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Article type : Original

HIF1α-mediated RelB/APOBEC3B downregulation allows Hepatitis B Virus persistence

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Key words: Hepatitis B virus, NF-κB, cccDNA, reservoir, HIF1α

Electronic word count: 5858

Number of Figures: 7

Number of Supplementary Figures: 6

Number of Supplementary Tables: 2

Conflict of interest statement: TR, SFD, MR, SS, ZH, SC, XZ, JW, MAL, RB,VM, SP, DH, CG, MSc, BH, BS, IMW, PS, JM, UP, DD, JL, ED, and MH declare no conflict of interest.

Abbreviations:

HBV: hepatitis B virus; cccDNA: covalently closed circular DNA; TLR: toll-like receptor; APOBEC3B/A3B: apolipoprotein B mRNA editing catalytic polypeptide-like B; LTβR: lymphotoxin beta receptor; HIF1α: hypoxia inducible factor 1 alpha; NF-κB: nuclear factor kappa B; NEMO: NF-kB Essential Modulator; RelA: NF-kappa-B p65 subunit; IKKα/β: IκB kinase alpha/beta; NIK: NF-κB

inducing kinase; RNA: ribonucleic acid; RT-qPCR: reverse transcription-quantitative polymerase chain reaction ; DNA: desoxyribonucleic acid; siCTRL: siRNA control; siHIF1α: siRNA HIF1alpha; BCA: Bicinchoninic acid assay; IHC: immunohistochemistry; ChIP: chromatin immune precipitation; CHB: chronic hepatitis B; HBcAg: hepatitis B core antigen; NO: normoxia; HO: hypoxia; DMOG: dimethyloxallyl glycin; FG-4592: roxadustat; NT: non treated; BS1: antibody agonising LTβR; d.p.i: days post infection;APOBEC3A/A3A: apolipoprotein B mRNA editing catalytic polypeptide-like A; APOBEC3G/A3G: apolipoprotein B mRNA editing catalytic polypeptide-like G; IFNα/γ: interferon alpha/gamma; IL-17: interleukin 17; LPS: lipopolysaccharide; TNF: tumour necrosis factor; HIF2α: hypoxia inducible factor 2 alpha; CAIX: Carbonic anhydrase IX; CoCl2: cobalt chloride; AhR: Aryl hydrocarbon receptor; ARNT: aryl hydrocarbon receptor nuclear translocator

Financial support statement: This work was funded in part by the European Union's Horizon 2020 program under the agreements 667273 (HEPCAR) (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) (M.H.), the ERC POC (Faith) (M.H.), the Helmholtz Future topic Inflammation and Immunology (M.H.), Zukunftssthema 'Immunology and Inflammation' (ZT-0027) and the Rainer Hoenig Stiftung. ED and MH were supported by the FNRS/FWO under EOS project n° 30826052. ED received financial support from the FSR and the Fondation Léon Fredericq of the University of Liege. MH, ED, DD, JL and MR were supported by an FP7-Infect-Era grant. DD and JL 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). JAM 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 number: 2018-I2M-2-002).

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- Formal analysis: TR, SFD, MR,
- Investigation: TR, SFD, MR, SS, ZH, SC, XZ, JW, MAL, RB, VM, SP, DH, CG, MSc, BH, BS, KR, KU, DT, PS, JL
- Resources: MSc, BH. KU, BS, IMW, PS, JM, UP, DD, JL, ED, MH
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- Supervision: ED, MH
- Project administration: ED, MH
- Funding acquisition: DD, JL, ED, MH

Abstract

New therapeutic strategies against Hepatitis B virus (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) stabilisation 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 stabilisation. We addressed whether HIF1α interferes with immune-mediated induction of the cytidine deaminase APOBEC3B and subsequent covalently closed circular DNA (cccDNA) decay. Liver biopsies of chronic HBV patients (CHB) were analysed by IHC, and *in situ* hybridization. The effect of HIF1α induction/stabilisation on differentiated HepaRG or mice +/- HBV +/- LTβR-agonist (BS1) was assessed *in vitro* and *in vivo*. Induction of A3B and subsequent effects were analysed by RT-qPCR, immunoblotting, ChIP, ICC, and mass-spectrometry. Analysing 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 stabilisation, strongly impaired A3B expression and anti-HBV effect. Interestingly, HIF1α knock-down was sufficient to rescue the inhibition of A3B-upregulation and -mediated antiviral effects, whereas HIF2α knock-down had no effect. HIF1α stabilisation decreased the level of RelB protein but not its mRNA, which was confirmed *in vivo.* Noteworthy, this function of HIF1α was independent of its partner ARNT. In conclusion, inhibiting HIF1α expression or stabilisation represents a novel 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 novel immune therapies.

Introduction

Hepatitis B virus (HBV) chronically infects more than 250 million people worldwide who are at high risk of developing end-stage liver disease and hepatocellular carcinoma (WHO, 2017). Current treatments allow the control of the infection but not its complete eradication due to the persistence of the viral DNA matrix, called covalently-closed-circular DNA (cccDNA) (1). Upon treatment arrest, the infection can relapse (1). Therefore, new treatments are urgently needed to progress towards a cure for chronic HBV infection.

New therapeutics developed for the treatment of HBV focus on the activation of the adaptive and innate immune system. Several toll-like receptors (TLR) agonists have offered promising results both *in vitro* and *in vivo* (2–4). 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) agonisation (e.g. by T-cells) leads to cccDNA decay (5,6).

Most immune receptors such as LTβR are described to signal through the nuclear factor-kappa B (NF-κB) pathways (7,8). NF-κB-signalling is divided into two arms: the classical/canonical and the alternative/non-canonical pathway (9). The canonical pathway 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 (9). The non-canonical pathway signals through the NF-κB inducing kinase (NIK), leading to the phosphorylation of IKKα and p100, which is subjected to processing into p52 forming p52/RelB heterodimers that activate target-genes such as immune mediators (10).

To reduce the extent of chronic inflammation and its deleterious effects, NF-κB signalling has to be tightly regulated (11). Among the factors involved in this regulation, hypoxia induced factor 1 alpha (HIF1α) has been shown to (i) be stabilised or induced by and (ii) regulate NF-κB signalling (12), in addition to its canonical induction by low oxygen levels (13). HIF1α is constantly produced, and is targeted to the proteasome in absence of stabilising conditions (13).

Here we identify HIF1α stabilization and the concomitant decrease of RelB protein level as a restricting factor for immune-mediated antiviral strategies 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 (14). Cells under hypoxia were cultured under 1% or 3% oxygen (InVivO2, Baker Ruskinn), 5% CO_2, in a humidified atmosphere.

Transgenic cell line preparation

HIFs overexpressing cell lines were generated from HepaRG-TR (15). HIFs ORFs were excised from HA-HIF1alpha P402A/P564A-pcDNA3 (Addgene #18955), or HA-HIF2alpha-pcDNA3 (Addgene #18950) using BamHI and XbaI (New England Biolabs). The P402A/P564A double mutation prevents HIF1α hydroxylation and degradation. The ORFs were then inserted into the BamHI/XhoI digested pLenti CMV/TO Hygro empty (w214-1) (Addgene #17484) using T4 DNA ligase (New England Biolabs). All HIFs vectors were a gift from William Kaelin and pLenti CMV/TO Hygro empty (w214-1) was a gift from Eric Campeau & Paul Kaufman.

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) and puromycin (Sigma Aldrich; 10 µg/mL; sgRNAs) until non-transduced cells have fully died.

Treatments and transfections

dHepaRG were treated with 0.5 µg/ml BS1 (generous gift from Dr. Jeffrey Browning, Biogen/Idec). Additionally, dHepaRG, not infected with HBV, were stimulated either with 10 ng/mL of TNFα, 50 ng/mL of IL-17, 100 ng/mL of LPS, or left untreated. dHepaRG infected with HBV were treated with 1000 IU interferon alpha 2A (Roferon, Roche), 800 IU tumour necrosis factor alpha (RnD systems, 210-TA) or 200 IU interferon gamma (RnD systems, 285-IF). All inhibitors and molecules used are presented in the ***Table S1.*** dHepaRG were transfected with 10 nM of siRNAs against HIF1α (Assay ID: s6539, Ambion), HIF2α (Assay ID: s4698, Ambion), AhR (Sigma), ARNT (Sigma), RelB

(Dharmacon), or non-targeting control siRNAs (4390843, Ambion) using Dharmafect 4 (Dharmacon, 1:1.000) (**Table S2**).

HBV preparation and inocula

HBV was purified and concentrated from the culture medium of HepAD38 cells via heparin columns and sucrose gradient ultracentrifugation as previously described (16). dHepaRG were infected with 200 vge/cell (viral genome equivalent/cell), in medium supplemented with 4% PEG-8000 (Sigma). 24 hours after infection, cells were washed 3 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. CHB patients were all in immune-active phase of the disease and presented F3/4 fibrosis grading and A3 activity (METAVIR scoring). Sections were cut to be 2 µM or 5 µM thick. Work with patient material was approved by the Heidelberg ethics committee under the following number: S206/2005.

Statistical analysis

Two-way ANOVA, Spearman correlation, and the unpaired Student 2-tailed t test were performed using Prism 8. (GraphPad, La Jolla, CA). Data are shown as mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.001$.

Additional "Material & Method" information can be found in Supplementary material.

Results

HIF1α stabilisation 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 (13). Inflammatory cytokines and/or ligands have been shown to efficiently inhibit HBV infection (2,17,18). Thus, we wanted to decipher if 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 immune-active phase, were stained for HIF1α and HBV core antigen (HBcAg). Highly oxygenated/low inflammation zones, highlighted by an absence of HIF1α staining, were also low for HBcAg staining in these CHB patients (**Figures 1A and 1B**). 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 (**Figure 1C**).

We have previously shown that, on the one hand, LTβR agonisation by an agonistic antibody (BS1) leads to cccDNA decay and HBV clearance, whereas, on the other hand, LTα/β are upregulated in CHB patients (5,19). Therefore, induction of LTβ in CHB patients should clear the infection given its anti-viral effect. To assess if the correlation of HIF1α and HBc observed *in vivo* (**Figure 1C**) could be due 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 co-staining of mRNA and protein. High HIF1α staining was found in areas with low A3B expression, whereas low HIF1α staining was found in area with strong A3B expression (**Figures 1D, 1E, and S1**).

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

HIF1α stabilisation decreases anti-cccDNA properties of LTβR agonisation

To confirm our findings *in vitro,* we used several HIF1α stabilising conditions, namely hypoxia (canonical HIF1α stabiliser and inducer; i.e. 1% oxygen), DMOG, or FG-4592 (two molecules

described to stabilise HIF1α through the inhibition of proline hydroxylases (PHDs), enzymes that, if active, hydroxylate HIF-α's in the presence of oxygen to address it for degradation). Schematic representation of the experiment timeline is presented in **Figure 2A**. Treatment with BS1 induced A3B, leading to cccDNA decrease, as previously described (**Figures 2B-G, siCtrl NO/BS1 or siCtrl DMSO/BS1**). Upon HIF1α stabilisation, A3B induction was decreased, impairing its antiviral effects on cccDNA (**Figures 2B-G, siCtrl HO/BS1, siCtrl DMOG/BS1, or siCtrl FG-4592/BS1**). A3B induction and anti-cccDNA activity was partially rescued by HIF1α knock-down (**Figures 2B-G, 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 blot analysis (**Figure 2H**). Of note, HIF1α knock-down under normoxia was sufficient to (i) increase A3B mRNA levels and (ii) decrease cccDNA levels as compared to siCtrl (**Figures 2A-B**). This effect was due to BS1-induced HIF1α stabilisation, as confirmed by immuno-precipitation of HIF1α under normoxia-BS1 condition (**Figure S2A**). Like A3B, the upregulation of NF-κB2, a NF-κB target gene, was attenuated in cells upon HIF1α stabilisation, which was rescued by HIF1α knock-down (**Figures S2B-D**). CAIX, a direct target gene of HIF1α, was upregulated upon HIF1α stabilisation, and showed a strong reduction when HIF1α was depleted (**Figures S2B-D**). LTβR mRNA expression was slightly reduced under hypoxia, which could be rescued by HIF1α knock-down, and was unchanged by DMOG or FG-4592 treatments (**Figures S2B-D**). Of note, HIF1α knock-down was confirmed by immuno-blotting (**Figures 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 (**Figures S2E**).

Altogether, these data highlight that HIF1α stabilisation impairs the upregulation 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 stabilisation of both HIF1α and HIF2α. Although we show that HIF1α knock-down can rescue A3B expression and antiviral effects of BS1 under HIF stabilising conditions (**Figure 2**), we aimed to investigate a potential additional role of HIF2α. Therefore, cell lines doxycycline-inducible for the over-expression 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 over-expressing cell line (**Figures 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 (**Figures S3A-D**). Overexpression of HIF1α or HIF2α alone inhibited A3B upregulation induced by BS1 (**Figure 3A**). However, under hypoxia, only siRNAs against HIF1α, but not HIF2α, rescued A3B upregulation, 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 condition (**Figure 3B**). HIF1α and HIF2α knock-down efficiencies were confirmed by RT-qPCR (**Figure S3E**). Moreover, inhibition of A3B by HIF1α and rescue by HIF1α knock-down were confirmed using different HIF1α stabilizers (DMOG, CoCl2, and VH298) (**Figures 3C-D and S3F**). Of note, LTβR surface expression remained unchanged under hypoxia, with a mild increase after HIF1α knock-down, highlighting that the effect of HIF1α stabilisation was not due to a decreased receptor expression (**Figures S4G-H**). Moreover, A3B repression was not due to cell death under hypoxia (**Figure S3I**).

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

HIF1α stabilisation inhibits NF-κB-induced A3B transcription by decreasing RelB protein expression level

The main signalling pathways activated upon LTβR-agonisation are related to NF-κB, suggesting that A3B is an NF-κB target gene. To confirm this hypothesis, we used two kinase inhibitors (ML120B and TPCA1) that target the IKK complex (IKKα/β). We observed that inhibition of IKKα/β reduces BS1-induced A3B in dHepaRG cells (**Figure S4A**). As we showed that HIF1α stabilisation prevents BS1-induced A3B, we anticipated that HIF1α inhibits NF-κB target genes. Indeed, the induction of the well-known NF-κB target genes *ηfkb2* and *nik* upon BS1 treatment in normoxia is highly reduced in hypoxic condition, and this effect was confirmed for A3B (**Figures S4B-D**). We also extended our

analysis with other activators of NF-κB (TNFα, IL-17, 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 cross-road of both NF-κB pathways, *relb* transcription is dependent on the canonical, while RelB protein is part of the non-canonical NF-κB dimer p52/RelB (9). We confirmed that, while BS1 increased RelB protein expression and A3B transcription, depletion of RelB drastically reduces BS1-induced A3B expression (**Figures S5B-C**). Therefore, we addressed whether the inhibitory effect of HIF1α stabilisation on BS1-induced A3B upregulation 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, while RelA expression and nuclear translocation were not strongly affected (**Figure 4A**). More importantly, the decrease of RelB protein levels in the DMOG/BS1 condition was completely rescued in HIF1α-depleted cells (**Figure 4B**). HIF1α stabilisation did not repress BS1-induced RelB mRNA upregulation (**Figure 4C**). These results were confirmed using longer DMOG treatment, different level of hypoxia, and other HIF1α stabilizers (**Figures S5D-G**). By immunostaining, we also confirmed that RelA nuclear translocation remained unchanged under hypoxia (**Figures S5H-I**), whereas hypoxia impaired RelB induction (**Figures 4D**). Interestingly, hypoxia also prevented BS1-induced p52 (the main binding partner of RelB) recruitment to the A3B promoter (**Figure 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 sacrificed 6 hours post injection. *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 (**Figure 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 (Aryl Hydrocarbon Receptor Nuclear Translocator) and AhR (Aryl hydrocarbon receptor) (20). 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 (21,22). Moreover, cross-talks between these proteins can occur through competition for common partners (e.g. HIF1α/ARNT versus AhR /ARNT) (23). Thus, we investigated if such processes could control RelB activity in our model. A schematic timeline of the experiments is depicted in **Figure 5A**.

In dHepaRG, AhR knock-down did not interfere with BS1-induced RelB expression, highlighting that AhR was dispensable for RelB stability (**Figure 5B**). Interestingly, contrary to HIF1α knock-down, RelB protein levels were not rescued in ARNT-depleted cells treated with DMOG/BS1 (**Figure 5C**). It was reported that ARNT represses the transcription of particular NF-κB target genes (22), as confirmed by the elevated expression of CXCL10 in ARNT-depleted cells (**Figure S6A**). However, ARNT knock-down had no impact on RelB mRNA expression, while VEGFα expression (a target gene of the HIF1α/ARNT heterodimer) was reduced (**Figures S6B-C**). In addition, neither AhR nor ARNT knock-down rescued A3B levels in DMOG treated cells (**Figures 5D-E)**. These results indicate that HIF1α/ARNT dimerisation, which is necessary for the canonical function of HIF1α as transcription factor, is not the cause of decreased RelB protein and A3B mRNA expression.

In summary, our results demonstrate that the HIF1α/RelB cross-talk 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 siRNAs transfected dHepaRG cells treated with or without BS1 under normoxia (NO) or hypoxia (HO). A schematic timeline of the experiment is depicted in **Figure 6A**. Interestingly, whereas 418 proteins were significantly dysregulated in BS1-treated versus non-treated cells under normoxia (NO/NT vs. NO/BS1), only 2 proteins were found dysregulated when comparing the same treatments under hypoxia (HO/NT vs. HO/BS1), indicating a global inhibition of responses to BS1 treatment (**Figure 6B**). Pathways were grouped into 4 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 and cellular transcriptional and translational machinery were among the most upregulated pathways, leading to production of immune response pathway effectors (**Figure 6C**).

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

"NO/NT/siCtrl vs. NO/BS1/siCtrl" comparison confirmed the results obtained in non-transfected conditions (**Figure 6D**). However, the "NO/NT/siCtrl vs. HO/BS1/siCtrl" comparison highlighted a significant downregulation of pathways implicated in RNA transcription and translation (e.g. ribosome, mRNA surveillance), preventing the increase of immune response pathway effectors (**Figure 6E**). While being upregulated under NO/BS1 condition (**Figure 6D**), the "NF-kB signalling pathway" was downregulated under hypoxia (**Figure 6E**). Interestingly, "NO/NT/siCtrl vs. HO/BS1/siHIF1α" comparison showed a partial rescue of some of these pathways upon HIF1α knock-down, namely RNA processing (i.e. Spliceosome) and transport, as well as NF-κB- and NOD-like receptor-signalling pathways (**Figure 6F**). Importantly, the "Ribosome" pathway returned to a level similar to normoxia upon HIF1α knock-down (**Figure 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 knock-down 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, as they have shown promising results both *in vitro* and *in vivo* (2–4). 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 non-cytolytic degradation of nuclear HBV cccDNA, enabling long-term inhibition of HBV-replication without rebound, even after treatment arrest (5,6).

HIF1α has been shown to impair immune responses (12,24). Inflammatory signalling 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 stabilisation. In the liver of CHB patients in immune-active (i.e. patients that potentially could clear the infection as they likely express high levels of cytokines), we found a positive correlation between HIF1α expression and HBcAg-positive areas. Since 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 hypothesised 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 hypothesise that blocking HIF1α stabilisation during immune-active phase of CHB patients could indeed be sufficient to allow more potent immune responses, among which induction of A3B, and viral elimination.

In vitro, we confirmed using 1% oxygen, DMOG, and a number of other molecules inducing HIF1α stabilisation, as well as HIF1α overexpressing cell lines, that HIF1α stabilisation mediates a strong impairment of LTβR-dependent A3B induction. However, impairment of immune responses was not limited to A3B as a NF-κB target genes, neither to BS1 as an NF-κB inducer, highlighting that HIF1α modulated NF-κB and other immune-signalling pathways (e.g. interferon alpha and gamma 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 post-translational modifications associated to proteasomal/lysosomal protein degradation (25). We also found that the inhibitory activity of HIF1α towards RelB was independent of its partner ARNT. A new ARNT-independent function of HIF1α starts to emerge (26) and the HIF1α/RelB cross-talk we discovered could bring more insights into the immune-metabolism of the liver.

The global inhibition of immune responses observed under HIF1α stabilisation, 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 anti-viral targets, as well as *in vivo*/in a therapeutic set-up. Mass spectrometry revealed that even though HIF1α knock-down partially rescued pathways implicated in RNA and protein production and processing, it could not fully reactivate the immune response in the 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 (13,24). Moreover, as inflammation can trigger HIF1α stabilisation, it will be mandatory to inhibit HIF1α to insure potent immune responses. Recently investigated HIF inhibitors have shown encouraging results in cancer therapies (27). 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 (2,4) to insure potent immune activation in the whole liver.

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

Acknowledgments: We thank the Genomics and Proteomics core facility and the Biostatistics core facility of the DKFZ for their support on the proteomics dataset 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).

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Figures Legends

Figure 1 – HIF1α stabilisation allows HBV persistence *in vivo*. (A-E) Paraffin sections of chronic HBV patients were consecutively cut and stained for HIF1α, HBcAg, or APOBEC3B mRNA *in situ* or co-stained for HIF1α and APOBEC3B mRNA *in situ*. **(A-B)** Regions were classified in three types: (i)no HIF1α positive cells; (ii) 1-5 HIF1α positive cells; (iii) >5 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 3 different zones. Every data point represents the mean of 2 view fields and the bars represent the mean +/- SD of eight patients. **(C)** Correlation between HIF1α- and HBcAg-positivity per view field. **(D)** Representative pictures of patients stained for A3B. Upper 3 pictures show a representative HIF1α high area, lower 3 pictures show A3B high area of the same patient sample. **(E)** Representative images of a patient stained for HIF1α and A3B. Upper 3 pictures show a representative HIF1α high area, lower 3 pictures show 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 **(E)** one-way ANOVA. *: $p < 0.05$; **: $p < 0.01$; ns: not significant.

Figure 2 – HIF1α stabilisation prevents the anti-viral effects of APOBEC3B *in vitro*. (A) Schematic representation of the experiments**. (B-C)** dHepaRG were infected with HBV. 6 d.p.i., cells were transfected with either 10 nM HIF1α-targeting or control siRNAs. On the next day, cells were subjected to 1% or 20% oxygen for 3 days and treated +/- 0.5 µg/mL of BS1. Transfection and treatments were repeated once. **(D-E)** dHepaRG were infected with HBV. At 10 and 13 d.p.i., cells were transfected either 10 nM HIF1α-targeting or control siRNAs. Cells were then treated +/- 0.5 µg/mL of BS1, and +/- 100 µM of DMOG. **(F-G)** dHepaRG were infected with HBV. At 10 and 13 d.p.i., cells were transfected either 10 nM HIF1α-targeting or control siRNAs. One day after the second transfection, cells were treated or not with 0.5 µg/mL BS1, either under presence of 30 µM of FG-4592 or DMSO. 6 days later, **(B, D, F)** mRNAs and **(C, E, G)** DNA were extracted and analysed

by RT-qPCR and qPCR. Bars represent the mean +/- SD of **(B, C)** one, or **(D-G)** three independent experiment performed in quadruplicates. Data were submitted to **(C, E, G)** unpaired student's t-test, or **(B, D, F)** one-way ANOVA. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.0001$; ns: not significant. **(H)** dHepaRG were infected with HBV. At 10 d.p.i., cells were treated +/- 0.5 µg/mL of BS1, and +/- 100 µM of DMOG for 12 days. Episomal DNA was extracted and analysed by Southern blot.

Figure 3: HIF1α but not HIF2α stabilisation inhibits APOBEC3s. (A-D) Schematic representation of the experiments. **(A)** Inducible dHepaRG over expressing the HIF1α degradation resistant mutant P402A/P564A or HIF2α treated for 3 days with increasing dose of doxycycline in presence of 0.5 µg/mL of BS1. **(B)** dHepaRG were transfected with 10 nM of either HIF1α-targeting, HIF2α-targeting, or both siRNAs or control siRNAs. The next day cells were treated +/- 0.5 µg/mL of BS1 under 1% oxygen. mRNAs were extracted and analysed by RT-qPCR. **(C)** dHepaRG were transfected with either 10 nM HIF1α-targeting or control siRNAs. One day after the second transfection, cells were treated or not, for 24h, with 0.5 µg/mL of BS1, either under presence of 100 µM of DMOG or DMSO. mRNAs were analysed by RT-qPCR. Bars represent the mean +/- SD of three independent experiments performed in triplicates. **(D)** dHepaRG were incubated for 3 days +/- 100 µM of CoCl2 or VH298 in presence or absence of 0.5 µg/mL of BS1. mRNAs and proteins were extracted and analysed 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. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

Figure 4 – HIF1α stabilisation decreases RelB level *in vitro* and *in vivo*. (A-F) Schematic representation of the experiments. **(A)** dHepaRG were treated for 24h with DMSO or 100 µM of DMOG +/- 0.5 µg/mL of BS1. Cytoplasm and nuclei were separated. **(B-C)** dHepaRG transfected with either 10 nM HIF1α-targeting siRNAs or control siRNAs. Two days after transfection, cells were treated for 24h with DMSO or 100 µM of DMOG +/- 0.5 µg/mL of BS1 for 24 hours. **(D)** dHepaRG were seeded into 4-well chamber slides. 3 days after seeding, cells were cultured under either 1%

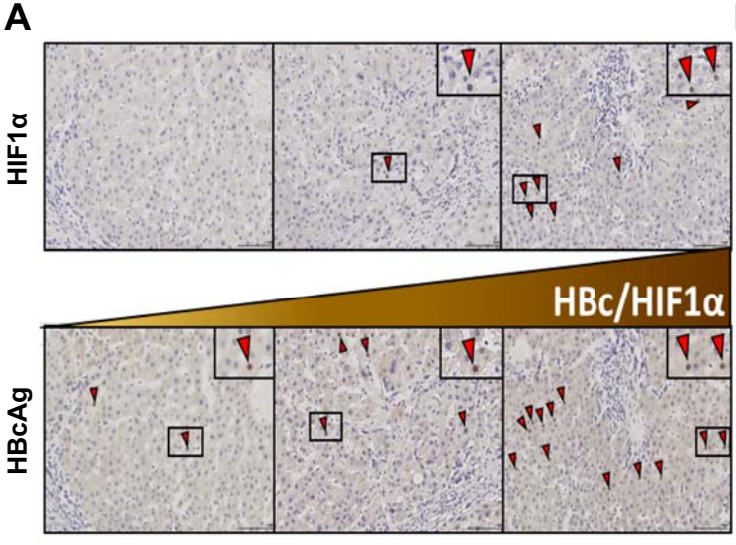
(Hypoxia) or 20% (Normoxia) oxygen for 3 days, either in the presence or absence of 0.5 µg/mL of BS1. Cells were then prepared for immunocytochemistry and stained for RelB. Representative pictures and quantification of RelB positive nuclei. Data represent the mean of 5 pictures per condition of two experiments. **(E)** dHepaRG were cultured under 1% or 20% oxygen +/- 0.5 µg/mL of BS1. 6 days post treatment, protein and nucleic acids were cross-linked and submitted to ChIP. DNA was extracted and binding of p52 to APOBEC3B promoter was analysed by qPCR. **(F)** Mice were injected i.p. with 300 mg/kg DMOG or the equal amount of DMSO for 6h. **(A, B, F)** Proteins were analysed by immunoblotting. **(C, F)** mRNAs were analysed by RT-qPCR. Bars represent the mean +/- SD of **(C, R)** three independent experiment. Data were submitted to **(D, E)** one-way ANOVA. *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$; ns: not significant.

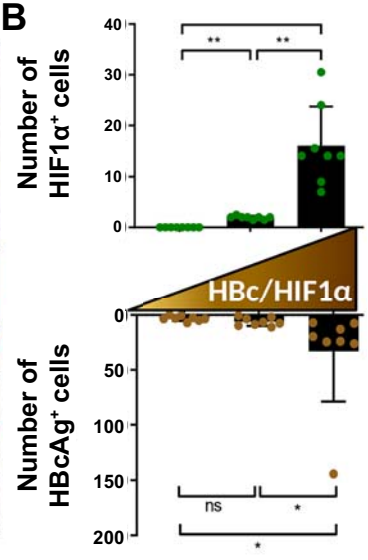
Figure 5 – ARNT knock-down does not rescue RelB and A3B level. (A) Schematic representation of the experiment. **(B-E)** dHepaRG cells were transfected with either 10 nM AhR-targeting, ARNT-targeting or control siRNAs. Two days after transfection, the cells were treated for 24h with DMSO or 100 µM of 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. ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

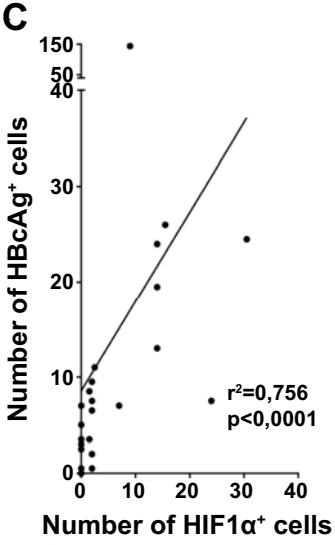
Figure 6 – HIF1α knock-down rescues "mRNA processing" and "ribosomes" pathways. (A) Schematic representation of the experiment. **(B-F)** dHepaRG were **(B-C)** either left untransfected or **(D-F)** transfected with either 10 nM HIF1α-targeting or control siRNAs. On the next day, cells were subjected 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 non-treated (NO/NT) vs. 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 which 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 pre-selected KEGG pathways using the ROAST algorithm. The pathways are

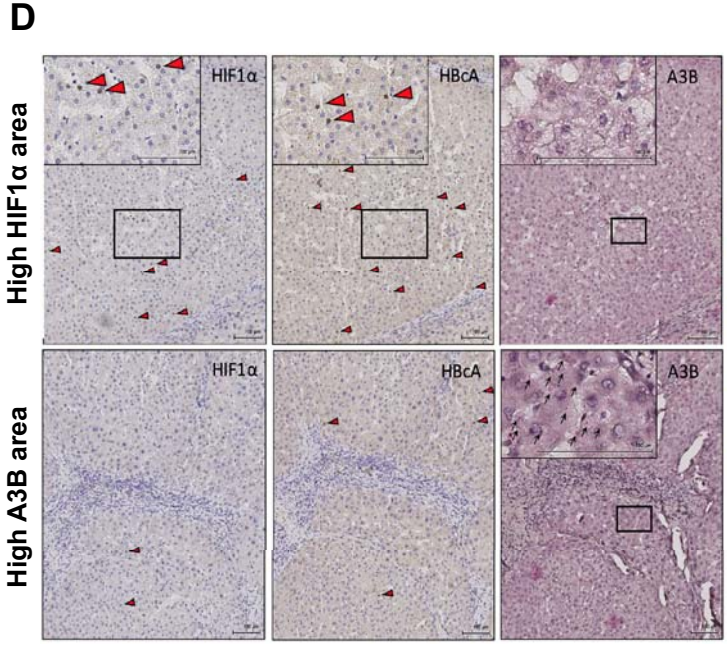
represented for **(C)** NO/NT vs. NO/BS1, **(D)** NO/NT/siCtrl vs. NO/BS1/siCtrl, **(E)** NO/BS1/siCtrl vs. HO/BS1/siCtrl, and **(F)** HO/BS1/siCtrl vs. HO/BS1/siHIF1α. The significantly (respectively, non-significant) upregulated (dark red bar; respectively, light red bar) or downregulated (dark blue bar; respectively, light blue bar) pathways are presented as the percentage of proteins analysed in the pathways. Of note, black bars represent the number of significantly dysregulated proteins in the pathway. Data were submitted to LIMMA algorithm for selection of significantly changed proteins. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

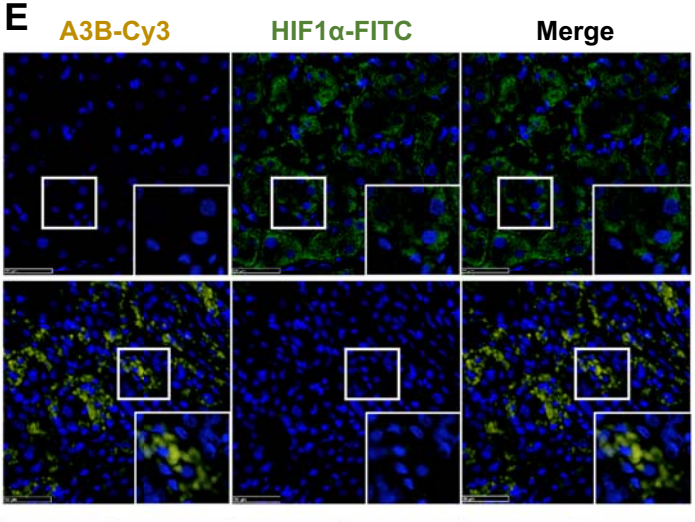
Figure 7- HIF1α stabilization prevents APOBEC3B-mediated anti-cccDNA effect by decreasing RelB protein. Graphical representation of the main proposed mechanism. Shortly, HIF1α stabilization under hypoxia or stabilizing molecules treatment decreases RelB protein levels but not its mRNA. The decrease of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





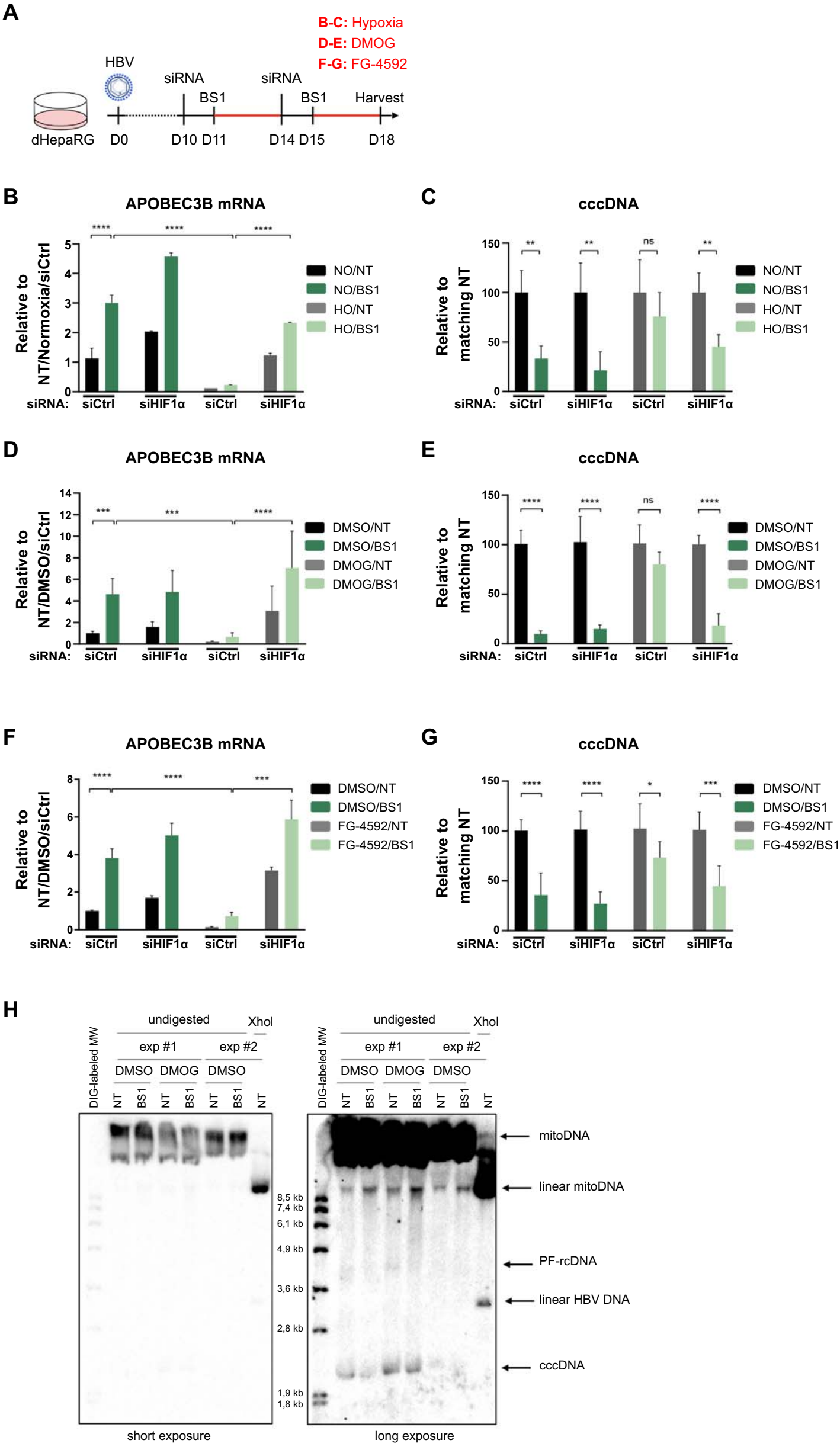




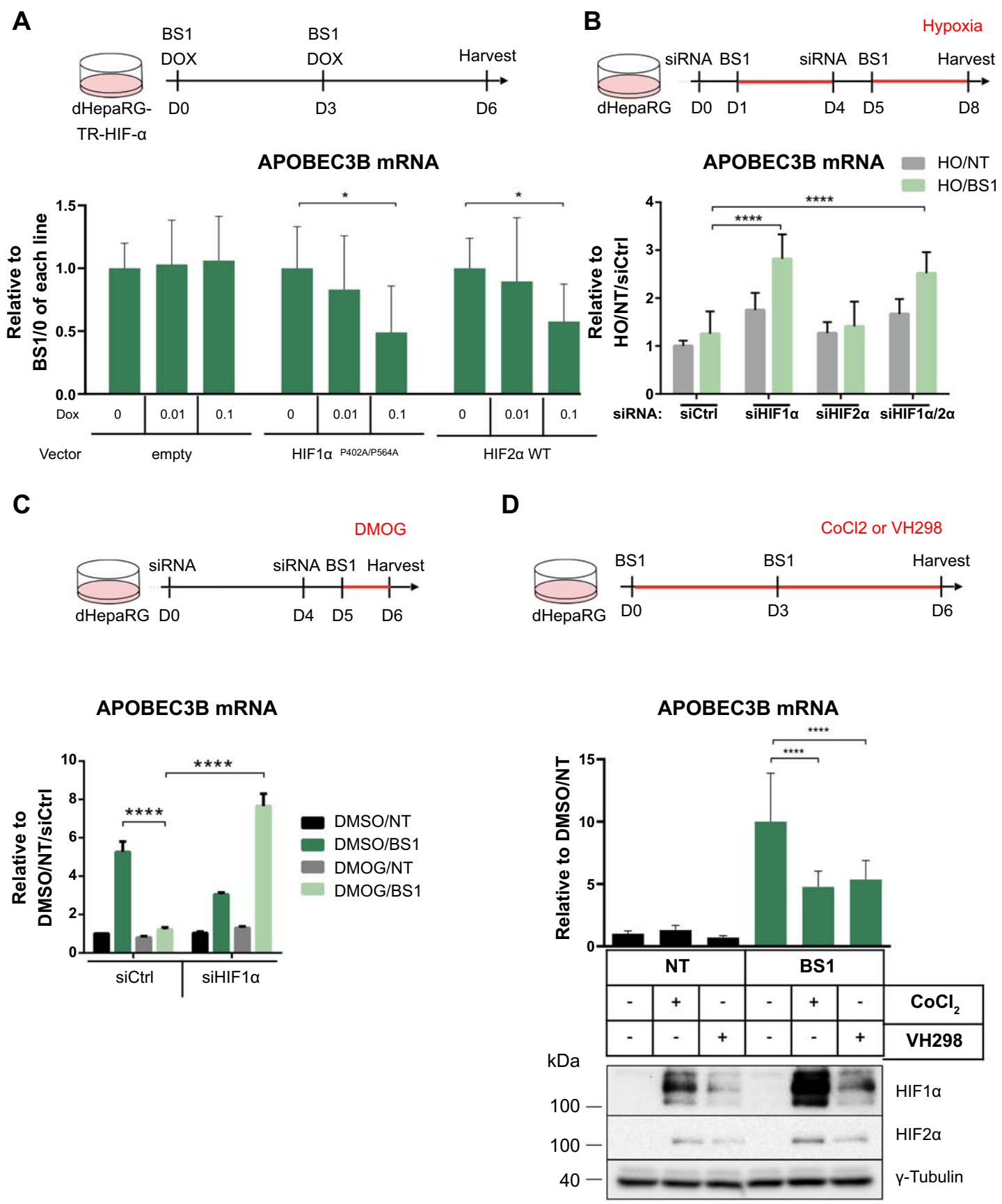


High A3B area	Mean % of stained area	*p*	High HIF1α area	Mean % of stained area	*p*
A3B signal	4,14 (±5,69)	p=0,0524	A3B signal	0,028 (±0,06)	** (p=0,0085)
HIF1α signal	0,158 (±0,19)		HIF1α signal	2,731 (±2,73)	

Riedl *et al.*, Figure 1



Riedl *et al.*, Figure 2



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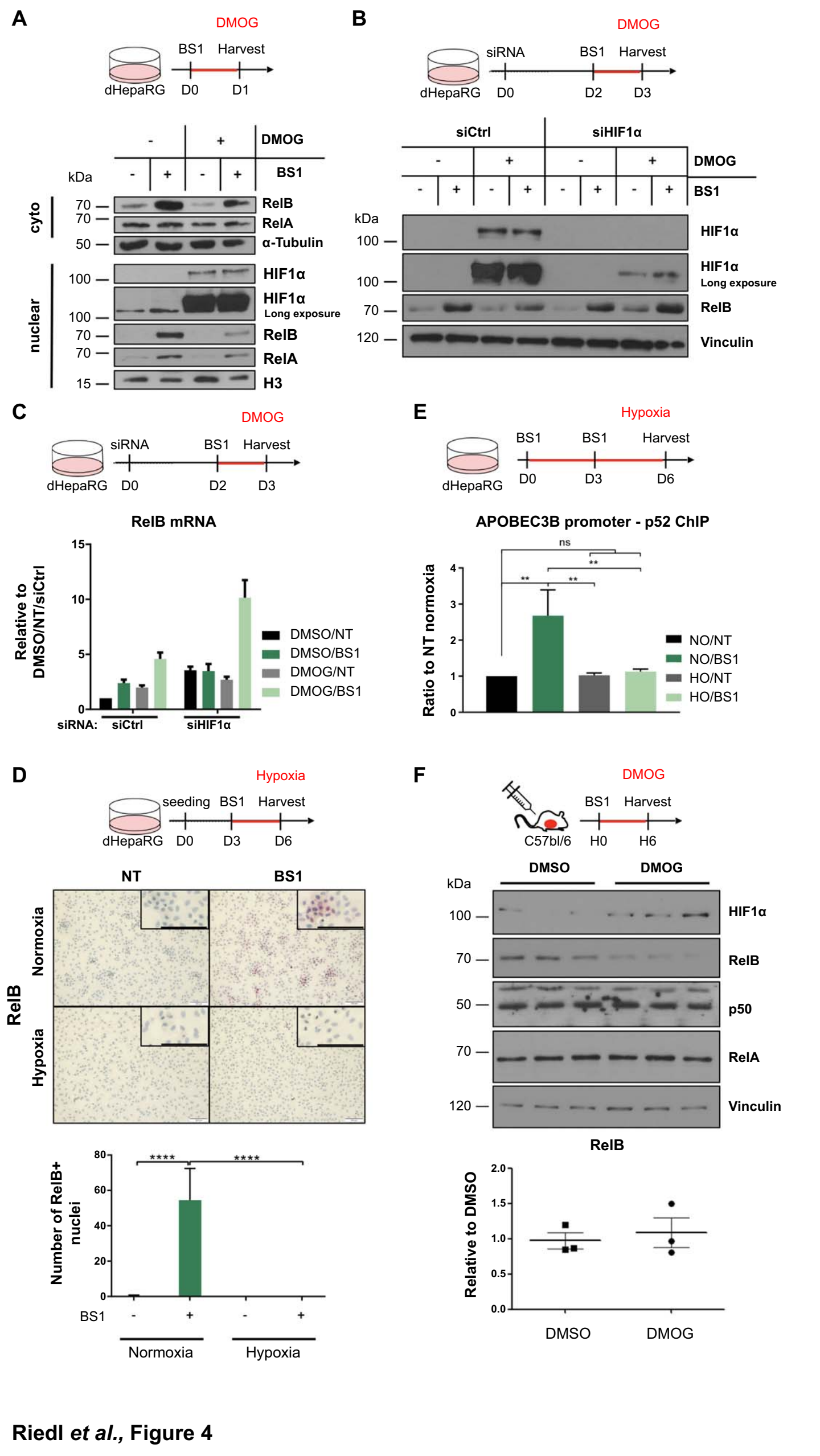

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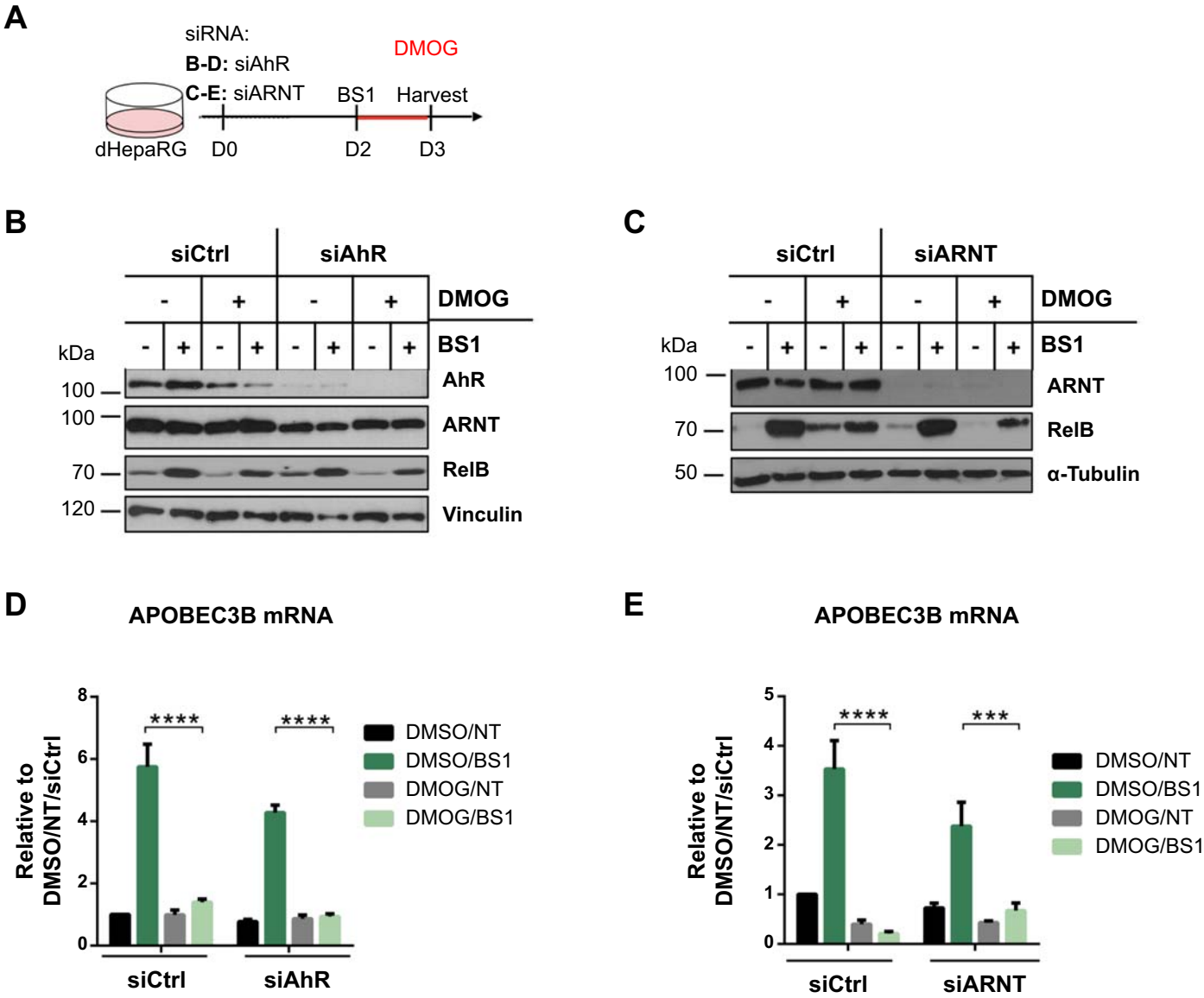

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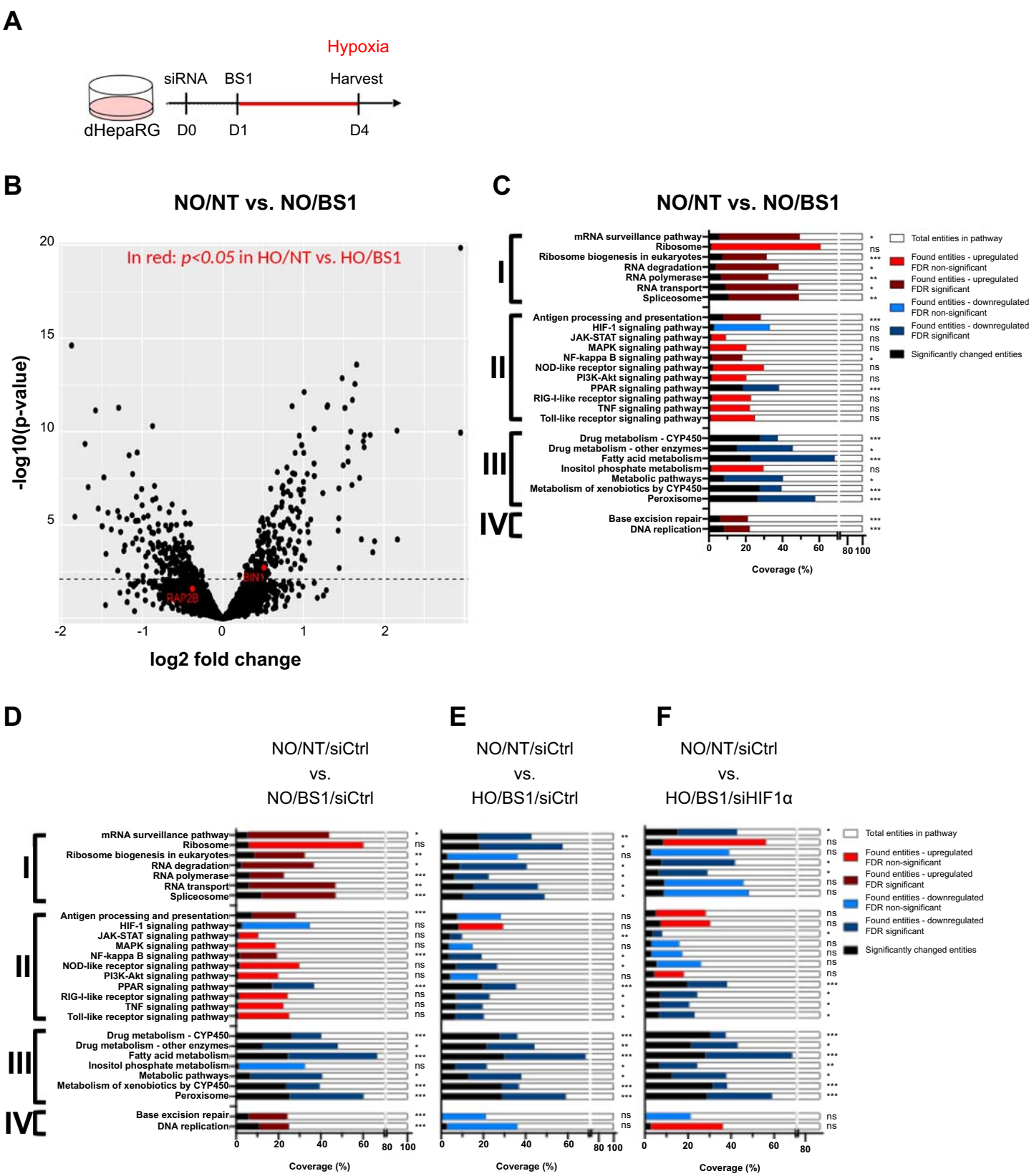
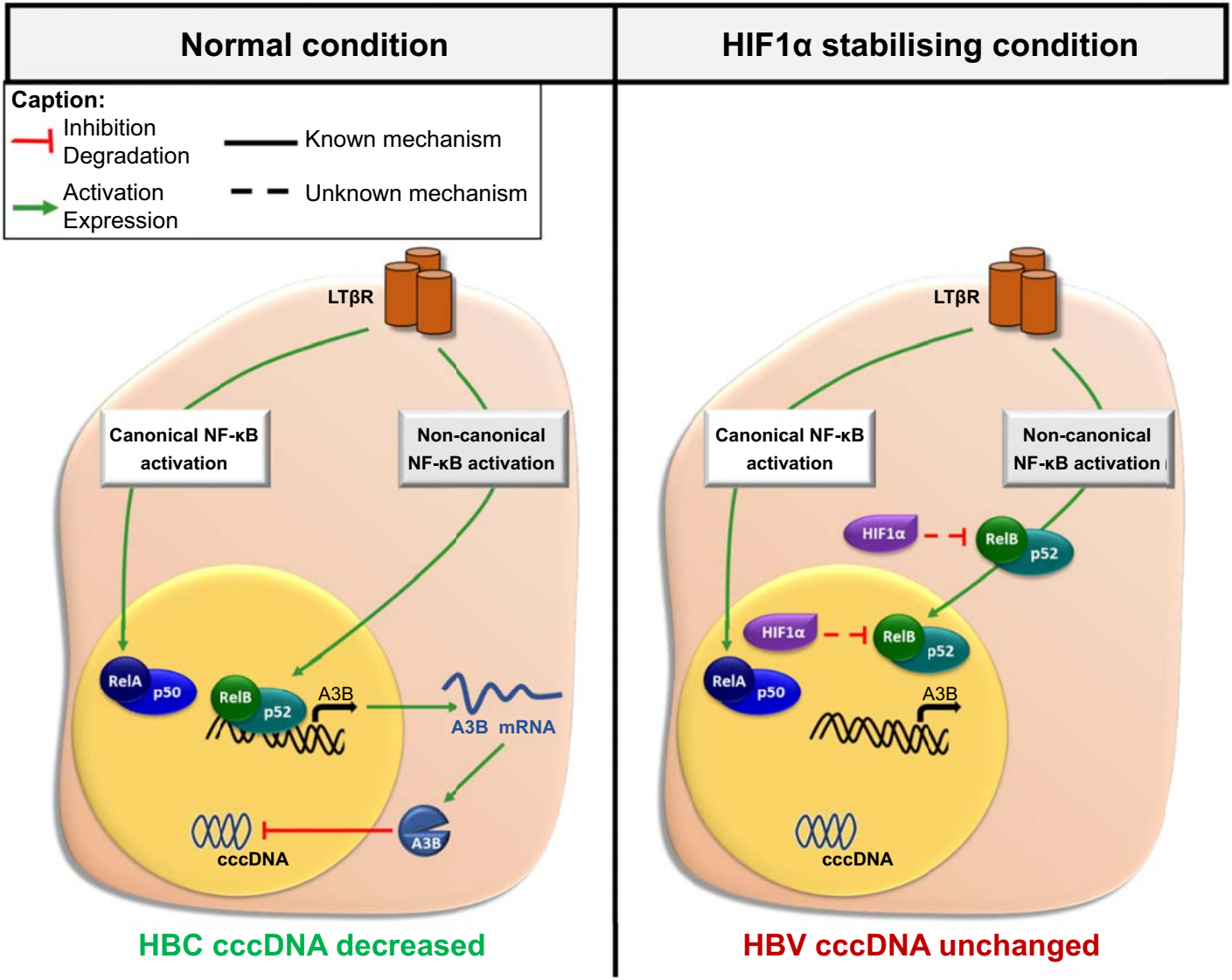


Riedl *et al.*, Figure 6



Riedl *et al.*, Figure 7