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Gut virome-colonising *Orthohepadnavirus* genus is associated with ulcerative colitis pathogenesis and induces intestinal inflammation *in vivo*

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ABSTRACT

Objectives Ulcerative colitis (UC) is a chronic inflammatory disorder of unknown aetiology. Gut virome dysbiosis is fundamental in UC progression, although its role in the early phases of the disease is far from fully understood. Therefore, we sought to investigate the role of a virome-associated protein encoded by the *Orthohepadnavirus* genus, the hepatitis B virus X protein (HBx), in UC aetiopathogenesis.

Design HBx positivity of UC patient-derived blood and gut mucosa was assessed by RT-PCR and Sanger sequencing and correlated with clinical characteristics by multivariate analysis. Transcriptomics was performed on HBx-overexpressing endoscopic biopsies from healthy donors.

C57BL/6 mice underwent intramucosal injections of liposome-conjugated HBx-encoding plasmids or the control, with or without antibiotic treatment. Multidimensional flow cytometry analysis was performed on colonic samples from HBx-treated and control animals. Transepithelial electrical resistance measurement, proliferation assay, chromatin immunoprecipitation assay with sequencing and RNA-sequencing were performed on *in vitro* models of the gut barrier. HBx-silencing experiments were performed *in vitro* and *in vivo*.

Results HBx was detected in about 45% of patients with UC and found to induce colonic inflammation in mice, while its silencing reverted the colitis phenotype *in vivo*. HBx acted as a transcriptional regulator in epithelial cells, provoking barrier leakage and altering both innate and adaptive mucosal immunity *ex vivo* and *in vivo*.

Conclusion This study described HBx as a contributor to the UC pathogenesis and provides a new perspective on the virome as a target for tailored treatments.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Gut virome dysbiosis is fundamental to ulcerative colitis (UC) progression, although it remains unclear how it may be involved in the early phases of chronic inflammation.

WHAT THIS STUDY ADDS

⇒ The hepatitis B virus X protein-harbouring *Orthohepadnavirus* genus, detected in a subcohort of patients with UC, affects intestinal mucosal barrier integrity by impacting the immune cell transcriptional state and induces intestinal inflammation *in vivo*.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study proposes the virome as a plausible target for tailored treatments in UC, possibly leading to an entirely new approach to therapeutic intervention.

INTRODUCTION

Ulcerative colitis (UC), one of the major forms of inflammatory bowel disease (IBD), is a chronic inflammatory condition of the colon characterised by a continuous pattern of epithelial ulceration causing bleeding and abdominal pain in patients, for which definite treatment is still lacking.[1] Despite UC's aetiology remains unknown, intestinal dysbiosis has been recognised as pivotal for its progression.[1] Among several intestinal commensals, the contribution of viruses to the pathogenesis of IBD has gained interest during the last years.[2 3] On one hand, bacteriophages can shape the bacterial composition of the intestinal communities,[4] on the other eukaryotic-targeting viruses have been proven to interact with the host and contribute to intestinal inflammation.[5] In this regard, Adiliaghdam *et al* have recently shown an outstanding proof-of-concept depicting the virome, isolated from patients with long-lasting IBD who underwent surgery, as an autonomous contributor to the disease phenotype

at the level of the innate immunity *in vitro* and in experimental colitis models.[6] However, the paramount question that remains unanswered is whether and which specific gut virome-associated factors contribute to IBD aetiopathogenesis interacting with the host's mucosal immunity.[7]

Previous studies have highlighted the hepatitis B viral protein X (HBx), belonging to the eukaryotic-targeting *Orthohepadnavirus* genus, to be upregulated specifically in the gut virome of early diagnosed, treatment-naïve paediatric patients with UC by comparison with healthy and Crohn's disease (CD) mucosae,[8] shedding light on the possible involvement of this viral entity in UC aetiopathogenesis.

Based on these premises, we here aim to understand the role(s) and function(s) of HBx in UC pathogenesis. We show that HBx impairs the gut mucosal immune defence, particularly at the level of the epithelial barrier, inducing *per se* colitis-like symptoms in mice. We also observe that HBx shapes the transcriptomic state of intestinal cells, acting as a DNA-binding protein and transcriptional regulator of genes known to be involved in intestinal inflammation and epithelial cell biology.

Our results identify HBx as a novel key orchestrator of UC pathogenesis, proving the concept that specific virome-derived factors may be directly involved in initiating and sustaining chronic intestinal inflammation and therefore become reasonable targets of innovative therapies implementing the current protocols with tailored disease management.

METHODS

Please refer to the online supplemental material 1 for comprehensive details.

RESULTS

The *Orthohepadnavirus* genus-belonging HBx transcript is detected in a cohort of patients with UC

Hepadnaviridae is a family of small enveloped double-stranded DNA viruses with mainly hepatotropic features that provoke either transient or persistent infections, such as hepatitis.[9] They include five genera, among which *Orthohepadnavirus* is the sole genus having mammals as a natural host.[9] While all genera share the expression of three major sets of proteins (precore/core, polymerase and preS/S), only *Orthohepadnavirus* carries an additional open reading frame, known as HBx.[9] HBx, largely studied in the context of persistent hepatitis B virus (HBV) infection in the liver,[10] has been described as interfering with the cellular transcriptional machinery and impacting different biological processes, which range from DNA damage repair to immune system activation.[11 12]

By metatranscriptomics, we have recently shown that HBx was specifically enriched in the gut virome of a cohort of paediatric patients with UC and absent in patients with CD and healthy subjects, pinpointing this viral entity as a possible direct contributor to intestinal inflammation onset.[8] Of note, during this study, HBx transcript was detected in both immune and non-immune cell populations isolated from HBx-positive UC patient-derived colonic mucosae (online supplemental figures 1A–C).

We then confirmed the HBx positivity in colonic biopsies in two independent cohorts of adult patients with UC, where we detected the transcript in 41%–57% of cases (figure 1A,B, and online supplemental figure 1). No differences were found between HBx-positive and HBx-negative patients with UC in terms of clinical characteristics and disease manifestation/progression. Indeed, by multivariate analysis, no direct associations were found between HBx transcript positivity and standard clinical management data including, but not limited to, age, gender, treatment protocols and faecal calprotectin levels as a proxy for disease severity[13] (figure 1C and online supplemental figure 2).

Since HBx is a protein encoded also by human HBV, its presence in the gut could be due to ongoing or former HBV infections of these patients. To exclude this option, anti-HBV serum positivity (anti-HBc) was correlated with HBx positivity, but no statistically significant correlation was found (figure 1C). We thus wondered whether the HBx encoding sequence might be part of the genetic background. Surprisingly, only 4% of patients with UC (cohort 1, figure 1B) showed the HBx encoding sequence in their genomic DNA. This finding might be explained by the fact that the *Hepadnaviridae* viruses only occasionally and accidentally integrate into the host's genome,[14] thus not being part of the genetic background of these individuals. To further support this hypothesis, we screened DNA samples from 52 UC trio probands (figure 1D) and the same percentage of HBx positivity (4%) was confirmed in the peripheral blood, composed of circulating immune and non-immune cells, which can derive also from the intestine.[15] Moreover, no vertical transmission was observed (figure 1E).

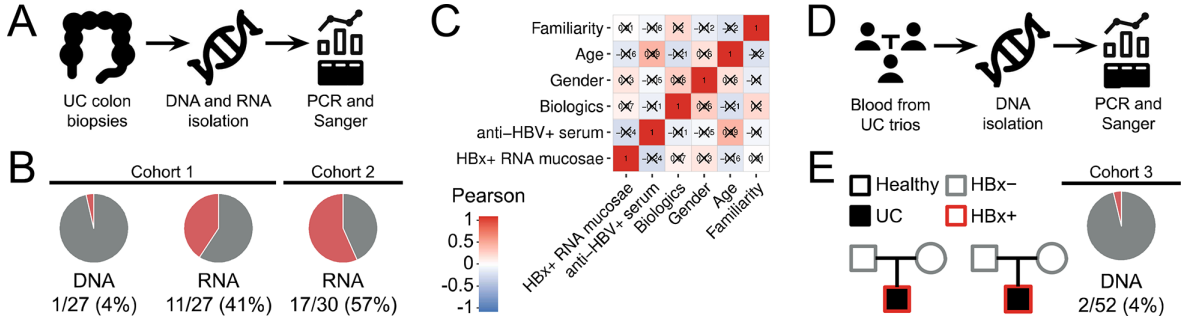

Figure 1 Hepatitis B virus X protein (HBx) transcript positivity characterises a cohort of patients with UC. (A) Schematic representation of the experimental workflow for HBx transcript detection in patients with UC. (B) Pie charts summarising HBx positivity in genomic DNA or RNA of UC cohort 1 (Humanitas Research and Clinical Institute, Milan, Italy), and RNA in UC cohort 2 (Fondazione Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy). (C) Correlation heatmap showing multivariate analysis results expressed as Pearson's coefficients (from 1 to −1). X marks non-significant at p<0.05 (adjustment: Holm). (D) Schematic representation of the experimental workflow for assessing HBx positivity in genomic DNA of patients with UC. (E) On the left, the inheritance tree shows the absence of vertical transmission of HBx genomic DNA in UC cohort 3-derived blood samples (Casa Sollievo della Sofferenza). On the right, is a pie chart summarising the results. Icons from Streamline (https://app.streamlinehq.com).

Collectively, these results highlighted the intestinal HBx transcript positivity to define a cohort of patients with UC and to be not related to anti-HBV serological reactivity nor part of the host's genetics.

HBx impacts the physiological functions of the intestinal mucosa both *ex vivo* and *in vivo*

Although HBx was detected in specific cell compartments (online supplemental figure 1), its impact on the gut mucosa was not elucidated yet. Therefore, to understand how a gut virome-derived factor may contribute to UC pathogenesis, we performed a transcriptomic analysis of healthy donor-derived mucosal biopsies transduced with lentiviruses carrying either the HBx-IRES-GFP encoding sequence or the GFP as control (online supplemental figure 3A). HBx overexpression was found to impact the gene expression profile of intestinal tissues by upregulating 1361 and downregulating 204 genes (online supplemental figure 3B), resulting in the enrichment of biological processes related to antimicrobial response (including the toll-like receptor-mediated signalling), pro-inflammatory pathways (tumour necrosis factor (TNF)-α, interleukins and chemokines), epithelial cell regeneration (Wnt pathway) and the downregulation of interferons (IFN)-related and response to virus-related pathways (online supplemental figure 3C), indicating HBx to interfere with the correct physiology of the tissue and to contribute to UC-specific cellular and molecular alterations.[1]

These results prompted us to further investigate the HBx functions directly *in vivo* to support the pathogenic role of HBx in intestinal inflammation. To this end, C57BL/6 wild-type mice were intrarectally injected with liposomes conjugated with either HBx-IRES-GFP-encoding (hereafter referred to as HBx) or GFP-encoding plasmid as control (hereafter referred to as GFP) (figure 2A). After 15 days, HBx-treated mice started developing clinical symptoms of colitis, as indicated by the increased Disease Activity Index (DAI) (figure 2B), a higher endoscopic score (figure 2C) and reduced colon length (figure 2D), by comparison with the GFP-treated animals. The endoscopic score combines various parameters, including colon translucency, mucosal granularity (representing oedema and small erosions) and changes in the vascular pattern. While in GFP-treated mice the mucosa was translucent, with low granularity and visible blood vessels, in HBx-induced animals we observed a significant increase in colon thickness and granularity, with clear signs of mucosal ulcerations and merely visible blood vessels (online supplemental figures 4A–D).

To evaluate whether HBx-induced colitis is influenced by microbiota, mice were treated for 2 weeks with a broad-spectrum antibiotic cocktail and then administered with either GFP-carrying or HBx-carrying liposomes for 15 days to evaluate the early effects of the viral factor in these conditions. Groups without antibiotics were used as control (online supplemental figure 5A). Interestingly, antibiotic treatment did not impact the overall DAI (online supplemental figure 5B), indicating that the HBx-induced effects did not depend on the microbiota composition.

Similar results were observed at the tissue level. In fact, both the colon lengths and endoscopic scores of HBx-treated mice did not change with statistical significance on antibiotic administration (online supplemental figures 5C–E), indicating that microbiota did not influence the HBx-induced colitis-like tissue hallmarks in mice.

Notably, the increased diarrhoea and faecal blood observed in HBx-treated mice (online supplemental figures 4C,D), along with the mucosal ulceration, led us to propose HBx as a factor that impairs barrier function in the intestinal mucosa, thus promoting gut inflammation. The barrier alteration then induces the translocation of commensal bacteria and microbial products from the gut lumen into the bowel wall, leading to acute mucosal inflammation that, if not resolved by an adequately mounting immune response, leads to chronic intestinal inflammation. This is in line with the leading theory explaining UC pathogenesis.[16]

To evaluate the immunological changes in our model, we performed a FACS analysis of murine colonic tissues. This revealed that the number of dendritic cells (DCs), $CD8^+$ T cells and neutrophils was significantly reduced in HBx-treated versus GFP-treated mice, while macrophages (MΦ) and endothelial cells were only slightly affected (figure 2E,F and online supplemental figure 6). Since the first defence against pathogens (innate immunity and T cells) was impaired and the low number of neutrophils may hamper the correct resolution of inflammation,[17] such an immune state of the colonic mucosa on HBx stimulation may explain why mice developed colitis-like symptoms.[17] This immune landscape is in line with UC-specific pathological traits.[17 18] In contrast with the higher levels of ulcerations (a typical sign of epithelial cell loss and crypt damage[19 20]), intestinal epithelial cell number was significantly increased by HBx compared with controls (figure 2E,F, and online supplemental figure 6).

Since the epithelial barrier alteration is crucial in initiating UC pathogenesis,[16] we sought to better elucidate this point by exploiting the Caco-2 cell line as an *in vitro* model of the intestinal epithelial barrier.[21] HBx-overexpressing Caco-2 cells displayed an increased proliferation rate by comparison with the GFP-transduced cells (figure 3A) and a reduced transepithelial electrical resistance (TEER) (figure 3B), indicating the barrier leakage. These data were also confirmed *in vitro* in epithelial organoid cultures (figure 3C,D) transduced with either GFP or HBx-IRES-GFP lentiviruses (figure 3E), which showed upregulation of stemness markers (*LRG5* and *OLFM4*) and downregulation of epithelial barrier markers (*MUC1*, *MUC13* and *TJP1*) in HBx-transduced cells compared with GFP (figure 3F,G), suggesting the viral protein to increase proliferation capabilities and to affect barrier function in the intestinal epithelium.

Collectively, these results demonstrate that HBx drives colitis *in vivo* by acting at the level of the epithelial barrier and, in parallel, affects the gut mucosal immune cells, likely not mounting an adequate defence against the invading microorganisms and thus fostering a persistent inflammation unable to self-resolve.

HBx acts as a transcriptional regulator in the intestinal epithelium

The findings collected so far prompted us to study more in detail the effect of HBx on the gut epithelium. Since HBx was previously found to bind the host's DNA and regulate transcription during HBV infection,[10 11 22 23] we performed a chromatin immunoprecipitation assay with sequencing (ChIP-Seq) analysis on V5-tagged HBx-transduced Caco-2 cells to understand the early effects of HBx (figure 4A). For comprehensive chromatin profiling, we took advantage of the open-source data available at ENCODE (https://www.encodeproject.org/)[24] and analysed several other epigenetic markers together with HBx-V5 occupancy. HBx ChIP-Seq profile closely resembled that of a typical transcription factor with discrete narrow peaks (online supplemental figures 7A,B) and was enriched for specific DNA motifs (figure 4B), demonstrating that HBx retained its chromatin-binding behaviour even in non-hepatic cells. To better

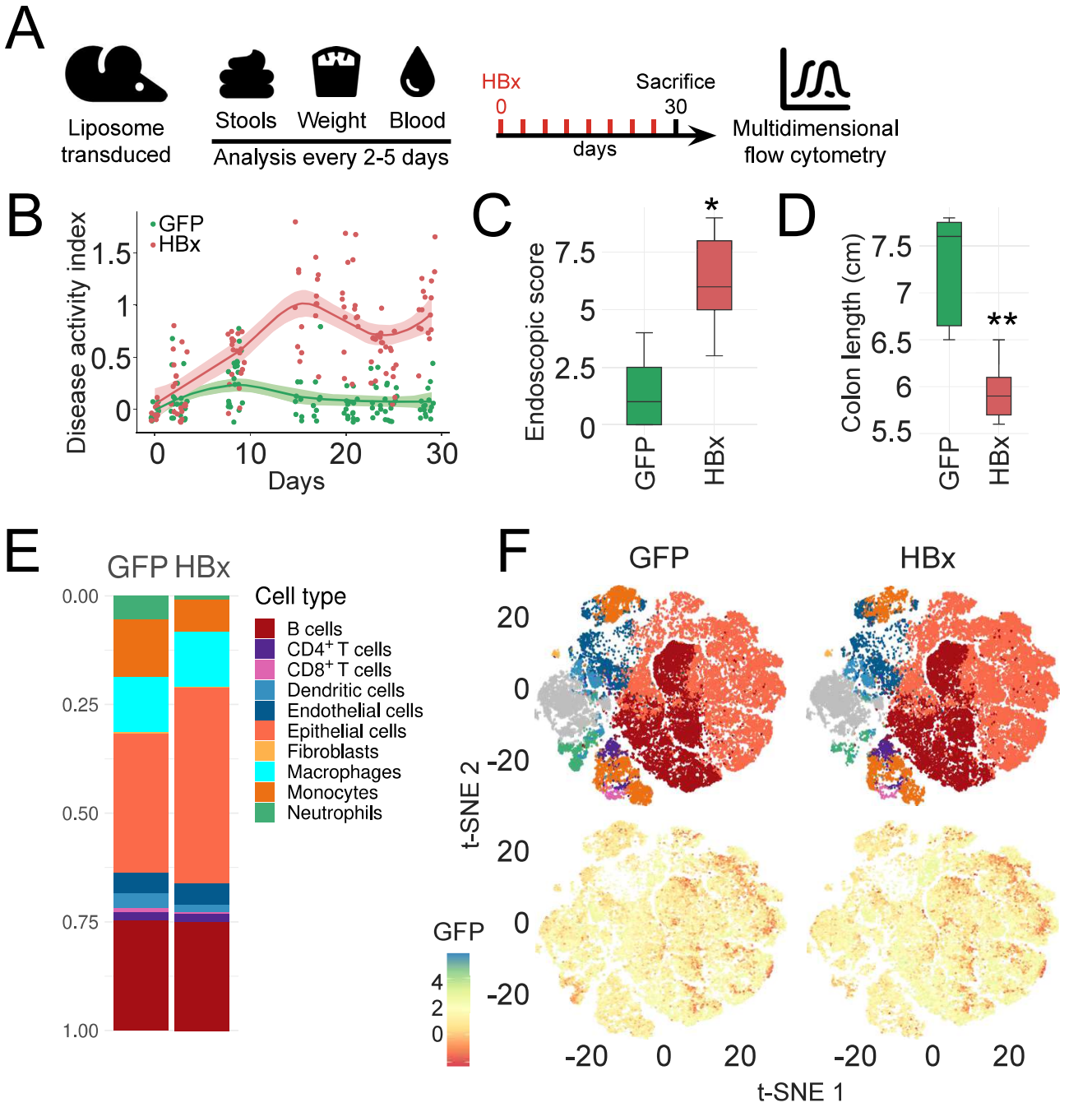

Figure 2 Hepatitis B virus X protein (HBx) induces colitis symptoms in mice and shapes the colonic mucosal immunity. (A) Schematic experimental workflow of *in vivo* HBx-induced colitis (n=6/group, two independent experiments). (B) Disease Activity Index (DAI) of mice treated with either GFP-carrying or HBx-carrying liposomes. Differences between groups are statistically significant. Statistical analysis was performed with two-way analysis of variance, with Bonferroni's postcorrection. Results with a p value <0.05 were considered significant. * P<.05; ** P<.01. (C,D) Box plots showing endoscopic score (C) and colon length (D) of HBx-induced colitic mice. (E, F) Relative cell population abundance (E) and t-distributed stochastic neighbour embedding (t-SNE) multidimensional scaling (F) in HBx-induced colitis mice versus the GFP control. Immunophenotyping was performed on n=3 mice/group. Icons from Streamline (https://app.streamlinehq.com).

characterise these regions, peaks were mapped to the nearest genes and annotated accordingly. Interestingly, the great majority of the peaks were found within intergenic regions (figure 4C), typically not accessible to transcription factors (low DNAse I hypersensitivity), and far from canonical promoter markers (ie, H3K4me3 and H3K27me3), as assessed by Pearson's correlation and Hidden-Markov chromatin modelling (figure 4D,E). These data depicted HBx as a possible pioneering factor,[25] binding putative enhancers in a heterochromatin state and therefore not commonly used by Caco-2 cells. To understand the

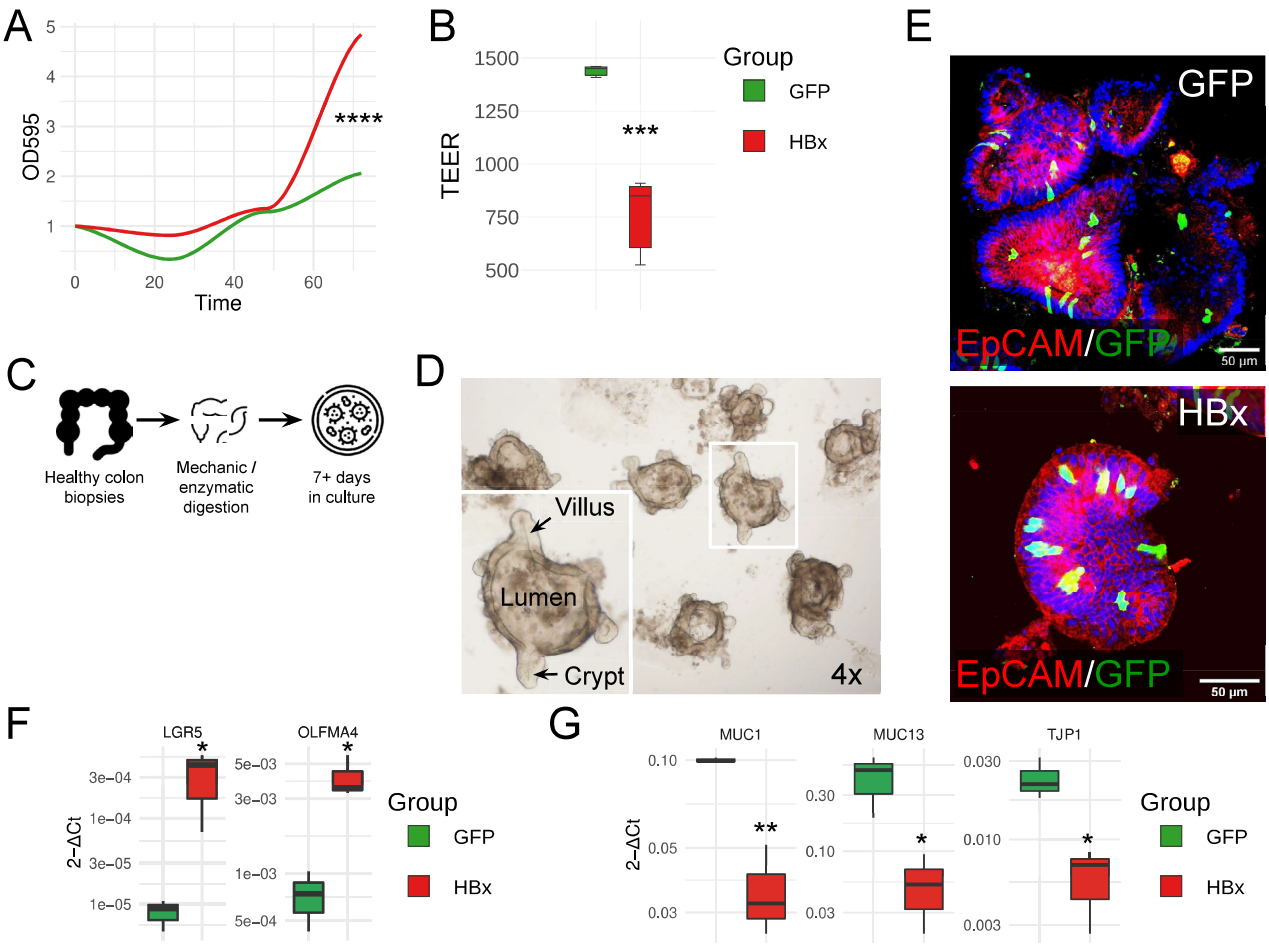

Figure 3 Hepatitis B virus X protein (HBx) alters epithelial barrier functions. (A) Graph showing cellular growth rate between HBx-transduced and GFP-transduced epithelial cell line. Experiments were performed in triplicates in three independent experiments. Statistical analysis was performed with two-way analysis of variance with Bonferroni's postcorrection. (B) Box plot showing transepithelial electrical resistance (TEER, expressed as Ω/cm²) measurements on HBx-transduced and GFP-transduced epithelial cell lines. Statistical analysis was performed with Student's t-test (C) Schematic representation of organoid isolation. (D) After 7 days in culture, organoids become mature and structured, with crypt and villus domains. (E) Immunofluorescence images showing GFP-transduced and HBx-transduced intestinal organoid structures and the positivity for the epithelial marker EpCAM (red) and the GFP (green). (F, G) Box plots showing real-time PCR results for the stem cell (F) and epithelial barrier markers (G) in organoids transduced with either the GFP-carrying or HBx-carrying lentiviruses, expressed as 2-ΔCT (GAPDH was used as the housekeeping genes). Statistical analysis was performed with Student's t-test. *P<0.05; **p<0.01; ***p<0.005; ****p<0.001. Icons from Streamline (https://app.streamlinehq.com).

consequences of such chromatin binding, we performed RNA-sequencing (RNA-Seq) and transcriptome profiling of the same cells. We observed a modest but specific dysregulation of gene expression, with 468 upregulated and only 11 downregulated genes on HBx-V5 overexpression (figure 4F), with the functional enrichment of Gene Ontology categories related to cell proliferation and tissue regeneration, pro-inflammatory signals, epithelial-to-mesenchymal transition and transcriptional regulation (figure 4G), supporting the hypothesis that HBx shapes the overall transcriptional state of epithelial cells towards a pro-inflammatory phenotype.[26] Furthermore, when the differentially expressed genes were intersected with HBx ChIP targets, 20 genes were found to be modulated while being directly bound by HBx, mainly within the intergenic regions (figure 4H–J). These genes were found to be involved in the regulation of epithelial barrier functions (FERM domain containing 4B (FRMD4B); fibulin 7 (FBLN7); mucin 17 cell surface-associated (MUC17)), intracellular signalling (platelet-derived growth factor receptor alpha (PDGFRA); sonic Hedgehog signalling molecule (SHH); phosphodiesterase 4D (PDE4D); transmembrane protein 242 (TMEM242)), cell division (establishment of sister chromatid cohesion N-acetyltransferase 1 (ESCO1); leucine-rich repeat and coiled-coil centrosomal protein 1 (LRRCC1); thyroid hormone receptor interactor 13 (TRIP13); tubulin beta 2B class IIb (TUBB2B), zinc finger CCHC-type containing 7 (ZCCHC7)) and transcription (cAMP-responsive element-binding protein 5 (CREB5); LUC7 like 2 (LUC7L2), pre-mRNA splicing factor) (online supplemental table 3). Their altered expression may explain the increased proliferation rate and epithelial barrier leakage shown by the HBx-overexpressing epithelial Caco-2 cell line. Interestingly, many of these genes were already found to be associated with UC pathogenesis.[27–33]

Conclusively, HBx was shown to impair the functions of epithelial cells by directly binding to intergenic DNA regions,

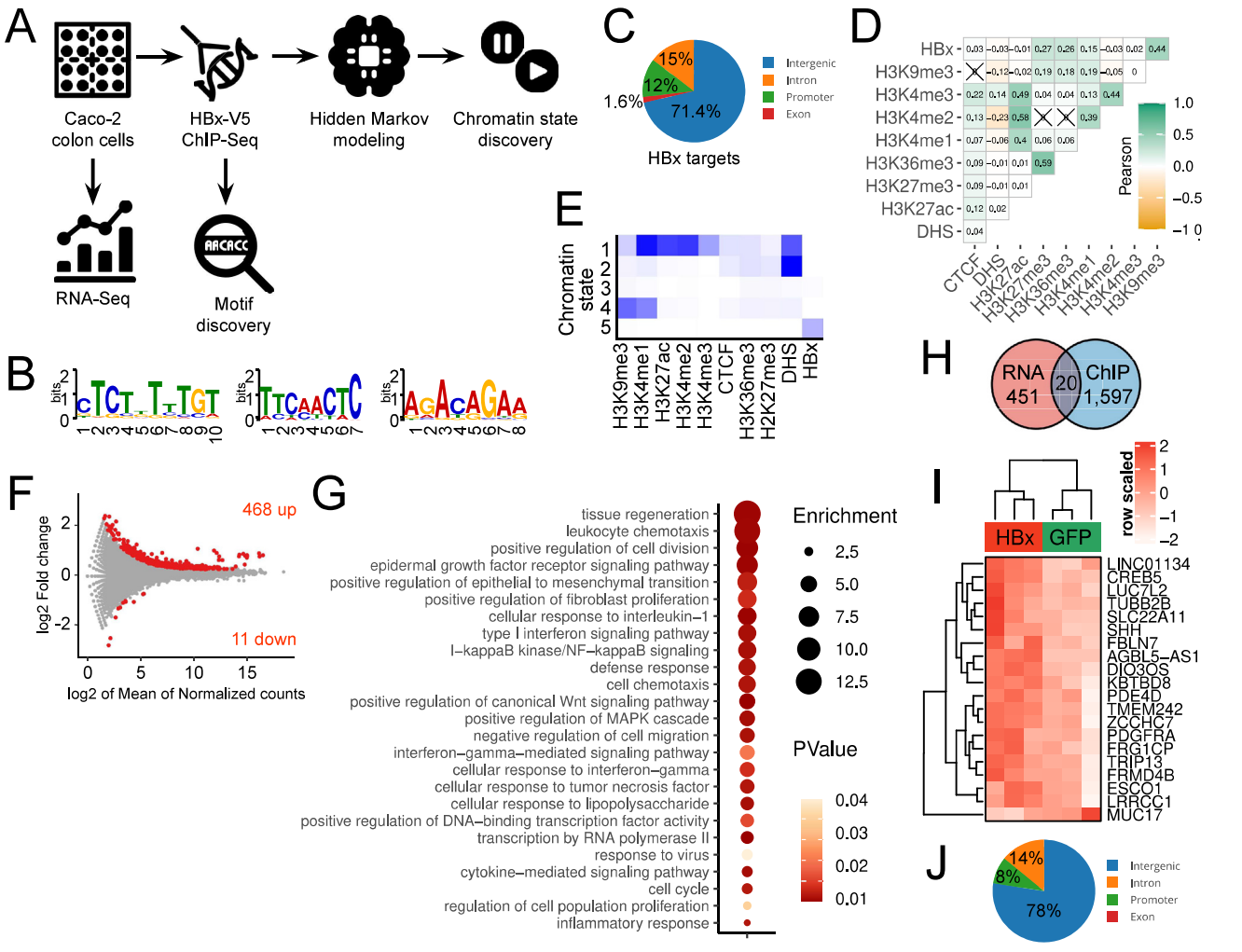

Figure 4 Hepatitis B virus X protein (HBx) regulates the expression of ulcerative colitis (UC)-related genes by binding to enhancer regions. (A) Schematic experimental workflow of the chromatin immunoprecipitation assay with sequencing (ChIP-Seq) and RNA-sequencing (RNA-Seq) analyses of HBx-V5-transduced and GFP-transduced cells. (B) Top three representative DNA motifs enriched in HBx peaks. (C) Pie chart showing gene body-centric annotation of all HBx peaks. (D) Correlation heatmap showing ChIP-Seq coverage multivariate analysis results expressed as Pearson's coefficients (from 1 to −1). (E) Heatmap showing the relative abundance of the different ChIP-Seq signals within the chromatins states found by hidden Markov modelling. (F, G) MA-plot (F) and Gene Ontology plot (G) showing differentially expressed genes and the resulting dysregulated biological processes, respectively, in HBx-expressing versus GFP-expressing Caco-2 cells. (H) Venn diagram showing the intersection between the differentially expressed genes and HBx putative targets. (I, J) Gene expression heatmap (I) showing the 20 genes found to be differentially expressed while directly bound by HBx within their respective enhancers, summarised in the pie chart (J) showing gene body-centric annotation. Icons from Streamline (https://app.streamlinehq.com).

affecting gene expression in epithelial cells that concomitantly acquired a stem-cell-like phenotype (by SHH overexpression) and reduced mucin expression (MUC17), together with the activation of other genes already reported to be involved in UC pathogenesis.

In vitro and *in vivo* silencing of HBx reverts colitis-like symptoms and restores the gut barrier functions

To pinpoint the relevance of HBx as a possible therapeutic target, we assessed the efficacy of HBx inhibition *in vitro*. To this aim, HBx-overexpressing cells were treated with three different HBx-targeting small interfering RNAs (siRNAs) (online supplemental figure 8A). HBx-overexpressing Caco-2 reduced their proliferation rate and recovered the TEER values by comparison with the scramble-treated cells (online supplemental figures 8B,C). Also, tight junction-related marker expression was recovered on HBx-targeting siRNA administration as compared with the control cells (online supplemental figure 8D).

We then tested siRNA HBx-targeting efficacy *in vivo*. HBx-induced colitic mice were treated with the viral factor-targeting siRNAs after colitis induction (figure 5A). Interestingly, the treatment with HBx-targeting siRNAs caused a prompt reversion of the inflammatory symptoms in terms of reduced DAI, bleeding, stool consistency, endoscopic scores and recovery of the colon length (figure 5B–D and online supplemental figures 9A–D), in contrast with animals receiving scrambled siRNAs. These results were paralleled by the recovery of the expression of the epithelial barrier integrity indicators Muc13 and *Tjp1* (online

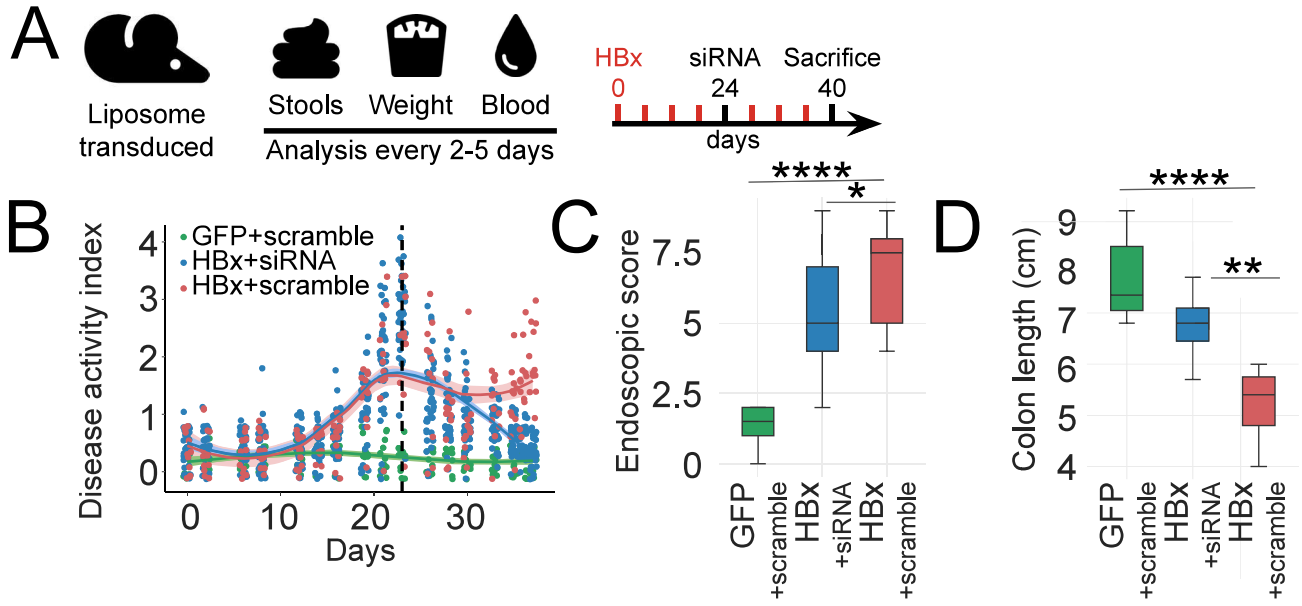

Figure 5 Hepatitis B virus X protein (HBx)-induced phenotype is reversible *in vivo*. (A) Schematic experimental workflow of *in vivo* HBx-induced colitis and small interfering RNA (siRNA)-mediated rescue treatment (n=6 mice/group, 2 independent experiments). (B) Disease Activity Index (DAI) of mice treated with either GFP-carrying or HBx-carrying liposomes and HBx-targeting siRNAs or the scramble. The black dotted line indicates the commencement of siRNA administration. Differences between groups are statistically significant. Statistical analysis was performed with two-way analysis of variance, with Bonferroni's postcorrection. Results with a p value <0.05 were considered significant. (C, D) Box plots showing endoscopic score (C) and colon length (D) of GFP-carrying or HBx-carrying liposomes and HBx-targeting siRNAs or the scramble. Statistical analysis was performed with Student's t-test. *P<0.05; **p<0.01; ****p<0.001. Icons from Streamline (https://app.streamlinehq.com).

supplemental figure 10A), the modulation of Notch signalling (online supplemental figure 10B) and the decreased expression of the pro-inflammatory *Tnf* and *Il1b* (online supplemental figure 10C), demonstrating that HBx inhibition reverted the pro-inflammatory phenotype, reestablishing the barrier integrity and the inflammation-related defence response.[34]

DISCUSSION

The two main types of IBD, UC and CD, differ in localisation, pattern of inflammation, type of immune cell infiltrate and complications developed.[35] We recently proposed the two diseases to be different also in terms of virome composition, pointing out HBx to be specific for patients with UC (treatment-naïve and early diagnosed) and absent in the gut mucosa of patients with CD and healthy patients.[8] Given these premises, it is reasonable to speculate that HBx could be associated with UC pathogenesis and not involved in CD.

The human microbiota is a densely populated community of different entities important for maintaining tissue homeostasis and stimulating the host's immunity.[36] While many studies have already investigated and assessed the roles and functions of bacteria in the gut, few notions are available for the viral commensals of the intestine, although they have been previously proposed to influence the overall intestinal microbiota composition.[4 5 37] Importantly, eukaryotic viruses colonising the mucosal surfaces may infect host cells without symptoms, and, even if asymptomatic, a virus-carrying host may harbour a persistent immune response with a 'continuum' of inflammatory mediators that might increase host susceptibility to disease.[7] Moreover, viruses are sensed by the host's specific molecules, thus evoking a specific transcriptional response in both infected and bystander cells with the consequent release of inflammatory signals, ultimately influencing systemic immunity.[7] For these reasons, eukaryotic-targeting viruses have recently attracted considerable interest in intestinal diseases, including UC.[5 38] Importantly, viruses isolated from patients with long-lasting inflammation have been demonstrated to autonomously induce intestinal inflammation in experimental models of colitis.[6]

As a step further, our work identifies for the first time a specific *Orthohepadnavirus*-derived factor, namely HBx, as being associated with UC early pathogenesis[8] and being a direct inducer of colitis-like symptoms in mice by provoking epithelial ulceration and mucosal barrier leakage, leading to the impairment of gut defence. This is an additional piece to the puzzle of IBD pathogenesis that putatively identifies one of the early causes of intestinal inflammation.

Of note, compelling evidence has recently pointed out viruses to be strongly associated with the onset of autoimmune diseases, such as the Epstein-Barr virus in the pathogenesis of multiple sclerosis.[39] A similar scenario may be that of the *Orthohepadnaviruses* found within the intestine that latently stimulate the immune system through the expression of HBx, which may predispose an individual to develop UC by directly regulating the expression of specific genes known to participate in UC pathogenesis.[27–33]

Furthermore, since HBx is a protein encoded by a hepatotropic viral family, its presence in the colon was largely unexpected, although non-human HBV lymphotropism and its ability to use lymphoid cells as extrahepatic reservoirs have been recently described also in lymphoid tissues, including spleen and

lymph nodes.[40–44] These findings support our and other studies reporting the virome to be made up of a large plethora of entities not necessarily colonising their preferential tissues but residing on the mucosal surfaces while expressing antigenic molecules and stimulating tissue immunity without activating the canonical infection cycle.[7 8]

Most likely, HBx might exert its detrimental role in the intestinal mucosa by affecting the host's defence against pathogens, altering mucosal homeostasis and ultimately causing the perpetuation of intestinal inflammation in patients with UC.

This possible scenario is corroborated by *in vitro* and *in vivo* experiments. Indeed, epithelial cells *in vitro* were affected in their transcriptional state and lost their capability to maintain the barrier on HBx administration. Likewise, mice showed colitis-like symptoms and barrier leakage with an altered immune milieu where DCs, $CD8^+$ T cells and neutrophils were reduced in number. Notably, DCs are a unique cell subset specialised in the production of type I IFNs. They promote antiviral immune responses and are implicated in the pathogenesis of autoimmune diseases characterised by a type I IFN signature.[45] Neutrophils are regarded as the first line of defence in the innate arm of the immune system. They capture and destroy invading microorganisms through phagocytic mechanisms and the formation of neutrophil extracellular traps after detecting pathogens.[46] $CD8^+$ T cells include cytotoxic T cells, important for killing virally infected cells, and $CD8^+$ suppressor T cells, which restrain certain types of the immune response.[47] Therefore, it is reasonable that, in UC pathogenesis, the HBx-induced impairment of these populations in the gut facilitates a persistent inflammation in the mucosa after the translocation of invading microorganisms across the ulcerated epithelium.

Notably, our data showed that the microbiota did not influence the HBx-mediated colitis in mice, at least during the induction phase, indicating that HBx does not cooperate with other commensals to exert its action. This is in line with the concept that antibiotic treatment is not effective in inducing remission in patients with UC.[48]

HBx was extensively described in the context of HBV-associated hepatocellular carcinoma for its ability to induce epigenetic modifications in the host's cells.[49] Whereas, to the best of our knowledge, no evidence is available in the setting of UC-associated colorectal carcinogenesis except for some association studies.[50]

Given HBx's prominent role in liver carcinogenesis, a similar effect could be speculated/hypothesised in colitis-associated colorectal cancer and investigated in future studies.

Interestingly, in colons derived from HBx-positive patients with UC, HBx transcript is detected, despite the absence of HBx DNA integration in the majority of the cases tested. This might be consistent with the notion that the Hepadnaviridae life cycle consists of its entry into the host cells, the release of the genome as a relaxed-circular DNA into the host cell's nucleus and conversion to covalently closed circular DNA, which ultimately serves as a template for the transcription of the viral RNAs, including HBx.[51] Occasionally, a part of the incoming Hepadnaviridae DNA is integrated into the host's genome, and, while remaining replication-incompetent, it can act as a template for the production of proteins, possibly related to viral-specific immune tolerance or the development of viral infection-related pathogenesis.[51] Additionally, in our study, the rare HBx DNA integration (4% of the cases tested) was demonstrated to be not inherited. In this regard, we do not exclude that HBx genomic DNA detected in the peripheral blood may derive from immune and non-immune cells with an intestinal origin.[15] Further studies will be required to better elucidate the source of this genomic integration.

HBx positivity was found to not correlate with anti-HBV reactivity. These results suggest, on one hand, the lack of correlation between active HBV infection and the symptomatology observed in our patients, on the other hand, they may suggest that HBx comes from non-human *Orthohepadnaviruses* other than human HBV, raising concerns about how this viral factor was acquired. We speculate that HBx positivity may occur after environmental exposure, for example, during an event of zoonotic spillover. Curiously, when the *Hepadnaviridae* family was searched within the Serratus viral discovery database, the majority of the hits were found in *Trichobilharzia* samples, a notorious human zoonotic agent colonising water during its life cycle (online supplemental results and online supplemental figures 11 and 12), suggesting that exposure to contaminated waters might be proposed as a risk for acquiring the viral factor. This scenario would be in line with previous association studies suggesting environmental factors among the triggers of chronic inflammatory conditions, including UC.[52]

Despite the lack of longitudinal studies demonstrating the association of zoonotic spillover with the increased incidence of UC in the population, we do not exclude that exposure to Hepadnaviridae could happen after contact with specific environmental agents carrying HBx, which may chronically stimulate the immune and non-immune gut compartments. Of note, zoonotic spillover was already shown for other disease outbreaks ('waterborne zoonoses: identification, causes, and control', https://www.who.int/publications/i/item/9241562730). Nevertheless, we are fully aware that this hypothesis needs to be tested in future studies aiming at the discovery of the real source of HBx positivity in the population.

Another worthwhile aspect of our study is the absence of clinical differences between HBx-positive and HBx-negative patients with UC in the cohorts analysed, while HBx was found to induce marked colitis-like symptoms in mice. This might be explained considering two main pieces of evidence: (i) these patients were not paediatric (as in our first study) and (ii) they were suffering from long-lasting inflammation while receiving anti-inflammatory treatments. All these factors might have impacted differential HBx-related clinical manifestations because of other insults intervening during the course of the disease. By contrast, mouse models were performed on healthy wild-type animals, where the first hit was the viral protein only, thus mimicking the early mechanisms leading to intestinal inflammation independently of other actors.

The currently available treatments for UC are all designed to block single factors involved in the mucosal inflammatory process, such as monoclonal antibodies against cytokines (anti-TNF) or small molecules directed at signalling molecules (JAKs). All these approaches are partially effective as these agents lose effectiveness and patients have disease relapse. The main reason for these failures is that UC is a complex disease where numerous biological functions (eg, protein translation, pro-inflammatory molecule secretion, cell proliferation, apoptosis, etc) exert integrated and complementary roles that foster disease persistence. As a result, blocking one single pathway (ie, JAK/STAT) or one single cytokine (ie, TNF) may not be enough to permanently inhibit an overwhelmingly complex inflammatory process.

Our study sheds light on the possibility that a viral genus may induce colitis in humans and the chance that targeting virome-derived factors in specific cohorts of patients with UC may offer a whole new therapeutic potential. First evidence has been

provided by the siRNA-mediated silencing of HBx, effective in recovering the pro-inflammatory phenotype *in vivo*.

Although the gaps between the *in vitro*, *ex vivo* and in vivo experimental results shown in the current study do exist and the path up to the clinical applications is long, the reality is that the treatment of UC is far from satisfactory and has reached a therapeutic 'ceiling'. The paradigm shift in UC pathogenesis that we propose here might result in alternative therapeutic approaches, at least for HBx-positive patients with UC, ultimately ameliorating patients' quality of life.

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Competing interests SD has served as a speaker, consultant and advisory board member for Schering Plough, Abbott (AbbVie) Laboratories, Merck and Co, UCB Pharma, Ferring, Cellerix, Millenium Takeda, Nycomed, Pharmacosmos, Actelion, Alfa Wasserman, Genentech, Grunenthal, Pfizer, AstraZeneca, Novo Nordisk, Vifor and Johnson and Johnson. LP-B has served as consultant for Merck, AbbVie, Janssen, Genentech, Ferring, Tillots, Vifor, Pharmacosmos, Celltrion, Takeda, Biogaran, Boerhinger-Ingelheim, Lilly, Pfizer, Jndex Pharmaceuticals, Amgen, Sandoz, Celgene, Biogen, Samsung Bioepis, Alma, Sterna, Nestlé, Enterome, Mylan, HAC-Pharma, Tigenix, and has served as speaker for Merck, AbbVie, Janssen, Genentech, Ferring, Tillots, Vifor, Pharmacosmos, Celltrion, Takeda, Boerhinger-Ingelheim, Pfizer, Amgen, Biogen, Samsung Bioepis. VJ has received has received consulting/advisory board fees from AbbVie, Alimentiv Inc (formerly Robarts Clinical Trials), Arena Pharmaceuticals, Asahi Kasei Pharma, Asieris, Bristol Myers Squibb, Celltrion, Eli Lilly, Ferring, Flagship Pioneering, Fresenius Kabi, Galapagos, GlaxoSmithKline, Genentech, Gilead, Janssen, Merck, Mylan, Pandion, Pendopharm, Pfizer, Protagonist, Reistone Biopharma, Roche, Sandoz, Second Genome,Takeda, Teva, Topivert, Vividion; speaker's fees from, AbbVie, Ferring, Galapagos, Janssen Pfizer Shire, Takeda, Fresenius Kabi. The other authors declare no conflicts of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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Data availability statement Data are available in a public, open access repository. Raw and processed data were deposited into the NBCI GEO repository with accession number GSE204665. Sanger sequencing results are available at OP978007-OP978010 in NCBI GenBank Caco-2 raw sequencing data for ChIP-Seq analysis were downloaded from ENCODE and they were mentioned as a reference in the text. Results, analytic methods and study materials will be made available on request to the authors.

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