bright field and fluorescent microscopy of the different treatments at 6 d.p.i. **(F)** Quantification
695 of the number of RFP positive cells per view field. **(G, I)** RNA and **(H)** DNA were extracted and
696 quantified by RT-qPCR and qPCR. Bars represent the mean +/- SD of **(F-I)** two or **(B)** four
697 independent experiments performed in triplicates. Data were submitted to **(B)** unpaired student's
698 t-test or **(F-I)** one-way ANOVA. ***: $p < 0.001$; ****: $p < 0.001$; ns: not significant.
699
700 **Figure 7 – APOBEC3B induction and subsequent cccDNA decay depend on NF-κB signalling**
701 **and miR-138-5p decrease.** Graphical representation of the main proposed mechanism(s). Upon
702 short-time agonization of the LTβR, NF-κB signalling induces weak APOBEC3B mRNA
703 expression because of the inhibitory activity of miR-138-5p, thereby preventing cccDNA decay.
704 Upon a prolonged agonization of LTβR, the miR-138-5p levels is decreased allowing potent
705 induction of APOBEC3B mRNA, and subsequently cccDNA decay that is independent of cccDNA
706 transcriptional activity.

Control of APOBEC3B induction and cccDNA decay by NF-κB and miR-138-5p

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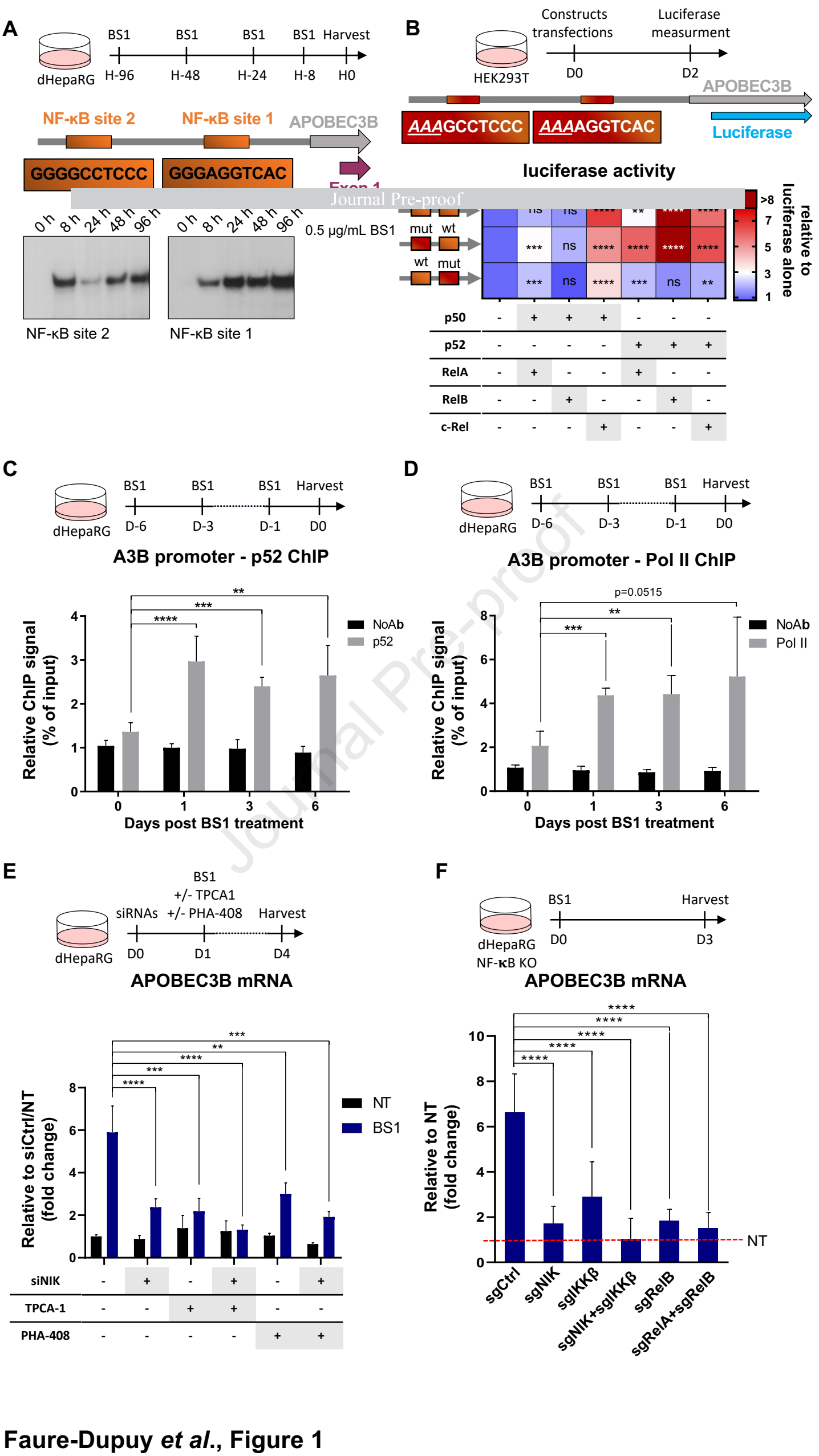
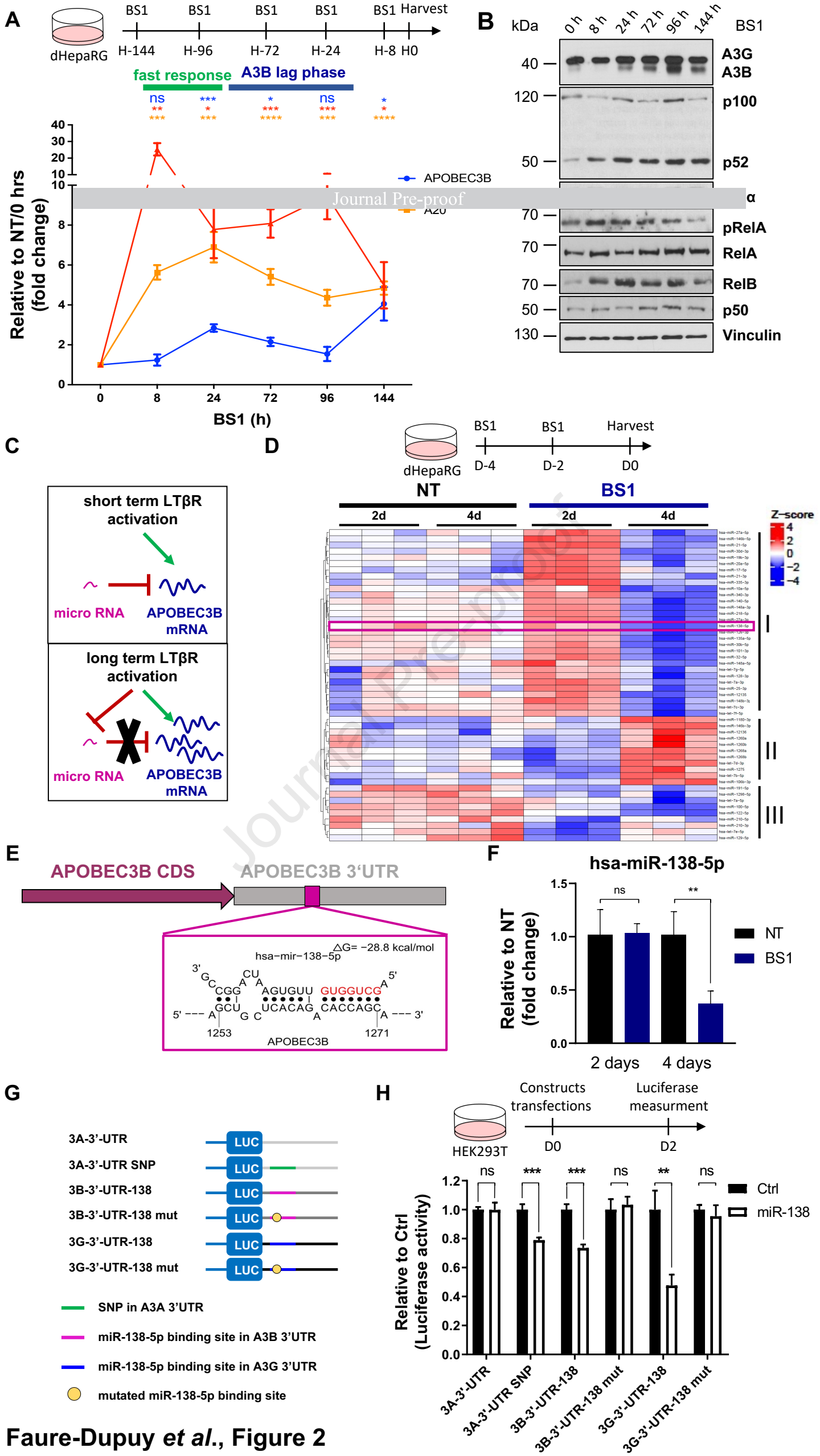
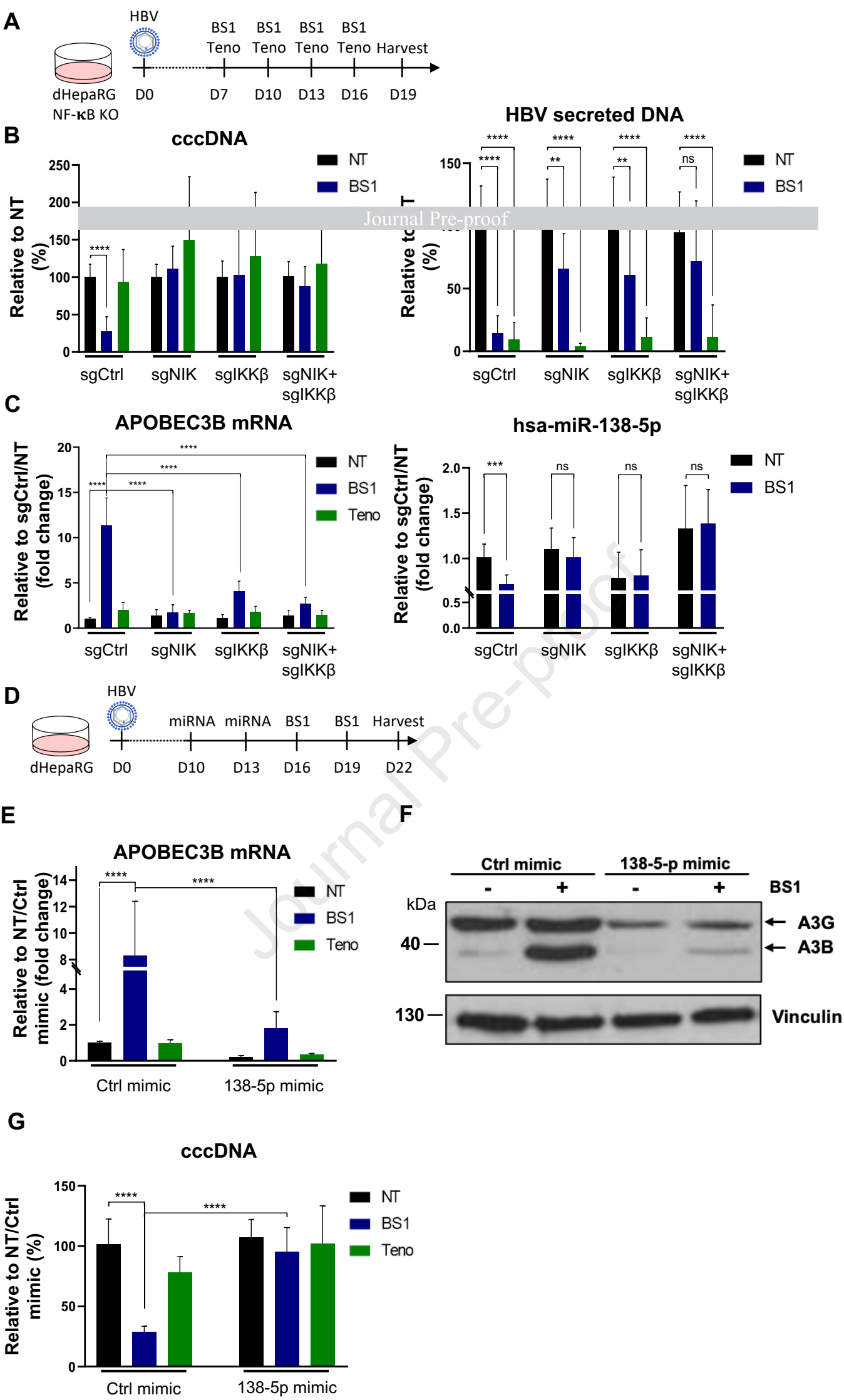


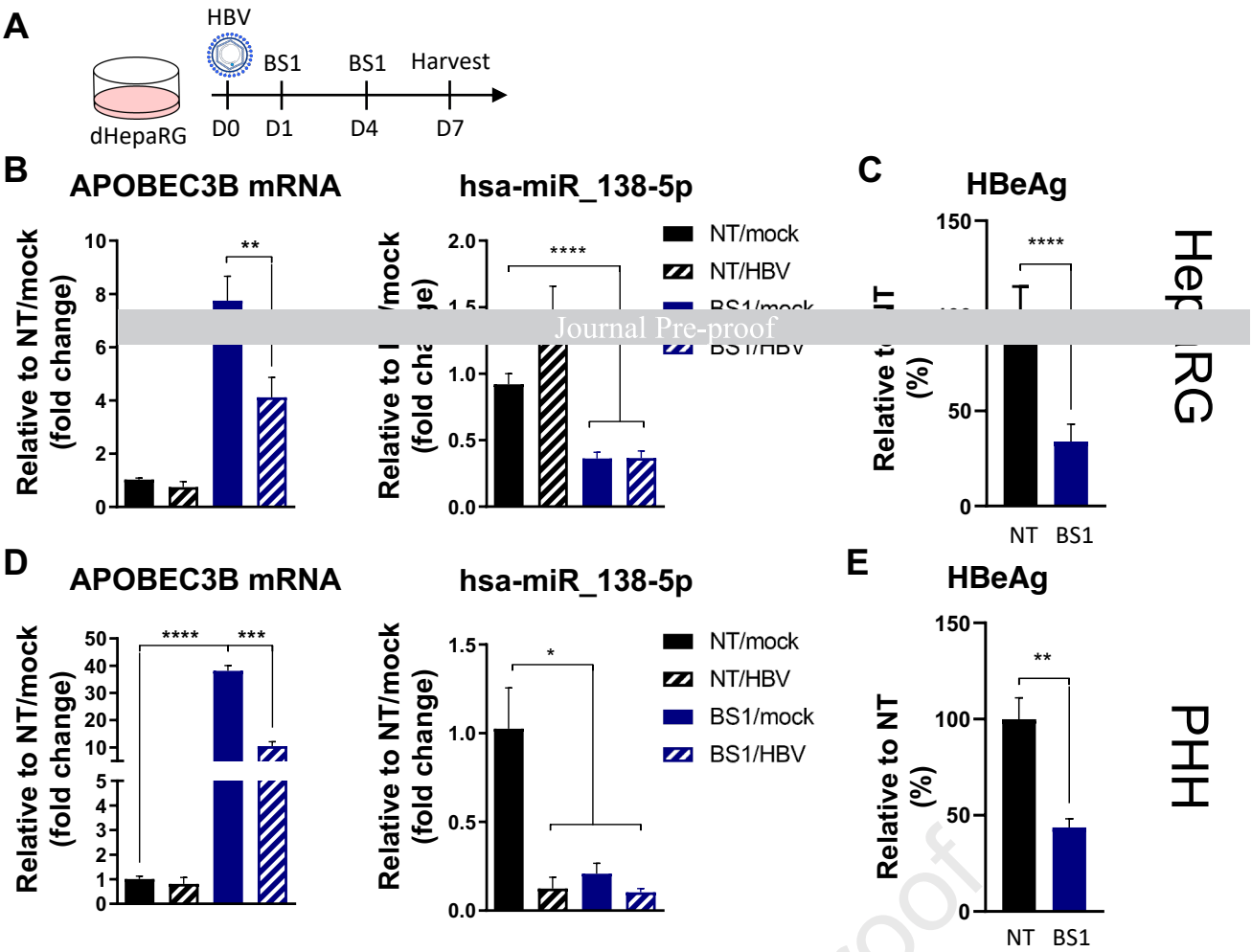
Faure-Dupuy *et al.*, Figure 1



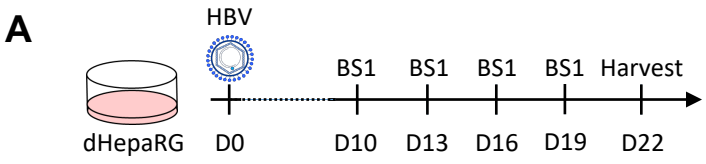
Faure-Dupuy *et al.*, Figure 2



Faure-Dupuy *et al.*, Figure 3

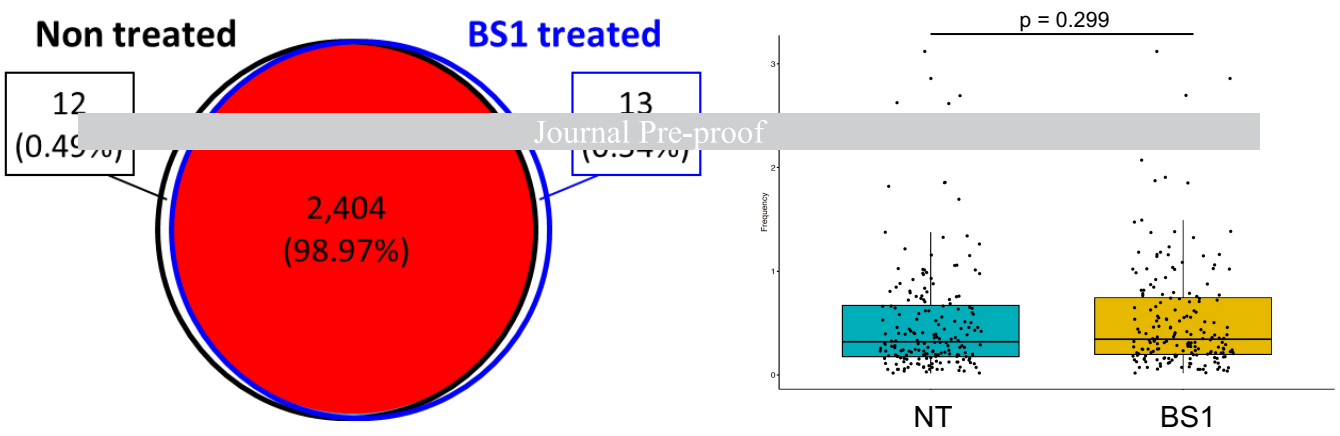


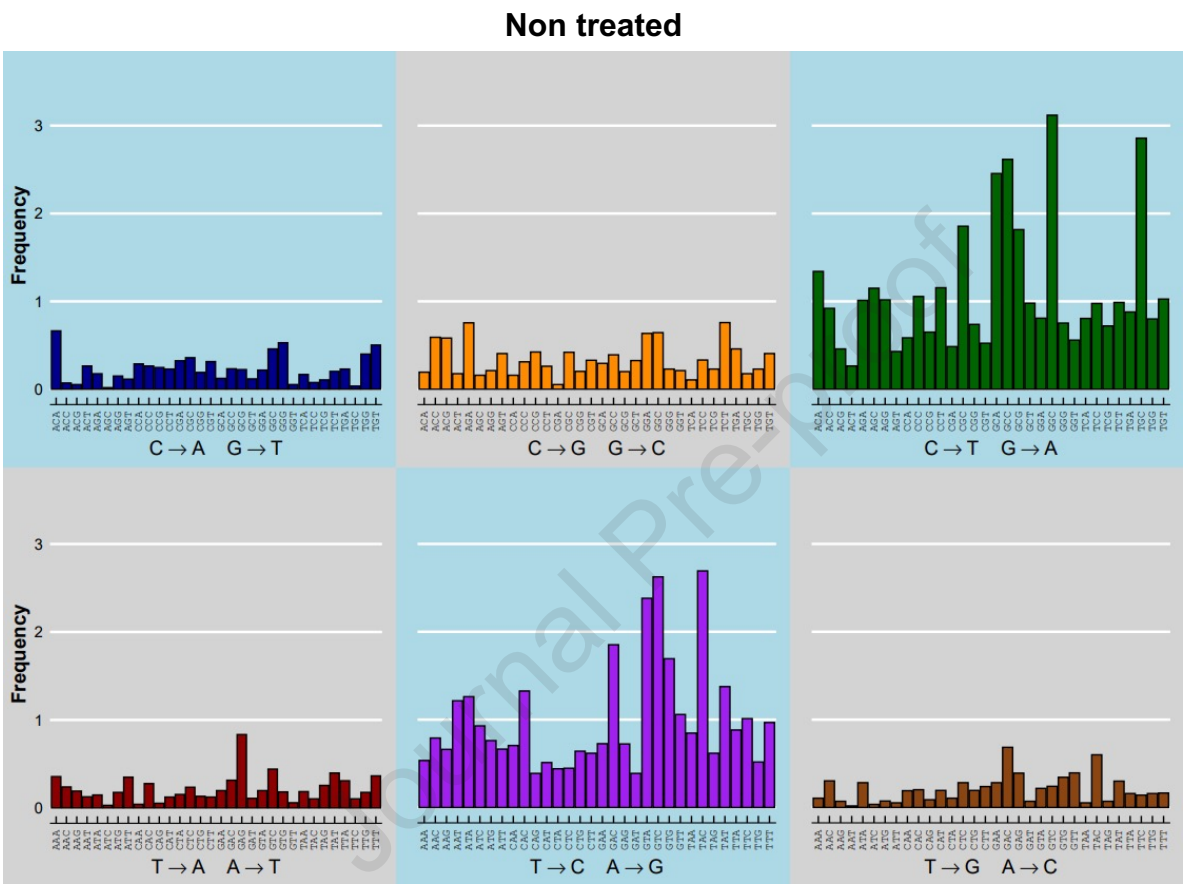
Faure-Dupuy *et al.*, Figure 4

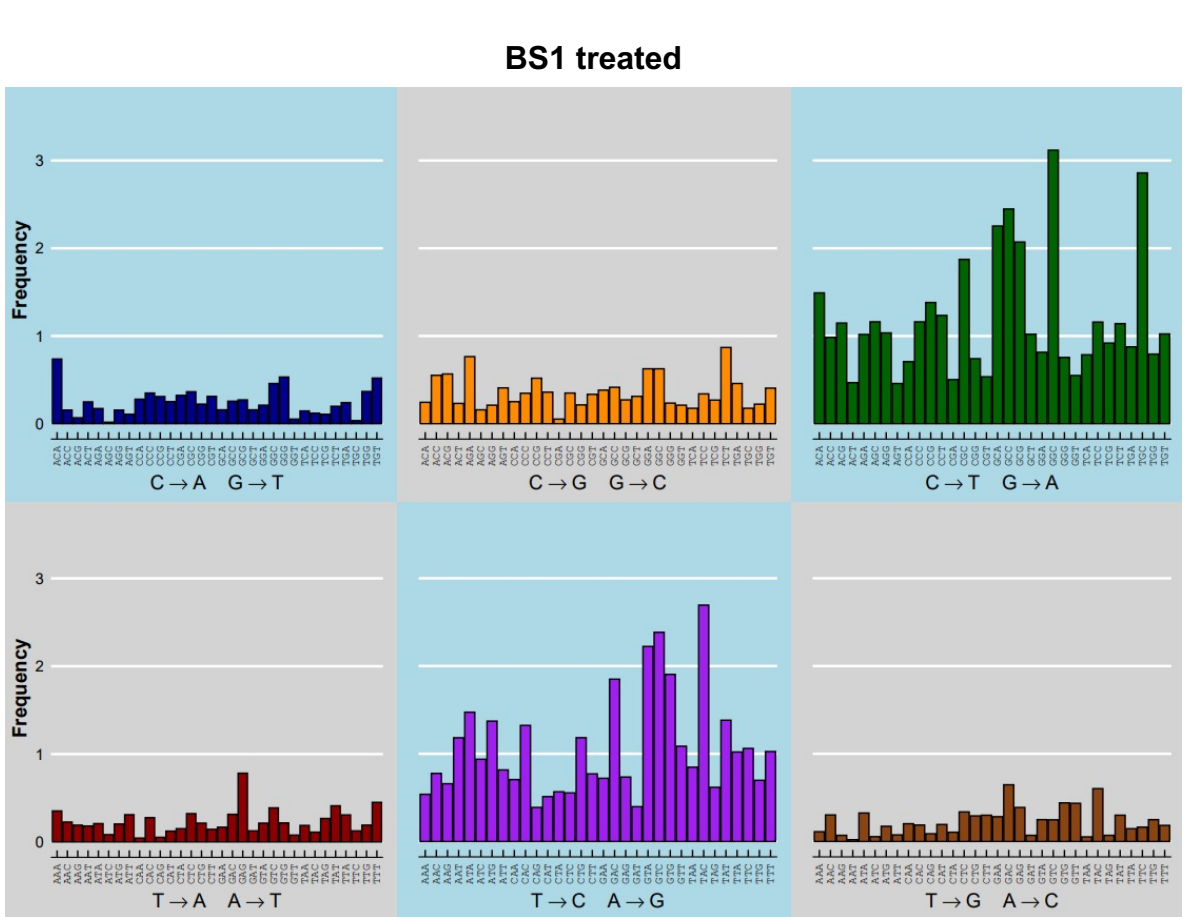


C

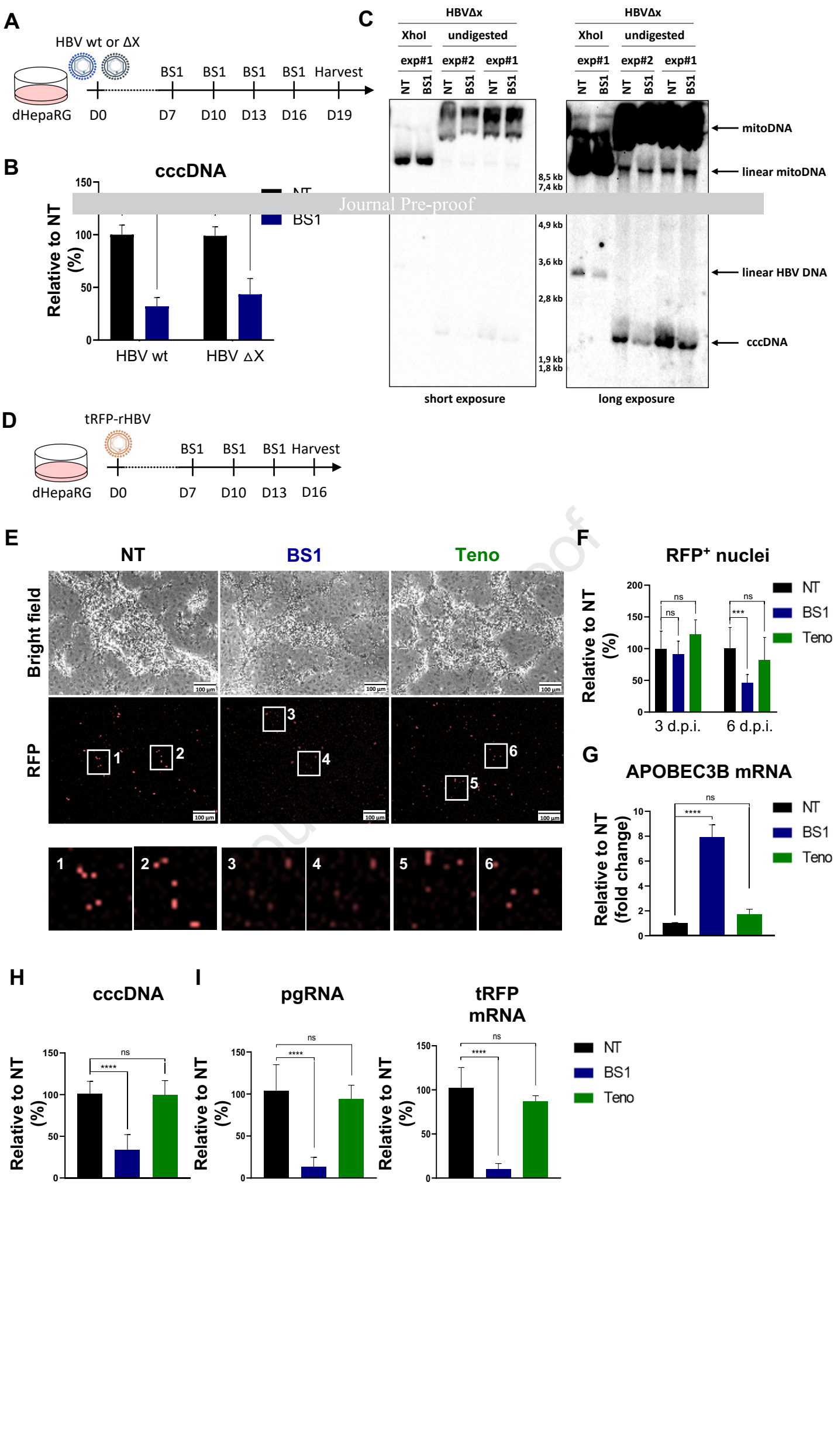
Treat-ment	N	Median	IQR
NT	192	0.319	0.493
BS1	192	0.344	0.547



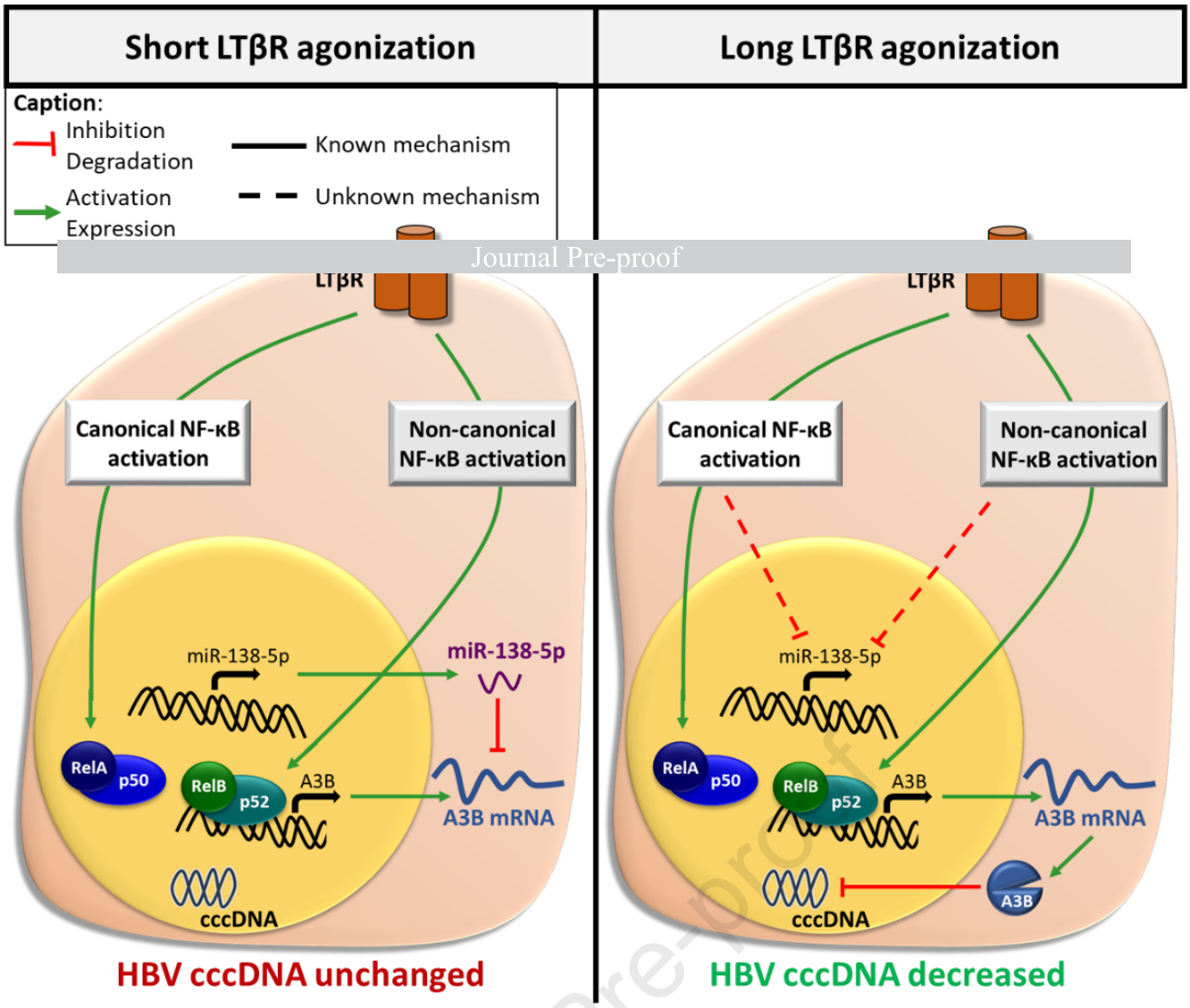




Faure-Dupuy ***et al.*, Figure 5**



Faure-Dupuy *et al.*, Figure 6



Faure-Dupuy *et al.*, Figure 7

Highlights:

- Impairment of NF-κB signalling prevents APOBEC3B induction and cccDNA decay.
- APOBEC3B is post-transcriptionally regulated by the hsa-miR-138-5p.
- Over-expression of the hsa-miR-138-5p inhibits APOBEC3B expression and cccDNA decay.
- A3B timely induces cccDNA decay without damages to cancer-related genes.
- APOBEC3B-mediated cccDNA decay is independent of cccDNA transcriptional activity.