Molecular determinants within the C-termini of L-HDAg that regulate hepatitis D virus replication and assembly

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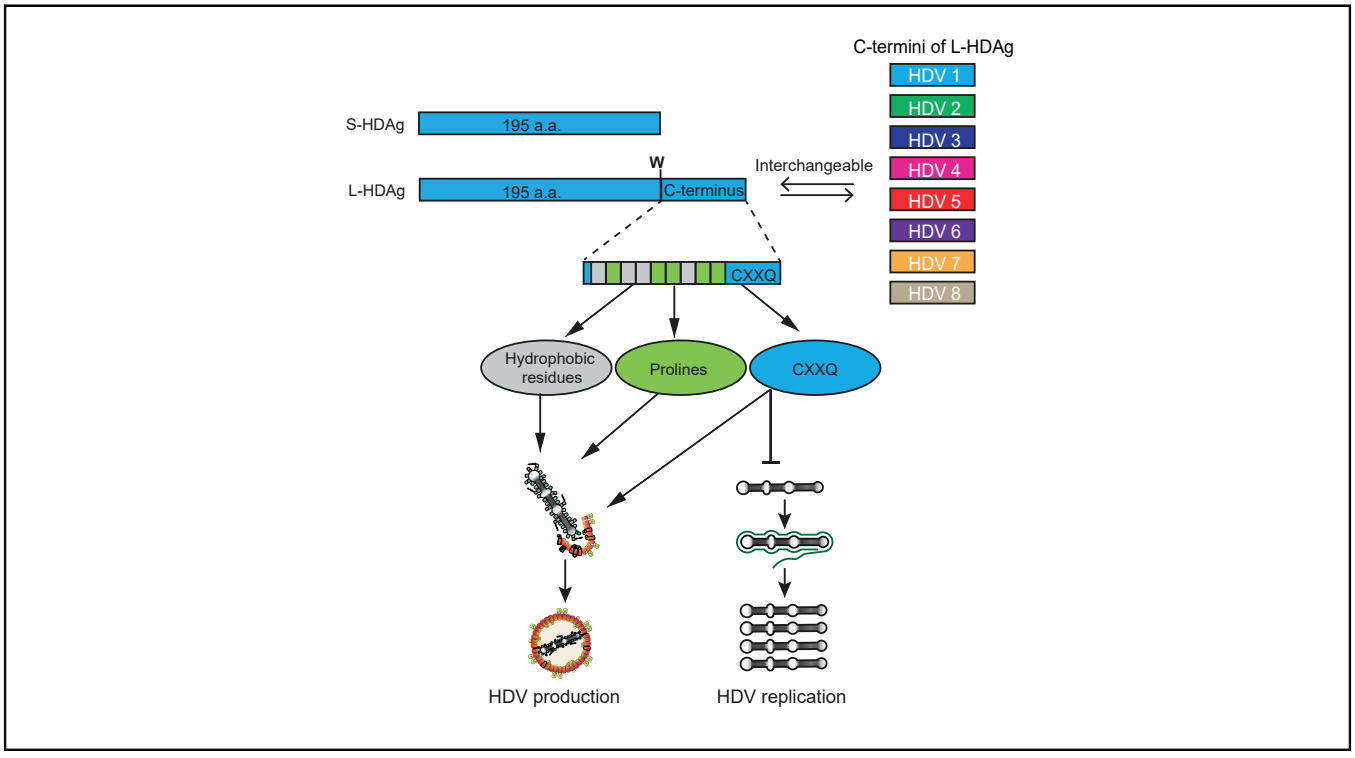
Graphical abstract



Highlights

- C-termini of L-HDAg are interchangeable among different HDV genotypes.
- C-termini of L-HDAg feature a conserved prenylation motif, enriched proline and hydrophobic residues.
- Inhibition of L-HDAg prenylation attenuates the trans-inhibitory function of L-HDAg.
- The enriched prolines and hydrophobic residues *per se* do not affect the trans-inhibitory function of L-HDAg.
- The enriched proline and hydrophobic residues contribute to HD virion production independently of L-HDAg prenylation.

Impact and implications

Hepatitis D virus (HDV) encodes one protein, hepatitis delta antigen (HDAg), in two isoforms: S- and L-HDAg. They are identical in sequence except that L-HDAg contains an additional 19-20 amino acids at its C-terminus. This C-terminal extension in L-HDAg confers regulatory roles in the HDV life cycle that are distinct from those of S-HDAg. Herein, we found that C-termini of L-HDAg, although with high sequence variation, are interchangeable among different HDV genotypes. Within the C-terminus of L-HDAg, the prenylation motif, and the enrichment of proline and hydrophobic residues are all essential determinants of L-HDAg's regulatory roles in HDV replication and assembly.

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Research article

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Molecular determinants within the C-termini of L-HDAg that regulate hepatitis D virus replication and assembly

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Background & Aims: Hepatitis D virus (HDV) is the causative agent of chronic hepatitis delta, the most severe form of viral hepatitis. HDV encodes one protein, hepatitis delta antigen (HDAg), in two isoforms: S- and L-HDAg. They are identical in sequence except that L-HDAg contains an additional 19-20 amino acids at its C-terminus, which confer regulatory roles that are distinct from those of S-HDAg. Notably, these residues are divergent between different genotypes. We aimed to elucidate the molecular determinants within the C-termini that are essential for the regulatory role of L-HDAg in HDV replication and assembly.

Methods: Northern blot, reverse-transcription quantitative PCR, and a newly established HDV trans-complementary system were used in this study.

Results: C-termini of L-HDAg, albeit with high sequence variation among different genotypes, are interchangeable with respect to the trans-inhibitory function of L-HDAg and HDV assembly. The C-terminus of L-HDAg features a conserved prenylation CXXQ motif and is enriched with proline and hydrophobic residues. Abolishment of the CXXQ motif attenuated the inhibitory effect of L-HDAg on HDV replication. In contrast, the enrichment of proline and hydrophobic residues *per se* does not modify the trans-inhibitory function of L-HDAg. Nevertheless, these residues are essential for HDV assembly. Mechanistically, prolines and hydrophobic residues contribute to HDV assembly via a mode of action independent of the prenylated CXXQ motif.

Conclusions: Within the C-terminus of L-HDAg, the CXXQ motif and the enrichment of proline and hydrophobic residues are all essential determinants of L-HDAg's regulatory roles in HDV replication and assembly. This intrinsic viral regulatory mechanism we elucidated deepens our understanding of the unique life cycle of HDV.

Impact and implications: Hepatitis D virus (HDV) encodes one protein, hepatitis delta antigen (HDAg), in two isoforms: S- and L-HDAg. They are identical in sequence except that L-HDAg contains an additional 19-20 amino acids at its C-terminus. This C-terminal extension in L-HDAg confers regulatory roles in the HDV life cycle that are distinct from those of S-HDAg. Herein, we found that C-termini of L-HDAg, although with high sequence variation, are interchangeable among different HDV genotypes. Within the C-terminus of L-HDAg, the prenylation motif, and the enrichment of proline and hydrophobic residues are all essential determinants of L-HDAg's regulatory roles in HDV replication and assembly.

Introduction

Approximately 250 million people worldwide are chronically infected with hepatitis B virus (HBV), 5–10% of these individuals are co-infected with hepatitis D virus (HDV).[1,2] Chronic HBV and HDV coinfection is considered the most severe form of viral hepatitis and can rapidly progress, with increased risks of cirrhosis, hepatic decompensation, and hepatocellular carcinoma.[3] In the last 30 years, standard and pegylated interferon-α treatments have been widely used as the only anti-HDV therapy but were characterized by poor tolerability and modest clinical benefit. Bulevirtide, the first-in-class entry inhibitor for HBV/HDV, was approved in Europe in 2020, defining the beginning of a new era for this difficult-to-treat/cure disease.[4]

HDV is a small defective RNA virus that requires HBV envelope proteins for virion assembly, secretion and *de novo* entry into hepatocytes. Its circular single-stranded RNA genome comprises 1,672–1,697 nucleotides. Owing to its high GC content, the HDV genome is characterized by an intramolecular base pairing of 74%, which confers the genome with a unique unbranched rod-like structure.[5] This RNA structure is a prerequisite for HDV replication driven by the host RNA polymerases.[6] HDV encodes only one protein, the hepatitis delta antigen (HDAg), which exists

Keywords: Hepatitis D virus; Hepatitis B virus; Virus replication; Virion assembly and production; L-HDAg.

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in two isoforms: small and large HDAgs (S-HDAg and L-HDAg).[7] S-HDAg is expressed early during infection and is required to initiate and promote HDV replication. In contrast, L-HDAg has been described to inhibit intracellular HDV RNA replication and is required for HDV assembly and secretion through its interaction with the self-assembly competent hepatitis B surface antigen (HBsAg).

Notably, S-HDAg and L-HDAg are identical in sequence except that L-HDAg contains an additional 19-20 amino acids at its C-terminus. This suggests that additional residues at the C-terminus are responsible for the differences in the functions of S-HDAg and L-HDAg. These residues, although highly conserved within HDV genotypes, are divergent among different genotypes.[8] This raised the question of whether the low conservation of the L-HDAg-specific extension is the major contributor to the markedly different replication and assembly kinetics of HDV genotypes 1-8 we reported previously.9 One highly conserved motif within the L-HDAg extension is the recognition sequence of the cellular enzyme farnesyl transferase. Prenylation of the C-terminal CXXQ motif is essential in the context of HDV assembly.[10] The farnesyl transferase inhibitor (FTI) lonafarnib, which is currently being tested in clinical trials,[11] inhibits this modification, thereby interfering with the release of virions but also other possible functions related to L-HDAg.[12,13]

Herein, by using northern blot, reverse-transcription quantitative PCR, in-cell ELISA, and a newly established HDV trans-complementary system, we aimed to identify the molecular determinants within the C-termini that are essential for the regulatory role of L-HDAg in HDV replication and assembly. The knowledge gained is essential for interpreting the intrinsic viral self-regulatory actions of L-HDAg in the unique life cycle of HDV.

Materials and methods

Plasmids

The plasmid pcDNA3.1-L-HDAg derived from the pJC126 plasmid was named pcDNA3.1-L-HDAg-1+1. The other seven plasmids (pcDNA3.1-L-HDAg-1+2 to pcDNA3.1-L-HDAg-1+8) were constructed based on the site-directed mutagenesis method using pcDNA3.1-L-HDAg-1+1 as the template.[14] To construct Tet-off expression vectors (pLVX-tight-L-HDAg-1+1 to pLVX-tight-L-HDAg-1+8), the corresponding L-HDAg open-reading frames were amplified by PCR from pcDNA3.1-L-HDAg-1+1 to pcDNA3.1-L-HDAg-1+8 and inserted into the pLVX-tight vector. pLVX-tight-L-HDAg-1+1-C211S, pLVX-tight-L-HDAg-1+1-Q214stop, and all other mutant plasmids were generated using the site-directed mutagenesis method. The sequences of all constructs were verified by Sanger sequencing. For further details regarding the materials and methods used, please refer to the supplementary information.

Results

C-termini of L-HDAg from different genotypes are interchangeably related to the trans-inhibitory function of L-HDAg

Although the C-terminal extension containing a prenylation CXXQ motif is present in the L-HDAg of all eight different genotypes, there is little additional sequence similarity in this region across HDV genotypes (Fig.1A). This prompted us to investigate whether divergent C-termini result in differences in the trans-inhibitory function of L-HDAg. Thus, vectors expressing chimeric L-HDAg were constructed as indicated (Fig.1B), where the C-termini of HDV 2-8 were fused to the S-HDAg of genotype 1. Western blot analysis indicated successful expression of all chimeric L-HDAg proteins (Fig. S1A). Next, the same amount of chimeric L-HDAg constructs were co-transfected with an HDV-1 clone, pJC126.[9,15] Northern blot analysis indicated that the S-HDAg-expressing construct exerted no marked effect on HDV replication, probably because adequate S-HDAg was expressed endogenously from pJC126. Nevertheless, the chimeric L-HDAg constructs profoundly suppressed HDV RNA replication even below the detection limit (Fig. 1C). The pJC126 clone initiated HDV-1 replication with the expression of both S- and L-HDAg (Fig. S1C). Therefore, to exclude the possible interference of intrinsically produced L-HDAg, a mutant clone named pJC126-L (del) was constructed (Fig. S1B). As expected, the pJC126-L (del) clone supported HDV RNA replication and S-HDAg expression, while abolishing the production of L-HDAg (Fig. S1C, D). Consistent with Fig. 1C, chimeric L-HDAg constructs, when co-transfected with pJC126-L (del), efficiently inhibited HDV replication (Fig. 1D). Collectively, these results suggest that the C-termini of L-HDAg derived from HDV 1-8, albeit with high sequence variation, are interchangeably related to the trans-inhibitory function of L-HDAg.

C-termini of L-HDAg from different genotypes are interchangeably related to hepatitis D virion production

The contributions of the C-termini to HDV assembly were explored based on the newly established trans-complementation system (Fig. S2). In this system, HuH7-Tet-off cells were transfected with pJC126-L (def), pLX304-HB2.7-B and a Tet-off inducible L-HDAg expression vector (Fig. S2A). When L-HDAg was expressed from day 2, 4 or 6 post transfection (p.t.), hepatitis D (HD) virions were efficiently produced (Fig. S2B, C). This system decouples the expression of L-HDAg from HDV while recapitulating viral replication and assembly, serving as a useful tool set for studying the contributions of the C-termini to HDV assembly. Eight chimeric L-HDAg expression constructs were cloned into a Tet-off inducible expression vector (Fig. S3A). Western blot analysis indicated successful expression of all chimeric L-HDAg proteins (Fig. S3B). Subsequently, HuH7-Tet-off cells were transfected with pJC126-L (def), pLX304-HB2.7-B, and the same amounts of L-HDAg constructs. The supernatant (SN) was collected after the removal of tetracycline 2 days p.t.. HD virions were detected by in-cell ELISA (Fig. 1E). Strikingly, despite the high sequence variation between the C-termini, we only observed slightly lower levels of HD virions produced in the L-HDAg-1+2 setting at relatively later time points (day 13-22). Generally, all eight chimeric L-HDAg proteins efficiently supported the production of HD virions with no marked differences (Fig. 1E). In conclusion, the C-termini of L-HDAg from different genotypes, albeit with high sequence variation, are interchangeably related to HD virion assembly.

Inhibition of L-HDAg prenylation attenuates the trans-inhibitory function of L-HDAg

Within the L-HDAg C-termini, the prenylation CXXQ motif is highly conserved. Its contribution to the life cycle of HDV was investigated. Firstly, based on the L-HDAg-1+1 construct, we abolished the prenylation CXXQ motif by mutating C_{211} to S_{211} or Q_{214} to a stop codon (Fig. 2A). Western blot analysis indicated successful expression of these two mutant L-HDAg proteins (Fig. 2B). As expected, neither the C211S nor the Q214stop mutants supported HD virion production (Fig. 2C). Notably, when

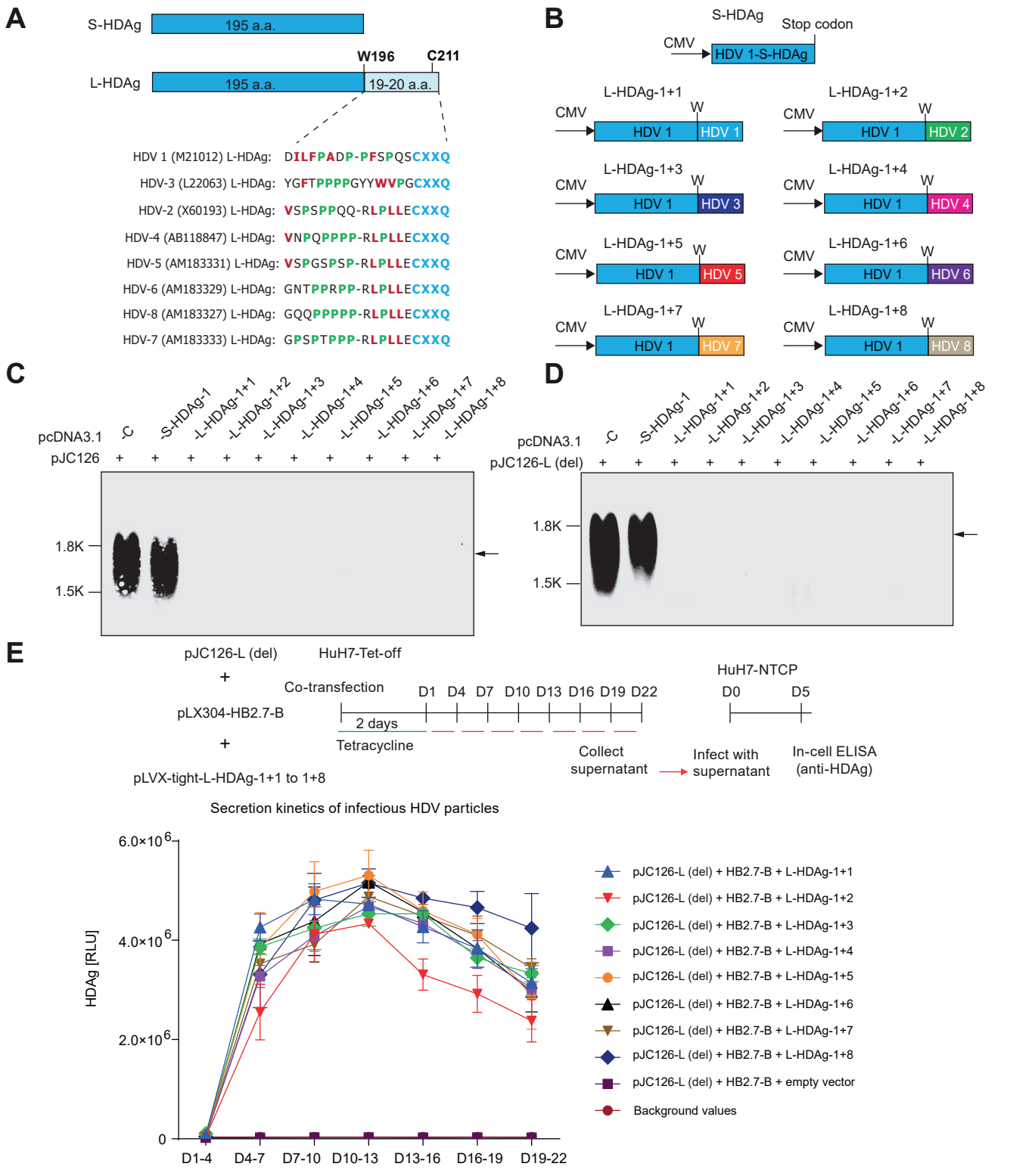

Fig. 1. C-termini of L-HDAg from different genotypes are interchangeably related to the trans-inhibitory function of L-HDAg and HDV production. (A) The amino acid sequences of L-HDAg C-termini from HDV 1-8 were illustrated. The hydrophobic residues were labeled red, the prolines were labeled green, and the isoprenylation site (CXXQ) was labeled blue. (B) Schematic illustration of the plasmids: based on the pcDNA3.1-L-HDAg-1+1 (HDV-1) plasmid, seven chimeric L-HDAg expression constructs were created in which the C-termini of HDV 2-8 were fused with the S-HDAg of genotype 1. All constructs were under the control of the CMV promoter. (C) Equal amounts of pcDNA3.1-C (empty vector), pcDNA3.1-S-HDAg-1 or pcDNA3.1-L-HDAg-1+1 to -1+8, together with pJC126 plasmid, were transfected into HuH7 cells. The levels of HDV RNA were analyzed by northern blot at day 6 p.t.. (D) Equal amounts of pcDNA3.1-C, pcDNA3.1-S-HDAg-1 or pcDNA3.1-L-HDAg-1+1 to 1+8, together with pJC126-L (del) plasmid, were transfected into HuH7 cells. The levels of HDV RNA were analyzed by northern blot at day 6 p.t.. (E) HuH7-Tet-off cells were co-transfected with pJC126-L (del), pLX304-HB2.7-B and pLVX-tight-L-HDAg-1+1 to -1+8. Tetracycline was removed from day 2 p.t.. The SN was collected at indicated time intervals to infect HuH7-NTCP. HDAg was quantified by in-cell ELISA based on two independent assays (with two replicates each). HDAg, hepatitis D antigen; HDV, hepatitis D virus; p.t., post transfection; SN, supernatant.

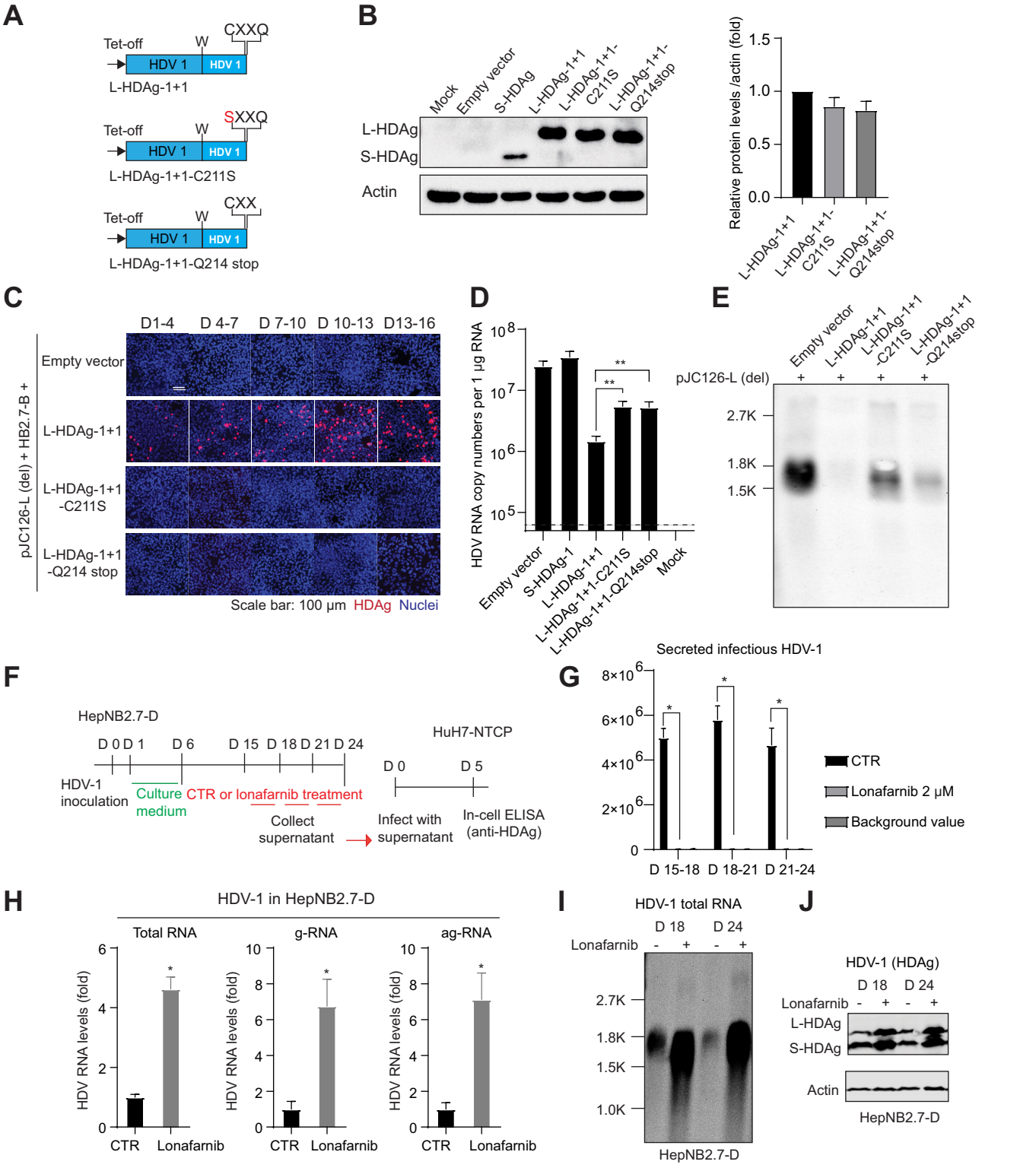

Fig. 2. Inhibition of L-HDAg prenylation attenuates the trans-inhibitory function of L-HDAg. (A) Schematic representation of the plasmids: based on the pLVX-tight-L-HDAg-1+1 plasmid, the Cysteine (C_{211}) or the Glutamine (Q_{214}) of the isoprenylation site (CXXQ) was mutated to Serine (S_{211}) or stop codon and named pLVX-tight-L-HDAg-1+1-C211S or pLVX-tight-L-HDAg-1+1-Q214stop, respectively. (B) The expression of wild-type or mutant L-HDAg proteins was detected by western blot at day 3 p.t.. The relative protein levels of wild-type and mutant L-HDAg proteins were quantified by ImageJ based on four independent repeats. (C) Same amounts of pLVX-tight-C, pLVX-tight-L-HDAg-1+1, pLVX-tight-L-HDAg-1+1-C211S or pLVX-tight-L-HDAg-1+1-Q214stop, together with pJC126-L (del) and pLX304-HB2.7-B, were transfected into HuH7-Tet-off cells. Tetracycline was removed from day 2 p.t.. The SN was collected at indicated time intervals to infect HuH7-NTCP. HDAg was stained at day 5 p.i. Images are representative of two independent assays. (D,E) Equal amounts of pLVX-tight –C (empty vector), pLVX-tight-S-HDAg-1, pLVX-tight-L-HDAg-1+1, pLVX-tight-L-HDAg-1+1-C211S or pLVX-tight-L-HDAg-1+1-Q214stop, together with pJC126-L (del), were transfected into HuH7-Tet-off cells. The levels of HDV RNA were analyzed by RT-qPCR or northern blot at day 8 p.t.. (F) Schematic representation of the experimental

co-transfected with the pJC126-L (del) clone, the two mutants still exhibited potent trans-inhibitory activity. Nevertheless, their trans-inhibitory activity was attenuated compared to that of wild-type (WT) L-HDAg (Fig. 2E). In this setting, all L-HDAg constructs were expressed at the onset of HDV replication. In fact, because of ADAR1 (adenosine deaminase acting on RNA 1) editing, L-HDAg is expressed later in the natural life cycle of HDV. Therefore, to further validate whether the prenylation motif supports the trans-inhibitory function of L-HDAg in a natural setting, we inoculated HepNB2.7-B or HepNB2.7-D cells[9,12] with HDV-1 or HDV-8. Lonafarnib was added only from day 6 post infection (Fig. 2F). This ensured that the prenylation of L-HDAg was inhibited after HDV replication was well established, but not at the beginning of replication. As expected, lonafarnib inhibited secretion of infectious HDV particles even if added post establishment of HDV replication (Fig. 2F, G). This is consistent with the clinical efficacy of lonafarnib, which inhibits HDV virion production when added in the setting of a previously established infection. Still, lonafarnib treatment increased the levels of intracellular HDV RNA and HDAg (Figs 2G-J and Fig. S4). Similar results were obtained in HuH7-END[16] and HuH7-NTCP[17] cells (Figs S5 and S6). In conclusion, in addition to its essential role in HDV assembly, the conserved prenylation CXXQ motif contributes to the trans-inhibitory function of L-HDAg.

FTIs accumulate intracellular HDV RNA solely via the inhibition of L-HDAg prenylation

Lonafarnib exerts dual effects on HDV: blocking virus assembly and increasing intracellular HDV RNA.[12,13] Notably, farnesyltransferases are expressed in different tissues as housekeeping enzymes, and many host proteins (*e.g.*, Ras, Ras-related GTP binding proteins, the subunits of trimeric G proteins and protein kinases) with significant functions in cellular events are prenylated.[18] To investigate whether the increased intracellular HDV RNA induced by lonafarnib was achieved solely via the inhibition of L-HDAg prenylation, while not an off-target effect on host prenylated proteins, we generated three cell lines: HuH7-pJC126, pJC126-L (del), and pJC126-L (C211S). HuH7-pJC126 (del) supported continuous HDV replication with no expression of L-HDAg, whereas HuH7-pJC126-L (C211S) supported HDV replication with the expression of L-HDAg that could not be prenylated (Figs. 3A and S7). Notably, lonafarnib treatment increased the levels of HDV RNA in HuH7-pJC126 cells but not in HuH7-pJC126 (del) and HuH7-pJC126-L (C211S) cells (Fig. 3B, C). Similar results were observed with the treatment of two additional FTIs (FTI 277 HCL and LB42708) (Fig. 3D-G). Collectively, these results demonstrated that FTIs increased the levels of intracellular HDV RNA solely via the inhibition of L-HDAg prenylation, but not through an off-target effect on host prenylated proteins.

Within the C-termini of L-HDAg, proline and hydrophobic residues *per se* do not affect the trans-inhibitory function of L-HDAg

The C-termini of the different HDV genotypes, albeit divergent, are enriched with proline and hydrophobic residues (Fig. 1A). Proline's side-chain is cyclized back onto the backbone amide position, making it an unusual amino acid with maximum rigidity. Therefore, based on the conformational flexibility scale of amino acids (Gly > Ser > Asp; Asn; Ala > Thr; Leu > Phe; Glu; Gln> His; Arg > Lys > Val > Ile > Pro),[19] we mutated the prolines (P, with maximum rigidity) into glycine (G, with the highest flexibility) in L-HDAg-1+1 (Fig. 4A, left panel). In parallel, the hydrophobic residues were mutated to non-hydrophobic glycine (Fig. 4A, right panel). Western blot analysis confirmed the successful expression of the two mutant L-HDAg proteins (Fig. 4B). Subsequently, their possible contributions to the trans-inhibitory function of L-HDAg were investigated. Compared to WT L-HDAg-1+1, these two mutants exhibited comparable trans-inhibitory effects against HDV replication (Fig. 4C). To further validate this observation, we chose the C-terminus of HDV-3 L-HDAg, which is different from other genotypes both at the amino acid level and in length (Fig. 1A). We mutated proline or hydrophobic residues at the C-terminus of L-HDAg-1+3 (Fig. 4D, E). Similar results were observed (Fig. 4F). Collectively, the proline and hydrophobic residues *per se* do not affect the trans-inhibitory activity of L-HDAg.

Within the C-termini of L-HDAg, proline and hydrophobic residues contribute to HD virion production via a mode of action that is independent of L-HDAg prenylation

We investigated the possible contributions of proline and hydrophobic residues to HDV assembly. Equal amounts of WT or mutant L-HDAg-1+1 constructs, together with pJC126-L (def) and pLX304-HB2.7-B, were transfected into HuH7-Tet-off cells. Tetracycline was removed on day 2 p.t., and the SN was collected at the indicated time points (Fig. 5A). The production of HD virions was measured using immunofluorescence. Interestingly, the mutation of prolines or hydrophobic residues of the L-HDAg-1+1 C-terminus abrogated HD virion production (Fig. 5B). To further validate this observation in other genotypes, we mutated prolines or hydrophobic residues at the C-terminus of L-HDAg-1+3. Similar results were observed (Fig. 5C). To investigate whether the abrogation of HD virion assembly was the result of a possible defect in L-HDAg prenylation, we transfected WT or mutant L-HDAg constructs with the pJC126-L (del) clone, followed by treatment with mock or lonafarnib. Notably, lonafarnib treatment increased HDV RNA levels in cells transfected with WT or mutant L-HDAg constructs (Fig. 5D, E). This indicates that the mutation of prolines or hydrophobic residues of the C-termini would not prevent L-HDAg prenylation. In summary, within the C-termini of L-HDAg, proline and hydrophobic residues contribute to HDV assembly via a mode of action independent of L-HDAg prenylation.

Discussion

Due to the high sequence divergence among the eight HDV genotypes, the exact molecular determinants of L-HDAg C-termini that play intrinsic regulatory roles in the HDV life cycle have not been fully elucidated. In this study, we successfully established a trans-complementation system based on a Tet-off inducible expression vector. It decouples the expression of L-HDAg from

layout: HepNB2.7-D cells were infected with HDV-1 (1IU/cell), then treated with lonafarnib (2 μM) from day 6-24. SN was collected at the indicated time intervals to infect HuH7-NTCP. (G) HDAg was quantified by in-cell ELISA based on two independent assays (with two replicates each). HepNB2.7-D cells were infected with HDV-1, then treated with lonafarnib (2 μM) from day 6 p.i.. (H) The levels of total HDV RNA, g-RNA) or ag-RNA were analyzed by RT-qPCR at day 18 p.i.. (I) The levels of total HDV RNA were analyzed by northern blot at day 18 and 24 p.i.. (J) HDAg was detected by western blot at day 18 and 24 p.i.. Statistical significance was determined using unpaired two-tailed Student's *t* test in the software GraphPad Prism 9 ($^{*}p < 0.05$, $^{**}p < 0.01$, n.s. not significant, $p > 0.05$). ag-RNA, antigenomic RNA; g-RNA, genomic RNA; HDAg, hepatitis D antigen; HDV, hepatitis D virus; p.i., post infection; p.t., post transfection; RT-qPCR, reverse-transcription quantitative PCR; SN, supernatant.

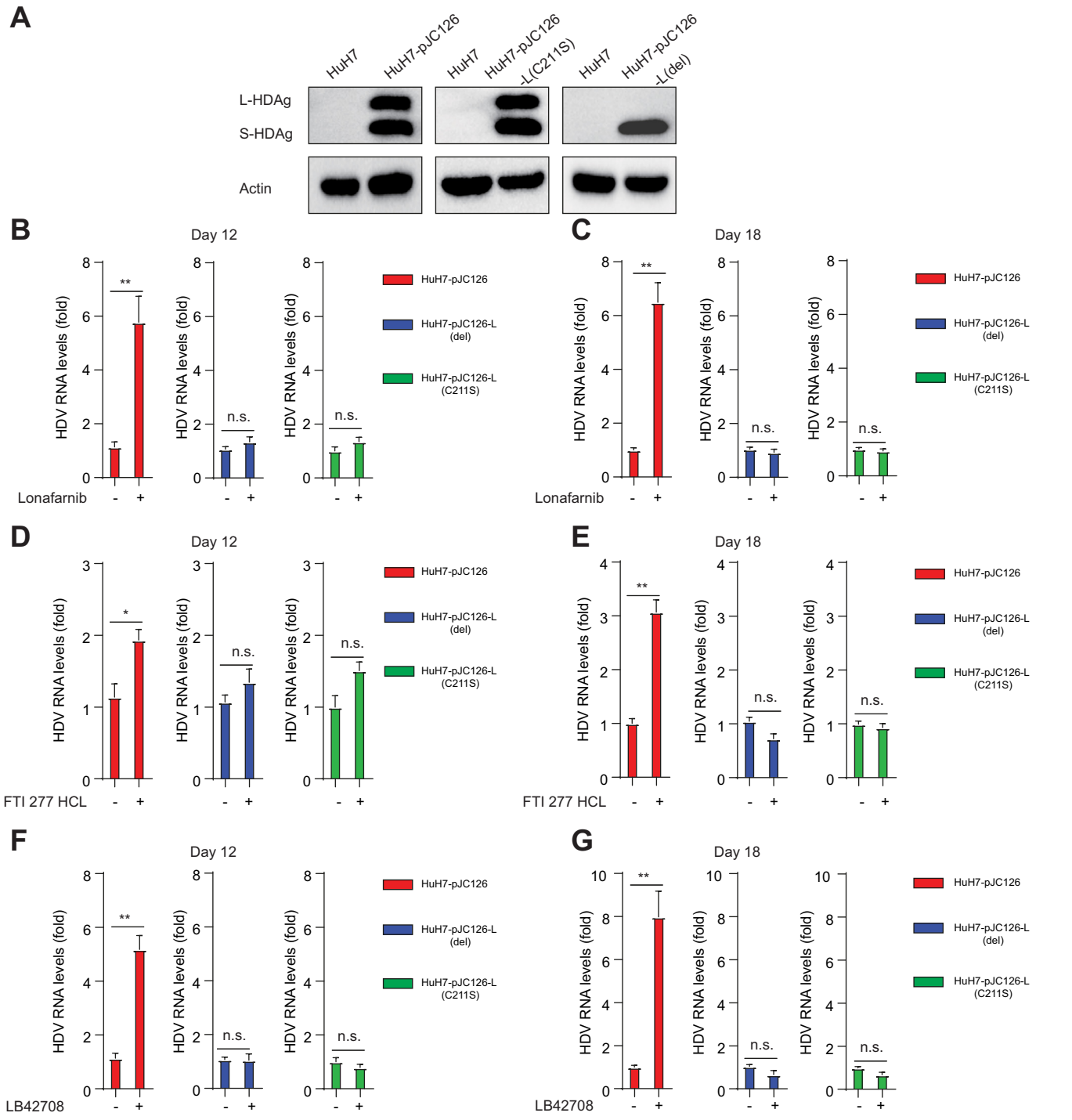

Fig. 3. FTIs accumulate intracellular HDV RNA solely via the inhibition of L-HDAg prenylation. (A) HuH7, HuH7-pJC126, HuH7-pJC126-L (del), and HuH7-pJC126-L (C211S) cells were seeded and cultured. HDAg was detected by western blot on day 6 after seeding. (B,C) HuH7-pJC126, HuH7-pJC126-L (del) or HuH7-pJC126-L (C211S) cells were treated with mock or lonafarnib (2 μM). The levels of intracellular HDV RNAs were analyzed by RT-qPCR on day 12 or 18 based on three independent assays (with two replicates each). (D,E) Same as (B,C) for FTI 277 HCL. (F,G) Same as (B,C) for LB42708. Statistical significance was determined using unpaired two-tailed Student's *t* test in the software GraphPad Prism 9 (*$p < 0.05$, **$p < 0.01$, n.s. not significant, $p > 0.05$). HDAg, hepatitis D antigen; HDV, hepatitis D virus; RT-qPCR, reverse-transcription quantitative PCR.

HDV while recapitulating authentic viral replication and assembly. Hence, this system provides a valuable tool set for dissecting the molecular determinants of L-HDAg that are essential for HD virion assembly and production.

The C-termini of L-HDAg are composed of the conserved prenylation CXXQ motif, and the remaining residues that are divergent among genotypes but are enriched with proline and hydrophobic residues (Fig. 1A).[20] Our study shows that the C-termini derived from HDV 1-8 are interchangeable with respect to the trans-inhibitory function of L-HDAg and HD virion assembly and production. Hence, the divergent C-termini of L-HDAg derived from different genotypes were not the cause of the

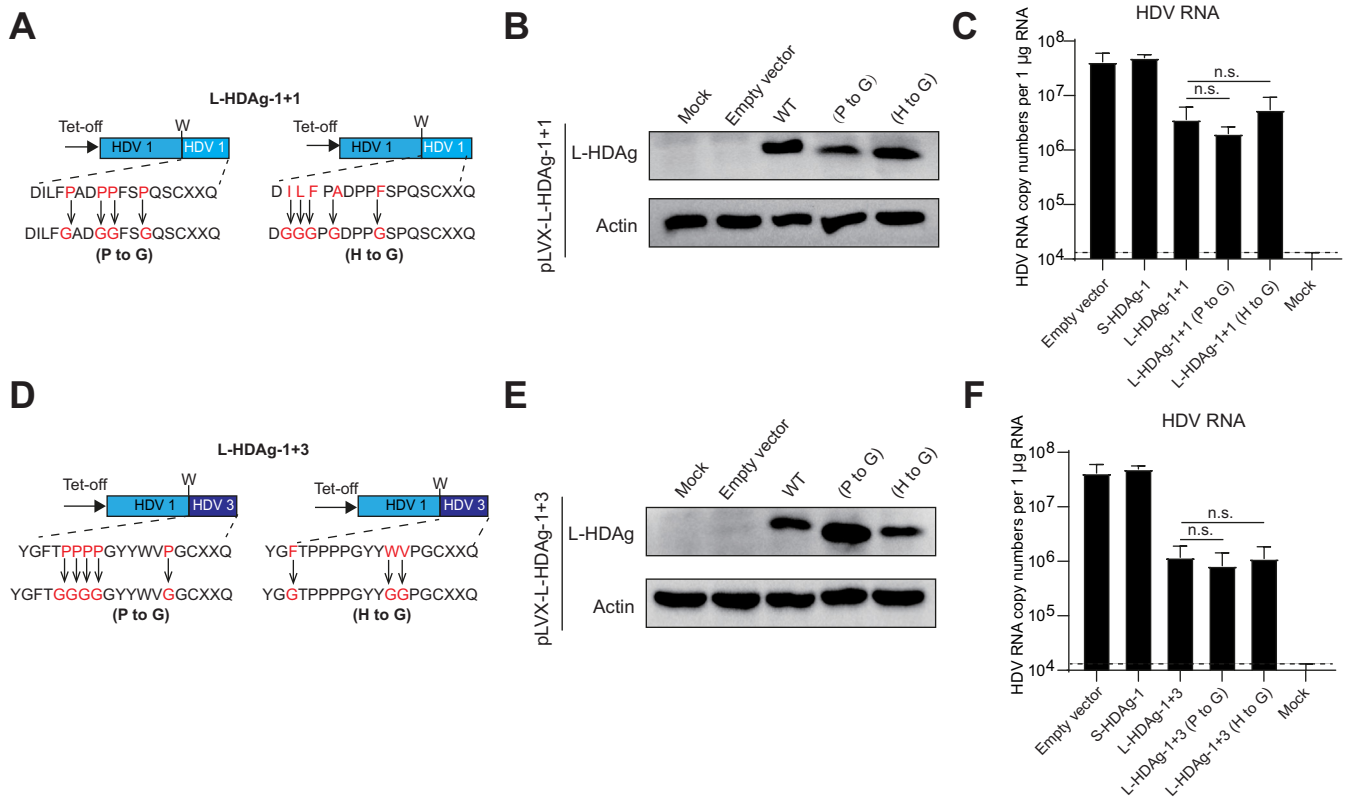

Fig. 4. Within the C-termini of L-HDAg, proline and hydrophobic residues *per se* do not affect the trans-inhibitory function of L-HDAg. (A) Schematic representation of the plasmids: based on the pLVX-tight-L-HDAg 1+1 plasmid, prolines (P) or hydrophobic amino acids (H) within the C-terminus of the L-HDAg were mutated to Glycine (G) and named pLVX-tight-L-HDAg-1+1 (P to G) or pLVX-tight-L-HDAg-1+1 (H to G), respectively. (B) The expression of wild-type or mutant L-HDAg-1+1 proteins was detected by western blot at day 3 p.t.. (C) Equal amounts of pLVX-tight –C (empty vector), pLVX-tight-S-HDAg-1, pLVX-tight-L-HDAg-1+1, pLVX-tight-L-HDAg-1+1 (P to G) or pLVX-tight-L-HDAg-1+1 (H to G), together with pJC126-L (del), were transfected into HuH7-Tet-off cells. The levels of HDV RNA were analyzed by RT-qPCR at day 8 p.t.. (D) Same as (A) for pLVX-tight-L-HDAg-1+3 plasmid and its mutants. (E) The expression of wild-type or mutant L-HDAg-1+3 proteins was detected by western blot at day 3 p.t.. (F) Same as (C) for L-HDAg-1+3 and its mutants. Statistical significance was determined using unpaired two-tailed Student's *t* test in the software GraphPad Prism 9 (*$p < 0.05$, **$p < 0.01$, n.s. not significant, $p > 0.05$). HDAg, hepatitis D antigen; HDV, hepatitis D virus; p.t., post transfection; RT-qPCR, reverse-transcription quantitative PCR.

different kinetics of HDV 1-8 we reported previously.[9] Instead, three key components (i. CXXQ motif, ii. proline residues, iii. hydrophobic residues) within the C-termini of L-HDAg ensure the conserved functions of L-HDAg of different genotypes in the regulation of the HDV life cycle.

HDV production largely relies on the specific interactions between S-HBsAg and L-HDAg. Importantly, in addition to the prenylation CXXQ motif, proline and hydrophobic residues of the C-termini are also essential for HDV assembly and production. Mechanistically, their contribution to HDV production is independent of the prenylated CXXQ motif. Of note, the C-terminus of S-HBsAg is highly hydrophobic and vital to HDV assembly.[21,22] To date, the exact protein-protein interactions between L-HDAg and S-HBsAg that mediate HDV assembly remain largely unknown. Based on the fact that prenylation could substantially increase protein hydrophobicity,[23] we propose a dynamic protein-protein interaction (PPI) model as follows. Due to conformational rigidity, prolines extend the C-termini out from the rest of the L-HDAg protein as a unique "sticky arm" structure[24] to facilitate the close contact between L-HDAg and S-HBsAg. Subsequently, within the C-termini of L-HDAg, the prenylated CXXQ motif and the hydrophobic residues exert collective hydrophobic interactions with the highly hydrophobic C-terminus of S-HBsAg to mediate HD virion assembly and release. This PPI model may help explain why the abolishment of any of these three key determinants inhibits HDV assembly and production. Notably, the proposed PPI model was based only on the limited knowledge we obtained at this stage. The development of accurate and complete repertoires of PPIs requires three-dimensional structural information on both proteins and the structural insights of their PPI interfaces.[25]

In contrast, only the prenylated CXXQ motif, but not the proline and hydrophobic residues *per se* contributed to the trans-inhibitory function of L-HDAg. Notably, L-HDAg mutants lacking the prenylation motif still possessed partial trans-negative effects on HDV replication (Fig. 2D,E). Thus, apart from the prenylation motif, the remaining C-terminal extension, but not the prolines or hydrophobic residues *per se*, contributed to the trans-negative function of L-HDAg. Nevertheless, the exact mechanism of action remains elusive.[26,27] The prenylation motif is required for HDV assembly and the trans-inhibitory function of L-HDAg. This finding explains the observation that FTI treatment blocks HDV assembly and simultaneously increases the levels of intracellular HDV RNA.[12] We further found that the dual effects of FTIs on HDV were solely achieved via the inhibition of L-HDAg prenylation and not through an off-target effect on host prenylated proteins (Fig. 3). Considering the great potential of lonafarnib for

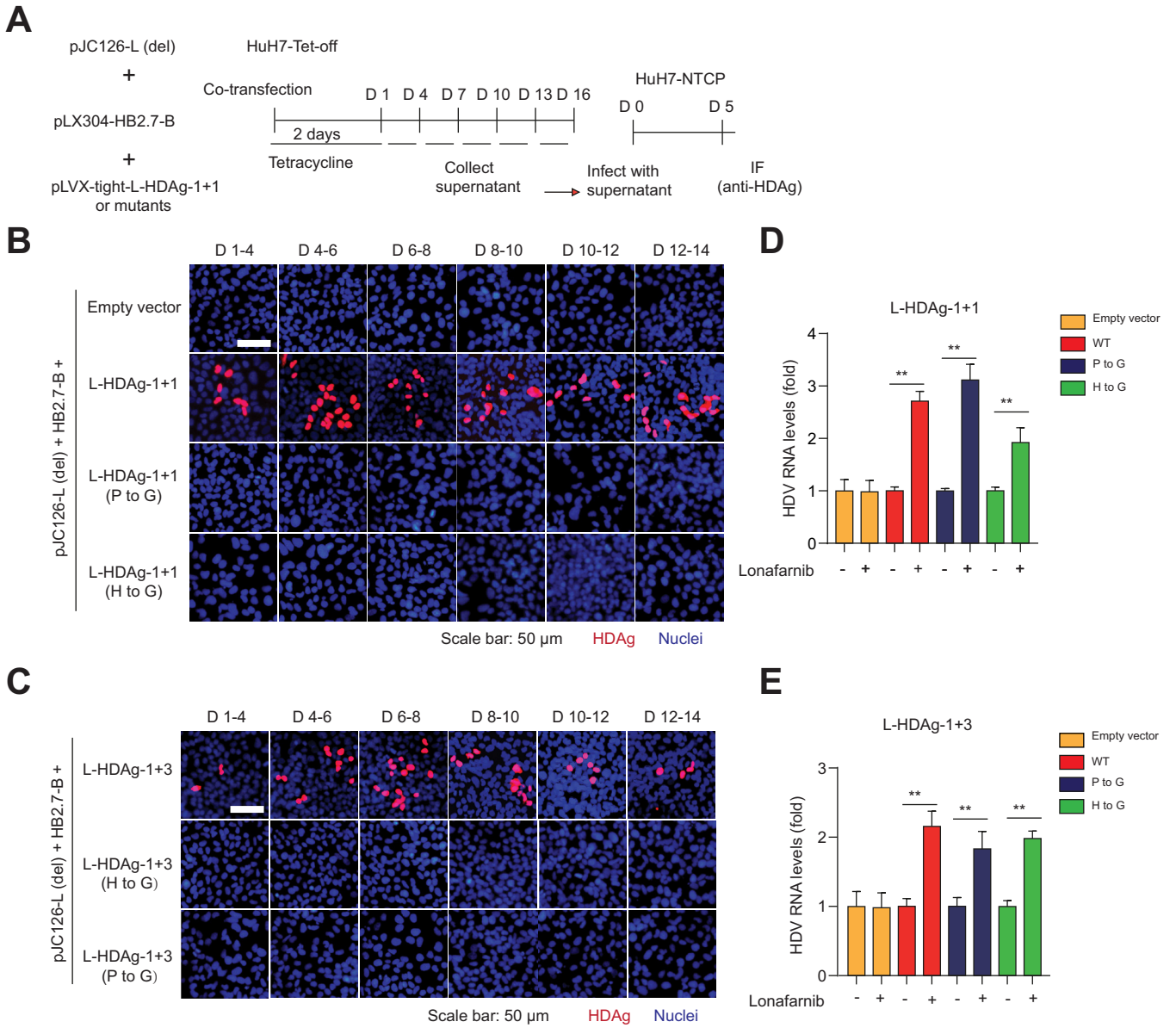

Fig. 5. Within the C-termini of L-HDAg, proline and hydrophobic residues are essential for HDV assembly. (A) Schematic representation of the experimental layout: same amounts of pLVX-tight-C (empty vector), pLVX-tight-L-HDAg-1+1, pLVX-tight-L-HDAg-1+1 (P to G) or pLVX-tight-L-HDAg-1+1 (H to G), together with pJC126-L (del) and pLX304-HB2.7-B plasmids, were transfected into HuH7-Tet-off cells. Tetracycline was removed from day 2 p.t.. The SN was collected at indicated time intervals to infect HuH7-NTCP. (B) HDAg was stained at day 5 p.i. Images are representative of two independent assays. (C) Same as (B) for pLVX-tight-L-HDAg-1+3 plasmid and its mutants. (D) pLVX-tight –C (empty vector), pLVX-tight-L-HDAg-1+1 (WT), pLVX-tight-L-HDAg-1+1 (P to G) or pLVX-tight-L-HDAg-1+1 (H to G), together with pJC126-L (del), were transfected into HuH7-Tet-off cells. Cells were treated with mock or lonafarnib until day 8. The levels of HDV RNA were analyzed by RT-qPCR at day 8 p.t.. (E) Same as (D) for pLVX-tight-L-HDAg-1+3 plasmid and its mutants. Statistical significance was determined using unpaired two-tailed Student's *t* test in the software GraphPad Prism 9 (*$p < 0.05$, **$p < 0.01$, n.s. not significant, $p > 0.05$). HDAg, hepatitis D antigen; HDV, hepatitis D virus; p.i., post infection; p.t., post transfection; RT-qPCR, reverse-transcription quantitative PCR; SN, supernatant.

the treatment of chronic HDV infections in the clinic, the elucidation of all possible modes of actions of this drug would be of great value.

When L-HDAg was expressed at the onset of replication, viral replication was strongly inhibited (Fig. 1C, D). However, there are some controversies concerning whether L-HDAg expressed naturally at later times (because of ADAR1 editing) can still inhibit HDV replication, especially when HDV replication is well established. Macnaughton *et al.* reported that the level of HDV RNA did not increase when a mutant HDV genome unable to make L-HDAg was used and HDV RNA synthesis was resistant to L-HDAg when overexpressed 3 days after the initiation of HDV RNA replication.[28] In contrast, Sato *et al.* found that a replication-competent HDV mutant that exhibited higher levels of editing than WT HDV prematurely aborted replication, while a mutant L-HDAg lacking the prenylation site or treatment with FTIs rescued replication defects.[29] In this study, we inoculated two different genotypes of HDV (HDV-1 and HDV-8) in HepNB2.7-B or HepNB2.7-D cells, which supported the complete HD viral replication cycle.[9,12] Lonafarnib treatment was initiated 6 days after viral inoculation.

This ensured that the inhibition of L-HDAg prenylation was initiated after HDV replication was well established, but not at the early stage of replication. Nevertheless, lonafarnib increased the levels of intracellular HDV RNA (Figs 2F–J, and Figs S4). This indicates that L-HDAg trans-inhibits HDV replication during its natural life cycle.

In summary, although the C-termini of L-HDAg exhibited high sequence variation between different genotypes, we identified three conserved determinants (i. prenylation CXXQ motif, ii. proline residues, iii. hydrophobic residues) that are essential for the regulatory function of L-HDAg in the HDV life cycle. The prenylation motif is required for HDV assembly and the trans-inhibitory function of L-HDAg. In contrast, the enrichment of proline and hydrophobic residues *per se* does not affect the trans-inhibitory function of L-HDAg. Nevertheless, they contributed to HDV assembly via a mode of action independent of the prenylated motif. The unique intrinsic viral self-regulatory mechanism we elucidated deepens our understanding of the full life cycle of HDV.

Abbreviations

FTIs, farnesyltransferase inhibitors; HBsAg, hepatitis B surface antigen; HD, hepatitis D; HDAg, hepatitis D antigen; HDV, hepatitis D virus; p.t., post transfection; SN, supernatant.

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Conflict of interest

Prof. Dr. Stephan Urban holds patents on bulevirtide/Hepcludex. Other authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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

Authors' contributions

Conception and design of the study: H. G., and W. W.; generation, collection, assembly, analysis and interpretation of data: H. G., Q. L., C. L., Y. H., D. L., R. T., and W. W.; drafting of the manuscript: H. G., and W.W.; discussion and critical revision of the manuscript: H. G., Y. N., R. T., K. Z., S. U., and W. W..

Data availability statement

Details related to materials and methods are provided in the the article along with Supplementary documents and CTAT table. Further data supporting the findings of this study are available upon reasonable request.

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Supplementary data

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References

Author names in bold designate shared co-first authorship

[1] Miao Z, Zhang S, Ou X, et al. Estimating the global prevalence, disease progression, and clinical outcome of hepatitis delta virus infection. J Infect Dis 2020;221:1677–1687.
[2] Stockdale AJ, Kreuels B, Henrion MYR, et al. The global prevalence of hepatitis D virus infection: systematic review and meta-analysis. J Hepatol 2020;73:523–532.
[3] **Urban S**, **Neumann-Haefelin C**, **Lampertico P**. Hepatitis D virus in 2021: virology, immunology and new treatment approaches for a difficult-to-treat disease. Gut 2021;70:1782–1794.
[4] Kang C, Syed YY. Bulevirtide: first approval. Drugs 2020;80:1601–1605.
[5] Kos A, Dijkema R, Arnberg AC, et al. The hepatitis delta (delta) virus possesses a circular RNA. Nature 1986;323:558–560.
[6] Lai MM. RNA replication without RNA-dependent RNA polymerase: surprises from hepatitis delta virus. J Virol 2005;79:7951–7958.
[7] Weiner AJ, Choo QL, Wang KS, et al. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24 delta and p27 delta. J Virol 1988;62:594–599.
[8] Le Gal F, Brichler S, Drugan T, et al. Genetic diversity and worldwide distribution of the deltavirus genus: a study of 2,152 clinical strains. Hepatology 2017;66:1826–1841.
[9] **Wang W**, **Lempp FA**, Schlund F, et al. Assembly and infection efficacy of hepatitis B virus surface protein exchanges in 8 hepatitis D virus genotype isolates. J Hepatol 2021;75:311–323.
[10] Glenn JS, Watson JA, Havel CM, et al. Identification of a prenylation site in delta virus large antigen. Science 1992;256:1331–1333.
[11] Yurdaydin C, Keskin O, Yurdcu E, et al. A phase 2 dose-finding study of lonafarnib and ritonavir with or without interferon alpha for chronic delta hepatitis. Hepatology 2022;75:1551–1565.
[12] Lempp FA, Schlund F, Rieble L, et al. Recapitulation of HDV infection in a fully permissive hepatoma cell line allows efficient drug evaluation. Nat Commun 2019;10:2265.
[13] Verrier ER, Salvetti A, Pons C, et al. Loss of hepatitis D virus infectivity upon farnesyl transferase inhibitor treatment associates with increasing RNA editing rates revealed by a new RT-ddPCR method. Antivir Res 2022;198:105250.
[14] Kunkel TA. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci U S A 1985;82:488–492.
[15] Kuo MY, Goldberg J, Coates L, et al. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: sequence, structure, and applications. J Virol 1988;62:1855–1861.
[16] Ni Y, Zhang Z, Engelskircher L, et al. Generation and characterization of a stable cell line persistently replicating and secreting the human hepatitis delta virus. Sci Rep 2019;9:10021.
[17] Guo H, Urban S, Wang W. In vitro cell culture models to study hepatitis B and D virus infection. Front Microbiol 2023;14:1169770.
[18] Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 1996;65:241–269.
[19] Huang F, Nau WM. A conformational flexibility scale for amino acids in peptides. Angew Chem Int Ed Engl 2003;42:2269–2272.
[20] Hwang SB, Lai MM. Isoprenylation mediates direct protein-protein interactions between hepatitis large delta antigen and hepatitis B virus surface antigen. J Virol 1993;67:7659–7662.
[21] Jenna S, Sureau C. Mutations in the carboxyl-terminal domain of the small hepatitis B virus envelope protein impair the assembly of hepatitis delta virus particles. J Virol 1999;73:3351–3358.
[22] Komla-Soukha I, Sureau C. A tryptophan-rich motif in the carboxyl terminus of the small envelope protein of hepatitis B virus is central to the assembly of hepatitis delta virus particles. J Virol 2006;80:4648–4655.
[23] Amaya M, Baranova A, van Hoek ML. Protein prenylation: a new mode of host-pathogen interaction. Biochem Biophys Res Commun 2011;416:1–6.
[24] Williamson MP. The structure and function of proline-rich regions in proteins. Biochem J 1994;297(Pt 2):249–260.

[25] **Zhang QC**, **Petrey D**, Deng L, et al. Structure-based prediction of protein-protein interactions on a genome-wide scale. Nature 2012;490:556–560.
[26] Glenn JS, White JM. trans-dominant inhibition of human hepatitis delta virus genome replication. J Virol 1991;65:2357–2361.
[27] Lazinski DW, Taylor JM. Relating structure to function in the hepatitis delta virus antigen. J Virol 1993;67:2672–2680.
[28] Macnaughton TB, Lai MM. Large hepatitis delta antigen is not a suppressor of hepatitis delta virus RNA synthesis once RNA replication is established. J Virol 2002;76:9910–9919.
[29] Sato S, Cornillez-Ty C, Lazinski DW. By inhibiting replication, the large hepatitis delta antigen can indirectly regulate amber/W editing and its own expression. J Virol 2004;78:8120–8134.