HEPATOLOGY

HEPATOLOGY, VOL. 74, NO. 4, 2021

Hypoxia-Inducible Factor 1 Alpha–Mediated RelB/APOBEC3B Down-regulation Allows Hepatitis B Virus Persistence

Tobias Riedl ,[1,2*] Suzanne Faure-Dupuy ,[1,3*] Maude Rolland ,[4*] Svenja Schuehle,[1,2] Zohier Hizir,[4] Silvia Calderazzo,[5] Xiaodong Zhuang,[6,7] Jochen Wettengel,[8] Martin Alexander Lopez,[4] Romain Barnault,[9] Valbona Mirakaj,[9] Sandra Prokosch,[1] Danijela Heide,[1] Corinna Leuchtenberger,[1] Martin Schneider,[10] Bernd Heßling,[10] Benjamin Stottmeier,[11,12] Isabel M. Wessbecher ,[11,13] Peter Schirmacher,[11,13] Jane A McKeating,[6,7] Ulrike Protzer ,[8] David Durantel ,[14] Julie Lucifora ,[14] Emmanuel Dejardin ,[4**] and Mathias Heikenwalder [1,3**]

BACKGROUND AND AIMS: Therapeutic strategies against HBV focus, among others, on the activation of the immune system to enable the infected host to eliminate HBV. Hypoxia-inducible factor 1 alpha (HIF1α) stabilization has been associated with impaired immune responses. HBV pathogenesis triggers chronic hepatitis-related scaring, leading *inter alia* to modulation of liver oxygenation and transient immune activation, both factors playing a role in HIF1α stabilization.

APPROACH AND RESULTS: We addressed whether HIF1α interferes with immune-mediated induction of the cytidine deaminase, apolipoprotein B mRNA editing enzyme catalytic subunit 3B (APOBEC3B; A3B), and subsequent covalently closed circular DNA (cccDNA) decay. Liver biopsies of chronic HBV (CHB) patients were analyzed by immunohistochemistry and *in situ* hybridization. The effect of HIF1α induction/stabilization on differentiated HepaRG or mice ± HBV ± LTβR-agonist (BS1) was assessed *in vitro* and *in vivo*. Induction of A3B and subsequent effects were analyzed by RT-qPCR, immunoblotting, chromatin immunoprecipitation, immunocytochemistry, and mass spectrometry. Analyzing CHB highlighted that areas with high HIF1α levels and low A3B expression correlated with high HBcAg, potentially representing a reservoir for HBV survival in immune-active patients. *In vitro*, HIF1α stabilization strongly impaired A3B expression and anti-HBV effect. Interestingly, HIF1α knockdown was sufficient to rescue the inhibition of A3B up-regulation and -mediated antiviral effects, whereas HIF2α knockdown had no effect. HIF1α stabilization decreased the level of v-rel reticuloendotheliosis viral oncogene homolog B protein, but not its mRNA, which was confirmed *in vivo*. Noteworthy, this function of HIF1α was independent of its partner, aryl hydrocarbon receptor nuclear translocator.

CONCLUSIONS: In conclusion, inhibiting HIF1α expression or stabilization represents an anti-HBV strategy in the context of immune-mediated A3B induction. High HIF1α, mediated by hypoxia or inflammation, offers a reservoir for HBV survival *in vivo* and should be considered as a restricting factor in the development of immune therapies. (Hepatology 2021;74:1766-1781).

HBV chronically infects >250 million persons worldwide who are at high risk of developing end-stage liver disease and HCC.[1] Current treatments allow control of the infection, but

Abbreviations: AhR, aryl hydrocarbon receptor; APOBEC3B/A3B, apolipoprotein B mRNA editing catalytic polypeptide-like B; ARNT, aryl hydrocarbon receptor nuclear translocator; BS1, antibody agonizing LTβR; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DMOG, dimethyloxallyl glycin; FG-4592, roxadustat; HIF1α, hypoxia-inducible factor 1 alpha; HIF2α, hypoxia-inducible factor 2 alpha; HO, hypoxia; IFNα/γ, interferon alpha/gamma; IKKα/β, IκB kinase alpha/beta; LTβR, lymphotoxin beta receptor; NF-κB, nuclear factor kappa B; NO, normoxia; RelA, NF-κB p65 subunit; RelB, v-rel reticuloendotheliosis viral oncogene homolog B; siCTRL, siRNA control; ssiHIF1α, siRNA HIF1α; siRNA, small interfering RNAs.

Received January 23, 2021; accepted April 30, 2021.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31902/suppinfo.

**Contributed equally as co-first authors.*

***Contributed equally as co-senior authors.*

not its complete eradication because of the persistence of the viral DNA matrix, called covalently closed circular DNA (cccDNA).[2] Upon treatment arrest, the infection can relapse.[2] Therefore, treatments are urgently needed to progress toward a cure for chronic HBV infection.

Therapeutics developed for the treatment of HBV focus on activation of the adaptive and innate immune system. Several Toll-like receptor agonists have offered promising results both *in vitro* and *in vivo*.[3–5] Among these treatments, we and others have shown that induction of the cytidine deaminase, apolipoprotein B mRNA editing enzyme catalytic subunit 3B (APOBEC3B; A3B), upon immune-mediated lymphotoxin-β receptor (LTβR) agonization (e.g., by T cells) leads to cccDNA decay.[6,7]

Most immune receptors such as LTβR are described to signal through the nuclear factor-kappa B (NF-κB) pathways.[8,9] NF-κB signaling is divided into two arms: the classical/canonical and the alternative/noncanonical pathway.[10] The canonical pathway signals through the IκB kinase (IKK) complex (inhibitor of nuclear factor κB kinase complex, consisting of NF-κB essential modulator/IKKα/IKKβ), triggering the phosphorylation and ubiquitination of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha and the release of p50/RelA (NF-κB p65 subunit) heterodimer.[10]

Supported, in part, by the European Union's Horizon 2020 program under the agreements 667273 (HEPCAR; to M.H.), by the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) Project ID 272983813-SFBTR 179, project ID 360372040-SFB 1335, and project ID 314905040-SFBTR 209, the ERC CoG (HepatoMetabopath; to M.H.), the ERC POC (Faith; to M.H.), the Helmholtz Future topic Inflammation and Immunology (to M.H.), Zukunftssthema 'Immunology and Inflammation' (ZT-0027), and the Rainer Hoenig Stiftung. E.D. and M.H. were supported by the FNRS/FWO under EOS project no. 30826052. E.D. received financial support from the FSR and the Fondation Léon Fredericq of the University of Liege. M.H., E.D., D.D., J.L., and M.R. were supported by an FP7-Infect-Era grant. D.D. and J.L. were supported by INSERM (Institut National de la Santé et de la Recherche Médicale; salaries and core-fundings), ANRS (Agence Nationale de Recherche sur le Sida et les hépatites virales, several grants from study section 12 [CSS12]), and EU-Infect Era (ANR 16-IFEC-0005-01). J.A.M.'s laboratory is funded by Wellcome Trust IA 200838/Z/16/Z, MRC project grant MR/R022011/1, and Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Science (CIFMS), China (grant no.: 2018-I2M-2-002).

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.31902

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the [1]Division of Chronic Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany; [2]Faculty of Biosciences, Heidelberg University, Heidelberg, Germany; [3]Department of Infectious Diseases, Molecular Virology, Heidelberg University, Heidelberg, Germany; [4]Laboratory of Molecular Immunology and Signal Transduction, GIGA-Institute, University of Liège, Liège, Belgium; [5]Division of Biostatistics, German Cancer Research Center (DKFZ), Heidelberg, Germany; [6]Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom; [7]Chinese Academy of Medical Sciences (CAMS) Oxford Institute (COI), University of Oxford, Oxford, United Kingdom; [8]Institute of Virology, Helmholtz Zentrum München, Munich, Germany; [9]Departement of Anesthesiology and Intensive Care Medicine, Molecular Intensive Care Medicine, University Hospital Tübingen, Eberhard-Karls-University, Tuebingen, Germany; [10]Mass Spectrometry Based Protein Analysis Unit, German Cancer Research Center (DKFZ), Heidelberg, Germany; [11]Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany; [12]German Center for Infection Research (DZIF), partner site Heidelberg, Heidelberg, Germany; [13]Tissue Bank of the German Center for Infection Research (DZIF), Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany; [14]INSERM, U1052, Cancer Research Center of Lyon (CRCL), University of Lyon (UCBL1), CNRS UMR_5286, Centre Léon Bérard (CLB), Lyon, France.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Mathias Heikenwälder, Ph.D.
Division Chronic Inflammation and Cancer (F180)
German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 242
69120 Heidelberg, Germany
E-mail: m.heikenwaelder@dkfz-heidelberg.de
Tel.: +49 6221 42-3891
or

Dejardin Emmanuel, Ph.D.
Laboratory of Molecular Immunology and Signal Transduction,
University of Liège, GIGA-Institute
Avenue de l'Hôpital, 1
CHU, B34
4000 Liege, Belgium
E-mail: e.dejardin@uliege.be
Tel.: +32 4 3664472

The noncanonical pathway signals through NF-κB-inducing kinase (NIK), leading to the phosphorylation of IKKα and p100, which is subjected to processing into p52 forming p52/RelB (v-rel reticuloendotheliosis viral oncogene homolog B) heterodimers that activate target genes such as immune mediators.[11]

To reduce the extent of chronic inflammation and its deleterious effects, NF-κB signaling has to be tightly regulated.[12] Among the factors involved in this regulation, hypoxia-inducible factor 1 alpha (HIF1α) has been shown to (1) be stabilized or induced by and (2) regulate NF-κB signaling,[13] in addition to its canonical induction by low oxygen levels.[14] HIF1α is constantly produced and is targeted to the proteasome in the absence of stabilizing conditions.[14]

Here, we identify HIF1α stabilization and the concomitant decrease of RelB protein level as a restricting factor for immune-mediated antiviral strategies against HBV.

Materials and Methods

CELL CULTURE

HepaRG, a nontransformed progenitor cell line that can be differentiated into hepatocytes, was cultured as described.[15] Cells under hypoxia were cultured under 1% or 3% oxygen (InVivO2; Baker Ruskinn, Sanford, ME), 5% CO_2, in a humidified atmosphere.

TRANSGENIC CELL-LINE PREPARATION

HIF-overexpressing cell lines were generated from HepaRG-TR.[16] HIF open reading frames (ORFs) were excised from HA-HIF1α P402A/P564A-pcDNA3 (#18955; Addgene, Teddington, United-Kingdom), or HA-HIF2α-pcDNA3 (#18950; Addgene), using BamHI and XbaI (New England Biolabs, Ipswich, MA). The P402A/P564A double mutation prevents HIF1α hydroxylation and degradation. ORFs were then inserted into the BamHI/XhoI digested pLenti CMV/TO Hygro empty (w214-1; #17484; Addgene) using T4 DNA ligase (New England Biolabs). All HIF vectors were a gift from William Kaelin, and pLenti CMV/TO Hygro empty (w214-1) was a gift from Eric Campeau and Paul Kaufman.

Preparation of lentiviral particles and transduction of HepaRG cells were performed based on protocols from Addgene. After each transduction step, HepaRG cells were selected with blasticidin (5 μg/mL; TetR; Invitrogen, Waltham, MA) and puromycin (10 μg/mL; single-guide RNA; Sigma-Aldrich, Saint-Louis, MO) until nontransduced cells had fully died.

TREATMENTS AND TRANSFECTIONS

dHepaRG cells were treated with 0.5 μg/mL of BS1 (generous gift from Dr. Jeffrey Browning, Biogen/Idec, Cambridge, MA). Additionally, dHepaRG cells, not infected with HBV, were stimulated either with 10 ng/mL of TNFα, 50 ng/mL of IL-17, or 100 ng/mL of lipopolysaccharide (LPS), or left untreated. dHepaRG cells infected with HBV were treated with 1,000 IU of interferon alpha (IFNα) 2A (Roferon; Roche, Mannheim, Germany), 800 IU of TNFα (210-TA; R&D Systems, Abingdon, United-Kingdom), or 200 IU of interferon gamma (IFNγ; 285-IF; R&D Systems). All inhibitors and molecules used are presented in Supporting Table S1. dHepaRG cells were transfected with 10 nM of small interfering RNAs (siRNAs) against HIF1α (Assay ID: s6539; Ambion, Oberursel, Germany), hypoxia-inducible factor 2 alpha (HIF2α; Assay ID: s4698; Ambion), aryl hydrocarbon receptor (AhR; Sigma-Aldrich), aryl hydrocarbon receptor nuclear translocator (ARNT; Sigma-Aldrich), v-rel reticuloendotheliosis viral oncogene homolog B (RelB; Dharmacon, Lafayette, CO), or nontargeting control siRNAs (siCtrl; 4390843; Ambion), using Dharmafect 4 (1:1,000; Dharmacon; Supporting Table S2).

HBV PREPARATION AND INOCULA

HBV was purified and concentrated from the culture medium of HepAD38 cells by heparin columns and sucrose gradient ultracentrifugation as described.[17] dHepaRG cells were infected with 200 viral genome equivalents per cell, in medium supplemented with 4% PEG-8000 (Sigma-Aldrich). Twenty-four hours after infection, cells were washed three times with PBS.

HUMAN LIVER SPECIMEN

Sections of formalin-fixed, paraffin-embedded liver resections of 15 patients chronically infected with

HBV were obtained from the DZIF partner site in Heidelberg/Institute of Pathology of the Medical University Heidelberg. Chronic hepatitis B (CHB) patients were all in the immune-active phase of the disease and presented F3/F4 fibrosis grading and A3 activity (METAVIR scoring). Sections were cut to be 2 or 5 μM thick. Work with patient material was approved by the Heidelberg ethics committee under the following number: S206/2005. We confirmed that informed consent was collected from all co authors for the manuscript.

STATISTICAL ANALYSIS

Two-way ANOVA, Spearman correlation, and the unpaired Student two-tailed *t* test were performed using Prism software (version 8; GraphPad Software Inc., La Jolla, CA). Data are shown as mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.001$).

Additional materials and methods information can be found in the Supporting Information.

Results

HIF1α STABILIZATION OFFERS A RESERVOIR FOR HBV IN IMMUNE-ACTIVE PATIENTS

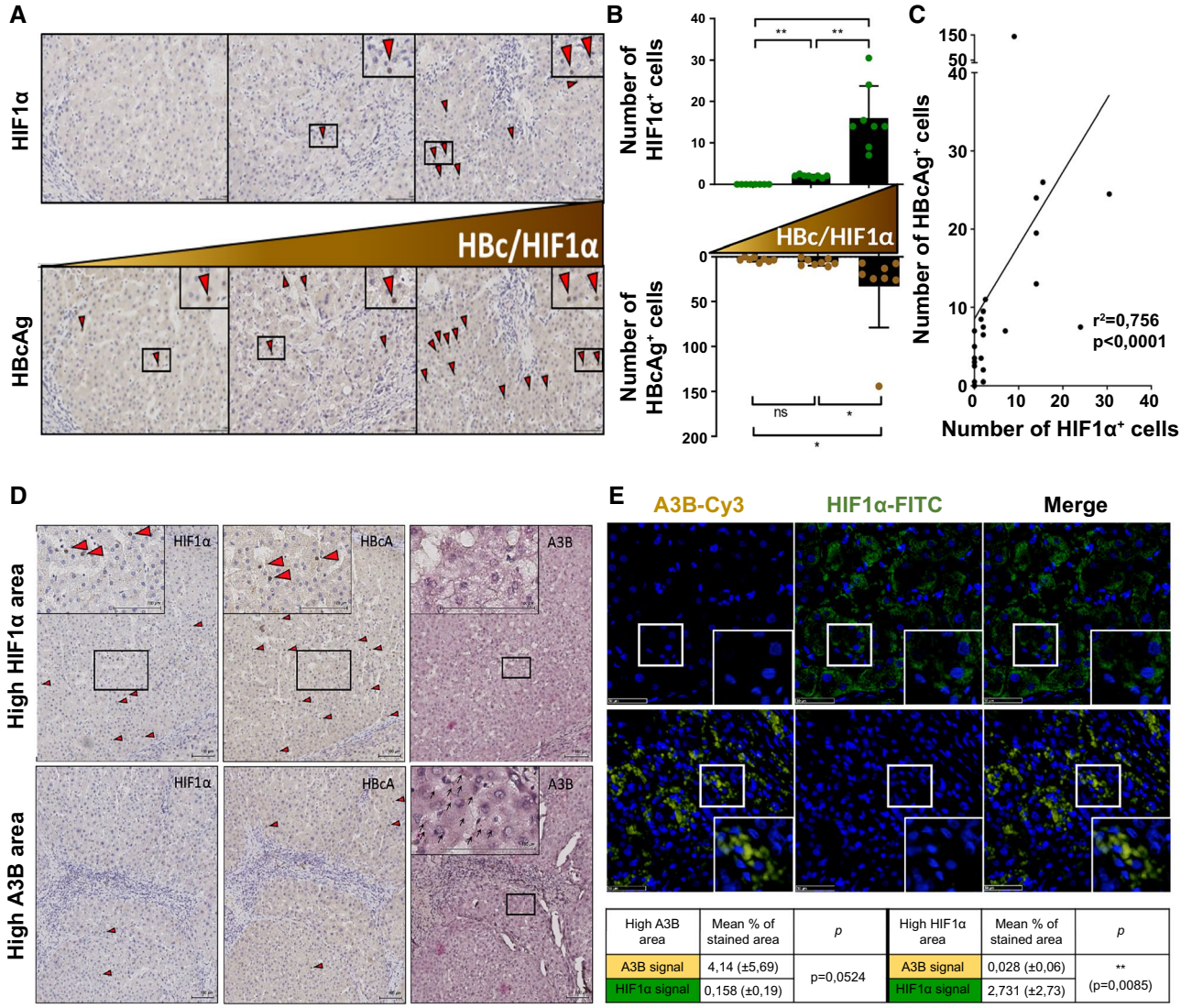
Hypoxia has been shown to strongly modulate immune responses, both positively and negatively, depending on the cells and the immune mechanisms involved.[14] Inflammatory cytokines and/or ligands have been shown to efficiently inhibit HBV infection.[3,18,19] Thus, we wanted to decipher whether HIF1α might be involved in HBV persistence in chronically infected patients by preventing immune activation. Consecutive cuts of livers from CHB patients with end-stage CHB, also considered as an immune-active phase, were stained for HIF1α and HBcAg. Highly oxygenated/low inflammation zones, highlighted by an absence of HIF1α staining, were also low for HBcAg staining in these CHB patients (Fig. 1A,B). In contrast, zones with low oxygen level or with inflammation (i.e., strong HIF1α staining) presented an increased number of HBcAg-positive nuclei. A correlation was found between the numbers of HIF1α- and HBcAg-positive cells (Fig. 1C).

We have previously shown that, on the one hand, LTβR agonization by an agonistic antibody (BS1) leads to cccDNA decay and HBV clearance, whereas, on the other hand, LTα/β are up-regulated in CHB patients.[6,20] Therefore, induction of LTβ in CHB patients should clear the infection given its antiviral effect. To assess whether the correlation of HIF1α and HBc observed *in vivo* (Fig. 1C) could be attributable to lower immune response in this area, liver of CHB patients were either stained for HIF1α and A3B by *in situ* mRNA hybridization on consecutive slides, or by costaining of mRNA and protein. High HIF1α staining was found in areas with low A3B expression, whereas low HIF1α staining was found in areas with strong A3B expression (Fig. 1D,E and Supporting Fig. S1).

Altogether, these data highlight that in areas with high HIF1α stabilization, A3B expression is impaired, allowing viral persistence even during liver inflammation. Therefore, high HIF1α areas provide a reservoir for HBV persistence *in vivo*.

HIF1α STABILIZATION DECREASES ANTI-cccDNA PROPERTIES OF LTβR AGONISATION

To confirm our findings *in vitro*, we used several HIF1α stabilizing conditions, namely hypoxia (canonical HIF1α stabilizer and inducer; i.e., 1% oxygen), dimethyloxallyl glycin (DMOG), or roxadustat (FG-4592; two molecules described to stabilize HIF1α through the inhibition of proline hydroxylases, enzymes that, if active, hydroxylate HIF-αs in the presence of oxygen to address it for degradation). A schematic representation of the experiment timeline is presented in Fig. 2A. Treatment with BS1 induced A3B, leading to cccDNA decrease, as described (Fig. 2B-G, siCtrl NO/BS1 or siCtrl DMSO/BS1). Upon HIF1α stabilization, A3B induction was decreased, impairing its antiviral effects on cccDNA (Fig. 2B-G, siCtrl HO/BS1, siCtrl DMOG/BS1, or siCtrl FG-4592/BS1). A3B induction and anti-cccDNA activity was partially rescued by HIF1α knockdown (Fig. 2B-G, siRNA HIF1α [siHIF1α] HO/BS1, siHIF1α DMOG/BS1, or siHIF1α FG-4592/BS1). BS1-induced decrease of cccDNA quantity and impairment thereof by DMOG treatment was also confirmed by Southern blotting analysis (Fig. 2H). Of note, HIF1α knockdown under normoxia was sufficient to (1) increase A3B mRNA levels and (2) decrease cccDNA levels as compared to siCtrl (Fig. 2A,B). This effect was attributable



High A3B area	Mean % of stained area	p	High HIF1α area	Mean % of stained area	p
A3B signal	4,14 (±5,69)	p=0,0524	A3B signal	0,028 (±0,06)	** (p=0,0085)
HIF1α signal	0,158 (±0,19)		HIF1α signal	2,731 (±2,73)	

FIG. 1. HIF1α stabilization allows HBV persistence *in vivo*. (A-E) Paraffin sections of CHB patients were consecutively cut and stained for HIF1α, HBcAg, or APOBEC3B mRNA *in situ* or costained for HIF1α and APOBEC3B mRNA *in situ*. (A,B) Regions were classified in three types: (1) no HIF1α-positive cells; (2) one to five HIF1α-positive cells; and (iii) greater than five HIF1α-positive cells. Arrowheads show positive nuclei. (A) Representative pictures of the three zones of HIF1α (upper panels) and HBcAg (lower panels) from the same patient. (B) Quantification of the number of HIF1α- and HBcAg-positive cells in the three different zones. Every data point represents the mean of two view fields, and the bars represent the mean ± SD of 8 patients. (C) Correlation between HIF1α and HBcAg positivity per view field. (D) Representative pictures of patients stained for A3B. Upper three pictures show a representative HIF1α-high area, and the lower three pictures show an A3B-high area of the same patient sample. (E) Representative images of a patient stained for HIF1α and A3B. Upper three pictures show a representative HIF1α-high area, and the lower three pictures show an A3B-high area of the same patient sample. Percentage of stained area for A3B and HIF1α was quantified and is presented in the table ± SD of 9 different patients. Data were submitted to (A) Pearson's correlation analysis and (E) one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations: Cy3, cyanine 3; FITC, fluorescein isothiocyanate.

to BS1-induced HIF1α stabilization, as confirmed by immunoprecipitation of HIF1α under normoxia-BS1 conditions (Supporting Fig. S2A). Like A3B, the up-regulation of nuclear factor kappa B subunit 2 (NF-κB2), a NF-κB target gene, was attenuated in cells upon HIF1α stabilization, which was rescued by HIF1α knockdown (Supporting Fig. S2B-D). Carbonic anhydrase IX, a direct target gene of HIF1α, was up-regulated upon HIF1α stabilization and showed a strong reduction when HIF1α was depleted

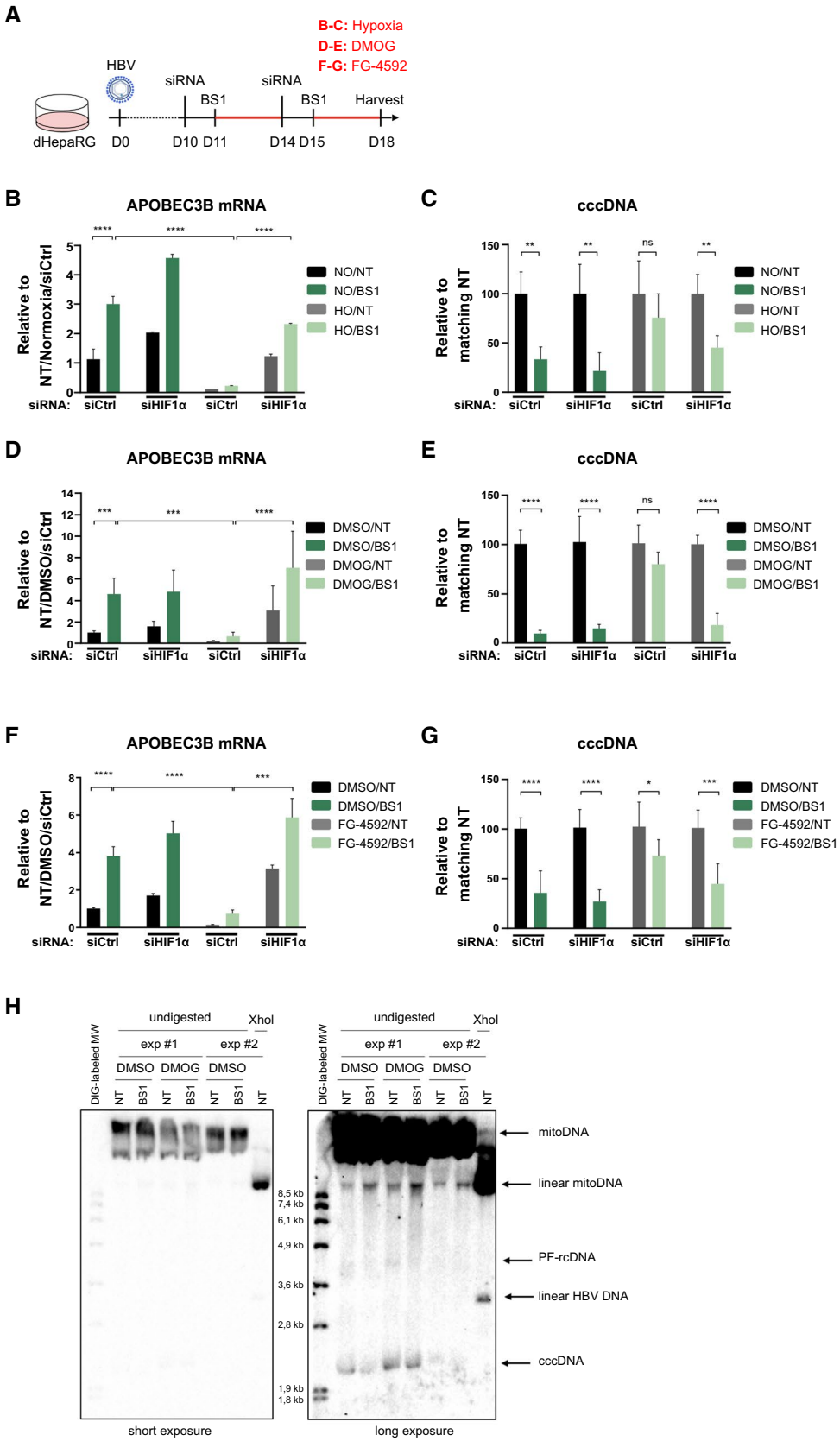

A
B-C: Hypoxia
D-E: DMOG
F-G: FG-4592
HBV
siRNA
BS1
siRNA
BS1
Harvest
dHepaRG
D0
D10 D11
D14 D15
D18
B
APOBEC3B mRNA
Relative to NT/Normoxia/siCtrl
NO/NT
NO/BS1
HO/NT
HO/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
C
cccDNA
Relative to matching NT
NO/NT
NO/BS1
HO/NT
HO/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
D
APOBEC3B mRNA
Relative to NT/DMSO/siCtrl
DMSO/NT
DMSO/BS1
DMOG/NT
DMOG/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
E
cccDNA
Relative to matching NT
DMSO/NT
DMSO/BS1
DMOG/NT
DMOG/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
F
APOBEC3B mRNA
Relative to NT/DMSO/siCtrl
DMSO/NT
DMSO/BS1
FG-4592/NT
FG-4592/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
G
cccDNA
Relative to matching NT
DMSO/NT
DMSO/BS1
FG-4592/NT
FG-4592/BS1
siRNA: siCtrl siHIF1α siCtrl siHIF1α
H
undigested
XhoI
exp #1
exp #2
DMSO DMOG DMSO
DIG-labeled MW
NT BS1 NT BS1 NT BS1 NT
8,5 kb
7,4 kb
6,1 kb
4,9 kb
3,6 kb
2,8 kb
1,9 kb
1,8 kb
mitoDNA
linear mitoDNA
PF-rcDNA
linear HBV DNA
cccDNA
short exposure
long exposure

FIG. 2. HIF1α stabilization prevents the antiviral effects of APOBEC3B *in vitro*. (A) Schematic representation of the experiments. (B,C) dHepaRG cells were infected with HBV. Six d.p.i., cells were transfected with either 10 nM of HIF1α-targeting or control siRNAs. On the next day, cells were subjected to 1% or 20% oxygen for 3 days and treated with ±0.5 μg/mL of BS1. Transfection and treatments were repeated once. (D,E) dHepaRG cells were infected with HBV. At 10 and 13 d.p.i., cells were transfected with either 10 nM of HIF1α-targeting or control siRNAs. Cells were then treated with ±0.5 μg/mL of BS1 and with ±100 μM of DMOG. (F,G) dHepaRG cells were infected with HBV. At 10 and 13 d.p.i., cells were transfected with either 10 nM of HIF1α-targeting or control siRNAs. One day after the second transfection, cells were treated or not with 0.5 μg/mL of BS1, either under the presence of 30 μM of FG-4592 or DMSO. Six days later, (B,D,F) mRNAs and (C,E,G) DNA were extracted and analyzed by RT-qPCR and qPCR. Bars represent the mean ± SD of (B,C) one or (D-G) three independent experiments performed in quadruplicates. Data were submitted to (C,E,G) an unpaired Student *t* test or (B,D,F) one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.0001$. (H) dHepaRG cells were infected with HBV. At 10 d.p.i., cells were treated with ±0.5 μg/mL of BS1 and with ±100 μM of DMOG for 12 days. Episomal DNA was extracted and analyzed by Southern blotting. Abbreviations: DIG, digoxigenin; d.p.i., days postinfection; mitoDNA, mitochondrial DNA; MW, molecular weight; NT, nontreated; PF, protein-free; rcDNA, relaxed circular DNA.

(Supporting Fig. S2B-D). LTβR mRNA expression was slightly reduced under hypoxia, which could be rescued by HIF1α knockdown and was unchanged by DMOG or FG-4592 treatments (Supporting Fig. S2B-D). Of note, HIF1α knockdown was confirmed by immunoblotting (Supporting Fig. S2B-D). Notably, cccDNA degradation induced by other treatments (e.g., IFNα [Roferon], IFNγ, or TNFα) was also prevented by HIF1α stabilization induced by DMOG (Supporting Fig. S2E).

Altogether, these data highlight that HIF1α stabilization impairs the up-regulation of A3B and anti-cccDNA activity of BS1 treatment, which can be efficiently rescued by HIF1α depletion.

HIF1α, BUT NOT HIF2α, IS INVOLVED IN HYPOXIA-MEDIATED APOBEC3B REPRESSION

Hypoxia can induce the stabilization of both HIF1α and HIF2α. Although we show that HIF1α knockdown can rescue A3B expression and antiviral effects of BS1 under HIF-stabilizing conditions (Fig. 2), we aimed to investigate a potential additional role of HIF2α. Therefore, cell lines doxycycline inducible for the overexpression of wild-type HIF1α, degradation-resistant HIF1α, or wild-type HIF2α were generated. Of note, only a degradation-resistant HIF1α (carrying a P402A and a P564A mutation, eliminating the sites that, when hydroxylated, target HIF1α for degradation) was detected in the overexpressing cell line (Supporting Fig. S3A). Consequently, subsequent experiments were only performed with the degradation-resistant HIF1α. Transcriptional activity and expression of mutated HIF1α and HIF2α were confirmed by RT-qPCR and immunoblotting, respectively (Supporting Fig. S3A-D). Overexpression of HIF1α or HIF2α alone inhibited A3B up-regulation induced by BS1 (Fig. 3A). However, under hypoxia, only siRNAs against HIF1α, but not HIF2α, rescued A3B up-regulation, and no cumulative effect was observed when knocking down both HIF1α and HIF2α, highlighting that HIF2α only plays a minor role in A3B inhibition under hypoxic conditions (Fig. 3B). HIF1α and HIF2α knock-down efficiencies were confirmed by RT-qPCR (Supporting Fig. S3E). Moreover, inhibition of A3B by HIF1α and rescue by HIF1α knockdown were confirmed using different HIF1α stabilizers (DMOG, $CoCl_2$, and VH298; Fig. 3C,D and Supporting S3F). Of note, LTβR surface expression remained unchanged under hypoxia, with a mild increase after HIF1α knockdown, highlighting that the effect of HIF1α stabilization was not attributable to a decreased receptor expression (Supporting Fig. S4G,H). Moreover, A3B repression was not attributable to cell death under hypoxia (Supporting Fig. S3I).

Altogether, these data show that under hypoxic conditions, HIF1α—but not HIF2α—impairs the induction of A3B.

HIF1α STABILIZATION INHIBITS NF-κB-INDUCED A3B TRANSCRIPTION BY DECREASING RelB PROTEIN EXPRESSION LEVEL

The main signaling pathways activated upon LTβR agonization are related to NF-κB, suggesting that A3B is an NF-κB target gene. To confirm this hypothesis, we used two kinase inhibitors ([N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2-methylnicotinamide]

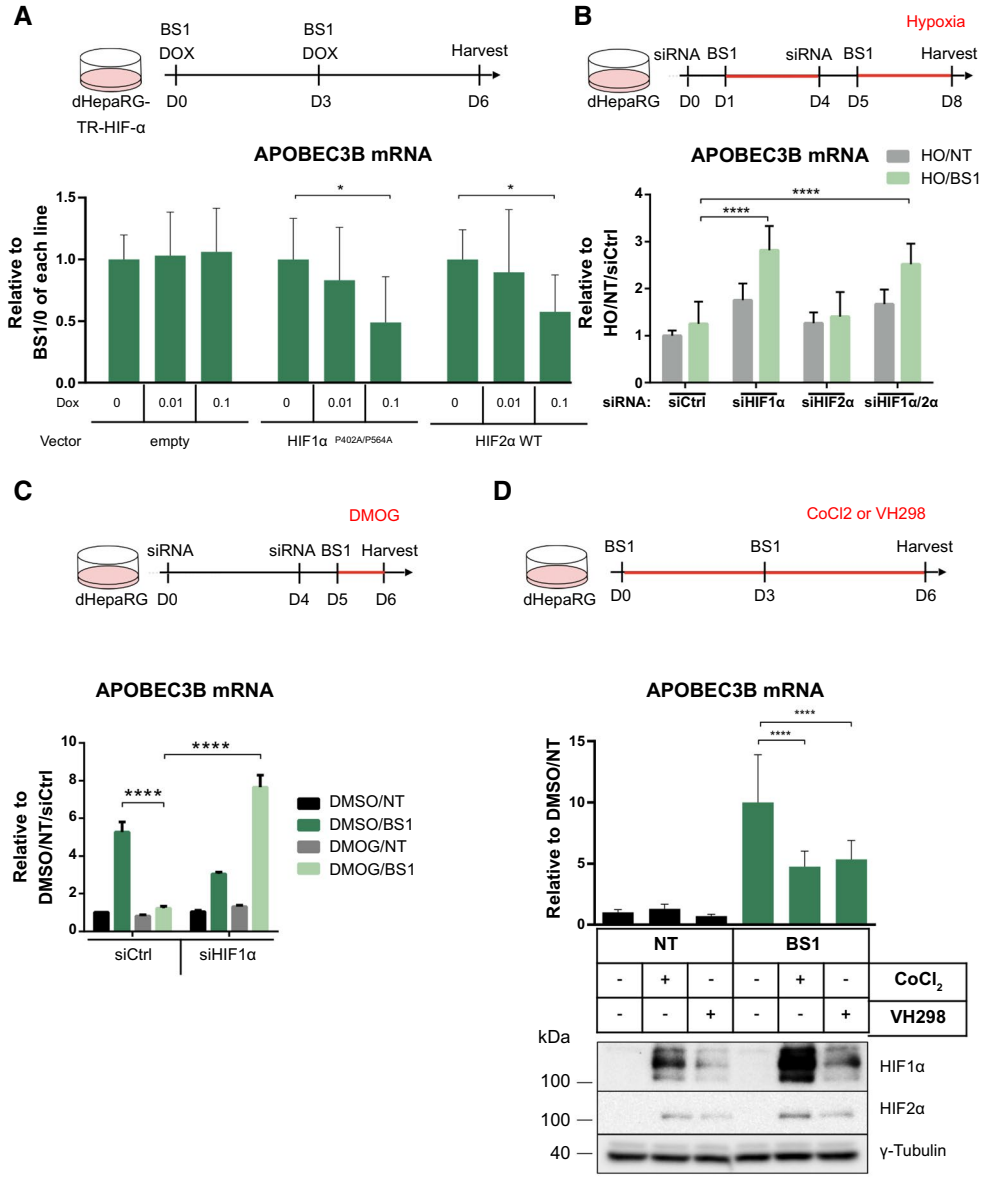


FIG. 3. HIF1α, but not HIF2α, stabilization inhibits APOBEC3s. (A-D) Schematic representation of the experiments. (A) Inducible dHepaRG cells overexpressing the HIF1α degradation-resistant mutant, P402A/P564A, or HIF2α treated for 3 days with an increasing dose of doxycycline in the presence of 0.5 μg/mL of BS1. (B) dHepaRG cells were transfected with 10 nM of either HIF1α-targeting, HIF2α-targeting, or both siRNAs or control siRNAs. The next day, cells were treated with ±0.5 μg/mL of BS1 under 1% oxygen. mRNAs were extracted and analyzed by RT-qPCR. (C) dHepaRG cells were transfected with either 10 nM of HIF1α-targeting or control siRNAs. One day after the second transfection, cells were treated or not, for 24 hours, with 0.5 μg/mL of BS1, either under the presence of 100 μM of DMOG or DMSO. mRNAs were analyzed by RT-qPCR. Bars represent the mean ± SD of three independent experiments performed in triplicates. (D) dHepaRG cells were incubated for 3 days with ±100 μM of $CoCl_2$ or VH298 in the presence or absence of 0.5 μg/mL of BS1. mRNAs and proteins were extracted and analyzed by RT-qPCR and immunoblotting with the indicated antibodies, respectively. (A-D) Data represent the mean ± SD of three independent experiments performed in triplicates. Data were submitted to one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Abbreviations: DOX, doxycycline; NT, nontreated.

and [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide) that target the IKK complex (IKKα/β). We observed that inhibition of IKKα/β reduces BS1-induced A3B in dHepaRG cells (Supporting Fig. S4A). Given that we showed that HIF1α stabilization prevents BS1-induced A3B, we anticipated that

HIF1α would inhibit NF-κB target genes. Indeed, the induction of the well-known NF-κB target genes, *nfkb2* and *nik*, upon BS1 treatment in normoxia is highly reduced in hypoxic conditions, and this effect was confirmed for A3B (Supporting Fig. S4B-D). We also extended our analysis with other activators of NF-κB (TNFα, IL-17, and LPS) and observed the same trend on the tested NF-κB target genes.

Therefore, our results indicate a hypoxia-related impairment of the NF-κB signaling pathways. Interestingly, RelB is at the crossroad of both NF-κB pathways; *relb* transcription is dependent on the canonical, whereas RelB protein is part of the noncanonical, NF-κB dimer, p52/RelB.[10] We confirmed that, whereas BS1 increased RelB protein expression and A3B transcription, depletion of RelB drastically reduces BS1-induced A3B expression (Supporting Fig. S5B,C). Therefore, we addressed whether the inhibitory effect of HIF1α stabilization on BS1-induced A3B up-regulation was a consequence of RelB inactivation.

Cell fractionation highlighted that DMOG strongly reduces BS1-induced RelB protein in both the cytosolic and the nuclear compartments, whereas RelA expression and nuclear translocation were not strongly affected (Fig. 4A). More important, the decrease of RelB protein levels in the DMOG/BS1 condition was completely rescued in HIF1α-depleted cells (Fig. 4B). HIF1α stabilization did not repress BS1-induced RelB mRNA up-regulation (Fig. 4C). These results were confirmed using longer DMOG treatment, a different level of hypoxia, and other HIF1α stabilizers (Supporting Fig. S5D-G). By immunostaining, we also confirmed that RelA nuclear translocation remained unchanged under hypoxia (Supporting Fig. S5H,I), whereas hypoxia impaired RelB induction (Fig. 4D). Interestingly, hypoxia also prevented BS1-induced p52 (the main binding partner of RelB) recruitment to the A3B promoter (Fig. 4E).

To investigate whether our *in vitro* findings would also be of relevance *in vivo*, C57BL6/J mice were injected either with DMSO or DMOG and euthanized 6 hours postinjection. *In vivo*, DMOG triggered HIF1α stabilization and a strong reduction of RelB protein expression in the liver, without affecting RelB mRNA. No change was observed for RelA or p50 (Fig. 4F).

Altogether, our *in vitro* and *in vivo* results identified a strong reduction of RelB protein, but not mRNA expression, as the main driver of HIF1α-induced impairment of A3B expression.

HIF1α-MEDIATED INHIBITION OF RelB/A3B EXPRESSION IS INDEPENDENT OF ITS TRANSCRIPTIONAL ACTIVITY

HIF1α belongs to a large family of proteins, including ARNT and AhR.[21] It has been reported that RelB can dimerize with AhR or ARNT (RelB/AhR or RelB/ARNT), either controlling RelB protein stability and/or RelB transcriptional activity.[22,23] Moreover, crosstalks between these proteins can occur through competition for common partners (e.g., HIF1α/ARNT vs. AhR/ARNT).[24] Thus, we investigated whether such processes could control RelB activity in our model. A schematic timeline of the experiments is depicted in Fig. 5A.

In dHepaRG cells, AhR knockdown did not interfere with BS1-induced RelB expression, highlighting that AhR was dispensable for RelB stability (Fig. 5B). Interestingly, contrary to HIF1α knockdown, RelB protein levels were not rescued in ARNT-depleted cells treated with DMOG/BS1 (Fig. 5C). It was reported that ARNT represses the transcription of particular NF-κB target genes,[23] as confirmed by the elevated expression of C-X-C motif chemokine ligand 10 in ARNT-depleted cells (Supporting Fig. S6A). However, ARNT knockdown had no impact on RelB mRNA expression, whereas vascular endothelial growth factor alpha expression (a target gene of the HIF1α/ARNT heterodimer) was reduced (Supporting Fig. S6B,C). In addition, neither AhR nor ARNT knockdown rescued A3B levels in DMOG-treated cells (Fig. 5D,E). These results indicate that HIF1α/ARNT dimerization, which is necessary for the canonical function of HIF1α as a transcription factor, is not the cause of decreased RelB protein and A3B mRNA expression.

In summary, our results demonstrate that HIF1α/RelB crosstalk prevents BS1-mediated A3B expression through an unconventional HIF1α-dependent mechanism.

HYPOXIA PREVENTS IMMUNE INDUCTION BY DYSREGULATING EXECUTING PATHWAYS

To investigate the global effect of hypoxia, mass spectrometry was performed on control or HIF1α-targeting siRNA-transfected dHepaRG cells treated

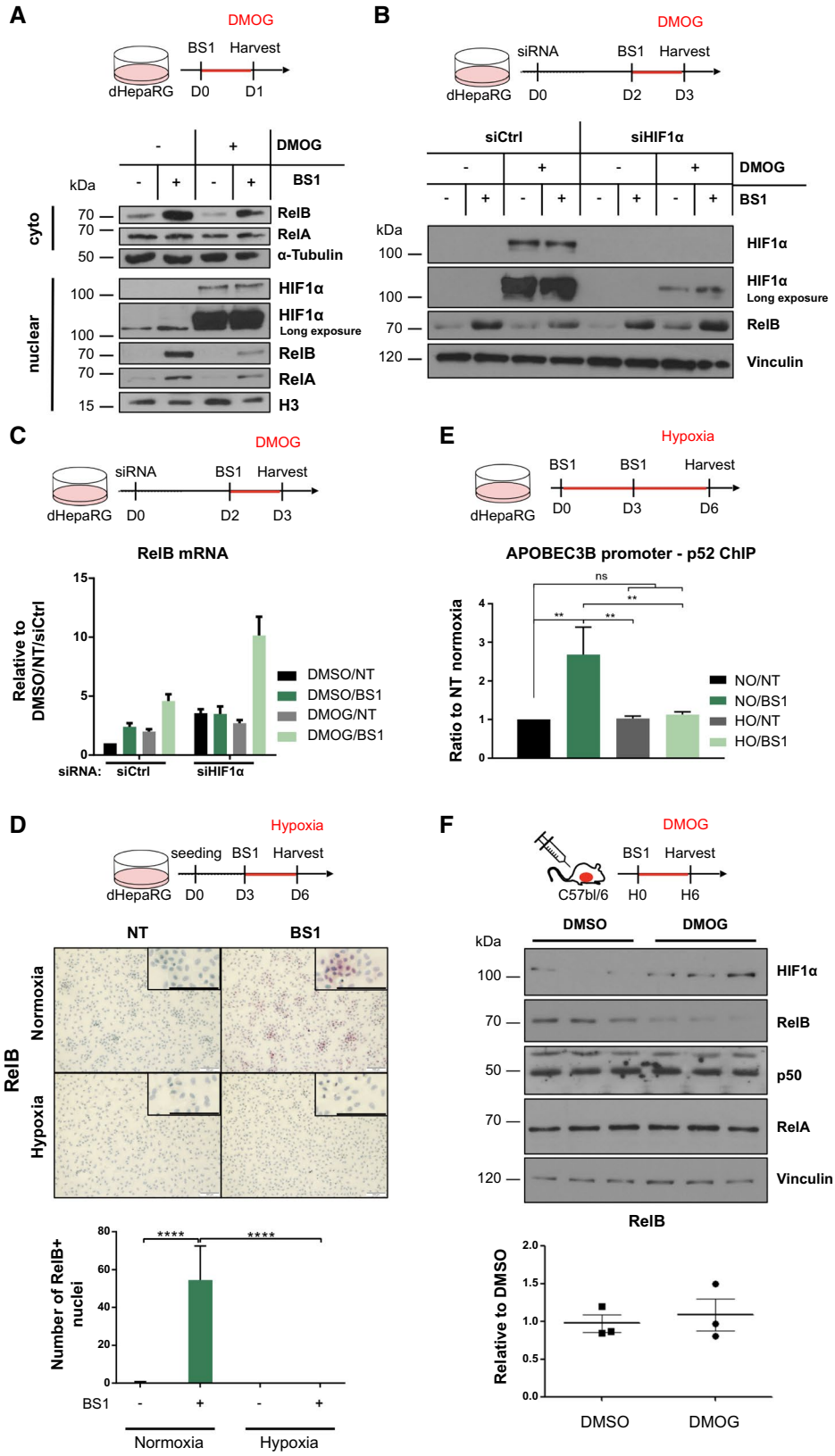

A
DMOG
BS1 Harvest
dHepaRG D0 D1
DMOG
BS1
kDa
cyto
nuclear
RelB
RelA
α-Tubulin
HIF1α
HIF1α Long exposure
RelB
RelA
H3
B
DMOG
siRNA BS1 Harvest
dHepaRG D0 D2 D3
siCtrl
siHIF1α
DMOG
BS1
kDa
HIF1α
HIF1α Long exposure
RelB
Vinculin
C
DMOG
siRNA BS1 Harvest
dHepaRG D0 D2 D3
RelB mRNA
Relative to DMSO/NT/siCtrl
DMSO/NT
DMSO/BS1
DMOG/NT
DMOG/BS1
siRNA: siCtrl siHIF1α
E
Hypoxia
BS1 BS1 Harvest
dHepaRG D0 D3 D6
APOBEC3B promoter - p52 ChIP
Ratio to NT normoxia
ns
NO/NT
NO/BS1
HO/NT
HO/BS1
D
Hypoxia
seeding BS1 Harvest
dHepaRG D0 D3 D6
NT
BS1
RelB
Normoxia
Hypoxia
Number of RelB+ nuclei
BS1
Normoxia
Hypoxia
F
DMOG
BS1 Harvest
C57bl/6 H0 H6
DMSO
DMOG
kDa
HIF1α
RelB
p50
RelA
Vinculin
RelB
Relative to DMSO
DMSO
DMOG

FIG. 4. HIF1α stabilization decreases RelB level *in vitro* and *in vivo*. (A-F) Schematic representation of the experiments. (A) dHepaRG cells were treated for 24 hours with DMSO or 100 μM of DMOG ± 0.5 μg/mL of BS1. Cytoplasm and nuclei were separated. (B,C) dHepaRG cells transfected with either 10 nM of HIF1α-targeting siRNAs or control siRNAs. Two days after transfection, cells were treated for 24 hours with DMSO or 100 μM of DMOG ± 0.5 μg/mL of BS1 for 24 hours. (D) dHepaRG cells were seeded into four-well chamber slides. Three days after seeding, cells were cultured under either 1% (Hypoxia) or 20% (Normoxia) oxygen for 3 days, either in the presence or absence of 0.5 μg/mL of BS1. Cells were then prepared for immunocytochemistry and stained for RelB. Representative pictures and quantification of RelB-positive nuclei. Data represent the mean of five pictures per condition of two experiments. (E) dHepaRG cells were cultured under 1% or 20% oxygen ± 0.5 μg/mL of BS1. Six days posttreatment, protein and nucleic acids were cross-linked and submitted to ChIP. DNA was extracted, and binding of p52 to APOBEC3B promoter was analyzed by qPCR. (F) Mice were injected i.p. with 300 mg/kg of DMOG or the equal amount of DMSO for 6 hours. (A,B,F) Proteins were analyzed by immunoblotting. (C,F) mRNAs were analyzed by RT-qPCR. Bars represent the mean ± SD of (C,R) three independent experiments. Data were submitted to (D,E) one-way ANOVA. **P* < 0.05; ***P* < 0.01; *****P* < 0.0001. Abbreviations: ChIP, chromatin immunoprecipitation; NT, nontreated.

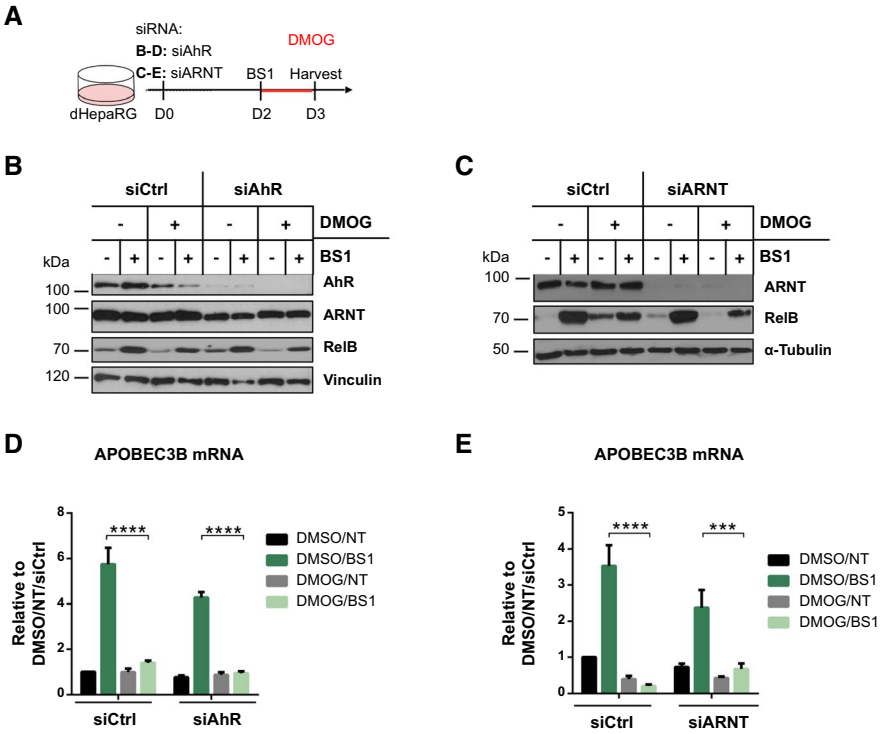


FIG. 5. ARNT knockdown does not rescue RelB and A3B level. (A) Schematic representation of the experiment. (B-E) dHepaRG cells were transfected with either 10 nM of AhR-targeting (siAhR), ARNT-targeting (siARNT), or control siRNAs (siCtrl). Two days after transfection, cells were treated for 24 hours with DMSO or 100 μM of DMOG ± 0.5 μg/mL of BS1. (B,C) Proteins were analysed by immunoblotting. (D, E) mRNAs were analysed by RT-qPCR. Bars represent the mean ± SD of (D, E) three independent experiments. Data were submitted to (D, E) one-way ANOVA. ****P* < 0.001; *****P* < 0.0001. Abbreviation: NT, nontreated.

with or without BS1 under normoxia (NO) or hypoxia (HO). A schematic timeline of the experiment is depicted in Fig. 6A. Interestingly, whereas 418 proteins were significantly dysregulated in BS1-treated versus nontreated cells under normoxia (NO/NT vs. NO/BS1), only two proteins were found to be dysregulated when comparing the same treatments under hypoxia (HO/NT vs. HO/BS1), indicating a global inhibition of responses to BS1 treatment (Fig. 6B). Pathways were grouped into four different clusters: I, transcription and translation; II, signal transduction and immune response; III, metabolism; and IV, DNA replication and repair. Results highlighted that BS1 treatment impaired the metabolism (e.g., drug and fatty acid metabolism) of dHepaRG cells and cellular transcriptional and translational machinery were among the most up-regulated pathways, leading to production of immune response pathway effectors (Fig. 6C).

Additional pathway analyses were conducted for the following comparisons: nontreated normoxia, siRNA control-transfected versus BS1-treated normoxia, siRNA control-transfected (NO/NT/siCtrl vs. NO/BS1/siCtrl); nontreated normoxia, siRNA control-transfected versus BS1-treated hypoxia, siRNA control-transfected (NO/NT/siCtrl vs. HO/BS1/siCtrl); nontreated hypoxia, siRNA control-transfected versus BS1-treated hypoxia, siHIF1α-transfected (NO/NT/siCtrl vs. HO/BS1/siHIF1α).

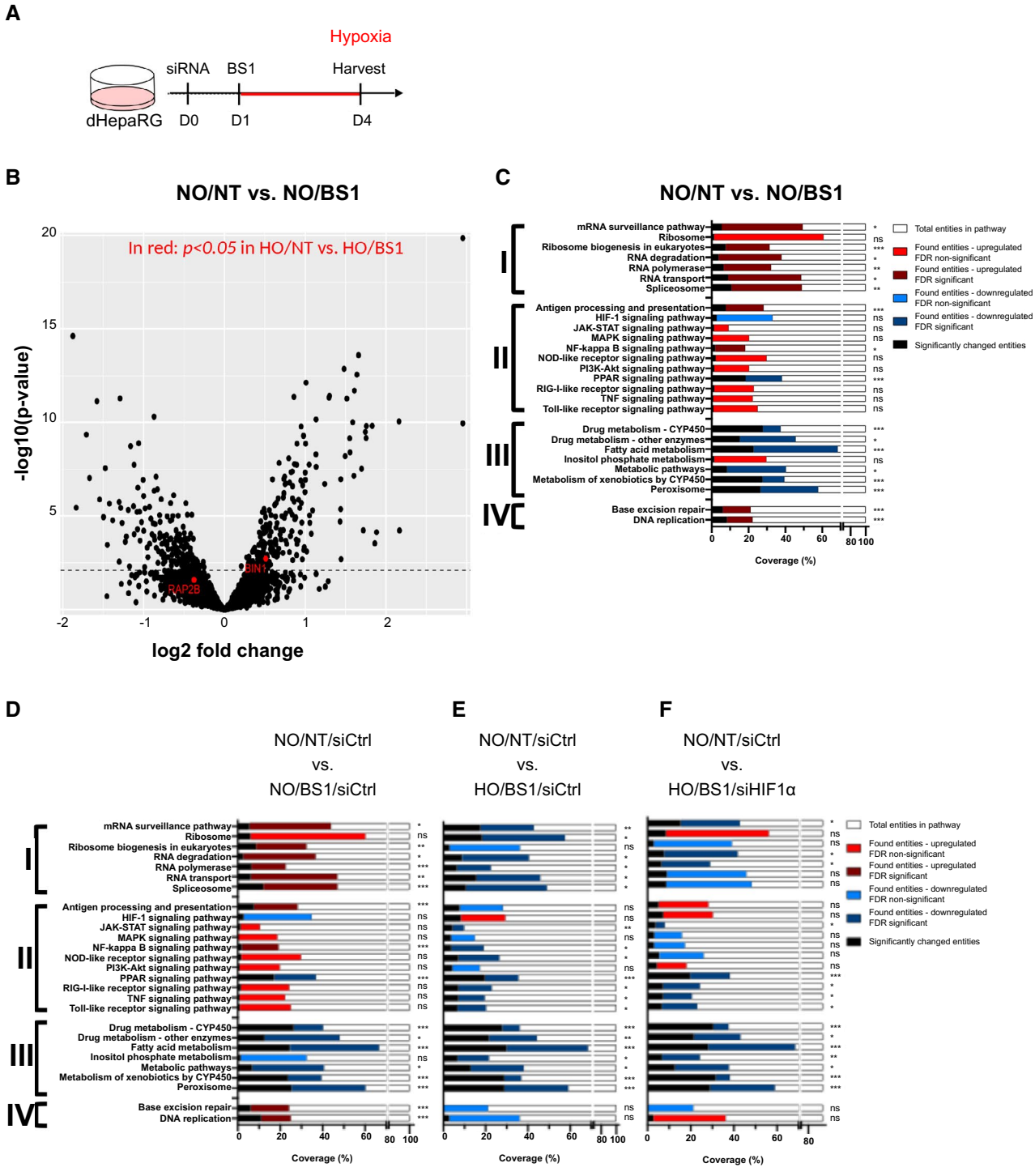


FIG. 6. HIF1α knockdown rescues mRNA-processing and ribosomes pathways. (A) Schematic representation of the experiment. (B-F) dHepaRG were (B,C) either left untransfected or (D-F) transfected with either 10 nM of HIF1α-targeting or control siRNAs. On the next day, cells were subjected to 1% (Hypoxia) or 20% (Normoxia) oxygen for 3 days ± 0.5 μg/mL of BS1. Proteins were submitted to unbiased mass spectrometry analysis. (B) Data are presented as volcano plot of normoxia nontreated (NO/NT) versus normoxia BS1-treated (NO/BS1) comparison. Dotted line represents the limit of significance (adjusted *P* value, <0.05). Red dots represent the only two proteins that are still significantly dysregulated (i.e., adjusted *P* value, <0.05) in similar comparison under hypoxia (HO/NT vs. HO/BS1). (C-F) Pathway analysis of significantly changed proteins was conducted with preselected KEGG pathways using the ROAST algorithm. The pathways are represented for (C) NO/NT versus NO/BS1, (D) NO/NT/siCtrl versus NO/BS1/siCtrl, (E) NO/BS1/siCtrl versus HO/BS1/siCtrl, and (F) HO/BS1/siCtrl versus HO/BS1/siHIF1α. The significantly (respectively, nonsignificant) up-regulated (dark red bar; respectively, light red bar) or down-regulated (dark blue bar; respectively, light blue bar) pathways are presented as the percentage of proteins analyzed in the pathways. Of note, black bars represent the number of significantly dysregulated proteins in the pathway. Data were submitted to a LIMMA algorithm for selection of significantly changed proteins. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Abbreviations: Akt, protein kinase B; CYP450, cytochrome P450; FDR, false discovery rate; JAK, Janus kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; NT, nontreated; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; RIG-I, retinoic-acid–inducible gene I; ROAST, rotation gene set testing; STAT, signal transducer and activator of transcription.

NO/NT/siCtrl versus NO/BS1/siCtrl comparison confirmed the results obtained in nontransfected conditions (Fig. 6D). However, the NO/NT/siCtrl versus HO/BS1/siCtrl comparison highlighted a significant down-regulation of pathways implicated in RNA transcription and translation (e.g., ribosome, mRNA surveillance), preventing the increase of immune response pathway effectors (Fig. 6E). While being up-regulated under NO/BS1 conditions (Fig. 6D), the NF-kB signaling pathway was down-regulated under hypoxia (Fig. 6E). Interestingly, NO/NT/siCtrl versus HO/BS1/siHIF1α comparison showed a partial rescue of some of these pathways upon HIF1α knockdown, namely RNA processing (i.e., Spliceosome) and transport, as well as NF-κB- and NOD-like receptor-signaling pathways (Fig. 6F). Importantly, the ribosome pathway returned to a level similar to normoxia upon HIF1α knockdown (Fig. 6F). Surprisingly, several metabolisms (i.e., drug, fatty acid, and xenobiotics metabolism) were similarly impaired by BS1 treatment under hypoxia and normoxia.

Altogether, these data showed that hypoxia globally impaired immune responses by inhibiting cellular pathways implicated in RNA processing and surveillance, as well as protein production, independently of the target gene or the stimulus. Interestingly, HIF1α knockdown rescued A3B induction, most probably by rescuing RNA processing and ribosome pathways, although it was not sufficient to completely revert the hypoxic state of the cells.

Discussion

Development of new therapeutics against HBV have largely focused on the use of immune mediators, given that they have shown promising results both *in vitro* and *in vivo*.[3–5] We and others have previously shown that immune-mediated induction of A3B by LTβR agonization (i.e., with the LTβR agonist, BS1, or $LT\alpha_1\beta_2$-expressing T cells) leads to noncytolytic degradation of nuclear HBV cccDNA, enabling long-term inhibition of HBV replication without rebound, even after treatment arrest.[6,7]

HIF1α has been shown to impair immune responses.[13,25] Inflammatory signaling has been shown to induce HIF1α, which we confirmed in our current study. Moreover, HBV pathogenesis and resulting fibrotic scaring processes will influence liver oxygenation, therefore modulation of HIF1α induction and stabilization. In the liver of CHB patients in immune-active (i.e., patients who potentially could clear the infection given that they likely express high levels of cytokines), we found a positive correlation between HIF1α expression and HBcAg-positive areas. Given that A3B mRNA was low in areas with high HIF1α, it can be expected that, *in vivo*, HBV might escape the immune responses in areas with elevated HIF1α staining.

We hypothesized that the correlation observed between HIF1α, HBcAg, and A3B mRNA highlights that low immune responses in HIF1α-high areas allow viral persistence, creating a viral reservoir. Therefore, we can hypothesize that blocking HIF1α stabilization during the immune-active phase of CHB patients could indeed be sufficient to allow more-potent immune responses, among which is induction of A3B, and viral elimination.

In vitro, we confirmed, using 1% oxygen, DMOG, and a number of other molecules inducing HIF1α stabilization, as well as HIF1α-overexpressing cell

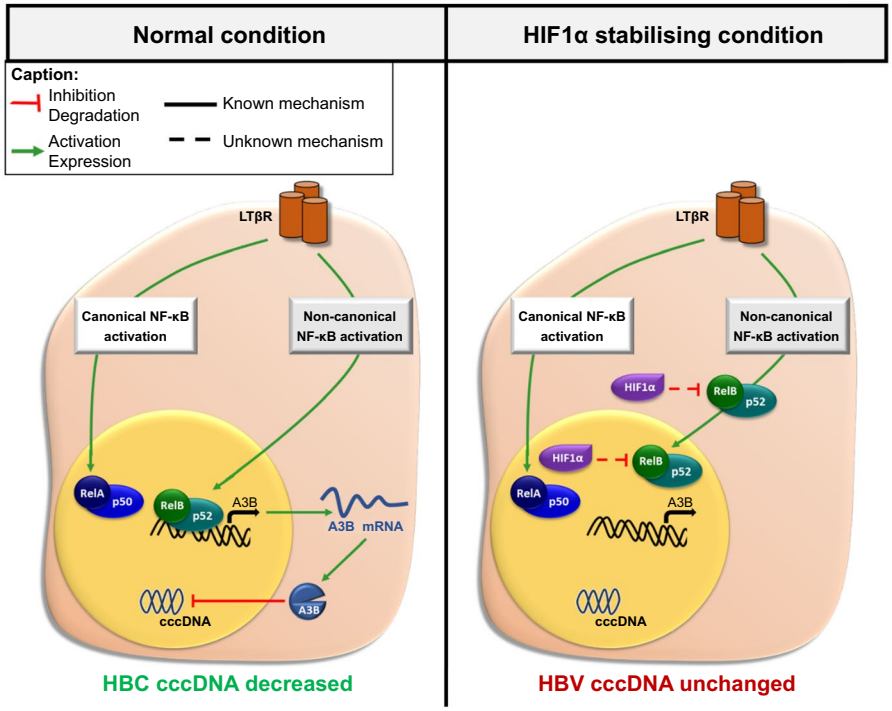


FIG. 7. HIF1α stabilization prevents APOBEC3B-mediated anti-cccDNA effect by decreasing RelB protein. Graphical representation of the main proposed mechanism. Briefly, HIF1α stabilization under hypoxia or stabilizing molecule treatment decreases RelB protein levels, but not its mRNA. The decrease of RelB protein prevents the induction of APOBEC3B by LTβR agonization and, subsequently, cccDNA decay.

lines, that HIF1α stabilization mediates a strong impairment of LTβR-dependent A3B induction. However, impairment of immune responses was not limited to A3B as an NF-κB target gene, neither to BS1 as an NF-κB inducer, highlighting that HIF1α modulated NF-κB and other immune-signaling pathways (e.g., IFNα/γ-induced cccDNA degradation) to prevent the induction of immune mediators. Indeed, we identified that HIF1α impairs RelB protein, but not RelB mRNA level, *in vitro* and *in vivo*. This suggests that either RelB mRNA is not properly exported from the nucleus and/or is not efficiently translated, as confirmed by our proteomic data, which showed an impairment of RNA-processing and ribosome pathways under hypoxia. Alternatively, RelB stability is subjected to posttranslational modifications associated with proteasomal/lysosomal protein degradation.[26] We also found that the inhibitory activity of HIF1α toward RelB was independent of its partner, ARNT. An ARNT-independent function of HIF1α starts to emerge,[27] and the HIF1α/RelB crosstalk we discovered could bring more insights into the immune metabolism of the liver.

The global inhibition of immune responses observed under HIF1α stabilization, with different ligands and on several targets, suggests the need to modulate HIF1α to obtain optimal immune activation and thus an antiviral response during immune therapies administration. However, it will be important to confirm the effect of HIF1α on other immune therapies and antiviral targets, as well as *in vivo*, in a therapeutic setup. Mass spectrometry revealed that even though HIF1α knockdown partially rescued pathways implicated in RNA and protein production and processing, it could not fully reactivate the immune response in cells. Interestingly, although the rescue of the "hypoxic state" of the proteome was only partial, it was sufficient to rescue A3B induction and thereby restore the anti-cccDNA effects of BS1

treatment. From a clinical perspective, this could have severe consequences for the outcome of immune-stimulatory approaches for the treatment of CHB patients. The oxygen status of the liver microenvironment is not only important for parenchymal cells to be able to integrate external stimuli, but also for immune cells to exert their function properly.[14,25] Moreover, given that inflammation can trigger HIF1α stabilization, it will be mandatory to inhibit HIF1α to insure potent immune responses. Recently investigated HIF inhibitors have shown encouraging results in cancer therapies.[28] These molecules should be tested in the treatment of CHB, especially in patients with fibrosis, and thus with compromised liver oxygenation. In the context of immune-mediated A3B activation, a focus should be made on HIF1α inhibitors. Additionally, HIF1α inhibitors could be combined with immune therapies[3,5] to insure potent immune activation in the whole liver.

In summary, we have shown that HIF1α stabilization impairs NF-κB-mediated A3B induction, which is important for HBV cccDNA purging (Fig. 7). We believe that preventing the inhibitory activity of HIF1α toward RelB might represent a therapeutic window that should be considered as a support of combinatory immune therapies, to ensure a better efficacy of the treatment.

Acknowledgment: We thank the Genomics and Proteomics core facility and the Biostatistics core facility of the DKFZ for their support on the proteomics data set presented in this study. We thank Prof. Jeff Browning for generously sharing BS1 antibody with us. Tissue samples were provided by the tissue bank of the German Center for Infection Research (DZIF; Heidelberg, Germany).

Author Contributions: Conceptualization: T.R., S.F.D., M.R., D.D., J.L., E.D., M.H. Methodology: T.R., S.F.D., M.R., D.D., J.L., E.D., M.H. Formal analysis: T.R., S.F.D., M.R. Investigation: T.R., S.F.D., M.R., S.S., Z.H., S.C., X.Z., J.W., M.A.L., R.B., V.M., S.P., D.H., C.G., M.Sc., B.H., B.S., K.R., K.U., D.T., P.S., J.L. Resources: M.Sc., B.H., K.U., B.S., I.M.W., P.S., J.M., U.P., D.D., J.L., E.D., M.H. Data curation: T.R., S.F.D., M.R. Writing, original draft: T.R., S.F.D., D.D., J.L., E.D., M.H. Visualization: T.R., S.F.D., M.R., E.D., M.H. Supervision: E.D., M.H. Project administration: E.D., M.H. Funding acquisition: D.D., J.L., E.D., M.H.

REFERENCES

1) https://www.who.int/news-room/fact-sheets/detail/hepatitis-b. Accessed August 2021.
2) Fanning GC, Zoulim F, Hou J, Bertoletti A. Therapeutic strategies for hepatitis B virus infection: towards a cure. Nat Rev Drug Discov 2019;18:827-844.
3) **Lucifora J**, **Bonnin M**, Aillot L, Fusil F, Maadadi S, Dimier L, et al. Direct antiviral properties of TLR ligands against HBV replication in immune-competent hepatocytes. Sci Rep 2018;8:5390.
4) Du K, Liu J, Broering R, Zhang X, Yang D, Dittmer U, et al. Recent advances in the discovery and development of TLR ligands as novel therapeutics for chronic HBV and HIV infections. Expert Opin Drug Discov 2018;13:661-670.
5) Niu C, Li LI, Daffis S, Lucifora J, Bonnin M, Maadadi S, et al. Toll-like receptor 7 agonist GS-9620 induces prolonged inhibition of HBV via a type I interferon-dependent mechanism. J Hepatol 2018;68:922-931.
6) **Lucifora J**, **Xia Y**, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 2014;343:1221-1228.
7) Koh S, Kah J, Tham CYL, Yang N, Ceccarello E, Chia A, et al. Nonlytic lymphocytes engineered to express virus-specific T-cell receptors limit HBV infection by activating APOBEC3. Gastroenterology 2018;155:180-193.e6.
8) Covino DA, Gauzzi MC, Fantuzzi L. Understanding the regulation of APOBEC3 expression: current evidence and much to learn. J Leukoc Biol 2018;103:433-444.
9) **Wang D**, **Li X**, **Li J**, **Lu Y**, Zhao S, Tang X, et al. APOBEC3B interaction with PRC2 modulates microenvironment to promote HCC progression. Gut 2019;68:1846-1857.
10) Dejardin E. The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. Biochem Pharmacol 2006;72:1161-1179.
11) Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, et al. The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. Immunity 2002;17:525-535.
12) Mitchell S, Vargas J, Hoffmann A. Signaling via the NFκB system. Wiley Interdiscip Rev Syst Biol Med 2016;8:227-241.
13) D'Ignazio L, Bandarra D, Rocha S. NF-κB and HIF crosstalk in immune responses. FEBS J 2016;283:413-424.
14) Balamurugan K. HIF-1 at the crossroads of hypoxia, inflammation, and cancer. Int J Cancer 2016;138:1058-1066.
15) Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 2002;99:15655-15660.
16) **Lucifora J**, **Arzberger S**, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol 2011;55:996-1003.
17) Seitz S, Iancu C, Volz T, Mier W, Dandri M, Urban S, et al. A slow maturation process renders hepatitis B virus infectious. Cell Host Microbe 2016;20:25-35.
18) **Faure-Dupuy S**, **Delphin M**, Aillot L, Dimier L, Lebossé F, Fresquet J, et al. Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection. J Hepatol 2019;71:1086-1098.
19) Isorce N, Testoni B, Locatelli M, Fresquet J, Rivoire M, Luangsay S, et al. Antiviral activity of various interferons and pro-inflammatory cytokines in non-transformed cultured hepatocytes infected with hepatitis B virus. Antiviral Res 2016;130:36-45.
20) **Haybaeck J**, **Zeller N**, Wolf MJ, Weber A, Wagner U, Kurrer MO, et al. A lymphotoxin-driven pathway to hepatocellular carcinoma. Cancer Cell 2009;16:295-308.

21) Bersten DC, Sullivan AE, Peet DJ, Whitelaw ML. bHLH-PAS proteins in cancer. Nat Rev Cancer 2013;13:827-841.
22) Millet P, McCall C, Yoza B. RelB: an outlier in leukocyte biology. J Leukoc Biol 2013;94:941-951.
23) Wright CW, Duckett CS. The aryl hydrocarbon nuclear translocator alters CD30-mediated NF-kappaB-dependent transcription. Science 2009;323:251-255.
24) Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgård R, et al. Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. Mol Cell Biol 1996;16:5221-5231.
25) Palazon A, Goldrath AW, Nizet V, Johnson RS. HIF transcription factors, inflammation, and immunity. Immunity 2014;41:518-528.
26) Baud V, Collares D. Post-translational modifications of RelB NF-κB subunit and associated functions. Cells 2016;5:22.
27) Villa J, Chiu D, Brandes A, Escorcia F, Villa C, Maguire W, et al. Nontranscriptional role of Hif-1α in activation of γ-secretase and notch signaling in breast cancer. Cell Rep 2014;8:1077-1092.
28) Fallah J, Rini BI. HIF inhibitors: status of current clinical development. Curr Oncol Rep 2019;21:6.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31902/suppinfo.