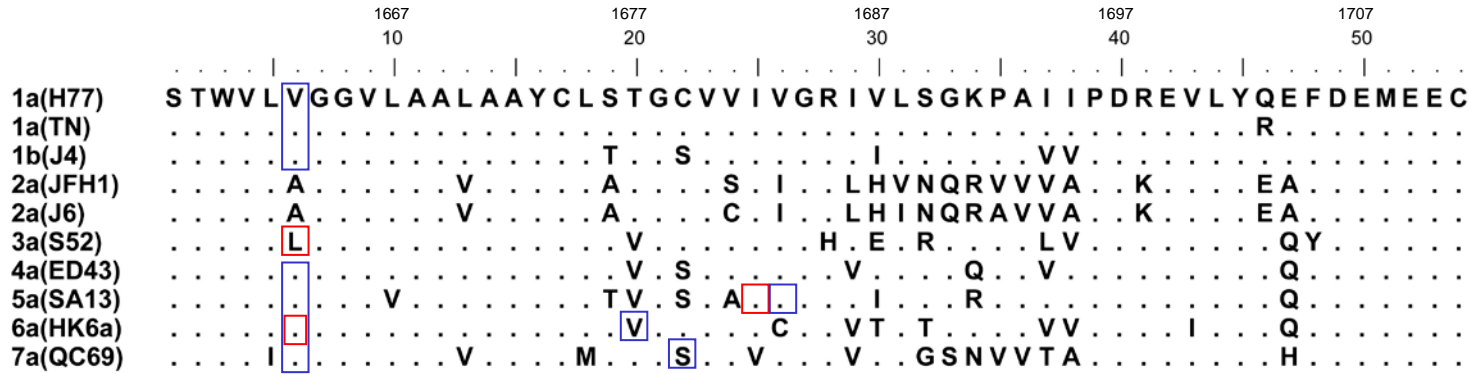


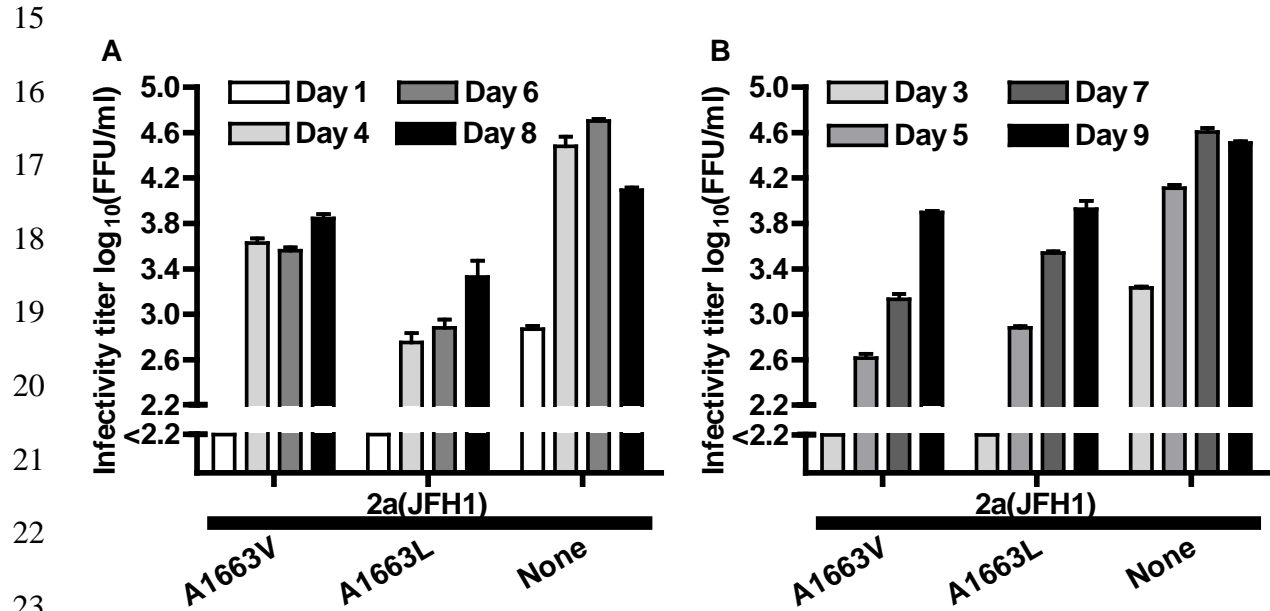
Supplemental Material

1
2
3
4
5
6
7
8
9
10
11
12
13
14

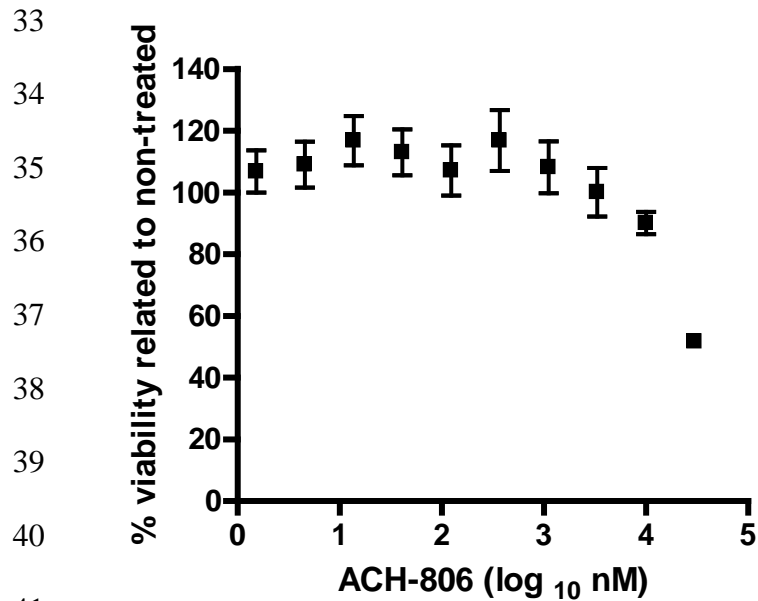
Supplemental Figures



Supplemental Figure 1. Alignment of NS4A genotype (isolate) sequences used in this study. H77 (AF009606) absolute and relative reference numbers are given. Red box; position, at which J6/JFH1-based recombinants with NS3P/NS4A of the respective genotype (isolate) acquired cell culture adaptive amino acid substitutions (1). Blue box; position, at which J6/JFH1-based recombinants with NS4A of the respective genotype (isolate) acquired cell culture adaptive amino acid substitutions.



25 **Supplemental Figure 2. Substitution of genotype 2 specific alanine at aa position 1663 led to attenuation of J6/JFH1.** (A) Shown are
 26 supernatant infectivity titers of cultures transfected with RNA transcripts of 2a(JFH1) and 2a(JFH1) recombinants with valine or leucine at
 27 aa position 1663 (V is found for most genotype 1, 4, 5, 6, and 7 isolates; L is found for most genotype 3 isolates) (Supplemental Figure 1).
 28 Titers are means of 3 replicates with SEM; lower cut-off 2.2 \log_{10} FFU/ml, indicated by column brake. (B) Supernatant infectivity titers of
 29 a subsequent 2nd passage kinetic experiment inoculated at an MOI of 0.01. Titers are means of 3 replicates with SEM; lower cut-off 2.2
 30 \log_{10} FFU/ml. 2a(JFH1)A1663V was genetically stable, while for 2a(JFH1)A1663L, L had reverted to A. All aa positions are annotated
 31 with H77 (AF009606) absolute reference numbers.



43 **Supplemental Figure 3. Cytotoxicity profile of NS4A inhibitor ACH-806.** 5000 Huh7.5 cells were plated per well of 96 well plates.
 44 After 1 day incubation, culture medium was exchanged. 24 and 48 hrs thereafter, ACH-806 at the indicated doses was administered; each
 45 dose was tested in triplicate. 24 hrs thereafter, CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to evaluate
 46 cytotoxic effects (see Materials and Methods). Percentages of viability were calculated by comparison of optical density (490 nm) values of
 47 treated cultures to the mean value of six non-treated cultures. Error bars indicate SEM.

48

49 **Supplemental Tables**

50 **Supplemental Table 1.** Coding nucleotide changes of J6/JFH1-J6(NS4A) recombinant in Huh7.5 cells

51

HCV gene		NS5B	NS5B
Nucleotide position †			
J6/JFH1-J6(NS4A)		8369	9066
H77 abs ref		8304	9001
pJ6JFH-J6(NS4A)		A	A
<i>ORIGINAL CONSTRUCT</i>	Passage (Day)	Infectivity Titer Log10 (FFU/ml)	
<i>1st Transfection</i>	1st (3)	4.3	G/A G/A
<i>2nd Transfection</i>	1st (7)	4.6	• •
Amino acid position †			
J6/JFH1-J6(NS4A)		2677	2909
H77 abs ref		2655	2887
Amino acid change		T→A	H→R

52

53

54

55

56

57

58

59 † Positions are numbered according to the HCV sequence of J6/JFH1-J6(NS4A) and with H77 (AF009606) absolute reference numbers.

60 H77 absolute nt or aa reference numbers were determined by entering the plasmid nt sequence or the derived polyprotein sequence in the

61 eu HCV database number tool (2). Positions with quasispecies are written with two capital letters in case of a 50/50 quasispecies and with

62 capital letter/lower case letter in case of a dominant and a minor sequence. Positions were included, at which coding nucleotide changes

63 occurred at least as a 50/50 quasispecies in at least one ORF. Dots indicate identity with the original plasmid sequence. Highlighted

64 positions are mutations engineered into the original construct. Indicated are number of viral passage (day of the passage experiment, at

65 which supernatant was obtained) as well as the peak infectivity titer recorded in the respective passage experiment. NS, non-structural
66 protein. NS3P, NS3 protease domain. NS3H, NS3 helicase domain.

67

68 **Supplemental Table 2.** Coding nucleotide changes of original and adapted J6/JFH1-H77(NS4A) recombinants in Huh7.5 cells

69	HCV gene	E2	p7	NS3H	NS4A	NS5A	NS5A	NS5B	
	Nucleotide position †								
70	J6/JFH1-H77(NS4A)	1679	2667	4458	5340	6284	6677	8970	
	H77 abs ref	1680	2656	4447	5329	6273	6666	8905	
71	pJ6/JFH1-H77(NS4A)	T	T	G	T	C	A	T	
	ORIGINAL CONSTRUCT	Passage	Infectivity						
72		(Day)	Log10 (FFU/ml)						
	<i>1st Transfection</i>	1st (6)	4.1	•	C/t	•	C	•	
73	<i>2nd Transfection</i>	1st (4)	4.2	C/t	C	A	C	T/C	
	CONSTRUCTS WITH INDICATED AMINO ACID CHANGES								
74	F772S	1st (6)	4.7	•	C	A/g	C	•	G/A
	F772S,V1663A	1st (6)	3.5	•	C	•	C	•	•
75	Amino acid position †								
	J6/JFH1-H77(NS4A)	447	776	1373	1667	1982	2113	2877	
76	H77 abs ref	447	772	1369	1663	1978	2109	2855	
	Amino acid change	F→L	F→S	R→Q	V→A	R→C	N→D	V→A	

78 † Positions are numbered according to the HCV sequence of J6/JFH1-H77(NS4A) and with H77 (AF009606) absolute reference numbers.

79 For detailed description see Supplemental Table 1.

80

81 **Supplemental Table 3.** Coding nucleotide changes of original and adapted J6/JFH1-TN(NS4A) recombinants in Huh7.5 cells

82	HCV gene	p7	p7	NS2	NS3P	NS3H	NS3H	NS3H	NS3H	NS4A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5B		
83	Nucleotide position †																	
	J6/JFH1-TN(NS4A)	2667	2668	2835	3533	4286	4613	4626	4982	5340	6683	6948	7035	7402	7652	7919		
	H77 abs ref	2656	2657	2824	3522	4275	4602	4615	4971	5329	6672	6937	7024	7402	7590	7854		
84	pJ6/JFH1-TN(NS4A)	T	C	T	A	A	T	T	A	T	A	C	A	G	A	T		
85	ORIGINAL CONSTRUCT	Passage	Infectivity															
		(Day)	Log10 (FFU/ml)															
	<i>1st Transfection</i>	1st (4)	3.6	C	•	•	•	•	A	C	•	C	•	•	•	C	G	•
86	<i>2nd Transfection</i>	1st (4)	4.2	C	T	A	G	G	•	•	•	C	•	•	T	•	•	G
	CONSTRUCTS WITH INDICATED AMINO ACID CHANGES																	
87	F772S	1st (6)	4.3	C	•	•	•	G/A	•	•	G/A	C	G/A	T/C	•	•	•	•
	F772S,V1663A	1st (10)	3.4	C	•	•	•	•	•	•	•	C	•	•	•	•	•	•
88	Amino acid position †																	
	J6/JFH1-TN(NS4A)	776	832	1065	1316	1425	1429	1548	1667	2115	2203	2232	2354	2438	2527			
89	H77 abs ref	772	828	1061	1312	1421	1425	1544	1663	2111	2199	2228	2354	2414	2505			
	Amino acid change	F→S	L→H	I→V	I→V	L→M	I→T	N→D	V→A	K→E	A→V	D→V	Q→H	T→A	L→V			

91 † Positions are numbered according to the HCV sequence of J6/JFH1-TN(NS4A) and with H77 (AF009606) absolute reference numbers.

92 For detailed description see Supplemental Table 1.

93

94 **Supplemental Table 4.** Coding nucleotide changes of original and adapted J6/JFH1-J4(NS4A) recombinants in Huh7.5 cells

95	HCV gene		E1	p7	NS2	NS2	NS4A	NS5A
	Nucleotide position †							
96	J6/JFH1-J4(NS4A)		962	2667	2948	2952	5340	6872
	H77 abs ref		963	2656	2937	2941	5329	6861
97	pJ6/JFH1-J4(NS4A)		A	T	G	G	T	A
	<i>ORIGINAL CONSTRUCT</i>	Passage	Infectivity Titer					
98		(Day)	Log10 (FFU/ml)					
	<i>1st Transfection</i>	1st (6)	4.2	G/A	•	A	•	C C
99	<i>2nd Transfection</i>	1st (3)	4.3	•	C/t	•	T/G	C •
	CONSTRUCTS WITH INDICATED AMINO ACID CHANGES							
100	F772S	1st (6)	4.6	•	C	•	•	C •
	F772S,V1663A	1st (3)	4.0	•	C	•	•	C •
101	Amino acid position †							
	J6/JFH1-J4(NS4A)		208	776	870	871	1667	2178
102	H77 abs ref		208	772	866	867	1663	2174
	Amino acid change		T→A	F→S	V→M	R→L	V→A	M→L

103

104 † Positions are numbered according to the HCV sequence of J6/JFH1-J4(NS4A) and with H77 (AF009606) absolute reference numbers.

105 For detailed description see Supplemental Table 1.

106

107 **Supplemental Table 5.** Coding nucleotide changes of original and adapted J6/JFH1-ED43(NS4A) recombinants in Huh7.5 cells

108

HCV gene		p7	NS2	NS2	NS3P	NS4A	NS4A	NS4B	NS5A	NS5A	NS5A	
Nucleotide position †												
pJ6/JFH1-ED43(NS4A)		2667	2859	2988	3533	5340	5349	5856	7149	7164	7611	
H77 abs ref		2656	2848	2977	3522	5329	5338	5845	7150	7162	7559	
pJ6/JFH1-ED43(NS4A)		T	A	T	A	T	T	T	T	T	C	
ORIGINAL CONSTRUCT	Passage (Day)	Infectivity Titer Log10 (FFU/ml)										
<i>1st Transfection</i>	T (57)	2.8	C	G/A	•	G	•	C	C	•	A	T/C
	1st (10)	4.6	C	•	•	G	C/T	C/T	C	•	A	•
	3rd (6)	4.2	C	•	•	G	C	•	C/T	•	A	•
<i>2nd Transfection</i>	1st (4)	4.2	•	•	C	•	C	•	•	C/T	•	•
CONSTRUCTS WITH INDICATED AMINO ACID CHANGES												
F772S												
<i>1st Transfection</i>	1st (7)	4.0	C	•	•	•	C	•	•	•	•	•
<i>2nd Transfection</i>	1st (6)	3.9	C	•	•	•	C	•	•	•	•	•
V1663A	1st (18)	4.2	C	•	•	•	C	•	•	•	•	•
F772S,V1663A	1st (6)	3.9	C	•	•	•	C	•	•	•	•	•
I879T,V1663A	1st (9)	4.3	•	•	C	•	C	•	•	•	•	•
Amino acid position †												
J6JFH-ED43(NS4A)		776	840	883	1065	1667	1670	1839	2270	2275	2424	
H77 abs ref		772	836	879	1061	1663	1666	1835	2270	2275	2402q	
Amino acid change		F→S	K→R	I→T	I→V	V→A	V→A	V→A	I→T	M→K	S→L	

111

112

113

114

115

116

117

118

119

120

121 † Positions are numbered according to the HCV sequence of J6/JFH1-ED43(NS4A) and with H77 (AF009606) absolute reference
 122 numbers. T, indicates that viruses from transfection culture were analysed. For detailed description see Supplemental Table 1.

123

124 **Supplemental Table 6.** Coding nucleotide changes of original and adapted J6/JFH1-SA13(NS4A) recombinants in Huh7.5 cells

125	HCV gene	E2	p7	p7	NS3P	NS3H	NS3H	NS4A	NS4A	NS5A	NS5A	NS5A		
	Nucleotide position †													
126	J6/JFH1-SA13(NS4A)	1554	2600	2667	3533	4431	4458	5340	5399	6558	6809	7150		
	H77 abs ref	1555	2589	2656	3522	4420	4447	5329	5388	6547	6798	7151		
127	pJ6/JFH1-SA13(NS4A)	T	A	T	A	C	G	T	G	A	T	A		
	ORIGINAL CONSTRUCT	Passage	Infectivity Titer											
128		(Day)	Log10 (FFU/ml)											
	1st Transfection	1st (3)	3.6	C/t	G	C/t	•	•	•	C	T	•	•	G
129	CONSTRUCTS WITH INDICATED AMINO ACID CHANGES													
	F772S,V1663A	1st (8)	4.3	T/c	•	C	G	T/C	T	C	•	G	A	•
130	F772S,V1663A,V1683F	1st (9)	3.7	•	•	C	•	•	•	C	T	•	•	•
	Amino acid position †													
131	J6/JFH1-SA13(NS4A)	405	754	776	1065	1364	1373	1667	1687	2073	2157	2270		
	H77 abs ref	405	750	772	1061	1360	1369	1663	1683	2069	2153	2270		
132	Amino acid change	M→T	K→E	F→S	I→V	P→L	R→L	V→A	V→F	Q→R	S→T	I→M		

133

134 † Positions are numbered according to the HCV sequence of J6/JFH1-SA13(NS4A) and with H77 (AF009606) absolute reference numbers.

135 For detailed description see Supplemental Table 1.

136

137 **Supplemental Table 7.** Coding nucleotide changes of original and adapted J6/JFH1-HK6a(NS4A) recombinants in Huh7.5 cells

138

HCV gene		E2	p7	NS2	NS2	NS3P	NS3P	NS3P	NS3H	NS4A	NS4A	NS4A	NS5A	NS5A	NS5B	NS5B	NS5B	
Nucleotide position †																		
J6/JFH1-HK6a(NS4A)		2291	2667	2843	3408	3630	3645	3666	4017	5340	5382	5412	6678	7142	8605	8751	8985	
H77 abs ref		2280	2656	2832	3397	3619	3634	3655	4006	5329	5371	5401	6667	7143	8540	8686	8920	
pJ6/JFH1-HK6a(NS4A)		A	T	C	C	T	C	A	C	T	T	C	A	C	G	T	A	
CONSTRUCTS WITH INDICATED Passage Infectivity Titer																		
AMINO ACID CHANGES	(Day)	Log10 (FFU/ml)																
F772S,V1663A																		
1st Transfection	1st (3)	4.0	•	C	•	•	•	•	C	•	C	C	•	G	T	A/G	C/T	G/A
2nd Transfection	1st (13)	4.0	T/A	C	A/C	T/C	C/T	T	•	T/c	C	C/T	•	•	•	•	•	•
F772S,E1105A,V1663A	1st (20)	3.7	•	C	•	•	•	•	C	•	C	T/c	•	•	•	•	•	•
F772S,V1663A,V1677A	1st (15)	4.1	•	C	•	•	•	C/t	A/g	•	C	C	C/T	•	•	•	•	•
F772S,E1105A,V1663A,V1677A	1st (8)	3.6	•	C	•	•	•	•	C	•	C	C	•	•	•	•	•	•
Amino acid position †																		
J6JFH-HK6a(NS4A)		651	776	835	1023	1097	1102	1109	1226	1667	1681	1691	2113	2268	2755	2804	2882	
H77 abs ref		647	772	831	1019	1093	1098	1105	1222	1663	1677	1687	2109	2268	2733	2782	2860	
Amino acid change		T→S	F→S	L→I	T→I	L→S	T→M	*	T→I	V→A	V→A	T→I	N→S	P→S	M→I	L→P	D→G	

141

142

143

144

146

147 † Positions are numbered according to the HCV sequence of J6/JFH1-HK6a(NS4A) and with H77 (AF009606) absolute reference

148 numbers. For detailed description see Supplemental Table 1.

149 *Amino acid change E→A/G encoded by nucleotide change A→C/G.

150

151 **Supplemental Table 8.** Coding nucleotide changes of original and adapted J6/JFH1-QC69(NS4A) recombinants in Huh7.5 cells

152 HCV gene		E1	E1	E2	p7	p7	NS2	NS2	NS2	NS3P	NS4A	NS4A	NS4B	NS5A	NS5B	NS5B	
Nucleotide position †																	
153 J6/JFH1-QC69(NS4A)		1124	1469	2512	2667	2700	2975	3062	3158	3533	5340	5387	6132	6390	8721	8722	
H77 abs ref		1125	1470	2501	2656	2689	2964	3051	3147	3522	5329	5376	6121	6379	8656	8657	
154 pJ6/JFH1-QC69(NS4A)		A	T	A	T	T	T	G	A	A	T	A	A	A	A	T	
<i>CONSTRUCTS WITH INDICATED AMINO ACID CHANGES</i>	Passage (Day)	Infectivity Titer															
		Log10 (FFU/ml)															
155 F772S,V1663A	1st (10)	3.7	•	•	G/A	C	•	•	A/G	G/A	G/A	C	T/a	•	•	•	•
F772S,V1663A,S1679C	1st (25)	3.6	C/a	•	•	C	C/T	•	•	•	G	C	T	•	G	C	C
156 F772S,V1663A,S1679C	1st (22)	3.8	•	G/T	•	C	•	C/T	•	•	•	C	T	G/A	•	•	•
F772S,I1061V,V1663A,S1679C	1st (13)	3.8	•	•	•	C	•	•	•	•	G	C	T	•	•	•	•
Amino acid position †																	
J6JFH-QC69(NS4A)		262	377	724	776	787	879	908	940	1065	1667	1683	1931	2017	2794		
H77 abs ref		262	377	720	772	783	875	904	936	1061	1663	1679	1927	2013	2772		
Amino acid change		I→L	L→V	I→M	F→S	V→A	W→R	G→S	M→V	I→V	V→A	S→C	N→S	K→R	D→A		

159

160 † Positions are numbered according to the HCV sequence of J6/JFH1-QC69(NS4A) and with H77 (AF009606) absolute reference

161 numbers. For detailed description see Supplemental Table 1.

162

163 Reference List

164

165 1. **Gottwein JM, Scheel TK, Jensen TB, Ghanem L, Bukh J.** 2011. Differential efficacy of protease inhibitors against HCV

166 genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. *Gastroenterology* **141**:1067-1079.

167 2. **Combet C, Garnier N, Charavay C, Grando D, Crisan D, Lopez J, hne-Garcia A, Geourjon C, Bettler E, Hulo C, Le MP,**
168 **Bartenschlager R, Diepolder H, Moradpour D, Pawlotsky JM, Rice CM, Trepo C, Penin F, Deleage G.** 2007. euHCVdb: the
169 European hepatitis C virus database. *Nucleic Acids Res* **35**:D363-D366.