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Hepatitis C virus-mediated Aurora B kinase inhibition modulates inflammatory pathway and viral infectivity

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Abstract

Background & Aims: Chronic hepatitis C is a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma. DNA methylation and histone covalent modifications constitute crucial mechanisms of genomic instability in human disease, including liver fibrosis and hepatocellular carcinoma. The present work studies the consequences of HCV-induced histone modifications in early stages of infection.

Methods: Human primary hepatocytes and HuH7.5 cells were transiently transfected with the core protein of hepatitis C virus (HCV) genotypes 1a, 1b, and 2a. Infectious genotype 2a HCV in culture was also used.

Results: We show that HCV and core protein inhibit the phosphorylation of Serine 10 in histone 3. The inhibition is due to the direct interaction between HCV core and Aurora B kinase (AURKB) that results in a decrease of AURKB activity. HCV and core significantly downregulate NF- κ B and COX-2 transcription, two proteins with antiapoptotic and proliferative effects implicated in the control of the inflammatory response. AURKB depletion reduced HCV and core repression of NF- κ B and COX-2 gene transcription and AURKB overexpression reversed the viral effect. AURKB abrogation increased HCV specific infectivity which was decreased when AURKB was overexpressed.

Conclusions: The core-mediated decrease of AURKB activity may play a role in the inflammatory pathway during the initial steps of viral infection, while ensuring HCV infectivity.

Keywords: Hepatitis C; Inflammation; NF- κ B; COX-2; Histone modifications.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; AURKB, Aurora B kinase; PP2, protein phosphatase 2; Chk1, checkpoint kinase 1; NF- κ B, nuclear factor- κ B; COX-2, cyclooxygenase-2.

Introduction

Chronic hepatitis C virus (HCV) infection is a leading cause of progression of liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) by mechanisms which are not fully understood. In recent years the role of epigenetic processes on gene expression related to fibrosis and carcinogenesis has been demonstrated [4,20,23,36]. These epigenetic changes are regulated by enzymatic activities such as DNA methyltransferases, histone acetyltransferases, methylases and kinases, among others [29].

Ser10 and Ser28 phosphorylation in histone H3 is induced by Aurora B kinase (AURKB) [13]. This kinase belongs to the Aurora family of Ser/Thr kinases, and defects in function and expression have been linked to tumorigenesis [11]. AURKB, together with Aurora A kinase (AURKA) have been found to be overexpressed in human cancers, including HCC, and several AURK inhibitors have been designed as potential anticancer molecules [19].

The role of AURKB in HCV-related disease progression has not been investigated. HCV infection has been shown to cause over- expression of protein phosphatase 2A (PP2A) [8], which potentially can produce acetylation and methylation of histone 4 and phosphorylation of histone 2AX [9]. A reduction of Ser10 histone H3 phosphorylation associated with cell cycle arrest has been reported in hepatoma cells infected with HCV [18], although the underlying mechanism has not been described.

The HCV core protein is the major component of the viral nucleocapsid and has been implicated in multiple functions, such as cellular proliferation and transformation, transcription regulation, cell cycle modulation, and apoptosis [26]. Therefore, it is a good candidate to mediate epigenetic changes induced by HCV infection.

Herein we report the impact of HCV in some epigenetic modifications of primary hepatocytes and hepatoma cells in culture. We show that inhibition of AURKB activity by HCV infection

is associated with histone modifications and that the viral core protein is sufficient to trigger this effect. At the same time, HCV regulates pro-inflammatory response through the downregulation of nuclear factor- κ B (NF- κ B) and cyclooxygenase-2 (COX-2) gene expression. Both proteins are critical mediators of inducible gene transcription involved in innate immunity [1]. Implications for liver disease and HCV survival are discussed.

Materials and methods

Cell culture and transfections

Human primary hepatocytes were prepared from liver biopsies of two patients submitted to a surgical resection for liver tumors (with the patients' written consent). Hepatocytes were isolated using the two step collagenase procedure [32]. HuH7.5 cells were cultured as previously described [30]. The complete coding regions of the core and NS5A HCV proteins of genotypes 1a, 1b, and 2a (clones H77, Con1, and Jc1 respectively, courtesy of Professor Charles Rice) were transferred to the pCMV expression vector (Clontech Laboratories, Inc); the integrity and orientation of the coding regions was confirmed by sequence analysis.

Cells were transfected using FuGeneHD (Promega). When indicated, the AURKB inhibitor ZM-447439 (2 μ M) (Tocris) was added 30 min after transfection. The following amounts of plasmid were used per well (60 mm): NF- κ B-luc (0.25 μ g) or COX-2-luc (0.25 μ g) + pTK-Ren (renilla) (0.01 μ g), together with pCMV0 (1 μ g) or pCMV-core (1a, 1b, or 2a) (1 μ g) or pCMV-NS5A (1a, 1b, or 2a) (1 μ g). pcDNA3-FLAG-AURKB (1–5 μ g) were used in the indicated experiments. Protein expression was monitored by Western blot assays and quantitated by densitometry. Luciferase and renilla activities were assayed with a dual luciferase assay system after 72 h of

transfection (Promega). Most of the experiments were repeated at least three times in duplicate, and all data are presented as the mean of at least six determinations \pm S.D.

HCV infection

The HCVcc virus stock was produced in HuH7.5 cells as previously described [30,33]. HuH7.5 reporter cells were infected with HCVcc at a multiplicity of infection (MOI) of 0.03 TCID₅₀/cell. Extracellular virus titers were determined as described [30]. All the experiments were processed 72 h after HCV infection, unless indicated in the figure legends. Under these conditions 80–90% of the cells were infected as determined by live imaging [16].

RNA interference

AURKB siRNAs (50 or 100 nM, Dharmacon) were transfected with FuGeneHD (Promega). At 72 h post-transfection, the cells were harvested. AURKB mRNA and protein levels were determined by qRT-PCR and Western blot, respectively.

Immunoprecipitation and Western blot

Western blot assays were performed as previously described [38]. The following antibodies were used: anti-H3Ser10ph, anti-H3 (Upstate Biotechnology), anti-AURKB (Abcam), anti-phosphorylated Aurora-BpT232 (Rockland), anti-H3K9me2, anti-H3K9me3, anti-H3K4me3 (Upstate Biotechnology) anti-HepcAg (1868) (Santa Cruz Biotechnologies, CA), anti-NS5A (9E10, courtesy of Professor Charles Rice) and anti-HA (Sigma). 5–30 μ g of total protein was used in Western blot assays depending on the antibody used. b-actin, GAPDH or H3 levels were used as loading control in Western blot assays. For IP, HuH7.5 cells were transfected with core and 72 h later, cells were lysed in buffer B [38]. 2 mg of cell extracts were pre-cleared for 2 h with 40 μ l of protein A agarose beads and incubated overnight with 60 μ l of protein A agarose

beads previously coupled with 2 µg of the specific antibody or rabbit IgG. The immunoprecipitated proteins and 20 µg of total extract (input control) were analysed by Western blot using the antibodies indicated in each figure.

Chromatin immunoprecipitation

Chromatin IP (ChIP) was performed as described in The Chromatin Immunoprecipitation Assay kit (Upstate, Cat. 17-295). Sonication was carried out using a Bioruptor UCD-200TM (Diagenode) following the manufacturer's instructions. IP was quantified by real-time PCR and the Ct values of the target sequences in the immunoprecipitate were calculated in relation to the input (INP) fractions by the comparative Ct method using the equation $2^{-(Ct(IP)-Ct(INP))}$. Primers are described in Supplementary Table 1.

Metabolic activity and cell viability studies

At 24, 48, 96, and 144 h post-infection cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (final concentration 0.2 mg/ml) at 37 °C for 3 h and subsequently with 100 µl of DMSO. Absorbance was measured at 570 nm. Cell proliferation was determined by counting cells with 0.4% Trypan blue/PBS solution (1:1).

Immunofluorescence and microscopy

Fixed and permeabilized HuH7.5 cells were incubated with anti-H3Ser10ph (1:100), then with goat-anti-rabbit IgG Alexa-Fluor 488 or 546 (1:200), respectively, for 1 h at 37 °C. Images were captured with Axiophot (Zeiss) microscope with a Plan-NEOFLUAR 20x. Images were processed with ZEN 2011 software (Zeiss) and Adobe Photoshop CS.

GST constructions and pull-down assays

pGEX-4T1-AURKB/1-75 and pGEX-4T1-AURKB/1-250 vectors were generated by

PCR from pGEX-4T1-AURKB using primers indicated in Supplementary Table 1. The PCR products were inserted into pGEX-4T1 vectors. The core coding region was inserted into the pcDNA3.1HisA vector (Invitrogen) to produce pcDNA3.1HisA-core. The pcDNA3.1HisA-core/1-122 vector was generated by PCR from pcDNA3.1HisA-core using primers indicated in Supplementary Table 1. Recombinant proteins were purified on glutathione–sepharose resin and analysed by SDS–PAGE. ³⁵S-labelled core and core 1/122 were *in vitro* translated with TNT T7 Quick Kit and used in pull-down assays with 0.5 µg of GST0 or GST-fused proteins as previously described [38].

Quantitative RT-PCR (qRT-PCR) of cellular mRNAs and viral RNA

DNAse-treated RNA (2 µg), isolated from hepatoma cells using TRI Reagent (Sigma), was reverse transcribed with the SuperScript™ First Strand Synthesis System (Invitrogen Life Technologies) according to the manufacturer's instructions. The resulting cDNA template (2 µl) was amplified using the 2x Brilliant SYBR Green QPCR Kit (Stratagene). Experiments were carried out in triplicate. Each value was normalized against the GAPDH gene and expressed as relative RNA abundance over time zero. HCV qRT-PCR was previously described [30]. Primers are described in Supplementary Table 1.

Statistical analysis

Student's t and Wilcoxon test were performed using the SSC-Stat software (version 2.18; University of Reading, Reading, UK) and the IBM SPSS Statistic 19 software. The statistical significance of differences between groups was expressed by asterisks or hashes (*, #, p < 0.05; **, ##, p < 0.01; ***, ###, p < 0.001).

Results

HCV effect on histone modifications

To determine the possible effect of HCV infection in epigenetic modifications of the host cells, we first examined histone covalent modifications in human hepatoma HuH7.5 cells infected with HCV. HCV infection markedly decreased phosphorylation of Serine 10 in histone H3 (H3Ser10ph), and slightly increased the di-methylation of Lysine 9 in H3 (H3K9me2); the other epigenetic markers analysed were not altered (Fig. 1A). To investigate whether the H3Ser10 phosphorylation was mediated by HCV proteins, core and NS5A were expressed in HuH7.5 cells. Expression of the core protein from HCV genotypes 1a, 1b, and 2a (Fig. 1B) inhibited H3Ser10ph, although the decrease was only statistically significant for core 1b and core 2a expression. Expression of the NS5A protein from the same genotypes did not alter H3Ser10ph (Fig. 1C). Levels of expression of core and H3Ser10ph in HCV infected and in core 2a transfected HuH7.5 cells were also tested (Supplementary Fig. 1A). In both cases core expression was sufficient to reduce H3Ser10ph levels. The decrease in H3Ser10ph was also observed in human primary hepatocytes expressing the core protein (Fig. 1D).

Role of AURKB in decreased phosphorylation of H3Ser10 by HCV and core

AURKB is one of the kinases that phosphorylates Ser10 residue of H3. Therefore, we tested whether HCV infection modified the expression of this kinase. Neither HCV nor core altered mRNA or protein AURKB expression (Fig. 2A and B, respectively). Since AURKB is active when it is phosphorylated [40], we examined whether phosphorylation of AURKB in the Thr-232 residue could be modified by HCV and core. Interestingly, HCV infection and core protein expression decreased the levels of phosphorylated AURKB, concomitant with a decrease in phosphorylation of H3Ser10 (Fig. 2B). Then, the AURKB activity was inhibited by ZM447439 treatment and the levels of Ser10 phosphorylation in H3 were determined.

As expected, inhibition of AURKB decreased the levels of H3Ser10ph in uninfected and empty vector transfected cells, relative to the levels of this marker in untreated cells (lanes 1-2 and 3-4 in Fig. 2C and lanes 1 and 3 in Fig. 2D). ZM447439 treatment abolished the core-mediated decrease of phosphorylation of H3Ser10 which were further reduced by HCV infection (lanes 3 and 4 in Fig. 2D and lanes 3-4 and 7-8 in Fig. 2C). Thus, HCV and its core protein decrease AURKB activity and reduce H3Ser10ph levels in the host cells.

Interaction between AURKB and HCV core

HCV infection significantly induces the levels of PP2A phosphatase [2], an enzyme which dephosphorylates AURKB resulting in inhibition of its kinase activity [37]. Thus, HCV core might abolish AURKB activity through PP2A activation. To test this hypothesis, HuH7.5 cells were treated with the PP2A inhibitor, okadaic acid. Inactivation of PP2A phosphatase did not abolish the inhibitory effect of core protein, while in mock infected cells the levels of Ser10 phosphorylation were increased after treatment with okadaic acid, confirming that inhibition of PP2A activates AURKB (Supplementary Fig. 1B). AURKB is also phosphorylated at Ser331 by checkpoint kinase 1 (Chk1), to fully induce AURKB activity in mitosis [31]. In turn, Chk1 is activated by phosphorylation of Ser317 [7]. Thus, we tested whether HCV infection regulates Chk1 activity and consequently the AURKB activity. HCV infection decreased the phosphorylation of Ser317 in Chk1, while core protein expression in HuH7.5 cells produced no effect (Supplementary Fig. 1C and D). Since core protein expression did not regulate Chk1 or PP2A activities, we analysed whether core interacts with AURKB. *In vitro*, pull-down assays indicated that core interacts with AURKB, an interaction confirmed by co-IP assays using extracts of HuH7.5 cells previously transfected with HCV core (Fig. 2E and F). Supplementary Fig. 2 shows that the catalytic domain of AURKB and the first 122 aa of core are essential to establish this

interaction.

Alteration of cellular proliferation and viability after HCV infection and core expression

Since AURKB is essential for chromosome stability and cell division [12], we investigated the effect of AURKB inhibition induced by HCV in cell division. Uninfected and HCV infected mitotic cells were labelled with H3Ser10ph antibody. The number of dividing cells decreased upon infection by HCV (Fig. 3A). Both infected and uninfected cells steadily increased the proliferation rate in a similar manner up to 72 h post-infection; however, at 144 h post-infection, HCV infected cultures showed approximately 37% decrease in cell number, as compared to uninfected cells (Fig. 3B). Although cell viability was decreased, cells continued being metabolically active at least until 144 h post-infection, as judged by a high metabolic activity at 144 h (Fig. 3C). A similar effect on cell proliferation a small decrease in viability were obtained upon expression of HCV core (Fig. 3D and E). The overexpression of AURKB slightly increased basal HuH7.5 cell proliferation partially recovered the reduction in cell number induced by HCV infection (Supplementary Fig. 3B). On the other hand, ZM447939 treatment, reduced basal HuH7.5 cell proliferation and further decreased the cell number observed in HCV infected cells (Supplementary Fig. 3A), indicating that HCV decreases proliferation by additional mechanism. These results indicate that the inhibition of AURKB could be one of the mechanisms by which HCV decreases cell proliferation and viability.

Effect of HCV and core expression on NF- κ B and COX-2 transcriptional regulation

As HCV replication and core expression produced a decrease in cell proliferation and viability, it was interesting to analyse whether the NF- κ B pathway was affected. Five hours post-infection, HuH7.5 cells were transfected with a luciferase reporter plasmid that contains three copies of NF- κ B binding sites (3xNF- κ B-luc) (Fig. 4A). HuH7.5 cells and primary

hepatocytes were also transfected with core expression vectors, together with 3xNF-kB-luc (Fig. 4B). Noteworthy, luciferase reporter activity was lower in both HCV infected and core expressing cells (Fig. 4A and B). Since NF-kB is a major anti-apoptotic agent in the liver, levels of the active truncated and the inactive precursor forms of poly (ADP-ribose) polymerase (PARP), a specific caspase substrate, were measured. HCV infection induced PARP cleavage, indicating an increase in apoptosis with a decrease in NF-kB dependent transcription (Supplementary Fig. 4A).

AURKB presents a role in regulation of transcription by increasing H3Ser10 phosphorylation. Since core decreases AURKB activity, it was interesting to investigate whether the inhibition of AURKB expression abolished core-mediated repression of NF-kB transcription. Therefore, HuH7.5 cells were infected with HCV or transfected with core together with siRNA of AURKB (Fig. 4C). A 60% inhibition of AURKB mRNA and protein levels (Supplementary Fig. 4B) were sufficient to decrease significantly the NF-kB basal transcription, resulting in a reduction of the inhibitory effect of HCV or core on NF-kB transcriptional regulation (Fig. 4C). Likewise, treatment of HuH7.5 cells with ZM447439 reduced HCV and core inhibitory regulation of NF-kB transcription (Fig. 4D).

NF-kB, as well as the COX-2 gene, are involved in inflammation, anti-apoptosis and carcinogenesis [1]. The expression of endogenous COX-2 mRNA was decreased by HCV infection in HuH7.5 cells, and also after core expression in human primary hepatocytes (Fig. 5A). To confirm the effect of HCV in COX-2 transcription, HuH7.5 cells were transfected with the COX-2 promoter luciferase reporter plasmid (pCOX-2-luc). HCV infected cells showed decreased COX-2 transcription, compared to uninfected cells (Fig. 5B). COX-2 promoter activity was also decreased by the expression of core in both HuH7.5 cells and human primary hepatocytes (Fig. 5B). The depletion of AURKB expression (Fig. 5C)

decreased basal COX-2 transcription and abolished HCV- and core-induced repression, in agreement with the results obtained in NF- κ B-dependent transcription promoter assay (Fig. 4C).

This result, together with the decrease of H3Ser10ph protein levels, suggests that HCV may regulate COX-2 expression through epigenetic modifications. To test this possibility, we performed ChIP assays to examine whether HCV infection modified the H3Ser10ph bound to the COX-2 promoter region. We also tested histone H3 acetylation, an epigenetic marker associated with transcriptional activation. ChIP analyses revealed that the levels of H3Ser10ph and acetylated histone H3 bound to COX-2 promoter were reduced in infected HuH7.5 cells, coincidental with the inhibition of COX-2 transcription. As expected, core was not recruited to the promoter (Fig. 5D). Since H3Ser10ph is induced by AURKB, we also checked AURKB recruitment to the COX-2 promoter. AURKB was associated with the promoter in uninfected cells and this association was reduced in HCV infected cells. Similar experiments were performed in HuH7.5 cells transfected with core (Fig. 5E). The levels of acetylated and Ser10 phosphorylated H3, and AURKB were reduced in the promoter region as a result of core expression. These data suggest that HCV and core regulate transcription of important genes implicated in inflammatory response and that the regulation is mediated by AURKB.

Effect of AURKB activity on infectious progeny production

Due to the fact that transcription of genes involved in the inflammatory response was inhibited in the early stage of HCV infection, and was significantly reduced by AURKB inhibition, we examined whether the absence of AURKB expression modifies the production of infectious HCV. The specific infectivity of HCV increased by 66% at 72 h after silencing of AURKB ($p = 0.023$) (Fig. 6A). However, the overexpression of AURKB significantly decreased HCV specific infectivity by 57% ($p = 0.03$) (Fig. 6B). These results suggest that the core-AURKB

interaction may inhibit particle formation. In the same experiment we observed that AURKB transitory transfection was able to reverse the transcriptional inhibitory effect induced by HCV infection in COX-2 and stimulated NF- κ B-dependent gene transcription (Fig. 6C and D). These results indicate an important role of AURKB in the expression of both genes regulated by HCV infection.

Discussion

Liver fibrosis and HCC development have been related to accumulation of genetic and epigenetic defects, which can modify numerous signaling pathways that control cellular proliferation, survival, differentiation and angiogenesis [28]. Some specialized viral functions modulate epigenetic processes that promote viral genome propagation and host-cell survival [6]. The study of how epigenetic events are initiated and established after HCV infection is of special interest to establish whether these processes might contribute to HCV-related disease. Using the HCV-HuH7.5 cell culture system [3] and human hepatocytes we have documented that HCV infection and core expression reduce phosphorylation of Ser10 in histone H3. This observation is in agreement with previous results that suggest a block in mitotic entry after HCV infection [18] demonstrated by a decrease in H3Ser10ph. Transient transfection of core from different genotypes reduced H3Ser10ph at similar levels. Interestingly, the low expression levels of core 2a, in both HCV infected and core 2a transfected cells, produced a significant reduction in H3Ser10ph (Supplementary Fig. 1A). These results indicate that low infection states could drive to the repression of this epigenetic marker. Although AURKB inhibitor, ZM447439, abolished core-mediated inhibition of H3Ser10 phosphorylation, other HCV proteins might also display the capacity to repress H3Ser10 phosphorylation, although the overexpression of NS5A did not decrease the levels of total H3Ser10ph (Fig. 1C). AURKB activity at the centromere is increased by Chk1 [41]. In contrast, protein

phosphatases PP1 or PP2A interact with AURKB inhibiting its activity [37]. It has been reported that HCV infection significantly induces upregulation of PP2A [2], and that NS5B expression reduces the phosphorylation levels of Chk1 [39]. In spite of Chk1 and PP2A regulating AURKB activity, our results indicate that these activities are not responsible for inhibiting H3Ser10 phosphorylation mediated by core. Using protein-protein interaction assays, we have shown that core binds the catalytic domain of AURKB. This interaction provides an interpretation that AURKB activity is precluded as a consequence of HCV infection and core expression, supported by the decrease in the Thr-232 phosphorylation site of AURKB which is indispensable for the AURKB activity [40] accompanied by a decrease in H3Ser10ph levels.

Some viral infections induce inflammation, which could trigger fibrosis progression, cirrhosis and lastly development of HCC [25]. However, our results show that NF- κ B transcription is inhibited by HCV infection and core expression in human primary hepatocytes and in HuH7.5 cells, despite NF- κ B playing a role in the pro-inflammatory response [1]. Similarly, we have observed an inhibition of COX-2 gene transcription, despite COX-2 also playing an important role in inflammatory signaling.

In agreement with our results, DNA microarray analyses showed that expression of core represses pro-inflammatory signals in HepG2 cells [27]. In macrophages, HEK293 and HeLa cells, core also suppressed NF- κ B activation and COX-2 expression by direct interaction with IKK- β [17].

Aberrant expression of AURKB is associated with tumor invasion and intrahepatic metastasis of HCC, leading to chromosomal instability and imbalance of multiple tumor suppressor and oncogene regulated pathways [22]. One of the most relevant results in our study is that AURKB is also a target of HCV during the infection process. It is known that the absence or

dysregulation of AURKB can give rise to chromosome instability and aneuploidy [12]. However, the effect of HCV infection in chromosomal alterations warrants additional studies. It may also be significant that the oncogenic potential of Epstein-Barr virus has been related with AURKB activity, although the underlined molecular mechanisms are different from those identified until now in our study [15].

ChiP experiments revealed that HCV core triggers epigenetic silencing of COX-2 gene promoter, thereby reducing levels of AURKB and H3Ser10ph which could be due to the core-AURKB interaction (Fig. 5D and E). In this context, the inhibition of AURKB activity could be viewed as a viral mechanism to inhibit pro-inflammatory gene transcription, because the overexpression of AURKB reverses the inhibitory effect of HCV and core on both NF- κ B and COX-2 transcription. These results agree with previous reports that demonstrated that AURKB is not only a mitotic kinase but can also play a role as a transcription regulator [14,35,38]. AURKB abrogation also decreases the basal transcription of both NF- κ B and COX-2 luciferase reporters (Figs. 4C and 5C). These results suggest a positive regulatory effect of AURKB on NF- κ B and COX-2 transcription, a point that requires further investigation.

Several reports have emphasized the relationship between viral infection and replication rate with the inflammatory response where activation of NF- κ B is essential to counteract several bacterial and viral infections including HCV infection [21,24,34]. If AURKB overexpression has the capacity to reactivate the pro-inflammatory pathway and to decrease the specific infectivity of HCV, the inhibition of AURKB activity mediated by core can be a mechanism to increase HCV infectivity as we have demonstrated by AURKB silencing assays. Supporting our results, two recent reports have emphasized the importance of other epigenetic activities as key modulators of the viral infectivity [5,10]. Taken together, our

results show a new mechanism by which HCV induces epigenetic changes by direct interaction between core and AURKB. The inhibition of AURKB activity increases HCV infectivity through modulation of pro-inflammatory genes. In summary, AURKB activity regulation could be a novel HCV strategy to ensure the persistence of viral infection.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

Data acquisition, experimental design and analysis, interpretation of data and critical revision of the manuscript: A. Madejón and J. Sheldon. Data acquisition: I. Francisco-Recuero, M. Dominguez-Beato and J. Muntané: Analysis, interpretation of data and critical revision of the manuscript: E. Domingo, J. Garcia-Samaniego and C. Perales. Critical revision of the manuscript: M. Lasa, I. Sanchez-Perez.

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Figure legends

Fig. 1. HCV infection decreases H3Ser10 phosphorylation. (A) Western blot assays of the levels of H3Ser10ph, H3K9me2, H3K9me3, H3K4me3 and core in HCV infected cells. H3 antibody represents the loading control. (B) After pCMV0 (M) or pCMV-core expression of different genotypes in HuH7.5 cells, H3Ser10ph levels and core protein expression were detected by specific antibodies. (C) H3Ser10ph and NS5A levels after expression of CMV-NS5A of different genotypes. (D) H3Ser10ph and core expression tested in human hepatocytes transfected with core. Band intensities from three different assays were quantified and represented as fold induction relative to the control. Protein extracts were prepared 72 h after HCV infection or core transfection.

Fig. 2. HCV infection decreases AURKB auto-phosphorylation by direct interaction between core and AURKB. (A) *AURKB* mRNA levels by qRT-PCR in HCV infected or core transfected cells for 72 h. (B) Western blot assay of phosphorylated AURKB (pAURKB), total AURKB and H3Ser10ph protein levels in HCV infected and core expressing cells. (C) NS5A, AURKB, H3Ser10ph and H3 levels in HCV infected cells treated with ZM447439. (D) H3Ser10ph levels after 72 h of core expression and ZM447439 treatment in HuH7.5 cells. (E) GST-AURKB or GST-0 fusion proteins interaction with S³⁵-labelled core 1b and 2a. (F) 2 mg of protein from core transfected HuH7.5 cells were immunoprecipitated with AURKB or IgG antibodies. Co-immunoprecipitated proteins were analysed by Western blot.

Fig. 3. HCV infection decrease cell proliferation and viability. (A) After 72 h of HCV infection, the total number of cells were labelled with Dapi and the

dividing cells visualised by immunofluorescence staining with H3Ser10ph. 1000 inter- phase and mitotic cells were counted from three independent experiments and the mitotic index expressed in percentage was represented. (B and D) The number of cells per well were counted at indicated times in control cells and HCV infected or core transfected HuH7.5 cells. (C and E) Optical density at 570 nm obtained by MTT cleavage in metabolically active HCV infected and core transfected HuH7.5 cells. Statistical analyses were performed from three independent experiments.

Fig. 4. HCV and core inhibit NF-kB-stimulated transcription and AURKB expression activity inhibition abolishes this effect. (A) NF-kB response element (3xNF-kB- Luc) was evaluated in HCV infected HuH7.5 cells. (B) The transcriptional activity of 3xNF-kB-Luc was measured in the presence of core protein in HuH7.5 cells (left panel) and in primary hepatocytes cultures (right panel). (C) Luciferase activity of the 3xNF-kB-Luc plasmid was evaluated after AURKB depletion in HCV infected (left panel) and core expressing HuH7.5 cells (right panel). (D) 3xNF-kB transcriptional activity was evaluated in HCV infected (left panel) or in core expressing HuH7.5 cells (right panel) in the absence or presence of 2 μ M ZM447439. Statistical analyses were performed from at least three independent experiments.

Fig. 5. HCV and core inhibits COX-2 transcription. (A) *COX-2* mRNA levels measured by qRT-PCR in HCV infected cells and in human primary hepatocyte cultures expressing core. (B) COX-2-Luc reporter activity was measured in HCV infected HuH7.5 cells and in human primary hepatocyte cells 72 h after transfection with pCMV-Core. (C) COX-2-Luciferase activity was measured in HCV infected and core expressing cells after AURKB depletion. (D, E) ChIP assays with the

antibodies indicated in HCV infected or core transfected HuH7.5 cells. The immunoprecipitated -230 bp DNA sequence in the COX-2 promoter was quantified by qPCR and visualised in agarose gels. Bars represent the mean \pm S.D. (n > 3).

Fig. 6. AURKB expression regulates HCV extracellular infectivity. (A) AURKB was depleted by transient transfection with siAURKB (150 nM) and the HCV specific infectivity was evaluated by titration from two experiments, with a total number of 13 samples per group. (B) Virus specific infectivity was evaluated by titration after AURKB overexpression from four experiments with a total number of 17 samples per group. (C, D) COX-2-Luc or 3xNF-kB-Luc vectors were transfected in HCV infected HuH7.5 cells together with AURKB (pcDNA3-FLAG-AURKB) expression vector. Luciferase activity was measured. Statistical analyses were performed from three independent experiments.

Figure 1

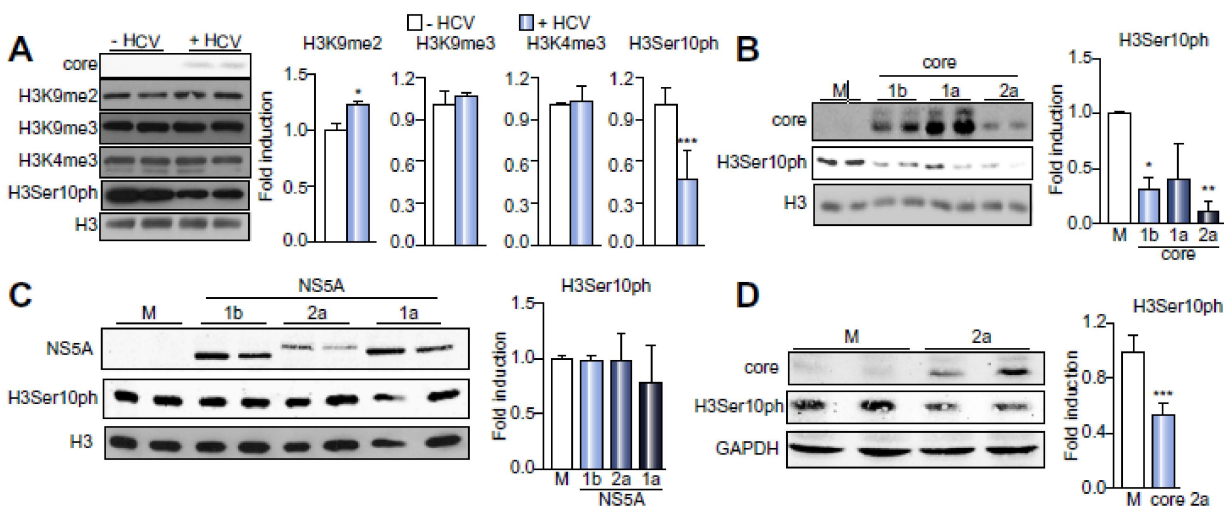


Figure 2

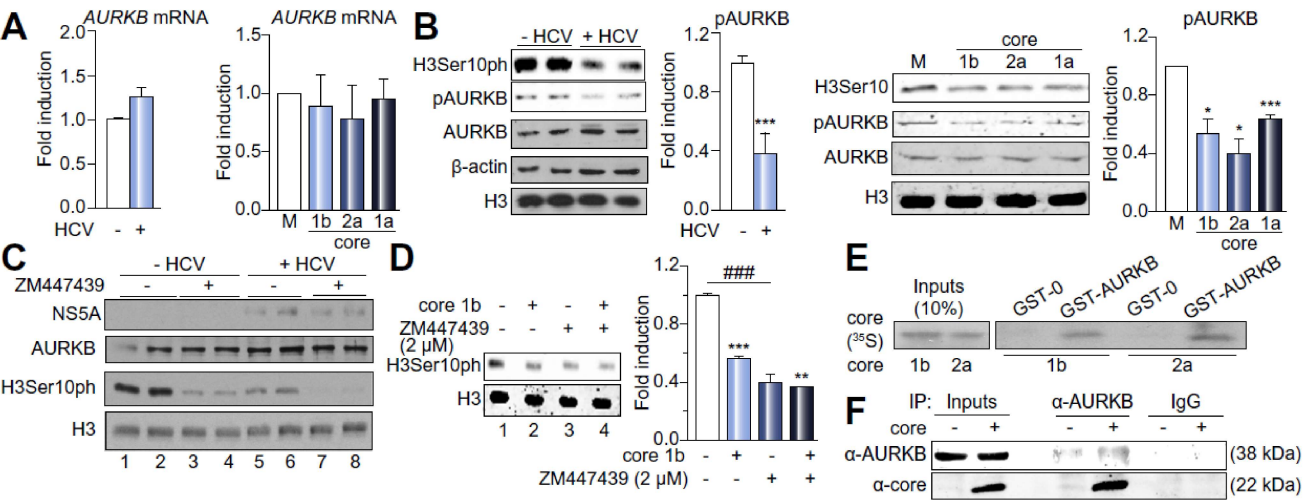


Figure 3

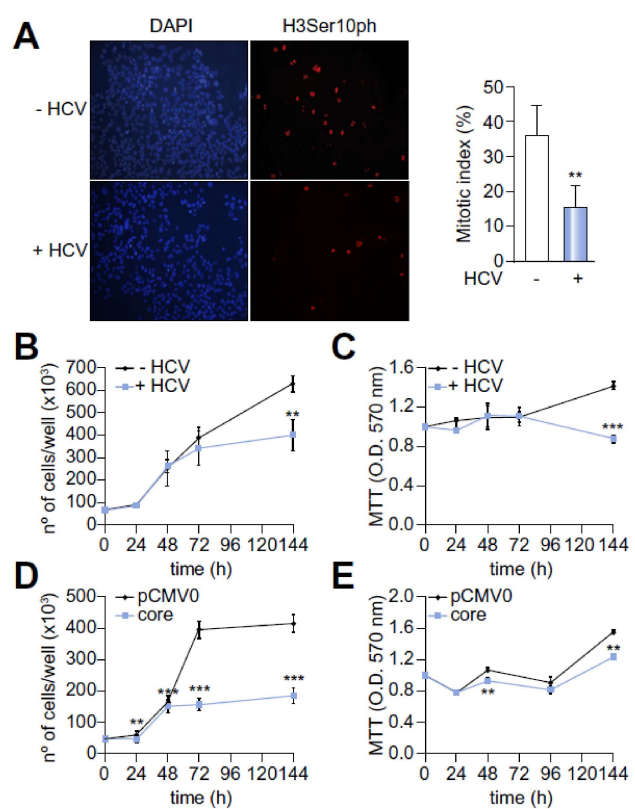


Figure 4

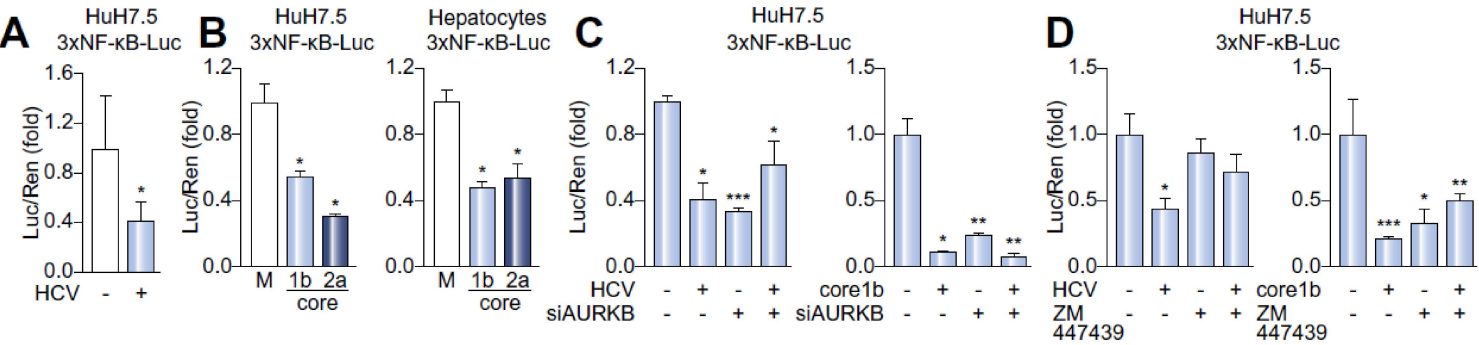


Figure 5

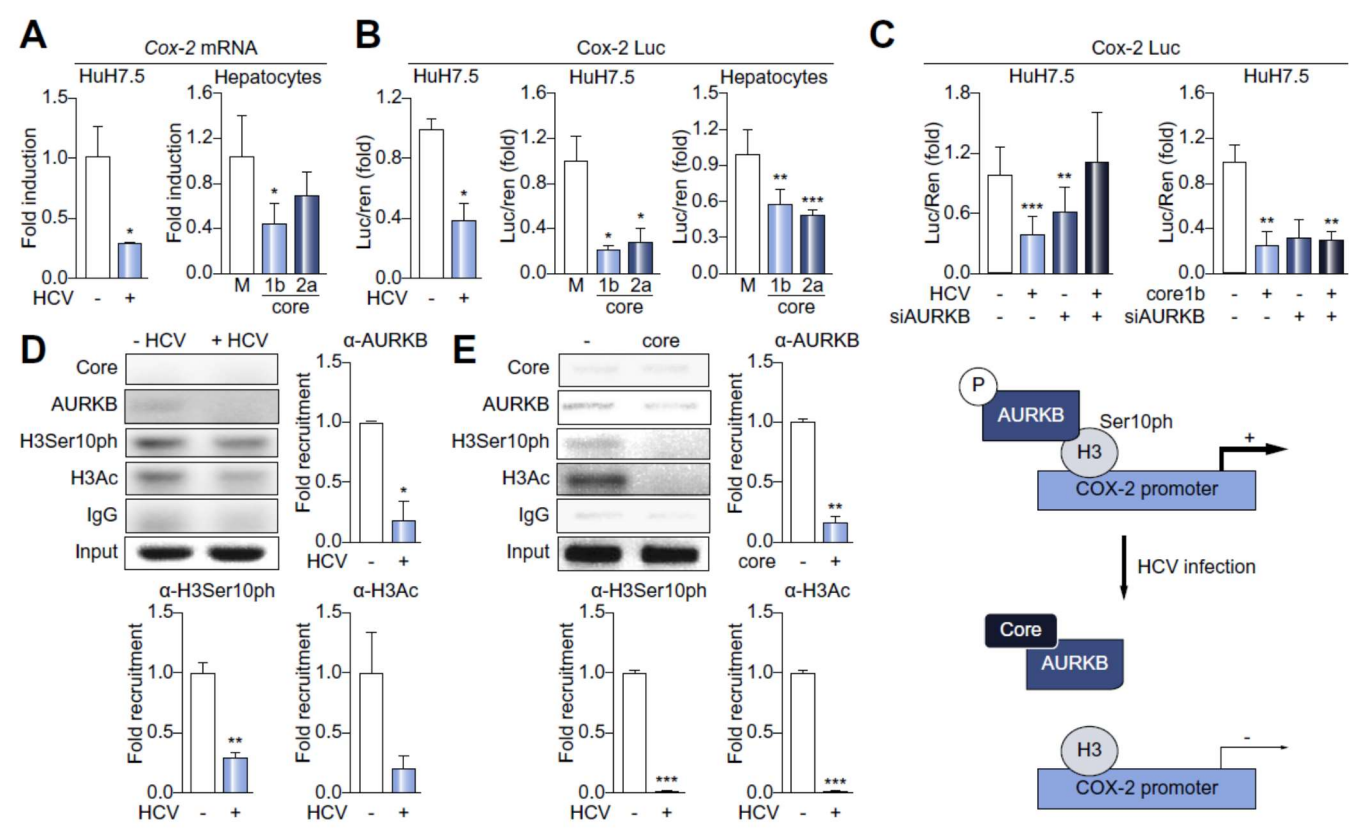


Figure 6

