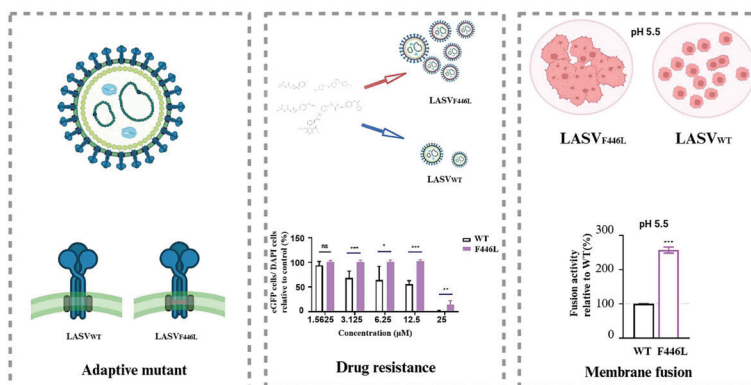




ORIGINAL ARTICLE

Functional Characterization of a Lassa Virus Fusion Inhibitor Adaptive Mutant

Graphical abstract



Highlights

- LASV GPC F446L conferred cross-resistance to structurally distinct inhibitors.
- F446L mutant increased the fusion activities of LASV and MOPV GPC.
- LASV F446L exerted little effect on the pseudotype viral growth profile or thermostability.
- LASV F446L was less compatible with a similar tyrosine residue and was intolerable to charged residues.

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Brief statement

In the current work, we aimed to characterize the effects of the F446L mutant on GPC-mediated membrane fusion, receptor binding, virus thermostability, growth kinetics, and fitness. The results should provide important context for investigating the relationship between viral variants and their virulence, which will contribute to the advancement of drug and vaccine design.

Functional Characterization of a Lassa Virus Fusion Inhibitor Adaptive Mutant

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Abstract

Objective: Lassa virus (LASV) glycoprotein complex (GPC) contains retained stable-signal peptide (SSP), glycoprotein 1 (GP1), and glycoprotein 2 (GP2). Through serial passaging of LASV with inhibitors, adaptive mutants were obtained, most of which had mutations in the transmembrane (TM) domain of GP2. Characterizing the fusion inhibitor target within the TM domain of GP2 provided insights for the development of drugs and vaccines.

Methods: We conducted membrane fusion, IIH6 inhibition, thermostability, and viral growth kinetics assays to characterize the effects of the F446L mutation on GPC-mediated membrane fusion, receptor binding, thermostability, growth kinetics, and fitness.

Results: F446L conferred cross-resistance to structurally distinct inhibitors. Additionally, F446L increased the fusion activity of LASV and Mopeia virus (MOPV) GPC, thus elevating the pH threshold for LASV fusion and promoting MOPV fusion at neutral pH. However, F446L exerted little effect on the pseudotype viral growth profile or thermostability. Introduction of other residues at the conserved F446 locus indicated that this site showed low compatibility with similar retained aromatic cyclic tyrosine residues and did not tolerate charged residues.

Conclusions: We characterized the effects of the F446L mutation on LASV, thus providing useful information for the development of vaccines and drugs.

Key words: Lassa virus (LASV), glycoprotein complex (GPC), fusion inhibitor, adaptive mutant, membrane fusion

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INTRODUCTION

Lassa virus (LASV) is an enveloped virus with a bi-segmented, negative-sense RNA genome that belongs to the genus *Mammarenavirus* (family *Arenaviridae*). Mammarenaviruses include 39 species, which are divided into New World and Old World viruses according to viral genetics, serology, antigenic properties, and geographical relationships [1,2]. The Old World viruses LASV and Lujo virus, and the New World viruses Junín

virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Chapare virus (CHAPV), and Sabiá virus (SBAV), can cause severe hemorrhagic fever and are categorized as biosafety level 4 pathogens [3,4].

The bi-segmented RNA genomes contain small (S) and large (L) segments. The S segment encodes the glycoprotein complex (GPC) and nucleoprotein, whereas the L segment encodes the matrix protein (Z) and viral polymerase.

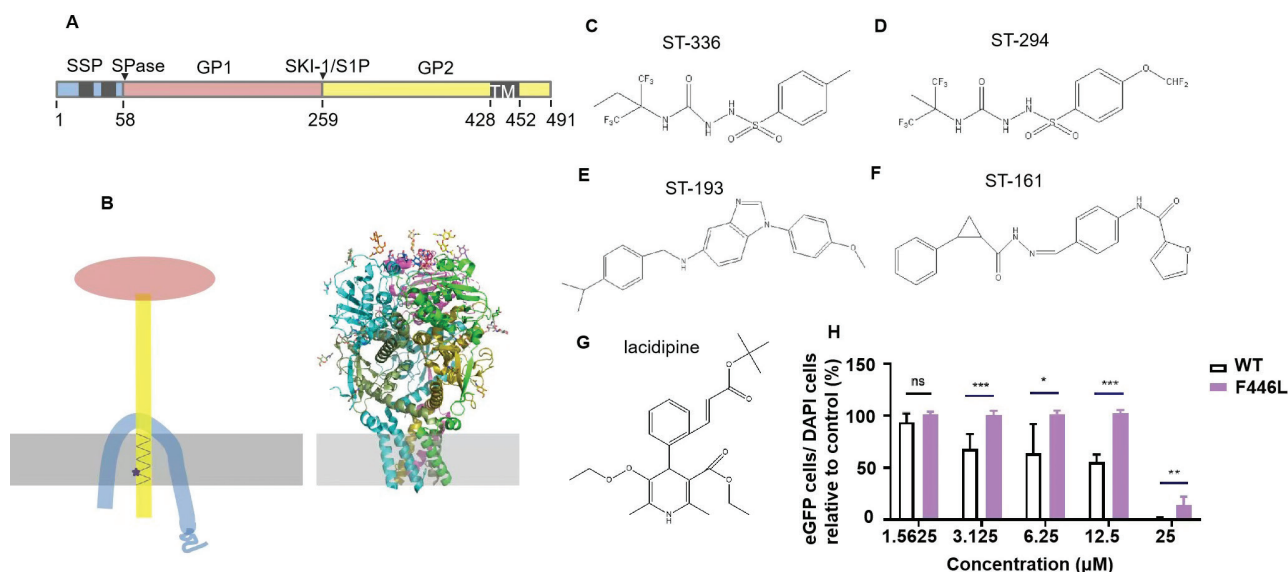


FIGURE 1 | LASV F446L confers resistance to distinct inhibitors. **(A)** Schematic diagram of the LASV GPC open reading frame. TM domains in SSP and GP2 are shown in light gray. F446 is labeled with an asterisk. The drawing is not to scale. **(B)** Left, schematic diagram of the SSP/GP1/GP2 heterotrimer. Right, cartoon diagram of LASV SSP/GP1/GP2 heterotrimers generated with PyMOL [8]. F446 is shown in stick representation with differently colored atoms. Structures of ST-336 **(C)**, ST-294 **(D)**, ST-193 **(E)**, ST-161 **(F)**, and lacidipine **(G)**. **(H)** The F446L mutation conferred resistance to lacidipine. Vero cells were incubated with lacidipine at the indicated concentration; 1 h later, LASV_{WT} and LASV_{F446L} were added to the cells (MOI 0.1) and adsorbed for 1 h. The eGFP signal was detected 23 h later with an Operetta High-Content Imaging System. Data are presented as means $\pm$ SDs from four to six independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

The mature GPC is present on the virion surface and consists of three subunits: the stable-signal peptide (SSP), receptor-binding subunit GP1, and membrane fusion subunit GP2 [5–7]. The three subunits are non-covalently bound and form an (SSP/GP1/GP2)₃ trimeric complex (Fig 1A and 1B). Notably, the uniquely retained SSP plays an essential role in GPC-mediated membrane fusion and cell entry. SSP anchors to the viral membrane by interacting with the membrane proximal external region, the transmembrane (TM) domain, and the cytoplasmic tail of GP2, thereby stabilizing the pre-fusion conformation of GPC and forming an interface that is targeted by fusion inhibitors [5–7]. Mammarenaviruses have been widely reported to generate adaptive mutants against entry inhibitors, primarily involving mutations at the SSP–GP2 interface. Intriguingly, some adaptive mutants show cross-resistance to other entry inhibitors [9]. To date, insight into the mechanisms underlying resistance remains limited. Whether the adaptive mutant de-stabilizes the pre-fusion conformation of GPC, or the native residue of the adaptive mutant is a viral target that interacts directly with the inhibitors, is unclear.

Herein, we aimed to characterize the effects of the F446L mutant on GPC-mediated membrane fusion, receptor binding, viral thermostability, growth kinetics, and fitness. The results provide valuable context for investigating the relationship between viral variants and their virulence, and might contribute to the advancement of drug and vaccine design.

MATERIAL AND METHODS

Cells, plasmids, and viruses

BHK-21 (ATCC number CCL-10), HEK 293T (ATCC number CRL-3216), Vero (ATCC number CRL-1586), and A549 (ATCC number CCL-185) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The GPC genes of LASV (Josiah strain, GenBank HQ688673.1), MOPV (GenBank AY772170.1), LCMV (Armstrong strain, GenBank AY847350.1), GTOV (GenBank NC_005077.1), JUNV (XJ13 strain, GenBank NC_005081.1), MACV (Carvalho strain, GenBank NC_005078.1), SBAV (GenBank U41071.1), and CHAPV (GenBank NC_010562.1) were synthesized (Sangon Biotech, Shanghai, China) and cloned into the pCAGGS vector. The primers used for generating the point mutation are listed in Table 1.

Plasmids encoding each protein component of the vesicular stomatitis virus (VSV), pBS-N, pBS-P, pBS-L, and pBS-G, were purchased from Kerafast (Boston, USA). The plasmid pVSVΔG-eGFP (plasmid 31842, Addgene) was modified to construct pVSVΔG-eGFP-GPC, which was used to generate LASVrv. Briefly, LASVrv virus was produced by transfection of BHK-21 cells in six-well plates that had been previously infected at a multiplicity of infection (MOI) of 5 with a recombinant vaccinia virus (vTF7-3) encoding T7 RNA polymerase. After a 45-min adsorption period, the infected cells were transfected with 11 μg of a mixture of plasmids including

TABLE 1 | Primers for point mutations.

Primer name	Sequence (5'-3')
LASV-F446A-F	TTCTATCTTATTAGCATCGCCCTTCACCTAGTC
LASV-F446A-R	GCGATGCTAATAAGATAGAACTTGTACTGAAC
LASV-F446D-F	TTCTATCTTATTAGCATCGACCTTCACCTAGTC
LASV-F446D-R	TCGATGCTAATAAGATAGAACTTGTACTGAACAC
LASV-F446L-F	TATCTTATTAGCATCTCCTTCACCTAGTC
LASV-F446L-R	GGATGCTAATAAGATAGAACTTGT
LASV-F446R-F	TTCTATCTTATTAGCATCCGCCTTCACCTAGTC
LASV-F446R-R	CGGATGCTAATAAGATAGAACTTGTACTGAACAC
LASV-F446Y-F	TCTATCTTATTAGCATCTACCTTCACCTAGT
LASV-F446Y-R	TAGATGCTAATAAGATAGAACTTGTACTGAA
LCMV-F452L-F	TCTAGTCAGCATCTTACTGCACCTTG
LCMV-F452L-R	TAAGATGCTGACTAGATATGCAGATG
MOPV-F444L-F	TTTGATCTCCGTGTTACTCCACCTAA
MOPV-F444L-R	TAACACGGAGATCAATAAAAGCTAG
GTOV-F432L-F	CACCACAAGTCTCTTATTACACCTGG
GTOV-F432L-R	TAAGAGACTTGTGGTGAAAAAGATTG
JUNV-F438L-F	TACCGCCAGCCTGTTACTGCACCTCG
JUNV-F438L-R	TAACAGGCTGGCGGTAAAGAACACGG
MACV-F449L-F	CACCGCTCCCTCTTACTGCACCTGG
MACV-F449L-R	TAAGAGGGAGGCGGTGAAGAAAATGG
SBAV-F441L-F	CACAACAACACTGTTACTTCACCTGG
SBAV-F441L-R	TAACAGTGTGTTGTGAAAAACAAAG
CHAPV-F437L-F	CACAACAACATTGTTACTCCATCTAG
CHAPV-F437L-R	TAACAATGTTGTTGTGAAGAACAGTG

pVSVAG-eGFP-GPC, pBS-N, pBS-P, pBS-G, and pBS-L in a 5:3:5:8:1 ratio [10,11]. At 48 h post transfection, the supernatants were collected and filtered to remove the vaccinia virus, then used to inoculate BHK-21 cells that had been transfected with pCAGGS-VSV-G 24 h prior. The supernatant was collected 36 h later, filtered, divided into aliquots, and frozen at -80°C until further use.

Membrane fusion assays

For qualitative evaluation of membrane fusion activity, 293T cells co-transfected with WT or mutant pCAGGS-GPC and pEGFP-N1 were incubated for 15 min with citric acid and sodium citrate buffers at the indicated pH. Syncytium formation was visualized 1 h later. For quantitative evaluation of membrane fusion activity, 293T cells in 24-well plates were co-transfected with pCAGGS-GPC (0.25 $\mu\text{g}/\text{well}$) and pCAGT7 (0.25 $\mu\text{g}/\text{well}$). Additional 293T cells in six-well plates were transfected with pT7EMCVLuc (2.5 $\mu\text{g}/\text{well}$) and pRL-CMV (0.1 $\mu\text{g}/\text{well}$). The transfected cells were then co-cultured at a ratio of 3:1. The plasmids used in this reporter

assay were kindly provided by Yoshiharu Matsuura (Osaka University, Osaka, Japan). Twelve hours after co-culturing, the cells were gently treated with acidic buffer for 15 min, and incubation continued. Luciferase activity was measured 24 h later with a Dual-Glo Luciferase Assay (Promega, Madison, WI, USA) [12–14]. Cell fusion activity was determined according to firefly luciferase (Fluc) activity standardized to *Renilla* luciferase (Rluc) activity.

IIH6 inhibition assays

To determine whether mutant recombinant LASV affected LASV binding to the cell surface receptor, we pretreated A549 cells with IIH6 (sc-53987; Santa Cruz Biotechnology, Dallas, TX, USA) or control IgM (sc-53347; Santa Cruz Biotechnology) at 37°C for 1 h [15,16]. The presence of functionally glycosylated alpha-dystroglycan (α -DG) was identified with the monoclonal antibody IIH6, which recognizes LARGE-derived sugar polymers that have been implicated in viral and ECM binding. The cells were then incubated with LASV_{WT} or LASV_{F446L} (MOI = 0.1) at 4°C for 1 h. After extensive washing with cold phosphate-buffered saline to remove unbound virus particles, the cells were incubated at 37°C for a further 24 h. They were then fixed with 4% paraformaldehyde and stained with DAPI. The eGFP-positive and DAPI-positive cells were imaged with an Operetta High-Content Imaging System (PerkinElmer, USA), and the percentage of infected cells was calculated in Harmony 3.5 software.

Thermostability assays

LASV_{WT} or LASV_{F446L} was resuspended in DMEM in the absence of serum and incubated for the indicated time at 37°C or 25°C , or incubated for 30 min at the indicated temperature [17,18]. The viruses were then used to infect Vero cells, which were evaluated for infection 23 h later with an Operetta High-Content Imaging System, as described above.

Viral growth kinetics

Vero cells in six-well plates were infected with WT or mutant recombinant LASV at an MOI of 0.01 and allowed to adsorb for 1 h at 37°C . The infected cells were then incubated, and at each indicated time point, 200 μL tissue culture supernatant was removed for plaque assays. After collection of each sample, 200 μL fresh medium was added to the wells to maintain the culture medium volume.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8 software (GraphPad Inc., La Jolla, CA, USA). All statistical analyses were performed with Student's t-test.

RESULTS

LASV GPC F446L exhibits resistance to structurally distinct inhibitors

In previous studies, passaging LASV with the ST-161 derivative led to the isolation of the F446L mutant,

whereas passaging Tacaribe virus (TCRV) in the presence of ST-336 and ST-193 led to isolation of the F436I mutant, corresponding to LASV GPC F446 mutants [19–21]. These findings suggest that the F446L mutation is resistant to these ST-series inhibitors and structurally similar inhibitors (Fig 1C–1F) [9,21,22]. Therefore, we asked whether F446L might confer resistance to other mammarenavirus entry inhibitors that selected for adaptive mutants with changes at locations other than that of F446L. To this end, we investigated the sensitivity of LASV_{F446Lrv} to lacidipine, given our previous report that lacidipine caused the adaptive T40K mutant in LASV SSP [6]. Lacidipine led to dose-dependent inhibition of LASV_{WTrv} infection (Fig 1H). Lacidipine (25 μ M) almost completely abolished the LASV_{WTrv} infection, in agreement with our previous findings [6]. Meanwhile, LASV_{F446Lrv} exhibited robust resistance to lacidipine. LASV_{F446Lrv} was able to establish approximately 100% infection, even after treatment with 12.5 μ M lacidipine. After treatment with 25 μ M lacidipine, LASV_{F446Lrv} was capable of establishing approximately 15% infection, a percentage significantly higher than that of wild-type (WT) LASV ($P = 0.00359$).

LASV GPC F446L promotes efficient fusion

F446 is located in the transmembrane domain of GP2 (Fig 1A and 1B). Because GP2 is the fusion subunit of the glycoprotein, and the TM domain plays an essential role in GPC-mediated fusion activity [21], we further evaluated the effect of F446L on GPC-mediated fusion. We

transfected 293T cells with WT or F446L GPC; 24 h later, we treated the cells with buffers with various pH values for 15 min. Syncytium formation was observed 4 h later. Both WT and F446L GP2 led to complete fusion after treatment with buffer at either pH 4.5 or 5.0 (Fig 2A). In comparison, WT GP2 induced only partial syncytium formation after exposure to pH 5.5, whereas F446L still demonstrated complete fusion. To confirm these findings, we performed quantitative evaluation with dual-luciferase assays. In agreement with the qualitative assay results, the quantitative assays indicated that F446L achieved much greater fusion activity than the WT after treatment with either pH 5.5 or pH 6.0 buffer (Fig 2B). Because pH values of 6.0 and pH 7.0 scarcely triggered GPC-mediated fusion for either WT or F446L, we did not conduct qualitative assays at these pH values. On the basis of our finding that F446L raised the pH threshold for GPC-mediated fusion, we investigated whether F446L might enter cells through the pH-dependent endocytic pathway. Ammonium chloride, a heteropolar compound regulating pH, can cause intracellular alkalinization [23]. LASV_{F446Lrv} remained sensitive to NH_4Cl treatment, similarly to LASV_{WTrv} and VSV_{rv}, thus indicating that the entry of LASV_{F446Lrv} was pH-dependent (Fig 2C).

F446L retains alpha-dystroglycan binding ability

LASV GP1 binds the α -DG receptor [24,25]. We expected that a TM mutant would have little effect on the receptor binding of the glycoprotein. To investigate the

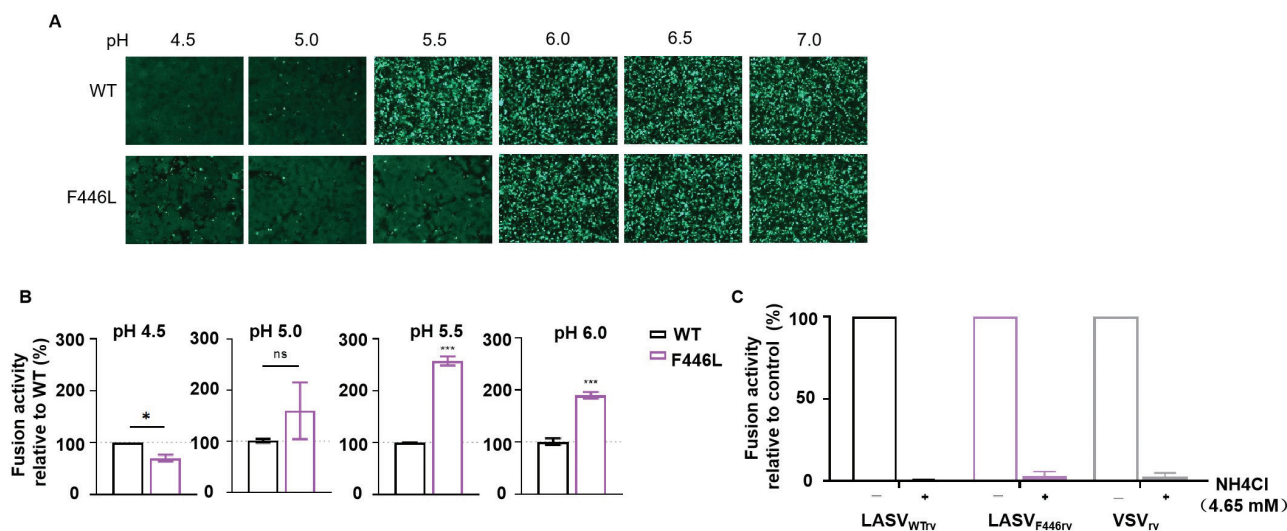


FIGURE 2 | Membrane fusion activity of the F446L mutant. **(A)** Qualitative evaluation of WT and F446L fusion activity. The 293T cells were transfected with WT or F446L GPC; 24 h later, the cells were treated with acidified buffer (pH 4.5–7) for 15 min. The cells were then placed in DMEM at neutral pH. Syncytium formation was visualized 1 h later with fluorescence microscopy. Images are representative fields from three independent experiments. **(B)** Quantitative evaluation of LASV WT and F446L fusion activity. The 293T cells were transfected with both GPC and pCAG-T7, then co-cultured at a ratio of 3:1 with cells transfected with pT7EMCVLuc and the pRL-CMV control vector. Fusion activity was evaluated according to Fluc activity, which was standardized to RLuc activity. **(C)** Acidification antagonist NH_4Cl assay. A549 cells were infected with LASV_{WTrv}, LASV_{F446Lrv}, and VSV_{rv} (MOI, 0.01) at 4°C for 1 h each, then washed with cold phosphate-buffered saline and incubated at 37°C for 90 min in the presence of NH_4Cl (4.65 mM). The eGFP-positive and DAPI-positive cells were imaged with an Operetta High-Content Imaging System, and the percentage of infected cells was calculated in Harmony 3.5 software. Data are presented as means $\pm$ SDs from three independent experiments. *** $P < 0.001$, * $P < 0.05$.

binding ability of F446L, we inoculated A549 cells with LASV_{WTrv} and LASV_{F446Lrv} at an MOI of 0.1 and allowed adsorption for 1 h; eGFP-positive cells were counted 23 h later. As a control, we used the monoclonal antibody IIH6, which recognizes a functional glycan epitope on α -DG and thus competes with LASV binding [15,16]. We observed 1420 ± 359 eGFP-positive A549 cells per 96-well plate in the LASV_{WTrv} infected group, whereas this number decreased to approximately 388 after IIH6 treatment (Fig 3). Similarly, we observed 1169 ± 322 positive cells per 96-well plate in the LASV_{F446Lrv} group without treatment with IIH6, whereas this number decreased to approximately 301 after IIH6 treatment. No significant difference was observed in the number of eGFP-positive cells between LASV_{WTrv} and LASV_{F446Lrv}, in either the absence ($P = 0.0729$) or presence ($P = 0.151$) of IIH6, thus indicating that LASV_{F446Lrv} retained its ability to bind the primary receptor on the cell surface.

LASV_{F446Lrv} has thermostability similar to that of LASV_{WTrv}

Because F446L might alter the fusion activity of GPC, we investigated whether F446L impaired the stability of the LASV GPC. LASV_{F446Lrv}, in parallel with LASV_{WTrv},

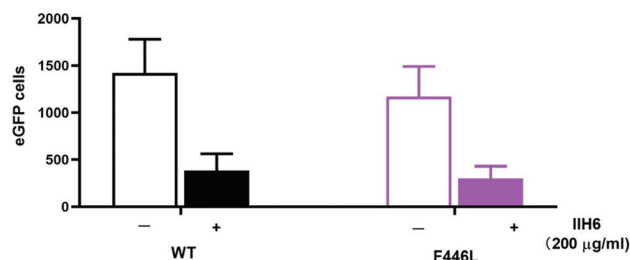


FIGURE 3 | LASV_{F446Lrv} continues to use α -DG as the primary receptor. A549 cells were incubated with the monoclonal antibody IIH6 (200 µg/mL) at 37°C for 1 h. LASV_{WTrv} and LASV_{F446Lrv} were added (MOI 0.1) and incubated at 4°C for an additional 1 h. The eGFP-positive cells were counted with an Operetta High-Content Imaging System 24 h later. Data are presented as means $\pm$ SDs from two or three independent experiments.

was incubated at various temperatures between 25°C and 55°C, then inoculated onto Vero cells. The eGFP-positive cells were analyzed with a high-content imaging system. The transduction activity of both LASV_{F446Lrv} and LASV_{WTrv} decreased with increasing temperature. A dramatic decrease occurred at 47°C, and both viruses lost transduction activity at 55°C. Notably, LASV_{F446Lrv} and LASV_{WTrv} exhibited similar thermal inactivation profiles (Fig 4A). Likewise, when both viruses were incubated at 37°C and 25°C, the transduction activity decreased over time, but no differences were observed between LASV_{F446Lrv} and LASV_{WTrv} (Fig 4B and 4C). These results suggested that the contribution of F446L to the increase in fusogenic activity was not associated with the thermostability of the recombinant virus.

Residue 446 in the TM domain regulates viral growth kinetics

Because F446L did not alter viral thermostability, we compared the growth kinetics of LASV_{F446Lrv} and LASV_{WTrv}. LASV_{F446Lrv} exhibited a multi-step growth curve similar to that of LASV_{WTrv} during the first 72 h when Vero cells were inoculated at an MOI of 0.01. The multi-step growth curve is a mathematical model used to describe the growth pattern of the virus, which usually includes four stages: exponential growth, reduced growth, stable growth, and decline. We measured the multi-step growth curve to aid in understanding of the influence of mutation sites on the viral growth cycle. Both viruses reached similar maximum titers at 24 h post-infection of approximately 10^8 plaque forming units (PFU)/mL. However, the titer of LASV_{F446Lrv} markedly decreased after 72 h, whereas that of LASV_{WTrv} began to decrease starting at 120 h (Fig 5).

To investigate whether residue 446 in the TM domain tolerated other residue changes, we introduced alanine (A), a positively charged arginine (R), a negatively charged aspartic acid (D), or a benzene containing tyrosine (Y) at this site, then evaluated the fitness of the mutant recombinant viruses. Intriguingly, we were unable to isolate stable infectious viral particles for either the F446R or

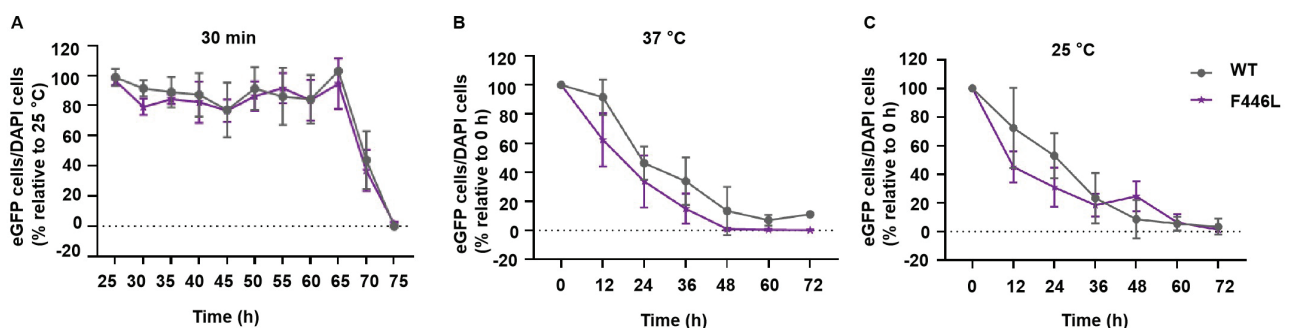


FIGURE 4 | Thermostability of LASV_{WTrv} and LASV_{F446Lrv}. LASV_{WTrv} and LASV_{F446Lrv} were incubated at the indicated temperature (25–55°C) for 30 min (A), or the indicated time (0–72 h) at 37°C (B) or 25°C (C), and plaques were assayed with Vero cells. Data are presented as means $\pm$ SDs for four independent experiments.

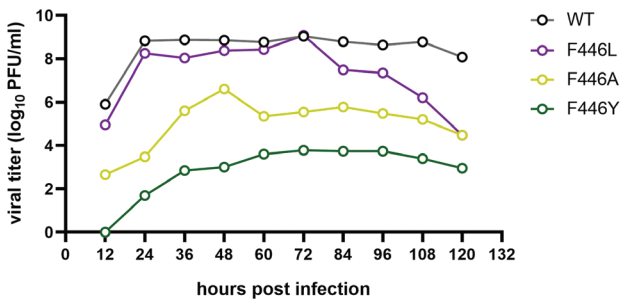


FIGURE 5 | Recombinant virus multi-step growth curves. Vero cells were infected with WT or mutant recombinant viruses at an MOI of 0.01 and allowed to adsorb for 1 h. Supernatant samples were collected at the indicated time points after infection and subjected to plaque assays for viral titer.

F446D mutant, thus suggesting that charged residues were incompatible with this site. F446A and F446Y mutants generated infectious viral particles, but the viral titers were much lower for both recombinant viruses than the WT. Notably, the F446Y mutant, with a structure similar to that of WT at position 446 in the TM domain, achieved a viral titer of 10^3 – 10^4 PFU/mL.

MOPV with a mutation corresponding to F444L shows fusion at neutral pH

Because the TM domain is highly conserved in mammarenavirus (Fig 6A), we introduced mutations corresponding to F446 into other mammarenavirus GPCs, then investigated the effects on GPC-mediated fusion. Unexpectedly, the mutation corresponding to F446 in

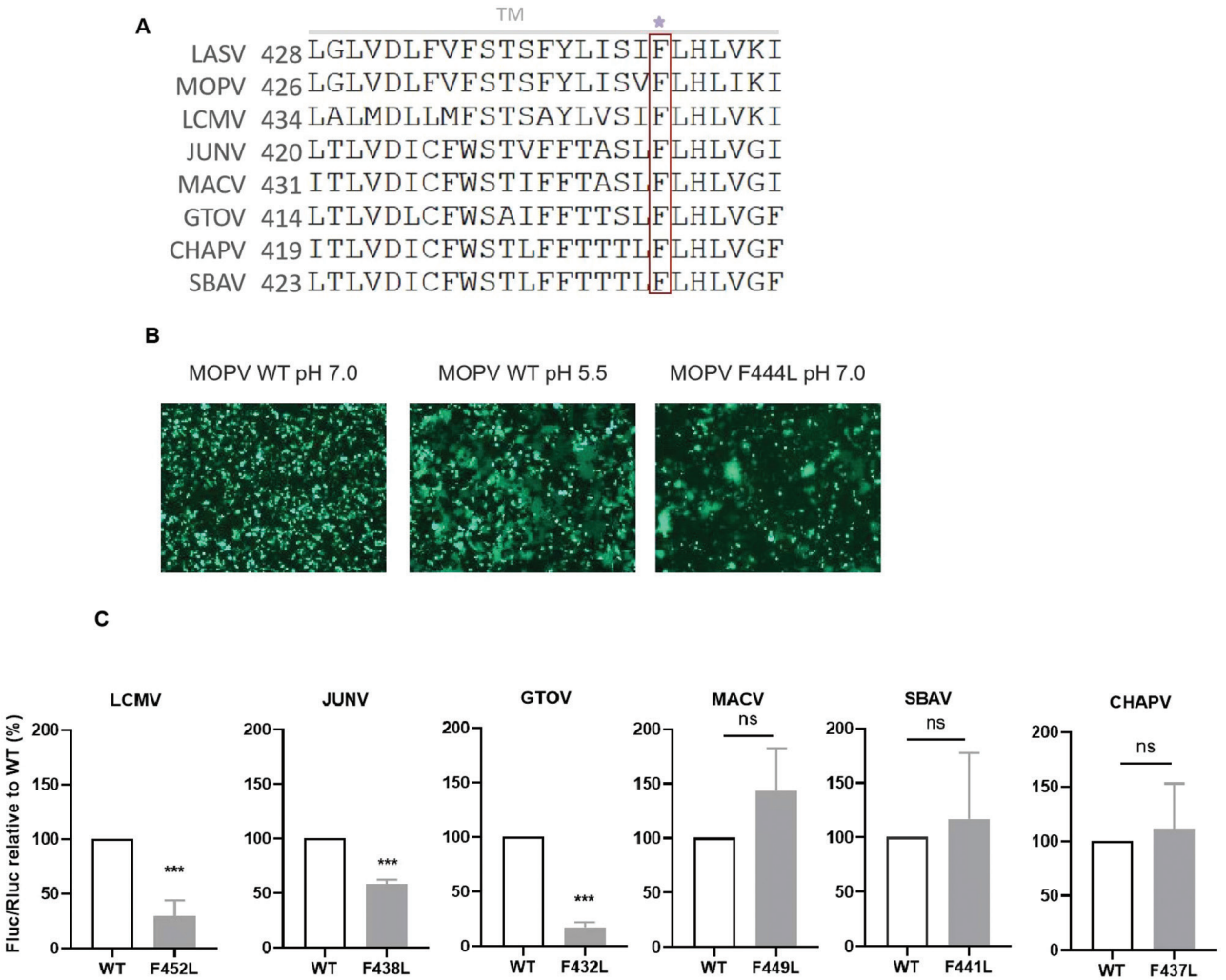


FIGURE 6 | Effects of corresponding mutations in other mammarenavirus on GPC-mediated fusion activity. **(A)** Sequence alignment of the TM domain of mammarenavirus GPCs. LASS F446 is highlighted with a purple asterisk. **(B)** The mutation corresponding to F446 in MOPV increased viral fusion activity. At 24 h after 293T cells were transfected with MOPV WT or F444L GPC, the cells were treated with acidic or neutral buffer for 15 min. Syncytium formation was visualized 1 h later with fluorescence microscopy. Images are representative fields from five independent experiments. **(C)** Quantitative evaluation of the effects of corresponding mutations on other mammarenavirus GPC-mediated fusion activity. The 293T cells were transfected with both GPC (WT or mutant) and pCAG-T7, then co-cultured at a ratio of 3:1 with cells transfected with pT7EMCVLuc and the pRL-CMV control vector. Fusion activity was evaluated by measurement of Fluc activity, which was standardized to Rluc activity. Data are presented as means $\pm$ SDs for three to six independent experiments. ***P < 0.001.

MOPV led to membrane fusion, even in the absence of acidic conditions. At 16 h post-infection with MOPV GPC F446L, clear syncytium formation was observed in 293T cells; the results were similar to those observed for cells infected with the MOPV GPC WT virus at pH 5.5 (Fig 6B). We also introduced the corresponding mutations into other mammarenaviruses and evaluated whether the pH threshold for GPC-mediated fusion increased. The corresponding mutations in LCMV, JUNV, and GTOV diminished GPC-mediated fusion activity but had little effect in MACV, SBAV, and CHAPV (Fig 6C). These results indicated that the effects of this mutation on increasing fusogenicity were limited largely to LASV and were closely associated with MOPV.

DISCUSSION

The specific retention of SSP and its interaction with GP2 provides an interface target for numerous structurally distinct inhibitors [5–7]. However, given the high mutation rate of arenaviruses [26], drug resistance is a major persistent concern in the context of direct antiviral agents. Among the reported drug-resistant variants, F446 in the TM domain of LASV GP2 and its corresponding mutants in other arenaviruses have been extensively reported. Among these adaptive mutants, TCRV F436I and JUNV F438I have been identified to be resistant to multiple small-molecule inhibitors [9,19–21]. In the current study, F446L conferred cross-resistance to not only the ST series of inhibitors but also lacidipine, which we have previously reported to result in the emergence of an adaptive mutant with a T40K change in SSP [6]. We suspected that the molecular mechanism of LASV F446L resistance might involve direct blocking of inhibitor binding to the GP2–SSP interface; allosteric effects in GP2 induced by the mutation, thereby altering its conformation and decreasing inhibitor binding affinity; or increased virulence, potentially through enhanced fusion activity, leading to decreased inhibitor efficacy.

Mutations in glycoproteins identified during pandemic outbreaks are usually accompanied by enhanced pathogenesis and transmission. The spike protein mutant D614G that emerged during the Coronavirus Disease 2019 pandemic has been reported to increase the infectivity and transmission of SARS-CoV-2, the etiological virus [27–29]. Similarly, the Ebola glycoprotein A82V mutant in the 2013–2016 epidemics increased infectivity in human cells together with fusion activity [17,30]. Accordingly, we evaluated the sequences of clinical isolates reported for the 2018 LASV outbreak in Nigeria [31] and found that the F446L mutant was absent.

Glycoprotein mutants that emerge during serial passaging for vaccine development are accompanied by attenuated virulence. The only currently approved arenavirus vaccine strain for JUNV, Candid #1, has six amino acid substitutions, and the major determinant of attenuation is GPC F427I, located in the TM domain [32–34]. Notably,

MACV F438I and TCRV F425L, corresponding to the JUNV F427I substitution, resulted in significantly less virulence in mouse models than observed for the WT parent viruses. The corresponding LASV V434I mutant promotes fusion activity at neutral pH and also confers resistance to a fusion inhibitor [33–35]. In the current study, LASV F446L also increased fusion activity, and the MOPV mutation corresponding to F444L led to membrane fusion at neutral pH. These adaptive mutants all replaced a bulky aromatic amino acid (F) with a nonaromatic residue (I, V, and L) in the TM domain, and were likely to result in a structural defect that was unable to sustain the high-energy prefusion conformation and spontaneously adopted the postfusion conformation, even under neutral pH conditions. This change might also have led to deregulation of the pH-sensing machinery of the protein, potentially by loosening side chain interactions within the GP2 trimer after protonation [33]. These findings may inform the identification of a conserved inhibitor-binding site and the development of pan-arenavirus inhibitors, or support basic research in the preparation of attenuated authentic vaccines.

F446L had little effect on the growth kinetics of LASV, in agreement with our previous findings that adaptive mutants generally do not impair viral fitness [6,36]. However, this position in the TM domain of GPC tolerated only phenylalanine and leucine, but not charged residues or tyrosine with the most structural similarity to that of WT at position 446 in the TM domain, thus suggesting that the highly conserved phenylalanine is critical for virion assembly and GPC function. Typically, glycoprotein mutations increase fusion activity at the cost of viral stability [17,18,37–39]. In the current study, because the F446L mutant showed little effect on the thermostability of LASVrv, a sensitive gap might exist between energy thresholds of fusion and thermostability, and GPC_{F446L} might maintain a metastable state. The viral titer of LASV_{F446Lrv} decreased sharply after 72 h, possibly because of decreased cell viability. We sequenced the LASV_{F446Lrv} genome after more than eight serial passages and observed no additional mutations or any reversion mutations, thus indicating that F446L generated stable progeny virions, even in the absence of inhibitor selection pressure. Importantly, whether F446L in native LASV exhibits similar thermostability, and whether LASV_{F446Lrv} might be less virulent during infection *in vivo* must be further investigated.

CONCLUSION

Currently no effective drugs or vaccines are available for the treatment of LASV. Continuous passage of LASV and inhibitors enabled adaptive mutants to be obtained, most of which bore mutations in the TM domain of GP2. These mutations might affect virulence; provide useful information for the development of authentic attenuated vaccines; or avoid the use of such inhibitors, thereby providing a theoretical basis for drug combinations.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

- Wang W, Zhou Z, Zhang L, Wang S, Xiao G. Structure-function relationship of the mammarenavirus envelope glycoprotein. *Virol Sin.* 2016;31:380-394.
- Maes P, Alkhovsky SV, Bao Y, Beer M, Birkhead M, Briese T, et al. Taxonomy of the family Arenaviridae and the order Bunyavirales: update 2018. *Arch Virol.* 2018;163:2295-2310.
- Buchmeier MJ, de la Torre JC, Peters CJ. *Fields Virology*. 4th edition. Philadelphia: Lippincott-Raven; 2007:1791-1828.
- Vela E. Animal models, prophylaxis, and therapeutics for arenavirus infections. *Viruses.* 2012;4:1802-1829.
- Shankar S, Whitby LR, Casquilho-Gray HE, York J, Boger DL, Nunberg JH. Small-molecule fusion inhibitors bind the pH-sensing stable signal peptide-GP2 subunit interface of the Lassa virus envelope glycoprotein. *J Virol.* 2016;90:6799-6807.
- Wang P, Liu Y, Zhang G, Wang S, Guo J, Cao J, et al. Screening and identification of Lassa virus entry inhibitors from an FDA-approved drugs library. *J Virol.* 2018;92:e00954-18.
- Tang K, Zhang X, Guo Y. Identification of the dietary supplement capsaicin as an inhibitor of Lassa virus entry. *Acta Pharm Sin B.* 2020;10:789-798.
- Katz M, Weinstein J, Eilon-Ashkenazy M, Gehring K, Cohen-Dvashi H, Elad N, et al. Structure and receptor recognition by the Lassa virus spike complex. *Nature.* 2022;603:174-179.
- York J, Dai D, Amberg SM, Nunberg JH. pH-induced activation of arenavirus membrane fusion is antagonized by small-molecule inhibitors. *J Virol.* 2008;82:10932-10939.
- Geisbert TW, Jones S, Fritz EA, Shurtleff AC, Geisbert JB, Liebscher R, et al. Development of a new vaccine for the prevention of Lassa fever. *PLoS Med.* 2005;2:e183.
- Safronetz D, Mire C, Rosenke K, Feldmann F, Haddock E, Geisbert T, et al. A recombinant vesicular stomatitis virus-based Lassa fever vaccine protects guinea pigs and macaques against challenge with geographically and genetically distinct Lassa viruses. *PLoS Negl Trop Dis.* 2015;9:e0003736.
- Takikawa S, Ishii K, Aizaki H, Suzuki T, Asakura H, Matsuura Y, et al. Cell fusion activity of hepatitis C virus envelope proteins. *J Virol.* 2000;74:5066-5074.
- Thomas CJ, Casquilho-Gray HE, York J, DeCamp DL, Dai D, Petrilli EB, et al. A specific interaction of small molecule entry inhibitors with the envelope glycoprotein complex of the Junin hemorrhagic fever arenavirus. *J Biol Chem.* 2011;286:6192-6200.
- Cao J, Zhang G, Zhou M, Liu Y, Xiao G, Wang W. Characterizing the Lassa virus envelope glycoprotein membrane proximal external region for its role in fusogenicity. *Virol Sin.* 2020;36:273-280.
- Moraz ML, Pythoud C, Turk R, Rothenberger S, Pasquato A, Campbell KP, et al. Cell entry of Lassa virus induces tyrosine phosphorylation of dystroglycan. *Cell Microbiol.* 2013;15:689-700.
- Oppliger J, Torriani G, Herrador A, Kunz S. Lassa virus cell entry via dystroglycan involves an unusual pathway of macropinocytosis. *J Virol.* 2016;90:6412-6429.
- Wang MK, Lim SY, Lee SM, Cunningham JM. Biochemical basis for increased activity of Ebola glycoprotein in the 2013-16 epidemic. *Cell Host Microbe.* 2017;21:367-375.
- Li P, Shan Y, Zheng W, Ou X, Mi D, Mu Z, et al. Identification of H209 as essential for pH 8-triggered receptor-independent syncytium formation by S protein of mouse hepatitis virus A59. *J Virol.* 2018;92:e00209-18.
- Bolken TC, Laquerre S, Zhang Y, Bailey TR, Pevear DC, Kickner SS, et al. Identification and characterization of potent small molecule inhibitor of hemorrhagic fever new world arenaviruses. *Antiviral Res.* 2006;69:86-97.
- Larson RA, Dai D, Hosack VT, Tan Y, Bolken TC, Hruby DE, et al. Identification of a broad-spectrum arenavirus entry inhibitor. *J Virol.* 2008;82:10768-10775.
- Zhang G, Cao J, Cai Y, Liu Y, Li Y, Wang P, et al. Structure-activity relationship optimization for Lassa virus fusion inhibitors targeting the transmembrane domain of GP2. *Protein Cell.* 2019;10:137-142.
- Zhang Z, Takenaga T, Fehling SK, Igarashi M, Hirokawa T, Muramoto Y, et al. Hexestrol, an estrogen receptor agonist, inhibits Lassa virus entry. *J Virol.* 2024;98:e0071424.
- Glushakova SE, Lukashevich IS. Early events in arenavirus replication are sensitive to lysosomotropic compounds. *Arch Virol.* 1989;104:157-161.
- Cao W, Henry MD, Borrow P, Yamada H, Elder JH, Ravkov EV, et al. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science.* 1998;282:2079-2081.
- Jae LT, Raaben M, Herbert AS, Kuehne AI, Wirchnianski AS, Soh TK, et al. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. *Science.* 2014;344:1506-1510.
- Garry RF. Lassa fever – the road ahead. *Nat Rev Microbiol.* 2023;21:87-96.
- Hou YJ, Chiba S, Halfmann P, Ehre C, Kuroda M, Dinnon KH 3rd, et al. SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo. *Science.* 2020;370:1464-1468.
- Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. *Cell.* 2020;182:812-827.e19.
- Plante JA, Liu Y, Liu J, Xia H, Johnson BA, Lokugamage KG, et al. Spike mutation D614G alters SARS-CoV-2 fitness. *Nature.* 2020;592:116-121.
- Diehl WE, Lin AE, Grubaugh ND, Carvalho LM, Kim K, Kyaw PP, et al. Ebola virus glycoprotein with increased infectivity dominated the 2013-2016 epidemic. *Cell.* 2016;167:1088-1098.e6.
- Siddle KJ, Eromon P, Barnes KG, Mehta S, Oguzie JU, Odia I, et al. Genomic analysis of Lassa virus during an increase in cases in Nigeria in 2018. *N Engl J Med.* 2018;379:1745-1753.
- Albariño CG, Bird BH, Chakrabarti AK, Dodd KA, Flint M, Bergeron E, et al. The major determinant of attenuation in mice of the Candid1 vaccine for Argentine hemorrhagic fever is located in the G2 glycoprotein transmembrane domain. *J Virol.* 2011;85:10404-10408.
- Droniou-Bonzom ME, Reignier T, Oldenburg JE, Cox AU, Exline CM, Rathbun JY, et al. Substitutions in the glycoprotein (GP) of the Candid#1 vaccine strain of Junin virus increase dependence on human transferrin receptor 1 for entry and destabilize the metastable conformation of GP. *J Virol.* 2011;85:13457-13462.

34. Madu IG, Files M, Gharaibeh DN, Moore AL, Jung KH, Gowen BB, et al. A potent Lassa virus antiviral targets an arenavirus virulence determinant. *PLoS Pathog.* 2018;14:e1007439.
35. Patterson M, Koma T, Seregin A, Huang C, Miller M, Smith J, et al. A substitution in the transmembrane region of the glycoprotein leads to an unstable attenuation of Machupo virus. *J Virol.* 2014;88:10995-10999.
36. Wang S, Liu Y, Guo J, Wang P, Zhang L, Xiao G, et al. Screening of FDA-approved drugs for inhibitors of Japanese Encephalitis virus infection. *J Virol.* 2017;91:e01055-17.
37. Cotter CR, Jin H, Chen Z. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. *PLoS Pathog.* 2014;10:e1003831.
38. Di Lella S, Herrmann A, Mair CM. Modulation of the pH stability of influenza virus hemagglutinin: a host cell adaptation strategy. *Biophys J.* 2016;110:2293-2301.
39. Wang W, Song HS, Keller PW, Alvarado-Facundo E, Vassell R, Weiss CD. Conformational stability of the hemagglutinin of H5N1 influenza A viruses influences susceptibility to broadly neutralizing stem antibodies. *J Virol.* 2018;92:e00247-18.