Hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) in the Old World and a highly fatal hantavirus cardiopulmonary syndrome (HCPS) in the New World. No vaccines or antiviral therapies are currently available to prevent or treat hantavirus disease, and gaps in our understanding of how hantaviruses enter cells challenge the search for therapeutics. We performed a haploid genetic screen in human cells to identify host factors required for entry by Andes virus, a highly virulent New World hantavirus. We found that multiple genes involved in cholesterol sensing, regulation, and biosynthesis, including key components of the sterol response element-binding protein (SREBP) pathway, are critical for Andes virus entry. Genetic or pharmacological disruption of the membrane-bound transcription factor peptidase/site-1 protease (MBTPS1/S1P), an SREBP control element, dramatically reduced infection by virulent hantaviruses of both the Old World and New World clades but not by rhabdoviruses or alphaviruses, indicating that this pathway is broadly, but selectively, required by hantaviruses. These results could be fully explained as arising from the modest depletion of cellular membrane cholesterol that accompanied S1P disruption. Mechanistic studies of cells and with protein-free liposomes suggested that high levels of cholesterol are specifically needed for hantavirus membrane fusion. Taken together, our results indicate that the profound dependence on target membrane cholesterol is a fundamental, and unusual, biophysical property of hantavirus glycoprotein-membrane interactions during entry.

**IMPORTANCE** Although hantaviruses cause important human diseases worldwide, no specific antiviral treatments are available. One of the major obstacles to the development of new therapies is a lack of understanding of how hantaviruses hijack our own host factors to enter cells. Here, we identified multiple cellular genes that control the levels of cholesterol in cellular membranes to be important for hantavirus entry. Our findings suggest that high concentrations of cholesterol in cellular membranes are required at a specific step in the entry process—fusion between viral and cellular membranes—that allows escape of the hantavirus genome into the host cell cytoplasm to initiate infection. Our findings uncover a fundamental feature of the hantavirus infection mechanism and point to cholesterol-lowering drugs as a potential new treatment of hantaviral infections.
Further, a glycosylphosphatidylinositol (GPI)-anchored protein, complement decay-activating factor (DAF/CD55) (18), and GC1QR (globular head of the complement C1q receptor) (19) have been implicated in hantavirus entry in cell culture. A recent study proposed roles for β2 integrin (CD18) heterodimers with CD11b (complement receptor 3 [CR3]) and CD11c (complement receptor 4 [CR4]) in HTNV entry and pathogenesis (20). However, the mechanistic roles of all of these host factors in hantavirus cell entry remain incompletely defined. Moreover, despite the identification of these host factors and their proposed implications for virulence, other host factors that influence hantavirus host range, tissue tropism, and pathogenesis likely await discovery.

To identify human genes required for ANDV entry and infection, we performed a genome-wide loss-of-function genetic screen in haploid human cells. While this work was in progress, Petersen et al. in 2014 published results from a similar screen and identified the host sterol regulatory element-binding protein (SREBP) pathway as a requirement for hantavirus entry (21). Our work confirms this finding and extends it by elucidating the mechanistic basis of the SREBP signaling requirement in hantavirus entry. We show that hantavirus membrane fusion and cytoplasmic escape are specifically and exquisitely sensitive to relatively small reductions in cellular membrane cholesterol that accompany disruption of the SREBP regulatory circuit. Studies with cells and purified liposomes reveal that this profound dependence on target membrane cholesterol is a fundamental, and unusual, biophysical property of hantavirus glycoprotein-membrane interaction during entry.

RESULTS
Multiple genes involved in cholesterol regulation are required for Andes virus entry. To study hantavirus entry in a biosafety level 2 (BSL-2) setting, we engineered a recombinant vesicular stomatitis virus (rVSV) in which the VSV glycoprotein (G) was replaced with that of Andes virus (ANDV), a prototypic New World hantavirus. Using this agent (rVSV-ANDV GP), we performed a loss-of-function genetic screen in haploid human (HAP1) cells, as described previously (22–25). The screen identified seven genes that regulate cellular cholesterol metabolism (Fig. 1A). Four of these genes are critical components of the SREBP (sterol regulatory element-binding protein) cholesterol regulatory pathway (Fig. 1B): (i) SREBF2 (sterol regulatory element-binding transcription factor 2), here termed SREBP2 with 929 disruptive gene trap insertions (see Materials and Methods); (ii) MBTPS1 (membrane-bound transcription factor peptide-dase site 1, here termed site 1 protease [SIP]) with 273 disruptive insertions; (iii) MBTPS2 (membrane-bound transcription factor peptidase site 2, here termed site 2 protease [SIP2]) with 32 disruptive insertions (Fig. 1B). Most of the gene trap insertions were located toward the 5' end of each gene and enriched for sense orientation insertions, which are more likely to impair gene function due to orientation-dependent activity of the splice acceptor. These findings suggest that ANDV entry is dependent on cellular cholesterol homeostasis.

SIP is required for cell entry and infection by prototypic New World and Old World hantaviruses. The SIP and S2P proteases play crucial roles in the SREBP signaling cascade that is initiated in response to cellular cholesterol depletion (26–28). Stepwise cleavage of SREBP2 by these proteases in the Golgi complex liberates the N-terminal transcription factor domain of SREBP2, allowing it to translocate to the nucleus and increase the expression of genes involved in cholesterol uptake and biosynthesis (29, 30) (Fig. 1B). Conversely, the inactivation of SIP or S2P promotes cellular cholesterol depletion by downregulating both extrinsic and intrinsic pathways for cholesterol acquisition (27, 28). Because SIP is a central regulator of the SREBP pathway for which a well-characterized small-molecule inhibitor exists, we focused our mechanistic studies on this enzyme.

We disrupted the SIP-encoding gene, MBTPSI, in the human osteosarcoma U2OS cell line by clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated genome editing (31). We obtained a single-cell clone (SIP-#1) in which both alleles bore deletions at the expected site (Fig. 2A, top). These deletions were predicted to frameshift the SIP open reading frame (ORF) at amino acid positions 401 (allele 1) and 394 (allele 2), generating truncated polypeptides of 428 (allele 1) and 400 (allele 2) amino acid residues that terminate at downstream in-frame stop codons. Because these truncation mutants lack both Ser 414, which forms a part of the catalytic triad of the SIP serine protease, and the transmembrane anchor (residues 999 to 1021), which positions SIP to cleave SREBP2, they are predicted to be inactive. Finally, PCR with primers specific to the guide RNA (gRNA) editing site amplified SIP cDNA from wild-type (WT) but not SIP-#1 cells (Fig. 2A, bottom left), providing evidence that no WT MBTPS1 alleles are present in the latter cells. Therefore, SIP-#1 is an SIP-null cell line. To rule out any off-target effects of the CRISPR/Cas9-mediated knockout, we reconstituted these cells with a Flag-tagged version of the SIP protein (Fig. 2B, bottom right). Both the SIP-null cells and the reconstituted cells resembled WT U2OS cells in morphology and growth characteristics (data not shown).

We next exposed WT and SIP-#1 U2OS cells to rVSVs encoding GPs from ANDV, Hantaan virus (HTNV), or VSV. ANDV GP- and HTNV GP-mediated infections were reduced >95% in the SIP-null cells and could be fully rescued by ectopic expression of the SIP cDNA. In contrast, VSV G-mediated infection was only modestly inhibited in the SIP-null cells (Fig. 2B and C). Crucially, authentic ANDV resembled rVSV-ANDV GP in its dependence on SIP for infection (Fig. 2C, right). Taken together, these results indicate that SIP is required for cell entry and infection by the prototypic New World and Old World hantaviruses, ANDV and HTNV.

A small-molecule inhibitor of SIP selectively inhibits cell entry and infection by hantaviruses. To corroborate the role of SIP in hantavirus entry, we tested the effect of a specific small-molecule inhibitor of SIP, PF-429242 (herein termed the SIP inhibitor) (32, 33), on infection by rVSVs bearing hantavirus glycoproteins. SIP inhibitor treatment reduced infection by rVSV-ANDV GP and rVSV-HTNV GP in a dose-dependent manner in U2OS cells. In contrast, rVSV-G, which fuses in early endosomes (34), and rVSV-LASV GP (Lassa virus, an Old World arenavirus), which fuses in late endosomes (35), were only modestly inhibited by the SIP inhibitor.
affected, suggesting that S1P inhibitor does not cause gross perturbations in viral trafficking to early and late endosomal compartments (Fig. 3A).

To assess whether S1P is also required for hantavirus infection in a human cell type relevant to in vivo pathogenesis, we pretreated primary human umbilical vein endothelial cells (HUVECs) with the S1P inhibitor and exposed them to authentic hantaviruses (Fig. 3B). We found that the S1P inhibitor substantially inhibited...
S1P is required for hantavirus infection. (A) The S1P gene was knocked out in U2OS cells by using the CRISPR/Cas9 system. (Top) WT S1P gene sequence aligned with the sequences of both of the alleles in the S1P knockout (S1P-#1) cell clone. The gRNA target sequence is marked in red. The protospacer-adjacent motif (PAM) sequence of the gRNA target site is underlined. (Lower left) RT-PCR results for WT and S1P knockout (S1P-#1) cells using gRNA target site-specific primers. Human NPC1-specific primers were used as a control. (Lower right) S1P protein expression by anti-Flag immunostaining in S1P-#1 cells stably expressing Flag-tagged S1P. (B) WT and S1P knockout (S1P-#1) and S1P-reconstituted (S1P-#1) cells stably expressing Flag-tagged S1P) U2OS cells were exposed to the indicated rVSVs (Fig. 4A). Thus, cells lacking S1P activity suffered a reduction in cholesterol-rich low-density lipoproteins. A likely explanation for this apparent paradox is that interruption of the SREBP2 signaling pathway downregulates not only the biosynthesis of cholesterol but also its uptake (37, 38; see Discussion for details).

To directly evaluate the requirement for membrane cholesterol in hantavirus entry, cells pretreated with the S1P inhibitor were incubated with increasing concentrations of chol:CD for 1 h to restore cholesterol levels and then exposed to rVSV-ANDV GP. Cholesterol depletion of S1P inhibitor-treated cells rescued viral entry in a concentration-dependent manner; the addition of as little as 62 μM cholesterol restored ANDV GP-mediated infection to essentially full levels (Fig. 4B). Additional time course experiments demonstrated that the effect of cholesterol loading on viral entry was also time dependent, with the highest tested dose of cholesterol (1 mM) effecting full rescue of infection in as little as 5 min (Fig. 4C). The rapid and complete rescue of infection effected by exogenous cholesterol in these experiments strongly suggests that hantaviruses have a profound and direct requirement for cellular membrane cholesterol during entry.

Cellular membrane cholesterol depletion does not affect viral attachment to cells. We next sought to identify the step(s) in hantavirus entry that is strongly dependent on the levels of cellular membrane cholesterol. To determine if attachment of viral particles to the cell surface is affected by depletion of membrane cholesterol, we pretreated cells with the S1P inhibitor and then exposed them to VSV-ANDV GP particles in which the viral phosphoprotein, P, was fused to the fluorescent protein mNeonGreen [VSV(mNG-P)-ANDV GP] at 4°C (Fig. 5A) (see Materials and Methods for details). Cells were washed extensively in the cold, and flow cytometry was used to measure the fluorescence associated with cell surface-bound viral particles. S1P inhibitor treatment did not significantly diminish viral attachment to cells, suggesting that cholesterol depletion does not affect this entry step. Unexpectedly, however, incubation of cells with chol:CD prior to
After 2 h of incubation, NH₄Cl (20 mM, final concentration) was added to the medium to prevent subsequent rounds of infection. Infected cells were enumerated PF-429242 or the 1% DMSO vehicle for 24 h were exposed to the indicated viruses (0.1 to 0.2 IU per cell) (Fig. 3; representative of 3 independent experiments).

In contrast, S1P inhibitor treatment (25 μM) did not significantly affect internalization of VSV(mNG-P) particles bearing the VSV glycoprotein (G) at any time point. A similar kinetic delay in internalization of viral particles containing ANDV GP, but not VSV G, was observed in S1P-null U2OS cells (Fig. S2). Thus, S1P inhibitor treatment slows, but does not block, viral internalization. We conclude, therefore, that cholesterol depletion acts predominantly at a step downstream of viral internalization.

**Cholesterol depletion inhibits ANDV GP-mediated virus-plasma membrane fusion.** We postulated that cholesterol depletion affects viral fusion with endosomal membranes and escape into the cytoplasm. To test this hypothesis, we established a “fusion infection” assay, in which viral fusion with the plasma membrane (and ensuing cytoplasmic release) is induced by extracellular acid pH. We found that ANDV GP-mediated fusion infection was triggered at a threshold pH between 5.8 and 6.1 but occurred optimally at pH 5.5 (see Fig. S3 in the supplemental material). Accordingly, cells were exposed to rVSV-ANDV GP at 4°C and then briefly incubated at 37°C, with the growth medium adjusted to pH 7.0 or pH 5.5. This medium was then replaced with standard growth medium (pH 7.0) containing NH₄Cl to block viral membrane fusion via the normal endocytic route. We found that extracellular acid pH, but not neutral pH, could rapidly trigger viral entry into the cytoplasm (Fig. 7A), consistent with acid-dependent viral fusion with the plasma membrane. Similar levels of viral infectivity were obtained with the standard infection and fusion infection protocols carried out in parallel (Fig. 7A). Fusion infection was essentially complete at 1 min after exposure of virus-bound cells to acid pH (Fig. 7B). Thus, acid-induced fusion infection mediated by ANDV GP is both efficient and rapid.

We next pretreated cells with the S1P inhibitor and examined their capacity to support fusion infection by rVSV-ANDV GP. Inhibitor treatment reduced viral fusion infection by >90%, in a manner that could be fully reversed by cholesterol replenishment (Fig. 7C). In contrast, S1P inhibitor treatment had little effect on alphavirus fusion infection and reduced alphavirus infection more modestly than rVSV-ANDV GP infection via the endocytic route (Fig. S4). This result was expected from previous work (42, 43) which showed that alphaviruses specifically require cholesterol for membrane fusion, but only at low concentrations (also see Fig. 8B). These findings strongly suggest that cholesterol depletion interferes directly with hantavirus membrane fusion. They also indicate that the membrane fusion mechanism of hantaviruses is more profoundly sensitive to reductions in membrane cholesterol than that of alphaviruses.

**Depletion in cellular membrane cholesterol inhibits hantavirus membrane fusion at or near the lipid mixing step and blocks cytoplasmic delivery of viral matrix protein.** Fusion between viral and cellular membranes is postulated to occur in a stepwise manner, with initial mixing of the outer membrane leaflets (hemifusion), followed by coalescence of the inner membrane leaflets to form a fusion pore, and expansion of this pore to allow passage of the viral core into the cytoplasm (44, 45). We sought to determine if cholesterol depletion inhibits hantavirus membrane fusion by abolishing the initial lipid mixing step or, instead, by...

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**FIG 3** A small-molecule inhibitor of S1P selectively inhibits hantavirus entry. (A) U2OS cells pretreated with the indicated concentrations of the S1P inhibitor PF-429242 or the 1% DMSO vehicle for 24 h were exposed to the indicated viruses (0.1 to 0.2 IU per cell) (n = 3; representative of 3 independent experiments). After 2 h of incubation, NH₄Cl (20 mM, final concentration) was added to the medium to prevent subsequent rounds of infection. Infected cells were enumerated as described for Fig. 2. (B) HUVECs were pretreated with the indicated concentrations of the S1P inhibitor or the 1% DMSO vehicle for 24 h and then exposed to authentic hantaviruses (1 PFU per cell). Viral-antigen-positive cells were enumerated by 24 h postinfection.
acting at a later step in the fusion reaction. Accordingly, we developed a fluorescence dequenching assay to directly interrogate virus-plasma membrane lipid mixing. rSVS particles were labeled with self-quenching concentrations of the lipophilic fluorescent dye 4-chlorobenzenesulfonate (DiD), which intercalates into viral membranes and fluoresces upon dilution following membrane merger or detergent solubilization (Fig. S5). Cells were exposed to DiD-labeled rSVS-ANDV GP at 4°C and then shifted briefly to 37°C in neutral or acidic medium, as described above for the fusion infection assay. Incubation of virus-bound cells at acid pH, but not neutral pH, greatly increased the cell-associated DiD signal, as monitored by fluorescence microscopy (Fig. 7D and E), providing evidence for ANDV GP-mediated fusion between the viral lipid bilayer and the plasma membrane. We used the DiD dequenching assay to assess the consequence of S1P inhibitor treatment on virus-cell lipid mixing during fusion infection. Inhibitor treatment reduced lipid mixing almost to background levels (Fig. 7D and E). Strikingly, cholesterol replenishment not only restored lipid mixing but greatly enhanced it, even relative to that in untreated controls (Fig. 7D and E).

Finally, we used the intracellular distribution of the virion-associated VSV M (matrix) protein as a marker for productive endosomal membrane penetration and cytoplasmic escape, as described previously (22, 46). WT and S1P-null U2OS cells were exposed to rVSV-G or rSVS-ANDV GP in the presence of cycloheximide (to block new M protein synthesis) and then immunostained to visualize the incoming M protein (Fig. S6). Entry by either virus into WT cells caused the redistribution of M protein throughout the cytoplasm, as shown previously (22, 46). In contrast, perinuclear M puncta were extensively observed in S1P-null cells exposed to rVSV-ANDV GP but not rVSV-G, indicative of a membrane penetration block (Fig. S6). Taken together, these findings suggest that cellular cholesterol depletion induced by genetic or pharmacological disruption of the SREBP2 signaling circuit blocks hantavirus entry at the membrane fusion step, consequently preventing virus delivery into the cytoplasm.

**Virus-liposome fusion mediated by ANDV GP is highly cholesterol dependent.** We considered two possible (nonexclusive) explanations for the preceding findings. First, ANDV GP may require high levels of cholesterol in the target membrane to carry out the fusion reaction. Alternatively, cholesterol depletion may affect the distribution and/or function of a cellular protein (e.g., a receptor) that is required for ANDV membrane fusion. To test the first hypothesis, we developed an *in vitro* assay for membrane fusion between viral particles and protein-free unilamellar membrane vesicles (liposomes). DiD-labeled rSVS-ANDV GP particles were incubated at neutral or acid pH with liposomes containing 50% cholesterol (mol/mol), and DiD dequenching concomitant with virus-liposome fusion was monitored by fluorimetry. A membrane fusion signal was detected at acid pH but not at neutral pH, as expected (Fig. 8A). Experiments with liposome preparations containing a range of cholesterol concentrations (Fig. 8B) revealed that ANDV GP-dependent virus-liposome fusion was strikingly dependent on target membrane cholesterol; lipid mixing was inhibited by 60% when cholesterol was lowered from 50% to 30% and fell to baseline levels when cholesterol was reduced further. In contrast, target membrane cholesterol was dispensable for VSV G-liposome fusion, which occurred efficiently under all conditions tested. Alphavirus fusion displayed a pattern dissimilar from those of ANDV and VSV; it was relatively unaffected at the higher cholesterol concentrations but was completely abolished in the absence of cholesterol, as shown previously (42, 43, 47) (Fig. 8B). Moreover, the different cholesterol dependencies of these three enveloped viruses for fusion with protein-free membranes were concordant with their sensitivity to S1P inhibition and cholesterol depletion in cell-based assays (Fig. 7). We conclude that the unusually strong dependence on target membrane cholesterol is a distinctive property of the hantavirus fusion mechanism that derives primarily from the interactions of its glycoproteins with host membranes and not host proteins.
DISCUSSION

Using a loss-of-function genetic screen in haploid human cells, we show that gene networks involved in the sensing, regulation, and biosynthesis of cellular cholesterol are required for hantavirus entry (Fig. 1A and B). We have implicated three genes that encode cholesterol-biosynthetic enzymes (LSS, SQLE, and ACAT2) (Fig. 1A), in addition to the four genes (SREBP2, S1P, S2P, and SCAP) that play key roles in the SREBP pathway, also previously identified by Petersen et al. (21). The identification of several components of these cholesterol pathways hinted strongly at a requirement for the metabolite cholesterol rather than for the individual gene products. Strikingly, these networks did not appear as hits in numerous published haploid genetic screens for host factors involved in viral infection and toxin-mediated cytotoxicity (22–25, 48). Results described previously and herein provide a likely explanation for this observation: hantavirus entry and infection ap-

FIG 5 Reduction in cellular membrane cholesterol does not affect viral attachment to cells. (A) Schematic of the virus attachment assay. (B) U2OS cells were treated with the S1P inhibitor (25 μM) or the 1% DMSO vehicle for 24 h and then with 62.5 μM cholesterol for the indicated amounts of time. To assess viral attachment, cells were exposed to fluorescein-labeled rVSV-ANDV GP at 4°C (1.5 IU per cell). Bound virus was quantified by flow cytometry. rVSV-ANDV GP binding and infection were performed in parallel, and values are normalized to binding and infection, respectively, in vehicle-treated cells (n = 4; two independent experiments).

FIG 6 Reduction in cellular membrane cholesterol delays, but does not block, viral internalization. (A) Schematic of the virus internalization assay. (B) U2OS cells were treated with the S1P inhibitor (25 μM) or the 1% DMSO vehicle for 24 h and exposed to VSV(mNG-P)-ANDV GP particles at 4°C. Following internalization of virus at 37°C for the indicated times (in minutes), remaining surface-bound virus particles were removed by a brief trypsin treatment. Cells were harvested and fixed with 4% PFA, and internalized virus was measured by microscopy (B) and flow cytometry (C and D) (n = 4; two independent experiments).
pear to be unusually sensitive to the perturbation of cellular membrane cholesterol. ANDV GP-mediated entry of retrovirus particles and HTNV infection were previously shown to be sensitive to treatment of cells with methyl-β-cyclodextrin, which greatly depletes cellular cholesterol levels (18, 49). However, the mechanistic basis of these effects was not determined.

Genetic or pharmacological disruption of S1P activity in a human osteosarcoma cell line, as well as in primary human endothelial cells (HUVECs), strongly inhibited infection by hantaviruses with zoonotic potential belonging to both the Old World and New World clades (Fig. 2 and 3). This blockade could be fully overcome by brief supplementation of cells with cholesterol (Fig. 4). Independently of its role in cholesterol regulation, S1P enzymatic activity is required for proteolytic processing and maturation of glycoproteins from some bunyaviruses (e.g., Crimean-Congo hemorrhagic fever virus) (50) and arenaviruses (e.g., Lassa virus and lymphocytic choriomeningitis virus) (51, 52). However, our findings argue against such a direct role for S1P in hantavirus entry; the kinetics and extent of S1P inhibitor bypass obtained with cholesterol supplementation (Fig. 4) show that reductions in hantavirus entry and infection can be fully explained by the depletion of membrane cholesterol secondary to S1P loss.

Why is an intact cholesterol homeostatic network required for hantavirus entry when target cells have been cultured in serum-containing medium replete with cholesterol-laden low-density lipoproteins (LDL)? Several considerations may resolve this apparent paradox. First, because functional SREBP signaling is required for continued transcription of the LDL receptor gene (LDLR), disruption of this regulatory circuit is predicted to greatly reduce the levels of cell surface LDLR, thereby limiting cholesterol acquisition (37, 38). Moreover, because mutant cells cannot sense that they are becoming cholesterol depleted, they cannot respond by upregulating cholesterol biosynthesis. It is therefore expected that cells will become cholesterol deficient even "in midst of plenty." Second, previous work offers a different possible explanation for why the biosynthetic genes SQLE, LSS, and ACAT2 hit in our
sensitive probe, filipin III complex from cholesterol in S1P inhibitor-treated cells (Fig. 4A), whereas a less inhibition, likely requires that cells be serum starved and/or treated with exogenous cholesterol is available in the form of LDL and suggested that loss of endogenous cholesterol synthesis selectively reduces endo/lysosomal cholesterol levels. Accordingly, we postulate that the disruption of cholesterol-biosynthetic genes inhibits acid-dependent hantavirus membrane fusion in endocytic compartments. Third, our findings, together with those of Petersen and coworkers (21), suggest that membrane cholesterol levels are only modestly reduced under these conditions. The cholesterol-binding domain of the *Clostridium perfringens* θ toxin, a probe that is especially sensitive to small perturbations in membrane cholesterol, reported a significant reduction in plasma membrane cholesterol in S1P inhibitor-treated cells (Fig. 4A), whereas a less sensitive probe, filipin III complex from *Streptomyces filipinensis*, did not (data not shown). Similarly, Petersen et al. measured an approximately 30% total cholesterol depletion in S1P inhibitor-treated cells (see Fig. S5 in reference 21). More-profound cholesterol depletion, typically associated with broad-spectrum viral inhibition, likely requires that cells be serum starved and/or treated with agents (e.g., cyclodextrins) that extract membrane cholesterol (54). These observations suggest that hantaviruses—more than most viruses and toxins—require high levels of cellular membrane cholesterol for optimal cell entry and infection.

How does modest cholesterol depletion attendant to S1P inhibitor treatment block hantavirus entry? Our results are in partial agreement with those of Petersen et al., who reported that these conditions did not affect ANDV GP-dependent viral attachment to cells but inhibited viral internalization into endosomes, the presumptive site(s) of hantavirus membrane fusion (21). In our hands, however, S1P inhibition slowed, but did not block, rVSV-ANDV GP internalization into endosomes. Instead, using assays for virus-membrane fusion, we provide evidence that cholesterol depletion selectively and profoundly inhibits GP-catalyzed fusion between viral and cellular membranes, thus blocking subsequent escape of viral cores into the cytoplasm (Fig. 7; see also Fig. S6 in the supplemental material). We therefore propose that S1P inhibition and attendant cellular cholesterol depletion act predominantly by blocking hantavirus membrane fusion, with smaller contributions from one or more upstream steps, including viral attachment and internalization.

Previous work has implicated multiple protein receptors in hantavirus infection and pathogenesis, but an understanding of their mechanistic roles in entry remains limited. Here, we show, for the first time, that ANDV GP can drive lipid mixing between viral and model membranes in a manner that requires acid pH but not cellular proteins (Fig. 8). This observation suggests that cellular receptors are not critically required to trigger at least some hantavirus membrane fusion machines (but see below).

Various target membrane cholesterol compositions in the virus-liposome assay allowed us to directly assess the effect of cholesterol on hantavirus membrane fusion. We found that ANDV GP required much higher levels of cholesterol for efficient lipid mixing than did the divergent glycoproteins from a rhabdovirus and an alphavirus (Fig. 8). These results closely mirrored the relative sensitivities of all three viral glycoproteins to cholesterol perturbation in cells, strongly suggesting that target membrane cholesterol is a biophysical requirement for hantavirus membrane fusion that is independent of cellular proteins.

We observed that the pH optimum for ANDV GP-mediated fusion with the plasma membrane is ~5.5 (Fig. S3), raising the possibility that ANDV penetrates from late endosomes. Interestingly, if this hypothesis is correct, our results from the liposome fusion assay (Fig. 8B) suggest that ANDV GP requires a higher concentration of cholesterol than might be available in late endosomes; the limiting membranes of these compartments are thought to contain less cholesterol than the plasma membrane (~35 mol%) (55–58). More work is thus needed (i) to identify the subcellular sites of hantavirus membrane fusion to assess whether cellular membrane constituents, including proteins (e.g., receptors) and lipids, “tune” the precise viral requirement for acid pH and membrane cholesterol and (ii) to determine if hantaviruses might exploit heterogeneities in the distribution of endosomal membrane cholesterol (59), such as cholesterol-rich microdomains, for fusion. The hantavirus-membrane fusion assays described herein may provide useful experimental systems to assess these hypotheses, as well as to identify additional cellular factors that modulate hantavirus glycoprotein fusogenicity, lipid mixing, and events downstream of the initial membrane merger, such as the formation of fusion pores.

Why do hantaviruses require high levels of target membrane cholesterol for fusion? Alphaviruses, which also require target membrane cholesterol, albeit at lower levels, provide one potentially applicable paradigm (43). Thus, like alphavirus E1, ANDV GP may need to bind specifically to cholesterol in order to insert into the target membrane. Previous work with synthetic peptides and liposomes corresponding to the putative ANDV fusion loop may support this hypothesis (60). Alternatively or in addition, it is conceivable that cholesterol modulates hantavirus membrane fu-
sion via its effects on target membrane fluidity and rigidity (61, 62). These properties may directly affect the insertion of a viral fusion peptide(s) into the cellular lipid bilayer, the conformation and disposition of that membrane-inserted segment(s), and the propensity of the outer bilayer leaflet to deform prior to hemifusion with its viral counterpart. More studies are required to differ-
entiate among these and other possible explanations for the unusual cholesterol dependence of hantavirus membrane fusion and to determine if this feature is shared by other bunyaviruses. Finally, it would be interesting to explore the possibility that cho-
lesterol use by hantaviruses (or changes in that use) influences viral virulence or transmissibility.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney Vero cells and 293T hu-
man embryonic kidney cells were cultured at 37°C with 5% CO₂ in high-
glucose Dulbecco’s modified Eagle medium (DMEM) (Life Technologies) supple-
mented with 10% fetal bovine serum (Atlanta Biologicals), 1% GlutaMAX (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). Human osteosarcoma U2OS cells were similarly main-
tained in modified McCoy’s 5A medium (Life Technologies) supple-
mented with the above-named reagents. HAP1 were cultured as described previously (22). Human umbilical vein endothelial cells (HUVECs; Lonza) were maintained in endothelial cell growth medium (EGM) supple-
mented with EGM-SingleQuots (Lonza) at 37°C with 5% CO₂. A re-
combinant VSV bearing the VSV G glycoprotein was propagated on Vero cells, as described previously (67). HTNV strain 76-118 (63), SNV strain CC107 (64) and ANDV strain Chile-9717869 (65) were kindly provided by J. Hooper (USAMRMD) and propagated in Vero E6 cells as previously described (63–65). Sindbis and Semliki Forest viruses were propagated as described previously (66). Propagation of rVSV-LASV GP has been pre-
viously described (24).

Cloning and rescue of VSVs bearing hantavirus glycoproteins. DNA se-
quences for the open reading frames (ORFs) of hantavirus glycopro-
teins, namely, Andes virus (ANDV) GP from the Chilean field strain R123 (GenBank accession number NP_604472.1), Sin Nombre virus (SNV) GP from a human field isolate (GenBank accession number NP_941974.1), and Hantaan virus (HTNV) GP from a cell culture-adapted strain, 76-118 (GenBank accession number NP_941978.1), were codon optimized for expression in human cells and synthesized by GenScript or Epoch Life Sciences Inc. The Ebola virus GP sequences in the vector encoding the hantavirus glycoproteins were amplified by PCR using the 2× Phusion high-fidelity (HF) PCR master mix with HF buffer (Thermo Scientific) and cycling conditions of 98°C for 1 min, followed by 35 cycles of 98°C for 15 s, 65°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 3 min with the following primers: 5’ AATCACAGTCTGACCATAAAATGG 3’ (forward) and 5’ CAGAC TAAACCAGTACCTCC 3’ (reverse). Amplified PCR products were tested for indels (insertion-deletions) at the target site by using a Surveyor mutation detection kit for standard gel electrophoresis (Transgenomic Inc.) as per the manufacturer’s instructions. At 24 h post-transfection, transfected cells were treated with 50 μg/ml of blasticidin for 24 to 36 h to kill the untransfected cells, and survivors were allowed to recover.

Genomic DNA was isolated from the surviving cells by using AquaPre-
serve and ProSink (MultiTarget Pharmaceuticals) according to the manu-
facturer’s directions. Genomic DNA flanking the gRNA target site was amplified by PCR using the 2× Phusion high-fidelity (HF) PCR master mix with HF buffer (Thermo Scientific) and cycling conditions of 98°C for 1 min, followed by 35 cycles of 98°C for 15 s, 65°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 3 min with the following primers: 5’ AATCACAGTCTGACCATAAAATGG 3’ (forward) and 5’ CAGAC TAAACCAGTACCTCC 3’ (reverse). Amplified PCR products were tested for indels (insertion-deletions) at the target site by using a Surveyor mutation detection kit for standard gel electrophoresis (Transgenomic Inc.) as per the manufacturer’s instructions. Once indels were confirmed, genomic DNA from multiple single-cell clones was isolated and the gRNA-targeted region was amplified as described previously (24). After cloning of the amplified PCR product into a TOPO-TA vector followed by Sanger sequencing of multiple TOPO-TA clones, we found that both the alleles were mutated.

Knockout of the S1P gene was confirmed by reverse transcription (RT)PCR using primers S1P-F (5’ CATGTTGTGCTGGCAGATGGG 3’) and S1P-R (5’ TTTCCCCAGCAAGACCCCGG 3’), specific for the deleted se-
quence in the S1P-#1 knockout cell clone.

Reconstitution of S1P expression in S1P knockout U2OS cells. The full-length ORF of the S1P gene was amplified by PCR from the cDNA of the U2OS cells generated by using the SuperScript III first-strand synthet-
sis system for RT-PCR (Life Technologies). The 2× Phusion high-fidelity PCR master mix with HF buffer (Thermo Scientific) was used to amplify the S1P ORF using the primers 5’ CAGATGTTGTGCTGGCAGATGGG 3’ (forward) and 5’ GATGTGCTCTGGCAGATGGG 3’ (reverse). Cycling conditions were 98°C for 1 min, followed by 35 cycles of 98°C for 15 s, 65°C for 15 s, and 72°C for 2 min, and a final extension at 72°C for 5 min. The amplified product was cloned into a modified pHb-Puro vector to express a C-terminal cMYC-DDK
tagged S1P. Stable cells expressing S1P in the S1P-#1 background were generated as described earlier (46). Expression of S1P in the reconstituted cells was confirmed by immunostaining with an anti-Flag M2 antibody (Sigma).

**Authentic hantavirus infections.** Wild-type, S1P knockout (S1P-#1) and S1P-reconstituted (S1P knockout cells stably expressing Flag-tagged S1P) U2OS cells were seeded in 96-well plates and incubated overnight at 37°C. Cells were inoculated with ANDV (1 IU per cell) for 2 h at 37°C. Virus inoculum was removed, and cells were washed with phosphate-buffered saline (PBS). Fresh culture medium was added, and cells were incubated at 37°C in 5% CO2. At 72 h postinfection (hpi), cells were washed with PBS and fixed with 10% formalin. Cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature and blocked with 1% bovine serum albumin (BSA) at 37°C for 2 h. Cells were incubated sequentially with ANDV nucleocapsid-specific rabbit polyclonal antibody NR-9673 (2.5 μg/ml for 2 h at room temperature; BEI Resources) and goat anti-rabbit IgG-Alexa 488 (1 mg/ml for 1 h at room temperature; Life Technologies). Nuclei were stained with Hoechst 33424 and then washed with PBS and stored at 4°C. Images were acquired at 20 fields/well with a 20× objective lens on an Operetta high-content imaging device (PerkinElmer). Images were analyzed with a customized scheme built from image analysis functions present in Harmony software, and the percentage of infected cells was determined using the analysis functions.

HUVECs were seeded in 96-well plates and incubated overnight at 37°C in 5% CO2. Cells were treated with 5 or 10 μM of the S1P inhibitor (PF-429242) or with the 1% DMSO vehicle for 24 h. Culture medium was removed, and fresh compound was added prior to inoculation with HTNV or SNV (1 IU per cell). Cells were incubated at 37°C for 24 h and immunostained for HTNV (HTNV nucleocapsid-specific rabbit polyclonal antibody NR-12152 [BEI Resources]) or SNV (SNV nucleocapsid-specific rabbit polyclonal antibody NR-9674 [BEI Resources]) antigen expression, and images were analyzed as described above.

**Cytotoxicity assay.** Cytotoxicity of the S1P inhibitor PF-429242 (Tocris Bioscience) on the HUVECs was estimated by using the WST-1 assay (Roche) as per the manufacturer’s instructions.

**Preparation of cholesterol:cyclodextrin complexes.** Cholesterol (Sigma-Aldrich) and 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) complexes were prepared as described previously (71). After formation, the solution was filtered through a 0.45-μm syringe filter to remove any residual crystals.

**Clostridium perfringens θ toxin-based cholesterol staining assay.** The cholesterol-binding domain (D4) of the *Clostridium perfringens θ* toxin was fused in frame to a hexahistidine tag and to enhanced blue fluorescent protein 2 (eBFP2), essentially as described for a D4-GFP fusion protein (36). The resulting eBFP2-θD4 construct was expressed in *Escherichia coli*, and protein was partially purified by nickel chelation chromatography, as described previously (36). After elution from nickel beads, the protein was further purified on a PD-10 desalting column (GE Healthcare). For cholesterol staining, U2OS cells were treated with 25 μM PF-429242 or the 1% DMSO vehicle (Fisher Scientific) for 24 h, followed by mock treatment or cholesterol supplementation for 5 min with 1 mM cholesterol:CD complex. Cells were washed three times with Hank’s balanced salt solution (HBSS) (Corning) and then incubated with eBFP2-θD4 in HBSS containing 0.1% BSA for 45 to 60 min in the dark to stain the plasma membrane and/or endosomal cholesterol. Cells were washed with HBSS before being imaged by fluorescence microscopy under a 63× oil immersion objective (numerical aperture, 1.4; Carl Zeiss).

**SIP inhibitor treatment and virus infection.** U2OS cells were pretreated with various concentrations of PF-429242 or the 1% DMSO vehicle for 24 h. Cells were inoculated with rSVV-G, rSVV-ANDV GP, rSVV-LASV GP, rSVV-HTNV GP, or Sindbis virus and incubated at 37°C in 5% CO2. At 2 hpi, 20 mM NH4Cl was added to stop subsequent rounds of infection. At 12 to 16 hpi, cell nuclei were counterstained with Hoechst 33342 dye (Life Technologies) and cells were imaged by fluorescence microscopy on an Axio Observer inverted microscope. Enhanced GFP (eGFP) expression was used to score infection. For experiments testing the effect of cholesterol supplementation on infection, cells received a cholesterol or mock treatment, as described above, prior to infection.

For all the infection experiments, individual channel images were exported as TIFF files, and infected (eGFP-positive) cells and nuclei were enumerated using CellProfiler (72). Infected cells were normalized to per-cell nuclear counts. For each technical replicate, 5,000 to 10,000 cells were counted.

**Virus attachment and infection assay.** For both of these assays, U2OS cells were treated with 25 μM PE-429242 or the 1% DMSO vehicle for 24 h before being supplemented with a 6.25 μM cholesterol:CD complex or nothing for 0 to 60 min at 37°C. rSVV-ANDV GP was labeled with 5 μg/ml of a lipophilic dye, functional-component spacer diacyl lipid (FSL)-fluorescein (Sigma-Aldrich), and bound to the cell surface by centrifugation (2,500 rpm for 60 min at 4°C) in serum-free medium. Cells were placed on ice and washed with cold PBS. For the attachment experiment, cells were then fixed with 4% paraformaldehyde (PFA), and surface-bound virus was analyzed by flow cytometry. For the infection experiments, cells were placed at 37°C for 1 h, followed by the addition of 20 mM NH4Cl to stop further rounds of infection. At 12 to 16 hpi, cells were scored for infection as described above.

**Internalization assay.** U2OS cells were pretreated with the 1% DMSO vehicle or 25 μM SIP inhibitor (PF-429242) for 24 h. SVV(mNP-ANDV GP or VSV(mNP-G)-G particles containing mNeonGreen-tagged phosphoproteins were bound to the cell surface by centrifugation as described above. Cells were washed with cold PBS while on ice and incubated at 37°C for 0 to 60 min in the presence of the drug and 20 mM NH4Cl. Cells were then placed back on ice and treated with 0.5% trypsin (Life Technologies) for 10 min (ANDV GP) or 40 min (VSV G) to remove surface-bound virus. Cells were washed with cold PBS again and fixed with 4% PFA, and the fluorescence from internalized virus was measured by flow cytometry.

**Fusion infection assay.** U2OS cells were pretreated with the 1% DMSO vehicle or 25 μM SIP inhibitor, followed by supplementation with cholesterol (1 mM for 5 min) or a mock treatment. rSVV-ANDV GP or Sindbis virus particles encoding eGFP were bound to the cell surface as described above. Cells were washed with cold PBS while on ice. The PBS was replaced with DMEM-F12 medium supplemented with 10 mM HEPES at pH 5.5 or 7.0. Cells were incubated at 37°C for 1 min and then returned to ice. Cells were incubated overnight at 37°C in 5% CO2 in medium containing drug and 20 mM NH4Cl. Cells were scored for infection as described above at 12 to 16 hpi.

**Virus cell-liquid mixing assay.** The virus cell-liquid mixing assay was performed as for the fusion infection assay except that virus was labeled with a lipophilic dye, 1,1’-diocadecyl-3,3,3’,3’-tetramethylindodicarbocyanine 4-chlorobenzensulfonate (DiD) (15 μM), by mixing and incubating the dye with virus at room temperature for 10 min. After binding of the virus (2.6 IU per cell) at 4°C and incubation of virus-bound cells to pH 5.5 or 7.0, cells were incubated at 37°C for 1 min and then returned to ice. Cells were incubated overnight at 37°C in 5% CO2 in medium containing drug and 20 mM NH4Cl. Cells were scored for infection as described above at 12 to 16 hpi.

**Liposome preparation.** 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), sphingomyelin (brain, porcine), and cholesterol (ovine wool, >98%) were purchased from Avanti Polar Lipids, Inc. The DOPE, DOPC, sphingomyelin, and cholesterol were dissolved in chloroform and methanol (2:1) and mixed in ratios of 1:1:1:0, 1:1:0:55, 1:1:1:5, and 1:1:1:3, respectively. Liposomes were generated by the freeze-thawing and extrusion method published in the work of Chatterjee et al. (73) using filters with a 0.4-μm pore.
pore size. The lipid films were hydrated in morpholineethanesulfonic acid (MES)-saline buffer (0.1 M MES and 0.9% NaCl) (pH 8) and stored under nitrogen gas at 4°C.

**Virus liposome-lipid mixing assay.** rVSVs (30 μg of virus, based on total protein) and Semliki Forest virus (7.5 μg of virus, based on total protein) were labeled with 15 μM and 7.5 μM DiD, respectively. Lipid mixing with liposomes was measured by DiD dequequencing and recorded at excitation and emission wavelengths of 620 nm and 665 nm, respectively, on an Aminco Bowman series 2 luminescence spectrometer. Each assay mixture contained 2 ml of HNE buffer (5 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA, pH 7.3) along with 0.2 μM liposomes and pretitrated amounts of DiD-labeled virus. The experiments were performed under constant stirring in a quartz cuvette at 37°C. Pretitrated amounts of 0.25 M MES (pH 4.8) were injected into the samples to achieve pH 7.3 or 5.5. After fluorescence was recorded for several minutes, Triton X-100 was added to a final concentration of 0.1%. For normalization, the minimum fluorescence value was taken as zero, and the maximum fluorescence obtained by adding Triton X-100 was set to 100.

**VSV M protein release assay.** This assay was performed using rVSV-G or rVSV-ANDV GP on WT or S1P-null U2OS cells as described previously (22, 46). Briefly, U2OS cells were pretreated with 20 μg/ml of cycloheximide (Acros Organics) for 30 min and infected with rVSV-ANDV GP or rVSV-G (200 IU per cell) in the presence of cycloheximide for 3 h at 37°C. Cells were washed with PBS and fixed and immunostained for VSV M antigen. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) and visualized by fluorescence microscopy under a 20× air objective on an Axios Observer inverted microscope (Zeiss). Individual channel images were exported as TIFF files, and puncta containing VSV M protein and cell nuclei were enumerated using CellProfiler. For each sample, approximately 1,000 cells from 11 to 12 microscopic fields were counted.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/ suppl/doi:10.1128/mBio.00801-15/-/DCSupplemental.

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**REFERENCES**

1. Peters CJ, Simpson GL, Levy H. 1999. Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Annu Rev Med 50:531–545. http://dx.doi.org/10.1146/annurev.med.50.1.531.
2. Schmaljohann C, Hjelle B. 1997. Hantaviruses: a global disease problem. Emerg Infect Dis 3:95–104. http://dx.doi.org/10.3201/eid0302.970202.
3. Vaheri A, Strandin T, Hepojoki J, Sironen T, Henonen H, Mäkelä S, Mustonen J. 2013. Uncovering the mysteries of hantavirus infections. Nat Rev Microbiol 11:539–550. http://dx.doi.org/10.1038/nrmicro3066.
4. Lee HW. 1996. Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome, p 253–267. In Elliott RM (ed.), The Bunyaviridae. Springer US, Boston, MA.
5. MacNeil A, Nicholson ST, Spiropoulou CF. 2011. Hantavirus pulmonary syndrome. Virus Res 162:138–147. http://dx.doi.org/10.1016/j.virusres.2011.09.017.
6. Núñez JJ, Fritz CL, Knust B, Buttke D, Enge B, Novak MG, Kramer V, Osadebe L, Messenger S, Albariño CG, Ströher U, Niemela M, Amman BR, Dong W, Manning CR, Nicholson ST, Rollin PE, Xia D, Watt JP, Vugia DJ, Yosemite Hantavirus Outbreak Investigation Team. 2014. Hantavirus infections among overnight visitors to Yosemite. National Park, California, USA, 2012. Emerg Infect Dis 20:386–393. http://dx.doi.org/10.3201/eid2003.131.581.
7. Elliott RM, Schmaljohann CS. 2014. Bunyaviridae, p 1284–1288. In Fields virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
8. Schmaljohann CS, Schmaljohann AL, Dalrymple JM. 1987. Hantavirus M RNA: coding strategy, nucleotide sequence, and gene order. Virology 157:31–39. http://dx.doi.org/10.1016/0042-6822(87)90310-2.
9. Kamrud KI, Schmaljohann CS. 1994. Expression strategy of the M genome segment of Hantaan virus. Virus Res 31:109–121. http://dx.doi.org/10.1016/0168-1702(94)90074-4.
10. Löber C, Anheier B, Lindow S, Klenk HD, Feldmann H. 2001. The Hantavirus virus glycoprotein precursor is cleaved at the conserved pentapeptide WAASA. Virology 299:224–229. http://dx.doi.org/10.1006/viro.2001.1171.
11. Ruusala A, Persson R, Schmaljohann CS, Pettersson RF. 1992. Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. Virology 186:53–64. http://dx.doi.org/10.1016/0042-6822(92)90060-3.
12. Deyde YM, Rizvanov AA, Chase J, Otteson EW, St Jeor SC. 2005. Interactions and trafficking of Andes and sin Nombre hantavirus glycoproteins G1 and G2. Virology 331:307–315. http://dx.doi.org/10.1016/j.virol.2004.11.003.
13. Antic D, Wright KE, Kang CY. 1992. Maturation of Hantaan virus glycoproteins G1 and G2. Virology 199:324–328. http://dx.doi.org/10.1006/viro.1992.9070.
14. Cifuentes-Muñoz N, Salazar-Quiroz N, Tischler ND. 2014. Hantavirus Gn and Gc envelope glycoproteins: key structural units for virus cell entry and virus assembly. Viruses 6:1801–1822. http://dx.doi.org/10.3390/ v6041801.
15. Gavrilovskaya IN, Shepley M, Shaw R, Ginsberg MH, Mackow ER. 1998. β3 Integrins mediate the cellular entry of hantaviruses that cause respiratory failure. Proc Natl Acad Sci U S A 95:7074–7079. http://dx.doi.org/10.1073/pnas.95.12.7074.
16. Gavrilovskaya IN, Brown EJ, Ginsberg MH, Mackow ER. 1999. Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by β3 integrins. J Virol 73:3951–3959.
17. Mackow ER, Gavrilovskaya IN. 2001. Cellular receptors and hantavirus pathogenesis. Curr Top Microbiol Immunol 256:91–115. http://dx.doi.org/10.1007/978-3-642-65753-7_6.
18. Krautkrämer E, Zeier M. 2008. Hantavirus causing hemorrhagic fever with renal syndrome enters from the apical surface and requires decay-accelerating factor (DAF/CD55). J Virol 82:4257–4264. http://dx.doi.org/10.1128/JVI.02210-07.
19. Choi Y, Kwon YC, Kim SI, Park JM, Lee KH, Ahn BY. 2008. A hantavirus causing hemorrhagic fever with renal syndrome requires gC1qR/p32 for efficient cell binding and infection. Virology 381:178–183. http://dx.doi.org/10.1016/j.virol.2008.08.035.
20. Raftery MJ, Lalwani P, Krautkrämer E, Peters T, Scharffetter-Kochanek K, Krüger R, Hofmann J, Seeger K, Krüger DH, Schönrich G. 2014. β2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. J Exp Med 211:1485–1497. http://dx.doi.org/10.1084/jem.20131092.
21. Petersen J, Drake MJ, Bruce EA, Riblett AM, Didigu CA, Wilen CB, Malani N, Male F, Lee F-H, Bushman FD, Cherry S, Doms RW, Bates P, Briley K. 2014. The major cellular sterol regulatory pathway is required for Andes virus infection. PLoS Pathog 10:e1003911. http://dx.doi.org/10.1371/journal.ppat.1003911.
22. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuchne AH, Kranzusch PJ, Griffin AM, Ruthel G, Dal Cin P,
Dye JM, Whelan SP, Chandran K, Brummelkamp TR. 2011. Ebola virus entry requires the cholesterol transporter Niemann–Pick C1. Nature 477: 340–343. http://dx.doi.org/10.1038/nature10348.

23. Carette JE, Guimaraes CP, Varadarajan M, Park AS, Wuethrich I, Godarova A, Kotekci M, Cochran BH, Spooner E, Ploegh HL, Brummelkamp TR. 2007. PFV and genetic host defense: human cells identify cholesterol factors used by pathogens. Science 326:1213–1235. http://dx.doi.org/10.1126/science.1178955.

24. Jae LT, Raaben M, Riemersma M, van Beusekom E, Blomen VA, Velds A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, Dye JM, Brummelkamp TR. 2013. Disrupting the glycosylation of dystroglycanopathies using haploid screens for Lassa virus entry. Science 340:479–483. http://dx.doi.org/10.1126/science.1233675.

25. Jae LT, Raaben M, Herbert AS, Kuehne AI, Wirchanski AS, Soh TK, Stubbs SH, Janssen H, Damme M, Saftig P, Ploegh SP, Dye JM, Brummelkamp TR. 2014. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. Science 344:1506–1510. http://dx.doi.org/10.1126/science.1252480.

26. Sakai J, Nothdurft A, Goldstein JL, Brown MS. 1998. Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein. Evidence from in vivo competition studies. J Biol Chem 273:5785–5793. http://dx.doi.org/10.1074/jbc.273.10.5785.

27. Brown MS, Goldstein JL. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A 96:11041–11048. http://dx.doi.org/10.1073/pnas.96.20.11041.

28. Brown MS, Goldstein JL. 1997. The SREBP pathway: regulation review of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89:331–340. http://dx.doi.org/10.1016/S0092-8674(00)02013-5.

29. Sakai J, Duncan EA, Rawson RB, Hua X, Brown MS, Goldstein JL. 1996. Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. Cell 85: 1037–1046. http://dx.doi.org/10.1016/S0092-8674(00)81304-5.

30. Hua X, Sakai J, Brown MS, Goldstein JL. 1996. Regulated cleavage of sterol regulatory element binding proteins requires sequences on both sides of the endoplasmic reticulum membrane. J Biol Chem 271: 10379–10384. http://dx.doi.org/10.1074/jbc.271.17.10379.

31. Malí P, Wang Y, Lee YH, Ji L, Gueli M, DiCarlo JE, Norville JE, Cifuentes-Munoz N, Darlix J-L, Tischler ND, Varghese AH, Wang I-K, Subashi TA, Shelly LD, Hay BA, Landschulz KT, Geoghegan KF, Harwood HJ. 2008. Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. J Pharmacol Exp Ther 326:1–8. http://dx.doi.org/10.1124/jpet.107.139926.

32. Hay BA, Abrams B, Zumbrenny AY, Valentine JJ, Warren LC, Pettersson RF, Helenius A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, van Bokhoven H, Brummelkamp TR, Kielian M, Ahn K. 2010. Haploid genetic screens in human cells identify host factors involved in cholesterol dependence. J Cell Biol. 190:171–189. http://dx.doi.org/10.1083/jcb.200910007.

33. Lu YE, Cassee T, Kielian M. 1999. The cholesterol requirement for Sindbis virus entry and exit and characterization of a spike protein region involved in cholesterol dependence. J Virol 73:4272–4278.

34. Kielian M, Chavel-Vos C, Liao M. 2010. Alphavirus entry and membrane fusion. Viruses 2:796–825. http://dx.doi.org/10.1006/viro.2001.1303.

35. Chernomordik LV, Kozlov MM. 2008. Mechanics of membrane fusion. Nat Struct Mol Biol 15:675–683. http://dx.doi.org/10.1038/nsmb.1455.

36. Kielian M. 2014. Mechanisms of virus membrane fusion processes. Annu Rev Virol 1:171–189. http://dx.doi.org/10.1146/annurev-virology-031413-085521.

37. Miller EH, Obernosterer G, Raaben M, Herbert AS, Deffieu MS, Krishnan A, Nudgingo E, Sandesara RG, Carette JE, Kuehne AI, Ruthel G, Pfeffer SR, Dye JM, Whelan SP, Brummelkamp TR, Chandran K. 2012. Sindbis virus entry requires the host-programmed recognition of an intracellular receptor. EMBO J. 31:1947–1960. http://dx.doi.org/10.1038/emboj.2012.53.

38. Phalen T, Kielian M. 1991. Cholesterol is required for infection by Semliki forest virus. J Cell Biol 112:615–623. http://dx.doi.org/10.1083/jcb.112.4.615.

39. Carette JE, Guimaraes CP, Wuethrich I, Blomen VA, Varadarajan M, Sakai J, Duncan EA, Rawson RB, Hua X, Brown MS, Goldstein JL. 2011. Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. Nat Biotechnol 29:542–546. http://dx.doi.org/10.1038/nbt.1857.

40. Cifuentes-Muñoz N, Darlix J-L, Tischder NC. 2010. Development of a lentiviral vector system to study the role of the Andes virus glycoproteins. Virus Res 153:29–35. http://dx.doi.org/10.1016/j.virusres.2010.07.001.

41. Bergeron E, Vincent MJ, Nichol ST. 2007.Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/SIP is critical for virus infectivity. J Virol 81:13271–13276. http://dx.doi.org/10.1128/JVI.01647-07.

42. Lenz O, ter Meulen J, Klenk HD, Seidah NG, Garten W. 2001. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. J Cell Biol 150:1211–12120. http://dx.doi.org/10.1083/jcb.200008154.

43. Sugii S, Lin S, Ohgami N, Ohashi M, Chang CC, Chang T-Y. 2006. Roles of endogenously synthesized sterols in the endocytic pathway. J Biol Chem 281:23191–23206. http://dx.doi.org/10.1074/jbc.M603215200.

44. Danthi P, Chow M. 2004. Cholesterol removal by methyl-beta-cyclodextrin inhibits poliovirus entry. J Virol 78:33–41. http://dx.doi.org/10.1128/JVI.78.1.33-41.2004.

45. Hulin-Matsuda F, Taguchi T, Greimel P, Kobayashi T. 2010. Alphavirus entry and membrane fusion. Viruses 2:615–623. http://dx.doi.org/10.1083/jcb.200008154.

46. Van Meer G, Voelcker DR, Feigenson GW. 2008. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124. http://dx.doi.org/10.1038/nrnm2330.

47. Kobayashi T, Beuchat M-H, Chevallier J, Makino A, Mayran N, Escola J-M, Lebrard C, Cosson P, Kobayashi T, Greublen J. 2002. Separation and characterization of late endosome membrane domains. J Biol Chem 277:32157–32164. http://dx.doi.org/10.1074/jbc.M202838200.

48. Möbius W, van Donselaar E, Ohno-Iwashita Y, Shimada Y, Heijnen HF, Slot JW, Geuze HJ. 2003. Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. Traffic 4:222–231. http://dx.doi.org/10.1046/j.1600-0855.2003.00072.x.
59. Sobo K, Le Blanc I, Luyet P-P, Fivaz M, Ferguson C, Parton RG, Gruenberg J, van der Goot FG. 2007. Late endosomal cholesterol accumulation leads to impaired intra-endosomal trafficking. PLoS One 2:e851. http://dx.doi.org/10.1371/journal.pone.0000851.

60. Tischler ND, Gonzalez A, Perez-Acle T, Rosemblatt M, Valenzuela PD. 2005. Hantavirus Gc glycoprotein: evidence for a class II fusion protein. J Gen Virol 86:2937–2947. http://dx.doi.org/10.1099/vir.0.81083-0.

61. Needham D, Nunn RS. 1990. Elastic deformation and failure of lipid bilayer membranes containing cholesterol. Biophys J 58:997–1009. http://dx.doi.org/10.1016/S0006-3495(90)82444-9.

62. Papanikolaou A, Papafotika A, Murphy C, Papamarcaki T, Tsolas O, Drab M, Kurzchalia TV, Kasper M, Christoforidis S. 2005. Cholesterol-dependent lipid assemblies regulate the activity of the ecto-nucleotidase CD39. J Biol Chem 280:26406–26414. http://dx.doi.org/10.1074/jbc.M413927200.

63. Lee HW, Lee PW, Johnson KM. 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. J Infect Dis 137:298–308. http://dx.doi.org/10.1093/infdis/137.3.298.

64. Schmaljohn AL, Li D, Negley DL, Bressler DS, Turell MJ, Korch GW, Ascher MS, Schmaljohn CS. 2005. Isolation and initial characterization of a newfound hantavirus from California. Virology 206:963–972. http://dx.doi.org/10.1006/viro.1995.1019.

65. Hooper JW, Larsen T, Custer DM, Schmaljohn CS. 2001. A lethal disease model for hantavirus pulmonary syndrome. Virology 289:6–14. http://dx.doi.org/10.1006/viro.2001.1133.

66. Glomb-Reinmund S, Kielian M. 1998. The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. Virology 248:372–381. http://dx.doi.org/10.1006/viro.1998.9275.

67. Wong AC, Sandesara RG, Mulherkar N, Whelan SP, Chandran K. 2010. A forward genetic strategy reveals destabilizing mutations in the Ebolavirus glycoprotein that alter its protease dependence during cell entry. J Virol 84:163–175. http://dx.doi.org/10.1128/JVI.01832-09.

68. Whelan SP, Ball LA, Barr JN, Wertz GT. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc Natl Acad Sci USA 92:8388–8392. http://dx.doi.org/10.1073/pnas.92.18.8388.

69. Shaner NC, Lambert GG, Chammas A, Ni Y, Cranfill PJ, Baird MA, Sell BR, Allen JR, Day RN, Israelsson M, Davidson MW, Wang J. 2013. A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. Nat Methods 10:407–409. http://dx.doi.org/10.1038/nmeth.2413.

70. Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM. 2005. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. Science 308:1643–1645. http://dx.doi.org/10.1126/science.1110656.

71. Klein U, Gimp l, Fahrenholz F. 1995. Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry 34:13784–13793. http://dx.doi.org/10.1021/bi00042a009.

72. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang JH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. 2006. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol 7:R100. http://dx.doi.org/10.1186/gb-2006-7-10-r100.

73. Chatterjee PK, Vashishtha M, Kielian M. 2000. Biochemical consequences of a mutation that controls the cholesterol dependence of Semliki Forest virus fusion. J Virol 74:1623–1631. http://dx.doi.org/10.1128/JVI.74.4.1623-1631.2000.