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25 **List of abbreviations:** HCV, hepatitis C virus; IFITM, interferon-induced transmembrane
26 protein; HCVpp, HCV pseudoparticle; HMAb, human monoclonal antibody; HCVcc, cell culture
27 derived HCV; MLV, murine leukemia virus ; TCID₅₀, tissue culture infectious dose 50 % ;
28 nAb, neutralizing antibody.

29

30 **Author contributions.** FW designed, performed and analyzed experiments. LH and GL
31 performed and analyzed experiments. TFB, MBZ and CS designed and analyzed experiments
32 and edited the MS and figures. BK, AT and JB helped design and carry out the study through
33 generation and testing of novel E1E2 clones and HCV chimeras and provided important critique
34 and intellectual content to the manuscript, MW and SP provided IFITM expression plasmids,
35 analyzed IFITM expression and localization by IF and edited the MS. ZK and SKHF provided
36 anti-HCV HMAbs and edited the MS text. PP provided liver tissue resections for isolation of
37 primary human hepatocytes, FH provided serum from chronically infected patients. FW and TFB
38 wrote the MS. TFB initiated and coordinated the study.

39

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49

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60

61 Abstract

62 While adaptive immune responses against hepatitis C virus (HCV) infection have been studied
63 in great detail, the role of innate immunity in protection against HCV infection and immune
64 evasion is only partially understood. Interferon-induced transmembrane proteins (IFITMs) are
65 innate effector proteins restricting host cell entry of many enveloped viruses, including HCV.
66 However, the clinical impact of IFITMs on HCV immune escape remains to be determined. Here,
67 we show that IFITMs promote viral escape from the neutralizing antibody response in clinical
68 cohorts of HCV-infected patients. Using pseudoparticles bearing HCV envelope proteins from
69 acutely infected patients, we show that HCV variants isolated pre-seroconversion are more
70 sensitive to the antiviral activity of IFITMs than variants from patients isolated during chronic
71 infection post-seroconversion. Furthermore, HCV variants escaping neutralizing antibody
72 responses during liver transplantation exhibited a significantly higher resistance to IFITMs than
73 variants that were eliminated post-transplantation. Gain-of-function and mechanistic studies
74 revealed that IFITMs markedly enhance the antiviral activity of neutralizing antibodies and
75 suggest a cooperative effect of human monoclonal antibodies and IFITMs for antibody-mediated
76 neutralization driving the selection pressure in viral evasion. Perturbation studies with the IFITM
77 antagonist amphotericin B revealed that modulation of membrane properties by IFITM proteins
78 is responsible for the IFITM-mediated blockade of viral entry and enhancement of antibody-
79 mediated neutralization. **Conclusion:** Our results identify IFITM proteins as a previously
80 unknown driver of viral immune escape and antibody-mediated HCV neutralization in acute and

81 chronic HCV infection. These findings are of clinical relevance for the design of urgently needed
82 HCV B cell vaccines and might help to increase the efficacy of future vaccine candidates.

83

84 **Introduction**

85 It is estimated that more than 71 million patients are chronically infected with hepatitis C virus
86 (HCV) (1). HCV infection is a leading cause of liver disease and cancer worldwide. The
87 development of direct acting antivirals markedly improved the outcome of antiviral treatment with
88 cure of the majority of treated patients (2). However, several challenges remain (3). High
89 treatment costs prevent or limit access of patients to therapy in resource-poor countries and may
90 lead to selective use even in industrialized countries. Moreover, in the majority of cases HCV
91 infection remains undiagnosed or is diagnosed at a late stage due to the limited efficacy of
92 current HCV screening programs. Furthermore, direct acting antivirals will not cure virus-induced
93 end-stage liver disease such as hepatocellular carcinoma and certain patient subgroups do not
94 respond to or cannot tolerate direct acting antiviral-based treatment strategies (4, 5). Finally,
95 reinfection remains possible, making control of HCV infection difficult in people at risk, such as
96 drug abusers. These unmet medical needs warrant the development of an effective vaccine,
97 protecting from chronic HCV infection as a means to impact the epidemic on a global scale (3).

98 Both cellular and humoral immune responses have been suggested to play a key role in
99 protection against infection in humans and nonhuman primates. Thus, vaccine development has
100 focused on eliciting both B and T cell responses (3). Indeed, a B cell vaccine consisting of
101 recombinant E1E2 viral envelope glycoprotein was shown to provide partial protection against

102 chronic HCV infection (6), to induce virus neutralizing antibodies and to be safe in healthy
103 volunteers (7). Furthermore, broadly virus neutralizing antibodies have been shown to confer
104 protection against HCV in humanized mouse models (8, 9) and are considered a promising
105 strategy to fight emerging infectious diseases (10). While adaptive immune responses have
106 been studied in great detail, the role of innate immune responses in HCV infection is only partially
107 understood.

108 The innate immune response constitutes the first line of defense against viral infections.
109 Interferons stimulate the expression of a set of more than 300 interferon-stimulated genes,
110 several of which have been shown to exert antiviral activity against HCV (11). A family of these
111 genes, the interferon-induced transmembrane (IFITM) proteins are potent inhibitors of host cell
112 entry of a broad range of enveloped viruses, including HCV (12-15). While IFITM1 is primarily
113 located at the cytoplasmic membrane and restricts HCV entry by interacting with the HCV co-
114 receptor CD81 (13), IFITM2 and 3 localize to endosomal compartments and potentially restrict
115 viral infection by blocking virus entry at the stage of hemifusion (16) or fusion pore formation
116 (17).

117 While the antiviral activity of the IFITM proteins against HCV has been studied in cell
118 culture models (13, 14, 18), the role of the IFITM proteins in viral pathogenesis during clinical
119 HCV infection is unknown. It is unclear whether inhibition of virus entry by IFITM proteins
120 contributes to viral clearance, whether IFITM-HCV interactions impact viral persistence in
121 chronic infection and whether IFITM proteins and antibodies cooperate to inhibit viral entry.

122 Clinical cohorts for the study of acute and chronic HCV infection have been a valuable
123 tool to investigate the mechanisms of HCV persistence and escape. These include cohorts
124 comparing early and late stage infection (19, 20). Furthermore, liver graft infection is a unique
125 model since it allows the study of HCV infection and viral escape in a very well defined timeframe
126 and detailed patient material (21-23).

127 To address the clinical role of IFITMs for viral escape and B cell responses, we
128 investigated virus-host interactions of IFITM proteins and neutralizing antibodies during HCV cell
129 entry. For this, we used HCV pseudoparticles (HCVpp) bearing envelopes from patients with
130 acute infection prior to seroconversion or patients undergoing liver transplantation due to chronic
131 hepatitis C. Moreover, we employed neutralizing human monoclonal antibodies (HMAbs) derived
132 from patients with chronic HCV infection.

133

134 **Material and Methods**

135 **Human material.** Human material, including sera and liver tissues from patients
136 undergoing surgical resection for isolation of human hepatocytes and followed at Strasbourg
137 University Hospital, was obtained with informed consent from all patients. The protocol was
138 approved by the Ethics Committee of Strasbourg University Hospital (CPP 10-17).

139 **Cell lines and primary human hepatocytes.** 293T cells, Huh7.5.1, Huh7.5.1-NTCP and
140 HepG2-CD81 cells were isolated and cultured as described previously (18, 19, 24). Primary
141 human hepatocytes (PHH) were isolated from liver resections as described previously (25).

142 **Antibodies.** The anti-E2 HMABs (CBH-20, CBH-7, CBH-22, HC84.26.WH.5DL) and
143 human anti-HCV sera have been described previously (19, 24, 26-29). The antibodies directed
144 against IFITM1, IFITM2/3 (Proteintech) and β -actin (Sigma) and the protocols for detection of
145 IFITM proteins by western blot and immune fluorescence have been described in (30).

146 **Plasmids.** The plasmids for the generation of HCVpp and cell culture derived HCV
147 (HCVcc) (Jc1 (genotype 2a) chimera Luc-Jc1 and Con1 (genotype 1b) chimera Con1R2A) have
148 been described in (21), The plasmids coding for the envelope proteins and HCVcc chimera
149 bearing envelope proteins isolated from patients undergoing liver transplantation were described
150 in (21). The plasmids encoding the envelope variants of acute patients have been described in
151 (31) (accession numbers KU285163, KU285164 and KU285165). Plasmid UKN1a.16.16
152 (accession number MK124622) was generated as described in (32). The plasmids encoding the
153 envelope proteins of the chronic variants are described in (32) (UKN1A14.38 and UKN3A1.28.),
154 (33) (HCV-J) and in (34) (gt3SXB1). The full-length chimeric clone incorporating the UKN1.5.3
155 E1E2 genes was generated in the H77/JFH-1 virus background, using previously described
156 methods (35).

157 **Statistics.** Data are shown as mean \pm SEM if $n \geq 3$. Representative experiments are
158 shown as mean \pm SD. Normality was assessed using the Shapiro-Wilk test. The 1-tailed
159 Student's t test was used for single comparisons. A p-value of less than 0.05 was considered
160 statistically significant.

161 **Vector production, transduction and selection of stable cells.** Retroviral vectors for
162 transduction were generated by transfection of 293T cells as described previously (30) using the

163 CMV-Gag-Pol MLV (mouse leukemia virus) packaging construct, a vesicular stomatitis virus-G-
164 encoding plasmid and plasmids coding for the IFITM proteins (pQCXIP) or empty vector as a
165 control (30). For transduction, cells were seeded at subconfluent density and spin-inoculated
166 with the retroviral vectors at 4000 x g for 30 min. Cells were then incubated at 37°C for 48 hours
167 to allow efficient transgene expression. Cells stably expressing IFITM proteins were
168 subsequently selected with 1.8 µg/mL puromycin.

169 **HCVpp production, infection, and neutralization.** HCVpp were generated by
170 transfection of 293T cells as described previously (19). To study HCV entry, HCVpp were added
171 to IFITM-transduced Huh7.5.1, Huh7.5.1-NTCP cells or PHH in triplicate and incubated for 72 h
172 at 37°C. HCV entry was determined by analysis of luciferase reporter gene expression as
173 described previously (24). For the study of antibody-mediated neutralization, HCVpp were mixed
174 with autologous anti-HCV serum, control serum, anti-E2 HMAbs or irrelevant isotype control IgG,
175 preincubated for 30 min at 37°C and added to Huh7.5.1, Huh7.5.1-NTCP cells or PHH in
176 triplicate for 72 h at 37°C (21, 24). To assess the effect of amphotericin B on the cooperative
177 inhibition of HCV entry by IFITM proteins and neutralizing antibodies, Huh7.5.1 cells were
178 treated with 5 µmol/L amphotericin B (Sigma Aldrich) for 1 hour at 37 °C prior to infection with
179 antibody-treated HCVpps.

180 **HCVcc production, infection, and neutralization.** Plasmids for cell culture-derived
181 HCV (HCVcc) production of Jc1 and Con1 chimera with luciferase reporter (Luc-Jc1 and Con1-
182 R2A) have been described previously (22, 36-39). HCVcc were produced in Huh7.5.1 cells as
183 described previously (39). Infectivity was quantified by luciferase activity, or by determining the

184 tissue culture infectious dose 50% (TCID50) (22). HCVcc neutralization using patient serum,
185 IgG, and mAbs was analyzed as described previously (22).

186

187 **Results**

188 **IFITM proteins inhibit cell entry of HCVpp and HCVcc.** To characterize the role of
189 IFITMs in clinical HCV infection, we first investigated inhibition of viral entry into cells. For this,
190 we transduced Huh7.5.1 or Huh7.5.1-NTCP cells with retroviral vectors encoding the antivirally
191 active IFITM proteins (IFITM1, IFITM2 and IFITM3) and then infected the cells with HCVpp
192 bearing the envelope proteins of HCV genotype 1b. Huh7.5.1-NTCP cells were used since
193 NTCP has been described to have a functional role in regulation of interferon stimulated gene
194 expression (18). Since no differences in IFITM antiviral activity on HCV entry and infection were
195 observed between Huh7.5.1 and Huh7.5.1-NTCP cells, when IFITMs were exogenously
196 expressed, Huh7.5.1 cells were then used for all subsequent experiments. Entry of HCVpp was
197 restricted by all three IFITMs, with IFITM2 and 3 showing a slightly higher restriction than IFITM1
198 (Figure 1A). Pseudoparticles bearing the envelope protein of the IFITM resistant retrovirus MLV
199 (40) were used as negative control (Figure 1B). Entry driven by the MLV-envelope protein was
200 not modulated by IFITM proteins, as expected. To analyze the impact of cell polarization, which
201 might affect IFITM activity due an altered subcellular localization of IFITM1 in hepatocytes, as
202 reported by Wilkins et al (13), we studied the effect of IFITMs on HCVpp entry in polarized
203 HepG2-CD81 cells in side-by-side experiments. The inhibition pattern observed upon IFITM
204 expression was very similar to that seen for nonpolarized Huh7.5.1 cells (Figure 1C and Figure

205 S1), suggesting that polarization appears not to modulate the ability of IFITM proteins to block
206 HCV entry. This is in line with the finding that IFITM-expression did not alter CD81 surface
207 expression (Figure S2) or distribution (Figure S3). We next assessed the effect of IFITM proteins
208 on HCV entry in the context of authentic virus using infectious HCVcc. The sensitivity of HCVcc
209 infection to inhibition by IFITM proteins was assessed in Huh7.5.1 cells stably expressing
210 IFITM1, 2 or 3. Similar to results observed for HCVpp, infection of Huh7.5.1 cells by HCVcc was
211 inhibited by all three IFITMs (Figure 1D). Expression of IFITM proteins was confirmed by
212 immunoblot (Figure 1E).

213 Finally, we studied the subcellular localization of IFITMs in Huh7.5.1 cells.
214 Immunohistochemistry studies (Figure 1F, Figure S3) showed that IFITM1 was located at the
215 plasma membrane, as shown by colocalization with SYFP tagged with a membrane-targeting
216 signal, while IFITM2 and 3 were found in endosomal compartments, as shown by partial
217 colocalization with the endosomal marker Rab7a (Figure S3). These observations are similar to
218 previous results observed in Huh7 cells (14). In summary, these results demonstrate that
219 infection of Huh7.5.1 with HCVpp or HCVcc is a suitable model to study the molecular
220 mechanisms of inhibition of HCV infection by IFITM proteins.

221 **Clinical variants isolated during acute HCV infection before seroconversion were**
222 **more sensitive to anti-viral activity of IFITMs than variants from chronic infection.**

223 To understand the role of IFITMs in the acute phase of infection, we analyzed the IFITM-
224 sensitivity of HCVpp expressing viral envelopes of three HCV variants isolated from the same
225 patient at three different time points post infection (UKNP1.5.1 pre-seroconversion; UKNP1.5.2

226 acute phase, two months later; UKNP1.5.3 chronic phase, 7 months later; Table 1). These
227 variants vary at key residues, including residues near or within the CD81 binding sites (aa312;
228 439; 500; 536; 626; 742) (31) (Figure 2E). As shown in Figure 2A and Figure S4, HCVpp
229 expressing envelopes of all variants were comparably susceptible to inhibition by all three tested
230 IFITM proteins. Interestingly, the analysis of the HCVpp bearing sequential HCVpp envelope
231 proteins revealed a marked and significant decrease of IFITM-sensitivity over time (Figure 2A).
232 This decrease was not due to differences in the relative infectivities of the HCVpp (Figure 4A).
233 Thus, infection mediated by the envelope proteins of variant UKNP1.5.1, isolated before
234 seroconversion, and variant UKNP1.5.2, isolated right after seroconversion, was inhibited by
235 98% and 85% respectively. In contrast, transduction driven by the envelope proteins of variant
236 UKNP1.5.3, which was isolated six months after seroconversion during the chronic phase of
237 infection, was inhibited by only 60% upon directed expression of IFITM proteins (Figure 2A). The
238 results obtained for UKNP1.5.3 were confirmed using an HCVcc chimera (Figure 2B), whereas
239 the infectivity of HCVcc derived from the two other strains was too low to obtain conclusive
240 results. Next, we investigated whether the differential IFITM-sensitivity of HCV envelope proteins
241 obtained pre-seroconversion and during chronic infection could be confirmed with a larger panel
242 of samples. For this, we analyzed the envelope proteins from six different early acute HCV
243 patients and four variants derived from chronically infected HCV patients. Among the pre-
244 seroconversion isolates, the highest susceptibility was observed for UKNP1.3.1 with more than
245 98-99 % inhibition of entry upon IFITM protein expression (Figure S4). UKNP1.6.1 was the most
246 resistant with about 85 % inhibition (Figure S4), which correlated with the sensitivity to

247 neutralizing antibodies that was published previously (31). The neutralization sensitivity of the
248 E1E2 proteins of this cohort was shown to be consistent in HCVpp and HCVcc models of
249 infection (31), indicating the same holds true for their IFITM-sensitivity. When we compared the
250 entry of HCVpp expressing pre-seroconversion envelope glycoproteins to entry of HCVpp
251 bearing envelope glycoproteins derived from independent chronic samples of the same
252 genotypes, we observed a significant and unexpected difference in IFITM-susceptibility. The
253 HCVpp bearing envelopes from variants isolated from chronic infection post-seroconversion
254 were much more resistant to inhibition by IFITM proteins (Figure 2C, 2D and Figure S4),
255 independent of the genotype of the variants. Taken together, these results suggest that IFITMs
256 may pose significant selective pressure on HCV during the acute phase of infection that can
257 result in viral evasion. The identification of mutations unique to variants during chronic infection
258 suggest their possible involvement in these interactions.

259 **Clinical HCV variants associated with viral immune escape during liver**
260 **transplantation are more resistant to inhibition by IFITM proteins than non-escape**
261 **variants.** To investigate the contribution of IFITM proteins to viral escape in chronic HCV
262 infection, we took advantage of a well-characterized clinical cohort of patients undergoing liver
263 transplantation with *de novo* infection of the liver graft (21, 22) (Table 1). In this cohort, variants
264 selected post-transplantation are characterized by more efficient viral entry and escape from
265 neutralizing antibodies (21, 22). We produced HCVpp bearing the full length E1/E2 proteins of
266 variants differing in sensitivity to neutralizing antibodies and subsequently infected transiently
267 transduced IFITM-expressing Huh7.5.1 cells. We observed that all patient-derived envelope

268 proteins were sensitive to inhibition by IFITM proteins. However, variants that were characterized
269 by escape from the neutralizing antibody (nAb) response were less affected by expression of
270 IFITM proteins than those that were sensitive to neutralizing antibodies, as shown for variants
271 derived from two different patients (Figure 3A). This was confirmed by TCID₅₀ analyses on
272 IFITM2 and 3 expressing cells using HCVcc chimeras expressing the envelope proteins of two
273 representative variants (variant VL with nAb escape phenotype and variant VA with nAb
274 sensitivity) isolated from the same patient (Figure 3B) (22). Next, we extended our analysis to
275 19 envelope variants (nine non-nAb escape and ten nAb escape-variants (Figure S5)) derived
276 from five different patients and observed a significantly higher sensitivity to inhibition by IFITM2
277 (Figure 3C) and IFITM3 (Figure 3D) for non-nAb escape variants and a significantly higher
278 resistance of nAb escape variants to inhibition by IFITM proteins. The direct comparison of entry
279 efficiency and IFITM-sensitivity revealed no apparent correlation of these two variables (Figure
280 4B). This shows that indeed selection for IFITM-sensitivity post-liver transplantation and not just
281 more efficient entry of the escape variants is responsible for the differential inhibition by IFITM
282 proteins.

283 In summary, these results suggest that IFITM proteins are important determinants for viral
284 escape and that escape from IFITM proteins is associated with resistance to antibody-mediated
285 neutralization.

286 **Neutralizing antibodies and IFITM proteins cooperatively block HCV entry.** The
287 differential inhibition of antibody escape and non-escape HCV strains by IFITM proteins in
288 chronic infection as well as the enhanced IFITM-sensitivity of viral strains in the acute phase

289 prior to antibody development prompted us to analyze the interplay between the antiviral
290 activities of IFITM proteins and the neutralizing B cell response in detail. For this, HCVpp were
291 incubated with low concentrations of neutralizing sera prior to infection of transiently transduced
292 IFITM2 expressing cells. Treatment with low concentrations of neutralizing patient serum (1:200)
293 did not significantly reduce viral entry into IFITM-negative control cells (Figure 5A). IFITM2
294 expression reduced virus entry in the absence of neutralizing sera by ten-fold for the non-escape
295 variant and five-fold for the escape variant, respectively (Figure 5A). When serum-treated
296 HCVpp were used to infect IFITM2-expressing cells, we observed a marked increase in
297 neutralization. The neutralization was about three- to four-fold higher compared to the control-
298 treated HCVpp, although the same serum-treatment had no effect on IFITM-negative control
299 cells. Furthermore, the increase in neutralization was significantly and markedly higher for the
300 non-escape variants compared to the escape variants (Figure 5A), suggesting a potential role
301 of the IFITM-mediated enhancement of neutralization as a determinant for viral escape. Titration
302 of the neutralizing serum corroborated our finding that neutralizing antibodies and IFITM proteins
303 cooperatively block virus entry. Inhibition correlated with the concentration of the neutralizing
304 serum, as shown by the slope of the regression curves for neutralization on control or IFITM2-
305 expressing cells (Figure 5B). The slope on IFITM2-expressing cells was more than ten-fold
306 higher as compared to the IFITM-negative control cells confirming a marked enhancement of
307 neutralization by IFITM2. Furthermore, the IFITM-mediated enhancement of neutralization was
308 confirmed using HCVcc of genotype 1b (Con1). As shown in Figure 5C, expression of IFITM2

309 enhanced the neutralization of HCVcc Con1 by a weakly neutralizing heterologous serum (1:100
310 dilution) from less than two-fold to 60-fold (Figure 5C).

311 To assess which serum component was responsible for the enhanced neutralizing
312 capacity of the sera in the presence of IFITM proteins we used HMABs directed against different
313 epitopes of the HCV E2 protein, some of which overlap with the polymorphic sites in acute patient
314 variants. Similar as in experiments using sera, the HMABs were used at sub-neutralizing
315 concentrations (15 µg/mL) that in our model only had a low effect on virus entry inhibition (at
316 maximum about 40 % or 1.67 fold inhibition by HC84.26.WH.5DL, lower for the other HMABs)
317 (Figure 5D). When IFITM2-expressing cells were infected with the HMAb-treated HCVpp we
318 again observed a marked cooperative effect that directly correlated with the neutralizing
319 properties of the antibody. Indeed, while the non-neutralizing HMAb CBH-20 did not exert a
320 cooperative effect, the affinity matured anti-E2 antibody HC84.26.WH.5DL with potent
321 neutralizing properties increased the inhibition of virus entry following IFITM2 expression to more
322 than 100-fold (Figure 5D). The antibodies CBH-7, HC11 and CBH-2 had intermediate effects
323 (Figure 5D).

324 Next, we confirmed these findings in the most physiologically relevant cell culture system:
325 infection of PHH (Figure 5E). Similar to the Huh7.5.1 cells, treatment of the HCVpp with low
326 concentrations of antibodies only had a minor influence on virus entry into naive PHH. As shown
327 for Huh7.5.1 cells, IFITM2 expression blocked HCV entry into PHH, with the non-escape variant
328 being more susceptible. Treatment with the neutralizing antibodies HC-11 and CBH-2 increased
329 the neutralization of the non-escape variant on IFITM-expressing cells by about 3-fold. Again,

330 the increase in neutralization was markedly lower in the escape variants (Figure 5E), confirming
331 the results that were obtained with Huh7.5.1 cells. Taken together, these data show that innate
332 and adaptive immune responses targeting viral entry cooperate to inhibit HCV infection and drive
333 viral immune evasion in acute and chronic HCV infection.

334 **Cooperative inhibition of HCV entry by IFITM proteins and neutralizing antibodies**
335 **is attenuated by treatment with amphotericin B.** It is known that IFITM proteins restrict virus
336 entry at the stage of hemifusion (16) or fusion pore formation (17) by altering curvature and
337 fluidity of host cell membranes through direct or indirect mechanisms, which render virus-host
338 cell fusion less energetically favorable (41, 42). Notably, the antiviral effect of IFITM2 and 3 on
339 influenza virus infection is attenuated by incubation of host cells with amphotericin B, an
340 amphiphilic antifungal drug that integrates into endosomal membranes (43), which can be
341 regarded as an IFITM antagonist. Mechanistic studies revealed that the compound decreases
342 the curvature and increases the fluidity of the endosomal membrane, which counteracts the
343 IFITM-mediated antiviral effects within the endosomal membrane in an indirect manner (43), as
344 illustrated in Figure 6D. We thus used amphotericin B to analyze whether IFITM-mediated
345 modulation of membrane properties and the resulting inhibition of viral entry is required for the
346 cooperative antiviral activity of antibodies and IFITM proteins. Treatment of cells with
347 amphotericin B reduced HCVpp entry into the host cells by about three-fold (Figure 6A) and
348 markedly reduced the antiviral activity of all IFITM proteins (Figure 6B). Moreover, amphotericin
349 B treatment largely abrogated the cooperation of IFITMs and neutralizing sera in inhibition of
350 HCVpp cellular entry (Figure 6C), indicating that IFITM-mediated modulation of cellular

351 membranes and the resulting antiviral activity are responsible for the cooperative inhibition of
352 virus entry by IFITM proteins and antibodies (illustrated in Figure 6D). Interestingly, a similar
353 enhancement of neutralization was observed using interferon-treatment of Huh7 cells (Figure
354 S6) supporting our conclusion that the IFITM-mediated antiviral effect and not a direct interaction
355 with the IFITM proteins is responsible for the enhancement of neutralization.

356

357 **Discussion**

358 In this study we provide conclusive evidence that IFITM proteins are important determinants of
359 viral escape from antiviral B cell responses in patients. This is supported by our finding that viral
360 envelope proteins obtained from acute pre-seroconversion patients showed an increased IFITM-
361 sensitivity as compared to envelope proteins obtained from chronic patients. Furthermore, the
362 functional analysis of HCV variants from patients escaping viral neutralizing responses during
363 liver transplantation compared with variants that are eliminated post-transplantation revealed a
364 direct correlation of escape from neutralizing responses and resistance to inhibition by IFITM
365 proteins.

366 Our finding that HCV variants of acute patients isolated pre-seroconversion were more
367 susceptible to inhibition by IFITM proteins than variants derived from chronically-infected
368 patients indicates that IFITM proteins drive immune evasion. This is supported by our analysis
369 of sequentially isolated envelope proteins from one patient. IFITM resistance increased over
370 time, with the envelope proteins isolated during chronic infection showing the highest IFITM
371 resistance. Furthermore, the acquisition of mutations within epitope II and the CD81 binding

372 domain that are targeted by neutralizing antibodies suggests that the IFITMs might modulate
373 interactions with the adaptive immune response. Indeed, the analysis of post-transplant variants
374 revealed a direct association of resistance to inhibition by IFITM proteins and escape from the
375 nAb response. Escape of the virus from host responses is critical for viral spread and survival
376 (44). In part, these findings could explain the low efficacy of innate immune activation in chronic
377 HCV-infected patients (45). Escape from innate responses does not only prevent the immune
378 system from clearing the viral infection but also limits the response to interferon-based therapies
379 (44). Furthermore, the finding that variants selected post-liver transplantation and characterized
380 by viral escape were significantly more resistant to inhibition by IFITM proteins than variants that
381 were eliminated post-transplantation (with sensitivity to antibody-mediated neutralization) could
382 also explain the rapid selection of these resistant variants. In addition, the distribution of the
383 IFITM-sensitivity of the escape variants appeared to be less dispersed than that of the non-
384 escape variants, potentially reflecting a bottleneck during the selection process. Differences in
385 IFITM expression levels between host and graft tissue might drive the selection of highly
386 infectious IFITM resistant variants, subsequently leading to reinfection of the graft, as universally
387 observed.

388 How can mutations present in HCV in chronic patients confer relative IFITM resistance?
389 For one, HCV variants that escape immune control frequently exhibit enhanced binding to the
390 HCV co-receptor CD81 (22) and IFITM1 has been suggested to exert antiviral activity in part by
391 interacting with CD81 (13). However, there are no reports of direct interactions of IFITM2 and 3
392 with CD81 and other HCV (co-)receptors (14), although IFITM3 seems to partially colocalize with

393 CD81 (14). Additionally, IFITM resistant HCV, like IFITM resistant influenza A viruses (46), might
394 exhibit an altered pH-optimum for virus entry thereby avoiding the need to fuse with IFITM-rich
395 internal membranes. Finally, IFITM-sensitivity might be linked to the number of viral
396 glycoproteins incorporated into the viral membrane, as previously demonstrated for simian
397 immunodeficiency virus (47) or to the composition of the viral membrane itself, however our
398 previous characterization of HCV envelope glycoprotein variants proteins in liver transplantation
399 indicate that an increased amount of incorporation of envelope proteins is most likely not
400 responsible for the observed changes in IFITM-sensitivity (21). It remains to be determined
401 whether IFITM-induced changes in membrane composition or altered interaction with
402 lipoproteins contribute to the IFITM-mediated escape from the nAb response.

403 The proteins of the IFITM family are potent inhibitors of host cell entry of a wide range of
404 enveloped viruses (11), including HCV (13, 14, 18). A single-cell analysis of clinical human liver
405 samples by laser capture microdissection and qRT-PCR revealed that IFITM3 expression and
406 HCV RNA were largely mutually exclusive (48), indicating an important role of the IFITM proteins
407 in HCV cell tropism. A very recent publication indicates that stem cells, that do not respond to
408 interferon, express high levels of interferon stimulated genes, including IFITM3, to protect them
409 from viral infection (49). Constitutive expression of interferon stimulated genes is lost upon
410 differentiation into hepatocyte-like cells (49) but becomes interferon-inducible, highlighting an
411 important contribution of the IFITM proteins to the innate defenses against HCV and other
412 pathogens.

413 Perturbation studies with amphotericin B that acts as an IFITM-antagonist in the context
414 of influenza virus infections (43) revealed that modulated membrane-properties are responsible
415 for the IFITM-mediated enhancement of neutralization, indicating that other innate entry
416 effectors, as shown for pretreatment with interferon-alpha, might exploit similar inhibitory
417 mechanisms to block virus entry.

418 Interestingly, a recent study has suggested a different role for IFITMs in clinical HIV-1
419 infection: While transmitted founder viruses were almost resistant to inhibition by IFITM
420 proteins, the virus became more susceptible over time, as it escaped from the nAb response
421 (50). This observation could reflect different roles of innate immune responses in HCV and HIV
422 infections with our findings supporting a much more prominent role of the IFITMs during acute
423 HCV infection compared to HIV. Furthermore, the increased IFITM-sensitivity could in part
424 explain the high susceptibility of acute HCV infection to interferon treatment. On a mechanistic
425 point of view, IFITM-sensitivity of HIV was associated with receptor-usage, with CCR5-tropic
426 viruses being generally more resistant to IFITM proteins than CXCR4-tropic viruses (50). This
427 suggests that the differential sensitivity of HIV to inhibition by the IFITM proteins might be due
428 to receptor-mediated targeting to subcellular compartments with differential IFITM expression,
429 or due to changes in envelope structure and electrochemical properties due to the switch of
430 receptor tropism from CCR5 to CXCR5, which was not observed for HCV infection.

431 Taken together, our findings show that IFITMs are important drivers of viral immune
432 evasion in acute and chronic HCV infection by enhancing antibody—mediated neutralization.
433 Harnessing these effects will help to facilitate the design of protective B cell HCV vaccines.

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573

574 Author names in bold designate shared first authorship

575 **Figure Legends**

576 **Figure 1: Directed IFITM expression inhibits HCV infection in cell culture.** Huh7.5.1 (A and
577 B) or HepG2-CD81 (C) cells were transduced with vectors encoding the indicated IFITM proteins
578 or were transduced with empty vector as control. Transduced cells were then infected with
579 HCVpp of GT1b (A), GT1a (C) or with MLVpp (B). Infection was assessed after 72 h by
580 measuring luciferase activity. Individual representative experiments are shown. Error bars
581 represent SD. Similar results were obtained in more than three independent experiments. (D)
582 Huh7.5.1 cells stably expressing IFITMs were infected with HCVcc Luc-Jc1. Infection efficiency
583 was assessed after 72 h by measuring luciferase activity. Shown is a representative of three
584 independent experiments, error bars indicate SD. (E) Expression of IFITM proteins in Huh7.5.1
585 cells was assessed by immunoblot using IFITM-specific antibodies. β -Actin is used as loading
586 control. One representative western blot is shown (F). Huh7.5.1 cells transduced with IFITM1-3
587 or a control vector were stained with a mouse anti-IFITM1 or a rabbit anti-IFITM2 antibody
588 (IFITM2 and 3).

589

590 **Figure 2: Differential sensitivity of acute and chronic HCV variants to inhibition by IFITM**
591 **proteins.** Huh7.5.1 cells were transduced to express the indicated IFITM proteins and then
592 infected with HCVpp bearing envelope proteins derived from acute or chronic patients. (A)
593 Analysis of the IFITM-sensitivity of sequential envelope variants (UKNP1.5.1 pre-
594 seroconversion; UKNP1.5.2 acute phase, two months later; UKNP1.5.3 chronic phase, 7 months
595 later), isolated from a single HCV patient. (B) TCID₅₀ analysis of UNKP1.5.3 HCVcc infection of

596 transduced IFITM-expressing Huh7.5.1 cells. Shown are means of two experiments performed
597 in sextuplicates. Error bars represent SD. (C) and (D) E1E2 patient variants isolated from
598 patients with acute or chronic HCV infection of genotype 1A and 3. Each dot represents the
599 result for a single envelope variant and IFITM2- (C) or IFITM3-expressing (D) Huh7.5.1 cells.
600 Shown are the means of three experiments conducted in triplicates. Error bars represent SEM.
601 Control was set to 100%. *P < 0.05, ** P < 0.01 using one-sided students T-test. (E) Clustal O
602 Alignment of the protein sequences of H77c, UKNP1.5.1, UKNP1.5.2 and UKNP1.5.3. The
603 sequences were obtained from GenBank. Changes highlighted in yellow are unique for
604 UKNP1.5.1, changes in green are only present in UKNP1.5.2 and changes highlighted in red
605 are unique for UKNP1.5.3. HVR1 and 2 are indicated in blue, red letters mark key positions for
606 CD81-binding.

607

608 **Figure 3: IFITMs differentially restrict HCV variants isolated from patients undergoing**
609 **liver transplantation.** Huh7.5.1 cells were transduced to express the indicated IFITM proteins
610 and then infected with HCVpp pseudotyped with HCV E1E2 patient variants isolated from
611 patients undergoing liver transplantation. Infection was assessed after 72 h by measuring
612 luciferase activity. (A) Results for variants from two patients are expressed as means \pm SEM
613 percentage HCVpp infection compared to control cells (set at 100%) from three independent
614 experiments performed in triplicate. (B) TCID50 analysis of HCVcc infection of IFITM-expressing
615 Huh7.5.1 cells. Shown are means of three experiments performed in sextuplicates. Error bars
616 represent SEM. Control was set to 100 %. (C and D) Each dot represents the result for a single

617 variant. Results for % Infection of IFITM2 (C) and IFITM3 (D) positive cells compared to the
618 control of HCV variants from five different patients. *P < 0.05; ** P < 0.01 one-sided Students
619 T-test. Shown are the means of three experiments performed in triplicates.

620

621 **Figure 4: The antiviral effect of the IFITMs is independent of virus infectivity.** Huh7.5.1
622 cells were transduced to express IFITM proteins and infected with HCVpp bearing the envelope
623 proteins of the indicated variants. (A) Shown are three individual datasets of the experiments
624 that are featured in figure 2A. (B) Correlation of relative infectivity (highest infectivity set to 1)
625 and relative IFITM-sensitivity (highest IFITM-sensitivity set to 1, lowest to 0). Each dot represents
626 one of the escape variants shown in Figure 3C and 3D. Shown are the results of one
627 representative experiment performed in triplicate (n=9).

628

629 **Figure 5: IFITM2 enhances antibody-mediated neutralization of HCV cell entry.** Huh7.5.1
630 cells (A, B, C, D) or primary human hepatocytes (E) were transduced by retroviral vectors coding
631 for IFITM2 or empty vector as control. Forty-eight hours after transduction cells were infected
632 with HCVpps expressing the envelope of variants selected during liver graft infection associated
633 or not-associated with viral escape (A,B,D,E), or with HCVcc of genotype 1b (Con1) (C). Before
634 infection, the particles were coincubated with serum derived from chronically HCV infected
635 patients (A, B, C) or 15 µg/ml of the patient-derived HMAbs CBH-20 CBH-7, HC11, CBH-2 and
636 HC84.26.WH.5DL or control antibody R04 (D, E) at 37 °C for 1 h. Entry of HCVpp was assessed
637 72 h post infection by measuring luciferase activity. Results are shown as fold inhibition of virus

638 entry. Inhibition of entry by control vector in combination with the control antibody R04 or with
639 control serum was set to 1. (B) The equation of the regression curve of anti-HCV serum and
640 IFITM2 expressing cells was calculated as $48748 x^2 + 640 x + 1.9$ The corresponding curve for
641 anti-HCV serum on naïve cells was $4380 x^2 + 149 x + 1.1$ (C) Infection with HCVcc was analyzed
642 72 h post infection by measuring luciferase activity. The graph represents means of three (D) or
643 four (A, B) experiments that were performed in triplicates. Error bars represent SEM. (C) Shows
644 a representative experiment performed in triplicates (n=6). (E) Represents a single experiment
645 performed in PHH. Error bars show SD.

646

647 **Figure 6: Cooperative inhibition of HCV entry by IFITM proteins and neutralizing**
648 **antibodies can be attenuated by treatment with amphotericin B.** Huh7.5.1 cells were
649 transduced by retroviral vectors coding for an empty control vector (A), with vectors coding for
650 IFITM1, 2 and 3 and empty vector as control (B) or only IFITM2 plus control (C) Forty-eight h
651 after transduction cells were treated with vehicle control or 5 µg/ml amphotericin B for 1 h.
652 Afterwards, the cells were infected with HCVpp expressing the envelope of a variant not
653 associated with viral escape and sensitive to antibody-mediated neutralization (P1VA). (C) Cells
654 were infected with HCVpp pretreated with serum derived from chronically HCV infected patients
655 or with control serum at 37 °C for 1 h. Entry of HCVpp was assessed 72 h post infection by
656 measuring luciferase activity. (A) Results are shown in RLU. (B,C) Results were normalized for
657 the vector control and are shown as fold inhibition compared to the respective controls. Shown
658 are the means of representative experiments performed in triplicates (n=6) ± SD. (D) Model of

659 cooperative inhibition of HCV entry by IFITMs and neutralizing antibodies and the antagonistic
660 effect of amphotericin B. The interaction between infectious particles and cell surface receptors
661 triggers endocytosis. Entry is blocked by IFITM proteins and neutralizing antibodies.
662 Amphotericin B (AmphoB) is believed to rescue virus entry by antagonizing the IFITM-mediated
663 increase of membrane rigidity and curvature.

664

665

Figure 1

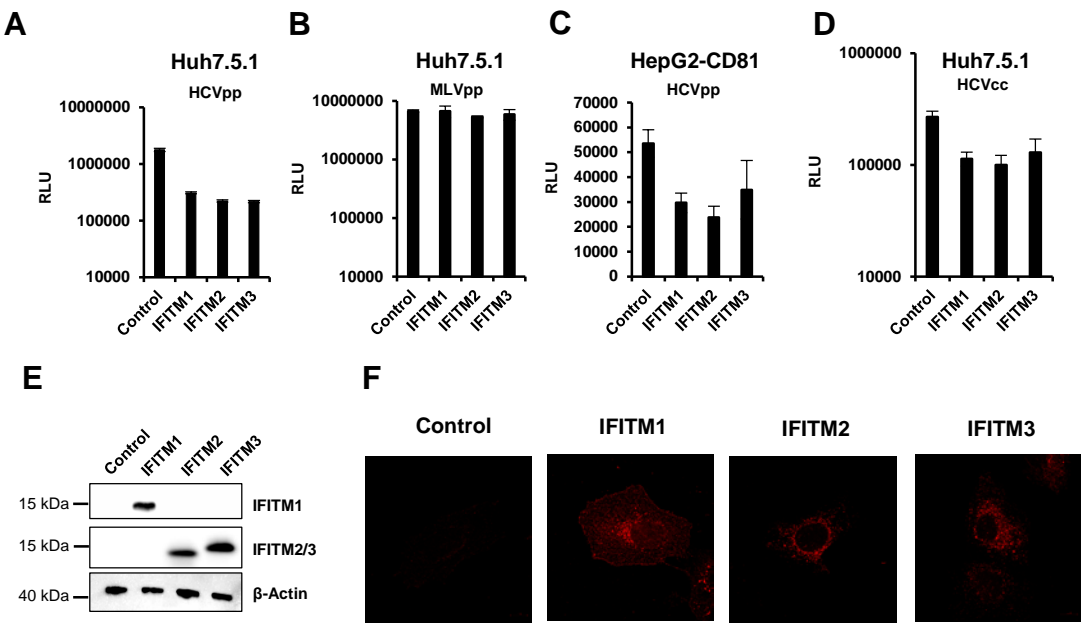


Figure 2

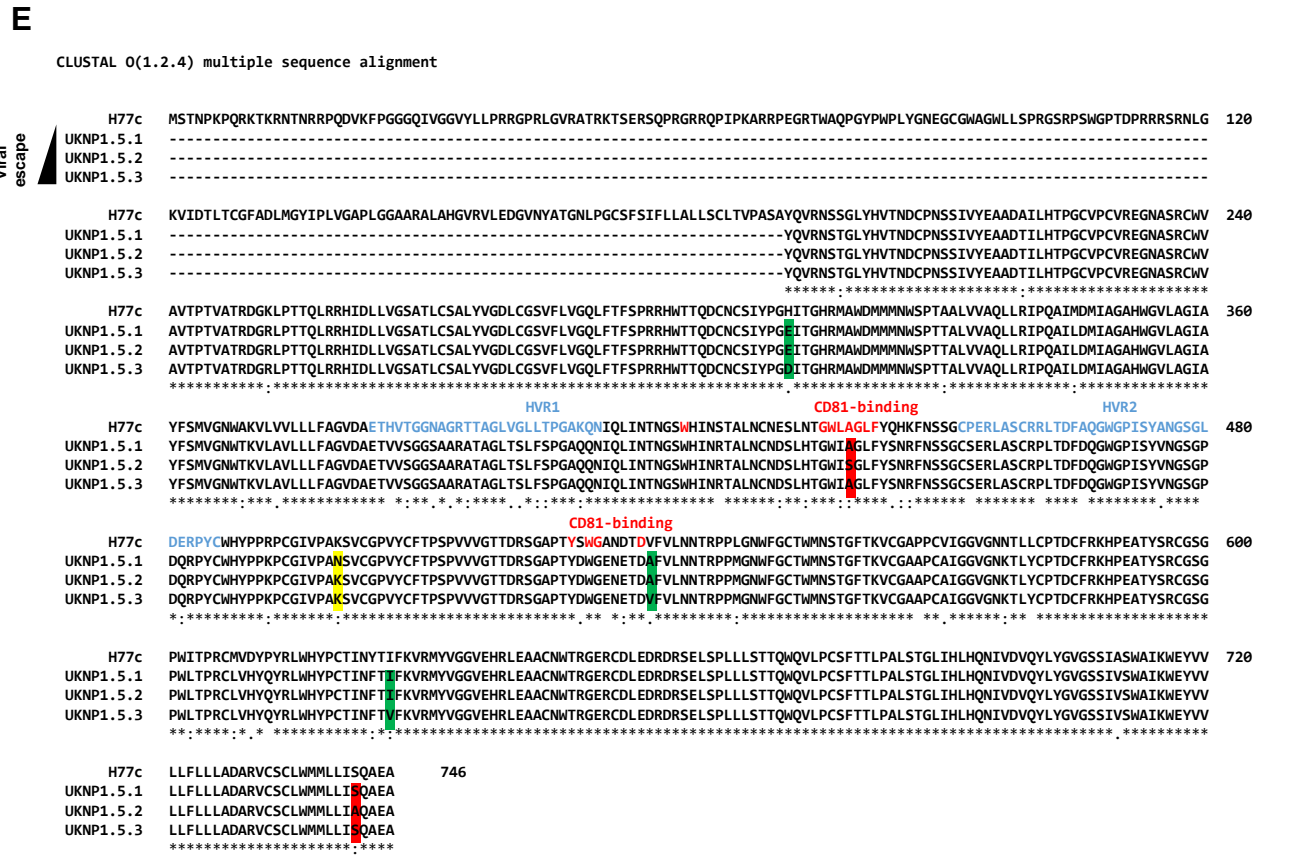
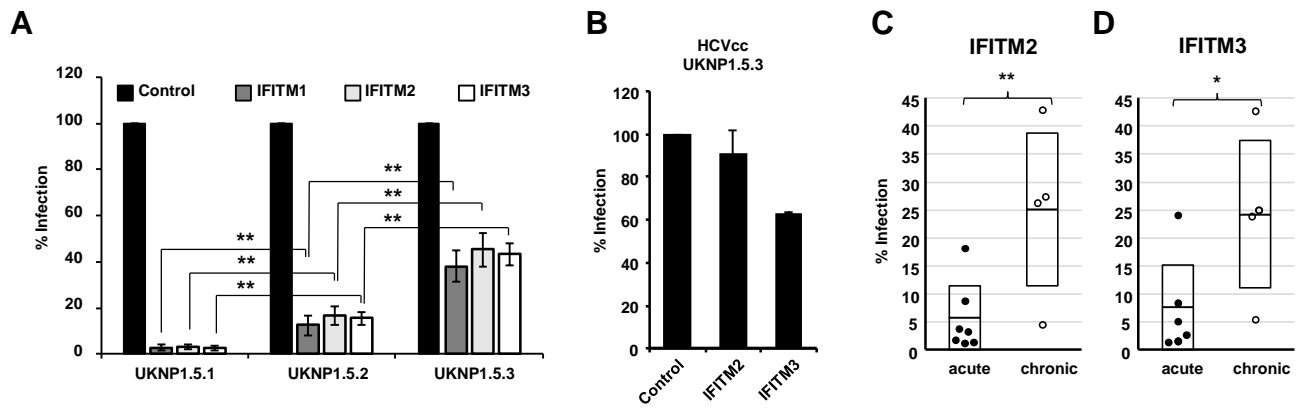


Figure 3

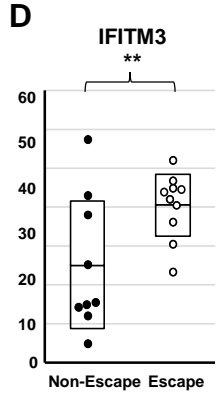
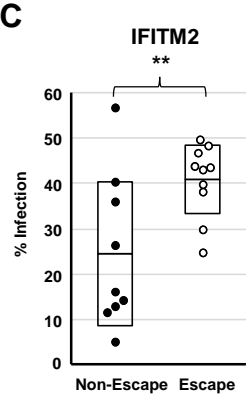
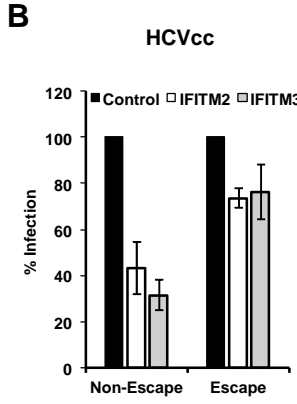
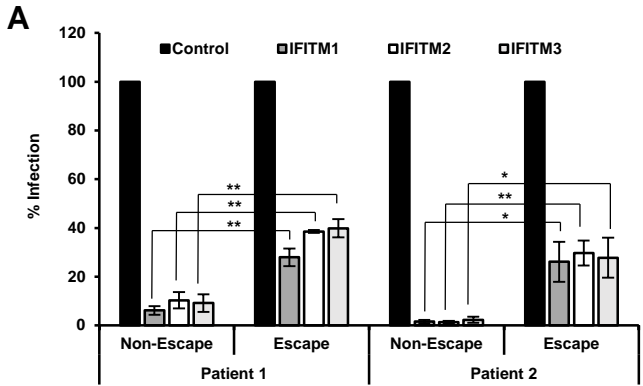


Figure 4

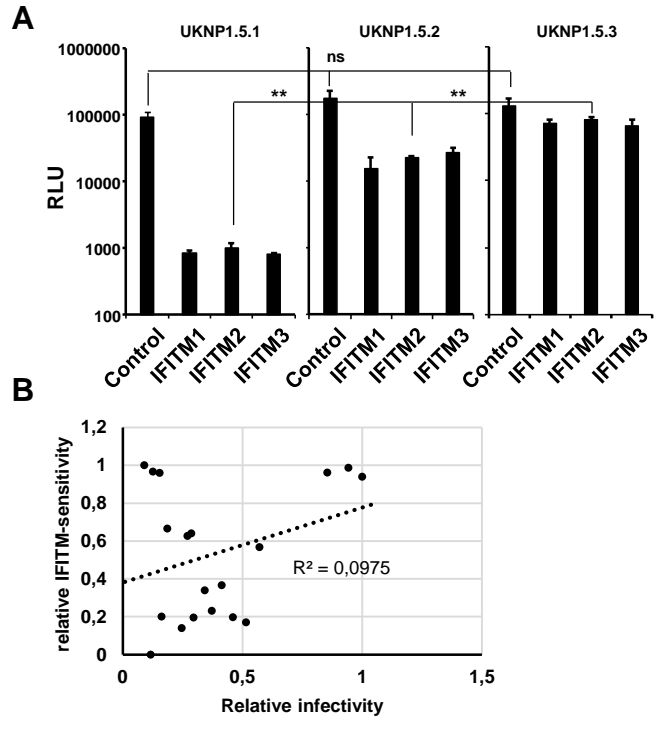


Figure 5

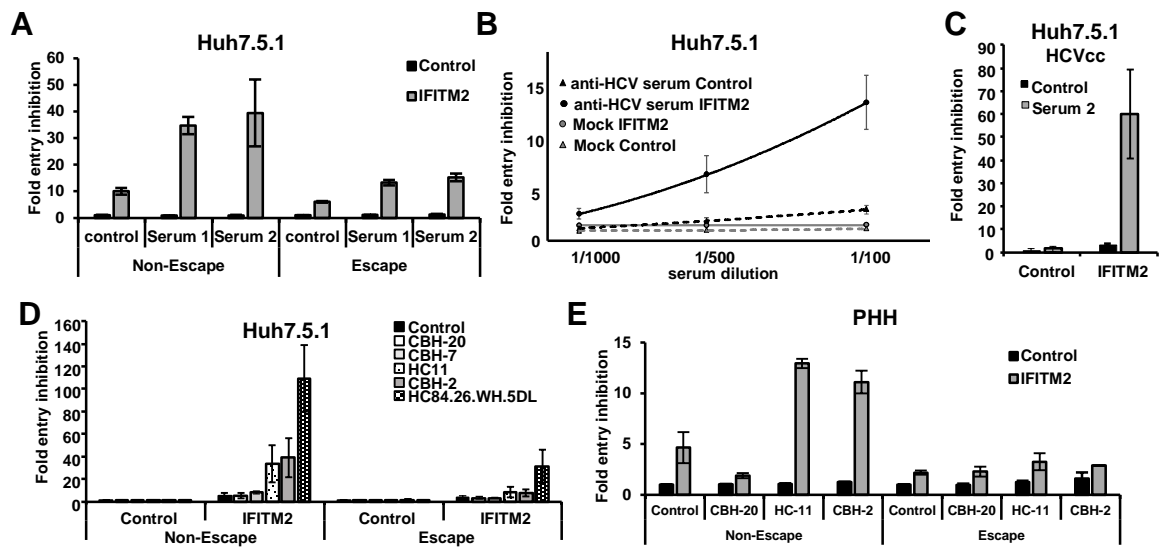


Figure 6

