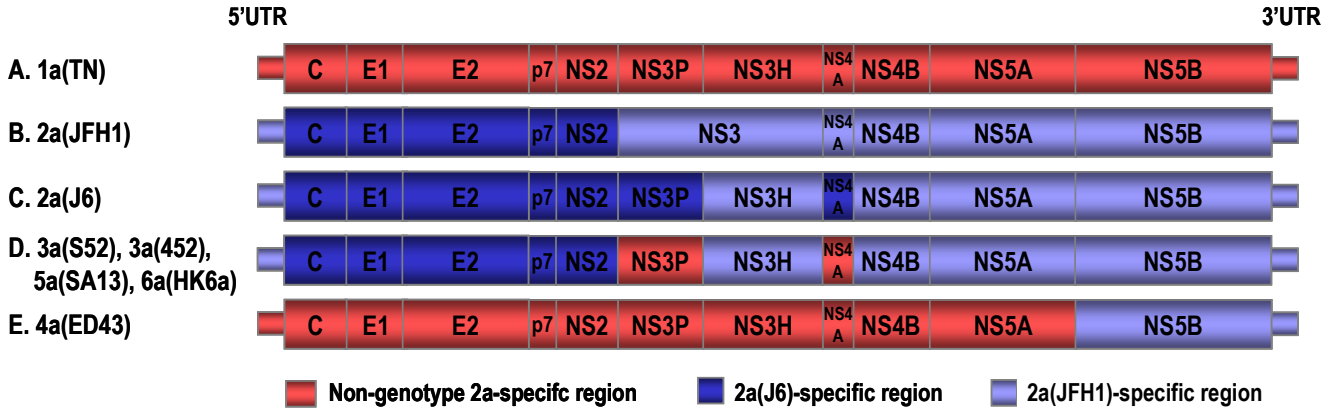


**Fig. S1. Schematic overview of HCV recombinants used in this study.** The recombinants used were designated according to the genotype(isolate) of their NS3P/NS4A sequence, as indicated on the left. The HCV capsid protein Core (C), envelope proteins E1 and E2, p7, and non-structural proteins (NS) NS2-NS5B are indicated on the genomes; 5´and 3´untranslated regions (UTRs) are indicated above the genomes. Genomic regions derived from genotype(isolate) 1a(TN), 3a(S52), 3a(452), 5a(SA13), 6a(HK6a), or 4a(ED43) are shown in red; genomic regions derived from genotype 2a isolate J6 are indicated in dark blue; and genome regions derived from genotype 2a isolate JFH1 are indicated in light blue. (*A*) 1a(TN) is identical with TN full-length +LSGF/A1226G/Q1773H (Table S1 in (3)). *(B)* 2a(JFH1) is identical with J6/JFH1 (4) and contains Core-NS2 of genotype(isolate) 2a(J6) as well as 5´UTR and NS3-3´UTR of 2a(JFH1). (*C*) 2a(J6) is identical with 2a(J6) described in (1) and contains 2a(J6) specific Core-NS3P and NS4A, while remaining genome regions are from 2a(JFH1). (*D*) 3a(S52), 3a(452), 5a(SA13), and 6a(HK6a) are identical with 3a(S52)mut7, 3a(452), 5a(SA13)mut7, and 6a(HK6a)mut6, described in (1) and contain genotype(isolate)-specific NS3P/NS4A, while remaining genome regions are derived from 2a(J6) or 2a(JFH1), as indicated. (*E*) 4a(ED43) is identical with 4a(ED43)5-5A_LS/R781W/A1309P/A1786V (2) and contains 5´UTR-NS5A of genotype(isolate) 4a(ED43) and NS5B-3´UTR of 2a(JFH1).

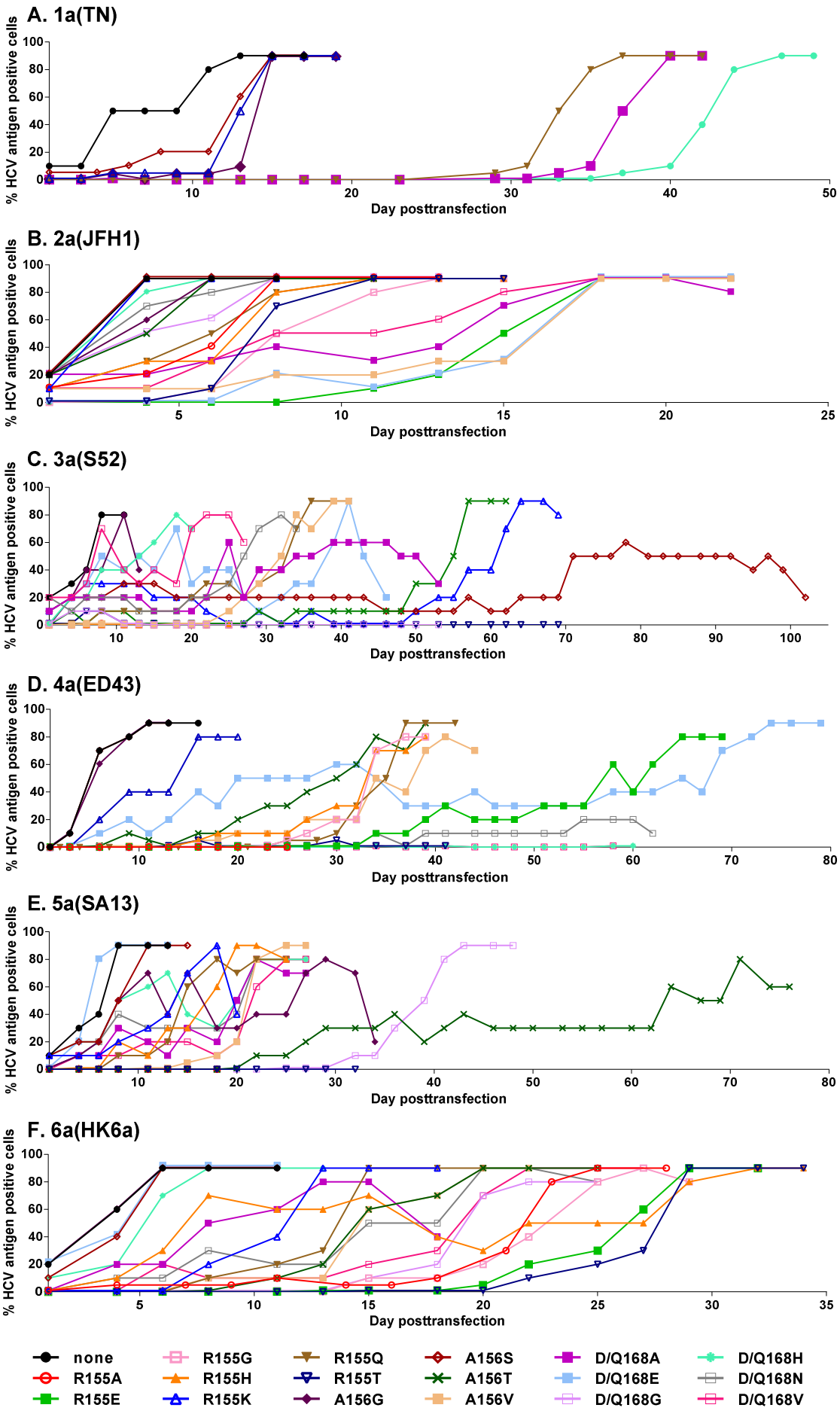
A. 1a(TN)
B. 2a(JFH1)
C. 3a(S52)
D. 4a(ED43)
E. 5a(SA13)
F. 6a(HK6a)
% HCV antigen positive cells
Day posttransfection
none
R155A
R155E
R155G
R155H
R155K
R155Q
R155T
A156G
A156S
A156T
A156V
D/Q168A
D/Q168E
D/Q168G
D/Q168H
D/Q168N
D/Q168V

**Fig. S2. Viral spread kinetics of HCV genotype 1-6 original recombinants and NS3P variants in Huh7.5 cells.** HCV RNA transcripts of original recombinants and recombinants with NS3P substitutions were transfected into Huh7.5 cells and viral spread was monitored by HCV-specific immunostaining as described in Materials and Methods. Graphs show percentage of HCV antigen positive cells (y-axis) as a function of the number of days posttransfection (x-axis) for recombinants with NS3P/NS4A of genotype(isolate) *(A)* 1a(TN), *(B)* 2a(JFH1), *(C)* 3a(S52), *(D)* 4a(ED43), *(E)* 5a(SA13), and *(F)* 6a(HK6a). Data from representative transfections are shown. In addition, we generated and tested 2a(J6)R155K, 2a(J6)R155T, 2a(J6)A156S, 2a(J6)A156T, and 3a(452)R155K, 3a(452)R155T, 3a(452)A156S, 3a(452)A156T (Table S1).

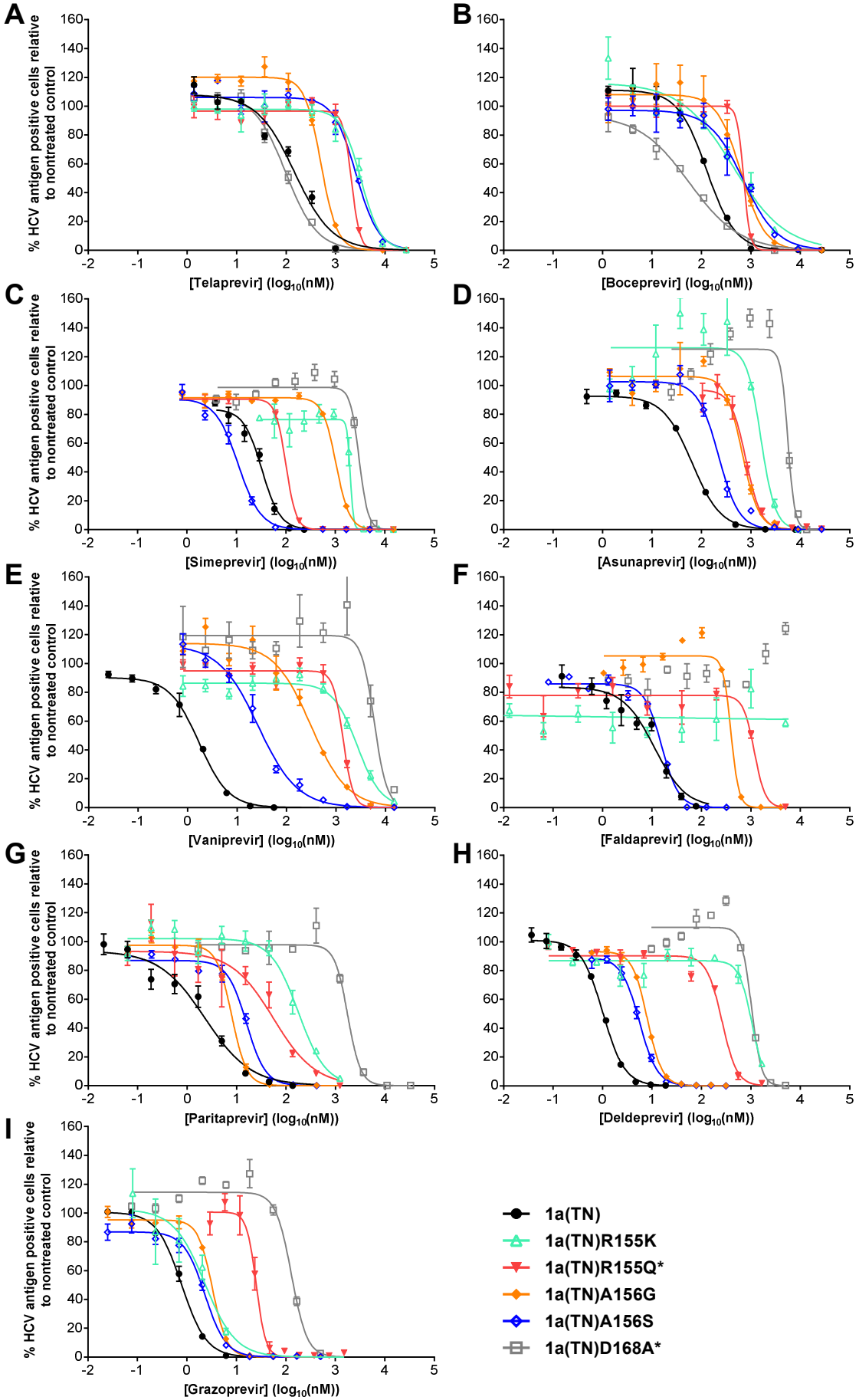
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
1a(TN)
1a(TN)R155K
1a(TN)R155Q*
1a(TN)A156G
1a(TN)A156S
1a(TN)D168A*

**Fig. S3. Efficacy of nine clinically relevant PIs against HCV genotype 1a(TN) original recombinant and NS3P variants.** The original 1a(TN) recombinant as well as 1a(TN)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

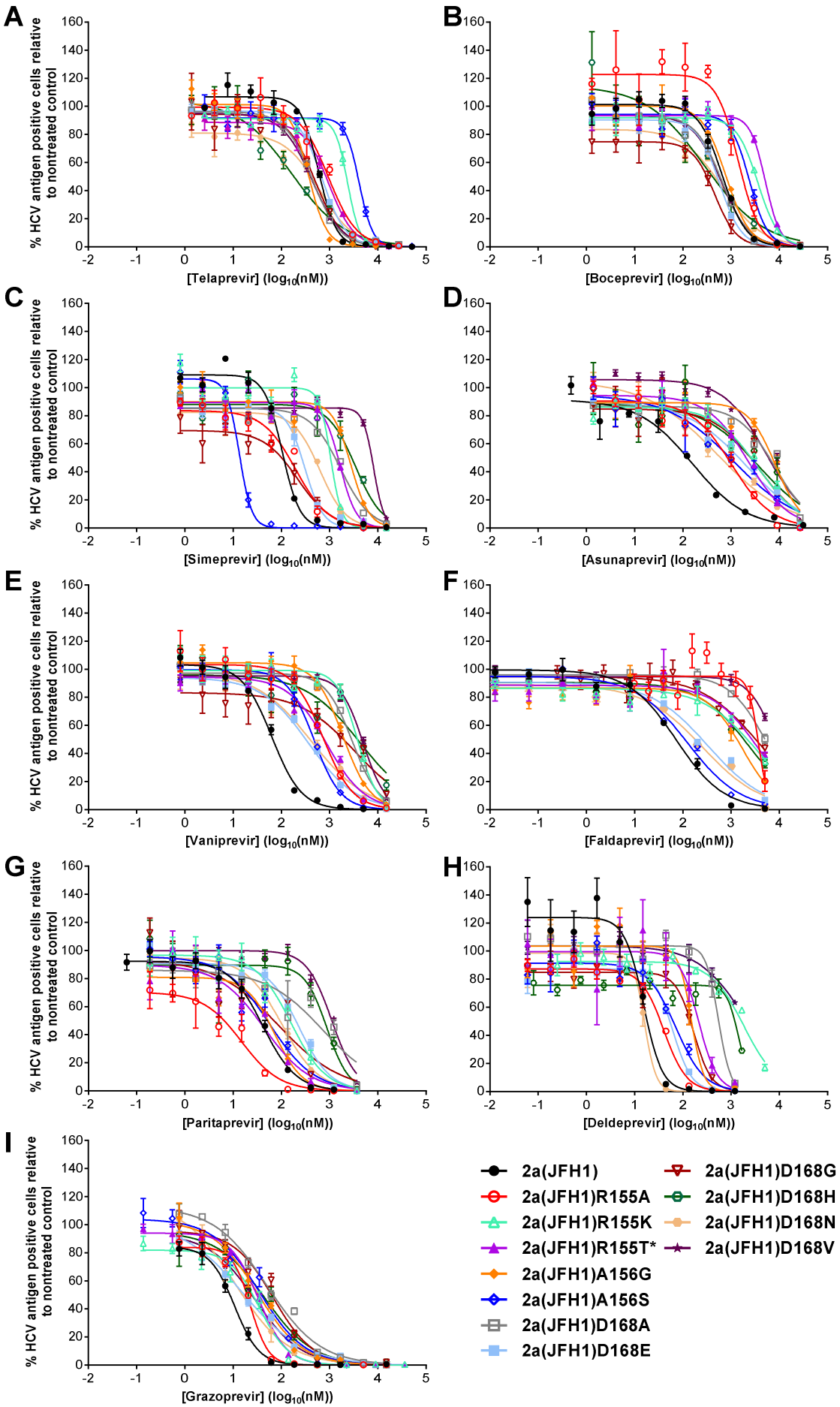
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
2a(JFH1)
2a(JFH1)R155A
2a(JFH1)R155K
2a(JFH1)R155T*
2a(JFH1)A156G
2a(JFH1)A156S
2a(JFH1)D168A
2a(JFH1)D168E
2a(JFH1)D168G
2a(JFH1)D168H
2a(JFH1)D168N
2a(JFH1)D168V

**Fig. S4. Efficacy of nine clinically relevant PIs against HCV genotype 2a(JFH1) original recombinant and NS3P variants.** The original 2a(JFH1) recombinant as well as 2a(JFH1)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

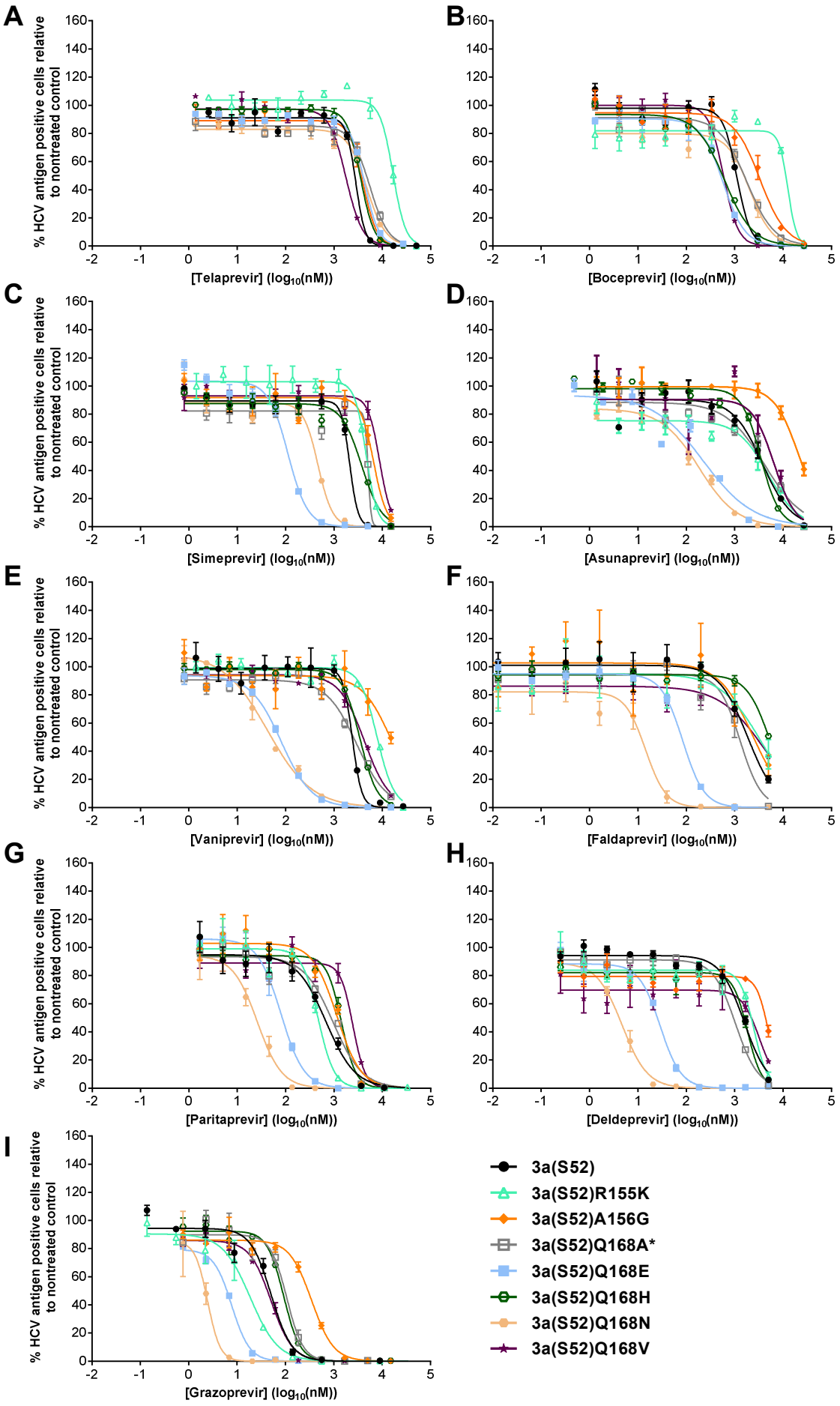
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
3a(S52)
3a(S52)R155K
3a(S52)A156G
3a(S52)Q168A*
3a(S52)Q168E
3a(S52)Q168H
3a(S52)Q168N
3a(S52)Q168V

**Fig. S5. Efficacy of nine clinically relevant PIs against HCV genotype 3a(S52) original recombinant and NS3P variants.** The original 3a(S52) recombinant as well as 3a(S52)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

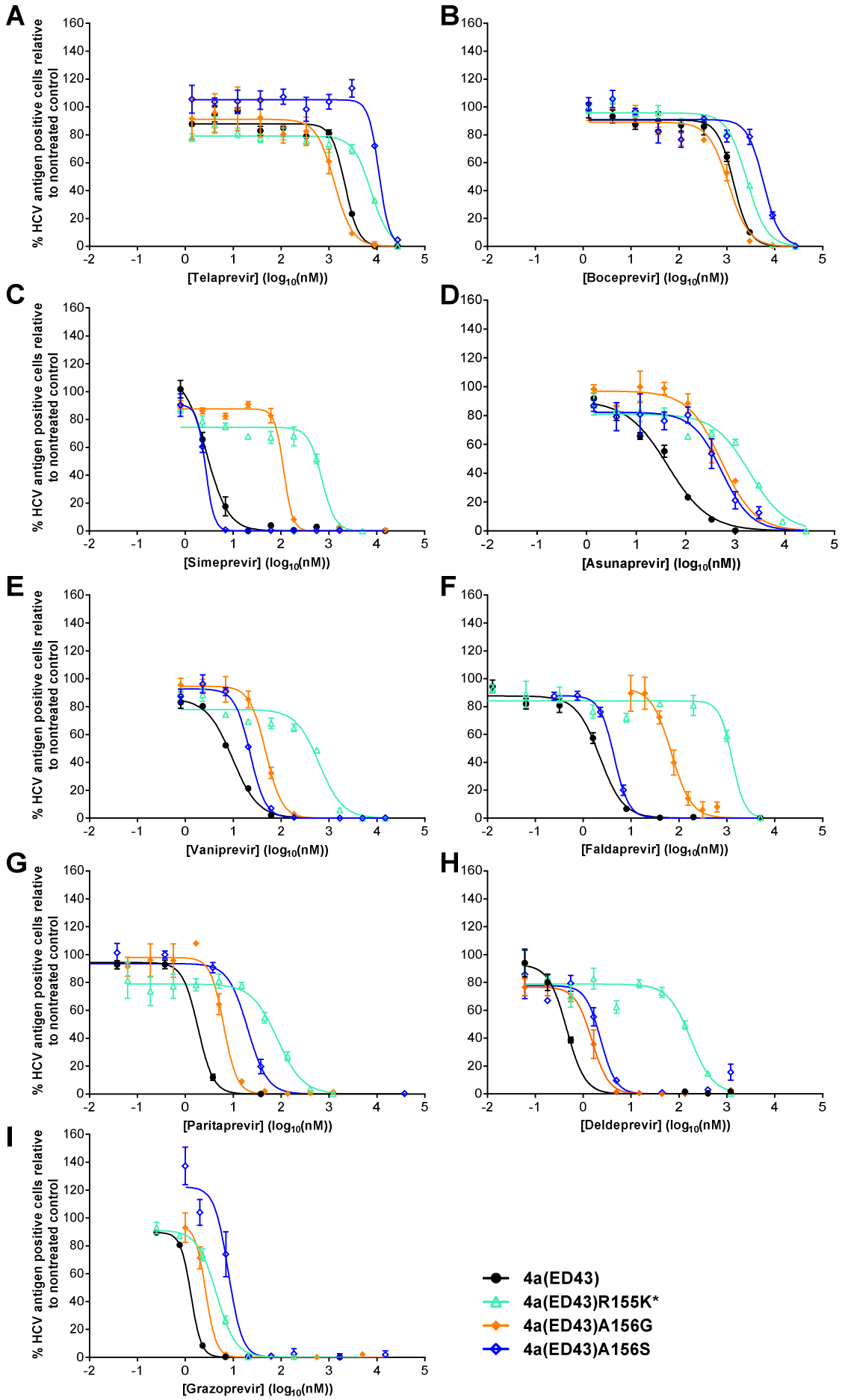
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
4a(ED43)
4a(ED43)R155K*
4a(ED43)A156G
4a(ED43)A156S

**Fig. S6. Efficacy of nine clinically relevant PIs against HCV genotype 4a(ED43) original recombinant and NS3P variants.** The original 4a(ED43) recombinant as well as 4a(ED43)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

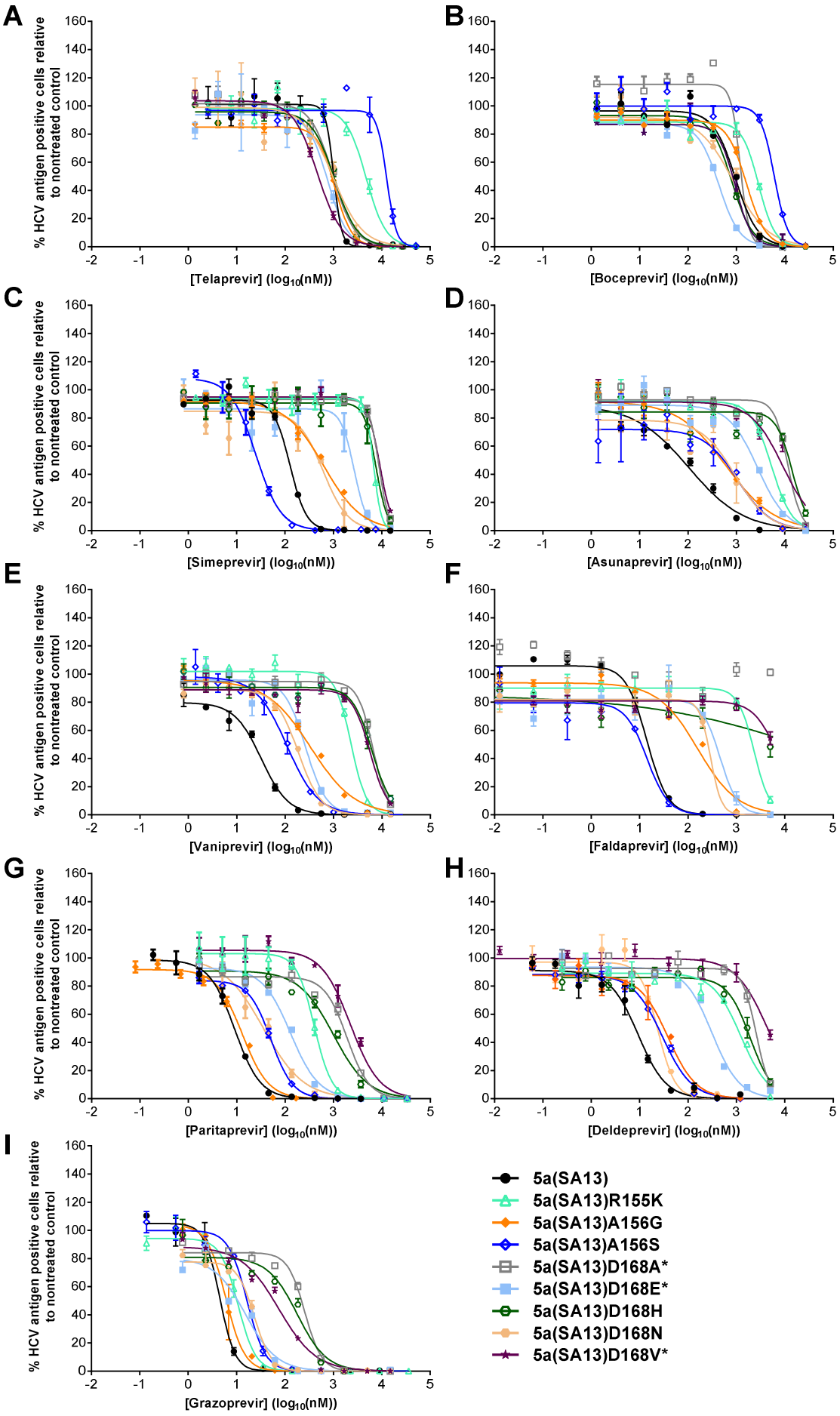
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
5a(SA13)
5a(SA13)R155K
5a(SA13)A156G
5a(SA13)A156S
5a(SA13)D168A*
5a(SA13)D168E*
5a(SA13)D168H
5a(SA13)D168N
5a(SA13)D168V*

**Fig. S7. Efficacy of nine clinically relevant PIs against HCV genotype 5a(SA13) original recombinant and NS3P variants.** The original 5a(SA13) recombinant as well as 5a(SA13)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM except for 5a(SA13)D168N treated with vaniprevir as well as 5a(SA13)R155K and 5a(SA13)D168A treated with deldeprevir, where data points present means of duplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

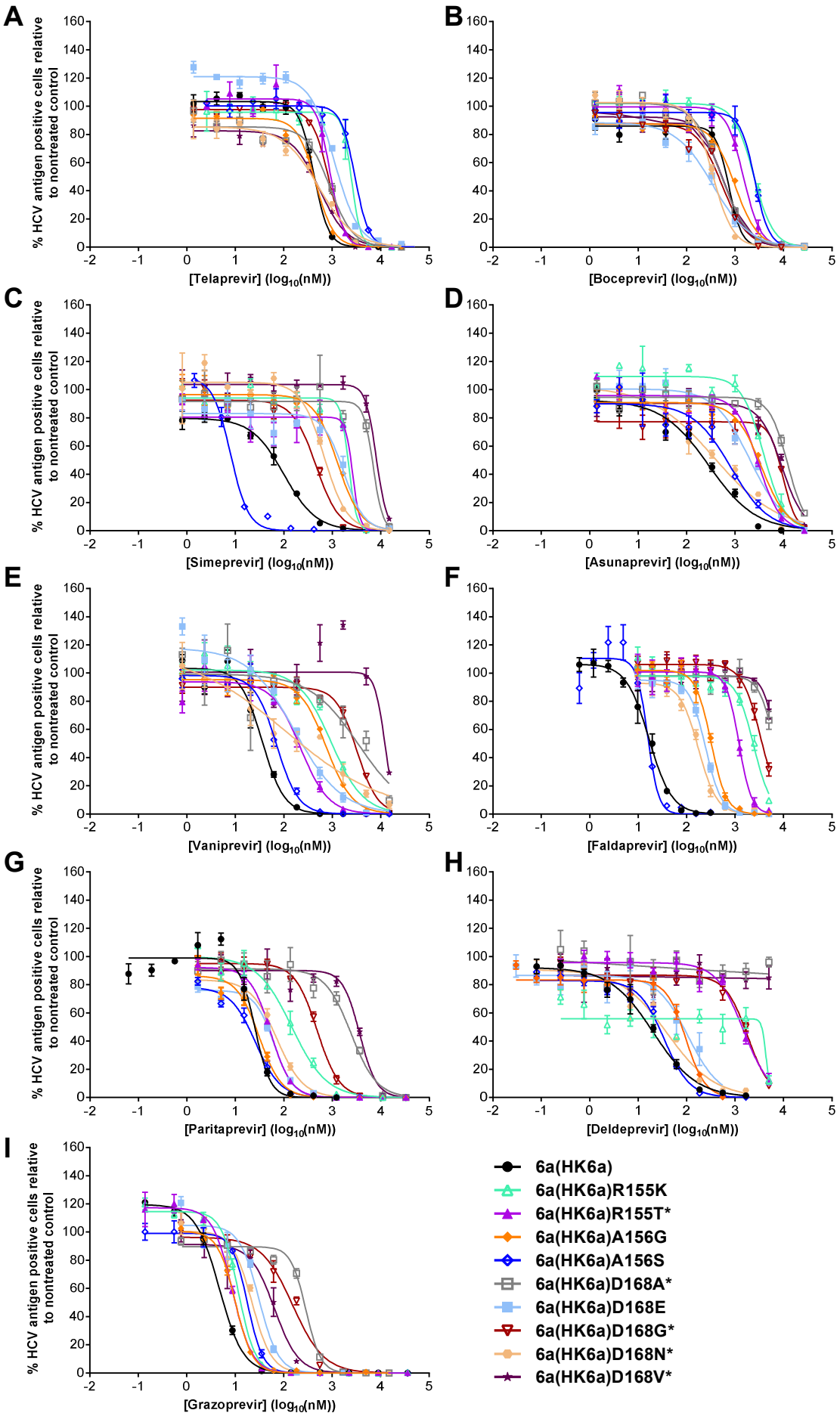
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
6a(HK6a)
6a(HK6a)R155K
6a(HK6a)R155T*
6a(HK6a)A156G
6a(HK6a)A156S
6a(HK6a)D168A*
6a(HK6a)D168E
6a(HK6a)D168G*
6a(HK6a)D168N*
6a(HK6a)D168V*

**Fig. S8. Efficacy of nine clinically relevant PIs against HCV genotype 6a(HK6a) original recombinant and NS3P variants.** The original 6a(HK6a) recombinant as well as 6a(HK6a)-variants with the indicated NS3P substitutions were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM except for 6a(HK6a)D168A treated with grazoprevir, where data points present means of duplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend); the 6a(HK6a)A156S stock used for asunaprevir treatment only had acquired C72S in NS3P.

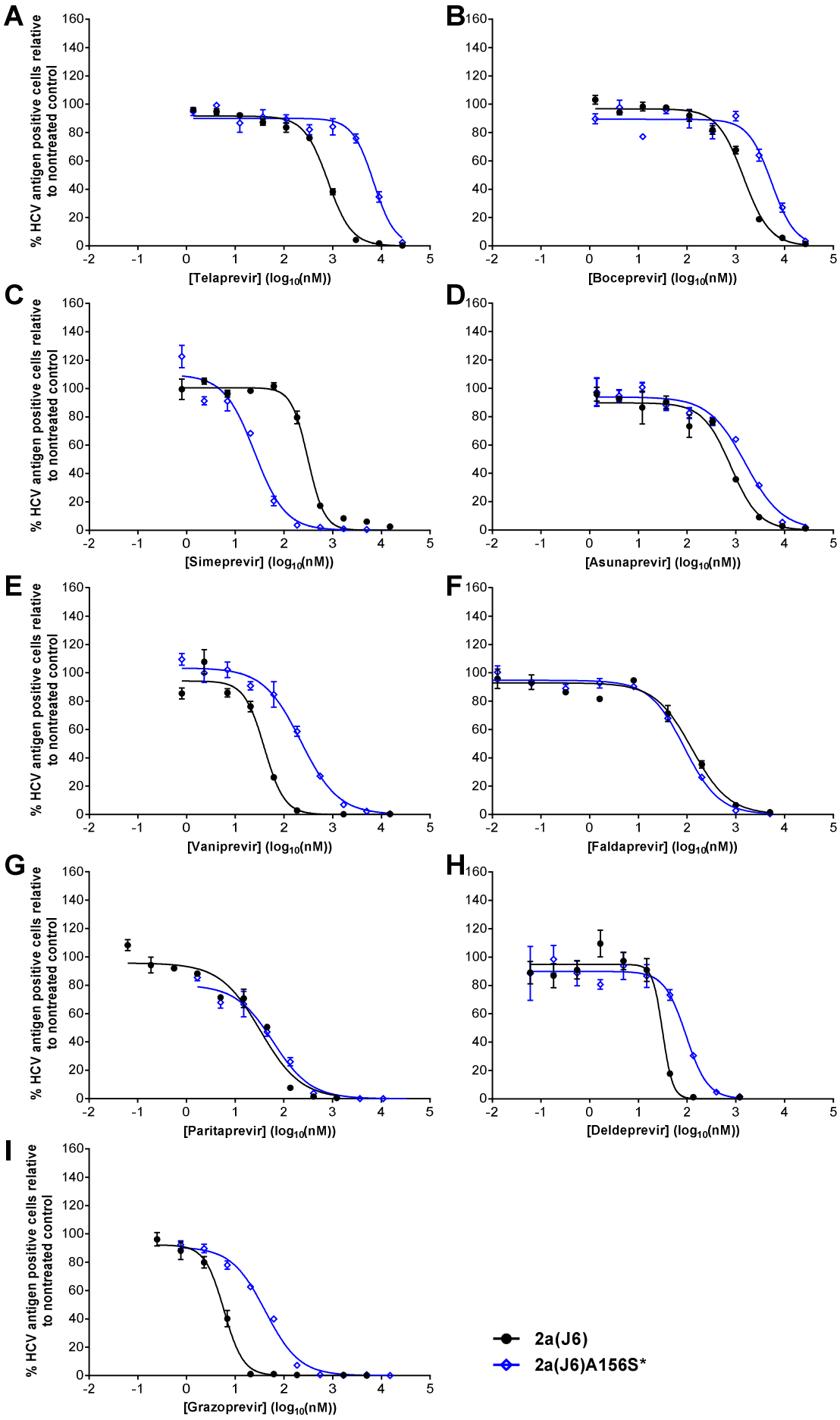
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
2a(J6)
2a(J6)A156S*

**Figure S9. Efficacy of nine clinically relevant PIs against HCV genotype 2a(J6) original recombinant and NS3P variant.** The original 2a(J6) recombinant as well as the 2a(J6)-variant with the indicated NS3P substitution were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods. *, in addition to the engineered substitution, the NS3P variant had acquired one or more additional substitutions in NS3P/NS4A (see Table 2 legend).

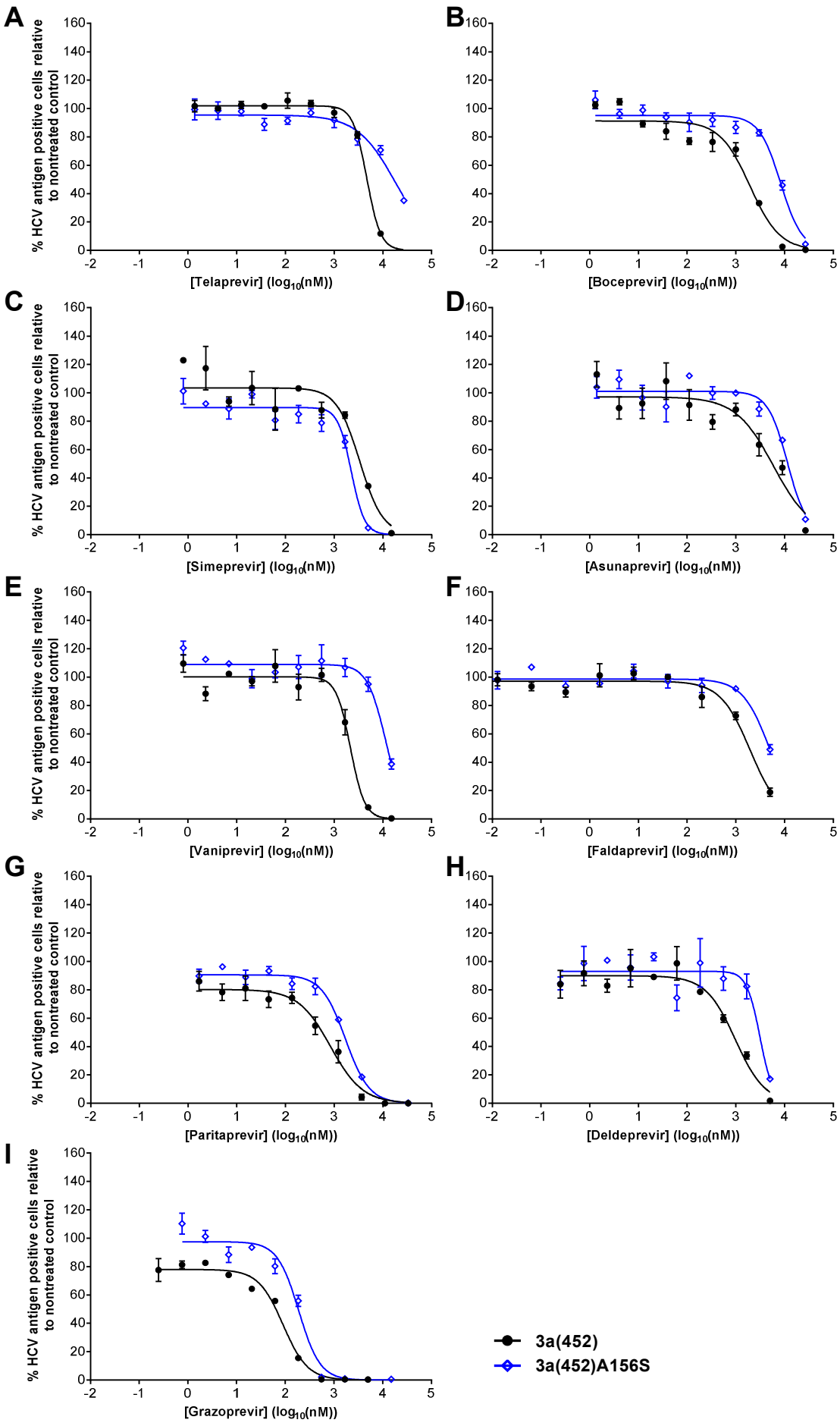
A
B
C
D
E
F
G
H
I
% HCV antigen positive cells relative to nontreated control
[Telaprevir] (log10(nM))
[Boceprevir] (log10(nM))
[Simeprevir] (log10(nM))
[Asunaprevir] (log10(nM))
[Vaniprevir] (log10(nM))
[Faldaprevir] (log10(nM))
[Paritaprevir] (log10(nM))
[Deldeprevir] (log10(nM))
[Grazoprevir] (log10(nM))
3a(452)
3a(452)A156S

**Fig. S10. Efficacy of nine clinically relevant PIs against HCV genotype 3a(452) original recombinant and NS3P variant.** The original 3a(452) recombinant as well as the 3a(452)-variant with the indicated NS3P substitution were tested in concentration-response assays against the nine PIs (*A*) telaprevir, (*B*) boceprevir, (*C*) simeprevir, (*D*) asunaprevir, (*E*) vaniprevir, (*F*) faldaprevir, (*G*) paritaprevir, (*H*) deldeprevir, and (*I*) grazoprevir. Data are from representative experiments; data points represent means of triplicates±SEM; curves were fitted as described in Materials and Methods.

# Table S1. Fitness and genetic stability of HCV original recombinants and NS3P variants

**Genotype 1a(TN)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none[a] | 11 | 3.2 | na | na | na | - |
| R155K[a] | 15 | 4.3 | 4.0 | AGG (R) --> AAA (K) | AAA (K) | - |
| R155Q[a] | 35 | 3.4 | nd | AGG (R) --> CAA (Q) | CAA (Q) | NS4A: V6A |
| A156G[a] | 15 | 4.2 | 3.3 | GCC (A) --> GGA (G) | GGA (G) | - |
| A156S[a] | 15 | 3.3 | 2.8 | GCC (A) --> AGT (S) | AGT (S) | - |
| D168A[a] | 40 | 3.4 | nd | GAC (D) --> GCA (A) | GCA (A) | NS3P: Y134C |
| D168H[a] | 44 | 3.8 | nd | GAC (D) --> CAT (H) | GAT (D) | - |

**Genotype 2a(JFH1)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none[a] | 4 | 4.3 | na | na | na | - |
| R155A | 8 | 2.9 | 2.8 | CGA (R) --> GCC (A) | GCC (A) | - |
| R155E | 18 | 4.9 | < 2.2 | CGA (R) --> GAG (E) | AAG (K) | - |
| R155G | 11 | 4.9 | < 2.2 | CGA (R) --> GGC (G) | CGC (R) | - |
| R155H | 8 | 3.5 | 3.3 | CGA (R) --> CAC (H) | CGC (R) | - |
| R155K[a] | 4 | 4.5 | 4.5 | CGA (R) --> AAG (K) | AAG (K) | - |
| R155Q | 8 | 3.5 | 3.2 | CGA (R) --> CAG (Q) | CGG (R) | NS3P: N110T |
| R155T | 11 | 3.3 | 2.8 | CGA (R) --> ACT (T) | ACT (T) | NS3P: D168A |
| A156G | 6 | 3.8 | 3.8 | GCA (A) --> GGC (G) | GGC (G) | - |
| A156S | 4 | 4.1 | 4.1 | GCA (A) --> AGT (S) | AGT (S) | - |
| A156T | 6 | 3.4 | 3.1 | GCA (A) --> ACT (T) | GCT (A) | - |
| A156V | 18 | 4.1 | < 2.2 | GCA (A) --> GTC (V) | GCC (A) | - |
| D168A | 18 | 4.0 | < 2.2 | GAT (D) --> GCG (A) | GCG (A) | - |
| D168E | 18 | 4.1 | < 2.2 | GAT (D) --> GAG (E) | GAG (E) | - |
| D168G | 8 | 3.8 | 3.8 | GAT (D) --> GGG (G) | GGG (G) | - |
| D168H | 4 | 4.3 | 4.3 | GAT (D) --> CAC (H) | CAC (H) | - |
| D168N | 6 | 4.3 | 4.3 | GAT (D) --> AAC (N) | AAC (N) | - |
| D168V | 15 | 3.9 | < 2.2 | GAT (D) --> GTG (V) | GTG (V) | - |

**Genotype 3a(S52)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none[a] | 8 | 3.8 | na | na | na | - |
| R155A | ns | nd | < 2.2 | AGG (R) --> GCT (A) | na | na |
| R155E | nv | nd | nd | AGG (R) --> GAA (E) | na | na |
| R155G | nv | nd | nd | AGG (R) --> GGT (G) | na | na |
| R155H | nv | nd | nd | AGG (R) --> CAT (H) | na | na |
| R155K | 64 | 3.0 | < 2.2 | AGG (R) --> AAA (K) | AAA (K) | - |
| R155Q | 36 | 3.2 | < 2.2 | AGG (R) --> CAA (Q) | CGA (R) | - |
| R155T | ns | nd | < 2.2 | AGG (R) --> ACA (T) | na | na |
| A156G | 11 | 2.7 | 2.7 | GCT (A) --> GGC (G) | GGC (G) | - |
| A156S | ns | nd | < 2.2 | GCT (A) --> AGC (S) | na | na |
| A156T | 57 | 3.2 | < 2.2 | GCT (A) --> ACA (T) | GCA (A) | - |
| A156V | 34 | 3.1 | < 2.2 | GCT (A) --> GTG (V) | GCG (A) | - |
| Q168A | ns | nd | < 2.2 | CAG (Q) --> GCT (A) | GCT (A) | NS3P: K62R |
| Q168E | 41 | 3.1 | 2.9 | CAG (Q) --> GAA (E) | GAA (E) | - |
| Q168G | ns | nd | < 2.2 | CAG (Q) --> GGA (G) | na | na |
| Q168H | 18 | 3.0 | 2.8 | CAG (Q) --> CAT (H) | CAT (H) | - |
| Q168N | 32 | 3.4 | < 2.2 | CAG (Q) --> AAT (N) | AAT (N) | - |
| Q168V | 22 | 3.5 | 2.9 | CAG (Q) --> GTA (V) | GTA (V) | - |

**Genotype 4a(ED43)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none | 9 | 3.2 | na | na | na | - |
| R155A | ns | nd | < 2.2 | CGT (R) --> GCA (A) | na | na |
| R155E | 65 | 2.5 | < 2.2 | CGT (R) --> GAA (E) | AAA (K) | NS3P: I114V and A151A/V |
| R155G | 37 | 2.8 | < 2.2 | CGT (R) --> GGA (G) | AGA (R) | NS3P: T98t/A |
| R155H | 39 | 2.7 | < 2.2 | CGT (R) --> CAC (H) | CGC (R) | - |
| R155K | 16 | 2.3 | < 2.2 | CGT (R) --> AAG (K) | AAG (K) | NS3P: V113V/i |
| R155Q | 37 | 3.5 | < 2.2 | CGT (R) --> CAG (Q) | CGG (R) | - |
| R155T | ns | nd | < 2.2 | CGT (R) --> ACA (T) | na | na |
| A156G | 9 | 2.8 | 2.8 | GCG (A) --> GGC (G) | GGC (G) | - |
| A156S | 9 | 2.7 | 2.7 | GCG (A) --> AGC (S) | AGC (S) | - |
| A156T | 34 | 2.4 | < 2.2 | GCG (A) --> ACT (T) | gct (a)/AGT (S)[b] | NS3P: T98T/A |
| A156V | 41 | < 2.2 | < 2.2 | GCG (A) --> GTC (V) | GCC (A)/tcc (s) | - |
| D168A | nv | nd | nd | GAC (D) --> GCA (A) | na | na |
| D168E | 72 | 3.1 | < 2.2 | GAC (D) --> GAG (E) | GAT (D) | - |
| D168G | nv | nd | nd | GAC (D) --> GGA (G) | na | na |
| D168H | ns | nd | < 2.2 | GAC (D) --> CAT (H) | na | na |
| D168N | ns | nd | < 2.2 | GAC (D) --> AAT (N) | na | na |
| D168V | ns | nd | < 2.2 | GAC (D) --> GTA (V) | na | na |

**Genotype 5a(SA13)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none[a,c] | 8 | 3.6 | na | na | na | - |
| R155A | nv | nd | nd | AGG (R) --> GCT (A) | na | na |
| R155E | nv | nd | nd | AGG (R) --> GAA (E) | na | na |
| R155G | nv | nd | nd | AGG (R) --> GGT (G) | na | na |
| R155H | 20 | 3.4 | < 2.2 | AGG (R) --> CAT (H) | CGT (R) | - |
| R155K | 18 | 2.9 | 2.8 | AGG (R) --> AAA (K) | AAA (K) | - |
| R155Q | 18 | 3.2 | < 2.2 | AGG (R) --> CAA (Q) | CGC (R) | NS3P: I18V/i |
| R155T | nv | nd | nd | AGG (R) --> ACA (T) | na | na |
| A156G | 29 | 2.5 | 2.4 | GCT (A) --> GGC (G) | GGC (G) | - |
| A156S | 11 | 2.9 | 2.6 | GCT (A) --> AGC (S) | AGC (S) | - |
| A156T | 71 | 2.5 | < 2.2 | GCT (A) --> ACA (T) | GCA (A) | V6V/a |
| A156V | 22 | 4.2 | < 2.2 | GCT (A) --> GTC (V) | GCC (A) | |
| D168A | 22 | 2.9 | < 2.2 | GAT (D) --> GCC (A) | GCC (A) | NS4A: L25V |
| D168E | 6 | 4.0 | 4.0 | GAT (D) --> GAG (E) | GAG (E) | NS4A: V6V/I |
| D168G | 41 | 3.3 | < 2.2 | GAT (D) --> GGC (G) | GAC (D) | - |
| D168H | 22 | 3.2 | 3.0 | GAT (D) --> CAC (H) | CAC (H) | - |
| D168N | 22 | 3.2 | < 2.2 | GAT (D) --> AAC (N) | AAC (N) | - |
| D168V | 25 | 3.2 | < 2.2 | GAT (D) --> GTC (V) | GTC (V) | NS3P: I17M |

**Genotype 6a(HK6a)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none[a] | 6 | 4.2 | na | na | na | - |
| R155A | 23 | 4.1 | < 2.2 | CGA (R) --> GCC (A) | GCC (A) | NS3P: D168A |
| R155E | 29 | 4.1 | < 2.2 | CGA (R) --> GAG (E) | AAG (K) | - |
| R155G[d] | 25 | 4.2 | < 2.2 | CGA (R) --> GGC (G) | CGC (R) | - |
| R155H | 29 | 3.5 | < 2.2 | CGA (R) --> CAC (H) | CGC (R) | - |
| R155K | 13 | 3.0 | < 2.2 | CGA (R) --> AAG (K) | AAG (K) | - |
| R155Q | 15 | 4.5 | < 2.2 | CGA (R) --> CAG (Q) | CGG (R) | - |
| R155T[a] | 29 | 3.6 | < 2.2 | CGA (R) --> ACT (T) | ACT (T) | NS3P: D168A |
| A156G | 6 | 3.9 | 3.9 | GCT (A) --> GGC (G) | GGC (G) | - |
| A156S | 6 | 3.8 | 3.5 | GCT (A) --> AGC (S) | AGC (S) | - |
| A156T | 20 | 4.2 | < 2.2 | GCT (A) --> ACA (T) | GCA (A) | |
| A156V | 20 | 4.2 | < 2.2 | GCT (A) --> GTC (V) | GCC (A) | - |
| D168A | 13 | 4.1 | < 2.2 | GAT (D) --> GCC (A) | GCC (A) | NS3P: K62R |
| D168E | 6 | 4.6 | 4.6 | GAT (D) --> GAG (E) | GAG (E) | - |
| D168G | 22 | 4.0 | < 2.2 | GAT (D) --> GGC (G) | GGC (G) | NS3P: K62R |
| D168H | 8 | 4.3 | 4.1 | GAT (D) --> CAC (H) | CAC (H) | - |
| D168N | 20 | 4.6 | < 2.2 | GAT (D) --> AAC (N) | AAC (N) | NS3P: K62R |
| D168V | 22 | 4.8 | < 2.2 | GAT (D) --> GTC (V) | GTC (V) | NS3P: K62R |

**Genotype 2a(J6)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none | 8 | 3.5 | na | na | na | - |
| R155K | 29 | 3.5 | < 2.2 | CGG (R) --> AAA (K) | AGA (R)/aaa (k) | - |
| R155T | 36 | 4.3 | < 2.2 | CGG (R) --> ACT (T) | AGA (R) | - |
| A156S | 48 | 3.5 | 2.5 | GCA (A) --> AGC (S) | AGC (S) | NS3P: T72T/a, P86L |
| A156T | 43 | 3.8 | < 2.2 | GCA (A) --> ACC (T) | GCC (A) | - |

**Genotype 3a(452)**

| | Transfection | | | | Viral passage | |
|---|---|---|---|---|---|---|
| NS3P substitution | Day ≥80% | Peak infectivity titer $log_{10}$ (FFU/ml) | Comparative titer $log_{10}$ (FFU/ml) | Engineered codon change nt (aa) | nt (aa) present at engineered codon in viral passage | Additional NS3P/4A substitution in 1st passage virus |
| none | 8 | 3.5 | na | na | na | - |
| R155K | 27 | 3.1 | < 2.2 | AGG (R) --> AAA (K) | AAA (K)/AGA (R) | NS3P: T7T/a |
| R155T | nv | nd | nd | AGG (R) --> ACT (T) | na | na |
| A156S | 8 | 3.6 | 2.9 | GCC (A) --> AGT (S) | AGT (S) | - |
| A156T | 20 | 3.6 | < 2.2 | GCC (A) --> ACT (T) | GCT (A) | - |

The indicated NS3P substitutions were introduced in the 1a(TN), 2a(JFH1), 3a(S52), 4a(ED43), 5a(SA13), 6a(HK6a), 2a(J6), and 3a(452) recombinants. HCV RNA transcripts of the original recombinants and NS3P variants were transfected into Huh7.5 cells. Viral spread was monitored by HCV-specific immunostaining; the day, at which viral infection had spread to ≥80% of culture cells, is indicated. Supernatant infectivity titers were determined. The peak supernatant infectivity titer was defined as the highest representative titer at the peak of infection. The comparative titer was defined as the infectivity titer of the variant at the day, at which the original recombinant achieved peak infectivity titer. In the few instances, where the variant achieved peak infectivity titer prior to the original recombinant, the peak infectivity titer of the variant is given. Transfections for recombinants in each panel were not necessarily done in the same experiment. However, in each transfection experiment the original virus was included and showed similar spread kinetics. Comparative titers of variants were always obtained on the day at which the original recombinant in the respective transfection experiment reached peak titer. na, not applicable. nv, recombinants, for which transfection cultures did not show any HCV antigen-positive cells during at least 3 weeks of follow-up were defined as non-viable and no titer was determined. ns, no viral spread despite presence of HCV antigen-positive cells (virus did not infect ≥80% of culture cells); peak infectivity titer was not determined. nd, not determined.

Supernatants derived from transfection cultures at the peak of infection were used to inoculate first viral passage cultures. Supernatants from passage cultures were subjected to extraction of viral RNA and direct sequencing of NS3P/NS4A. The nt sequences of the original codons and of the engineered codons used to generate the indicated aa changes are indicated. Further, the nt sequences present at the engineered codons and the encoded aa following viral passage are indicated. At last, substitutions acquired in the NS3P/NS4A by passaged viruses in addition to the engineered substitution are indicated. Color shadings indicate the overall result of direct sequence analysis of the NS3P/NS4A. Green shading: NS3P variants were genetically stable, meaning that the engineered NS3P substitution was maintained and no additional substitutions were acquired in NS3P/NS4A. Yellow shading: The engineered NS3P substitution was maintained, but one or more additional substitutions were found in NS3P/NS4A as specified in the far right column (capital letters indicate presence in the majority of viral genomes, while small letters indicate presence in a minority of viral genomes). Orange shading: The engineered NS3P substitution had reverted to the original aa but not to the original nt sequence, or had changed to another residue than the original aa or the engineered substitution; in some instances also additional substitutions were acquired. No shading indicates that recombinants with the respective substitutions were non-viable or viral spread was not observed resulting in first viral passage attempts remaining unsuccessful. Nt, nucleotide; aa, amino acid; -, no additional substitution was found in passaged virus. [a], data on genetic stability were obtained from the first passage from replicate transfections which showed similar kinetics as the transfections shown. [b], for 4a(ED43)A156T, subcloning analysis showed seven clones with AGT (S) and one clone with GCT (A). [c], for 5a(SA13), in certain passage stocks, substitutions at aa position 6 in NS4A were observed. [d], in a replicate experiment, 6a(HK6a)R155G maintained the engineered substitution and acquired the additional NS3P substitution D168V.

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