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**The acidic domain of the hepatitis C virus NS4A protein is required for viral assembly and envelopment through interactions with the viral E1 glycoprotein**

Short title: The role of hepatitis C virus NS4A in viral envelopment

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## 21 **Abstract**

22           Hepatitis C virus (HCV) assembly and envelopment are coordinated by a complex  
23 protein interaction network that includes most of the viral structural and nonstructural proteins.  
24 While the nonstructural protein 4A (NS4A) is known to be important for viral particle production,  
25 the specific function of NS4A in this process is not well understood. We performed mutagenesis  
26 of the C-terminal acidic domain of NS4A and found that mutation of several of these amino  
27 acids prevented the formation of the viral envelope, and therefore the production of infectious  
28 virions, without affecting viral RNA replication. In an overexpression system, we found that  
29 NS4A interacted with several viral proteins known to coordinate envelopment, including the viral  
30 E1 glycoprotein. One of the NS4A C-terminal mutations, Y45F, disrupted the interaction of  
31 NS4A with E1. Specifically, NS4A interacted with the first hydrophobic region of E1, a region  
32 previously described as regulating viral particle production. Supernatants from HCV NS4A Y45F  
33 transfected cells had significantly reduced levels of HCV RNA, however they contained  
34 equivalent levels of Core protein. Interestingly, the Core protein secreted from these cells  
35 formed high order oligomers with a density matching the infectious virus secreted from WT cells.  
36 These results suggest that this Y45F mutation in NS4A causes secretion of low density Core  
37 particles devoid of genomic HCV RNA. These results corroborate previous findings showing that  
38 mutation of the first hydrophobic region of E1 also causes secretion of Core complexes lacking  
39 RNA, and therefore suggest that the interaction between NS4A and E1 is involved in the  
40 incorporation of viral RNA into infectious HCV particles. Our findings define a new role for NS4A  
41 in the HCV lifecycle and help elucidate the protein interactions necessary for production of  
42 infectious virus.

## 43 **Author Summary**

44 RNA viruses, which encompass both established and emerging pathogens, pose  
45 significant public health challenges. Viruses in the family *Flaviviridae*, including Dengue virus,  
46 Zika virus and hepatitis C virus (HCV), continue to cause morbidity and mortality worldwide.  
47 One HCV protein, NS4A, has known functions in several steps of the viral lifecycle, however,  
48 how it contributes to viral particle production is not understood. Here, we investigated the role of  
49 one region of NS4A, the C-terminal acidic domain, in regulating the viral lifecycle. We found that  
50 some of the amino acids within this domain are important for viral envelopment to make  
51 infectious particles, specifically through interaction with the E1 glycoprotein. NS4A interacts with  
52 the first hydrophobic domain of E1. Disruption of this interaction prevents the production of  
53 infectious virus particles and instead results in release of low density Core protein complexes  
54 that lack HCV RNA into the cellular supernatant. Overall, our results reveal that NS4A is  
55 important for late stages of the HCV lifecycle and suggest that the interaction between NS4A  
56 and E1 may regulate the incorporation of viral RNA into the virion for the formation of infectious  
57 HCV particles.

## 58 Introduction

59 Hepatitis C virus (HCV) is a positive-sense RNA virus of the genus *Hepacivirus* in the  
60 *Flaviviridae* family. Over 70 million people worldwide are chronically infected with HCV and this  
61 chronic infection can lead to liver cirrhosis and hepatocellular cancer [1]. In the years spanning  
62 2003-2013, HCV-related deaths numbered more than any other CDC-reported infectious  
63 disease [2]. Despite the availability of newly designed, highly effective direct-acting antivirals,  
64 disease prevalence remains high, and no vaccine exists for the virus [3-5].

65 HCV encodes a single stranded, positive-sense RNA genome of approximately 9.6  
66 kilobases in length. Upon virus entry into hepatocytes, the viral genome is translated to form a  
67 single polyprotein. The polyprotein is co- and post-translationally cleaved by both host and viral  
68 proteases, including the NS3-NS4A viral protein complex, to form ten individual proteins. These  
69 ten proteins include both structural proteins, which eventually make up the virion, and non-  
70 structural proteins, which coordinate RNA replication and the other steps in the viral lifecycle,  
71 including virion assembly and envelopment (reviewed in [6]).

72 The late stages of the viral lifecycle, including assembly and envelopment, are just  
73 beginning to be dissected. While many details of these processes are not understood, recent  
74 work has uncovered several key steps that lead to production of infectious virus. Following RNA  
75 replication, HCV RNA is shuttled to the cytosolic lipid droplet where Core protein accumulates,  
76 oligomerizes, and recruits the NS3 and NS5A proteins [7-10]. NS5A is thought to play a role in  
77 RNA recruitment to the lipid droplet, whereas NS3 binds to Core and likely aids in movement of  
78 Core bound to RNA from the lipid droplet to nearby sites on the endoplasmic reticulum (ER) [11-  
79 16]. This process is coordinated by the NS2 protein which acts as a bridge between the non-  
80 structural protein NS3, and the structural protein E2, to link virion assembly at the lipid droplet to  
81 envelopment at the ER [17-22]. The role of NS2 in these steps is supported by the actions of the  
82 p7 protein [23, 24]. Lastly, Core oligomers bound to RNA bud into the ER lumen, acquiring an

83 ER-derived lipid bilayer envelope that contains the viral E1 and E2 transmembrane  
84 glycoproteins [25, 26]. It is unclear what signals are necessary for the membrane curvature that  
85 results in budding but it is clear that E1 and E2 are necessary for successful envelopment, as  
86 deletion of E1 and E2 prevents formation of the viral envelope and production of infectious  
87 virions [24]. Following virion budding into the ER lumen, the virion is transported through the  
88 very-low-density lipoprotein (VLDL) secretory pathway acquiring apolipoproteins and other  
89 lipids, and is ultimately released from the cell in a noncytolytic manner as a lipovirion [27-  
90 30]. In addition to the viral proteins mentioned here, a number of host proteins also play a role in  
91 HCV morphogenesis [31](reviewed in [32]).

92 In addition to its roles in viral assembly and envelopment, the NS3-NS4A protein  
93 complex has several other well-established functions in the HCV lifecycle. It is essential for viral  
94 polyprotein processing, viral RNA replication, and negative regulation of antiviral innate  
95 immunity (reviewed in [33]). NS3 functions as both a serine protease and an RNA helicase and  
96 requires its cofactor NS4A to enhance these activities and to target the protease complex to  
97 intracellular membranes [34, 35]. NS4A is 54 amino acids long and contains three domains, an  
98 N-terminal transmembrane domain that anchors NS3 to intracellular membranes, a central NS3-  
99 interaction domain required for proper folding of NS3, and a C-terminal domain that contains a  
100 kink region followed by an acidic region with a high number of acidic amino acids [36-39]. While  
101 the specific roles of NS4A in the HCV lifecycle are largely thought to occur indirectly through its  
102 function as a cofactor for NS3, mutation-based studies of the NS4A acidic domain suggest  
103 some independent roles for NS4A in regulating the HCV lifecycle, including during assembly  
104 and envelopment, as described below [36, 40].

105 There is strong evidence that NS3 and NS4A are each involved in the steps of virion  
106 assembly and envelopment. Specifically, NS3 has been shown to be involved in viral particle  
107 production through interactions with both Core and NS2 [15, 21, 22, 41]. In addition, culture  
108 adaptive amino acid mutations in the  $\alpha_0$  helix of NS3 have been shown to promote viral

109 assembly [16]. Separately, HCV particle production is also regulated by specific amino acids in  
110 the NS4A acidic domain such that when mutated, infectious virus formation is inhibited without  
111 affecting RNA replication. Some of these mutations can be partially rescued by compensatory  
112 amino acid substitutions in NS3, suggesting that NS3 and NS4A together can cooperate to  
113 regulate HCV particle production [40]. However, both the extent of the role of NS4A in assembly  
114 and envelopment and the specific function of NS4A in regulating the production of infectious  
115 HCV remain unclear.

116 Here, we define a new role for the NS4A protein in regulating HCV envelopment. We  
117 have identified amino acids in the acidic domain of NS4A that are required for the formation of  
118 the viral envelope. Further, we have found that NS4A alone can interact with a number of viral  
119 proteins that coordinate viral envelopment, including Core, E1, E2 and NS5A. Interestingly,  
120 disruption of the NS4A-E1 interaction prevents envelopment of the HCV particle and results in  
121 secretion of Core particles that are not associated with viral RNA. Taken together, our findings  
122 reveal a new role for NS4A in coordinating the HCV lifecycle and define new viral interactions  
123 that lead to successful HCV particle envelopment.

## 124 **Results**

### 125 **A Y45F mutation in the hepatitis C virus NS4A protein causes a decrease in** 126 **infectious viral titer.**

127         The acidic domain of NS4A has significant sequence homology between all HCV  
128 genotypes, with amino acids 40-54 in the acidic domain differing by at most 3 amino acids (Fig  
129 1A). In particular, the tyrosine residue at position 45 is conserved in all seven genotypes of  
130 HCV. While previous studies have implicated the acidic domain of NS4A in regulating HCV RNA  
131 replication and particle production, the mechanism of this regulation was not explored [40]. We  
132 sought to investigate how the NS4A acidic domain contributes to HCV particle production. We  
133 engineered a structurally conservative amino acid substitution, changing the tyrosine residue (Y,  
134 TAT) at position 45 to a phenylalanine (F, TTT) in the genotype 2a strain of HCV, Japanese  
135 fulminant hepatitis-1 (JFH1) [42]. We then generated WT or NS4A Y45F *in vitro* transcribed  
136 RNA and transfected it into Huh7.5 cells. At 3 days post-transfection, while the WT RNA  
137 produced more than 3 logs of infectious virus, no titer was detected from cells transfected with  
138 the RNA containing the Y45F mutation, as measured by focus forming assay (Fig 1B). After  
139 several passages, Y45F RNA began to produce infectious virus, and after 14 days it produced  
140 equivalent titers to that of the WT virus (Fig 1B). Sequencing of the NS4A region of HCV RNA  
141 extracted from cells at 1, 3, and 14 days post-transfection revealed that the Y45F mutation had  
142 reverted back to WT by day 14, with some reversion detected as early as day 3 (Fig 1C). These  
143 results reveal that substitution of the tyrosine at position 45 with phenylalanine in NS4A  
144 prevents production of infectious HCV, indicating that Tyr-45 is required for the production of  
145 infectious virus.

### 146 **The NS4A Y45F mutation in HCV does not alter viral RNA replication.**

147 To determine if the loss of infectious HCV production by the NS4A Y45F amino acid  
148 change was due to altered HCV RNA replication, we engineered the Y45F mutation into an  
149 HCV subgenomic replicon construct containing a luciferase reporter and measured luciferase  
150 production over time following transfection of Huh7.5 cells with *in vitro* transcribed HCV RNA.  
151 We found that the HCV replicon RNA with the Y45F mutation in NS4A replicated as efficiently  
152 as WT, while the HCV RNA with a lethal mutation in the NS5B RNA dependent RNA  
153 polymerase (GND) did not replicate (Fig 2A). Additionally, the HCV proteins NS3, NS4A and  
154 NS5A were expressed in lysates harvested at 48 hours post-transfection of either WT or Y45F  
155 RNA, indicating that the Y45F mutation did not affect the production of these viral proteins (Fig  
156 2B). Of note, the epitope of the NS4A antibody is in the C-terminal domain of NS4A that  
157 contains Tyr-45. Therefore, the lack of a detectable NS4A band by immunoblotting in the mutant  
158 condition suggests that the Y45F mutation prevents NS4A recognition by this antibody (Fig 2B).  
159 Indeed, the fact that HCV RNA replication is not altered by the Y45F mutation indicates that the  
160 NS4A protein must be stably expressed, as NS4A is required for HCV RNA replication [36, 40,  
161 43]. Since the interaction of NS3 with NS4A is essential for viral replication, we tested if the  
162 Y45F mutation impacted this interaction using a co-immunoprecipitation experiment with  
163 overexpressed proteins. The results show that the Y45F mutation does not alter NS3-NS4A  
164 complex formation (Fig 2C). Together, these results indicate that the Y45F mutation in NS4A  
165 does not alter HCV RNA replication, HCV protein expression, or NS3-NS4A complex formation.  
166 Therefore, the NS4A Y45F mutation in HCV must cause a defect at a later stage of the viral  
167 lifecycle.

### 168 **The NS4A Y45F mutation inhibits viral envelopment.**

169 As the NS4A Y45F mutation did not alter HCV RNA replication but did prevent infectious  
170 virus production, we next tested if this mutation affected viral assembly and envelopment or viral  
171 release. We first examined if the Y45F mutation caused a viral release defect by measuring



172 both intracellular and extracellular titer. We transfected Huh7.5 cells with WT, Y45F, or GND  
173 HCV RNA, and measured the viral titer from the supernatant (extracellular titer) or from lysates  
174 generated by freeze-thaw cycles (intracellular titer) by using a focus forming assay. As before,  
175 HCV NS4A Y45F RNA did not produce extracellular titer (Fig 3A), and here we found that it also  
176 did not produce intracellular titer (Fig 3B). Taken together, these results indicate that the Y45F  
177 mutation impairs viral particle production prior to the formation of fully infectious virions.

178 An infectious HCV virion contains viral RNA, encapsidated by the viral Core protein,  
179 surrounded by an outer lipid envelope containing the viral glycoproteins, E1 and E2, and cellular  
180 lipids and lipoproteins [32, 44, 45]. Taking advantage of these structural properties of the HCV  
181 virion, we next tested if the Y45F mutation prevented viral envelopment by using a proteinase K  
182 protection assay. The HCV Core protein in enveloped virions, which have an outer lipid  
183 envelope, is protected from degradation following proteinase K treatment [24]. Because the  
184 HCV glycoproteins are required for acquisition of the lipid bilayer membrane, a viral RNA with a  
185 deletion of the E1 and E2 coding region (amino acids (aa) 192-720,  $\Delta$ E1/E2) can be used a  
186 negative control for envelopment [24]. Lysates were harvested from HCV RNA (WT, Y45F, or  
187  $\Delta$ E1/E2) transfected Huh7.5 cells, incubated with proteinase K, and analyzed by immunoblot for  
188 Core. We found that while Core was protected from proteinase K digestion in WT, it was not  
189 protected in lysates containing the Y45F mutation, similar to  $\Delta$ E1/E2 (Fig 3C). These data  
190 indicate that the Y45F mutation prevents envelopment of the virion, resulting in a lack of both  
191 intracellular and extracellular viral titer, suggesting that Tyr-45 may be an important residue for  
192 HCV envelopment.

### 193 **The acidic domain of NS4A is required for HCV envelopment.**

194 Based on our findings that HCV RNA with the NS4A Y45F mutation has a defect in viral  
195 envelopment, we hypothesized that other amino acids in the NS4A C-terminal acidic domain  
196 may also be required. To test this, we introduced several mutations into the acidic domain of

197 NS4A that were previously found to be important for production of infectious HCV particles  
198 (K41A, L44A, and E52A) and tested their effects on viral envelopment [40]. We performed a  
199 proteinase K protection assay, as in Figure 3, and found that the K41A, L44A and E52A mutants  
200 all resulted in a quantifiable decrease in protease-resistant Core as compared to WT,  
201 suggesting that these mutations also caused a defect in envelope formation (Figs 4A and 4B).

202 We additionally tested the impact of these amino acids on RNA replication, HCV protein  
203 expression, and production of both intracellular and extracellular titer; and also tested two  
204 additional mutations with known replication defects, Y45A and D49A, as controls [40]. HCV  
205 RNA containing the NS4A K41A, L44A, and E52A mutations all replicated and expressed HCV  
206 proteins to a similar extent as WT, while NS4A D49A and Y45A showed mild to severe  
207 replication defects (Figs 4C and 4D). These mutations all prevented intracellular and  
208 extracellular infectious virus from being produced, as seen previously by others (Figs 4E and  
209 4F) [40]. Taken together, these data show that multiple amino acids within the acidic domain of  
210 NS4A are important for formation of the viral envelope and production of infectious virus.

### 211 **NS4A Y45 is required for NS4A interaction with the E1 glycoprotein**

212 Because a complex network of HCV proteins regulates HCV assembly and  
213 envelopment, we hypothesized that NS4A may facilitate an interaction between either structural  
214 (Core, E1 or E2) or non-structural (p7, NS2 or NS5A) proteins to regulate these processes.  
215 Therefore, we first tested if overexpressed NS4A WT or Y45F interacted with Core, E1, or E2  
216 using co-immunoprecipitation in Huh7.5 cells. We found that overexpressed NS4A WT interacts  
217 with Core, E1 and E2 (Figs 5A, S1A-B). While Core and E2 interactions with NS4A were  
218 equivalent for WT and Y45F (Figs S1A-B), the NS4A Y45F mutation greatly decreased NS4A  
219 and E1 interaction (Figs 5A-B). To determine if NS4A WT interacts with E1 also in the context of  
220 HCV infection we transfected Huh7.5 cells with an infectious clone of HCV containing an N-  
221 terminal HA tag on E1 [41]. We then immunoprecipitated E1 using the HA epitope and found

222 that, indeed, NS4A and E1 can interact during HCV infection (Fig 5C). We also tested the  
223 interactions of NS4A with p7, NS2, and NS5A, non-structural proteins that all have roles in HCV  
224 envelopment [10-12, 17-21, 23, 24]. NS4A did interact with NS5A and this interaction was not  
225 altered by the Y45F mutation in NS4A (Fig. S1D). We found no interaction between  
226 overexpressed NS4A WT or Y45F with either NS2 or p7 (Fig S1C). Together, these data show  
227 that NS4A can bind to Core, E1, E2, and NS5A, and that mutation of NS4A at Tyr-45 disrupts its  
228 binding to the E1 protein.

### 229 **NS4A binds to the first hydrophobic region of E1**

230 To investigate the mechanism of how the NS4A-E1 interaction might facilitate viral  
231 envelopment, we mapped the binding site of NS4A on E1. The E1 and E2 glycoproteins are  
232 translated in the ER membrane and are cleaved from the viral polyprotein by a host protease.  
233 After cleavage, E1 and E2 form a stable heterodimer and are retained in the ER. E1 has an N-  
234 terminal ectodomain, two hydrophobic regions and a C-terminal transmembrane domain (Fig  
235 6A) (reviewed in [46]). We therefore created a series of E1 truncation mutants based on these  
236 known domains of E1, containing N-terminal Flag tags (Fig 6A). We overexpressed the  
237 truncation mutants and NS4A-HA in Huh7.5 cells and then performed Flag  
238 immunoprecipitations followed by immunoblotting for NS4A-HA. We found that NS4A co-  
239 immunoprecipitated with E1 aa1-106, aa1-138, and aa67-192, all of which contain the first  
240 hydrophobic region of E1, but did not interact with aa1-66 or aa107-192, which lack this region  
241 (Fig 6B). These data suggest that NS4A binds to E1 via the first hydrophobic region of the  
242 protein.

### 243 **NS4A Y45F results in release of Core oligomers devoid of HCV RNA**

244 The first hydrophobic region of E1 has previously been implicated in viral particle  
245 production, and several amino acids within this region are important for viral infectivity [47, 48].

246 Additionally, one specific E1 mutation, D263A (E1 aa71), attenuated viral infectivity and resulted  
247 in secreted Core protein but not HCV RNA [48]. Taken together with our findings that NS4A  
248 binds to the E1 the hydrophobic region, which contains aaD263, we hypothesized that Y45F  
249 may have a similar phenotype as the E1 D263A mutant. Therefore, we measured the secretion  
250 of HCV RNA and Core protein into supernatants from cells replicating HCV NS4A WT or Y45F  
251 RNA. We found that while the Y45F mutation resulted in lower levels of extracellular HCV RNA,  
252 as measured by RT-qPCR, secretion of Core into the supernatant was unaltered as compared  
253 to WT (Figs 7A-B). These results were surprising as we did not expect to detect Core protein in  
254 the supernatant secreted from HCV NS4A Y45F transfected cells. Because of these unexpected  
255 data, we sought to profile the viral components in supernatants from Y45F cells. We collected  
256 and concentrated cellular supernatants from HCV NS4A WT or Y45F transfected cells and then  
257 ultracentrifuged these samples over iodixanol gradients. We collected 10 equal fractions from  
258 the top, with fraction 1 having the lowest density and fraction 10 having the highest density.  
259 Each fraction was analyzed by RT-qPCR for HCV RNA, by focus forming assay for viral titer,  
260 and by immunoblot for Core protein. In the WT samples, fractions 2 and 3 had the highest levels  
261 of both HCV RNA and viral infectivity (Fig 7C). These fractions also contained high molecular  
262 weight complexes of Core protein (Fig 7D, lanes 2 and 3). We observed a second peak of HCV  
263 RNA in fractions 7, 8, and 9, along with a small amount of higher density Core protein, however  
264 these fractions had significantly less viral infectivity (Fig 7D, lanes 7-9). Therefore, the HCV  
265 RNA in these higher density fractions is likely non-infectious and may represent secreted  
266 membrane-associated RNA from replication complexes [49]. However, we saw little to no  
267 infectious viral titer from any fraction in the Y45F samples and found the majority of HCV RNA  
268 present in fractions 7-9, while the expected infectious fractions (2-4) had little RNA (Fig 7C).  
269 Interestingly, high molecular weight complexes of Core protein were still observed in fractions 2-  
270 4, similar to the distribution of Core in WT samples (Fig 7D). The fact that Core forms oligomers  
271 and that these were in a different fraction than the peak of HCV RNA suggests that the Y45F

272 mutation results in release of partially formed virions containing Core protein oligomers but  
273 devoid of HCV RNA.

## 274 **Discussion**

275           Our results define a new role for NS4A in the late stages of the HCV lifecycle.  
276 Specifically, we have found that the acidic domain of NS4A is important for regulating assembly  
277 and that mutation of specific amino acids within this domain prevents formation of the viral  
278 envelope. Further, we have identified new interactions between NS4A and both structural and  
279 non-structural viral proteins. This suggests that NS4A may act as a bridge, linking virion  
280 assembly at the lipid droplet and envelopment at the ER, similar to the actions of the NS2  
281 protein. Importantly, we found that NS4A binds to E1 and that antagonizing this interaction with  
282 one amino acid change in NS4A prevents viral envelopment. We mapped the binding site of  
283 NS4A on E1 and found that it interacts with the first hydrophobic region, a region that is known  
284 to be important for viral particle production [47, 48]. Finally, we found that the Y45F mutation in  
285 NS4A, which prevents envelopment, also results in secretion of noninfectious, incompletely  
286 formed virions that are composed of low density Core protein oligomers that lack HCV RNA.  
287 Together our results reveal a new role for NS4A in coordinating the proper assembly and  
288 envelopment of HCV particles to make infectious virus.

289           The NS4A protein contains only 54 amino acids and yet has three distinct domains with  
290 specific functions in the HCV lifecycle. While the functions of the NS4A transmembrane domain  
291 and the NS3-interaction domain are largely defined [33], much less is known about the function  
292 of the C-terminal region, which contains a high number of acidic amino acids from aa40 to aa54  
293 (Fig 1A). We found that mutation of several amino acids in the acidic domain, including Y45F,  
294 disrupted the formation of the viral envelope and therefore prevented production of infectious  
295 virus, without affecting viral RNA replication (Figs 2A, 3A, 3C, and 4). Indeed, the presence of a  
296 Tyr at aa45 of NS4A was so essential for the viral lifecycle that a viral RNA containing the Y45F  
297 mutation reverted back to the WT sequence after only a few days of passage in cell culture (Fig  
298 1B-C). Given that changing a Tyr to Phe is a structurally conservative mutation, removing only

299 the hydroxyl group, it is formally possible that NS4A could be phosphorylated at this tyrosine.  
300 However, it is unlikely that lack of phosphorylation of this tyrosine would be the sole contributor  
301 to the envelopment defects, as several other amino acids within the region displayed the same  
302 phenotypes when mutated (Fig 4) and a Y45A mutation prevented HCV RNA replication  
303 altogether (Fig 4C) [40]. Therefore, the acidic domain likely regulates envelopment through the  
304 concerted actions of the amino acids in this acidic region of NS4A.

305 Changing the amino acids in NS4A at K41, L44, Y45, and E52 to alanine all resulted in  
306 loss of viral titer due to defects in envelopment (Fig 4). The acidic domain of NS4A, which has  
307 been proposed to have an alpha helical structure, is important for replication, and indeed the  
308 Y45A change results in less replication [36, 40]. However, because mutation of the other amino  
309 acids within this C-terminal domain did not alter viral RNA replication, it is unlikely that they  
310 disrupt the conformation of the alpha helix. Structural predictions of this alpha helix suggest that  
311 K41, Y45 and E52 all lie on one face of the helix while L44 and D49 would face the opposite  
312 direction [36]. Therefore, it is possible that the amino acids we studied in this region could  
313 facilitate different protein interactions on opposite faces of the protein, each contributing to HCV  
314 envelopment. In support of this hypothesis, previous work has shown that an adaptive mutation  
315 in NS3 partially rescues an assembly defect resulting from the K41A mutation, suggesting that  
316 NS4A can cooperate with NS3 via K41 for viral particle production [40]. We found that NS4A  
317 WT and Y45F bound NS3 equivalently (Fig 2C) but that the Y45F mutation prevented NS4A  
318 interaction with E1 (Fig 5A). This suggests that while both the K41A and Y45F RNAs are  
319 defective in HCV envelopment, they may function in NS4A to facilitate different protein-protein  
320 interactions that regulate envelopment.

321 Supporting the hypothesis that NS4A interacts with several HCV proteins to coordinate  
322 virion envelopment, we did identify several previously unknown interactions of NS4A with both  
323 structural and non-structural proteins including Core, E1, E2, and NS5A. Others have shown

324 that compensatory mutations within NS4A rescue assembly and envelopment defects caused  
325 by mutations in NS2, however we found that NS4A did not interact with NS2 during  
326 overexpression. Therefore, these data suggest that during infection, NS2 and NS4A likely work  
327 together through a multi-protein complex or to perform similar roles in the lifecycle [19]. Indeed,  
328 NS2 is considered to be the main organizer of envelopment, binding both structural and  
329 nonstructural proteins to link viral assembly steps at the lipid droplet to envelopment steps at the  
330 ER [17-21, 23, 24]. NS4A also binds to proteins involved in both early (Core, NS3, and NS5A)  
331 and later (E1 and E2) steps of assembly and envelopment which could suggest that NS4A may  
332 also serve as a link between virion production steps at the lipid droplet and the ER, similar to  
333 NS2. Overall, these results suggest that NS2 and NS4A could play similar roles in organizing  
334 and facilitating viral envelopment.

335 We found that NS4A binds to E1 and that this interaction is disrupted by the Y45F  
336 mutation (Fig 5), suggesting that the NS4A-E1 interaction is important for envelopment of the  
337 virion. The E1 protein has an N-terminal ectodomain, two internal hydrophobic domains, a  
338 transmembrane domain and a very short, 2 amino acid cytoplasmic, C-terminal tail (Fig 6A) [46].  
339 Surprisingly, we found that NS4A binds to the first hydrophobic region of the protein (Fig 6B).  
340 This region has also previously been shown to bind to Core, and mutations within this domain  
341 diminish viral particle production [47, 48, 50-52]. Curiously, this E1 domain is hydrophobic and is  
342 proposed to associate with the lipid membrane bilayer while the NS4A acidic domain is  
343 cytoplasmic and not known to have membrane interactions. It is possible that the acidic domain  
344 of NS4A could associate with the ER membrane to interact with this region of E1. However, it is  
345 equally likely that NS4A and E1 are linked by a host protein. Future studies designed to  
346 determine how NS4A interacts with E1 would yield further insights into the HCV envelopment  
347 process.



348           The finding that NS4A binds to the first hydrophobic region of E1 is particularly  
349 interesting, as this region in E1 has recently been shown to regulate viral particle production [47,  
350 48]. In fact, a D263A mutation at the start of hydrophobic region 1 in E1 (E1 aa71), resulted in  
351 decreased viral titer and secretion of Core particles that were devoid of genomic RNA. Further,  
352 this mutation disrupted the localization of E1 with HCV RNA in fluorescence *in situ* hybridization  
353 experiments [48]. In our studies, fractionation of supernatant from cells replicating HCV NS4A  
354 Y45F RNA revealed that low density fractions contained little to no HCV RNA, similar to E1  
355 D263A (Fig. 7). However, these low density fractions contained secreted Core oligomers,  
356 suggesting that these oligomers were associated with cellular lipids or apolipoproteins. Indeed,  
357 transfected Core protein has been shown to self-assemble into higher order complexes and  
358 non-enveloped particles have been found in the serum of HCV infected patients [53, 54].  
359 Transfection of Core alone can also alter VLDL secretion, and therefore it is possible that  
360 secreted Core may be associated with cellular lipids and lipoproteins [55]. Taken together, these  
361 data suggest that the NS4A acidic domain and the E1 first hydrophobic domain cooperate  
362 during envelopment, perhaps to aid in the incorporation of viral RNA into the virion. While NS4A  
363 itself does not have RNA binding capability, it is linked to NS3, which contains an RNA binding  
364 helicase domain, and thus, NS3-NS4A together could cooperate via E1 interactions for  
365 incorporation of RNA into the developing virion.

366           Our study contributes new insights into the steps required for HCV to form infectious  
367 viral particles. As the viral particle lifecycle stages that occur in association with lipid droplets  
368 and the ER are tightly linked and likely occur nearly simultaneously, it is unclear if nucleocapsid  
369 intermediates (Core protein assembled around HCV RNA) exist separate from fully enveloped  
370 nucleocapsids [32]. Our data show that Core protein assembles into oligomers prior to  
371 envelopment and suggest that the function of NS4A in viral assembly and envelopment is after  
372 this Core oligomerization step. Further, the fact that we identified Core protein oligomers that did  
373 not contain a protective envelope or HCV RNA suggests that RNA incorporation into the virion

374 at or near envelopment sites could be a necessary signal for virion budding events to occur. Our  
375 data therefore support a model by which NS4A interacts with E1 to link viral RNA to Core  
376 oligomers in the forming virion and signal the envelopment of the Core-RNA complex.

## 377 **Materials and Methods**

### 378 **Cell lines and culture conditions.**

379 Huh7.5 cells, which have been previously described [56], were maintained in Dulbecco's  
380 modification of eagle's medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum  
381 (FBS; HyClone) and 25mM HEPES (Thermo-Fisher) at 37°C with 5% CO<sub>2</sub>. The identity of the  
382 Huh7.5 cells used in this study was verified by using the Promega GenePrint STR kit (DNA  
383 Analysis Facility, Duke University), and cells were verified as mycoplasma free by the LookOut  
384 Mycoplasma PCR detection kit (Sigma).

### 385 **Plasmids and site-directed mutagenesis.**

386 These plasmids have been described previously: psJFH1-p7+NS [57] and HJ3-E1/HA-NS2/YFP  
387 ([41], gift of Dr. MinKyung Yi). psJFH1-p7+NS is a culture adapted strain of JFH1 containing 7  
388 mutations within p7 and the nonstructural proteins [57]. pJFH1-SGR-luc contains a bicistronic  
389 replicon as follows: [JFH1-derived untranslated region (UTR; nt 1-397)]-[in frame *Renilla*  
390 luciferase reporter]-[EMCV IRES-nonstructural genes (NS3-NS5B)]. To make this plasmid, a  
391 DNA fragment encoding *Renilla* luciferase was fused between the T7 promoter sequence-5'  
392 UTR of JFH1 and the EMCV IRES-nonstructural genes from pSGR-JFH1 [58] following PCR (for  
393 oligonucleotide sequence see Table 1), digestion (with inserted *Bgl*III site between 5'UTR and  
394 5'end of *Renilla*, a *Pme*I site between the 3'end of *Renilla* and 5'end of the ECMV IRES, and an  
395 existing *Age*I site in the 5'UTR of pSGR-JFH1), and a 3-piece ligation. Mutagenesis of  
396 constructs was performed using the QuikChange lightning site-directed mutagenesis kit  
397 (Stratagene) on pJFH1-SGR-luc or psJFH1-p7+NS using the indicated oligonucleotides (Table  
398 1). psJFH1-p7+NS ΔE1/E2 was constructed by removing amino acids 192-720 from the psJFH1  
399 p7+NS background [24]. HCV over-expression constructs (noted below) were constructed by  
400 PCR amplification of the gene of interest from psJFH1-p7+NS and insertion of the *Pme*I-*Not*I  
401 digested fragment into pEF-Tak-Flag [59] or the *Eco*RI-*Xba*I digested fragment into pEF1. pEF-

402 Tak Flag-NS2 was created using InFusion (Clontech) after PCR. Table 1 provides the sequence  
 403 of all oligonucleotides used. Bold letters in the oligonucleotide sequences indicate overlap with  
 404 vector sequence, and the sequence of the HA tag within the oligonucleotides is underlined. All  
 405 nucleotide and amino acid positions refer to the JFH1 genome (GenBank accession number:  
 406 AB047639). The sequences of all plasmids were verified by DNA sequencing and are available  
 407 upon request.

408 **Table 1: Oligonucleotides used in this study**

Construct Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Oligonucleotides used for site-directed mutagenesis		
pJFH1-SGR-luc (5'UTR to <i>BglII</i> )	CATGAATCACTCCCCTGTGA	<b>TAGATCTTGGGCGACGGTTGGTG</b>
pJFH1-SGR-luc ( <i>BglII</i> - <i>Renilla</i> - <i>PmeI</i> )	<b>AAGATCTATGACTTCGAAAGTTTATG</b> A TC	<b>TGTTTAAAC</b> TTATTGTTCAATTTTTG AGAACTC
pJFH1-SGR-luc Y45F	CCATCTCATCAAAGCCTCGAACAGG ACCTCCTTATCCGG	CCGGATAAGGAGGTCCTGTTCGA GGCTTTTGATGAGATGG
psJFH1-p7+NS Y45F	CCATCTCATCAAAGCCTCGAACAGG ACCTCCTTATCCGG	CCGGATAAGGAGGTCCTGTTCGA GGCTTTTGATGAGATGG
pJFH1-SGR-luc K41A	CGTCGTTGCGCCGGATGCGGAGGTC CTGTATG	CATACAGGACCTCCGCATCCGGC GCAACGACG
psJFH1-p7+NS K41A	CGTCGTTGCGCCGGATGCGGAGGTC CTGTATG	CATACAGGACCTCCGCATCCGGC GCAACGACG 3
pJFH1-SGR-luc L44A	CGCCGGATAAGGAGGTCGCGTATGA GGCTTTTGATG	CATCAAAGCCTCATACGCGACC TCCTTATCCGGCG
psJFH1-p7+NS L44A	CGCCGGATAAGGAGGTCGCGTATGA GGCTTTTGATG	CATCAAAGCCTCATACGCGACC TCCTTATCCGGCG
pJFH1-SGR-luc Y45A	TTCCTCCATCTCATCAACAGCCTCAT ACAGGACCT	CATCTCATCAAAGCCTCAGCCA GGACCTCCTTATCCGGC
pJFH1-SGR-luc D49A	CCTGTATGAGGCTTTTGCTGAGATGG AGGAATGCG	CGCATTCCCTCATCTCAGCAAAG CCTCATACAGG
pJFH1-SGR-luc E52A	GCTTTTGATGAGATGGCGGAATGCG CCTCTAGG	CCTAGAGGCGCATTCCGCCATCT CATCAAAGC
psJFH1-p7+NS E52A	GCTTTTGATGAGATGGCGGAATGCG CCTCTAGG	CCTAGAGGCGCATTCCGCCATCT CATCAAAGC
Oligonucleotides used for cloning into expression vectors		
pEF-Tak Flag-Core	<b>GATGATAAAGCGGCCGCT</b> AGCACAA ATCCTAAACCTCAAAG	<b>CTGATCAGCGGGTTTAAAC</b> CCTAA GCAGAGACCGGAACGG
pEF-Tak Flag-E1	<b>GATAAAGCGGCCGCT</b> GCCCAGGTGA AGAATAC	<b>CGGGTTTAAAC</b> CGCGTCCACCCC AGCGG
pEF-Tak Flag-E2	<b>ATGATAAAGCGGCCGCT</b> TGGCACCAC CACCGTT	<b>GGGTTTAAAC</b> TTCCGGCCTGGCCC ACAAGA
pEF-Tak Flag-p7	<b>TGATAAAGCGGCCGCT</b> GCAGCATTG GAGAAG	<b>CGGGTTTAAAC</b> GGCATAAGCCTG CCGGG
pEF-Tak Flag-NS3	<b>ATAAAGCGGCCGCT</b> TGCTCCCATCAC TGCT	<b>CGGGTTTAAAC</b> GGTCATGACCTC AAGGTCA
pEF-Tak Flag-NS5A	<b>GATAAAGCGGCCGCT</b> TCCGGATCCT GGC	<b>CGGGTTTAAAC</b> GCAGCACACGGT GGTATCG

pEF NS4A-HA	<b>AATTCTGCAGATAGCTTATGAGCAGG TGGTCCT</b>	<b><u>CTCTAGACTAAGCGTAGTCTGGG ACGTCGTATGGGTAGCATTCTC CATC</u></b>
pEF NS4A-HA Y45F	<b>AATTCTGCAGATAGCTTATGAGCAGG TGGTCCT</b>	<b><u>CTCTAGACTAAGCGTAGTCTGGG ACGTCGTATGGGTAGCATTCTC CATC</u></b>
pEF-Tak Flag-NS2	<b>TGATGATGATAAAGCGGCCGCTTAT GACGCACCT</b>	<b><u>CTGATCAGCGGGTTTAAACAAGG AGCTTCCACCCCT</u></b>
pEF-Tak Flag-E1 1-66	<b>GATGATGATGATAAAGCGGCCGCTG CCCAGGTGAAGAATA</b>	<b><u>GGCTGATCAGCGGGTTTAAACCT AACCCCTGCGTGAGGGCA</u></b>
pEF-Tak Flag-E1 1-106	<b>GATGATGATGATAAAGCGGCCGCTG CCCAGGTGAAGAATA</b>	<b><u>GGCTGATCAGCGGGTTTAAACCT AGTACTGCGGCGAGACG</u></b>
pEF-Tak Flag-E1 1-138	<b>GATGATGATGATAAAGCGGCCGCTG CCCAGGTGAAGAATA</b>	<b><u>GGCTGATCAGCGGGTTTAAACCT ACGTGGGCGACCAAGTTC</u></b>
pEF-Tak Flag-E1 67-192	<b>GATGATGATGATAAAGCGGCCGCTC TGCGGACGC</b>	<b><u>GGCTGATCAGCGGGTTTAAACCT ACGCGTCCACCCAGCGG</u></b>
pEF-Tak Flag-E1 107-192	<b>GATGATGATGATAAAGCGGCCGCTC ACTGTTTTGTGCAAG</b>	<b><u>GGCTGATCAGCGGGTTTAAACCT ACGCGTCCACCCAGCG</u></b>

409

#### 410 ***In vitro* transcription of HCV RNA and electroporation.**

411 Plasmid DNA encoding the described HCV constructs was linearized using the *Xba*I restriction  
412 enzyme. Purified linearized DNA was used as a template for *in vitro* transcription with a  
413 MEGAscript T7 transcription kit (Thermo-Fisher). The *in vitro* transcribed RNA was treated with  
414 DNase (Thermo-Fisher) and then purified by phenol-chloroform extraction. The quality of the  
415 RNA was verified on a denaturing gel. For electroporation, 1µg (HCV Replicon RNA) or 5µg  
416 (HCV Infectious Clone RNA) was electroporated into 4x10<sup>6</sup> Huh7.5 cells in Cytomix  
417 electroporation buffer (120mM KCl, 10mM Potassium Phosphate Buffer, 5mM MgCl<sub>2</sub>, 25mM  
418 HEPES, 0.15mM CaCl<sub>2</sub>, 2mM EGTA, pH 7.6) at 250V and 950µF in a 4mm cuvette with a Gene  
419 Pulser Xcell system (Bio-Rad). Four hours post electroporation, cells were washed extensively  
420 with Phosphate Buffered Saline (PBS) and cDMEM.

#### 421 **Focus forming assay.**

422 *Extracellular titer:* Supernatants were harvested from Huh7.5 cells electroporated with HCV  
423 RNA at indicated time points, serially diluted, and used to infect naïve Huh7.5 cells in triplicate  
424 wells of a 48-well plate for 2 hours. Plates were harvested at 48hpi and fixed with 4%  
425 paraformaldehyde. Cells were permeabilized (0.2% Triton-X-100 in PBS), blocked (10% FBS in

426 PBS) and immunostained with mouse anti-HCV NS5A antibody (9e10, 1:500, gift of Dr. Charles  
427 Rice). Infected cells were visualized following incubation with horseradish peroxidase (HRP)-  
428 conjugated secondary antibody (1:500; Jackson ImmunoResearch) and VIP Peroxidase  
429 Substrate Kit (Vector Laboratories). Foci were counted at 40X magnification. Titer (FFU/mL)  
430 was determined as previously described [60]. *Intracellular titer*. Cell pellets were washed with  
431 PBS and resuspended in serum-free DMEM. Cells were then lysed using a series of  
432 freeze/thaw cycles in a dry ice/Ethanol bath. Post-nuclear supernatants were used to infect  
433 naïve Huh7.5 cells, and a focus forming assay was performed as described above.

#### 434 **Luciferase assays.**

435 JFH1 SGR-luc *in vitro* transcribed RNA (1µg) was electroporated into Huh7.5 cells. Cells were  
436 suspended in 20mL cDMEM and plated in 12-well plates. Cells were harvested after a PBS  
437 wash by incubation in *Renilla* lysis buffer (Promega). *Renilla* luciferase values were measured  
438 according to manufacturer's instructions (Renilla Luciferase Assay System, Promega) using a  
439 BioTek Synergy 2 microplate reader.

#### 440 **HCV NS4A sequencing.**

441 RNA was extracted from cells by using the Qiagen RNeasy kit according to manufacturer's  
442 instructions and then used as a template for cDNA synthesis with the iScript cDNA synthesis kit  
443 (BioRad). The NS4A region of the HCV genome was amplified by nested PCR with the following  
444 oligonucleotides: Round 1: 5' - CAGTCCGATGGAGAAGAAGG - 3', 5' -  
445 GCATGGGATGGGGCAGTC - 3', Round 2: 5' - ACACATAGACGCCCCACTTCC - 3', 5' -  
446 GTATGTCCTGGGCCTGCTTA - 3', and then the 542bp PCR product was purified and  
447 sequenced by Sanger sequencing.

#### 448 **Quantification of HCV RNA by RT-qPCR.**

449 RNA from cells was isolated using the RNeasy kit (Qiagen) and RNA from infected supernatants  
450 was isolated using the QIAamp viral RNA kit (Qiagen), both according to manufacturer's  
451 instructions. The RNA copy number of harvested RNA was measured in triplicate by RT-qPCR

452 using the TaqMan Fast Virus 1-Step Mix (Qiagen) with an HCV-specific probe targeting the 5'  
453 untranslated region of HCV (Assay ID: Pa03453408\_s1). The copy number was calculated by  
454 comparison to a standard curve of a full-length *in vitro* transcribed HCV RNA, as described [57].

#### 455 **Immunoblotting.**

456 Cells were lysed in a modified radio immunoprecipitation assay (RIPA) buffer (10mM Tris pH  
457 7.5, 150mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with protease  
458 inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Millipore), and post-nuclear  
459 supernatants were harvested by centrifugation. Quantified protein was resolved by SDS/PAGE,  
460 transferred to PVDF membranes using the Turbo-transfer system (BioRad) and blocked with  
461 StartingBlock (Thermo-Fisher) or 3% bovine serum albumin (Sigma) in PBS with 0.1% Tween  
462 (PBS-T). Membranes were probed with specific antibodies, washed with PBS-T and incubated  
463 with species-specific HRP conjugated antibodies (Jackson ImmunoResearch, 1:5000), washed  
464 again with PBS-T, and treated with Pico PLUS enhanced chemiluminescent (ECL) reagent  
465 (Thermo-Fisher). The signal was then captured on X-ray film or by using a LICOR Odyssey FC.  
466 Antibodies used for immunoblot include mouse anti-HCV Core (1:250, Abcam), mouse anti-HCV  
467 NS3 (1:500, Abcam), rabbit anti-HCV NS4A (1:1000, Genscript [61]), mouse anti-HCV NS5A  
468 (1:500, 9e10, gift of Dr. Charles Rice), anti-Flag HRP (1:2500, Sigma), and rabbit anti-HA  
469 (1:500, Sigma).

#### 470 **Proteolytic Protection Assay.**

471 This protocol was adapted from the manuscript by Gentsch and colleagues [24]. Briefly, cells  
472 electroporated with JFH1-p7+NS *in vitro* transcribed RNA were harvested at 48 hours post  
473 electroporation by scraping into cold proteinase K buffer (50mM Tris-HCl pH 8.0, 10 mM CaCl<sub>2</sub>,  
474 1mM DTT). Cells were then lysed by five freeze/thaw cycles and aliquots of lysate (50µL) were  
475 either (i) left untreated (ii) pretreated with 5µL of 10% Triton-X-100 followed by proteinase K  
476 treatment (50µg/mL) for 30 minutes on ice or (iii) treated with proteinase K only. Proteinase K  
477 treatment was terminated by incubation with 10mM phenylmethane sulfonyl fluoride. The

478 samples were mixed with 4X SDS sample buffer (1M Tris (pH 6.8), 60% glycerol, 0.06%  
479 Bromophenol Blue, 12% SDS)), incubated at 50°C for 5 minutes, and immunoblotted for HCV  
480 Core protein, as above.

#### 481 **Immunoprecipitations.**

482 300-500µg of protein extracted as above was incubated with 50µL anti-Flag M2 magnetic beads  
483 (Sigma) in 1X Tris buffered saline (TBS) at 4°C overnight with head over tail rotation. Beads  
484 were washed 3X in modified 1X RIPA buffer and eluted in 2X Laemmli Buffer (BioRad) at 50°C  
485 for 5 minutes. Protein was resolved by SDS/PAGE and immunoblotting, as above.

#### 486 **Biochemical subcellular fractionation.**

487 Concentrated supernatants were purified over a 10-50% iodixanol gradient, as previously  
488 described [48]. Briefly, at 48 hours post electroporation of HCV RNA in Huh7.5 cells,  
489 supernatant was collected, mixed with polyethylene glycol (PEG) 8000 to a final concentration  
490 of 8% and incubated with rocking at 4°C overnight. PEG supernatants were centrifuged at  
491 11,000 X g for 30 minutes, supernatant was removed, and remaining pellets were suspended in  
492 cold 1X PBS. These resuspensions were layered over a 10-50% iodixanol gradient and  
493 centrifuged at 222,000 X g in a SW41 rotor in a Beckman Coulter ultracentrifuge. 10 equal  
494 fractions (1ml) were collected with a BioComp piston gradient fractionator, and then viral titer  
495 (FFU/ml), HCV RNA copy number, and HCV Core protein (immunoblotting) was measured from  
496 each fraction.

#### 497 **Statistical Analysis.**

498 Student's unpaired t-tests and one-way analysis of variance (ANOVA) were used for statistical  
499 analysis of data. Values are presented as mean ± standard error of the mean (n=3 or as  
500 indicated). \* - P < 0.05, \*\* - P < 0.01, \*\*\* - P < 0.001, \*\*\*\* - P < 0.0001.



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## 667 **Figure Captions**

668 **Fig 1. A Y45F mutation in hepatitis C virus NS4A causes a decrease in infectious viral**  
669 **titer.** (A) Schematic of the viral NS4A protein. \* indicates location of Y45F mutation. Numbers  
670 correspond with aa position within the NS4A protein (aa1-54) or the full-length polyprotein  
671 (aa1690-1711) (B) Focus forming assay of supernatants harvested from Huh7.5 cells at  
672 indicated days post electroporation with HCV WT or NS4A Y45F *in vitro* transcribed RNA.  
673 FFU/mL = focus forming units/milliliter. Values are presented as mean  $\pm$  SD (n = 3). Data is  
674 representative of three independent experiments. (C) Sequencing of the NS4A region (nt 4710-  
675 5251) of cDNA amplified from Huh7.5 cells transfected with HCV WT or NS4A Y45F RNA in (B)  
676 at indicated days.

677 **Fig 2. The NS4A Y45F mutation in HCV does not alter viral RNA replication.** (A) Renilla  
678 luciferase assay to measure HCV replicon luciferase reporter (JFH1-SGR-luc) activity from  
679 Huh7.5 cells following electroporation. GND = lethal mutation in HCV NS5B RNA-dependent  
680 RNA polymerase. RLU = *Renilla* luciferase units. Data is presented as mean  $\pm$  SEM, n=3. (B)  
681 Immunoblot analysis of extracts of Huh7.5 cells at 72 hours post transfection with indicated HCV  
682 RNA. (C) Immunoblot analysis of anti-Flag immunoprecipitated extracts and whole cell lysates  
683 (WCL) from Huh7.5 cells transfected with indicated tagged HCV proteins or vector.

684 **Fig 3. The NS4A Y45F mutation inhibits HCV envelopment.** (A, B) Focus forming assay of  
685 supernatants for extracellular titer, or cellular lysates for intracellular titer, respectively, from  
686 Huh7.5 cells at 48 hours post electroporation with HCV WT, Y45F, or GND *in vitro* transcribed  
687 RNA. (C) Immunoblot analysis for HCV Core protein of cell lysates of Huh7.5 cells at 48 hours  
688 post electroporation with *in vitro* transcribed HCV RNA subjected to the indicated treatments in  
689 a proteinase K protection assay.  $\Delta$ E1/E2 = complete deletion of E1 and E2 coding regions (aa  
690 192 to 720 of JFH1). For panels A and B, data is presented as mean  $\pm$  SEM, (n=3). C is  
691 representative of 3 independent experiments.

692 **Fig 4. The acidic domain of NS4A is important for HCV envelopment.** (A) Immunoblot  
693 analysis for HCV Core protein of cell lysates of Huh7.5 cells at 48 hours post electroporation  
694 with *in vitro* transcribed HCV RNA containing the indicated mutations in the NS4A region,  
695 subjected to the indicated treatments in a proteinase K protection assay. (B) Quantification of  
696 ratio of protease resistant Core to untreated Core from (A).  $\Delta$ E1/E2 = complete deletion of E1  
697 and E2 coding regions (aa 192 to 720 of JFH1). Data is presented as mean  $\pm$  SEM (n=3). \* - P  
698 < 0.05. \*\* - P < 0.01. (C) *Renilla* luciferase assay to measure HCV replicon luciferase reporter  
699 (JFH1-SGR-luc) activity from Huh7.5 cells following electroporation of indicated constructs.  
700 GND = lethal mutation in HCV NS5B RNA-dependent RNA polymerase. RLU = *Renilla*  
701 luciferase units. (D) Immunoblot analysis of lysates from 72 hour time point from (C). (E, F)  
702 Focus forming assay of supernatants for extracellular titer or cellular lysates for intracellular titer,  
703 respectively, from Huh7.5 cells electroporated *in vitro* transcribed HCV RNA containing the  
704 indicated mutations in the NS4A region. For (B, C, E, F), data is presented as mean  $\pm$  SEM,  
705 (n=3). Panel A is representative of three independent experiments.

706 **Fig 5. NS4A Y45 is required for NS4A interaction with the E1 glycoprotein.** (A) Immunoblot  
707 analysis of anti-Flag immunoprecipitated extracts from Huh7.5 cells transfected with NS4A-HA  
708 WT, NS4A-HA Y45F and Flag-tagged E1. (B) Quantification of the NS4A:E1 signal seen in the  
709 immunoprecipitation from (A). Data is presented as mean  $\pm$  SEM, (n=3). \*\*\* - P < 0.001. (C)  
710 Immunoblot analysis of anti-HA immunoprecipitated extracts from Huh7.5 cells at 48 hours post  
711 electroporation with *in vitro* transcribed HCV HJ3-E1/HA-NS2/YFP [41] which contains an N-  
712 terminal HA tag on the E1 protein.

713 **Fig 6. NS4A binds the first hydrophobic region of E1.** (A) Schematic of the HCV E1 protein  
714 with functional domains indicated. aa locations are based on the JFH1 genome, within the E1  
715 coding region. (B) Immunoblot analysis of anti-Flag immunoprecipitated extracts from Huh7.5  
716 cells transfected with NS4A-HA WT and Flag-tagged E1, either full length (WT) or as indicated.

717 **Fig 7. NS4A Y45F causes release of Core oligomers devoid of HCV RNA.** Huh7.5 cells were  
718 electroporated with either WT or NS4A Y45F *in vitro* transcribed HCV RNA. Supernatants were  
719 harvested and analyzed for HCV RNA by RT-qPCR (A) or Core protein by immunoblot (B). Data  
720 in A are presented as mean  $\pm$  SEM (n=4). \*\*\*\* - P < 0.0001. Supernatant from Huh7.5 cells  
721 electroporated with *in vitro* transcribed HCV WT or NS4A Y45F RNA was concentrated and  
722 fractionated over a 10-50% iodixanol gradient and 10 equal fractions were collected. Fractions  
723 were analyzed by focus-forming assay for infectivity and RT-qPCR for HCV RNA (C) and also  
724 for HCV Core protein by immunoblot (D). Fraction 1-10 correspond with fractions running from  
725 top to bottom of the gradient. B-D are representative of 2 independent experiments.

726 **Supporting information**

727 **Fig S1. NS4A binds to Core and NS5A but not p7 or NS2.** Immunoblot analysis of anti-Flag  
728 immunoprecipitated extracts from Huh7.5 cells transfected with NS4A-HA WT, NS4A-HA Y45F  
729 and Flag-tagged Core (A), E2 (B), p7/NS2 (C), or NS5A (D).

Figure 1

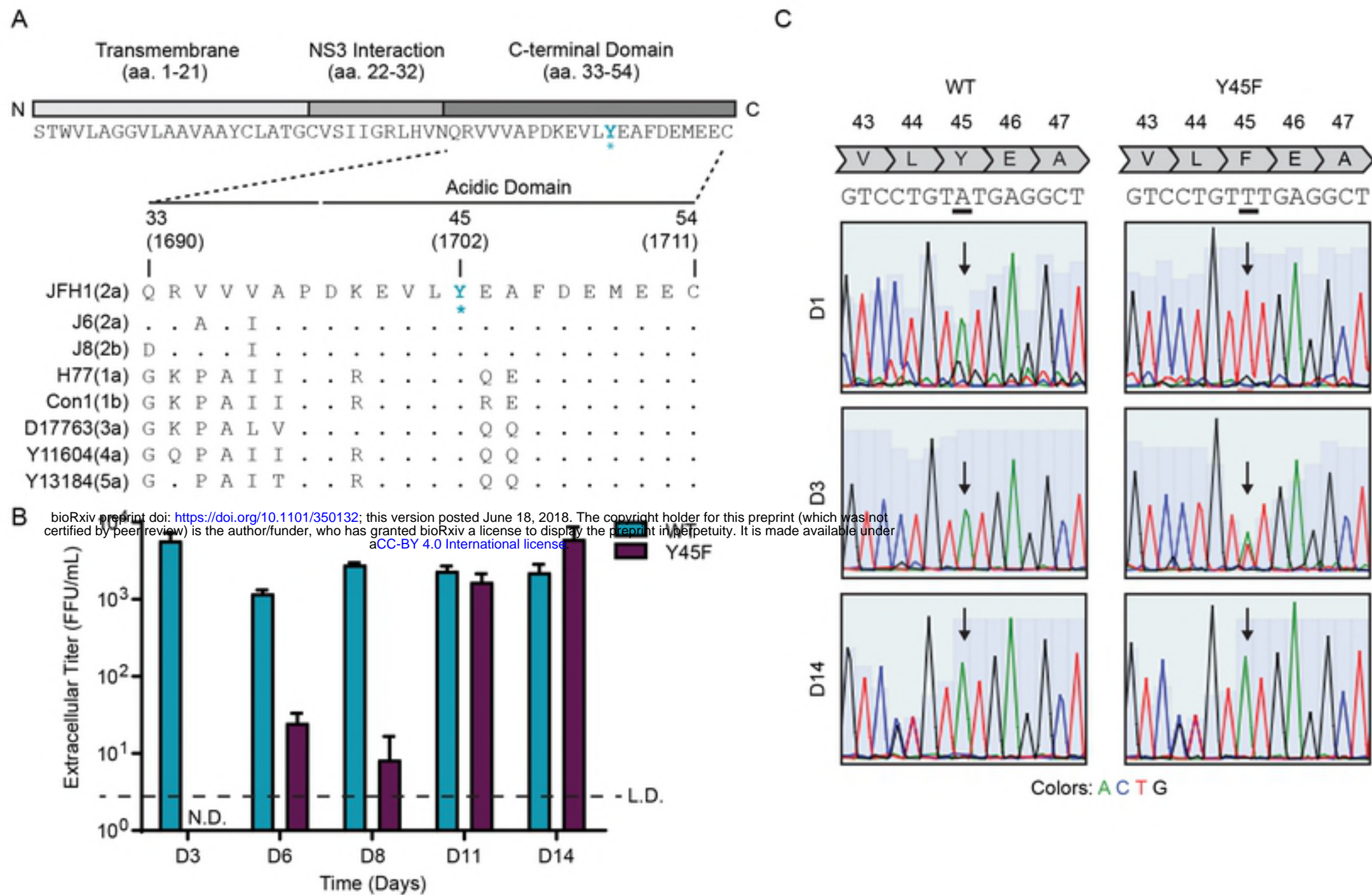
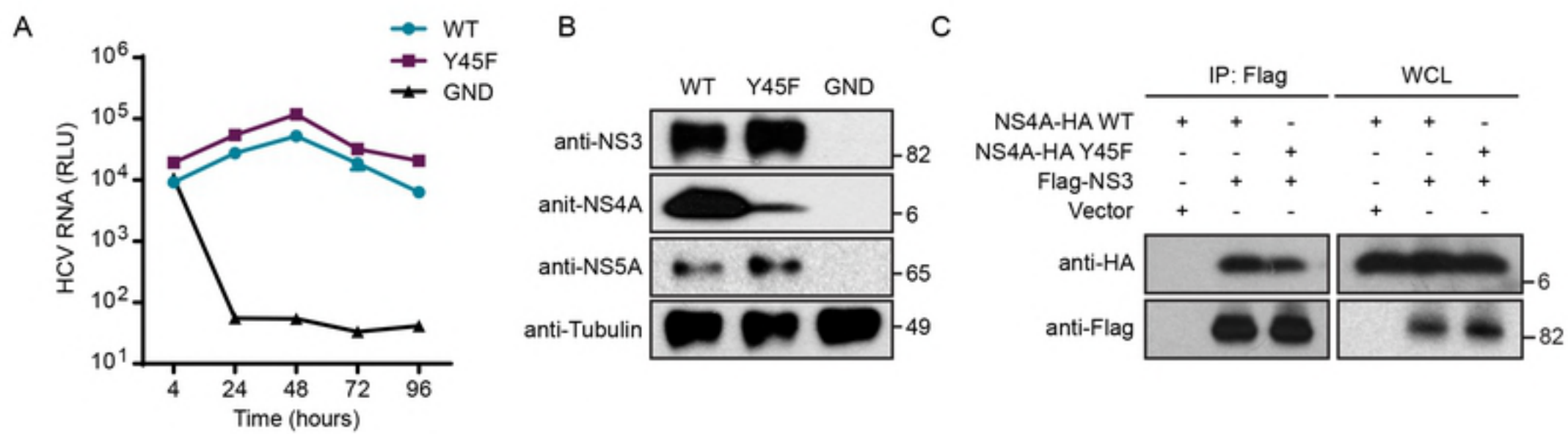
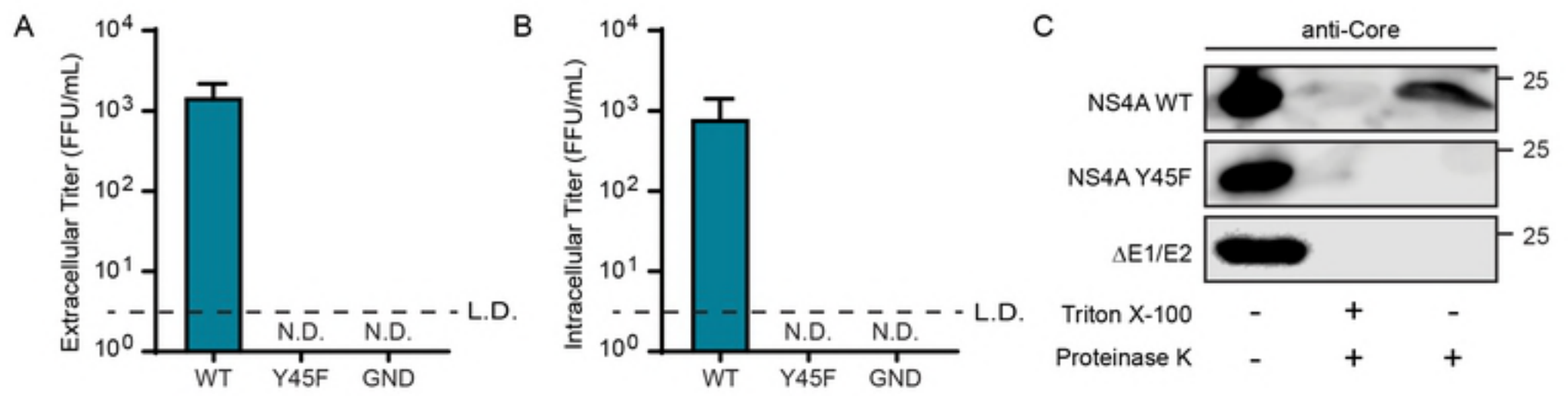


Figure 2



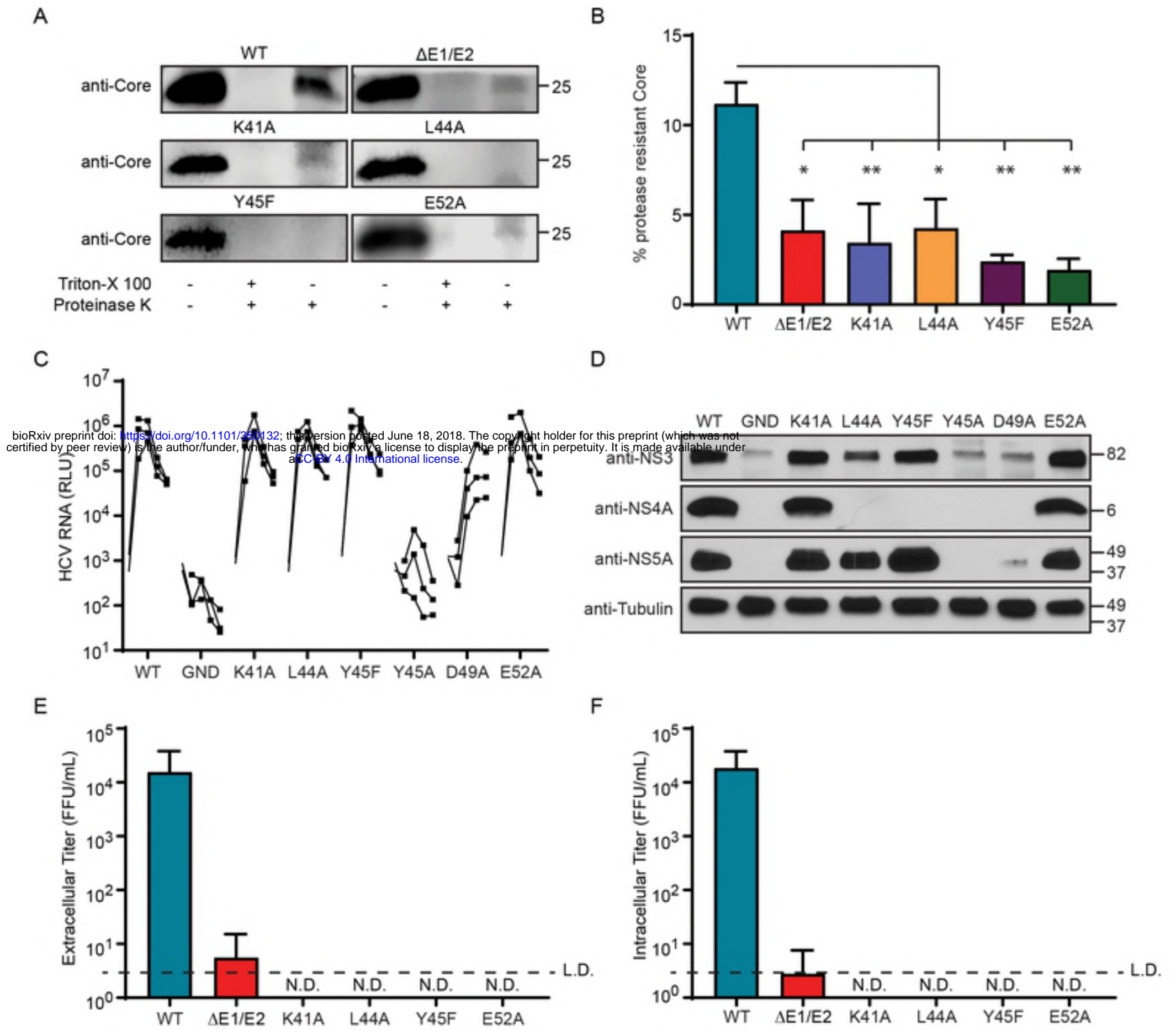
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Figure 3



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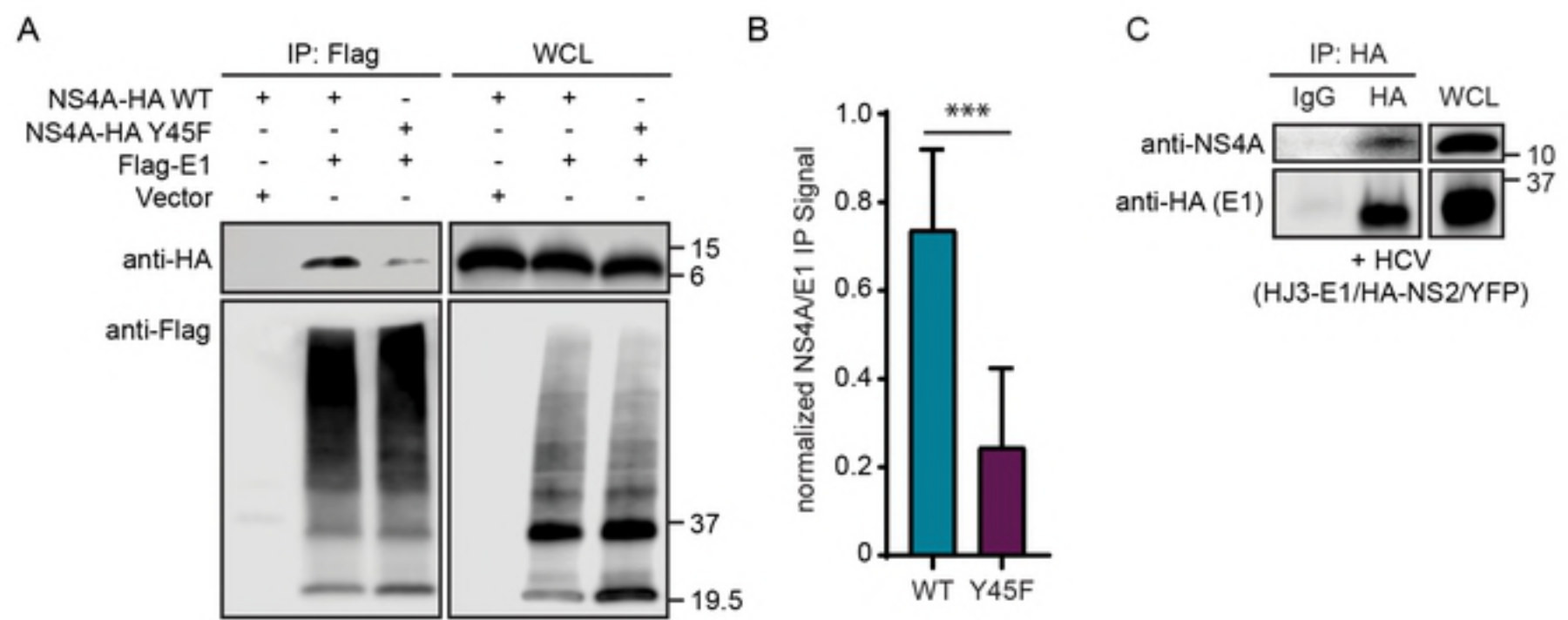
Figure 4



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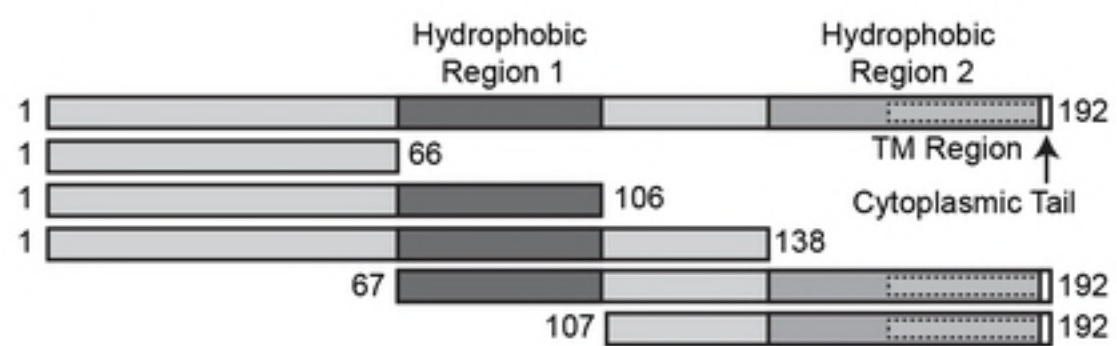
Figure 5



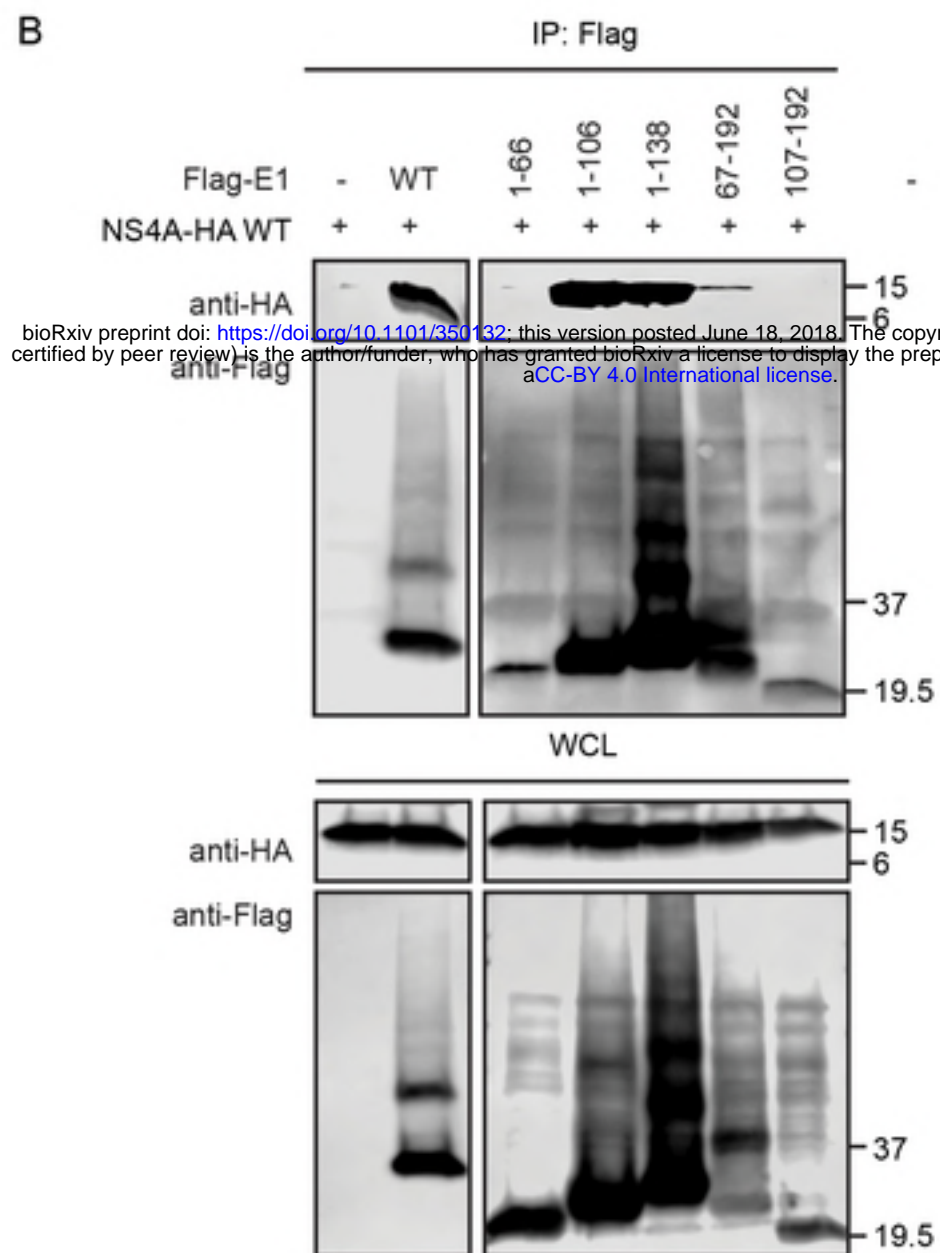
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Figure 6

A



B



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Figure 7

