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1 **Single nucleotide polymorphisms in human NPC1 influence filovirus entry into cells**

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50 **ABSTRACT**

51 Niemann-Pick C1 (NPC1), a host receptor involved in the envelope glycoprotein
52 (GP)-mediated entry of filoviruses into cells, is believed to be a major determinant of cell
53 susceptibility to filovirus infection. It is known that proteolytically digested Ebola virus
54 (EBOV) GP interacts with two protruding loops in domain C of NPC1. Using previously
55 published structural data and the single nucleotide polymorphisms (SNPs) database, we
56 identified ten naturally occurring, missense SNPs in human NPC1. To investigate whether
57 these SNPs affect cell susceptibility to filovirus infection, we generated Vero E6 cell lines
58 stably expressing NPC1 with SNP substitutions and compared their susceptibility to
59 vesicular stomatitis virus pseudotyped with filovirus GPs and infectious EBOV. We found
60 that some of the substitutions resulted in reduced susceptibility to filoviruses as indicated
61 by the lower titers and smaller plaque/focus sizes of the viruses. Our data suggest that
62 human NPC1 SNPs may likely affect host susceptibility to filoviruses.

63

64 Key words: Ebolavirus, Marburgvirus, Niemann-Pick C1 (NPC1), single nucleotide
65 polymorphism (SNP), NPC1 knockout Vero E6 (Vero E6/NPC1-KO)

66

67 **INTRODUCTION**

68 Ebolaviruses and marburgviruses are members of the family *Filoviridae* and
69 cause severe hemorrhagic fever in humans and nonhuman primates. *Filoviridae* consists
70 of three genera, encompassing seven species (5, 1, and 1 species in the genera *Ebolavirus*,
71 *Marburgvirus*, and *Cuevavirus*, respectively) [1]. Currently, eight distinct viruses are
72 assigned to these seven species: Ebola virus (EBOV), Bundibugyo virus (BDBV), Tai
73 Forest virus (TAFV), Sudan virus (SUDV), and Reston virus (RESTV) in the genus
74 *Ebolavirus*; Marburg virus (MARV) and Ravn virus (RAVV) in the genus *Marburgvirus*;
75 and Lloviu virus (LLOV) in the genus *Cuevavirus*. The biological properties of LLOV
76 are uncharacterized since infectious LLOV has not been isolated yet. Filoviruses have
77 continuously produced sporadic outbreaks with increased frequency in Central and West
78 Africa and are considered a significant public health concern.

79 The envelope glycoprotein (GP) is the only viral surface protein of filoviruses
80 and mediates viral entry into cells [2]. After binding to attachment factors such as C-type
81 lectins [3, 4], virus particles are internalized into host cells via macropinocytosis [5-7].
82 Late endosomal low pH leads to cysteine protease-mediated proteolysis of the viral GP
83 [8, 9]. This digested GP (dGP) can then interact with the host endosomal fusion receptor,
84 Niemann-Pick C1 (NPC1), allowing fusion between the viral envelope and the host
85 endosomal membrane [10-12]. Thus, NPC1 represents a major determinant of cell
86 susceptibility to filovirus infection [13, 14].

87 The recently published co-structures of EBOV dGP in complex with NPC1
88 revealed that two surface-exposed loops on NPC1 mediate the interaction with dGP [15,
89 16]. Biochemical analysis of purified NPC1 and dGP has further shown that amino acid
90 substitutions in these two loops (e.g., P424A, F503G and F504G) reduce the binding to

91 dGP [15]. This finding led us to hypothesize that single nucleotide polymorphisms (SNPs)
92 in human NPC1 might influence host susceptibility to filovirus infection. Mutations tested
93 in previous studies [13-15] were not linked to human NPC1 SNPs and are unregistered in
94 the SNP database (<https://www.ncbi.nlm.nih.gov/snp/>); thus it remains unknown whether
95 naturally occurring SNPs found in human NPC1 can affect the efficiency of filovirus
96 infection.

97 Using a structural-guided approach to the SNP database, we identified ten
98 missense SNPs in the GP-interacting loop regions of NPC1. To investigate the potential
99 effects of these substitutions on filovirus infection in vitro, we generated an NPC1
100 knockout Vero E6 cell line and established stable cell lines expressing the NPC1 SNP
101 mutants. Cell susceptibility was examined using vesicular stomatitis virus (VSV)
102 pseudotyped with filovirus GPs [2, 17] as well as a replication-competent EBOV
103 expressing green fluorescent protein (GFP) [18]. Here, we report human NPC1 SNPs that
104 influence the susceptibility of cells to filovirus entry and infection.

105

106 **MATERIALS AND METHODS**

107 **Viruses**

108 VSV containing the GFP gene instead of the receptor-binding VSV-G protein
109 gene (VSV Δ G) and pseudotyped viruses with GPs of EBOV (Mayinga), BDBV (Butalya),
110 TAFV (Pauléoula), SUDV (Boniface), RESTV (Pennsylvania), MARV (Angola), RAVV
111 (Kitum Cave), and LLOV (Asturias), designated by VSV Δ G-EBOV, -BDBV, -TAFV, -
112 SUDV, - RESTV, - MARV, - RAVV, and - LLOV, respectively, were generated as
113 described previously [2, 19]. Infectious units (IUs) of these pseudotyped VSVs were
114 determined in Vero E6 cells as previously described [2, 20]. Replication-competent

115 recombinant VSVs (rVSV-EBOV [Mayinga] and rVSV-MARV [Angola]) were generated
116 as described previously [17]. rVSV-EBOV, rVSV-MARV, and recombinant EBOV
117 expressing GFP (EBOV-GFP) [18] were propagated in Vero E6 cells and stored at -80°C.
118 Infectivity of rVSV-EBOV, rVSV-MARV, and EBOV-GFP in each cell line was
119 determined by plaque- and focus-forming assays, respectively, as described previously
120 [21, 22]. Relative infectivity in each cell line was calculated by setting IU, plaque forming
121 unit, or focus forming unit values given by Vero E6 cells expressing 293T-NPC1 to 100%.

122

123 **Biosafety**

124 All infectious work with EBOV-GFP was performed in the biosafety level-4
125 laboratory (BSL-4) at the Integrated Research Facility of the Rocky Mountain
126 Laboratories, Division of Intramural Research, National Institute of Allergy and
127 Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA. All Standard
128 Operating Procedures (SOPs) were approved by the Institutional Biosafety Committee
129 (IBC)

130

131 **Cell lines**

132 Vero E6, NPC1 knockout Vero E6 (Vero E6/NPC1-KO) and HEK293T-derived
133 Platinum-GP (Plat-GP) cells [23] (Cell Biolabs) were grown in Dulbecco's modified
134 Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum. Vero
135 E6/NPC1-KO cells were generated by previously described methods [24, 25] with a few
136 modifications and transduced with retroviruses expressing HEK293T-derived NPC1 and
137 its mutant genes (see Supplementary Materials and Methods).

138

139 **Cloning of the NPC1 gene into plasmids**

140 The coding region of the HEK293T NPC1 gene was polymerase chain reaction
141 (PCR)-amplified from cDNA prepared from total RNA extracted from HEK293T cells
142 according to a previous study [26]. The PCR product was cloned into a murine leukemia
143 virus-based retroviral vector, pMXs-puro [23]. Ten SNPs found in the two loops:
144 rs772847092 (I419V and I419F), rs77815278 (Y420S), rs771644708 (Y420H),
145 rs143797098 (P424A), rs140149624 (S425L), rs749078710 (A427T), rs748246747
146 (G500E), rs191537721 (D502E), and rs756587493 (D508N), were produced by site-
147 directed mutagenesis using a KOD-Plus-Neo polymerase (TOYOBO) with primers
148 containing the desired nucleotide substitutions. All mutations were confirmed by DNA
149 sequencing.

150

151 **Statistical analysis**

152 All statistical analyses were performed using R software (version 3.2.3) [27].
153 Correlations between transcription and expression levels of NPC1 were estimated using
154 the rank correlation coefficient of Spearman (r_s). For comparison of viral infectivity and
155 plaque/focus size, the one-way ANOVA followed by Dunnett's tests and Welch's t-test
156 were used. *P*-values of less than 0.05 were considered to be statistically significant.

157

158

159 **RESULTS**

160 **Structure-guided approach to identifying candidate NPC1 SNPs**

161 Human NPC1 has at least 254 unique SNPs listed in the SNP database
162 (<https://www.ncbi.nlm.nih.gov/snp/>). Of these SNPs, 222 are missense, resulting in amino
163 acid changes, and they are widely distributed throughout the structure of NPC1 (Figure
164 1A). Using the co-structure of full-length NPC1 and EBOV dGP [16], we identified 10
165 missense SNPs in 8 amino acid positions located in the interface (i.e., 2 loop structures at
166 amino acid positions 419-428 and 500-508) with EBOV GP (Figure 1B).

167

168 **Human NPC1 polymorphisms affecting filovirus entry into cells**

169 We next established an NPC1 knockout Vero E6 cell line, Vero E6/NPC1-KO
170 cl.19 (Supplementary Figure 1A and B), and confirmed that its susceptibility to VSVΔG-
171 EBOV, rVSV-EBOV, and EBOV-GFP was completely abolished (Supplementary Figure
172 1C-E). The Vero E6/NPC1-KO cl.19 cells were then transduced with a retroviral vector
173 resulting in eleven different cell lines stably expressing wild-type human NPC1 (293T-
174 NPC1) or NPC1 SNP mutants (I419V, I419F, Y420S, Y420H, P424A, S425L, A427T,
175 G500E, D502E, and D508N). Introducing the wild-type NPC1 gene into the knockout
176 cells completely restored the susceptibility to filovirus entry and infection
177 (Supplementary Figure 1C-E), as has been shown with NPC1-deficient CHO and reptile-
178 derived cells [10, 12]. Transcription and expression levels of the transduced NPC1 genes
179 were examined by quantitative PCR and western blot analyses, respectively (see
180 Supplementary Materials and Methods). In all of the NPC1 SNP mutant cell lines,
181 expression levels of NPC1 were equivalent to or higher than those of 293T-NPC1-
182 expressing cells (Supplementary Figure 2). We also confirmed that these NPC1s were

183 similarly localized at endosomal vesicles (data not shown).

184 To evaluate whether these SNP substitutions affected the efficiency of viral entry,
185 each cell line was infected with replication-incompetent VSV pseudotyped with filovirus
186 GPs, and relative IUs were compared among the cell lines (Figure 2). We found that
187 P424A, S425L, D502E, and D508N substitutions in NPC1 reduced the entry of VSV
188 pseudotyped with GPs from multiple filovirus species. P424A and D508N substitutions
189 significantly affected the entry of VSVΔG- EBOV, - BDBV, - TAFV, - SUDV, - RESTV,
190 and - LLOV, but not MARV and/or -RAVV. On the other hand, S425L and D502E
191 substitutions reduced the entry of VSVΔG-MARV, and some of the pseudotyped VSV
192 with ebolavirus GPs. Y420S substitution only reduced the entry of VSVΔG-LLOV. It was
193 noted that these substitutions resulted in different patterns of reduction corresponding to
194 the filovirus genera (i.e., *Ebolavirus*, *Marburgvirus*, and *Cuevavirus*). In particular,
195 P424A/D508N and S425L/D502E substitutions seemed to be important for the reduced
196 entry of VSVs pseudotyped with ebolavirus and marburgvirus GPs, respectively. In some
197 cell lines, infectivity of the viruses was enhanced and it was correlated with relatively
198 high expression levels of NPC1 (Supplementary Figure 2).

199

200 **Reduced plaque-forming ability of rVSV-EBOV in Vero E6 cells expressing NPC1** 201 **with P424A or D508N substitution**

202 We then compared the plaque-forming abilities of rVSV-EBOV and rVSV-
203 MARV using the same cell lines (Figure 3). Although statistically significant difference
204 was not observed, the P424A and S425L substitutions moderately reduced the plaque
205 number of rVSV-EBOV and rVSV-MARV, respectively (Figure 3A). As expected, the
206 average plaque sizes of rVSV-EBOV in cells expressing NPC1 with the D508N

207 substitution were significantly smaller than in 293T-NPC1-expressing cells and the
208 P424A substitution also slightly reduced the plaque size (Figure 3B). Consistent with the
209 data shown in Figure 2, rVSV-MARV formed slightly, but not significantly, smaller
210 plaques in the cell line expressing S425L NPC1 (Figure 3B).

211

212 **Reduced focus-forming ability of EBOV in Vero E6 cells with the P424A substitution**

213 To examine the effects of substitutions associated with NPC1 SNPs on actual
214 filovirus infection, we used an infectious EBOV expressing GFP and determined the viral
215 focus numbers and average focus size in each cell line. Consistent with our pseudotyped
216 VSV data, the P424A substitution resulted in reduced focus numbers of EBOV-GFP
217 (Figure 3C). The average size of foci in this cell line was also smaller than in cells
218 expressing 293T-NPC1 (Figure 3D). Although these differences were not significant in a
219 multiple comparison analysis, Welch's t-test gave significant differences compared to
220 293T-NPC1-expressing cells. The D508N substitution slightly reduced the focus size of
221 EBOV-GFP but there was no statistically significant difference compared to 293T-NPC1-
222 expressing cells.

223

224

225 **DISCUSSION**

226 With human immunodeficiency virus type 1 (HIV-1) infection, there is a subset
227 of people who are inherently resistant to the virus [28-31]. In particular, genetic variation
228 in the host viral receptors has been shown to reduce the susceptibility to HIV infection in
229 humans [28, 29]. Though asymptomatic filovirus infection is less studied, Baron et al.
230 [32] reported potential asymptomatic cases during the outbreak in Sudan in 1979, and
231 Dean et al. [33] estimated the proportion of asymptomatic transmissions during the recent
232 epidemic in West Africa. Although host genetics and Ebola virus disease progression and
233 resistance have been studied in mice [34, 35], the details of genetic polymorphisms that
234 may affect susceptibility of humans to filoviruses have not been elucidated yet.

235 In the present study, we focused on ten reported NPC1 SNP substitutions that
236 interface with GP. Although none of the SNPs tested here completely ablated viral entry
237 or infectivity, some SNPs were nevertheless found to be important for filovirus infection.
238 In our experiments, P424A substitution reduced entry of VSV pseudotyped with
239 ebolavirus but not marburgvirus GPs. Moreover, focus sizes of EBOV-GFP were also
240 reduced in this cell line. Interestingly, Wang et al. [15] demonstrated that the P424A NPC1
241 mutant retained binding capacity to dGP but with reduced affinity compared to wild-type
242 NPC1. Taken together, these findings suggest that the P424A substitution in NPC1 lowers
243 its binding capacity to dGP, resulting in reduced viral entry and infection.

244 S425L substitution also reduced the infection with some pseudotyped VSVs,
245 including VSV Δ G-MARV and -RAVV. The serine residue at position 425 in NPC1 was
246 shown to be involved in the interaction with the amino acid residue at position 142 of the
247 EBOV GP molecule [15]. However, since amino acid residues at this position are different
248 in EBOV and MARV GPs (serine at 142 of EBOV GP and glutamine at 126 of MARV

249 GP, respectively), the effects of S425L substitution might be different between EBOV and
250 MARV. It has also been demonstrated that single amino acid mutations at positions 502
251 or 503 in NPC1 can reduce viral infection [13, 14]. Our experiments confirmed this
252 finding as D502E substitution reduced the entry of some of the pseudotyped viruses we
253 tested and also reduced infection with EBOV-GFP (although not significantly). Notably,
254 while the previous study introduced a significant loss of charge at residue 502 by mutating
255 the aspartic acid to phenylalanine [13], our study shows that even minimal variation at
256 this residue (D to E) can have an effect on viral entry.

257 Some other amino acids that are also important for binding to dGP have not been
258 reported as SNP sites in the NPC1 loops [15]. The rapidly increasing sequence data in
259 public databases may hereafter provide additional information on NPC1 SNP
260 substitutions not evaluated in this study. It is also of interest to investigate multiple SNP
261 substituins in the loops. Although more sensitive studies are needed to further assess the
262 capacity for NPC1 variation to influence filovirus infection, identifying genetic variations
263 that affect the susceptibility of hosts to filoviruses will be crucial in understanding
264 filovirus disease progression and host cell-restriction.

265

266

267 **Supplementary Data**

268 Supplementary materials are available at The Journal of Infectious Diseases online.
269 Consisting of data provided by the authors to benefit the reader, the posted materials are
270 not copyedited and are the sole responsibility of the authors, so questions or comments
271 should be addressed to the corresponding author.

272

273 **Notes**

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280

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370 35.
- 371
- 372

373 **Figure Legends**

374

375 **Figure 1. Structural-guided approach to human NPC1 SNP selection.**

376 A, 222 missense SNPs are represented in black on the structure from NPC1. B, Co-
377 structure of EBOV GP (blue) and NPC1 (tan) with amino acid positions of SNPs indicated
378 in red (PDB: 5JNX) [16].

379

380 **Figure 2. Infectivities of VSV pseudotyped with filovirus GPs in Vero E6 cell lines.**

381 Confluent monolayers of each cell line grown in 96-well plates were infected with
382 VSV Δ G-EBOV, VSV Δ G-BDBV, VSV Δ G-TAFV, VSV Δ G-SUDV, VSV Δ G-RESTV,
383 VSV Δ G-MARV, VSV Δ G-RAVV, and VSV Δ G-LLOV. Twenty hours later, the virus
384 infectious unit (IU) in each cell line was determined by counting the number of GFP-
385 expressing cells with an IN Cell Analyzer 2000 (GE Healthcare). Relative infectivity in
386 each cell line was calculated by setting the IU value given by Vero E6 cells expressing
387 293T-NPC1 (approximately 1000-3000 IU/well for each assay) to 100%. Means and
388 standard errors of thirty replicates are shown. Values significantly lower than for 293T-
389 NPC1 are indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

390

391 **Figure 3. Plaque/focus formation of rVSV-EBOV, rVSV-MARV, and EBOV-GFP in**

392 **Vero E6 cell lines**

393 A, rVSV-EBOV and rVSV-MARV (multiplicity of infection = 0.0005 in Vero E6 cells)
394 were inoculated into Vero E6 cells grown in 6-well tissue culture plates. After adsorption
395 for 1 hour, the inoculum was removed and the cells were overlaid with Eagle's minimal
396 essential medium containing 1.0% Bacto Agar (BD) and then incubated for 3 days at

397 37°C. Cells were stained with 0.5 % crystal violet in 10% formalin and the plaque-
398 forming unit (PFU) in each cell line was determined by counting the number of all visible
399 plaques per well (i.e., 50-100 plaques for each assay). Data represent means and standard
400 errors of at least three independent experiments. *B*, Plaque sizes were measured with
401 Image-J software. Means and standard errors of 50-100 plaques per well are shown. *C*,
402 EBOV-GFP (multiplicity of infection = 0.002 in Vero E6 cells) were inoculated into
403 confluent monolayers of Vero E6 cells grown in 96-well tissue culture plates. After
404 adsorption for 1 hour, the inoculum was replaced with Eagle's minimal essential medium
405 containing 1.2% carboxymethyl cellulose. After incubation for 3 days at 37°C, the cells
406 were fixed and GFP foci in each cell line were counted under a fluorescence microscope.
407 Data represent means and standard errors of triplicate assays. *D*, Focus sizes were
408 measured with Image-J software. Means and standard errors of 60-150 foci per well are
409 shown. (*A-D*) Values significantly lower than for 293T-NPC1 are indicated (* $P < 0.05$,
410 ** $P < 0.01$, *** $P < 0.001$).

1 **Single nucleotide polymorphisms in human NPC1 influence filovirus entry into cells**

2

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50 **ABSTRACT**

51 Niemann-Pick C1 (NPC1), a host receptor involved in the envelope glycoprotein
52 (GP)-mediated entry of filoviruses into cells, is believed to be a major determinant of cell
53 susceptibility to filovirus infection. It is known that proteolytically digested Ebola virus
54 (EBOV) GP interacts with two protruding loops in domain C of NPC1. Using previously
55 published structural data and the single nucleotide polymorphisms (SNPs) database, we
56 identified ten naturally occurring, missense SNPs in human NPC1. To investigate whether
57 these SNPs affect cell susceptibility to filovirus infection, we generated Vero E6 cell lines
58 stably expressing NPC1 with SNP substitutions and compared their susceptibility to
59 vesicular stomatitis virus pseudotyped with filovirus GPs and infectious EBOV. We found
60 that some of the substitutions resulted in reduced susceptibility to filoviruses as indicated
61 by the lower titers and smaller plaque/focus sizes of the viruses. Our data suggest that
62 human NPC1 SNPs may likely affect host susceptibility to filoviruses.

63

64 Key words: Ebolavirus, Marburgvirus, Niemann-Pick C1 (NPC1), single nucleotide
65 polymorphism (SNP), NPC1 knockout Vero E6 (Vero E6/NPC1-KO)

66

67 **INTRODUCTION**

68 Ebolaviruses and marburgviruses are members of the family *Filoviridae* and
69 cause severe hemorrhagic fever in humans and nonhuman primates. *Filoviridae* consists
70 of three genera, encompassing seven species (5, 1, and 1 species in the genera *Ebolavirus*,
71 *Marburgvirus*, and *Cuevavirus*, respectively) [1]. Currently, eight distinct viruses are
72 assigned to these seven species: Ebola virus (EBOV), Bundibugyo virus (BDBV), Tai
73 Forest virus (TAFV), Sudan virus (SUDV), and Reston virus (RESTV) in the genus
74 *Ebolavirus*; Marburg virus (MARV) and Ravn virus (RAVV) in the genus *Marburgvirus*;
75 and Lloviu virus (LLOV) in the genus *Cuevavirus*. The biological properties of LLOV
76 are uncharacterized since infectious LLOV has not been isolated yet. Filoviruses have
77 continuously produced sporadic outbreaks with increased frequency in Central and West
78 Africa and are considered a significant public health concern.

79 The envelope glycoprotein (GP) is the only viral surface protein of filoviruses
80 and mediates viral entry into cells [2]. After binding to attachment factors such as C-type
81 lectins [3, 4], virus particles are internalized into host cells via macropinocytosis [5-7].
82 Late endosomal low pH leads to cysteine protease-mediated proteolysis of the viral GP
83 [8, 9]. This digested GP (dGP) can then interact with the host endosomal fusion receptor,
84 Niemann-Pick C1 (NPC1), allowing fusion between the viral envelope and the host
85 endosomal membrane [10-12]. Thus, NPC1 represents a major determinant of cell
86 susceptibility to filovirus infection [13, 14].

87 The recently published co-structures of EBOV dGP in complex with NPC1
88 revealed that two surface-exposed loops on NPC1 mediate the interaction with dGP [15,
89 16]. Biochemical analysis of purified NPC1 and dGP has further shown that amino acid
90 substitutions in these two loops (e.g., P424A, F503G and F504G) reduce the binding to

91 dGP [15]. This finding led us to hypothesize that single nucleotide polymorphisms (SNPs)
92 in human NPC1 might influence host susceptibility to filovirus infection. Mutations tested
93 in previous studies [13-15] were not linked to human NPC1 SNPs and are unregistered in
94 the SNP database (<https://www.ncbi.nlm.nih.gov/snp/>); thus it remains unknown whether
95 naturally occurring SNPs found in human NPC1 can affect the efficiency of filovirus
96 infection.

97 Using a structural-guided approach to the SNP database, we identified ten
98 missense SNPs in the GP-interacting loop regions of NPC1. To investigate the potential
99 effects of these substitutions on filovirus infection in vitro, we generated an NPC1
100 knockout Vero E6 cell line and established stable cell lines expressing the NPC1 SNP
101 mutants. Cell susceptibility was examined using vesicular stomatitis virus (VSV)
102 pseudotyped with filovirus GPs [2, 17] as well as a replication-competent EBOV
103 expressing green fluorescent protein (GFP) [18]. Here, we report human NPC1 SNPs that
104 influence the susceptibility of cells to filovirus entry and infection.

105

106 **MATERIALS AND METHODS**

107 **Viruses**

108 VSV containing the GFP gene instead of the receptor-binding VSV-G protein
109 gene (VSV Δ G) and pseudotyped viruses with GPs of EBOV (Mayinga), BDBV (Butalya),
110 TAFV (Pauléoula), SUDV (Boniface), RESTV (Pennsylvania), MARV (Angola), RAVV
111 (Kitum Cave), and LLOV (Asturias), designated by VSV Δ G-EBOV, -BDBV, -TAFV, -
112 SUDV, - RESTV, - MARV, - RAVV, and - LLOV, respectively, were generated as
113 described previously [2, 19]. Infectious units (IUs) of these pseudotyped VSVs were
114 determined in Vero E6 cells as previously described [2, 20]. Replication-competent

115 recombinant VSVs (rVSV-EBOV [Mayinga] and rVSV-MARV [Angola]) were generated
116 as described previously [17]. rVSV-EBOV, rVSV-MARV, and recombinant EBOV
117 expressing GFP (EBOV-GFP) [18] were propagated in Vero E6 cells and stored at -80°C.
118 Infectivity of rVSV-EBOV, rVSV-MARV, and EBOV-GFP in each cell line was
119 determined by plaque- and focus-forming assays, respectively, as described previously
120 [21, 22]. Relative infectivity in each cell line was calculated by setting IU, plaque forming
121 unit, or focus forming unit values given by Vero E6 cells expressing 293T-NPC1 to 100%.

122

123 **Biosafety**

124 All infectious work with EBOV-GFP was performed in the biosafety level-4
125 laboratory (BSL-4) at the Integrated Research Facility of the Rocky Mountain
126 Laboratories, Division of Intramural Research, National Institute of Allergy and
127 Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA. All Standard
128 Operating Procedures (SOPs) were approved by the Institutional Biosafety Committee
129 (IBC)

130

131 **Cell lines**

132 Vero E6, NPC1 knockout Vero E6 (Vero E6/NPC1-KO) and HEK293T-derived
133 Platinum-GP (Plat-GP) cells [23] (Cell Biolabs) were grown in Dulbecco's modified
134 Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum. Vero
135 E6/NPC1-KO cells were generated by previously described methods [24, 25] with a few
136 modifications and transduced with retroviruses expressing HEK293T-derived NPC1 and
137 its mutant genes (see Supplementary Materials and Methods).

138

139 **Cloning of the NPC1 gene into plasmids**

140 The coding region of the HEK293T NPC1 gene was polymerase chain reaction
141 (PCR)-amplified from cDNA prepared from total RNA extracted from HEK293T cells
142 according to a previous study [26]. The PCR product was cloned into a murine leukemia
143 virus-based retroviral vector, pMXs-puro [23]. Ten SNPs found in the two loops:
144 rs772847092 (I419V and I419F), rs77815278 (Y420S), rs771644708 (Y420H),
145 rs143797098 (P424A), rs140149624 (S425L), rs749078710 (A427T), rs748246747
146 (G500E), rs191537721 (D502E), and rs756587493 (D508N), were produced by site-
147 directed mutagenesis using a KOD-Plus-Neo polymerase (TOYOBO) with primers
148 containing the desired nucleotide substitutions. All mutations were confirmed by DNA
149 sequencing.

150

151 **Statistical analysis**

152 All statistical analyses were performed using R software (version 3.2.3) [27].
153 Correlations between transcription and expression levels of NPC1 were estimated using
154 the rank correlation coefficient of Spearman (r_s). For comparison of viral infectivity and
155 plaque/focus size, the one-way ANOVA followed by Dunnett's tests and Welch's t-test
156 were used. *P*-values of less than 0.05 were considered to be statistically significant.

157

158

159

160 **RESULTS**

161 **Structure-guided approach to identifying candidate NPC1 SNPs**

162 Human NPC1 has at least 254 unique SNPs listed in the SNP database
163 (<https://www.ncbi.nlm.nih.gov/snp/>). Of these SNPs, 222 are missense, resulting in amino
164 acid changes, and they are widely distributed throughout the structure of NPC1 (Figure
165 1A). Using the co-structure of full-length NPC1 and EBOV dGP [16], we identified 10
166 missense SNPs in 8 amino acid positions located in the interface (i.e., 2 loop structures at
167 amino acid positions 419-428 and 500-508) with EBOV GP (Figure 1B).

168

169 **Human NPC1 polymorphisms affecting filovirus entry into cells**

170 We next established an NPC1 knockout Vero E6 cell line, Vero E6/NPC1-KO
171 cl.19 (Supplementary Figure 1A and B), and confirmed that its susceptibility to VSV Δ G-
172 EBOV, rVSV-EBOV, and EBOV-GFP was completely abolished (Supplementary Figure
173 1C-E). The Vero E6/NPC1-KO cl.19 cells were then transduced with a retroviral vector
174 resulting in eleven different cell lines stably expressing wild-type human NPC1 (293T-
175 NPC1) or NPC1 SNP mutants (I419V, I419F, Y420S, Y420H, P424A, S425L, A427T,
176 G500E, D502E, and D508N). Introducing the wild-type NPC1 gene into the knockout
177 cells completely restored the susceptibility to filovirus entry and infection
178 (Supplementary Figure 1C-E), as has been shown with NPC1-deficient CHO and reptile-
179 derived cells [10, 12]. Transcription and expression levels of the transduced NPC1 genes
180 were examined by quantitative PCR and western blot analyses, respectively (see
181 Supplementary Materials and Methods). In all of the NPC1 SNP mutant cell lines,
182 expression levels of NPC1 were equivalent to or higher than those of 293T-NPC1-
183 expressing cells (Supplementary Figure 2). We also confirmed that these NPC1s were

184 similarly localized at endosomal vesicles (data not shown).

185 To evaluate whether these SNP substitutions affected the efficiency of viral entry,
186 each cell line was infected with replication-incompetent VSV pseudotyped with filovirus
187 GPs, and relative IUs were compared among the cell lines (Figure 2). We found that
188 P424A, S425L, D502E, and D508N substitutions in NPC1 reduced the entry of VSV
189 pseudotyped with GPs from multiple filovirus species. P424A and D508N substitutions
190 significantly affected the entry of VSV Δ G- EBOV, - BDBV, - TAFV, - SUDV, - RESTV,
191 and - LLOV, but not MARV and/or -RAVV. On the other hand, S425L and D502E
192 substitutions reduced the entry of VSV Δ G-MARV, -and some of the pseudotyped VSV
193 with ebolavirus GPs. Y420S substitution only reduced the entry of VSV Δ G-LLOV. It
194 was noted that these substitutions resulted in different patterns of reduction
195 corresponding to the filovirus genera (i.e., *Ebolavirus*, *Marburgvirus*, and *Cuevavirus*).
196 In particular, P424A/D508N and S425L/D502E substitutions seemed to be important for
197 the reduced entry of VSVs pseudotyped with ebolavirus and marburgvirus GPs,
198 respectively. In some cell lines, infectivity of the viruses was enhanced and it was
199 correlated with relatively high expression levels of NPC1 (Supplementary Figure 2).

200 201 **Reduced plaque-forming ability of rVSV-EBOV in Vero E6 cells expressing NPC1** 202 **with P424A or D508N substitution**

203 We then compared the plaque-forming abilities of rVSV-EBOV and
204 rVSV-MARV using the same cell lines (Figure 3). Although statistically significant
205 difference was not observed, the P424A and S425L substitutions moderately reduced the
206 plaque number of rVSV-EBOV and rVSV-MARV, respectively (Figure
207

208 3A). As expected, the average plaque sizes of rVSV-EBOV in cells expressing NPC1
209 with the D508N substitution were significantly smaller than in 293T-NPC1-
210 expressing cells and the P424A substitution also slightly reduced the plaque size (Figure
211 3B). Consistent with the data shown in Figure 2, rVSV-MARV formed slightly, but not
212 significantly, smaller plaques in the cell line expressing S425L NPC1 (Figure 3B).

213

214 **Reduced focus-forming ability of EBOV in Vero E6 cells with the P424A substitution**

215 To examine the effects of substitutions associated with NPC1 SNPs on
216 actual filovirus infection, we used an infectious EBOV expressing GFP and determined
217 the viral focus numbers and average focus size in each cell line. Consistent with our
218 pseudotyped VSV data, the P424A substitution resulted in reduced focus numbers
219 of EBOV-GFP (Figure 3C). The average size of foci in this cell line was also
220 significantly smaller than in cells expressing 293T-NPC1 (Figure 3D). Although
221 these differences were not significant in a multiple comparison analysis, Welch's t-test
222 gave significant differences compared to 293T-NPC1-expressing cells. The D508N
223 substitution slightly reduced the focus size of EBOV-GFP but there was no statistically
224 significant difference compared to 293T-NPC1-expressing cells.

225

226

227

228 **DISCUSSION**

229 With human immunodeficiency virus type 1 (HIV-1) infection, there is a subset
230 of people who are inherently resistant to the virus [28-31]. In particular, genetic variation
231 in the host viral receptors has been shown to reduce the susceptibility to HIV infection in
232 humans [28, 29]. Though asymptomatic filovirus infection is less studied, Baron et al.
233 [32] reported potential asymptomatic cases during the outbreak in Sudan in 1979, and
234 Dean et al. [33] estimated the proportion of asymptomatic transmissions during the recent
235 epidemic in West Africa. Although host genetics and Ebola virus disease progression and
236 resistance have been studied in mice [34, 35], the details of genetic polymorphisms that
237 may affect susceptibility of humans to filoviruses have not been elucidated yet.

238 In the present study, we focused on ten reported NPC1 SNP substitutions that
239 interface with GP. Although none of the SNPs tested here completely ablated viral entry
240 or infectivity, some SNPs were nevertheless found to be important for filovirus infection.
241 In our experiments, P424A substitution reduced entry of VSV pseudotyped with
242 ebolavirus but not marburgvirus GPs. Moreover, focus sizes of EBOV-GFP were also
243 reduced in this cell line. Interestingly, Wang et al. [15] demonstrated that the P424A
244 NPC1 mutant retained binding capacity to dGP but with reduced affinity compared to
245 wild-type NPC1. Taken together, these findings suggest that the P424A substitution in
246 NPC1 lowers its binding capacity to dGP, resulting in reduced viral entry and infection.

247 S425L substitution also reduced the infection with some pseudotyped VSVs,
248 including VSV Δ G-MARV and -RAVV. The serine residue at position 425 in NPC1 was
249 shown to be involved in the interaction with the amino acid residue at position 142 of the
250 EBOV GP molecule [15]. However, since amino acid residues at this position are different
251 in EBOV and MARV GPs (serine at 142 of EBOV GP and glutamine at 126 of MARV

252 GP, respectively), the effects of S425L substitution might be different between EBOV and
253 MARV. It has also been demonstrated that single amino acid mutations at positions 502
254 or 503 in NPC1 can reduce viral infection [13, 14]. Our experiments confirmed this
255 finding as D502E substitution reduced the entry of some of the pseudotyped viruses we
256 tested and also reduced infection with EBOV-GFP (although not significantly). Notably,
257 while the previous study introduced a significant loss of charge at residue 502 by mutating
258 the aspartic acid to phenylalanine [13], our study shows that even minimal variation at
259 this residue (D to E) can have an effect on viral entry.

260 Some other amino acids that are also important for binding to dGP have not been
261 reported as SNP sites in the NPC1 loops [15]. The rapidly increasing sequence data in
262 public databases may hereafter provide additional information on NPC1 SNP
263 substitutions not evaluated in this study. It is also of interest to investigate multiple SNP
264 substituins in the loops. Although more sensitive studies are needed to further assess the
265 capacity for NPC1 variation to influence filovirus infection, identifying genetic variations
266 that affect the susceptibility of hosts to filoviruses will be crucial in understanding
267 filovirus disease progression and host cell-restriction.

268

269

270 **Supplementary Data**

271 Supplementary materials are available at The Journal of Infectious Diseases online.
272 Consisting of data provided by the authors to benefit the reader, the posted materials are
273 not copyedited and are the sole responsibility of the authors, so questions or comments
274 should be addressed to the corresponding author.

275

276 **Notes**

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283

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373 35.
- 374
- 375

376 **Figure Legends**

377

378 **Figure 1. Structural-guided approach to human NPC1 SNP selection.**

379 A, 222 missense SNPs are represented in black on the structure from NPC1. B, Co-
380 structure of EBOV GP (blue) and NPC1 (tan) with amino acid positions of SNPs indicated
381 in red (PDB: 5JNX) [16].

382

383 **Figure 2. Infectivities of VSV pseudotyped with filovirus GPs in Vero E6 cell lines.**

384 Confluent monolayers of each cell line grown in 96-well plates were infected with
385 VSV Δ G-EBOV, VSV Δ G-BDBV, VSV Δ G-TAFV, VSV Δ G-SUDV, VSV Δ G-RESTV,
386 VSV Δ G-MARV, VSV Δ G-RAVV, and VSV Δ G-LLOV. Twenty hours later, the virus
387 infectious unit (IU) in each cell line was determined by counting the number of GFP-
388 expressing cells with an IN Cell Analyzer 2000 (GE Healthcare). Relative infectivity in
389 each cell line was calculated by setting the IU value given by Vero E6 cells expressing
390 293T-NPC1 (approximately 1000-3000 IU/well for each assay) to 100%. Means and
391 standard errors of thirty replicates are shown. Values significantly lower than for 293T-
392 NPC1 are indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

393

394 **Figure 3. Plaque/focus formation of rVSV-EBOV, rVSV-MARV, and EBOV-GFP in**
395 **Vero E6 cell lines**

396 A, rVSV-EBOV and rVSV-MARV (multiplicity of infection = 0.0005 in Vero E6 cells)
397 were inoculated into Vero E6 cells grown in 6-well tissue culture plates. After adsorption
398 for 1 hour, the inoculum was removed and the cells were overlaid with Eagle's minimal
399 essential medium containing 1.0% Bacto Agar (BD) and then incubated for 3 days at

400 37°C. Cells were stained with 0.5 % crystal violet in 10% formalin and the plaque-
401 forming unit (PFU) in each cell line was determined by counting the number of all visible
402 plaques per well (i.e., 50-100 plaques for each assay). Data represent means and standard
403 errors of at least three independent experiments. *B*, Plaque sizes were measured with
404 Image-J software. Means and standard errors of 50-100 plaques per well are shown. *C*,
405 EBOV-GFP (multiplicity of infection = 0.002 in Vero E6 cells) were inoculated into
406 confluent monolayers of Vero E6 cells grown in 96-well tissue culture plates. After
407 adsorption for 1 hour, the inoculum was replaced with Eagle's minimal essential medium
408 containing 1.2% carboxymethyl cellulose. After incubation for 3 days at 37°C, the cells
409 were fixed and GFP foci in each cell line were counted under a fluorescence microscope.
410 Data represent means and standard errors of triplicate assays. *D*, Focus sizes were
411 measured with Image-J software. Means and standard errors of 60-150 foci per well are
412 shown. (*A-D*) Values significantly lower than for 293T-NPC1 are indicated (* $P < 0.05$,
413 ** $P < 0.01$, *** $P < 0.001$).

414

Supplementary Materials and Methods

Establishment of Vero E6/NPC1-KO

We found a candidate gRNA target sequence in the genomic region of Vero E6 NPC1 and designed oligonucleotides (CRISPR-VeroNPC1-F1: 5'-TAATACGACTCACTATAGCTGCTACTGTGTCCGGCGC-3' and CRISPR-VeroNPC1-R1: 5'-TTCTAGCTCTAAAACGCGCCGGACACAGTAGCAG-3') by using the CRISPRdirect web tool [1] (<https://crispr.dbcls.jp/>). Synthesis of a gRNA template, in vitro transcription of gRNA, and purification of gRNA products were performed using the GeneArt precision gRNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Vero E6 cells were transfected with gRNA products and the Platinum Cas9 nuclease (Invitrogen) mixture using Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Invitrogen) according to the manufacturer's instructions, followed by incubation for 3 days at 37°C. By genomic cleavage detection assay with a specific primer set (VeroNPC1-367F: 5'-CTGAGGAGAAGGGCAAAG-3' and VeroNPC1-846R: 5'-CATCGCTAGACCAACTTCC-3') using a GeneArt Genomic Cleavage Detection Kit (Invitrogen), we confirmed a cleaved DNA product of 426 base pairs induced by the gRNA introduction into cells. We retreated the transfected Vero E6 cells with Cas9 nuclease solution and the gRNA to generate double knockout cells. For clonal isolation, the transfected cell suspension (5 cells/ml) was seeded on 96-well plates precoated with poly D-lysine (Nunc). After clonal expansion for 3 weeks, we obtained 97 clones. Cells from each well were harvested, followed by western blotting to confirm that there was no expression of NPC1. Of the 97 clones examined, we selected 2 (Vero E6/NPC1-KO cl. 19 and cl. 89) and used cl. 19 for the following experiments.

Generation of each NPC1-expressing cell line

To generate the retrovirus, Plat-GP cells were cotransfected with pMXs-puro [2] encoding HEK293T NPC1 or cDNA of each NPC1 mutant and the expression plasmid pCAGGS [3] encoding VSV-G cDNA with FuGENE HD transfection reagent (Promega) according to the manufacturer's recommendations. Two days later, culture supernatants containing retroviruses were collected, clarified through 0.45- μ m filters, and inoculated into Vero E6/NPC1-KO cl. 19 cells. Cell lines stably expressing each NPC1 SNP were selected with DMEM containing 10% FCS and 20 μ g/ml puromycin (Sigma-Aldrich).

SDS-PAGE and western blotting

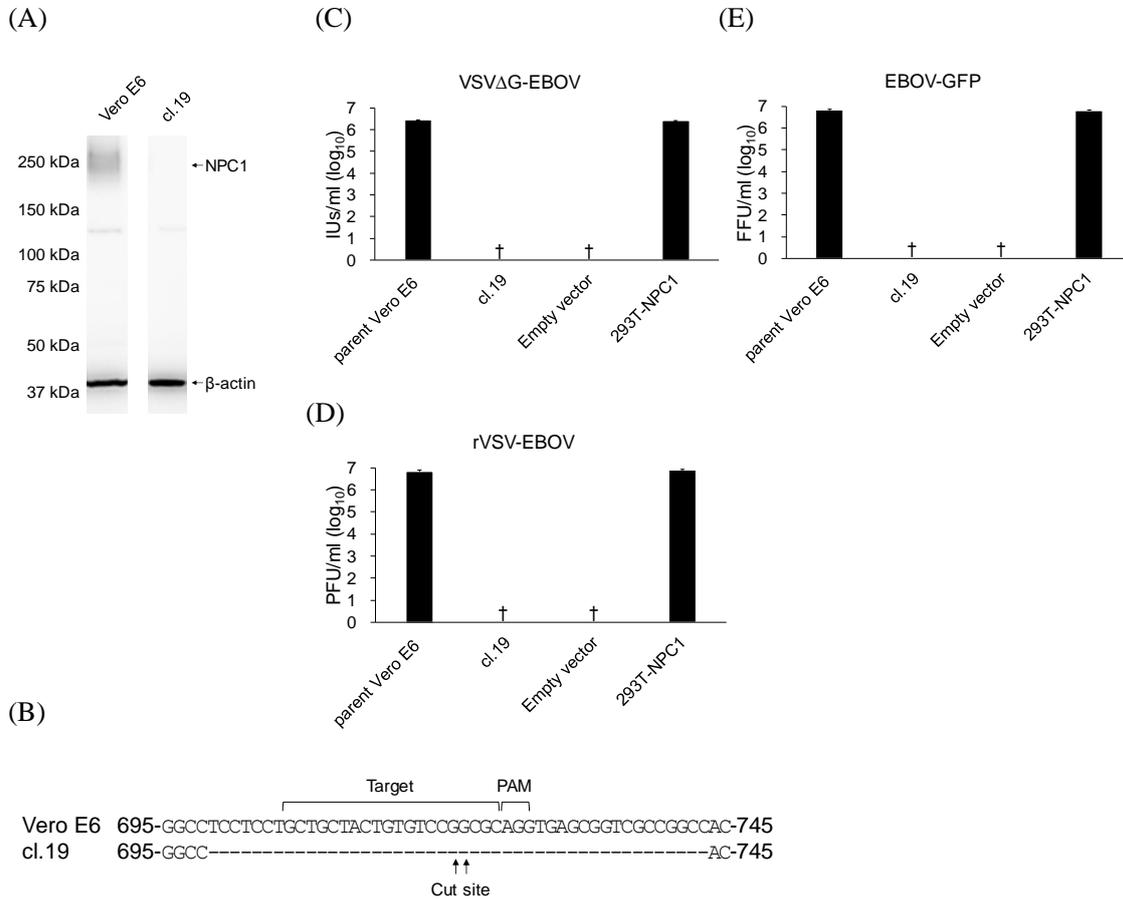
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buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA, 0.5% wt/vol CHAPS) [4] containing a protease inhibitor mixture (Roche). To facilitate disruption of cells, cell suspensions were frozen at -80°C. Samples were centrifuged at 4°C, 10000×g for 10 min. Supernatants were mixed with sodium dodecyl sulfate (SDS)-PAGE sample buffer (Bio-Rad) with 10% 2-mercaptoethanol and incubated at 65°C for 15 min. Expressed proteins were separated in SDS-polyacrylamide gels (SuperSep Ace 5-20%, Wako) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck). PBS containing 3% (wt/vol) skim milk (BD) and PBS containing 0.05% (vol/vol) Tween 20 (PBST) were used as blocking and wash buffers, respectively. The PVDF membranes were incubated with an anti-Niemann Pick C1 rabbit antibody (abcam, ab108921) recognizing the polypeptide containing amino acid residues from position 1250 to the C-terminus of human NPC1 and anti-β actin mouse monoclonal antibody (abcam, ab6276) for 60 min, washed with PBST, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115-035-062) and HRP-conjugated goat anti-rabbit IgG (KPL, 074-1506) for 60 min. After washing with PBST, the bound antibodies were visualized with Immobilon Western (Millipore). Relative expression levels were analyzed with Amersham Imager 600 Software (GE Healthcare).

Real-time PCR

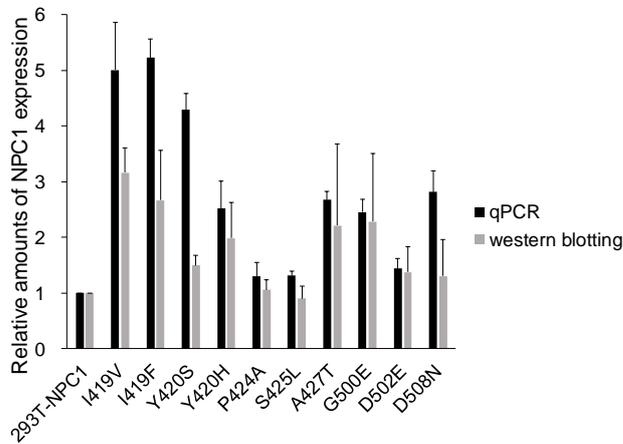
To compare the transcription levels of the NPC1 genes in the stable cell lines, the copy numbers of NPC1 mRNA were analyzed with real-time PCR using the comparative CT method in a Quant Studio 3 (Applied Biosystems). RNA extraction and reverse transcription from cultured cell lysates were conducted using a Power SYBR® Green Cells-to-CT Kit (Applied Biosystems) and real-time PCR mixtures were prepared with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) and 0.4 μM primers (HEK293T-NPC1-2987F: 5'-TGAGATTCCTGCCCATGTTC-3'; HEK293T-NPC1-3086R: 5'-TGGCCAAGGAGGATGTAAAC-3'; hum_b-actin-270F: 5'-TTCTACAATGAGCTGCGTGTG-3'; hum_b-actin-389R: 5'-GGGGTGTTGAAGGTCTCAAA-3'). PCR reactions were performed according to the manufacturer's instructions.

Supplementary Figures



Supplementary Figure 1. Generation of Vero E6/NPC1-KO cells and its susceptibility to VSV Δ G-EBOV, rVSV-EBOV, and EBOV-GFP

(A) Expression of NPC1 in parent Vero E6 and Vero E6/NPC1-KO clone 19 (cl. 19) cells was analyzed by western blotting. The experiment was performed three times and representative data are shown. (B) Deletion of the desired region of the NPC1 gene in cl. 19 cells were confirmed by DNA sequencing. (C-E) Parent Vero E6, cl. 19, cl. 19 transduced with an empty vector, and cl. 19 stably expressing 293T-NPC1 were infected with VSV Δ G-EBOV (C), rVSV-EBOV (D), or EBOV-GFP (E). Data represent means and standard errors of triplicate assays. †: Not detected.



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Transcription levels of NPC1 mRNAs were analyzed with qPCR and western blotting. Data represent means and standard deviations of at least three independent experiments. The rank correlation coefficient of spearman (r_s) showed that transcription and expression levels of NPC1 were significantly correlated ($r_s = 0.63$, P -value = 0.000073).

References

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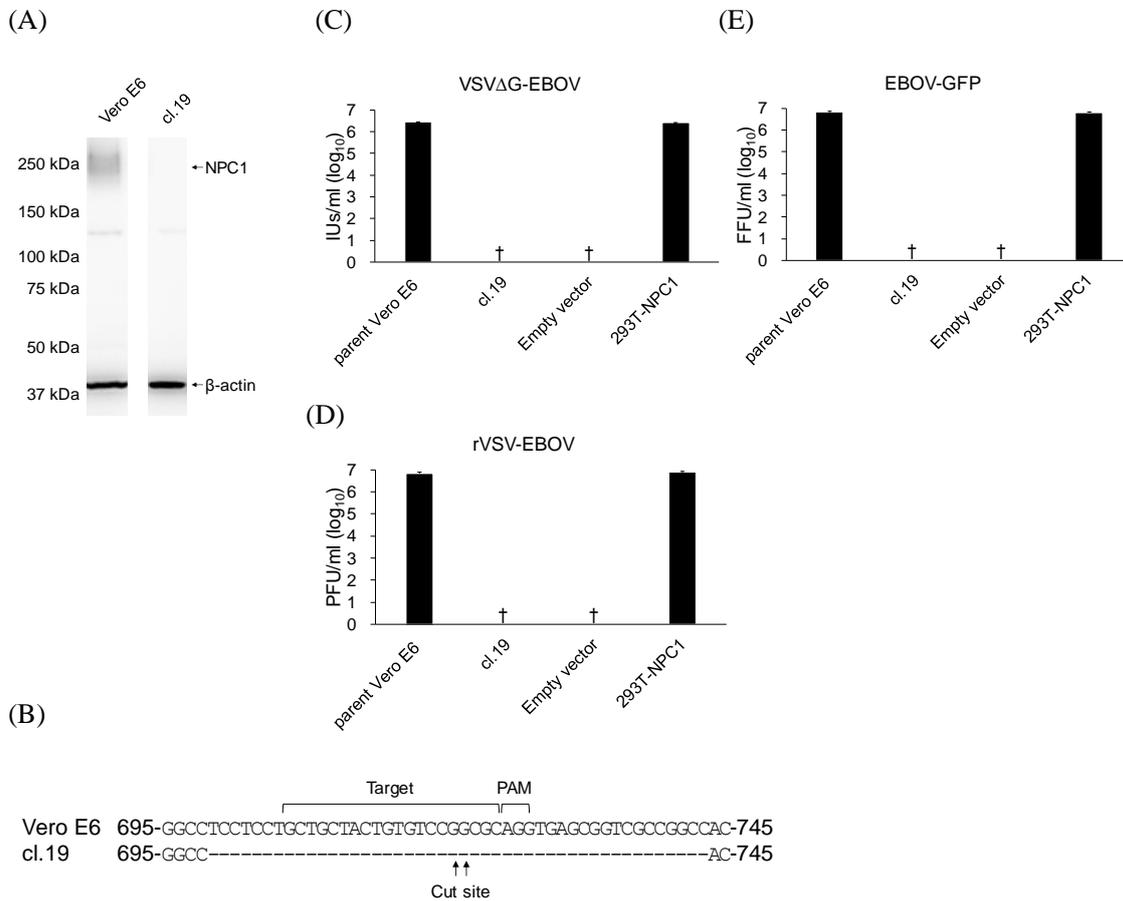
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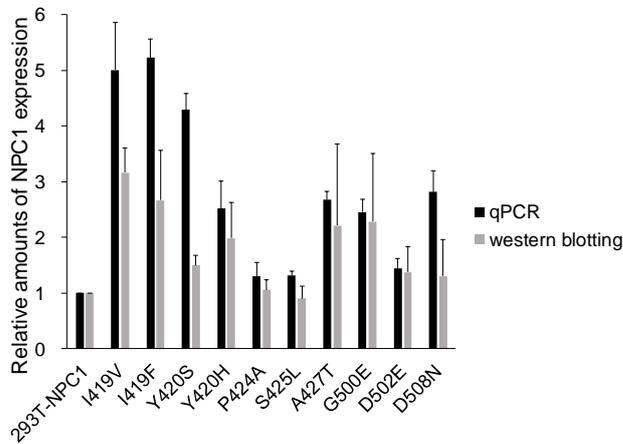
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Figure 1

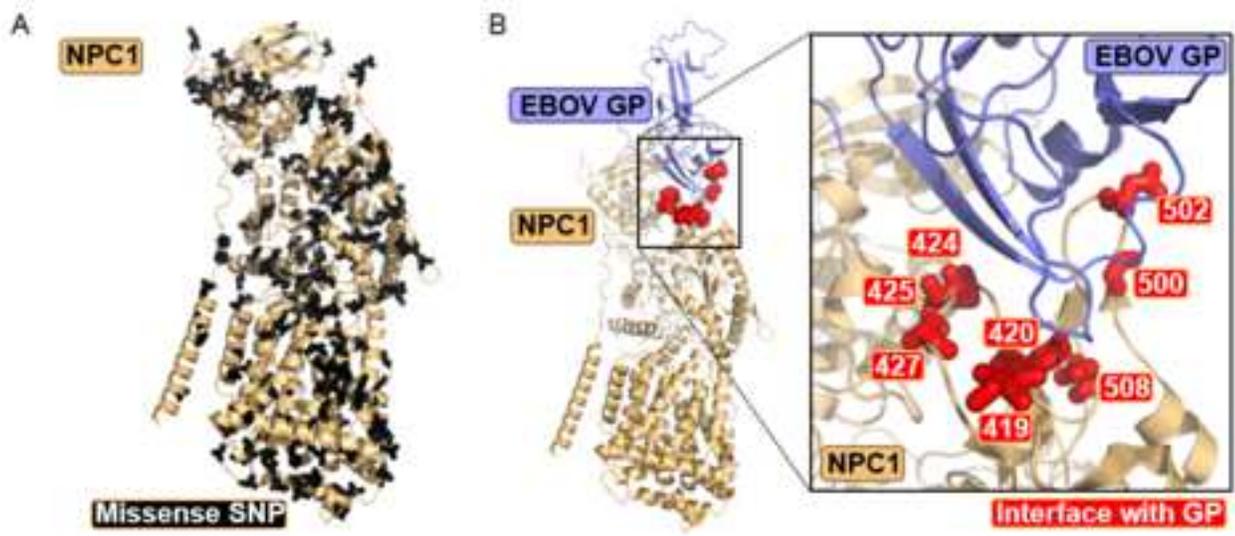


Figure 1

Figure 2

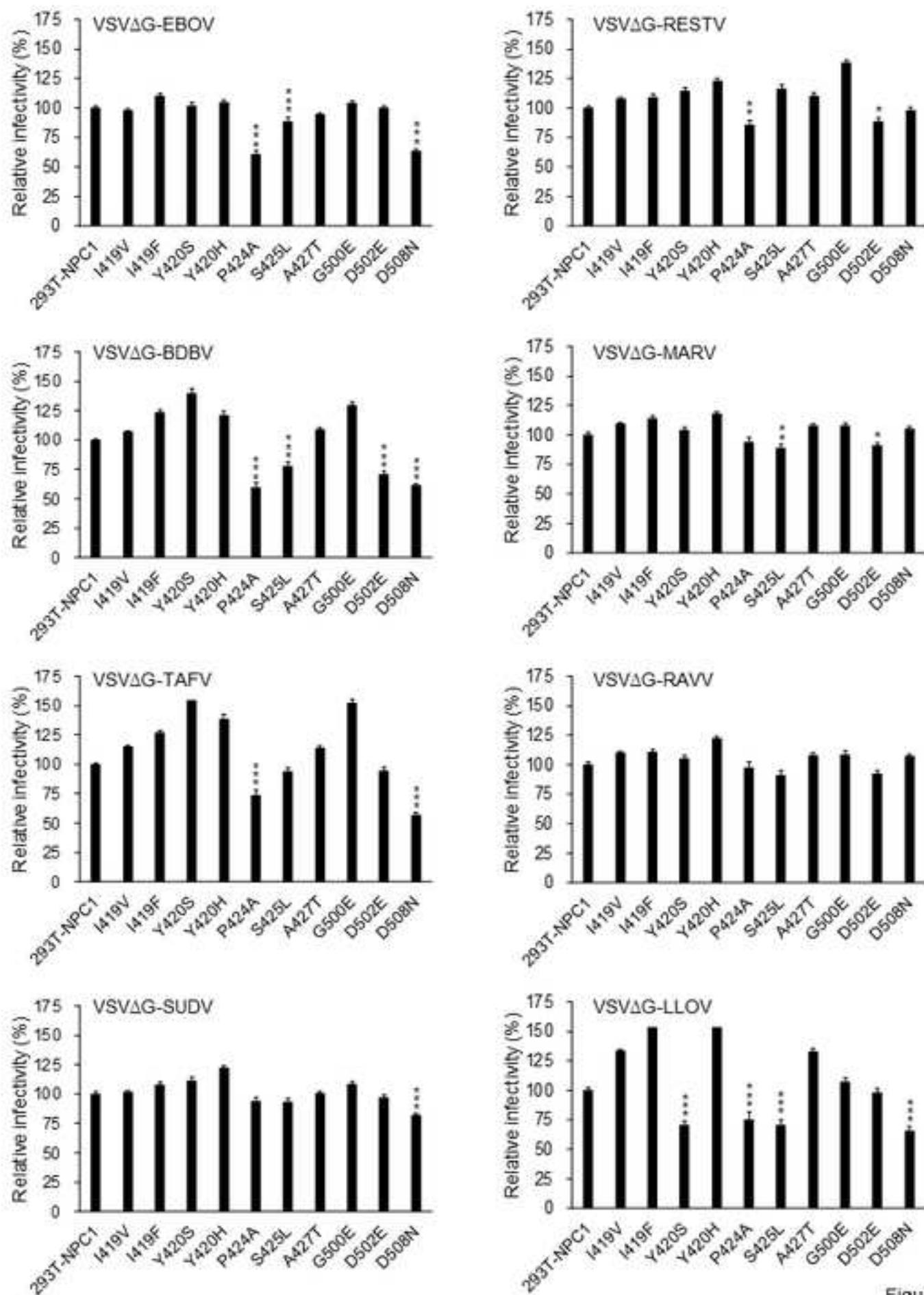


Figure 2

Figure 3

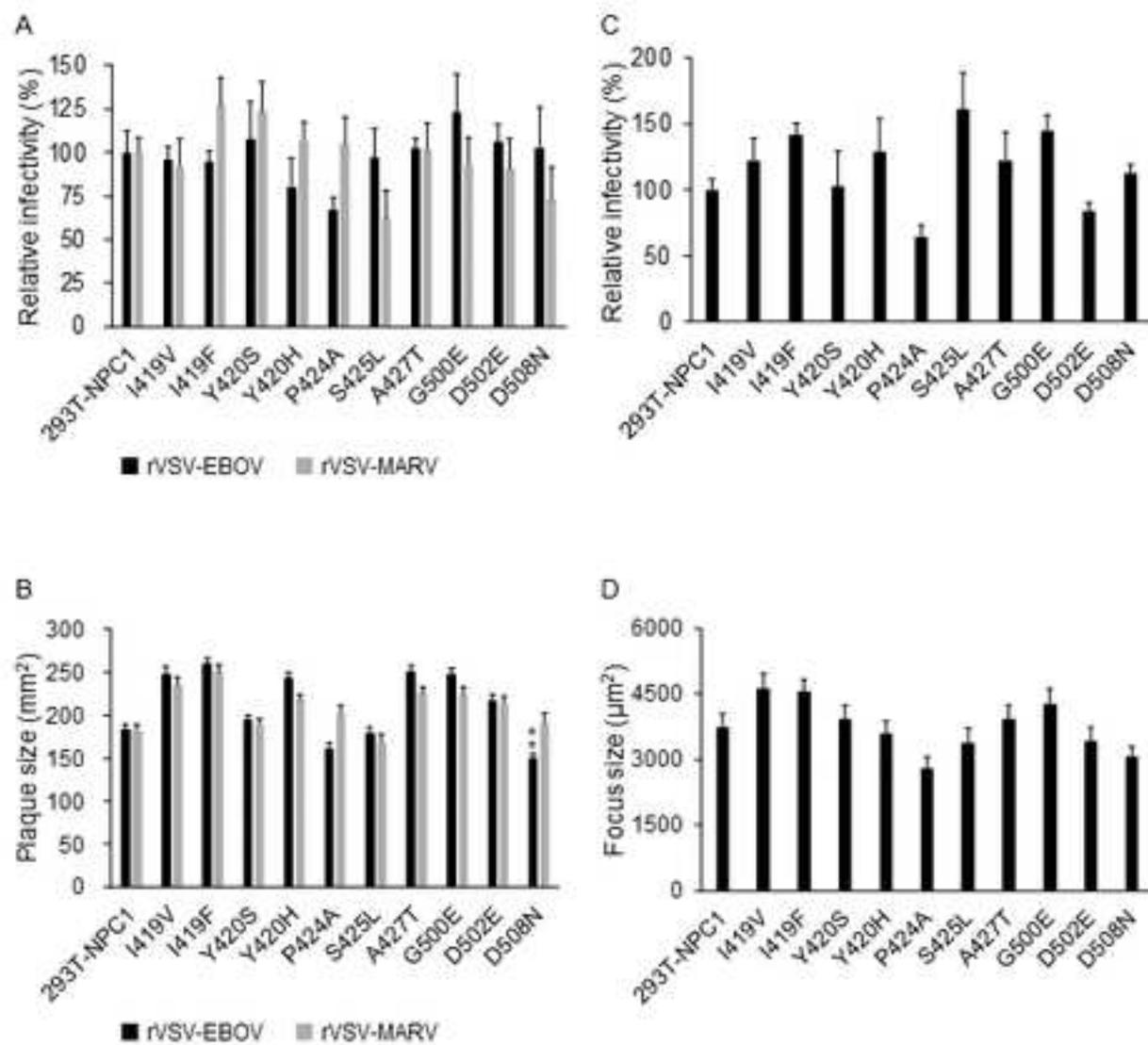


Figure 3