CELL-TO-CELL INFECTION, CELL-CELL
FUSION AND PRODUCTION OF EBOLAVIRUS: MECHANISMS OF
ACTION AND CELLULAR MODULATORS

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by

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CELL-TO-CELL INFECTION, CELL-CELL
FUSION AND PRODUCTION OF EBOLAVIRUS: MECHANISMS OF
ACTION AND CELLULAR MODULATORS

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“I am a slow walker, but I never walk backwards.” - Abraham Lincoln

“Clear Head, Clever Hand and Clean Habit, C3H3”- The Motto of the Liu Lab
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ABSTRACT

Ebolavirus (EBOV), a member of the Filoviridae family, is a deadly non-segment negative-sense RNA virus that causes hemorrhagic fever among human and other species; the fatality rate can be up to 80%. In 2014, a re-emergent and serious outbreak of EBOV occurred in West Africa, which had claimed more than 11,000 human deaths. Although antibody cocktails and life supports were available, many people, including scientists and clinicians, still have had suffered and died of EBOV infection. Hence, a better understanding of EBOV biology, in particular its interaction with the host, is of critical importance. The purpose of my Ph.D. thesis project is to gain a better understanding of EBOV glycoprotein (GP)-mediated fusion, entry, and cell-to-cell transmission, and VP40-mediated production, with particular focus on the interplay between EBOV proteins and host factors that modulate this process.

We first established a cell-to-cell transmission assay by co-culturing viral producer cells and target cells. We found that EBOV utilizes cell-cell contact for efficient spread between cells, the process of which is dependent on GP. The cell-to-cell transmission of EBOV is more efficient than the cell-free infection performed in parallel. By applying inhibitors known to block cell-to-cell transmission and neutralizing antibody, we found that cell-to-cell transmission mediated by EBOV GP is sensitive to these inhibitors but relatively resistant to KZ52. Interestingly, we found that, even without the expression of viral core (e.g. MLV-Gag protein or EBOV VP40), EBOV GP is sufficient to support
transfer of Tet-off protein from donor cells to target cells. Consistent results are also
gained in the context of replication competent rVSV bearing GP as well as EBOV
VP40-based VLPs.

In collaboration with Fredric Cohen’s lab at Rush University, we established a cell-cell
fusion assay for EBOV and investigated the role of potential triggers involved in the viral
fusion process. We found that low pH is not a direct trigger of GP-mediated fusion yet the
activities of Cathepsin are required. We observed that binding of EBOV GP to the
endosomal receptor NPC1 is critical for enlargement of fusion pore and fusion kinetics.

We performed a series of experiments to determine the role of an antiviral protein,
Viperin, in the replication of EBOV. We found that Viperin efficiently inhibits the
replication of replication-competent virus-like particles (trVLPs). Consistently,
knockdown of endogenous Viperin increases the replication of EBOV trVLPs in human
lung cancer A549 cells. Mechanistically, we found that Viperin specifically reduces the
expression of EBOV VP40 by inducing autophagy-mediated lysosomal degradation.
Consistent with this finding, autophagy induction by Rapamycin significantly inhibits the
replication of EBOV trVLPs, in association with reduced viral production. We examined
the effect of some non-human primate Viperin proteins, and found that the antiviral
activity against EBOV is conserved in these species, suggesting that Viperin may have
contributed to the evolution and transmission of EBOV in primates.

In summary, we have determined the mechanisms of action of EBOV GP-mediated
cell-cell transmission and cell-cell fusion, and defined a novel role of Viperin in inhibiting EBOV replication and production. Our work will help better understand the EBOV biology, its interactions with the host, as well as the development of new therapeutics against EBOV diseases.
I. INTRODUCTION

1.1 EbolaVirus (EBOV) biology and disease

1.1.1 The origin of EBOV

In 1976, a teacher in Yambuku in Africa suffered from a disease with symptoms of fever similar to malaria infection. After the first treatment with a shot of chloroquine, the patient felt better at early stage, but soon got worse and continued to suffer from pain, fever, headache, and bloody diarrhea; he died three days later. Soon after, more cases surrounding him were reported with similar symptoms, suggesting that the disease was spreading. Due to the lack of sterilizing facility, people used the shared needles in hospital of Yambuku among the patients, which eventually led to the death of 151 people to this terrifying disease. This disease is later known as Ebola hemorrhagic fever directly linked to viral infection.

The name of “Ebola” comes from the name of the Ebola river of Yambuku, where the first reported outbreak was recorded. Since viral infection develops symptoms such as high body temperatures and inner organ bleeding, the disease caused by such a virus infection has been called as Ebola hemorrhagic fever. 

Ebolavirus was later categorized as a genus of negative sense ssRNA viruses in Filoviridae family in the order of Mononegavirales, together with Marburgvirus. The family name of “Filovirus” is derived from “filum” in Latin, reflecting the fact that filoviruses have unique filamentous morphology as thread-like shape under an electron microscope. Similar to other members
in the *Mononegavirales* order, such as *Rhabdoviridae*, *Paramyxoviridae*, and
*Bornaviridae* families, *Filoviridae* share the typical features of these viruses in viral
replication and genomic expression, suggesting they have shared a common ancestor (1).

### 1.1.2 The epidemic of EBOV

So far, most of EBOV outbreaks occurred in countries of Africa, such as Dem. Rep. of
Congo, South Sudan, Gabon, Uganda, Liberia, Sierra Leone and Guinea, while just a few
but increasing cases are also found in other area such as Europe (most at United Kingdom
and Italy) and United States. The recent outbreak in 2014 was the largest one in history,
which caused accumulative 11316 deaths out of 28638 recorded cases over the world.
Although the medical condition is better in certain areas, the averaged death rate was
about 50%.

EBOV is categorized as BSL-4 agent and has the highest fatality rate among human
pathogens. Up to now, there are five species of EBOV indentified that cause serious and
species-specific diseases in primates and other animals. These viruses were named based
on the location where they were first discovered, which include Zaire Ebolavirus, Sudan
Ebolavirus, Bundibugyo Ebolavirus, Côte d’Ivoire Ebolavirus and Reston Ebolavirus. Of
note, the first four strains of Ebolavirues cause hemorrhagic fever and death in humans
and other primates. By contrast, Reston Ebolavirus is harmless to human and appears to
be transmitted from swine. Currently, it is thought that bats are likely the natural hosts of
EBOV.
In order to control the spread of the outbreak, at the end of 2014 FDA fast-track proved two anti-EBOV drugs, one antibody cocktail called Zmapp and one RNA-interference drug called TKM-Ebola. However, neither of the clinic trials tested in Africa for these two showed overall significant effects on EBOV infection. More details will be discussed in section below.

1.1.3 Genome and proteins of EBOV

EBOV virion consists of lipid bi-layer envelope and a complex formed by viral nucleocapsid, polymerase, matrix protein and viral genomic RNA genome. The entire EBOV genome is about 19 Kb long and encodes seven conserved viral proteins, i.e. nucleoprotein (NP), viral protein 35 (VP35), matrix viral protein 40 (VP40), the envelope glycoprotein GP, viral protein 30 (VP30), small matrix viral protein 24 (VP24) and viral polymerase L (Figure 1-1). The genes encoding these proteins are individually flanked by non-translated regions up to 684 nucleotides, remarkably long at its 3’ or 5’ ends that are only observed in Filoviruses and Henipaviruses in the context of nonsegmented negative-strand RNA viruses (2, 3) (Figure 1-2). The function of these non-translated regions remains elusive, but appears to be important for EBOV transcription initiation and termination. Although other Filoviruses such as Marburgvirus (MARV) encode counterparts of these proteins and share the overall mode of genomic replication and transcription, EBOV has distinct mechanisms, including the formation of nucleocapsid, the recognition of replication promoter and distinct requirements and functions of the
transcription factors (2).

EBOV genome encodes two nucleoproteins, NP and VP30. NP is the major viral nucleoprotein encoded by the first gene of EBOV, and is responsible for the viral genomic encapsidation that is known to be critical for the subsequent viral genomic replication, transcription and virions assembly (2).

The minor nucleoprotein is VP30, which is also known as a transcription activator required for both early initiation and later reinitiation during viral transcription (2, 4). VP30 is also involved in viral nucleocapsid assembly, possibly via binding of NP-formed helical structure. Although the details of the mechanisms of VP30 in transcription remains unclear, recent report showed that the phosphorylations of VP30, particularly at serine and threonine residues, are critical in EBOV transcription as well as in the incorporation of VP30 into EBOV virions (5). When VP30 is phosphorylated, it can bind to viral genomic RNA in association of NP, polymerase L and VP35 to form the viral ribonucleoprotein (RNP) complex (5, 6). The viral RNP complex is later packaged into virions at the plasma membrane, through interactions with VP40 (7, 8).

Similar to VP30, VP35 is another common phosphoprotein (P) in filoviruses as its counterparts are often present in many other negative-sense ssRNA viruses. For EBOV, VP35 is multifunctional protein: first, it is known as a linker to facilitate the binding of polymerase L to the promoter binding site located at the start of NP gene in viral mRNA; second, VP35 can interact with both VP40 and NP to facilitate the packaging of viral
RNA into viral particles (7); third, it participates in viral antagonisms that block type I IFN-mediated immune responses and enhance viral replication. After EBOV enters the host cells, the double-strands RNA (dsRNA) binding activity of VP35 can block the sensing activity of the pathogen recognition receptor PRR (i.e. RIG-I, PKR) and its down-stream signaling, which in turn inhibits the interferon regulation factor 3 (IRF3)-mediated production of type I IFN (9). Alternatively, VP35 can interact with RIG-I activator PACT to antagonize the RIG-I-mediated signaling and type I IFN responses (Figure 1-3). Of note, the VP35-PACT interaction is mutually antagonistic since it competes the binding of viral RNA with VP35 thus hindering the initiation of viral replication (10). This ability of viral antagonism of VP35 is supported by the observation that knockdown of VP35 strongly activates immune response and blocks EBOV amplification in mice model (11).

EBOV genome encodes two viral matrix proteins. One is VP40, the major matrix protein of EBOV that mediates the budding of VLPs at the plasma membrane, where it associates EBOV GP (12, 13). VP40 is known to be synthesized in ER and transported towards plasma membranes. However, the exact route of VP40 trafficking before budding and release is still not known. So far, evidence has suggested that the trafficking of VP40 is linked to several factors and pathways such as L domain-dependent ESCRT machinery, endosomal pathway, the Sec24C-mediated COPII transport system and the cytoskeleton-related microtubules (14).
VP24 is a unique minor matrix protein of filoviruses, which is important for the packaging of viral genome into viral assembly (2, 15, 16). VP24 overexpression in 239T cells has been shown to negatively regulating viral genomic replication in EBOV monocistronic minigenome-based system (17). Similar to VP35, VP24 has been identified as another suppressor of the type I and II IFN production as well as STAT-1 related down-stream signaling. Mechanistic studies showed that expression of VP24 impairs the accumulation of activated STAT-1 in the nuclear via competing its binding of nuclear receptor karyopherin α1 (18). Other evidence has shown that defective VP24 enhances IFN responses and reduces EBOV release.

The gene encoding EBOV glycoprotein (GP) is the most complex one; it has three different forms, secreted GP (sGP), membrane-associated GP (GP) and small secreted GP (ssGP). sGP contains a stop codon at the middle of gene, and it is the major product transcribed from this gene. Since it has no membrane-associated structure, sGP is efficiently secreted into extracellular compartment, where it plays a role in EBOV virulence (19). However, the exact function of sGP is still unclear. Although early reports argued that sGP can disrupt the formation of immune complex, possibly via binding to neutrophils and subsequent signaling receptors occupation, the conclusion that it can inactivate neutrophils was later challenged by others and appeared as experimental artifact. Other possible roles of sGP in EBOV pathogenesis have been recently proposed, such as restoration of the barrier of epithelial cells as well as increasing apoptosis of T
and B lymphocytes. Nonetheless, there is no clear evidence for a role sGP in vivo. Further investigation of how sGP affects immune subversion is needed.

The full length of GP that mediates viral entry is another product generated from the mRNA editing of GP gene, consisting of GP1 and GP2 linked by a single disulfide bond. One of the major functions of full length GP is to facilitate viral receptor binding and triggering of viral-host membrane fusion, although the underlying mechanism is still unknown (20-26). Recent report showed that GP can also promote the release of VLPs at the plasma membrane via antagonizing the host restriction mechanism (27, 28). Similar to sGP, the full length GP is enzymatically cleaved at plasma membrane by TNF-α converting enzyme (TACE) of ADAM protein family, and is released to the extracellular space as shed GP. The shed GP has been recently shown to activate immune cells such as DCs and macrophages, resulting in increased vascular permeability that contributes to EBOV pathogenicity (29) (Figure 1-4).

EBOV polymerase L is the largest but the least transcribed protein of EBOV. It contains the activity of RNA-dependent RNA polymerase, which is responsible for EBOV genomic replication and transcription. The protein is inactive until it interacts with VP35 to forms a polymerase complex (2).
Figure 1-1: The model of EBOV structure.

EBOV consists of seven proteins, including NP, VP35, VP40, GP (encodes sGP, full length GP and ssGP), VP30, VP24 and L. Image is adapted from (30) with permission (https://creativecommons.org/licenses/by/4.0/).
Figure 1-1: The model of EBOV structure.
Figure 1-2: Schematic diagram of EBOV RNA genome.

The coding and non-coding regions of EBOV RNA are sequentially shown as boxes and black bars, respectively. Note that the 3’ and 5’ ends at EBOV non-coding leader and trailer regions are unusually long, which is distinct from other RNA viruses. Green triangle: transcription start signal; Red bar: transcription stop signal; Black arrows: gene overlaps; Asterisk: gene editing.
Figure 1-2: Schematic diagram of EBOV RNA genome.
While VP35 plays a role in EBOV genomic replication and transcription, it also serves as viral factor that inhibits RIG-I mediated signaling and type-1 IFN production, which is thought to be achieved via binding to either viral dsRNA or host factor PACT. Note that the interaction between VP35 and PACT has mutually effects on both host immune response and EBOV replication. Image is adapted from (10) (permission number: 3907200779940).
Figure 1-3: Role of VP35 in RIG-I mediated signaling.
Figure 1-4: Role of shed GP during EBOV infection.

Upon release, shed GP binds and actsives dendritic cells and macrophages via TLR-4, resulting in increased release of inflammatory-related cytokines. These released cytokines contribute to EBOV. The image is adapted from (29) with permission (https://creativecommons.org/licenses/by/4.0/).
Figure 1-4: Role of shed GP during EBOV infection.
1.1.4 The life cycle of EBOV

The life cycle of EBOV can be divided into three steps: viral entry, genomic replication/transcription/translation, and assembly/budding (Figure 1-5).

1.1.4.1 Entry

The life cycle of EBOV begins with the binding of virions to the target cell surface, the process of which is mediated by viral glycoprotein GP and its receptors. Although efforts have been made to identify the receptors of EBOV on the plasma membrane of the target cell, the progress of search is slow and the number of identified authentic receptors is limited. In early reports, several cellular factors have been shown to non-specifically support the attachment of the virions into the cells (31), such as C-type lectin family proteins (i.e. dendritic cell- and liver/lymph node-specific ICAM-3-grabbing non-integrin (DC/L-SIGN) (32, 33), human macrophage galactose-type C-type lectin (hMGL) (34), liver and lymph node sinusoidal endothelial cell C-type lectin (LSECTin/CLEC4G) (35, 36), β1 integrins (37), Tyro3 family receptors (38) and TIM-1 (39, 40). None of them is sufficient for EBOV entry. Therefore, these adhesion molecules and co-factors only serve as binding molecules. In contrast, a recently reported endosomal protein, Niemann Pick C1 (NPC1) has been identified as a specific receptor of EBOV that promotes viral-host fusion at the membrane of endosomes (23, 40-42), where the binding of EBOV GP occurs. The specific receptors of EBOV at the outer plasma membrane that promote viral internalization into endosomes have yet to be identified.
Upon binding to the surface of target cells, EBOV virions are endocytosed into endosomal compartments of target cells. The endocytosis pathways of EBOV are currently controversial. Early reports using chemical inhibitors have been shown that retroviral pseudotypes bearing GP utilize clathrin- and caveolae-dependent pathways to enter the cells (43-45). The conclusion has been challenged by other research groups, as they found that overexpression of caveolae does not affect EBOV entry, and that the caveolae-deficient cell lines are still permissive for EBOV infection (46). In addition, more arguments have been made as retroviral pseudotypes bearing GP have distinct morphology of virions, which would not represent the exact property of authentic EBOV in viral entry. Instead, recent reports involving authentic EBOV have shown that macropinocytosis serves as the key pathway of EBOV entry (44, 47-49). EBOV VLPs have been shown to be co-localized with the mark protein, sorting nexin (SNX) 5 in the macropinocytosis-specific endosomes. Knockdown of Pak1 and CtBP/BARS, the essential macropinocytosis regulators, reduces authentic EBOV infection (47, 48).

Following entry, EBOV virions traffic through early endosomes to late endosomes/lysosomes, where virus-host fusion would occur. The involvement of endosomal compartment has been demonstrated by the co-localization of EBOV virions with endosomes-specific markers such as the Early Endosome Antigen 1 (EEA1) and SNX5 as well as Rab 5 and Rab 7, the early and late endosomal GTPase, which have been demonstrated to be essential for EBOV infection.
Under the acidic environment of late endosomes, GP on the surface of the virion is processed by cysteine proteases such as Cathepsin B/L, thus becoming metastable for fusion (22, 24, 50). Unlike other pH-dependent viruses, EBOV does not directly undergo conformational changes at low pH. Instead, the acidic pH merely drives the priming of GP into relatively smaller form; the process replies on the activity of Cathepsin B/L (51). Although exact size of the cleaved form is controversial, smaller GP with ~19 kDa has been observed in EBOV VLPs under the treatments of Cathepsin B (22, 52, 53). The treatment of Cathepsin as well as thermolysin, an enzyme that fully mimics the cleavage of GP by Cathepsin, promotes EBOV entry. In contrast, inhibitors of Cathepsin B/L significantly attenuate EBOV infection (53). Upon priming, cleaved GP is able to bind the endosomal receptor, Niemann Pick C1 (NPC1) at the endosomal membrane (23), where the fusion occurs by unknown mechanism. The crystal structure of bound cleaved GP onto NPC1 has been recently resolved, showing direct evidence of interactions between protruding loops of NPC1 domain C and hydrophobic cavity of cleaved GP (42). The inhibition of NPC1 significantly reduces EBOV infection in several cell lines. In contrast, NPC1 expression sufficiently supports EBOV infection in non-permissive CHO cells (54). Calcium-dependent two-pore channels (TPCs) have been recently reported to be essential for the late stage of viral entry (55). Upon the viral-host membrane fusion, viral components are released into the cytoplasm, and viral genomic replication starts.

1.1.4.2 Genomic replication and transcription
After entry, the EBOV genome is released in the cytoplasm, when viral genomic replication and transcription occur. For viral transcription (2), EBOV nucleocapsid proteins, i.e. VP35 and L, initially bind to the encapsidated viral genome at the promoter sites of the leader region ahead of gene NP, and transcribe viral mRNA by the activity of RNA-dependent polymerase L. The movement of polymerase L during transcription can be blocked by a stable secondary structure formed by the first 23 amino acids of nascent mRNA, while VP30 acts as a transcription activator that resolves this blockage. The elongation and re-initiation of each gene are achieved independently of VP30, although a RNA stem-loop structure is formed at the start of each transcript. Seven mRNAs are individually transcribed, and their corresponding proteins are subsequently produced. Newly synthesized nucleoproteins, including NP, VP35 and L, would continuously synthesize viral genome and support genomic replication.

The genomic replication begins with synthesis of the complementary positive-strand RNA (cRNA), which in turn serves as the template for synthesis of negative strands. Both positive and negative strands need to be encapsidated by NP in transcription and replication. Since the promoter of cRNA seems to be stronger than negative strand, negative strands are more efficiently produced. At the late stage, nascent viral RNA is packaged into virions.

1.1.4.3 Assembly, budding and release

The assembly begins with the accumulation of viral nuleocapsids at the inclusion
bodies in the perinuclear area. The minimal requirement for EBOV nucleocapsid assembly includes NP, VP35 and VP24. NP serves as core protein that interacts with VP35 and VP24 to form the nucleocapsid-like structure (8). The interaction of VP35 and VP40 facilitates the packaging of EBOV RNA into VLPs (7). VP24 is known to be critical for the nucleocapsid assembly as knockdown of VP24 largely impairs the nucleocapsid formation in EBOV infected Vero cells (15). Other report proposes that VP24 is essential for condensation of EBOV RNA during packaging (56).

Like other viruses, filoviruses hijack the cellular machinery for trafficking and budding. Transport of EBOV assembly likely involves the mechanisms of cytoskeleton, microtubules and endosomes, by which EBOV VP40 drives the movement of EBOV nucleocapsid-like structures at perinuclear area toward the budding sites (8). Overexpression of VP40 is shown to sufficiently induce the localization of nucleocapsid-like structures at the plasma membrane without other viral proteins (i.e. GP, L and VP30). Treatments of microtubule inhibitors, such as nocodazole for polymerization and taxol for depolymerization, reduce the transport of EBOV VLPs (8), and this suggests that microtubule is required for the VP40 transport. By live-cell imaging assay, the transport of GFP-labeled nucleocapsid has been recently examined within authentic EBOV infected cells. Movement of nucleocapsid is shown to be arrested by the treatments of cytoskeleton inhibitors, such as F-actin depolymerizing agent cytochalasin D, and actin nucleating Arp2/3 complex inhibitor CK869 (57). This suggests
that cytoskeleton play a role in the transport of nucleocapsid. Furthermore, two overlapping late domains (i.e. PTAP/PPEY) have been identified in VP40 sequence, indicating that EBOV may recruit host ESCRT machinery for assembly and budding (58). Similar to lentiviruses, the interactions of EBOV VP40 with Tsg101 and Nedd4 are shown to be critical for EBOV release (59). A third L-domain YPx(n)L/I has been recently identified in VP40 as it binds to ALIX that affects EBOV egress (60). The late endosomal pathway has been shown to be possibly utilized at the late stage of the EBOV assembly as VP40 interacts with nucleocapsid and the endosomal membrane (13, 61).

Two modes of EBOV budding have been detected at the plasma membrane; EBOV virions can be released from the cell surface horizontally or vertically. It has been shown that mature EBOV particle buds at the plasma membrane horizontally, most of which incorporates viral nucleocapsid. In contrast, most of the virions released vertically are empty and appear smaller (8). Other report has suggested that EBOV enters the filopodia of infected cells and buds at the tip of cellular protrusions (57).

The C-terminus of VP40 has been shown to be critical for EBOV release (62). Truncation of C-terminus of VP40 induces spontaneous self-oligomerization (63) and reduces the VP40 release from cells (64). Furthermore, VP40 interacts with lipid rafts in vitro, which sufficiently leads to the oligomerization of VP40 as well as the recruitment of Tsg101 to lipid rafts (65). This suggests a possible role of lipid rafts in EBOV release. Like other viral envelope, EBOV GP utilizes classical host secretory pathway for
transport, and is then incorporated into virions at the plasma membrane (66). Overexpression of GP significantly enhances the release of EBOV VLPs (28), possibly via counteracting with the restriction molecules at the plasma membrane (27).
Figure 1-5: Schematics of the EBOV life cycle.

The interactions between EBOV and infected cells in different phases are depicted.

Image is adapted from (67) (permission number: 3907210419574).
Figure 1-5: Schematics of the EBOV life cycle.
1.1.5 Antiviral treatment of EBOV.

The large Ebola outbreak during 2014-15 occurred in multiple countries in West Africa with varied fatality rates from 21% to 60% (68). However, in EBOV-endemic area, there are no effective FDA-proved antiviral treatments or vaccine available for EBOV. The demands for antiviral treatments have required the development of new EBOV therapeutic strategies. Currently, researches have shown promising results in animal experiments with cocktails of antibodies, small RNA interference and small molecule inhibitors. With the overall goal of cure and prevention of EBOV disease, these numbers of potential drugs have been accelerated into clinical trials in EBOV affected area. As of the end of 2015, some of the drugs have shown certain effects on EBOV.

Due to a number of practical concerns and issues, including dosage adjustments for geriatrics and pediatrics, different formulations for delivering drugs as well as concerns of frequency and timing of prophylaxis, the therapeutics for EBOV becomes challenging and demanding. Better strategies are suggested according to the time-course of EBOV infection and disease (68).

In the past few years, another challenging part for the development of anti-EBOV drugs is that the authentic virus requires the facility of BSL-4. The studies of EBOV are restricted to a small number of laboratories worldwide until reverse genetics has been applied into this field. Reverse genetics provides a number of valuable tools for the studies of EBOV without the requirement of BSL-4, such as the development of
recombinant filovirus, pseudotypes bearing EBOV GP and EBOV virus-like particles. These novel techniques effectively support the development of antiviral therapeutics and vaccines against EBOV infection along with the better understanding of the nature of EBOV.

The current ongoing therapies in clinical trials for prevention of EBOV could be classified to three types; they are nucleoside analogs, RNA-silencing molecules and immune therapy.

Examples of nucleoside analogs therapies are: Favipiravir, also named as T-705, has been examined in animal trials with a great potential against EBOV in vitro and vivo; Brincidofovir (BCV), an common antiviral against double-stranded viruses, has also been considered by FDA as a candidate treatment for EBOV due to its great efficacy against EBOV; JK-05 is a novel anti-EBOV compound approved for emergency use in China, and now it has been tested in animal trials for its activity of inhibiting viral RNA polymerase; The adenosine analog, BCX4430, has also been planned for clinical trials due to its ability of being incorporated into newly generated viral RNA and disrupting the function of RNA polymerase.

Other promising antiviral drugs for EBOV are RNA interference-based drugs, such as TKM-Ebola and phosphorodiamidate morpholino oligomers (PMOs), due to their well tolerance in trials. Both of them are designed to target three of viral key genes, VP35, VP24 and polymerase L, while the former two EBOV proteins are known to act as
immune suppressors that antagonize host immune response. TKM-Ebola has been given fast-track approval by FDA in the early 2014; in the meantime it also tested in human clinical trials in Guinea led by WHO. As of the end of 2015, this drug has been stopped as it appeared not to work, but its statistics analysis is still ongoing. The specific PMO (AVI-6002) has undergone phase I clinical trial but still require in-depth investigation. It was also found to be well tolerant in patients.

The immune therapy for EBOV is also promising and thus developed in the use of passive immunity under the guideline by WHO. Zmapp is a novel one designed for EBOV, which consists of three chimeric monoclonal antibodies (mAb) generated from mouse hybridomas modified with human components. Currently, Zmapp has been undergone NIH-led PREVAIL II clinical trial in Liberia, Sierra Leone and Guinea.

There are also ongoing vaccine developments for EBOV, such as cAd3-EBOZ and rVSV-ZEBOV. Both of the vaccine candidates have been tested in PREVAIL I in Liberia but stopped at phase III clinical trials due to the decline in new Ebola cases. Vaccine cAd3-EBOZ is developed by the use of chimpanzee cold virus vector bearing EBOV gene insert as a non-replicating virus. Vaccine rVSV-ZEBOV uses a vesicular stomatitis virus vector carrying an EBOV gene insert.

1.2 Cell-to-cell transmission

Viral entry is the most critical step that initiates virus to invade host cells and facilitate viral replication. Recently, the mode of virus spreading among cells, so called cell-to-cell
transmission, has been shown to contribute to efficient viral infection and pathogenesis. Several reports in retroviral and lentiviral infection have shown that the cell-to-cell transmission of virus is up to 10000 fold more efficient than the cell-free virus infection (69, 70). Similar phenomenon has also been observed in other viruses, such as influenza virus, vesicular stomatitis virus, and EBOV (51, 71, 72).

1.2.1 Modes of viral transmission

Growing evidence shows that many viruses have distinct modes to enter target cells, such as cell-to-cell transmission and cell-free infection, as suggested in earlier studies of retroviruses, and orthomyxoviruses (Figure 1-6). The cell-cell contact between infected and uninfected cells provides advantage of higher efficiency for viral spread compared to cell-free infection (e.g. from 100 to 1000 folds more in HIV) among a small population of closely connected cells. In contrast, cell-free infection has relatively less efficiency but could infect distant cells without the restriction of contact. Either or both of these mechanisms of viral infections could be utilized by viruses during viral spread, depending on the types of viruses. The advantages of viral cell-to-cell transmission are studied and generally summarized as follows:

1. Avoiding the barriers that hinder cell-free infection. Fewer steps are needed for viral cell-to-cell transmission, which makes viruses less sensitive to antiviral factors at both intracellular and extracellular compartments. Cellular factors that often affect viral protein expression, assemble and release are also less effective for cell-to-cell
transmission.

2. Formation of biological synapse in cell-cell contact efficiently orchestrates and delivers viral particles into target cells. Viral adhesion on target cells can be supported by a high local concentration of viral particles and receptors on cell surface, thus increasing the local multiplicity of infection (MOI) and providing a favorable environment for viral endocytosis.

3. Cell-to-cell transmission circumvents some restrictions of cell-free infection. One classic example is that the extracellular Vaccinia virus remains on the surface of viral producer cells and spreads by cell-to-cell transmission, which is deliberately enhanced by virus-stimulated actin polymerization. Recently, studies in IAV also showed that the infected cells can form nanotubes protruding into neighbor cells and promote cell-to-cell spread.

4. Cell-to-cell transmission has a lower threshold for a successful viral spread, compared to cell-free infection. It has fewer requirements and less cellular cost for cell-free infection, such as sufficient gene expression, sufficient level of cellular factors for viral particles assembly and release, sufficient viral stability and efficient viral entry.
Figure 1-6: Modes of transmission between cells.

Viral transmission could occur either by cell-free infection (a) or via the junctions between cells (b, c) (73) (permission number: 3907211161323).
Figure 1-6: Modes of transmission between cells.
1.2.2 Roles of biological synapses in viral cell-to-cell transmission

The word “synapse” means “point of junction” in Greek, as the combination of meaning “with” and “to join” for “syn” and “aptein”. It is recently used in neurology, immunology and virology, describing distinct features of connected cells: First, cells in contact with others are still individual; second, an unique junction is stably formed at cell-cell contact; Third, transmit of materials occurs at the junctions (i.e. neurotransmitters for neural synapses (NSs); signaling or secretion of cytokines for immunological synapses (ISs); viral materials for virological synapses (VSs)).
1.2.2.1 Role of neural synapses (NSs)

Neural synapses (NSs) were first described 100 years ago as entities for intracellular signaling. Recently NSs have also been shown to play a role in neurotropic viruses spread (74). As to relay information (i.e. neurotransmitters) across neurons, axons at NSs support the microtubule-mediated transmission of viruses (Figure 1-7). Literatures showed that capsids of Alpha Herpesviruses (HSV) were accumulated at axon terminus, suggesting a basic requirement of cell-cell contact for viral spread.
Figure 1-7: The connecting microtubules between neurons have been utilized by transmission of herpesviruses.

The HSV infected cell is depicted as blue, while the uninfected target cell is depicted as green. The block arrow indicates one direction of viral transmission. Of note, the viral transmission could mutually occur for both directions (73) (permission number: 3907211161323).
Figure 1-7: The connecting microtubules between neurons have been utilized by transmission of herpesviruses.
1.2.2.2 Role of immunological synapses (ISs)

Immunological synapses (ISs) were documented 30 years ago, when they were named as the observation of a functional and structured molecular architecture between immune cells (i.e. between DCs and T cells; between T cells and antigen-presenting cells (APCs)). It was found that the formation of ISs could either activate the differentiation of naïve T cells by engagement of T cells receptor (TCR), resulting in the raise of immune response against, or in contrast, facilitates viral spread in secondary lymphoid organs (SLOs) through the interactions between T cells and APCs. Recent reports showed that IS also plays an important role in HIV persistence in resting memory CD4+ T cells, where HIV production is inactivated but proviruses are retained as forming a long-live reservoir for HIV latency. Thus, the clearance of HIV becomes challenging due to the contribution of ISs for HIV persistence, as well as the insufficient restriction of viral replication that saves a small population of infected cells.

The spatial organization of ISs is composed of concentric rings of membrane receptors at cell surface, which are detectable under confocal microscopy and named as supramolecular activation clusters (SMACs) (75). During T cells activation, three types of SMACs are formed at the cell surface, including the central SMAC (cSMAC), the peripheral SMAC (pSMAC) as well as the distal SMAC (dSMAC) (Figure 1-8). The composition of ISs is dynamic during the priming of naïve T cells, initiated by interacting with DCs in SLO. At early stage of ISs formation, the TCR microclusters composed by
TCR and major histocompatibility complex class II (MHCII) complexes, are accumulated at dSMAC, and then move toward cSMAC along with signaling molecules for T cells activation, such as Zeta-chain-associated protein kinase 70 (ZAP-70), lymphocyte-specific protein tyrosine kinase (Lck), the Linker for Activation of T cells (LAT) and Lymphocyte cytosolic protein 2 (SLP-76). Large molecules (i.e. CD43 and CD45), are retained far from the center of IS and make up dSMAC. The signaling activity of these molecules is lost during the migration toward cSMAC, and the microclusters are further surrounded and stabilized by pSMAC that are rich in adhesion molecule LFA-1 and its ligand Intercellular Adhesion Molecule 1 (ICAM-1). Critical receptors such as CD4 as well as CXCR4 and CCR5, are recruited in the formation of cSMAC. ISs are terminated by down-regulation of TCRs via GTPase-mediated internalization. In addition to the formation of SMAC, the polarization of adhesion molecule talin, as well as microtubule organization center (MTOC) are also organized at the cell-cell junction. Taken together with remodeling of actin skeleton, these features become the characteristics of ISs (76, 77).
Figure 1-8: The comparison of VSs and ISs formed between T cells in the context of HIV and HTLV-1 infection.

The infected cells and target cells are depicted as green and blue, respectively. The gag proteins or granules are directed to the sites of synaptic junction associated with indicated viral or host proteins. Distinct features for VSs (A) and ISs (B) are highlighted at sides. The image is adapted from (75) (license ID: 3910320932001).
Figure 1-8: The comparison of VSs and ISs formed between T cells in the context of HIV and HTLV-1 infection.
1.2.2.3 Role of virological synapses (VSs)

Although the cell-to-cell spread of viruses was documented for many years (78, 79), Virological Synapse (VSs) was just recently reported in the context of Human T-lymphotropic virus type 1 (HTLV-1) infection between connected lymphocytes, as it meets the minimum criteria for the definition of synapse previously described (80). Instead of transmitting through cell-free route, the spread of HTLV-1 is directed between infected and uninfected lymphocytes across viruses-induced “synapse” with 10000 folds higher efficiency. Under confocal microscope, the accumulation of HTLV-1 Gag and Env proteins were observed shortly after T cells conjugates are formed, when the transmit of HTLV-1 proteins as well as viral genome into uninfected cells would occur (81).

VS formed during HIV cell-to-cell transmission is another comprehensively studied example, which occurs at junctions of DC-T cell or T-T cell (77, 82, 83). There are currently three described models of HIV transmission in which VSs may play a role (75). In the first model, HIV is captured by antigen presenting cells (APCs) via interactions between HIV Env proteins and specific receptors (e.g. CD4 for T cells; DC-SIGN for DCs) at the surface, followed by subsequent internalization and accumulation of viruses in the intracellular compartment. Currently for DCs, a question of whether the cells are infected or not during the delivery of HIV is still controversial. Viral particles are further redistributed to VSs (also called “infectious synapse” in DCs (83)), which leads to the secondary infection of target cells that express HIV receptors, such as CD4.
The second model is cell-to-cell spread between infected T cells and targeting HIV permissive epithelial cells which are in a close contact at mucosa barrier. The target cells include macrophage and epithelial cells expressing receptors of HIV (e.g. galactosylceramide). The spread of HIV via VSs has been only observed in vitro system, and thus whether or not the transmission occurs via VSs in vivo is uncertain.

The third model is in the context of sexual transmission of HIV, where contact between HIV infected cells and non-permissive epithelial barrier cells facilitates a rapid transfer and replication of HIV in the mononuclear cells at the basolateral side (84). The process so-called “transcytosis” facilitates the delivery of viral particles through the epithelial barrier without the barrier cells becoming infected.
1.2.3 Experimental approaches for study of viral cell-to-cell transmission

Several experimental techniques have been explored to study viral cell-to-cell transmission. One is the development of the transwell plates, in which a porous membrane prevents the contact of the infected cells from the uninfected targets. This membrane sufficiently blocks the cell-to-cell transmission, while it allows cell-free infection to occur. For certain viruses that infect suspension cells, such as HIV, shaking of the infected cells would also help to break newly formed cell-cell contact during viral inoculation. By contrast, the treatments of viscous agent (i.e. methylcellulose) would specifically and sufficiently restrict the diffusion of cell-free viruses but not of cell-to-cell, which allows us to individually measure viral infection as either cell-to-cell or cell-free.

One common method for cell-to-cell transmission has been described by Walther Mothes’ lab at Yale University School of Medicine, which provides a useful tool that allows the measurement of viral cell-to-cell transmission separated from cell-free infection (85) (Figure 1-9).
**Figure 1-9: Schematics of the HIV cell-to-cell transmission assay.**

In this experiment, a specifically signed plasmid that encodes both full length of HIV\textsubscript{NL4-3} and intron-regulated Gaussia Luciferase reporter has been used to transiently transfect 293T cells to produce HIV viruses. Since the intron suppresses the expression of the Gaussia Luciferase construct in the viral producer cells, the Luciferase signals can be detected only in the infected cells. As shown in Figure 1-9, viral producer cells were co-cultured with uninfected target cells for an indicated period of time, when the combined Luciferase signals were measured in the supernatant, which reflecting both cell-to-cell transmission and cell-free infection. For the signal induced by cell-free infection of HIV, same amount of viral producer cells were separately cultured to generate viruses within the same period of time, and the viruses were titered by the cell-free infection. Therefore, the Luciferase expression induced by cell-to-cell transmission is determined by the comparison of cell-free and total signals. The image is adapted from (85) with permission (https://creativecommons.org/licenses/by/4.0/).
Figure 1-9: Schematics of the HIV cell-to-cell transmission assay.
1.2.4 Pathogenesis of viral cell-to-cell transmission

The fast kinetics of viral cell-to-cell transmission always conjugates their clinical pathogenesis. Early study showed that the viral spread of retroviruses via cell-cell contact is less sensitive to neutralizing antibodies as well as other antiviral factors, including Tetherin and TRIM5α (85), which provides a viral antagonism that evades host immune response. It is also possible that rapid spread of viral cell-to-cell transmission may contribute to the cytokine storms of innate immune responses, as observed from EBOV pathogenesis (86).

Besides supporting cell-to-cell transmission of HIV, cell-cell contact can also drive the syncytium formation of HIV permissive cells, resulting in the depletion of immune cells during the development of AIDS. Recently, resting HIV non-permissive CD4 T cells have also been shown to be triggered to induce caspase-1-dependent pyroptosis by cell-to-cell transmission of HIV but not cell-free infection, resulting in increased cell death (87). The high content of provirus could also be observed during the cell-to-cell transmission of HIV, which would increase the incidence of recombination variants, resulting in diversity of viral genome and eventual viral persistence (85).
1.3 Interferon-stimulated genes (ISGs)

1.3.1 Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (Viperin)

The cDNA clone of Viperin was first obtained from IFN-γ-treated primary human macrophages, while homology analysis indicates a high similarity between Viperin and two genes (cig5 and cig33), which are obtained from HCMV-infected human primary foreskin fibroblast (88). A further analysis showed that Viperin is induced in IFN-treated immune cells, suggesting that it may acts as an antiviral factor. In study of Herpesviruses, a set of interferon-stimulated genes in HCMV infected human fibroblast were identified and studied for their antiviral functions. Among these factors, Viperin was found to be largely expressed during the infection of HCMV and greatly inhibits viral replication via downregulating viral structure proteins such as gB, pp28, pp65 (89).

So far, Viperin has been widely studied and shown to significantly inhibit the replication of HCMV and many other viruses (90-92). Interestingly, viruses such as HCMV can redistribute Viperin from endoplasmic reticulum to Golgi apparatus and other intracellular vesicular compartment, reflecting mechanisms that viruses may evolve strategies for developing antagonism (89, 93).
1.3.1 Domains of Viperin

Early research on Viperin indicated that the homologues of Viperin are highly expressed in the response of either IFN stimulation or viral infection among a wide variety of mammals and fishes (i.e. best5 in rat and vig-1 in fish), while their biological functions seem diverse and the effects on viruses remains unclear (89, 94, 95) (Table 1-1).

Viperin is highly conserved among different species, containing 361 amino-acids (in human) with a predicted molecular mass of 42 kDa. Three distinct domains of Viperin have been identified with signatures of conserved functional motifs. The exact amino acid lengths for each domain of Viperin are diverse but could be generally divided as N-terminal domain, central domains (or called SAM domains) and c-terminal domains (Figure 1-10).

The N-terminal domain of Viperin includes the first 70 amino acids, which are the most varied in length and sequence across species. It contains both leucine zipper motif and an amphipathic alpha-helix at the first 42 amino acids, the latter of which mediates its membrane association and subcellular localization (e.g. ER and lipid droplets). The leucine zipper motif is responsible for the proper protein folding and membrane anchoring (96, 97). Of note, the interaction of ER with whole Viperin but not just the amphipathic alpha-helix induces the crystalloid of ER and blocks the secretion of soluble proteins (96). However, whether or not this is relative to Viperin’s antiviral activity
remains unclear. So far, the N-terminus of Viperin is known to be important in inhibition of hepatitis C virus (HCV) and chikungunya virus (CHIKV) (98, 99).

The central domain of Viperin, also named as radical S-adenosylmethionine (SAM) domain, is highly conserved between species and contains the amino acids from residues 71 to 182. This sequence carries 4 characteristic CxxxCxxC motifs of a radical SAM superfamily of MoaA enzymes, which are responsible for binding [4Fe-4S]$^{1+}$ cluster (92). Two recent reports have provided evidence that Viperin reductively cleaves SAM in vitro to generate products of 5’-deoxyadenosine (5’-dAdo), S-(5’-adenosyl)-L-homocysteine and methylthioadenosine (97, 100). In addition, mutagenesis studies suggested that the motifs of Fe-S binding SAM domain are critical for the limitation of the viral replication, such as human immunodeficiency virus (HIV), Bunyamwera virus, hepatitis C virus (HCV) and tick-borne encephalitis virus (TBEV) (101-104). Interestingly, these motifs have also been shown essential for enhancing viral entry of HCMV (93). However, no specific enzymatic activity has been described to Viperin, and the detailed mechanism of how SAM domain contributes its antiviral activity remains unresolved.

The C-terminal of Viperin spans from residues 183 to 361, which are also highly conserved among different species. So far, the exact role of Viperin C-terminus in the context of antiviral activity is not clear. Early studies of Flavivirus showed that the last C-terminal residues of Viperin are critical for the interactions of Viperin with specific viral non-structural proteins (e.g. NS5A for HCV; NS3A for DENV), leading to reduced
viral replication (103, 105). In TBEV infection, the last residue W361 in the C-terminus of Viperin is essential for binding the cytosolic iron-sulfurprotein assembly (CIA) factor CIAO1 to form the assembly of Fe-S cluster, which results in the inhibition of THBV RNA synthesis (101). Altogether, it suggests that the C-terminus of Viperin may play a role in protein-protein interaction.
Figure 1-10: Schematic diagram of Viperin gene.

The major domains are shown in the figure with their known functions. The image is adapted from (106) (permission number: 3907230256115).
Figure 1-10: Schematic diagram of Viperin gene.

N-terminal domain | Central domain | C-terminal domain
(mediates ER and lipid droplet association) | (contains functionally important Fe-S cluster binding motif) | (highly conserved but functionally undefined)
Table 1-1: Summary of Viperin’s antiviral effects and functional domains.

Viruses that are currently known to be affected by Viperin are summarized and shown with their possible mechanisms and critical domains. This table is modified version from (92) (permission number 3907230576074).
Table 1-1: Summary of Viperin’s antiviral effects and functional domains.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Mechanisms</th>
<th>Critical Domains</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV</td>
<td>Inhibits replication via down-regulating viral</td>
<td>nd</td>
<td>(89)</td>
</tr>
<tr>
<td></td>
<td>structural protein gB, pp28 and pp65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCMV</td>
<td>Enhances entry via interaction with cellular SAM</td>
<td>domain</td>
<td>(93)</td>
</tr>
<tr>
<td></td>
<td>mitochondrial protein TFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>Inhibits replication via localization in RC with C’-terminus and amphipathic helix</td>
<td>(103, 107)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCV NS5A and VAP-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV</td>
<td>Inhibits replication via interaction with DENV</td>
<td>C’-terminus</td>
<td>(105)</td>
</tr>
<tr>
<td></td>
<td>NS3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>Inhibits viral egress</td>
<td>SAM domain</td>
<td>(102)</td>
</tr>
<tr>
<td>IAV</td>
<td>Inhibits viral budding from the plasma</td>
<td>nd</td>
<td>(108)</td>
</tr>
<tr>
<td></td>
<td>membrane possibly via interacting with FPPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHIKV</td>
<td>Higher viremia in knockout mice</td>
<td>Amphipathic helix</td>
<td>(98)</td>
</tr>
<tr>
<td>TBEV</td>
<td>Inhibits viral RNA synthesis</td>
<td>SAM domain and C’-terminus</td>
<td>(101)</td>
</tr>
<tr>
<td>WNV</td>
<td>Increased replication in CNS of knockout mice</td>
<td>nd</td>
<td>(109)</td>
</tr>
<tr>
<td>Bunyamwera</td>
<td>Inhibits replication</td>
<td>SAM domain?</td>
<td>(104)</td>
</tr>
<tr>
<td>JEV</td>
<td>Inhibits viral production</td>
<td>nd</td>
<td>(110)</td>
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</tbody>
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53
<table>
<thead>
<tr>
<th>Virus/Infection</th>
<th>Effect</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>Inhibits replication</td>
<td>nd (111)</td>
</tr>
<tr>
<td>RSV</td>
<td>Inhibits replication via inhibiting viral filament formation</td>
<td>nd (112, 113)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Increased replication in knockdown cells</td>
<td>nd (114)</td>
</tr>
<tr>
<td>SINV</td>
<td>Inhibits viral production</td>
<td>nd (110)</td>
</tr>
<tr>
<td>HSV</td>
<td>Inhibits replication of UL41-null HSV-1</td>
<td>nd (115)</td>
</tr>
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</table>

nd: not done
1.3.2 Regulation of Viperin expression

The pathways of Viperin induction in mammalian cells remain incompletely explained (90, 92, 111, 116, 117). Viperin, first identified as cig5 (cytomegalovirus inducible gene 5), is strongly induced in the human cytomegalovirus (HCMV)-infected fibroblasts. So far, Viperin has been demonstrated to be induced by viral infections, IFN-stimulation, such as type I (α and β), II (γ) and III (λ) IFNs, polyinosinic polycytidylic acid (poly (I: C)), Lipopolysaccharides (LPS) as well as double-stranded DNA or RNA (dsRNA) analogues.

As described in the Figure 1-11, Viperin can be induced through the IFN-mediated signaling cascades activated by binding of the pattern recognition receptors, such as RIG-1, MDA5 and TLRs, in either the cytosolic compartments or at the cell surface (92). The IFN-mediated induction pathways of Viperin are diverse and often cell-type dependent during viral infections. For example, HCMV can induce Viperin expression in macrophages, human monocyctic cell lines such as U937 and MonoMac 6, but not in the epithelial cell line such as HeLa cells (89). Among these cell lines, the inductions of Viperin are also varied due to different types of IFNs, as it has been shown to be less or no expressed by the stimulation of type II IFN (γ) compared with type I (α and β) (89). In addition, the IFN-dependent induction of Viperin replies on the binding of different receptors or regulation factors expressed in certain cell lines. For examples, in HCMV-infected trophoblast Viperin is induced by IFN-β through a toll-like receptor 3.
(TLR-3)-mediated signaling (118). Similarly, fish has a Viperin homolog, which is induced by activation of retinoic acid-inducing gene I (RIG-I)-like receptors (RLR)-triggered IFN signaling pathway and exhibits protection against fish viruses, such as grass carp reovirus (GCRV) (119). In mice model, Viperin is also potently induced by TLR-7 and TLR-9 stimulations in plasmacytoid dendritic cells (pDCs) (117). In the lipid bodies of pDCs, Viperin interacts with and recruits the signal mediators IRAK1 and TRAF6 to facilitate the translocation of transcription factor IRF7, resulting in the type I IFN production. Report in Sendai virus infection showed that Viperin expression during viral infection is specifically mediated through a key regulator IFN-stimulated gene factor-3 (ISGF3) complex, independent of RIG-I and mitochondrial anti-viral signaling (MAVS). The host factor, positive regulatory domain I-binding factor 1 (PRDI-BF1, also called BLIMP1) competes binding of ISGF3 and negatively regulates Viperin expression.

Alternatively, during some viral infections (i.e. VSV) Viperin can be induced independently of IFN stimulation via IFN regulatory factor (IRF)-1 or (IRF)-3-mediated pathways, providing mechanisms that evade viral antagonisms of suppressing IFN system (92, 111). Upon recognition by RLR receptors, viral RNA interacts with the adaptor protein MAVS (also known as CARD adaptor inducing IFN-β (Cardif) (120), IFN-β promoter stimulator-1 (IPS-1) (121) or virus-induced signaling adaptor (VISA) (122)) at mitochondria to activate immune response. Recent reports showed that MAVS is also localized at peroxisomes and is able to mediate a rapid, transient and IFN-independent
expression of ISGs upon virus infection, in which IRF1 and IRF3 play an important role (123). This is different from the mitochondrial localized MAVS, which activates type I IFN-dependent immune responses and establishes a stable antiviral state. Moreover, the peroxisomal MAVS has also been shown to selectively induce an IRF-1-mediated IFN-λ response during RNA virus infection (124).
Figure 1-11: Schematic diagram of Viperin’s IFN-dependent and -independent induction pathways.

Upon viral infections, Viperin is induced via multiple pathways and exhibits antiviral functions. The activation of different Pattern Recognition Receptors (PRR) up-regulates the IRF-1 and IRF-3-mediated signaling, which can either directly induce Viperin expression or via type-I IFN signal cascades. The image is adapted from (92) (permission number 3907230576074).
Figure 1-11: Schematic diagram of Viperin’s IFN-dependent and -independent induction pathways.
1.3.3 Biological functions of Viperin

Recently, the mRNA of Viperin has been shown to serve as a specific substrate of human RNase MRP, which is a conserved endoribonuclease for autosomal recessive skeletal dysplasia such as cartilage-hair hypoplasia (CHH) (125). The analysis of DNA microarray indicates an up-regulated Viperin mRNA in a response to siRNA-mediated knock down of human RNase MRP. Two cleavage sites for human RNase MRP in the Viperin mRNA have been indentified, which together provides first evidence that human RNase MRP cleaves an mRNA in human cells.

Viperin has also been shown to affect T cells activation and differentiation in mice. Knockout of Viperin in splenic CD4+ T cells significantly reduces the secretion of T helper 2 (Th2) cytokines, such as IL-4, IL-5 and IL-13, and exhibits attenuated GATA activation. In addition, Viperin is also required for the DNA binding of NF-κB1/p50 and AP-1/JunB at downstream of TCR-mediated signaling (126).

Based on functional and structural analysis, Viperin belongs to the Radical SAM superfamily of MoaA enzymes, as it contains four conserved CxxxCxxC motifs in its central domain and binds to Fe-S cluster (97, 127). The catalytic function of MoaA enzymes is required for the early steps of Molybdopterin biosynthesis, which is essential for enzymatic reactions in life (127).
1.3.4 Virological functions of Viperin

So far, Viperin has been reported to inhibit infections by a number of RNA viruses, including human immunodeficiency virus (HIV), influenza A virus (IAV), Sendai virus (SV), vesicular stomatitis virus (VSV), West Nile virus (WNV), hepatitis C virus (HCV), Chikungunya virus (CHIKV) and etc (89, 91, 98, 102, 103, 105-107, 109, 115, 128) (Figure 1-12). However, the underlying mechanism of how Viperin inhibits viral replication remains elusive.

Besides HCMV, one recent report showed that Viperin could restrict influenza A virus (IAV) release by disrupting lipid rafts through inhibiting farnesyl diphosphate synthase (FPPS), an enzyme essential for lipid biosynthesis (108). This mechanism suggests that Viperin may interfere other viruses’ budding that also requires lipid rafts, such as EBOV and Marburg virus (MARV) of the filovirus family.

In addition, Viperin could inhibit the replication of Flavivirus, such as HCV, DENV and TBEV (99, 101, 103, 105, 107, 129). The C-terminus of Viperin is required for HCV inhibition. Under confocal microscope, Viperin has been shown to be co-localized at the interface of lipid droplets, in association with HCV non-structure protein NS5A and cellular factor VAP-A. Consequently, it blocks the formation of viral replication complex and therefore reduces HCV replication (103). Similarly, Viperin has also been shown to interact with DENV non-structure protein NS3A at viral replication complex via its C-terminus, although the localization of lipid droplets via its interaction of
N-terminus seems somehow not related (105). Different from DENV, the replication of TBEV has been shown to be inhibited by Viperin mainly via its SAM domain. The significance of Viperin’s interaction with cytosolic Fe/S protein assembly factor CIAO1 is highlighted in reference (101).

Interestingly, one early report in HCMV showed that Viperin can enhance HCMV entry (130). This is considered as a viral antagonism, which will be discussed in the next section.
Figure 1-12: Viperin is a multiple-functional protein.

As shown in this figure, Viperin acts as wither antiviral factor or proviral factor. The image is adapted from (92) (permission number 3907230576074).
Figure 1-12: Viperin is a multiple-functional protein.
1.3.5 Escape of Viperin by virus

As an ISG, Viperin has been reported to regulate a broad-spectrum of RNA and DNA viruses, such as HCMV, JEV and HSV (89, 110, 115). The antiviral activity of Viperin likely serves as the driven force that promotes viruses to develop new strategies for antagonism (131-133). So far, Viperin has been shown to be antagonized by some viruses (92).

The first known example is observed during the study of HCMV (89). Viperin was shown to inhibit the replication of HCMV but also conversely been redistributed by viral structural proteins from ER to other vesicular compartments, such as Golgi apparatus. This early report suggests a potential strategy that HCMV evades its antiviral activity. Later the same group demonstrated that the viral mitochondrial inhibitor of apoptosis protein (vMIA) of HCMV can interact with and re-localize Viperin at mitochondria to bind the mitochondrial trifunctional protein (TFP). This interaction of Viperin with TFP interferes the ATP generation and thus disrupts actin cytoskeletons of host cells, resulting in enhanced HCMV entry (93).

JEV has been reported to evade the antiviral activity of Viperin (110). It was shown that the mRNA of Viperin is highly expressed in Japanese encephalitis virus infection (JEV)-infected human A549 cells but with no detectable Viperin expression. Viperin expression is restored by the treatment of MG132 and thus inhibits the replication of JEV, suggesting that JEV induces Viperin expression but counteracts its antiviral function.
though proteasome-mediated degradation.

Recently, Viperin has been reported to be counteracted by HSV virion host shutoff protein, named UL41 (115). Results indicated that Viperin expression in infected cells was continuously reduced when MOI of HSV was increased. A high throughput screening analysis further indicated that the ectopically expressed viral protein UL41 reduces the mRNA level of Viperin. This was thus confirmed that the replication of UL41-null HSV, but not WT, can be inhibited by Viperin overexpression.
1.3.6 Positive selection of Viperin

Similar to viral evolution, antiviral factors also evolve adaptively against viral antagonisms within different species, providing new mechanisms that benefit hosts for surviving. Since viruses have broad but specific tropism, homologs of ISGs indentified in different species often manifest distinct antiviral functions. Notably, some of positively-selected ISGs in non-human primates show stronger antiviral effects, such as PKR, TRIM5α and APOBEC3G, than that of human counterparts (134-137).

Viperin has been shown to evolve under positive selection in primates (134). 20 primates Viperin sequences from hominoids, Old world Monkey and New world monkey were used to construct a cladogram of Viperin phylogeny, followed by free-ratio analysis of dN/dS ratios for each branch. Results indicated that several branches of Viperin with radios > 1 were under positive selection. The relationship between the antiviral activity of Viperin and its positive selection in evolution has so far been only tested in the context of lentiviruses and retroviruses (134). However, no significant differences of Viperin orthologs against different lentiviruses and retroviruses were observed. Therefore, the actual pressure of Viperin’s evolution still remains uncovered, which requires further experimental analyses in the future.
II. CELL-CELL CONTACT PROMOTES EBOV

GP-MEDIATED INFECTION

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2.1 Abstract

EBOV is a highly pathogenic filovirus that causes hemorrhagic fever in humans and animals. Here we provide evidence that cell-cell contact promotes infection mediated by the glycoprotein (GP) of EBOV. Interestingly, expression of EBOV GP alone, even in the absence of retroviral Gag-Pol, is sufficient to transfer a retroviral vector encoding Tet-off from cell to cell. Cell-to-cell infection mediated by EBOV GP is blocked by inhibitors of actin polymerization, but appears to be less sensitive to KZ52 neutralization. Treatment of co-cultured cells with cathepsin B/L inhibitors, or an entry inhibitor 3.47 that targets the virus binding to receptor NPC1, also blocks cell-to-cell infection. Cell-cell contact also enhances spread of rVSV bearing GP in monocytes and macrophages, the primary targets of natural EBOV infection. Altogether, our study reveals that cell-cell contact promotes EBOV GP-mediated infection, and provides new insight into understanding of EBOV spread and viral pathogenesis.

2.2 Introduction
EBOV belongs to the filovirus family and causes severe hemorrhagic fever in humans and animals. The fatality rates of the disease induced by EBOV can reach up to 90%, with currently no effective antiviral drug or FDA-licensed vaccine available (138, 139). A better understanding of EBOV infection, especially the early stage of viral transmission, would facilitate the development of novel therapeutic approaches to combat this deadly disease.

EBOV infection of host cell is mediated by its sole glycoprotein, known as GP. GP is synthesized as a precursor (GP0) in the endoplasmic reticulum, cleaved into GP1 and GP2 in the Golgi apparatus, and eventually targeted to the plasma membrane for viral incorporation (140-142). During this process, EBOV GP is modified by N- and O-linked glycosylation, the exact functions of which are still not well understood (143, 144). In mature virions, EBOV GP exists as a homotrimer, with each monomer composed of GP1 and GP2 subunits that are linked by a disulfide bond and non-covalent interactions (145, 146). As is the case for many class I viral fusion proteins, GP1 is responsible for interacting with cellular receptors or cofactors, whereas GP2 is directly involved in fusion of EBOV with target cell membranes (141, 145, 147-152).

The detailed molecular mechanism underlying EBOV GP-mediated infection is currently unknown (31). However, sufficient evidence has indicated that EBOV enters host cells through macropinocytosis (47, 48, 153), a process that is initiated by binding of EBOV GP to attachment factors, such as DC-SIGN and TIM-1 (48, 154-157). Following
the uptake of viral particles into late endosomes and lysosomes, GP1 is cleaved by cellular cysteine proteases, especially cathepsins B (CatB) and L (CatL), resulting in the production of a fusion-competent intermediate (158-160) that binds to the recently identified intracellular receptor, Niemann-Pick type C1 (NPC1) (41, 54, 161). Studies from several groups have shown or suggested that NPC1, low pH, and possibly mild reduction of GP are important for EBOV GP-mediated infection (24, 53, 54, 148, 158).

Cell-to-cell transmission has been shown to play important roles in the dissemination and pathogenesis of many pathogenic viruses, including HIV and HCV (71, 73, 162, 163). In this work, we provide evidence that cell-cell contact facilitates infection mediated by EBOV GP and this process requires cellular cathepsins and NPC1. Our work supports the idea that cell-to-cell infection may be another means of EBOV spread and could serve as a potential target of viral therapeutics.

2.3 Results

**Cell-cell contact promotes EBOV GP-mediated retroviral infection.** EBOV is a BSL-4 agent; thus, in this study we employed several systems, including retroviral pseudotypes, virus-like particles (VLPs), as well as rVSV that bear GP to determine cell-to-cell infection. For the retroviral system, we co-transfected 293T cells with the pQCXIP retroviral vector encoding a tetracycline-controlled transcription factor (tTA, referred to as Tet-off hereafter), along with plasmids that encode EBOV GP and murine leukemia virus (MLV) Gag-Pol. Following 24-h transfection, donor 293T cells producing
pseudovirions were co-cultured with target 293FT cells stably expressing tetracycline-responsive element (TRE)-driven *Gaussia* luciferase (293FT/TRE-GLuc); cell-to-cell infection efficiency was assayed by measuring the GLuc activity of media after a 24-48 h co-culture (Figure 2-1A, top). To control cell-free viral infection, we cultured the same numbers of transfected donor cells for the same period of time (24 h), and the collected supernatants were used to infect target 293FT/TRE-GLuc cells that had been pre-mixed with parental untransfected 293T cells; this procedure would ensure the same numbers of cells to be used for cell-free infection (Figure 2-1A, bottom). Donor 293T cells expressing VSV-G or no envelope served as controls for cell-to-cell and cell-free infections.

Expression of EBOV GP in donor 293T cells led to a ~130-fold higher GLuc activity compared to the mock control (“No-Env”) (Figure 2-1B). In contrast, the cell-free infection was only 3~5-fold above the background (Figure 2-1B), thus resulting in a 40-fold difference between cell-to-cell and cell-free infections. Similarly, the cell-to-cell infection efficiency mediated by VSV-G was much more higher than the cell-free infection, i. e, ~70-fold, although VSV-G exhibited much a generally higher Gluc activities than EBOV GP in both cell-to-cell and cell-free infections (Figure 2-1B). To confirm the greater cell-to-cell vs. cell-free infection mediated by EBOV GP, we next applied a *Transwell* culture system, where cell-to-cell infection was measured by co-culturing donor and target cells on the bottom of the Transwell plates, and the cell-free
infection was achieved by seeding the same number of donor cells on the top and target cells on the bottom, allowing cell-free virions to migrate through a 0.45-μM membrane. In this system, we observed a 70-fold and 40-fold difference between cell-to-cell and cell-free infection for EBOV and VSV, respectively (Figure 2-1C).

We treated co-cultured cells with KZ52, a broadly neutralizing antibody against EBOV, and observed that while KZ52 still inhibited cell-to-cell infection mediated by EBOV GP (Figure 2-1D), the efficiency was consistently lower than that of cell-free infection; specificity was confirmed by the absence of an effect of KZ52 on VSV-G (Figure 2-1D). We also treated co-cultured cells with inhibitors of actin polymerization, such as latrunculin B (LAT-B) and cytochalasin D (CytoD), which are known to block cell-to-cell transmission of other viruses (162), and we found that both drugs strongly inhibited, in a dose-dependent manner, cell-to-cell infection mediated by EBOV GP and VSV-G (about 5~10 fold, Figure 2-1E and F). Interestingly, cell-free infection of EBOV was also inhibited by LAT-B and CytoD, but only ~2 fold; noticeably, the effect of CytoD on cell-free infection was not dose-dependent, suggesting possible cytotoxicity at higher doses (data not shown). Collectively, these results revealed that cell-cell contact can promote EBOV GP-mediated infection, a phenomenon that has recently been reported for HIV, hepatitis C virus (HCV) and influenza A virus (IAV) (71, 162-164).

Ebola virus-like particles (VLPs) bearing GP are efficiently transferred from cell to cell. We next evaluated if cell-cell contact can also promote EBOV GP-mediated
transfer of VP40 VLPs by using VP40-Blam-based virion-fusion and VP40-GFP-based uptake assays. Upon a 2 h co-culture, approximately 8.5% of 293FT/tdTomato target cells exhibited beta-lactamase activity, the effect of which was inhibited by KZ52, LAT-B and CytoD (Figure 2-2A and B). In contrast, no beta-lactamase activity was detected for cell-free infection performed in parallel (Figure 2-2C, second bar in “EBOV” group). Similar results were also obtained for VSV-G, although the cell-to-cell transfer efficiency was higher for VSV-G and it was not inhibited by KZ52 (Figure 2-2A and B). Spinoculation of the same cell-free VLP stock to infect the same number of target cells resulted in ~4.7% of fusion-positive cells (FACS plots not shown), suggesting that the inability to detect beta-lactamase activity in the cell-free infection without spinoculation was not due to a lack of cell-free virions produced from the donor cells (Figure 2-2C).

To directly measure VP40 protein transfer, we transfected 293T donor cells with VP40-GFP in the presence or absence of GP, and co-cultured the transfected cells with target 293FT/tdTomato cells. Similar to the results obtained for VP40-Blam, cell-to-cell transfer mediated by EBOV GP and VSV-G was detected after a 2 h co-incubation and the levels increased after 6 h (Figure 2-2D and E). Of note, VP40-GFP alone exhibited some levels of transfer (possibly due to lipid-mediated non-specific transfer reported for EBOV), albeit at lower levels as compared to cells co-expressing GP or VSV-G (Figure 2-2D and E). Once again, cell-cell transfer mediated by EBOV GP, but not by VSV-G or VP40 alone, was inhibited by KZ52, although LAT-B and CytoD both effectively blocked
cell-to-cell transfer mediated of EBOV GP or VSV-G, but not by VP40 alone (Figure 2-2D and E).

Cell-to-cell transfer mediated by EBOV GP can occur without retroviral Gag-Pol. Because of high sensitivity, we decided to use the Tet-off-based retroviral pseudotype system to interrogate the cellular and viral determinants required for cell-to-cell transfer mediated by EBOV GP. We first explored if EBOV GP alone is sufficient to mediate transfer of retroviral vector encoding Tet-off in the absence of MLV Gag-Pol, and found that, indeed, expression of EBOV GP in 293T donor cells (along with Tet-off but without MLV Gag-Pol) consistently led to a significant level of Gluc activity compared to the mock Tet-off control (~53 fold, compare bar 3 with bar 4; \( p < 0.001 \)). Notably, inclusion of MLV Gag-Pol in donor cells only further increased the Gluc activity by ~2-fold (compare bar 1 with bar 3; Figure 2-3A). Similarly, expression of VSV-G in the absence of MLV Gag-Pol also resulted in a marked increase in Gluc activity, again with less than a 2-fold enhancement by Gag-Pol in donor cells (Figure 2-3A). To assess if EBOV GP-mediated transfer of Tet-off from cell to cell is due to a direct cell-cell fusion on the plasma membrane, we treated co-cultured cells with either neutral or low pH; however, no apparent differences between neutral and low pH in Gluc activity were found (Figure 2-3B), nor syncytia formation was observed (data not shown). In contrast, Gluc activity was significantly increased (20-fold) for VSV-G upon a low pH pulse (Figure 2-3B). To further confirm that the increased Gluc activity of EBOV GP was not due to a direct
cell-cell fusion on the plasma membrane, we expressed GP in target cells and Tet-off in donor cells; again, we observed no increase in Gluc activity compared to mock control regardless of pH treatment (Figure 2-3C). The ability of this EBOV GP, which is tagged with a FLAG at the N-terminus and has no mucin domain, to mediate cell-to-cell transfer without MLV Gag-Pol was also confirmed in additional EBOV GP constructs: full length GP WT without any tag (“Native-GP”) (141), full length GP with an N-terminal FLAG tag (“F-GP”), and mucin-deleted GP without an N-terminal FLAG (“Δmuc-GP”) (Figure 2-3D).

A previous study has shown that Tet-off is present in exosomes or nanovesicles that can be secreted into culture media and transferred to adjacent cells (165). We therefore evaluated if the Gluc signal we detected might be due to secretion of exosomes containing GP and Tet-off from donor cells. We thus treated co-cultured cells with a chemical inhibitor of exosomal production, i. e, GW4869 (166), and we observed that GW4869 indeed reduced the Gluc activity induced by EBOV GP or VSV-G in a dose-dependent manner (2, 5, and 10 μM) (Figure 2-3E). Western blotting analysis showed that purified exosomes contained an abundant level of GP as well as the common exosome marker CD63 (167, 168) (Figure 2-3F). We then treated target 293FT/Gluc cells with purified exosomes, and observed increased Gluc activity in target 293FT/gluc cells compared to exosome purified from mock cells not expressing GP (Figure 2-3G). Taken
together, these results suggest that exosomes secreted from donor cells contributed to the increased Gluc activity of EBOV GP (see Discussion).

**Cleavage of EBOV GP by cathepsins is required for mediating cell-to-cell transfer.**

We next determined if GP-mediated enhancement of cell-to-cell transfer, even in the absence of a retroviral core, requires some of the essential factors known to be necessary for EBOV infection. Cleavage of EBOV GP by cathepsins B and L is required for EBOV infection, possibly by priming conformational changes of GP1 needed for receptor binding prior to membrane fusion (158-161, 169). We applied a panel of protease inhibitors, including cathepsins B and L (referred to as CatB and CatL hereafter), and evaluated their effects on EBOV GP-mediated Tet-off transfer from donor cell to target cell. As shown in Figure 2-4A, the pan-spectrum protease inhibitor leupeptin, the pan-cysteine cathepsin inhibitor E64d, as well as the CatB specific inhibitor CA074 all greatly inhibited Tet-off transfer mediated by EBOV \((p < 0.001)\). In sharp contrast, none of these inhibitors had any effect on IAV (Figure 2-4A). The reductions in EBOV GP-mediated cell-to-cell transfer caused by E64d and CA074 were dose dependent, with roughly comparable efficiencies (Figure 2-4B; \(p < 0.01\) or 0.001). We also treated co-cultured cells with cathepsin L inhibitor III, which inhibited EBOV GP-mediated but not IAV HA-mediated cell-to-cell transfer (Figure 2-4C). To distinguish if the effects of these protease inhibitors were on donor cells or target cells, we treated donor cells with leupeptin for 24 h before and during co-culture, or treated both donor and target cells
during co-culture. Treatment of donor cells alone had no effect on Gluc activity; however, treatment of target cells alone or in combination with donor cells significantly decreased Gluc activity (Figure 2-4D). These results indicate that cathepsin activity in target cells, rather than in donor cells, is crucial for GP to mediate cell-to-cell transfer.

Given that the protease activities of CatB and CatL are dependent on the acidification of late endosomes and lysosomes (158), we next treated co-cultured cells with NH₄Cl, a lysosomotropic agent, which neutralizes the acidic pH of late endosomes and lysosomes and thereby blocks EBOV infection (140). Cell-to-cell transfer mediated by EBOV GP and VSV-G was both strongly inhibited by NH₄Cl in a dose-dependent manner (p < 0.001) (Figure 2-4E). These results together demonstrated that pH-dependent cathepsin activity is required for cell-to-cell transfer, a property that is in line with cell-free EBOV entry.

**NPC1 and TIM-1 promote cell-to-cell transfer mediated by EBOV GP.** Several cellular factors have recently been reported to function as receptors or cofactors for EBOV, with NPC1 and TIM-1 being the most prominent (23, 41, 54, 154). We evaluated the possible roles of these two cellular factors in EBOV GP-mediated infection by cell-cell contact. To facilitate our analyses, we established target 293FT/Gluc cell lines stably expressing full length NPC1, or the NPC1 domain C without the cytoplasmic tail (gift of Kartik Chandran referred to as NPC1 CT hereafter) (54), or human TIM-1 (170). We found that full-length NPC1, NPC1 CT, as well as TIM-1 all enhanced cell-to-cell transfer mediated by EBOV GP (p < 0.01 or 0.001), with no differences apparent between
neutral and low pH (Figure 2-5A). The expression of these cellular proteins in target cells was confirmed by Western blotting (Figure 2-5B). Immunofluorescence staining revealed that TIM-1 was predominantly expressed on the cell surface, as expected; NPC1 CT was also detectable on the cell surface, similar to a previous report (54), but with much less fluorescence intensity than found for TIM-1 (Figure 2-5C). The full length NPC1 protein was primarily expressed in intracellular vesicles (Figure 2-5C).

We next knocked down NPC1 in 293FT/Gluc target cells by transducing cells with shRNA lentiviral vectors, and examined their effects on cell-to-cell transfer mediated by EBOV GP. Among the 5 shRNA clones tested, one clone (clone 1, Sigma #5428) consistently exhibited the strongest inhibition of cell-to-cell transfer mediated by EBOV GP ($p < 0.01$ or 0.001) (Figure 2-5D). The level of NPC1 expression in 293FT/Gluc target cells expressing this shRNA clone was most reduced compared to cells expressing other shRNAs and mock control (Figure 2-5E). We also treated co-cultured cell with 3.47, a small molecule inhibitor that specifically blocks EBOV GP to bind to NPC1, and found that it significantly inhibited EBOV GP-mediated cell-to-cell transfer ($p < 0.001$) in a dose-dependent manner (Figure 2-5F). Altogether, these results demonstrate that endogenous NPC1 does play a role in EBOV GP-mediated cell-to-cell transfer, consistent with its reported role in EBOV cell-free infection (23, 41, 54).

The receptor-binding property of EBOV GP is critical for cell-to-cell transfer. We further investigated the role of receptor binding in EBOV GP mediated infection by
cell-cell contact by making a series of GP mutants in GP1—F88A, K95A, K114A, K115A and K140A—which have been previously shown to impair the binding of EBOV GP to putative receptors and NPC1 (54, 145, 147, 150-152, 160, 171). We found that all of these mutants exhibited significantly decreased cell-to-cell transfer efficiency; F88A and K95A had the most dramatic phenotype, showing an almost complete loss in Tet-off transfer ($p < 0.001$) (Figure 2-6A). Consistent with the cell-to-cell transfer data, the cell-free infection of these mutants encoding alkaline phosphatase (AP), especially F88A and K95A, was also greatly reduced compared to the parental GP, albeit to different extents ($p < 0.05, 0.01$ or $0.001$) (Figure 2-6B). Western blotting analysis showed that all these GP mutants were well expressed and processed into GP1 and GP2 in viral producer cells, although to somewhat different levels or efficiencies (Figure 2-6C). While the relatively low level of expression of F88A could have contributed to its low cell-to-cell transmission activity, most mutants, including K95A, were well expressed in transfected cells, including on the cell surface (Figure 2-6D and E), indicating that the reduced cell-to-cell and cell-free infection efficiencies observed for most of these GP mutants likely reflect their intrinsic low receptor binding and/or fusion potential.

We next evaluated two additional GP mutants, D47A and I584F, which have been recently shown to mediate EBOV entry in a somewhat CatB-independent manner (50, 169). Indeed, adding the CatB inhibitor CA074 to co-cultured cells did not reduce cell-to-cell transfer for either mutant, especially I584F, to the same extent as for the
parental GP at all concentrations tested ($p < 0.01$ or $0.001$) (Figure 2-6F). The relative insensitivity of mutants GP1 D47A and GP2 I584F to CA074 is consistent with their relative independence from CatB-mediated priming in membrane fusion and EBOV entry.

Co-culturing of epithelial cells and macrophages promotes spread of recombinant VSV (rVSV) bearing EBOV GP. The above data suggest that EBOV GP can efficiently mediate transfer of VP40 or Tet-off from cell to cell. We next determined if this could also occur in a replication-competent viral system, especially in monocytes and macrophages, which are the primary targets of EBOV infection in vivo. For this purpose, we employed a replication-competent rVSV-GFP system that expresses EBOV GP (rVSV-GFP-GP). We first infected 293T donor cells with appropriate doses of rVSV-GFP-GP so that optimal numbers of GFP positive (green) donor cells would be produced. We then co-cultured these donor cells with target 293FT/Gluc cells that were pre-labeled with CMTMR, and cell-to-cell infection was measured by gating the CMTMR-positive cells for GFP signals. In parallel, we also determined cell-free infection by infecting the same CMTMR-labeled target cells with rVSV-GFP-GP produced from donor cells (see Methods for details).

After 2 h of co-culture, the infection rate of rVSV-GFP-GP, which likely reflected a combination of cell-to-cell and cell-free infection, was low (i.e., 0.86%). Infection was, however, significantly increased at 6 h (9.9%) and 12 h (47.1%) (Figure 2-7A).
contrast, cell-free infection of rVSV-GFP-GP was virtually undetectable before 6 h following co-culture (0.708%), only became quite apparent after 12 h (4.58%); this was 10-fold lower than infection via cell-cell contact. By treating the co-cultured cells with KZ52, LAT-B, or CytoD, as we had done for retroviral pseudoviral and VLP systems, we observed that rVSV-GFP-GP infection via cell-cell contact was strongly inhibited by all three agents (Figure 2-7B). Of note, cell-free infection of rVSV-GFP-GP was also affected by LAT-B and Cyto-D, consistent with a previous report (172).

We then performed a similar cell-to-cell infection assay by using THP-1 or THP-1 cells treated with PMA, which served as either donor or target cells; PMA is known to differentiate THP-1 monocytes to macrophages. In both cases, the rVSV-GFP-GP infection efficiency mediated in co-cultured cells was much greater than was cell-free infection (Figure 2-7C and D). Notably, rVSV-GFP-GP transfer from PMA-treated THP-1 cells to 293FT/Gluc cells was about 10-fold more efficient than transmission from parental THP-1 cells to 293FT/Gluc (Figure 2-7C and D), which likely reflects the low infection capability of donor THP-1 cells by rVSV-GFP-GP as compared to PMA-treated THP-1 cells. However, there was no significant difference between PMA-treated or untreated THP-1 cells that served as target cells (compare bar 1 and bar 3, p > 0.05; Figure 2-7E). Similar to the results shown for 293T-to-293FT, both cell-to-cell infection and cell-free infection of rVSV-GFP-GP were strongly inhibited by KZ52, LAT-B and CytoD (Figure 2-7C and D).
To confirm the results of cell-to-cell spread infection of rVSV-GFP-GP between 293T and 293FT cells, we applied a viscous 1% methylcellulose solution to co-cultured 293T and 293FT/Tomato cells (low MOI infection of donor cells); in parallel, this solution was also applied to cell-free viral infection (higher MOIs were used). Previously, methylcellulose has been used to distinguish between cell-cell and cell-free viral infection (173). As shown in Figure 2-7F and G, methylcellulose almost completely blocked the cell-free infection of rVSV-GFP-GP, yet had no apparent effect on cell-to-cell infection. Taken all above results together, we conclude that coculturing monocytes/macrophages and epithelial cells enhances spread of replication-competent rVSV-GFP-GP in vitro.

2.4 Discussion

Using retroviral pseudotypes, EBOV VP40-based VLPs, as well as rVSVs encoding GP, we demonstrate in this work that cell-cell contact promotes EBOV GP-mediated infection. Interestingly, we found that, the EBOV GP protein alone, even in the absence of the retroviral Gag-Pol, is sufficient to transfer Tet-off from cell to cell, and that the key molecules involved in cell-to-cell transfer parallel that of authentic EBOV infection. We further showed that the cellular cathepsins B/L and NPC1 are essential for cell-to-cell transfer to occur. We ruled out the possibility that cell-cell fusion on the plasma membrane is responsible for the increased Gluc activity by separately expressing EBOV GP in target cells and Tet-off in donor cells, with no Gluc activity detected.
Several lines of evidence support that cell-to-cell transfer of Tet-off from donor cells to target cells mediated by EBOV GP is specific and not due to experimental artifacts. First, the increase in Gluc activity is only detected in cells co-expressing viral glycoprotein GP; it is not detected for cells expressing Tet-off alone. Second, the increased Gluc signal in the culture media of GP-expressing cells, but not that of VSV-G-expressing cells, is diminished by KZ52, a broadly neutralizing antibody against EBOV GP, as well as by 3.47, a small molecule inhibitor that specifically blocks EBOV GP from binding to its receptor NPC1. Third, EBOV GP mutants deficient for NPC1 binding or infection exhibit decreased activities of cell-to-cell transfer efficiency. In addition, we tested an EBOV GP mutant engineered to harbor a furin-recognizing sequence in place of the putative cathepsin L cleavage site and found that this GP exhibits significantly increased cell-to-cell transfer efficiency (data not shown). It is noteworthy that other viral glycoproteins, such as VSV-G and IAV HA, can also efficiently mediate cell-to-cell transfer of Tet-off at neutral pH. However, the Gluc activities induced by these two viral fusion proteins are dramatically enhanced by an extracellular low pH pulse, indicating that cell-cell fusion at the plasma membrane can contribute to cell-to-cell transfer, although this is not the case for EBOV, nor for VSV-G and IAV HA, which all require low pH directly or indirectly for fusion. Because no infectious EBOV virions or virus-like particles are involved in this very sensitive Tet-off-based cell-to-cell infection assay, we suggest that this system will be extremely useful for study of highly contagious
BL4 agents such as EBOV and Hanta virus, including screens for potential entry inhibitors.

What are the possible mechanisms of the enhancement of EBOV GP-mediated by cell-to-cell infection compared to cell-free infection? One obvious possibility is that cell-cell contact can increase the local concentrations of retroviral pseudovirions, EBOV VLPs, and rVSV-GP, resulting in relatively high MOIs thus enhanced infection and spread. In contrast, viral particles in the cell-free infection system may be relatively unstable, or more susceptible to inactivation by serum and other culture conditions. Evidence supporting this scenario is that spinoculation of cell-free virions produced from donor cells did increase cell-free infection efficiency. Additionally, we provide data showing that exosomes, which may or may not be concentrated in the cell-cell contact area, are enriched in EBOV GP, and that inhibition of exosome release by a chemical inhibitor led to decreased Tet-off transfer from cell to cell. Indeed, exosomes have recently been shown to play roles in viral cell-to-cell transmission and pathogenesis, although the underlying mechanism remains murky (174); more work is needed to dissect the exact roles of exosomes in EBOV infection. It is formally possible that different forms of EBOV GP, including full-length GP as well as secreted GP (sGP), may function through exosomes that modulate EBOV spread in a VP40-independent fashion.

Our study strongly suggests, though does not definitely prove, that EBOV GP promotes viral cell-to-cell transmission in addition to mediating cell-free infection; not
surprisingly, both modes of infection require cathepsin and NPC1. Strong similarities between cell-to-cell and cell-free infection, including sensitivities to the neutralizing antibodies, have also been demonstrated for HIV (175). It is thus possible that EBOV GP may associate with some cell surface molecules, including TIM-1 and DC-SIGN, thereby creating a structure known as virological synapse (VS) to facilitate cell-to-cell transmission. It is also possible that EBOV particles may travel from donor cell to target cell through nanotubular or related structures. Hence, detailed analyses of EBOV infection using high-resolution live-cell imaging would be desirable and informative to address these possibilities. Ultimately, we will need to address if authentic live EBOV, or EBOV derived from reverse genetics systems (176), can spread via cell-cell contact in humans and animal infections as well as their implications for viral pathogenesis and antiviral therapy (177, 178).

2.5 Materials and Methods

DNA constructs and plasmids. The native full-length EBOV GP construct was originally provided by Gary Kobinger (National Microbiology Laboratory, Winnipeg, Canada). The mucin-deleted EBOV GP (pcDNA- Δmuc-GP) was originally provided by David Sanders (Purdue University). The N-terminal FLAG-tagged full length EBOV GP construct (F-GP) was made by replacing the signal peptide of GP with that of preprotrypsin followed by a FLAG sequence (inserted between the signal peptide and mature GP); this F-GP was subcloned into the backbone of pCIneo (Promega). The
N-terminal FLAG-tagged GP present in pCIneo-F-GP was subcloned into the mucin-deleted pcDNA-Δmuc-GP construct, resulting in Δmuc-F-GP, which was primarily used in this study. All EBOV GP mutants were generated by overlapping PCR-based mutagenesis using pcDNA-F-Δmuc construct as the template. All constructs were confirmed by DNA sequencing.

The pQCXIP-Tet-off construct was provided by Marc Johnson (University of Missouri) (179). The 3 × FLAG-tagged NPC1 and NPC1 domain C proteins with the cytoplasmic tail deleted constructs (referred to as NPC1 CT in this work) were from Kartik Chandran (Albert Einstein College of Medicine) (54). VP40 was provided by Yoshihiro Kawaoka (University of Wisconsin, Madison), VP40-Blam was provided by Lijun Rong (University of Illinois, Chicago) and VP40-GFP was provided by Kartik Chandran. The influenza HA and NA constructs were offered by Gary Nabel (Vaccine Research Center, NIH).

**Cell lines and reagents.** 293T, 293GP/LAPSN (expressing MLV Gag-Pol and transfer vector encoding alkaline phosphatase) and HTX (a subclone of HT1080) have been previously described (180). THP-1 cells were obtained from ATCC. The 293FT/Gluc cell line that stably expresses Gluc was provided by Marc Johnson (179). The 293FT/Gluc cell lines stably expressing NPC1 or NPC1 CT or TIM-1 were generated by transducing 293FT/Gluc cells with pBabe (for NPC1 and NPC1 CT) or with a pQCXIP (for TIM-1) retroviral vector expressing the individual proteins, followed by puromycin selection.
(Sigma, 2 µg/ml). All cells were grown in Dulbecco’s modified Eagle’s (DMEM) medium, supplemented with 0.5% penicillin/streptomycin plus 10% fetal bovine serum (FBS).

The human KZ52 antibody was provided by Dennis Burton and Erica Saphire (The Scripps Research Institute) (145, 181). The rabbit antibody against EBOV GP1 and 3.47 were obtained from James Cunningham (Harvard Medical School) (41). The anti-NPC1 antibody was purchased from Abcam (Cambridge, MA). The anti-TIM-1 antibody was purchased from R&D Systems. The anti-CD63 antibody was purchased from Invitrogen (Carlsbad, CA). The anti-MLV Gag antibody was purified from R187 hybridoma cell line (ATCC). Leupeptin, E64d, CA074, and cathepsin L inhibitor III (Z-Phe-Tyr(t-Bu)-diazomethylketone, also known as Z-FY(t-Bu)-DMK) were all purchased from EMD Millipore (Billerica, MA). Ammonium chloride (NH4Cl), 12-O-Tetradecanoylphorbol 13-acetate (PMA), methylcellulose, GW4869, shRNA lentiviral vectors targeting NPC1, anti-FLAG antibody, anti-β-actin antibody, and secondary anti-mouse immunoglobulin G conjugated to FITC or HRP were purchased from Sigma (St Louis, MO). The Gaussia luciferase activity was measured by following the manufacturer’s instructions (Promega, Madison, WI) with minor modifications.

**Cell-to-cell infection.** For the retroviral vector-based system, 293T cells were seeded onto six-well plates and transfected with 1 µg pQCXIP-Tet-off vector, 1 µg MLV Gag-Pol and 0.5 µg of plasmids encoding EBOV GP, IAV HA or VSV-G. Alternatively,
MLV Gag-Pol was omitted in the transfection. The next day, $2 \times 10^5$ of the transfected 293T donor cells were thoroughly washed and trypsinized in order to remove bound virus and also residual plasmid DNA, followed by co-culturing with $4 \times 10^5$ 293FT/Gluc target cells or its derivatives in 24-well plates for 24 to 48 h. Inhibitors were added during co-culture unless otherwise specified. For Transwell settings, cell-to-cell infection were determined by co-culturing donor and target cells on the bottom (without insert) and cell-free infection was measured by seeding donor cells on the top well and target cells mixed with the same number of untransfected donor cells on the bottom. Approximately 2 µl of co-cultured media were assayed for Gluc activity in 10 µl buffer containing 10 μM coelenterazine in 0.1 M Tris, pH 7.4 and 0.3 M sodium ascorbate. In all experiments, 293T donor cells transfected with pQCXIP-Tet-off alone were co-cultured with target cells to serve as background control.

For the VP40-based VLP assay, we transfected 293T cells with VP40-Blam or VP40-GFP in the presence or absence of EBOV GP. The transfected cells were co-cultured with 293FT/tdTomato cells, and cell-to-cell transfer efficiency was determined by measuring either fusion activity using a Blam-based virion-fusion assay (182) or by detecting a GFP signal using flow cytometry. Inhibitors were added during co-culture if applicable.

For the rVSV-based assay, 293T, THP-1, or PMA-treated THP-1 cells were infected with appropriate amounts of rVSV expressing EBOV GP or VSV-G (kindly provided by
Kartik Chandran), and the infected cells were co-cultured with CMTMR-labeled 293FT, THP-1, or THP-1 cells treated with PMA, or co-cultured with 293FT/tdTomato cells (red). If desirable, 1% methylcellulose was laid on top of co-cultured cells. Cell-to-cell infection was measured by flow cytometry by measuring the GFP signal in red-cell populations or visualized by fluorescence microscope. Inhibitors were applied during co-cultured as needed.

Cell-free infection. When counting donor cells for cell-to-cell infection, an equivalent number (2 ×10^5) of transfected or infected donor cells were seeded onto a new 6-well plate and cultured for the same time period as the duration of cell-to-cell infection. The total volumes of supernatants were harvested, and used to infect target cells that were mixed with un-transfected 293T cells; this ensured that cell numbers used for cell-to-cell and cell-free assays were absolutely comparable. If needed, spinoculation or 1% methylcellulose was applied during infection. The cell-free infectivity was determined at 24-48 h for Gluc activity or 2~12 h for GFP detection (rVSV-GFP and VP40-GFP) after infection. For experiments involving inhibitors or the KZ52 antibody, cells were pre-treated with appropriate concentrations of these agents for 2 h and subsequently infected with viruses in the presence of the agents throughout infection. In some cases, 293GP/LAPSN packaging cells were used to produce MLV pseudovirions bearing EBOV GP and HTX cells were used as target cells for infection. The viral titer was determined by alkaline phosphatase (AP) staining.
Isolation and purification of exosomes. Isolation of exosomes was carried out by using a referenced protocol, with minor modifications (167). Briefly, 293T donor cells were co-transfected, using a calcium phosphate method, with pQCXIP-Tet-off vector and a plasmid encoding EBOVGP. The supernatants of transfected cells were harvested 24–48 h post-transfection. Supernatants containing the exosomes were clarified by centrifugation for 10 min at 300 × g and 4°C to remove cells, followed by centrifugation for 10 min at 2,000×g and 4°C to remove cell debris. Supernatants containing the exosomes were further clarified by ultracentrifugation for 30 min at 10,000 × g at 4°C. Exosomes were then concentrated by ultracentrifugation for 70 min at 100,000 × g at 4°C, and re-suspended in PBS buffer. Contaminating proteins in exosome suspensions were removed by an additional ultracentrifugation for 70 min at 100,000 × g at 4°C. Purified exosomes were boiled in a sodium dodecyl sulfate (SDS) sample buffer for 10 min before loading to 10% SDS-PAGE electrophoresis and Western blotting. An anti-FLAG was used to detect EBOV GP and anti-CD63.

Western blotting. Cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% Triton) containing freshly added PMSF and a protease inhibitor cocktail (Sigma) for 20 min on ice. The lysates were clarified by centrifugation at 13,000 × g and 4°C for 10 min, followed by boiling for 10 min in SDS sample buffer. Cell lysates were subjected to 7.5 or 10% SDS-PAGE electrophoresis, followed by transfer onto polyvinylidene difluoride membranes. Western blotting was performed by using specific
primary antibodies, followed by appropriate secondary antibodies conjugated to horseradish peroxidase. The signals were detected by the chemiluminescence image analyzer LAS3000 (GE Healthcare Bio-Sciences, Pittsburgh, PA).

**Flow cytometry.** Cells were detached from dishes by adding PBS plus 5 mM EDTA, and re-suspended in PBS containing 2% FBS. Cells were then incubated with mouse anti-FLAG antibody on ice for 1 h, washed 3 times with cold PBS containing 2% FBS, and incubated with FITC conjugated anti-mouse IgG antibody for an additional 45 min. Cells were fixed and analyzed by flow cytometry.

**Immunofluorescence staining, 3D de-convolution and confocal microscopy.** 293FT/Gluc cells stably expressing NPC1, NPC1 CT or TIM-1 were fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100 for 10 min. Cells were blocked with 5% BSA, and stained with anti-FLAG antibody for 1 h. After 3 washes with PBS, cells were incubated with anti-mouse FITC for 1h. Cells were stained with DAPI and Z-stack images were collected using a Leica DMI6000 B inverted deconvolution microscope with a 60 × oil immersion lens.

**Statistical analysis.** One-way ANOVA analysis of variance, with Dunnett's multiple comparison methods, was used to perform all statistical tests. Unless otherwise specified, results from 3 to 6 independent experiments were used for the analysis.
Figure 2-1: EBOV GP mediates cell-to-cell infection of retroviral pseudotypes.

(A) Schematic representation of cell-to-cell vs. cell-free infections. See details in Methods and Results. (B) Comparisons between cell-to-cell and cell-free infections mediated by EBOV GP and VSV-G. Results shown are averages of three independent experiments measured 24 and 48 h after co-culture. “No Env” indicates the background Gluc activity derived from co-culture of 293T donor cells transfected with “Tet-off” alone. The fold differences in Gluc activity above the corresponding “No Env” background are indicated. (C) Comparisons between cell-to-cell and cell-free infections in Transwell plates. The fold differences in Gluc activity above the corresponding “No Env” background are indicated. (D) Effect of KZ52 on cell-to-cell and cell-free infections. For cell-to-cell infection, KZ52 was added during co-culture. For cell-free infection, KZ52 was incubated with viral supernatants for 2 h at 37°C prior to infection and maintained during infection. The efficiencies of cell-to-cell or cell-free infection of EBOV GP and VSV-G without KZ52 were set to 100%, respectively, and relative activities at different doses were calculated and plotted. (E) Effect of LAT-B on cell-to-cell infection mediated by EBOV GP or VSV-G. Two concentrations of LAT-B (i. e., 2 µM and 5 µM) were applied to co-culture, and Gluc activity was measured 24-48 h later. (F) Effect of CytoD on cell-to-cell infection mediated by EBOV GP or VSV-G. Three concentrations of CytoD were used during cell co-culture. Results are from at least three independent experiments. * p < 0.05; *** p < 0.001.
Figure 2-1: EBOV GP mediates cell-to-cell infection of retroviral pseudotypes.
Figure 2-2: EBOV virus-like particles (VLPs) are efficiently transmitted from cell to cell.

293T donor cells expressing VP40-Blam (A-C) or VP40-GFP (D-E) in the presence or absence of EBOV GP were co-cultured with 293FT/tdTomato target cells for different periods of time, and percentages of VP40-positive cells in target cells (red) were determined by a Blam-based fusion assay or by quantifying GFP signals using flow cytometry. (A) Cell-to-cell transfer of VP40-Blam in the presence of KZ52 or action polymerization inhibitors (LAT-B and CytoD) was measured by a Blam-based virion-cell fusion assay. The cleaved CCF2 signals (blue) represent fusion-positive cells; the percentages are indicated inside the boxes. (B) Effect of KZ52, LAT-B, and CytoD on virion fusion following cell-to-cell transmission. The level of cell-to-cell infection with DMSO (“Mock”) was set to 100 % for plotting and comparison. (C) Summary of comparisons between cell-to-cell infection, cell-free infection without spinoculation, and cell-free infection with spinoculation, which are mediated by EBOV GP or VSV-G. Note that cell-free infection with spinoculation confirms the presence of virions in the supernatants of donor cells, which shows minimal levels of cell-free infection without spinoculation. (D and E) Cell-to-cell infection of VP40-GFP and the effect of KZ52 (20 µg/ml), LAT-B (1 µM), and CytoD (1 µM) after 2 h and 6 h of co-culture. Results are averages and standard deviations of 3-5 independent experiments. In all figures, * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 2-2: EBOV virus-like particles (VLPs) are efficiently transmitted from cell to cell.
Figure 2-3: Cell-to-cell transfer of retroviral pseudovirions occurs in the absence of MLV Gag-Pol and involves exosomes.

(A) Comparisons of cell-to-cell and cell-free infections in the presence or absence of MLV Gag-Pol. The fold differences in Gluc activity above the corresponding “No Env” background are indicated. Note that a ~2-fold increase in cell-to-cell transfer resulted from the presence of MLV Gag-Pol. (B) Low pH treatment increases cell-to-cell transfer mediated by VSV-G but not by EBOV GP. A pH 5.0 pulse was applied after a 2 h co-culture, and Gluc activity was measured at 24 and 48 h, respectively. The fold differences in Gluc activity between neutral and low pH are indicated. (C) Expression of EBOV GP (and Tet-off) in donor cells, but not in target cells, led to efficient cell-to-cell transfer. (D) The ability of EBOV GP to mediate cell-to-cell transfer is confirmed by testing different versions of EBOV GP constructs. Δmuc-F-GP: a mucin-deleted GP with a FLAG tag at the N-terminus, which is mainly used in this study; F-GP: a full length GP with the FLAG tag at the N-terminus; Δmuc-GP: a mucin-deleted GP without any tag; Native-GP: a full length GP without any tag. Relative cell-to-cell transmission of EBOV GP was calculated by setting that of Δmuc-F-GP to 100 %. (E) Effect of the exosome inhibitor GW4869 on cell-to-cell transfer. Different concentrations of GW4869 were tested. (F) Western blotting reveals the presence of EBOV GP (anti-FLAG) in concentrated exosomes. CD63 serves as a marker of exosomes. (G) Infection of 293FT/Gluc target cells with purified exosomes containing GP results in increased Gluc
activity. Absolute Gluc read-outs are shown. In all figures, ** p < 0.01; *** p < 0.001.
Figure 2-3: Cell-to-cell transfer of retroviral pseudovirions occurs in the absence of MLV Gag-Pol and involves exosomes.
Figure 2-4: Cellular cathepsin activity is essential for cell-to-cell transfer of EBOV GP.

(A) Effects of leupeptin, CA074, and E64d on cell-to-cell transfer of EBOV GP and IAV HA. Co-cultured cells were treated with 50 µM leupeptin, 5 µM E64d, or 5 µM CA074 throughout the assay, and Gluc activity was measured 24-48 h after co-culture. (B and C) Effects of different doses of E64d, CA074, or CatL III inhibitor on cell-to-cell transfer mediated by EBOV GP or IAV HA. For comparison, cell-to-cell transfer efficiencies of EBOV GP and IAV HA in the absence of inhibitors are set to 100%, respectively. (D) Effects of leupeptin added to donor cell, target cells, and both on cell-to-cell transfer mediated by EBOV GP. (E) Effects of NH4Cl on cell-to-cell transfer mediated by EBOV GP and VSV-G. Unless otherwise specified, results represent the averages ± standard deviations of at least three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 2-4: Cellular cathepsin activity is essential for cell-to-cell transfer of EBOV GP.
Figure 2-5: Overexpression of NPC1 or TIM-1 increases cell-to-cell transfer mediated by EBOV GP.

(A) Cell-to-cell transfer by EBOV GP was examined in parental 293FT/Gluc (Mock, set to 100%) or 293FT/Gluc cells overexpressing full length NPC1 (NPC1) or the NPC1 domain C with the cytoplasmic tail truncated (NPC1 CT) at neutral or low pH. (B) Western blotting analysis of the expression of NPC1 and NPC1 CT using anti-FLAG. β-actin served as the loading control. (C) Immunostaining of 293FT/Gluc cells overexpressing NPC1 and NPC1 CT (tailless). Cells were permeabilized and stained with an anti-FLAG antibody, and the fluorescence signal was visualized and analyzed by a 3D deconvoluted fluorescence microscope (Leica). Arrows indicate fluorescence signals on the cell surface. (D) Relative cell-to-cell transfer mediated by EBOV GP and IAV HA examined in target cells stably expressing lentiviral vectors encoding NPC1 shRNA. Five stable cell lines expressing different clones of NPC1 shRNAs were tested, with clone 1 (Sigma #5428) consistently exhibiting significant reduction in cell-to-cell transfer. The cell-to-cell transfer mediated by EBOV GP in parental 293FT/Gluc was set to 100% for comparison. (E) Western blotting analysis of NPC1 in 293FT/Gluc cells expressing NPC1 shRNA. An anti-NPC1 antibody was used for probing NPC1 expression; β-actin served as the loading control. (F) Effects of 3.47 on cell-to-cell transfer mediated by EBOV GP and IAV HA. Indicated concentrations of 3.47 were added to the co-culture media for 24-48 h before Gluc activity was measured. In all cases, ** * p < 0.01; *** * p <
0.001.
Figure 2-5: Overexpression of NPC1 or TIM-1 increases cell-to-cell transfer mediated by EBOV GP.
Figure 2-6: Cell-to-cell transfer mediated by EBOV GP requires receptor-binding.

(A) Comparisons of cell-to-cell transfer by EBOV GP mutants. The transfer efficiency of parental GP was set to 100%. (B) Relative cell-free infection of EBOV GP mutants in HTX cells. Note that in this case, pseudovirions were generated from 293GP/LAPSN cells (normal transfection scale in 6-well plate) and used to infect HTX; AP positive foci in HTX cells were counted 72 h post-infection. The infectivity of parental GP was set to 100% for comparison. (C) Western blotting analysis of EBOV GP mutants using an anti-FLAG antibody. β-actin served as the loading control. (D-E) Flow cytometry analysis of the surface expression of parental EBOV GP and mutants. 293T cells were transiently transfected with plasmids encoding EBOV GPs and stained using an anti-FLAG antibody. Representative flow cytometry profiles from one typical experiment are shown (D). Quantification of the fluorescence intensities of EBOV GPs (E). The geometric mean (Geom) of each GP mutant was compared to that of parental GP, the latter of which was set to 100%. Results were summarized from three independent experiments. (F) Differential sensitivities of EBOV GP-mediated cell-to-cell transfer to the CatB inhibitor, CA074. Cell-to-cell transfer mediated by each GP mutant without being treated with CA074 was set to 100%. The p values indicate results of comparisons between D47V or I584F and parental GP at each equivalent concentration of CA074. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 2-6: Cell-to-cell transfer mediated by EBOV GP requires receptor-binding.
Figure 2-7: Cell-to-cell transmission occurs in epithelial cells and macrophages for replication-competent rVSV encoding EBOV GP.

(A-D) Cell-to-cell transmission was performed by co-culturing donor cells (293T, THP-1 or PMA-treated THP-1 cells) infected with appropriate amounts of rVSV-GFP encoding EBOV GP (green) and target 293FT cells that were pre-labeled with CMTMR (red). The efficiency of cell-to-cell transmission was determined by gating the GFP positive cell population in the CMTMR-labeled cells using flow cytometry. (A) Representative flow cytometry files show the infection rates of rVSV-GFP-EBOV GP via cell-to-cell transmission or cell-free infection at 2, 6 and 12 h following co-culture. (B) Summary of the transmission of rVSV-GFP encoding EBOV GP from 293T to 293FT cells in comparison with that of cell-free infection; the effects of KZ52, LAT-B, and CytoD on these two modes of infection are also shown. (C-D) Transmission of rVSV-GFP encoding EBOV GP from THP-1 cells or PMA-treated THP-1 (macrophages) to 293FT cells. In all cases, 20 µg/ml of KZ52, 1 µM of LAT-B, or 1 µM of CytoD were used to treat co-cultured cells. Results shown are from at least three independent experiments performed in duplicate. (E) Transmission of rVSV-GFP encoding EBOV GP from 293FT cells to THP-1 cells or THP-1 cells treated with PMA (macrophages). (F) Effects of methylcellulose on cell-to-cell transmission or cell-free infection of rVSV-GFP encoding EBOV GP. 293T cells were infected with appropriate amounts of rVSV expressing EBOV GP; 24 h post infection, the infected 293T cells were co-cultured with
293FT/tdTomato cells at a 1:3 ratio in the presence or absence of 1 % methylcellulose. GFP signals in the Tomato-positive cell populations were analyzed by flow cytometry. For cell-free virus infection, 293FT/tdTomato cells were directly infected with appropriate amounts of rVSV expressing EBOV GP in the presence or absence of 1 % methylcellulose. (G) Representative images showing cell-to-cell transmission or cell-free infection of rVSV-GFP encoding EBOV GP in the presence or absence of methylcellulose (mock). Note that the rVSV-GFP-GP viral stocks used for cell-free infection here were not from the donor cells used for cell-to-cell infection. Images shown were taken after 18-24 h after infection. In all cases, results were from at least three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 2-7: Cell-to-cell transmission occurs in epithelial cells and macrophages for replication-competent rVSV encoding EBOV GP.
III. INDUCTION OF CELL-CELL FUSION BY EBOV GLYCOPROTEIN: LOW PH IS NOT A TRIGGER

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Note: The work presented in chapter III was contributed equally by Ruben M. Markosyan and Chunhui Miao. The manuscript was written by Shan-Lu Liu and Fredric S. Cohen and approved by all authors. This work has been published in PLoS Pathogens (183).

3.1 Abstract

EBOV is a highly pathogenic filovirus that causes hemorrhagic fever in humans and animals. Currently, how EBOV fuses its envelope membrane within an endosomal membrane to cause infection is poorly understood. We successfully measure cell-cell fusion mediated by the EBOV fusion protein, GP, assayed by the transfer of both cytoplasmic and membrane dyes. A small molecule fusion inhibitor, a neutralizing antibody, as well as mutations in EBOV GP known to reduce viral infection, all greatly reduce fusion. By monitoring redistribution of small aqueous dyes between cells and by electrical capacitance measurements, we discovered that EBOV GP-mediated fusion pores do not readily enlarge—a marked difference from the behavior of other viral fusion
proteins. EBOV GP must be cleaved by late endosome-resident cathepsins B or L in order to become fusion-competent. Cleavage of cell surface-expressed GP appears to occur in endosomes, as evidenced by the fusion block imposed by cathepsin inhibitors, agents that raise endosomal pH, or an inhibitor of anterograde trafficking. Treating effector cells with a recombinant soluble cathepsin B or thermolysin, which cleaves GP into an active form, increases the extent of fusion, suggesting that a fraction of surface-expressed GP is not cleaved. Whereas the rate of fusion is increased by a brief exposure to acidic pH, fusion does occur at neutral pH. Importantly, the extent of fusion is independent of external pH in experiments in which cathepsin activity is blocked and EBOV GP is cleaved by thermolysin. These results imply that low pH promotes fusion through the well-known pH-dependent activity of cathepsins; fusion induced by cleaved EBOV GP is a process that is fundamentally independent of pH. The cell-cell fusion system has revealed some previously unappreciated features of EBOV entry, which could not be readily elucidated in the context of endosomal entry.

3.2 Introduction

EBOV outbreaks continually occur and up to 90% of those infected die; currently there are no approved vaccines or antiviral therapeutics against the virus (138, 184). EBOV initiates infection by fusion from within endosomes. Experimentally, endosomal interiors are difficult to control, but systems that track the entry of several other viruses into cells have been developed and employed (185-188). Historically, these methods have relied on
fusion of infectious virus or pseudovirus within cells; cell-cell fusion has not been among the systems in use for EBOV. It is surprising that a cell-cell fusion system has not been developed, as the processing of the Ebola fusion protein, GP, and other conditions necessary for fusion have been elaborated (53). (Some years ago there was an isolated report of EBOV GP-mediated cell-cell fusion, but this study has not been followed up by any other laboratory, including the original (20)). Cell-cell fusion has several important advantages over intracellular fusion assays, including complete control of the aqueous solution bathing the ectodomain of the fusion protein. In the present study we describe a direct and sensitive system to measure EBOV GP-mediated cell-cell fusion with high time resolution, thereby providing fusion kinetics. The system exhibits the well-known central properties of EBOV entry, providing strong support for the utility of the cell-cell fusion system to explore mechanisms of EBOV entry that are not possible or practical with whole infectious virus.

EBOV GP is a prototypic class I viral fusion protein (22). It is synthesized as a homotrimer; each monomer is cleaved into GP1-GP2 subunits by proteases within the Golgi apparatus (140, 141). The GP1 subunit is responsible for binding to the intracellular receptor Niemann Pick type C1, (NPC1) and possibly to other molecules (189), and the GP2 subunit is responsible for membrane fusion (23, 41, 54, 147, 149, 151, 190). The two subunits of each monomer remain linked through a disulfide bond and a multitude of weak interactions (22, 24, 191, 192). After endocytosis of the virus, the GP1
subunit is cleaved by the endosomal proteases cathepsin B and/or L (52, 53, 193-195), while remaining attached to GP2 (22), and then binds to NPC1 (23, 41). The low pH within endosomes is necessary for viral fusion. But it has not been known whether low pH directly triggers fusion by causing conformational changes in GP or whether it augments fusion by increasing the activities of the cathepsins (52, 53).

After developing our system, we discovered that an EBOV GP-induced fusion pore that connects two plasma membranes does not readily enlarge over time, in contrast to the pores of other viral fusion proteins. This anomalous lack of growth may be the reason cell-cell fusion has not been successfully observed in many prior attempts that used less sensitive assays to detect fusion.

On the question of low pH, we found that activation of cathepsins by acidity is the sole cause for augmentation of fusion: if EBOV GP on the cell surface is artificially cleaved by thermolysin in the presence of cathepsin inhibitors, the extent of fusion is independent of pH.

3.3 Results

**EBOV GP mediates cell-cell fusion.** We utilize fluorescent dye spread assays to monitor cell-cell fusion. Effector COS7 cells transfected to express EBOV GP were loaded with calcein-AM (CaAM, green) and pretreated with thermolysin (Th) and. It has been shown that thermolysin treatment cleaves GP1 on the viral membrane into a fusion-competent 18-19 kDa subunit (22, 193, 194). Within the laboratory, thermolysin is
therefore often used in lieu of membrane-bound cathepsins to cleave the GP1 subunit into a fusion-competent form. The COS7 cells were bound to 293T target cells that were either unlabeled or, for purposes of microscopic identification, loaded with the aqueous dye CMAC (blue). We lowered the external pH for 10 min at room temperature, reneutralized, raised temperature to 37°C, and monitored dye spread at various times. We tracked the transfer of calcein between cells to quantify the extents of fusion; CMAC was used solely to identify the target cells. The fraction of cells that were stained by both calcein and CMAC, 2 hr after a 10-min low pH pulse, was comparable for cell-cell fusion mediated by EBOV GP, by Jaagsiekte sheep retrovirus (JSRV) Env, and influenza A virus (IAV) hemagglutinin (HA)—all requiring low pH for fusion to proceed (Figure 3-1). Fusion did not occur for effector cells that were mock transfected, establishing that CaAM transferred only due to fusion (top row).

It is often thought that EBOV fusion requires acidic pH (52, 53, 148). But we found that thermolysin-treated effector cells expressing EBOV GP also fused to target cells at neutral pH (7.2) (Figure 3-2A, bar 2), albeit to a smaller extent than occurred 2 hrs after a 10 min exposure to an acidic pH of 5.7 at room temperature (bar 1). Representative images for dye transfer are shown to the right of the bar graphs (Figure 3-2A). In its natural cellular setting, EBOV GP is cleaved not by thermolysin but by endosomal cathepsins B and L. In measuring fusion without prior thermolysin treatment of effector cells, we found that fusion still occurred, albeit to smaller extents (Figure 3-2A, bars 3
and 4). Again, a 10-min acidification (Figure 3-2A, bar 3) led to greater amounts of fusion than occurred at neutral pH when measured after a 2-h reneutralization (Figure 3-2A, bar 4). Mock transfected effector cells, with or without thermolysin treatment, did not support any dye transfer at neutral or low pH, verifying that fusion required EBOV GP (e.g., see Figure 3-1).

The observed differences in extents of fusion between cells that were treated with thermolysin and those not were eliminated by long times of incubation after reneutralization (Figure 3-2B). When EBOV GP was not cleaved by thermolysin, there was a 30 min latency between the fusion trigger (acidification and raising temperature from 10°C to 37°C, Figure 3-2B, dark yellow circles) and the occurrence of fusion. There was no latency when thermolysin cleaved the protein (dark red squares, same fusion trigger as for dark yellow circles), suggesting that the 30 min latency when thermolysin was not used was due to the time it takes for a sufficient number of copies of cleaved GP to accumulate at a potential fusion site. The extent of fusion for non-treated effector cells (dark yellow circles) 2 hrs after reneutralization was almost equal to that observed after a 1 hr reneutralization for thermolysin-treated cells. But 4 hrs after a pH 5.7 pulse, the extent of fusion was independent of whether EBOV GP was cleaved by thermolysin. The kinetic difference is, to a large extent, likely due to the ~30 min latency until fusion occurs. The slopes of the linear portion for rates of fusion are comparable, suggesting that, after the latency, the kinetics of fusion are the same at low and neutral pH. The latency
for EBOV GP-mediated fusion is much longer than for some viral fusion proteins, such as IAV HA (196), but comparable to others, such as HIV Env in some studies (197). We thus tested, at various times, whether some of the cell pairs that had not yet fully fused had hemifused: the addition of 0.5 mM CPZ to cell pairs ruptures hemifusion diaphragms that have formed between cell pairs, and this is a standard means to test for hemifusion (198-200). We used thermolysin-treated effector cells to maximize cleavage of EBOV GP and found that adding CPZ either 30, 45, or 60 min after reneutralization did not induce any dye spread above that already observed (data not shown), strongly indicating that a negligible percentage of cells were hemifused, but not fused, at any given time.

NPC1 is an intracellular receptor for EBOV GP (23, 201). We compared extents of fusion for target parental HEK 293T cells versus target HEK 293T cells that stably overexpressed NPC1. Effector cells that were not treated with thermolysin yielded fusion at pH 7.2 (Figure 3-3A, bar 2), and a greater extent of fusion after a 10-min acidic pH 5.7 pulse (Figure 3-3A, bar 1). The extent of calcein spread was greater for target cells overexpressing NPC1 (Figure 3-3A, bars 3 and 4) than for parental 293T cells (Figure 3-3A, bars 1 and 2) for matching conditions. Fusion was still pH-dependent for target cells overexpressing NPC1: calcein spread was greater 2 hr after a 10-min pH 5.7 pulse (Figure 3-3A, bar 3) than in the absence of the pulse (Figure 3-3A, bar 4). We confirmed that fusion was dependent on the presence of NPC1 by generating and purifying a recombinant soluble protein consisting of domain C of NPC1 fused to GST (denoted
sNPC1). The purity and size of sNPC1 was confirmed (Figure 3-3B, inset). We added sNPC1 to the external solution and found that the extent of fusion increased monotonically with the amount of sNPC1 added (Figure 3-3B), in accord with the prior demonstration that by binding NPC1, EBOV GP undergoes conformational changes favorable for fusion (54). The augmentation of fusion by sNPC1 indicated that, although there was a sufficient amount of NPC1 on cell surfaces to stimulate fusion, this amount was relatively small and fusion was consequently limited.

NPC1 is an endosomal protein (202), but a small fraction of NPC1 may be present on the plasma membrane of a cell. We assessed this possibility by using flow cytometry to measure binding with an antibody against NPC1 (from LifeSpan Biosciences) on parental 293 cells; shRNA that targeted NPC1 was stably expressed in one set of these 293 cells, and NPC1 was overexpressed in another set (Figure 3-3C and 3D). The level of binding of the secondary FITC-labeled antibody against endogenous NPC1 (as measured by mean fluorescence intensity, MFI) was 3-fold greater than in the absence of the primary Ab (Figure 3-3C, bar 1 vs. bar 4, and Figure 3-3D). Expression was reduced for cells in which NPC1 was knocked down by shRNA (bar 2), and was greater for cells in which NPC1 was overexpressed (bar 3). These results demonstrate that copies of NPC1 reside in the plasma membrane of the 293 cells we used as targets in cell-cell fusion experiments.

EBOV GP is certainly cleaved within endosomes as part of viral infection (195).
Because we observed cell-cell fusion at acidic pH without adding thermolysin, it is extremely likely that a fraction of GP on the cell surface was cleaved into a fusion-competent form. An antibody that only recognizes cleaved GP has not been reported, so we had to devise an alternate means to quantitatively measure the extent of cleavage. We were able to distinguish between the two forms of GP by using the property that NPC1 binds to cleaved, but not uncleaved, EBOV GP. We used a sNPC1 to examine cleaved GP by flow cytometry; in parallel, we measured the total amount of GP on cell surfaces by using an anti-FLAG antibody that bound to the FLAG tag on our GP construct. We also created a GP construct that was intrinsically more likely to be cleaved on the cell surface: we inserted the furin recognition site RRKR at amino acids 203-206 of GP1 (referred to as GPfurin), the putative cleavage site for CatL in GP1 (149, 203). We reasoned that because exogenous expression of furin facilitates cleavage at this inserted site, generating the fusion-active 18-19 kDa subunit, the extent of cleavage of GP on the plasma membrane as well as the extent of cell-cell fusion would be greater for this construct than for WT.

We experimentally confirmed our expectations: We determined the amount of cleaved GP on the cell surface by adding sNPC1 (fused with GST) to cells expressing either EBOV GP or GPfurin, and measuring their binding to an anti-GST antibody. This antibody was detected by a FITC-conjugated secondary antibody (Figure 3-4). The fraction of WT GP cleaved on parental cells (Figure 3-4A, bar 1) was the same for cells that were
transfected with both GP and furin (bar 2). The specificities of sNPC1 and antibody binding were confirmed by the 4-5 fold higher fluorescence than was seen for cells that did not express GP (bar 5). It is notable that cotransfection of cells by GP\textsuperscript{furin} and furin resulted in greater cleavage (bar 4 vs bar 3). We found that the expression of total WT GP as measured by the anti-FLAG antibody was not significantly altered by coexpression of furin (Figure 3-4B, columns 1 and 2), but cells that coexpressed GP\textsuperscript{furin} and furin consistently showed a decreased total GP (compare bar 3 and 4), possibly due to non-specific degradation of GP\textsuperscript{furin}. To determine the relative percentage of cleaved GP, we normalized cleaved GP by total GP. (These are relative and not absolute percentages because different antibodies were used to detect cleaved vs. total GP.) We found that a higher percentage of GP on the plasma membrane was cleaved for cells coexpressing GP\textsuperscript{furin} and furin than for cells expressing WT GP or GP\textsuperscript{furin} alone (Figure 3-4C). Western blot analyses, using an anti-FLAG or an anti-GP1 antibody (kind gift of James Cunningham), showed that the addition of furin increased the amount of cleaved GP\textsuperscript{furin} construct as compared to GP\textsuperscript{furin} alone (Figure 3-4D, lanes 4 and 5 in left and right panels). Furin did not cleave any WT GP (lanes 1). We used these constructs to verify that an increased cleavage of EBOV GP led to a greater extent of fusion (Figure 3-4E). Cotransfecting cells with GP and furin (bar 2) led to the same extent of fusion as did transfection of GP alone (bar 1). In contrast, cotransfecting with GP\textsuperscript{furin} and furin led to more fusion (bar 4) than transfecting only GP\textsuperscript{furin} (bar 3). Control experiments of
transfecting only furin showed that furin, per se, did not promote fusion (bar 5). These experiments, taken together, establish that EBOV GP does appear on the cell surface, that some of it is cleaved, and that for the GP$^{\text{furin}}$ construct, cleavage is augmented by coexpression of furin.

To further confirm that the observed fusion was indeed mediated by EBOV GP, we utilized mutations that had previously been shown to greatly reduce viral infection (204). We used MLV pseudovirus expressing GP, and observed that, indeed, the level of infection caused by the point mutant W597A (Figure 3-5, bar 2), the double mutant G598A/G599A (bar3), and the point mutant I610A (bar 4) were all substantially less than for WT GP (Bar 1). We then measured the extents of cell-cell fusion mediated by each of the mutant proteins. The extent of fusion in absence of thermolysin treatment supported by all three of the mutants (Figure 3-5B, bars 2, 3, and 4) was much less than for WT GP (bar 1). Flow cytometry measurements, using the same cells as for fusion experiments, showed that each of the mutant GPs was well expressed on the cell surface (Figure 3-5C). These experiments provide support that reduced infectivity by EBOV correlates with reduced GP-mediated fusion.

We next tested 3.47, a small molecule inhibitor against NPC1, which prevents EBOV entry, as well as testing a neutralizing antibody (KZ52) against EBOV GP. We found that both significantly reduced EBOV GP-mediated cell-cell fusion (Figure 3-6A and B). The inhibitor 3.47 greatly reduced EBOV GP-mediated fusion but did not significantly alter
cell-cell fusion induced by either Semliki Forest Virus (SFV) E1/E2 or IAV HA (Figure 3-6A, 3.47 at 1 µM). Similarly, the neutralizing antibody KZ52, which recognizes the interface between GP1 and GP2 (145), reduced EBOV GP-mediated fusion, but not SFV-E1/E2 or IAV HA-induced fusion (Figure 3-6B, KZ52 at 5 µg/ml). Higher concentrations of 3.47 completely inhibited fusion (Figure 3-S1A), but fusion was not further reduced by increasing the concentration of KZ52 beyond that employed in Figure 3-6B (Figure 3-S1B).

Another central fingerprint of GP-mediated fusion is inhibition of EBOV infectivity by Bafilomycin A1 (BafA1). By neutralizing endosomes, BafA1 inhibits infection, at least in part, by reducing cathepsin activity which in turn results is reduced cleavage of GP1. We found that addition of BafA1 (25 or 100 nM) reduced the amount of cleaved GP that appeared on the cell surface (Figure 3-6C, bar 2 vs bar 1). This occurred despite a consistently greater amount of total GP in the plasma membrane after the addition of BafA1 (Figure 3-6D). (This greater amount was unexpected. Possibly, BafA1 prevented lysosomal degradation of GP.) Normalizing the amount of cleaved GP by the total shows that cleavage of cell surface GP was significantly reduced by BafA1 (Figure 3-6E). Thus, all data support the conclusion that the aqueous dye spread we observe is due to fusion induced by EBOV GP.

**EBOV GP-mediated cell-cell fusion is maximal at mildly acidic pH.** Many of the effects of pH on kinetics and extents of EBOV GP-induced fusion we found were
unexpected and quite different than those of pH-dependent fusion for other viral proteins. Notably, the extents of fusion did not monotonically increase as pH was progressively lowered, and the apparent pH dependence qualitatively varied with the times of reneutralization (Figure 3-4). After a pH 5.7 pulse, the extents of fusion were always greater than those achieved after more acidic pulses; following a pH 5.7 pulse (at short incubation times (i.e., 30 min) after the shift to neutral pH), more fusion was observed than for a less acidic pulse (Figure 3-7A). However, for pH pulses of 5.7 and above, as the reneutralization time was increased, the extents of fusion became less dependent on pH; fusion was independent of pH for 5.7 and above after a 1 h reneutralization (Figure 3-7A). In contrast, effector cells expressing IAV HA showed the typical and expected response of greater extents of fusion for lower pH values at all times after reneutralization; fusion events reached their full extents after a 30 min reneutralization (Figure 3-7B, using the same protocol as for EBOV GP experiments). Thus, IAV HA induces fusion more rapidly than does EBOV GP.

In separate experiments, we compared extents of EBOV GP-mediated fusion after a 4 h and 1 h reneutralization that followed 10 min, room temperature, acidic pH pulses (Figure 3-7C). After the 4 h reneutralization, fusion was relatively independent of the acidity of the pH pulse, and a low pH pulse did not greatly augment fusion (compare filled bars to open bars, Figure 3-7C). Equality in final extents of fusion at pH 5.7 and 7.2 could be a consequence of all cell pairs quickly fusing at low pH, thereby eliminating the
possibility of further fusion, although we consider this unlikely.

In addition to single cell measurements of aqueous dye transfer, we also monitored lipid dye continuity between effector cells (treated with thermolysin) and target cells. We labeled effector cells with the lipophilic fluorescent dye DiO and labeled target cells with DiI and determined extents of fusion by flow cytometry (FACS). The double positive cells (i.e., the third quadrant) are clearly products of hemifusion or cell-cell fusion (Figure 3-7D). For effector cells treated with thermolysin, the percentage of fusion for the representative experiment was 18.5% at pH 5.7, the optimal pH for fusion (Figure 3-7D, second panel) and only 1.5% at pH 5.0 (first panel). Averaging six separate experiments for each condition, after a 2-h reneutralization, lipid mixing was greatest for a 10-min pH 5.7 pulse, and less for a pH 5.0 pulse than for cells maintained at neutral pH (Figure 3-7E). The approximately two-fold greater fusion determined by flow cytometry at pH 5.7 than at 7.2 is also in agreement with the data for spread of calcein (Figure 3-2). For mock-transfected effector cells, virtually no lipid dye spread was observed between effector and target cells (Figure 3-7E), in agreement with the aqueous dye spread measurements. Therefore it is clear that EBOV GP mediates a considerable amount of cell-cell fusion, and does so at an optimal pH of 5.7.

**EBOV GP-mediated fusion pores do not readily enlarge.** Once calcein movement from effector to target cell commenced, it continued for EBOV GP-mediated fusion, but at an extremely slow rate. In general, the fluorescence due to calcein never equalized
between target and effector cells for EBOV GP-induced fusion (Figure 3-8). In contrast, for fusion pores created by other viral fusion proteins (201, 205), such as JSRV Env (Figure 3-8A, upper images), the fluorescence did equalize. It is possible that the EBOV GP pores eventually closed, preventing calcein from attaining the same concentration in effector and target cells. We therefore quantified the rate of transfer of calcein by plotting calcein fluorescence of effector and target cells as a function of time (Figure 3-8B). For EBOV GP-induced pores (red curve), the transfer occurred over a time course of tens of minutes, and over this period the increasing fluorescence of a target cell never equalized the decreasing fluorescence of an effector cell (Figure 3-8B). The fluorescence of the effector and target cells, on the other hand, equalized within a minute or so for JSRV Env-mediated pores (Figure 3-8B, blue curve). The exceedingly slow transfer of calcein is a further indicator that EBOV GP-mediated pores remained extremely small. The fact that calcein transferred, albeit slowly over long times, shows that the fusion pores did not irreversibly close (or if they did, new pores opened) within tens of minutes of formation. As a control, we added saponin to effector cells and measured release of calcein to be sure that the dye did not become compartmentalized and therefore failed to transfer for reasons unrelated to the size of the fusion pore. Release was fast from the saponin-treated cells and was almost complete within 10 s (data not shown), demonstrating that the overwhelming majority of calcein was, in fact, free and mobile.

We further studied the size and rate of growth of EBOV GP-mediated fusion pores by
assessing the size of dyes that can permeate these pores over time. We loaded effector cells with CMTMR in addition to calcein. CMTMR forms disulfide bonds with the tri-peptide glutathione, and these complexes are somewhat larger than calcein. The complexed glutathione can also form disulfide bonds with cytosolic proteins and hence CMTMR fluorescently labels proteins that are much larger than calcein. As a consequence, the size distribution of molecules labeled by CMTMR is expected to be quite diverse, some only somewhat larger than calcein and others very much larger. We found that CMTMR transferred for only 2-3% of the cell pairs for which calcein exchange occurred (Figure 3-8C). The relative inability of CMTMR to spread indicates that fusion pores typically did not enlarge sufficiently to allow passage of a molecule of the size of the nucleocapsid of EBOV. In actual viral infection, factors absent in our model system are probably promoting expansion of the fusion pore connecting an envelope and endosomal membrane.

We used electrical capacitance measurements to directly and quantitatively assess fusion pore size. The slow time course for EBOV GP-mediated fusion necessitated that the tight electrical seal between the patch pipette and plasma membrane be maintained for long times. This proved difficult in practice. We were able, however, to electrically observe pores between cell pairs in three cases, and in these cases the pores never enlarged within 30 s of formation and generally fluctuated within small values of conductance (Figure 3-9A). The conductance of the fluctuating pores did not return to
baseline, showing that the pores did not close, but instead remained restricted to a small size. By way of comparison, it can be readily seen from representative traces of electrically measured fusion pores created by other viral proteins (Figure 3-9B) that fusion pores generally significantly enlarge over time. The absence of pore enlargement for EBOV GP suggests that many of the prior attempts at monitoring cell-cell fusion mediated by this fusion protein did not succeed because the reporter molecules that needed to permeate the fusion pore for detection of fusion were too large to pass through the pore. Although only three pores were electrophysiologically measured, the finding that each of them did not exhibit increased conductance over time implies that the slow passage of fluorescent dyes through them was not due to structures that prevent their access to the pores. Slow pore enlargement could be due to a number of factors, including slow recruitment and incorporation of additional copies of cleaved GP into the wall of the pore, or slow accumulation of lipids into the wall.

**Neutralization of intracellular compartments eliminates EBOV GP-mediated fusion.** We added NH4Cl to external media to test whether acidic intracellular compartments were essential for EBOV GP-mediated cell-cell fusion. The addition of 10 mM NH4Cl greatly reduced fusion after a 10-min pH 5.7 pulse in the absence of thermolysin treatment, so as to avoid activating uncleaved EBOV GP on the cell surface (Figure 3-10A). In contrast, the addition of 10 mM NH4Cl did not affect fusion induced by an optimal pH pulse for either SFV E1/E2 or IAV HA (Figure 3-10A). Similarly, 100
µM chloroquine inhibited cell-cell fusion mediated by the fusion protein of EBOV, but not by the proteins from either SFV or IAV (Figure 3-10A). The elimination of fusion by the addition of 10 mM NH4Cl (bar 2; same conditions as in Figure 3-10A) was most likely caused by reducing cathepsin activity through neutralization of intracellular compartments: it was largely reversed by adding a recombinant cathepsin B to the external solution (Figure 3-10B, bar 3). Because the normally acidic intracellular compartments were neutralized by NH4Cl, the pool of EBOV GP on the cell surface that was previously uncleaved must have been cleaved by the added membrane-impermeant recombinant protease. The dose-response curves for inhibition of fusion by chloroquine (Figure 3-10C) or NH4Cl (Figure 3-10D) verified that inhibition of fusion is increased with increasing concentration of the neutralizing agent. Therefore, even if the external solution is acidified, EBOV GP-mediated cell-cell fusion does not occur unless the acidity of intracellular organelles is maintained. We conclude that EBOV GP present on the cell surface requires an intracellular compartment for cleavage, as is consistent with previous reports. It is possible, however, that there are copies of cathepsins in the plasma membrane, and acidification of the external solution activates them to cleave EBOV GP.

**EBOV GP cycling between plasma and intracellular membranes affects cell-cell fusion.** Proteinase K (PK) has proved useful for assessing conformational changes that viral proteins undergo at different stages of fusion (198, 206). We found that EBOV GP was PK-sensitive for all steps of the fusion process (Figure 3-S2A), that fusion was
restored over time after removing PK (Figure 3-S2B), and that normal cellular trafficking of protein led to replacement of proteolytically digested GP with newly delivered intact GP (Figure 3-S3).

We also used Brefeldin A (BFA, 50 μM)—an inhibitor of trafficking from endoplasmic reticulum to Golgi—to further characterize the consequences for fusion of altering intracellular trafficking of EBOV GP. Here we found that treatments expected to increase the amounts of cleaved EBOV GP on the cell surface led to greater extents of fusion (Figure 3-S4).

**pH-dependent cathepsin activity is essential for GP-mediated fusion.** For virus internalized in endosomes, EBOV GP is believed to be cleaved by cathepsins B and L, but not by cathepsins A or D. We prevented cathepsin-induced cleavage by treating bound effector and target cells with a cathepsin B inhibitor (CA-074) or a cathepsin L inhibitor (Z-FY-CHO). In the absence of thermolysin treatment, the inhibitors led to significantly reduced fusion at both neutral and low pH (Figure 3-11A, compare “untreated” and “treated”: as always, changes of solutions containing membrane-impermeant buffers were used to control pH). Using inhibitors against cathepsin A (lactacystin) or cathepsin D (pepstatin A)—neither of which is thought to cleave EBOV GP—did not lead to reduced fusion using the same protocol as for the cathepsin B and L inhibition experiments (Figure 3-11A). These results provide strong support that fusion observed in our experiments in the absence of thermolysin treatment
is due to, at least in part, copies of EBOV GP on the cell surface that have their GP1 subunits cleaved by cathepsins. These results also document that neither cathepsin A or D cleaves EBOV into a fusion-competent form. From the results as a whole, it is clear that low pH does not induce fusion unless the GP1 subunit has been cleaved. It is known that cathepsin activity is increased by acidity. We suggest that low pH acts, at least in part, by augmenting cathepsin activity on the cell surface. The same pattern of pH-dependence of fusion was observed for effector cells treated with thermolysin while cathepsin activity was continually inhibited: fusion was dependent on pH and was significantly reduced by the cathepsin B inhibitor or the cathepsin L inhibitor (Figure 3-11A, thermolysin-treated, bars 2 and 3 of each set of columns), but was relatively unaffected by cathepsin A or D inhibitors (bars 4 and 5). Cell-cell fusion exhibits a maximum at pH 5.7 (column 4 compared to column 1-3). Several cathepsins exhibit maximal activity in the pH range of 5.5 to 6.8 (207), so the maximum extent of fusion at pH 5.7 would likely be due to the pH dependence of cathepsin activity on the cell surface.

Control experiments provide additional support for the conclusion that cathepsins aid EBOV GP-mediated fusion between cells. Blocking cathepsin B (by adding the cathepsin B inhibitor) immediately after application of an acidic pH pulse resulted in a substantial reduction in the extent of fusion after a 2-h reneutralization (Figure 3-11B, effector cells were thermolysin-treated). The reduction from the control was ~2-fold; a 2-fold reduction also occurred when the cathepsin B inhibitor was constantly present (see Figure 3-12B).
The nearly equal percentages of inhibition of fusion are expected, since in the presence of the cathepsin inhibitor, uncleaved copies of EBOV GP would not be cleaved during the period of reneutralization. Thus, low pH appears to promote cleavage of EBOV GP by cathepsins on the cell surface. Incubating effector cells that were not treated with thermolysin with a recombinant human cathepsin B (rhCat B) (Figure 3-11C, bar 2) increased fusion significantly over the control (bar 1). The simplest explanation for this increase is that the recombinant protein led to a higher level of GP1 cleavage than that induced by endogenous cellular cathepsins. To explicitly test whether increasing the activity of cathepsin increased the likelihood that GP on the cell surface was cleaved, we cotransfected cells to express GP and cathepsin B and used sNPC1 to measure the percentage of GP in the plasma membrane that was cleaved (as described for Figure 3-4). This percentage was greater (Figure 3-11, column 2) than the control (column 1) in the presence of cathepsin B transfection. Using the same techniques, we also showed that adding thermolysin to solution did indeed increase cleavage of cell surface GP (Figure 3-11D).

**Altering EBOV GP cycling and inhibiting cathepsin activity show that once GP is cleaved, fusion is independent of pH.** Does low pH directly cause conformational changes in EBOV GP to induce fusion, or does it work via increasing the activity of cathepsins, or both (24, 53)? We were able to approach these questions by using the ability of BFA to effectively block delivery of EBOV GP to the cell surface and,
independently, by using cathepsin inhibitors to prevent GP cleavage. We incubated effector cells with BFA for 45 min to prevent further delivery of EBOV GP to the plasma membrane prior to a thermolysin-treatment, and maintained the presence of the drug during all solution changes. The extent of fusion was independent of pH, and considerably less than when the trafficking inhibitor was not employed (Figure 3-12A). The clear conclusion is that, with BFA present, all fusion was caused by copies of EBOV GP that had been cleaved by thermolysin and that remained on the cell surface. The finding that pH pulses did not affect the extent of fusion at all shows that acidity did not promote the conformational changes in cleaved EBOV GP that would lead to fusion.

We inhibited cathepsin activity to further test the conclusion that once cleaved, EBOV GP no longer requires low pH to induce fusion. We performed experiments in which CA-074, a cathepsin B inhibitor, was continually present (Figure 3-12B). The inhibitor was added to isolated effector cells and maintained for 45 min to ensure that all EBOV GP delivered to the plasma membrane was not cleaved. Effector cells were then thermolysin-treated, always maintaining the inhibitor. These cells were bound to target cells, and the external solution was acidified to pH 5.7; after reneutralization, cells were maintained for 1 h at 37ºC, with all manipulations performed in the presence of the inhibitor (Figure 3-12B, experimental protocol illustrated on top). The extent of fusion was greater when the inhibitor was not added (control): this indicates that delivery to the cell surface of EBOV GP cleaved by endosomal cathepsin (subsequent to thermolysin
treatment) significantly contributes to fusion. More importantly—and central to the mechanism of EBOV GP-mediated fusion—the extent of fusion was independent of pH. This finding strongly implies that acidic conditions have no direct effect on EBOV GP-mediated fusion. The pH dependence of fusion is solely due to the ability of cathepsin to cleave EBOV GP; once cleaved, acidic conditions directly induce conformational changes in cleaved EBOV GP that lead to fusion.

3.4 Discussion

Using both cytoplasmic and membrane dye transfer assays, we established that known properties of EBOV fusion occurring within endosomes are replicated by our cell-cell fusion system and that specific inhibitors of EBOV infection—the small molecule inhibitor 3.47 and a neutralizing antibody KZ52—block fusion. The inhibition of EBOV GP-mediated cell-cell fusion (but not IAV HA or SFV E1/E2 fusion) by the lysosomotropic agents NH4Cl and chloroquine is expected: EBOV GP cleavage is eliminated because cathepsin activity is greatly reduced by neutralization of endosomes; inhibiting cathepsin activity reduces cleavage of EBOV GP (208, 209). We also showed that copies of NPC1 reside in the target membrane and some GP resides in the plasma membrane, and that a fraction of the GP is properly cleaved. It is virtually certain that the cell-cell fusion process investigated in the present study is mediated by EBOV GP.

It is likely that past lack of success in observing cell-cell fusion is attributable to the fact that the fusion pore mediated by EBOV GP on the cell surface remains small. Over
the time scales of electrical measurements, the pore does not enlarge at all. Based on fluorescence dye spread measurements, it enlarges more slowly and to a lesser extent than any other pore mediated by a viral fusion protein of which we are aware, and it may even tend to close. The EBOV GP fusion pore is large enough to allow the passage of calcein, but just barely. There is little doubt that the fluorescent dye CMTMR does not permeate the pore because virtually all of it complexes with proteins; the complex becomes permeable only after a pore enlarges. Electrical measurements directly demonstrate that the EBOV GP-induced pore remains small. Over the course of time in our cell-cell fusion experiments, EBOV GP-mediated fusion pores do not significantly enlarge.

The question now becomes: how readily does a fusion pore enlarge when connecting an EBOV envelope with an endosomal membrane? This fusion pore must expand to sizes that permit passage of the large viral nucleocapsid. Four major possibilities present themselves: (i) the necessary enlargement is extremely slow for the endosome-viral pores; (ii) elements engaging plasma but not endosomal membranes, such as cytoskeleton, retard the growth of fusion pores; (iii) a protein (such as the two-pore calcium channel, present in the endosomal compartments that support EBOV fusion (55)) is required for pore enlargement; (iv) control of calcium concentrations (e.g., through the two-pore channels) regulates fusion pore formation or enlargement in endosomes. Methods to monitor the formation and growth of fusion pores of EBOV GP-bearing viral particles
within endosomes will be needed to answer these questions (210).

We have unambiguously shown that a fraction of EBOV GP on the cell surface is cleaved. By using the GP\textsuperscript{furin} construct we also demonstrated that increased cleavage correlates with greater fusion. In addition, we functionally evaluated the cleavage status of EBOV GP on the cell surface by adding a water-soluble recombinant cathepsin B or thermolysin to solution and found that these proteases promoted fusion. Late endosomes and lysosomes are generally thought to be the cellular site of cleavage of EBOV GP by cathepsins (211); it is likely that a fraction of EBOV GP is cleaved within endosomes and then recycled to the plasma membrane where it mediates cell-cell fusion, independent of pH (Figure 3-12). We suggest that uncleaved EBOV GP that reaches the surface is cleaved upon acidification of the external solution by cathepsins within the plasma membrane. Thermolysin cleaves uncleaved copies of EBOV GP that are delivered to the cell surface, accounting for the enhancement of fusion by the addition of the protease. Regardless of the site of GP cleavage, an appreciable fraction of the GP1 subunit is indeed cleaved into its fusion-competent form after the addition of thermolysin.

NPC1 serves as receptor for EBOV GP in endosomes, and is essential for the virus to infect a cell. We have now shown that NPC1 is not confined only to intracellular membranes, but rather that some copies reside in plasma membranes. Our finding that sNPC1 promotes EBOV GP-mediated cell-cell fusion suggests that domain C of NPC1 alone is sufficient to induce the needed conformational changes in the fusion protein.
It is well established that the extents of cell-cell fusion correlate with the levels of viral fusion protein expression on cell surfaces (212, 213). Thus, it is not surprising that the extents of cell-cell fusion induced by EBOV GP are affected by its delivery to, and loss from, plasma membranes. For some viral fusion proteins, such as the paramyxovirus Hendra and Nipah virus F proteins, and SARS coronavirus S protein, cell-cell fusion is sensitive to protein cycling (214-216). These proteins require acidic intracellular compartments for cleavage: endosomes for Nipah virus (217), and endosomes and the Trans-Golgi Network for Hendra virus (218). But the dependence of cell-cell fusion on protein trafficking is unusual for typical pH-dependent viral fusion proteins; for these proteins acidity does not promote cleavage, but instead directly induces conformational changes (219). Once activated, these typical low-pH-dependent fusion proteins quickly inactivate if they do not promote fusion (220). Hence, protein delivered to the cell surface subsequent to an acidic pulse will not be able to promote fusion. In contrast, proteins that induce fusion at neutral pH will promote fusion once they are delivered to the cell surface. Our results show that EBOV GP cleaved by endosomal cathepsins are no longer sensitive to pH and therefore can induce fusion once they arrive at the plasma membrane.

Infectivity is subject to processes other than fusion, and so infectivity need not always correlate with extents of cell-cell fusion. For example, it has recently been shown that the activity of two-pore calcium channels in endosomes is required for EBOV infection (55), and that EBOV infects by fusing to endosomal membranes that contain both NPC1 and
the two-pore channel (210). Tetrandrine blocks these channels and inhibits EBOV infection. We found that tetrandrine (150 nM) did not affect EBOV GP-mediated cell-cell fusion (data not shown).

It is notable that the extent of fusion that occurs after 4 h at neutral pH was roughly equal to the extent that follows a pH 5.7 pulse. Because fusion induced by cleaved GP is pH-independent, we interpret the continual increase in fusion at neutral pH over time to be a consequence of intracellular trafficking: new copies of EBOV GP continually replace or supplement old copies and these new/supplemented, cleaved copies can cause fusion between cells that had not previously fused. Thus, acidification likely promotes more fusion at early times through activation of surface cathepsins that cleave EBOV GP. However, it is not presently clear why fusion kinetics is faster after a pH 5.7 pulse than after a pH 5.4 (or more acidic) pulse. The pH dependence of cathepsin activity is complicated (221). While activity generally increases with acidification, some cathepsins exhibit the same activity in the range of pH 7 as at lower values of pH (222). For others, activities are maximal at an intermediate pH, such as 5.7 (207). Also, the pH dependence of cathepsin activity varies with environment and conditions, such as redox potentials on each side of the membrane in which a cathepsin resides (223, 224). Any relevant cathepsins (e.g., B or L) on the cell surface can, at their optimal pH, cleave EBOV GP. A direct test of whether EBOV GP on the cell surface is maximally cleaved by cathepsins at pH 5.7 will require methods to measure the percentage of EBOV GP that is cleaved as
well as cathepsin activity at the cell surface.

The role of acidic pH in EBOV fusion has been debated in the field (24, 31, 53). Our data unambiguously show that cell-cell fusion is regulated by extracellular pH. The acidity of the extracellular solution can, in principle, augment both the activity of cathepsins that reside in the plasma membrane and directly promote conformational changes of cleaved EBOV GP on the cell surface. (Although cathepsins are regarded as endosomal membrane proteins, some copies also likely reside in plasma membranes from which many endosomes derive (225)). The great reductions in fusion caused by inhibition of cathepsin activity and recovery of fusion by addition of a recombinant cathepsin establish that cathepsins’ activity at the cell surface is consequential. Independent experiments in which cathepsin activity was inhibited or delivery of protein to cell surface was blocked show that the pH-dependence of fusion is eliminated once EBOV GP is cleaved. This demonstrates that fusion mediated by the cleaved form is intrinsically pH-independent. That is, cleaved EBOV GP is essentially a neutral pH fusion-inducing protein; all the experimentally observed and biological relevant pH-dependence is a consequence of cathepsin activity. The faster kinetics of cell-cell fusion after a pH 5.7 pulse than for pulses at higher values can be accounted for by greater cleavage of EBOV at the cells surface at pH 5.7. A previous study used model peptides to mimic the six-helix bundle of EBOV GP2 and found that low pH increased bundle stability (226). The stage of fusion in which bundle formation occurs has not been identified for EBOV
GP. It may be, for example, that the bundles form subsequent to pore formation, as occurs for HIV Env (197), and that increased bundle stability aids pore enlargement, but not fusion itself (227). Alternatively, the model peptide may not mimic bundle stability within a full length, structurally intact GP.

As a general rule, fusion kinetics for viral proteins that induce fusion at neutral pH are slower than for proteins that utilize low pH as a trigger. This could explain the slow fusion kinetics of EBOV GP mediated fusion, despite classification as a low pH-requiring process. Alternatively, a need to continually deliver EBOV GP could be the reason EBOV GP-mediated cell-cell fusion is slow. The development of an experimentally convenient system of EBOV GP-mediated fusion should make it possible to determine molecular mechanisms by which EBOV releases its genome into infected cells.

Our results and conclusions are diagrammatically summarized in Figure 3-13. NPC1 is an intracellular receptor for EBOV GP within endosomes. But, as we have shown, NPC1 can also reside in the plasma membrane. Endosomal cathepsins cleave EBOV GP, and any cathepsins that reside in plasma membranes will also cleave surface GP upon acidification of the external solution. Both cleaved and uncleaved copies of EBOV GP are continually delivered to and retrieved from the surface, and hence intracellular trafficking contributes to extents and kinetics of fusion. But binding of EBOV GP to the target membrane should inhibit endocytotic retrieval. Consequently, EBOV GP (both cleaved and uncleaved) should accumulate at potential fusion sites, leading to more
fusion over time. Preventing acidification of endosomes to block cleavage of EBOV GP, or inhibiting delivery of the protein to the cell surface, greatly reduces fusion. Acidification of the external solution to pH 5.7 increases the activity of cathepsins that reside in the cell surface, and this results in additional cleavage of EBOV GP. The addition of thermolysin converts all surface GP to the cleaved form, thereby resulting in the maximal extent of fusion. That cell-cell fusion induced by cleaved EBOV GP does not depend on pH, provides critical insight into the mechanism of EBOV entry and infection.

3.5 Materials and Methods

**Reagents and constructs.** Purchased reagents were: Lactacystin (a cathepsin A inhibitor, Santa Cruz Biotechnology, Dallas, TX); pepstatin A (a cathepsin D inhibitor, Santa Cruz Biotechnology); Cathepsin L inhibitor (catalog no. sc-3132, Santa Cruz Biotechnology); CA-074 (Cathespin B inhibitor, Calbiochem); Recombinant human cathepsin B (R &D Systems, Fisher Scientific), Brefeldin A (Cayman Chemicals, Ann Arbor, MI), poly-lysine (M.W. 70 kD, Sigma); bovine serum albumin (BSA, Sigma), Thermolysin (Sigma); Proteinase K, chlorpromazine (CPZ) (Sigma), lentiviral shRNA targeting NPC1 (Sigma), anti-NPC1 (LifeSpan BioSciences, Seattle, WA), anti-FLAG and anti-ß-actin antibody (Sigma); anti-GP1 antibody (gift of James Cunningham, Harvard Medical School, Boston, MA). PBS++ and DMEM were obtained from Gibco (Grand Island, NY). All fluorescent probes were purchased from Molecular Probes (Life Technologies,
Eugene, OR). The mucin-deleted EBOV GP construct was originally obtained from David Sanders (Purdue University, West Lafayette, IN). For this work, we mainly used an N-terminal FLAG-tagged, mucin-deleted EBOV GP construct by replacing the signal peptide of GP with that of preprotrypsin followed by a FLAG sequence. All GP mutants, including GP\textsuperscript{furin}, were made by overlapping PCR using the FLAG-tagged GP construct as the template. The plasmid to express JSRV Env has been described (205). To express IAV HA for dye spread experiments, we used the X31 strain (plasmid provided by Judith White, University of Virginia, Charlottesville, VA). A standard calcium phosphate method was used to express SFV E1/E2 via transfection of the pCB3-wt vector, plasmid provided by Margaret Kielian, Albert Einstein College of Medicine, Bronx, NY (228). A small molecular inhibitor, 3.47, was a gift of James Cunningham (Harvard Medical School, Boston, MA).

**Cell lines.** HEK 293T and COS7 cells employed have been previously described (229). The HEK 293T cells stably expressing were generated by transducing cells with pBabe retroviral vector expressing NPC1 (gift of Kartik Chandran) followed by puromycin selection (Sigma, 2 µg/ml). All cells were grown in Dulbecco’s modified Eagle’s (DMEM) medium, supplemented with 0.5% penicillin/streptomycin plus 10% fetal bovine serum (FBS).

**Fusion experiments using aqueous dye transfer.** For all experiments using EBOV GP, COS7 cells were maintained in Eagle’s Medium with glucose, L-glutamine, and sodium
pyruvate, supplemented with 10% Cosmic Calf Serum (HyClone, Logan, Utah), Pen Strep (Gibco), and 0.5 mg/ml G418 Sulfate (Cell Gro, Manassas, VA), and transfected to express EBOV GP by a standard calcium phosphate procedure (230). About \( \sim 2 \times 10^6 \) cells were loaded with 1.5 \( \mu \)M calcein-AM as previously described (197) and sometimes coloaded with 1 \( \mu \)M 5-(and-6)\(((4\text{-chloromethyl})\text{benzoyl})\text{amino})\text{tetramethylrhodamine}) (CMTMR) (197). If these effector cells were thermolysin-treated to cleave EBOV GP, 200 \( \mu \)g/ml thermolysin was incubated with the cells for 20 min at room temperature. Exchanging the solution with DMEM removed thermolysin; residual thermolysin was further removed by spinning down the cells three times and replacing the aqueous solution. HEK 293T cells were maintained in the same media and in the same way as COS7 cells and were used as targets. \( \sim 2 \times 10^6 \) cells were loaded with 20 \( \mu \)M CMAC. Effector cells were mixed, including a gentle vortex—in a tube containing either PBS\( ^{++} \) (sometimes supplemented with 1 mg/ml BSA) or DMEM—with the labeled target HEK 293T cells. The cells were added into polylysine-coated (1 mg/ml) wells of an 8-well slide (Thermo Fisher) (197) and allowed to settle and adhere to the bottom for 30 min at room temperature. The pH was lowered (or not) for 10 min at room temperature to the indicated value (pH 5.7 unless stated otherwise, using an exchange solution consisting of 100 mM NaCl, 1.5 mM KCl, 2.5 mM MgCl\(_2\), 2.5 mM CaCl\(_2\), 20 mM MES), the solution was then reneutralized to pH 7.2 by an exchange of solutions, and the temperature raised to 37°C. After this reneutralization for the indicated time, generally 2 h, fusion was
scored as a function of time by the transfer of calcein into target cells, as described (229). For fusion experiments utilizing IVA HA as control, the expressed HA in effector cells were was cleaved into HA1-HA2 subunits with trypsin, as previously described (196).

**Lipid dye mixing.** To label effector cells, ~ 2x10^6 cells/ml were incubated with 10 µM DiO for 30 min at 37°C. The day before an experiment, target cells were split and plated on glass cover slips placed in culture dishes so as to allow convenient transfer. These target cells were labeled by 100 µM DiI for 30 min at 37°C. Labeled effector cells were thermolysin treated (200 µg/ml) and added above labeled target cells. Binding was allowed to occur for 40 min at room temperature before washing out unbound effector cells. The solutions bathing the cover slips (one cover slip per culture dish) was acidified to the indicated pH for 10 min at room temperature, and the culture dish placed in a 37°C incubator for 2 h. Cells were detached from cover slips by adding 10 µg/ml trypsin to a divalent-free solution containing 0.5 mM EDTA for 10 min at room temperature, followed by vigorous, repeated pipetting to dissociate bound (i.e., neither hemifused or fused) cells. Colocalization of the two lipid dyes was monitored by by flow cytometry (Guava Easy Cite, Guava Technology, Millipore), using two channels emission, one for each dye (515 nm for DiO and 560 nm for DiI; both excited by a 488 nm laser). The same protocols were followed for mock-transfected COS7 cells; this data was subtracted from data obtain for COS7 cells expressing EBOV GP to obtain percentages of fused cells.

**Production of sNPC1 and measurements of NPC1 expression on the cell surface.** The
domain C of NPC1 was cloned into a pGEX-4T1 vector that had a GST tag on the N-terminus (GE Healthcare Life Sciences, Pittsburgh, PA). The expression of fusion protein was induced in E. Coli. by IPTG (0.5 mM) and purified by glutathione sepharose 4B (GE Healthcare Life Sciences). Protein was quantified by a Bradford assay and used for cell-cell fusion and for measurements of cleaved GP. The expression of NPC1 on 293T cell surfaces was determined by using anti-NPC1 (against N-terminus 34-174 aa; LifeSpan BioSciences).

**Determination of EBOV GP cleavage.** HEK293T cells were transfected with EBOV GP or Gp\textsuperscript{furin} in the presence or absence of a plasmid that encodes furin (kind gift of Paul Bates, University of Pennsylvania). Transfected cells were detached by PBS containing 5 mM EDTA. One portion (1 million cells) was used to measure the cleaved GP by incubating cells with 2 µg sNPC1 for 2 h on ice, followed by adding a mouse monoclonal anti-GST antibody; the fluorescence signal was quantified by adding a FITC-conjugated secondary anti-mouse antibody using flow cytometry. Another portion of the transfected cells (also 1 million) was used to measure the total GP expression on the cell surface, using an anti-FLAG antibody. The total GP, as determined by mean fluorescence intensity (MFI), was used to determine the percentage of GP on the plasma membrane that was cleaved: the amount of cleaved normalized by total GP. Cleavage of Gp\textsuperscript{furin} in cell lysates was determined by Western blotting using anti-FLAG or an anti-GP1 antibody.

**Viral infection.** Production of MLV retroviral pseudotypes bearing EBOV GP and viral
infection were as described previously (231). Briefly, 293 GP/LAPSN packaging cells stably expressing MLV Gag-Pol and alkaline phosphatase (AP) were transfected with plasmids encoding the EBOV GP or mutants, and the viruses produced were used to infect HTX cells (a subclone of HT10180). Viral infectivity was determined by counting AP+ foci 72 h after infection.

Proteolysis, altering intracellular trafficking, and inhibition of protein synthesis. We proteolytically determined the stages of fusion at which EBOV GP was proteinase K-sensitive by treating cells with 0.2 mg/ml proteinase K for 20 min, and maintaining or removing it as indicated, at various points in our protocol (Figure 3-S1). We cleaved EBOV GP with thermolysin and used our standard protocol (a 10-min pH 5.7 pulse), incubated the cells for 2 h at pH 7.2, and then measured fusion. All experiments were performed in parallel on the same days; as controls, proteinase K was not employed and extents of fusion were measured. Brefeldin A (50 µM) was used to inhibit anterograde protein trafficking as described in the experiments of Figure 3-S4.

Kinetics of fusion and pore enlargement determined by dye spread. We determined the latency between lowering pH and fusion by using cells mixed within a tube, placed over poly-lysine coated cover slips within a culture dish maintained at 10°C and allowed to settle for 30 min. The pH was lowered to the indicated value for 10 min at room temperature through an exchange of solutions. The cover slips were then transferred into dishes at 37°C, neutral pH, for indicated times. The time of transfer is defined as time = 0.
For thermolysin-treated cells, the effector cells were treated prior to binding to target cells. The extents of fusion were quantified at varied times by spread of calcein.

We used the rate of accumulation of calcein into the target cell and its depletion in effector cells to access the size of the fusion pore as a function of time (227). The fluorescence of both effector and target cells was proportional to calcein concentration, as verified by the procedure detailed in (227).

**Neutralizing intracellular compartments.** NH₄Cl (10 mM unless otherwise noted) was added to the bathing solution and maintained throughout the course of an experiment, including during any low pH pulses, to obtain neutralization. The same procedure was used with chloroquine (100 µM unless stated otherwise) or BafA1 (25 or 100 nM) to cause endosome neutralization. The ionic and buffering contents of the NH₄Cl-containing low pH solutions were pre-adjusted to the required pH so as to maintain osmotic strength at 290 mOsM during low pH pulses.

**Immunostaining.** To monitor EBOV GP expression and its recovery after proteinase K treatment, cells were treated with 0.2 mg/ml of the protease for 20 min at room temperature, and the staining protocol now described was used immediately or after cells were maintained for 3 h in DMEM at 37°C, as indicated. EBOV GP-expressing COS7 cells were transferred to 15 ml tubes and incubated for 1 h at room temperature with a primary antibody (human anti-EBOV GP-KZ52 stock at 1.3 mg/ml, IBT Bioservices, Gaithersburg, MD) that was diluted 1:200 in PBS++ that was supplemented with 10 %
fetal bovine serum. After three washes with the 10% FBS-PBS++ solutions, a secondary FITC-conjugated goat anti-human antibody (Fisher Scientific) was added at a final concentration of 100 μg/ml and maintained for 45 min at room temperature in the dark. After cells were washed twice, they were added to 8-well slides that had been treated with poly-L-lysine (M.W. 70,000 - 150,000, Sigma Aldrich, St. Louis, MO). The cells were then fixed for 20 min at room temperature with 2 % paraformaldehyde, and washed twice with DMEM.

**Patch clamp.** Pore size over time was also quantified by using patch clamp time-resolved admittance measurements (229), often referred to as capacitance measurements. Fusion was promoted by a 10-min pH 5.7 pulse at room temperature. Because the fusion pore took considerable time to form, and a seal between the patch pipette and cell could only be maintained once solutions and temperature were established, temperature was raised to 37°C for ~15 min before attempting electrical measurements. (This procedure reduced the time between establishing the seal and fusion pore formation). The cover slip was then placed in a temperature-controlled chamber on a microscope stage, and the seal established. The external solution consisted of 135 mM N-methyl-glucamine aspartate-5 mM MgCl₂-2 mM HEPES (pH 7.2); the solution within the patch pipette was 135 mM cesium glutamate-5 mM MgCl₂-5 mM BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetate]-10 mM HEPES (pH 7.2). To electrically characterize fusion pores created by IAV HA, ASLV Env, and HIV Env, we
used cell lines that stably express the fusion protein and target cells that stably express the
cognate receptor: HAb2 cells that express IAV HA as effectors and 293T cells as targets
(229); NIH 3T3 EnvA cells that express ASLV Env and 293T TVA cells as targets (229, 232); and TF228 cells that express HIV Env and Hela T4 cells as targets (197).

**Statistics.** Pair-wise Student t-tests were used to compare the outcome of a manipulation
on fusion as compared to the control. In figures, unless otherwise indicated, a single
asterisk (*) denotes $p < 0.05$, two asterisks (**) denotes $p < 0.01$, and three asterisks (***)
denotes $p < 0.001$.

**Consequences of membrane trafficking of EBOV GP on cell-cell fusion.**

**Proteinase K (PK) treatment.** We used digestion of cell surface EBOV GP by PK to
show that cell-cell fusion was strongly dependent on membrane trafficking of GP.
Incubating effector cells with PK either before (Figure 3-S2A, bar 2) or after (bar 3)
thermolysin treatment each led to minimal extents of fusion, showing that cleaved and
uncleaved GP is effectively degraded by PK. Adding (and then maintaining) PK prior to
the low pH pulse, but after binding effector and target cells, led to significantly less
fusion after reneutralization (bar 4) than did the control (bar 1). The addition of PK
immediately after the low pH pulse led to the same extent of fusion (bar 5) as did the
control (bar 1), strongly indicating that low pH induced conformational changes in GP
(through cleavage by cathepsins). Insensitivity of fusion to PK after, but not before, an
acidic pulse has previously been found for IAV HA-induced fusion [30].
We found that the elimination of fusion by PK was reversed over time (Figure 3-S2B). Effector cells were incubated with PK, washed, and then treated with thermolysin. Immediately carrying out our fusion protocol led to negligible extents of fusion, independent of whether a low pH pulse was employed (first set of two columns; bar 1 in absence of pulse, bar 2 with low pH pulse). If effector cells were allowed to recover for 2 h at 37°C before thermolysin treatment and fusion then initiated, extents of fusion were comparable to those recorded when PK had not been used. This was the case in the absence of a low-pH pulse (bar 2 of second set of columns) or with a pH-5.7 pulse (bar 2 of second set of columns). Increasing the time of recovery to 3 h after removing proteinase K led to extents of fusion comparable to those in which PK was never added (compare bar for low pH pulse after a two or three hour recovery (Figure 3-S2B) to bar 1 of Figure 3-S2A). The high level of fusion with recovery after protein degradation was likely due to new copies of EBOV GP that reached the cell surface.

We therefore used immunofluorescence to verify that cycling of EBOV GP was the reason fusion was restored over time. The fluorescence of effector cells expressing EBOV GP is shown (Figure 3-S3B). Background fluorescence for mock-transfected cells was negligible (Figure 3-S3A). Incubating cells with PK under the same conditions as used for fusion experiments and immediately staining led to a markedly reduced fluorescence (Figure 3-S3C and left bar of Figure 3-S3E). This shows that the PK had destroyed the integrity of EBOV GP on the cell surface. The fluorescence levels were
comparable to control when waiting 3 h after treating cells with PK (including the washout) before performing immunostaining (Figure 3-S3D and right bar of Figure 3-S3E). This demonstrates that the expression level of intact EBOV GP recover over time after PK treatment, and accounts for the observed time course of cell-cell fusion subsequent to removing PK. In conclusion, cell-cell fusion is dynamic, subject to EBOV GP retrieval from and delivery to the cell surface by normal cellular processes during the course of a fusion experiment.

**Brefeldin A (BFA) treatment.** Effector cells were incubated with the inhibitor (30 min at room temperature) prior to our standard thermolysin treatment, then bound to target cells, and fusion was induced by pH 5.7. BFA was constantly maintained to inhibit delivery of EBOV GP to the cell surface throughout an experiment (Figure 3-S4). The treatment led to significantly reduced fusion (Figure 3-S4, bar 2) compared to the control (bar 1) in which BFA was not present. This reduced fusion is consistent with removal of thermolysin-treated GP from the cell surface and the blockage of GP (both uncleaved and cathepsin-cleaved) transport from intracellular stores to the plasma membrane during the time course of our experiments: BFA prevented the replenishment of GP to the plasma membrane. In contrast, washing out BFA before thermolysin treatment led to a significantly restored extent of fusion (Figure 3-S4, bottom panel, bar 3). Allowing the effector cells to recover for an additional 30 min after the BFA washout, but before the thermolysin treatment, led to full restoration of fusion (Figure 3-S4, bar 4). After cleaving
EBOV GP with thermolysin, adding BFA to inhibit intracellular trafficking, and then binding these effector cells (always maintaining the concentration of BFA) to target cells, low amounts of fusion were observed two hours after a pH 5.7 pulse (Figure 3-S4, bar 5). The reduction in fusion is again consistent with the removal of thermolysin-cleaved GP from the cell surface and the prevention of GP delivery to the surface by BFA. Each process, alone or in combination, would lead to an insufficient density of GP on the cell surface. A greater extent of fusion occurred when the pH 5.7 pulse was applied after adding BFA (bar 6); this is expected because a low-pH pulse induces significant fusion, consistent with activation of cathepsins on the cell surface (see Figure 3-2B, thermolysin) before EBOV GP is presumed to be removed from the plasma membranes. Taken together, the results of these experiments clearly indicate that the extents of fusion correlate with the expected amount of cleaved EBOV GP on the cell surface.
Figure 3-1: Images of fused effector and target cells.

Effector (COS7) cells were loaded with calcein-AM (column 1, green), target cells were loaded with CMAC (column 2, blue) and both dyes are shown in column 3 (merged). The viral proteins expressed by transfecting effector cells are shown to the right of the images. Cells expressing EBOV GP were treated with 200 µg/ml thermolysin for 20 min; fusion was augmented with a 10-min pH 5.7 pulse. For cells expressing JSRV Env, a 10-min pH 5.0 pulse was used to trigger fusion. Effector cells expressing influenza virus (IAV) HA were treated with trypsin and neuraminidase as described (196), bound to HEK 293T cells, and fusion was triggered with a 10-min pH 4.8 pulse. For mock-transfected effector cells, a 10-min pH 5.7 pulse was employed. Fused cells are marked by arrowheads. For this set of experiments, the extent of fusion 1 hr after reneutralization was about 80% for EBOV GP, 50% for JSRV Env, and 70% for IAV HA.
Figure 3-1: Images of fused effector and target cells.
Figure 3-2: Thermolysin treatment results in greater extents of fusion between cells.

(A) Schematic of the experimental protocol is shown above the bar graph. E, effector cells; T, target cells, Th, thermolysin. Bar graph: Fusion of thermolysin-treated effector cells expressing EBOV GP (columns 1 and 2, dark red) was greater than for untreated cells (columns 3 and 4, dark yellow). For both thermolysin-treated and non-treated cells, a 10-min pH 5.7 pulse applied at room temperature augmented fusion, measured after an additional 2 h incubation at neutral pH. For each condition, at least 7 experiments were performed. Typical images used to obtain the data of the bar graph are shown on the right: in top images, cells were treated with 200 µg/ml thermolysin; in bottom images, cells were not treated. Cells that have fused are marked by arrows. (B) The kinetics of fusion for thermolysin-treated (dark red squares) and untreated (dark yellow circles) effector cells. Cleaving EBOV GP by thermolysin speeds fusion kinetics, but extents of fusion are the same for treated and untreated cells after a pH 5.7 pulse at 10°C is followed by a 4 h reneutralization. * p <0.05; *** p < 0.001.
Figure 3-2: Thermolysin treatment results in greater extents of fusion between cells.
Figure 3-3: Extents of fusion increased by overexpressing the receptor for EBOV GP.

(A) Overexpression of NPC1 (second set of two bars) led to greater fusion with effector cells than did mock-transfected target cells (first set of bars). For these experiments, the effector cells were not thermolysin-treated (i.e., these experiments relied on endogenous levels of GP cleavage). For each set of experiments, a 10-min pH 5.7 pulse (labeled “pulse pH +”) led to more fusion than when pH was never lowered (-). For each condition, n = 4. (B) The addition of sNPC1 to the external solution leads to a greater extent of fusion. Inset: Coomassie staining verification of sNPC1. BSA serves as a loading control. (C) Reducing and increasing the expression levels of NPC1 results in changes in the amount of NPC1 on the plasma membrane. (D) Fluorescence profiles of NPC1 from flow cytometry.
Figure 3-3: Extents of fusion increased by overexpressing the receptor for EBOV GP.
Figure 3-4: Detection of cleaved EBOV GP on the cell surface.

(A) The amount of cleaved GP or GP\textsuperscript{furin} on the cell surface with and without cotransfection of furin was determined by using sNPC1. Mean fluorescence intensity (MFI) was acquired by flow cytometry. Relative MFI was calculated by setting the WT GP without furin to 100. “None”: 293T cells that were not transfected with GP. Averages with standard deviations of at least 3 independent experiments are shown in each bar. (B) The expression of total GP, cleaved and uncleaved, on the cell surface was determined using an anti-FLAG antibody; in parallel, the same number of transfected cells were employed to measure cleaved GP using sNPC1. Relative MFI values are shown by setting the WT GP without furin to 100. “None”: 293T cells not expressing GP. (C) Relative cleaved GP on the plasma membrane measured in (A) was normalized by total GP measured in (B). (D) Western blots demonstrating cleavage of GP in the cell lysate of transfected cells used in panels (A), (B), and (C). (E) Extents of cell-cell fusion using the transfection protocols of (A), (B), and (C).
Figure 3-4: Detection of cleaved EBOV GP on the cell surface.
Figure 3-5: Reduced infection caused by mutations within EBOV GP correlates with reduced fusion.

(A) The reduction in retroviral MLV pseudotyped infection is shown for a series of EBOV GP mutants. (B) The mutations that resulted in reduced infection also led to reduced cell-cell fusion. (C) Each of the mutants was expressed well on the cell surface as determined by flow cytometry using an anti-FLAG antibody.
Figure 3-5: Reduced infection caused by mutations within EBOV GP correlates with reduced fusion.
Figure 3-6: The small molecule inhibitor 3.47 and the neutralizing antibody KZ52 against EBOV GP blocked GP mediated fusion.

(A) The inhibitor 3.47 (1 µM) was specific for EBOV GP, not affecting fusion mediated by SFV E1/E2 or IAV HA. (B) The inhibition of fusion by KZ52 (5.0 µg/ml) was also specific to EBOV GP. In all experiments of (A) and (B) a 10-min low pH pulse (pH 5.7 for EBOV GP, pH 5.4 for SFV E1-E2, pH 4.8 for IAV HA cleaved by trypsin) was employed. Results are at least four independent experiments. A 10-min pH 5.7 pulse augmented fusion for each protein. A comparison was made between pH 5.7 and 7.2 in each column. (C) Fluorescence intensity of the FITC-conjugated antibody measured by FACS showed that the presence of BafA1 reduced cleavage of plasma membrane GP. (D) The addition of BafA1 appeared to result in an increase of total GP in the plasma membrane. (E) BafA1 reduced the normalized cleaved GP on cell surface. In all figures, error bars are SEM; * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 3-6: The small molecule inhibitor 3.47 and the neutralizing antibody KZ52 against EBOV GP blocked GP mediated fusion.
Figure 3-7: The extent of fusion mediated by EBOV GP is maximal at pH 5.7.

(A) The extent of fusion between EBOV GP-expressing cells that were thermolysin-treated and target cells as a function of a 10-min, 37°C, low-pH pulse of the indicated pH values at varied times after reneutralization (30 min, black circle; 45 min, blue triangle; 1 h, red square). (B) The extent of cell-cell fusion induced by IAV HA is shown for the same conditions as in panel (A). Fusion progressively increased for lower pH pulses. (C) The extent of fusion as a function of pH (pH pulse applied at room temperature) after a 1 h (open bars) and 4 h (closed bars) reneutralization. (D) Effector cells were labeled with DiO and target cells with DiI. Both dyes were excited by a 488 nm laser; DiO emission was detected at 515 nm and DiI emission was recorded for 560 nm. For FACS measurements, trypsin and EDTA were added to cells prior to assaying; this treatment separates bound cells back into individual cells, but does not separate fused cells. Representative data for lipid dye mixing is shown for a 10-min pH 5.0 pulse (left panel), and a pH 5.7 pulse (right panel). (E) Average percentage of fusion as determined by lipid dye mixing as a function of pH (bars 2, 3, and 4). Lipid dye spread was negligible when using effector cells that were mock-transfected (bar 1). Thresholds for DiO and DiI were the same for all experiments and indicated on the two panels. Fusion was scored as the percentage of fluorescent particles above both thresholds (i.e., the third quadrant). Error bars are SEM (n = 6, for each bar) and extents of fusion were statistically compared to the extent for a pH 5.7 pulse. * p <0.05; *** p < 0.001.
Figure 3-7: The extent of fusion mediated by EBOV GP is maximal at pH 5.7.
Figure 3-8: EBOV GP-induced fusion pores enlarge slowly.

(A) The slow spread of calcein through EBOV GP-induced pores (upper panels) compared to JSRV Env-induced (lower panels) pores is shown. Calcein-AM was loaded into effector cells, and target cells were unlabeled. The dotted circle encloses the target cell receiving calcein. The moment that calcein first appears in the target cell is defined as time = 0. The right panels show calcein within the cells at t = 10 min. (B) A plot of calcein fluorescence in the effector and target cells as a function of time. The red line is the trace for an EBOV GP pore; the blue line is the trace for a JSRV Env pore. The upper time scale in units of hundreds of seconds refers to the JSRV Env pore; the lower time scale in units of minutes refers to the EBOV GP pore. The small difference in fluorescence of effector and target cells connected by the JSRV Env pore indicates that a small percentage of calcein is not free to transfer, possibly because it is bound to cellular elements. (C) Images of effector cells loaded with calcein AM (green) and CMTMR (red) and target cells loaded with CMAC (blue) taken after a 2 hr reneutralization at 37°C that followed a 10-min pH 5.7 pulse. Calcein has transferred (white arrows, left panel) from most of the effector cells in contact with target cells. CMTMR (middle panel) mixed with CMAC for only one cell (brown arrow). An overlay of calcein and CMTMR is shown in the third panel, with calcein (white arrows) and CMTMR (brown arrow) transfer shown.
Figure 3-8: EBOV GP-induced fusion pores enlarge slowly.
Figure 3-9: Electrical measurements demonstrate the slow, limited growth of EBOV GP-mediated pores.

(A) Conductance traces of the three fusion pores, as detected by capacitance measurements are shown. None appreciably enlarged. (B) Representative pores induced by fusion proteins of different viruses are compared to the EBOV GP pore. HIV: Human Immunodeficiency Virus 1; ASLV: Avian Sarcoma and Leukosis Virus. The illustrated representative EBOV GP pore is the same pore shown in the first trace of panel A.
Figure 3-9: Electrical measurements demonstrate the slow, limited growth of EBOV GP-mediated pores.
Figure 3-10: Neutralization of endosomes by ammonium chloride reduces fusion induced by EBOV GP.

(A) Adding chloroquine (100 μM) or NH₄Cl (10 mM) to the bathing solution greatly reduced EBOV GP-induced fusion after a pH 5.7 pulse (first set of three bars, endogenous cleavage (i.e., without thermolysin treatment) was employed. For fusion induced by SFV E1/E2 (with a pH 5.4 pulse, second set of three bars) or IAV HA (cleaved into HA1-HA2 subunits by a standard trypsin procedure, and employing a pH 4.8 pulse, third set of three bars), neither chloroquine nor NH₄Cl greatly reduced fusion. (The addition of NH₄Cl did, however, result in a slight decrease in SFV E1/E2-induced fusion.) Chloroquine or NH₄Cl was added 20 min prior to acidification. Low pH was maintained for the standard 10 min, and fusion was measured 1 h after reneutralization.

(B) The majority of the blockage of EBOV GP-induced fusion by NH₄Cl was restored by the addition of recombinant cathepsins B (rh CatB) to the external solution immediately after a 10-min pH 5.7 pulse. The recombinant enzyme was present during the subsequent 1 hr reneutralization, with fusion then measured. (C) Dose-response curve for inhibition of fusion by chloroquine. (D) Dose-response curve for inhibition of fusion by NH₄Cl. (C) and (D) are plotted semi-logarithmically
Figure 3-10: Neutralization of endosomes by ammonium chloride reduces fusion induced by EBOV GP.
Figure 3-11: Blocking cathepsins that cleave EBOV GP reduces fusion.

(A) An inhibitor of cathepsin L (second bar of each set of bars), cathepsin B (third bar of each set of bars), cathepsin A (fourth bar of each set) and cathepsin D (fifth bar of each set) are shown and compared to the case in which the inhibitor was not added (first bar of each set, control). For effector cells not thermolysin-treated, fusion experiments were performed in the absence of a low-pH pulse (first set of columns) and with a pH 5.7 pulse (second set of columns). For cells treated with thermolysin, cathepsin inhibitors were added prior to the thermolysin treatment and maintained throughout the experiments. For thermolysin-treated cells, fusion was measured for the case without (third set of bars) and with a low pH pulse (fourth set). Only inhibitors of cathepsin L or B diminished fusion, and they did so for all conditions. Each of the four cathepsin inhibitors was added (separately) at the time of mixing effector and target cells. The cathepsin L and cathepsin B inhibitors reduced fusion to the same extent at pH 5.7 and pH 7.2. The cathepsin B and D inhibitors were without effect. Fusion was measured 2 hr after the low pH pulse. The concentration of all inhibitors was 100 μM, a high concentration to ensure maximal inhibition. (B) In contrast to the experiments in (A), the cathepsin B inhibitor was added subsequent to the low pH 5.7 pulse (second bar). Less fusion occurred than for control (filled bar). (C) A recombinant human cathepsin B (rh CatB, 200 μM) was added to effector cells that had not been treated with thermolysin. The addition of rh CatB (hashed bar) led to substantially increased fusion (filled bar, control). A 10-min pH 5.7 pulse was
applied to promote fusion. The extents of fusion were measured after a 2-hr reneutralization for all experiments of this figure. Error bars are SEM. (D) 293T cells were co-transfected with plasmids encoding EBOV GP and cathepsin B. Cleaved GP on the plasma membrane was measured by flow cytometry using sNPC1, and the cleaved form was normalized by the total GP (measured by anti-FLAG), as described in Figure 3-4. Alternatively, GP-expressing cells were treated with thermolysin, and cleaved GP was measured and normalized as described. **p<0.01; ***p<0.001.
Figure 3-11: Blocking cathepsins that cleave EBOV GP reduces fusion.
Figure 3-12: After EBOV GP is cleaved, GP-mediated fusion is independent of pH.

(A) Blocking delivery of EBOV GP to the cell surface yields pH-independent fusion: BFA was added to effector cells 45 min prior to mixing them with target cells, and then maintained to prevent EBOV GP trafficking to the plasma membrane. This eliminated the pH-dependence of fusion for thermolysin-treated effector cells. (B) Utilizing the standard fusion protocol, but with a cathepsin B inhibitor (100 µM) used to pretreat effector cells for 45 min and maintained at all times, fusion of thermolysin-treated effector cells with target cells was independent of pH. Fusion was much greater in the absence of the inhibitor. More importantly, inhibition of cathepsin activity eliminates the pH-dependence of fusion.
Figure 3-12: After EBOV GP is cleaved, GP-mediated fusion is independent of pH.
Figure 3-13: Schematic diagram illustrating control of fusion by cleavage of EBOV GP and its transport to the cell surface.

EBOV GP is synthesized in ER, transported to Golgi complexes where it is processed into GP1 and GP2 subunits, and ultimately targeted to the plasma membrane. EBOV GP can undergo endocytosis from the plasma membrane and eventually reach late endosomes and lysosomes. It is then further cleaved by cellular cathepsins, bound by NPC1, and recycled back to plasma membrane. Alternatively, EBOV GP is directly cleaved by cathepsins on the plasma membrane or by thermolysin treatment. EBOV GP proteins do not permanently remain on the surface, but rather undergo continual delivery and removal. Cleaved GP accumulates at potential fusion sites, leading to the observed increased fusion over time. Solid arrows denote pathways definitively established in the present study. Dashed arrows denote pathways that are likely to occur based on data of the present study. Light dashed arrows denote pathways suggested by data of the present study.
Figure 3-13: Schematic diagram illustrating control of fusion by cleavage of EBOV GP and its transport to the cell surface.
Figure 3-S1: Dose-response curves for inhibition of EBOV GP-mediated fusion by 3.47 (A) and KZ52 (B), plotted semi-logarithmically.
Figure 3-S1: Dose-response curves for inhibition of EBOV GP-mediated fusion by 3.47 (A) and KZ52 (B), plotted semi-logarithmically.
Figure 3-S2: Proteinase K treatment demonstrates that protein synthesis and trafficking of EBOV GP to the cell surface contributes to the extents of fusion.

(A) The periods in which proteinase K (PK, 200 μg/ml) was present are marked in the schematic protocol, and numbers correspond to bar numbers below. Regardless of whether PK was present prior (bar 2) or subsequent (bar 3) to treating effector cells with thermolysin, the presence of PK virtually eliminated fusion. Similarly, adding and then maintaining PK to effector cells as they were bound with target cells led to greatly reduced fusion (bar 4). But adding PK immediately after the low pH pulse (bar 5) hardly affected fusion. (B) EBOV GP-mediated fusion recovered over time after proteinase K treatment: Left-hand bars of each pair denote that a pH pulse was not applied; a pH 5.7 pulse was applied for the right hand bars. Adding proteinase K and washing out immediately prior to thermolysin treatment virtually abolished fusion (first set of two bars). Allowing 2 hr between proteinase K removal and thermolysin treatment restored most of the fusion (second set of bars). Waiting 3 h completely restored fusion (third set of bars).
Figure 3-S2: Proteinase K treatment demonstrates that protein synthesis and trafficking of EBOV GP to the cell surface contributes to the extents of fusion.
Figure 3-S3: Immunostaining of cells demonstrates recovery of cell surface EBOV GP after proteinase K treatment.

Left hand panels of each pair show confocal images of FITC fluorescence alone; right hand panels show fluorescence and cells in differential interference contrast. An anti-EBOV GP antibody (KZ52) was used for staining EBOV GP. A secondary FITC-labeled antibody was used to immunostain. (A) Immunostaining showed that mock-transfected cells did not react with the antibody. (B) Cells transfected with EBOV GP did show significant staining (upper right images). (C) The staining protocol was used without delay after treating cells with PK. (D) Maintaining the cells for 3 h in DMEM at 37°C before immunostaining. (E) The effect of PK treatment on EBOV GP expression was assessed using Volocity imaging software (Perkin Elmer). Integral fluorescence per field (3 image fields per datum point) was calculated after subtracting the fluorescence background determined from the mock-transfected images. This quantification shows that expression of EBOV GP was greatly reduced by the proteinase K treatment and significantly recovered after the protease was absent for 3 h. This demonstrates that the EBOV GP expression levels were steady over time.
Figure 3-S3: Immunostaining of cells demonstrates recovery of cell surface EBOV GP after proteinase K treatment.
Figure 3-S4: Inhibitors of trafficking show that EBOV GP is dynamically exchanged between plasma and intracellular membranes.

The presence of Brefeldin A (BFA, 50 μM) at all points of the fusion protocol that utilizes thermolysin-treated effector cells and a pH 5.7 pulse reduced fusion greatly (bar 2) compared to the control (bar 1, BFA was not included). Washing out BFA and immediately treating effector cells with thermolysin led to greater fusion (bar 3). Waiting 30 min after the washout before thermolysin treatment led to fusion (bar 4) comparable to control. Adding and maintaining BFA after binding effector and target cells, but before applying a low pH pulse led to substantially reduced fusion (bar 5). Applying BFA after the low pH pulse led to less fusion than the control (bar 1), but to greater fusion than when the drug was added prior to the low pH pulse (bar 6, extent of fusion higher than for bar 5). Thermolysin was used to cleave EBOV GP just prior to measuring fusion for all conditions of, allowing meaningful comparisons.
Figure 3-S4: Inhibitors of trafficking show that EBOV GP is dynamically exchanged between plasma and intracellular membranes.
IV. VIPERIN RESTRICTS EBOV PRODUCTION BY INDUCING AUTOPHAGY-MEDIATED VP40 DEGRADATION

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This manuscript is in preparation.

4.1 Abstract

EBOV is a deadly RNA-virus that causes hemorrhagic fever in humans and animals. Here, we show that interferon-inducible Viperin protein potently inhibits EBOV production and replication. By using a replication-competent EBOV tetracistronic minigenome system (trVLP), we demonstrate that EBOV infection induces expression of Viperin in human A549 cells, which in turn inhibits the EBOV replication. Consistent with this finding, knockdown of Viperin in A549 cells enhances EBOV replication. Further experiments show that Viperin severely impairs EBOV production but has modest effect on viral entry. By using EBOV VP40-based virus-like particle (VLP) and minigenome (trVLP) systems, we show that Viperin strongly blocks EBOV production by decreasing VP40 levels in viral producer cells. Interestingly, we find that the down-regulation of VP40 can be effectively rescued by treating cells with two lysosomal degradation inhibitors, NH₄Cl and BafA1, suggesting that autophagy might be involved.
Indeed, overexpression of Viperin induces LC3 lipidation, upregulates Beclin-1 and ATG5, as well as increases autophagy-related puncta formation by LC3-GFP. Consistent with the role of autophagy, induction of autophagy by Rapamycin blocks EBOV production. Altogether, our results indicate that Viperin inhibits EBOV production by autophagy-mediated degradation of VP40, suggesting that IFN-stimulated Viperin interacts with the cellular autophagy pathway to restrict EBOV infection.

4.2 Introduction

EBOV is a deadly ssRNA virus that belongs to *Filoviridae* family in the order of *Mononegavirales* (1). EBOV causes hemorrhagic fever in human and other species, with a fatality rate up to 90%. So far, there are no FDA-licensed vaccines available against EBOV infection. While adaptive immune response is crucial for ultimate control of EBOV infection, innate immunity also plays important role, especially in the early stage of viral infection. Indeed, several IFN-stimulated genes (ISGs), including ISG15, IFITMs and Tetherin, have been shown to potently inhibit EBOV replication (131, 233, 234). However, many viruses, including EBOV, have evolved various strategies to counteract the effects of antiviral factors by encoding viral proteins. For example, EBOV polymerase cofactor (VP35), the small matrix protein (VP24), and glycoprotein (GP) have been shown to antagonize ISG15 and Tetherin (235, 236). Limited understanding of host innate immunity, especially IFN-mediated immune response, has hampered development of novel antiviral therapeutics against EBOV and other viral diseases.
Viperin, originally known as cytomegalovirus inducible gene 5 (cig5), is an antiviral factor initially identified from human cytomegalovirus (HCMV) infected fibroblasts (89). Viperin consists of 361 amino acids in length, with a predicted molecular weight of 42 kDa. While Viperin is IFN-inducible, it is also expressed in some tissues at a basal level. Primate Viperin has been shown to evolve under positive selection as many other antiviral factors, such as PKR, TRIM5α and APOBEC3G (134-137), which together shape the co-evolution of virus and host. In addition to type I IFN, poly (I: C), Lipopolysaccharides (LPS), and viral infection have also been shown to upregulate Viperin expression.

Viperin has been shown to restrict a variety of DNA and RNA viruses, such as HCMV, human immunodeficiency virus (HIV), influenza A virus (IAV), hepatitis C virus (HCV), and Japanese encephalitis virus (JEV), etc. (89, 91, 98, 102, 103, 105-107, 109, 115, 128). However, the mechanisms of action by Viperin are still poorly understood. A previous study on IAV infection indicates that Viperin can restrict its release by disrupting lipid rafts through the interference of farnesyl diphosphate synthase (FPPS), an enzyme essential for cholesterol and isoprenoid biosynthesis (108). Viperin may also restrict viral infection by other mechanisms (92). A deeper understanding of the antiviral effect of Viperin as well as the underlying mechanisms shall contribute to development of therapeutic agents against pathogenic viral diseases, including Ebola.

In this study, we examined the antiviral activity of Viperin on EBOV. Our results show
that Viperin potently blocks EBOV replication and production by inducing autophagy, resulting in degradation of EBOV VP40. The effects of Viperin on other steps of EBOV infection are also investigated, which together support the notion that Viperin is an important ISG to restrict EBOV.

4.3 Results

EBOV induces Viperin expression in cultured epithelial cells, which in turn restricts viral replication. Viperin can be induced by viral infection, either dependent or independent of IFN (92). We assessed if EBOV replication can induce Viperin expression by using a tetracistronic minigenome virus-like particles (trVLPs) system. We transfected 293T cells with plasmids encoding 4 essential EBOV proteins (pCAGGS-NP, pCAGGS-VP35, pCAGGS-VP30, pCAGGS-L) and a Renilla-coding minigenome, all of which are required for trVLP production, and infected A549 cells with the produced trVLPs. Note that A549 cells were pre-transfected with four essential plasmids so as to support EBOV replication, and the multiple rounds of EBOV replication was measured by luciferase activity and the effect on Viperin expression was quantified by real-time polymerase chain reaction (qRT-PCR). We found that EBOV replication in A549 cells strongly induced Viperin expression, in a time-dependent manner following infection, although the extent of induction was relatively less compared to IFN-α treatment (Figure 4-1A).

We next knocked down Viperin in A549 cell lines by stably transducing cells with
lentiviral vectors encoding Viperin shRNA, and infected these cells with trVLPs produced from 293T cells; A549 cells are known to express an endogenous level of Viperin (89, 110). The knockdown efficiency of Viperin in A549 cells was confirmed by western blotting, with clone 1 showing greater reduction of Viperin compared to clone 2 (Figure 4-1B). Consistent with its higher knockdown efficiency, clone 1 exhibited significantly enhanced replication of EBOV trVLPs (by ~ 5000 folds) compared to clone 2 and shRNA control (Figure 4-1C). To further confirm these results, we overexpressed Viperin in HEK293T cells, either by transient transfection or by establishing a stable cell line, and we observed a significantly decreased EBOV trVLP replication (Figure 4-1D). The expressing of Viperin in 293T stable cells was confirmed by western blotting (data was not shown).

To determine if Viperin inhibits EBOV RNA genomic replication, we applied an EBOV monocistronic minigenome system in 293T cells overexpressing Viperin. The activity of luciferase, which is an indicative of RNA replication, was determined at 24 and 48 hours post-transfection. However, we only found modest reduction of luciferase activity in Viperin-expressing cells compared to control, roughly by ~ 2-fold, (Figure 4-1E), indicating that Viperin does not significantly affect EBOV RNA replication, implying that the restriction of EBOV by Viperin likely occurs at other steps of the viral life cycle.

**Viperin modestly affects EBOV entry.** We next examined if Viperin affects EBOV
entry. To this end, we infected A549 cells stably expressing shRNA against Viperin, or 293 cells stably overexpressing Viperin, with EBOV trVLPs without pre-transfecting these cells with required plasmids for multiple rounds of viral replication; the luciferase activity in infected cells was determined at 24 hours post-infection. We observed that knockdown of Viperin in A549 cells led to a less-than 2-fold increase in luciferase activity (Figure 4-2A), in sharp contrast to its dramatic effect on the multiple rounds of EBOV replication (Figure 4-1C). Consistent with this result, overexpression of Viperin in 293 did not show a significant effect on EBOV entry, again by 25% (Figure 4-2B). To confirm these results, we generated EBOV VP40-based VLPs, and infected these cells with VLPs containing VP40-Blam; in this case, EBOV entry was determined by measuring the β-lactamase activity of infected cells by flow cytometry. Alternatively, MLV-pseudotypes bearing EBOV GP and or recombinant vesicular stomatitis virus (rVSV) bearing GP were produced and used for infection. In all cases, no more than 2-fold increase in EBOV entry was observed in Viperin knockdown cells (Figure 4-2C, D, and E), again supporting the notion that Viperin has no significant effect on EBOV entry.

Viperin severely impairs EBOV production. Given that Viperin has no significant effect on EBOV entry and its genomic replication, we evaluated if Viperin reduces viral production. HEK293T cells were transfected with plasmids encoding Viperin for EBOV trVLP production, followed by ultracentrifugation to concentrate the viral particles. The levels of VP40 in the purified virions and cell lysates were determined by western
blotting using an anti-VP40 antibody. The results showed that VP40 proteins in both purified virions and viral producer cells were significantly decreased, in a Viperin-dose dependent manner (Figure 4-3A). Accordingly, the infectivity of the produced virions in 293T cells was reduced, also in a Viperin-dose dependent manner (Figure 4-3B). To confirm these results, we applied an EBOV VP40-Blam system, which does not involve EBOV RNA genome replication but allows measurement of virus uptake. To this end, we transfected 293T cells with increasing doses of Viperin, along with plasmids encoding EBOV GP and VP40-GFP; equal volumes of culture supernatants were used for infection of HTX cells and virus uptake was determined by detecting GFP signal using flow cytometry. As shown in Figure 4-3C and D, Viperin significantly reduced the uptake of EBOV VP40-GFP VLPs in HTX cells in a dose-dependent manner. We noted low levels of GFP signals for VP40-GFP VLPs without expressing GP, which were also reduced by Viperin, possibly reflecting a non-specific binding and uptake of VP40-GFP VLPs by cell membrane (Figure 4-3C and D). Altogether, these results demonstrate that Viperin inhibits EBOV production, which contributes to the reduced viral replication shown in Figure 4-1.

The ability of Viperin to reduce VP40 expression can be rescued by treating cells with lysosomotropic agents. As shown above, one of the most obvious effects of Viperin on EBOV was the decreased VP40 level in viral producer cells. To investigate the underlying mechanism, we transiently transfected 293T cells with VP40-GFP, along with
different amounts human or Rhesus Viperin-coding plasmids, in the presence of EBOV GP; cell lysates and purified viral particles were examined by western blotting. We found that not only human but also Rhesus Viperin proteins decreased VP40 levels, similar to the results obtained from the trVLP system shown in Figure 4-3A (Figure 4-4A). Interestingly, we found that EBOV GP level in cell lysates was also slightly reduced by Viperin (Figure 4-4A), which had likely contributed to the reduced infectivity of VL40-VLP virions. To assess if the effect of Viperin on VP40 and GP was specific to EBOV, we performed similar transfection procedure using MLV pseudovirions bearing EBOV GP, and we observed that, while MLV Gag was not affected by Viperin, EBOV GP was modestly decreased by Viperin in a dose-dependent manner (Figure 4-4B). We also observed a low level of Viperin being incorporated into viral particles, which could have had additional effects on EBOV infectivity and replication.

Given that Viperin impairs the protein levels of GP and VP40 in viral producer cells, we next sought to investigate if proteasomal pathway and lysosomal degradation pathways are involved. We treated 293T cells coexpressing Viperin and EBOV VP40-GFP with BafA1, NH₄Cl, or MG132 – the first two belong to lysosomal inhibitors and the last one is a proteasomal inhibitor. Western blotting results showed that treatment of cells with BafA1 and NH₄Cl, but not MG132, substantially rescued the expression of VP40-GFP (Figure 4-4C), suggesting that lysosome-dependent pathway is involved in Viperin-mediated degradation of VP40.
Expression of Viperin induces autophagy that decreases VP40 expression. Viperin is a cytosolic protein, which is normally localized in lipid droplets and vesicular compartments. One possibility that Viperin reduces VP40 and GP is through autophagy, a normal cell process that degrades cytoplasmic materials including viral proteins. Autophagy-mediated degradation involves fusion between autophagosome and lysosomes, a process that can be inhibited by lysosomotropic agents, such as BafA1 and NH4Cl used above (237). To investigate if autophagy is involved in Viperin-mediated degradation of VP40, we transfected 293T cells with increasing doses of Viperin, along with plasmids encoding EBOV VP40-GFP and GP; the expression levels of the autophagy-specific proteins, i.e., ATG5 and beclin-1, were determined by western blotting. Results showed that the expression levels of ATG5 and beclin-1 were increased, as the amount of Viperin increased (Figure 4-5A), suggesting that Viperin likely induces autophagy.

To further investigate the possible role of Viperin in autophagy, we determined if expression of Viperin induces lipidation of microtubule-associated protein 1 light chain 3 (LC3). LC3 is an essential component of autophagosome, and its lipidated form, known as LC3-II, is often used as a marker of autophagic process (238, 239). Induction of autophagy often leads to increased LC3-II expression and associated puncta formation in the cell (237, 240). We thus transfected 293T cells with plasmid encoding LC3-GFP along with Viperin, and upon examination by fluorescent microscope we observed
increased numbers of puncta formed by LC3-GFP, with greater size, in Viperin-overexpressing cells compared to mock control (Figure 4-5B). As expected, Rapamycin greatly induced puncta formation formed by LC3-GFP (Figure 4-5B and C). Western blotting results showed an increased level of LC3-II in Viperin-expressing cells compared to mock (Figure 4-5D), confirming that expression of Viperin does induce autophagy.

We next examined if Rapamycin-induced autophagy diminishes EBOV VP40 production. To this end, we transfected 293T cells with VP40-GFP plasmid and treated cells with different concentrations of Rapamycin; cells were lysed 36 hours post-transfection and western blotting was performed. We observed that Rapamycin strongly increased the expression of ATG5, as would be expected, yet decreased the levels of EBOV VP40 (Figure 4-5E), indicating that autophagy is linked to the degradation of EBOV VP40.

4.4 Discussion

Previous studies have shown that Viperin acts as a multifunctional protein exhibiting both antiviral and proviral functions depending on specific viruses. One of its notable antiviral functions is that Viperin blocks the release of influenza virus by disturbing lipid rafts on the plasma membrane (106, 108). This mechanism has prompted us to investigate whether Viperin has a similar function to restrict EBOV. Indeed, we find that Viperin potently inhibits EBOV replication. Surprisingly, however, we did not find a
potent effect of Viperin on EBOV release but EBOV production is impaired. Specifically, we reveal that EBOV VP40 is downregulated by Viperin in viral producer cells, likely by inducing autophagy, leading to diminished viral production.

Several lines of evidence support the conclusion that Viperin impairs EBOV production by decreasing VP40 levels during viral production. First, we show that overexpression of Viperin in 293T cells dramatically reduces VP40 protein levels in cells producing virions, leading to significantly decreased viral production; this was demonstrated in both VP40-GFP-based VLP and the replication-competent trVLP systems. Second, we find that knockdown of Viperin in A549 cells significantly increases EBOV production, resulting in enhanced viral infection in target cells. Third, in addition to human Viperin, Viperin protein derived from rhesus monkey also impairs viral production by downregulating EBOV VP40 levels in viral producer cells. Fourth, the effect of Viperin on EBOV production appears to be filovirus VP40-specific, as MLV pseudotypes bearing EBOV GP is marginally affected by Viperin. Last, we demonstrate that the replication of EBOV trVLPs in human A549 epithelial cells induces Viperin expression, suggesting that induction of Viperin is physiologically relevant to EBOV replication. Because Viperin can also be induced by type I IFN, we cannot rule out the possibility that the induction of Viperin during EBOV trVLP replication is mediated by type I IFN. Regardless, our results demonstrate that Viperin contributes to and likely plays an important role in host suppression of EBOV replication at the early stage of the
viral life cycle.

One crucial finding of this study is that expression of Viperin induces autophagy, which mediates the degradation of EBOV VP40 in viral producer cells and is therefore responsible for the impaired viral production. Autophagy is a “self-eating” process that is critical for maintenance of normal cellular homeostasis by engulfing cytosolic cargos through fusion of autophagosomes and lysosomes (241). Autophagy has been shown to modulate many viral infections, although depending on specific viruses the effects can be either antiviral or proviral (92). In particular, fusion between autophagosomes and lysosomes efficiently has been shown to induce the activation of interferon regulatory factor 7 (IRF7), which up-regulates type I production therefore promoting antiviral activities (242). Notably, many viruses, including influenza A virus and herpesviruses, have evolved distinct mechanisms to counteract the autophagy-mediated inhibition of viral infection, hence benefiting their replication (243-247). Some other viruses, such as paramyxoviruses, can induce autophagy and usurp this pathway to enhance their viral production and replication (248).

We show in this study that the ability of Viperin to reduce EBOV VP40 in viral producer cells can be effectively rescued by treating the cells with lysosomal inhibitors, NH₄Cl and BafA1, providing the first piece of evidence that autophagy-mediated lysosomal degradation is likely involved in the impairment of EBOV production by Viperin. Indeed, our additional experiments demonstrate that induction of autophagy by
Rapamycin potently degrades VP40. While it remains to be determined how exactly Viperin triggers autophagy, our results open a new avenue of research towards a better understanding the interplay between Viperin and cellular autophagy pathway during EBOV infection.

A previous study from HIV shows that Viperin is under positive selection among primate species, which has been likely been driven by viral infection (134). However, overexpressions of primate Viperin proteins failed to significantly restrict all lentiviruses tested (134). As EBOV is thought to infect primates, which may have facilitated transmission to humans, we have examined some primate Viperin proteins and found that rhesus monkey Viperin also decreases EBOV VP40 levels in viral produces and impairs viral production. It will be interesting to determine if Viperin proteins derived other nonhuman primate species would have similar or distinct anti-EBOV activities. Indeed, for HIV and primate lentiviruses, differential antiviral activities of TRIM5α, Tetherin and APOBEC3G between human and nonhuman primate species have been documented to explain the current epidemic of AIDS, which is likely due to relative weak or nonfunctional antiviral activities of some of these cellular factors, or alternatively, the potent antagonisms rendered by lentiviral accessory proteins. In this sense, it will be interesting and important to explore the antiviral functions of Viperin proteins of other nonhuman primate species, including gorillas and chimpanzees, as well as explore how EBOV and other filoviruses may counteract Viperin restriction. Overall, a better
understanding of the EBOV-host interaction, as described in this study, could offer new approaches to development of novel antiviral strategies.

4.5 Materials and Methods

Reagents and plasmids. The plasmid encoding VP40-Blam was from Lijun Rong (University of Illinois, Chicago). The plasmid encoding VP40-GFP was provided by Kartik Chandran (Albert Einstein College of Medicine). The plasmid encoding FLAG-tagged mucin-deleted EBOV GP was from David Sanders (Purdue University). The plasmids required for production of EBOV trVLPs, i.e., pCAGGS-NP, pCAGGS-VP35, pCAGGS-VP30, pCAGGS-L, pCAGGS-T7 and p4cis-vRNA-Rluc (encoding VP40, GP and VP24), were provided by Heinz Feldmann (National Institute of Allergy and infectious Diseases). LC3-GFP was purchased from Addgene (Cambridge, MA). The rabbit antibody against EBOV VP40 was purchased from IBT Bioservices (Gaithersburg, MD). The mouse antibody against GFP was purchased from Santa Cruz. The mouse antibody against Flag tag was purchased from Sigma (St Louis, MO). The rabbit antibody against Viperin was purchased from Sigma (St Louis, MO).

Cell lines. Cells were maintained in Dulbecco’s modified Eagle’s (DMEM) medium with 10% fetal bovine serum (FBS) plus 1% penicillin/streptomycin (CORNING). HEK293T and HTX (a subclone of HT1080) have been previously described (205). HEK293 cell lines stably expressing Viperin were generated by transducing cells with pLPCX or LNCX retroviral vectors expressing individual proteins (gifts of Michael Emerman).
followed by puromycin (Sigma, 1 ug/ml) or neomycin selection (Sigma, 1 mg/ml). A549 cells with Viperin knockdown and control shRNA were generated by transducing cells with Viperin-specific shRNA or scramble-shRNA, respectively, followed by puromycin selection (Sigma, 2 ug/ml).

**Production and infection of EBOV tetracistronic minigenome virus-like particles (trVLPs).** HEK293T cells were seeded onto 6-well plates and transfected with plasmids pCAGGS-NP, pCAGGS-VP35, pCAGGS-VP30, pCAGGS-L, pCAGGS-T7 and p4cis-vRNA-Rluc as previously described (249), in the presence or absence of Viperin. The supernatant containing EBOV trVLPs was centrifuged to remove cell debris and used to infect pre-transfected target cells expressing NP, VP35, VP30 and L. At 24 to 48 hours post infection, the infected cells were lysed for detecting *Renilla* luciferase activity by following providers’ instructions (Promega, Madison, WI).

**Production and infection of MLV pseudotypes bearing EBOV GP or EBOV-VP40 VLPs.** Production of MLV retroviral pseudotypes bearing EBOV GP and viral infection were as described previously (231). For production of VP40-based VLPs, HEK293T cells were transfected with plasmids encoding EBOV GP and VP40-GFP or VP40-Blam in the presence or absence of Viperin. The supernatant containing EBOV VLPs was collected from 24 to 72 hours, followed by centrifugation to remove cell debris. Viruses were used to infect HTX cells for 6 hours, and the viral uptake was determined by flow cytometry.

**Western blotting.** Cells were washed by PBS and lysed in radioimmunoprecipitation
assay (RIPA) lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS and 1% NP40) containing freshly added PMSF and a protease inhibitor cocktail (Sigma) for 20 min on ice. Cell lysates were centrifuged at 13,000 × g and 4°C for 10 min to remove cell debris. Cell lysates were boiled for 10 min in SDS sample buffer, subjected to 7.5 % or 10 % SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. The proteins in cell lysate were probed with indicated antibodies, followed by analysis of the chemiluminescence image analyzer LAS3000.

Immunofluorescence microscope and induction of autophagy. HEK293T cells were transfected with plasmids encoding LC3-GFP in the presence or absence of Viperin. Autophagy inhibitors or inducers were added into culture medium at 6 hours post-transfection. At 24 to 48 hours post-transfection, puncta formed by LC3-GFP were detected and counted by fluorescent microscope. The expression and lipidation of LC3-GFP was detected in cell lysate of transfected cells by western blotting.

Statistical analysis. One-way ANOVA (and nonparametric) analysis was used in all statistics analysis. In all figures, at least results from 3 independent experiments were used in statistical tests unless otherwise indicated.
Figure 4-1: Replication of EBOV trVLPs in cultured epithelial cells induces Viperin expression, which inhibits viral infection.

(A) Induction of Viperin by EBOV replication. A549 cells were pre-transfected with plasmids required for replication of EBOV trVLPs, followed by infection of EBOV trVLPs or treatment with IFN-α (1000 U/ml) at 24 hour post-transfection. At indicated time points, the mRNA level of Viperin was determined by qRT-PCR. The results shown are from one representative experiment. (B) Western blotting analysis of Viperin expression in A549 cell lines stably expressing Viperin shRNA or control shRNA. Viperin expression in cell lysate was detected by using a specific antibody against Viperin. (C) Infection of EBOV trVLPs in A549 cell lines stably expressing Viperin- or control-shRNA. All A549 cell lines were pre-transfected with plasmids required for replication and subjected to EBOV trVLPs infection. At 48 hours post-infection, luciferase activity was determined in infected cells. (D) Infection of EBOV trVLPs in 293T cells transiently-transfected with Viperin plasmids or in 293 cells stably expressing Viperin. (E) Effect of Viperin on EBOV RNA replication using a monocistronic minigenome system. HEK293T cells were transfected with plasmids encoding EBOV NP, VP35, VP30, L and monocistronic minigenome, along with Viperin; at 48 hours post-transfection, luciferase activity was determined in transfected cells.
Figure 4-1: Replication of EBOV trVLPs in cultured epithelial cells induces Viperin expression, which inhibits viral infection.
Figure 4-2: Viperin modestly affects EBOV entry.

(A and B) One-round infection of EBOV trVLPs in A549 cells stably expressing shRNA against Viperin (A), or in 293 cells stably expressing Viperin (B). Cells were infected with EBOV trVLPs without being pre-transfected with plasmids that support viral replication. At 24 hours post-infection, luciferase activity was determined in infected cells. (C, D and E) A549 cells stably expressing shRNA against Viperin or control shRNA were infected with EBOV VP40-Blam VLPs (C), MLV-pseudotypes bearing EBOV GP (D), or rVSV bearing GP (E). Cells were infected for different periods of time, and GFP signals in infected cells were analyzed by flow cytometry.
Figure 4-2: Viperin modestly affects EBOV entry.
Figure 4-3: Viperin severely impairs EBOV production.

HEK293T cells were transfected with plasmids encoding Viperin, along with plasmids required for the production of EBOV trVLPs (A and B), of EBOV VP40-GFP-based EBOV VLPs (C and D) or of MLV-pseudotypes bearing EBOV GP (E). Viruses were harvested from 24 to 72 hours post-transfection, and subjected to viral infection. (A) The levels of VP40 in purified EBOV trVLPs and cell lysates were determined by western blotting with an anti-VP40 antibody. (B) The infectivity of virions produced from (A) was determined by infecting HEK293T cells. (C) Representative flow cytometry profiles showing the infection rates of EBOV VLPs generated from Viperin-expressing HEK293T cells. (D) Summary of results of multiple experiments from (C). (E) The infection rates of MLV-pseudotypes bearing GP produced from Viperin-expressing HEK293T cells.
Figure 4-3: Viperin severely impairs EBOV production.
Figure 4-4: The ability of Viperin to reduce VP40 can be rescued by treating cells with lysosomotropic agents.

(A) Western blotting analysis of expression of VP40-GFP in cell lysates or purified VLPs from Viperin-expressing HEK293T. HEK293T cells were transfected with plasmids encoding Viperin, EBOV GP, and EBOV VP40-GFP. Supernatants containing EBOV VLPs were harvested from 24 to 72 hours post-transfection. An anti-GFP antibody was used for probing VP40-GFP expression; an anti-flag antibody was used for probing flag-tagged GP expression; β-actin serves as the loading control. (B) Effects of Viperin on the expression of MLV-pseudotypes bearing GP. HEK293T cells were transfected with plasmids encoding Viperin, MLV-gag-pol and EBOV GP. An anti-MLV-gag antibody was used for probing MLV-gag expression; an anti-GP antibody was used for probing GP expression; GAPDH serves as the loading control. (C) Effects of lysosomotropic agents on the expression of VP40-GFP that was decreased by Viperin. HEK293T cells were transfected with plasmids encoding Viperin and EBOV VP40-GFP. Lysosomotropic agents, NH₄Cl (10 mM) and BafA1 (25 nM), were added when medium was changed 6 hour post-transfection. An anti-GFP antibody was used for probing VP40-GFP expression; β-actin serves as the loading control.
Figure 4-4: The ability of Viperin to reduce VP40 can be rescued by treating cells with lysosomotropic agents.
Figure 4-5: Expression of Viperin induces autophagy, resulting in decrease of EBOV VP40

(A) Western blotting analysis of expression of ATG5 and Beclin-1 in Viperin-expressing 293T cells. HEK293T cells were transfected with plasmids encoding EBOV VP40-GFP, along with different doses of human Viperin or Rhesus Viperin. A rabbit anti-ATG5 antibody was used for probing ATG5 expression; a rabbit anti-Beclin-1 antibody was used for probing Beclin-1 expression; β-actin serves as the loading control.

(B, C) Effects of Viperin on puncta formation of LC3-GFP. HEK293T cells were transfected with plasmids encoding LC3-GFP and human Viperin. Rapamycin (2 uM) was added when medium was changed at 6 hour post-transfection. Images were taken at 24 hours post-transfection. (B) Quantitative analysis of puncta formation of LC3-GFP shown in (C). The numbers of LC3-GFP puncta in ~400 cells in each group were counted and averaged. (D) Western blotting analysis of expression of LC3-GFP in Viperin-expressing 293T cells. HEK293T cells were transfected with plasmids encoding LC3-GFP, human Viperin or Rhesus Viperin. A mouse anti-GFP antibody was used for probing LC3-GFP expression; a rabbit anti-Viperin antibody was used for probing Viperin expression; β-actin serves as the loading control. (E) Effects of Rapamycin (2 µM) on EBOV VP40 (anti-GFP) and ATG5 (anti-ATG5).
Figure 4-5: Expression of Viperin induces autophagy, resulting in decrease of EBOV VP40.
V. OVERALL SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

In this PhD thesis, I described three projects, two of which have been published in peer-reviewed journals (Chapter II in Virology and Chapter III in PLoS Pathogens) and the other is currently under preparation (Chapter IV) and will be submitted for publication shortly. In Chapter II, I focused on the elucidation of the mechanism of action by which EBOV GP mediates cell-to-cell transmission. In Chapter III, I provided evidence that low pH is not a trigger of EBOV GP-mediated cell-cell fusion. In Chapter IV, I investigated the role of an IFN-inducible Viperin in inhibiting EBOV replication.

The main goal of Chapter II is to study if and how EBOV GP mediates viral infection through cell-to-cell transmission. I show that cell-cell contact promotes viral infection of MLV pseudotypes bearing GP, of EBOV VLPs expressing GP or rVSVs expressing GP, and that this process is solely mediated by EBOV GP. Interestingly, I find that, without viral core proteins (i.e. MLV gag or EBOV VP40), EBOV GP alone is sufficient to induce the transfer of Tet-off protein from donor cell to target cells. This cell-cell contact-dependent infection of EBOV is more efficient than the cell-free infection; it is significantly inhibited by inhibitors of actin polymerization, such as LAT-B and CytoD, but exhibits relative resistant to the broad neutralizing antibody KZ52. Given that NPC1 and cathepsin B/L are essential for EBOV entry, I also investigate if they play a role in GP-mediated cell-to-cell infection. I show that knockdown of NPC1, or treatment of
NPC1-binding inhibitor 3.47, or treatment of cathepsin inhibitors blocks EBOV cell-to-cell infection. To be more physiological relevant, I examine the spread of rVSV bearing GP in monocytes and macrophages, which are the primary targets of EBOV in nature, and I find that infection of rVSV bearing GP is also enhanced by cell-cell contact and cannot be blocked by methylcellulose, a viscous solution that specifically inhibits the cell-free infection. We conclude that EBOV GP is necessary and sufficient to promote viral cell-to-cell infection.

In Chapter III, in collaboration with Fredric Cohen’s lab at Rush University I interrogate the possible triggers of EBOV GP-mediated cell-cell fusion. To achieve this, we adapted an aqueous dye-based cell-cell fusion assay and measured EBOV GP-mediated cell-cell fusion. We provide evidence that GP does mediate cell-cell fusion at the plasma membrane, which is sensitive to a small molecule inhibitor 3.47 and a neutralizing antibody. We also examined the role of low pH, cathepsin cleavage, and NPC1 binding in the EBOV GP-mediated cell-cell fusion process. We show that the EBOV GP-mediated cell-cell fusion does occur at neutral pH. However, knockdown of NPC1, or treatment of cells with cathepsin inhibitors or lysosomotropic agents, all reduce cell-cell fusion. In contrast, addition of soluble NPC1 greatly enhances GP-mediated cell-cell fusion. We also investigate the size of fusion pores created by GP, and show that GP-mediated fusion pores do not easily enlarge as by many other viral fusion proteins. We conclude that low pH is not the trigger for EBOV fusion, but only serves as acidic
environment that is required for the activity of cathepsin.

In addition to the studies of EBOV GP-mediated cell-cell infection and cell-cell fusion, I also expand my research scope to other cellular factors, in particular Viperin, that modulate EBOV infection. I demonstrate that Viperin diminishes EBOV production via autophagy-mediated degradation of VP40. Specifically, Viperin reduces the expression of EBOV VP40 in viral producer cells, which is abrogated by treatment of lysosomotropic inhibitors, NH$_4$Cl and BafA1. I further demonstrate that Viperin induces the expression of autophagy-specific proteins, ATG5 and beclin-1, correlating with the puncta formation of autophagy lipidation marker LC3. Perhaps one of the most compelling pieces of evidence is that induction of autophagy by Rapamycin significantly reduces EBOV production and viral replication. We therefore conclude that Viperin induces autophagy, which degrades VP40 and possibly other viral proteins, thereby impairing EBOV production.

**EBOV GP-mediated cell-to-cell infection**

For HIV cell-to-cell transmission, the HIV gag proteins are accumulated at virological synapses (VSs), thus promoting spread into adjacent cells (77, 250). It is therefore possible that EBOV GP may interact with some binding-receptors, co-factors or adhesion molecules, such as TIM-1, C-type lectin or β-integrins, to form VS-like structures and facilitate viral spread. In many cases, polarization of virions increases the local density of virions at the cell-cell contact, resulting in enhanced viral spreading as infection with higher MOI does (81, 251). In theory, cell-free virions are unstable and dispersed in
culture medium, which may have less infectivity. Consistent with this hypothesis, our results show that spinoculation of EBOV VLPs does enhance EBOV infection. We are currently in the process of examining accumulation of EBOV virions at cell-cell contact during cell-to-cell infection.

Studies on influenza A virus (IAV) have shown that viral core proteins can traffic to neighboring cells via filamentous intracellular connection, and thereby establish productive infection (71). Previous studies show that EBOV VP40 can form hexamers or octamers, as being observed as filamentous structure at the surface of infected cells. EBOV virions have been reported to traffic into filopodia for budding (57); thus, filamentous protrusions from viral producer cells could serve as “bridges” to support the cell-to-cell infection of EBOV. However, it is currently unclear whether or not EBOV infection induces the formation of structures as filopodia, or there are pre-existing structures in host cells (i.e. microtubule or nanotubes) that allow EBOV to travel from cell to cell. Application of fluorescence-labeled EBOV virions in live-cell imaging shall be useful for tracking the movement of viruses in GP-mediated cell-to-cell infection.

Exosomes secreted from cells can transfer viral materials into recipient cells. Precedents have been observed in infections of HCV and VSV (72, 165). Viral proteins and genome can be packaged into exosomes and released into extracellular compartment, followed by uptake by other cells. In immune responses, exosomes derived from viral non-permissive cells are rich in antiviral proteins (i.e. ISGs) and can transfer viral
resistance to permissive cell lines, resulting in the blockage of viral production (252). In contrast, exosomes containing viral materials can facilitate viral spread, and are often less sensitive to neutralizing antibodies (168, 253). We show that treatment of cells with exosome production inhibitor GW4869 reduces signals induced by GP-mediated cell-to-cell transfer, yet addition of purified exosome leads to increased Gluc activity in recipient cells. Our finding may imply that at the early stage of viral replication, when viral core proteins or mature viral particles are limited, exosomes could facilitate initial viral spreading. It is also possible that sGP or shed GP mediates viral spread via similar mechanisms. Future work should focus on the molecular characterization of the contents of exosomes secreted from authentic EBOV-infected cells.

It is currently unknown if cell-to-cell infection occurs in authentic EBOV infection and, if so, the underlying mechanisms; this will require a BSL-4 facility, which is not accessible to most laboratories. As EBOV tetracistronic minigenome system has been shown to faithfully reflect authentic EBOV, it is possible to apply this EBOV minigenome system (described in Chapter IV) containing mCherry-tagged polymerase L. By tracking the movement of fluorescence-labeled EBOV virions, we shall be able to determine if replication-competent EBOV can spread through cell-to-cell and also the underlying mechanisms.

Lipid rafts are involved in EBOV release and entry. It is possible that lipid rafts play a role in GP-mediated cell-to-cell infection. We will examine the effects of lipid rafts on
GP-mediated cell-to-cell infection by modulating lipid compositions, including cholesterol, on the plasma membrane. Given that phosphatidylserine (PS) is important for VP40 binding at plasma membrane and the oligomerization of VP40 hexamers, it will be interesting to examine the role of PS and TIM-1 in EBOV GP-mediated cell-to-cell infection.

**Triggers of EBOV GP-mediated cell-cell fusion**

One of the key questions about EBOV entry is how EBOV fuses with the host cell membrane during infection, in particularly if low pH itself serves as a direct trigger for fusion as EBOV is a pH-dependent virus. In our study, we attempt to dissect the triggers of EBOV fusion. In collaboration with Fredric Cohen's lab at Rush University, we developed an aqueous cytoplasmic and membrane dye-based cell-cell fusion assay and determined if factors known to be essential for EBOV entry, such as low pH, cleavage activity of cathepsin, NPC1 binding, is essential for triggering EBOV fusion. Our conclusion is that low pH is not a trigger for EBOV fusion, but merely serves as acidic requirement of cathepsins’ enzymatic activity.

The key contributions of mine in this study are summarized as follows. First, I provide evidence that key factors in EBOV fusion, such as cleaved GP, are detectable at the cell surface, which allows the cell-cell fusion to occur. Second, I made a soluble form of NPC1 (sNPC1) and applied it to cell-cell fusion assay. I show that sNPC1 increases GP-mediated cell-cell fusion in dose-dependent manner. Third, by establishing NPC1
knockdown or overexpressing cells, I demonstrate expression of NPC1 at cell surface. Forth, I reveal that a small fraction of GP is cleaved at the plasma membrane, responsible for GP-mediated cell-cell fusion. Fifth, I engineered a GP mutant (GP\textsuperscript{furin}) that contains a furin cleavage site at the position of cathepsin L and show that in the presence of furin, GP\textsuperscript{furin} has an increased level of cleaved GP at the plasma membrane, correlating with enhanced cell-cell fusion. Last, I made a series of GP constructs known to be binding-deficient, and show that these GP constructs exhibit reduced cell-cell fusion, suggesting that NPC1-binding is important for GP-mediated fusion.

Another important question is how to explain the slow enlargement of fusion pore by EBOV GP, which is not consistent with the rapid spread of EBOV in vivo. The relatively small fusion pore induced by EBOV GP shown in electrical measurements would not be appreciated in authentic EBOV containing viral genome and other proteins. Future work will focus on the identification of differences between plasma membrane and endosomal membrane following EBOV infection, including specific proteins, lipids, cytoskeleton arrangement, and possibly the environmental calcium concentration.

**Role of Viperin in EBOV replication**

The main goal in our Viperin study is to determine if Viperin restricts EBOV replication and how it does so. Early evidence shows that Viperin restricts the release of IAV by disrupting lipid rafts (108). Similar inhibitory effect has been observed in the production of HIV\textsubscript{BAL} when Viperin is induced in monocytes-derived macrophage (254).
Given that lipid rafts is essential for EBOV budding, as it interacts with EBOV VP40 to induce proper structural changes for EBOV budding as well as it serves as the viral budding site, it is possible that Viperin affects EBOV via similar mechanisms (65, 255).

However, our initial finding does not follow this hypothesis. We first observed that Viperin reduces the production of EBOV trVLPs. The decreasing trends of VP40 between cell lysate and purified VLPs are similar, suggesting that Viperin unlikely affects EBOV release but viral gene expression. Similar effects have been observed on equine infectious anemia virus (EIAV) where equine Viperin reduces the expression of viral envelope and receptor via distortion of ER (256). However, human Viperin differs from its equine counterpart because its effect on ER has not been reported. Early study in HCMV shows that Viperin reduces the expression of HCMV structural proteins, such as glycoprotein (gB), tegument proteins (pp28 and pp65), although the exact mechanism remains unclear (89). In our study, we show that the impaired expression of VP40 can be rescued by inhibitors of lysosomotropic agents, pointing to the possibility of autophagy. Indeed, we show that expression of Viperin upregulates autophagy-specific proteins, ATG5 and Beclin-1. Moreover, expression of Viperin increases punta formation of LC3-GFP and its lipidation. By treatment of Rapamycin, the well-known autophagy inducer, we observed that Rapamycin reduces the expression of EBOV VP40 and the replication of EBOV trVLPs. Our future work will be concentrated on the link between Viperin and autophagy, particularly if Viperin interacts with factors of the autophagy pathway.
It is currently unknown if Viperin solely depends on autophagy to regulate EBOV replication/production. It is possible that Viperin interacts with autophagy-related proteins, or serves as a platform for autophagy assembly, as observed from TRIM proteins (240). This is partially supported by our finding, as we show that Viperin increases autophagy-specific proteins as well as puncta formation of LC3-GFP. However, in some cases, antiviral factors are still potent in autophagy-deficient cells, suggesting that their antiviral effects are independent of autophagy (239). Therefore, to determine the requirement for autophagy in the restriction of EBOV by Viperin, we will establish autophagy-deficient cell lines, such as ATG5 or Beclin-1 knockdown cells, and examine effects of Viperin on EBOV production in these cell lines. Results from these experiments will offer us a better understanding of the interplay between Viperin, autophagy and EBOV infection.
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VITA

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