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## Combined small molecule and loss-of-function screen uncovers estrogen receptor alpha and CAD as host factors for HDV infection and antiviral targets

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1     **A combined small molecule and loss-of-function screen uncovers estrogen**  
2     **receptor alpha and CAD as host factors for HDV infection and antiviral targets**

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4

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23

24 **ABSTRACT**

25 **Objective:** Hepatitis D virus (HDV) is a circular RNA virus co-infecting hepatocytes with  
26 hepatitis B virus. Chronic hepatitis D results in severe liver disease and an increased risk of  
27 liver cancer. Efficient therapeutic approaches against HDV are absent.

28 **Design:** Here, we combined an RNAi loss-of-function and small molecule screen to uncover  
29 host-dependency factors for HDV infection.

30 **Results:** Functional screening unraveled the HIF-signaling and insulin resistance pathways,  
31 RNA polymerase II, glycosaminoglycan biosynthesis and the pyrimidine metabolism as virus-  
32 hepatocyte dependency networks. Validation studies in primary human hepatocytes  
33 identified the CAD enzyme and estrogen receptor 1 (ESR1) as key host factors for HDV life  
34 cycle. Mechanistic studies revealed that the two host factors are required for viral replication.  
35 Inhibition studies using PALA and Fulvestrant, specific CAD and ESR1 inhibitors  
36 respectively, uncovered their impact as antiviral targets.

37 **Conclusion:** The discovery of HDV host-dependency factors elucidates the pathogenesis of  
38 viral disease biology and opens therapeutic strategies for HDV cure.

39

40 **KEYWORDS:** Antiviral therapy; Hepatitis D; Screening; Liver

41

42 **Word count:** 3989; **References:** 50

43

44 **Abbreviations:** HDV: hepatitis D virus; HBV: hepatitis B virus; HSPG: heparan sulfate  
45 proteoglycan; NTCP: sodium taurocholate co-transporting polypeptide; HD-Ag: delta antigen;  
46 RNP: ribonucleoprotein; EBV: Epstein-Barr virus; CAD: carbamoyl-phosphate synthetase 2,  
47 aspartate transcarbamylase, and dihydroorotase; ESR1: estrogen receptor 1; PHH: primary  
48 human hepatocytes; DHODH: dihydroorotate dehydrogenase; PALA: N-(Phosphonoacetyl)-  
49 L-aspartic acid; TFV: tenofovir;

50

51

52 **SUMMARY BOX**

53 What is already known about this subject?

- 54 • Chronic hepatitis D is the most severe form of viral hepatitis
- 55 • Efficient therapeutic strategies are absent
- 56 • Hepatitis D virus is a small HBV satellite virus
- 57 • Knowledge about HDV-hepatocyte interactions is limited
- 58 • HDV host-dependency factors are largely unknown

59 What are the new findings?

- 60 • A RNAi screen identified ESR1 and CAD as novel host factors for HDV infection
- 61 • The inhibition of CAD restricts HDV infection through uridine depletion
- 62 • ESR1 and CAD are functionally linked by transcriptional activation of gene expression
- 63 • ESR1 and CAD inhibitors Fulvestrant and PALA, respectively, specifically inhibit HDV
- 64 replication in a dose-dependent manner in human hepatocytes
- 65 • CAD and ESR1 are previously undiscovered targets for antiviral therapies

66

67 **Significance of the study**

68 How might it impact on clinical practice in the foreseeable future?

- 69 • The discovery of HDV host-dependency factors opens the door for novel therapeutic
- 70 strategies against chronic hepatitis D – a major unmet medical need
- 71 • Fulvestrant and PALA-like molecules are candidate compounds for HDV antivirals to
- 72 enter preclinical development

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## 79 INTRODUCTION

80 Hepatitis delta virus (HDV) infects human hepatocytes and causes acute acerbation of liver  
81 disease in patients chronically infected with hepatitis B virus (HBV)[1]. Among HBV infected  
82 patients, 5 to 10% are co-infected with HDV[2]. HDV co- or sur-infection in HBV carriers  
83 induces more severe disease compared to infection with HBV alone. This includes a greater  
84 likelihood of experiencing liver failure and a more rapid progression to liver cirrhosis, with a  
85 markedly increased risk of developing hepatocellular carcinoma[3]. Consequently, chronic  
86 hepatitis D is considered as one of the most severe forms of viral hepatitis. Treatment with  
87 HBV nucleos(t)ide analogues is not effective at reducing HDV replication or disease.  
88 Moreover, the virus is only poorly controlled by the current IFN-based therapies[3] and to  
89 date, no treatment allows efficient cure of hepatitis D with eradication of the virus and its  
90 associated disease[4-6]. Thus, there is a major unmet medical need for curative therapies.

91 HDV is a small, circular RNA HBV satellite virus related to plant viroids using HBV  
92 envelope proteins to assemble its infectious particles[1, 5, 6]. Consequently, it can only be  
93 propagated by HBV-infected hepatocytes producing HBV envelope proteins (HBsAg). The  
94 HDV genome is a circular 1.7 kb single-stranded negative-sense RNA molecule (for a review  
95 on HDV virology and pathogenesis, see [5]). The HDV life cycle starts with virus entry into  
96 hepatocyte after attachment to heparan sulfate proteoglycans (HSPG) at the cell surface[5,  
97 7]. As HBV and HDV share the same envelope proteins, they appear to share the same entry  
98 pathway and receptor(s), including the sodium taurocholate co-transporting polypeptide  
99 (NTCP) and Glypican 5 (reviewed in [5, 6, 8]). The HDV genome is then imported in the  
100 nucleus, and three types of RNAs are transcribed by the host RNA polymerase II: replication  
101 is initiated by synthesis of an anti-genomic RNA that serves as a template for the production  
102 of new HDV genomic RNA, and HDV mRNA from genomic RNA[5]. mRNA transcripts  
103 contain one unique open reading frame encoding the delta antigen (HDAg) which exists in  
104 two forms (small version S-HDAg and large version L-HDAg) which play differential roles in  
105 the HDV life cycle[5, 6]. Indeed, S-HDAg is essential to HDV replication, while prenylation of  
106 L-HDAg leads to the inhibition of HDV replication and is crucial for binding to HBV envelope

107 proteins and virion assembly[6]. In the nucleus, L-HDAgs bind to the *de novo* HDV genomic  
108 RNA to form a new ribonucleoprotein (RNPs), which is exported to the cytoplasm and  
109 interacts with HBsAgs at the endoplasmic reticulum to form new infectious virions, which are  
110 then secreted to propagate further rounds of HDV infection[6]. While the molecular virology  
111 of HDV has been elucidated, the role of host-dependency factors for HDV infection remains  
112 largely unknown[5].

113 A unique feature of HDV is the apparent lack of non-structural protein encoded by the  
114 viral genome (including viral polymerase), making it highly dependent of the host machinery  
115 for its replication. Targeting host factors is an emerging concept in the treatment of infectious  
116 diseases including hepatitis viruses[9-12]. Two host compounds targeting HDV factors have  
117 been developed up to clinical proof-of-concept: These include Myrcludex B, a small peptide  
118 targeting HBV/HDV entry factor NTCP and the prenylation inhibitor Lonafrinib[4, 13, 14].  
119 Both compounds demonstrated a significant clinical antiviral effect in chronically infected  
120 patients[4, 13, 14], supporting the validity of the concept of host-targeting antivirals for HDV  
121 infection. However, data on long-term safety, potential resistance in subsets of patients and  
122 sustained virological response remain to be determined. Thus, complementary approaches  
123 targeting other host factors and/or steps of the HDV life cycle are needed for curative  
124 therapies.

125 Taking advantage of our recently developed robust HDV infection system for the study  
126 of HDV-host factor interactions[7] combined with a dual screening approach, we aimed to  
127 uncover HDV host-dependency factors as antiviral targets.

128

## 129 **RESULTS**

130 **A high-throughput RNAi loss-of-function screen uncovers hepatocyte host-**  
131 **dependency factors required for HDV infection.** To identify host factors required for HDV  
132 infection, we performed a high-throughput loss-of-function screen using the HDV-susceptible  
133 cell line Huh-106[7]. Since one key goal was the discovery of druggable host targets, we  
134 used a library comprising siRNAs targeting 7567 genes which have been selected as targets

135 for therapeutics, including kinases, proteases, phosphatases, G protein–coupled receptor,  
136 ion channels, ubiquitin ligases, protein involved in senescence, autophagy, DNA repair, and  
137 targetable nuclear receptors. Huh-106 cells were transfected with siRNAs pools 48 hours  
138 before inoculation with HDV (Figure 1A) with an optical immunostaining-based viral protein  
139 (HDAg) readout 7 days after infection. The silencing of expression of HDV host factor NTCP  
140 was used as functional positive control (Figure 1B-C). The results of the primary screen are  
141 presented in Table S1.

142 Using the hit selection algorithm described in Methods and Figure 1E, we identified  
143 191 host factor genes showing a robust correlation between gene silencing and level of HDV  
144 infection, moderate toxicity of gene-specific siRNA and their expression in the liver (Figure  
145 1D-E and Table S2). The threshold of hit selection (described in Figure 1E) was based on  
146 the functional effect of *SLC10A1* silencing (approximately 45% decrease in HDV infection,  
147 Table S1). The hit rate of 191 genes corresponds to 2.5% of total genes, which is similar to  
148 screens for other virus host factor-dependency screens[15, 16].

149 Functional pathway analysis using KEGG pathways identified a network of host  
150 factors belonging to HSPG biosynthesis, including *EXT1* and *EXT2* (Figure 2A-B). These  
151 genes encode two major exostosins, key HSPG-related enzymes exhibiting  
152 glycosyltransferase activities and involved in the elongation of HS chains[17]. Moreover,  
153 several subunits of the RNA polymerase II were identified in our screen (Figure 2B),  
154 including specific subunits such as *POLR2G* or *POLR2I*, encoding Rpb7 and Rpb9, which  
155 play key roles in the initiation of transcription and binding to DNA template for an accurate  
156 selection of the transcription start sites, respectively[18, 19]. The identification of HSPG  
157 enzymes and RNA polymerase II as known HDV-related pathways[5, 7] validates our  
158 approach for host factor discovery (Figure 2). Importantly, the screen identified several  
159 previously unknown HDV-related networks, such as the hypoxia inducible factor 1 alpha  
160 (HIF-1 $\alpha$ ) signaling pathway, known to enhance the replication of several RNA and DNA  
161 viruses such as Epstein-Barr virus (EBV) and HIV[20, 21]. Interestingly, the HIF-1 $\alpha$  signaling  
162 pathway also plays a key role in hepatocarcinogenesis and liver tumor progression[22, 23]

163 through its ability to target the expression of oncogenic genes such as the proliferation-  
164 specific transcription factor Forkhead box M1[24]. HIF-1 $\alpha$  overexpression in HCC has been  
165 correlated with worse clinical outcomes and is considered as a poor prognosis factor and  
166 molecular target for liver disease therapy[24]. Interestingly, the highly significant scoring of  
167 insulin resistance-related pathways highlights the importance of hepatocyte metabolism as  
168 host-dependency factors on HDV infection (Figure 2).

169 Finally, we identified several host factors involved in the biosynthesis of pyrimidine,  
170 including *CANT1*, *ENTPD5*, and *CAD*.

171

172 **The pyrimidine biosynthesis enzyme CAD is a key host factor for HDV infection and**  
173 **antiviral target in human hepatocytes.** Given its high impact on HDV infection, functional  
174 relevance, liver expression and targetability, we focused on *CAD*, encoding the carbamoyl-  
175 phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), an enzyme  
176 playing a key role in the pyrimidine biosynthesis (Table S2; Figure 2)[25]. Silencing of *CAD*  
177 led to a robust decrease in HDV infection (Figure 3A). *CAD* is a trifunctional enzyme  
178 catalyzing the first steps of pyrimidine biosynthesis[26]. Interestingly, *CAD* expression is  
179 known to be regulated by activated estrogen receptor 1 (ESR1) which binds to the  
180 transcription factor SP1[27], two other host-dependency factors uncovered by the RNAi  
181 screen (Figure 3A). These data suggest a key role of the pyrimidine biosynthesis pathway  
182 and its associated nuclear proteins for the HDV life cycle.

183 We next investigated the functional role of *CAD* in HDV infection. We first performed  
184 additional silencing studies using individual *CAD*-specific siRNA. As shown in Figure 3B-D, a  
185 strong reduction of HDV RNA were observed after transfection of Huh-106 cells with the 4  
186 individual siRNAs composing the siRNA pool, validating the key role of *CAD* expression in  
187 HDV infection. Notably, *CAD* expression was not significantly affected by HDV infection  
188 (Figure 3E).

189 Aiming to characterize the potential of *CAD* as an antiviral target, we synthesized a  
190 specific inhibitor of *CAD*, sparfosic acid or PALA (N-(Phosphonoacetyl)-L-aspartic acid)[26].



191 PALA is an aspartic acid derivative, which inhibits the aspartate carbamoyltransferase  
192 activity of the enzyme. We then investigated the antiviral activity of PALA in HDV-infected  
193 Huh-106 cells. As shown in Figure 3F, PALA dose dependently inhibits HDV infection with an  
194  $IC_{50} = 1.2 \mu\text{M}$ . Notably, a decrease in cell viability was observed after 7 days of culture.

195 To validate this result in the most physiological model for HDV infection, we  
196 investigated the antiviral activity of PALA using HDV infection of primary human hepatocytes  
197 (PHH). Interestingly, while no toxicity was observed in PHH even at high doses (100  $\mu\text{M}$ ), the  
198  $IC_{50}$  of PALA was slightly higher in PHH (Figure 3G), which may reflect differences in uridine  
199 pools and/or CAD expression in rapidly dividing Huh7-based cells versus non-dividing PHH  
200 in cell culture[28].

201 To investigate the mechanism of action of CAD-HDV interaction, we performed a  
202 rescue experiment using PALA and uridine-supplemented medium. Northern blot detection of  
203 HDV genomic RNA in infected cells revealed that the addition of uridine in PALA-treated cells  
204 restored HDV infection, validating the functional relevance of pyrimidine pathway for HDV  
205 replication (Figure 4A). Notably, PALA at a concentration of 2.5  $\mu\text{M}$  induced a strong  
206 decrease in HDV infection which was efficiently rescued by uridine treatment (Figure 4B-C).  
207 This dose was then selected for further functional studies. Importantly, no direct interaction  
208 was observed between CAD and the HDV antigens (Figure 4D). As the inhibition of  
209 pyrimidine pathway has been shown to stimulate the innate antiviral response with  
210 subsequent inhibition of viral propagation [29], we next assessed the expression of *IFNB1*  
211 following PALA treatment in Huh-106 cells. As shown in Figure 4E, no *IFNB1* induction was  
212 observed after treatment with either PALA or Brequinar, targeting dihydroorotate  
213 dehydrogenase (DHODH), suggesting an absence of innate immune stimulation by  
214 pyrimidine inhibitors in hepatocyte-derived cells.

215 To further validate the involvement of the biological function of CAD in HDV infection,  
216 cell culture medium was then complemented using either glutamine (Glu, the initial substrate  
217 of CAD), or dihydroorotate (DHO, the final product of CAD) (Figure 5A, as described in[25]).  
218 As shown in Figure 5B and Figure S1, while glutamine treatment had no effect on

219 intracellular HDV RNA, DHO treatment in PALA-treated cells restored HDV RNA to non-  
220 treated levels, suggesting that PALA antiviral activity is linked to CAD physiological function.

221 To map the replication steps targeted mediated by CAD and targeted by PALA, we  
222 performed Northern blot analyses of HDV RNA allowing to quantify the HDV genome and  
223 anti-genome in the presence or absence of PALA. As shown in Figure 5C, PALA treatment  
224 effectively decreased the levels of both HDV genomic and antigenomic RNAs. Moreover, this  
225 inhibition was already observed during the early stages of viral replication (Figure S2). These  
226 data demonstrate that PALA inhibits all the steps of HDV replication including the synthesis  
227 of antigenomic RNA (Figure 5C).

228 Next, we performed kinetic studies adding PALA pre-, peri- and HDV post-infection  
229 (Figure 5D). PALA potently inhibited HDV infection given at any time before, during and  
230 following initiation of replication. Interestingly, the inhibitory effect was most pronounced  
231 when PALA was added prior or during initiation of replication. Finally, to exclude effects on  
232 other steps of the early HDV life cycle, we studied the effect of PALA treatment on HDV  
233 binding to the Huh-106 cell surface. The absent effect on HDV binding further confirms HDV  
234 replication as the life cycle step targeted by PALA (Figure 5E).

235 To test the specificity of the pyrimidine pathway for HDV infection, we treated HBV-  
236 producing HepAD38 cells with PALA. As shown in Figure 5F, PALA did not modulate HBV  
237 antigen production as shown by an absent effect on HBeAg and HBsAg secretion. While  
238 tenofovir (TFV) treatment dose-dependently inhibited HBV replication, PALA had no effect on  
239 HBV DNA in the supernatant of HepAD38 cells (Figure 5G). Finally, the silencing of *CAD*  
240 expression did not affect HBV infection in HepG2-NTCP cells (Figure 5H). These results  
241 suggest that the pyrimidine/CAD pathway is relevant for HDV but not HBV replication.  
242 Collectively, our results identify CAD as a key HDV host factor involved in HDV replication  
243 and target for antiviral therapy.

244

245 **A small molecule screen uncovers ESR1 inhibitor Fulvestrant as host targeting agent.**

246 Next, we performed a small molecule screen using the Prestwick library, containing 1284

247 FDA-approved drugs (Figure 6A). Among the molecules exhibiting an antiviral activity against  
248 HDV we identified Fluvastatin and Cyclosporine A, two well described NTCP inhibitors (Table  
249 S3), confirming the validity of the screen. Interestingly, Ribavirin, a broad antiviral nucleoside  
250 analog[30] previously used for the treatment of chronic hepatitis C[31] exhibits a marked  
251 antiviral activity against HDV (Table S3). In the same vein, Nelfinavir, a protease inhibitor  
252 presenting anti-retroviral activity[32] was also identified in the drug screen (Table S3).

253         Given our discovery of ESR1 as HDV host-dependency factor we next focused on  
254 ESR1-targeting agents. The library includes several agonists and antagonists of ESR1, as  
255 well as characterized targets for endocrine therapy in breast cancer[33]. Interestingly, the  
256 ESR1 antagonist Fulvestrant exhibited a marked, significant and dose-dependent antiviral  
257 effect against HDV, whereas Tamoxifen and Toremifen, two ESR1 modulators with  
258 estrogenic effects in the liver[34] increased HDV infection, validating the importance of ESR1  
259 in HDV infection (Figure 6B-C). Interestingly, a 48 h-treatment started after virus inoculation  
260 induced a marked decrease in HDV-infected cells (Figure 5D), suggesting an effect on HDV  
261 replication.

262         Next, we aimed investigate the mechanism of action of the antiviral activity of  
263 Fulvestrant by investigating a functional link of ESR1 and CAD suggested by protein/protein  
264 interaction studies[27]. To address this question, CAD expression was analyzed in Huh-106  
265 cells and PHH in the presence or absence of the ESR1-antagonist (Figure 6E-G). As shown  
266 in Figure 6G, Fulvestrant treatment resulted in a loss of CAD expression in both Huh-106 cell  
267 line and PHH, suggesting that the antiviral activity of ESR1-inhibitor Fulvestrant is mediated  
268 by ESR1-mediated down-regulation of *CAD* expression, which in turn is required for HDV  
269 replication, confirming the previous observations with PALA.

270         Taken together, our dual screening approach uncovers the pyrimidine pathway and  
271 its associated and regulatory proteins as host factors for HDV infection and targets for  
272 antiviral therapy.

273

274

275 **DISCUSSION**

276 Chronic hepatitis D is the most severe form of viral hepatitis and at present time no treatment  
277 allows robust viral clearance. Using a dual screening approach we identified *ESR1* and *CAD*  
278 as host factors and antiviral targets for HDV infection. The functional impact of the pyrimidine  
279 biosynthesis for HDV replication is corroborated by (i) a marked decrease in HDV infection  
280 after *CAD* and *ESR1* silencing; (ii) a significant antiviral effect of *ESR1* and *CAD* inhibitors  
281 *PALA* and *Fulvestrant* and (iii) kinetic experiments mapping viral replication as the step of the  
282 viral life cycle mediated by these host factors and targeted by the antivirals (vi) validation  
283 studies in primary human hepatocytes.

284         Whereas the molecular virology of HDV is well described, the molecular interactions  
285 between the virus and liver host factors are still largely unknown[5]. In mammalian cells, the  
286 pyrimidine biosynthesis pathway leads to the production of *de novo* nucleotides and plays a  
287 key role in RNA and DNA production, as well as protein glycosylation or cell membrane  
288 assembly[25]. Moreover, uridine nucleotides and derivatives regulate key physiological  
289 processes, such as lipid metabolism, regulation of normal central nervous system activity or  
290 modulation of reproduction[35], making this pathway a major actor of important metabolic  
291 processes. As a source of nucleotides for HDV RNA, this pathway is likely to be required for  
292 optimal virus replication. Moreover, as a key regulator of viral glycosylation[25], an alteration  
293 of this pathway may alter posttranslational modification of host or viral proteins, disrupting  
294 viral infection.

295         *CAD* is a multifunctional enzyme exhibiting carbamoylphosphate synthetase,  
296 aspartate transcarbamoylase, and dihydroorotase activities catalyzing the three first steps of  
297 the pyrimidine biosynthesis pathway[36], playing a key role in HDV infection as such. The  
298 observed antiviral activity of the *CAD*-specific inhibitor *PALA* validates the importance of this  
299 factor and pathway in HDV infection. It is of interest to note that another enzyme of the  
300 pyrimidine biosynthesis pathway *DHODH*, which catalyzes the production of orotate from  
301 *DHO*, has been described as an antiviral target by small molecule *GSK983*[37]. Here, we  
302 confirmed that the *CAD*-mediated *DHO* and uridine starvation is responsible for the observed

303 antiviral effect (Figures 4 & 5). Interestingly, HSPG synthesis has been shown to be  
304 dependent on the pyrimidine synthesis pathway in a CAD-defective animal model[38].  
305 However, we did not observe any effect of CAD inhibition on either HBV infection or HDV  
306 entry (Figure 5 E-H). While recent studies have suggested that the inhibition of pyrimidine  
307 biosynthesis can stimulate innate antiviral responses[29], no induction of IFN expression was  
308 observed in our model (Figure 4E). Thus, it is likely that the modulation of CAD activity or  
309 expression affects HDV infection through its classical enzymatic activity linked to pyrimidine  
310 metabolism, mainly affecting viral replication through uridine starvation, affecting both  
311 genomic and antigenomic HDV RNAs. In this context, our study suggests that the first steps  
312 of replication are particularly susceptible to PALA. These results are consistent with the  
313 timing of HDV RNA amplification, much more pronounced during the early phase of  
314 replication, before L-HDAg-mediated slow-down of replication[5].

315         Among the genes regulating the pyrimidine pathway, we identified *ESR1* as a HDV  
316 host-dependency factor and antiviral target. *ESR1* is a nuclear hormone receptor expressed  
317 in the mammary gland and female reproductive track, but also in lung and liver[39]. In our  
318 mechanistic analyses, we show that *ESR1* inhibition by Fulvestrant led to a decrease in CAD  
319 protein expression which most likely explains the antiviral effect of the molecule.

320         Our screening strategy also identified small molecules exhibiting significant antiviral  
321 activity against HDV infection. Among them was found ribavirin, a nucleoside analog  
322 previously used for the treatment of chronic HCV infection[40]. Given its concentration in the  
323 primary screen (10  $\mu$ M), HDV inhibition is most likely due to the ability of ribavirin to inhibit *de*  
324 *novo* synthesis of GTP as recently suggested as a possible mechanism for the inhibition of  
325 hepatitis E virus (HEV) replication in Huh7 cells ( $IC_{50}$  of 18.9  $\mu$ M for GTP depletion;  $EC_{50}$  of 3  
326  $\mu$ M for HEV replication[41]). However, clinical attempts to treat hepatitis D using ribavirin  
327 were largely unsuccessful[4].

328         Collectively, we identified previously undiscovered pathways and host factors for HDV  
329 infection and antiviral targets. Host targeting agents are a promising approach for the  
330 development of new antiviral treatments, notably because they limit the emergence of

331 resistant variants[8-10, 42]. In particular for RNA viruses, which do not integrate into the  
332 genome, host targeting agents hold promise for viral cure[43]. Indeed, in chronic HCV  
333 infection antibodies targeting a viral host entry factor Claudin-1 cure viral infection in a state-  
334 of-the-art mouse model[12], and a miRNA-122 antagonist has been shown to cure chronic  
335 HCV infection in monotherapy in a subset of patients[44]. Two host compounds targeting  
336 HDV factors have been developed up to clinical proof-of-concept: these include Myrcludex B,  
337 a small peptide targeting HBV/HDV entry factor NTCP and the prenylation inhibitor  
338 Lonafranib[4, 13, 14]. In randomized clinical trials, both compounds demonstrated a  
339 significant and robust antiviral effect in chronically infected patients[4, 13, 14], supporting the  
340 validity of the concept of host-targeting antivirals for HDV infection. However, long-term  
341 safety, potential resistance in subsets of patients and data on sustained virological response  
342 remain to be determined. The discovery of novel host factors described in this study may  
343 overcome these limitations by complementary, safer and more efficient approaches.  
344 Compounds targeting essential host factors of the viral replication step may be conceptually  
345 superior to entry or prenylation inhibitors by targeting the most vulnerable step of the viral life  
346 cycle. Indeed, viral replication is the target of many effective clinically licensed antiviral  
347 therapies such as nucleoside analogues for HBV infection or polymerase/protease inhibitors  
348 for HIV and HCV infection.

349         A theoretical disadvantage of HTA is their putative adverse effects on physiological  
350 processes mediated by the host factors. In this context, given that an HDV treatment will  
351 occur in a context of diseased liver, assessment of safety will be a major parameter in  
352 development. In this regard, it is of interest to note that pyrimidine synthesis inhibitors, such  
353 as Leflunomide or Teriflunomide, are currently used for the treatment of rheumatoid arthritis  
354 and multiple sclerosis[45, 46]. The safety profile of these compounds suggests that  
355 pyrimidine biosynthesis is a targetable pathway for antiviral strategies. Indeed, the FDA-  
356 approved Leflunomide, targeting DHODH, induces uridine starvation at concentrations  
357 similar to PALA concentrations we used in this study (0-100  $\mu$ M)[47]. Although detailed  
358 toxicity analyses in animal model and human tissues will be required to assess the

359 therapeutic window of PALA, the dose-response effects of Leflunomide suggests that PALA  
360 doses required to inhibit HDV infection are most likely in a range that will have an acceptable  
361 clinical safety profile.

362 In conclusion, by uncovering key host factors for HDV infection our results  
363 significantly improve the understanding of the HDV life cycle and contribute to the  
364 development of novel antiviral strategies for HDV cure.

365

366

## 367 **MATERIAL AND METHODS**

368 **Human subjects.** Human serum from patients with chronic HBV/HDV infection followed at  
369 the Strasbourg University Hospitals, Strasbourg, France was obtained with informed consent.  
370 PHH were obtained from liver tissue from patients undergoing liver resection for liver  
371 metastasis at the Strasbourg University Hospitals with informed consent. Protocols were  
372 approved by the local Ethics Committee of the Strasbourg University Hospitals (CPP) and  
373 the Ministry of Higher Education and Research of France (DC 2016 2616).

374

375 **Cell lines and human hepatocytes.** Huh-106[7], HepAD38[7] and HepG2-NTCP[7] cells  
376 have been described. PHH were isolated and cultured as described[48].

377

378 **Key Reagents.** Fulvestrant (I4409), DL-dihydroorotic acid (DHO, D7003), L-glutamine  
379 (59202C), uridine (U3003), and tenofovir (1643601) were purchased from Sigma-Aldrich®  
380 (Merck). Sparfocid acid (L-Aspartic acid, N-(phosphonoacetyl)-, disodium salt (9CI) or PALA,  
381 NSC: 224131) was first obtained from the Drug Synthesis and Chemistry Branch,  
382 Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National  
383 Cancer Institute, National Institute of Health, Bethesda, Maryland, USA. For further analyses,  
384 PALA was synthesized at the Institute of Chemistry, University of Strasbourg, as described in  
385 Supplementary Material and Methods. PreS1 peptide was synthesized by Bachem[7].

386

387 **HDV and HBV production and infection.** The production of recombinant HDV and HBV  
388 infectious virus as well as the protocol for HDV infection of Huh-106 cells and PHHs and for  
389 HBV infection of HepG2-NTCP cells have been described[7, 49, 50]. A detailed protocol is  
390 presented in Supplementary Material and Methods.

391

392 **RNAi loss-of-function and small molecule screens.** Screening was performed at the High  
393 Throughput Screening platform of the Institut de Génétique et de Biologie Moléculaire et  
394 Cellulaire (IGBMC) in Illkirch, France. The Human ON-TARGETplus “Druggable Genome”  
395 siRNA Library was used for gene expression silencing. For the small molecule screen, the  
396 Chemical Library® containing 1280 FDA-approved molecules was obtained from Prestwick.  
397 Detailed information about both screening strategies as well as the algorithm for selection of  
398 HDV-dependency candidates are presented in Supplementary Material and Methods.

399

400 **Validation of CAD as a HDV host factor using individual siRNAs.** Huh-106 cells were  
401 reverse-transfected with the 4 individual siRNAs from the siCAD pool, a pool of siRNA  
402 targeting SLC10A1 (siNTCP), or a non-targeting siRNA control (siCtrl) using Lipofectamin  
403 RNAi max as described[7, 50]. Gene expression was assessed, two days after transfection,  
404 by Western blot detection of CAD protein using a Rabbit monoclonal anti-CAD antibody  
405 (Abcam ab40800) as previously described[7, 50].  $\beta$ -actin expression was assessed as a  
406 loading control using a mouse monoclonal anti- $\beta$ -actin antibody (Sigma, A5441). Two days  
407 after transfection, cells were infected by HDV and infection was assessed after 7 days as  
408 described in Supplementary Material and Methods.

409

410 **Statistical analysis.** All experiments were performed at least twice in an independent  
411 manner. Statistical analyses were performed using a two-tailed Mann-Whitney U test unless  
412 otherwise stated;  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*) were considered statistically  
413 significant. Significant  $p$ -values are indicated by asterisks in the individual figures and figure  
414 legends. The number of biological replicates is indicated in the figure legends (n).



415

416 Additional methodological information are available in Online Supplementary Material.

417

418

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420 We thank J. Taylor (Fox Chase Cancer Center) for the gift of the HDV expression plasmid

421 (pSVL(D3)) and our colleagues S. Durand and M. Renaud for excellent technical support.

422

#### 423 **AUTHOR CONTRIBUTIONS**

424 TFB initiated the study. TFB and ERV designed and supervised research. ERV, AW, LB and

425 TFB set up, designed and performed the siRNA and small molecule screens. TG and PPa

426 produced the Sparfocic acid. ERV, CB, LH, VTL and EC performed the validation

427 experiments. CSu performed the HDV Northern blots. PPe provided human hepatocytes.

428 ERV, AW, CB, LH, EC, MBZ, CSu, CSc, LB, TFB analyzed the data. ERV, HES and AK

429 performed the bioinformatical analyses of the screens. ERV and TFB wrote the manuscript.

430 All the authors approved the study.

431

#### 432 **COMPETING INTEREST**

433 The authors have no competing interest to disclose.

434

#### 435 **DATA AVAILABILITY**

436 The datasets generated in this study, including the results from both RNAi and small

437 molecule primary screens are available within Online Supplementary Material files. The rest

438 of the data is available from the corresponding authors upon reasonable request.

439

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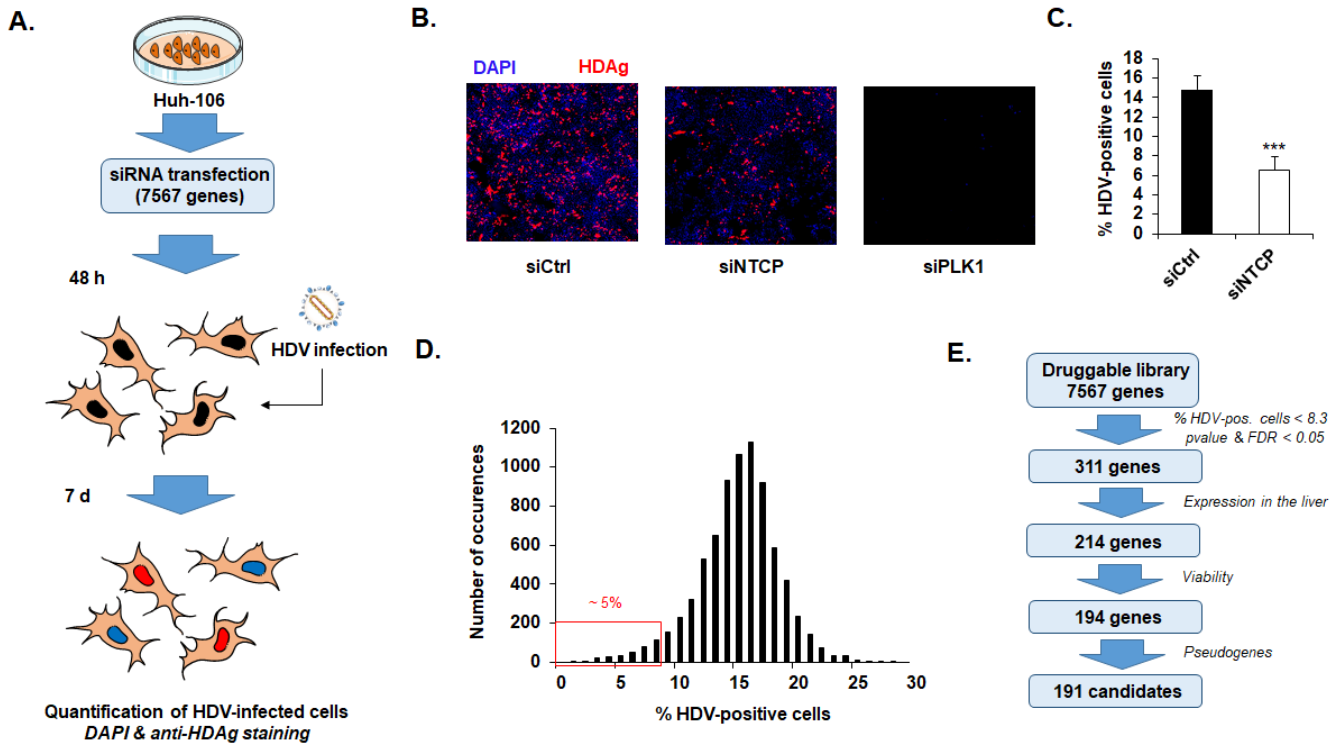
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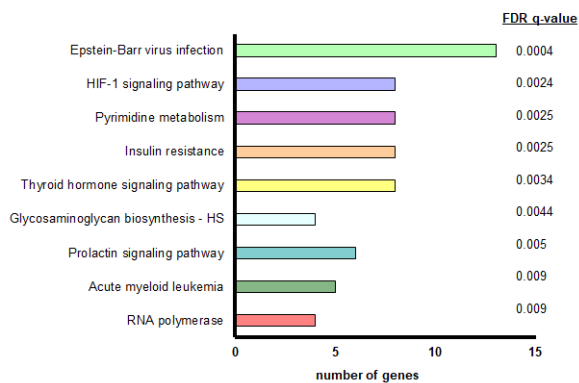
576 **FIGURE LEGENDS**  
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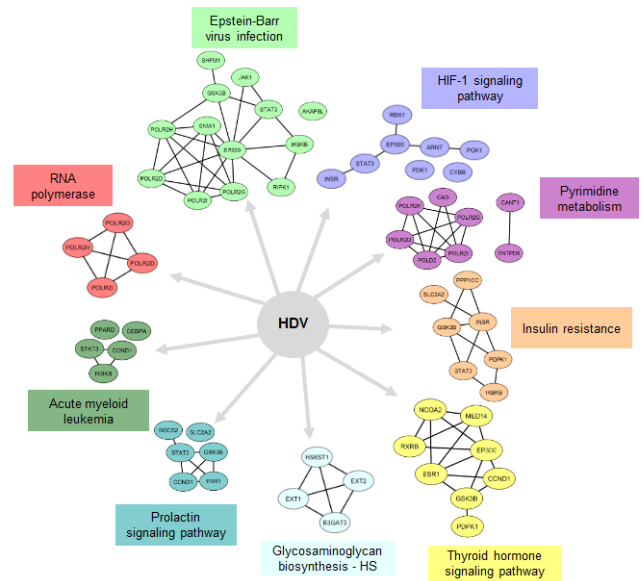
579  
 580 **Figure 1. Identification of host-dependency factors for HDV infection using a high-**  
 581 **throughput RNAi screen. A.** Approach with flow chart of the screen. NTCP-overexpressing  
 582 Huh7 cells (termed Huh-106) were transfected with pools of 4 siRNAs per target of the  
 583 Dharmacon “Druggable genome” library 48 h before HDV infection. Infection was assessed  
 584 after 7 days by immunofluorescence. Each siRNA pool was tested in triplicate. As controls,  
 585 Huh-106 cells were transfected with a non-targeting siRNA control (siCtrl), siRNA targeting  
 586 *SLC10A1* (siNTCP) and *PLK1* (siPLK1, lethal for the cells) expression. Representative  
 587 images of HDV infection in Huh-106 cells from the primary screen are shown in **B**. As  
 588 readout for infection, cells were stained with an anti-HDAg antibody and cell nuclei were  
 589 stained with DAPI. **C.** General effect of the siNTCP on HDV infection at the screen level.  
 590 Results are expressed as means  $\pm$  SD % HDV positive cells from siCtrl-treated cells ( $n =$   
 591 318) and siNTCP-treated cells ( $n = 265$ ). \*\*\*  $p$ value  $< 0.001$  (unpaired Student’s t-test). **D.**  
 592 Distribution of host-factor candidates according to their inhibitory effect on HDV infection.  
 593 The top 5% (% HDV positive cells  $< 8.3$ ) were selected as candidates for further work-up. **E.**  
 594 Selection of candidate genes from the primary screen. From the 311 genes inducing a  $> 45\%$   
 595 decrease in HDV infection after silencing, candidate genes were selected depending on their  
 596 robustness ( $p$ -value & FDR  $< 0.05$ ), their expression in the liver (Illumina Body Map tool)  
 597 and their toxicity. Toxicity was evaluated as the percentage of viability compared to the siCtrl,  
 598 quantified by counting the DAPI-positive nuclei in the wells at the end of infection. From the  
 599 194 selected candidates, *RNF130* was present twice (subset 8 and subset 10) and 2  
 600 pseudogenes (*tAKR/AKR1C6P* and *LOC402164*) were removed. 191 candidates (top 2.5%)  
 601 were further worked-up.



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B.



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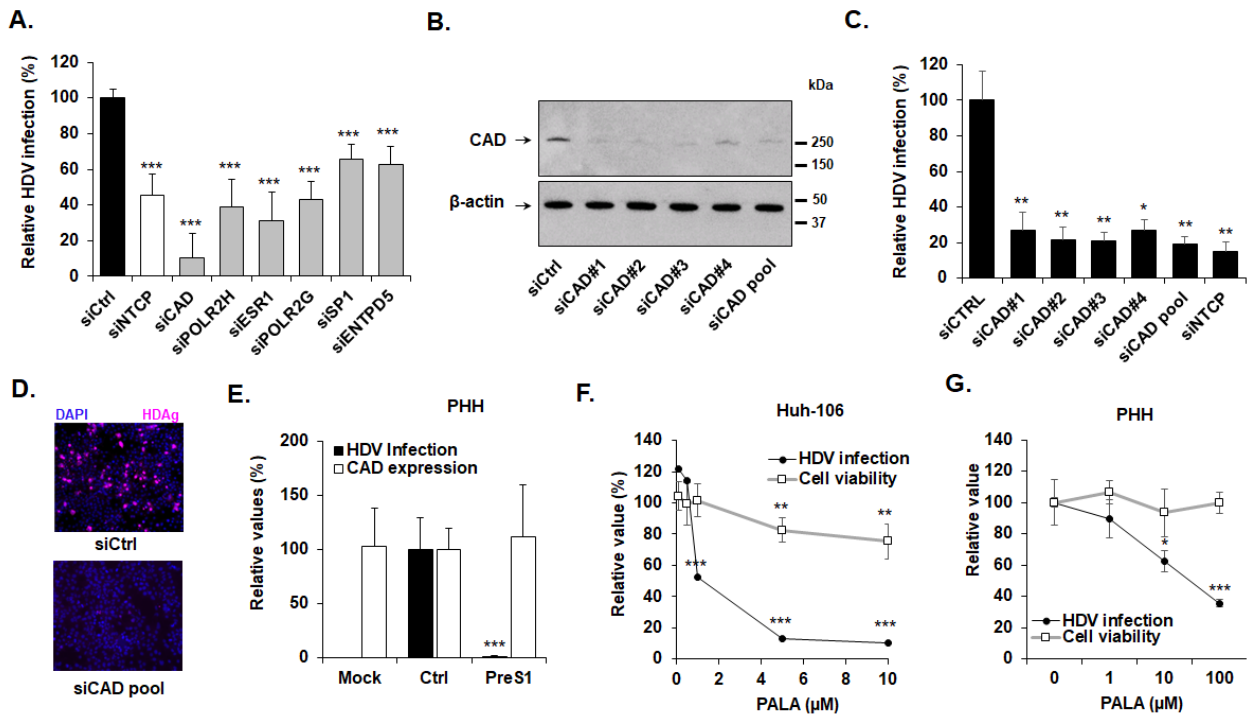
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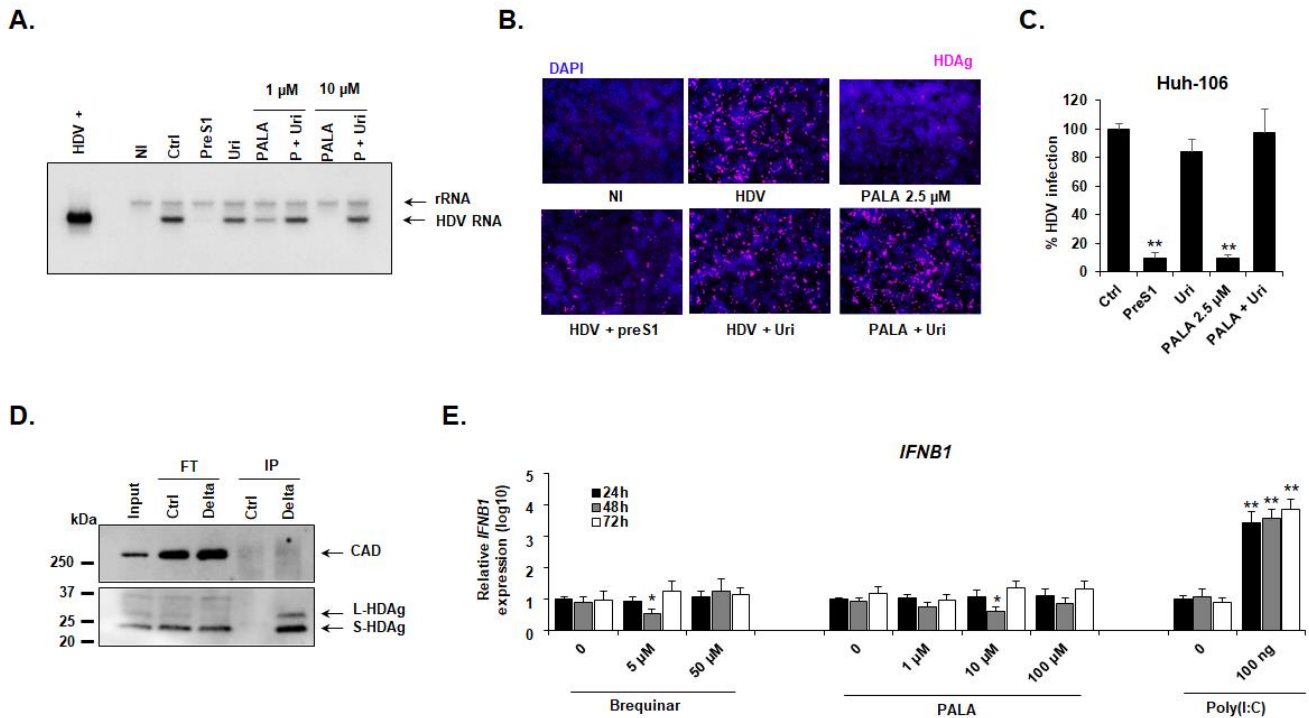
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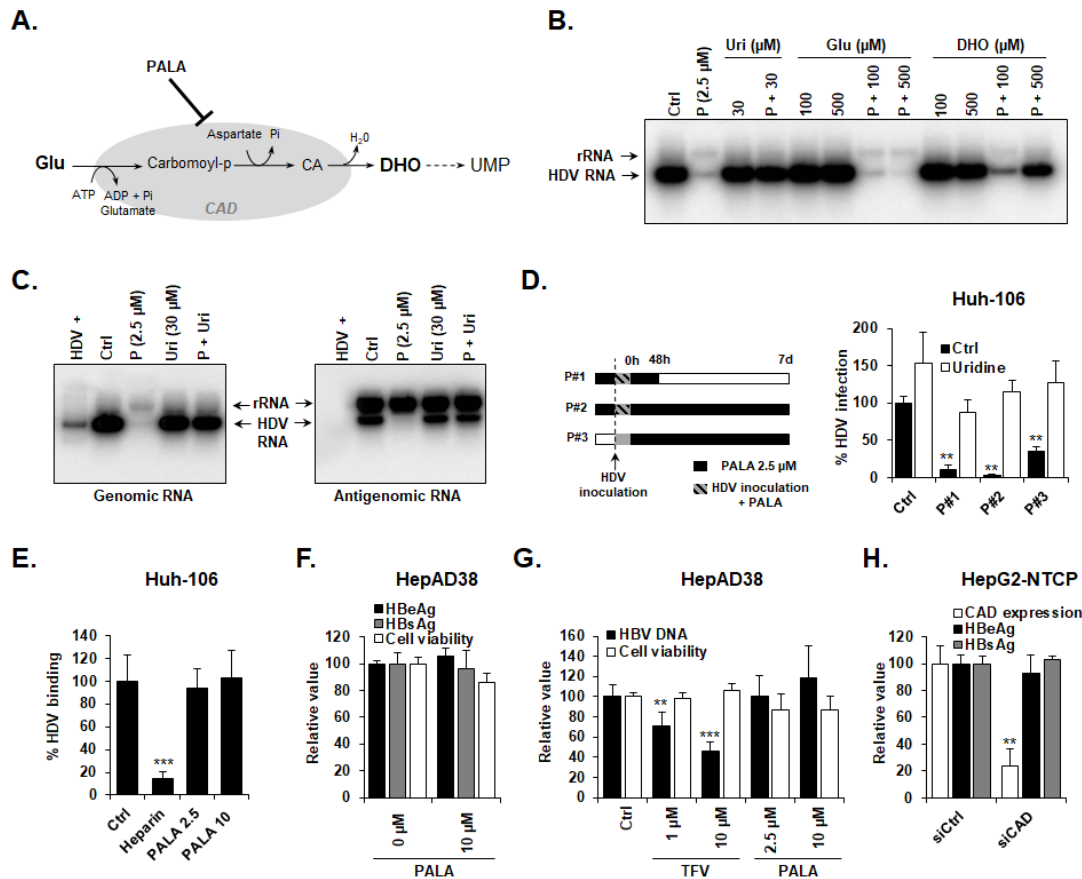
**Figure 2. Pathway analysis of HDV host factors identified within the RNAi screen.** The identified host genes were subjected to functional enrichment pathway analysis through ToppGene Suite (<https://toppgene.cchmc.org>) using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Pathways scoring at a FDR value of < 0.05 were considered significant. **A.** Representation of the number of genes and FDR values of different KEGG pathways significantly enriched within the primary siRNA screen candidates. **B.** Individual genes contributing to the enrichment of the significant KEGG pathways were further analyzed for protein-protein interactions using STRING database. Interaction networks were represented using Cytoscape 3.6.0.



612  
 613 **Figure 3. CAD is a key host factor required for HDV infection.** A. Functional validation of  
 614 host factors belonging to the pyrimidine biosynthesis using perturbation studies. Results are  
 615 presented as means  $\pm$  SD % HDV infection compared to control siRNA (siCtrl, set at 100%)  
 616 from three independent screens (n = 9, primary screen and two validation screens performed  
 617 in triplicate). \*\*\* pvalue < 0.001 (unpaired Student's t-test compared to siCtrl samples). B-D.  
 618 CAD is required for HDV infection. Huh-106 cells were reverse-transfected with 4 individual  
 619 siRNAs targeting CAD mRNA or with the pool of 4 siRNAs. Silencing efficacy was assessed  
 620 by Western blot after two days (B). One representative experiment is shown. Cells were then  
 621 infected with HDV and virus infection was assessed after 7 days by qRT-PCR. Results are  
 622 expressed as means  $\pm$  SEM % HDV infection compared to siCtrl (set at 100%) from three  
 623 independent experiments (n = 8) (C). Alternatively, HDV infection was assessed by  
 624 immunofluorescence using a patient-derived anti-HDAg antibody (D). One representative  
 625 experiment using the pool of siCAD is shown. E. HDV infection has no effect on CAD  
 626 expression in PHH. PHH were treated with preS1 peptide (preS1) or a peptide control (Ctrl)  
 627 for one hour prior to infection with HDV for seven days. Results are expressed as means  $\pm$   
 628 SD % HDV infection (assessed by HDV RNA levels) or CAD expression compared to control  
 629 peptide-treated cells (Ctrl, set at 100%) from four independent experiments (n = 8). F-G. The  
 630 CAD inhibitor PALA dose-dependently inhibits HDV infection in Huh-106 cells (F) and PHH  
 631 (G). Cells were treated with PALA at the indicated concentrations 24 h before infection with  
 632 HDV. Cells were then cultured for 7 days in presence of PALA. HDV infection was assessed  
 633 by qRT-PCR. Cell viability was assessed by Presto Blue. Results are expressed as means  $\pm$   
 634 SD % HDV infection or cell viability compared to untreated cells (0, set at 100%) from three  
 635 independent experiments (Huh-106, F, n = 9) or as means  $\pm$  SEM % HDV infection or cell  
 636 viability from three independent experiments (PHH, G, n = 8).  
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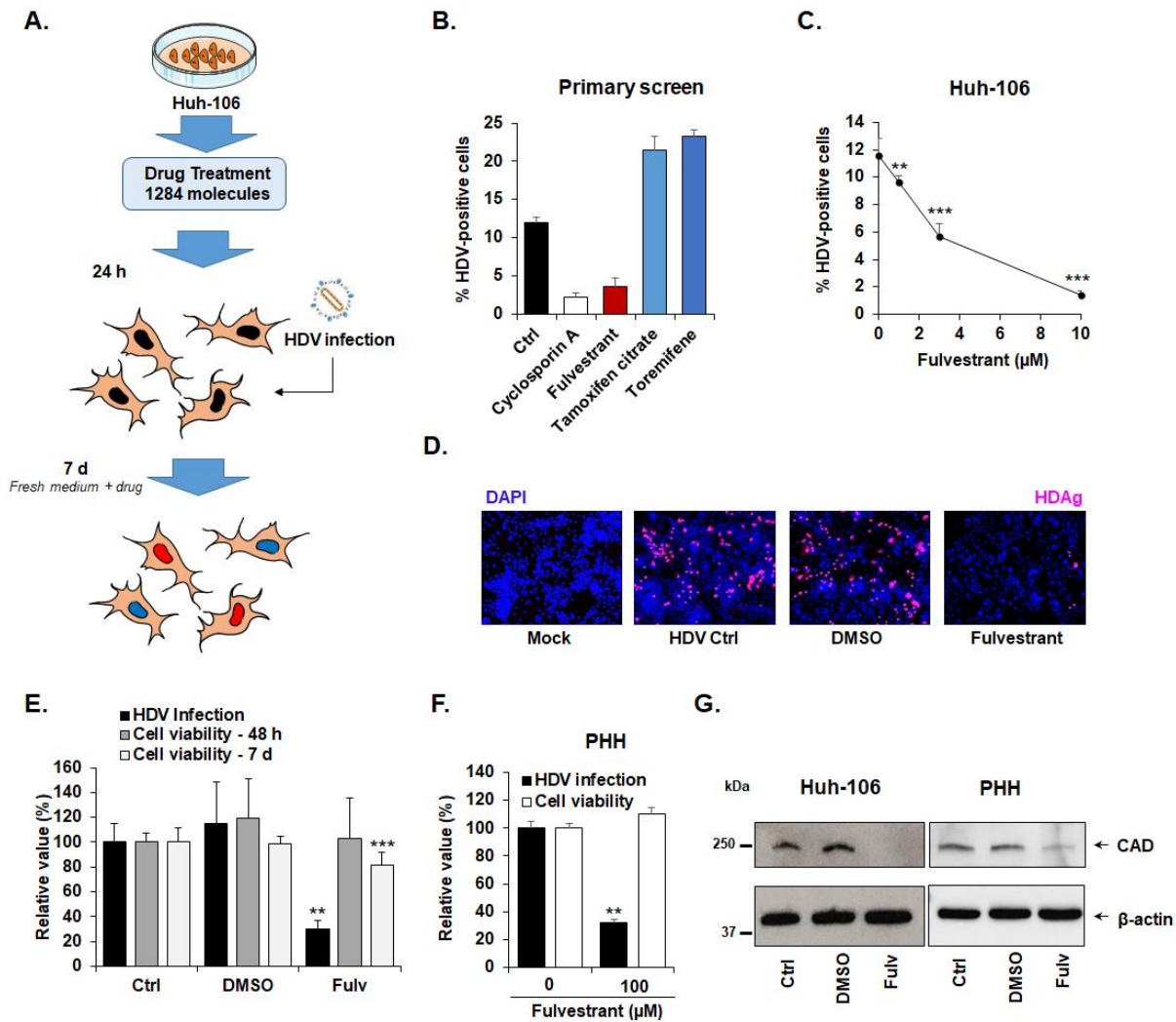
638 **Figure 4. Characterization of CAD-HDV functional interaction.** **A-B-C.** Uridine  
639 complementation restores HDV infection in PALA-treated cells. Huh-106 cells were treated  
640 with PALA (1 μM or 10 μM) in presence of absence of 30 μM uridine 24 h prior to infection  
641 with HDV and compound treatment was maintained for 7 days. HDV infection was assessed  
642 by Northern blot (**A**). HDV+ corresponds to approximately  $5.10^7$  HDV RNA genome  
643 equivalents extracted from HDV particles produced in Huh7 cells. rRNA corresponds to  
644 ribosomal RNA. One representative experiment is shown. Alternatively, Huh-106 cells were  
645 treated with PALA 2.5 μM in presence of absence of 30 μM uridine. HDV infection was  
646 assessed after 7 days by IF (**B**) or qRT-PCR (**C**) Results are expressed as means ± SEM %  
647 HDV infection compared to HDV-infected untreated cells (Ctrl, set at 100%) from three  
648 independent experiments (n = 6). **D.** Absent direct interaction between HDV antigens and  
649 CAD. Huh-106 were transfected with pSVL(D3) plasmid encoding the HDV genome. Three  
650 days after transfection, cells were lysed and HDAg-specific co-IP was performed using an  
651 anti-HDAg antibody (Delta) or with a control antibody (Ctrl). HDV antigens and CAD  
652 expression in the original cell lysate (Input), in Flow-through control samples (FT) and in IP  
653 eluates (IP) were assessed by Western blot. One experiment is shown. **E.** No IFN  
654 induction after inhibition of pyrimidine biosynthesis in Huh-106 cells. Huh-106 cells were  
655 treated with Brequinar or PALA at the indicated concentrations. Alternatively, Huh-106 cells were  
656 reverse-transfected with Poly(I:C) (100 ng). Cells were then lysed every day for three days,  
657 and *IFNB1* expression was assessed by qRT-PCR. Results are expressed as means ± SD  
658 relative *IFNB1* expression (log10) compared to untreated or non-transfected cells (0, all set  
659 at 1) from three independent experiments (n = 6).  
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**Figure 5. PALA specifically inhibits HDV replication by targeting CAD biological function.** **A.** Schematic representation of reactions catalyzed by CAD enzyme. Glu: glutamine; Carbomoyl-p: carbomoyl phosphate; Pi: inorganic phosphate; CA: Carbamoyl aspartic acid; DHO: dihydroorotate; UMP: uridine mono-phosphate. **B.** DHO treatment restores HDV infection in PALA-treated cells. Huh-106 cells with treated with PALA 2.5  $\mu\text{M}$  and infected with HDV in presence or absence of L-glutamine (Glu) or DHO at the indicated concentrations. Infection was assessed after 7 days by Northern blot detection of HDV genomic RNA. rRNA corresponds to ribosomal RNA. One experiment is shown. **C.** PALA inhibits both HDV genomic and antigenomic production. Huh-106 cells were treated with PALA 2.5  $\mu\text{M}$  24 h prior to infection with HDV. HDV genomic and antigenomic RNAs were detected by Northern blot using specific probes 7 days after infection. HDV+ corresponds to approximately  $5 \cdot 10^7$  HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells. One experiment is shown. **D.** Kinetics of HDV infection by PALA. Huh-106 cells were pretreated with PALA at 2.5  $\mu\text{M}$  and infected with HDV (conditions P#1 and P#2). Cells were then cultured in presence of PALA for 48 h (P#1) or for 7 days (P#2) in presence or absence of uridine (30  $\mu\text{M}$ ). Alternatively, Huh-106 cells were infected with HDV with no PALA pre-treatment (P#3). 16 h after viral inoculation, cells were cultured in presence of PALA 2.5  $\mu\text{M}$  with or without uridine (30  $\mu\text{M}$ ). HDV infection was assessed after 7 days by qRT-PCR. Results are expressed as means  $\pm$  SEM % HDV infection compared to HDV-infected untreated cells (Ctrl, set at 100%) from three independent experiments (n = 6). **E.** PALA does not affect HDV binding. Huh-106 cells were culture for 24 h à 16°C in presence of HDV particles which were pre-treated or not with heparin (30  $\mu\text{g}/\text{mL}$ ). HDV binding was measured by qRT-PCR quantification of total HDV RNA bound to cells after 24h. Results are expressed as means  $\pm$  SD % HDV binding relative to control untreated cells (Ctrl, set at 100%) from three independent experiments (n=9). **F-H.** CAD inhibition or silencing does not affect HBV infection and replication. **F.** HBV-producing HepAD38 cells were treated with PALA (10  $\mu\text{M}$ ) for three days. HBeAg and HBsAg secretion in culture supernatant was then quantified by chemiluminescent immunoassay (CLIA). Cell viability was assessed by Presto

690 Blue. Results are expressed as means  $\pm$  SEM % HBeAg production, HBsAg production or  
691 cell viability compared to untreated cells (0  $\mu$ M, all set at 100%) from three independent  
692 experiments (n = 6). **G.** HepAD38 cells were treated with either tenofovir (TFV) or PALA at  
693 the indicated concentrations for three days. HBV replication was assessed by quantification  
694 of HBV DNA copies in the supernatant of treated cells by qPCR. Results are expressed as  
695 means  $\pm$  SD % HBV DNA in the supernatant or cell viability compared to untreated cells (Ctrl,  
696 all set at 100%) from three independent experiments (n = 9) **H.** HepG2-NTCP cells were  
697 reverse-transfected with siCAD or siCtrl for two days prior to infection with HBV. HBV  
698 infection was assessed after 10 days by quantification of HBeAg and HBsAg production  
699 (CLIA). Results are expressed as means  $\pm$  SD % CAD expression, HBeAg production or  
700 HBsAg production compared to siCtrl-transfected cells (siCtrl, all set at 100%) from three  
701 independent experiments (n = 6).  
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**Figure 6. ESR1 inhibitor Fulvestrant inhibits HDV replication by suppression of CAD expression.** **A.** Small molecule screen flow chart. Huh-106 cells were treated with individual compounds belonging to the Prestwick Chemical library (10  $\mu\text{M}$ ) one day prior to infection with HDV. Infection was assessed after 7 days by immunofluorescence. Every compound was tested in triplicate. **B.** ESR1 modulators affect HDV infection. From the primary screen, Fulvestrant (an ESR1 inhibitor) from the one hand, Tamoxifen and Toremifene from the other hand (two agonists/modulators of ESR1) exhibited antiviral or proviral activity against HDV, respectively. Results are presented as means  $\pm$  SD % HDV infected cells ( $n = 3$ ). **C.** Fulvestrant dose-dependently inhibits HDV infection. Huh-106 cells were treated with Fulvestrant at the indicated concentrations and then infected with HDV for seven days. Results are expressed as means  $\pm$  SD % HDV infected cells from three independent experiments ( $n = 9$ ). **D.** Short Fulvestrant treatment for 48 h following virus inoculation inhibits HDV infection. Huh-106 cells were infected with HDV and then treated with Fulvestrant (10  $\mu\text{M}$ ) or DMSO for 48 h. Cells were then culture in absence of drug and infection was assessed after 7 days by IF. One representative experiment is shown. **E.** Fulvestrant antiviral activity is associated with slight cytotoxicity. Cell viability was assessed after 48 h and after 7 days by Presto blue. HDV infection was assessed after 7 days by qRT-PCR. Results are expressed as means  $\pm$  SD % HDV infection (HDV RNA) or cell viability compared to untreated HDV-infected cells (Ctrl, set at 100%) from three independent experiments ( $n = 9$ ). **F.** Fulvestrant antiviral activity in PHH. PHH were infected with HDV and then treated with Fulvestrant for 48 h at the indicated concentrations. Results are expressed as means  $\pm$  SD % HDV infection (HDV RNA) or cell viability compared to untreated HDV-infected PHH (Ctrl, set at 100%) from two independent experiments ( $n = 6$ ). **G.** Fulvestrant inhibits CAD expression. Huh-106 cells and PHH were treated with Fulvestrant (Fulv, 10  $\mu\text{M}$ ) or DMSO for 72h. CAD expression was assessed by Western blot.