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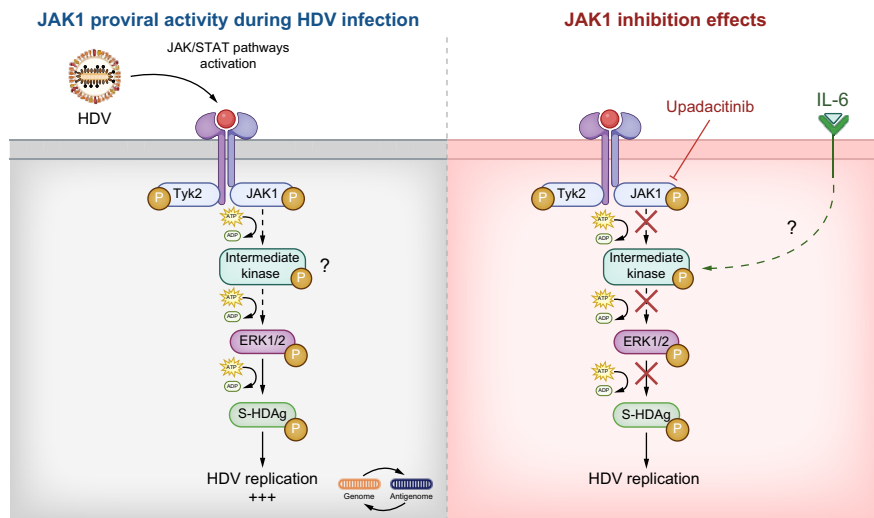
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Graphical abstract



Highlights

- JAK1 exhibits an unexpected proviral activity during HDV infection in various *in vitro* models, including primary human hepatocytes.
- JAK1 proviral activity is independent of the MDA5-mediated innate immune response and the activation of STAT3.
- JAK1 depletion is associated with inhibition of HDV replication via modulation of ERK1/2 and S-HDAg phosphorylation levels.
- FDA-approved JAK1-specific inhibitors such as upadacitinib are efficient antivirals for the treatment of hepatitis D.

Impact and implications

Chronic hepatitis D is the most aggressive form of chronic viral hepatitis. As no curative treatment is currently available, new therapeutic strategies based on host-targeting agents are urgently needed. Here, using loss-of-function strategies, we uncover an unexpected interaction between JAK1, a major player in the innate antiviral response, and HDV infection. We demonstrated that JAK1 kinase activity is crucial for both the phosphorylation of the delta antigen and the replication of the virus. By demonstrating the antiviral potential of several FDA-approved JAK1 inhibitors, our results could pave the way for the development of innovative therapeutic strategies to tackle this global health threat.

JAK1 promotes HDV replication and is a potential target for antiviral therapy

Margaux J. Heuschkel¹, Charlotte Bach¹, Laura Meiss-Heydmann¹, Emma Gerges¹, Emanuele Felli², Fabio Giannone², Patrick Pessaux², Catherine Schuster¹, Julie Lucifora³, Thomas F. Baumert^{1,2,4}, Eloi R. Verrier^{1,*}

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Background & Aims: Chronic co-infection with HBV and HDV leads to the most aggressive form of chronic viral hepatitis. To date, no treatment induces efficient viral clearance, and a better characterization of virus-host interactions is required to develop new therapeutic strategies.

Methods: Using loss-of-function strategies, we validated the unexpected proviral activity of Janus kinase 1 (JAK1) – a key player in innate immunity – in the HDV life cycle and determined its mechanism of action on HDV through various functional analyses including co-immunoprecipitation assays.

Results: We confirmed the key role of JAK1 kinase activity in HDV infection. Moreover, our results suggest that JAK1 inhibition is associated with a modulation of ERK1/2 activation and S-HDAg phosphorylation, which is crucial for viral replication. Finally, we showed that FDA-approved JAK1-specific inhibitors are efficient antivirals in relevant *in vitro* models including primary human hepatocytes.

Conclusions: Taken together, we uncovered JAK1 as a key host factor for HDV replication and a potential target for new antiviral treatment.

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Introduction

Chronic hepatitis D is the most severe form of chronic viral hepatitis and is caused by HDV, a small defective hepatotropic virus identified in 1977.¹ This satellite virus of HBV, the leading cause of hepatocellular carcinoma (HCC) worldwide, is characterized by a small genome that does not encode its own envelope proteins and is dependent on the expression of hepatitis B surface antigens (HBsAg) for the completion of its replicative cycle and the secretion of infectious viral particles.² Hepatitis D is therefore the result of either HBV and HDV co-infection or HDV/HBV superinfection in patients chronically infected with HBV.³ At least twenty-five million people are chronically coinfecting with HBV and HDV⁴ but this number remains unclear and fluctuates between studies, some of them estimating the number of chronically HDV-infected patients at seventy-two million individuals worldwide with a variable and increasing prevalence across regions, especially in developed countries, despite the availability of a preventive HBV vaccine that also protects against HDV infection.^{5,6}

Although recent progress has been made in the understanding of the molecular virology of HDV, therapeutic options for chronic hepatitis D are still limited. Current antiviral

treatments against HBV infection are generally not effective against HDV, and the absence of a HDV-specific polymerase complicates attempts to identify appropriate therapeutic targets. For decades, the main treatment consisted of the use of pegylated-interferon alpha (PEG-IFN- α) but only a limited number of patients achieve a sustained response, which is the foundation of eventual improvement in related liver fibrosis.⁷ In this context, host-targeting agents, which target cellular factors that are essential for the viral cycle, have been successfully explored for the development of new antivirals. This is exemplified by the development of the entry inhibitor bulevirtide, which received approval for medical use in the European Union in July 2020.⁸ While the main steps of the HDV life cycle have been characterized, notably through the development of adapted study models based on NTCP-expressing cells,⁹ the underlying molecular mechanisms of hepatocyte-HDV interactions still need to be more deeply investigated.

HDV is a small spherical virus that possesses a lipidic envelope in which the three HBsAg (L-, M-, and S-) are anchored. This envelope contains the ribonucleoprotein complex that includes the viral genome associated with the two isoforms of the hepatitis delta antigen (HDAg), S-HDAg for the short form and

Keywords: Hepatitis D virus; host factor; replication; antiviral treatment; HDAg; kinase.

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L-HDAg for the long form.¹⁰ These two viral proteins undergo numerous modifications and play a key role in the viral replication cycle. Notably, phosphorylation of S-HDAg is important for viral replication while farnesylation of L-HDAg is essential to negatively regulate replication and promote the assembly of new virions.^{2,10}

HDV is a non-cytopathic virus strongly activating the type I and III interferon (IFN) response upon recognition of viral RNA in the cytoplasm by the cytosolic sensor melanoma differentiation associated gene 5 (MDA5).¹¹ This recognition leads to increased expression of IFN-stimulated genes (ISGs), such as *MX2* and radical S-adenosyl methionine domain containing 2 (*RSAD2*), thus contributing to the inhibition of HBV replication in coinfecting hepatocytes.^{12,13} The virus seems sensitive to a limited degree to this endogenous response,¹¹ while stages in the early life cycle of HDV are moderately affected by exogenous type I IFN.^{11,14,15} In addition to the interaction of viral RNA with the RNA sensing machinery, L-HDAg has been suggested

to activate the transcription factors NF- κ B and signal transducer and activator of transcription (STAT)3 through oxidative stress,¹⁶ putatively contributing to the persistence of the virus in hepatocytes.¹⁷

In the context of a complex interaction between HDV and innate immune factors and the need of new antiviral targets, we previously performed a loss-of-function screen using pools of four small-interfering RNAs (siRNAs) targeting more than 7,500 genes belonging to the druggable genome and identified 191 candidates potentially important for the HDV cycle. Among the primary candidates were key members of the pyrimidine biosynthesis pathway but also, surprisingly, Janus kinase 1 (JAK1).¹⁸ Although JAK1 plays a key role in the innate immune response against viral infection,¹⁹ we observed an unexpected proviral activity of this kinase in a bona fide infection system. The aim of this study was the validation of JAK1 as a key host factor in HDV infection and the evaluation of its potential as an antiviral target for innovative therapies against HDV.

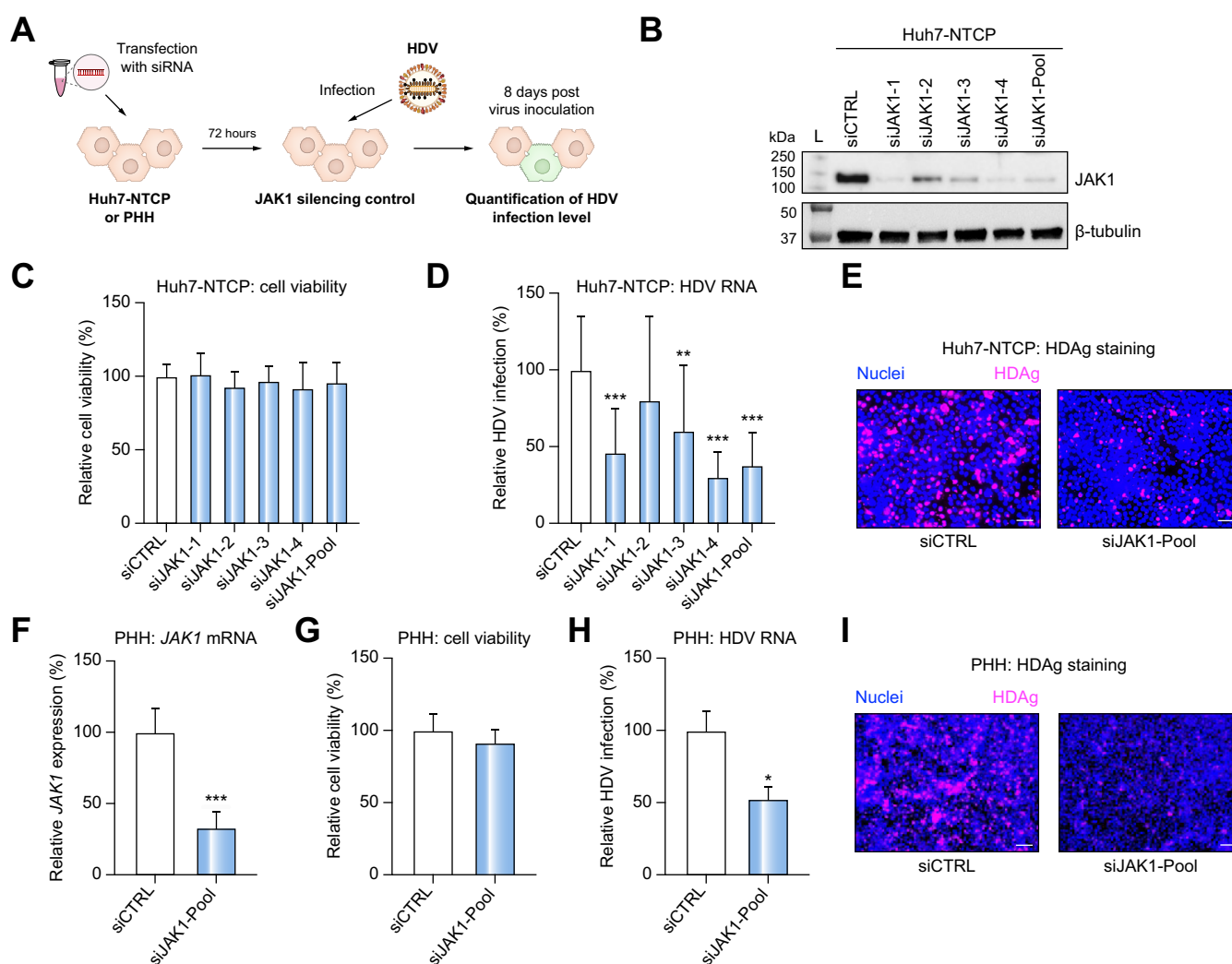


Fig. 1. JAK1-KD is associated with decreased HDV infection. (A) Experimental strategy. (B-E) JAK1-KD in Huh7-NTP cells. JAK1 expression was controlled by WB from one representative experiment 72 h after siRNA transfection (B). (C-E) Cells were then infected by HDV for 8 days, cell viability was assessed (C), and HDV infection was quantified by RT-qPCR from six independent experiments. Alternatively, (E) HDV infection was assessed by IF. (F-I) JAK1-KD inhibits HDV infection in PHHs. JAK1-KD was assessed by RT-qPCR (F), and cell viability (G) and HDV infection (H-I) were assessed after 8 days of infection as described above from four independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney U test). Scale bar: 100 μ m. IF, immunofluorescence; KD, knockdown; PHHs, primary human hepatocytes; RT-qPCR, reverse-transcription quantitative PCR; siRNA, small-interfering RNA.

Materials and methods

Materials and methods are fully presented in the CTAT table and supplementary information.

Results

Validation of JAK1 as a cellular host factor for HDV infection

To confirm the results from the primary screen,¹⁸ we first performed the deconvolution of the siRNA pool targeting *JAK1* expression prior to HDV infection in Huh7-NTCP cells. As shown in Fig. 1A-E, the silencing efficacy of the four individual siRNAs correlated with the inhibition of HDV infection. The inhibitory impact of *JAK1* knockdown (KD) on HDV infection when the siRNA pool was transfected after viral inoculation further suggests a role for JAK1 in the replication of the virus (Fig. S1). We next validated this result by knocking down *JAK1*

expression in primary human hepatocytes (PHHs) and observed a significant inhibition of HDV infection associated with a decrease in *JAK1* expression in siJAK1-Pool-transfected PHHs (Fig. 1F-I). We next engineered a stable Huh7-NTCP-derived *JAK1* knockout (KO) clone named Huh7-NTCP-JAK1-KO, and two derived cell lines named JAK1^{res} and JAK1^{mut}, expressing a wild-type version of JAK1 resistant to the single-guide RNA used to produce the KO cell line or a mutant version of the protein unable to bind ATP²⁰ carrying L633 K and K622A mutations in the pseudo-kinase domain, respectively (Fig. 2A). The previously described Huh7-NTCP-YBX1-KO cell line²¹ was used as a control as the loss of YBX1 (Y-box binding protein 1) protein expression had no effect on HDV infection. To assess the kinase activity of JAK1 in these cell lines, cells were stimulated with IFN- α -2a and the level of phosphorylated STAT1 and STAT3 (pSTAT1 and pSTAT3) proteins was analyzed by western blot. As shown in Fig. 2B, both JAK1-KO cells and JAK1^{mut} cells were unable to phosphorylate STAT1 and STAT3

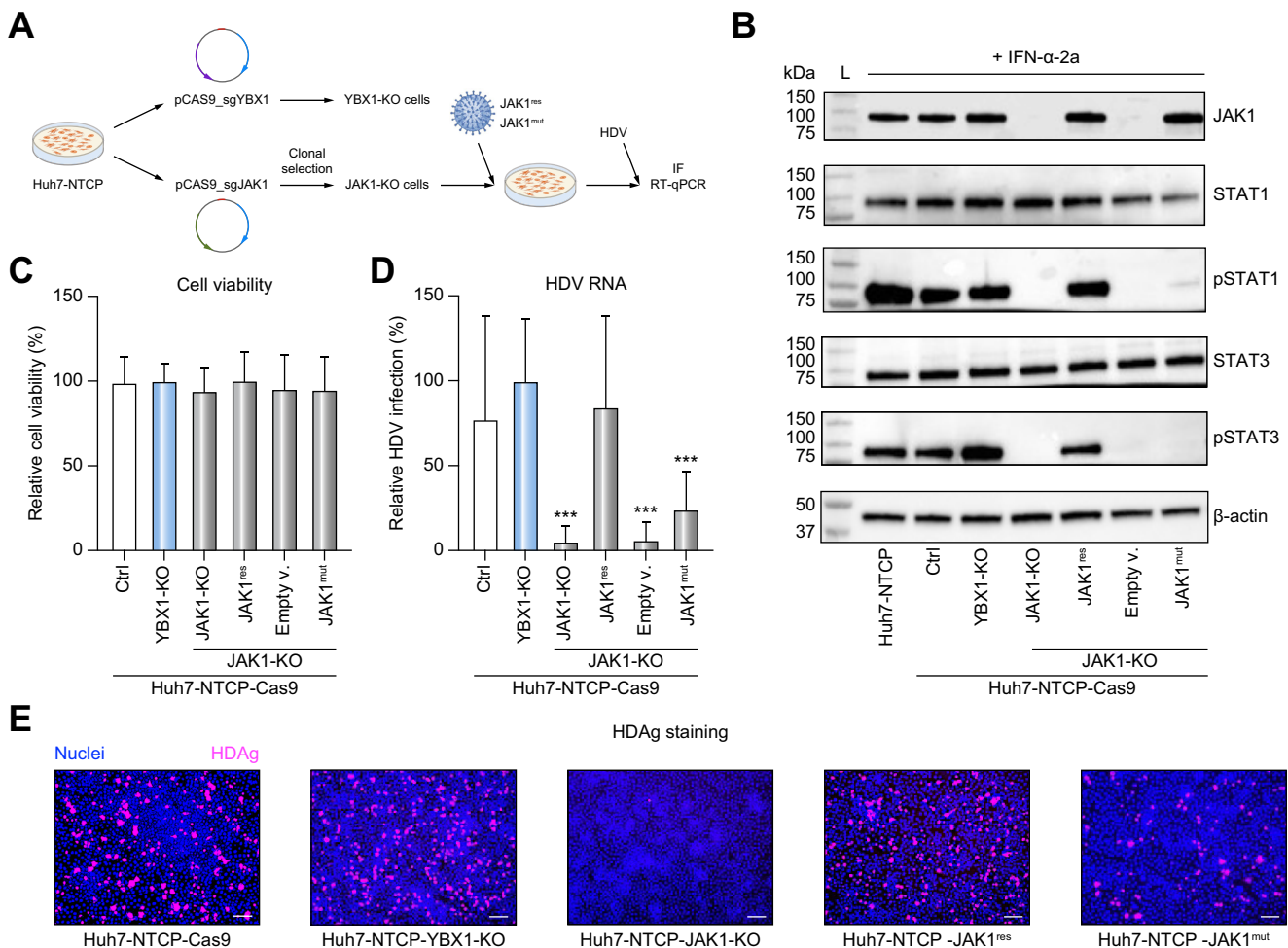


Fig. 2. JAK1 is a key host factor for HDV infection. (A) Generation of the cell lines. (B) Cells were stimulated with IFN- α -2a for 30 min. Expression levels were assessed by western blot. One representative experiment is shown. (C-E) JAK1 kinase activity is crucial for HDV infection. Cells were infected with HDV at 100 Vge/cell. After 8 days, cell viability was controlled (C) and HDV infection was assessed by RT-qPCR quantification of HDV RNA (D). The results from four independent experiments are presented. Alternatively, HDV infection was assessed by IF (E). One representative experiment is shown. ****p* < 0.001 (Mann-Whitney *U* test). Scale bar: 100 μ m. IF, immunofluorescence; KO, knockout; RT-qPCR, reverse-transcription quantitative PCR.

upon IFN- α -2a stimulation. To validate the role of JAK1 in the viral life cycle, these cell lines were then infected with HDV. HDV infection level was strongly reduced in JAK1-KO cells while the infectious phenotype was rescued in the JAK1^{res} cell line (Fig. 2C-E). Furthermore, no rescue was observed in JAK1^{mut} cells, thus demonstrating the key role of JAK1 kinase activity in the HDV life cycle. To test the impact of clonal selection on cellular susceptibility to HDV infection, Huh7-NTCP-Cas9 and JAK1-KO cells were differentiated for 1 week in DMSO-containing medium (to induce the susceptibility to HBV infection). As shown in Fig. 3B, DMSO-mediated differentiation had no impact on JAK1 expression levels. Cells were then infected with HBV or HDV. As shown in Fig. 3C-F, HDV infection was markedly reduced in JAK1-KO cells compared to control cells while HBV infection level remained similar between the two cell lines, suggesting a comparable susceptibility to HBV and HDV entry in JAK1-KO and control

cells. Taken together, we demonstrated the key role of JAK1 kinase activity in HDV replication.

JAK1 inhibitors exhibit antiviral activity against HDV infection

To investigate the potential of JAK1 as an antiviral target, we tested an FDA-approved JAK1-inhibitor, upadacitinib, used for the treatment of rheumatoid arthritis,²² in comparison with a JAK2-specific inhibitor, fedratinib, another FDA-approved molecule notably used for the treatment of myelofibrosis.²³ Interestingly, only upadacitinib inhibits the phosphorylation of STAT1 and STAT3 upon IFN- α -2a stimulation in Huh7-NTCP cells (Fig. 4A), suggesting no impact of fedratinib on JAK1 activity at the tested concentrations. We next assessed the effect of these compounds on HDV infection by treating cells with the inhibitors 24 h prior to viral inoculation and for 8 days

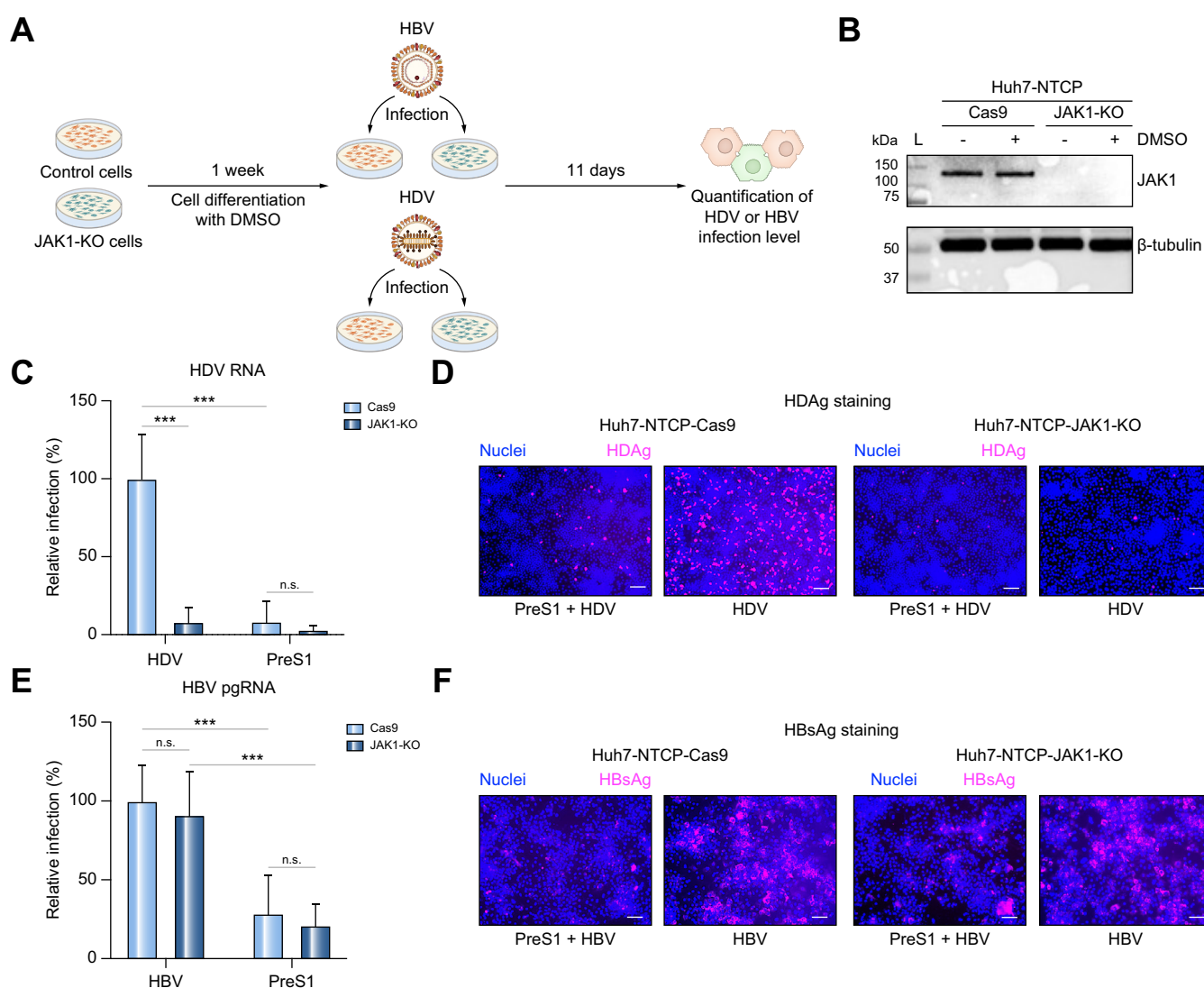


Fig. 3. JAK1-KO does not affect viral entry. (A) Experimental strategy. (B) Control of JAK1 expression by western blot. (C-D) JAK1-KO inhibits HDV infection. HDV infection was quantified by RT-qPCR from three independent experiments (C) or by IF (D). (E-F) JAK1-KO does not affect HBV infection. HBV infection was assessed by RT-qPCR from three independent experiments (E) or by IF (F). *** $p < 0.001$ (Mann-Whitney U test). Scale bar: 100 μ m. IF, immunofluorescence; KO, knockout; pgRNA, pregenomic RNA; RT-qPCR, reverse-transcription quantitative PCR.

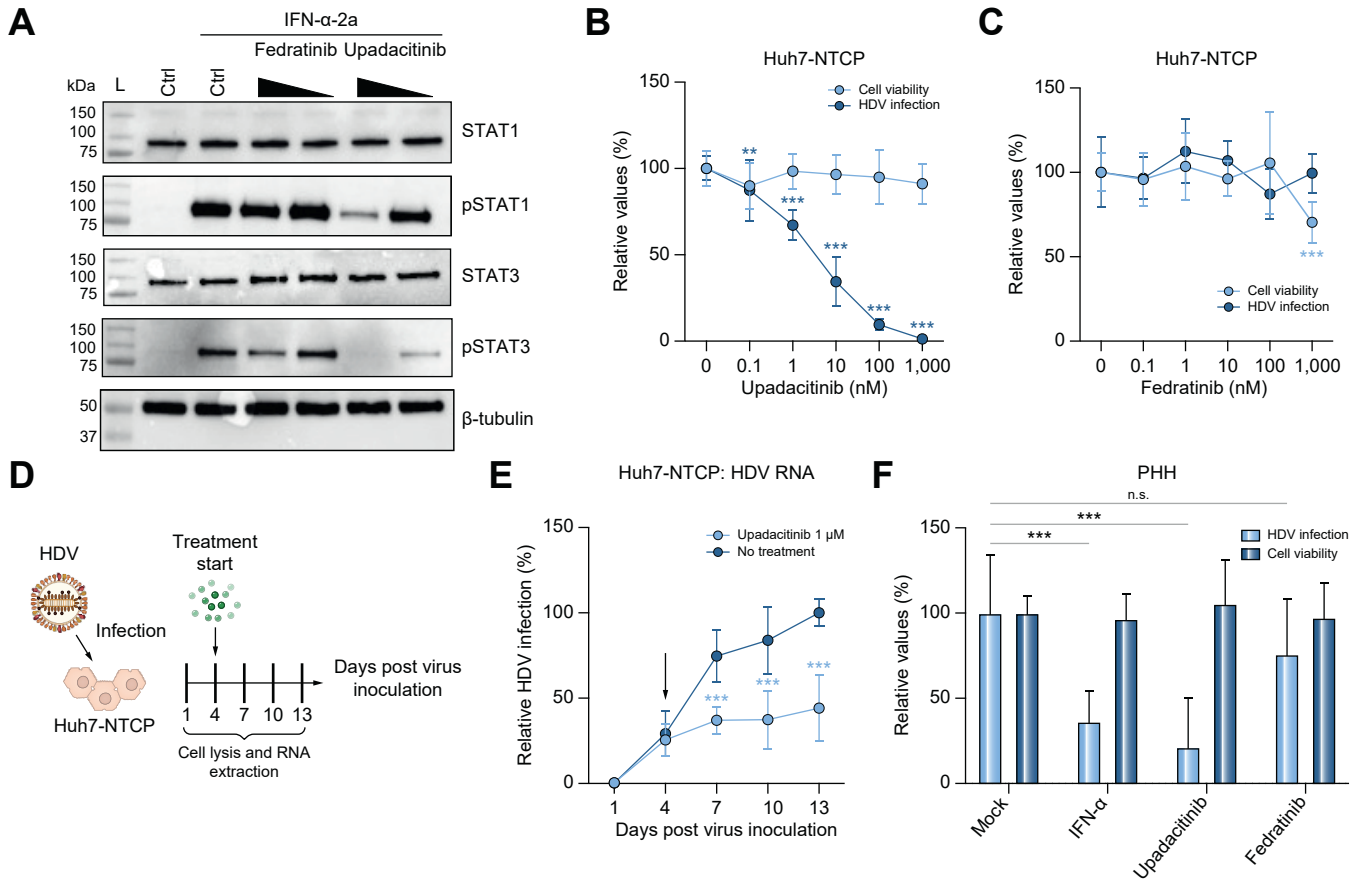


Fig. 4. Upadacitinib inhibits HDV replication. (A) STAT1 and STAT3 phosphorylation levels upon 30 min treatment with IFN- α -2a and either upadacitinib or fedratinib (100 nM or 1 μ M) were assessed by western blot. (B–C) Antiviral activity of JAK inhibitors. Cell viability and HDV infection were assessed after 8 days in infected Huh7-NTCP pretreated for 24 h with increasing doses of either upadacitinib (B) or fedratinib (C) from four independent experiments. Treatment duration: d(-)1-8. (D–E) Upadacitinib inhibits HDV infection after initiation of viral replication. Huh7-NTCP cells were infected with HDV and treated with 1 μ M upadacitinib after inoculation. Treatment duration: d4-13 after virus inoculation. HDV infection was assessed by RT-qPCR after 1, 4, 7, 10 and 13 days from three independent experiments. (F) Upadacitinib inhibits HDV infection level in PHHs. PHHs were infected with HDV and treated with 1 μ M upadacitinib or fedratinib. Treatment duration: d1-8. After 8 days, HDV infection was assessed by RT-qPCR from four independent experiments. *** p < 0.001, ** p < 0.01 (Mann-Whitney U test). PHHs, primary human hepatocytes; RT-qPCR, reverse-transcription quantitative PCR.

of infection. As shown in Fig. 4B,C, only upadacitinib dose-dependently inhibited HDV infection by contrast with fedratinib. Moreover, upadacitinib treatment at various stages post viral inoculation dose-dependently inhibits HDV infection levels (Fig. S2A–C). This is notably illustrated by the marked inhibition of viral replication by 1 μ M upadacitinib administered 3 days post infection (Fig. 4D,E), suggesting an impact on the replication of the virus even after its initiation. To eliminate any non-specific effect in the observed antiviral activity of upadacitinib, Huh7-NTCP-Cas9, JAK1-KO, and JAK1^{Res} cells were infected with HDV (Fig. S2D,E) and treated with increasing doses of upadacitinib. Upadacitinib dose-dependently inhibited HDV infection in the control and the JAK1^{Res} cells. By contrast, upadacitinib treatment had no effect on the relative levels of HDV infection observed in JAK1-KO cells, thus confirming that upadacitinib’s antiviral activity against HDV infection was JAK1 dependent. Interestingly, the antiviral potential of upadacitinib was also validated in HDV-infected PHHs (Fig. 4F). Of note, two other JAK1-specific inhibitors, oclacitinib and filgotinib (Fig. 5A), also inhibited HDV infection in both HDV-infected Huh7-NTCP cells (Fig. 5B) and PHHs (Fig. 5C,D). Finally, since JAK/STAT signaling pathways are known to be modulated by

several viruses,^{19,24} we evaluated the impact of JAK1 inhibition in additional infection models. As shown in Fig. S3, contrary to its effect on HDV infection, upadacitinib treatment exhibits no antiviral activity against HCV, HBV, nor SARS-CoV-2, suggesting a specific interaction between HDV and the kinase. Taken together, our results highlight the specific antiviral potential of JAK1 inhibitors on HDV infection.

JAK1 proviral activity is independent of the MDA5-mediated innate immune response and the activation of STAT3

JAK1 plays a key role in the transduction of the type I IFN signal. In this context, HDV RNA is recognized by the innate immune sensor MDA5 leading to the activation of the type I and III IFN pathways and the JAK1-mediated induction of ISG expression.¹¹ We first hypothesized that the proviral activity of JAK1 may be linked to the induction of proviral ISG upon infection, like MOV10, known to be involved in the HDV life cycle.²⁵ Thus, we first aimed to assess the innate immune response after HDV infection in two cellular models, Huh7-NTCP and HepG2-NTCP cells, the latest being known to express ISG upon HDV infection.¹¹ *RSAD2* was selected as the

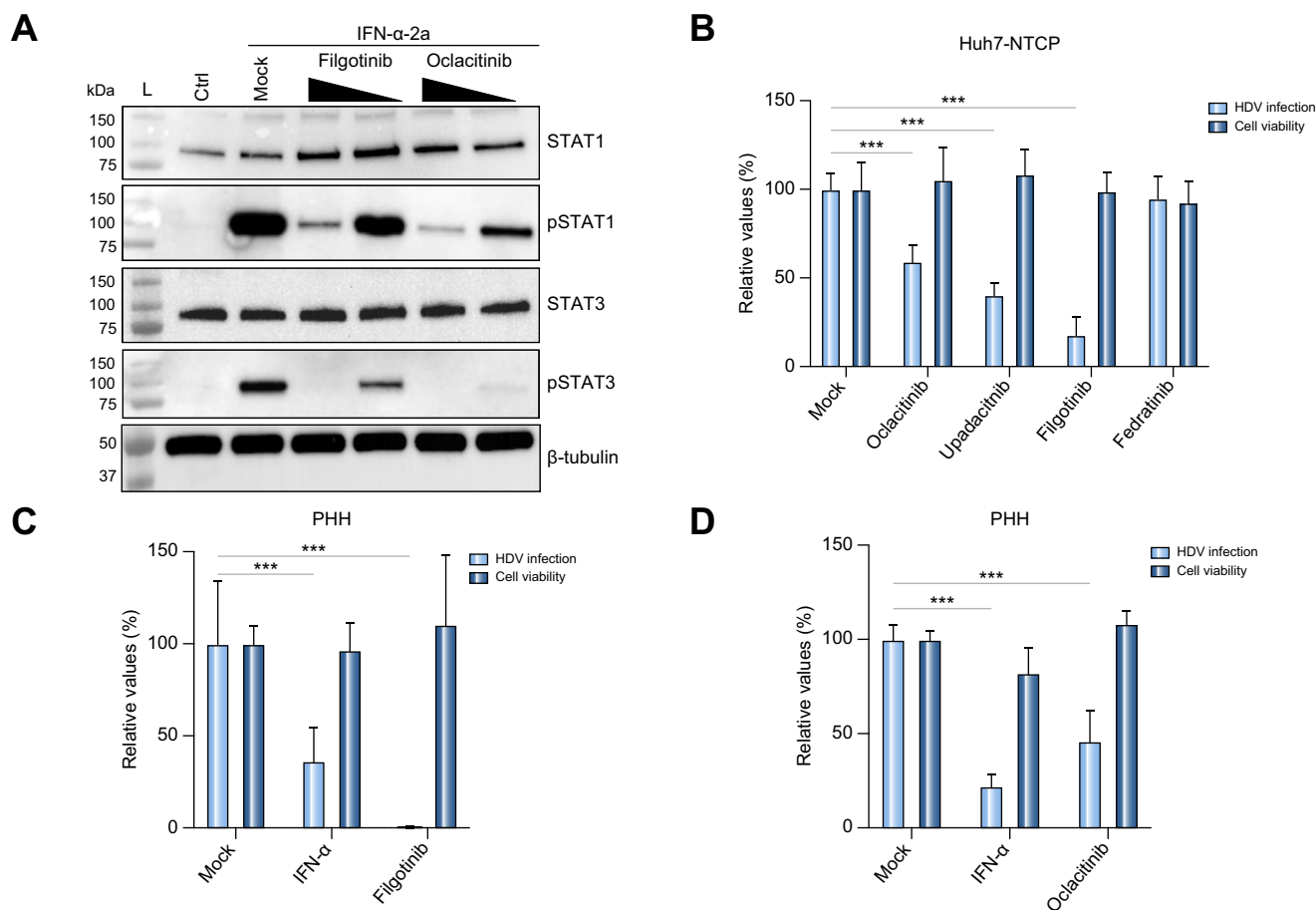


Fig. 5. JAK1 inhibitors specifically inhibit HDV infection. (A) STAT1 and STAT3 phosphorylation levels upon 30 min treatment with IFN- α -2a and either filgotinib or oclacitinib (100 nM or 1 μ M) were assessed by western blot. (B) Cells were infected with HDV and treated with the different inhibitors at 1 μ M. HDV infection was quantified by RT-qPCR after 8 days from four independent experiments. (C-D) PHHs were infected with HDV and treated with 1 μ M of inhibitor or with IFN- α -2a as a control. Treatment duration: d1-8. HDV infection was quantified by RT-qPCR from four (filgotinib, panel C) and three (oclacitinib, panel D) independent experiments. *** p < 0.001 (Mann-Whitney U test). PHHs, primary human hepatocytes; RT-qPCR, reverse-transcription quantitative PCR.

most upregulated ISG upon HDV infection *in vitro*.¹³ As shown in Fig. 6A,B, no induction of *RSAD2* expression was observed in Huh7-NTCP cells in contrast to HepG2-NTCP cells, confirming a previous result suggesting no type I IFN response upon HDV infection in Huh7-NTCP cells.¹¹ Interestingly, *JAK1*-KD in HepG2-NTCP cells is also associated with inhibition of HDV infection (Fig. 6C-F) despite a differential innate immune activation compared to Huh7-NTCP cells. In the same manner, upadacitinib inhibits HDV infection in HepG2-NTCP with an IC_{50} lower than 1 nM (Fig. S4A), suggesting the existence of an innate immunity-independent proviral activity of JAK1. We next aimed to validate this observation in a more relevant model for innate immune pathways and took advantage of differentiated HepaRG (dHepaRG) cells, with *IFIH1* (encoding MDA5) KO.¹³ We first confirm the importance of MDA5 for the expression of ISG upon HDV infection (Fig. 6G). Interestingly, upadacitinib treatment in HDV-infected dHepaRG wild-type cells and dHepaRG-MDA5-KO cells revealed a comparable antiviral activity of this inhibitor (Fig. 6H), reinforcing the hypothesis of an independence between JAK1 proviral activity and the HDV-induced innate immune response. Thus, we hypothesized that the proviral activity of JAK1 may be associated with STAT3 activation, as it was previously demonstrated that L-HDAg can

activate STAT3 through oxidative stress independently of the IFN-mediated response.¹⁶ Although we confirmed STAT3 activation in HDV-infected Huh7-NTCP cells and to a lesser extent in HDV-infected HepG2-NTCP cells (Fig. S4B,C), STAT3 activation was no longer observed in HDV-infected dHepaRG-MDA5-KO cells (Fig. 6I), suggesting that the IFN-independent STAT3 activation by HDV was specific to the Huh7 or HepG2 background. As upadacitinib still exhibits an antiviral activity in absence of ISG expression and STAT3 activation in the dHepaRG model, our results highlight a proviral activity of JAK1 independent of the innate immune response and pro-inflammatory factors activated by the virus upon infection.

JAK1 promotes HDAG phosphorylation through ERK1/2 activation

As a major kinase involved in many cellular processes, we hypothesized that JAK1 could affect HDAG phosphorylation, especially S-HDAg phosphorylation at serine 177 (Ser177), which is known to be crucial for HDV replication.²⁶ To assess the impact of *JAK1* activity on HDAG phosphorylation, Huh7-NTCP cells with altered *JAK1* expression were transfected with a plasmid expressing S-HDAg to avoid the effect of JAK1

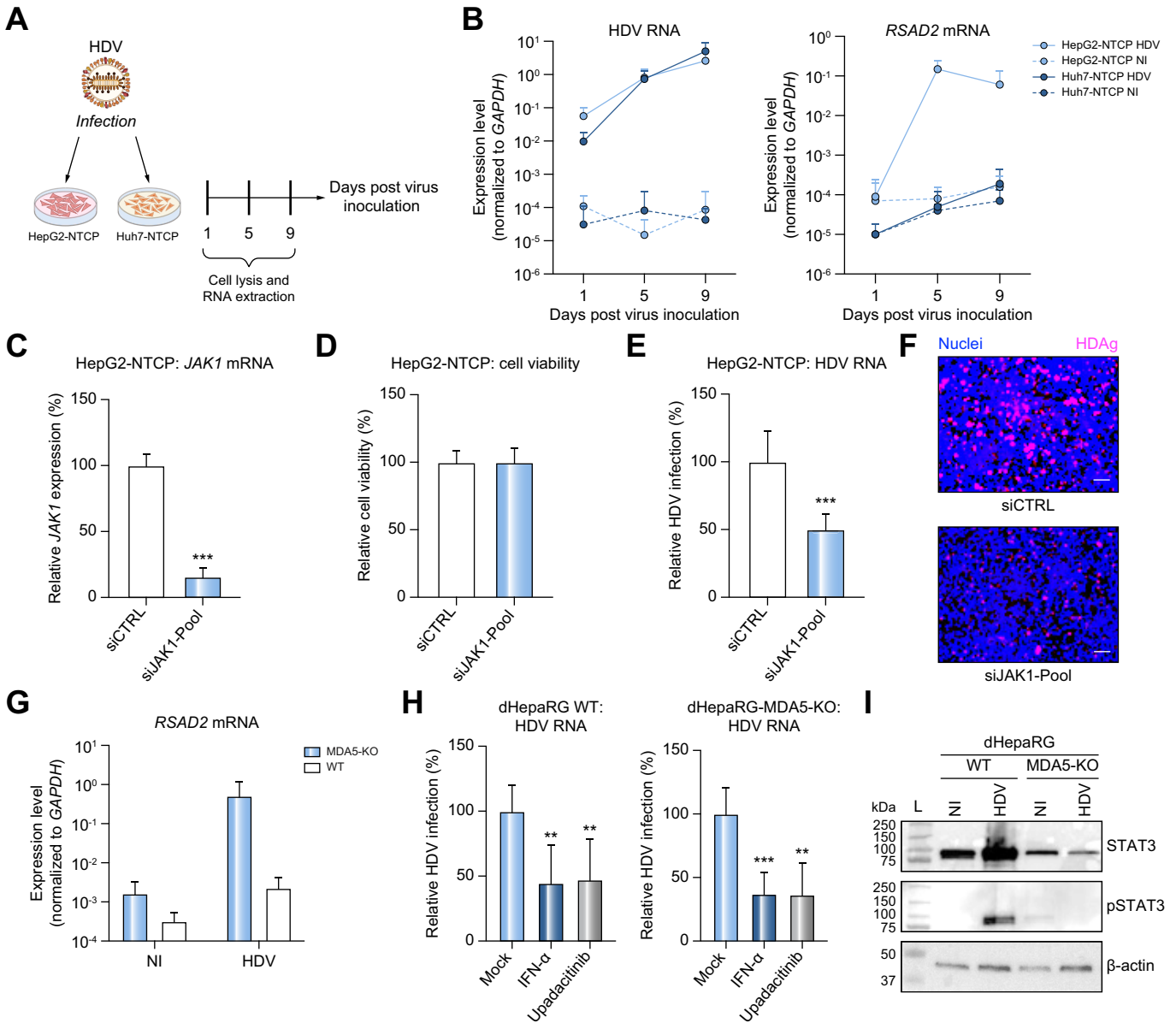


Fig. 6. Provirial activity of JAK1 is independent of the innate immune response and STAT3 activation. (A-B) ISG expression in HDV-infected cells. Huh7-NTCP and HepG2-NTCP cells were infected with HDV; *RSAD2* expression as well as HDV RNA levels were quantified by RT-qPCR at the indicated time points post infection. Results are expressed as means + SD log10 relative expression level normalized to *GAPDH* expression from three independent experiments. (C-F) *JAK1*-KD in HepG2-NTCP cells was performed as described above. *JAK1* expression (C) and cell viability (D) were assessed and HDV infection was quantified by RT-qPCR from three independent experiments (E) or by IF (F). (G-I) Upadacitinib inhibits HDV infection in dHepaRG WT- and MDA5-KO cells. HDV-infected cells were treated with 1 μ M of upadacitinib or with IFN- α -2a. Treatment duration: d1-8. *RSAD2* expression (G) and HDV infection (H) were quantified in both cell lines by RT-qPCR from four independent experiments. pSTAT3 expression was assessed by western blot in HDV-infected cells 8 days after virus inoculation (I). *** $p < 0.001$ and ** $p < 0.01$ (Mann-Whitney *U* test). Scale bar: 100 μ m. IF, immunofluorescence; KO, knockout; RT-qPCR, reverse-transcription quantitative PCR; SD, standard deviation; WT, wild-type.

on HDV replication that would affect HDAg expression levels in an infectious context. HDAg phosphorylation was assessed by co-immunoprecipitation using an anti-phospho-serine antibody (Fig. 7A). Analysis of HDAg phosphorylation indicated that *JAK1* depletion was associated with a loss of S-HDAg phosphorylation; either in *JAK1*-KO (Fig. 7B) or *JAK1*-KD (Fig. 7C) cells. Similar results were obtained by co-immunoprecipitation with an anti-HDAg antibody followed by detection of phosphorylated peptides by western blotting using the anti-phospho-serine antibody (Fig. 7D-F). As a technical control,

no phosphorylated S177A S-HDAg was detected (Fig. 7G-H), strongly suggesting that the pS177 is the major phosphorylation site detectable through our approach. To validate this observation in an infection model, we treated HDV-infected Huh7-NTCP cells with 1 μ M upadacitinib 4 days after viral inoculation, as described in Fig. 4E and Fig. S2B, to avoid too pronounced an inhibition of HDAg synthesis by the drug. Upadacitinib treatment was associated with a decrease in S-HDAg phosphorylation levels at day 7 and 10 after viral inoculation, confirming our previous observations (Fig. S5).

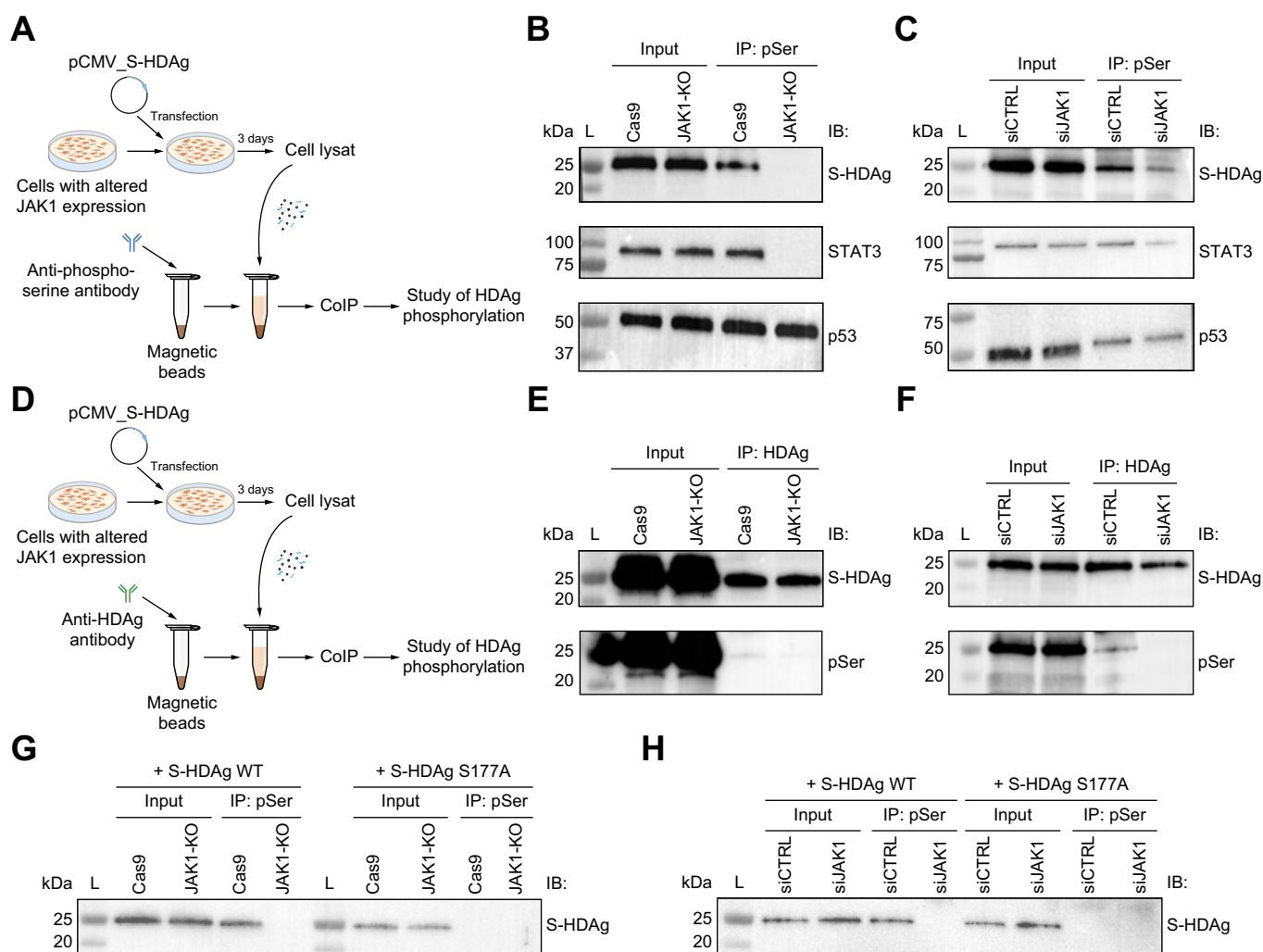


Fig. 7. Provirial activity of JAK1 is associated with a modulation of S-HDAg phosphorylation. (A-C). HDAg phosphorylation in HDAg-expressing cells determined by co-IP with an anti-phosphoserine antibody (pSer). Experimental strategy is presented in (A). (B-C) S-HDAg expression in the original cell lysate (input) and in the IP eluates (IP) was assessed by western blot in JAK1-KO (B) or KD (C) cells. STAT3 (positive control) or P53 (negative control) expression was determined for each sample. (D-F) Similar assays with co-IP performed using an anti-HDAg antibody. (G-H) Similar assays in cells expressing S177A HDAg. Co-IP assays were performed using an anti-phosphoserine antibody. S-HDAg expression was assessed by IB. Co-IP, co-immunoprecipitation; IB, immunoblot; IP, immunoprecipitation; KD, knockdown; KO, knockout; L-HDAg, large delta antigen isoform; S-HDAg, small delta antigen isoform.

Given that JAK1 is a tyrosine kinase and due to the absence of tyrosine residues in the sequence of HDAg, we then hypothesized the involvement of an intermediate kinase that could phosphorylate HDAg upon a JAK1-dependent activation. To assess the impact of JAK1 depletion on the activity of cellular kinases, we performed a human phospho-kinase array using control and JAK1-KO cells. We had a particular focus on ERK1/2 and p38 α that have been described as major kinases phosphorylating HDAg Ser177.²⁷ As shown in Fig. 8A and in Fig. S6A, among the analyzed kinases, no phosphorylated ERK1/2 (pERK1/2) was detected in JAK1-KO cells, while a slight decrease was observed in phosphorylated p38 α levels. No other major variation between the tested kinases was observed, except AKT1/2/3 in Array B. We then focused on pERK1/2 for further analyses. To link ERK1/2 activity and HDV infection in a JAK1-KO context, we first tested whether interleukin-6 (IL-6) stimulation could rescue pERK1/2 levels, as IL-6 is a documented activator of mitogen-activated protein

kinases.²⁸ As shown in Fig. 8A, IL-6 treatment does restore pERK1/2 levels in JAK1-KO cells, a result confirmed by western blot (Fig. 8B and Fig. S6A), suggesting a regulation of ERK1/2 phosphorylation by JAK1. We then confirmed this result with the JAK inhibitors. Interestingly, upadacitinib, but not fedratinib induced a loss of ERK1/2 phosphorylation in both Huh7-NTCP cells and PHHs (Fig. 8C,D), confirming the importance of JAK1 in the phosphorylation of ERK1/2. More importantly, we showed that the restoration of pERK1/2 levels by IL-6 treatment was associated with a rescue of the HDV infectious phenotype in infected JAK1-KO cells (Fig. 8E,F), suggesting a JAK1-dependent association between pERK1/2 and HDV replication. In line with a previous result linking HDAg Ser177 phosphorylation by ERK1/2 and HDV RNA-RNA polymerase II interaction,²⁷ we observed that a suboptimal dose of upadacitinib (to avoid too pronounced a decrease in HDV RNA levels) was associated with a slight decrease in the interaction between HDV RNA and RNA polymerase II (Fig. S6B-C),

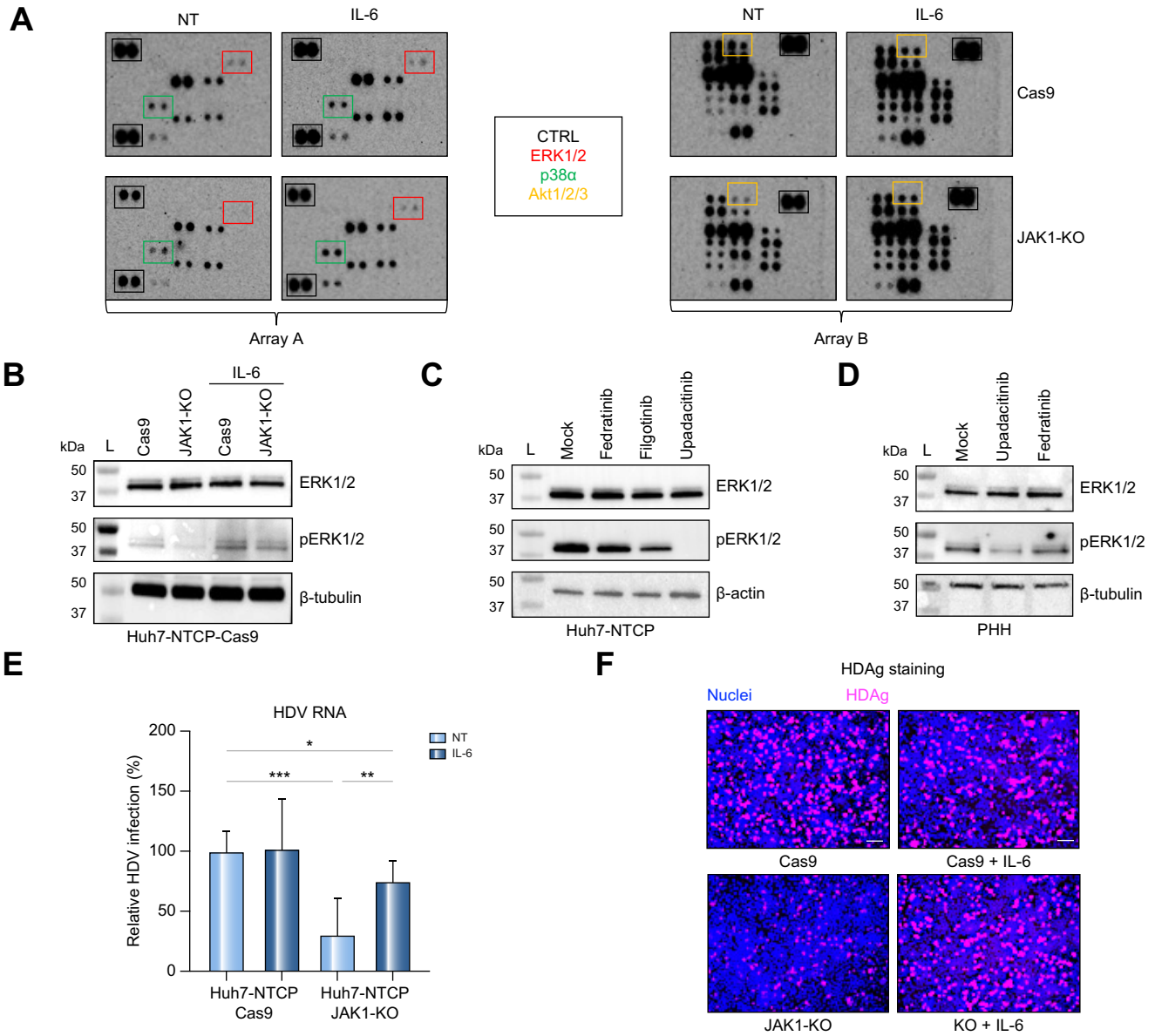


Fig. 8. JAK1 proviral activity is linked to ERK1/2. (A) Human phospho-kinase array in JAK1-KO cells and control cells (Cas9) with or without treatment with IL-6 for 30 min. (B) Validation of pERK1/2 modulation in Cas9 or JAK1-KO cells by western blot. (C-D) Inhibition of JAK1 activity is associated with decreased phosphorylation levels of ERK1/2 *in vitro*. Huh7-NTCP cells (C) or PHHs (D) were treated with 1 μM of fedratinib, filgotinib or upadacitinib. ERK1/2, pERK1/2 and β-actin or β-tubulin expression levels were assessed by western blot. (E-F) IL-6 treatment restores HDV infection in JAK1-KO cells. Cells were infected with HDV and cultured for 8 days in the absence (NT) or presence of IL-6 (100 ng/ml). HDV infection was assessed by RT-qPCR from three independent experiments (E) or by IF (F) *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ (Mann-Whitney *U* test). Scale bar: 100 μm. IF, immunofluorescence; KO, knockout; PHHs, primary human hepatocytes; RT-qPCR, reverse-transcription quantitative PCR.

although this result would benefit from deeper investigations. Taken together, we demonstrated that the proviral activity of JAK1 is associated with the activation of ERK1/2 and the phosphorylation of S-HDAG, known to be crucial for HDV replication.

Discussion

HDV is responsible for the most severe and aggressive form of chronic viral hepatitis, and currently no treatment results in viral cure in HBV/HDV-coinfected patients. In this context, host-targeting agents have demonstrated great potential as

antivirals for innovative therapy in recent years.²⁹ Previously, we identified a pool of cellular factors important for HDV infection and potential antiviral targets.¹⁸ In this study, we confirmed the importance of one of these candidates, the Janus kinase JAK1 in the HDV life cycle. The unexpected proviral activity of JAK1 was highlighted by: (1) a marked decrease in HDV infection levels upon JAK1 depletion in numerous *in vitro* models including PHHs; (2) the modulation of pERK1/2 and phosphorylated levels of HDAG in JAK-KO cells; (3) the efficient antiviral activity of FDA-approved JAK1 inhibitors such as upadacitinib in *in vitro* infection models, including PHHs. Previously, a proviral activity of JAK1 was reported in the context

of HIV infection using JAK1-specific siRNA in HeLa-derived cell lines.^{30,31} However, during HIV infection, the proviral role of JAK1 was associated with the activation of STAT1 and STAT3 proteins, which promoted enhanced inflammation and viral replication.³² Thus, our study provides, for the first time, evidence of a virus hijacking a cellular kinase, which is normally involved in the antiviral response, to activate a viral protein and enhance viral replication.

Previous studies demonstrated the importance of S-HDAg in HDV replication.³³ The phosphorylation of S-HDAg mediated by cellular kinases, such as ERK1/2,²⁷ PKR (RNA-activated protein kinase),³⁴ PKC (protein kinase C) or CKII (casein kinase II),³⁵ is essential for the interaction between the RNA polymerase II and HDV RNA. Due to its specific genomic structure, HDV is the only documented virus that hijacks RNA polymerase II activity to synthesize RNA from an RNA matrix.³³ Although it has been demonstrated that S-HDAg participates in the formation of a transcription pre-initiation complex³⁶ and that S-HDAg interacts *in vitro* and *in vivo* with chromatin remodeling complexes, in particular with the bromodomain regulatory protein BAZ2B (bromodomain-associated to zinc finger protein 2B) to recruit the RNA polymerase II on HDV RNA,³³ the mechanisms by which HDAg and cellular factors interact in HDV replication is not yet fully understood. Here, the results of our study demonstrated that JAK1 inhibitors affect the ability of RNA polymerase II to bind HDV RNA, confirming the importance of HDAg phosphorylation in the recruitment of the polymerase for viral replication³⁷ and highlighting a new key cellular factor involved in that process. Interestingly, this result was associated with an apparent decrease in the interaction between HDV RNA and HDAg (Fig. S6). Deeper investigations are needed to determine whether this observation is either due to a lower immunoprecipitation efficacy/lower HDAg expression in the context of upadacitinib treatment or if HDAg phosphorylation status is also important for its interaction with genomic and antigenomic RNA. More generally, additional efforts should be made to determine whether JAK1 is directly responsible for the phosphorylation of ERK1/2 or if other intermediate kinases whose activation can be modulated by JAK1, such as p38- α or Akt1/2/3 (Fig. 8A), are involved in S-HDAg phosphorylation.

JAK1 is a major actor of the innate immune response, which acts as the first line of defense upon viral infection and can be activated in response to several cytokines, notably IFNs.^{38,39} In the case of HDV infection, IFN treatment moderately affects the first stages of the viral life cycle and suppresses HDV RNA amplification during hepatocyte proliferation. Nevertheless, IFN weakly impacts the intra-nuclear HDV replication in resting

cells,⁴⁰ highlighting the complex interaction between HDV and the innate immune response. Notably, the antiviral ISGs targeting HDV replication still need to be identified. So far, only proviral ISGs have been described for HDV (such as MOV10), and our study shed light on another actor of the innate immune response facilitating viral replication, putatively explaining the weak efficacy of IFN treatment in patients and the relative antiviral capacity of both endogenous and exogenous IFN *in vitro*. Interestingly, our results suggest that no activation of JAK1 is required to drive the phosphorylation of HDAg, suggesting the existence of a basal kinase activity of JAK1 in the absence of any stimulation by cytokines.

Recently, JAK inhibitors, already approved in the treatment of rheumatologic, dermatologic, hematologic, and gastrointestinal indications, were tested for the treatment of viral infections, notably for the prevention of cytokine storms during SARS-CoV-2 infection.⁴¹ JAK inhibitors like ruxolitinib and filgotinib have also been proposed as antiviral agents against HIV independently of their original clinical indications.²⁴ Their potential as putative inhibitors of HIV-1 transcription has also been investigated.⁴² Here, the use of such FDA-approved JAK1-inhibitors (upadacitinib, filgotinib), known to be generally safe and effective,²⁴ showed an antiviral potential in different *in vitro* models including PHHs. Our study demonstrated that JAK1 inhibitors are antiviral candidates for pre-clinical studies in the context of HDV infection. However, their investigation in *bona fide in vivo* models is needed to assess their potential for antiviral therapy. Moreover, their interaction with IFN co-treatment should be carefully analyzed, and of note, due to their immunosuppressive properties, JAK1 inhibitors may be associated with increased risk of viral infections in patients.⁴³ Therefore, the antiviral effects of upadacitinib should be evaluated in a meticulous way in the context of HBV/HDV co-infection in preclinical models. Thus, given the existence of intermediate kinases involved in the proviral activity of JAK1, the use of compounds inhibiting downstream pathways, such as mitogen-activated protein kinase inhibitors, should be explored to limit the putative side effects of JAK inhibitors.

In conclusion, by identifying and characterizing JAK1 as a new key host factor for HDV infection, our results significantly improve our understanding of the HDV life cycle and its interactions with the host innate immune response. We also propose a new function of the key innate immunity-related kinase in the absence of apparent stimulation by cytokines. Finally, our findings pave the way to the development of innovative therapeutic strategies to tackle hepatitis D, a global health threat.

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Abbreviations

HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HDAg, hepatitis delta antigen; IFNs, interferons; IL-6, interleukin 6; IP, immunoprecipitation; ISG, interferon-stimulated genes; JAK1, Janus kinase 1; KD, knockdown; KO, knockout; L-HDAg, large delta antigen isoform; MDA5, melanoma differentiation associated gene 5; PHHs, primary human hepatocytes; RSAD2, radical S-adenosyl methionine domain containing 2; S-HDAg, small delta antigen isoform; siRNA, small-interfering RNA; STAT, signal transducer and activator of transcription.

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Conflict of interest

The authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

E.R.V. designed the study and supervised research. M.J.H. performed the functional *in vitro* assays with the support of C.B., L.M.-H., and E.G. for the different infection assays and J.L. for the RIP assay. M.J.H., C.B., L.M.-H., E.G., J.L., C.S., T.F.B., and E.R.V. analyzed the data. E.F., F.G., and P.P. provided human hepatocytes. J.L. provided key material and protocols. M.J.H. and E.R.V. drafted the manuscript and J.L., C.S., and T.F.B. edited it. All the authors approved the manuscript.

Data availability statement

The original data from this study are available through the corresponding author upon reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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