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Dual role of neddylation in transcription of hepatitis B virus RNAs from cccDNA and production of viral surface antigen

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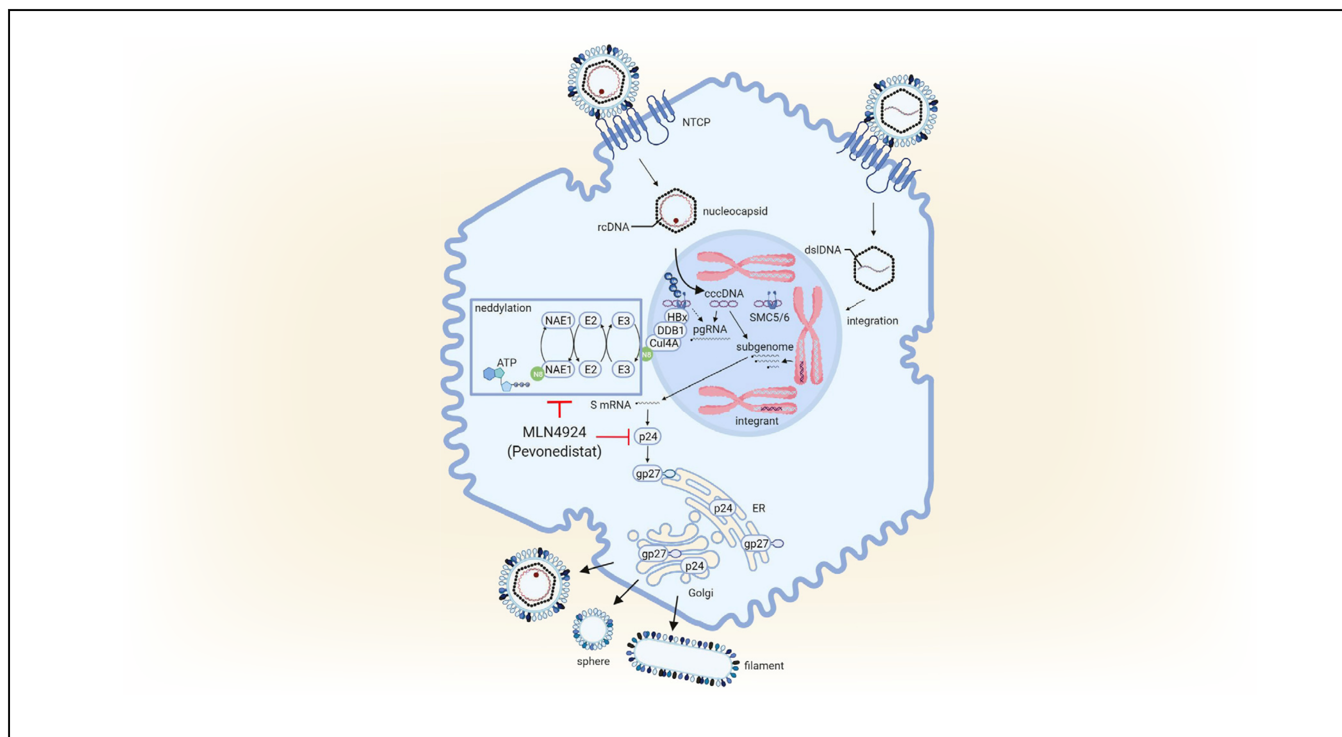
Graphical abstract



Highlights

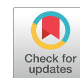
- Neddylation plays a dual role in HBV expression from viral integrants and episomal cccDNA.
- Impaired neddylation suppresses production of HBsAg expressed from viral integrants.
- Neddylation promotes HBsAg generation from viral integrants in an HBx-independent manner.
- MLN4924 also inhibits the synthesis of viral transcripts from episomal cccDNA.

Lay summary

Current treatments for chronic hepatitis B are rarely able to induce a functional cure. This is partly because of the presence of a pool of circular viral DNA in the host nucleus, as well as viral DNA fragments that are integrated into the host genome. Herein, we show that a host biological pathway called neddylation could play a key role in infection and viral DNA integration. Inhibiting this pathway could hold therapeutic promise for patients with chronic hepatitis B.

https://doi.org/10.1016/j.jhepr.2022.100551

Dual role of neddylation in transcription of hepatitis B virus RNAs from cccDNA and production of viral surface antigen



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JHEP Reports **2022.** https://doi.org/10.1016/j.jhepr.2022.100551

Background & Aims: HBV persistence is maintained by both an episomal covalently closed circular (ccc)DNA reservoir and genomic integration of HBV DNA fragments. While cccDNA transcription is regulated by Cullin4A-DDB1-HBx-mediated degradation of the SMC5/6 complex, HBsAg expression from integrants is largely SMC5/6 independent. Inhibiting neddylation of Cullin-RING ubiquitin ligases impairs degradation of substrates. Herein, we show that targeting neddylation pathway components by small-interfering (si)RNAs or the drug MLN4924 (pevonedistat) suppresses expression of HBV proteins from both cccDNA and integrants.
Methods: An siRNA screen targeting secretory pathway regulators and neddylation genes was performed. Activity of MLN4924 was assessed in infection and integration models. Trans-complementation assays were used to study HBx function in cccDNA-driven expression.
Results: siRNA screening uncovered neddylation pathway components (*Nedd8*, *Ube2m*) that promote HBsAg production post-transcriptionally. Likewise, MLN4924 inhibited production of HBsAg encoded by integrants and reduced intracellular HBsAg levels, independent of HBx. MLN4924 also profoundly inhibited cccDNA transcription in three infection models. Using the HBV inducible cell line HepAD38 as a model, we verified the dual action of MLN4924 on both cccDNA and integrants with sustained suppression of HBV markers during 42 days of treatment.
Conclusions: Neddylation is required both for transcription of a cccDNA reservoir and for the genomic integration of viral DNA. Therefore, blocking neddylation might offer an attractive approach towards functional cure of chronic hepatitis B.
Lay summary: Current treatments for chronic hepatitis B are rarely able to induce a functional cure. This is partly because of the presence of a pool of circular viral DNA in the host nucleus, as well as viral DNA fragments that are integrated into the host genome. Herein, we show that a host biological pathway called neddylation could play a key role in infection and viral DNA integration. Inhibiting this pathway could hold therapeutic promise for patients with chronic hepatitis B.
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Introduction

Chronic HBV infection is a major cause of liver failure, cirrhosis and hepatocellular carcinoma that affects over 296 million people worldwide.[1] Current therapeutic approaches (nucleos(t)ide analogues and occasionally interferon-α) are non-curative and aim at suppressing HBV DNA. Both regimens rarely lead to the loss of HBV surface antigen (HBsAg), the key event in immunological control of the virus which is defined as functional cure.[2] Persistent HBV infection is characterized by the failure of the immune system to eliminate covalently closed circular (ccc)DNA from the nucleus of infected hepatocytes. cccDNA constitutes the episomal template for both genomic and subgenomic viral RNAs. It recruits histones and other nuclear proteins in order to establish an episome with a long half-life time.[3,4] Like other episomes (*e.g.* plasmids), the transcriptional activity of cccDNA is repressed by the structural maintenance of chromosomes 5/6 (SMC5/6) complex.[5] To counteract this cellular downregulation, the HBV X protein (HBx) hijacks DNA damage-binding protein 1 (DDB1) and subsequently recruits NEDD8-Cullin4-RING ubiquitin E3 ligases to induce ubiquitination and proteasomal degradation of the SMC5/6 complex. This mechanism enables high levels of transcription from only a few cccDNA molecules in an actively HBV-replicating hepatocyte.[6] The interplay of SMC5/6-mediated down-modulation of cccDNA-dependent transcription and the HBx counteraction controls hepadnaviral

Keywords: HBsAg; integrants; cccDNA; transcription; HBx; SMC6; neddylation; MLN4924.
Received 18 August 2021; received in revised form 10 July 2022; accepted 14 July 2022; available online 7 August 2022
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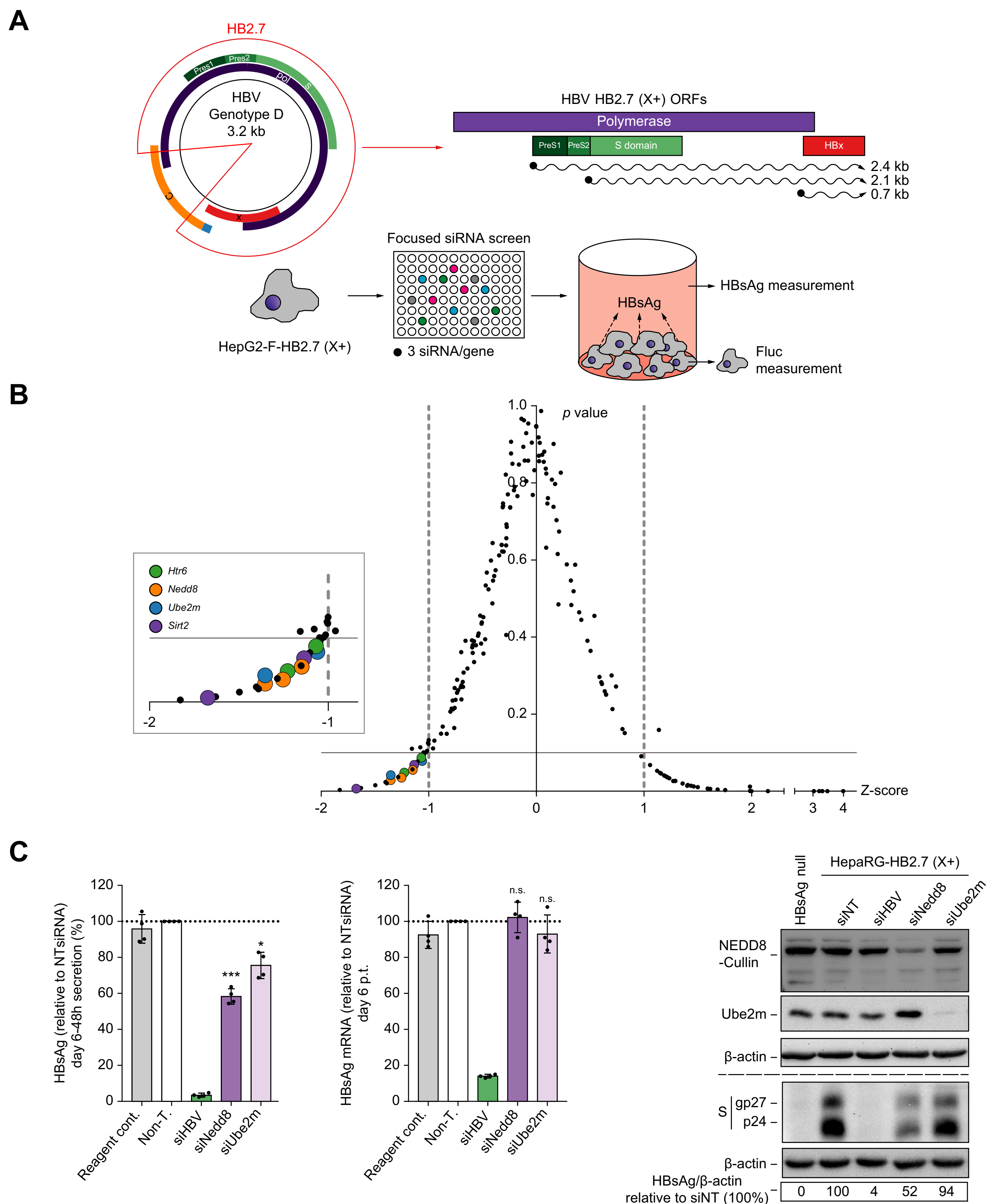

Fig. 1. Focused siRNA mini-screen identifies neddylation pathway genes involved in HBsAg secretion. (A) Overview of siRNA mini-screen for the identification of HBsAg secretion regulators in transfected HepG2-F-HB2.7(X+) cells using an Ambion siRNA library containing 3 siRNA sets for each candidate gene. The genes are listed in Table S1. HepG2-F-HB2.7(X+) cells were reverse transfected with siRNAs randomly pre-seeded in 96-well plates. Secreted HBsAg levels were measured in culture medium harvested between 48 and 96 hours post transfection. Firefly luciferase values were measured in the cells at 96 hours post transfection. (B) HBsAg readouts were normalized to NT siRNA and Z-score values of each siRNA are depicted: Z = (siRNA value - population mean value)/ population standard deviation (N≥3). Sirt2 (purple), Htr6 (green), Ube2m (blue) and Nedd8 (orange) genes with Z-score ≤-1 in at least 2 independent siRNA sets are marked. *P* values were calculated for each siRNA by using 2-tailed Student's *t* test, equal variance assumption. (C) Validation of Nedd8 and Ube2m genes was

gene expression and presumably plays an important role in immune escape and sustained persistence.[7–9]

Functional activation of Cullins, such as Cullin4 which is involved in regulating HBx function, requires neddylation.[6,10–12] Neddylation relies on the transfer of NEDD8 (neural precursor cell expressed, developmentally downregulated 8), a ubiquitin-like polypeptide of 81 amino acids, to specific lysine residues or the N-terminal end of the substrates.[13,14] Similar to ubiquitination, neddylation proceeds via a multi-step reaction requiring activating E1 enzyme (*i.e.* NAE1/UBA3), conjugating E2 proteins (Ubc12/UBE2F) and E3 ligases. This cascade of processes is important in the control of the cell cycle and inhibitors of specific steps involved in neddylation are attractive targets for cancer therapeutics. In 2019, MLN4924 (pevonedistat) and the thiazolide derivative nitazoxanide were identified as potent inhibitors of HBV replication.[15,16] Nitazoxanide has previously been reported to interfere with HBV replication in HepG2.2.15 cells[17] and shown to interfere with HBx-DDB1 binding and to suppress cccDNA-driven HBV transcription.[16]

A hallmark of acute and chronic HBV infection is the production of high levels of non-infectious subviral particles (SVPs). SVPs self-assemble into two distinct morphotypes: filaments and spheres. Both contain the small envelope protein (S), but differ in their content of the large envelope protein (L).[18] The spherical SVPs are 22 nm in diameter, have a low L:S ratio and are thought to be secreted via the constitute secretory pathway. Filamentous SVPs are heterogeneous in length, ranging between 100 nm to >1,000 nm, but have a rather constant diameter of 22 nm.[19] The filamentous SVPs have a high L:S protein ratio, similar to that of HBV virions. In contrast to spherical SVPs, filaments are reported to be released via the ESCRT/multivesicular body pathway, analogous to the secretion of HBV virions.[20]

Persistent expression of HBV proteins (especially the three HBV surface proteins or fragments thereof) is not exclusively driven by cccDNA, but may arise from subgenomic fragments that integrate during replication into cellular genomes of hepatocytes. Such integration arises from double-stranded linear HBV intermediates and occurs via non-homologous end joining. Under certain circumstances, cells carrying the integrants clonally expand and become the major source of HBsAg in the blood of chronically infected patients.[21,22] Since HBsAg production from integrants is independent of viral replication, it is not affected by nucleos(t)ide analogues.[23,24] This has been confirmed in HBeAg-negative patients and chimpanzees with chronic hepatitis B.[25] Of note, high levels of HBsAg most likely suppress the robust immune responses against this antigen which are required for functional cure, as defined by anti-HBsAg seroconversion.[26] Moreover, HBsAg produced from HBV integrants may complement the life cycle of hepatitis D virus (HDV), the satellite RNA virus of HBV, which uses the HBV surface glycoproteins for its own envelopment to disseminate via the extracellular route.[27,28]

Since none of the current therapies for HBV profoundly affect HBsAg levels in patients, we searched for host cell factors involved in the production and secretion of HBV surface proteins in order to identify novel drug targets. By using an RNAi-based screen and pharmacological inhibitor studies, we identified a dual role of neddylation in both HBsAg secretion and synthesis of HBV transcripts from the episomal cccDNA. Through application of MLN4924, a well characterized inhibitor of the neddylation pathway, we identified a key molecule for possible therapeutic interference with HBsAg production and secretion.

Materials and methods

Stable cell lines

All cells were incubated at 37°C, 5% CO_2 and 95% humidity. Primary human hepatocytes (PHHs) were obtained as previously described.[29] HepG2, HepG2-F-HB2.7, HepG2^{NTCP} and HepG2^{NTCP}-HBx cells were cultivated in Dulbecco's modified Eagle's medium (Life Technologies, Darmstadt, Germany) containing 10% fetal calf serum, 1× non-essential amino acids (Life Technologies), 100 units/ml penicillin, and 100 µg/ml streptomycin. Culture and differentiation protocols of HepaRG and HepaRG^{NTCP} cells have been described previously.[30] HepaRG-HB2.7(X+/-) cells were used in undifferentiated state. For HepAD38 cells, DMEM/F12, HEPES medium (Life Technologies) was supplemented with 10% fetal calf serum, 1× non-essential amino acids (Life Technologies), 100 µg/ml penicillin, and 100 µg/ml streptomycin (Life Technologies), 5 µg/ml insulin (SAFC Biosciences), 50 µM hydrocortisone (Sigma-Aldrich), 2 mM L-glutamine (Life Technologies) and 1 mM sodium pyruvate (Life Technologies).[31]

siRNA screen

A custom-made small-interfering (si)RNA library containing 192 siRNAs (Ambion Silencer Select) that target 64 human genes (Table S1 in Excel format) with three independent siRNAs per gene was used. HepG2-F-HB2.7(X+) cells were reverse transfected by seeding 10^4 cells per well of a 96-well plate in 200 µl of culture medium. Two to 4 days post transfection, cell culture supernatants were collected and the amount of HBsAg contained therein was determined by ELISA, while the cells were lysed in luciferase lysis buffer (1% Triton X-100, 25 mM glycyl-glycin, 15 mM $MgSO_4$, 4 mM EGTA and 10% glycerol) for measurement of firefly luciferase activity (an indicator of cell viability). Dharmacon On-TARGET plus pooled siRNAs targeting HBV transcripts, *Nedd8* and *Ube2m* were used for the validation experiments. The final concentration of the validation siRNAs was 30 nM for non-targeting control, *Nedd8*, *Ube2m*, and HBV siRNAs. The protocol for spotting siRNAs on 96-well plates can be found elsewhere.[32]

HBsAg measurement

Cell culture media of HepAD38, HepG2/HepaRG-HB2.7 and HBV-infected cells was collected and centrifuged at 400 g for 5 minutes using a tabletop centrifuge. Depending on the cell type, cleared supernatant was used undiluted or diluted up to 1:20 with PBS. For the primary siRNA screen, a home-made ELISA was used as described previously.[33] For all other experiments, absolute HBsAg values were measured using the HBsAg quantitative system (Architect, Abbott). Values >0.05 IU/ml were regarded as positive. Intracellular HBsAg was analysed by western blot and

performed using Dharmacon siRNAs sets in HepaRG-HB2.7(X+) cells. A siRNA targeting the 3'termini of HBV transcripts was used as positive control. Medium of transfected cells was refreshed at day 4 post transfection and amounts of secreted HBsAg as well as levels of cellular HBs transcripts were determined (mean ± SD; n = 4). Shown are the RNA levels normalized to those of cells treated with the non-targeting siRNA. $^*p < 0.05$, $^{***}p < 0.001$ compared to non-targeting siRNA (One-way ANOVA), n.s.: not significant. Intracellular levels of HBsAg and knockdown efficiency of siNedd8 and siUbe2m treatments were detemined. β-actin served as loading control. Shown are representative western blot images of 4 independent experiments. Fluc, firefly luciferase; non-T, non-targeting siRNA.

in-cell ELISA assays. Cells were lysed in Laemmli buffer and proteins contained in lysates were separated by electrophoresis using 12% SDS-polyacrylamide gel. After transfer of the proteins onto a PVDF membrane, it was blocked with 5% BSA and incubated with primary antibody at 4°C overnight. On the next day, the membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. The signal was developed by using an ECL chemiluminescence imager (INTAS) and band intensities were quantified with the Bio-Rad Image Lab Software package (version 6.0.1). All antibodies used in this study are listed in the CTAT document.

Results

An RNAi screen identifies neddylation pathway genes as key players in HBsAg secretion

To identify host factors involved in HBsAg secretion, we prepared a custom-made library of 64 genes, the majority of which are shown to regulate general secretory pathways.[34] The siRNA screening was performed with the HepG2-derived cell line HepG2-F-HB2.7(X+) stably expressing and secreting SVPs from an integrated HBV genome fragment (genotype D) encoding all three viral surface proteins under control of the authentic HBV enhancer and promoters, but independent from the episomal cccDNA.[35] In addition, this cell line stably expresses the firefly luciferase gene to monitor cytotoxic effects caused by a respective knockdown (Fig. 1A and Fig. S1A). Cells seeded into siRNA-precoated 96-well plates were reverse transfected and after 96 hours, the supernatant from each well was subjected to HBsAg quantification by ELISA. Statistical analysis of multiple replicates of the screening and hit ranking based on Z-scores identified 4 genes whose knockdown significantly reduced secreted HBsAg (Z-score ≤-1 for at least two independent siRNAs per gene without cytotoxic effects): *Nedd8*, *Ube2m*, *Sirt2* and *Htr6* (Fig. 1B and Fig. S1B). Two of these genes, *Nedd8* and *Ube2m*, are known as key factors in the neddylation pathway, supporting its involvement in HBsAg production or secretion.

To validate the primary screening results, we focused on dependency factors rather than on factors that increased HBsAg secretion. Two different approaches were chosen. In the case of Htr6 and Sirt2, small molecule inhibitors targeting these proteins were applied and their effects on HBsAg release from HepG2-F-HB2.7(X+) cells were determined. In addition, siRNAs obtained from a different supplier were used in a HepaRG-derived cell line stably expressing all viral surface proteins under authentic promoter control from an integrant (HepaRG-HB2.7(X+)). Although the knockdown validation experiments yielded the same phenotype of the primary screen, small molecule inhibitors of Sirt2 had no effect while one of the two Htr6 inhibitors caused a dose-dependent increase in extracellular HBsAg (Fig. S2 and Fig. S3). Since this increase was at variance to the siRNA screening data and the expression level of the *Htr6* gene in HepaRG-HB2.7(X+) cells was below the limit for reliable detection by quantitative reverse-transcription PCR, we focused on *Nedd8* and *Ube2m*. For these 2 genes, we validated the phenotype by using different siRNAs transfected into HepaRG-HB2.7(X+) cells. We observed a significant decrease of secreted HBsAg, indicating that Nedd8 and Ube2m are important for the production of extracellular HBsAg, independent of a preceding infection with HBV. Notably, knockdown of Nedd8 or Ube2m did not affect the level of HBs transcripts but led to a decline in the level of intracellular HBsAg, suggesting that the reduction of secreted HBsAg produced from the HBV subgenome integrant is due to impaired HBsAg production or stability (Fig. 1C).

The neddylation inhibitor MLN4924 suppresses HBsAg production from integrated HBV subgenomes in an HBx-independent manner

To corroborate this observation with a different approach, we employed the NAE1-specific inhibitor of neddylation MLN4924,[36] and determined its impact on extracellular HBsAg levels in HepaRG-HB2.7(X+) cells. These cells harbour a 2.7-kb integrant and encode HBsAg and HBx under authentic promoter control. To investigate whether HBx plays a role in regulating the expression of HBsAg, we used HepaRG-HB2.7(X-) cells wherein two stop codons had been inserted into the HBx open reading frame. Applying concentrations of MLN4924 (≤200 nM) that did not affect cell viability (Fig. S3), we found that HBsAg secretion was reduced in a dose-dependent manner in both HB2.7(X+) and (X-) cell lines (Fig. 2A). Amounts of intracellular p24 and glycosylated p27 (gp27) were reduced by MLN4924 treatment at 200 nM as determined by western blot (Fig. 2B). The ratio of intracellular to extracellular HBsAg ELISA values did not show any sign of HBsAg accumulation in the cells, consistent with the siRNA validation results (Fig. 2B,C). Moreover, the intracellular decrease of envelope proteins (Fig. 2B) was not due to impaired synthesis of envelope-encoding mRNAs (Fig. 2D). We therefore concluded that the antiviral effect of inhibiting neddylation in HepaRG-derived cells expressing HBsAg from integrants was mediated at a post-transcriptional level, *e.g.* HBsAg protein synthesis or stability in an HBx-independent manner.

MLN4924 potently and specifically inhibits HBV replication in various infection models

Based on the concept that neddylation of Cullin4 is a prerequisite for activation of the HBx-DDB1 complex to counteract silencing of cccDNA, we examined whether this modification, besides acting directly on HBsAg production, also affects cccDNA-dependent replication. To that aim, we infected differentiated HepaRG^{NTCP} cells (dHepaRG^{NTCP}) with HBV in the presence of MLN4924, the selective inhibitor of NAE1, blocking the complete downstream pathway. In this experimental setting of infection, we observed a potent and profound reduction of HBsAg at 2-digit nanomolar concentrations of MLN4924, with an almost complete loss of extracellular HBsAg at 250 nM. Notably, we also observed a reduction of HBeAg and total HBV transcripts (Figs. 3A and S5). This indicated that MLN4924 not only affects HBsAg secretion, but also impairs a pathway related to cccDNA. We therefore compared the levels of total HBV RNA to the levels of cccDNA (determined by quantitative PCR) at different concentrations of the inhibitor in dHepaRG^{NTCP} cells and found a stronger effect on mRNA at lower concentrations and only a moderate effect on cccDNA copy numbers at higher concentration. Notably, the inhibitory effect of MLN4924 treatment on HBsAg production and release was more profound in the dHepaRG^{NTCP} infection system compared to the effect observed in cells carrying an HBV integrant (Figs. 2A and 3A). Moreover, the EC_{50} value correlated with that observed for transcription inhibition. HepG2^{NTCP} cells were less responsive than dHepaRG^{NTCP} cells and PHHs (Fig. S5). A >50% reduction at the transcriptional level in HepG2^{NTCP} cells requires >1 μM of MLN4924, but HepG2^{NTCP} cells have a far

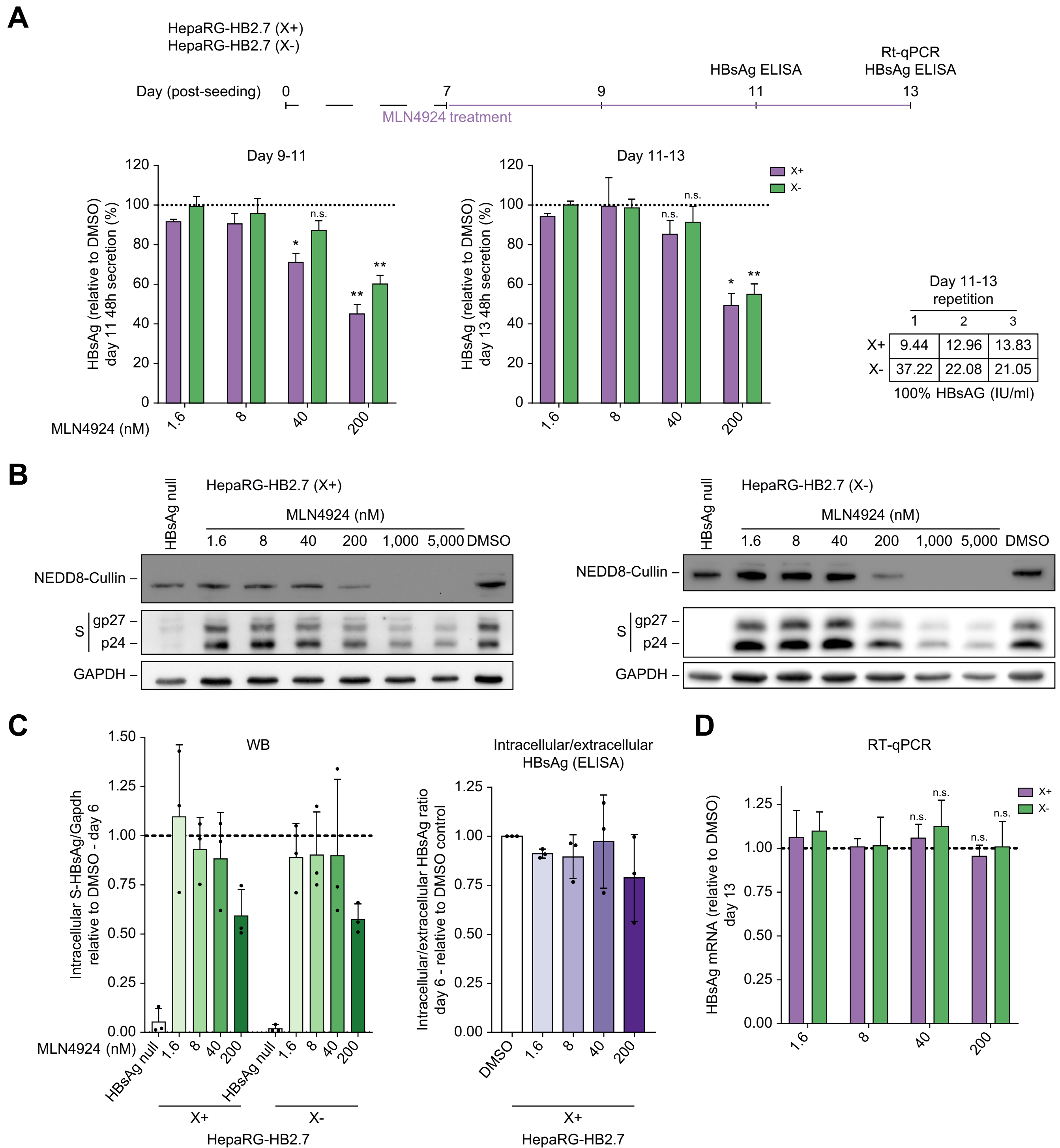

Fig. 2. Impact of neddylation pathway inhibitor MLN4924 on HBsAg expression. (A) HepaRG-HB2.7-X (+) and (X-) cells were treated with MLN4924 with 1:5 serial dilutions starting on day 7 post seeding for 6 days. Secreted HBsAg amounts accumulating in culture supernatants between day 9 to 11 (lower left) and day 11 to 13 (lower right) post seeding were determined. Values were normalized to those obtained with DMSO vehicle-treated cells (set to 100%) (mean ± SD, n = 3). Absolute values of secreted HBsAg are shown for each DMSO vehicle control (100%) between day 11 to 13. *p <0.05, **p <0.01 compared to vehicle control (One-way ANOVA), n.s.: not significant. (B) Shown are representative western blots of HBsAg and Nedd8-conjugated Cullin in cells harvested on day 13. GAPDH served as loading control. Lysates from empty-vector transduced cells served as negative control. (C) The relative ratios of total HBV S protein (p24 plus gp27) and GAPDH in 3 independent western blots were quantified (mean ± SD; n = 3). In addition, ratios of intracellular to extracellular HBsAg ELISA levels were calculated in treated HepaRG-HB2.7-(X+) (right panel in C). (D) The levels of HBs transcripts on day 13 were quantified by RT-qPCR and normalized to those of DMSO-treated cells (100%). n.s.: not significant compared to vehicle control (One-way ANOVA).

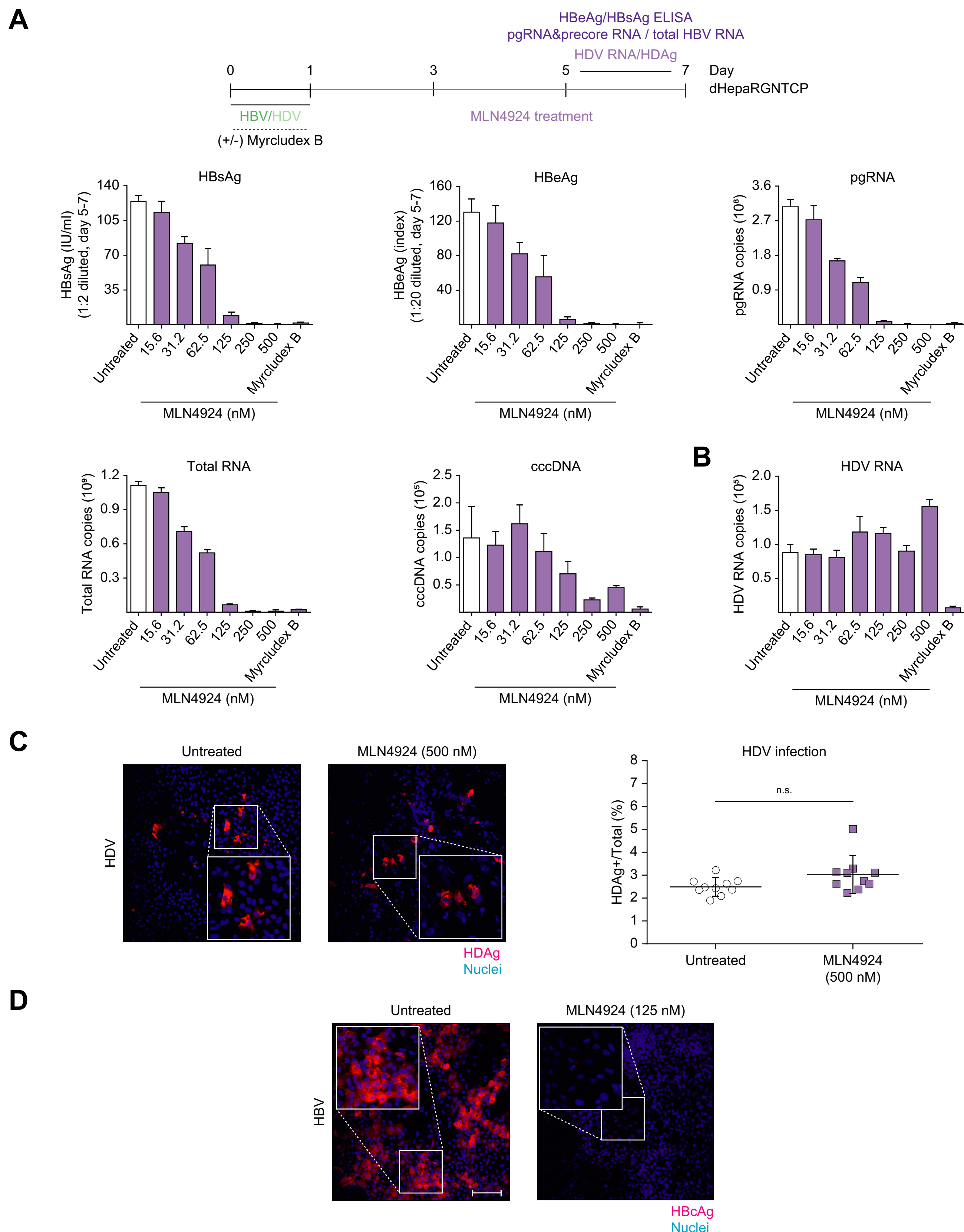

Fig. 3. MLN4924 selectively inhibits HBV but not HDV replication. (A) dHepaRG^{NTCP} cells were infected with HBV using a multiplicity of 500 genomic equivalents (mge). Infected cells were treated with 1:2 serial dilutions of MLN4924 from day 1 to day 7 as shown on the top of the panel. As control, the entry inhibitor Myrcludex B (0.5 μM) was added during infection. Secreted HBsAg and HBeAg levels from day 5 to day 7 were measured. Total transcripts, pgRNA and cccDNA on day 7 were quantified. cccDNA copies were normalized by β-globin as described in the methods (mean ± SD; n = 2). (B) dHepaRG^{NTCP} cells were

Table 1. EC$_{50}$ and CC$_{50}$ values of MLN4924 in various HBV infection models.

Name	PHH (nM)	dHepaRG^{NTCP} (nM)	HepG2^{NTCP} (nM)
EC$_{50}$ of HBsAg	293.4 [102~860]*	26.9 [9~66]	175.1 [40~910]
EC$_{50}$ of HBeAg	143.7 [68~298]	15.0 [5~33]	30.2 [0~235]
EC$_{50}$ of transcription	292.4 [18~11,596]	30.2 [5~113]	484.7 [>80]
CC$_{50}$	857.0 [245~3,607]	2,442 [1,025~6,545]	>50,000

CC$_{50}$, half maximum dose of cytotoxicity; EC$_{50}$, half maximal effective dose; PHH, primary human hepatocyte.
* Best-fit values [95% profile likelihood (Cl)].

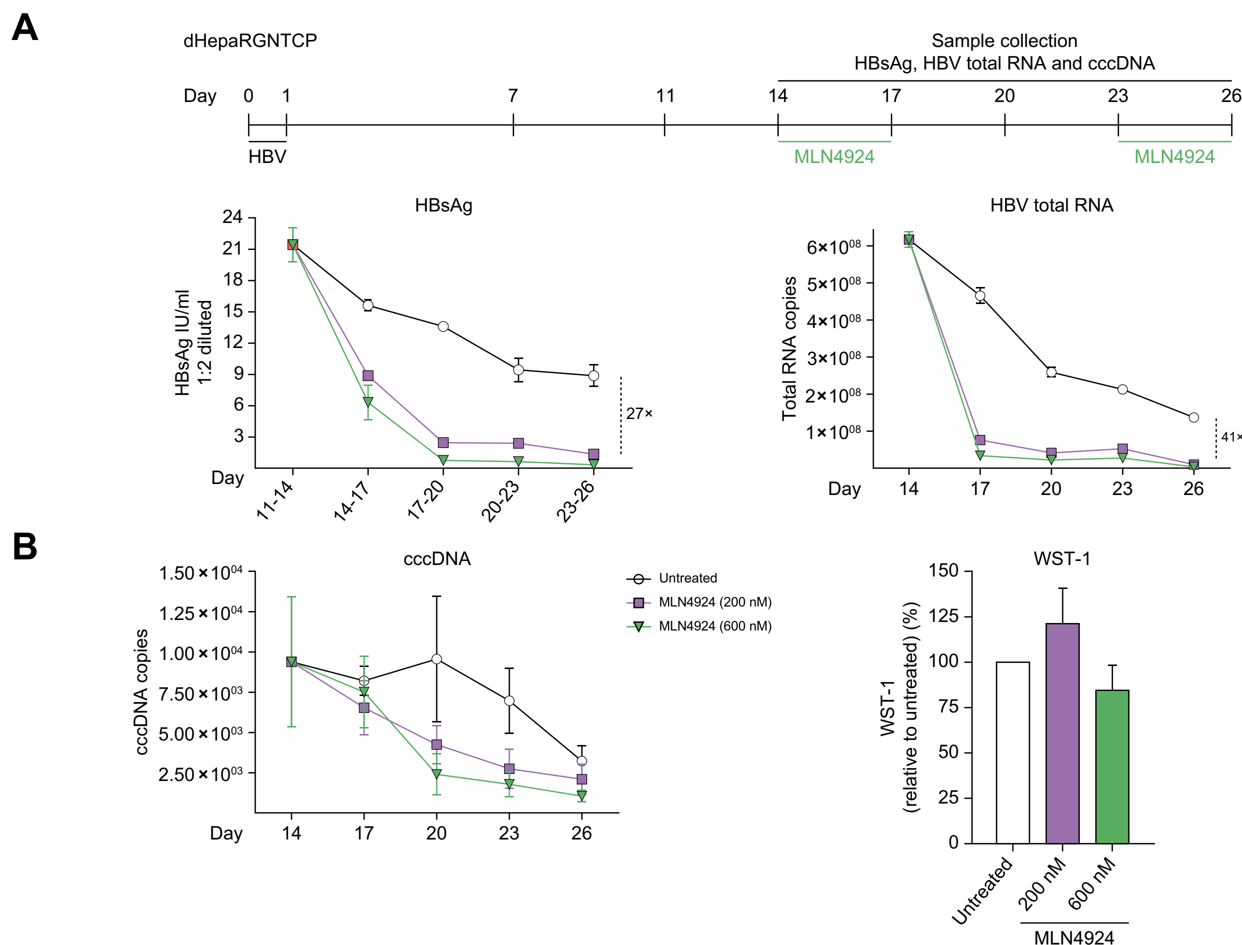

Fig. 4. MLN4924 potently inhibits transcription from static cccDNA in long-term culture. dHepaRG^{NTCP} cells were infected with HBV (mge = 500) and long-term maintained by changing medium twice per week during the first 2 weeks. MLN4924 treatment began at day 14 post infection and lasted for 3 days. Treatment was terminated, the drug was removed by extensive washes of the cells on day 17 and treatment was restarted on day 23. (A) At 3-day intervals, culture medium was harvested and used to measure HBsAg. Levels of total transcripts (A) and cccDNA (B) were quantified at every collection time point. Viability of the cells was determined by WST-1 assay on day 26. Data shown in this figure is from 1 of 2 independent experiments.

higher CC$_{50}$ value (>50 μM). EC$_{50}$ values in all infection models (dHepaRG^{NTCP}, HepG2^{NTCP} and PHH) are summarized in Table 1.

To investigate whether inhibition of neddylation is specific for HBV or also affects HDV, the satellite of HBV, dHepaRG^{NTCP} cells were infected with HDV and monitored for specific markers of replication like HDV RNA and HDAg. In contrast to HBV, treatment with MLN4924 led to a slight increase of intracellular HDV RNA (Fig. 3B). Furthermore, MLN4924 treatment did not reduce the number of HDAg-positive cells (Fig. 3C), whereas HBcAg was virtually undetectable (Fig. 3D).

These results suggested that neddylation plays a dual role in HBV replication: first, it impacts HBsAg synthesis/stability

infected with HDV (mge = 5). MLN4924 or Myrcludex B treatment was the same as described for (A). HDV RNA level, including genomic and antigenomic strands, was quantified on day 7 post infection (mean ± SD; n = 2). (C) Intracellular HDAg expression (red) on day 7 was detected in untreated or MLN4924 (500 nM) treated HDV-infected cells. Quantification of HDAg-positive cells in untreated and treated cells. For each condition, 10 images were analysed. Nuclear DNA stained with the Hoechst dye is shown in blue. The scale bar indicates 200 μm. n.s.: not significant compared to untreated control (Student's t test). (D) Intracellular HBcAg expression (red) on day 7 was visualized in untreated or MLN4924 (125 nM)-treated HBV-infected dHepaRG^{NTCP} cells.

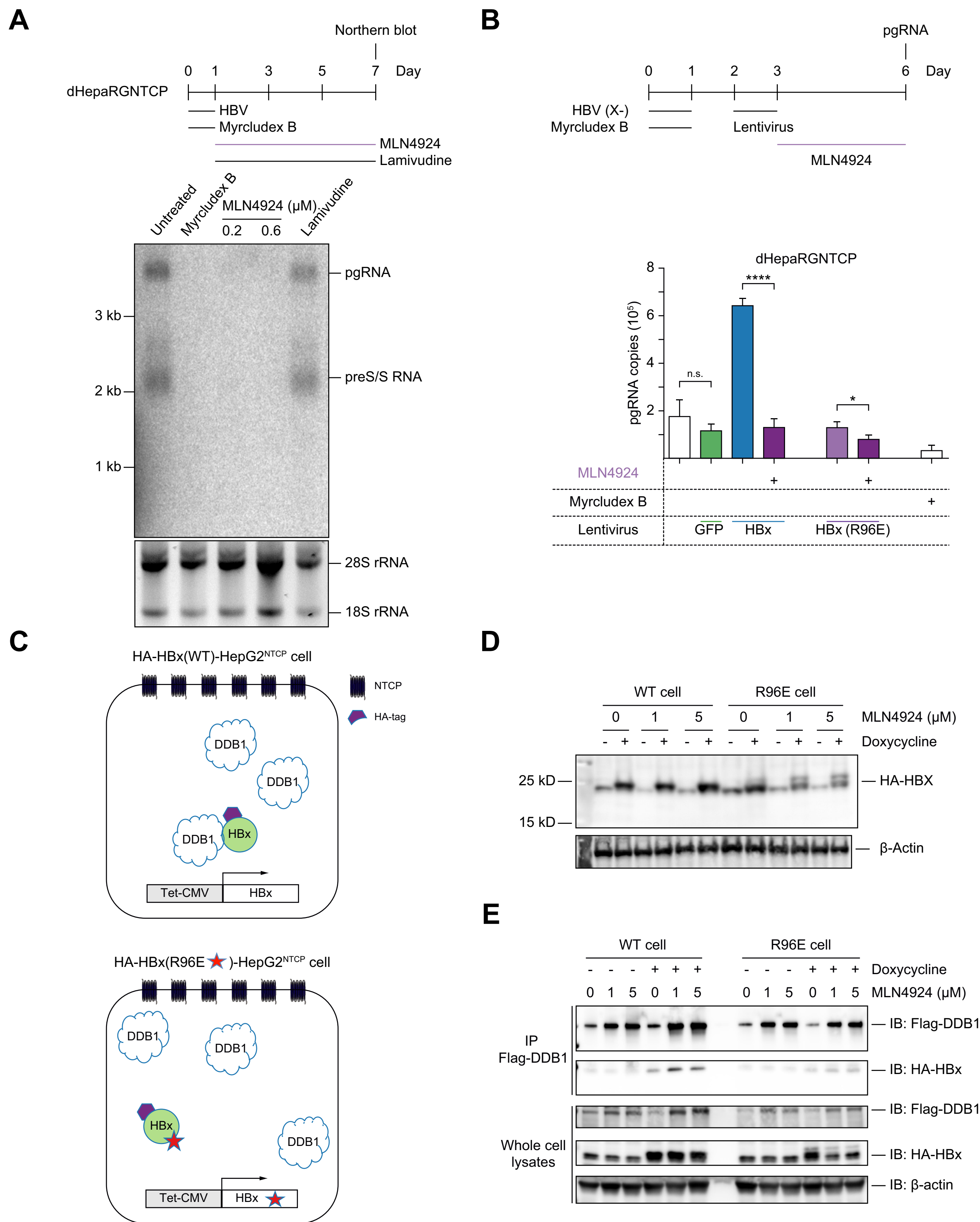

Fig. 5. MLN4924 inhibits transcription from cccDNA and does not alter the HBx-DDB1 interaction. (A) dHepaRG^{NTCP} cells were infected with HBV and treated with MLN4924 (200 and 600 nM) or lamivudine (2 µM) for 6 days post infection. Myrcludex B (500 nM) was applied as control. 3 µg total cellular RNA was subjected to northern blot, whereas 2 µg cellular RNA is shown in a native gel to visualize 28S/18S ribosomal RNA (rRNA) using GelRed dye (upper and lower panel, respectively). Shown is 1 of 2 independent experiments. (B) dHepaRG^{NTCP} cells were infected with HBx-deficient HBV (HBV X-) (mge = 100) and 2 days later transiently transduced with lentiviruses (mge = 50) encoding GFP, or WT HBx or HBx R96E mutant. On the next day, lentivirus inoculum was removed, cells were washed and treated with MLN4924 (200 nM) for 3 days. pgRNA levels were quantified on day 6. Representative data of 2 independent experiments is shown

directly when cccDNA is not the source of the viral antigen; second, neddylation is crucial for transcription of HBV RNAs or maintenance of RNA stability.

Effect of neddylation inhibition on long-term HBV replication

To mimic established HBV infection and reactivation of infection after treatment cessation, we performed long-term infection experiments on dHepaRG^{NTCP} cells that enabled monitoring of viral markers for 26 days. The cells were treated with 2 different non-cytotoxic concentrations of MLN4924 between day 14 and 17 followed by culturing in the absence of the drug until day 23; then cells were treated again for 3 days with the neddylation inhibitor (Fig. 4). Cells in duplicated wells were harvested every 3 days, starting on day 14. At the end of the first drug treatment phase (day 17), the level of HBsAg declined 27-fold, and total HBV RNA levels reached almost background levels (Fig. 4A). Of note, at this point in time, cccDNA copy numbers did not drop significantly indicating that the observed effect was not due to the loss of cccDNA. However, cccDNA levels steadily declined thereafter although no drug was present (Fig. 4B). During the second MLN4924 treatment (day 23-26), HBsAg and viral transcripts remained at the background, while cccDNA declined further. There was no correlation between the viability of cells and the dramatic decrease in the HBV parameters measured on day 26 upon MLN4924 treatment (Fig. 4B). These results demonstrated the potent antiviral effect of neddylation inhibition on long-term HBV replication.

MLN4924 prevents viral transcription from all HBV promoters

We next determined the impact of neddylation inhibition on the individual HBV transcripts upon HBV infection. Northern blot analysis revealed that pregenomic (pg)RNA and preS/S mRNA amounts were profoundly reduced by MLN4924 in infected dHepaRG^{NTCP} cells (Fig. 5A). Consistently, reduction of HBV RNAs, including the HBx mRNA, was also observed upon MLN4924 treatment of HBV-infected HepG2^{NTCP} cells in a dose-dependent manner. This was not due to altered stability of viral RNA (Fig. S6). In contrast, the nucleoside analogue lamivudine had no effect in either cell system (Fig. S7).

Given the effect of neddylation inhibition on cccDNA-dependent HBV transcription, we were wondering to what extent HBx is involved in the observed response. We therefore took advantage of HBV mutants unable to express HBx.[37,38] We used an HBV mutant encoding two stop codons in the HBx open reading frame[39] and infected dHepaRG^{NTCP} cells. Two days later, cells were transduced with lentiviruses encoding wild-type (WT) HBx, an HBx mutant with an R96E amino acid exchange rendering the protein deficient for DDB1 binding,[40] or GFP serving as a negative control (Fig. 5B). Twenty-four hours post transduction, cells were treated with MLN4924 and harvested at day 6 post HBV infection to quantify pgRNA by quantitative reverse-transcription PCR. Infection in the presence of the entry inhibitor ensured that all signals arose from virions that authentically entered the cells and produced cccDNA. As depicted in Fig 5B, pgRNA synthesis could be rescued through trans-complementation with EF1α promoter-driven WT HBx expression, but not with the DDB1 binding-deficient HBx.

To investigate the role of neddylation inhibition on HBx itself as well as on direct interference of HBx-DDB1 interactions, we generated HepG2-derived cell lines expressing WT or inactive (R96E) HBx in an inducible manner (Fig. 5C). This system was used to determine the possible impact of neddylation inhibition on HBx stability and its association with DDB1. We found that MLN4924 treatment did not alter the amount of WT HBx, but affected the mobility of the HBx R96E mutant by generating a slower migrating band (Fig. 5D). Compared to WT HBx, the interaction of the HBx R96E mutant with DDB1 was weaker as expected, but co-precipitated HBx amounts were not affected by MLN4924 treatment for both WT and mutant HBx (Fig. 5E). Moreover, infection experiments on these cells with HBx-deficient virions demonstrated that neddylation inhibition strongly reduced levels of HBV pgRNA and HBeAg, whereas cccDNA levels were unaffected (Fig. S8).

Next, we examined the fate of the SMC5/6 restriction complex under conditions of neddylation inhibition and performed SMC6-specific immunofluorescence on HBV-infected dHepaRG^{NTCP} cells and PHHs that were treated with MLN4924 (Fig. S9). We found that HBV infection induced the depletion of SMC6 in most infected cells, 90% and 85% for PHHs and dHepaRG^{NTCP} cells at day 5 and day 7 post infection, respectively, which was restored almost to the level of non-infected cells upon neddylation inhibition (Fig. S9B,D). These data are consistent with a recent finding and provide evidence that SMC5/6 restoration is one of the consequences of MLN4924 treatment.[41]

To elucidate the roles of HBx and SMC5/6, we knocked-down SMC5/6 and infected the cells with HBx-deficient virions. Indeed, MLN4924 inhibited transcription of WT and HBx-deficient viruses in the absence of SMC5/6, whereas transcription of WT virus was much more reduced upon treatment in the presence of SMC5/6 (Fig. S10). Collectively, these data suggest that the effect of MLN4924 is partially dependent of SMC5/6 and transcription of HBx-deficient virus is restricted by SMC5/6 in the absence of HBx.

Neddylation blockage affects both transcription from cccDNA and HBsAg production from viral integrants

The results reported so far indicate that neddylation is vital in both HBsAg production from viral integrants and in the regulation of transcription from cccDNA. To corroborate this dual function in the HBV life cycle in one system, we took advantage of HepAD38 cells.[31] This cell line encodes a single copy of overlength HBV genome transcribed under the control of a tetracycline/doxycycline inducible promoter (Fig. 6A). In the presence of the antibiotic (+doxycycline), the viral pgRNA is very inefficiently transcribed because of tight control of the tet-inducible promoter, and therefore, only very low amounts of HBeAg and HBcAg are expressed. In contrast, the viral surface proteins continue to be transcribed with high efficiency under control of

(mean ± SD). *p <0.05, ****p <0.0001 (Student's t test), n.s.: not significant. (C) HepG2^{NTCP} cells were stably transduced with lentiviruses (Tet-CMV-HA tagged HBx) encoding WT HBx or HBx (R96E) (red asterisk: DDB1-binding mutant). Cells (HBx (WT) and HBx (R96E)-HepG2^{NTCP}) were infected with HBV and used to evaluate MLN4924. (D) Both cell lines were co-treated with doxycycline and MLN4924 and after 72 hours, HBx expression levels were examined by immunoblot. (E) Both cell lines were transiently transfected with a pcDNA3.1 plasmid encoding a Flag-tagged DDB1 fusion protein. After 24 hours, cells were treated with MLN4924 for 48 hours in the presence or absence of doxycycline added to the culture medium. The cells were lysed in modified RIPA buffer and pre-cleared lysates were used for Flag-specific pull-down. Flag-DDB1 and co-captured HBx were analysed by western blot. β-actin served as a loading control of whole cell lysates. GFP, green fluorescent protein.

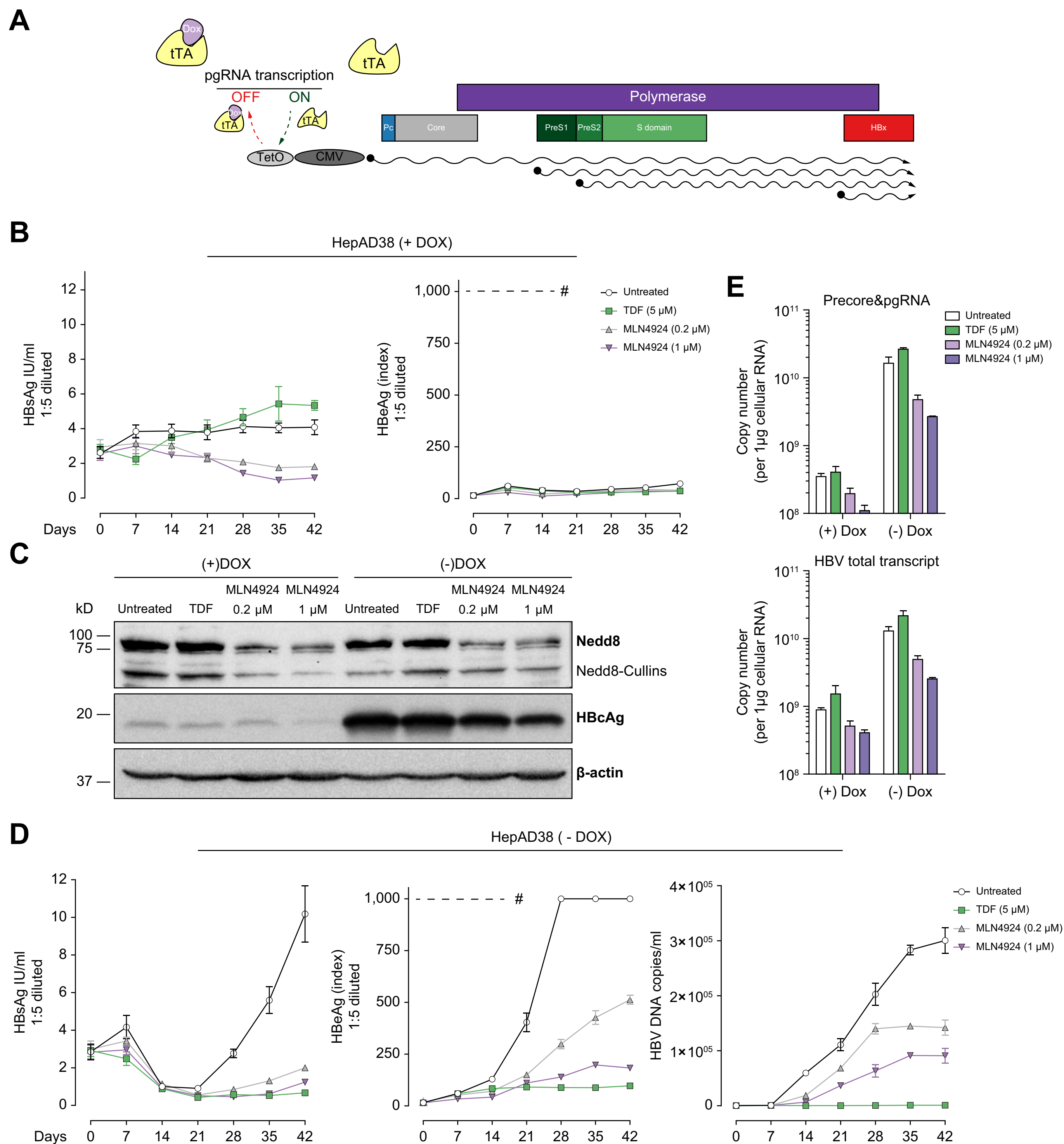

Fig. 6. Dual effect of neddylation inhibition on HBV replication as revealed in the HepAD38 model. (A) Schematic representation of 1.1-mer HBV integrant under DOX-off inducible CMV promoter. (B) Time-course of HBeAg and HBsAg levels. Values correspond to IU/ml for HBsAg. Index values of HBeAg were determined using internal calibrators of the ADVIA Centaur XP Immunoassay system. (C) Cells treated as specified on the top were harvested on day 42 post treatment and lysates were analysed by western blot to determine the levels of neddylated Cullin proteins and HBcAg. β-actin served as a loading control. (D) HBeAg, HBsAg and HBV DNA amounts detectable in supernatants of cells at day 42 of culture. #HBeAg values ≥1,000 index. (E) Levels of intracellular pgRNA/preCoreRNA and total HBV transcripts on day 42 are depicted for the untreated group. Drug-treated groups were analysed on day 42. Shown is 1 of 2 independent experiments. TDF, tenofovir; tTA, tetracycline-controlled transactivator.

their authentic preS1/S2 promoters mimicking the situation of hepatocytes carrying integrants (Fig. 6B). pgRNA synthesis requires induction by doxycycline withdrawal leading to efficient production of HBeAg and HBcAg (Fig. 6C,D). In addition, as a result of reverse transcription of pgRNA and nuclear reimport of nucleocapsids, cccDNA is formed in these cells,[42] which serves as template for the transcription of the RNA pregenome and thus, HBcAg and HBeAg, in addition to HBsAg (Fig. 6D). Therefore, upon prolonged induction of HBV pregenome synthesis, viral particles and cccDNA are produced, with viral DNA production being sensitive to treatment with tenofovir (Figs. 6D and S11).[31]

Transcription of the HBV pregenome was investigated for 42 days in the presence or absence of MLN4924 and doxycycline. Under conditions of HBV repression (+doxycycline), HBsAg was suppressed by the neddylation inhibitor in agreement with our previous findings in HepaRG-HB2.7 cells (Fig. 2). Tenofovir, as a potent inhibitor of reverse transcription of pgRNA, had no effect on the HBsAg expressed from viral integrants, excluding a possible contribution of pregenome synthesis and viral replication in HBsAg synthesis (Fig. 6B). Upon induction of HBV pregenome transcription, we observed an evident increase of secreted HBsAg (Fig. 6D), which is presumably caused by additional HBsAg production from the slowly increasing numbers of cccDNA. Depending on replication, this additional HBsAg (and HBeAg) can be suppressed by tenofovir (Fig. 6D). HBV transcription was lowered by MLN4924 but not tenofovir (Fig. 6E). Notably, neddylation inhibition strongly reduced the amounts of both antigens, consistent with transcription inhibition from cccDNA (Fig. 3).

Taken together, these results corroborate the dual role of the neddylation pathway in the HBV life cycle, *i.e.* maintaining transcription from cccDNA and promoting synthesis/release of HBsAg.

Discussion

Herein, we show that neddylation plays a dual role in the HBV life cycle, being (1) directly involved in the HBx-independent synthesis of HBsAg and (2) in the transcriptional regulation from cccDNA. Using MLN4924 (pevonedistat), a selective inhibitor of the NAE1 E1 enzyme in the neddylation pathway, we verified a previously reported antiviral activity of this drug against HBV[15,43] but not HDV replication. Furthermore, through functional analyses in various suitable cell culture systems, we demonstrated MLN4924's dual mode of antiviral action on HBV.

HBV integration causes genomic instability in infected hepatocytes, which contributes to carcinogenesis.[44,45] HBV integration plays an important role in this process and occurs early post infection.[46] While not directly required for HBV replication, integrants typically encode HBx-host chimeric genes and all HBV envelope proteins under control of the authentic promoter.[47,48] Recent clinical findings uncovered clonally expanded integrants as a major source of HBsAg in HBeAg-negative patients, which is a challenge for the development of novel drugs.[22,25,46] Moreover, high-level HBsAg expression is a key driver of insufficient immune control of HBV infection and therefore, a major target for curative therapeutic approaches.

In the present study, we disclose that perturbation of two neddylation pathway components, *Nedd8* and *Ube2m*, by siRNA-mediated depletion leads to a marked reduction of HBsAg production in a cell culture system that mimics hepatocytes with an integrant. Besides knockdown of *Nedd8* and *Ube2m*, the NAE1-specific inhibitor MLN4924 exhibits the same effect, indicating that the neddylation pathway plays a key role in the maintenance of HBsAg originating from integrants. Through mutational analyses we could show that this suppression is independent of HBx (Fig. 2). The direct effect of MLN4924 on HBsAg is moderate (~50% reduction in HBsAg with 200 nM – a concentration which is significantly higher than the reported EC_{50} of MLN4924 required to inhibit NAE1 *in vitro* of 4.7 nM).[36] We presently do not know how neddylation modulates HBsAg expression. However, since the levels of HBsAg-specific transcripts remained unchanged, whereas intra- and extracellular levels of HBsAg diminished, we hypothesize a post-transcriptional mode of HBsAg repression (*e.g.* production or stability; Figs. 1 and 2). Furthermore, we cannot exclude that a so far unknown neddylation substrate might be involved. These pathways and their key players have to be identified in the future and may become important targets for specific drugs that aim at reducing HBsAg.

With the identification of SMC5/6 as a key host restriction factor of cccDNA-dependent transcription and the understanding that HBx counteracts SMC5/6-mediated repression,[49] advanced insights into the regulation of HBV replication arose.[7,8] Substantiation that DDB1 plays the key role as a cellular adaptor protein to recruit SMC5/6 into the E3-Cullin4-RING ligase complex to promote ubiquitin-dependent degradation further opened new insights into cccDNA biology.[9] The discovery of this pathway reveals novel targets for drug intervention aiming at therapeutic shut down of all HBV transcripts, an important part of HBV cure. Taking advantage of the circumstance that the first step in HBx-dependent SMC5/6 degradation essentially depends on neddylation of the Cullin4-E3 ligase, we studied the impact of MLN4924 on transcription from cccDNA. Using different susceptible cell culture systems ($HepG2^{NTCP}$, $dHepaRG^{NTCP}$ and PHH), we consistently observed the potential of MLN4924 to shut down HBV gene expression from cccDNA already at low nanomolar concentrations (EC_{50} range, depending on the cell type, 15-290 nM). A more than 95% suppression of HBsAg and HBeAg could be achieved at concentrations <2 μM. Such profound reductions equated to the impairment observed for replication of an HBx-deficient HBV and indicates that addressing the neddylation pathway might be a suitable approach to shut down mRNA transcription from cccDNA with approved orally available therapeutics.[37,38] Supporting this notion, blocking neddylation supressed HBV, but not HDV replication (Fig. 3). Regarding the difference in the mode-of-action, neddylation inhibitors are expected to synergize with drugs that act post-transcriptionally (*e.g.* siRNAs or antisense oligonucleotides). Regarding the profound and sustained, but not complete, suppression of viral antigens, addition of neddylation inhibitors could therefore induce further reductions, *e.g.* of HBsAg.

A particular aspect of our study is related to the kinetics of suppression, namely the lack of reversibility of the effect of MLN4924 in $dHepaRG^{NTCP}$ cells. As depicted in Fig. 4, a three-day treatment at day 14-17 post infection resulted in an immediate reduction of HBsAg secretion, without affecting cccDNA levels. While SMC5/6 degradation occurred in almost all HBV-infected cells in the absence of MLN4924, MLN4924 treatment did prevent SMC5/6 degradation in infected cells. Since removal of the drug did not lead to an immediate rebound of HBV gene expression from cccDNA, it will be interesting to evaluate whether SMC5/6 can rebound in the long term once the drug is

removed. In a recent study, siRNAs targeting HBV transcripts or pegylated interferon-α treatment of humanized mice restored SMC5/6, however the follow-up analysis at off-treatment stage revealed rebound of HBV.[41] Quantitative analysis of cccDNA at later time points revealed a reduction upon MLN4924 treatment. The exact mechanism behind this observation is unclear. One possible explanation could be perturbation in proteostasis of cccDNA maintenance factors regulated by Cullin-RING ubiquitin ligases (*e.g.* Cullin5).[50]

It has been reported that the E3 ligase HDM2 promotes neddylation of HBx, thereby regulating its stability.[51] We cannot exclude that the levels of endogenous HBx may vary through application of the drug and thereby influence HBx functionality in addition to the described effect. However, in our inducible HBx overexpression system in HepG2^{NTCP} cells, without overexpression of NEDD8, MLN4924 did not result in changes in total intracellular levels of HBx, although we observed changes in the migration pattern of the HBx R96E mutant form (Fig. 5E). While the nature of the slower migrating HBx-specific band is not clear yet, HBx R96E, which is a DDB1 binding-deficient mutant, might become prone to post-translational modifications upon inhibition of the neddylation pathway.

MLN4924 is a chemotherapeutic drug used to treat solid tumours and haematological malignancies. It demonstrated good tolerability in phase II/III clinical trials and has received breakthrough status. As an antiviral drug its use might be limited, however a fast and profound reduction of HBsAg from cccDNA and integrants, *e.g.* combined with immune-modulators, could make MLN4924 an attractive candidate, especially when aiming at lead-in regimens that provoke restoration of the immune system to achieve viral clearance.

Abbreviations

cccDNA, covalently closed circular DNA; DDB1, DNA damage-binding protein 1; HBsAg, hepatitis B virus surface antigen; HBx, hepatitis B virus X protein; NAE1, NEDD8-activating enzyme E1 subunit 1; NEDD8, neural precursor cell expressed, developmentally downregulated 8; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; siRNA, small-interfering RNA; Smc5/6, structural maintenance of chromosomes 5/6; SVP, subviral particles; WT, wild-type.

Financial support

Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Projektnummer 272983813 – SFB/TRR 179; DZIF - TTU Hepatitis Projects 05.704. and 05.806.

Conflict of interest

Stephan Urban is co-inventor and applicant on patents protecting HBV preS1-derived lipopeptides (Myrcludex B/Bulevirtide/Hepcludex). All other authors declare no conflict of interest.

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

Authors' contributions

Conceptualization, B.Q., F.N., R.B. and S.U.; Methodology, P.M. J.B., H.E.; Investigation, B.Q., F.N., M.M.L. and Y.N.; Resources, F.W.R.V.; Software, B.Q.; Data analysis: B.Q., F.N., R.B. and S.U.; Writing-original draft, B.Q., F.N., R.B. and S.U.; Final draft: B.Q., F.N., R.B. and S.U.; Supervision, R.B. and S.U.; Funding, R.B. and S.U.

Data availability statement

All authors confirm that the data in this study are available within the article and supplementary materials. Any additional data are available from the corresponding authors upon reasonable request.

Acknowledgements

We acknowledge Dr. Vibor Laketa, head of the Infectious Diseases Imaging Platform (IDIP) at the University Hospital Heidelberg for expert support. We thank Prof. Dr. Bernd Wollscheid (ETH Zürich) for providing pInducer20-HA-HBx and pInducer20-HA-HBx (R96E) constructs. We thank Dr. Volker Lohmann for lentiviral packaging constructs, Prof. Dr. Ulrike Protzer for providing HepG2-H1.3-ΔX cell line and Franziska Schlund, Anja Rippert, Dr. Christa Kuhn and Dr. Stefan Seitz for routine laboratory support. We thank Ms. Saskia Mönch, Dr. Renate König, Dr. Alessia Ruggieri and Dr. Richard J.P. Brown for their help during paper revision. Bingqian Qu was a doctoral student of the International Research Training Group of the SFB/TRR179 program.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2022.100551.

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Author names in bold designate shared co-first authorship

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