Single-point mutations of hepatitis C virus NS3 that impair p53 interaction and anti-apoptotic activity of NS3

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17 Abbreviations: HCV, hepatitis C virus; aa, amino acid; GST, glutathione S transferase; PBS,

18 phosphate-buffered saline; wt, wild-type; mut, mutant-type.

19

19 **Abstract**

20

21   The N-terminal domain of NS3 of hepatitis C virus (HCV) possesses serine protease

22 activity, which is essential for virus replication. This portion is also implicated in malignant

23 transformation of hepatocytes. We previously demonstrated that an N-terminal portion of

24 NS3 formed a complex with the tumor suppressor p53 and suppressed actinomycin D-induced

25 apoptosis. We report here that single-point mutations of NS3 at position 106 from Leu to Ala

26 (L106A), and position 43 from Phe to Ala (F43A) to a lesser extent, significantly impaired

27 complex formation with p53. Moreover, the L106A mutation impaired an otherwise more

28 distinct anti-apoptotic activity of NS3. F43A and L106A mutations also inhibited serine

29 protease activity of NS3. These results collectively suggest the possibility that Leu^{106} and

30 Phe^{43} are involved in p53 interaction and serine protease activity, and therefore, can be a good

31 target for certain low-molecular-weight compound(s) to inhibit both oncogenic and

32 replicative abilities of HCV.

33

34 **Key words:** hepatitis C virus; NS3; p53; tumor suppressor; protein-protein interaction;

35 point-mutation; apoptosis; serine protease

Introduction

Hepatitis C virus (HCV) was identified as the major etiological agent of most transfusion-associated non-A, non-B hepatitis in 1989 [1,2]. It has been estimated that more than 170 million people are infected with HCV worldwide [3]. HCV easily establishes persistent infection, which may be mild, or sometimes even asymptomatic, in early phases of the disease, but after a decade or two, may cause liver cirrhosis and eventually hepatocellular carcinoma [4].

HCV, an enveloped RNA virus, belongs to genus *Hepacivirus*, family *Flaviviridae* [5]. The viral genome, single-stranded, positive-sense RNA of about 9.6 kb, consists of 5'- and 3'-untranslated regions, and a large open reading frame that encodes a polyprotein precursor of 3,010-3,030 amino acids (aa) [5,6]. The polyprotein is cleaved by the host signal peptidase and virus-encoded two proteases to generate mature viral proteins; four structural proteins, such as Core, E1, E2, p7, and six nonstructural proteins, such as NS2, NS3, NS4A, NS4B, NS5A and NS5B [5,7,8].

HCV NS3 is a multifunctional protein essential for virus replication. Its N-terminal one-third is responsible for chymotrypsin-like serine protease activity that cleaves at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions [9,10]. A minimal portion of the NS3 serine protease activity has been mapped to a region between aa 33 and 178 [11]. Its C-terminal half possesses RNA helicase activity [9,10]. In addition to its important roles in virus replication, NS3 is implicated in malignant transformation. For example, an N-terminal portion of NS3 was reported to transform NIH3T3 cells [12] and rat fibroblast cells [13]. We also observed that an N-terminal portion of NS3 inhibited apoptosis of NIH3T3 cells [14], which is a prerequisite for malignant transformation.

The tumor suppressor p53 is a key molecule in the signaling pathway of apoptosis

61   induced by DNA damage or deregulated cell cycle [15]. Some DNA tumor viruses are known

62   to interact with p53 to inactivate its function. For example, human papillomavirus E6 forms a

63   ternary complex with p53 and E6AP, an E3 ubiquitin ligase, and this complex formation

64   results in the ubiquitination and subsequent degradation of p53 [16,17]. It has also been

65   demonstrated that simian virus 40 T antigen binds to and inactivate the function of p53 [18].

66   Similarly, adenovirus E1B [19] and hepatitis B virus X protein [20] were reported to suppress

67   p53 function although their direct binding to p53 appears to be weak.

68   As for the possible interaction between HCV NS3 and p53, it was reported that NS3

69   repressed p53-dependent transcriptional activity of p21^{wafl} [21]. Also, we have demonstrated

70   that p53 enhances nuclear accumulation of NS3 [22, 23] and that NS3, through its N-terminal

71   portion, forms a complex with p53 [24]. In this study, we defined a minimum p53-binding

72   region(s) of NS3 and identified single-point mutations that impaired the interaction with p53.

73   We also examined possible biological significance of those mutations.

74

75   **Materials and Methods**

76

77   *Expression plasmids.* cDNA encoding an N-terminal region of NS3 (aa 1 to 198) of the

78   HCV isolate no. 43 [25] (GenBank accession no. **AB072085**) was amplified using primers

79   NS3/M/B and NS3/AS/H (Table 1), and subcloned into pcDNA3.1/Myc-His(-)C (pcDNA)

80   (Invitrogen) to generate pcDns3/1-198. Various deletion mutants of pcDns3/1-198 (see Fig.

81   1A) were constructed using appropriate sets of primers, NS3-del-S and NS3-del-AS. To

82   express the full-length NS3 (NS3-Full), the entire NS3 sequence was amplified from

83   pBSns3/1-631 [23] (GenBank accession no. **D45172**) using primers NS3/M/B and

84   NS3F/HindIII, and subcloned into pcDNA. The resultant plasmid was designated

85   pcDns3-Full. All the NS3 fragments expressed from these plasmids could be detected by

86　anti-Myc antibody. Expression plasmids for NS3 fragments tagged with glutathione S

87　transferase (GST) and Myc-His were also constructed. In brief, *Hinc*II-*Bam*HI fragment of

88　pGEX-4T-1 (Amersham Biosciences) containing GST sequence was subcloned into

89　pBlueScript II SK- to generate pBS-GST. An *EcoR*I–*BamH*I fragment of pBS-GST was

90　introduced to pcDNA to generate pcDgst. Various deletion mutants of NS3 were amplified

91　from pcDns3/1-198 and subcloned into pcDgst.

92　　　　Single-point mutations were introduced using QuikChange Site-Directed Mutagenesis

93　kit (Stratagene) according to the manufacturer's instruction. For plasmid-based expression,

94　the Myc-tagged NS3 fragments described above were subcloned into pSG5 vector

95　(Stratagene) downstream of simian virus 40 early promoter.

96　　　　An expression plasmid for Myc-tagged NS4B, designated pEFns4B, was constructed

97　by amplifying the corresponding sequence from pM094AJ (HCV-1b) [26] using primers

98　NS4B-1-S and NS4B-Xba-AS, and subcloned into pEF1/Myc-His (Invitrogen). A coding

99　region for NS5A/5BΔC polyprotein (aa 1973 to 2721 in the entire HCV polyprotein) was

100　amplified from pTMns2-5b(810-2721) [23] using primers 5A5BncoI and 5A5BpstI, and

101　subcloned into pTM1 vector to generate pTM-NS5A/5BΔC. This plasmid was used to express

102　a substrate for the NS3 serine protease.

103　　　　To express p53, *Xho*I fragments of pcDM8VAarg/neo and pcDM8A431/neo [22,23,27],

104　which harbor the entire sequences of wild-type (wt) and mutant-type (mut) p53, respectively,

105　were subcloned into pcDNA and pSG5 vectors. Resultant plasmids were designated

106　pcDwt-p53, pcDmut-p53 and pSGwt-p53. pSGwt-p53 was used for plasmid-based

107　expression.

108

109　　　　*Transient protein expression*. HeLa and Huh7 cells were maintained in Dulbecco's

110　modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at

111   37°C in 5% CO_2. For protein expression, vaccinia virus-T7 hybrid expression and

112   plasmid-based expression systems were used, as reported previously [22–24,28]. After

113   overnight cultivation, the protein expression was examined by the following analyses.

114

115   *Immunoprecipitation and immunoblotting.* Cells were lysed in a buffer consisting of 10

116   mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA. After being

117   clarified by centrifugation, cell lysates were incubated with anti-p53 rabbit antiserum (Santa

118   Cruz) for 1 h at 4°C and then further incubated with protein A sepharose beads (Amersham)

119   on a rotator for 30 min at 4°C. Normal rabbit serum served as a control. After being washed

120   with the buffer, the immunoprecipitates were subjected to SDS-PAGE and blotted onto a

121   polyvinylidene fluoride membrane (Immobilon-P, Millipore). After being blocked with 5%

122   skim milk, the membrane was incubated with mouse monoclonal antibody against either the

123   Myc peptide (Santa Cruz Biotechnol.) or p53 (Ab-1; Calbiochem), and subsequently with

124   peroxidase-labeled goat anti-mouse IgG (MBL, Nagoya, Japan). The protein bands were

125   visualized by using an enhanced chemiluminescence (Amersham) and their intensity

126   measured by LAS-1000 (Fuji Film).

127

128   *Double-staining immunofluorescence.* Cells were fixed with 3.7% paraformaldehyde

129   for 15 min at 37°C and cold methanol for 10 min. After being washed with

130   phosphate-buffered saline (PBS), the cells were incubated with anti-Myc mouse monoclonal

131   antibody (Santa Cruz) and anti-p53 rabbit antiserum (Santa Cruz) for 1 h. The cells were

132   washed with PBS and then incubated with FITC-conjugated goat anti-mouse IgG (MBL) and

133   Cy3-conjugated donkey anti-rabbit IgG (Chemicon) for another 1 h. The cells were observed

134   with a laser scanning confocal microscope (Carl Zeiss).

135

136   *NS3-expressing cell lines.* NIH3T3 cells were maintained in Dulbecco's modification of

137   Eagle's medium containing 10% heat-inactivated fetal calf serum at 37°C in a CO_2 incubator,

138   as described previously [14]. The cells were transfected with pSGns3/1-180,

139   pSGns3/1-180/L106A, or the pSG5 empty vector, together with pSV2-neo as a selection

140   marker, and cultivated in the presence of a neomycin derivative (G418, 600 μg/ml, GIBCO).

141   After 2 weeks, stable transformants were cloned using cloning cylinders and tested for NS3

142   expression by immunoblotting. Five clones each strongly expressing NS3/1-180 or

143   NS3/1-180/L106A, and the non-expressing control, were selected and mixed to avoid clonal

144   variation.

145

146   *Quantitative apoptosis assays.* Cells were seeded into 6-mm well of a 96-well plate at a

147   concentration of 5×10^3 cells/well and incubated overnight. The cells were then treated with

148   actinomycin D (10 ng/ml) for 3 days. The number of the living cells was monitored every day

149   by using cell proliferation reagent WST-1 (Roche), according to the manufacturer's protocol.

150   In brief, 100 μl of culture medium containing 10% WST-1 was added to each well. After 1 h,

151   the absorbance was measured at 450 nm with a microplate reader.

152

153   *Measurement of serine protease activity of NS3.* Various forms of NS3 were

154   co-expressed with NS5A/5BΔC polyprotein, a substrate for the NS3 serine protease. The cells

155   were lysed and probed with subjected to immunoblot analysis using anti-NS5A mouse

156   monoclonal antibody (kindly provided by I. Yoshida, Research Institute for Microbial

157   Diseases, Kan-Onji Branch, Kagawa, Japan) and peroxidase-conjugated goat anti-mouse IgG

158   (MBL). Intensity of the bands corresponding to the cleaved NS5A and the uncleaved

159   NS5A/5BΔC was measured. Arbitrary units of serine protease activity of each NS3 were

160   calculated by the following formula.

7

161   Protease activity (arbitrary unit) = NS5A ÷ (NS5A/5BΔC + NS5A)

162

163   **Results**

164

165   *Minimum regions of NS3 responsible for complex formation with p53.* We previously

166   demonstrated that an N-terminal portion of NS3 (aa 29 to 174) was involved in complex

167   formation with wt-p53 [24]. In this study we aimed to narrow down the p53-binding region(s)

168   and to determine a residue(s) responsible for the interaction. We expressed a number of

169   deletion mutants of NS3 (Fig. 1A). Initial immunoprecipitation experiments revealed that two

170   non-overlapping regions of NS3, one spanning from 1 to 90 and the other from 91 to 198,

171   each formed a complex with p53 (Fig. 1B, lanes 6 and 8). The specificity of the interaction

172   between NS3 and p53 was secured by control experiments (lanes 1 and 2), and also confirmed

173   by the lack of interaction between p53 and a control protein, HCV NS4B (Fig. 1C). These

174   results suggested the possible presence of at least two separate regions responsible for p53

175   binding. We then tried to express further deleted NS3 sequences. However, those shorter

176   fragments were quite unstable and only poorly detectable, which made the analysis practically

177   impossible. Therefore, we expressed NS3 fragments tagged with GST and Myc-His to

178   overcome this problem.

179   GST- and/or Myc-tagged NS3 fragments used in this study are shown in Fig. 1A. Using

180   these fragments, we identified three separate regions; NS3/41-90, /61-110 and /121-160

181   formed a complex with p53 (Fig. 1D, lanes 3, 9 and 15). On the other hand, NS3/46-90,

182   /61-105 and /121-155 fail to interact with p53 (lanes 4, 10 and 16), suggesting that residues at

183   positions from 41 to 45, from 106 to 110, and from 156 to 160 were likely to involved in the

184   interaction.

185   To identify the responsible residues, we introduced a single-point mutation and tested

186   the ability of each NS3 mutant to interact with p53. The results clearly demonstrated that a

187   single-point mutation of NS3 at position 43 from Phe to Ala (F43A) abolished the interaction

188   with p53 (Fig. 2, lane 5). Likewise, single-point mutations at position 106 from Leu to Ala

189   (L106A) and position 158 from Val to Ala (V158A) abolished the interaction with p53 (lanes

190   9 and 15). These results obtained with GST- and Myc-tagged short fragments of NS3

191   suggested the possible importance for Phe, Leu and Val at positions 43, 106 and 158,

192   respectively, in complex formation with p53.

193

194   *Residues of NS3 responsible for complex formation with p53.* We then introduced the

195   F43A and L106A mutations into Myc-tagged NS3/1-180 and NS3-Full, and examined their

196   possible interaction with p53. The results obtained demonstrated that the introduction of

197   L106A mutation into NS3/1-180 markedly inhibited interaction with p53 while F43A

198   mutation only weakly impaired it (Fig. 3A). On the other hand, V158A mutation did not

199   affect the interaction (data not shown). When introduced to NS3-Full, L106A mutation, again,

200   markedly inhibited interaction with p53 while F43A mutation only weakly (Fig. 3B). These

201   results suggested an important role for Leu at position 106, and Phe at position 43 to a lesser

202   extent, in the interaction between NS3 and p53.

203   We also examined the possible effect of those single-point mutations on the interaction

204   with mut-p53, which has a mutation at position 273 from Arg to Gln and, unlike wt-p53,

205   localizes exclusively in the cytoplasm [22]. The result showed that, when introduced to

206   NS3/1-180, L106A mutation markedly impaired the interaction with mut-p53 (Fig. 3C). F43A

207   mutation also impaired the interaction with mut-p53, though to a lesser extent than that

208   observed with the L106A mutation. These results are consistent with those obtained with

209   wt-p53 (see Fig. 3A).

210

211   *Effects of single-point mutations on subcellular localization of NS3 in the presence and*

212   *absence of wt-p53.* We have reported that NS3-Full and its C-terminally truncated form

213   (NS3/1-433) were each co-localized with wt-p53 almost exclusively in the nucleus when

214   transiently expressed in HeLa cells [22,23]. In this study, we investigated the subcellular

215   localization of NS3/1-180, NS3-Full, and their point-mutants both in the presence and

216   absence of wt-p53 in Huh7 cells using plasmid-based expression system. NS3/1-180 was

217   localized both in the cytoplasm and the nucleus when expressed alone (Fig. 4, panel *a*), and

218   was localized almost exclusively in the nucleus when co-expressed with wt-p53 (panel *b*),

219   with the results being consistent with our previous observations [22,23]. NS3/1-180/F43A

220   exhibited practically the same localization patterns as those of the parental NS3/1-180 both in

221   the absence and presence of wt-p53 co-expression (panels *e* and *f*). However,

222   NS3/1-180/L106A was localized exclusively in the cytoplasm, exhibiting a dot-like pattern,

223   irrespective of p53 co-expression (panels *i* and *j*). Similar results were obtained when L106A

224   mutation was introduced into NS3-Full; the L106A mutant showed exclusive cytoplasmic

225   localization, irrespective of wt-p53 co-expression (panels *q* and *r*). Similar localization

226   patterns of NS3/1-180, NS3-Full and their point-mutants in the presence and absence of

227   wt-p53 were consistently observed using vaccinia virus-T7 hybrid expression system in HeLa

228   cells (data not shown).

229

230   *Impairment of anti-apoptotic activity of NS3 by the L106A mutation.* Cells were treated

231   with actinomycin D for 3 days and examined for apoptosis. Consistent with our previous

232   observations with a longer NS3 sequence encoding aa 1 to 433 [14], cells stably expressing

233   NS3/1-180 showed a higher degree of resistance against actinomycin D-induced apoptosis

234   than the non-expressing control. The otherwise distinct anti-apoptotic activity of NS3 was

235   significantly impaired by the L106A mutation (Fig. 5). Significant impairment of NS3

236   anti-apoptotic activity by the L106A mutation was also evident 2 days after actinomycin D

237   treatment (data not shown). Similar results were reproducibly obtained in separate

238   experiments.

239

240   *Effects of single-point mutations on serine protease activity of NS3.* The NS3 serine

241   protease cleaves NS5A/5BΔC polyprotein into two fragments, NS5A and NS5BΔC. By using

242   this system, we compared serine protease activities of various single-point mutants of NS3.

243   As shown in Fig. 6A, introduction of either F43A or L106A mutation into NS3/1-180

244   markedly impaired the otherwise strong serine protease activity whereas the inhibitory effect

245   of V158A mutation was marginal. Similarly, when introduced into NS3-Full, L106A

246   mutation markedly impaired the serine protease activity while F43A mutation impaired it

247   moderately (Fig. 6B).

248

249   **Discussion**

250

251   We demonstrated in the present study that single-point mutations of NS3, either

252   NS3/1-180 or NS3-Full, at position 106 from Leu to Ala (L106A), and position 43 from Phe

253   to Ala (F43A) to a lesser extent, inhibited complex formation with p53 (Fig. 3). We

254   previously reported that a 146-residue fragment near the N-terminus of NS3 (aa 29 to 174)

255   was involved in the interaction with p53 [22–24]. We also noticed that secondary structure of

256   the N-terminal 120 residues of NS3 was well correlated with the risk for development of

257   hepatocellular carcinoma in HCV-infected patients [25]. These results suggest an important

258   role for the N-terminal portion of NS3, especially Phe and Leu at positions 43 and 106,

259   respectively, in the pathogenesis of HCV, such as anti-apoptotic status [14], a mutation-prone

260   phenotype [29, 30], malignant transformation of cultured cells [12,13] and eventually

261   development of hepatocellular carcinoma in patients [25]. We previously reported that

262   N-terminal two-thirds of NS3 (aa 1 to 433) inhibited actinomycin D-induced apoptosis [14].

263   In the present study, we observed that even a shorter NS3 fragment, NS3/1-180, inhibited

264   actinomycin D-induced apoptosis and that the L106A mutation significantly impaired

265   otherwise more distinct anti-apoptotic activity of NS3/1-180 (Fig. 5). Our results collectively

266   suggest the possibility that the L106A mutation impairs NS3 anti-apoptotic activity through

267   decreased p53 binding.

268   Inhibition of apoptosis is a prerequisite for malignant transformation of the cell.

269   However, another cellular event(s) is required for malignant transformation. In fact, we

270   observed that, despite distinct inhibition of apoptosis, NIH3T3 cells stably expressing the

271   N-terminal two-thirds of NS3 [14] or NS3/1-180 (data not shown) did not exhibit typical

272   malignant phenotypes, such as higher population density in anchorage-dependent culture,

273   colony formation in anchorage-independent culture and tumor formation in nude mice. Our

274   results appear to be inconsistent with a previous report by other researchers that an N-terminal

275   portion of NS3 flanked with a short stretch of a C-terminal portion of NS2 mediated

276   malignant transformation of NIH3T3 cells [12]. This discrepancy might be explained by the

277   differences in aa sequences and expression levels of NS3, and/or cellular characteristics, etc.

278   Further studies are needed to fully understand the mechanism of malignant transformation by

279   HCV.

280   L106A and F43A mutations also inhibited serine protease activity of NS3 (Fig. 6). Due

281   to the impairment of the enzymatic activity essential for virus replication [5], HCV strains

282   with such a mutation(s) would not replicate efficiently and, therefore, they would become less

283   prevalent in a patients population. Indeed, we have not found the F43A or L106A mutations

284   among ~200 HCV clinical isolates so far tested [25].

285   Crystal structure analysis of the serine protease domain of NS3 reported previously

286 [31,32] suggest that the residues at positions 43 and 106 are localized spatially in the close

287 proximity to Ser at position 139, one of the catalytic triad of the NS3 serine protease activity.

288 This may explain why a single-point mutation, such as L106A, impairs two unrelated NS3

289 properties, p53-binding and serine protease activity. These results collectively imply the

290 possibility that certain low-molecular-weight compound(s) that specifically interacts with the

291 residues at position 106 or 43 interferes with both oncogenic and replicative abilities of HCV.

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300 **References**

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396

Figure legends

397

398   **Fig. 1.** Minimum regions of NS3 responsible for complex formation with wt-p53. (A)

399   Schematic diagram of the HCV genome and NS3. NS3-Full, NS3/1-198 and various deletion

400   mutants are depicted. (B) Various deletion mutants of Myc-tagged NS3 were expressed

401   without (lane 2) or with wt-p53 (lanes 1 and 3 to 8) in HeLa cells by using vaccinia-T7 hybrid

402   expression method. Cell lysates were immunoprecipitated using anti-p53 rabbit antiserum

403   (lanes 2 to 8) or with normal rabbit IgG (lane 1), and the immunoprecipitates were probed

404   with anti-Myc antibody to detect NS3 (upper panel). The lysates were directly (without being

405   immunoprecipitated with anti-p53 antibody) probed with anti-Myc antibody to verify

406   comparable degrees of expression of the NS3 mutants (middle panel). Efficient

407   immunoprecipitation of wt-p53 with anti-p53 antibody was also verified (lower panel). (C)

408   Myc-tagged NS3/1-198 and full-length NS4B were each co-expressed with wt-p53. Cells

409   expressing NS3 alone (lane 1) were also prepared. Cell lysates were immunoprecipitated with

410   anti-p53 antibody, and the immunoprecipitates were probed with anti-Myc antibody (upper

411   panel). The lysates were directly probed with anti-Myc antibody to verify the expression of

412   NS3/1-198 and NS4B (middle panel). Efficient immunoprecipitation of wt-p53 was also

413   verified (lower panel). (D) NS3 mutants tagged with GST and Myc were expressed without

414   (lanes 1, 7 and 12) or with wt-p53 (lanes 2 to 6, 8 to 11, and 13 to 16). Cell lysates were

415   immunoprecipitated using anti-p53 antibody, and then probed with anti-Myc antibody (upper

416   panel). The lysates were directly probed with anti-Myc antibody (middle panel). Efficient

417   immunoprecipitation of wt-p53 was also verified (lower panel).

418

419   **Fig. 2.** Single-point mutations of NS3 responsible for complex formation with wt-p53.

420   GST- and Myc-tagged NS3/31-90, NS3/61-130, NS3/121-198 and their single-point-mutants

421 were expressed in HeLa cells without (lanes 1, 7 and 13) or with wt-p53 (lanes 2 to 6, 8 to 12,

422 and 14 to 17). Cell lysates were immunoprecipitated using anti-p53 antibody, and probed with

423 anti-Myc antibody to detect NS3 (upper panel). The lysates were directly probed with

424 anti-Myc antibody (middle panel). Efficient immunoprecipitation of wt-p53 was also verified

425 (lower panel). WT, wild-type (without point-mutation).

426

427   **Fig. 3.** F43A and L106A mutations in NS3/1-180 and NS3-Full impair complex

428 formation with wt-p53. (A) Myc-tagged NS3/1-180 and its point mutants were expressed in

429 HeLa cells without (lane 1) or with wt-p53 (lanes 2 to 5). Cell lysates were

430 immunoprecipitated using anti-p53 antibody, and probed with anti-Myc antibody to detect

431 NS3 (upper panel). The lysates were directly probed with anti-Myc antibody (middle panel).

432 Efficient immunoprecipitation of wt-p53 was also verified (lower panel). Amounts of NS3

433 co-immunoprecipitated with p53 were normalized to the total amounts of NS3 in the lysates.

434 The normalized values, considered as p53-binding capacity of NS3, are depicted in the graph

435 (bottom). (B) Similar analysis was performed using NS3-Full. (C) Similar analysis was

436 performed using mut-p53 and NS3/1-180. WT, wild-type (without point-mutation).

437

438   **Fig. 4.** L106A, but not F43A, mutation alters subcellular localization of NS3/1-180 and

439 NS3-Full in the presence of wt-p53. NS3/1-180, NS3-Full and their single-point mutants were

440 each expressed without or with wt-p53 in Huh7 cells by plasmid-based expression system.

441 The cells were stained with anti-Myc mouse monoclonal antibody and anti-p53 rabbit

442 antiserum, followed by FITC-conjugated anti-mouse IgG (green) and Cy3-conjugated

443 anti-rabbit IgG (red). Merged pictures are shown on the right. WT, wild-type (without

444 point-mutation).

445

446   **Fig. 5.** L106A mutation impairs anti-apoptotic activity of NS3/1-180. Percent cell

447   survival after actinomycin D treatment (10 ng/ml, 3 days) is shown. Data represent the mean

448   ± SD obtained from triplicate cultures of two independent experiments. Expression levels of

449   NS3/1-180 and its L106A mutant in mixed cultures of five clones each are shown at the

450   bottom.

451

452   **Fig. 6.** F43A and L106A mutations in NS3/1-180 and NS3-Full impair the serine

453   protease activity. (A) NS5A/5BΔC, a substrate for NS3 serine protease, was expressed in

454   HeLa cells without (lane 1) or with Myc-tagged NS3/1-180 (lane 2) and its point mutants

455   (lanes 3 to 6). Cell lysates were probed with anti-NS5A antibody to detect uncleaved and

456   cleaved NS5A fragments (upper panel). The lysates were also probed with anti-Myc antibody

457   to verify a comparable degree of NS3 expression in each lane (lower panel). Serine protease

458   activities were measured, as described in the Materials and methods, and depicted in the

459   graph (bottom). (B) Similar analysis was performed using NS3-Full.

Table 1.   Oligonucleotide primers used for PCR amplification.

Primer	Sequence [a]	Amplified direction
NS3/M/B	5'- GCAA<u>GGATCC</u>GCC**ATG**GCGCCTATCACGGCCTA -3'	Forward
NS3/AS/H	5'- CTCC<u>AAGCTT</u>GGAATGTTTGCGGTA -3'	Reverse
NS3FHindIII	5'- CTCC<u>AAGCTT</u>GAGTGACCTCTAGGT -3'	Reverse
NS3-del-S [b]	5'- GCAA<u>GAATCC</u>GCC**ATG** (N)$_{15\text{-}18}$–3'	Forward
NS3-del-AS [b]	5'- (n)$_4$<u>AAGCTT</u>C (N)$_{13\text{-}18}$ -3'	Reverse
NS4B-1-S	5'- CCGC<u>GAATTC</u>AAC**ATG**GCGTGGAGCAGTC -3'	Forward
NS4B-Xba-AS	5'- TATA<u>TCTAGA</u>CATGGCGTGGAGCAG -3'	Reverse
5A5BNcoI	5'- CCA<u>CCATGG</u>GCTCCGGCTCGTGGCTCAG -3'	Forward
5A5BpstI	5'- CAGG<u>CTGCAG</u>AGGCCTTCAAGTAACATGTG -3'	Reverse

[a] Restriction enzyme recognition sites are underlined. The translation initiation codon is shown in boldface letters.

[b] A group of primers used to amplify various deletion mutants of NS3 (see Fig. 1A). N, nucleotide residues either identical or complementary to those of the isolate no. 43 [25] (GenBank accession no. **AB072085**); n, arbitrarily added residues that help ensure the recognition of the following sequences by the respective restriction enzymes.
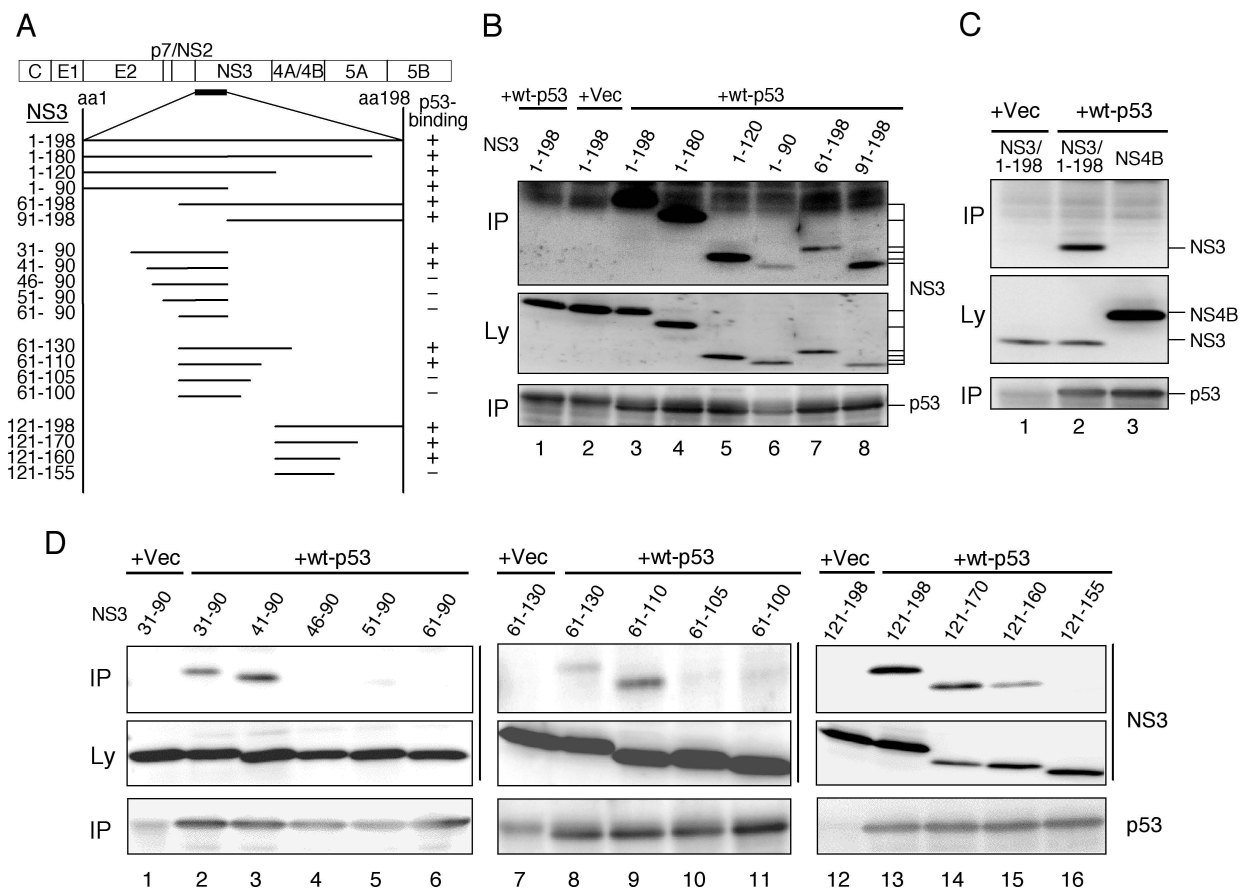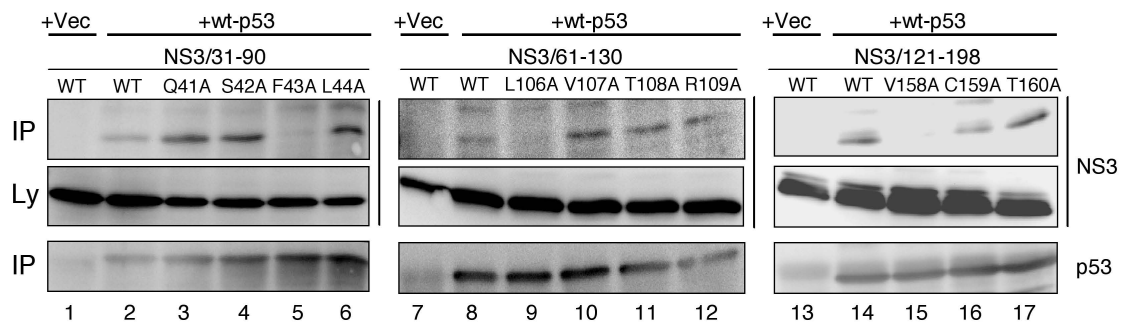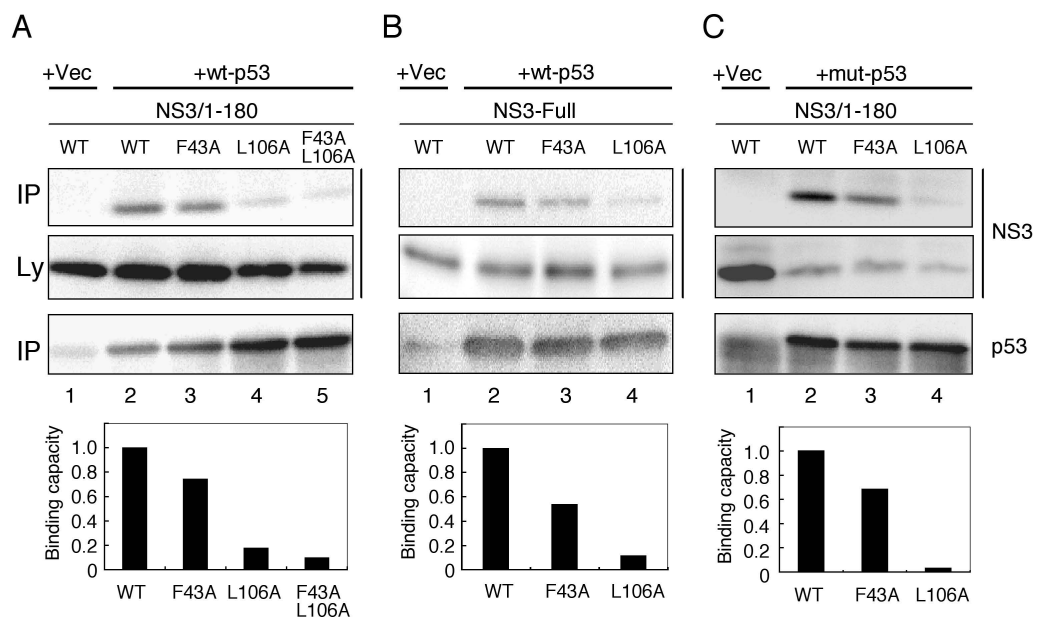
Fig. 1. Tanaka et al.

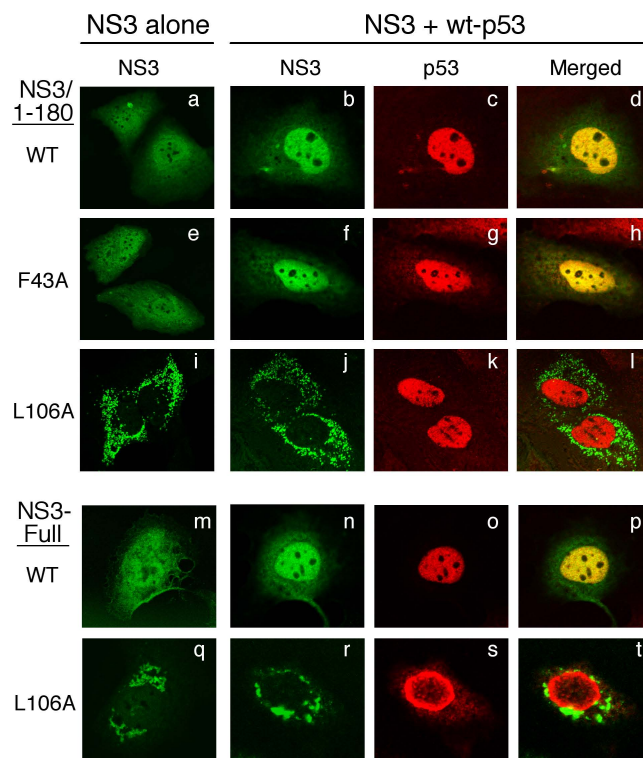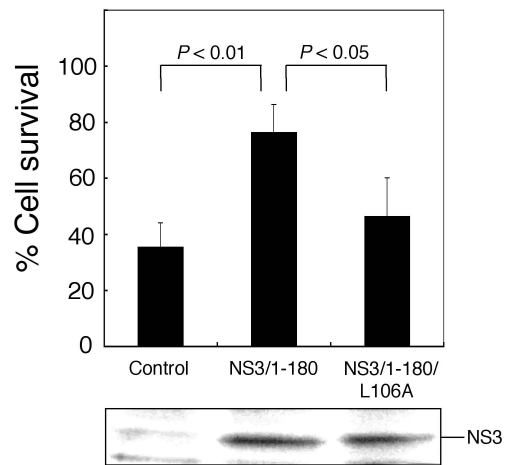Fig. 2. Tanaka et al.

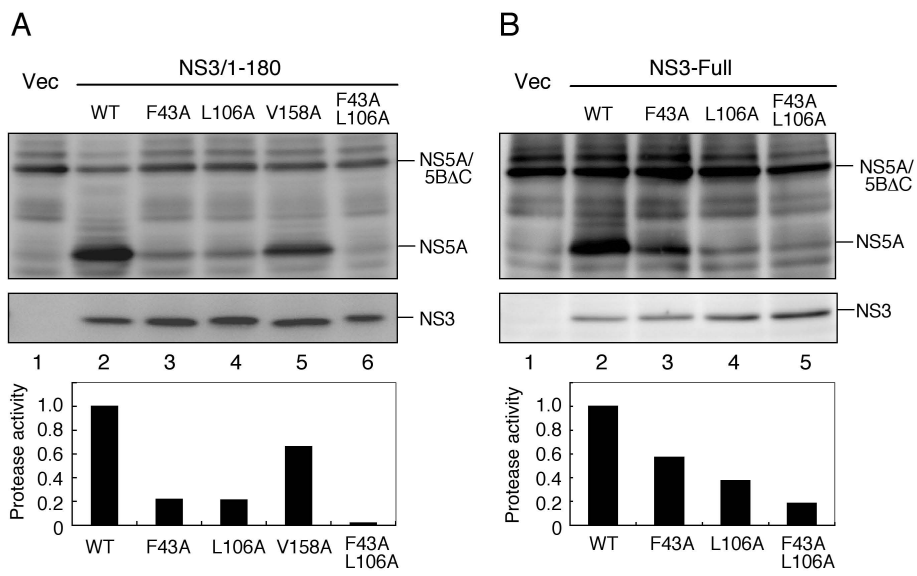Fig. 3. Tanaka et al.

Fig. 4. Tanaka et al.

Fig. 5. Tanaka et al.

Fig. 6. Tanaka et al.