



# Direct Intracellular Visualization of Ebola Virus-Receptor Interaction by *In Situ* Proximity Ligation

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**ABSTRACT** Ebola virus (EBOV) entry into host cells comprises stepwise and extensive interactions of the sole viral surface glycoprotein (GP) with multiple host factors. During the intricate process, following virus uptake and trafficking to late endosomal/lysosomal compartments, GP is proteolytically processed to cleaved GP (GP<sub>CL</sub>) by the endosomal proteases cathepsin B and L, unmasking GP's receptor-binding site. Engagement of GP<sub>CL</sub> with the universal filoviral intracellular receptor Niemann-Pick C1 (NPC1) eventually culminates in fusion between viral and cellular membranes, cytoplasmic escape of the viral nucleocapsid, and subsequent infection. Mechanistic delineation of the indispensable GP<sub>CL</sub>-NPC1-binding step has been severely hampered by the unavailability of a robust cell-based assay assessing interaction of GP<sub>CL</sub> with full-length endosomal NPC1. Here, we describe a novel *in situ* assay to monitor GP<sub>CL</sub>-NPC1 engagement in intact, infected cells. Visualization of the subcellular localization of binding complexes is based on the principle of DNA-assisted, antibody-mediated proximity ligation. Virus-receptor binding monitored by proximity ligation was contingent on GP's proteolytic cleavage and was sensitive to perturbations in the GP<sub>CL</sub>-NPC1 interface. Our assay also specifically decoupled detection of virus-receptor binding from steps post-receptor binding, such as membrane fusion and infection. Testing of multiple FDA-approved small-molecule inhibitors revealed that drug treatments inhibited virus entry and GP<sub>CL</sub>-NPC1 recognition by distinctive mechanisms. Together, here we present a newly established proximity ligation assay, which will allow us to dissect cellular and viral requirements for filovirus-receptor binding and to delineate the mechanisms of action of inhibitors on filovirus entry in a cell-based system.

**IMPORTANCE** Ebola virus causes episodic but increasingly frequent outbreaks of severe disease in Middle Africa, as shown by the recently overcome second largest outbreak on record in the Democratic Republic of Congo. Despite considerable effort, FDA-approved anti-filoviral therapeutics or targeted interventions are not available yet. Virus host-cell invasion represents an attractive target for antivirals; however, our understanding of the inhibitory mechanisms of novel therapeutics is often hampered by fragmented knowledge of the filovirus-host molecular interactions required for viral infection. To help close this critical knowledge gap, here, we report an *in situ* assay to monitor binding of the EBOV glycoprotein to its receptor NPC1 in intact, infected cells. We demonstrate that our *in situ* assay based on proximity ligation represents a powerful tool to delineate receptor-viral glycoprotein interactions. Similar assays can be utilized to examine receptor interactions of diverse viral surface proteins whose studies have been hampered until now by the lack of robust *in situ* assays.

**KEYWORDS** NPC1, Ebola virus, glycoprotein, inhibitors, proximity ligation, virus entry

Members of the family *Filoviridae*, including Ebola virus (EBOV), are emerging zoonotic pathogens that cause episodic but increasingly frequent outbreaks of a highly lethal disease in Middle Africa (1). The two largest outbreaks of EBOV disease on record, from 2014 to 2016 in Liberia, Guinea, and Sierra Leone and from 2018 to 2020

**Citation** Mittler E, Alkutkar T, Jangra RK, Chandran K. 2021. Direct intracellular visualization of Ebola virus-receptor interaction by *in situ* proximity ligation. *mBio* 12:e03100-20. <https://doi.org/10.1128/mBio.03100-20>.

**Editor** Carolyn B. Coyne, University of Pittsburgh School of Medicine

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**Received** 11 November 2020

**Accepted** 16 November 2020

**Published** 12 January 2021

in the Democratic Republic of Congo, which both included spillover cases to neighboring countries, highlight the potential of filoviruses to cause health emergencies of international scope (51). There is an urgent need for effective countermeasures; however, their development is hindered by our limited understanding of filovirus-host molecular interactions required for viral entry and infection.

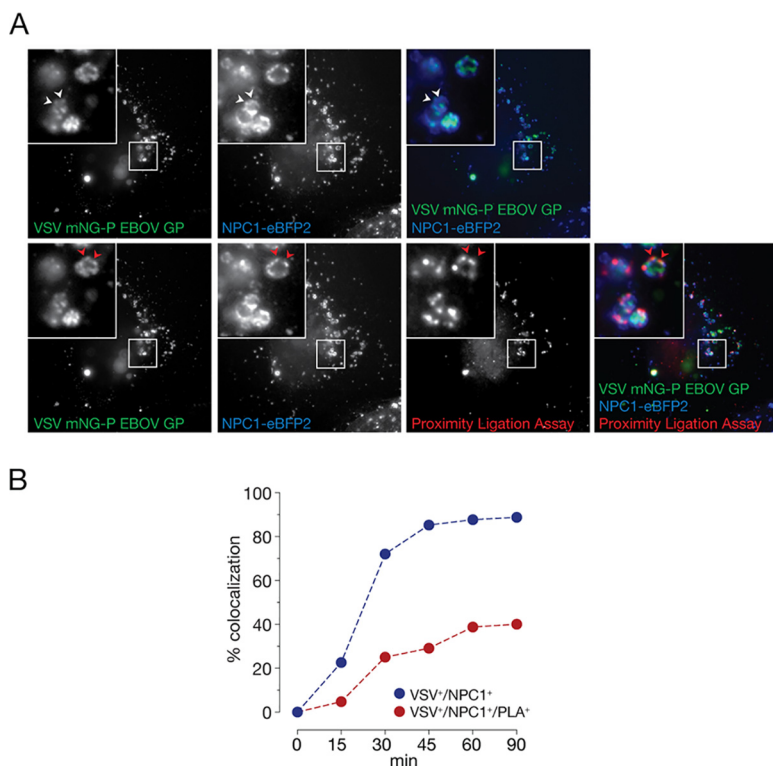
The single filovirus-encoded spike glycoprotein (GP) is necessary and sufficient to mediate all steps in viral entry into host cells, culminating in cytoplasmic escape of the viral nucleocapsid. Following endocytosis, virions traffic to late endosomes/lysosomes (LE/LY) (2–5), where GP gains access to multiple essential host factors. GP is proteolytically processed by endosomal cysteine cathepsins B and L (CatB and CatL, respectively), which remove the heavily glycosylated C-terminal glycan cap and mucin domain sequences in the GP<sub>1</sub> subunit (6–8), thereby exposing a recessed receptor-binding site (RBS). This cleaved GP species (GP<sub>CL</sub>) binds domain C of Niemann-Pick C1 (NPC1), a ubiquitously expressed cholesterol transporter embedded in endo-/lysosomal membranes that acts as a universal intracellular receptor for all filoviruses (9–13). Although GP<sub>CL</sub>-NPC1 recognition is a prerequisite for downstream steps in virus entry and infection, *in vitro* work suggests that NPC1 binding is not sufficient to trigger large-scale conformational changes in GP or to initiate a subsequent merger of viral and host membranes (14, 15). Indeed, NPC1's precise role beyond GP binding, which presumably provides a physical link between virus particles and host membranes, remains elusive to date.

Because a robust cell-based assay assessing the interaction of GP<sub>CL</sub> with full-length endosomal NPC1 in its native context has been unavailable, mechanistic studies of this indispensable virus-receptor interaction have been largely limited to *in vitro* assays. These assays are predominantly based on a truncated, soluble form of a single domain in NPC1, domain C, as well as on *in vitro*-cleaved GPs (10, 11). However, studies with NPC1-targeting inhibitors suggest that these assays do not fully recapitulate the authentic interaction(s) between *in situ*-cleaved GP and full-length NPC1 in its membrane context within late endosomes and/or lysosomes (10, 15). To address this gap, we describe herein an *in situ* assay to monitor GP<sub>CL</sub>-NPC1 binding in individual endosomal compartments of intact, infected cells by using DNA-guided, antibody-mediated proximity ligation. We employed this assay to show that GP<sub>CL</sub>-NPC1 interaction is restricted to the lumina of NPC1-positive (NPC1<sup>+</sup>) LE/LY, is contingent on the proteolytic cleavage of GP, and is sensitive to the mutational disruption of the GP<sub>CL</sub>-NPC1 interface. Testing of multiple FDA-approved small-molecule inhibitors in our assay revealed that drug treatments inhibited virus entry and GP<sub>CL</sub>-NPC1 recognition by distinct mechanisms. Application of this assay will allow us to dissect the cellular and viral requirements for filovirus-receptor interaction and to delineate the mechanisms of action of small-molecule inhibitors on filovirus entry.

## RESULTS

**Development of an assay visualizing EBOV GP-NPC1 binding in intact cells by *in situ* proximity ligation.** During viral entry, proteolytically cleaved forms of EBOV GP (GP<sub>CL</sub>) interact with their critical endosomal receptor NPC1. We postulated that an *in situ* proximity ligation assay (PLA) could be used to monitor this essential binding step in intact cells. To detect viral particles, we used a recombinant vesicular stomatitis virus (rVSV) containing the viral phosphoprotein P linked to a fluorescent monomeric NeonGreen (mNG-P) protein and bearing EBOV GP (16). Viral particles were allowed to attach at 4°C to U2OS human osteosarcoma cells stably expressing NPC1 tagged with a blue fluorophore, eBFP2 (16), and the cells were then shifted to 37°C to allow synchronized viral internalization. Visualization of fixed U2OS<sup>NPC1-eBFP2</sup> cells by fluorescence microscopy revealed that most internalized viral particles reached NPC1-containing late endosomes (NPC1<sup>+</sup> LE) within 60 min (Fig. 1).

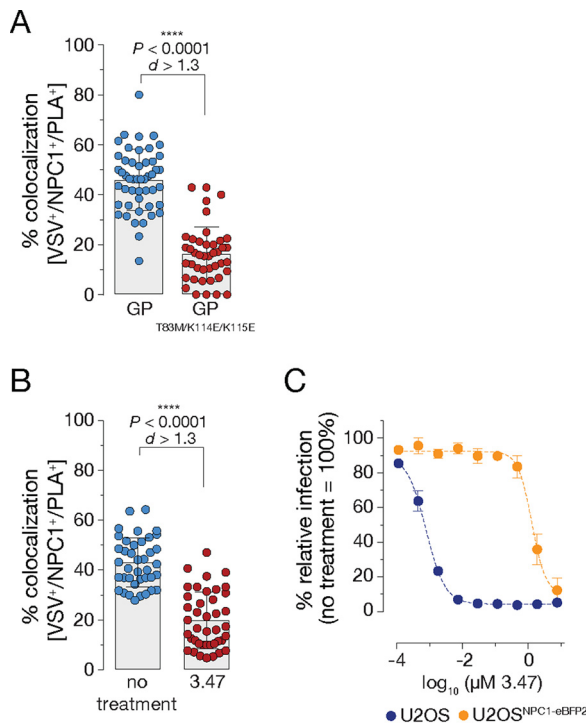
To detect closely apposed GP<sub>CL</sub> and NPC1 molecules in infected cells, we incubated them with oligonucleotide-linked monoclonal antibodies directed against each protein; GP's highly conserved receptor-binding site (RBS), unmasked by proteolytic cleavage to GP<sub>CL</sub> in endosomes, was detected with the RBS-specific antibody MR72 (16–18),



**FIG 1** Development of an EBOV GP-NPC1 binding assay in intact cells by *in situ* proximity ligation. (A) VSV mNG-P particles bearing EBOV GP were internalized into U2OS cells ectopically expressing NPC1-eBFP2 for 60 min. Cells were fixed, permeabilized, and subjected to proximity ligation assay (PLA) using GP<sub>CL</sub>- and NPC1-specific antibodies (MR72/MAb-548). During amplification, resulting PLA products were labeled with a detector oligonucleotide conjugated with a red fluorophore. White arrowheads, VSV/NPC1 colocalization; red arrowheads, VSV/NPC1/PLA colocalization. (B) VSVs bearing EBOV GP were endocytosed into U2OS cells as described in panel A, fixed at the indicated time points, and subjected to PLA. VSV-positive compartments (VSV<sup>+</sup>) were enumerated; the percentages of compartments also positive for NPC1 (VSV<sup>+</sup>/NPC1<sup>+</sup>) or NPC1 plus PLA (VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup>) were determined. Averages of pooled cells out of two independent experiments are shown ( $n \geq 30$  per time point).

and NPC1 was detected with the domain C-specific monoclonal antibody MAb-548 (16). Based on the rough size of the oligonucleotide-linked MABs used, we estimate that the maximum distance between GP<sub>CL</sub> and NPC1 molecules amounts to approximately 20 nm (19). Circular DNA molecules were generated by oligonucleotide-guided proximity ligation, amplified *in situ*, and visualized with a fluorophore-conjugated detector oligonucleotide (20, 21). The PLA signal required the addition of both MR72 and MAb-548 antibodies and was only observed in cells that were allowed to internalize viral particles (Fig. S1 in the supplemental material). Also, the PLA signal colocalized with VSV mNG-P particles bearing EBOV GP in the lumina of NPC1<sup>+</sup> LE (Fig. 1A). Proximity ligation and viral trafficking to NPC1<sup>+</sup> LE displayed similar kinetics; both peaked within 60 min post-viral uptake, followed by a plateau phase (Fig. 1A and B). Although most internalized viral particles trafficked to and colocalized with NPC1<sup>+</sup> compartments (Fig. 1B), only a subset of these VSV<sup>+</sup>/NPC1<sup>+</sup> vesicles also displayed a PLA signal (Fig. 1B). Accordingly, in the following studies aimed at determining the viral and cellular requirements for proximity-dependent ligation of GP<sub>CL</sub>- and NPC1-specific antibodies, we routinely enumerated the number of VSV<sup>+</sup>/NPC1<sup>+</sup> compartments per cell and reported the percentage of these compartments that were also positive for PLA signal (VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup>).

**Proximity ligation is sensitive to perturbations in the GP<sub>CL</sub>-NPC1 domain C interface.** Specific interactions of EBOV GP with NPC1 domain C have been mapped to amino acid residues in the GP<sub>1</sub> subunit that are exposed upon proteolytic cleavage (10,



**FIG 2** Proximity ligation is sensitive to perturbations in the GP<sub>CL</sub>-NPC1 domain C interface. (A) VSV mNG-P particles bearing EBOV GP or EBOV GP<sup>T83M/K114E/K115E</sup> were internalized into U2OS<sup>NPC1-eBFP2</sup> cells for 60 min followed by cell fixation, permeabilization, and PLA. Cells were analyzed by fluorescence microscopy; data points represent the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell; bars depict the pooled averages and standard deviations ( $\pm$  SD) for all cells from two independent experiments ( $n \geq 40$ ). An unpaired two-tailed *t* test was used to compare VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments of cells infected by VSV bearing either EBOV GP or EBOV GP<sup>T83M/K114E/K115E</sup> (\*\*\*\*,  $P < 0.0001$ ). Group means calculated from the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles were compared by Cohen's *d* effect size ( $d > 1.3$ ). (B) U2OS<sup>NPC1-eBFP2</sup> cells were preincubated with the inhibitor 3.47 (1  $\mu\text{M}$ ) for 60 min at 37°C followed by VSV mNG-P EBOV GP uptake for 60 min and PLA. Data points were acquired and analyzed as described in panel A. (C) After preincubation of U2OS<sup>NPC1-eBFP2</sup> and wild-type U2OS cells with increasing 3.47 concentrations, cells were infected with VSV mNG-P EBOV GP for 16 h. Infection was measured by automated counting of mNG<sup>+</sup> cells and normalized to infection obtained in the absence of 3.47. Averages  $\pm$  SD for six technical replicates pooled from two independent experiments are displayed. Data were subjected to nonlinear regression analysis to derive 3.47 concentration at half-maximal inhibition of infection ( $\text{IC}_{50} \pm 95\%$  confidence intervals for nonlinear curve fit).

17, 22). Mutations of the charged surface-exposed amino acids K114 and K115 and the polar amino acid T83 led to significant defects in NPC1 binding and viral entry (4, 17). To determine if these mutations impact proximity ligation, we exposed cells to VSVs bearing GP<sup>T83M/K114E/K115E</sup>. Virions were efficiently trafficked to NPC1<sup>+</sup> LE and underwent GP<sub>1</sub> cleavage, as indicated by detection of the GP<sub>1</sub> RBS with the MAb MR72. However, viral infectivity was greatly diminished (Fig. S2A and B). Concordantly, we observed a significant reduction of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> colocalization, suggesting that GP<sub>CL</sub>-NPC1 engagement in LE is necessary for PLA signal formation (Fig. 2A).

The small-molecule filovirus entry inhibitor 3.47 was described to block EBOV GP-dependent entry and infection potentially by interfering with GP<sub>CL</sub>-NPC1 recognition (11). Accordingly, we directly compared the effect of 3.47 on viral entry in wild-type U2OS cells expressing basal levels of NPC1 against that in U2OS<sup>NPC1-eBFP2</sup> cells used for the PLA (Fig. 2C). Consistent with its activity as an NPC1-targeting inhibitor, and as shown previously (11), 3.47's antiviral activity was substantially attenuated in cells over-expressing NPC1, but we were nevertheless able to identify a concentration (1  $\mu\text{M}$ ) that afforded  $\sim 50\%$  inhibition of viral entry; higher concentrations of 3.47 were cytotoxic. Pretreatment of U2OS<sup>NPC1-eBFP2</sup> cells with 3.47 at 1  $\mu\text{M}$  significantly reduced, but

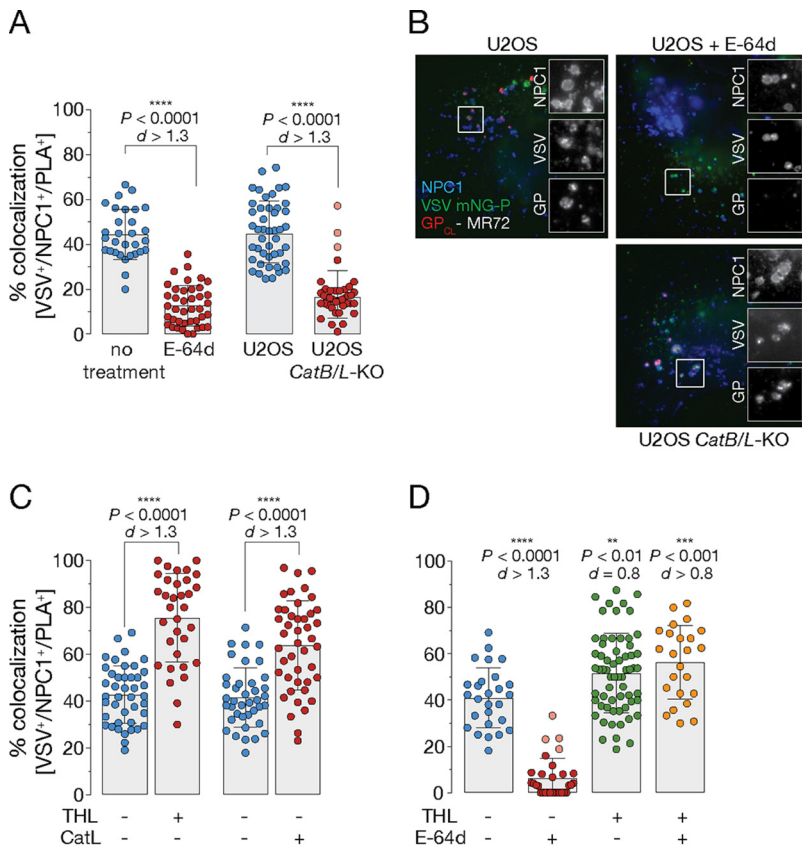
did not entirely block PLA signal; we suggest that the observed residual signal is based on that the applied 3.47 concentration is not sufficient to counteract cellular NPC1 entirely (Fig. 2B). However, the small-molecule incubation did not modify NPC1<sup>+</sup> LE morphology, block viral trafficking and GP cleavage, or influence detection by the assay antibodies, MR72 and MAb-548 (Fig. S2C). Our data that both genetic and pharmacological disruptions of the virus-receptor interface inhibit PLA strongly suggest that this assay indeed monitors GP<sub>CL</sub>-NPC1 domain C engagement in endosomal compartments.

#### Detection of GP<sub>CL</sub>-NPC1 binding by PLA requires endosomal cleavage of GP.

Endosomal host cysteine cathepsins B and L (CatB and CatL, respectively) are key mediators of the entry-related GP→GP<sub>CL</sub> cleavage that is required for GP<sub>CL</sub>-NPC1 binding and viral membrane fusion (4, 6–8). Their inactivation by pan-cysteine cathepsin inhibitors, including E-64 and E-64d, blocks filovirus entry (6, 7) (Fig. S3A). Concordantly, we found that pretreatment of cells with E-64d abolished both GP cleavage (and consequently exposure of the GP<sub>1</sub> NPC1-binding site recognized by MR72) (Fig. 3B) and GP<sub>CL</sub>-NPC1 engagement as measured by PLA (Fig. 3A). Intriguingly, previous work has also hinted at the existence of cellular target(s) of E-64 in addition to CatB and CatL, whose inhibition imposes one or more blocks to viral entry (6, 7, 23, 24). To further investigate the cysteine protease-dependent EBOV entry mechanism, we generated *CatB/L*-knockout (KO) U2OS cell lines by CRISPR/Cas9 genome engineering (Fig. S4A). As expected, the *CatB/L*-KO cells lacked CatB and CatL activity and were substantially resistant to EBOV GP-dependent entry (Fig. S4B and C). Surprisingly, however, and in contrast to our findings in E-64d treated cells, viral particles underwent efficient exposure of the NPC1-binding site (and MR72 epitope) in GP in *CatB/L*-KO cells (Fig. 3B). Despite this apparent capacity for GP cleavage in the absence of CatB and CatL, viral particles were nevertheless unable to engage NPC1 as measured by PLA (Fig. 3A).

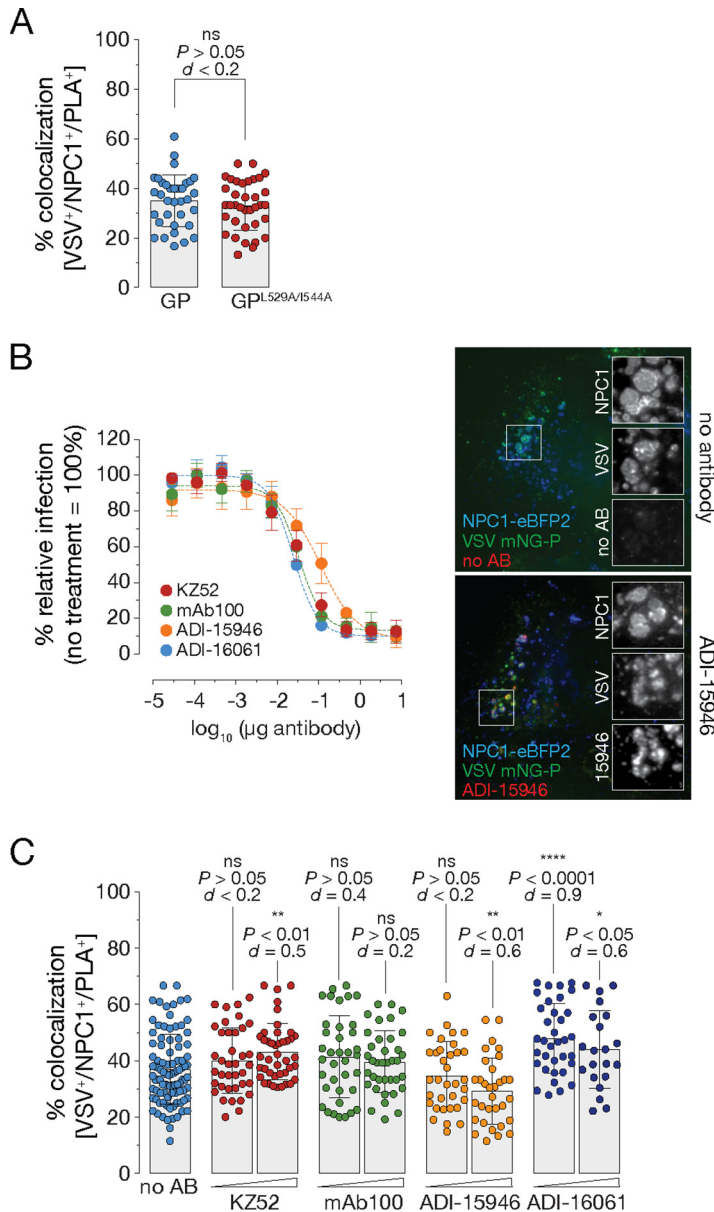
GP priming in viral entry can be recapitulated *in vitro* by incubating rVSV bearing full-length EBOV GP with CatL or the bacterial protease thermolysin (THL) which mimics CatL/B cleavage (7); both cleave off GP<sub>1</sub> sequences corresponding to the glycan cap and mucin domain. Increased infectivity of viral particles bearing THL/CatL-cleaved GPs was reported previously and can be attributed to improved cell binding of virions as well as increased accessibility of the GP's RBS for NPC1 domain C interaction (6, 10, 25) (Fig. S3B). *In vitro* cleavage of GP significantly enhanced GP<sub>CL</sub>-NPC1 domain C binding *in situ* by 1.5- to 2-fold, indicating that proteolytic processing of GP is indispensable for detection of virus-receptor interaction by proximity ligation (Fig. 3C). In order to determine possible requirements for additional cysteine protease activity pre-NPC1 binding, we evaluated proximity ligation with THL-cleaved GP in the presence of the pan-cysteine cathepsin inhibitor E-64d. Here, the absence of cysteine protease proteolytic activity did not affect GP<sub>CL</sub>-NPC1 engagement (Fig. 3D), suggesting the existence of an E-64d-sensitive downstream entry block post-NPC1 binding, as proposed previously (4, 23).

***In situ* proximity ligation decouples GP<sub>CL</sub>-NPC1 interaction from post-binding entry steps.** As one of the critical steps in viral entry, GP-NPC1 interaction is the starting point for subsequent post-binding entry processes, including, among others, membrane fusion triggering, cytoplasmic nucleocapsid escape, and infection (4, 9–11). To examine if the established *in situ* PLA selectively monitored GP<sub>CL</sub>-NPC1 engagement or, in addition, also downstream steps such as membrane fusion, we made use of an EBOV GP mutant harboring amino acid substitutions in the GP<sub>1</sub> internal fusion loop, GP<sup>L529A/I544A</sup>. Amino acids L529 and I544 were shown to form a fusogenic hydrophobic surface at the tip of the fusion loop and proposed to be crucial for insertion of the loop into host membranes and subsequent membrane fusion steps (26, 27). Late-endosomal delivery of VSV bearing GP<sup>L529A/I544A</sup> and exposure of GP's RBS did not differ substantially from those of VSV harboring wild-type GP. However, steps post NPC1-binding, including membrane fusion triggering and virus infection, were inhibited (4) (Fig. S5A). Consistent with these observations, *in situ* proximity ligation of VSV bearing GP<sup>L529A/I544A</sup> resembled that obtained with VSV decorated with wild-type GP (Fig. 4A).



**FIG 3** Detection of GP<sub>CL</sub>-NPC1 binding by PLA requires endosomal cleavage of GP. (A) VSV mNG-P particles bearing EBOV GP were incubated with either U2OS<sup>NPC1-eBFP2</sup> cells pretreated with E-64d (100 mM for 6 h at 37°C) (left) or U2OS *CatB/L*-KO cells ectopically overexpressing Flag-tagged NPC1 (1 h at 37°C), permeabilized, and subjected to PLA. Data points represent the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell; bars depict the average  $\pm$  SD for all data points pooled from two independent experiments ( $n \geq 30$ ). Points with reduced transparency represent values outside the 10th to 90th percentiles. An unpaired two-tailed *t* test was used to compare VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments of wild-type cells with either inhibitor-treated (left) or KO (right) cells infected by EBOV GP-decorated VSV (\*\*\*\*,  $P < 0.0001$ ). Group means calculated from the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles were compared by Cohen's *d* effect size. (B) Cells described in panel A were exposed to VSV mNG-P EBOV GP uptake for 1 h at 37°C. After fixation, viral particles, NPC1, and GP<sub>CL</sub> were visualized by fluorescence microscopy. Representative images from two independent experiments are shown. (C) VSV mNG-P particles bearing EBOV GP were treated *in vitro* with thermolysin (THL) or cathepsin L (CatL), respectively. Viral particles were taken up into U2OS<sup>NPC1-eBFP2</sup> cells followed by fixing of the cells and subjecting them to PLA. Data points represent the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell; bars depict the average  $\pm$  SD for all data points pooled from two independent experiments ( $n \geq 25$ ). To compare VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments of cells which endocytosed VSV studded with either uncleaved or THL/CatL-cleaved GP, an unpaired two-tailed *t* test was used (\*\*\*\*,  $P < 0.0001$ ). Cohen's *d* effect size was used to compare the group means calculated from the percentages of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles ( $d > 1.3$ ). (D) U2OS<sup>NPC1-eBFP2</sup> cells were (not) preincubated with E-64d (as described in panel A), and VSV mNG-P particles bearing EBOV GP were treated *in vitro* with THL. Following virus internalization into U2OS<sup>NPC1-eBFP2</sup> cells, cells were fixed and subjected to PLA. Data points represent the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell; bars depict the average  $\pm$  SD for all data points pooled from two independent experiments ( $n \geq 25$ ). Points with reduced transparency represent values outside the 10th to 90th percentiles. To compare VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments of E-64d-treated cells with those of untreated cells which endocytosed VSV studded with either uncleaved or THL-cleaved GP, an unpaired two-tailed *t* test was used (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ). Cohen's *d* effect size was used to compare the group means calculated from the percentages of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles.

Previous work identified a variety of antibodies with neutralizing activity against EBOV. Those displaying exceptional neutralizing potency were described to target conformational epitopes located in the base subdomain of the GP trimeric complex (28–32). It was proposed that these base-binding antibodies efficiently block fusogenic



**FIG 4** *In situ* proximity ligation decouples GP<sub>cl</sub>-NPC1 interaction from post-binding entry steps. (A) VSV mNG-P particles bearing EBOV GP or EBOV GP<sup>L529A/1544A</sup> were internalized into U2OS<sup>NPC1-eBFP2</sup> cells for 60 min followed by PLA. Data points represent the percentage of triple-positive compartments per individual cell; bars depict the average ± SD for all data points pooled from two independent experiments ( $n \geq 37$ ). Data analyses included an unpaired two-tailed *t* test to compare VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles of cells which internalized VSV decorated with wild-type or mutant GP (ns,  $P > 0.05$ ). Cohen's *d* effect size was used to compare the group means calculated from the percentages of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments. (B) ADI-15946 was incubated with VSV EBOV GP particles and exposed to U2OS<sup>NPC1-eBFP2</sup> (right). After virus uptake for 1 h at 37°C, cells were fixed, and viral particles, NPC1, and bound antibodies were visualized by fluorescence microscopy. Representative images from two independent experiments are shown. Virions were preincubated with increasing amounts of KZ52, mAb100, ADI-15946, or ADI-16061 and then exposed to U2OS<sup>NPC1-eBFP2</sup> cells for 16 h at 37°C (left). The number of infected cells was determined by automated counting of mNG<sup>+</sup> cells and normalized to infection obtained in the absence of antibodies. Averages ± SD for six technical replicates pooled from two independent experiments are shown. (C) VSV mNG-P EBOV GP virions were complexed with KZ52, mAb100, ADI-15946, or ADI-16061 (50 μg/ml and 100 μg/ml) for 1 h at room temperature. Following internalization into U2OS<sup>NPC1-eBFP2</sup> cells, cells were fixed and subjected to *in situ* PLA. Data points represent the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell; bars show the average ± SD for all data points pooled from two independent experiments ( $n \geq 22$ ). VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles were analyzed by unpaired two-tailed *t* test (ns,  $P > 0.05$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ ) comparing cells which were exposed to virion-antibody complexes to cells exposed to untreated virus. Group means calculated from the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles were also compared by Cohen's *d* effect size.

rearrangements of GP, thereby inhibiting viral entry steps downstream of NPC1 binding. We used these neutralizing antibodies to further investigate if *in situ* PLA monitored GP<sub>CL</sub>-NPC1 binding specifically or also integrated post-receptor binding events (e.g., viral membrane fusion). We tested antibodies targeting the GP<sub>1</sub>-GP<sub>2</sub> interface (KZ52, mAb100), the GP<sub>1</sub>-GP<sub>2</sub> interface plus the glycan cap (ADI-15946), or a subdomain of the fusion loop, the heptad repeat 2 (ADI-16061). Preincubation of VSVs with these neutralizing antibodies (50 µg/ml) afforded delivery of virus-antibody complexes to NPC1<sup>+</sup> LE but did not hinder virus trafficking to LEs itself or proteolytic processing to GP<sub>CL</sub> (Fig. 4B, right, and Fig. S5B and C). However, infection was blocked under these conditions, as expected (Fig. 4B, left). Despite their potent neutralizing activity, and consistent with the specified target epitopes as well as previously reported *in vitro* NPC1-binding studies (28–32), base-binding antibodies mAb100 and ADI-15946 had little or no effect on *in situ* PLA, consistent with our hypothesis that this assay selectively detects GP<sub>CL</sub>-NPC1 complex formation (Fig. 4C). Surprisingly, incubation with KZ52 and ADI-16061 slightly improved the PLA signal, possibly because they enhanced an upstream step, the delivery of VSV bearing GP<sub>CL</sub> to NPC1<sup>+</sup> LE (Fig. 4C).

Collectively, our results provide strong evidence that the *in situ* PLA specifically monitors GP<sub>CL</sub>-NPC1 binding in infected cells and decouples the detection of virus-receptor binding from post-receptor binding steps in viral entry.

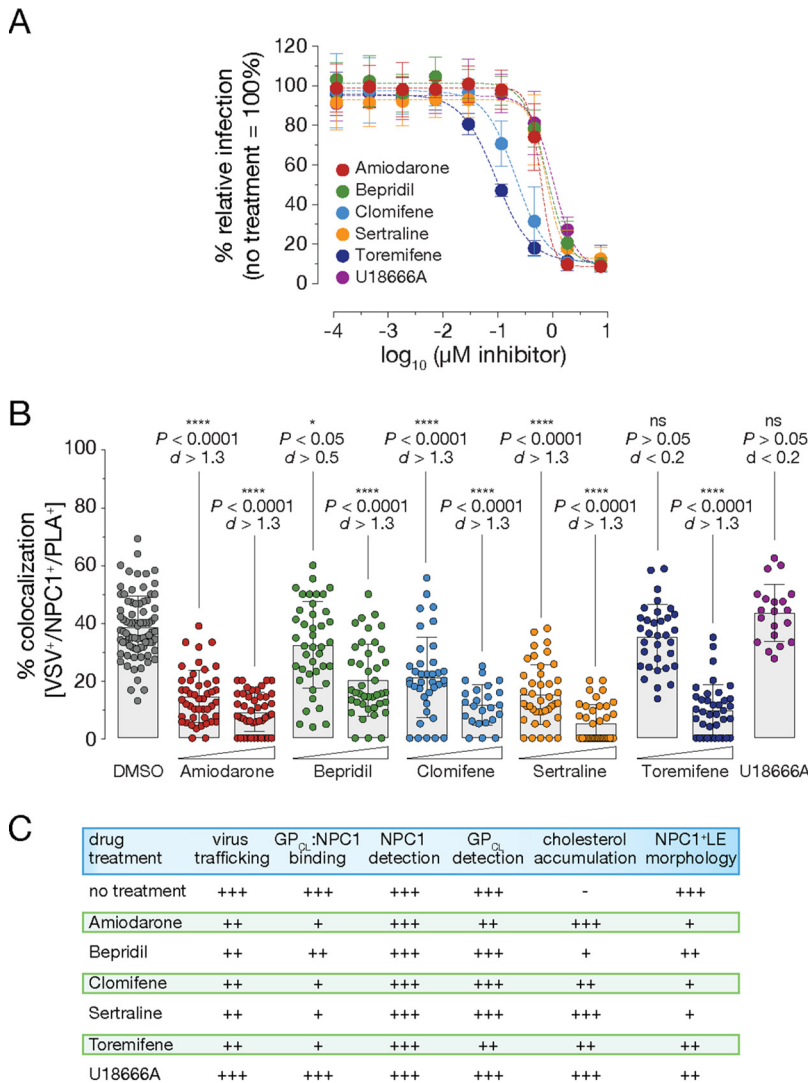
**Small-molecule inhibitor-mediated selective interference of GP-NPC1 binding delineated by *in situ* PLA.** The unprecedented EBOV outbreak in West Africa from 2013 to 2016 uncovered a pressing need for anti-EBOV therapeutics; since then, numerous studies screened for and characterized FDA-approved drugs with anti-filoviral activity. The most promising drug candidates, amiodarone, bepridil, clomifene, sertraline, and toremifene, were reported to block one or several steps during EBOV entry (15, 33–39). As their precise mechanisms of anti-EBOV activity still remain elusive, we examined the capacity of these inhibitors to alter intracellular GP<sub>CL</sub>-NPC1 interaction in the PLA. As a control, we also tested the well-characterized amphiphilic drug U18666A, which was described to block cholesterol export from lysosomes and shown to inhibit EBOV entry at higher concentrations, but not to impact GP<sub>CL</sub>-NPC1 binding *in vitro* (9, 15, 40).

We first titrated the drugs in an EBOV entry assay in wild-type U2OS and U2OS<sup>NPC1-eBFP2</sup> cells overexpressing NPC1 employed in the PLA (Fig. 5A and Fig. S6A). Several of the inhibitors were less potent in NPC1-overexpressing cells than in wild-type cells, as reported previously, suggesting they act via the GP<sub>CL</sub>-NPC1 axis (15) (Fig. S6A). Some inhibitors hampered VSV trafficking to NPC1<sup>+</sup> LE, whereas others had little or no effect on viral delivery to NPC1<sup>+</sup> LE compared to a dimethyl sulfoxide (DMSO)-treated control (Fig. S6B). Because these trafficking defects likely contribute to the observed inhibition in infection, we sought to discount these trafficking effects on GP<sub>CL</sub>-NPC1 binding by focusing exclusively on PLA activity in VSV<sup>+</sup>/NPC1<sup>+</sup> compartments. Importantly, these inhibitors exhibited little or no effect on GP cleavage (detected by MR72) or the accessibility of NPC1 domain C (detected by MA5-548) (Fig. 5C; data not shown).

Screening the inhibitory compounds via PLA revealed that amiodarone, bepridil, clomifene, sertraline, and toremifene all significantly interfered with GP<sub>CL</sub>-NPC1 interaction compared to a DMSO-treated control (Fig. 5B). In contrast, but consistent with previous *in vitro* data, U18666A did not block GP<sub>CL</sub>-NPC1 binding (Fig. 5B). The drug treatments also substantially modified NPC1 trafficking to LE and/or LE localization; we observed pronounced imbalances in NPC1 distribution, illustrated by LE vesicles with extensively elevated or decreased NPC1 levels accumulating in the perinuclear regions of cells (Fig. 5C and Fig. S6C, bottom). Further, NPC1's transporter activity was strongly impaired; although cells did not exhibit cholesterol accumulation in NPC1<sup>+</sup> LE following the short-term drug treatments carried out prior to PLA measurements, prolonged inhibitor incubations (16 h instead of 1 h of incubation prior to PLA) clearly blocked cholesterol clearance from LE (Fig. 5C and Fig. S6C, top).

In summary, our results imply that a number of FDA-approved small-molecule inhibitors interfere with GP<sub>CL</sub>-NPC1 engagement, and such block downstream viral





**FIG 5** Small-molecule inhibitor-mediated selective interference of GP-NPC1 binding delineated by *in situ* PLA. (A) U2OS<sup>NPC1-eBFP2</sup> cells were preincubated with increasing concentrations of amiodarone, bepridil, clomifene, sertraline, toremifene, and U18666A, respectively, before exposure to VSV mNG-P EBOV GP for 16 h at 37°C. Infection was measured by automated counting of mNG<sup>+</sup> cells and normalized to infection obtained in the presence of vehicle only. Averages ± SD for 9 to 18 technical replicates pooled from 3 to 6 independent experiments are displayed. (B) U2OS<sup>NPC1-eBFP2</sup> cells were incubated with amiodarone, bepridil, clomifene, and sertraline, respectively (2 µM or 5 µM, 1 h at 37°C), toremifene (2 µM or 10 µM, 1 h at 37°C), or U18666A (10 µM, 2 h at 37°C), followed by VSV mNG-P EBOV GP uptake for 1 h. Cells were subjected to *in situ* PLA and analyzed by fluorescence microscopy. The percentage of triple-positive VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments per individual cell is represented by data points; bars show the average ± SD for all data points pooled from two independent experiments ( $n \geq 20$ ). VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> vesicles were analyzed by unpaired two-tailed *t* test (ns,  $P > 0.05$ ; \*,  $P < 0.05$ ; \*\*\*\*,  $P < 0.0001$ ) comparing inhibitor-exposed to untreated cells. Group means calculated from the percentage of VSV<sup>+</sup>/NPC1<sup>+</sup>/PLA<sup>+</sup> compartments were also compared by Cohen's *d* effect size. (C) Table summarizing results from panel B and Fig. S6 in the supplemental material. Data for “virus trafficking” can be found in Fig. S6B, data for “GP<sub>CL</sub>-NPC1 binding” are shown in panel B, data for “NPC1 detection” and “GP<sub>CL</sub> detection” are not shown, exemplary images for “cholesterol accumulation” are depicted in Fig. S6C, top, and exemplary images for “NPC1<sup>+</sup> LE morphology” are shown in Fig. S6C, bottom.

entry steps. However, our findings and previously published work indicate that the inhibitory mode of action of these compounds is likely multifactorial. Although our data suggest that some of the compounds directly affect GP<sub>CL</sub>-NPC1 interaction in endosomal compartments during entry, they also uncover contributions from drug-induced changes in the morphology and distribution of NPC1<sup>+</sup> compartments.

## DISCUSSION

Interaction of the filovirus spike protein GP with its universal intracellular receptor NPC1 is indispensable for viral entry and infection (9–13). However, our understanding of the mechanism of GP-NPC1 recognition is largely derived from *in vitro* assays that use detergent-extracted NPC1 or a soluble form of the GP-interacting domain C of NPC1 together with *in vitro*-cleaved GP<sub>CL</sub> (10, 11). Although powerful, these assays likely do not fully recapitulate the authentic virus-receptor interaction in late endo-/lysosomal compartments. As a case in point, a number of small-molecule entry inhibitors that appear to target NPC1 in cells do not block GP<sub>CL</sub>-NPC1 domain C interaction *in vitro*, leaving open questions about their mechanism of action (15).

Here, we describe a novel assay that monitors the GP-NPC1 interaction in intact cells. Detection of virus-receptor complexes *in situ* is based on the principle of DNA-assisted, antibody-mediated proximity ligation (20, 21). Several studies have used PLAs to detect interaction of virus particles with host proteins at the plasma membrane (41), in the cytoplasm (42, 43), and in early endosomes (44). To our knowledge, our assay represents the first example to afford visualization of protein-protein interactions in late endosomal/lysosomal compartments.

Previous studies used *in vitro* protease treatments in conjunction with infectivity measurements in protease inhibitor-treated or genetically engineered cells to establish a requirement for GP cleavage in EBOV entry (6–8). Further, *in vitro* binding studies with soluble NPC1 domains demonstrated that GP cleavage is required for virus-receptor interaction (9–13). Here, we used the PLA to directly investigate the role of GP proteolytic cleavage in GP-NPC1 interaction within cellular endo-/lysosomal compartments. Concordant with our current understanding, we found that blockade of all endosomal cysteine cathepsins with the pan-cysteine cathepsin inhibitor E-64d abrogated both GP cleavage and GP-NPC1 association *in situ* and that *in vitro* pre-cleavage of EBOV GP to GP<sub>CL</sub> significantly boosted this interaction. We also observed that genetic knockout of the two key cysteine cathepsins identified previously, CatB and CatL, substantially inhibited GP-NPC1 association as measured by PLA. Unexpectedly, knocking out *CatB* and *CatL* did not prevent exposure of the RBS in endosomes, as judged by the continued capacity of RBS-specific MAb MR72 to detect viral particles. Thus, one or more non-CatB/CatL cysteine cathepsins appear to at least partially cleave GP in endosomes in a manner that, however, does not permit stable GP-NPC1 association. Alternatively, it is possible that CatB and/or CatL mediate additional yet undiscovered cleavage events that are required for GP-NPC1 binding.

Similar to published *in vitro* GP-NPC1 binding assays, our *in situ* PLA could also specifically detect GP-NPC1 interactions independent of downstream post-binding steps in entry, allowing us to focus on molecular mechanisms underlying the receptor-binding step. We employed fusion-blocking GP mutants and neutralizing antibodies targeting epitopes essential for GP's conformational rearrangements during membrane fusion; both interventions, however, left GP<sub>CL</sub>-NPC1 binding measured by PLA largely unaffected. Previous publications suggested a dual mechanism of action for several of these antibodies, KZ52, mAb100, and ADI-15946 (28, 30); to block EBOV GP-mediated infection, they hampered post-NPC1-binding steps and interfered with GP's proteolytic processing *in vitro*. Surprisingly, the latter *in vitro* findings were not recapitulated by the *in situ* PLA. Our results suggest that in the presence of neutralizing antibodies, the CatB/L cleavage sites located in GP's  $\beta$ 13- $\beta$ 14 loop were readily accessible in an *in situ* endosomal environment, while they did not appear to be in the *in vitro* cell-free system. Potential minor changes in the GP cleavage efficiency cannot be monitored via the RBS-specific MAb MR72; however, our PLA results suggest that the level of GP cleavage was sufficient to allow efficient NPC1 binding. Further, our experiments suggest that *in vitro* cleavage studies do not fully recapitulate the authentic interactions

within the trilectin of endosomal proteases, virus glycoproteins, and GP-targeting MABs in an acidic endosomal environment.

Lastly, by examining the effect of small-molecule inhibitors on GP<sub>CL</sub>-NPC1 binding, we further unraveled their multifaceted mechanisms of inhibitory action toward EBOV GP-mediated infection. All FDA-approved drugs tested, amiodarone, bepridil, clomifene, sertraline, and toremifene, significantly hampered GP<sub>CL</sub>-NPC1 interaction measured by *in situ* PLA. Previous reports focused on bepridil, sertraline, and toremifene, which were proposed to destabilize GP's prefusion conformation by binding into a cavity at the GP<sub>1</sub>-GP<sub>2</sub> interface *in silico* and *in vitro* (35, 38, 39). Our data also indicated changes in the intracellular distribution and morphology of NPC1<sup>+</sup> LE/LY, as well as NPC1's cholesterol transporter function following drug incubations. This was most notable for toremifene, clomifene, and sertraline, suggesting that they (in addition) also acted via the GP<sub>CL</sub>-NPC1 axis; overexpression of NPC1 clearly counteracted their inhibitory mechanism during infection. We propose that the loss of GP<sub>CL</sub>-NPC1 binding is most likely caused by adverse effects on both NPC1 and GP, leading to destabilization of GP and drastic modifications in the NPC1<sup>+</sup> LE/LY phenotype. Resolving the effects of small-molecule inhibitors on GP<sub>CL</sub>-NPC1 binding remains the focus of ongoing work and will help us to decode this interaction in more detail.

In summary, the newly established *in situ* proximity ligation assay provides a powerful new tool to delineate molecular mechanisms underlying receptor-filoviral glycoprotein interactions, to characterize host factors modulating EBOV entry, to unravel the mode of action of antibodies and small-molecule inhibitors, and to explore yet unidentified host proteins and their ability to affect formation of the multivalent GP<sub>CL</sub>-NPC1 interface in a cell-based system. In principle, it is also feasible that unknown host factors within the endosomal milieu might participate in establishing GP<sub>CL</sub>-NPC1 binding; we suggest that our *in situ* proximity ligation assay may aid in the identification of such entry host factors.

## MATERIALS AND METHODS

**Cells and viruses.** Human osteosarcoma U2OS cells were cultured in modified McCoy's 5A medium (Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin-streptomycin (Life Technologies), and 1% GlutaMax (Life Technologies). Cells were kept at 37°C with 5% CO<sub>2</sub> in a humidified incubator. U2OS cells stably overexpressing NPC1-eBFP2 were generated as described previously and maintained under the same conditions mentioned above (16). Propagation of recombinant vesicular stomatitis Indiana virus (rVSV) expressing enhanced green fluorescent protein (eGFP) in the first position and bearing the VSV G or EBOV GP glycoprotein, derived from EBOV/H.sap/COD/76/Yam-Mayinga (EBOV "Mayinga" isolate) as well as an rVSV bearing the EBOV GP glycoprotein, and a monomeric NeonGreen-phosphoprotein P (mNG-P) fusion protein has been described previously (4, 45–47). Pseudotyped VSVs bearing mNG-P and variant GPs, GP<sup>T83M/K114E/K115E</sup> and GP<sup>L529A/I544A</sup> (both also lacking the mucin-like domain), were prepared as previously reported (6, 24, 48).

For some experiments, cleaved viral particles bearing GP<sub>CL</sub> were first generated by incubation with thermolysin (THL; 1 mg/ml, pH 7.5, 37°C for 1 h; Sigma) or recombinant human cathepsin L (CatL, 2 ng/μl, pH 5.5, 37°C for 1 h; R&D Systems) as described previously (4). Reactions were stopped by removal onto ice and addition of phosphoramidon (1 mM; Peptides International) or E-64 (10 μM; Peptides International), respectively. While viral particles cleaved with CatL were used immediately, THL-cleaved virus was kept at –80°C until usage.

**Antibodies.** For immunofluorescence analysis, NPC1-eBFP2 was detected by a rabbit anti-BFP antibody (GeneTex) followed by a secondary anti-rabbit antibody -Alexa 405 or -Alexa 488 fluorophore (Thermo Scientific). During proximity ligation assay (PLA), NPC1 was detected by MAB-548, whose generation was described earlier (16). Detection of proteolytically cleaved GP was carried out with an RBS-specific human anti-GP antibody, MR72, either followed by a secondary anti-human antibody-Alexa 555 fluorophore or following the PLA protocol as described below. To detect EBOV GP by immunofluorescence analysis and to determine the effect of GP-targeting antibodies on GP<sub>CL</sub>-NPC1 interaction, we used the previously described human anti-GP antibodies KZ52, MAB100, ADI-15946, and ADI-16061 (28–32). For production of KZ52, ADI-15946, and ADI-16061, variable heavy- and light-chain domain sequences were cloned into the mammalian expression vectors pMAZ-IgL (encoding the expression cassette of human κ light-chain constant domains) and pMAZ-IgH (encoding the expression cassette of human γ1 chain constant domains). Antibody production in FreeStyle 293-F cells (Thermo Fisher) and subsequent purification were carried out as described earlier (16).

**Inhibitors.** Stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO) and stored as frozen aliquots until use. Cells were incubated with 3.47 (Microbiotix), amiodarone (Sigma), bepridil (Sigma), clomifene (Sigma), sertraline (Toronto Research Chemicals), or toremifene (Sigma) for 1 h or

16 h at 37°C with concentrations as indicated. Incubation of cells with E-64d (Peptides International) was extended to 6 h at 37°C, while cells were not preincubated with U18666A (Calbiochem) for PLA, but preincubated for 1 h or 16 h at 37°C for additional described experiments at concentrations indicated. Inhibitors were maintained at the same concentrations during virus spinoculation and virus entry into cells.

**Generation of U2OS *CatB/L* double-knockout cells overexpressing NPC1.** U2OS cells were transduced with a lentivirus carrying human codon-optimized *Streptomyces pyogenes* Cas9 (spCas9) and blasticidin resistance genes to generate U2OS-Cas9 cells expressing Cas9. Briefly, 293FT cells were cotransfected with Cas9-expressing plasmid lentiCas9-BLAST (Addgene plasmid no. 52962, a gift from Feng Zhang), the lentiviral packaging plasmid psPAX2 (Addgene plasmid no. 12260, a gift from Didier Trono), and a VSV G-expressing plasmid. The supernatant filtered through a 0.45- $\mu$ m filter was used to transduce U2OS cells in the presence of 6  $\mu$ g/ml of polybrene, and transduced cells were selected with 15  $\mu$ g/ml of blasticidin. A lentiGuide-Puro plasmid (Addgene plasmid no. 52963, a gift from Feng Zhang) expressing human *CatL*-targeting sgRNA (5'-CTTAGGGATGTCCACAAAGC-3'; targets anti-sense strand, nucleotides (nt) 1,021 to 1,040 of the *CatL* transcript variant 1 mRNA or nt 4,205 to 4,186 of *CatL*, NCBI gene ID 1514) was used to generate lentiviruses as described above. U2OS-Cas9 cells transduced with these lentiviruses were selected with 2  $\mu$ g/ml of puromycin. Editing of the *CatL* gene was confirmed by Sanger sequencing of a 673-bp amplicon using primers flanking the single guide RNA (sgRNA) target site. A single cell clone carrying a homozygous deletion of 15 nucleotides that is predicted to disrupt a critical N-glycosylation site (204-NDT-206) that is required for lysosomal targeting of CatL by causing a T206I change (along with a deletion of amino acids [aa] 207 to 210) was selected (49, 50). Absence of wild-type allele from the *CatL* knockout (KO) cells was confirmed by reverse transcriptase PCR (RT-PCR) using primers specific for the 15-nt deletion. To generate the *CatB/L* double KO, the *CatL*-KO cells were further transduced with a lentivirus encoding *CatB*-specific sgRNA (5'-TTGACCAGCTCATCCGACAG-3'; targets anti-sense strand, nt 251 to 270 of human *CatB* transcript variant 1 mRNA, nt 14,791 to 14,772 of human *CatB*, NCBI gene ID 1508). A single cell clone carrying an insertion of a single nucleotide (T) that is predicted to cause a frameshift leading to a truncated propeptide of 28 amino acids (frameshift at aa position 27) was selected. Absence of *CatB* and *CatL* activity in this single-cell clone was confirmed by cathepsin activity assays (described below). Stable U2OS cells expressing NPC1 C-terminally tagged with a triple flag sequence in the *CatB/L*-KO background were generated as described earlier. In short, retroviruses packaging the transgene were produced by triple transfection of 293T cells, and target U2OS cells were directly exposed to sterile-filtered retrovirus-laden supernatants in the presence of polybrene (6  $\mu$ g/ml). Transduced cell populations were selected with puromycin (2  $\mu$ g/ml), and expression of Flag-tagged NPC1 was confirmed by immunostaining with an anti-Flag M2 antibody (Sigma).

**Cathepsin L and B activity assay.** U2OS-Cas9, U2OS *CatL*-KO, U2OS *CatB*-KO, and U2OS *CatB/L*-KO cells stably expressing Flag-tagged NPC1 were lysed (50 mM morpholineethanesulfonic acid [MES] [pH 5.5], 135 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% Triton X-100) for 1 h on ice. As a control, cleared U2OS-Cas9 lysates were preincubated with the protease inhibitor E-64 (20  $\mu$ M; Peptides International) for 20 min at room temperature (when indicated). Then, lysates were mixed with reaction buffer (100 mM Na acetate [pH 5], 1 mM EDTA, and 4 mM dithiothreitol), incubated with the fluorogenic peptide substrate Z-FR-AMC (150  $\mu$ M; R&D Systems), and measured at a fluorometer following a 0- to 30-min incubation (excitation wavelength [ $\lambda_{ex}$ ], 390 nm; emission wavelength [ $\lambda_{em}$ ], 460 nm). Measurements of substrate hydrolysis were normalized to maximum hydrolysis on U2OS-Cas9 cells reached after 30 min; data represent the mean value and standard deviations of three independent experiments ( $n = 3$ ).

**Infection experiments.** Confluent U2OS cells were infected with pre-titrated amounts of pseudo-typed VSV particles bearing wild-type or mutant GPs. Prior to infection, VSVs were diluted in corresponding media, and infected cells were maintained at 37°C for 14 to 16 h post-infection before manual counting of eGFP<sup>+</sup> and mNG<sup>+</sup> cells or automated counting using a Cytation 5 cell imaging multimode reader (BioTek Instruments) and a CellInsight CX5 imager (Thermo Fisher) including onboard software. When indicated, cells were preincubated with small-molecule inhibitors diluted in corresponding media; for antibody neutralization experiments, VSV particles were incubated with increasing concentrations of test antibodies at room temperature for 1 h prior to addition to cell monolayers. Virus infectivities were measured as described above. Virus neutralization data were subjected to nonlinear regression analysis (4-parameter, variable slope sigmoidal dose-response equation; GraphPad Prism).

**Immunofluorescence microscopy and immunostaining.** To investigate determinants of VSV mNG-P EBOV GP internalization into endosomal compartments, pre-titrated amounts of VSV particles bearing mNG-P and either wild-type or mutant EBOV GP were diluted into imaging buffer (20 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, and 2% fetal bovine serum [FBS]) and spinoculated onto prechilled U2OS<sup>NPC1-eBFP2</sup> cells on coverslips. Unbound virus was removed by washing with cold phosphate-buffered saline (PBS). Cells were then placed in warm imaging buffer and allowed to internalize VSVs for 1 h at 37°C. Cells were fixed with 3.7% paraformaldehyde and permeabilized with PBS/0.1% Triton X-100. NPC1-eBFP2 was detected with either primary mouse anti-NPC1 MAb-548 or rabbit anti-BFP antibodies and secondary anti-mouse or anti-rabbit antibody-Alexa 405 fluorophore conjugates, respectively (Thermo Scientific). Cleaved GP was detected with the primary mouse RBS-specific anti-GP antibody MR72 followed by secondary anti-mouse antibody-Alexa 555 fluorophore conjugate (Thermo Scientific). To investigate transport of IgGs bound to VSV particles into NPC1<sup>+</sup> endosomal compartments, VSV particles bearing mNG-P were preincubated with antibodies (50 or 100  $\mu$ g/ml, respectively) for 1 h at room temperature, followed by internalization into target cells and detection by incubation with secondary anti-human-Alexa 555 fluorophore conjugates (Thermo Scientific). Cells were examined by immunofluorescence analysis performed on an Axio Observer Z1 widefield

epifluorescence microscope (Zeiss Inc.) equipped with an ORCA-Flash4.0 LT digital CMOS (complementary metal oxide semiconductor) camera (Hamamatsu Photonics), a 63×, 1.4 numerical aperture oil immersion objective, and a DAPI (4',6-diamidino-2-phenylindole)/fluorescein isothiocyanate (FITC)/tetramethyl rhodamine isocyanate (TRITC)/Cy5 filter set. Images were processed in Photoshop (Adobe Systems).

**Cholesterol accumulation assay.** Cholesterol accumulation following inhibitor treatment of U2OS<sup>NPC1-eBFP2</sup> cells was monitored by incubation with filipin (50 μg/ml; Sigma) for 1 h at room temperature. Cells were examined by immunofluorescence analysis as described in the Materials and Methods section "Immunofluorescence microscopy and immunostaining."

**Proximity ligation assay.** U2OS<sup>NPC1-eBFP2</sup> cells were allowed to internalize VSV particles (in the presence or absence of small-molecule inhibitors or antibodies), fixed, and permeabilized as described in the Materials and Methods section "Immunofluorescence microscopy and immunostaining." MR72, an RBS-targeting anti-GP MAb, and MAb-548, an NPC1 domain C-targeting MAb, were directly labeled with Duolink In Situ Probemaker Plus and Duolink In Situ Probemaker Minus (Sigma) following the manufacturer's instructions. Fixed cells were incubated with labeled antibodies in a humidity chamber at 37°C for 1 h. Excess antibody was removed by washing with Duolink in situ wash buffer (Sigma). GP-NPC1 interaction was detected by applying the Duolink in situ detection reagents red kit following the manufacturer's instructions (Sigma). After removing excess reagents, NPC1-eBFP2 was detected using a rabbit anti-BFP antibody followed by a secondary anti-rabbit antibody-Alexa 405 fluorophore conjugate (Thermo Fisher). Cells were examined by immunofluorescence analysis as described in the Materials and Methods section "Immunofluorescence microscopy and immunostaining."

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**FIG S1**, TIF file, 2.4 MB.

**FIG S2**, TIF file, 2.1 MB.

**FIG S3**, TIF file, 0.2 MB.

**FIG S4**, TIF file, 0.5 MB.

**FIG S5**, TIF file, 1.2 MB.

**FIG S6**, TIF file, 1.5 MB.

## ACKNOWLEDGMENTS

We acknowledge Isabel Gutierrez, Estefania Valencia, Laura Polanco, and Cecelia Harold for laboratory management and technical support.

This work was supported by National Institutes of Health (NIH) grant R01AI134824 (to K.C.).

K.C. is a member of the Scientific Advisory Board of Integrum Scientific, LLC.

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