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

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**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:** siRNAs 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 hepatic viral...
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: $Z = \left(\text{siRNA value} - \text{population mean value}\right)/\text{population standard deviation}$ ($N>3$). 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.\textsuperscript{13-19}

Functional activation of Culmins, such as Culmin4 which is involved in regulating HBx function, requires neddylation.\textsuperscript{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.\textsuperscript{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 thiazolidide derivative nitazoxanide were identified as potent inhibitors of HBV replication.\textsuperscript{15,16} Nitazoxanide has previously been reported to interfere with HBV replication in HepG2.2.15 cells\textsuperscript{17} and shown to interfere with HBx-DDB1 binding and to suppress cccDNA-driven HBV transcription.\textsuperscript{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).\textsuperscript{18} The spherical SVPs are 22 nm in diameter, have a low L:S ratio and are thought to be secreted via the constitutive 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{21,22} Since HBsAg production from integrants is independent of viral replication, it is not affected by nucleos(t)ide analogues.\textsuperscript{23,24} This has been confirmed in HBeAg-negative patients and chimpanzees with chronic hepatitis B.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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% CO2 and 95% humidity. Primary human hepatocytes (PHHs) were obtained as previously described.\textsuperscript{29} HepG2, HepG2-F-HB2.7, HepG2\textsuperscript{NTCP} and HepG2\textsuperscript{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\textsuperscript{NTCP} cells have been described previously.\textsuperscript{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).\textsuperscript{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 10⁴ 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 MgSO₄, 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.\textsuperscript{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.\textsuperscript{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 determined. 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. The siRNA screening was performed with the HepG2-derived cell line HepG2-F-HB2.7(X+) stably expressing and secreting SFPs 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. In this case, the 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 transcribed 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. To that aim, we infected differentiated HepaRGNTCP cells (dHepaRGNTCP) with HBV in the presence of MLN4924, the selective inhibitor of NAE1, blocking the complete neddylation 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 HBcAg 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 dHepaRGNTCP cells and found a stronger 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 dHepaRGNTCP infection system compared to the effect observed in cells carrying an HBV integrant (Figs. 2A and 3A). Moreover, the EC50 value correlated with that observed for transcription inhibition. HepG2NTCP cells were less responsive than dHepaRGNTCP cells and PHHs (Fig. S5). A >50% reduction at the transcriptional level in HepG2NTCP cells requires >1 µM of MLN4924, but HepG2NTCP cells have a far
decline in the level of intracellular HBsAg, suggesting that the reduction of secreted HBsAg produced from the HBV sub-genome 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, 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.
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) dHepaRGNTCP 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) dHepaRGNTCP cells were
higher CC<sub>50</sub> value (>50 nM). EC<sub>50</sub> values in all infection models (dHepaRGNTCP, HepG2NTCP 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, dHepaRGNTCP cells were 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 HBV-infected dHepaRGNTCP 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.

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

**Table 1.** EC<sub>50</sub> and CC<sub>50</sub> values of MLN4924 in various HBV infection models.

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

*Best-fit values [95% profile likelihood (CI)].

CC<sub>50</sub>, half maximum dose of cytotoxicity; EC<sub>50</sub>, half maximal effective dose; PHH, primary human hepatocyte.

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**Fig. 4.** MLN4924 potently inhibits transcription from static cccDNA in long-term culture. dHepaRG<sup>NTCP</sup> 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.

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higher CC<sub>50</sub> value (>50 nM). EC<sub>50</sub> values in all infection models (dHepaRG<sup>NTCP</sup>, HepG2<sup>NTCP</sup> 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<sup>NTCP</sup> 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.
Fig. 5. MLN4924 inhibits transcription from cccDNA and does not alter the HBx-DDB1 interaction. (A) dHepaRGNTCP 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) dHepaRGNTCP cells were infected with 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 dHepaRGNTCP 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 dHepaRCNTCP cells (Fig. 5A). Consistently, reduction of HBV RNAs, including the HBx mRNA, was also observed upon MLN4924 treatment of HBV-infected HepG2NTCP 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.27,28 We used an HBx mutant encoding two stop codons in the HBx open reading frame and infected dHepaRCNTCP 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,30 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 EF1x 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 HBsAg, whereas cccDNA levels were unaffected (Fig. S8).

Next, we examined the fate of the SMCS5/6 restriction complex under conditions of neddylation inhibition and performed SMCS6-specific immunofluorescence on HBV-infected dHepaRCNTCP cells and PHHs that were treated with MLN4924 (Fig. S9). We found that HBV infection induced the depletion of SMCS6 in most infected cells, 90% and 85% for PHHs and dHepaRCNTCP 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 SMCS5/6 restoration is one of the consequences of MLN4924 treatment.31

To elucidate the roles of HBx and SMCS6/5, we knocked-down SMCS6/5 and infected the cells with HBx-deficient virions. Indeed, MLN4924 inhibited transcription of WT and HBx-deficient viruses in the absence of SMCS5/6, whereas transcription of HBV virus was much more reduced upon treatment in the presence of SMCS5/6 (Fig. S10). Collectively, these data suggest that the effect of MLN4924 is partially dependent of SMCS5/6 and transcription of HBx-deficient virus is restricted by SMCS5/6 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 over-length HBV genome transcribed under the control of a tetracycline/doxycycline inducible promoter (Fig. S6A). 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 HBsAg and HBeAg are expressed. In contrast, the viral surface proteins continue to be transcribed with high efficiency under control of

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**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, 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 S1).m

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, 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. Through the process of integration, the viral genome inserts into the host genome at a specific site, where it is then replicated and expressed. This integration process can lead to the destruction of host genes, which can result in the development of liver cancer.

Our findings suggest that neddylation inhibition can be a potential therapeutic strategy for the treatment of HBV infection. Further studies are needed to explore the potential of neddylation inhibitors as antiviral agents in clinical trials.
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. 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).

It has been reported that the E3 ligase HDM2 promotes neddylation of HBx, thereby regulating its stability. However, 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 HepG2NTCP 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; PRRs, primary human hepatocytes; siRNA, small-interfering RNA; Smc5/6, structural maintenance of chromosomes 5/6; SVP, subviral particles; WT, wild-type.

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### 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.; 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.

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### Supplementary data

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