Single nucleotide polymorphisms in human NPC1 influence filovirus entry into cells

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Viruses

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

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

Biosafety

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

Cell lines

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

Cloning of the NPC1 gene into plasmids

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

Statistical analysis

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

RESULTS

Structure-guided approach to identifying candidate NPC1 SNPs

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

Human NPC1 polymorphisms affecting filovirus entry into cells

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

similarly localized at endosomal vesicles (data not shown).

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

Supplementary Data

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

Notes

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Figure Legends

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

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

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

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

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

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

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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Viruses

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

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

Biosafety

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

Cell lines

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

Cloning of the NPC1 gene into plasmids

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

Statistical analysis

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

RESULTS

Structure-guided approach to identifying candidate NPC1 SNPs

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

Human NPC1 polymorphisms affecting filovirus entry into cells

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

similarly localized at endosomal vesicles (data not shown).

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

Supplementary Data

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

Notes

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Figure Legends

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

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

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

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

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

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

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

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-μm 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 μg/ml puromycin (Sigma-Aldrich).

SDS-PAGE and western blotting

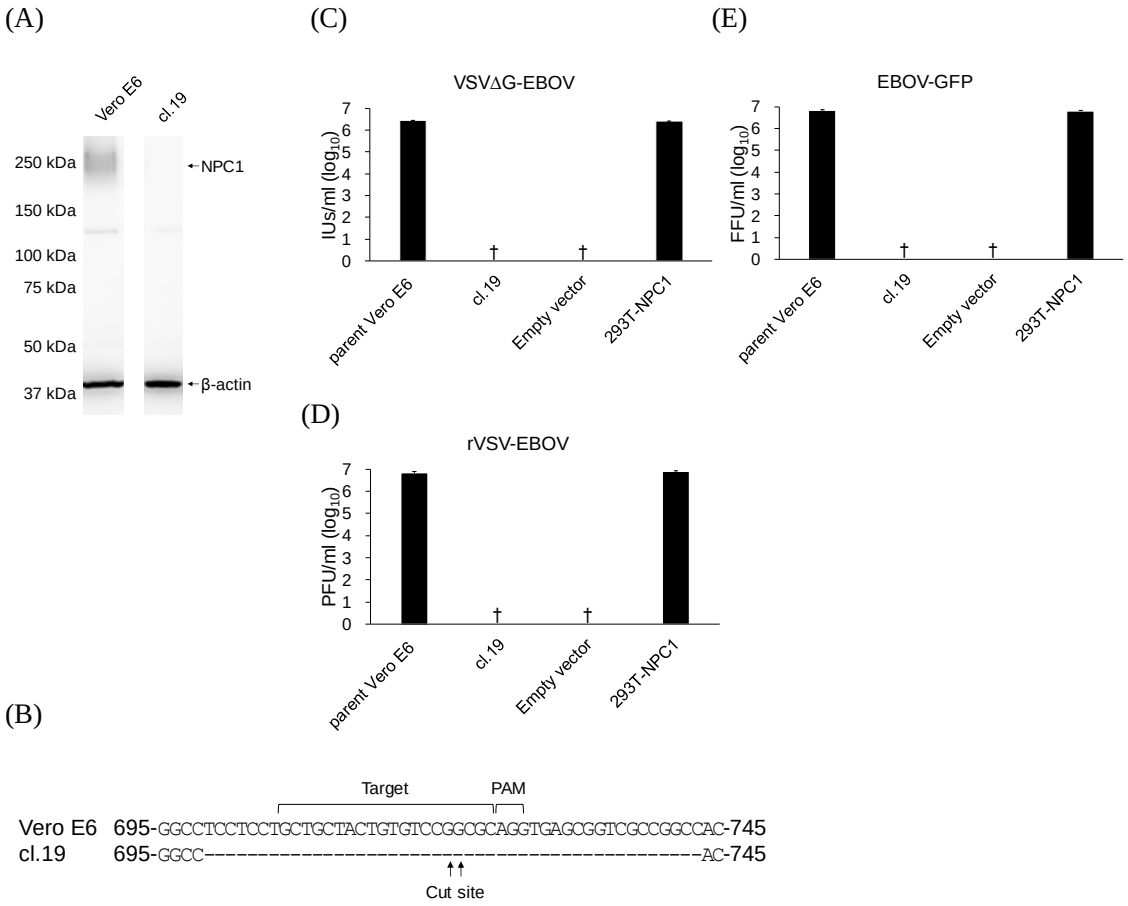
Cells (2×10^6 cells for each cell line) were lysed with 100 μl of NTE−CHAPS

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

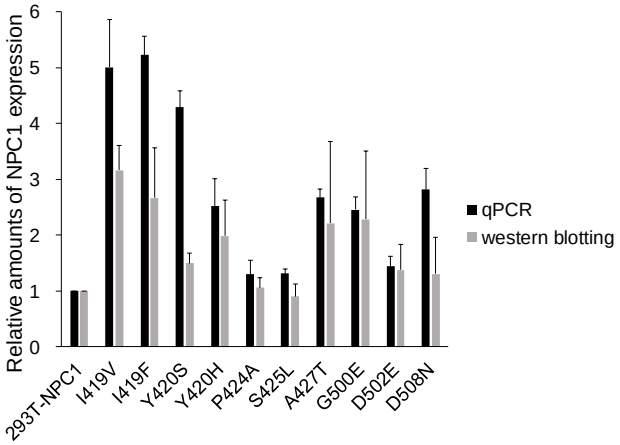
To compare the transcription levels of the NPC1 genes in the stable cell lines, the copy numbers of NPC1 mRNA were analyzed with real-time PCR using the comparative CT method in a Quant Studio 3 (Applied Biosystems). RNA extraction and reverse transcription from cultured cell lysates were conducted using 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’-TGGCCAAGGAGGATGTTAAC-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 VSVΔG-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 VSVΔG-EBOV (*C*), rVSV-EBOV (*D*), or EBOV-GFP (*E*). Data represent means and standard errors of triplicate assays. †: Not detected.



Supplementary Figure 2. Transcription and expression levels of NPC1 in each stable cell line

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).

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4. Miller EH, Obernosterer G, Raaben M, et al. Ebola virus entry requires the host-programmed recognition of an intracellular receptor. EMBO J **2012**; 31:1947-60.

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-μm 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 μg/ml puromycin (Sigma-Aldrich).

SDS-PAGE and western blotting

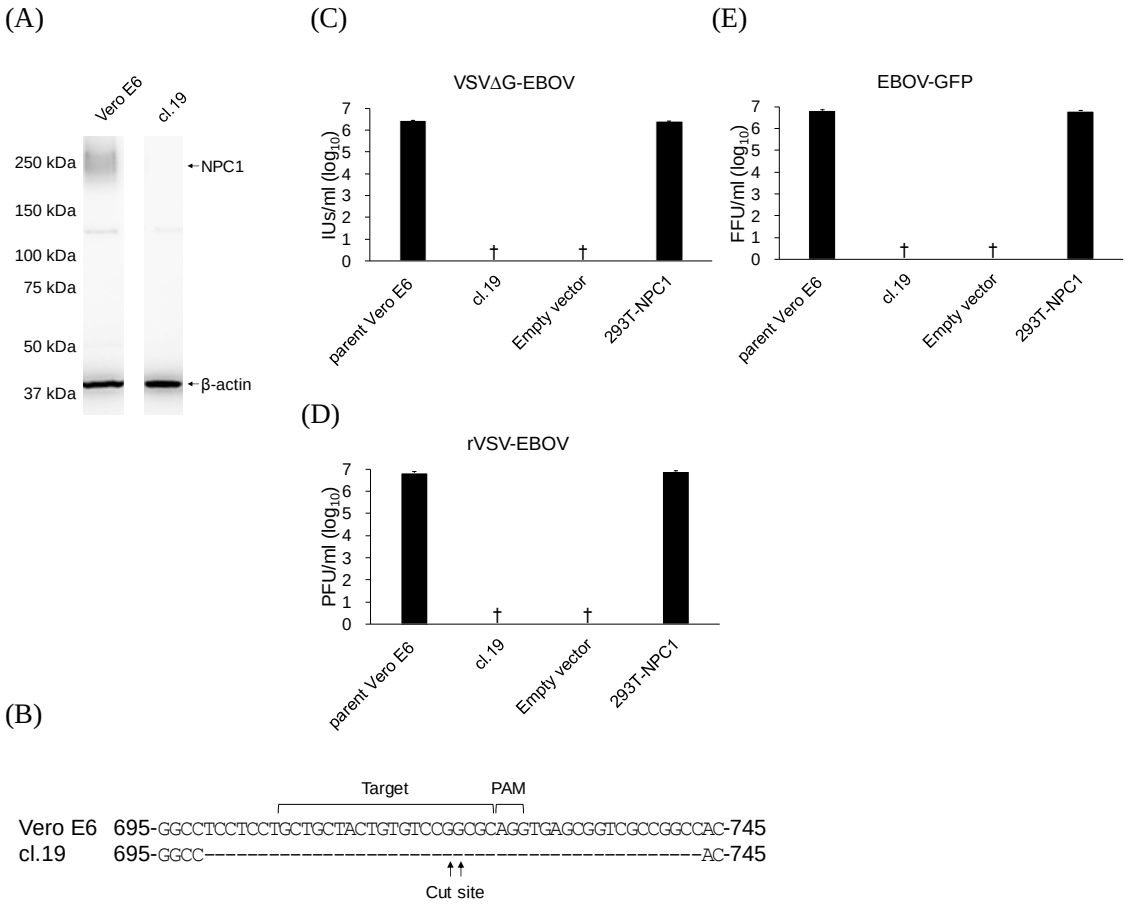
Cells (2×10^6 cells for each cell line) were lysed with 100 μl of NTE−CHAPS

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

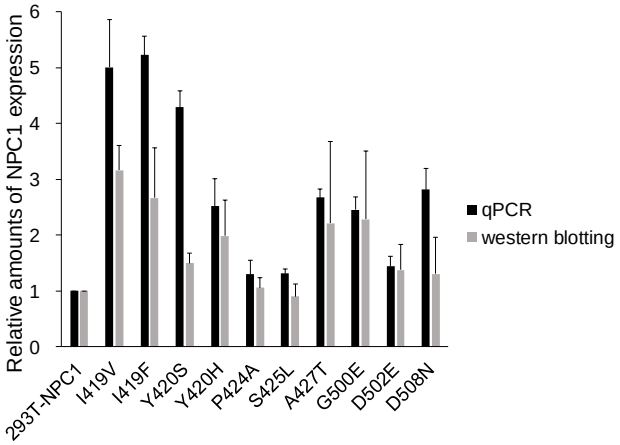
To compare the transcription levels of the NPC1 genes in the stable cell lines, the copy numbers of NPC1 mRNA were analyzed with real-time PCR using the comparative CT method in a Quant Studio 3 (Applied Biosystems). RNA extraction and reverse transcription from cultured cell lysates were conducted using 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’-TGGCCAAGGAGGATGTTAAC-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 VSVΔG-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 VSVΔG-EBOV (*C*), rVSV-EBOV (*D*), or EBOV-GFP (*E*). Data represent means and standard errors of triplicate assays. †: Not detected.



Supplementary Figure 2. Transcription and expression levels of NPC1 in each stable cell line

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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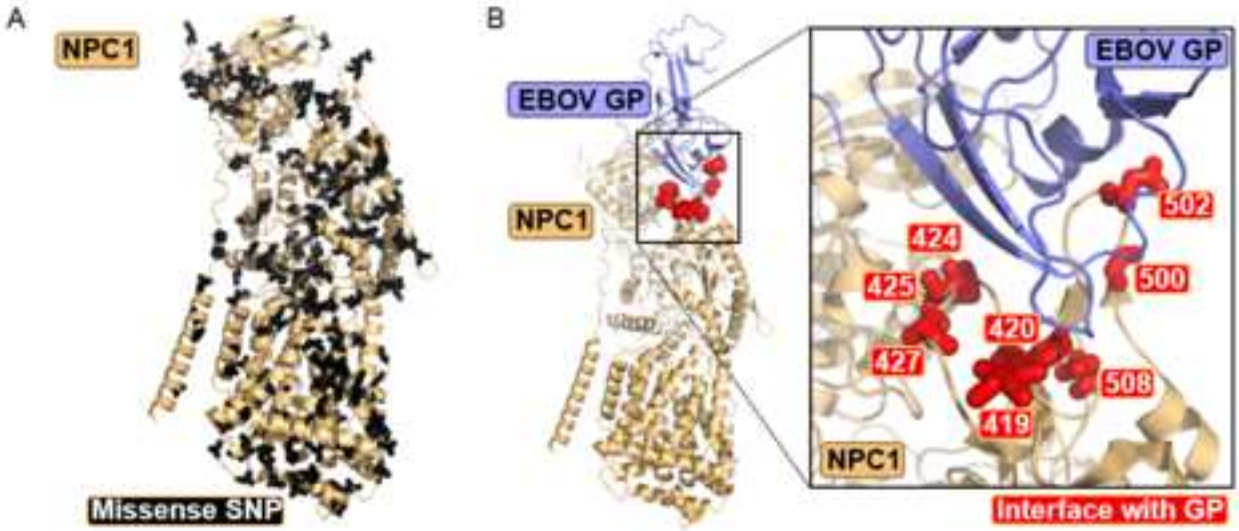

A
NPC1
Missense SNP
B
EBOV GP
NPC1
EBOV GP
502
424
500
425
420
427
508
419
NPC1
Interface with GP

Figure 2

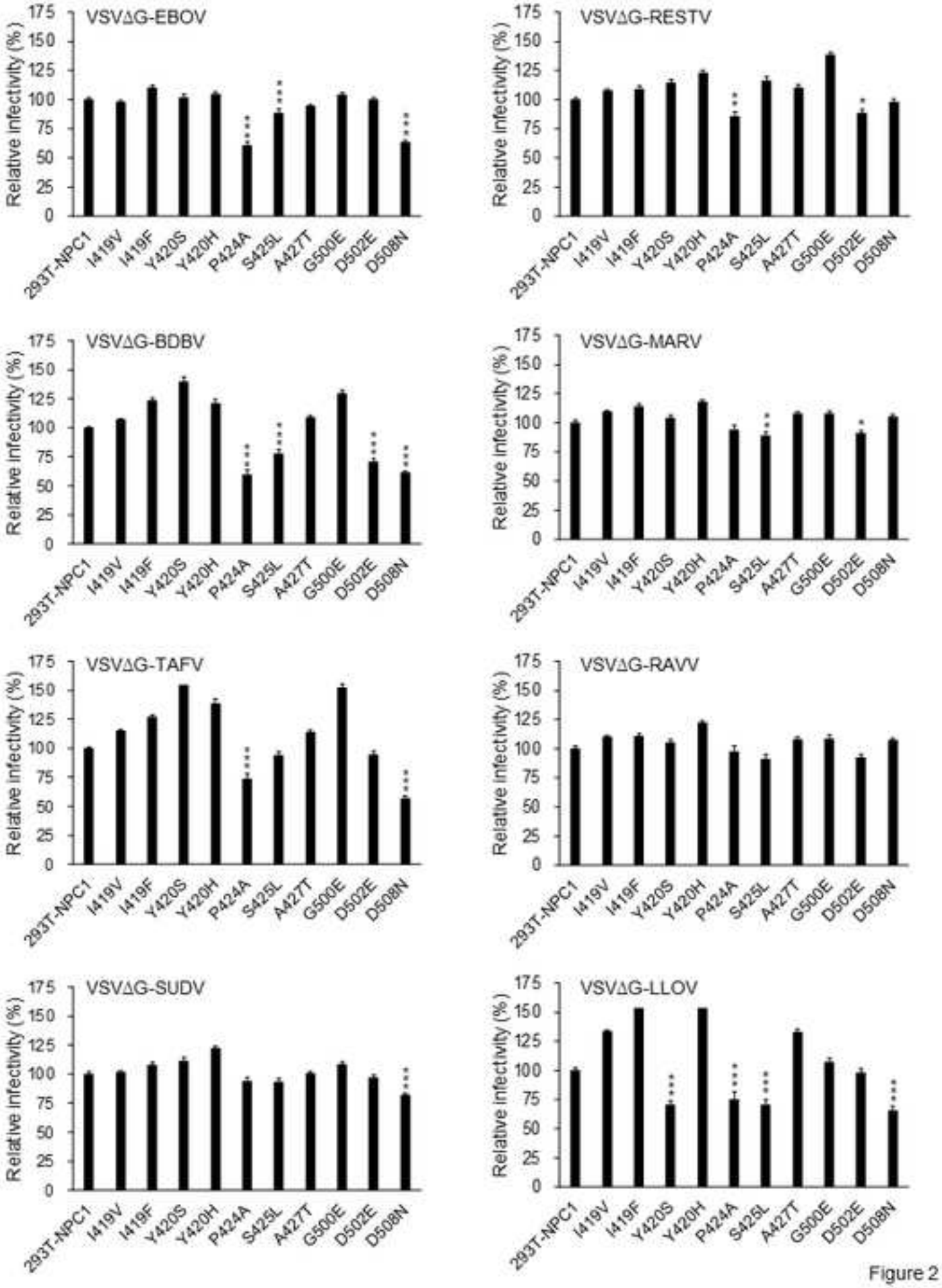
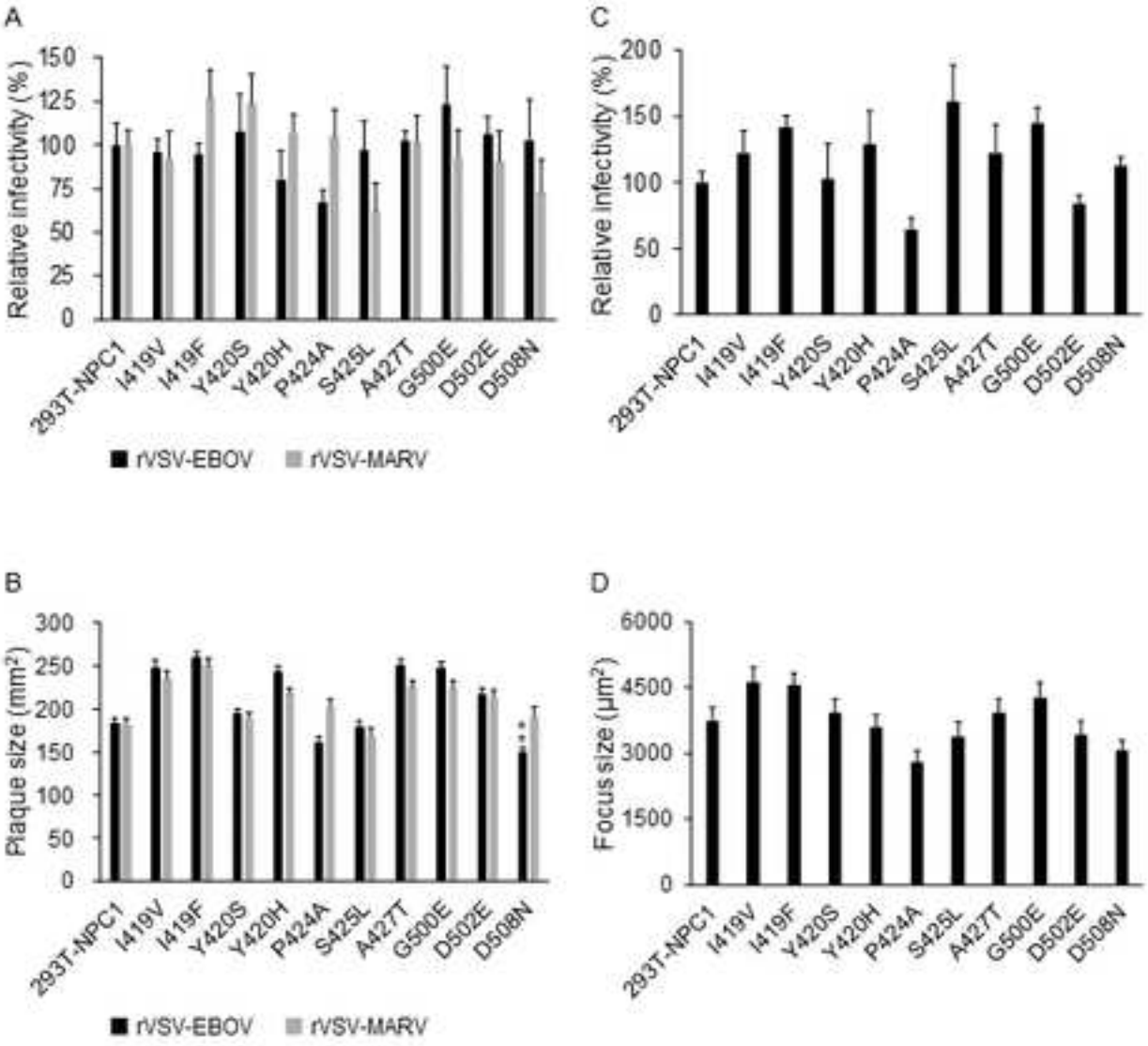


Figure 2


A
Relative infectivity (%)
150
125
100
75
50
25
0
293T-NPC1
I419V
I419F
Y420S
Y420H
P424A
S425L
A427T
G500E
D502E
D508N
rVSV-EBOV
rVSV-MARV
C
Relative infectivity (%)
200
150
100
50
0
293T-NPC1
I419V
I419F
Y420S
Y420H
P424A
S425L
A427T
G500E
D502E
D508N
B
Plaque size (mm²)
300
250
200
150
100
50
0
293T-NPC1
I419V
I419F
Y420S
Y420H
P424A
S425L
A427T
G500E
D502E
D508N
rVSV-EBOV
rVSV-MARV
D
Focus size (µm²)
6000
4500
3000
1500
0
293T-NPC1
I419V
I419F
Y420S
Y420H
P424A
S425L
A427T
G500E
D502E
D508N