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1 **A fusion peptide in preS1 and the human protein-disulfide isomerase ERp57 are involved in**
2 **hepatitis B virus membrane fusion process**

3
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28

29 **Short title:**

30 Hepatitis B virus membrane fusion determinants

31 **Summary**

32

33 Cell entry of enveloped viruses relies on the fusion between the viral and plasma or endosomal
34 membranes, through a mechanism that is triggered by a cellular signal. Here we used a combination
35 of computational and experimental approaches to unravel the main determinants of hepatitis B virus
36 (HBV) membrane fusion process. We discovered that ERp57 is a host factor critically involved in
37 triggering HBV fusion and infection. Then, through modelling approaches, we uncovered a putative
38 allosteric cross-strand disulfide (CSD) bond in the HBV S glycoprotein and we demonstrate that its
39 stabilization could prevent membrane fusion. Finally, we identified and characterized a potential fusion
40 peptide in the preS1 domain of the HBV L glycoprotein. These results underscore a membrane fusion
41 mechanism that could be triggered by ERp57, allowing a thiol/disulfide exchange reaction to occur and
42 regulate isomerization of a critical CSD, which ultimately leads to the exposition of the fusion peptide.

43

44

45 **Introduction**

46

47 Hepatitis B is a major public health problem; it affects over 250 million people worldwide and 850,000
48 deaths occur each year as a result of hepatitis B complications (WHO, March 2015). The structure of
49 its etiological agent, the hepatitis B virus (HBV), features a nucleocapsid that is surrounded by a lipid
50 bilayer containing the envelope glycoproteins (GPs) designated as the small (S), medium (M) and
51 large (L), which are the product of a single open reading frame. They share the C-terminal S-domain
52 that contains four putative transmembrane domains. The L and M proteins have N-terminal extensions
53 (preS1/prS2 and preS2, respectively) that mediate diverse functions in nucleocapsid binding and
54 receptor recognition (Baumert et al., 2014). The first 2-75 amino acids sequence of the preS1 domain
55 of the L protein (Blanchet and Sureau, 2007; Bremer et al., 2011; Le Seyec et al., 1999) and the
56 antigenic loop (AGL) of the S domain (Le Duff et al., 2009; Salisse and Sureau, 2009; Schulze et al.,
57 2007) have been identified as essential determinants for infectivity of HBV and hepatitis delta virus
58 (HDV).

59 Entry of enveloped viruses into cells can be defined as the sequence of events occurring from the
60 attachment of the virus to the host cell until the release of the genome in the cytoplasm, *via* fusion
61 between viral and cellular membrane. Like for most enveloped viruses, HBV entry into cells is a finely
62 regulated and complex process consisting in different steps, in which several viral and cellular factors
63 are involved. Its first step involves low-affinity binding to heparan sulfates proteoglycans (HSPGs)
64 residing on the hepatocytes' surface (Leistner et al., 2008; Schulze et al., 2007). This attachment is
65 mediated by the preS1 region of the L protein and/or the antigenic loop of the S protein (Ni et al.,
66 2014; Schulze et al., 2007). Afterwards, the virus interacts with its high-affinity receptor, the sodium
67 taurocholate-cotransporting polypeptide (NTCP) (Ni et al., 2014; Yan et al., 2012) through the amino-
68 terminal end of the L protein preS1 domain (Glebe et al., 2005a; Gripon et al., 2005; Yan et al., 2012).
69 NTCP is an integral membrane protein, expressed at the basolateral membrane of hepatocytes, which
70 explains the tropism of HBV for the liver.

71 The post-binding entry steps of HBV occur through endocytosis; however, the exact mechanism is still
72 unclear and somehow controversial. One early study showed that HBV in HepaRG cells is internalized
73 *via* caveolin-mediated endocytosis (Macovei et al., 2010). Nevertheless, inhibition of caveolin-
74 mediated endocytosis or silencing of caveolin-1 did not impair HBV infection in Tupaia hepatocytes
75 (Bremer et al., 2009) or in HepaG2-NTCP cells (Herrscher et al., 2020). Contrastingly, several other
76 studies presented evidence that HBV endocytosis is clathrin-dependent (Herrscher et al., 2020; Huang
77 et al., 2012; Umetsu et al., 2018). Recent studies reported that HBV infection of HepaRG cells
78 depends on Rab5 and Rab7 (Macovei et al., 2013), which are GTPases involved in the biogenesis of
79 endosomes, and that the epidermal growth factor receptor (EGFr) is a host-entry cofactor that
80 interacts with NTCP and mediates HBV internalization (Iwamoto et al., 2019). These findings support

81 the hypothesis that HBV is transported from early to mature endosomes. After the early endosome
82 stage, translocation is associated with a gradually decreasing pH, from about 6.2 in early endosomes
83 to close to 5.5 in late endosomes, which allows fusion of many enveloped viruses with the endosomal
84 membrane. However, in the case of HBV, pharmacological agents that raise or neutralize the pH in
85 the endocytic pathway do not affect infection (Macovei et al., 2010, 2013; Rigg and Schaller, 1992).
86 Furthermore, treatment with protease inhibitors have no effect on infection (Macovei et al., 2013),
87 suggesting that HBV transport into the degradative branch of the endocytic pathway is not required
88 *per se* to initiate this process.

89 Virus entry by membrane fusion involves interactions between viral fusion proteins and host receptors,
90 which results in conformational changes of the virus envelope proteins. However, the molecular
91 determinants and mechanism of membrane fusion of HBV remain to be defined. Previous results
92 indicated the essential role of the cysteine residues of the antigenic loop, as shown by the reduction of
93 virus entry levels by inhibitors of thiol/disulfide exchange reaction (Abou-Jaoudé and Sureau, 2007),
94 hence suggesting a redox state responsible for conformational changes that can have a role during
95 the fusion step.

96 Here, using a combination of computational and experimental approaches, we sought to better
97 understand how HBV induces the fusion of its lipid membrane with that of the infected cell.
98 Specifically, using a coevolution analysis of HBV GPs and molecular modelling combined with
99 experimental investigations *ex vivo* in molecular virology and *in vivo* in liver humanized mice, we
100 provide evidence that the mechanism triggering HBV membrane fusion involves ERp57, a cellular
101 protein disulfide isomerase. Furthermore, our results highlight the role of specific cysteines in the AGL
102 determinant and well as a sequence (aa 48 to 66) in the preS1 determinant that could ultimately act as
103 a fusion peptide mediating HBV membrane fusion.

104

105

106 **Results**

107

108 **HBV membrane fusion is independent of acid pH and receptor expression.** To investigate the
109 fusion activation mechanism and to identify the fusion determinants of HBV, we designed a cell-cell
110 fusion assay whereby Huh7 “donor” cells, expressing a luciferase reporter gene under control of the
111 HIV-1 promoter, were co-cultured with either Huh7-tat or Huh7-NTCP-tat “indicator” cells, expressing
112 the HIV-1 transactivator of transcription (Tat) protein, which induces luciferase expression only in
113 fused donor and indicator cells (Lavillette et al., 2007). We transfected donor cells with pT7HB2.7
114 (Sureau et al., 1994), an expression plasmid encoding the wild-type HBV glycoproteins L, M and S.
115 The transfected donor cells were then co-cultivated with Huh7-tat or Huh7-NTCP-tat indicator cells
116 for 1 day. The medium of the co-cultures was then acidified at pH4 for 3 min to trigger fusion and the

117 next day, the luciferase activity in the lysates of co-cultured cells was measured as a read-out of
118 membrane fusion (Figure 1A). The GPs of vesicular stomatitis virus (VSV) or of Crimean-Congo
119 hemorrhagic fever virus (CCHFV) were used as controls for viruses that need acidic pH to promote
120 membrane fusion. We found that HBV GPs induced similar levels of fusion in co-cultures that were
121 exposed to either acidic or neutral pH, as well as in co-cultures lacking or expressing the NTCP
122 receptor (Figure 1A; see raw data in Figure 1-figure supplement 1). Since HBV entry requires HSPG
123 to mediate the capture of its viral particles through HBsAg (Leistner et al., 2008; Schulze et al., 2007),
124 we addressed whether blocking of HBsAg/HSPG interaction could inhibit cell-cell fusion using heparin
125 as competitor. Yet, while the applied doses of heparin could prevent cell-free entry, as shown
126 previously (Schulze et al., 2007), addition of soluble heparin to the co-cultures did not prevent HBsAg
127 mediated fusion, whether the indicator cells expressed or not NTCP (Figure 1B). We confirmed these
128 results by using CHO and CHO-pgsB618 (Richard et al., 1995) cells as donor and/or indicator cells.
129 While both cell types do not express NTCP, only the former expresses HSPGs. We found that cell-cell
130 fusion could be detected for either indicator cell type to the same extent as for Huh7 cells (Figure 1C).
131 Altogether, these results indicated that cell-cell fusion mediated by HBV GPs is independent of acid
132 pH and requires neither HSPG nor NTCP receptor, which underscores an alternative fusion trigger.

133

134 **The preS1 domain of HBsAg harbors a critical determinant of membrane fusion.** The L, M and S
135 GPs of HBV are produced by a single open reading frame and share a common C-terminal S-domain.
136 M and L proteins harbor additional N-terminal extensions (preS2 and preS1/preS2, respectively), with
137 preS1 harboring the NTCP-binding determinant (Glebe et al., 2005b; Gripon et al., 2005). Noteworthy,
138 the fusion determinants of HBV GPs and, particularly, the fusion peptide that could induce merging of
139 viral and endosomal membranes has not yet been functionally identified in infection or cell-cell fusion
140 assays.

141 First, to address which GP is responsible for HBV membrane fusion, we evaluated the role of either
142 proteins in cell-cell fusion assays (Figure 1D). Huh7 cells were transfected with plasmids encoding wt
143 HBV GPs, *i.e.*, L, M and S (pT7HB2.7 plasmid), vs. only L, M or S (using pCiL, pCiM and pCiS
144 plasmids, respectively) (Komla-Soukha and Sureau, 2006). To analyze the expression of either protein
145 at the cell surface, transfected cells were labeled with sulfo-NHS-SS-biotin, a chemical compound that
146 is unable to penetrate biological membranes. After lysis and immuno-precipitation of biotinylated
147 proteins, we found that the individually expressed L, M or S proteins were detected at similar levels as
148 compared to HBV GPs (L, M and S) expressed simultaneously, as in cells transfected with the wt
149 pT7HB2.7 plasmid (Figure 1E, 1F). Then, to determine the fusion activity of either protein, we
150 performed cell-cell fusion assays as described above. We found that none of the L, M or S proteins
151 expressed alone were able to induce membrane fusion (Figure 1D). Furthermore, when we tested the
152 pT7HB2.7Mless plasmid, which induces co-expression of S and L only (“noM” in Figure 1D-1F), we

153 detected a cell-cell fusion activity at the same level than for wt HBV GPs (Figure 1D). This indicated
154 that M is not necessary for membrane fusion, in agreement with previous results (Ni et al., 2010;
155 Sureau et al., 1994) showing that M is dispensable for infectivity of viral particles (Figure 1-figure
156 supplement 3).

157 Altogether, these results suggested that the determinants of membrane fusion are harbored within L
158 and S GPs.

159

160 Next, aiming to identify a fusion peptide in either protein, we used a computational approach to
161 pinpoint regions of the HBV GPs that may potentially interact with membrane bilayers. Using
162 Membrane Protein Explorer (MPEx), a tool based on the Wimley-White Interfacial Hydrophobicity
163 Scale (Snider et al., 2009), five regions of high interfacial hydrophobicity were identified (Figure 2-
164 figure supplement 1A). Two out of the five hydrophobic regions did not correspond to HBV GP
165 transmembrane regions (TM1, TM2, and TM3/TM4), and therefore were considered as candidate
166 fusion peptides (Figure 2A, 2B). The first predicted segment comprised amino acids 48 to 66 that
167 partially overlap with the preS1 domain. The second segment, which includes amino acids 127 to 145,
168 is included in the preS2 region. Our prediction analyzes indicated that the first segment ($\Delta G = -3.38$)
169 was more likely to be a fusion peptide than the second one ($\Delta G = -0.85$) (Figure 2-figure supplement
170 1B). Considering the Wimley-White scale, a set of mutants was designed to alter the hydrophobicity of
171 the two predicted segments (Figure 2B and Figure 2-figure supplement 1B). In the first segment, three
172 mutants were studied by changing the aromatic residues to an alanine or glutamate: F52A, F56A,
173 W66A, F52A/W66A (FW/AA) and F52E/W66E (FW/EE), or a glycine to an alanine (G53A). In the
174 second segment, four mutants were considered: Y129A, F130A, S136E, L144A; while the first two
175 mutants targeted aromatic residues, S136 and L144 were also considered important because they are
176 at the center of the predicted region and have a relatively high hydrophobicity.

177 To evaluate the role of these two sequences in HBV fusion, we introduced these single or double
178 mutations in both regions and inserted them in the pT7HB2.7 HBV GP expression plasmid. Each
179 mutant was compared to wt HBV GPs in both infection assays, using HDV particles (Sureau, 2010;
180 Perez-Vargas et al., 2019), and cell-cell fusion assays, as above-described. We found that HDV
181 particles carrying these mutant GPs were produced by Huh7 cells at levels similar to those produced
182 with wt GPs (Figure 2C, 2D), hence ruling out gross misfolding induced by the mutations that would
183 otherwise prevent HBV GPs incorporation on viral particles (Abou-Jaoudé and Sureau, 2007).
184 Interestingly, no infectivity could be detected for most of the mutations introduced in the preS1 peptide
185 (Figure 2E), whereas the HDV particles with mutations in the preS2 peptide showed levels of
186 infectivity that were similar to those obtained with the wt GPs (Figure 2F). Correlating with the results
187 of these infection assays, we found that the mutants in the preS1 peptide that prevented HDV
188 infectivity also abrogated cell-cell fusion activity (Figure 2G) in a manner unrelated to the levels of GPs

189 cell surface expression (Figure 2I and Figure 2-figure supplement 2). In contrast, mutations in the
190 preS2 peptide displayed the same levels of cell-cell fusion activity as compared to wt (Figure 2H, 2J).
191 Altogether, these results indicated that the preS1 region harbors a potential fusion peptide.

192

193 **Stabilizing cross-strand disulfide exchanges in HBV S protein prevents membrane fusion.** Next,
194 we sought to investigate the mechanisms that could induce fusion-activating conformational changes
195 in the HBV GPs, leading to exposure of the fusion peptide. As neither the HBV receptor interaction nor
196 the acidic pH could trigger membrane fusion (Figure 1), we thought that conformational rearrangement
197 of HBV GPs might involve reshuffling of their disulfide bonds. Indeed, previous studies showed that
198 cysteine residues of the HBV S antigenic loop are essential for HDV infectivity and that viral entry is
199 blocked by inhibitors of thiol/disulfide exchange reactions, such as TCEP, DTT, DTNB or AMS (Abou-
200 Jaoudé and Sureau, 2007). Thus, to extend the notion that thiol/disulfide exchange reactions are
201 implicated during membrane fusion and entry, we performed HBV infection and fusion assays in the
202 presence of DTNB, an alkylator agent. First, using different DTNB concentrations that were added
203 either at the onset of infection or at 16h post-infection, we confirmed that DTNB could block HDV
204 infection in a dose-dependent manner, but only when it was added at the onset of infection (Abou-
205 Jaoudé and Sureau, 2007) (Figure 3-figure supplement 1). Second, using time-of-addition
206 experiments, we found that DTNB could inhibit infection only if added within the first 2 hours after
207 inoculation with HDV particles (Figure 3A). These results suggested that DTNB blocks a thiol/disulfide
208 exchange reaction that could be necessary at an early step of infection, such as a trigger of the fusion
209 mechanism, though not at a later stage of the entry process. Third, to evaluate the effect of DTNB in
210 membrane fusion, we performed cell-cell fusion assays in presence of DTNB, which was added at the
211 onset of cell co-cultures vs. at 16h after seeding the cell co-cultures. We showed that DTNB added
212 during the co-culture neither induced cytotoxicity (Figure 1-figure supplement 2) nor affected
213 expression of HBV glycoproteins on the cell surface (Figure 3C, 3D). Yet, we found a dose-dependent
214 reduction of the level of cell-cell fusion when DTNB was added immediately after cell-cell contact,
215 whereas we detected a much lower effect in fusion activity when DTNB was added at 16h after cell
216 contact (Figure 3B).

217 Altogether, these results suggested a role of the disulfide bonds network during HBV membrane
218 fusion steps, perhaps at the level of the fusion trigger.

219

220 To address this possibility and to identify potential mechanisms involved in fusion triggering, we
221 focused on the “a” determinant of protein S that exhibits eight conserved Cys, which, for some of
222 them, are in strong proximity in the sequence (Figure 4A). To avoid trivial contact predictions between
223 consecutive Cys, we defined four Cys-containing regions in a way that Cys pairs that are potentially in
224 contact should have a sequence separation of at least four amino acids. The first Cys-containing

225 region includes C270, the second C284 and 287, the third C300, C301 and C302, and the last one
226 C310 and C312 (Figure 4A). We applied secondary and tertiary structure prediction methods together
227 with the contact prediction method RaptorX (Ma et al., 2015; Wang et al., 2017), based on coevolution
228 signals, to predict disulfide connectivity in the “a” determinant, which may identify which Cys forms
229 disulfide bonds. Notably, RaptorX predicted structural contacts between either region (Figure 4-figure
230 supplement 1) and we highlighted pairs of residues in contact in the four Cys-regions, with the
231 strongest signal detected between the third and fourth regions (Figure 4B). Next, applying the JPred
232 secondary structure prediction method (Cole et al., 2008), we predicted two β -strands in the Cys-rich
233 regions delimited by the S segments 298-303 and 310-313 (Figure 4A). Then, considering the
234 secondary structure prediction and the contact prediction, we built a three-dimensional model for the
235 region 294-317 (Figure 4C), which indicated that this sequence is compatible with a β -hairpin
236 structural motif containing a cross-strand disulfide (CSD) between C301 and C310. Finally, through
237 the analysis of its five χ dihedral angles (Figure 4-figure supplement 2), this disulfide bond was
238 classified in a “-HStaple conformation”, which is a particular type of disulfide geometry associated with
239 allosteric functions by triggering a conformational change upon switching between the reduced and
240 oxidized states (Chiu and Hogg, 2019; Hogg, 2003).

241 We therefore hypothesized that the redox state of this disulfide may act as an allosteric switch that
242 could contribute to control conformational rearrangements of the S protein. Thus, we used our
243 structural model of the C301-C310 disulfide bond to design a mutant of S that could disrupt this
244 hypothetical allosteric function, *i.e.*, the T303C/G308C double mutant that induces an additional C303-
245 C308 disulfide bond (Figure 4C). Further molecular dynamics (MD) simulations (1,000 frames per MD
246 trajectory) carried out to differentiate between allosteric and structurally stabilizing disulfides, where
247 the disulfides can be classified based on their angles (Figure 4-figure supplement 2), showed that the
248 T303C/G308C mutant predominantly forms a structural disulfide bond.

249 Aiming to validate our prediction that an additional disulfide bond between the two β -strands could, by
250 stabilizing the 298-313 β -hairpin motif, prevent membrane fusion from occurring, we produced HDV
251 particles carrying the individual (T303C or G308C) and double (T303C/G308C) mutations in HBV
252 GPs. By measuring HDV RNAs in cell supernatants, we found that all mutants could produce
253 comparable levels of viral particles relative to wt virus (Figure 5A), suggesting absence of gross
254 alterations of HBV GP conformation that would otherwise preclude virion assembly (Abou-Jaoudé and
255 Sureau, 2007). Importantly, we found that while HDV particles generated with GPs harboring the
256 individual mutations were as infectious as wt, those that were produced with the T303C/G308C double
257 mutation (noted TG/CC in Figure 5) and the putative additional C303-C308 CSD bond were not
258 infectious (Figure 5B). Moreover, we found that HDV particles harboring GPs with this T303C/G308C
259 mutation had similar binding levels on Huh7 cells than those generated with wt GPs (Figure 6A),
260 underscoring a post-binding defect. Then, to address this possibility, we performed cell-cell fusion

261 assays with either HBV GP mutant, which were readily expressed at the cell surface (Figure 5C). We
262 found that whereas the single mutations displayed similar fusion activity as compared to wt HBV GPs,
263 the T303C/G308C double mutation completely prevented HBV GP-induced cell-cell fusion activity
264 (Figure 5D).

265 Altogether, these results suggested that the putative C303-C308 additional disulfide bond stabilizing
266 the loop containing the C301-C310 CSD bond inhibited HBV entry and fusion, perhaps by preventing
267 conformational rearrangements of HBV GPs that are required for promoting membrane fusion.

268

269 **ERp57 is a protein disulfide isomerase that promotes HBV entry and infectivity *in vivo*.** We
270 reasoned that isomerization of the C301-C310 CSD (Figure 4) or of another CSD of the AGL
271 determinant with allosteric functions could facilitate some conformational rearrangements required to
272 promote membrane fusion. We therefore hypothesized that such an isomerization could be induced by
273 a host factor from the Protein Disulfide Isomerase (PDI) family, which are enzymes that can both
274 reduce and oxidize disulfide bonds.

275 To address if PDIs are involved in HBV entry, we tested the effect of inhibitors (NTZ, EGCG, Rutin,
276 Bacitracin, PX-12) that target different PDI species (PDIA1, ERp5, ERp57, TMX1) for their effect in cell
277 entry of viral particles. First, through binding assays of viral particles to Huh7 or Huh7-NTCP cells
278 performed in the presence of either inhibitor, we found that none of these inhibitors affected binding of
279 HDV particles generated with either wt or T303C/G308C mutant GPs (Figure 6A). Second, using
280 infection assays with Huh7-NTCP cells pre-incubated with either inhibitor, we found that HDV particles
281 had strongly reduced infectivity in presence of NTZ and EGCG inhibitors that both target ERp57
282 (Figure 6B). Third, we confirmed these results using infection assays with authentic HBV particles
283 (Figure 6C). Finally, to demonstrate that the inhibitors acted at the level of membrane fusion, we
284 performed cell-cell fusion assays, as above-described, whereby either inhibitor was added at the onset
285 of co-cultures of HBV GP-expressing Huh7 donor and Huh7-NTCP-Tat indicator cells, and was kept
286 throughout the assay period. Remarkably, we found a strong reduction of the levels of cell-cell fusion
287 with the same drugs that inhibited HDV infection (Figure 6D). Hence, these results suggested a
288 potential role of ERp57 in HBV membrane fusion.

289

290 Next, aiming to confirm and extend these findings, we selected a subset of the above PDIs, *i.e.*,
291 ERp46, ERp57 and ERp72, that displayed low but significant expression at the surface of Huh7 cells
292 (Figure 7A), in agreement with a previous report (Turano et al., 2002). We down-regulated either PDI
293 in target cells *via* transduction of Huh7-NTCP cells with shRNA-expressing lentiviral vectors (Figure 7-
294 figure supplement 1 and Figure 7-figure supplement 2). We found that down-regulation of ERp57,
295 though not ERp46 or ERp72, strongly reduced the levels of HDV (Figure 7B) and HBV infection
296 (Figure 7C) and of cell-cell fusion (Figure 7D). Finally, through confocal microscopy analysis of Huh7-

297 NTCP cells (Figure 8A), we investigated the co-localization between ERp57 and Rab5 (early
298 endosomes), Rab7 (late endosomes), Rab11 (recycling endosomes) or Lamp1 (lysosomes). The
299 quantifications of these results showed that ERp57 could be detected in late endosomes but poorly in
300 the other above-tested locations (Figure 8B), in line with the notion that fusion of HBV particles occurs
301 in late endosomes (Macovei et al., 2013). Thus, ERp57 can be found at locations compatible for both
302 cell-cell fusion and cell-free entry by internalization.

303 Altogether, these results indicated that ERp57 is likely a protein disulfide isomerase that promotes
304 HBV entry at a membrane fusion step.

305

306 Finally, we sought to demonstrate that ERp57 inhibition may prevent HBV propagation *in vivo* using
307 NTZ, which has a short half-life of about 1.5h (Ruiz-Olmedo et al., 2017; Stockis et al., 1996). We
308 generated a cohort of liver humanized mice (HuHep-mice) derived from the NFRG mouse model
309 (Azuma et al., 2007) (Figure 9A). We retained the animals that displayed >15 mg/mL of human serum
310 albumin (HSA), which corresponds to 40-70% of human hepatocytes in the liver (Calattini et al., 2015).
311 In agreement with previous reports (Perez-Vargas et al., 2019), these animals supported HBV
312 infection (Group#1) for several weeks/months (Figure 9B; see Figure 9-figure supplement 1 for
313 individual mice). The second group of HuHep mice was treated with NTZ 30min prior to inoculation
314 with HBV, and then, treated again with NTZ 1h later. We found that viremia in this group was delayed
315 by about 4 weeks, as compared to Group#1 for which HBV could disseminate immediately after
316 inoculation. This indicated that the blocking of ERp57 could prevent HBV infection *in vivo*.

317

318

319 **Discussion**

320

321 The entry process of enveloped viruses into cells is the series of all events that take place from the
322 attachment of the virus to the host cell until the release of its genome in the cytoplasm. It is a finely
323 regulated and complex process with several steps, in which many viral and cellular factors are
324 involved. The first interaction often occurs with HSPGs. It may lack specificity but serves to give a
325 virus an initial catch hold from which it can recruit specific receptors and entry co-factors that drive the
326 reactions leading to entry. Fusion is the last step of enveloped virus entry and allows the release of the
327 viral capsid in the cytoplasm following the merging of the viral membrane with a membrane of the
328 infected cell. The interactions with the target cell that trigger conformational changes of the viral
329 surface glycoproteins, ultimately leading to the insertion of their fusion peptide into the cell membrane,
330 vary widely for enveloped viruses and can be divided into different scenarios. In a first one (*e.g.*, HIV),
331 fusion is triggered directly by the interaction of the viral glycoprotein with its cellular receptor, through
332 allosteric conformational rearrangements. In some cases, a sequential interaction with additional host

333 factors is required to trigger the conformational changes required for fusion. In a second scenario
334 (e.g., influenza virus), the interactions with the receptor at the cell surface leads to the endocytosis of
335 viral particles, which is followed by GP protonation in the low pH environment of the intracellular
336 endosomal organelles that triggers the fusogenic conformational change. In a third scenario (e.g.,
337 Ebola virus), the initial interactions of the virion with the cell surface trigger its endocytosis followed by
338 a second interaction with an internal receptor, often found in late endosomes, which is preceded by
339 proteolytic cleavage of the fusion protein by an endosomal protease, leading to fusion activation
340 (Harrison, 2015; White and Whittaker, 2016). Finally, for certain viruses (e.g., Sindbis virus), the fusion
341 protein requires an activating redox reaction involving disulfide bonds of their glycoproteins to induce
342 membrane fusion (Key et al., 2015; Rey and Lok, 2018).

343

344 Using a cell-cell fusion assay, we found that HBV fusion activity reached similar levels whether
345 indicator cells expressed or not HSPG and/or NTCP but was not increased when the cell co-cultures
346 were exposed at low pH, in contrast to *bona fide* pH-dependent GPs such as VSV-G or CCHFV
347 Gn/Gc (Figure 1). That both HSPG and NTCP, which are respectively HBV virion membrane capture
348 molecules (Leistner et al., 2008; Schulze et al., 2007) and specific entry factors (Ni et al., 2014; Yan et
349 al., 2015), are not required for cell-cell fusion highlights that this fusion assay reveals late entry events,
350 such as those occurring after virus interaction with either factor. Similarly, for other viruses such as
351 e.g., influenza virus or hepatitis C virus (HCV), binding to their cell entry receptor is not a requirement
352 in both cell-cell fusion (Lin and Cannon, 2002) and liposome fusion (Lavillette et al., 2006) assays
353 triggered by low pH treatment. Thus, while it is clear that cell-cell fusion does not recapitulate *per se*
354 all the events required to promote cell entry of viral particles since it bypasses the step of
355 internalization that subsequently allows membrane fusion in endosomes that are required for entry of
356 the above-mentioned viruses and of HBV (Macovei et al., 2013; Iwamoto et al., 2019), it a suitable
357 experimental tool for investigating some of the structural and functional determinants that promote
358 envelope glycoprotein membrane-fusion activity (Earp et al., 2004). Accordingly, our results indicate
359 that the trigger for the HBV membrane fusion mechanism not only is independent of an allosteric
360 interaction of its GPs with the NTCP receptor but also is independent of GPs protonation that is
361 induced by the low pH environment of endosomes. That low pH does not increase HBV cell-cell fusion
362 agrees with previous results indicating that pharmacological agents that raise or neutralize the pH of
363 the endocytic pathway had no effect on HBV infection (Macovei et al., 2010, 2013; Rigg and Schaller,
364 1992).

365 Previous results from the group of Camille Sureau showed that cysteine residues of the HBV antigenic
366 loop are essential for HDV infectivity and that viral entry is blocked by inhibitors of thiol/disulfide
367 exchange reaction (Abou-Jaoudé and Sureau, 2007). Our results extend this notion as they indicate
368 that such reactions seem to be necessary to mediate a critical early post-binding event but not at a

369 later stage of the infection process since no effect in virus infectivity could be detected when DTNB
370 was added at 4h post-infection (Figure 3). Since isomerization of disulfide bonds has been shown to
371 be crucial for conformational rearrangements of GPs from other enveloped viruses leading to fusion
372 (Fenouillet et al., 2007), we sought to investigate if and how such reactions could be implicated during
373 the membrane fusion step of HBV entry. Here, using our cell-cell fusion assay, we found that DTNB
374 blocked HBV GP-mediated membrane fusion (Figure 3B). Altogether, these results indicated a role of
375 disulfide bond network of S GP during HBV membrane fusion.

376

377 Capitalizing on the above-mentioned experimental information that inhibitors of thiol/disulfide
378 exchange reactions alter virus entry, we sought to examine how disulfide bonds of the HBV GPs, or
379 rather, how a potential reshuffling of its disulfide bonds profile, could be important for HBV entry.
380 Indeed, cross-strand disulfides occurring in some viral surface GPs are believed to play a role in virus
381 entry (Barbouche et al., 2003; Jain et al., 2007; Rosenthal et al., 1998; Wouters et al., 2004).
382 Particularly, allosteric disulfide bonds can modulate the activity of the proteins in which they reside by
383 mediating a structural change when they are reduced or oxidized (Hogg, 2003; Schmidt et al., 2006).
384 Allosteric control of protein function is defined as a change in one site (the allosteric site) that
385 influences another site by exploiting the protein's flexibility; an allosteric disulfide bond represents the
386 "allosteric site" and the conformational change triggered by cleavage of such bonds alters protein
387 function. For the HBV S protein, we used the contact prediction method RaptorX (Ma et al., 2015,
388 Wang et al., 2017) to predict contacts between four Cys-rich regions of the AGL determinant (Figure
389 4), which highlighted that two of these regions may likely interact: *i.e.*, the Cys-rich regions III and IV
390 (Figure 4-figure supplement 1). Using the secondary structure prediction method JPred (Cole et al.,
391 2008), we proposed that these regions organize in two β -strands and we constructed a three-
392 dimensional model of the region 294-317 of the HBV S GP, which indicated that this sequence is
393 compatible with a β -hairpin structural motif containing a CSD bond between C301 and C310 (Figure
394 4). Interestingly, the analysis of the signs of the five χ dihedral angles defined by the Cys residues
395 allowed to classify this particular disulfide bond in a -HStaple conformation, which is a particular type
396 of disulfide geometry associated with allosteric functions that is known to trigger conformational
397 changes upon switching between the reduced and oxidized states (Chiu and Hogg, 2019; Schmidt et
398 al., 2006). Hence, we hypothesized that the redox state of the C301-C310 disulfide bond may act as
399 an allosteric switch controlling conformational rearrangements of the HBV GP leading, ultimately, to
400 exposure of the fusion peptide. Of note, the β -hairpin region with the predicted CSD lies at the surface
401 of the S protein according to a three-dimensional *in silico* model (van Hemert et al., 2008), which may
402 allow interactions with other HBs subunits and/or cellular factors. Yet, to test our structural and
403 dynamic model involving a C301-C310 CSD bond in S GP (Figure 4), we reasoned that creating an
404 additional, neighboring disulfide bond between positions 303 and 308 may stabilize the β -hairpin motif

405 (Figure 4), which may prevent molecular rearrangements and thus, membrane fusion to occur. The *in*
406 *silico* analysis indicates that the T303C/G308C double mutant most probably generates two structural
407 CSD according to our JPred-based (Cole et al., 2008) structural modelling, which affects the structural
408 conformation of the C301-C310 CSD that is no longer classified as an allosteric bond. When we tested
409 the T303C/G308C mutation in functional assays, we found that the mutant HBV GPs induced an
410 almost complete loss of infection and fusion activity (Figure 5), hence suggesting that by stabilizing
411 cross-strand disulfide exchange, the putative additional disulfide bond prevented conformational
412 rearrangements of HBV GPs that are required for promoting membrane fusion. One possibility is that
413 such stabilization could prevent an isomerization of the C301-C310 CSD bond that generates
414 alternative disulfide bond(s) such as, for example, between C284 and C310 that was proposed in a
415 previous study (Mangold et al., 1995). Yet, the antigenic loss of S induced by these mutations did not
416 allow us to design an assay that would detect the additional disulfide bond in the T303C-G308C
417 mutant nor the block of conformational rearrangements that is suggested by its phenotype.

418

419 Assuming that the isomerization of the C301-C310 allosteric CSD or of other thiols/disulfides of the
420 AGL determinant could facilitate the conformational rearrangements of HBs required to promote HBV
421 membrane fusion, we hypothesized that such isomerization could be induced by a host factor from the
422 PDI family, which are enzymes that can both reduce and oxidize disulfide bonds. Protein disulfide
423 isomerases consist of a family of 21 structurally related proteins with a thioredoxin-like domain. Most
424 of these isomerases have a CXXC motif that catalyzes formation, reduction and rearrangement of the
425 disulfide bonds in proteins (Abell and Brown, 1993). These isomerases are primarily involved in the
426 folding of proteins in the ER, catalyzing formation of their disulfide bonds, and most of these
427 isomerases have ER retention signals. However, some isomerases from the PDI family have also
428 been shown to be present at the cell surface, both in functional and in biochemical assays (Turano et
429 al., 2002). Accordingly, cell surface-localized PDIs are involved in processes such as cell adhesion,
430 nitric oxide signaling, and in cell entry of different viruses (Diwaker et al., 2013; Fenouillet et al., 2007).
431 In support of the notion that PDIs are involved in HBV entry, we found that inhibitors that target
432 different PDI members could block infection and cell-cell fusion though not the binding of viral particles
433 to the cell surface. Of note, we found that bacitracin, which targets PDIA1, did not inhibit HBV entry
434 and membrane fusion, in line with a previous study showing that it could not inhibit HDV entry (Abou-
435 Jaoudé and Sureau, 2007). While the above ruled out PDIA1 as an entry co-factor of HBV, we found a
436 strong reduction of the levels of HBV and HDV infection as well as of HBV-induced cell-cell fusion
437 when we used the NTZ and EGCG inhibitors (Figure 6), which target ERp57 (Müller et al., 2008;
438 Pacello et al., 2016). Consistently, we detected a low but significant expression of ERp57 as well as of
439 ERp46 and ERp72 at the cell surface (Figure 7), in agreement with a previous study (Turano et al.,
440 2002). Furthermore, we detected ERp57 in late endosomes (Figure 8), which is meaningful since

441 previous reports showed that HBV infection of HepaRG cells depends on Rab5 and Rab7 (Macovei et
442 al., 2013), which are GTPases involved in the biogenesis of endosomes, and that the epidermal
443 growth factor receptor is a host-entry cofactor that interacts with NTCP and mediates HBV
444 internalization (Iwamoto et al., 2019). Using a gene silencing approach, we confirmed that down-
445 regulation of ERp57 but not of these alternative PDIs could decrease the levels of HDV and HBV
446 infection as well as of cell-cell fusion (Figure 7). Importantly, we showed that a short time treatment of
447 liver humanized mice with NTZ could delay HBV infection by ca. 2-4 weeks (Figure 9). Since NTZ has
448 a short half-life of about 1.5h *in vivo* (Ruiz-Olmedo et al., 2017; Stockis et al., 1996) and since NTZ
449 was administrated at very short times before and after HBV inoculation, we calculated that less than
450 10% of the drug was still present in those mice at 7h post-infection, which likely precludes an effect on
451 HBV post-entry steps (Korba et al., 2008). Altogether, these results support the role of ERp57 at early
452 steps of HBV infection and validate this PDI as a therapeutic target. Note that our results did not
453 discard the possibility that some other PDIs could also play a role during HBV entry into cells.

454

455 The fusion-mediating GPs of enveloped viruses contain a sequence, termed fusion peptide that
456 interacts with and destabilizes the cellular target membrane. Such an event is finely controlled so as to
457 occur at the appropriate time and location and to prevent fortuitous inactivation of GP fusion activity
458 and virus infectivity. Hence, a conformational change in these GPs is a requirement to induce the
459 accessibility and function of the fusion peptide segment. Candidate fusion peptides are generally
460 identified as hydrophobic sequences, of ca. 16 to 26 residues in length, that are conserved within a
461 virus family and that may adopt α -helical conformation with strongly hydrophobic faces. They can be
462 internal or located at the amino-terminus of fusion GP subunits (Apellániz et al., 2014; Epand, 2003;
463 Martin et al., 1999). There are a number of criteria that characterize fusion peptide segments and,
464 while none of these criteria taken individually are absolute to define a fusion peptide segment, they are
465 sufficiently restrictive to predict if a given region of a protein presents features of a fusion peptide
466 segment (Delos and White, 2000; Delos et al., 2000), which needs to be further functionally tested.

467 Previously, a conserved peptide comprising 23 amino acids at the N-terminal end of the HBV S protein
468 and overlapping its TM1 sequence was shown to interact with model membranes, causing liposome
469 destabilization in a pH-dependent manner (Berting et al., 2000; Rodríguez-Crespo et al., 1994, 1995,
470 1999). However, it was also demonstrated that hydrophobic residues in TM1 were critical for S protein
471 expression as well as for infectivity (Siegler and Bruss, 2013). An essential role of TM1 in fusion
472 mechanism, albeit in a pH-independent manner, could be shown for the duck hepatitis B virus (DHBV)
473 (Chojnacki et al., 2005; Grgacic and Schaller, 2000), although there is also evidence for the
474 involvement of the preS domain of DHBV at an early step of infection, likely during the fusion process
475 (Delgado et al., 2012).

476 Here, through a computational hydropathy analysis of the HBV GPs, we identified two potential short
 477 sequences within the preS1 and preS2 regions that may potentially interact with membrane bilayers.
 478 To validate these predictions, we characterized in both infection and cell-cell fusion assays HBV GP
 479 mutants in key positions in either sequence. We found that while none of the mutations in the preS2
 480 segment altered infection or membrane fusion activities, mutations in the preS1 sequence induced an
 481 almost complete loss of infectivity and cell-cell fusion (Figure 2). Note that these mutants had similar if
 482 not identical levels of cell surface expressed L, M and S proteins and/or capacity to induce the
 483 formation of HDV particles. These results suggested that the preS1 region harbors a fusion peptide in
 484 addition to the NTCP binding determinant.

485
 486 Overall, our study characterizes some crucial determinants of HBV entry and membrane fusion. The
 487 mechanism by which fusion proteins are activated and undergo conformational rearrangements or
 488 fusion intermediates is a particularly complex process involving several regions of viral surface GPs.
 489 Our results suggest that for HBV, this mechanism could be triggered by ERp57, allowing a
 490 thiol/disulfide exchange reaction to occur and regulate isomerization of critical CSD(s), which
 491 ultimately results in the exposition of the fusion peptide that seems to be located within the preS1
 492 region.

493
 494

495 **Material and Methods**

496

497 **Key Resources Table**

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|-------------------------|---|-------------|---|
| strain, strain background (<i>M. musculus</i> , females and males) | NOD-FRG mice | DOI: 10.1038/nbt1326 DOI : 10.1074/jbc.M115.662999 | | Breeding and experimentation in PBES – Originally purchased to YEcureis corporation |
| strain, strain background (HBV) | Hepatitis B virus (HBV) | This paper | | HBV, genotype D, produced by co-transfection of HepG2.2.15 cells with plasmids pCiHB(env-) and pT7HB2.7 |

| | | | | |
|-----------------------------------|--|-------------------------|--|---|
| strain, strain background (HDV) | Hepatitis D virus (HDV) | This paper | | HDV, genotype 1, produced by co-transfection of Huh7 cells with plasmids pSVLD3 and pT7HB2.7, or variant constructs |
| cell line (<i>Homo-sapiens</i>) | Huh7 - hepatocarcinoma cells | PMID: 6286115 | | |
| cell line (<i>Homo-sapiens</i>) | Huh7-NTCP | This paper | | Generated by transduction with pLX304NTCP retroviral vector and selection with blasticidin |
| cell line (<i>Homo-sapiens</i>) | Huh7-Tat (H-tat) cells | This paper | | Generated by transduction with LXSNTat retroviral vector and selection with G418 |
| cell line (<i>Homo-sapiens</i>) | H-tat cells down regulated for ERp46, ERp57 or ERp72 | This paper | | Generated by transduction of H-tat cells with shRNA lentiviral vectors against ERp46, ERp57 or ERp72 followed by selection with puromycin |
| cell line (<i>Homo-sapiens</i>) | Huh7-NTCP-Tat (N-tat) cells | This paper | | Generated by transduction of Huh7-NTCP cells with LXSNTat retroviral vector |
| cell line (<i>Homo-sapiens</i>) | N-tat cells down regulated for ERp46, ERp57 or ERp72 | This paper | | Generated by transduction of N-tat cells with shRNA lentiviral vectors against ERp46, ERp57 or ERp72 followed by selection with puromycin |
| cell line (<i>Homo-sapiens</i>) | HepG2.2.15 human hepatoma cells | From David Durantel lab | | Production of HBV particles |

| | | | | |
|---|------------------------------------|---------------------------------------|----------|--|
| cell line (<i>Homo-sapiens</i>) | 293T human kidney cells | ATCC | CRL-1573 | Production of retro- and lentiviral particles |
| cell line (<i>Cricetulus griseus</i> , female) | CHO-K1 Chinese hamster ovary cells | ATCC | CCL-61 | Cell-cell fusion assays |
| transfected construct (human) | pLX304NTCP | DNASU plasmid repository | HQ447437 | retroviral construct to transfect and express NTCP |
| transfected construct (HBV) | pSVLD3 | DOI: 10.1128/JVI.63.5.1945-1950.1989 | | Harbors a trimer of the HDV, genotype 1 genome. Used for production of HDV particles |
| transfected construct (HBV) | pT7HB2.7 | DOI: 10.1128/JVI.68.6.4063-4066.1994 | | Gift from Camille Sureau, used for production of HBV and HDV particles and expression of HBV envelope proteins |
| transfected construct (HBV) | pT7HB2.7Mles s (noM) | This paper | | Generated for expression of HBV L and S proteins (M protein is silenced) |
| transfected construct (HBV) | pCiL | doi: 10.1128/JVI.77.9.5519-5523.2003 | | Encodes only the L-HBsAg protein |
| transfected construct (HBV) | pCiS | doi: 10.1128/JVI.80.10.4648-4655.2006 | | Encodes only the S-HBsAg protein |
| transfected construct (CCHFV) | pCAGGS_GP/wt-M | DOI: 10.1128/JVI.03691-14 | | Major open reading frame of CCHFV M-segment subcloned into pCAGGS |
| transfected construct (HBV) | pCIHB(env-) | DOI: 10.1128/JVI.00621-06 | | Gift from Camille Sureau, used for production of HBV particles |
| transfected construct (HIV1-Tat) | LXSN-tat retroviral vector | DOI: 10.1128/JVI.73.3.1956-1963.1999 | | HIV-1 <i>tat</i> gene cloned into the LXSN retroviral vector |

| | | | | |
|----------------------------------|-------------------------------------|------------------------------------|--------------------------------------|---|
| transfected construct (HIV1-LTR) | pLTR-luc | DOI: 10.1016/0378-1119(90)90032-m | | gift from Olivier Schwartz, contains a 722-base pair <i>Xho</i> I (-644)- <i>Hind</i> III (+78) fragment from HIV-1 placed in front of the luciferase reporter gene |
| transfected construct (VSV) | phCMV-VSV-G | DOI: 10.1016/s0091-679x(08)60600-7 | | To express the envelope protein of VSV |
| transfected construct (human) | shRNA against ERp46 (ERp46-shRNA 1) | Sigma | NM_022085 / TRCN00000 64353 / PLKO.1 | Lentiviral construct to transfect and express the shRNA. |
| transfected construct (human) | shRNA against ERp46 (ERp46-shRNA 2) | Sigma | NM_022085 / TRCN00000 64354 / PLKO.1 | Lentiviral construct to transfect and express the shRNA. |
| transfected construct (human) | shRNA against ERp57 (ERp57-shRNA 3) | Sigma | NM_005313 / TRCN00003 19038 / PLKO | Lentiviral construct to transfect and express the shRNA |
| transfected construct (human) | shRNA against ERp57 (ERp57-shRNA 4) | Sigma | NM_005313 / TRCN00001 47738 / PLKO.1 | Lentiviral construct to transfect and express the shRNA |
| transfected construct (human) | shRNA against ERp72 (ERp72-shRNA 3) | Sigma | NM_004911 / TRCN00002 89676 / PLKO.1 | Lentiviral construct to transfect and express the shRNA |
| transfected construct (human) | shRNA against ERp72 (ERp72-shRNA 4) | Sigma | NM_004911 / TRCN00000 49334 / PLKO.1 | Lentiviral construct to transfect and express the shRNA |

| | | | | |
|--|---|--|--------------------------------------|---|
| transfected construct (human) | shRNA against ERp72 (ERp72-shRNA 5) | Sigma | NM_004911 / TRCN00003 07107 / PLKO.1 | Lentiviral construct to transfect and express the shRNA |
| biological sample (<i>M. musculus</i>) | Blood samples | PBES (Plateau de Biologie Experimentale de la Souris) SFR Biosciences Lyon | | isolated from NOD-FRG mice |
| antibody | anti-HBsAg antibody, HPR conjugated (goat polyclonal) | DiaSorin | 9F80-01 | WB(1:400) |
| antibody | anti-human calnexin (rabbit polyclonal) | Enzo | ADI-SPA-865-F | WB(1:1000) |
| antibody | anti-mouse TXNDC5/ERp46 (rabbit polyclonal) | Abcam | Ab10292 | FACS(1:20) WB(1:1000) |
| antibody | anti-human ERp57 (mouse monoclonal) | Abcam | Ab13506 | FACS(2 μ g/10 ⁶ cells) WB(1:10000) IF(1:100) |
| antibody | anti-human ERp72 (rabbit polyclonal) | Abcam | Ab155800 | FACS(1:100) WB(1:1000) |
| antibody | anti-human NTCP/SLC10A1 antibody, PE conjugated (rabbit polyclonal) | Bioss Antibodies | bs-1958R-PE | FACS(1:100) |
| antibody | anti-human Rab5 (rabbit monoclonal) | Cell Signaling Technology | (C8B1):3547 | IF(1:200) |

| | | | | |
|------------------------|--------------------------------------|---------------------------|-------------------------------|-------------------------------------|
| antibody | anti-human Rab7 (rabbit monoclonal) | Cell Signaling Technology | (D95F2):9367 | IF(1:100) |
| antibody | anti-human Rab11 (rabbit monoclonal) | Cell Signaling Technology | (D4F5):5589 | IF(1:50) |
| antibody | anti-human Lamp1 (rabbit monoclonal) | Cell Signaling Technology | (D2D11):9091 | IF(1:200) |
| sequence-based reagent | F52A | This paper | preS1 mutagenesis PCR primers | GTAGGAGCTGGAGCAG CCGGGCTGGGTTTAC |
| sequence-based reagent | F52E | This paper | preS1 mutagenesis PCR primers | GTAGGAGCTGGAGCAGA AGGGCTGGGTTTCA |
| sequence-based reagent | G53A | This paper | preS1 mutagenesis PCR primers | CTGGAGCATTGCGCT GGGTTTAC |
| sequence-based reagent | F56A | This paper | preS1 mutagenesis PCR primers | TTCGGGCTGGGTGCC ACCCACCGCA |
| sequence-based reagent | W66A | This paper | preS1 mutagenesis PCR primers | GAGGCCTTTTGGGGGCG AGCCCTCAGGCTC |
| sequence-based reagent | W66E | This paper | preS1 mutagenesis PCR primers | GAGGCCTTTTGGGGGAG AGCCCTCAGGCTC |
| sequence-based reagent | Y129A | This paper | preS2 mutagenesis primers | GAGTGAGAGGCCTGGCTT TCCCTGCTGGTG |

| | | | | |
|-------------------------|---|-------------|---------------------------|--|
| sequence-based reagent | F130A | This paper | preS2 mutagenesis primers | GAGAGGCCTGTATG CCCC TGCTGGTGG |
| sequence-based reagent | S136E | This paper | preS2 mutagenesis primers | CCCTGCTGGTGGCT CCGAA TCAGGAACAGTAA C |
| sequence-based reagent | L144A | This paper | preS2 mutagenesis primers | CAGTAAACCCTGTT GCGACT ACTGCCTCTCC |
| sequence-based reagent | T303C | This paper | CSD mutagenesis primers | CCTCCTGTTGCTGT TGCAA CCTTCGGACG |
| sequence-based reagent | G308C | This paper | CSD mutagenesis primers | GTACCAAACCTTCG GACTGT AATTGCACCTGTATT CCC |
| sequence-based reagent | TG/CC | This paper | CSD mutagenesis primers | GTTGCAAACCTTCG GACTGT AATTGCACCTGTATT CCC |
| commercial assay or kit | FuGENE HD Transfection Reagent | Promega | E2312 | Transfection reagent |
| commercial assay or kit | Dual-Luciferase® Reporter Assay System | Promega | E1910 | Quantification of luciferase activity |
| commercial assay or kit | iScript cDNA synthesis kit | Bio-Rad | 1708891 | cDNA synthesis |
| commercial assay or kit | FastStart Universal SYBR Green Master | Roche Sigma | 4913850001 | Real time qPCR assays |
| commercial assay or kit | CytoTox-ONE™ Homogen Membrn Integrity Assay | Promega | G7891 | Cytotoxicity assay |

| | | | | |
|-------------------------|--|---|-----------------|-------|
| chemical compound, drug | Bacitracin | Sigma | B0125-250KU | water |
| chemical compound, drug | NTZ (Nitazoxanide) | Sigma | N0290-50MG | DMSO |
| chemical compound, drug | EGCG (-)-Epigallocatechin gallate | Sigma | E4268-100MG | water |
| chemical compound, drug | RUTIN HYDRATE | Sigma | R5143-50G | DMSO |
| chemical compound, drug | PX-12 | Sigma | M5324-5MG | DMSO |
| chemical compound, drug | DTNB 5,5'-Dithiobis(2-nitrobenzoic acid) | Sigma | D218200-1G | DMSO |
| chemical compound, drug | EZ-Link Sulfo-NHS-LC-LC-Biotin | Life technologies | 21338 | |
| software, algorithm | ImaJ software | ImaJ | RRID:SCR_003070 | |
| software, algorithm | Membrane Protein eXplorer | http://blanco.biomol.uci.edu/mpex/ | RRID:SCR_014077 | |
| software, algorithm | RaptorX | http://raptorx.uchicago.edu/ | RRID:SCR_018118 | |
| software, algorithm | Jpred | http://www.compbio.dundee.ac.uk/jpred/ | RRID:SCR_016504 | |

| | | | | |
|---------------------|-----------------------------|---|-----------------|----------------|
| software, algorithm | MODELLER | http://salilab.org/modeller/modeller.html | RRID:SCR_008395 | |
| software, algorithm | Clustal X | http://www.clustal.org/clustal2/ | RRID:SCR_017055 | |
| software, algorithm | Molecular Modelling Toolkit | http://dirac.cnrs-orleans.fr/MMTK.html | | |
| software, algorithm | GROMACS | http://www.gromacs.org | RRID:SCR_014565 | |
| software, algorithm | UCSF Chimera | http://plato.cgl.ucsf.edu/chimera/ | RRID:SCR_004097 | |
| other | Hoechst 33342 stain | Thermo Fisher | H3570 | 10 µg/ml |
| other | STREPTAVIDIN AGAROSE RESIN | Thermo fisher | 20353 | |
| other | TRI-Reagent | Molecular Research Center Euromedex | TR118-200 | RNA extraction |

498

499 **Plasmids.** Plasmid pSVLD3 harboring a trimer of the HDV gt1 genome (accession number
500 M21012.1), pCiL encoding the L protein, pCiS encoding the S protein (Komla-Soukha and Sureau,
501 2006) and pT7HB2.7 encoding the three HBV envelope proteins were a gift from Camille Sureau
502 (Sureau, 2010; Sureau et al., 1994). To induce the expression of L and S only, the pT7HB2.7 plasmid
503 was modified at the M start codon and Kozak consensus sequence in order to silence the expression
504 of M protein, resulting in pT7HB2.7Mless construct. The pCiM plasmid encoding the M protein was
505 constructed by deleting the preS1 region from pCiL until the N-terminal methionine of preS2. All
506 mutations in pT7HB2.7 plasmid were introduced by point directed mutagenesis. The pHCMV-VSV-G
507 encoding the G protein from vesicular stomatitis virus (VSV) and pCAGGS-GP/wt-M encoding the Gn
508 and Gc glycoproteins from Crimean-Congo hemorrhagic fever virus (CCHFV) were described
509 previously (Freitas et al., 2020). The plasmid encoding the luciferase reporter under control of an HIV-
510 1 long terminal repeat internal promoter (pLTR-luc) was a gift from Olivier Schwartz and was used as

511 described before (Lavillette et al., 2007). Mission shRNAs plasmids (Sigma), shRNAs sequences and
512 oligonucleotides used for introducing mutations in HBV GPs are described in Supplementary file 1.

513

514 **Cells.** Huh7 human hepatocarcinoma cells and Huh7-NTCP cells, which were generated by
515 transduction of Huh7 cells with a retroviral vector transducing the NTCP plasmid (pLX304NTCP,
516 DNASU) and selected for blasticidin resistance, were grown in William's E medium (WME) (Gibco,
517 France) supplemented with non-essential amino acids, 2 mM L-Glutamine, 10 mM HEPES buffer, 100
518 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum. 293T human kidney cells
519 (ATCC CRL-1573), CHO-K1 (CHO) Chinese hamster ovary cells (ATCC CCL-61) and CHO-pgsB-618
520 cells (ATCC CRL-2241), which do not produce glycosaminoglycans, were grown in Dulbecco's
521 modified minimal essential medium (DMEM, Gibco) supplemented with 100 U/mL of penicillin, 100
522 µg/mL of streptomycin, and 10% fetal calf serum. Huh7-Tat and Huh7-NTCP-Tat indicator cells
523 expressing HIV Tat were generated by transduction of Huh7 and Huh7-NTCP cells, respectively, with
524 the LXSNT-tat retroviral vector and selected for G418 resistance. HepG2.2.15 human hepatoma cells
525 were used to produce HBV virus, there were maintained in WME medium complemented with 10%
526 fetal bovine serum. Authentication of purchased cell lines was performed by ATCC. Authentication of
527 Huh7 cells was based on expression of human transferrin and serum albumin. Authentication of
528 HepG2.2.15 cells was based on titration of released infectious HBV particles. All cell lines were
529 certified mycoplasma-free, as per our monthly contamination testing.

530

531 **PDI inhibitors.** 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB), Nitazoxanide (NTZ), (-)-Epigallocatechin 3-
532 gallate (EGCG), Rutin, Bacitracin and PX-12 were purchased from Sigma-Aldrich and dissolved in
533 DMSO, ethanol or water according to the manufacturer's instructions.

534

535 **Antibodies.** For western-blot analysis, HBs antigen and calnexin were detected with goat anti-HBV
536 polyclonal antibody (murex, DiaSorin) coupled to horseradish peroxidase (HRP) and rabbit calnexin
537 polyclonal antibody (Enzo), respectively. The rabbit anti-ERp46 (Abcam), mouse anti-ERp57 (Abcam),
538 and mouse anti-ERp72 (Santa Cruz Biotechnology) antibodies were used for detecting protein
539 disulfide isomerase proteins by flow cytometry and western blot. NTCP was detected with polyclonal
540 NTCP/SLC10A1 antibody (Bioss Antibodies) coupled to PE for flow cytometry. Mouse anti-ERp57
541 (Abcam), Rabbit anti-Rab5, anti-Rab7, anti-Rab11 and anti-Lamp1 (Cell Signaling Technology), and
542 Donkey anti-Rabbit-Alexa-Fluor-488 and Donkey anti-Mouse-Alexa-Fluor-568 (Thermo Fisher)
543 antibodies were used for immuno-fluorescence studies.

544

545 **shRNA-expressing stable cell lines.** 293T cells were seeded 24h prior to transfection with VSV-G
546 plasmid, psPAX2 packaging plasmid, and pLKO.1 expression vector carrying shRNA against ERp46,
547 ERp57, or ERp72 using calcium phosphate precipitation. Medium was replaced 16h post-transfection.
548 Vector supernatants were harvested 24h later, filtered through a 0.45 µm filter. Stable knockdown of
549 ERp72, ERp57 or ERp46 in Huh7-NTCP, Huh7-tat, and Huh7-NTCP-tat cells was performed by
550 selection with puromycin after lentiviral transduction. The knockdown was validated by flow cytometry
551 and western blot using antibodies against ERp46, ERp57 or ERp72.

552

553 **Cell-cell fusion assays.** Huh7 “donor” cells (2.5×10^5 cells/well seeded in six-well tissue culture dishes
554 24 h prior to transfection) were co-transfected using FuGENE 6 transfection reagent (Promega) with 3
555 µg of pT7HB2.7- wt or mutated glycoproteins and 50 ng of pLTR-luc reporter plasmid. For a positive
556 control, cells were co-transfected with 3 µg of either pCAGGS-GP/wt-M, expressing CCHFV GPs, or 1
557 µg of pHCMV-VSV-G and with 50 ng of the pLTR-luc plasmid. For negative controls, cells were co-
558 transfected with 2 µg of an empty pHCMV plasmid and 50 ng of the pLTR-luc plasmid. Twelve hours
559 later, transfected cells were detached with Versene (0.53 mM EDTA; Gibco), counted, and reseeded
560 at the same concentration (10^5 cells/well) in twelve-well plates. Huh7-Tat or Huh7-NTCP-Tat indicator
561 cells, detached with EDTA and washed, were then added to the transfected cells (3×10^5 cells per
562 well). After 24h of cocultivation, the cells were washed with PBS, incubated for 3 min in fusion buffer
563 (130 mM NaCl, 15 mM sodium citrate, 10 mM MES [2-(N-morpholino)ethanesulfonic acid], 5 mM
564 HEPES) at pH4, pH5 or pH7, and then washed three times with normal medium. The luciferase
565 activity was measured 24h later using a luciferase assay kit according to the manufacturer’s
566 instructions (Promega).

567

568 **HDV particles production and infection.** Huh7 cells were seeded in 10 cm plates at a density of 10^6
569 cells per plate and were transfected with a mixture of 2.5 µg of pSVLD3 plasmid and 10 µg of plasmid
570 allowing the expression of surface envelope glycoproteins of VSV or HBV using FuGENE 6
571 transfection reagent (Promega), as described previously (Perez-Vargas et al., 2019). Transfected cells
572 were grown for up to 9 days in primary hepatocyte maintenance medium containing 2% DMSO to slow
573 cell growth.

574 The supernatants of virus producer cells were filtrated through 0.45 nm-pore filters and were analyzed
575 by RTqPCR for detection of HDV RNA, using the primers described below. These supernatants were
576 also used for infection experiments in Huh7-NTCP cells or PDI-down-regulated Huh7-NTCP cells,
577 which were seeded in 48-well plates at a density of 1.5×10^4 cells per well. Infected cells were cultured
578 in primary hepatocyte maintenance medium containing 2% DMSO following infection. RTqPCR
579 assays were used to assess infectivity of viral particles at 7 days post-infection.

580 For inhibition assays, drugs were incubated with cells for 2h at 37°C before virus addition or at
581 different times post-infection and the infectivity was assessed 7 days post-infection by RT-qPCR.

582

583 **Binding assays.** HDV wt particles (10^7 GE) were added to Huh7-NTCP cells and incubated for 1h at
584 4°C. Unbound virions were removed by three washes with cold PBS, and RTqPCR was used to
585 assess the amount of bound viral particles.

586

587 **RTqPCR detection of HDV RNAs in virus-producer and in infected cells.** Cells were washed with
588 phosphate-buffer saline (PBS) and total RNA was extracted with TRI Reagent according to the
589 manufacturer's instructions (Molecular Research Center). RNAs were reverse transcribed using
590 random oligonucleotides primers with iScript (Bio-Rad). The following specific primers were used: for
591 HDV RNA quantification, forward primer 5'-GGACCCCTTCAGCGAACA and reverse primer 5'-
592 CCTAGCATCTCCTCTATCGCTAT. qPCR was performed using FastStart Universal SYBR Green
593 Master (Roche) on a StepOne Real-Time PCR System (Applied Biosystems). As an internal control of
594 extraction, *in vitro*-transcribed exogenous RNAs from the linearized Triplescript plasmid pTRI-Xef
595 (Invitrogen) were added to the samples prior to RNA extraction and quantified with specific primers (5'-
596 CGACGTTGTCACCGGGCACG and 5'-ACCAGGCATGGTGGTTACCTTTGC). All values of
597 intracellular HDV RNAs were normalized to GAPDH gene transcription. For GAPDH mRNAs
598 quantification, we used the forward 5'-AGGTGAAGGTCCGAGTCAACG and reverse 5'-
599 TGGAAGATGGTGGTGGATTTC primers.

600

601 **Western-blot analyses.** The proteins from pelleted cell supernatants or extracted from total cell
602 lysates were denatured in Lammeli buffer at 95°C for 5 min and were separated by sodium dodecyl
603 sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (GE
604 Healthcare). Membranes were blocked with 5% nonfat dried milk in PBS and incubated at 4°C with a
605 rabbit or mouse antibody diluted in PBS-0.01% milk, followed by incubation with a IRdye secondary
606 antibody (Li-Cor Biosciences). Membranes visualization was performed using an Odyssey infrared
607 imaging system CLx (LI-COR Biosciences).

608 For cell surface biotinylation, Huh7 cells were transfected into 10 cm plates with plasmid encoding wt
609 or mutant HBV GPs. After 48h, the cell monolayers were rinsed three times with ice-cold PBS and
610 overlaid with 0.5 ml biotin solution (0.5 mg sulpho-N-hydroxysuccinimide-biotin (Pierce) per ml of
611 PBS, pH 7.2). The cells were then labeled for 30 min at 4°C. The biotin solution was removed and the
612 cells were rinsed once with ice-cold 100 mM glycine solution and then incubated for 15 min with 100
613 mM glycine at 4 °C to stop the reaction. The last washing step was performed with ice-cold PBS.
614 Proteins were solubilized by the addition of 1 ml RIPA buffer and equivalent quantities of protein
615 lysates from each sample (Nanodrop quantification, Thermofisher) were immunoprecipitated with

616 Biotin-agarose beads. Proteins were electrophoresed under reducing conditions in SDS-PAGE
617 followed by electrophoretic transfer to nitrocellulose. Surface-biotinylated proteins were detected with
618 anti-HBV antibody (murex) coupled to horseradish peroxidase (HRP) and enhanced
619 chemiluminescence (ECL; Roche). The membranes of biotinylated samples were routinely re-probed
620 with anti-calnexin antibody to confirm the absence of the intracellular protein calnexin. In addition, 10%
621 of each lysate was denatured and loaded onto separate gels. Immunoblotting for calnexin on the
622 membranes of lysate was done to confirm uniform protein loading.

623 Densitometry analysis (Image Lab BioRad software) was used to estimate the relative total amount of
624 L, M and S mutant proteins, which were expressed relative of the wild type L, M and S total proteins.

625

626 **Flow cytometry.** The surface expression of NTCP, ERp46, ERp57 and ERp72 was quantified by
627 FACS analysis from 10^6 live cells using antibodies added to cells for 1h at 4°C. After washing, the
628 binding of antibody to the cell surface was detected using PE (Phycoerythrin)-conjugated anti-mouse
629 antibodies.

630

631 **Immuno-fluorescence (IF), confocal microscopy imaging and image analysis.** Huh7-NTCP cells
632 were grown on uncoated 14mm-diameter glass coverslips. Forty-eight hours after seeding, cells were
633 washed with PBS, fixed with 3% paraformaldehyde in PBS for 15 min, quenched with 50mM NH₄Cl
634 and permeabilized with 0.1% Triton X-100 for 7min. Fixed cells were then incubated for 1h with
635 primary antibodies in 1% BSA/PBS, washed and stained for 1 hr with the corresponding fluorescent
636 Alexa Fluor conjugated secondary antibody (Alexa-Fluor-488 and Alexa-Fluor-568, Thermo Fisher) in
637 1% BSA/PBS. Cells were washed three times with PBS, stained for nuclei with Hoechst 33342
638 (Thermo Fisher) for 5 min, washed two times with PBS and mounted in Mowiol 40-88 (Sigma-Aldrich)
639 prior to image acquisition with LSM-710 confocal microscope (Zeiss). Single section confocal images
640 of 0.6µm of thickness were analyzed with the ImageJ software. The Pearson's correlation coefficients
641 were calculated by using the JACoP plugin for ImageJ.

642

643 **Cytotoxicity assay.** The release of LDH from damaged cells was measured with CytoTox-ONE
644 (Promega, MA, USA) homogeneous membrane integrity assay. Cells were grown in a 96-well flat-
645 bottom culture plate in density of 3×10^3 cells per well and treated with the different drugs for 2h or 24h.
646 Maximum LDH release was determined by adding 2µl of CytoTox-ONE lysis solution to control wells
647 for 10min. The assay was performed in 96-well plates by adding 100 µl of the sample supernatant and
648 100 µl of CytoTox-ONE reagent, after which the plate was shaken for 10s. After 10min of incubation,
649 50 µl CytoTox-One stop solution was added and the plate was shaken again for 10s. The fluorescence
650 signal was measured at $\lambda_{EX}=560$ nm, $\lambda_{EM}=590$. LDH-release was calculated as percentage of LDH
651 released in the culture media of total LDH (media and lysates).

652

653 **Fusion peptide prediction.** The HBV surface sequence used was taken from the UniProt database,
654 with accession number P03138. Hydropathy plots were obtained with Membrane Protein eXplorer
655 software (Snider et al., 2009) using as input the reference sequence. Hydropathy plots were also used
656 to evaluate the effect of residue mutations. Sequences with a propensity to partition into the lipid
657 bilayer were identified using interfacial settings and pH=5.0.

658

659 **Contact prediction on the Cys rich region.** Contact prediction was performed using RaptorX (Wang
660 et al., 2017, Teppa et al., 2020). RaptorX integrates evolutionary coupling and sequence conservation
661 information through an ultra-deep neural network formed by two deep residual neural networks.
662 RaptorX predicts pairs of residues, whose mutations have arisen simultaneously during evolution.

663

664 **Structural models and molecular dynamic simulation studies.** The HBV surface protein sequence
665 was taken from the UniProt database, with accession number P03138. Secondary structure prediction
666 was performed with Jpred (Cole et al., 2008). The S protein region 294-317 was modelled using
667 MODELLER (Sali and Blundell, 1993). The template crystal structure of the Newcastle disease virus
668 fusion protein (PDB code: 1G5G) was retrieved from the PDB database (Berman et al., 2000).
669 Sequence alignment was generated with Clustal X (Larkin et al., 2007). The model evaluation was
670 conducted using the Ramachandran plot (Ramachandran et al., 1963). The model of the wild-type
671 sequence was further used to create two structural models with mutations using UCSF Chimera
672 package (Pettersen et al., 2004). One model contains the double mutations T303C/G308C, which may
673 create an extra disulfide bond. The overall effect of those mutations would be to "shift" the disulfide
674 bridge of two amino acids towards the turn of the β -hairpin motif. After mutations, the models were
675 energy minimized by applying Molecular Modelling Toolkit (MMTK) with Amber parameters for
676 standard residues, and 100 steepest descent minimization steps with a step size of 0.02 Å. To
677 investigate the stability of the disulfide bonds, molecular dynamic (MD) simulations of the three models
678 were carried out by GROMACS version 2020 (Abraham et al., 2015) in conjunction with OPLS-AA/L
679 all-atom force field. The models were immersed in the cubic boxes filled with water molecules with a
680 minimal distance of 1.0 nm between the peptide surface and box. Each system was equilibrated to the
681 desired temperature through a stepwise heating protocol in NVT ensemble followed by 100.0 ps
682 equilibration in NPT ensemble with position restraints on the protein molecule. The final productive MD
683 was performed for each system for 10 ns under periodic boundary conditions without any restraints on
684 the protein with a time step of 2 fs at constant pressure (1 atm) and temperature (300 K). Coordinates
685 were saved every 10 ps, yielding 1000 frames per MD trajectory. All the frames were further
686 investigated to differentiate between allosteric and structurally stabilizing disulfides. Disulfide bonds
687 were classified based on the five relevant torsion angles (χ_1 , χ_2 , χ_3 , χ_2' and χ_1') (see Figure 4-figure

688 supplement 2), disulfides were treated as symmetrical. In this system, twenty conformational
689 categories are possible (Marques et al., 2010; Schmidt and Hogg, 2007; Schmidt et al., 2006). The
690 three central angles (χ_2 , χ_3 and χ_2) define the basic shape: Spiral, Hook and Staple (Eklund et al.,
691 1984). The χ_3 defines the orientationally motif: left-handed (LH) or right-handed (RH) if the sign is
692 negative or positive, respectively (Eklund et al., 1984). The χ_1 and χ_1' determines the sign of the
693 nomenclature (Qi and Grishin, 2005).

694

695 ***In vivo* experiments.** All experiments were performed in accordance with the European Union
696 guidelines for approval of the protocols by the local ethics committee (Authorization Agreement C2EA-
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698 2020060810332115 v4). Primary human hepatocytes (PHH, Corning, BD Gentest) were
699 intrasplenically injected in NFRG mice (Azuma et al., 2007), a triple mutant mouse knocked-out for
700 fumarylacetoacetate hydrolase ($fah^{-/-}$), recombinase activating gene 2 ($rag2^{-/-}$), interleukin 2 receptor
701 gamma chain ($IL2rg^{-/-}$). 48h after adeno-uPA conditioning (Bissig et al., 2010; Calattini et al., 2015).
702 Mice were subjected to NTBC cycling during the liver repopulation process, as described previously
703 (Calattini et al., 2015). Mice with human serum albumin (HSA) levels >15 mg/mL, as determined using
704 a Cobas C501 analyzer (Roche Applied Science), were inoculated with virus preparations by intra-
705 peritoneal injection. Sera were collected at different time points before and after infection. Mice were
706 sacrificed 6 weeks post-infection.

707

708 **Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 5.02 for
709 Windows, GraphPad Software (San Diego California, USA). The Mann-Whitney or Wilcoxon tests
710 were used for statistical comparisons. A p-value of 0.05 or less was considered as significant. When
711 applicable, data are presented as mean \pm standard deviation and results of the statistical analysis are
712 shown as follows: ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

713

714

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716

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728

729

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738

739

740 **Competing interests**

741

742 The authors have declared that no competing interests exist.

743

744

745 **References**

746

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966

967 **Figure legends**

968

969 **Figure 1. HBV GP fusion trigger is independent of acid pH, HSPG and NTCP.** (A) Huh7 “donor”
970 cells transfected with the pT7HB2.7 plasmid allowing expression of HBV GPs (HBV) and a luciferase
971 marker gene driven by the HIV-1 promoter were co-cultured with either Huh7-tat (H-tat) or Huh7-
972 NTCP-tat (N-tat) “indicator” cells that express the HIV Tat protein. After 24h of co-culture, the cells
973 were treated at pH4 (or pH 5 for VSV-G) vs. pH7 for 3 minutes. The luciferase activity induced by
974 fusion between donor and indicator cells was then measured 24h later. A control plasmid that does not
975 allow GP expression (Empty) was used to determine the background of luciferase expression. The
976 CCHFV Gn/Gc (CCHFV) or VSV-G (VSV) GPs were used as positive controls for fusion at low pH.
977 Fusion mediated by HBV GPs with Huh7-tat cells was taken as 100%. The bars represent the means
978 (N=3). Error bars correspond to standard deviation. See the raw data of individual experiments in
979 Figure 1-figure supplement 1. (B) Results of cell-cell fusion assays performed as above-described in
980 the presence of heparin at the indicated concentrations throughout the co-coculture. No cytotoxicity
981 could be detected in these conditions (Figure 1-figure supplement 2). The graphs represent the
982 average of two independent experiments. Fusion mediated by HBV GPs with mock-treated Huh7 cells
983 was taken as 100%. (C) CHO “donor” cells transfected with the pT7HB2.7 plasmid and a luciferase
984 marker gene driven by the HIV-1 promoter were co-cultured with either Huh7-tat (H-tat), CHO-tat
985 (CHO wt), or CHO-pgsB618-tat (pgsB618) “indicator” cells that express the HIV Tat protein. The
986 luciferase activity induced by fusion between donor and indicator cells was then measured 24h later. A
987 control plasmid that does not allow GP expression (Empty) was used to determine the background of
988 luciferase expression. Fusion mediated by HBV GPs with Huh7-tat was taken as 100%. The graphs
989 represent the average of two independent experiments. (D) Huh7 “donor” cells transfected plasmids
990 allowing expression of L, M or S HBV GPs alone, both L and S GPs (noM) or all HBV GPs (Wt) and a
991 luciferase marker gene driven by the HIV-1 promoter were co-cultured with Huh7-tat or Huh7-NTCP-
992 tat “indicator” cells that express HIV Tat protein. Cell co-cultures were then processed as above
993 described to determine cell-cell fusion activity. Fusion mediated by HBV GP at pH7 with Huh7-tat cells
994 was taken as 100%. The bars represent the means (N=3). Error bars correspond to standard
995 deviation. (E) Detection of HBV GPs at the cell surface by biotinylation. Transfected Huh7 cells were
996 biotinylated for 30 min at 4°C and then processed biochemically. Cell lysates were subjected to
997 streptavidin pull-down prior to western blot analysis using anti-HBsAg antibody (Murex). The molecular
998 weight markers (kDa) are shown to the right. Calnexin detection was used as control for cytoplasmic
999 protein marker, showing the integrity of cell membrane, as shown in this representative western blot.
1000 (F) Relative GPs expression at the cell surface as compared to Wt, quantified by adding the L+M+S
1001 signals from western blot analyses. The results are expressed as mean \pm SD (N=3). No statistical

1002 differences could be found using the Mann-Whitney test (p-value >0.05). See also the quantification of
1003 total HBV GP expression in the Figure 1-figure supplement 4.

1004

1005 **Figure 2. Functional analysis of predicted HBV fusion peptides.** (A) Sequence of HBV L protein
1006 showing the amino acid color code and boxes for the localization of the two predicted fusion peptides
1007 in preS1 and in preS2. (B) Sequence of the two predicted fusion peptides showing the positions that
1008 were mutated (bold). (C, D) Huh7 cells were co-transfected with pSVLD3 plasmid coding for HDV
1009 RNPs and plasmids coding for wt or mutant HBV GPs. The FW/AA and FW/EE are double alanine
1010 mutants at position F52 and W66. As control, pSVLD3 was co-transfected with an empty plasmid
1011 (referred to as “noGP”). At day 9 post-transfection, the cell supernatants were harvested, filtered and
1012 the extracellular RNA was extracted and purified before quantifying HDV RNAs by RTqPCR HDV RNA
1013 levels in GE (genome equivalent) are expressed as means \pm SD (N=3) per mL of cell supernatants.
1014 (E, F) HDV particles were used to infect Huh7-NTCP cells, which were grown for 7 days before total
1015 intracellular RNA was purified. The results of HDV RNA quantification by RTqPCR are expressed after
1016 normalization with GAPDH RNAs as means \pm SD (N=3) per mL of cell lysates containing 10^6 cells. (G,
1017 H) Huh7 “donor” cells co-expressing wt or mutant HBV GPs and a luciferase marker gene driven by
1018 the HIV-1 promoter were co-cultured with either Huh7-tat (H-tat) or Huh7-NTCP-tat (N-tat) “indicator”
1019 cells that express HIV Tat protein. After 24h, the cells were treated at pH4 or pH7 for 3 minutes. The
1020 luciferase activity induced by the fusion between the donor and indicator cells was measured 24h
1021 later. Fusion mediated by wt GP at pH7 with Huh7-NTCP-tat cells was taken as 100%. The bars
1022 represent the means (N=5). Error bars correspond to standard deviations. (I, J) Quantification of wt
1023 and mutant GPs at cell surface by western blot analyses (see examples in Figure 2-figure supplement
1024 2). The results show the relative GPs expression of preS1 (I) and preS2 (J) mutants compared to Wt,
1025 as indicated, and are expressed as means \pm SD (N=3). No statistical differences could be found using
1026 the Mann-Whitney test (p-value >0.05).

1027

1028 **Figure 3. DTNB, a thiol-specific oxidizing reagent, inhibits HBV membrane fusion.** (A) DTNB (2
1029 mM) was added to the cell supernatant containing HDV particles at the onset of infection (0h) or at the
1030 indicated times post-infection and was removed 8hr later. VSV- Δ p, *i.e.*, HDV particles generated with
1031 VSV-G GP rather than HBV, were used as control for a virus entry process that is not affected by
1032 DNTB. As negative control, pSVLD3 was co-transfected with an empty plasmid (referred to as
1033 “noGP”). At 7 days post-infection, HDV RNAs were extracted from infected cells and quantified by
1034 RTqPCR. The results are expressed after normalization with GAPDH RNAs as means \pm SD (N=3) per
1035 mL of cell lysates containing 10^6 cells. The results of infection in the absence of DTNB are shown
1036 [DTNB(-)]. (B) Huh7 “donor” cells co-expressing HBV GPs and a luciferase marker gene driven by the
1037 HIV-1 promoter were co-cultured with Huh7-NTCP-tat “indicator” cells that express HIV Tat protein.

1038 Different concentrations of DTNB were added at 0h vs. at 16h after initiating the cell co-culture, as
1039 indicated. No cytotoxicity could be detected in these conditions (Figure 1-figure supplement 2). The
1040 luciferase activity induced by fusion between donor and indicator cells was then measured 24h later.
1041 Fusion mediated by HBV GPs without DTNB was taken as 100%. The graphs represent the average
1042 of four independent experiments. **(C, D)** Huh7 cells transfected with pUC19 (noGP) or the pT7HB2.7
1043 (HBV) plasmids were incubated with DMSO (0) or increasing doses of DTNB (0.5, 1 and 2 mM) for
1044 16h prior to incubation with biotin for 30 min at 4°C. Biotin was omitted from one sample (-) and served
1045 as a negative control for non-specific binding of proteins to streptavidin. Cells were subsequently lysed
1046 and the biotinylated surface proteins were captured by streptavidin agarose. Total **(C)** and biotin-
1047 labelled proteins **(D)** were then analyzed by western blot using anti-HBsAg (Murex) and anti-calnexin
1048 antibodies. Calnexin detection was used as control for cytoplasmic protein marker, showing the
1049 integrity of cell membrane, as shown in these representative western blots. The molecular weight
1050 markers (kDa) are shown to the left.

1051

1052 **Figure 4. Disulfides conformation models.** **(A)** Cysteine-rich regions on the “a” determinant
1053 (residues 261-324) of the HBV S GP. Four sub-regions that are rich in cysteine are coloured: I (blue),
1054 II (green), III (yellow) and IV (red). Jpred secondary structure prediction different from random-coil is
1055 indicated: β -strand (arrow) and α -helix (zigzag line). **(B)** Probability of contacts predicted by RaptorX
1056 between the four cysteine-rich regions. The probabilities higher than 0.7 are highlighted in red (see
1057 also Figure 4-figure supplement 1). **(C)** Predominant disulfide conformations obtained by molecular
1058 dynamics simulation of the modelled 294-317 region of the HBV surface protein. Note that the β -
1059 strand on the wt sequence (left) adopts a loop conformation with an allosteric disulfide conformer
1060 between the C301-C310 bond, which is specifically classified as a -/+RHHook conformation. The
1061 T303C/G308C double mutant (right) may generate an additional disulfide bond, resulting in two
1062 structural disulfides of +/-RHStaple and -/+LHSpiral conformations that form the C301-310 and C303-
1063 C308 bonds, respectively.

1064

1065 **Figure 5. Evidence for a functional role of the CSD in the region 294-317 of the HBV S GP.** **(A)**
1066 Huh7 cells were co-transfected with pSVLD3 plasmid coding for HDV RNPs and plasmids coding for
1067 wt, single or double mutant (TG/CC) HBV GPs. As control, pSVLD3 was co-transfected with an empty
1068 plasmid (referred to as “noGP”). At day 9 post-transfection, the cell supernatants were harvested,
1069 filtered and the extracellular RNA was extracted and purified before quantifying HDV RNAs by
1070 RTqPCR. HDV RNA levels in GE (genome equivalent) are expressed as means \pm SD (N=4) per mL of
1071 cell supernatants. **(B)** HDV particles were used to infect Huh7-NTCP cells, which were grown for 7
1072 days before total intracellular RNA was purified. The results of HDV RNA quantification by RTqPCR
1073 are expressed after normalization with GAPDH RNAs as means \pm SD (N=4) per mL of cell lysates

1074 containing 10^6 cells. **(C)** Detection of GP mutants at the cell surface by biotinylation. Huh7 cells
1075 expressing wt or mutant HBV GPs were biotinylated for 30 min at 4°C and then processed
1076 biochemically. Cell lysates were subjected to streptavidin pull-down prior to western blot analysis
1077 using anti-HBsAg antibody (Murex). The molecular weight markers (kDa) are shown to the left.
1078 Calnexin detection was used as control for cytoplasm protein marker, showing the integrity of cell
1079 membrane, as shown in this representative western blot. The relative quantification of cell surface GP
1080 expression compared to wt were quantified from western blot analyses (means \pm SD; N=3) are shown
1081 below. See the quantification of total HBV GP expression in the Figure 1-figure supplement 4. **(D)**
1082 Huh7 “donor” cells co-expressing wt or mutant HBV GPs and a luciferase marker gene driven by the
1083 HIV-1 promoter were co-cultured with either Huh7-tat (H-tat) or Huh7-NTCP-tat (N-tat) “indicator” cells
1084 that express HIV Tat protein. After 24h, the cells were treated at pH4 or pH7 for 3 minutes. The
1085 luciferase activity induced by the fusion between the donor and indicator cells was measured 24h
1086 later. Fusion mediated by wt GP at pH7 with Huh7-NTCP-tat cells was taken as 100%. The bars
1087 represent the means (N=4). Error bars correspond to standard deviations.
1088

1089 **Figure 6. PDI inhibitors in HBV entry.** **(A)** HDV particles harboring wt or TG/CC mutant
1090 (T330C/G308C) HBV GPs were incubated with Huh7 or Huh7-NTCP cells that were pre-treated for 2h
1091 with the indicated inhibitors that block different PDI proteins or with DMSO, used as vehicle. Binding of
1092 either virus particles to the cells was quantified by RTqPCR and expressed after normalization with
1093 GAPDH RNAs as mean \pm SD (N=3) per mL of cell lysates containing 10^6 cells. **(B)** HDV or **(C)** HBV
1094 particles were used to infect Huh7-NTCP cells that were pre-incubated for 2h with the indicated
1095 inhibitors that block different PDI proteins or with DMSO, used as vehicle. Infected cells were grown
1096 for 7 days before total intracellular RNA or DNA was purified. The results of HDV RNA and HBV DNA
1097 quantification by RTqPCR and qPCR, respectively, are expressed after normalization with GAPDH
1098 RNAs as means \pm SD (N=3) per mL of cell lysates containing 10^6 cells. **(D)** Huh7 “donor” cells co-
1099 expressing HBV GPs and a luciferase marker gene driven by the HIV-1 promoter were co-cultured
1100 with Huh7-NTCP-tat “indicator” cells that express HIV Tat protein. The indicated PDI inhibitors were
1101 added when “donor” and “indicator” cells were mixed for co-cultures and the luciferase activity induced
1102 by cell-cell fusion was measured 24h later. DMSO was used as vehicle. Fusion mediated by HBV GPs
1103 without inhibitor was taken as 100%. The graphs represent the average of four independent
1104 experiments. The PDI inhibitors were used at the following concentrations: NTZ, 30 $\mu\text{g}/\text{mL}$; EGCG, 5
1105 μM ; Rutin, 5 μM ; Bacitracin, 5 mM; PX-12, 30 $\mu\text{g}/\text{mL}$. No cytotoxicity could be detected in these
1106 conditions (Figure 1-figure supplement 2).

1107
1108 **Figure 7. ERp57 down-regulation inhibits in HBV entry.** **(A)** Intracellular (upper panels) and cell-
1109 surface (lower panels) staining of ERp46, ERp57 and ERp72 PDI members. Huh7-NTCP cells were

1110 subjected to flow cytometry analysis, in order to evaluate the expression of the indicated PDIs. Cells
1111 stained with secondary antibody only (No primary) were used to provide the background of flow
1112 cytometry analyses. **(B)** HDV or **(C)** HBV particles were used to infect Huh7-NTCP cells in which the
1113 indicated PDIs were down-regulated by lentiviral vectors carrying shRNA (see Figure 7-figure
1114 supplement 1 and Figure 7-figure supplement 2). Naïve Huh7-NTCP cells were used as controls.
1115 Infected cells were grown for 7 days before total intracellular RNA or DNA was purified. The results of
1116 HDV RNA and HBV DNA quantification by RTqPCR and qPCR, respectively, are expressed after
1117 normalization with GAPDH RNAs as means \pm SD (N=3) per mL of cell lysates containing 10^6 cells. **(D)**
1118 Huh7 “donor” cells co-expressing HBV GPs and a luciferase marker gene driven by the HIV-1
1119 promoter were co-cultured with Huh7-NTCP-tat “indicator” cells that express HIV Tat protein in which
1120 the indicated PDI were down-regulated by lentiviral vectors carrying shRNA. After 24h, the cells were
1121 treated at pH4 or pH7 for 3 minutes. The luciferase activity induced by the fusion between donor and
1122 indicator cells was measured 24h later. Fusion mediated by HBV GPs at pH7 with naïve Huh7-NTCP-
1123 tat cells (Ctrl) was taken as 100%. A control plasmid that does not allow GP expression (Empty) was
1124 used to determine the background of luciferase expression. The bars represent the means (N=3).
1125 Error bars correspond to standard deviations.

1126

1127 **Figure 8. Intracellular localization of ERp57 in Huh7-NTCP cells.** Huh7-NTCP cells were grown on
1128 glass cover slides and fixed 48hrs after seeding. **(A)** Endogenous ERp57 with Rab5, Rab7, Rab11 or
1129 Lamp1 were immune-stained, and the colocalization of ERp57 (red channels) with Rab5, Rab7,
1130 Rab11 or Lamp1 (green channels) was analyzed by confocal microscopy. Scale bars of panels and
1131 zooms from squared area represent 10 μ m and 2 μ m, respectively. **(B)** The degree of colocalization
1132 between ERp57 and the different cell markers was assessed by determining the Pearson’s correlation
1133 coefficients with the JACoP plugin of ImageJ. Results are expressed as the mean of 6 individual cells.
1134 Error bars correspond to standard deviations.

1135

1136 **Figure 9. *In vivo* assessment of ERp57 inhibition.** **(A)** 4-8 weeks old NOD-FRG mice were
1137 engrafted with primary human hepatocytes (PHH). After ca. 2-3 months, the animals displaying HSA
1138 levels >15 mg/mL were randomly split in 4 different groups (N=3 to N=5 animals, see Table in the
1139 inset) that were infected with HBV (10^8 GE/mouse), using the displayed NTZ treatment schedule. **(B)**
1140 At different time points post-infection, blood samples (50 μ l) were collected and the viremia in sera
1141 was monitored by qPCR (GE/mL of serum). The graphs show the results of viremia (means \pm SD) of
1142 HBV. See results of individual mice in Figure 9-figure supplement 1.

1143

1144 **Legends to figure supplements**

1145

1146 **Figure 1-figure supplement 1. HBV GP fusion trigger is independent of acid pH and NTCP.** Huh7
1147 “donor” cells transfected with the pT7HB2.7 plasmid allowing expression of HBV GPs (HBV) and a
1148 luciferase marker gene driven by the HIV-1 promoter were co-cultured with either Huh7-tat (H-tat) or
1149 Huh7-NTCP-tat (N-tat) “indicator” cells that express the HIV Tat protein. After 24h of co-culture, the
1150 cells were treated at pH4 (or pH5 for VSV-G) vs. pH7 for 3 minutes. The luciferase activity induced by
1151 fusion between donor and indicator cells was then measured 24h later. A control plasmid that does not
1152 allow GP expression (Empty) was used to determine the background of luciferase expression. The
1153 CCHFV Gn/Gc (CCHFV) or VSV-G GPs (VSV) were used as positive controls for fusion at low pH.
1154 Results from three independent experiments expressed as ratios of luciferase activities of the different
1155 conditions relative to that of the control conditions.

1156

1157 **Figure 1-figure supplement 2. Results of cell survival after drug treatments.** The indicated drugs
1158 were used as described in Figure 1B (Heparin), Figure 3 (DNTB), and Figure 6 (NTZ, EGCG, Rutin,
1159 Bacitracin and PX-12). Cell supernatants were collected immediately after treatment (Post-treatment)
1160 or after a further incubation at 37°C of the treated cells (Post-incubation). Cell toxicity assessment was
1161 performed with the LDH (CytoTox-ONE Promega) using the indicated positive and negative controls of
1162 the kit. Error bars correspond to standard deviations.

1163

1164 **Figure 1-figure supplement 3. Characterization of “noM” HDV particles.** (A) Huh7 cells were co-
1165 transfected with pSVLD3 plasmid coding for HDV RNPs and with plasmids encoding either the wt HBV
1166 GPs (Wt) or only L and S (noM). As control, pSVLD3 was co-transfected with an empty plasmid
1167 (referred to as “noGP”). At day 9 post-transfection, the cell supernatants were harvested, filtered and
1168 the extracellular RNA was extracted and purified before quantifying HDV RNAs by RTqPCR. HDV
1169 RNA levels in GE (genome equivalent) are expressed as means \pm SD (N=4) per mL of cell
1170 supernatants. (B) HDV or noM particles were used to infect Huh7-NTCP cells, which were grown for 7
1171 days before total intracellular RNA was purified. The results of HDV RNA quantification by RTqPCR,
1172 are expressed after normalization with GAPDH RNAs as means \pm SD (N=4) per mL of cell lysates
1173 containing 10^6 cells.

1174

1175 **Figure 1-figure supplement 4. Total protein expression.** (A) Cell lysates of Huh7 cells expressing
1176 the indicated wt or mutant GPs from Figure 1 (left) and Figure 5 (right) were subjected to western blot
1177 analysis, using anti-HBsAg antibody (Murex). The molecular weight markers (kDa) are shown to the
1178 left. Calnexin detection was used as control for cytoplasmic protein marker, as shown in these
1179 representative western blots. The black dots indicate dimers of S, as described in the literature

1180 (Huovila et al., 1992), which are formed in the pre-Golgi compartment (B) Relative GPs expression
1181 compared to Wt, quantified from western blot using anti-HBsAg antibody. The results are expressed
1182 as mean \pm SD (N=3).

1183

1184 **Figure 2-figure supplement 1. Prediction of fusion peptides within S protein by using Wimley-**
1185 **White Interfacial Hydrophobicity Scale. (A)** The hydropathy profile (black curve) and its smoothed
1186 approximation (green curve). The interface scale measures a residue's free energy of transfer within
1187 an unfolded polypeptide chain, from water to a phosphocholine bilayer. The five predicted regions with
1188 high propensity to interact with the lipid surface of the cell membrane are indicated with horizontal red
1189 bars, the four putative transmembrane regions are indicated with horizontal brown bars. The two
1190 regions indicated with red arrows were considered as putative fusogenic peptides. The preS1, preS2
1191 and S regions are represented above the curve. (B) Impact of mutations in predicted putative
1192 fusogenic segments. The table reports the Gibbs free energy (ΔG) of the two presumed fusogenic
1193 segments computed for WT and mutants. A negative ΔG indicates that a peptide is favoured for
1194 partitioning from water to lipid bilayer, so it may be suspected as fusogenic. A dash indicates that the
1195 region is no longer expected to interact with lipid bilayer and hence to be fusogenic.

1196

1197 **Figure 2-figure supplement 2. Cell surface and intracellular detection of preS1 and preS2 HBV**
1198 **GP mutants. (A, B)** Huh7 cells expressing wt or mutant HBV GPs from Figure 2 were biotinylated for
1199 30 min at 4°C and then processed biochemically. Cell lysates were subjected to streptavidin pull-down
1200 prior to western blot analysis using anti-HBsAg antibody (Murex). The molecular weight markers (kDa)
1201 are shown to the left. Calnexin detection was used as control for cytoplasm protein marker, showing
1202 the integrity of cell membrane. (C, D) Detection and quantification of total GP expression. Cell lysates
1203 of Huh7 cells expressing the indicated wt or mutant GPs from Figure 2 were subjected to western blot
1204 analysis using anti-HBsAg antibody (Murex). The molecular weight markers (kDa) are shown to the
1205 left. Calnexin detection was used as control for cytoplasmic protein marker, as shown in these
1206 representative western blots. The results show the relative GPs expression compared to Wt of preS1
1207 (C) and preS2 mutants (D), as indicated, and are expressed as mean \pm SD (N=3).

1208

1209 **Figure 3-figure supplement 1. Effect of DTNB on HDV entry.** Different concentrations of DTNB
1210 were added to the cell supernatant containing HDV particles at the onset of infection (0h) or at 16
1211 hours post-infection. At 7 days post-infection virus HDV RNA from cells were extracted and quantified
1212 by RTqPCR. The results are expressed after normalization with GAPDH RNAs as means \pm SD (N=3)
1213 per mL of cell lysates containing 10^6 cells.

1214

1215

1216 **Figure 4-figure supplement 1. Contact map prediction for the L protein by RaptorX.** (A) The
1217 symmetric NxN matrix, where N is the length of the L protein, represents the probability of two
1218 residues being in contact. Higher probabilities are represented by darker colors. The green square
1219 highlights the “a” determinant containing the four cysteine-reach regions illustrated in Figure 4A and in
1220 (C) on the sequence. (B) A zoom of the green square where contacts between residues in the four
1221 cysteine-reach regions are delimited by five distinguished boxes. (C) Colors of the boxes pair with the
1222 links connecting the cysteine-reach regions.

1223

1224 **Figure 4-figure supplement 2. Geometry of a disulphide bond.** The five χ angles used to classify a
1225 disulfide bond conformers are labelled across the bond. MD simulations (see Methods) confirmed the
1226 stability of the bond as an allosteric disulfide, specifically on a -/+RH Hook conformation. While only
1227 three out of the twenty possible configurations, namely -RH Staple, -LH Hook and -/+RH Hook, are
1228 identified as allosteric disulfide bonds (Hogg, 2013; Schmidt and Hogg, 2007), the -/+RH Hook
1229 conformation is more stressed than other geometries, due to stretching of the S-S bond and bending
1230 of the neighboring bond angles (Zhou et al., 2014).

1231

1232 **Figure 7-figure supplement 1. Down-regulation of PDI family members.** (A-C) Naïve Huh7-NTCP
1233 cells (Ctrl+) or shRNA-expressing Huh7-NTCP cells were subjected to flow cytometry (left) and
1234 western blot (right) analyses, in order to evaluate the expression levels of the indicated PDIs [(A),
1235 ERp46; (B), ERp57; (C), ERp72] before or after down-regulation. Huh7-NTCP cells stained with
1236 secondary antibody only (Neg) were used to provide the background of flow cytometry analyses.

1237

1238 **Figure 7-figure supplement 2. NTCP expression in target cells.** (A) Huh7-NTCP and Huh7-NTCP-
1239 tat cells were subjected to flow cytometry analysis, in order to evaluate the expression of NTCP at
1240 intracellular and cell surface levels, as indicated. Cells stained with secondary antibody only (Neg)
1241 were used to provide the background of flow cytometry analyses. (B) Huh7-NTCP and (C) Huh7-
1242 NTCP-tat cells were subjected to flow cytometry analysis, in order to evaluate the expression of NTCP
1243 after the stable expression of shRNAs targeting the indicated PDIs. Non-transduced cells were used
1244 as positive control (Ctrl+) and cells stained with secondary antibody only (Neg) were used to provide
1245 the background of flow cytometry analysis.

1246

1247 **Figure 9-figure supplement 1.** (A) NTZ at 30 $\mu\text{g}/\text{mL}$ was added to the cell supernatant 2h before
1248 infection (2h-) vs. at the onset of infection (0h), at 4h (4h+) or at 16h (16h+) post-infection. DMSO was
1249 used as control vehicle. At 7 days post-infection, HDV RNAs were extracted from cells and quantified
1250 by RTqPCR. The results are expressed after normalization with GAPDH RNAs as means \pm SD (N=4)

1251 per mL of cell lysates containing 10^6 cells. **(B-E)** Study of NTZ effect in HBV *in vivo*. 4-8 weeks old
1252 NOD-FRG mice were engrafted with primary human hepatocytes (PHH). After *ca.* 2-3 months, the
1253 animals displaying HSA levels >15 mg/mL were split in 4 groups that were infected with HBV (10^8
1254 GE/mouse) with or without NTZ. See schedule in Figure 9A. At different time points post-infection,
1255 blood samples (50 μ l) were collected and the viremia in sera was monitored by qPCR on the HBV
1256 genome (GE/mL of serum) The graphs show the results of viremia for individual mice within each
1257 group. **(B)** Group #1: mice were infected and inoculated with DMSO, **(C)** Group #2: mice were infected
1258 and inoculated with NTZ (100 mg/kg), **(D)** Group #3: mice were only inoculated with NTZ (100 mg/kg)
1259 and **(E)** Group #4: mice were only inoculated with DMSO (used as control vehicle of NTZ).
1260

1261 **Legend to Supplementary file**

1262

1263 **Supplementary file 1. Oligonucleotide sequences used for shRNAs and mutagenesis.** The
1264 sequences correspond to the oligonucleotides used to generate the lentiviral vectors carrying shRNA
1265 against the indicated PDIs in Figure 7 or the HBV GP mutants described in Figure 2 (preS1 and preS2
1266 mutants) and in Figures 4 and 5 (CSD mutants).

1267

1268 **Legends to the source data files**

1269

1270 **Figure 1-source data 1. HBV GP fusion trigger is independent of acid pH and NTCP.** The values
1271 correspond to the data expressed in the graphs displayed in Figure 1A, 1D and 1F.

1272

1273 **Figure 1-source data 2. HBV GP fusion trigger is independent of acid pH and NTCP.** These
1274 images are the original and uncropped gels that correspond to the blots displayed in Figure 1E. The
1275 vertical bars correspond to samples that were not described in the Results section.

1276

1277 **Figure 2-source data 1. Functional analysis of predicted HBV fusion peptides.** The values
1278 correspond to the data expressed in the graphs displayed in Figure 2C, 2D, 2E, 2F, 2I and 2J.

1279

1280 **Figure 2-source data 2. Functional analysis of predicted HBV fusion peptides.** The values
1281 correspond to the data expressed in the graphs displayed in Figure 2G.

1282

1283 **Figure 2-source data 3. Functional analysis of predicted HBV fusion peptides.** The values
1284 correspond to the data expressed in the graphs displayed in Figure 2H.

1285

1286 **Figure 3-source data 1. DTNB, a thiol-specific oxidizing reagent, inhibits HBV membrane
1287 fusion.** The values correspond to the data expressed in the graphs displayed in Figure 3A and 3B.

1288

1289 **Figure 3-source data 2. DTNB, a thiol-specific oxidizing reagent, inhibits HBV membrane
1290 fusion.** These images are the original and uncropped gels that correspond to the blots displayed in
1291 Figure 3C.

1292

1293 **Figure 3-source data 3. DTNB, a thiol-specific oxidizing reagent, inhibits HBV membrane
1294 fusion.** These images are the original and uncropped gels that correspond to the blots displayed in
1295 Figure 3D.

1296

1297 **Figure 5-source data 1. Evidence for a functional role of the CSD in the region 294-317 of the
1298 HBV S GP.** The values correspond to the data expressed in the graphs displayed in Figure 5A-5C.

1299

1300 **Figure 5-source data 2. Evidence for a functional role of the CSD in the region 294-317 of the
1301 HBV S GP.** The values correspond to the data expressed in the graphs displayed in Figure 5D.

1302

1303 **Figure 5-source data 3. Evidence for a functional role of the CSD in the region 294-317 of the**
1304 **HBV S GP.** These images are the original and uncropped gels that correspond to the blots displayed
1305 in Figure 5C. The vertical bars correspond to samples that were not described in the Results section.

1306

1307 **Figure 6-source data 1. PDI inhibitors in HBV entry.** The values correspond to the data expressed
1308 in the graphs displayed in Figure 6A-6D.

1309

1310 **Figure 7-source data 1. ERp57 down-regulation inhibits in HBV entry.** The values correspond to
1311 the data expressed in the graphs displayed in Figure 7B-7D.

1312

1313 **Figure 9-source data 1. *In vivo* assessment of ERp57 inhibition.** The values correspond to the
1314 data expressed in the graphs displayed in Figure 9B.

1315

1316

1317

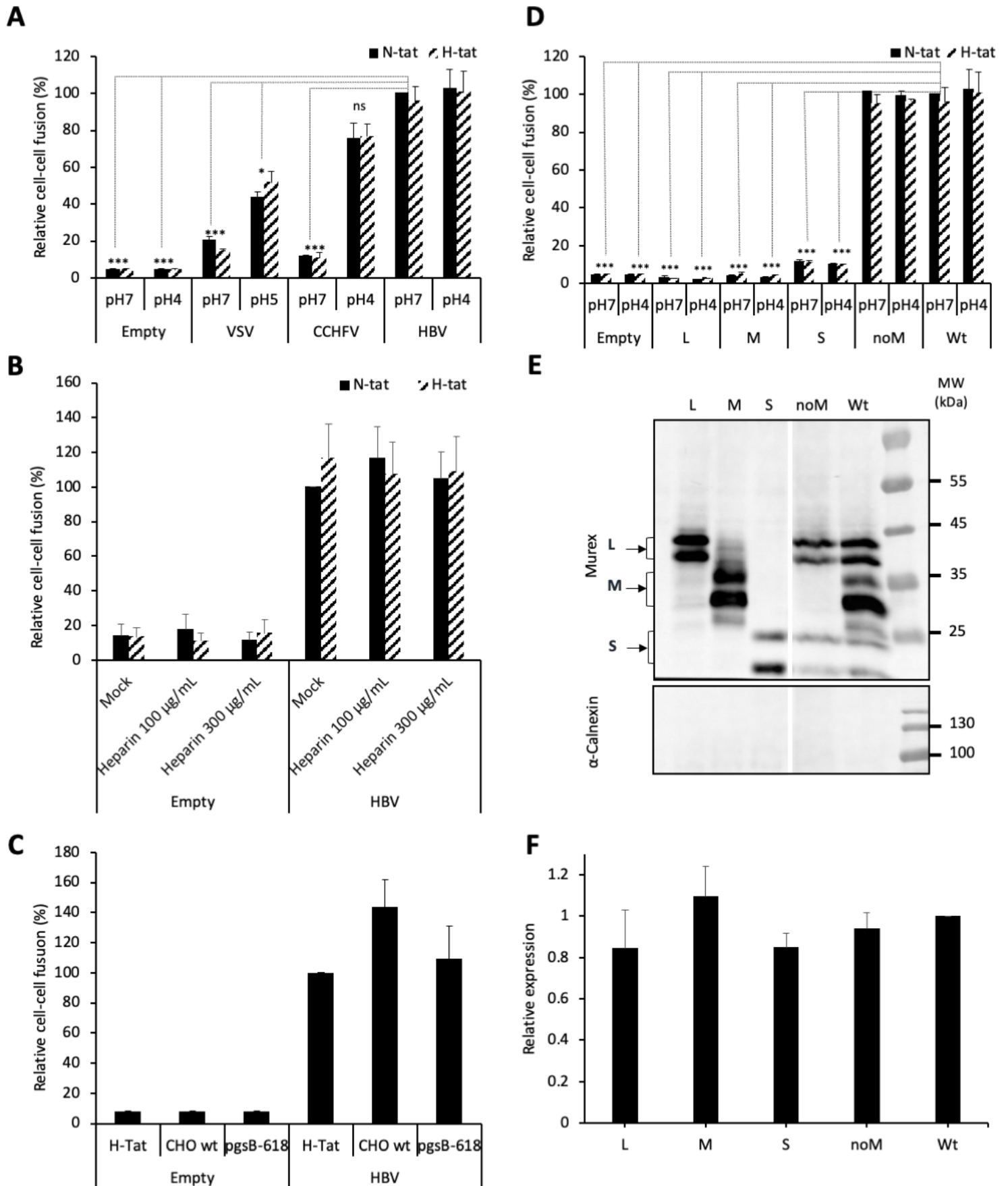


Figure 1. Pérez-Vargas et al.

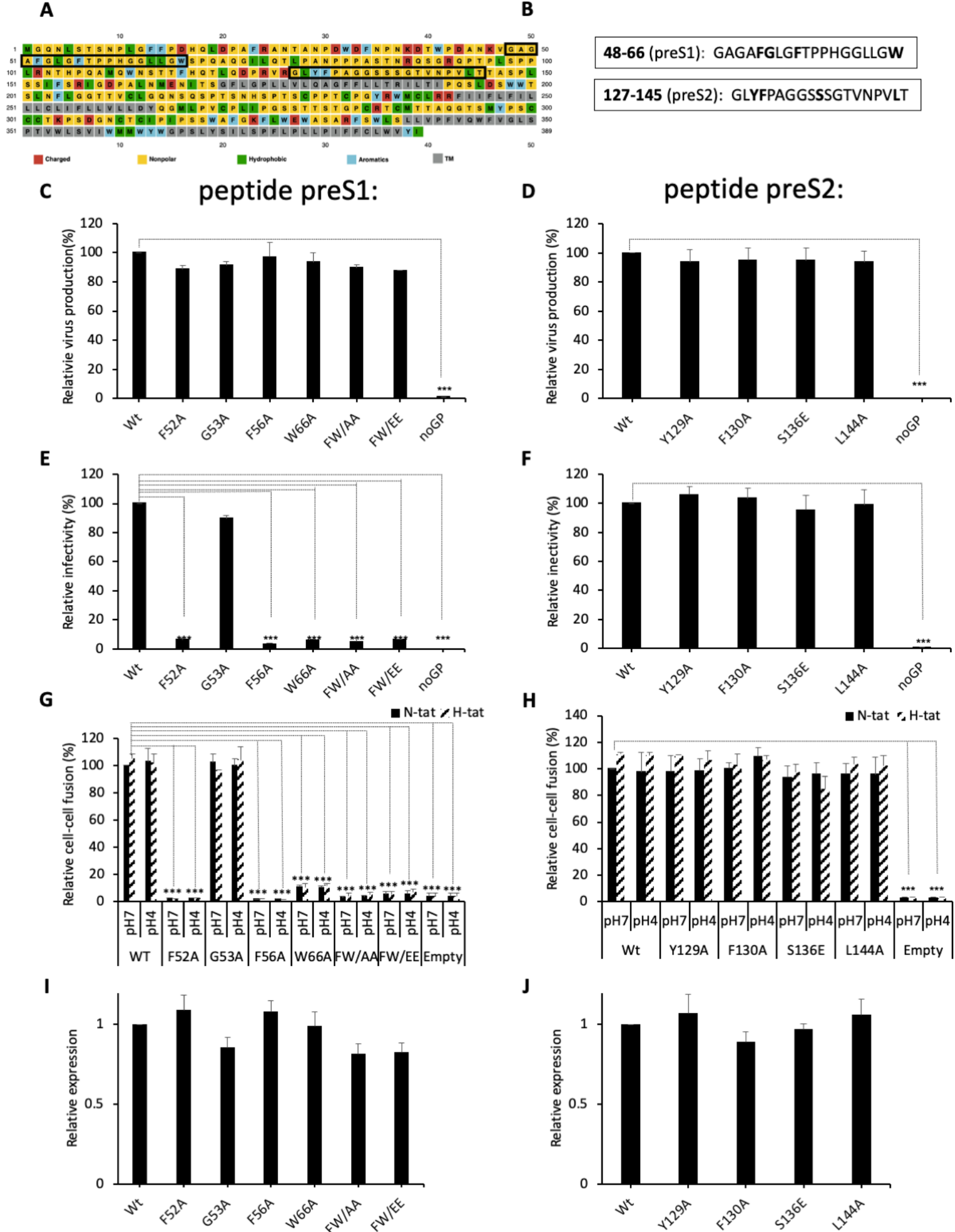


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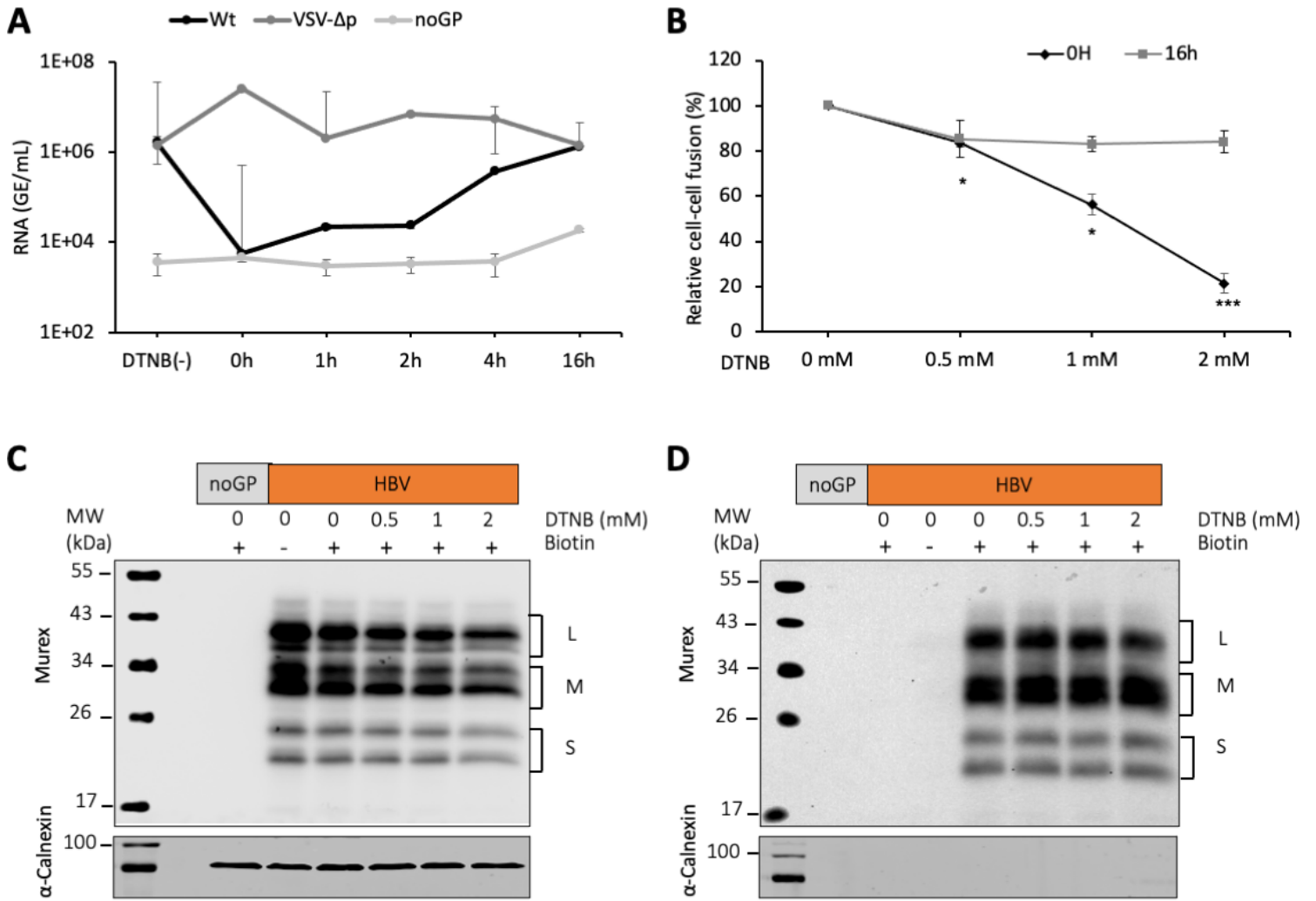
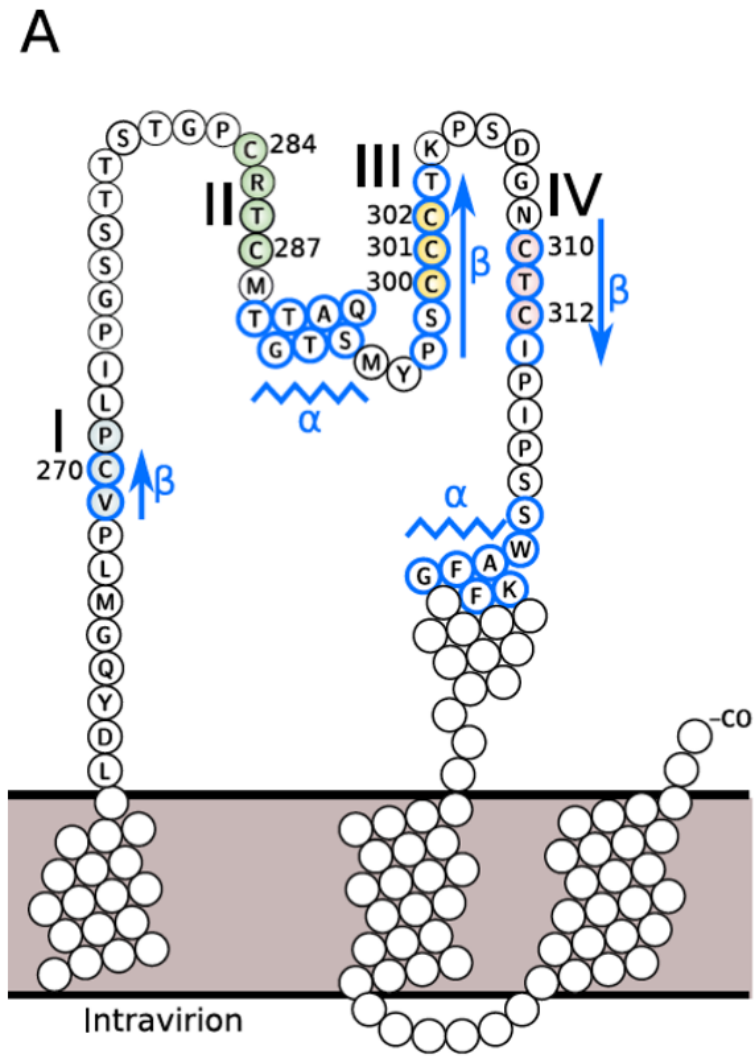


Figure 3. Pérez-Vargas et al.



B

| | II | | | | III | | | IV | | | |
|-----|------|------|------|------|------|------|------|------|-------------|-------------|-------------|
| | 284 | 285 | 286 | 287 | 300 | 301 | 302 | 310 | 311 | 312 | |
| I | 269 | 0.30 | 0.18 | 0.19 | 0.28 | 0.38 | 0.39 | 0.30 | 0.38 | 0.39 | 0.45 |
| | C270 | 0.47 | 0.23 | 0.26 | 0.43 | 0.53 | 0.58 | 0.47 | 0.57 | 0.40 | 0.57 |
| | 271 | 0.29 | 0.20 | 0.22 | 0.31 | 0.35 | 0.35 | 0.29 | 0.35 | 0.34 | 0.35 |
| II | C284 | | | | | 0.60 | 0.58 | 0.52 | 0.64 | 0.45 | 0.62 |
| | 285 | | | | | 0.40 | 0.38 | 0.30 | 0.36 | 0.27 | 0.34 |
| | 286 | | | | | 0.44 | 0.36 | 0.28 | 0.38 | 0.35 | 0.39 |
| | C287 | | | | | 0.58 | 0.50 | 0.40 | 0.49 | 0.35 | 0.58 |
| III | C300 | | | | | | | | 0.49 | 0.43 | 0.83 |
| | C301 | | | | | | | | 0.62 | 0.54 | 0.84 |
| | C302 | | | | | | | | 0.87 | 0.78 | 0.84 |

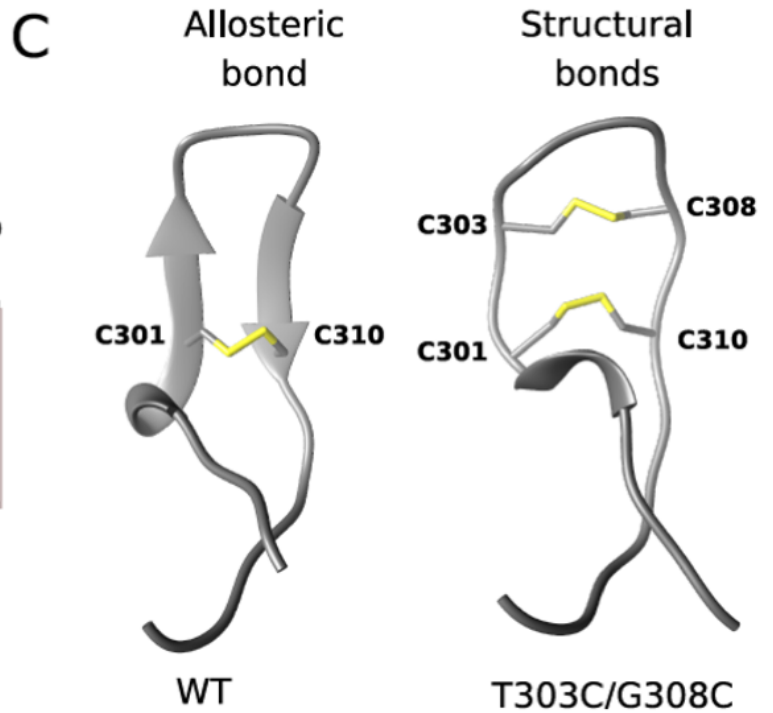


Figure 4. Pérez-Vargas et al.

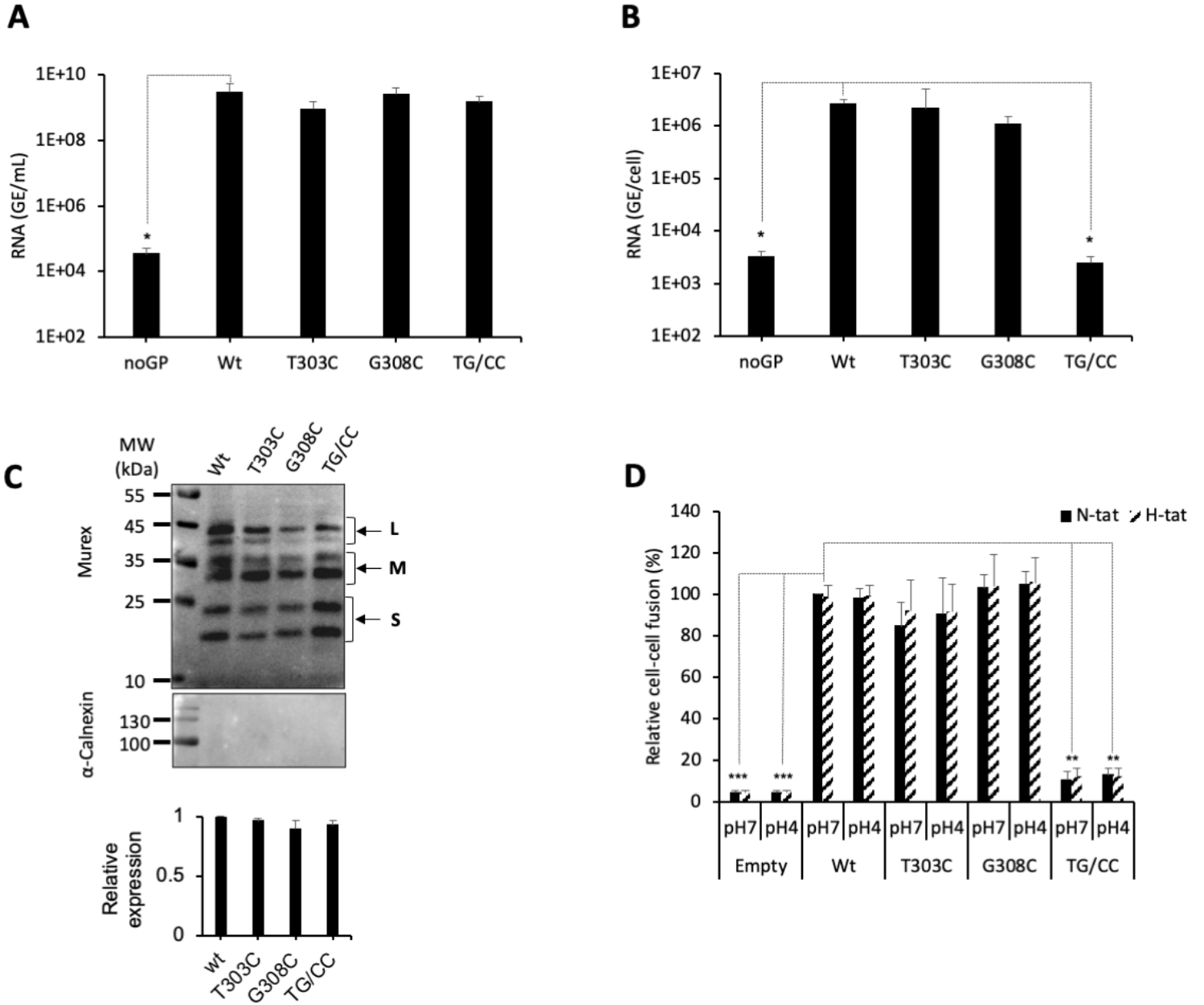


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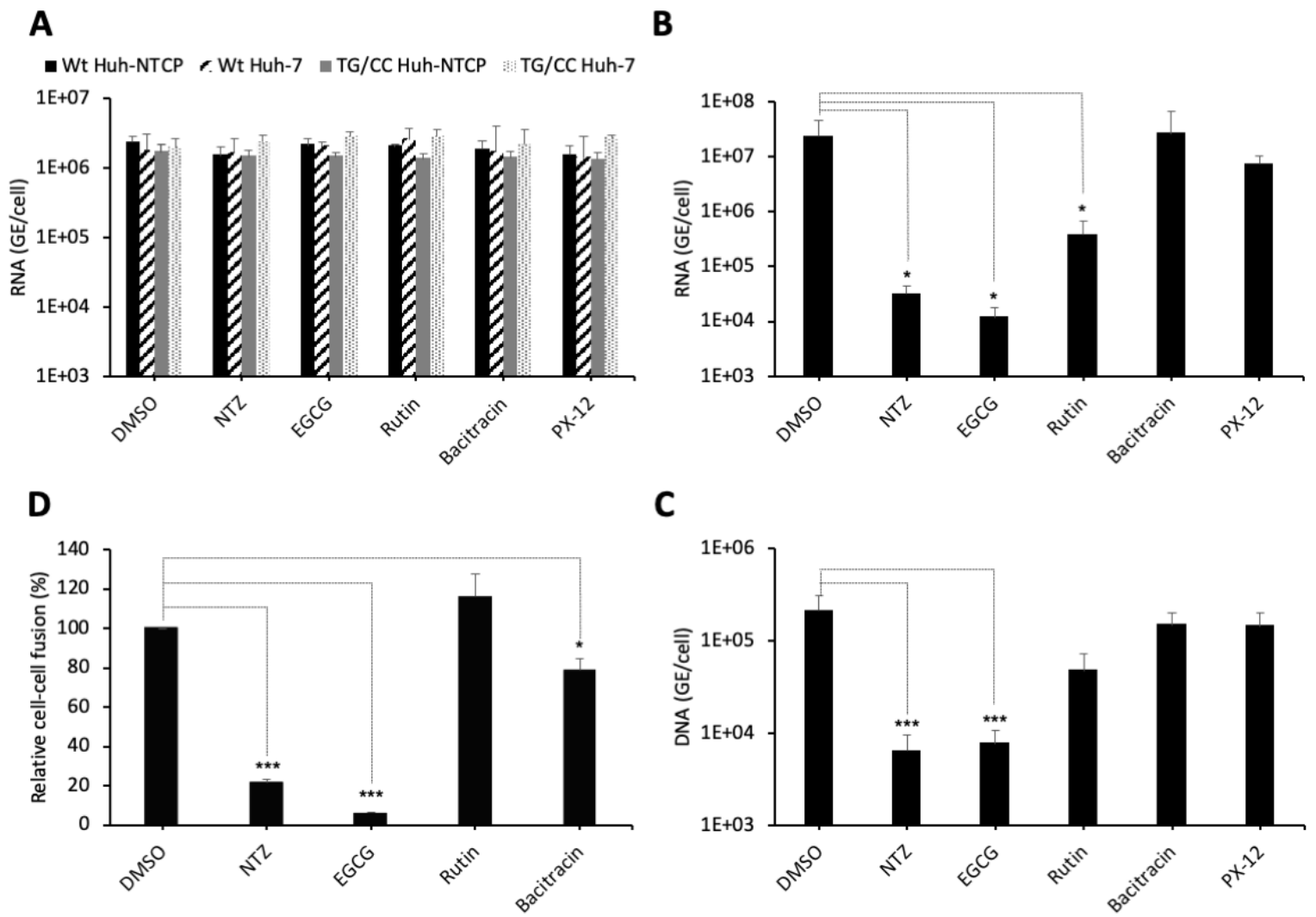


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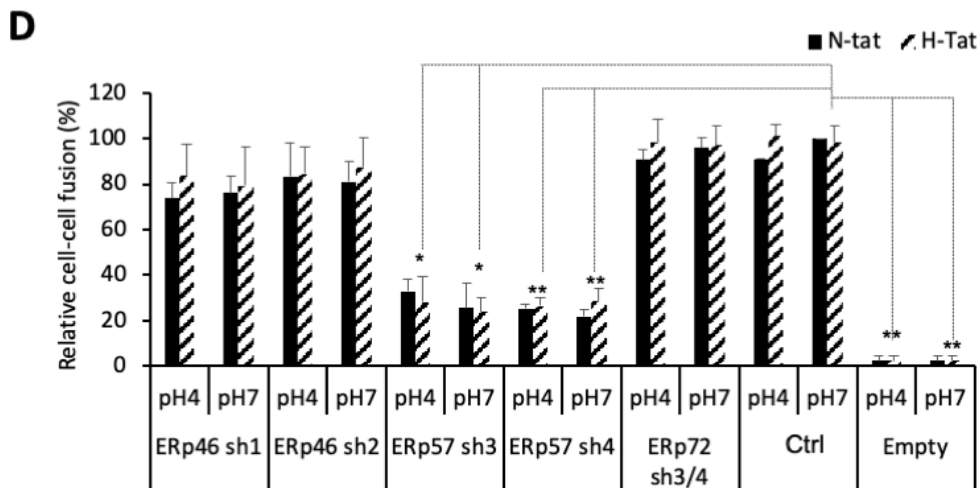
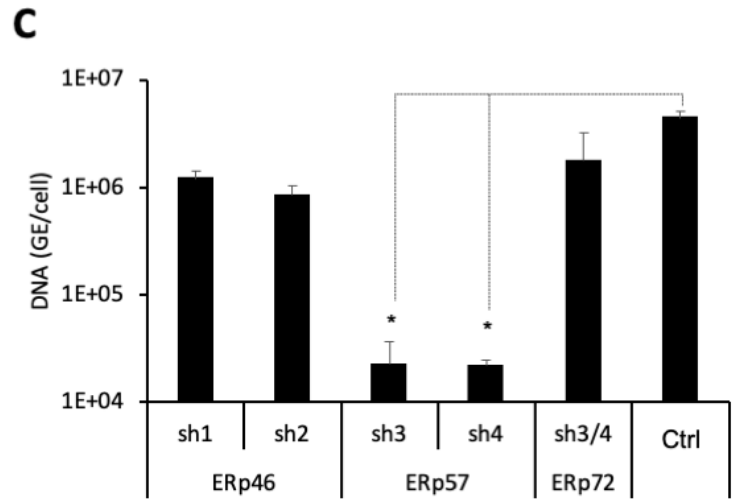
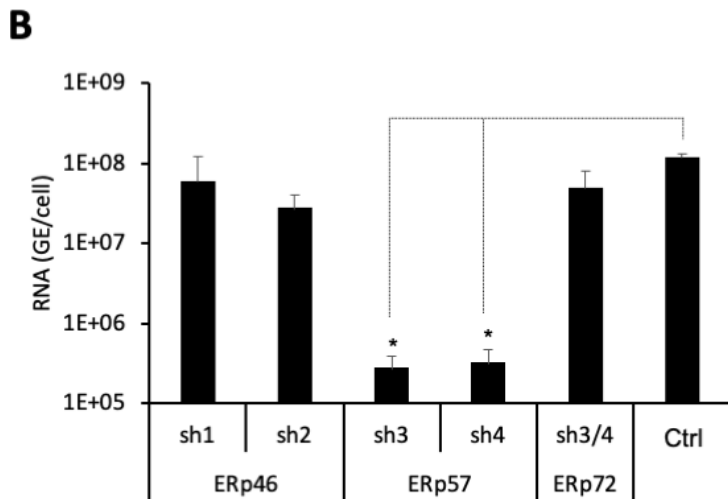
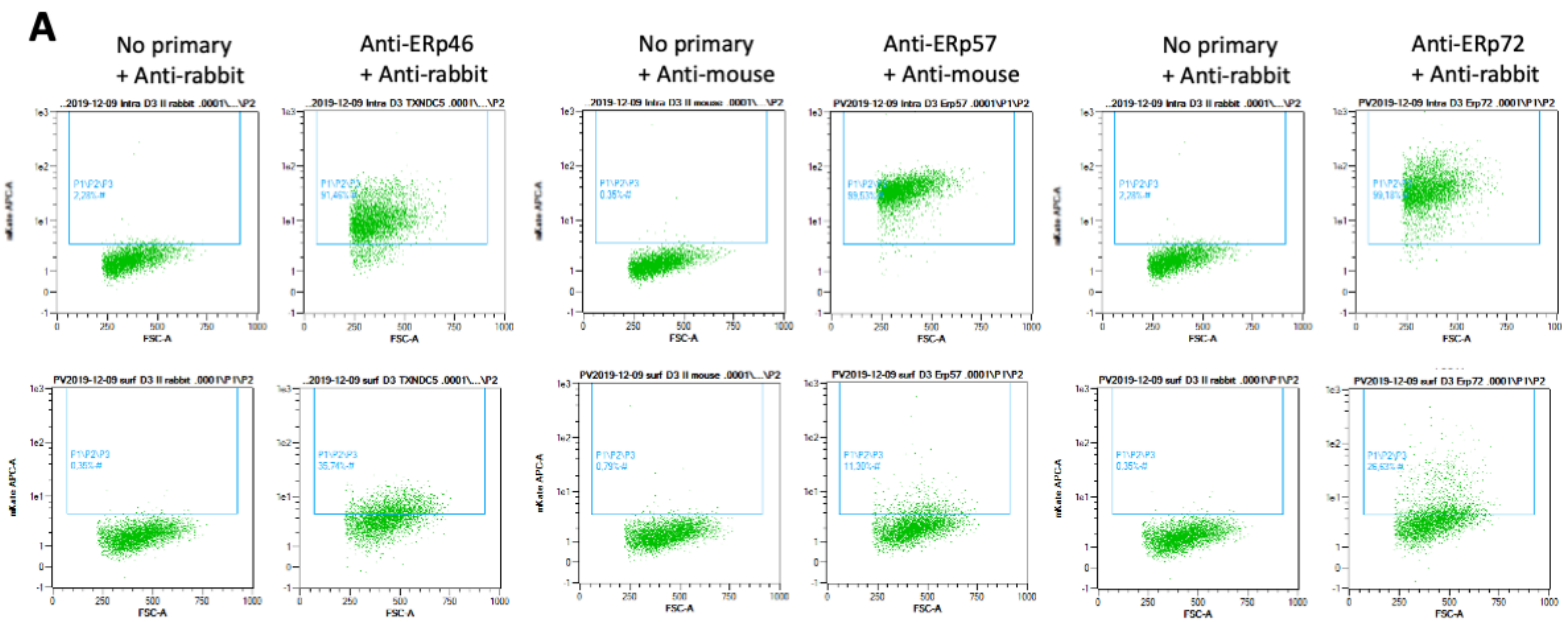


Figure 7. Pérez-Vargas et al.

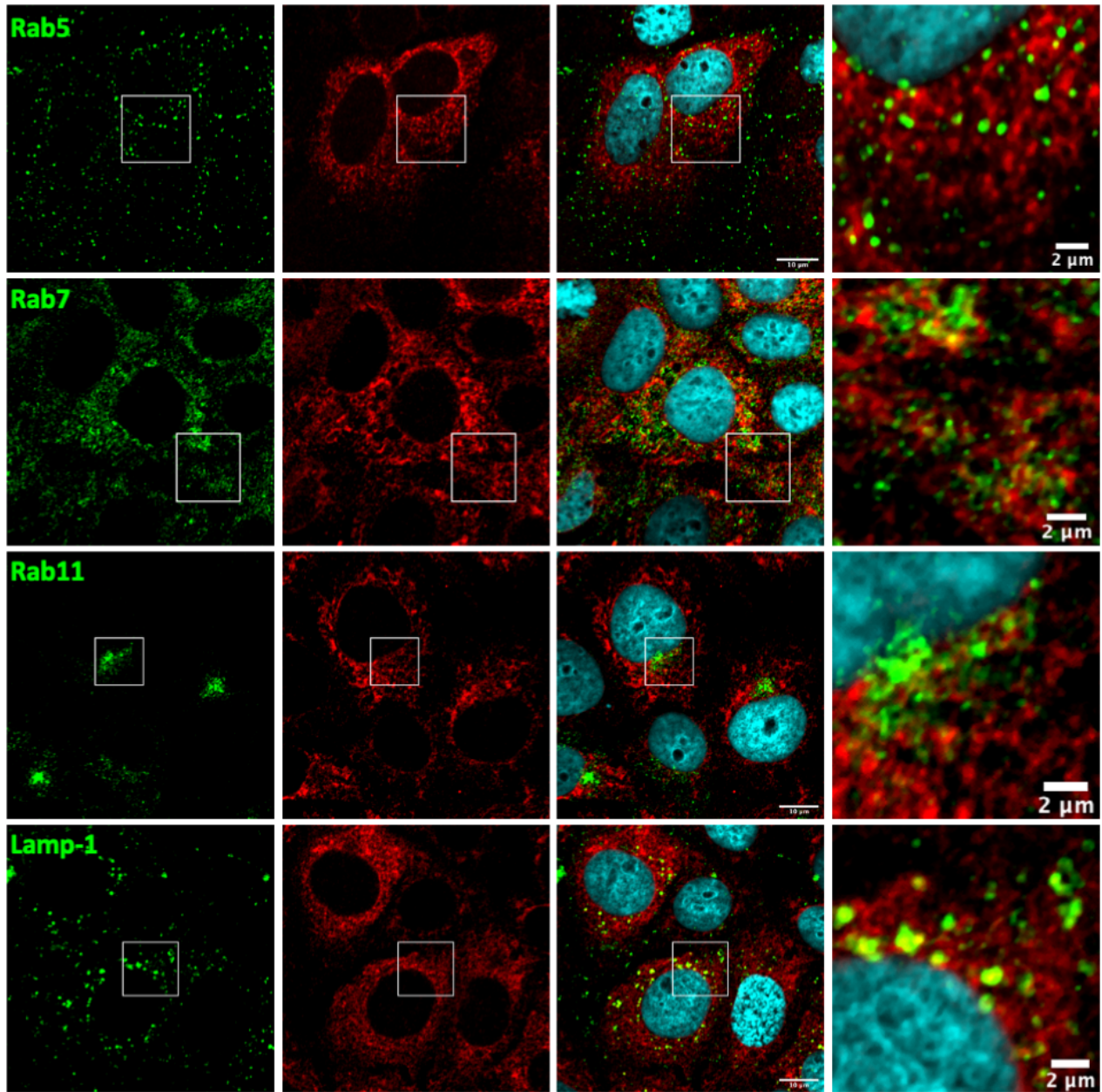
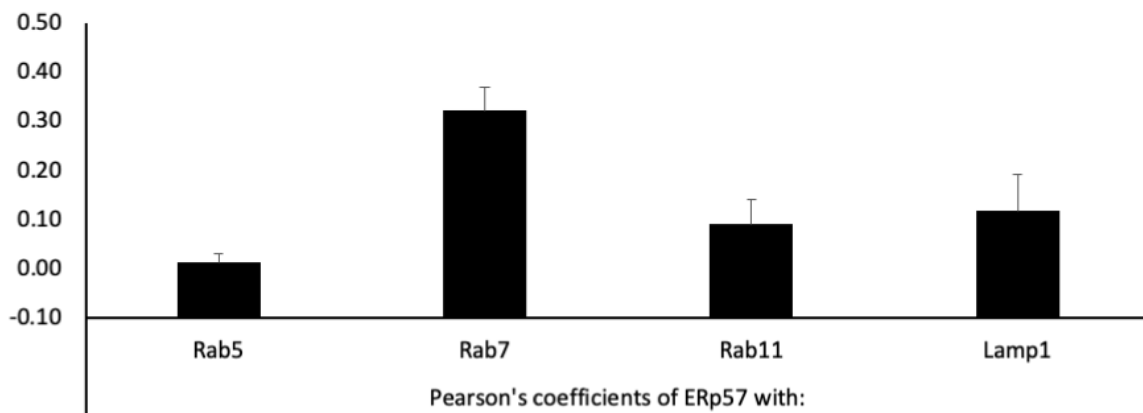
A**B**

Figure 8. Pérez-Vargas et al.

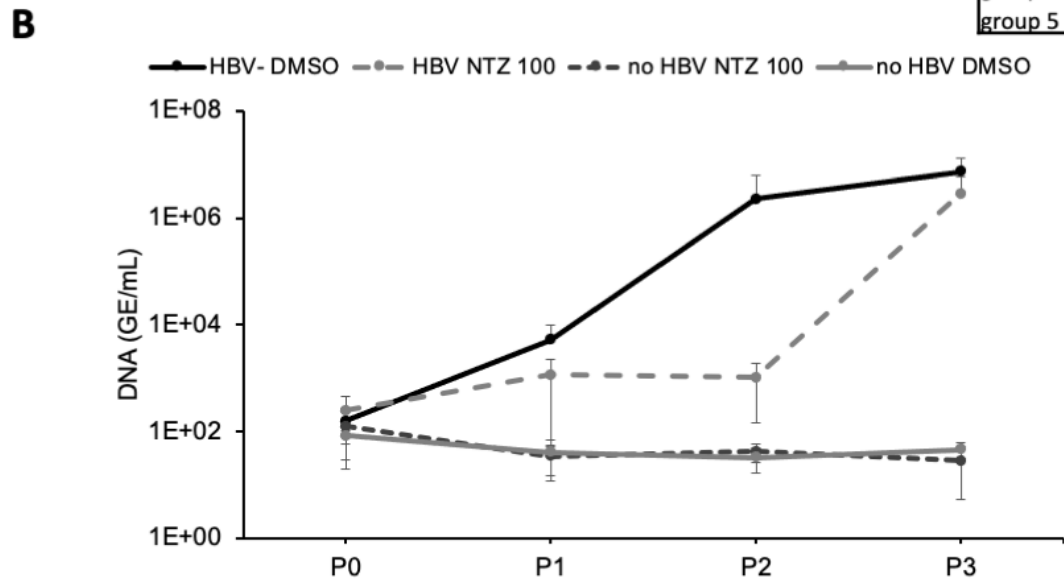
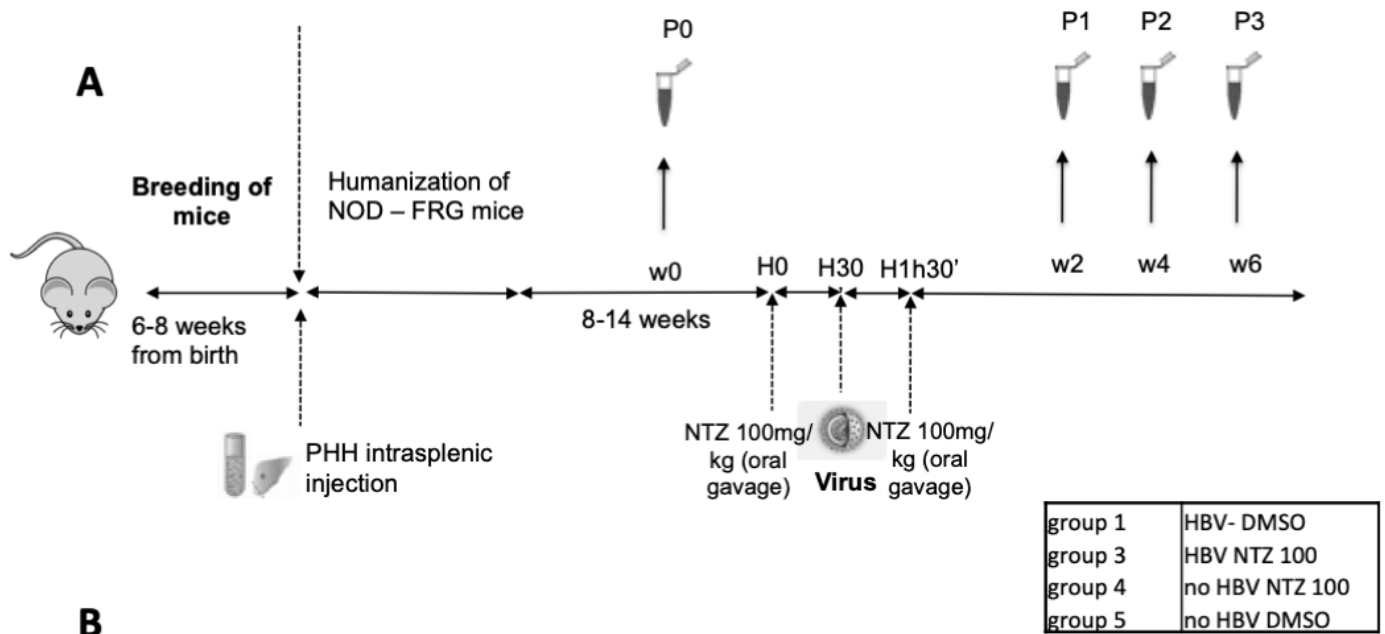


Figure 9. Pérez-Vargas et al.

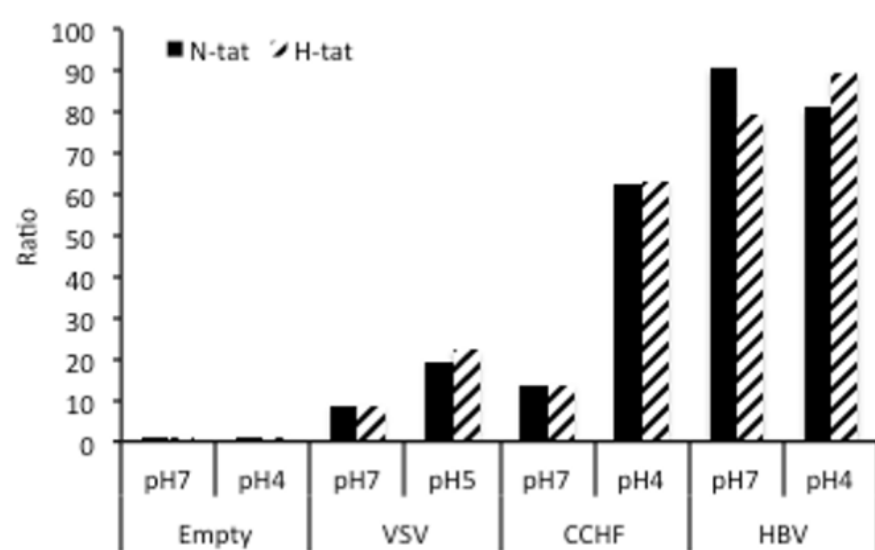
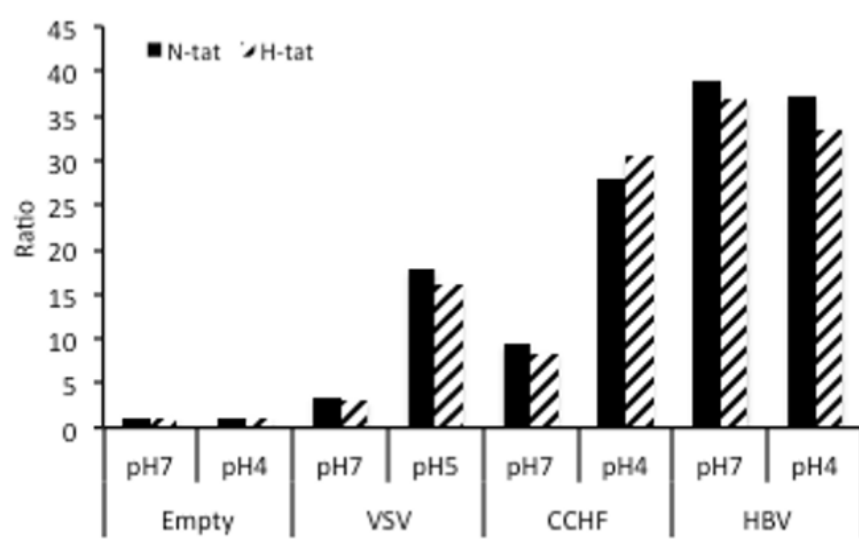
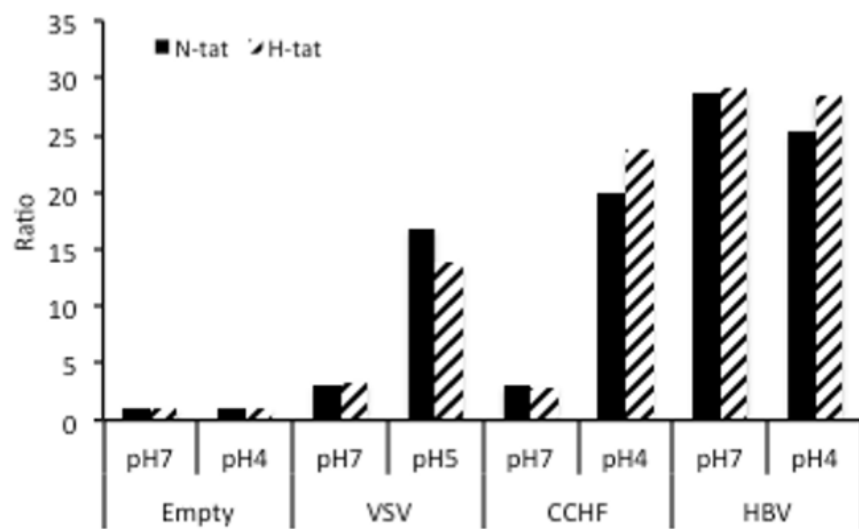


Figure 1-figure supplement 1

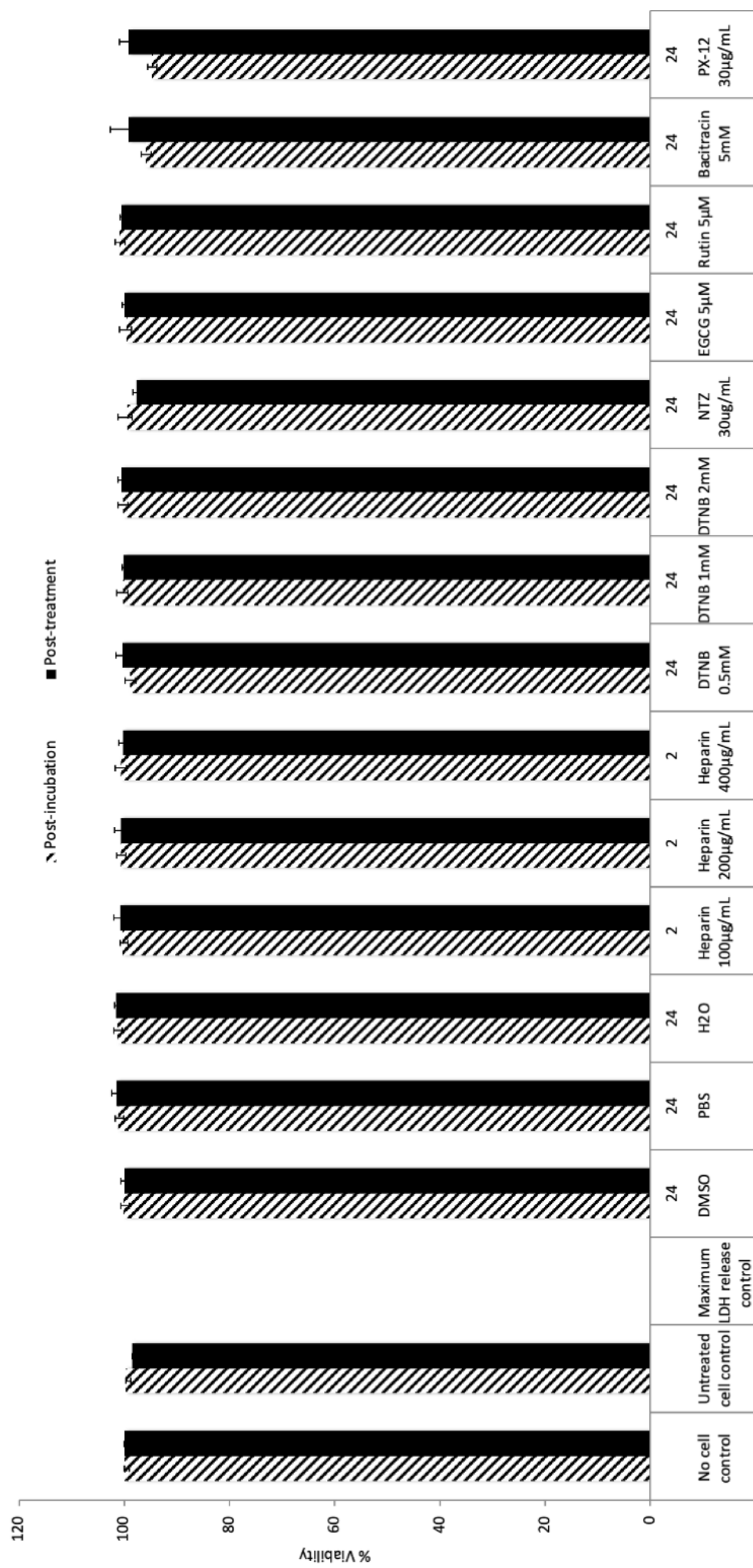
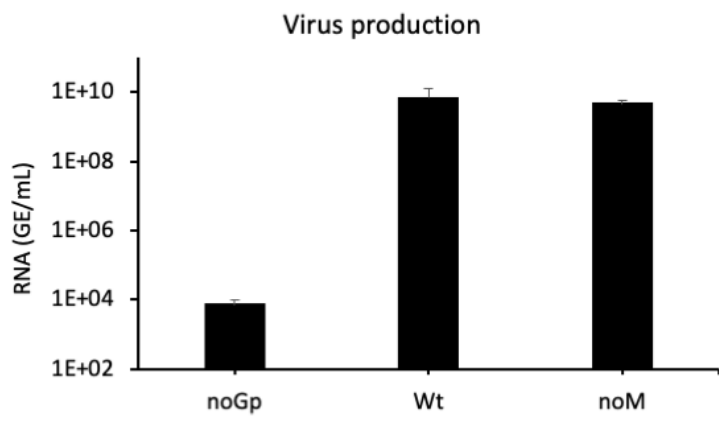
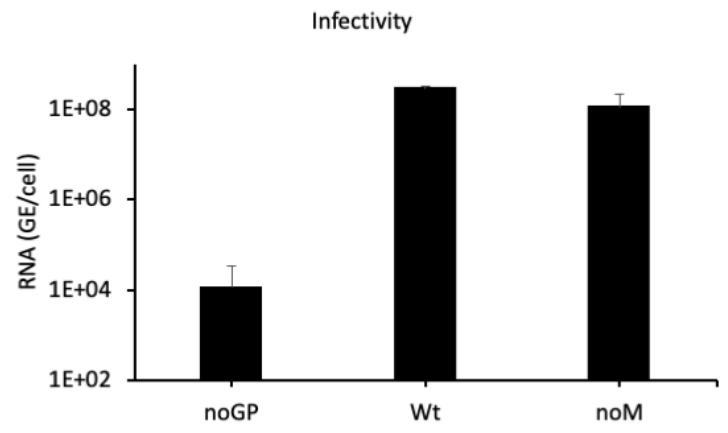


Figure 1-figure supplement 2

A**B**

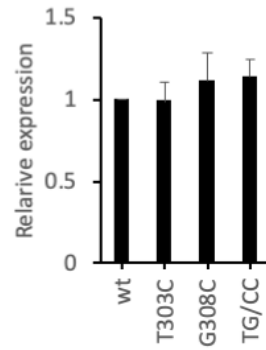
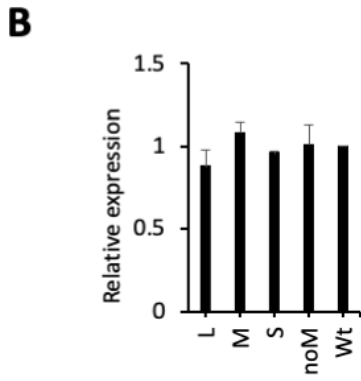
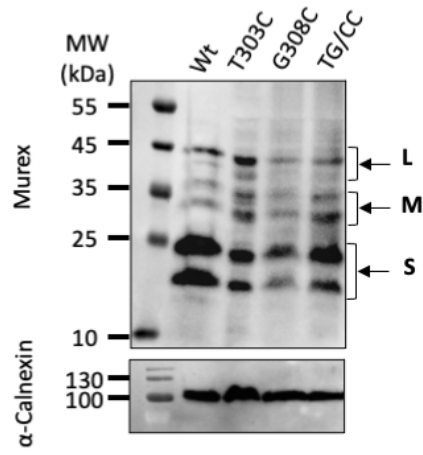
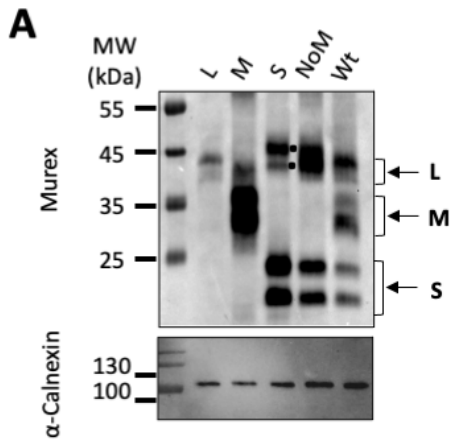
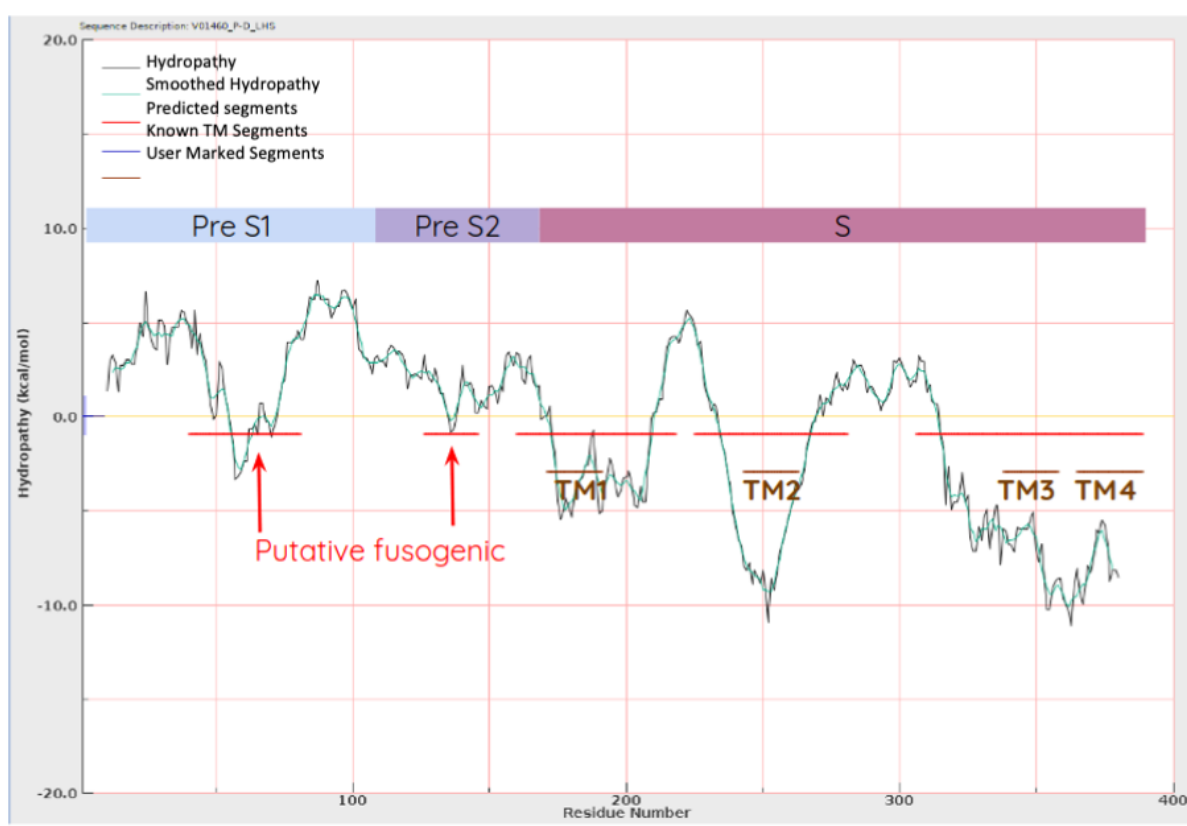


Figure 1-figure supplement 4

A**B**

| First fusion candidate | | | Second fusion candidate | | |
|------------------------|--------------------------|---------------------|-------------------------|--------------------------|---------------------|
| | ΔG (Kcal/mol) | Predicted region | | ΔG (Kcal/mol) | Predicted region |
| wt | -3.38 | 48-66 | wt | -0.85 | 127-145 |
| F52A | -2.08 | 48-66 | Y129A | - | - |
| G53A | -3.22 | 48-66 | F130A | - | - |
| F56A | -2.08 | 48-66 | S136E | - | - |
| W66A | -1.46 | 47-65 | L144A | -0.12 | 127-145 |
| F52A/W56A | -0.16 | 61-79 | | | |
| F52E/W56E | - | - | | | |

Figure 2-figure supplement 1

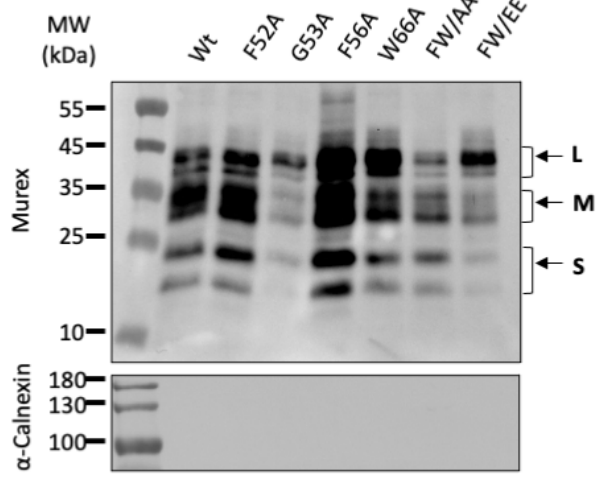
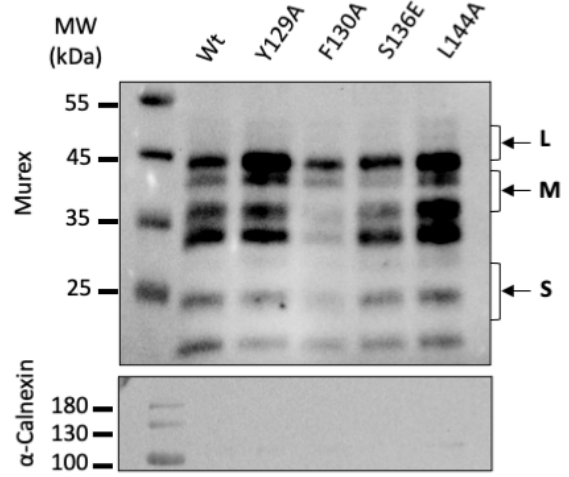
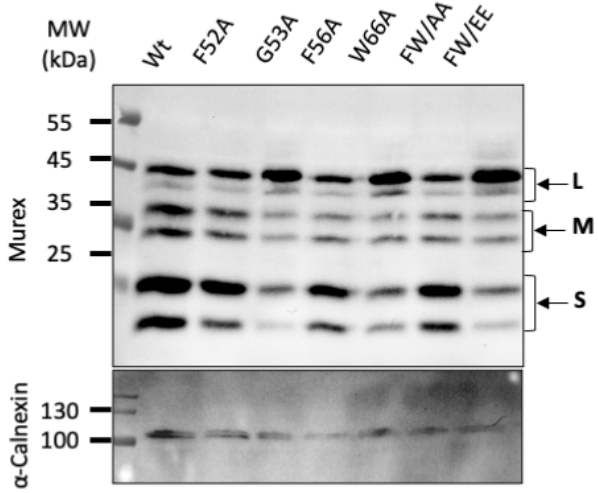
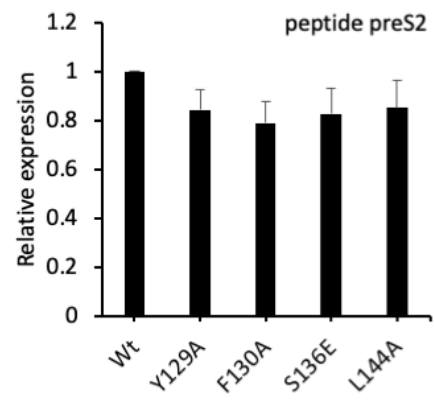
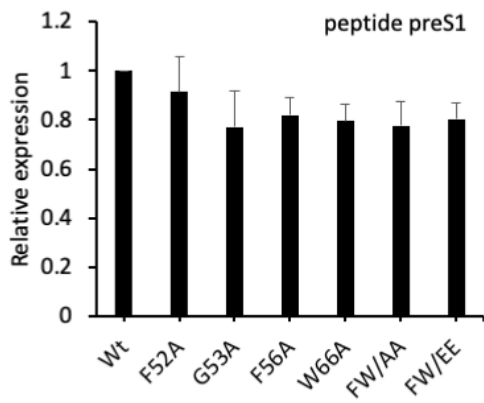
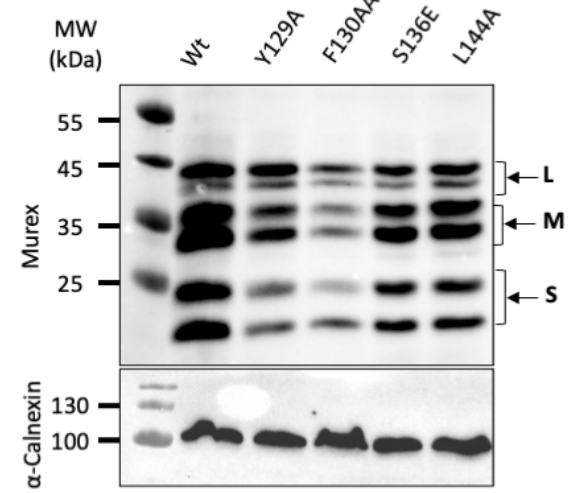
A**B****C****D**

Figure 2-figure supplement 2

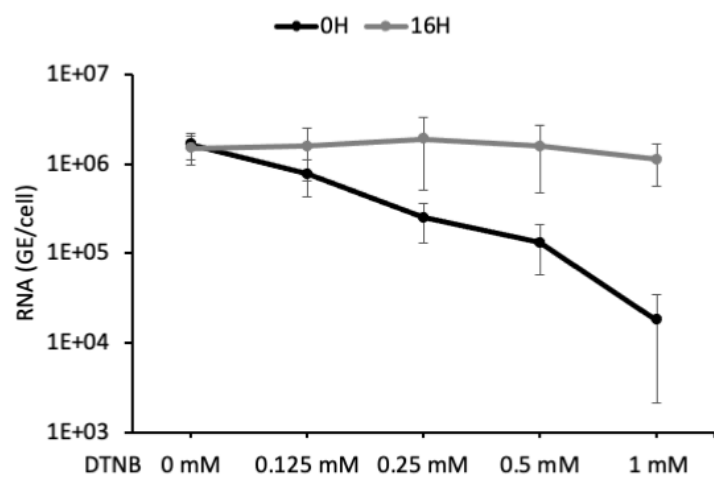


Figure 3-figure supplement 1

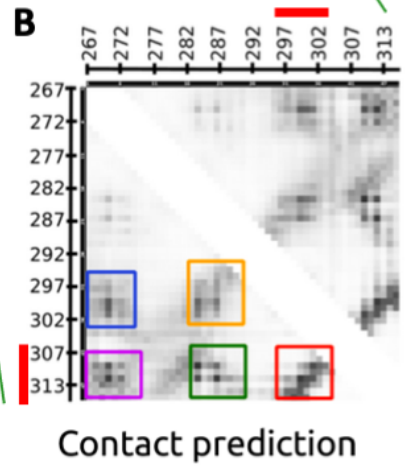
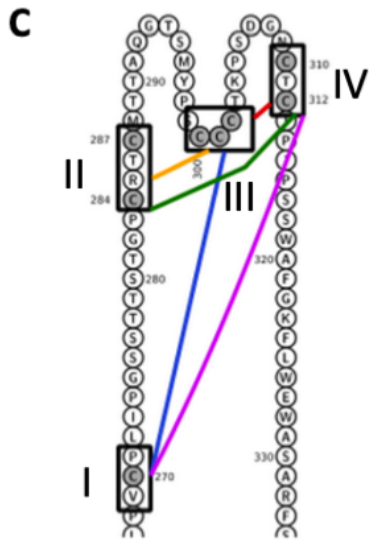
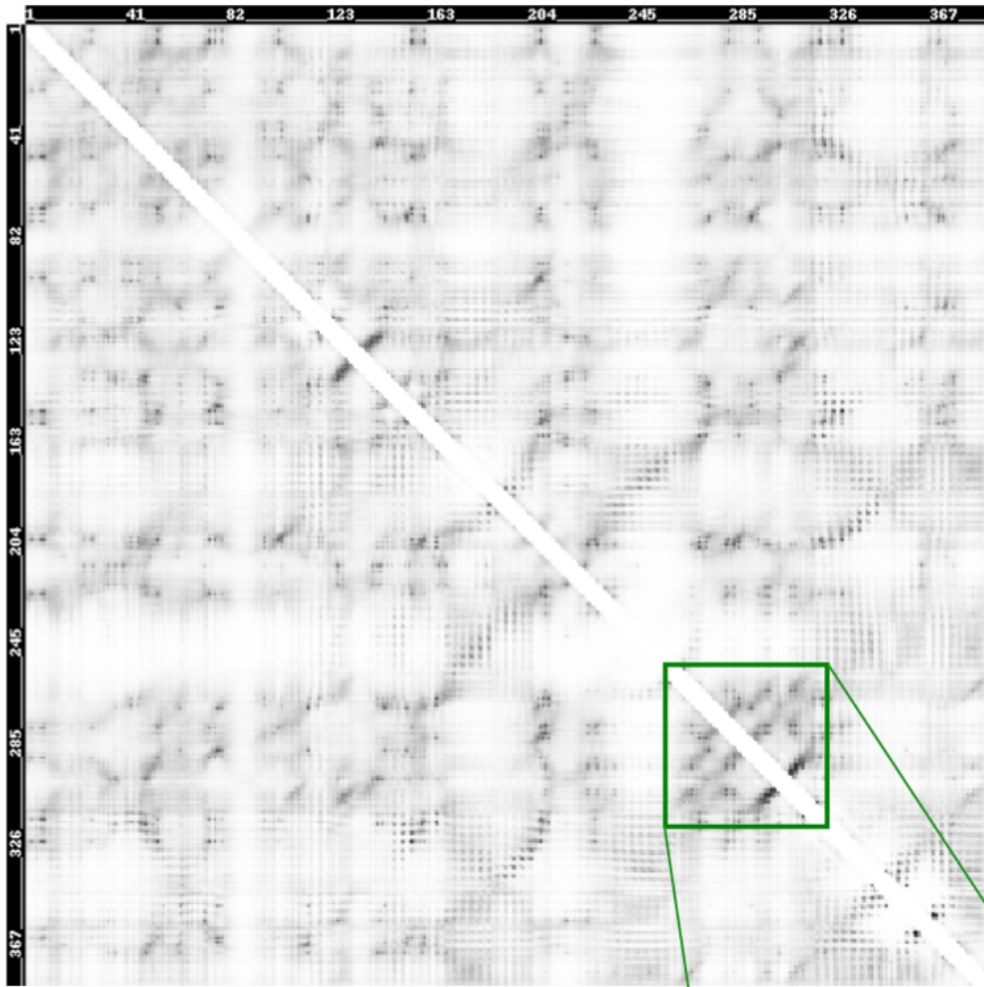
A

Figure 4-figure supplement 1

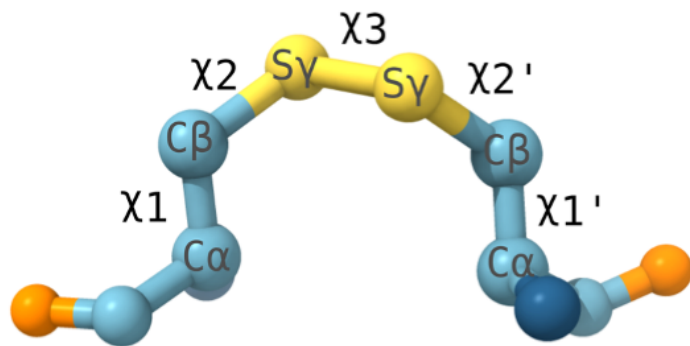


Figure 4-figure supplement 2

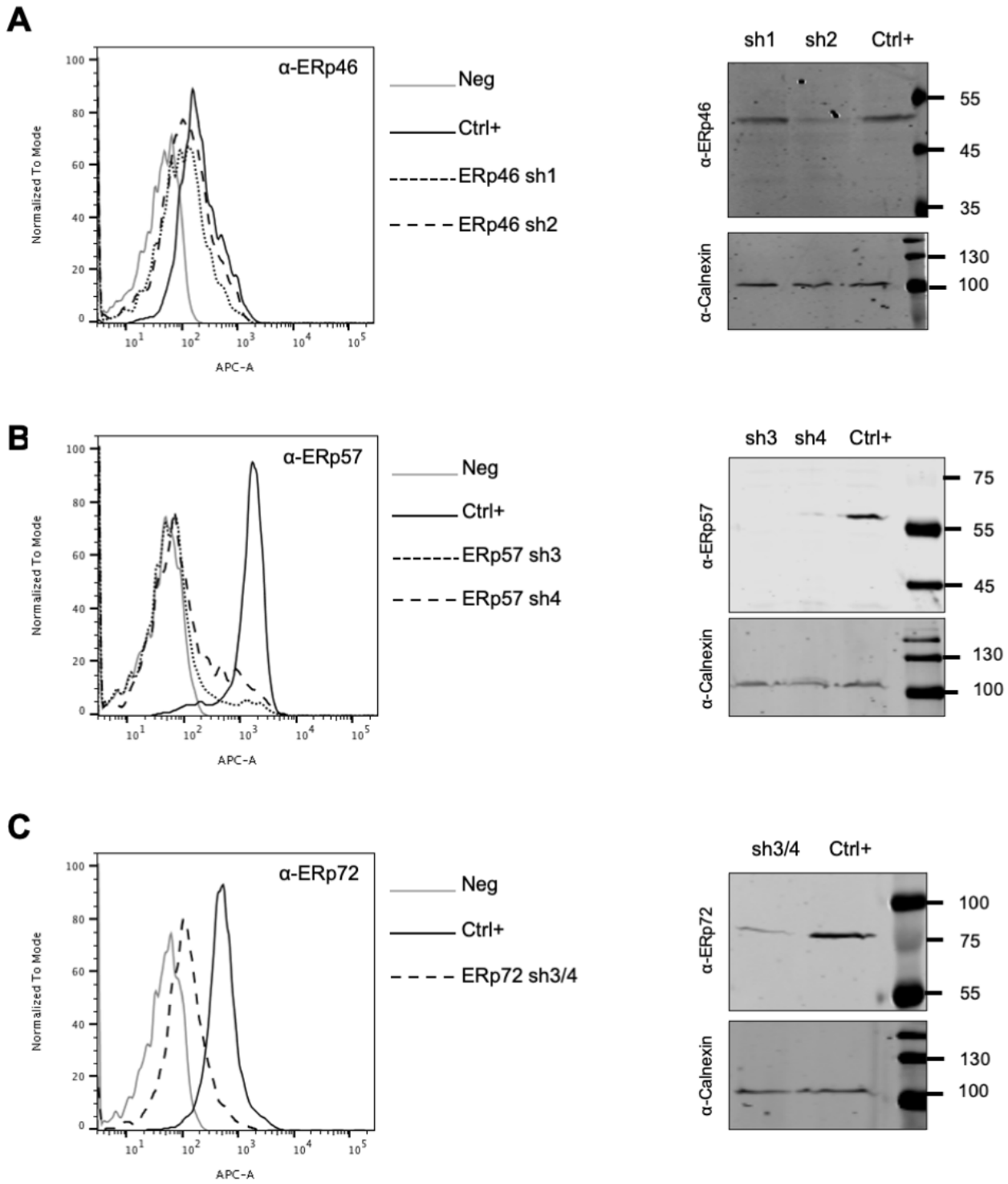


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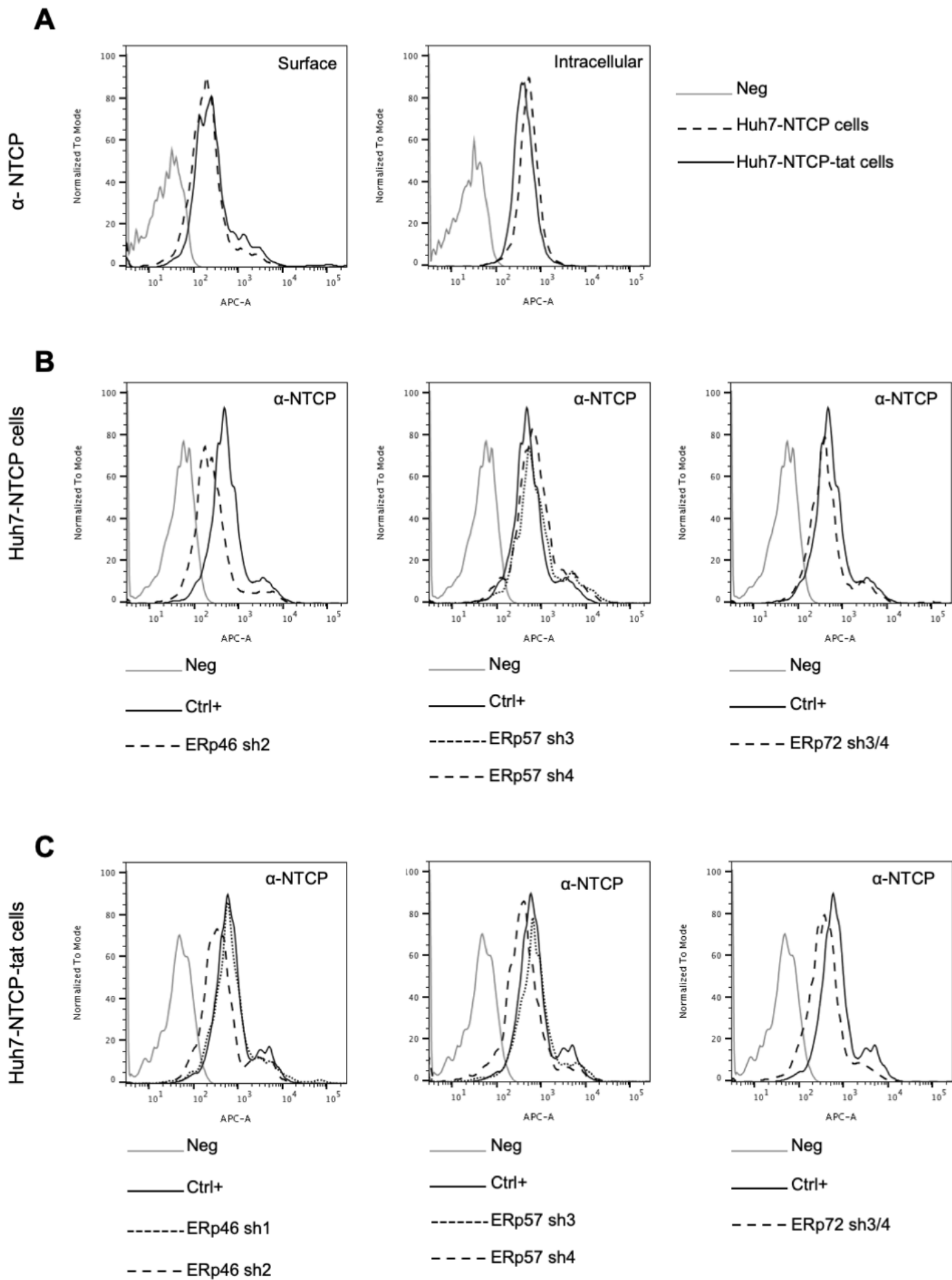


Figure 7-figure supplement 2

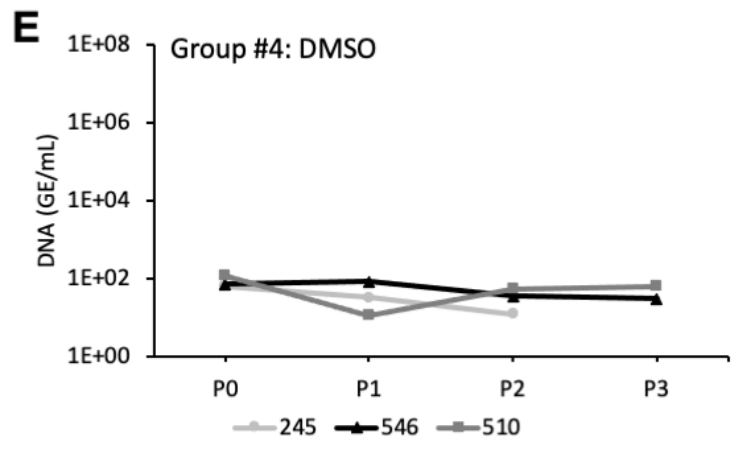
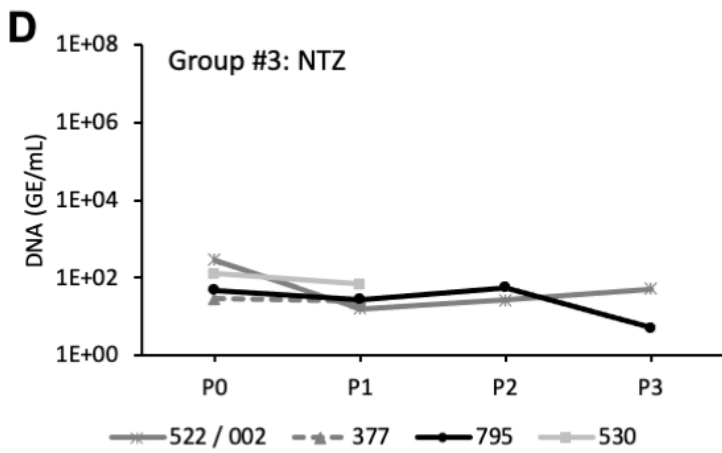
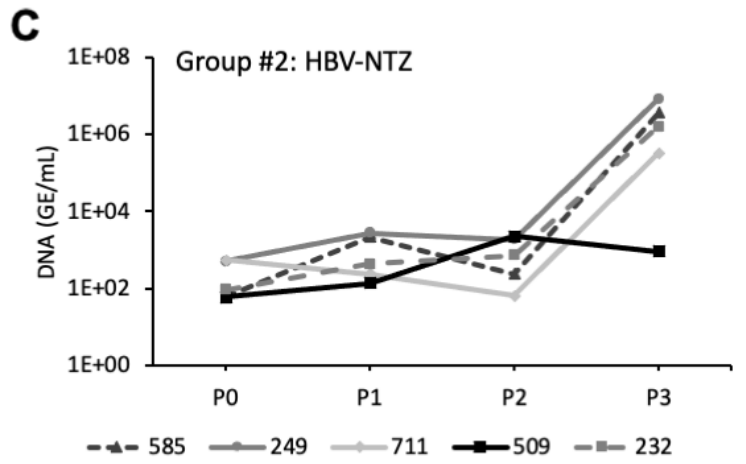
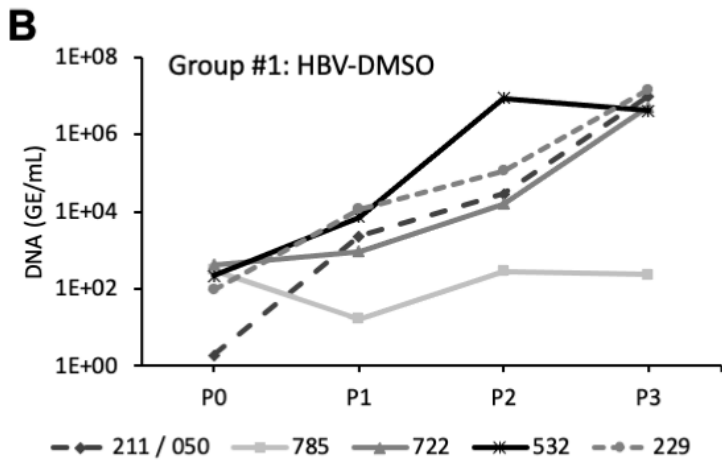
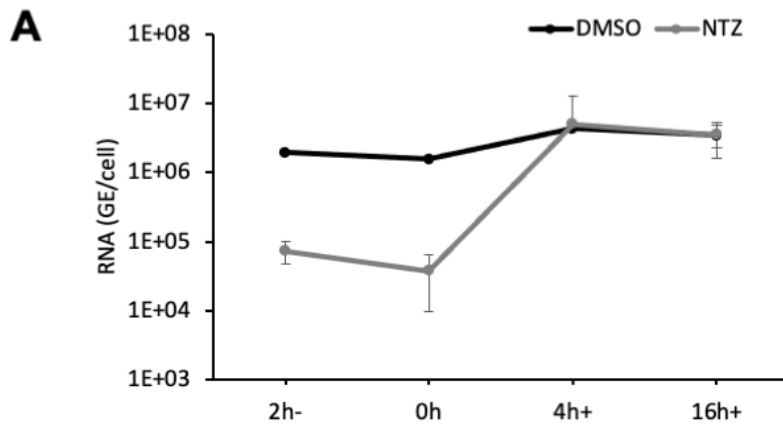


Figure 9-figure supplement 1