# Interactions of the Ebola virus glycoprotein with host cell factors during viral entry and release

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**Affidavit**

I hereby declare, that my doctoral thesis entitled “Interactions of the Ebola virus glycoprotein with host cell factors during viral entry and release” has been written independently with no other sources than quoted.

Göttingen, February 2019

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## 1. Abstract

Ebola virus disease (EVD) is a severe disease that affects humans and non-human primates. The recent EVD epidemic in West Africa shows that EVD poses a severe threat to human health. EVD is caused by Ebola virus (EBOV) and other ebolaviruses, which belong to the family *Filoviridae*. The EBOV glycoprotein (GP) is the only viral surface protein and mediates host cell entry. Processing of GP by host cell proteases (priming) is required for viral entry into target cells and cathepsin (Cat) B and L have been implicated in GP priming in cell culture. However, these enzymes may be dispensable for viral spread in the infected host and the determinants governing CatB/L dependence of viral entry are incompletely understood. Therefore, the first goal of this thesis was to identify such determinants. Apart from mediating viral entry, EBOV-GP also promotes viral release by antagonizing the interferon-induced antiviral host cell protein tetherin. However, the domains in GP that govern counteraction of tetherin and the contribution of this process to viral spread are incompletely understood. The second aim of the present thesis was thus to identify GP domains and amino acid motifs that control tetherin antagonism.

The results of the present thesis show that EBOV-GP-driven entry depends on CatB/L activity irrespective of the shape of the viral particle and the target cell type. Moreover, Calu-3, a human cell line with low endogenous CatL expression, was found to be largely resistant to entry driven by EBOV-GP and other filovirus GPs. Finally, entry was restored by directed expression of CatL or the attachment promoting factor DC-SIGN. Regarding tetherin counteraction by GP, the results obtained show that a GXXXA motif in the transmembrane domain of GP is largely dispensable for GP expression, particle incorporation and host cell entry but is required for tetherin antagonism. Lack of tetherin antagonism was observed in transfected cells and was confirmed in the context of an infectious vesicular stomatitis virus chimera encoding EBOV-GP. In summary, the present thesis identifies Calu-3 cells as one of the few cell lines largely resistant to filovirus GP-driven entry and shows that entry is limited at the stage of attachment and GP priming. Moreover, the results identify a GXXXA motif in GP as essential for tetherin antagonism and provide the first evidence that antagonism can promote viral spread, at least in the context of a surrogate system.

## 2. Introduction

Ebola virus disease (EVD) is a severe, frequently fatal disease that affects human and non-human primates (NHP). It is caused by ebolaviruses, is highly contagious and associated with a high case-fatality rate. The EVD epidemic in West Africa from 2013 to 2016 was the largest outbreak ever witnessed and caused more cases and deaths than all previous outbreaks combined. Furthermore, secondary cases outside Africa occurred [1]. This highlights that EVD poses a severe global threat to human health

### 2.1. Ebola virus disease

Ebola virus disease is a zoonotic disease which was first described in 1976 [2]. The main causative agent of EVD is Ebola virus (EBOV), which belongs to the family *Filoviridae*. It has been suggested that fruit bats are the natural reservoir of EBOV. Thus, serological analyses have shown that these animals harbor EBOV-specific antibodies or viral RNA [3,4]. However, infectious EBOV has so far not been isolated from bats. This is in contrast to other filoviruses like Marburg virus (MARV), for which infectious virus has been isolated from fruit bats [5–8]. Thus, fruit bats are most likely the reservoir of EBOV but contribution of other animals to maintenance in nature cannot be excluded [9,10].

Transmission of EBOV to humans occurs via contact with body fluids or organs of infected wild animals such as chimpanzees, gorillas, fruit bats, monkeys, or forest antelopes [11,12]. Once the virus has been introduced into the human population, human-to-human transmission may occur via direct contact with body fluids from infected patients or deceased people. In addition, recent studies suggest that EBOV can be sexually transmitted and that transmission can occur for up to 12 months after recovery of male survivors [1,13–17].

The incubation period of EVD ranges from 2 to 21 days in humans [2,18]. Symptoms start abruptly with fever, muscle pain, cough, headache and abdominal pain. During the next phase of the disease symptoms like vomiting, diarrhea, dyspnea, hypovolemic shock, and organ failure occur [19–21]. Studies conducted during the outbreak in West Africa revealed that hemorrhages occur in approximately 5 % of the patients but are

not associated with fatal outcome [20–23]. These studies confirm results obtained during the EBOV outbreaks in Kikwit 1995 [24] and Uganda 2000 [25]. Instead, hypovolemic shock and organ failure lead to death usually within 7 to 14 days after the onset of the symptoms [20,21,26]. The adequate supportive care, like administration of intravenous fluids, control of electrolytes, and prevention of shock associated complications, increases the chances of survival [21,26]. During the West African outbreak persistence of EBOV in immune-privileged sites (i.e. testicles [14,27], eye [28], central nervous system [29], and breast milk [30]) of EVD survivors was recorded. Moreover, persistently infected convalescent patients transmitted the virus to others raising the possibility that EVD might become endemic in the African population [31].

*Vaccination against EBOV.* To date, there are no approved vaccines or antiviral agents to combat EVD. However, several pre- and post-exposure treatments for EVD are under development, including vaccines based on inactivated EBOV, recombinant viruses, virus-like particles, DNA and plant-based antibodies. The first attempt to develop a vaccine against EVD was focused on heat- or formalin-inactivated EBOV preparations. Both showed protection in a guinea pig model. However, incomplete inactivation is always a concern [32,33]. In another trial, virus-like particles (VLPs), based on the EBOV glycoprotein (GP), viral protein (VP) 40 and nucleoprotein (NP), were protective in rodent models [34]. The most promising results in vaccine development have been obtained with recombinant viral vectors. Among these, the replication-competent vesicular stomatitis virus-EBOV chimera (VSV-EBOV), which harbors the gene for EBOV-GP, has been used in clinical trials which revealed that it is well tolerated when used in low doses [35,36]. However, when higher vaccine doses were applied, vaccinated volunteers developed arthralgia and arthritis [35–38]. Notably, VSV-EBOV conferred up to 100 % protection in NHP models [39] and was found to be highly efficient in preventing EVD in humans [38] as demonstrated during the epidemic in West Africa. Moreover, it is being used in the recent outbreak in the Democratic Republic of Congo (DRC) [40,41]. However, it has not been approved by regulatory agencies so far and is still in phase III clinical trials [42,43]. The vaccine GamEvac-Combi has been tested in clinical trials in Russia. This vaccine contains two EBOV-GP expression systems: live attenuated recombinant VSV and a recombinant replication defective adenovirus serotype-5 (Ad5) [44]. During the clinical trial

vaccinated patients did not show serious adverse effects and strong humoral and cellular immune response against EBOV-GP were detected [44]. Furthermore, vaccination with VSV-EBOV-prime Ad5-boost vaccine induced a significantly stronger antibody response than immunization with VSV-EBOV alone [44–46].

*EVD treatment*. Passive immunization using whole blood, plasma or serum from EVD survivors, is a treatment option recommended by the World Health Organization (WHO) in emergency conditions. Unfortunately, studies in NHP and in EVD patients showed no association of treatment with survival [47–53]. Therefore, application of purified antibodies is a preferable therapeutic option. Antibody cocktails such as ZMapp, ZMab or MIL77 confer full protection against EBOV infection in NHP. For instance, studies carried out during the 2013-2016 epidemic suggest that ZMapp improves survival of EVD patients [54]. Accordingly, the humanized version of ZMapp antibodies has been recommended as EVD treatment by WHO [55,56]. Another monoclonal antibody that has been under study is KZ52, a neutralizing antibody obtained from an EVD survivor [57]. This antibody prevents EBOV-GP interaction with host cell proteases and protected guinea pigs against EBOV challenge, but failed to protect rhesus macaques [57–59]. Despite the largely encouraging results discussed above, efficiency and cross-reactivity of the monoclonal antibodies with other ebolavirus species need to be improved.

For post-exposure treatment antiviral agents that can inhibit viral replication are available. Favipiravir (T-705), a drug first developed for influenza treatment, was found to inhibit the RNA polymerase of EBOV in cell culture and was administrated to humans during the EVD epidemic in West Africa. However, treatment had only a weak effect on viral replication and was not associated with a survival benefit [60]. Several other small molecules are currently under study for their possible use in EVD treatment. Interferons (IFN) are natural antivirals and IFN-γ has been approved by the Food and Drug Administration as a treatment for chronic diseases, such as Chronic Granulomatous Disease and osteopetrosis [61]. Furthermore, IFN-γ treatment can reduce the mortality rate in mice when administrated before or after EBOV challenge [62]. These results indicate that IFNs could be repurposed and adapted as a prophylactic treatment of EVD [41,62]. Toremifene and clomiphene can block EBOV

entry into target cells and exert antiviral activity in cell culture and in mice models [63,64]. Nafamostat mesylate is a protease inhibitor that reduces cathepsin (Cat) B levels in rat pancreas [65] and for EBOV entry the viral GP needs to be processed by CatB. Therefore, Nafamostat might be suitable for EVD treatment but its antiviral activity has not been assessed in animal models [66]. Another strategy to treat EVD are small interfering RNAs (siRNAs) that target viral mRNAs encoding for proteins essential for EBOV replication and assembly, such as the RNA polymerase (L), and the viral proteins VP35 and VP24. A study showed that a combination of siRNAs targeting these three proteins can provide post exposure protection against EBOV challenge in a NHP model [41]. In sum, treatment options against EVD are available. However, efficiency and activity against diverse ebolavirus species need to be improved.

### 2.1.1. Ebola virus disease outbreaks

The first recorded EVD outbreaks occurred in 1976 in the DRC (formerly Zaire), and in Sudan and were caused by members of two different ebolavirus species, EBOV (species *Zaire ebolavirus*) and Sudan virus (SUDV, species *Sudan ebolavirus*), respectively. Since then, several EVD outbreaks caused by EBOV (most frequently), SUDV, Tai Forest virus (TAFV, species *Taï Forest ebolavirus*), and Bundibugyo virus (BDBV, species *Bundibugyo ebolavirus*) have been recorded in remote areas within Central Africa and although case-fatality ratios were high, the case numbers were limited to a maximum several hundred per outbreak [1,18].

In March 2014, the WHO reported an EVD outbreak in Guinea that was caused by EBOV. The outbreak started in December 2013 and then turned into an epidemic that spread to Sierra Leone and Liberia. It was declared a Public Health Emergency of International Concern (PHEIC) in August 2014. During the 2013-2016 EVD epidemic in West Africa, the virus reached densely populated areas, partially due to a delayed response of local authorities and WHO. Furthermore, secondary cases outside Africa (Spain and the United States of America) were recorded. By the time the WHO lifted the PHEIC in March 2016, there were more than 28,000 cases and 11,000 deaths reported, which are more cases than in all previous outbreaks combined. In June 2016, the WHO declared the end of EVD transmission in Guinea and Liberia [18,67].

After the West African epidemic, a small EVD outbreak in the DRC was registered in May 2017 and declared over in July 2017. During this outbreak five confirmed EVD and three probable cases have been reported, four of which died [68]. An ongoing EVD outbreak in the DRC, again caused by EBOV, started in August 2018 and until January 22$^{th}$, 2019 there were 713 cases (664 laboratory confirmed cases by RT-PCR, positive IgM or virus isolation) and 439 deaths reported. So far, only north-eastern provinces of DRC including North Kivu and Ituri have been affected, but current outbreak hotspots (Butembo, Katwa) encompass an urban area with a population of roughly one million people. Containing the current outbreak has been challenging due to security conflicts, a long-term humanitarian crisis and other ongoing epidemics in the area. Currently, WHO's risk assessment is high at regional and national levels [18,69].

## 2.2. Ebola virus biology and classification

Ebola virus belongs to the family *Filoviridae*, which comprises three genera: *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. According to the International Committee on Taxonomy of Viruses there are five species in the genus *Ebolavirus*: *Zaire ebolavirus* (single member: Ebola virus, EBOV), *Sudan ebolavirus* (single member: Sudan virus, SUDV), *Taï Forest ebolavirus* (single member: Tai Forest virus, TAFV), *Bundibugyo ebolavirus* (single member: Bundibugyo virus, BDBV) and *Reston ebolavirus* (single member: Reston virus, RESTV) [70]. All members of the genus *Ebolavirus* are highly pathogenic for humans, except for RESTV. A recent study discovered a sixth species, *Bombali ebolavirus* (single member: Bombali virus, BOMV) [6]. BOMV has only been detected in bats but the viral glycoprotein can mediate entry into human cells. However, no human infections were documented so far. The genus *Marburgvirus* contains only one species *Marburg marburgvirus* (two members: Marburg virus, MARV; and Ravn virus, RAVV) [71], both are known to be pathogenic in humans, and MARV outbreaks have been reported in the past [72]. *Lloviu cuevavirus* (single member: Lloviu virus, LLOV) is the only species within the *Cuevavirus* genus, but no human infections have been reported so far [5].

Filovirus particles are filamentous (Figure 1A), with a fixed diameter of 80 nm and a variable length (up to 14,000 nm). They are enveloped and contain a negative sense

single-stranded RNA (ssRNA) genome of approximately 19 kb. EBOV is the most studied member within the genus *Ebolavirus*, consequently the subsequent discussion will focus on EBOV. The EBOV genome consists of seven open reading frames (ORFs) that encode for seven structural proteins: nucleoprotein (NP), viral protein 35, 40, 30, 24 (VP35; VP40; VP30; VP24), RNA polymerase (L), and glycoprotein (GP) (Figure 1B) [73–75]. NP encapsidates the viral genome, jointly with VP30, which acts as a transcriptional activator. The polymerase L is responsible for viral genome transcription and replication [76,77]. VP35 is a polymerase cofactor and along with NP, VP30 and L, forms the ribonucleocapsid. VP24 is required for nucleocapsid maturation [77–79]. VP40 forms the matrix of the virus and is responsible for virus assembly and budding [80,81]. Finally, GP is incorporated into the viral membrane and drives viral entry into target cells [82].

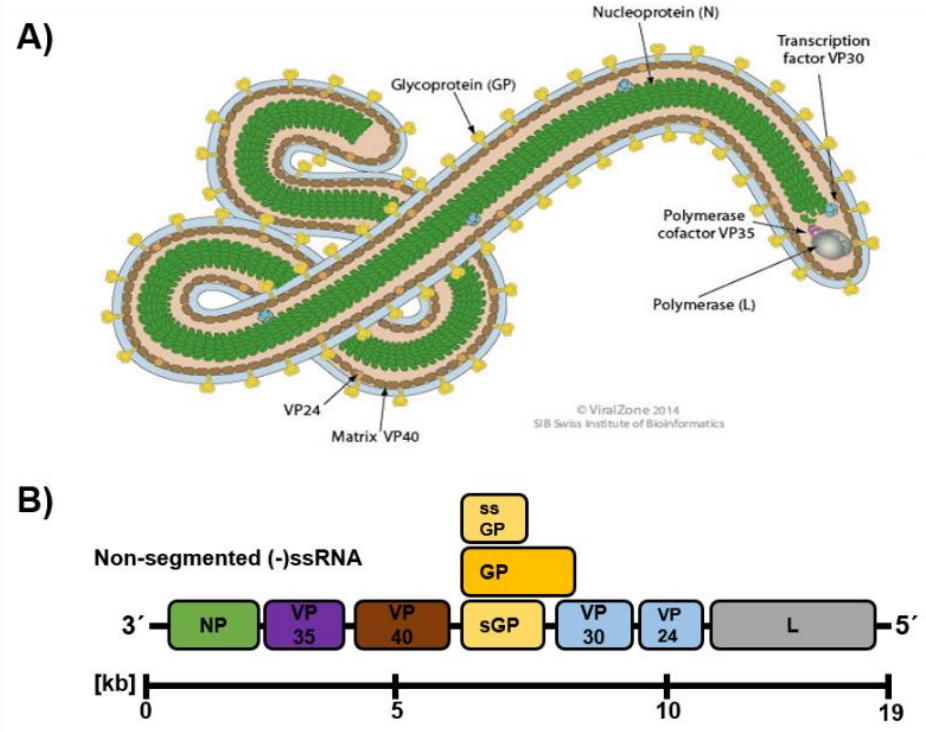


**Figure 1. Particle architecture and genome organization of Ebola virus**. A) EBOV particles are filamentous; GP is the only viral surface protein. VP35 is a polymerase cofactor and along with NP, VP30 and L, forms the ribonucleocapsid. VP40 forms the viral matrix along with VP24. B) Schematic representation of EBOV genome organization and the encoded proteins. (Adapted from ViralZone [83])

### 2.3. Ebola virus replication cycle

EBOV and other filoviruses replicate in the cytoplasm. First, EBOV-GP attaches to host cell surface factors and viral particles are internalized via macropinocytosis [84]. Subsequently, virions are trafficked to late endosomes where GP is processed (primed) by the cellular cysteine proteases CatB/ L. Primed EBOV-GP then interacts with the intracellular receptor Niemann-Pick C1 (NPC1) [85,86]. Engagement of NPC1 and a subsequent poorly defined stimulus triggers GP-driven fusion of the viral envelope with the endosomal membrane [87–89]. This requires large conformational changes in GP that are also observed with unrelated viral glycoproteins, collectively referred to a class I membrane fusion proteins [90]. In the course of these conformational rearrangements a fusion loop in GP2 is inserted into the host membrane and a thermostable six-helix bundle structure in GP2 is formed, which results in close approximation of the viral and the cellular membrane. Finally, a fusion pore is formed which allows the release of the nucleocapsid into the host cytoplasm [91–97]. Then, the viral polymerase L produces mRNAs and cRNA that will serve as template for generation of new genomic (-)ssRNA [98]. New viral ribonucleoprotein complexes (RNP) are assembled in the perinuclear region and, stimulated by VP40, transported to the budding sites via actin-dependent RNP trafficking [99,100]. GP is transported via the endoplasmic reticulum (ER) and Golgi apparatus to the site of viral budding where it colocalizes with VP40 in cholesterol-enriched microdomains [101–103]. At the plasma membrane, VP40 forms hexamers and facilitates the incorporation of the RNP into filamentous particles. In order to facilitate release, VP40 recruits cellular proteins including Nedd4/Rsp5, Tsg101 and Vps4 [104–106], which induce translocation of VP40 to the phosphatidylserine-enriched inner leaflet of the plasma membrane and catalyze fission of the plasma membrane during budding [107] (Figure 2).

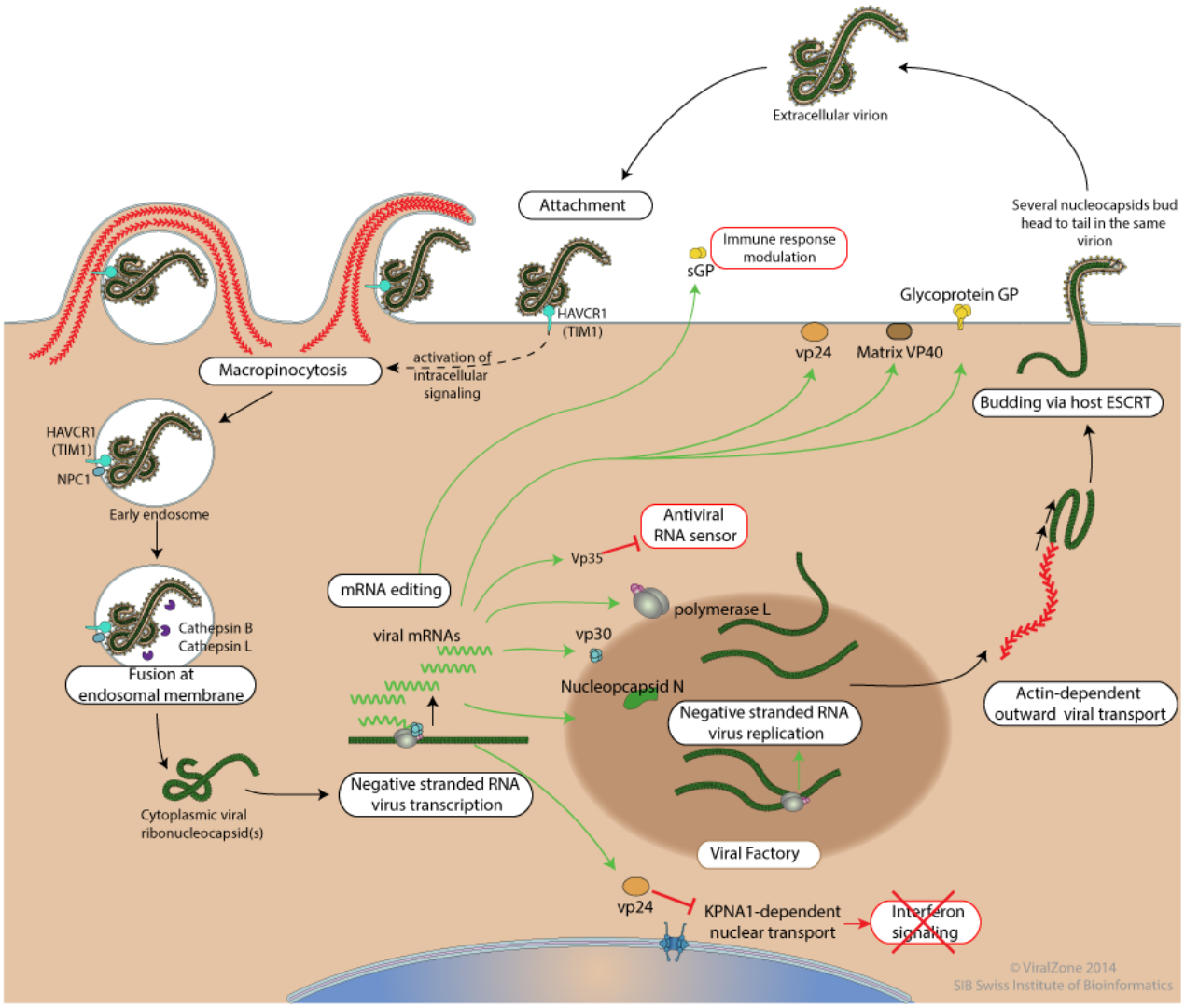


**Figure 2.** Ebola virus replication cycle. (Reprinted from [83])

## 2.4. Ebola virus glycoprotein

The GP gene is the only gene of EBOV able to encode for different proteins. It can produce three mRNAs encoding for pre-soluble GP, pre-GP ($GP_0$) and pre-small soluble GP proteins in approximately 14:5:1 ratio, although this ratio can be cell type dependent (Figure 3) [76,108].

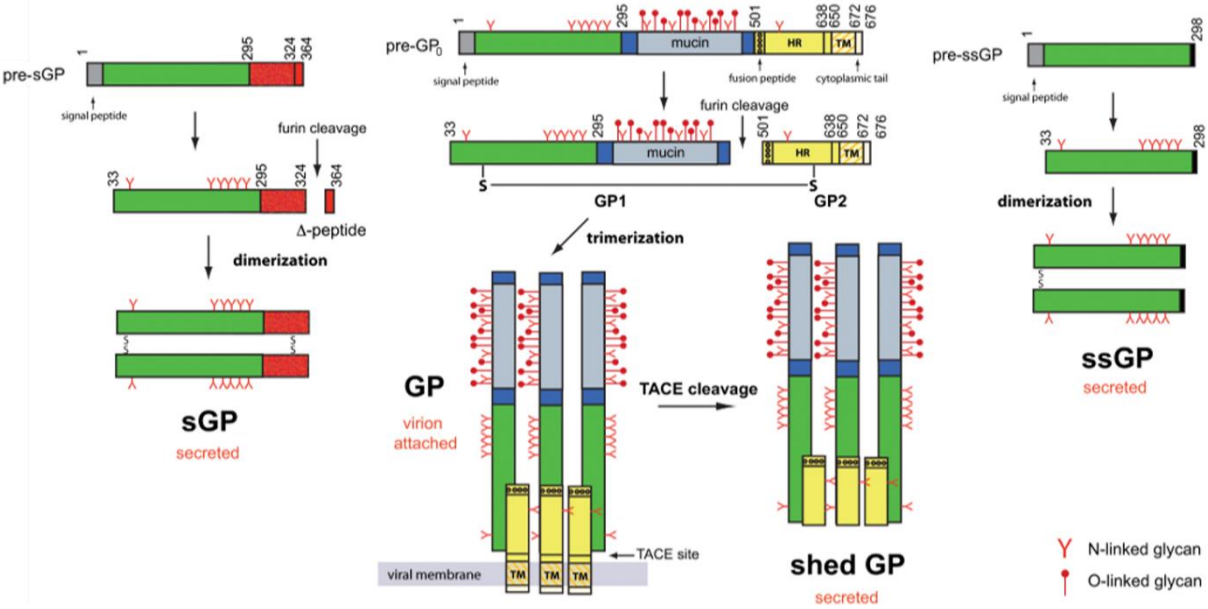


**Figure 3. Proteins encoded by the GP open reading frame.** Pre-sGP (left), is encoded by the primary transcript and is most abundant. Processing of pre-sGP leads to formation of mature N-glycosylated dimers of sGP and O-glycosylated monomers of Δ-peptide. The mRNA encoding for pre-$GP_0$ (middle) results from a +1 frameshift. Cleavage of $GP_0$ by furin separates the surface unit GP1 from the transmembrane unit GP2, which remain associated by a disulfide bond. GP trimers are incorporated into the viral membrane that decorate the viral surface. Cleavage of GP by ADAM17/TACE results in the production of shed GP. Pre-ssGP (right) is encoded by a +2 frameshift in the GP ORF and is the least abundant transcript. Mature ssGP is secreted as N-glycosylated dimer. (Adapted from [109])

The soluble GP (sGP) is encoded by unedited mRNA and is the most abundant product of the GP gene. It is synthesized as pre-sGP and post-translational modifications include proteolytic cleavage by furin resulting in two products, sGP and delta-peptide (Δ-peptide). Both proteins are secreted due to their lack of transmembrane domains [110]. Mature sGP is released extracellularly as N-glycosylated dimer [111,112]. The role that sGP plays in EBOV pathogenesis is not completely understood. Since it shares the N-terminal 295 aa with full-length GP, it has been suggested that sGP acts as a decoy antigen for antibodies directed against full-length GP [101]. Furthermore, it has been postulated that it can lead to antigenic subversion, i.e. high amounts of sGP divert the immune response away from full-length GP by acting as a target for neutralizing antibodies [113]. Studies performed in cell culture have shown that sGP

may exert anti-inflammatory activity, such as restoration of the barrier function of endothelial cells, and inactivation of neutrophils [114,115]. Finally, it has been suggested that sGP could reduce viral cytotoxicity by limiting the expression of EBOV-GP [116,117].

The Δ-peptide is the C-terminal cleavage product of the pre-sGP protein and is O-glycosylated [110]. It has been proposed that Δ-peptide may prevent virus superinfection by binding to permissive cells and interfering with viral entry [118]. However, the *in vivo* relevance of this process has not yet been determined. Another hypothesis, based on *in silico* analysis of Δ-peptide sequences, suggests that it acts as a viroporin, but experimental evidence is lacking [119].

The small soluble protein (ssGP) is produced as the result of a +2 frameshift in the GP ORF [76,120]. It is largely N-glycosylated and secreted as dimers. Despite having similar biochemical properties and primary sequence as sGP, ssGP does not exhibit the same anti-inflammatory function in endothelial cells as sGP. To date, ssGP function and its contribution to EBOV pathogenesis are still unknown [108,121].

The full-length glycoprotein (GP) is the only viral surface protein. It is synthesized as a precursor ($GP_0$) as the result of a +1 frameshift of the GP ORF [76,120]. $GP_0$ post-translational modifications start in the ER where it is N- and O-glycosylated. Then, it is transported into the Golgi apparatus where furin cleavage divides it into two subunits, the surface unit GP1 and the transmembrane unit GP2, remain associated via a disulfide bond between GP1 Cys53 and GP2 Cys609 [102,122]. The GP1-GP2 heterodimers assemble into trimers that are transported to the cell surface where they are incorporated into in the viral envelope [109,123]. The GP1 subunit contains the signal peptide, the receptor binding domain (RBD), and a heavily N- and O-glycosylated mucin-like domain (MLD) that is rich in serine, threonine and proline residues. The GP2 subunit comprises an internal fusion loop, two heptad repeat regions (HR1, HR2), a transmembrane domain (TMD) and a short cytoplasmic tail [93,102]. A schematic representation of EBOV-GP domains and trimeric structure is depicted in figure 4. Additionally, EBOV-GP located at the plasma membrane can be cleaved by tumor necrosis factor alpha converting enzyme (TACE), a member of the

disintegrin and metalloproteinase family, resulting in release of shed GP [124]. As well as sGP, shed GP can act as an antibody decoy capturing anti-EBOV-GP antibodies or stimulate antibody dependent enhancement of infection [124]. In addition, it has been suggested that shed GP might modulate the homeostasis of the endothelium, the secretion of pro-inflammatory cytokine [125,126], and may reduce the abundance of GP on the cell surface and in virions [117,127].

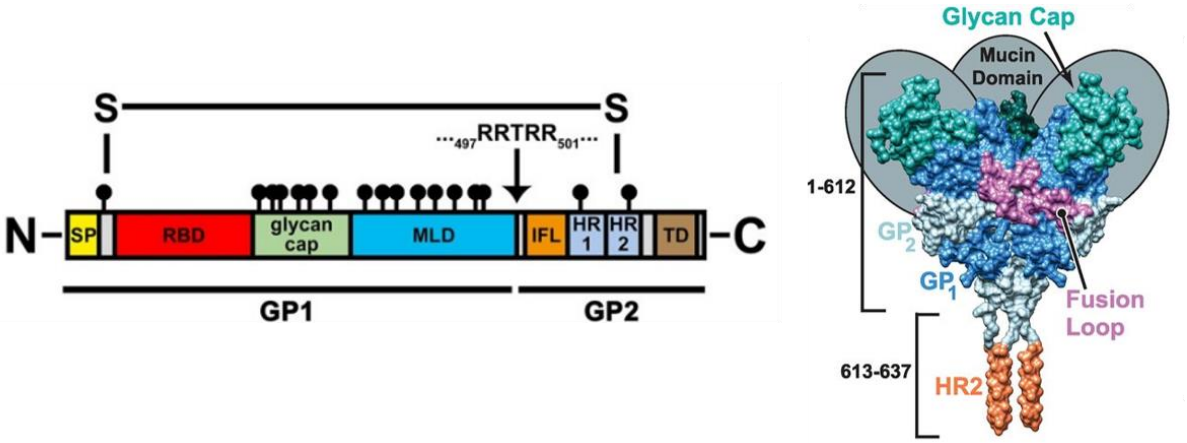


**Figure 4. Ebola virus glycoprotein domain organization and 3D structure.** Schematic representation of the domain organization of EBOV-GP is shown in the left panel. SP = signal peptide (yellow), RBD = receptor binding domain (red), glycan cap (green), MLD = mucin-like domain (blue), IFL = internal fusion loop (orange), HR = heptad repeat 1 and 2 (light blue), TD = Transmembrane domain (brown) (Adapted from [128]). The right panel shows the domain organization of the GP in the context of the 3D structure of the protein (Adapted from [129])

*Role of the Ebola virus glycoprotein in viral pathogenesis.* EBOV-GP mediates viral entry into host cells, the first step in the EBOV replication cycle. Entry comprises viral attachment to the cell surface, viral uptake into cells, binding of GP to its intracellular receptor NPC1 and membrane fusion, as outlined above and as described in detail below [82,130]. Several studies have suggested that GP is also involved in EBOV pathogenesis and in the activation of the inflammatory response [115,131,132]. Expression of EBOV-GP causes cytotoxic effects, cell rounding and detachment of adherent cells *in vitro*. Moreover, analogous observations have been reported in explanted blood vessels, which results in increased vascular permeability [133,134]. The MLD contributes to cellular cytotoxicity by masking adhesion molecules, including intercellular cell adhesion molecule-1 (ICAM-1), platelet/endothelial cell adhesion molecule-1 (PECAM-1), vascular cell adhesion molecule-1 (VCAM-1) and epidermal growth factor receptor (EGFR) of the host cell surface [135–137]. The steric shielding not only reduces accessibility and function of adhesion molecules but also of the major

histocompatibility complex class I, suggesting that the immune cell recruitment might be altered. Therefore, shielding might contribute to immune suppression and activation of the inflammatory response during EBOV infection [131,132,136,138,139]. Although it has been shown that the MLD plays a major role in EBOV-GP mediated cytotoxic effects, it is dispensable for EBOV-GP-driven entry [102,140]. It has been suggested that the MLD glycans cover antigenic epitopes within the RBD. This might be an immune evasion strategy mediated by the MLD since it blocks the activity of neutralizing antibodies directed against this domain [136,137,141]. Finally, sGP and shed GP might modulate immune responses and integrity of the vasculature as outlined above.

## 2.5. Ebola virus entry

Ebola virus entry can be divided in four steps: attachment, viral uptake and proteolytic priming of GP, receptor binding and membrane fusion.

### 2.5.1. Cellular attachment factors

Attachment is mediated by interactions of the GP1 subunit or phosphatidylserine (PtdSer) in the viral envelope with proteins located at the surface of host cells [142–144]. Engagement of these attachment promoting factors can augment virion uptake but is not essential for EBOV-GP- driven entry

#### 2.5.1.1. Phosphatidylserine-mediated virus entry enhancing receptors

PtdSer-mediated virus entry enhancing receptors (PVEERs) are a group of receptors and receptor complexes with the ability to bind PtdSer present on the viral envelope. The mechanism underlying augmentation of viral infectivity by PVEERs is called apoptotic mimicry. It was first postulated to be used by hepatitis B virus and was first experimentally confirmed in the context of vaccinia virus infection [145,146]. PVEERs bind to PtdSer, which is expressed on the inner leaflet of the plasma membrane of living cells. However, PtdSer is exposed on the surface of apoptotic or necrotic cells, allowing phagocytic cells to recognize and remove these cells [145,147]. PtdSer binding to PVEERs can occur through direct interaction or through a ligand that can bind both PtdSer and the receptor [148,149]. Several viruses, including members of the flavivirus, filoviruses, arenavirus, baculovirus and alphavirus families are known to

take advantage of apoptotic mimicry [143,144,149,150]. The PVERRs used by filoviruses for attachment are TIM-1, TIM-4, Axl, Dtk and MER [143,151,152].

*T-cell immunoglobulin and mucin (TIM) family*. The human TIM family proteins are type I cell surface glycoproteins with an N-terminal immunoglobulin variable (IgV)-like domain, a mucin-like domain (O-glycosylated), a transmembrane domain and a C-terminal cytoplasmic tail [153]. The ligand binding pocket of TIM-1 and TIM-4 is located between two loops of the IgV domain [154–156]. Aspartate and asparagine residues present in the upper loop coordinate calcium ions and form hydrogen bonds with the phosphate and serine groups of PtdSer [157]. Binding of PtdSer to TIM proteins is necessary for clearance of apoptotic bodies and immune cell regulation [158–162]. TIM-1 is mainly expressed on immune cells such as B cells, mast cells, Th2 $CD4^+$ T cells but is also found on epithelial cells of kidney, airway and eye mucosa [163–167]. TIM-4 is expressed on macrophages and mature dendritic cells (DCs) located in spleen, lymph node, and peritoneum [154,155,168]. Macrophages and DCs are known to be early and sustained target cells during EBOV infection [169,170]. It has been demonstrated that EBOV-GP can interact with TIM-1 and TIM-4 via its receptor binding domain, further leading to virus internalization and increased infectivity [167]. However, subsequent studies showed that TIM proteins recognize PtdSer displayed on the viral envelope and thereby augment viral entry [143,144].

*Tyro3/Axl/Mer (TAM) family*. Tyro3, Axl, and Mer (TAM) belong to the family of receptor tyrosine kinases. They possess two N-terminal immunoglobulin (Ig)-like domains, followed by two fibronectin type III domains, a single transmembrane domain, and a cytoplasmic protein tyrosine kinase (PTK) domain. TAM proteins interact via their Ig-like domains with their ligands Gas6 (Axl) and protein S (Tyro3 and Mer). This interaction activates the TAM receptors and results in autophosphorylation of tyrosines in the PTK domain [171,172]. TAM proteins are involved in cytokine release, cell proliferation, survival, and adhesion [173]. Similar to TIM proteins, they bind to PtdSer exposed on apoptotic cells, and binding is facilitated by the γ-carboxyglutaminc acid residues present in Gas6/Protein S [174,175]. PTK activity and signaling of TAM proteins is required for inhibition of inflammation and augmentation of viral entry [149,176,177]. TAM proteins are expressed on a broad spectrum of tissue and cells,

e.g. lung, brain, kidney, platelets, macrophages, natural killer (NK) cells, and DCs [173,178–181] and might thus promote EBOV entry into a broad spectrum of target cells. Finally, it is noteworthy that Axl may promote EBOV host cell entry by enhancing macropinocytosis [151,182–184], and that Axl, unlike TIM-1, requires proper ligand binding and kinase signaling to enhance EBOV entry [185,186].

The mechanism by which PtdSer is exposed on the outer leaflet of the membrane of enveloped viruses is unknown. A recent study by Nanbo and colleagues suggests that XK-related protein (Xkr) 8, a cellular scramblase responsible for exposure of PtdSer on apoptotic cells, plays an important role. Thus, Xkr8 and EBOV-GP are trafficked together to the viral budding sites and are incorporated into EBOV-like particles, and incorporation of Xkr8 is required for augmentation of viral entry in a PtdSer-dependent manner [187].

#### 2.5.1.2. C-type lectins

C-type lectins (CTLs) are one of the largest families of animal lectins that have been classified into 17 groups (I-XVII), based on phylogeny as well as structural and functional properties [188]. In vertebrates, CTLs functions include serum glycoprotein homeostasis, pathogen sensing, and the initiation of immune response [189]. CTLs depend on $Ca^{2+}$ to recognize glycan ligands. They recognize a wide range of ligands including proteins, lipids, inorganic molecules, and ice crystals [190].

*Myeloid C-type lectin*s. Myeloid CTLs are pattern recognition receptors (PRRs) specialized in glycolipid and glycoprotein recognition [191,192]. They are mainly expressed by antigen-presenting cells including DCs, macrophages and monocytes [189]. C-type lectin receptors (CLRs) are at the frontline of innate and adaptive antiviral immune responses, CLR-virus interaction triggers a signaling cascade that induces the production of pro-inflammatory cytokines [193,194]. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) also known as CD209, is a type II transmembrane receptor composed of a short N-terminal cytoplasmic tail, a transmembrane region, a flexible neck domain involved in oligomerization, and a $Ca^{2+}$-dependent carbohydrate recognition domain. DC-SIGN binds to high mannose glycans and fucose-containing glycans [195–197]. Several viruses, including human

immunodeficiency virus type 1 (HIV-1), EBOV, dengue virus (DENV) and severe acute respiratory syndrome coronavirus (SARS-CoV) are recognized by DC-SIGN [198]. Lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN) also known as DC-SIGNR has a similar structure as DC-SIGN. However, it has a polymorphic neck region and the length of the DC-SIGNR neck region may impact affinity for ligands and viral pathogen specificity [193,198]. L-SIGN binds to N-linked high mannose oligosaccharides, with preference for mannosylated residues [193,198,199]. EBOV [200], MARV [201], HIV-1 [202], hepatitis C virus (HCV) [203], hepatitis B [204], and SARS-CoV [205] exploit L-SIGN for glycoprotein-mediated attachment and internalization. Liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) recognizes glycans containing mannose, N-acetylglucosamine and fucose residues. LSECtin can enhance EBOV and SARS-CoV but not HIV-1 or HCV infection and may do so in a mannose-independent manner [206–208]. Macrophage galactose C-type lectin (MGL) binds galactose and N-acetylgalactosamine residues, and viruses like EBOV, MARV and influenza A virus (IAV) use it for cellular entry and for evasion of host immune responses [209–213].

*Asialoglycoprotein receptors.* Asialoglycoprotein receptors (ASGPRs) are $Ca^{2+}$ dependent type II transmembrane proteins, with a short cytoplasmic tail, a transmembrane domain and a carbohydrate binding domain. Their main function is to regulate glycoprotein levels in serum, thereby maintaining homeostasis. ASPGR-1 is expressed in liver and has been shown to enhance MARV entry [188,214,215].

Cellular lectins such as MGL, DC-SIGN, L-SIGN, LSECtin, and ASGPR-1 can increase filovirus attachment to susceptible cells [200,207,209,214,216,217] by binding to N- and/or O-linked glycans on GP. However, binding to these molecules is usually not sufficient to allow for infectious entry, although one study suggested that directed expression of DC-SIGN and L-SIGN on the T cell line Jurkat renders these cells susceptible to GP-driven entry [200,216,218,219].

### 2.5.2. Virus internalization and processing of EBOV-GP

The next steps of EBOV entry are particle uptake and proteolytic processing of GP. Initially it was thought that EBOV and filoviruses in general were internalized in a clathrin- and/or caveolin-dependent fashion [220–222]. However recent studies showed that macropinocytosis is the main mechanism for EBOV uptake and it depends on particle size, since particles bigger than 100 nm are usually internalized via macropinocytosis. Furthermore, it was shown that EBOV induces PI3K, Rac1, PKC, Cdc42 and Pak1 activity and triggers plasma membrane ruffling, which are factors required for macropinocytosis [84,182,184,223]. Moreover, it is known that macropinocytosis is active in macrophages [224] and DCs [225], primary targets of EBOV. After uptake, virions are trafficked to late endosomes where GP is primed by the cysteine proteases CatB and L.

*Cathepsin B and L*. Cathepsins are divided into three families according to the amino acid present in their active site: serine, aspartate and cysteine cathepsins. The latter are members of the family of papain-like cysteine proteases. At sequence level, there are 11 human cysteine cathepsins, B, C, F, H, K, L, O, S, V, X, and W [226–228]. Cathepsins are synthetized as preproenzymes. The signal peptide is cleaved by signal peptidase during the import of the nascent polypeptide chain into the ER. The propeptide maintains cathepsins in their inactive form while they are trafficked through the ER and Golgi apparatus. Once sorted into late endosomes they are proteolytically processed into their mature and proteolytically active form [229–232]. Most cathepsins have endopeptidase activity. However, CatB can act as carboxypeptidase [233,234]. They require a slightly acidic environment, such as the one found in endosomes and lysosomes, to have proper proteolytic activity [230,235]. Cathepsins are ubiquitously and redundantly expressed. Hence, cathepsins can be upregulated to take over the function of a related enzyme that has been eliminated by gene knockout or inhibited [227,229,236–238].

CatB and CatL are involved in antigen processing and presentation, and apoptosis [239,240]. Also, they have been identified as the proteases responsible for filovirus GP priming in endosomes [220,241–243]. Priming of EBOV-GP by CatB/L occurs in two steps: First, GP1 (130 kDa) is cleaved by CatB and/or CatL into a 50 kDa form, that is

further cleaved, again by CatB/L or probably by other host proteases into a 19 kDa form [242,244–246]. In this step, the glycan cap and the MLD are removed by these enzymes, exposing the RBD and potentiating the capacity for membrane fusion. The GP1-RBD remains associated with GP2 via a disulfide bond in a metastable conformation [82,244,245]. Schornberg and colleagues showed that virions bearing the 19 kDa GP1 (achieved by processing virions bearing EBOV-GP with thermolysin, a bacterial derived protease that mimics CatB/L cleavage, and recombinant CatB/L) depicted higher infectivity as compared to untreated virions which harbor unprocessed GP. Furthermore, it was demonstrated that entry of virions containing the 19 kDa GP1 was largely resistant to CatB/CatL inhibitors but remained sensitive to a lysosomotropic agent and a cysteine protease inhibitor [87,242,243], suggesting that GP is first cleaved by CatB/CatL and subsequently another lysosomal factor, likely a thiol reductase, is required to trigger membrane fusion. It is important to note that CatB/L dependence varies among filoviruses, for instance CatB activity is required for EBOV-, TAFV- and BDBV- but not for SUDV-, RESTV- or MARV-GP-driven entry [247]. Moreover, several but not all studies demonstrating a role of CatB/L in filovirus entry used vesicular stomatitis virus (VSV) [248] or murine leukemia virus (MLV) [249] vectors pseudotyped with filovirus GPs [247,250] and shape and size of these particles differs significantly from those of authentic filoviruses. Notably, Marzi and colleagues showed that $CatB^{-/-}$ or $CatL^{-/-}$ knockout mice supported EBOV spread as efficient as wild type mice [251]. These contradictory findings suggest that protease choice of EBOV in cell culture might not adequately reflect protease choice in the infected host or that EBOV can use proteases other than CatB/L to secure GP priming in the host.

### 2.5.3. Receptor binding

After GP priming by CatB/L, the primed EBOV-GP interacts with the intracellular receptor NPC1 [85,86], which, jointly with an unknown stimulus, triggers membrane fusion, the final step of viral entry.

*Niemann-Pick C1*. NPC1 is a late endosomal/lysosomal protein that is ubiquitously expressed. It has 13 transmembrane-spanning helices (sterol sensing domain, SSD), and 3 luminal domains, domain A (N-terminal domain, NTD), domain C and domain I [252–255]. NPC1 has been implicated in cholesterol homeostasis and absorption. To

accomplish this function, NPC1 (NTD domain and domain C loop 2) binds to Niemann-Pick C2 (NPC2) which in turn binds to cholesterol [256–259]. Abrogation of NPC1 or NPC2 function leads to cholesterol accumulation in lysosomes, causing the fatal Niemann-Pick disease [252,260–262]. NPC1 was first identified to be an important entry factor for EBOV by two studies: the first one, performed by Carette and colleagues, used genome-wide screening of haploid human cells to identify those that were not compatible with filovirus GP-driven entry [86]. The second study was done by Côté and coworkers, who screened a library of chemical compounds for filovirus entry inhibitors, including U18666A, which mimics Niemann-Pick disease [85]. Later it was shown that U18666A blocks EBOV-driven entry into host cells, despite the fact that it binds to a different domain (SSD) than EBOV-GP (domain C). However, the concentration of U18666A needed to block EBOV-driven entry is 100-fold higher [85,87,263,264] than the one needed to inhibit cholesterol trafficking. It was hypothesized that when applied at high concentrations, U18666A and other cationic amphiphiles may interact with the NPC1 C-loop 2 with low affinity but sufficient to block virus entry [265]. Further, studies revealed that primed EBOV-GP but not unprocessed EBOV-GP binds to loops 1 and 2 from domain C, indicating that the RBD has to be fully exposed in order to interact with NPC1 [255,266,267]. The *in vivo* relevance of NPC1 was demonstrated by Herbert and colleagues, using $NPC1^{-/-}$ mice [268]. They could show that these mice were completely resistant to mouse-adapted EBOV infection, which indicates that NPC1 expression is essential for filovirus replication and pathogenesis, making it a promising therapeutic target. In sum, NPC1 is an intracellular receptor of EBOV and interactions of primed GP with NPC1 in conjunction with low pH might help release the internal fusion loop (IFL) and facilitate the conformational changes in GP required for membrane fusion [93,255,269].

## 2.6. Ebola virus and the interferon system

### 2.6.1. Innate immune system

All live forms are constantly exposed to pathogens, and humans are no exception. However, the immune system allows us to prevent and or control infections. In vertebrates, the immune system is divided in an innate and an adaptive arm. Adaptive responses are highly specific but slow, taking between one or two weeks to develop upon the first exposure to a pathogen, since specific clones of B and T cells reactive against the new pathogen need to be activated and proliferate [270]. Subsequently, the adaptive but not the innate responses lead to the development of immunologic memory. Thus, memory B and T cells have the ability to respond more rapidly to subsequent encounters with the same antigen or pathogen and may provide lifelong protection [271].

The innate immune system is not only present in vertebrates, but also in invertebrates and plants. It is the first line of defense against pathogens, taking only minutes to activate and hours to remove the threat. It plays a critical role in activation and regulation of the adaptive immune system [272,273]. The innate immune system is known to be a non-specific response against pathogens. However, this concept is changing. New evidence suggests that NK cells and macrophages can develop specific immune memory based on recognition of certain PAMPs. After PAMP recognition, epigenetic changes at the level of histone methylation and acetylation reprogram the cells, thereby increasing their activity upon secondary stimulation with the same or similar stimuli and providing protection in a T/B-cell-independent manner [274–276]. The main components of the innate immunity include physical barriers (i.e. skin, mucosal membranes, and gut-associated lymphoid tissue), chemical barriers (i.e. low pH, hydrolytic enzymes, antimicrobial peptides and pro-inflammatory cytokines) present on the surface of physical barriers. The third component includes a cellular response, carried out by phagocytic cells (macrophages, neutrophils, monocytes and DCs), which is triggered upon invasion by a pathogen that was able to overcome the epithelial barriers [272,277].

Macrophages, neutrophils and DCs are able to recognize pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), peptidoglycans or

complex carbohydrates present on bacteria, fungi and other pathogens. PAMPs are sensed by PRRs and PRR binding triggers gene expression of proteins of the innate immune system (e.g. interferons, defensins, cytokines) [272,278,279].

#### 2.6.1.1. Interferon system

The interferon (IFN) system is an important innate defense against viral infections. IFNs are divided in type I, type II and type III, based on their structural homology, chromosomal location and interaction with their receptor chains. Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω and are essential for eliminating viral infections. Type II IFNs (IFN-γ) are also required for defense against viral infection. In addition, they regulate expression of major histocompatibility complex and modulate the adaptive immune response [272,277,280,281]. Type III IFNs comprise IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. They display similar functions as type I IFNs [282–284].

Expression of IFNs is induced in infected cells upon recognition of PAMPs by PRRs such as toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [285]. TLRs are membrane spanning proteins with a leucine-rich repeats (LRRs) extracellular region and toll-IL-1 receptor (TIR)-domains. So far, 10 TLRs have been identified in humans. They can be expressed in the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6) or in endosomes/lysosomes (TLR3, TLR7, TLR8, TLR9). The TLRs responsible for sensing viral PAMPs are: TLR3 (double stranded RNA, dsRNA), TLR7 and TLR8 (single stranded RNA, ssRNA), and TLR9 (unmethylated CpG) [272,286].

RLRs and NLRs receptors are cytosolic PRRs. RLRs are RNA helicases with caspase recruitment domains (CARD) that recognize different species of RNAs: RIG-I binds to short dsRNA and 5’-tri- or diphosphate ssRNA [287–289]. MDA5 recognizes long dsRNA and viral mRNAs lacking 2’-O-methylation [290,291]. The third RLR is LGP2, which can enhance MDA5 activation when binding to dsRNA in tandem with MDA5, and inhibit RIG-I by direct interaction or by competing for dsRNA [292–294]. NLRs induce production of immune and inflammatory responses. They are able to recognize flagellin, LPS, peptidoglycans, bacterial toxins, and nucleic acids from bacteria and

viruses. The best characterized NLRs are NOD1 and NOD2, similar to RLRs, they have a CARD domain and can recognize breakdown products formed during synthesis or degradation of the bacterial cell wall. NOD2 can also activate responses against viruses like IAV [272,295,296].

After viral PAMPs recognition, a signaling cascade to produce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), IFN-regulatory factors (IRFs) and type I and III IFNs is triggered. First, PRRs interact with their adapter proteins (TLRs: TRIF, MyD88; RLRs: MAVS). In the case of NLRs the cascade starts with NOD binding to the serine/threonine kinase RIP2. Adaptor proteins then recruit TRAF ubiquitin ligases, IKKα/β and TBK1 kinases. Subsequently, phosphorylation, dimerization, activation and nuclear translocation of IRF3, IRF7, and NF-κB takes place. Finally, these protein bind to the promoter region of IFN-stimulated elements inducing expression of type I and III IFNs and some IFN-stimulated genes (ISGs) [272,297–302]. IFNs are secreted into the extracellular space, where they bind to IFN receptors (IFNR1, IFNR2, IFNLR) on neighboring cells [303]. IFN receptor binding leads to the activation of the JAK/STAT pathway. In short, Janus-kinases (JAK-1 and TYK2) are activated upon IFN binding and phosphorylate signal transducer and activator of transcription (STAT) factors 1 and 2. This leads to the formation of the IFN-stimulated gene factor 3 complex via the interaction of STAT1 and STAT2 with IRF9. Finally, this complex translocates into the nucleus, binds to IFN-stimulated response elements and triggers expression of over 500 ISGs [300,301,304–306].

#### 2.6.1.2. Interferon induced genes

IFNs induce a so-called antiviral state, and synthesis of different ISGs helps to inhibit different stages of the viral replication cycle. In the following paragraphs some of the most characterized will be discussed.

*Myxovirus resistance (Mx) proteins.* Mx proteins belong to the family of dynamin-like large guanosine triphosphatases (GTPases). They have an N-terminal GTPase (G) domain, a middle domain (MD), and a C-terminal GTPase effector domain (GED). The MD and GED domains are important for Mx antiviral activity, self-oligomerization and formation of the ring-like structures. These ring-like structures bind to the viral

nucleocapsid, induce conformational changes and stimulate GTPase activity. In human cells two Mx proteins are expressed, Mx1 (MxA) and Mx2 (MxB) [307–310]. They inhibit early stages of the virus replication cycle and display broad antiviral activity and. For example, MxA traps incoming viral nucleocapsids from IAV or Thogoto viruses, preventing their import into the nucleus. Also, it can inhibit mRNA synthesis of VSV and human parainfluenza viruses [311–314]. Human MxB prevents integration of HIV-1 into the host genome by targeting the viral capsid and inhibiting uncoating, nuclear uptake or integration by the viral pre-integration complex [315–318].

*Human interferon-induced transmembrane (IFITM) proteins*. IFITMs comprise four proteins: IFITM1, IFITM2, IFITM3 and IFITM5. IFITM1, 2, and 3 are viral restriction factors with constitutive and ubiquitous expression. However, upon IFN stimulation their expression is enhanced [319,320]. IFITM1 is mainly located at the plasma membrane while IFITM2 and IFITM3 localize in late endosomes, lysosomes and autolysosomes [321,322]. IFITM proteins inhibit the fusion of the viral envelope with the membrane of the target cell [323]. IFITMs inhibit viruses like IAV, coronaviruses, filoviruses, flaviviruses and HIV-1 [321,324,325]. There are two models that could explain IFITMs antiviral mechanism. One suggests that IFITM proteins alter endosome acidification, inhibit cellular proteases required for priming of viral glycoproteins [326,327] or redirect viruses to a non-fusogenic pathway [322,327]. The second mechanism proposes that IFITMs may inhibit membrane fusion by altering the biological properties of cellular membranes such as curvature and fluidity [328].

*Tripartite motif (TRIM) family proteins.* Tripartite motif (TRIM) family proteins are highly conserved proteins with a N-terminal RBBC motif, one or two B-box domains and a C-terminal coiled-coil domain. They are a group of E3 ubiquitin ligases involved in cell functions including cell cycle progression and autophagy, and can display antiviral activity [329,330]. TRIM5α is known to inhibit HIV-1 uncoating. Thus, recognition of incoming capsids by TRIM5 α accelerates uncoating and resulting in premature exposure of the nucleoprotein complex [331]. TRIM22 is another protein that inhibits HIV-1. TRIM22 targets Gag trafficking to the plasma membrane, resulting in decreased particle production [332]. In addition, TRIM22 can inhibit hepatitis B virus by preventing the transcription of viral genes [333], encephalomyocarditis virus by promoting

ubiquitination of the viral C3 protease [334], and IAV by targeting the viral nucleoprotein for degradation [335].

*2'-5'-oligo-adenylate synthetase (OAS) proteins and the protein kinase R (PKR)*. Two examples of antiviral effector proteins that interfere with viral infection at the stage of protein synthesis are OAS proteins and PKR. The 2'-5'-OAS family in humans comprises four genes: OAS1, 2, 3 and OAS-like (OASL) and they are able to inhibit viral protein synthesis [336]. For this, they bind to dsRNA, synthesize 2',5'-linked phosphodiester bonds, which in turn form 2'-5'-olygoadenylates that activate the latent ribonuclease L (RNase L). RNase L then degrades cellular and viral RNAs [337,338]. Similar to OAS, PKR binds to viral dsRNA. PKR is constitutively expressed but its expression is upregulated upon IFN stimulation [339]. The antiviral activity of PKR relies on phosphorylation of the alpha unit of eukaryotic translation initiation factor 2. Phosphorylation leads to sequestration of a guanine nucleotide exchange factor called eIF2b which facilitates recycling of guanidine diphosphate (GDP) to guanidine triphosphate (GTP). This results in inhibition of both cellular and viral translation by preventing the conversion GDP to GTP [340,341].

*Virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (Viperin).* ISGs can also target late steps of the replication cycle. One well-known example is viperin. Viperin is a virus inhibitory protein associated with the ER that can inhibit farnesyl diphosphate synthase. Decreased expression of this enzyme interferes with cell membrane fluidity. By altering fluidity of lipid rafts, it affects budding of enveloped viruses that require these microdomains, such as IAV and HIV-1 [342–344]. Furthermore, it has been shown that viperin inhibits RNA replication of HCV by binding to the non-structural 5A protein and interfering with the stability and function of the viral replication complex [345]. Another ISG that inhibits a late step in viral replication is tetherin and this protein will be discussed in detail in the following chapter.

### 2.6.2. Tetherin

Tetherin, also known as BST-2, HM1.24 or CD317, is constitutively expressed and its expression is upregulated by type I and II IFNs [346,347]. It was first identified as a surface marker of terminally differentiated normal and neoplastic B cells [348,349]. Tetherin is a type II transmembrane protein composed of 180 amino acids and exhibits the following domain organization: A N-terminal cytoplasmic tail, a transmembrane domain (TMD), followed by a coiled-coil ectodomain and a C-terminal glycosylphosphatidylinositol (GPI)-anchor [350,351]. Tetherin forms homodimers via disulfide bonds between cysteine residues 53, 63, and 91 [352,353], which are located in the extracellular domain. It is mainly located at the plasma membrane within lipid rafts but it can also be found in the trans-Golgi network (TGN) and in vesicular compartments [350,354]. The GPI anchor of tetherin is essential for its transport through the ER and localization in lipid rafts, while the cytoplasmic tail, via a conserved double tyrosine motif, mediates endocytic recycling through the TGN in a clathrin-dependent manner [355,356]. Also, upon virus tethering the cytoplasmic tail of tetherin can induce activation of NF-κB, resulting in the expression of ISGs and thereby amplification of the innate immune response [357,358].

The unique structure and domain organization of tetherin, having two membrane anchors, allows it to inhibit release of budding virions from infected cells by forming a physical tether between them. This ability was discovered when HIV-1 virions, lacking viral protein U (Vpu) were retained at the cell surface [359–361]. Later it was shown that tetherin prevents release of several enveloped virus families such as rhabdo- [362], alpha- [363,364], arena-, paramyxo-, orthomyxo- [365,366], herpes- [367], orthohepadna- [368], flavi- [369–371], and filoviruses [372,373].

There were three proposed mechanism by which tetherin cross-links virions. First, one molecule within a tetherin dimer might insert into envelope of the budding virus, while the second molecule remains on the host cell membrane. In this scenario, the disulfide bonds between the ectodomains of the tetherin molecules are responsible for the tether effect [374]. A study performed by Fitzpatrick and colleagues provided evidence that supports this theory. Treatment with phosphatidyl inositol-specific phospholipase C, an enzyme that cleaves the GPI anchor, did not efficiently release tethered virions

from the cell surface [375]. The second mechanism suggests that both GPI-anchors of a tetherin homodimer are taken up by the virus, in a parallel orientation, and the TMDs remain inserted into the plasma membrane, or *vice versa* [375]. Findings that support this scenario are that dithiothreitol treatment, which reduces disulfide bonds, failed to release tethered particles [375]. Furthermore, data observed via cryo-electron tomography showed that expected and experimental distance between virions and cell membrane matched, when the coiled-coil domains of tetherin are extended, [376]. Finally, an antiparallel orientation to tether virions has been suggested. In this configuration, the GPI anchor of one tetherin molecule and the TMD of the second one are incorporated into the virion while their opposite membrane anchors remain inserted in the plasma membrane [376,377]. A study performed by Venkatesh and Bieniaz provided conclusive proof that the tetherin conformation associated with antiviral activity are homodimers in which tetherin monomers are assembled in a parallel fashion and in which the GPI anchors are preferably inserted into the viral membrane [378]. Studies to identify determinants important for tetherin antiviral activity concluded that domain organization, topology, and intracellular transport are more important than amino acid sequence. Most importantly, Perez-Caballero and colleagues found that a synthetic molecule that was engineered to have the same domain organization as tetherin but not sequence homology was able to restrict viral release [360].

The *in vivo* relevance of tetherin has been shown in tetherin-deficient mice. In Chikungunya infected mice, viral load at the inoculation site was higher in tetherin deficient animals than in wild type mice and more prominent infection of lymphoid tissue was observed in the absence of tetherin [379]. Similar results were found when tetherin knockout mice were infected with murine retroviruses including mouse mammary tumor virus and MLV [380,381]. Furthermore, studies conducted with Friend retrovirus showed that wild type mice had increased $CD4^+$ and $CD8^+$ T cell responses as compared to tetherin knockout mice. These responses were critical for recovery and are possibly driven by tetherin cytoplasmic tail signaling [382]. Thus, tetherin can inhibit viral spread in the host and modulate immune response *in vitro* and *in vivo*.

#### 2.6.2.1. Tetherin antagonists

Several viruses susceptible to tetherin restriction evolved countermeasures – viral proteins that interfere with tetherin expression or antiviral activity at the site of viral budding or antiviral activity ([361,383] table 1).

| Virus | Tetherin antagonist | Mechanism |
|---|---|---|
| HIV-1 | Vpu [359,384] | Downregulation, degradation, removal from lipid rafts |
| HIV-2 | Env [385,386] | Intracellular sequestration |
| SIV | Nef/Env [387–389] | Downregulation |
| KSHV | K5 [390] | Downregulation and degradation |
| HSV | gM [391] | Downregulation |
| SARS-CoV | ORF7a [392] | Glycosylation interference |
| CHIKV | nSP1 [363] | Downregulation |
| hPIV-2 | V protein [393] | Downregulation |
| Sendaivirus | GP [394] | Unknown |
| EBOV | GP [372,373,395] | Unknown |
| MARV | GP [365] | Unknown |

**Table 1.** Tetherin antagonists and mechanisms.

The best studied tetherin antagonists is HIV-1 Vpu protein. Vpu's antagonism, relies on interaction between its TMD with the TMD of tetherin [396,397]. An AXXXAXXXAXXXW motif within Vpu's TMD was found to be crucial for tetherin-Vpu interaction, mutation of the alanine residues in this motif impairs the ability of Vpu to counteract tetherin [396,398]. As mentioned above, the mechanisms employed by Vpu to counteract tetherin include cellular surface down-regulation and degradation of tetherin [384,399–401]. For degradation, tetherin is ubiquitinated and targeted for lysosomal degradation in the presence of Vpu [401,402]. For this, Vpu recruits E3 ubiquitin ligase via a conserved phosphoserine motif in its cytoplasmic tail. Mutation of this motif prevents Vpu's interaction with the ubiquitin ligase complex, ß-TrCP2, and hence the initiation of the ubiquitination cascade and ultimately tetherin antagonism [403,404]. Furthermore, Vpu inhibits the anterograde transport of tetherin by preventing proper cellular transport of newly synthetized tetherin from the ER and/or

from early endosomes to the cell surface [397,405]. In the case of tetherin removal from lipid rafts, Vpu forms a complex with tetherin via the tryptophan residue 76 of the cytoplasmic tail of Vpu [401,406]. This anchors the C-terminus of Vpu to the lipid bilayer and displaces tetherin from budding sites [406]. However, tetherin antagonism by several viral proteins other than Vpu has in not been fully elucidated and it is largely unclear how tetherin is antagonized by EBOV-GP.

#### 2.6.2.2. EBOV-GP as a tetherin antagonist

Kaletsky and colleagues demonstrated that EBOV-GP like HIV-1 Vpu can promote release of VP40-based, EBOV-like particles from tetherin-positive cells [395]. Moreover, they were able to prove that EBOV-GP interacts with tetherin, a finding confirmed by separate studies, without altering tetherin expression levels. Additionally, they provided initial insight on the domains within GP involved in tetherin antagonism. Thus, they demonstrated that sGP and shed GP, which lack the TMD and cytoplasmic tail of EBOV-GP, did not inhibit tetherin, indicating that full-length GP is required for tetherin antagonism. However, deletion of the MLD did not affect tetherin counteraction by GP. Later, Lopez and colleagues and other laboratories showed that Vpu requires a specific sequence within the tetherin TMD to counteract tetherin and target it for degradation while EBOV-GP was able to antagonize even artificial tetherin and did not remove tetherin from the cell surface [372]. Radoshitzky and colleagues confirmed previous findings showing that release of virus-like partices (VLPs) based on the matrix proteins of several enveloped viruses, including EBOV, HIV-1 and Lassa virus (LASV) are restricted by tetherin and that co-expression of several viral antagonist could rescue viral release. In contrast, EBOV-GP and MARV-GP failed to rescue LASV-like particles release from tetherin-positive cells [365] and the reasons for the failure of GP to promote particle release from tetherin-positive cells is at present unknown. Finally, they demonstrated that tetherin expression does not reduce EBOV infection [365], a finding confirmed by another study [407], and that GP and tetherin fail to colocalize in infected cells. Whether these findings indicate that GP-mediated tetherin antagonism promotes viral spread in tetherin-positive cells or that EBOV-release is intrinsically not sensitive to tetherin remains to be elucidated.

Kühl *et. al.* and subsequent studies showed that EBOV-GP can antagonize tetherin orthologues of diverse species [360,373,408], confirming that tetherin antagonism is sequence independent. Furthermore, they provided evidence that EBOV-GP interacts with tetherin via GP2 and confirmed that co-expression of GP does not modulate tetherin levels at the cell surface [372,373]. Lopez and colleagues investigated whether GP might remove tetherin from lipid rafts, which have been proposed as platform for EBOV budding [103,409]. However, they found that neither EBOV-GP nor Vpu affected localization of tetherin in lipid rafts and did not prevent colocalization of viral particles and tetherin at the budding sites. Furthermore, they did not find evidence of EBOV-GP being a lipid-raft protein, which contrasts previous findings [103].

Gustin and colleagues reported that EBOV-GP enhances egress of VP40-based VLPs by preventing the interaction of tetherin and VP40 [395,410]. Moreover, they found that GP can mask tetherin from recognition by antibodies and that tetherin is efficiently incorporated into VP40-based particles. A role for VP40 in tetherin sensitivity was also supported by a study conducted with MARV: It was reported that MARV-VP40 is largely resistant to inhibition by tetherin and that MARV adaptation to mice resulted in acquisition of mutations in VP40 that markedly increased sensitivity towards human but not mouse tetherin [411]. Collectively, these results suggest that both VP40 and GP can impact tetherin sensitivity and that GP might antagonize tetherin by disrupting VP40-tetherin interactions. Finally, it is noteworthy that tetherin antagonism by feline immunodeficiency virus (FIV) envelope (Env) glycoprotein was reported to entail Env-mediated exclusion of tetherin from virions or virion assembly sites and failed to promote release of HIV-based particles from tetherin-positive cells [412]. Unlike GP, FIV-Env can only counteract carnivore tetherins and only in the context of FIV particles. These findings suggest that Env affects tetherin sensitivity at the point of FIV budding and does not exert a cell-wide phenotype and the same might apply to EBOV-GP.

The studies discussed above demonstrated that the GP2 subunit interacts with tetherin [373] and a separate study showed that the TMD of EBOV-GP contributes to tetherin counteraction [413]. However, introducing the EBOV-TMD into LASV-GPC did not confer tetherin antagonism to LASV-GPC. This indicated that the TMD is not the only determinant in EBOV-GP that controls tetherin antagonism [413]. Indeed, during the

course of the present thesis Vande Burgt and colleagues confirmed the importance of the TMD for tetherin antagonism [413,414] and showed that also the glycan cap of GP is important [414]. Moreover, Brinkmann and colleagues showed that mutations in the EBOV-GP RBD can abrogate tetherin antagonism [395,415] and that tetherin antagonism requires adequate GP glycosylation [415]. In sum, the TMD, glycan cap and RBD as well as proper glycosylation of EBOV-GP are required for GP driven tetherin antagonism but it is unknown how they contribute to tetherin antagonism.

## 3. Aims

Ebola virus disease (EVD) is a severe disease that threatens human and animal health. Infection by Ebola virus (EBOV) is major cause of EVD and understanding how EBOV interacts with host cells and evades immune control might open novel avenues for antiviral intervention. The EBOV glycoprotein (GP) is the only viral surface protein and mediates the first step in EBOV infection, viral entry into target cells [82]. For this, EBOV-GP needs to be processed (primed) by the host cell proteases cathepsin B/L (CatB/L) [241], at least in cell culture. However, studies with $CatB^{-/-}$ or $CatL^{-/-}$ knockout mice indicated that these proteases are dispensable for viral spread in the infected host [251]. The reasons for the discrepancy between the *in vitro* and *in vivo* findings are poorly understood. Therefore, one goal of the present thesis was to identify determinants that control CatB/CatL dependence of EBOV entry.

Apart from mediating viral entry, EBOV-GP also counteracts the interferon-induced antiviral host cell factor tetherin [82,395]. It is conceivable that tetherin counteraction may allow EBOV to spread in tetherin-positive cells and tissues, and efficient spread of EBOV in tetherin transfected cells has been demonstrated. However, the determinants in GP that govern counteraction of tetherin and the contribution of this process to viral spread are incompletely understood [413–415]. Therefore, a second aim of this thesis was to identify determinants in EBOV-GP that contribute to tetherin antagonism and to obtain insights whether antagonism can promote viral spread in cell culture. A GXXXA motif within the transmembrane domain (TMD) of EBOV-GP was found to be required for the well-documented cytotoxic effects associated with expression of EBOV-GP in cell culture [416] and served as starting point for the thesis research. In summary, the aims of this thesis were to:

1) Identify determinants governing the CatB/L dependence of EBOV-GP-driven entry into host cells.
2) Identify the contribution of a GXXXA motif in EBOV-GP to tetherin antagonism and determine whether antagonism contributes to viral spread.

## 4. Manuscripts

**Calu-3 cells are largely resistant to entry driven by filovirus glycoproteins and the entry defect can be rescued by directed expression of DC-SIGN or cathepsin L.**

Submitted to Virology, January 2019

**A GXXXA Motif in the Transmembrane Domain of the Ebola Virus Glycoprotein Is Required for Tetherin Antagonism.**

Journal of Virology, 2018

## 4.1. First manuscript

# Calu-3 cells are largely resistant to entry driven by filovirus glycoproteins and the entry defect can be rescued by directed expression of DC-SIGN or cathepsin L.

González-Hernández, M., Hoffmann, M., Pöhlmann, S.

Virology, submitted on January 9th, 2019. Submission number: VIRO-19-3

Individual contributions:

I performed the experiments that resulted in the data shown in figure 1 (panels A, D, and E), figure 2 and figure 4 (panels A-E). Moreover, I analyzed all the data that I generated and I contributed to the writing of the manuscript.

*Manuscript
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# Calu-3 cells are largely resistant to entry driven by filovirus glycoproteins and the entry defect can be rescued by directed expression of DC-SIGN or cathepsin L

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## ABSTRACT

Priming of the viral glycoprotein (GP) by the cellular proteases cathepsin B and L (CatB, CatL) is believed to be essential for cell entry of filoviruses. However, non-filamentous particles have frequently been used to prove this concept. Here, we report that GP-mediated entry of spherical, bullet-shaped and filamentous particles depends on CatB/CatL activity and that this effect is cell line-independent. Moreover, we show that the human cell line Calu-3, which expresses low amounts of CatL, is largely resistant to entry driven by diverse filovirus GPs. Finally, we demonstrate that Calu-3 cell entry mediated by certain filovirus GPs can be rescued upon directed expression of CatL or DC-SIGN. Our results identify Calu-3 cells as largely resistant to filovirus GP-driven entry and demonstrate that entry is limited at the stage of virion attachment and GP priming.

## 1. Introduction

The family *Filoviridae* comprises three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. To date, there are five species of ebolaviruses, *Zaire ebolavirus* (only member: Ebola virus [EBOV]) *Sudan ebolavirus* (only member: Sudan virus [SUDV]), *Bundibugyo ebolavirus* (only member: Bundibugyo virus [BDBV]), *Tai Forest ebolavirus* (only member: Tai Forest virus [TAFV]), *Reston ebolavirus* (only member: Reston virus, [RESTV]), one species of marburgvirus, *Marburg marburgvirus* (two members: Marburg virus [MARV] and Ravn virus [RAVV]) and one species of cuevavirus, *Lloviu cuevavirus* (only member: Lloviu virus [LLOV]) (Kuhn, 2017). There is compelling evidence that all members of the three genera circulate in bats, which are believed to serve as their natural reservoir, and that zoonotic transmissions of most of these viruses to humans can induce severe disease (Mandl et al., 2018; Olival and Hayman, 2014). Multiple filovirus outbreaks in Africa were recorded during the last 52 years and usually entailed less than 500 cases (Centers for Disease Control and Prevention, 2018). In contrast, an outbreak of Ebola virus disease (EVD) in West Africa in 2013 resulted in an EVD epidemic with more than 10.000 deaths and secondary transmission events in Europe and the US (Lo et al., 2017). Further, a recent outbreak of EVD has started in the Democratic Republic of Congo, causing 543 confirmed cases and 309 deaths (WHO, 2018), and is still ongoing (as of January 2019). These examples show that filovirus infection constitutes a serious health threat that is not limited to African countries in which most filoviruses are endemic. Despite promising results of clinical trials, neither vaccines nor antiviral agents approved by regulatory agencies are currently available to combat filovirus infection. Therefore, the development of antiviral strategies remains a research focus and host cell factors required by diverse filoviruses for spread but dispensable for host cell survival are potential drug targets.

The filovirus glycoprotein (GP) mediates viral entry into target cells. It is a highly N- and O-glycosylated type I transmembrane protein and it is the sole viral protein incorporated into the viral envelope (Martin et al., 2016). Host cell entry of filoviruses is best studied for EBOV and we will therefore focus the subsequent discussion on factors important for EBOV GP-driven entry. To initiate infection, EBOV particles first attach to target cells, which can be facilitated by GP interactions with attachment factors like the C-type lectin DC-SIGN (Alvarez et al., 2002; Simmons et al., 2003). In addition or alternatively, attachment can be facilitated in a GP-independent fashion: Phosphatidylserine residues on the viral envelope can be recognized by TIM (T-Cell Immunoglobulin And Mucin Domain-Containing) proteins (Jemielity et al., 2013; Kondratowicz et al., 2011; Moller-Tank et al., 2013) or, via an adaptor, by tyro 3 kinases, including Axl, which are located on the cell surface (Shimojima et al., 2006). After attachment, virions are taken up into cells by macropinocytosis (Quinn et al., 2009; Saeed et al., 2010) and are trafficked into late endosomes, where GP is processed by the host cell cysteine proteases cathepsin B and L (CatB, CatL) (Chandran et al., 2005; Kaletsky et al., 2007; Schornberg et al., 2006), which are known to also process several other viral glycoproteins (Pager et al., 2006; Simmons et al., 2005). Proteolytically processed GP (also termed primed GP) then interacts with the cholesterol transporter Niemann-Pick C1 (NPC1) (Carette et al., 2011; Cote et al., 2011) and, upon low pH exposure and a poorly defined additional stimulus (Bale et al., 2011; Brecher et al., 2012; Schornberg et al., 2006) mediates the fusion of viral and late endosomal membranes, thereby allowing the release of the viral genetic information in the host cell cytoplasm. Priming of GP by CatB and to a lesser degree CatL is essential for host cell entry and results frequently obtained with surrogate systems indicate that entry driven by the GPs of all filoviruses might depend on CatB/CatL activity (Gnirss et al., 2012; Maruyama et al., 2014; Misasi et al., 2012).

However, the relative dependence varies, with RESTV showing generally modest CatB/CatL dependence and MARV exhibiting pronounced CatL (Misasi et al., 2012) but not CatB dependence (Gnirss et al., 2012; Misasi et al., 2012). Moreover, EBOV entry into dendritic cells was reported to be CatB but not CatL dependent (Martinez et al., 2010). Notably, a study by Marzi and colleagues conducted with authentic viruses suggested that EBOV and BDBV entry into cultured cells is CatB dependent while entry of several other ebolaviruses is not (Marzi et al., 2012). Moreover, the study showed that lack of CatB or CatL expression is compatible with full viral spread and pathogenesis in the rodent host (Marzi et al., 2012). The discrepancies between these results and the CatB/CatL dependence reported by several other studies are incompletely understood. However, one can speculate that particle shape - filoviruses are filamentous while bullet-shaped or spherical particles are frequently used to model filovirus entry - and target cell type may play a role.

Here, we show that EBOV-GP-driven entry is CatB/CatL dependent irrespective of the surrogate system, particle shape and target cell type. Moreover, we demonstrate that Calu-3 cells express very low levels of endogenous CatL, in keeping with a previous study (Park et al., 2016), and are largely refractory to entry driven by ebola-, marburg- and cuevavirus GPs. Finally, we show that entry into Calu-3 cells can be rescued by directed expression of DC-SIGN or CatL, suggesting that attachment and GP priming can limit entry into these cells.

## 2. Results

### *2.1. EBOV-GP-driven entry into cell lines requires CatB/CatL activity irrespective of the shape of the GP-bearing particle and the target cell type*

We first examined whether retroviral (spherical), rhabdoviral (bullet-shaped) and filoviral (filamentous) particles differ in their CatB/CatL-dependence for host cell entry. For this, we used retro- and rhabdoviral vectors previously used to analyze EBOV-GP-mediated host cell entry (Takada et al., 1997; Wool-Lewis and Bates, 1998) as well as replication competent vesicular stomatitis virus (VSV) encoding EBOV-GP (Gonzalez-Hernandez et al., 2018). Moreover, we employed two systems that depend exclusively on filovirus proteins. First, we produced EBOV-like particles harboring luciferase by directed expression of all viral proteins from plasmids, including a VP30 version that is fused to firefly luciferase (Dietzel et al., 2017). Second, we employed the EBOV trVLP system in which a truncated EBOV minigenome is packaged into filoviral particles due to expression of the missing open reading frames (EBOV-NP, -VP30, -VP35 and - L) *in trans* (Watt et al., 2014). While the VP30-Luc system allows measuring a single delivery of VP30-luciferase into cells, the trVLP system allows studying entry and spread of filovirus-like particles. Finally, we employed the endosomal cysteine protease inhibitors E-64d and MDL28170 to block CatB/CatL activity in target cells. These inhibitors were previously shown to interfere with CatB/CatL activity (Brana et al., 1999; Gewies and Grimm, 2003; Hoffmann et al., 2016; Simmons et al., 2005) and were previously used to inhibit EBOV-GP-driven entry (Gnirss et al., 2012; Kaletsky et al., 2007).

Analysis of GP-driven entry into 293T cells revealed that entry was blocked by E-64d and MDL28170 in a concentration-dependent manner, irrespective of whether GP was presented on retro-, rhabdo- or filovirus particles (Fig. 1). In contrast, entry driven by the glycoprotein (G) of vesicular stomatitis virus (VSV) was not affected by E-64d and MDL28170, as expected. Moreover, analysis of entry of EBOV-GP-bearing rhabdoviral particles into six different cell lines of human (accidental host; 293T, Huh-7, A549 and Calu-3), non-human primate (accidental host; Vero E6) and fruit bat (suspected reservoir host; EpoNi/22.1) origin revealed that entry was sensitive to inhibition by E-64d irrespective of the target cell type, although it should be stated that entry into Calu-3 was very close to background (Fig. 2). Collectively, our results show that, under the experimental conditions chosen, EBOV-GP-driven entry was CatB/CatL-dependent irrespective of the type of GP-bearing particle and target cell line. Moreover, our results identify Calu-3 cells as one of the very few cell lines that are largely resistant to GP-mediated entry.

*2.2. Calu-3 cells are largely resistant to entry driven by filoviral but not arena-, paramyxo- and rhabdoviral glycoproteins*

The inefficient EBOV-GP-driven entry into Calu-3 cells raised the question whether this phenotype was specific for EBOV-GP or could also be observed for other viral glycoproteins. In order to answer this question, we investigated whether glycoproteins from viruses of diverse viral families also fail to mediate efficient Calu-3 entry or whether this effect is rather specific for EBOV-GP. We found that glycoproteins of arena- (Lassa virus [LASV], lymphocytic choriomeningitis virus [LCMV] and Machupo virus [MACV]), paramyxo- (Mumps virus [MuV] and Nipah virus [NiV]) and rhabdoviruses (VSV) mediated entry into Calu-3 cells with high

efficiency and to roughly the same levels as measured for Vero E6 cells (Fig. 3A). EBOV-GP-driven entry into Vero E6 cells was highly efficient and comparable to that measured for the other glycoproteins tested, while entry into Calu-3 cells was severely attenuated (~200-fold compared to Vero E6 cells) (Fig. 3A). In order to investigate whether Calu-3 cells are largely refractory to entry driven by filovirus GPs in general, we performed the same experiment but tested GPs from viruses of all known filovirus species. All GPs tested mediated efficient entry into control Vero E6 cells, with entry driven by LLOV-GP being slightly less efficient than that observed for the other GPs (Fig. 3B). In contrast, Calu-3 cell entry was severely attenuated for all filovirus GPs with MARV-GP-driven entry being most efficient and ranging ~10-fold over background (Fig. 3B). These results reveal that Calu-3 cells are poorly susceptible to entry driven by filoviral glycoproteins, with the exception of MARV-GP, but are readily susceptible to entry driven by arena-, paramyxo- and rhabdoviral glycoproteins.

*2.3. Directed expression of DC-SIGN or CatL can rescue Calu-3 cells entry driven by several but not all filovirus GPs*

A previous study found that Calu-3 cells express very low amounts of endogenous CatL (Park et al., 2016). Quantitative RT-PCR analysis confirmed the presence of low amounts of CatL transcripts as compared to 293T (~183-fold higher levels than Calu-3) and Huh-7 cells (~264-fold higher levels than Calu-3) (Fig. 4A). In contrast, CatB transcript numbers in Calu-3 cells were comparable or only slightly lower than those in 293T and Huh7 cells (~3- and 13-fold), respectively (Fig. 4A). Therefore, we next asked whether the low endogenous CatL levels in Calu-3 cells were responsible for inefficient GP-mediated cell entry. In parallel, we examined

whether directed expression of other cellular factors known to promote EBOV entry could rescue entry efficiency. To address these questions, we transduced Calu-3 cells with vectors encoding CatB, CatL, Axl, DC-SIGN and NPC1, selected transduced cells via antibiotics and then examined protein expression by immunoblot. These analyses revealed robust expression of CatB, CatL, DC-SIGN, NPC1 and Axl, indicating that transduction and selection were successful (Fig. 4B).

Next, we determined whether the directed expression of the entry factors stated above modulated entry driven by EBOV-GP and other filovirus GPs. All particles examined were readily able to transduce Vero E6 control cells with high efficiency, whereas entry into Calu-3 cells was poor (Figs 3B and 4C). In comparison, directed expression of CatL in Calu-3 cells strongly increased transduction driven by MARV-GP (~51.6-fold) (Fig. 4C). In addition, a moderate increase was observed for EBOV-GP-, SUDV-GP-, TAFV-GP- and RESTV-GP-driven entry (~2.4-, ~3.3-, ~2.5- and ~5.6-fold, respectively), while no appreciable enhancement was observed for entry driven by BDBV-GP and LLOV-GP (~1.3-fold, both). Moreover, directed expression of DC-SIGN in Calu-3 cells robustly promoted entry driven by EBOV-GP (~15.9-fold), SUDV-GP (~24.1-fold), and MARV-GP (~81.9-fold). For particles bearing RESTV-GP or TAFV-GP entry efficiency was moderately increased (~3.7- and ~2.4-fold, respectively), whereas entry driven by BDBV-GP and LLOV-GP was again almost at background levels (~1.5- and ~1.8-fold increase, respectively) (Fig. 4C). Finally, expression of the other entry factors did not modulate transduction mediated by any of the filovirus GPs and none of the factors had an impact on transduction mediated by VSV-G, as expected (Fig. 4C). These results indicate that filovirus GP-driven entry into Calu-3 cells is limited at the stage of attachment and priming and that the severity of these limitations varies among the GPs of different filovirus species.

*2.4 Augmentation of filovirus GP-driven entry into Calu-3 cells via directed expression of CatL or DC-SIGN can be blocked by a cathepsin L inhibitor and a DC-SIGN antibody, respectively*

We next sought to provide formal proof that the increased filovirus GP-driven entry into Calu-3 cells expressing DC-SIGN and CatL as compared to Calu-3 control cells was a direct consequence of the expression of these entry factors. For this, we investigated whether entry augmentation could be blocked by the CatL inhibitor MDL28170 and an antibody directed against DC-SIGN. We selected particles bearing MARV-GP for these experiments, since MARV-GP-driven entry into CatL and DC-SIGN expressing cells was markedly more efficient than entry into Calu-3 control cells. Directed CatL expression in Calu-3 cells increased the efficiency of MARV-GP-driven entry as expected and pre-treatment with MDL28170 abolished this effect (Fig. 4D). Similarly, directed expression of DC-SIGN augmented entry, confirming our results discussed above, and augmentation was partially abrogated by pretreatment of target cells with a monoclonal antibody directed against the ectodomain of DC-SIGN (Fig. 4E). In contrast, entry of control particles bearing VSV-G was not sensitive to DC-SIGN antibody or MDL28170. These results indicate that directed expression of CatL or DC-SIGN augments MARV-GP-driven Calu-3 cell entry through direct effects.

## 3. Discussion

Controversial results regarding the requirement of CatB/CatL activity for filovirus entry have been reported (Chandran et al., 2005; Marzi et al., 2012) and differences in the experimental systems used to study GP-driven entry might account, at least in part, for the discrepant observations. Our study shows that EBOV-GP-driven entry into different cell lines depends on CatB/CatL activity and that CatB/CatL dependence is irrespective of the shape of the GP presenting particles. Moreover, we identify Calu-3 cells as largely resistant to entry driven EBOV-GP and other filovirus GPs and found that entry can be restored by promoting attachment and/or CatL-dependent GP priming.

CatB/CatL were identified as GP processing enzymes essential for EBOV entry in a study that relied on both surrogate systems and infectious EBOV (Chandran et al., 2005). Moreover, subsequent analyses performed with surrogate models and/or authentic filoviruses confirmed CatB/CatL dependence of EBOV-GP-driven entry but also provided evidence that CatB/CatL dependence of entry driven by other filoviral GPs was variable (Chandran et al., 2005; Gnirss et al., 2012; Martinez et al., 2010; Misasi et al., 2012; Sanchez, 2007). For instance, directed expression of CatB in CatB/CatL double knockout cells increased EBOV-GP- and TAFV-GP-driven entry while directed expression of CatL had only a moderate effect. Moreover, expression of both proteases only moderately increased SUDV-GP-driven entry. In contrast, directed expression of either CatB or CatL markedly increased RESTV-GP-driven entry and expression of CatL but not CatB efficiently promoted MARV-GP-driven entry (Misasi et al., 2012). The general requirement for CatB and/or CatL activity for entry driven by most ebola- and marburgvirus glycoproteins demonstrated by these studies was contrasted by a report by Marzi and colleagues, who showed that Vero cell infection by EBOV and to some degree BDBV

depends on CatB but not CatL activity while entry driven by TAFV, SUDV and RESTV is independent of CatB/CatL activities (Marzi et al., 2012). Finally, this study demonstrated that knockout of CatB or CatL does not interfere with EBOV spread and pathogenesis in mice (Marzi et al., 2012). The reason for the discrepancy between these results is largely unclear and differences in the particles used to study GP-driven entry and in the target cells might be accountable.

The present study shows that the shape of the particles used to study EBOV-GP-driven entry does not have a prominent impact on CatB/CatL dependence. Thus, 293T cell entry of spherical retroviral particles, bullet-shaped rhabdoviral particles and filoviral particles was inhibited by E-64d and MDL28170. In this context, it should be stated that particles produced using the trVLP system should adequately mimic virions produced in EBOV infected cells, since particle production in the trVLP system exclusively depends on viral proteins and all proteins encoded by EBOV are expressed in the particle producing cells (Watt et al., 2014). Importantly, the filamentous structure of these particles has been recently confirmed (Wang et al., 2018), thereby supporting our overall conclusion that particle shape is not a major determinant of CatB/CatL dependence of EBOV-GP-driven entry.

Analysis of lung- (A549, Calu-3), liver- (Huh-7) and kidney-derived human cell lines showed that CatB/CatL dependence was independent of the target cell type. Similarly, CatB/CatL dependence was not impacted by the donor species, since EBOV-GP-driven entry into human, non-human primate and fruit bat cell lines was CatB/CatL-dependent, confirming a previous study (Hoffmann et al., 2016). However, these experiments revealed that Calu-3 cell entry driven by EBOV-GP and all other filovirus GPs studied was inefficient. This finding was remarkable for two reasons: First, EBOV exhibits a broad cell tropism and EBOV-GP is known to mediate entry

into a diverse panel of cell lines, with only lymphoid cells being refractory for incompletely understood reasons (Dube et al., 2010; Wool-Lewis and Bates, 1998; Yang et al., 1998). Second, Calu-3 cells have previously been shown to express very low levels of endogenous CatL, which were incompatible with CatL-dependent entry of MERS-CoV into these cells (Park et al., 2016). This finding raised the question whether the low endogenous CatL expression might also limit EBOV-GP-driven entry into these cells.

We confirmed low CatL expression in Calu-3 cells and equipped these cells with an expression cassette for CatL in order to unravel whether CatL expression limits EBOV-GP dependent entry. In parallel, we examined whether directed expression of the filovirus entry factors NPC1, Axl and DC-SIGN increased entry efficiency, which would suggest that attachment (DC-SIGN, Axl) or receptor engagement (NPC1) limits entry into Calu-3 cells. Finally, cells were equipped with CatB as control. We found that both directed expression of DC-SIGN and CatL can promote Calu-3 cell entry driven by filoviral GPs. However, the entry promoting effect of these factors was dependent on the viral GP studied. Expression of DC-SIGN, a calcium-dependent lectin that binds to glycans on GP and promotes viral attachment to cells (Alvarez et al., 2002; Simmons et al., 2003), markedly increased entry driven by EBOV-GP, SUDV-GP and MARV-GP. Moreover, DC-SIGN expression moderately augmented entry driven by RESTV-GP and TAFV-GP, suggesting that viral attachment limits Calu-3 cell entry driven by most if not all GPs tested. In comparison, directed expression of CatL markedly increased MARV-GP- and moderately augmented EBOV-GP-, SUDV-GP-, TAFV-GP-, RESTV-GP-driven entry. These findings are largely in keeping with a previous report suggesting a pronounced contribution of CatL to RESTV- and MARV-GP-driven entry (Misasi et al., 2012) and demonstrate that priming can also limit GP-driven entry into Calu-3 cells. Finally, it is

notable that for at present unclear reasons entry driven by LLOV- and BDBV-GP was not appreciably rescued upon CatL or DC-SIGN expression, although previous studies showed that entry driven by these GPs depends on CatL activity (LLOV) and is augmented by DC-SIGN expression (LLOV, BDBV) (Maruyama et al., 2014; Marzi et al., 2006).

Collectively, our results show that low levels of endogenous CatL can limit susceptibility of cells to filovirus GP-driven entry and may thus constitute a determinant of filovirus cell tropism.

## 4. Materials and methods

### *4.1. Cell culture*

Vero E6 (African green monkey kidney cells), 293T (human kidney cells), Huh-7 (human liver cells), A549 (human lung adenocarcinoma cells), Calu-3 (human bronchial adenocarcinoma cells) and EpoNi/22.1 (Buettikofer's epauletted fruit bat kidney cells) were incubated in a humidified atmosphere at 37 °C and 5% $CO_2$. Vero E6, 293T, EpoNi/22.1, and Huh7 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, PAN-Biotech), A549 cells were grown in DMEM: Nutrient Mixture F-12 + GlutaMax medium (Gibco) and Calu-3 cells were maintained in minimum essential medium (MEM, Gibco). All media were supplemented with 10% fetal bovine serum (Biochrom) and 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (PAN-Biotech). Calu-3 cells stably expressing cMYC-tagged CatB (GenBank: XM_017013097.2), CatL (GenBank: XM_005251716.4), DC-SIGN (GenBank: AB527563.1), NPC1 (GenBank: AB971140.1), and Axl (GenBank: BC032229.1) were generated using retroviral transduction and vector pQCXIP. For further cultivation, cells were maintained in MEM supplemented with 0.5 µg/ml of puromycin, 10% fetal bovine serum, and 1% of penicillin-streptomycin. All cells were detached by either resuspension in fresh culture medium (293T cells) or by the use of trypsin/EDTA (PAN-Biotech) for further subcultivation and seeding.

### *4.2. Viruses*

We employed a previously described recombinant VSV (recVSV) containing a dual reporter consisting of eGFP and firefly luciferase (fLuc) that is located between the open reading frames

for VSV-G and VSV-L(Gonzalez-Hernandez et al., 2018). Similarly, a chimeric VSV was used in which the genetic information for VSV-G was replaced by that of EBOV-GP (Gonzalez-Hernandez et al., 2018). All viruses were propagated on Vero E6 cells.

*4.3. Plasmids*

Expression plasmids encoding for the envelope glycoproteins of EBOV, BDBV, TAFV, RESTV, SUDV, MARV, LLOV and VSV have been described previously (Hoffmann et al., 2016). The plasmids required for the trVLP system (Watt et al., 2014) and VP30-luciferase containing particles have also been described before (Hoffmann et al., 2017). For the generation of CatB and CatL encoding retroviral vectors, the respective open reading frames were amplified by PCR from cDNA prepared from A549 cells and inserted into the vector pQCXIP using NotI and BamHI restriction enzymes. The same strategy was applied for generation of NPC1, DC-SIGN and Axl encoding vectors, cDNA prepared from Huh-7 cells (NPC1) and previously described expressions plasmids (DC-SIGN, Axl, (Pohlmann et al., 2001); (Watt et al., 2014)) were used as PCR template. The integrity of all PCR amplified sequences was confirmed by automated sequence analysis.

*4.4. Production of rhabdoviral pseudotypes*

The pseudotypes were generated and used for transduction as described previously (Hoffmann et al., 2016). Briefly, 293T cells were seeded in 6-well plates and transfected using calcium phosphate precipitation with plasmids encoding the viral glycoproteins under study or empty

plasmid (pCAGGS) as a negative control. At 18 h post transfection, the cells were inoculated at a multiplicity of infection (MOI) of 3 with a replication-deficient VSV, in which the ORF for VSV-G had been exchanged by two separate ORFs for eGFP and firefly luciferase (fLuc) (Berger Rentsch and Zimmer, 2011) (kindly provided by G. Zimmer). After 1 h incubation at 37°C, the cells were washed with PBS and incubated for 1 h with a 1:1,000 dilution of I1 (an anti-VSV-G mouse hybridoma supernatant from CRL-2700; American Type Culture Collection) to neutralize residual input virus. Finally, fresh culture medium was added to the cells. At 18 to 20 h post transduction, supernatants were collected, clarified from cell debris by centrifugation at 4,700 x g for 10 min, aliquoted, and stored at -80°C until use.

*4.5. Production of MLV pseudotypes*

Transduction vectors based on murine leukemia virus (MLV, MLVpp) were generated according to an established protocol (Wrensch et al., 2014) with slight modifications. First, 293T cells grown in 6-well plates were transfected with MLV-Gag/Pol (4 µg), MLV-Luc (6 µg) (Wrensch et al., 2014)and expression plasmid for the respective glycoprotein (4 µg). At 16 h post transfection, the cell culture supernatant was replaced by fresh culture medium. 48 h later, supernatants were collected and clarified from debris by centrifugation (4,000 × g, 10 min).

*4.6. Production of EBOV-like particles*

Generation of EBOV-like particles containing VP30-Luc or the tetracistronic transcription and replication competent EBOV minigenome (trVLP) was performed according to previously published protocols(Hoffmann et al., 2017; Hoffmann et al., 2018; Watt et al., 2014):
Briefly, for production of VP30-Luc particles, 293T cells were grown in T-75 cell culture flasks and transfected with expression plasmids for EBOV-NP (1 µg), EBOV-VP35 (1 µg), EBOV-L (8.3 µg), EBOV-VP40 (2 µg), EBOV-GP or VSV-G (2 µg), and EBOV-VP30-Luc (12.5 µg). At 16 h post transfection, the cell culture supernatant was replaced by fresh culture medium. 24 h later, supernatants were collected, clarified from debris by centrifugation (4,000 × g, 10 min) and subsequently concentrated employing Vivaspin concentrator columns (molecular mass cutoff = 30,000 kDa, Sartorius). Finally, volumes were equilibrated to 500 µl by addition of DMEM.
For production of EBOV trVLPs, 293T cells were grown in 6-well plates and transfected with expression plasmids for EBOV-NP (125 ng), EBOV-VP35 (125 ng), EBOV-VP30 (75 ng) EBOV-L (1 µg), p4cis-vRNA-RLuc (250 ng) and T7-polymerase (250 ng). At 16 h post transfection, the cell culture supernatant was replaced by fresh culture medium. 48 h later, supernatants were collected and clarified from debris by centrifugation (4,000 × g, 10 min).

*4.7. Transduction/Infection of cell lines and its inhibition with CatB and CatL inhibitors*

For transduction and infection, target cells were seeded in 96-well plates. At 24 h after seeding, medium was removed and cells were inoculated with equal volumes of transduction vectors (VSVpp, MLVpp, VP30-luc particles or trVLPs) or recVSV (MOI of 0.1). If required, cells were previously treated with inhibitor or antibody (see below). In case of transduction using EBOV-like particles based on the trVLP system, target cells were transfected with expression plasmids

for EBOV-NP, -VP30, -VP35, -L and DC-SIGN prior (24 h) to transduction. In order to quantify transduction/infection efficiencies, firefly (fLuc, VSVpp, recVSV, MLVpp, VP30-Luc particles) or *Renilla* luciferase (rLuc, trVLP particles) activities in cell lysates were measured 24 (VSVpp, recVSV, VP30-Luc) or 48 h (MLVpp, trVLP) post transduction. For this, cell culture medium was aspirated, 50 µL of cell culture lysis reagent (Promega) was added and cells incubated for 30 min at room temperature. Lysates were then transferred to a white, opaque-walled 96-well plate (Thermo Fisher Scientific), luciferin (fLuc; Beetle-Juice, PJK) or coelenterazine (rLuc; Sigma-Aldrich) was added and luciferase activity was measured in a microplate reader (Hidex Sense Microplate Reader) using the PlateReader Software (version 0.5.41.0, Hidex).

*4.8. Treatment of target cells with cathepsin inhibitors or anti-DC-SIGN antibody*

To investigate entry inhibition by MDL28170 (Tocris), E-64d (Tocris) or DC-SIGN antibody (120526, NIH-AIDS Research and Reference Reagent Program), the inhibitors or medium was added to target cells and the cells were incubated for 3 h (MDL28170/E-64d) or 1 h ( DC-SIGN antibody) before transduction/infection was performed

*4.9. Quantification of CatB/CatL mRNA expression*

Total RNA from 293T, Huh-7, and Calu-3 cell lines was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer instructions. Afterwards, cDNA was produced using the SuperScript III First Strand Synthesis System (Invitrogen) with random hexamers. To determine the copy numbers CatB/CatL mRNA expression, quantitative PCR was performed using the

QuantiTect SYBR Green PCR Kit (Qiagen), 1 µl of cDNA as template and the following primers: Cathepsin B 5'-TACAGCCCGACCTACAAACA-3', 5'-CCATGATGTCCTTCTCGCTA-3'; Cathepsin L 5'-GCAGGTCATGAGTCCTTCCT-3', 5'-CTTTACGTAGCCACCCATGC-3'; β-actin 5'-GGCTCCCAGCACAATGAAGA-3', 5'-GGAGCCGCCGATCCA-3'. As standard, serial dilutions of expression plasmids for CatB, CatL, and β-actin were subjected to PCR analysis. Ct values were determined using the Rotor-Gene Q device along with the Rotor-Gene Q software (Qiagen) and used to calculate their respective copy numbers. Further, copy numbers for CatB and CatL were normalized against that of ß-actin.

*4.10. Immunoblotting*

To detect expression of entry and attachment factors in stably transduced Calu-3 cell lines, the cells were lysed using 200 µL 2x sodium dodecyl sulfate (SDS)-containing lysis buffer (50 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue, 1 mM EDTA), and boiled for 15 min at 95 °C. Subsequently, samples were separated by SDS-PAGE and blotted onto nitrocellulose membranes (GE Healthcare Life Sciences; 0.2 µm). Afterwards, membranes were blocked for 30 min in PBS containing 5% milk powder and 0.1% Tween 20. Finally, expression of cMYC tagged CatL, CatB, DC-SIGN, NPC1 and Axl was determined using undiluted supernatants of a hybridoma cell line that secretes anti-MYC antibody (9E10). Bound antibodies were detected using a horseradish peroxidase linked anti-mouse antibody (Dianova) at a 1:5,000 dilution. Signals were detected using the ChemoCam imaging system along with the ChemoStarProfessional software (Intas).

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## Figure legends

**Fig. 1.** EBOV-GP-driven entry into 293T cells requires CatB/CatL activity irrespective of the transduction system. 293T were seeded in 96-well plates and pre-incubated with DMSO or the indicated concentrations of E-64d or MDL28170 and then transduced with equal volumes of (A) single-cycle VSV particles (VSVpp) pseudotyped with the indicated GPs or no glycoprotein as negative control; (B) replication-competent VSV or VSV-chimera encoding EBOV-GP instead of VSV-G at a MOI of 0.1; (C) MLV particles pseudotyped with the indicated glycoproteins or no glycoprotein; (D) EBOV-like particles containing VP30-luciferase and bearing EBOV-GP, VSV-G or no glycoprotein; (E) EBOV-like particles generated in the trVLP system. Luciferase activities in cell lysates were determined at 24 h post transduction as indicator of transduction efficiency. In panels A-D, transduction mediated by EBOV-GP and VSV-G is shown relative to transduction mediated by control particles bearing no glycoprotein, which was set as 1. In panel E, transduction by particles produced in the absence of the viral polymerase, L, were set as 1. The average of three independent experiments is shown in each panel. Error bars indicate standard error of the mean (SEM).

**Fig. 2.** EBOV-GP-driven entry is CatB/CatL-dependent irrespective of the target cell line. Equal volumes of VSVpp bearing the indicated glycoproteins or bearing no glycoprotein were used for transduction of 293T, Huh-7, A549, Calu-3, Vero E6, and EpoNi/22.1 cell lines pre-incubated for 3 h with E-64d in the indicated concentrations or DMSO as control. At 24 h post transduction, luciferase activities in cell lysates were quantified as indicator of transduction efficiency. Transduction mediated by EBOV-GP and VSV-G is shown relative to transduction mediated by

control particles bearing no glycoprotein, which was set as 1. The average of four independent experiments conducted with separate pseudotype preparations is shown. Error bars indicate SEM.

**Fig. 3**. Calu-3 cells are largely resistant to entry driven by filoviral but not arena-, paramyxo-and rhabdoviral glycoproteins. Vero E6 and Calu-3 cells were transduced with equal volumes of VSVpp pseudotyped with the glycoproteins from diverse viruses (A) or pseudotyped with filovirus glycoproteins (B). Particles bearing no glycoprotein served as negative control. At 24 h post transduction, luciferase activities in cell lysates were quantified as indicator of transduction efficiency. Transduction mediated by the different glycoproteins is shown relative to transduction mediated by control particles bearing no glycoprotein, which was set as 1. The average of three independent experiments is shown. Error bars indicate SEM.

**Fig. 4.** Directed expression of Cat L and DC-SIGN can rescue filovirus GP-driven entry into Calu-3 cells. (A) CatB, CatL and β-actin mRNA expression in 293T, Huh-7 and Calu-3 cells was quantified by quantitative RT-PCR. CatB and CatL copy numbers were plotted in relation to β-actin copy numbers for each cell line. Shown is a single representative experiment with triplicate samples, error bars indicate standard deviation (SD). The results were confirmed in a second independent experiment. (B) Calu-3 cells engineered to stably express CatB, CatL, DC-SIGN, NPC1, and Axl with a cMYC antigenic tag were subjected to immunoblot analysis using an anti-cMYC antibody. Detection of β-actin expression served as loading control. Similar results were obtained in a separate experiment. (C) Equal volumes of VSVpp bearing the indicated filovirus glycoproteins were used to transduce Calu-3 cells stably expressing the indicated entry and attachment factors. At 24 h post transduction, luciferase activities in cell lysates were quantified

as indicator of transduction efficiency. Transduction driven by the different GPs in Calu-3 cells stably expressing the indicated cellular factors is shown relative to transduction of Calu-3 control cells which was set as 1. The average of five independent experiments is shown. Error bars indicate SEM. (D) Calu-3 control cells and Calu-3 CatL-cMYC cells were incubated for 3 h with DMSO or 50 µM of MDL28170 and transduced with same volume of VSV-pseudotyped with MARV-GP, VSV-G or bearing no glycoprotein. (E) Calu-3 control cells and Calu-3 DC-SIGN-cMYC cells were pre-incubated for 1 h with 20 µg/ml of anti-DC-SIGN monoclonal antibody or an isotype matched control antibody and then transduced with equal volumes of VSV particles bearing the indicated glycoproteins or bearing no GP. For panels (D) and (E) luciferase activity was quantified at 24 h post transduction. Entry into Calu-3 CatL and Calu-3 DC-SIGN cells is shown relative to entry into Calu-3 control cells, which was set as 1. The average of at least five independent experiments is shown. Error bars indicate SEM.

Figure 1
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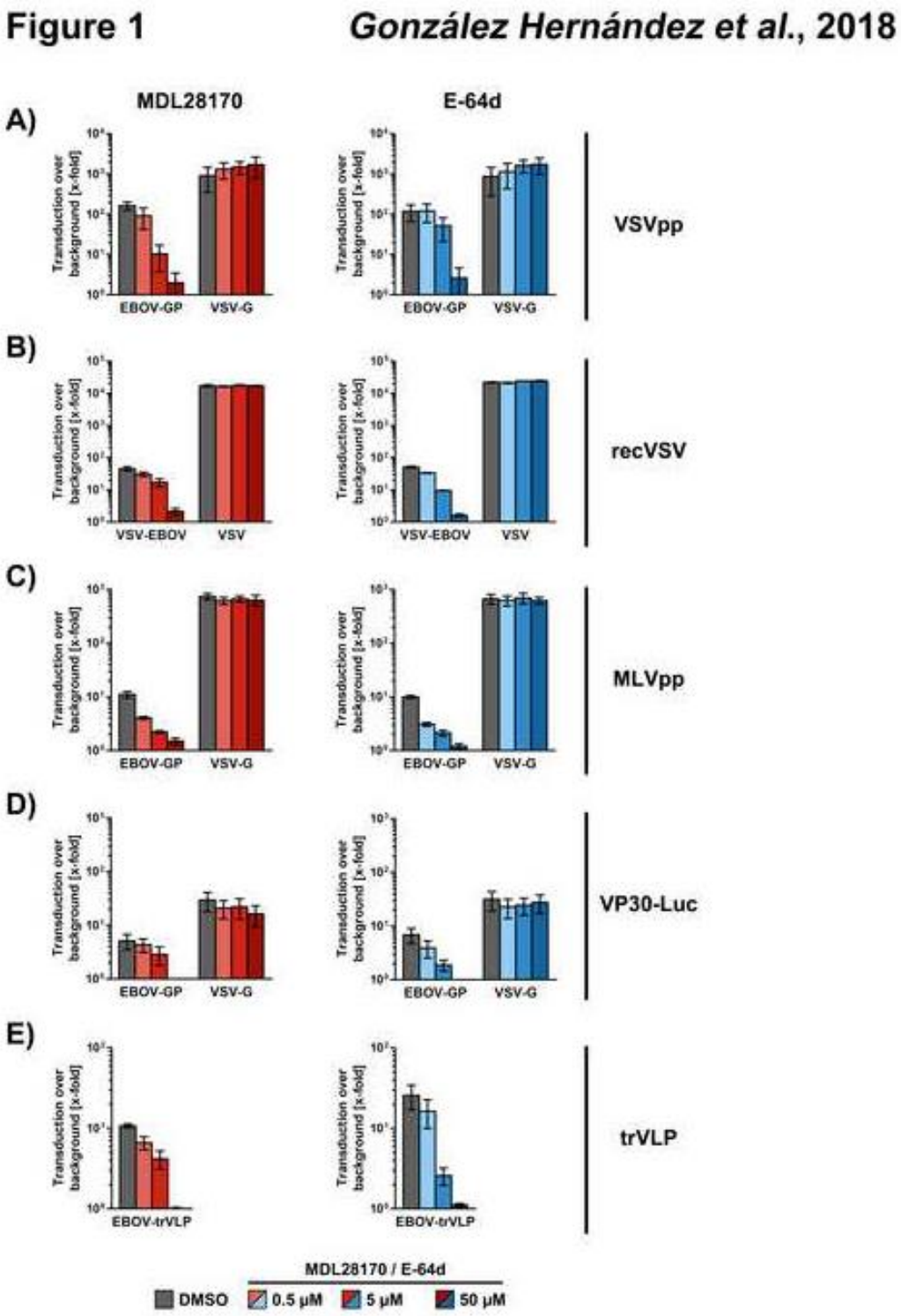

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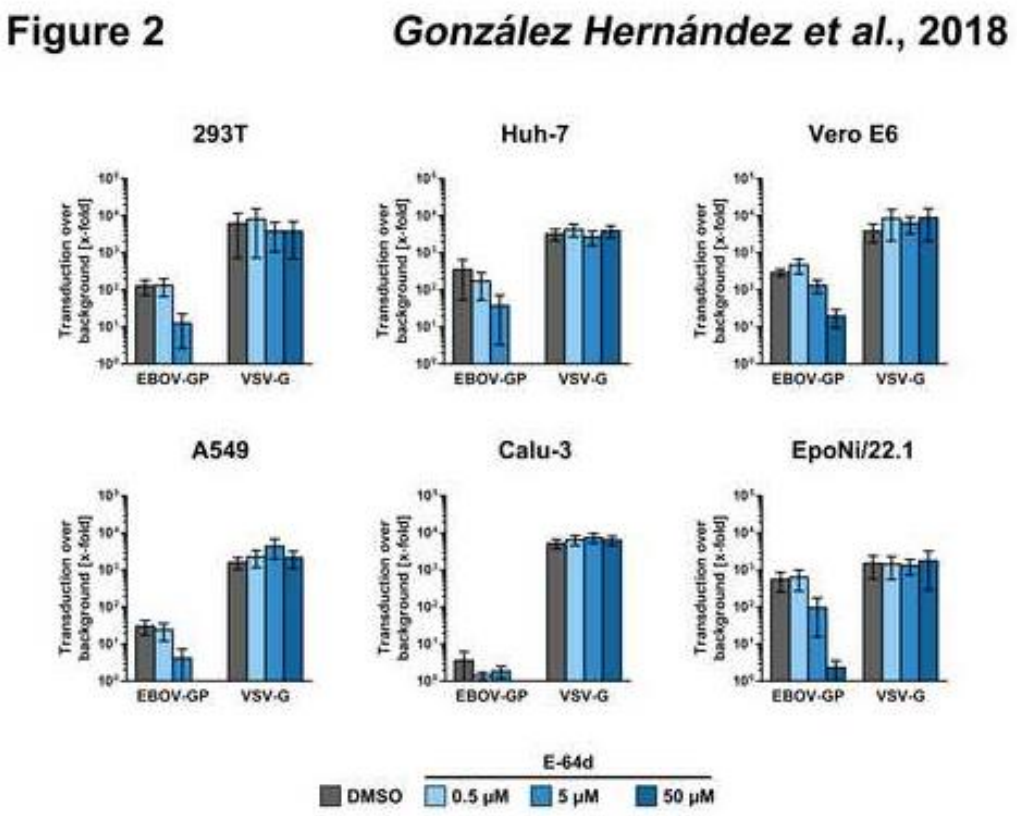

Figure 3
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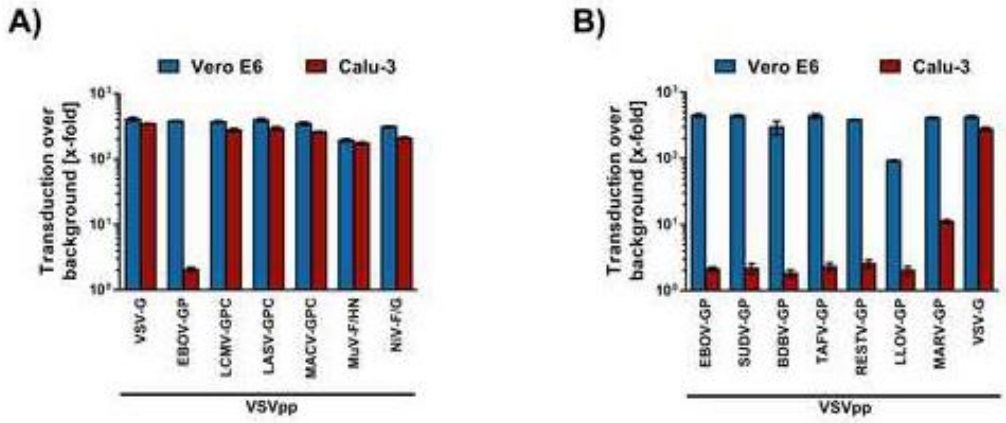

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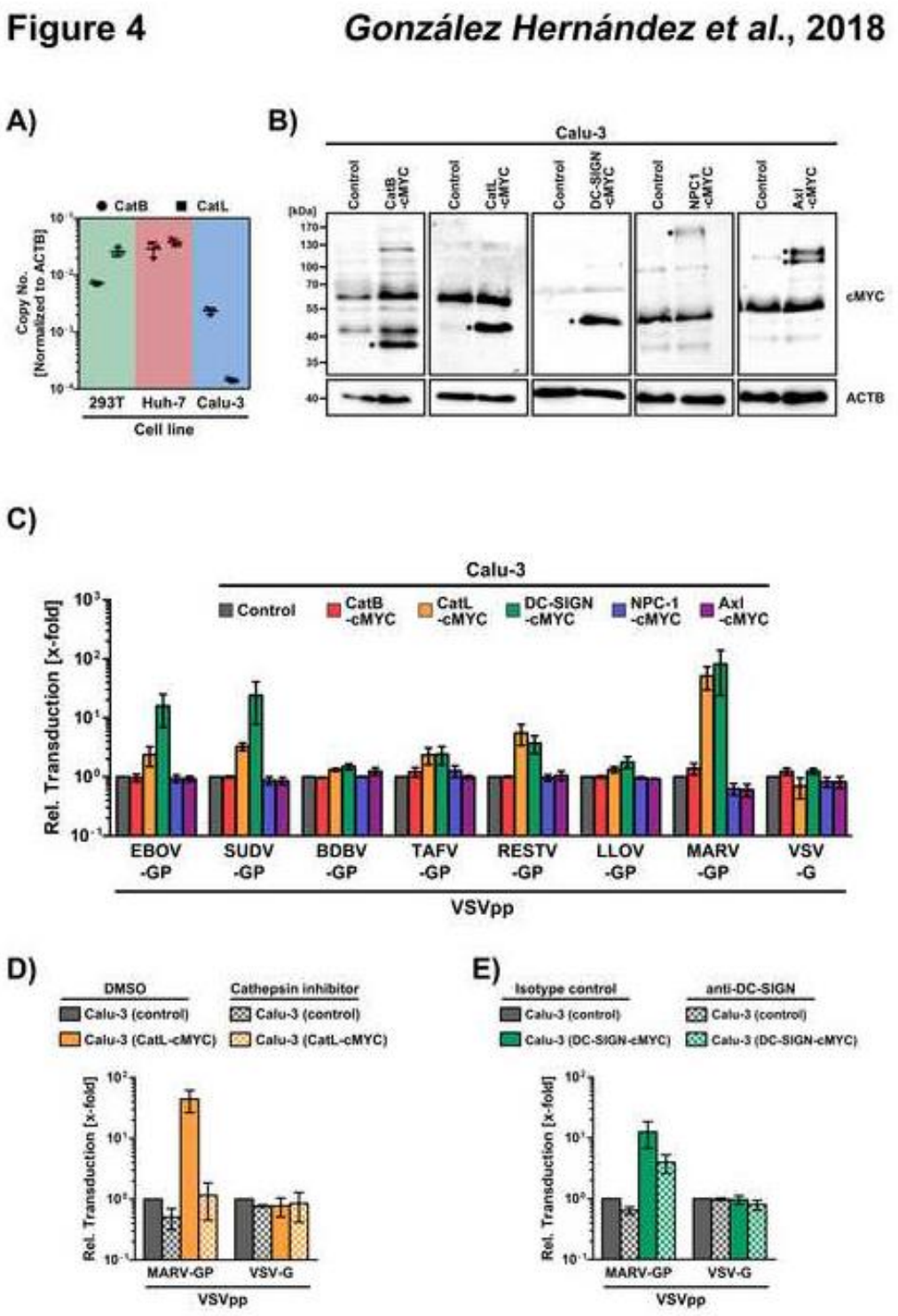

## 4.2. Second manuscript

# A GXXXA Motif in the Transmembrane Domain of the Ebola Virus Glycoprotein Is Required for Tetherin Antagonism.

González-Hernández, M., Hoffmann, M., Brinkmann, C., Nehls, J., Winkler, M., Schindler, M., Pöhlmann, S.

Journal of Virology, 2018. 92(13):e00403-18. doi: 10.1128/JVI.00403-18.

Individual contributions:

I conducted the experiments that resulted in the data presented in figure 1 (panels B-D), figure 2 (panels A-D), figure 3 (panels A-C) and figure 4 (panels C, D and E). Furthermore, I analyzed the data that I generated, quantified the immunoblots blots and wrote parts of the manuscript.

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VIRUS-CELL INTERACTIONS

# A GXXXA Motif in the Transmembrane Domain of the Ebola Virus Glycoprotein Is Required for Tetherin Antagonism

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**ABSTRACT** The interferon-induced antiviral host cell protein tetherin can inhibit the release of several enveloped viruses from infected cells. The Ebola virus (EBOV) glycoprotein (GP) antagonizes tetherin, but the domains and amino acids in GP that are required for tetherin antagonism have not been fully defined. A GXXXA motif within the transmembrane domain (TMD) of EBOV-GP was previously shown to be important for GP-mediated cellular detachment. Here, we investigated whether this motif also contributes to tetherin antagonism. Mutation of the GXXXA motif did not impact GP expression or particle incorporation and only modestly reduced EBOV-GP-driven entry. In contrast, the GXXXA motif was required for tetherin antagonism in transfected cells. Moreover, alteration of the GXXXA motif increased tetherin sensitivity of a replication-competent vesicular stomatitis virus (VSV) chimera encoding EBOV-GP. Although these results await confirmation with authentic EBOV, they indicate that a GXXXA motif in the TMD of EBOV-GP is important for tetherin antagonism. Moreover, they provide the first evidence that GP can antagonize tetherin in the context of an infectious EBOV surrogate.

**IMPORTANCE** The glycoprotein (GP) of Ebola virus (EBOV) inhibits the antiviral host cell protein tetherin and may promote viral spread in tetherin-positive cells. However, tetherin antagonism by GP has so far been demonstrated only with virus-like particles, and it is unknown whether GP can block tetherin in infected cells. Moreover, a mutation in GP that selectively abrogates tetherin antagonism is unknown. Here, we show that a GXXXA motif in the transmembrane domain of EBOV-GP, which was previously reported to be required for GP-mediated cell rounding, is also important for tetherin counteraction. Moreover, analysis of this mutation in the context of vesicular stomatitis virus chimeras encoding EBOV-GP revealed that GP-mediated tetherin counteraction is operative in infected cells. To our knowledge, these findings demonstrate for the first time that GP can antagonize tetherin in infected cells and provide a tool to study the impact of GP-dependent tetherin counteraction on EBOV spread.

**KEYWORDS** Ebola virus, glycoprotein, tetherin

The interferon (IFN) system is a component of innate immunity and constitutes the first line of defense against viral infection. The IFN response is characterized by three processes: the sensing of invading pathogens, the induction of signaling cascades, and the expression of IFN-stimulated genes (ISGs) (1–4). The entire spectrum of ISGs was first comprehensively characterized by Schoggins and colleagues (3), who showed that many of the 300 to 400 ISG-encoded proteins exert antiviral activity.

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Understanding how ISG-encoded proteins inhibit viruses will provide not only important insights into how virus infection can be controlled by innate immunity but also a possible basis for novel antiviral strategies.

The host cell protein tetherin is upregulated by IFN (i.e., is encoded by an ISG) and is also constitutively expressed in certain cells and tissues (5, 6). Tetherin inhibits release of progeny particles from infected cells by forming a physical tether between the virus and host cell (6, 7). This activity critically depends on tetherin's particular domain organization: the protein harbors an N-terminal transmembrane domain (TMD) and a C-terminal glycosylphosphatidylinositol (GPI) anchor, which permit simultaneous insertion of tetherin into a viral and a cellular membrane. In response to the antiviral pressure imposed by tetherin, several viruses have evolved tetherin antagonists (8), including the HIV-1 protein Vpu (6), and most of these proteins block tetherin's antiviral activity by reducing tetherin levels at the site of viral budding, the plasma membrane (8).

The Ebola virus (EBOV), a member of the family *Filoviridae*, causes severe and frequently fatal disease in humans and nonhuman primates. The virus blocks signaling pathways of the IFN system via its VP24 and VP35 proteins (9). In addition, EBOV and other filoviruses counteract tetherin via their only virus-encoded surface protein, the viral glycoprotein (GP) (10–12). In this context, it should be noted that tetherin antagonism by GP has so far exclusively been demonstrated in cells releasing EBOV-like particles, and it is unknown if GP antagonizes tetherin in the context of EBOV infection and whether such antagonism is required for viral spread. In contrast to Vpu, EBOV-GP interferes with tetherin's antiviral activity without altering tetherin expression levels or cellular localization (12–14), and the mechanism underlying tetherin counteraction by EBOV-GP is largely unclear. Moreover, it is incompletely understood which domains in GP contribute to tetherin antagonism. Vande Burgt and colleagues reported a role for the glycan cap (15), and our previous work demonstrated that a single amino acid change in the receptor binding domain (RBD) can abrogate tetherin counteraction (10). In addition, the transmembrane domain (TMD) of EBOV-GP was shown to contribute to tetherin antagonism (15, 16), but the responsible amino acid motifs remain to be elucidated.

Here, we employed EBOV surrogate systems to investigate the contribution of a GXXXA motif in the TMD of EBOV-GP to tetherin antagonism. Although confirmation with infectious EBOV is pending and conclusions are thus tentative, our results indicate that the GXXXA motif, which was previously reported to be important for GP-mediated cell detachment (17), is also required for tetherin antagonism. Moreover, these results provide the first evidence that GP can antagonize tetherin in the context of infected cells.

## RESULTS

**Mutation of the GXXXA motif in the EBOV-GP TMD is compatible with robust GP expression and only modestly reduces GP-driven host cell entry.** To address the role of the GXXXA motif in the EBOV-GP TMD in tetherin antagonism, we first changed the GXXXA motif to LXXXL using PCR-based mutagenesis (Fig. 1A). Next, we investigated GP expression and incorporation into viral particles, employing transiently transfected 293T cells. We found that EBOV-GP wild type (wt) and the LXXXL mutant were expressed and proteolytically processed at comparable levels (Fig. 1B). Moreover, both wt GP and the LXXXL mutant were comparably incorporated into vesicular stomatitis virus (VSV) pseudoparticles (Fig. 1C), which are frequently used to study EBOV-GP-driven entry, and the LXXXL mutant mediated entry into cell lines derived from accidental hosts (humans and nonhuman primates) and a natural reservoir (fruit bat) with about half the efficiency of wt GP (Fig. 1D). Thus, the GXXXA motif is dispensable for GP expression and particle incorporation, and its mutation has only a modest effect on GP-driven host cell entry.

**The integrity of GXXXA motif is essential for tetherin antagonism.** Having demonstrated that the GXXXA motif is dispensable for GP expression and, to some

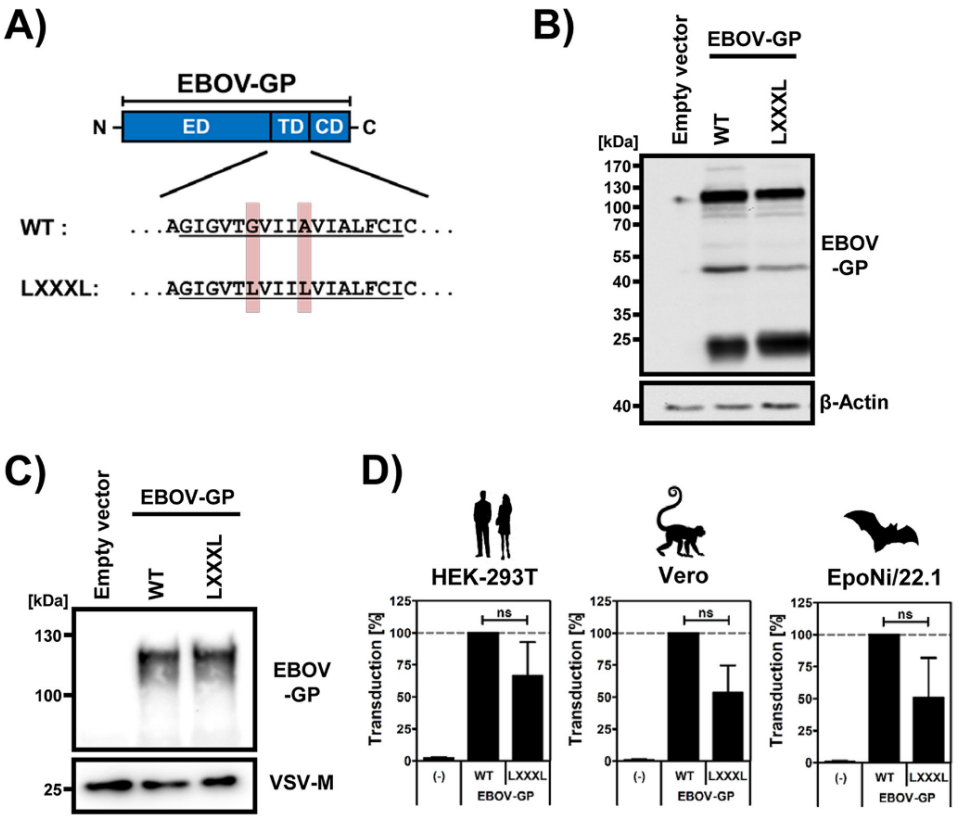


**FIG 1** Mutation of a GXXXA motif in the EBOV-GP TMD is dispensable for GP expression and particle incorporation but slightly reduces GP-driven host cell entry. (A) Sequence of the GXXXA motif in the EBOV-GP wt and LXXXL mutant (ED, ectodomain; TD, transmembrane domain [underlined]; CD, cytoplasmic domain). (B) Plasmids encoding V5-tagged versions of the indicated glycoproteins or empty plasmid (pCAGGS) as a negative control were transiently transfected into 293T cells. Expression of the GPs in cell lysates was detected using anti-V5 tag antibody. Detection of β-actin expression served as a loading control. Similar results were obtained in three independent experiments. (C) VSV pseudotypes harboring the indicated glycoproteins were concentrated from cell culture supernatants by centrifugation through a 20% sucrose cushion and then analyzed for the presence of EBOV-GP and VSV-M by Western blotting. The results were confirmed in three independent experiments. (D) The indicated human, nonhuman primate, and fruit bat cell lines were transduced with equal volumes of vesicular stomatitis virus (VSV) particles pseudotyped with the indicated GPs or with particles bearing no glycoprotein as negative controls. Luciferase activity in cell lysates, which was used as an indicator of transduction efficiency, was measured at 24 h postransduction. Shown are normalized data from four experiments performed with independent pseudotype preparations, in which transduction mediated by EBOV-GP wt was set as 100%. Error bars indicate standard errors of the means, and statistical significance was analyzed using paired two-tailed *t* tests (ns, not significant).

extent, for GP-driven host cell entry, we next investigated if the GXXXA motif is required for tetherin antagonism. For this endeavor, we first employed a previously documented virus-like particle (VLP) assay, in which release of VLPs is driven by the HIV-1 p55 Gag protein and is inhibited by tetherin (12). In the Gag-based assay, VLPs were readily released from tetherin-negative control cells, and release was markedly reduced upon expression of tetherin (Fig. 2A and B). The tetherin-mediated restriction of VLP release was rescued upon coexpression of HIV-1 Vpu and EBOV-GP wt (Fig. 2A and B), as expected. In contrast, the LXXXL mutant was largely unable to promote VLP release from tetherin-positive cells (Fig. 2A and B), and this defect could not be rescued by expressing large amounts of the mutant (data not shown). Thus, the GXXXA motif is essential for efficient tetherin counteraction, at least under the conditions studied.

We next studied whether the LXXXL motif is also required for rescue of the release of EBOV-like particles from blockade by tetherin. For this, the above-described VLP

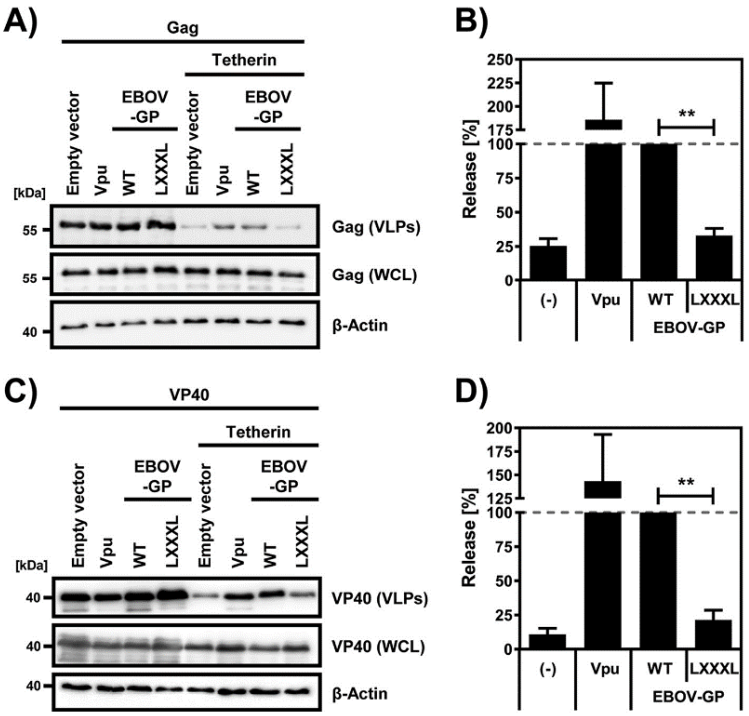


**FIG 2** The GXXXA motif is required for tetherin antagonism. (A) 293T cells were cotransfected with plasmids encoding HIV-Gag, the indicated glycoproteins or Vpu, and tetherin or empty plasmid. Cells and supernatants were harvested at 48 h posttransfection. Virus-like particles (VLPs) were pelleted by centrifugation through a 20% sucrose cushion. Whole-cell lysates (WCL) and VLPs were analyzed for the presence of Gag by Western blotting. Detection of β-actin expression served as a loading control. The results of a representative experiment are shown. (B) Three independent experiments conducted as described for panel A were quantified using the ImageJ program. VLP release from cells coexpressing EBOV-GP wt and tetherin was set as 100%. Error bars indicate standard errors of the means, and statistical significance was analyzed using a paired two-tailed *t* test (**, $P \leq 0.01$). (C) VLP release was examined as described for panel A, but EBOV-VP40 instead of HIV-Gag was used for particle production. (D) Four independent experiments conducted as described for panel C were quantified using the ImageJ program. VLP release from cells coexpressing EBOV-GP wt and tetherin was set as 100%. Error bars indicate standard errors of the means, and a paired two-tailed *t* test was used to determine statistical significance (**, $P \leq 0.01$).

assay was repeated using EBOV VP40 instead of HIV Gag. Expression of VP40 is sufficient for release of filamentous particles from cells (18, 19) and thus mimics release of EBOV from infected cells. In this assay, expression of EBOV-GP wt modestly increased the release of VLPs from tetherin-negative control cells (2-fold increase on average; $n = 4$), in keeping with previous studies (20, 21), and rescued particle release from blockade by tetherin (Fig. 2C and D). Notably, the LXXXL mutant also promoted VLP release from tetherin-negative cells (1.5-fold increase on average; $n = 4$) but failed to rescue particle release from blockade by tetherin (Fig. 2C and D). These results show that the GXXXA motif is also required for tetherin antagonism in the context of EBOV-like particles and that GP-mediated augmentation of particle release and GP-driven tetherin antagonism are genetically separable processes.

**The requirement of the GXXXA motif for tetherin antagonism is independent of membrane cholesterol levels.** The directed expression of GP in cells can induce cellular detachment (22, 23). A recent study reported that the mutation of the GXXXA motif to LXXXL or depletion of cholesterol reduces GP-driven cell detachment (17). We confirmed that mutation of GXXXA to LXXXL reduces cell detachment (Fig. 3A) and asked whether addition of cholesterol confers efficient tetherin antagonism to the

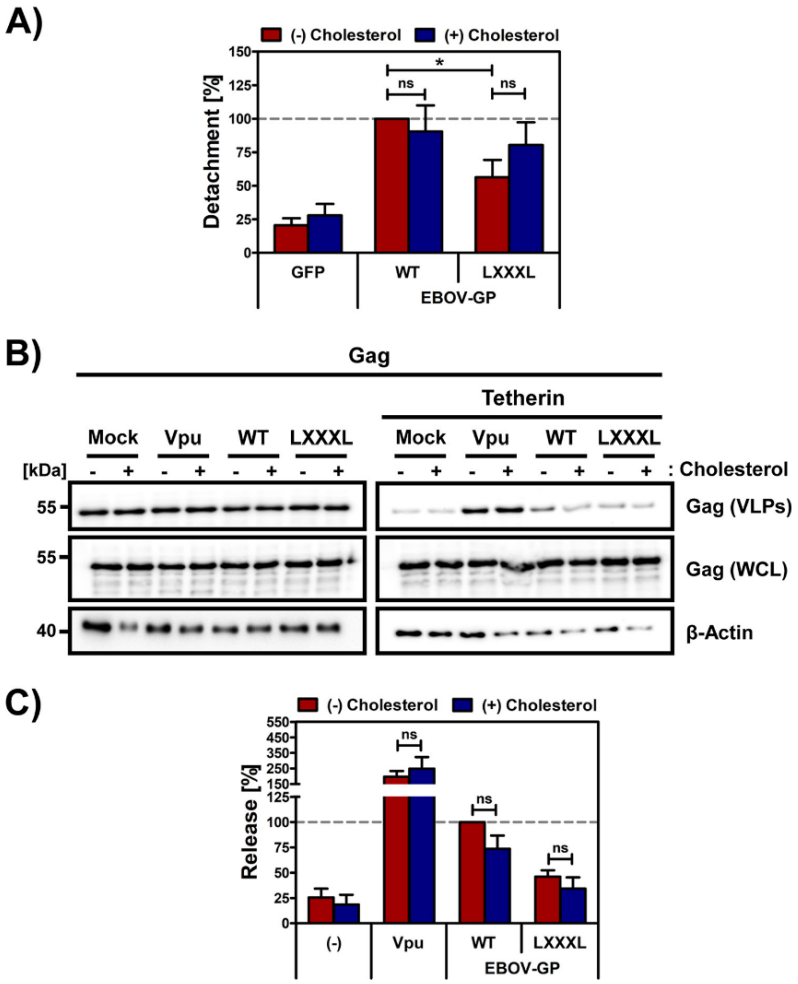


**FIG 3** Defective tetherin antagonism by mutant LXXXL cannot by restored with cholesterol. (A) HeLa cells were transiently transfected with plasmids encoding the indicated glycoproteins or GFP as a negative control. At 6 h posttransfection, cells were treated with water-soluble cholesterol at a concentration of 100 $\mu$M or water alone for 30 min. Subsequently, cells were washed with PBS, and fresh medium was added. Supernatants were collected at 48 h after transfection, and the number of detached cells was quantified using flow cytometry. Shown are normalized data from five independent experiments carried out with triplicates samples, for which detachment driven by EBOV-GP wt was set as 100%. Error bars indicate standard errors of the means, and statistical significance was analyzed using one-way ANOVA with Bonferroni posttest analysis. (B) The HIV-Gag release assay was performed as described in the legend of Fig. 2; but at 6 h posttransfection water-soluble cholesterol was added at a final concentration of 100 $\mu$M as indicated, and cells were incubated for 30 min. Afterwards, the cells were washed and incubated overnight. The results of a single representative experiment are shown. (C) Three independent experiments conducted as described for panel B were quantified using the ImageJ program. VLP release from cells coexpressing EBOV-GP wt and tetherin, but not treated with cholesterol, was set as 100%. For panels A and C, error bars indicate standard errors of the means, and statistical significance was analyzed using paired *t* test (ns, not significant; *, $P \leq 0.05$).

LXXXL mutant. However, cholesterol did not endow the LXXXL mutant with efficient tetherin antagonism and even reduced tetherin counteraction by wt GP (Fig. 3B and C), although not to a statistically significant degree. Thus, the GXXXA motif in the TMD of EBOV-GP is essential for efficient tetherin antagonism, and the requirement for this motif for tetherin counteraction is independent of membrane cholesterol.

**The GXXXA motif is required for robust viral spread in tetherin-positive cells.** Most previous analyses of tetherin counteraction by EBOV-GP were conducted with GP-transfected cells while infected cells were rarely studied. EBOV is a biosafety level 4 (BSL4) organism and could not be analyzed in the present study. However, VSV chimeras encoding EBOV-GP (VSV-EBOV-GP) afforded an opportunity to study EBOV-GP in the context of infected cells without the necessity of BSL4 conditions (24, 25). In order to analyze whether the GXXXA motif contributes to VSV-EBOV-GP spread in tetherin-positive cells, we generated VSV-EBOV-GP wt and VSV-EBOV-GP LXXXL via reverse genetics (Fig. 4A) and ensured by sequence analysis that the viruses had not acquired changes in GP during amplification in cell culture. Analysis of viral particles confirmed that the GP wt and LXXXL mutant were comparably incorporated into particles (Fig. 4B), as expected (Fig. 1C). Moreover, investigation of GP expression in infected cells showed that both wt and mutant GPs were expressed at similar levels (Fig. 4C). In order to analyze tetherin sensitivity of viruses bearing GP wt and mutant LXXXL, we infected Vero cells, which were stably transfected with empty vector (pQCXIP, control) or a tetherin-encoding vector and expressed high levels of tetherin (Fig. 4D). VSV wt was included as a control since this virus is highly tetherin sensitive (26, 27), and so far no VSV protein has been identified that antagonizes tetherin in infected cells. Infection of the Vero control cells (pQCXIP) revealed that VSV replicated more efficiently than VSV bearing EBOV-GP (Fig. 4E), as expected (24). Replication of VSV-EBOV-GP wt and mutant LXXXL was also readily detectable; but peak viral titers observed upon infection with the LXXXL mutant were about 10-fold reduced, and replication kinetics were delayed compared to the kinetics of infection with VSV-EBOV-GP wt virus (Fig. 4E). The expression of tetherin in target cells had a stronger inhibitory effect on peak viral titers (Fig. 4F) of VSV than of VSV-EBOV-GP, suggesting that GP-mediated tetherin antagonism might be operative in infected cells. More importantly, tetherin expression had a clearly more profound inhibitory effect on spread of the VSV-EBOV-GP LXXXL mutant than the VSV-EBOV-GP wt virus (Fig. 4E and F), indicating that the GXXXA motif was required for tetherin counteraction in VSV-EBOV-GP-infected cells.

## DISCUSSION

It remains largely enigmatic how EBOV-GP counteracts tetherin. Recent studies sought to obtain insights via mutagenic analyses. They showed that the glycan cap, the RBD, and the TMD contribute to tetherin antagonism (10, 15, 16). The observation regarding the TMD, jointly with the finding that a GXXXA motif within the EBOV-GP transmembrane domain is required for GP-mediated cellular detachment (17), prompted us to investigate whether the GXXXA motif contributes to tetherin antagonism. We show that the motif is dispensable for GP expression and particle incorporation but is required for efficient GP-driven tetherin antagonism. Our results underline an important contribution of the TMD to tetherin counteraction and provide, for the first time, evidence that GP can inhibit tetherin when expressed in the context of cells infected with an EBOV surrogate.

Our previous work provided the first hints toward a role of the EBOV-GP TMD in tetherin counteraction. We showed that the GP2 subunit of GP interacts with tetherin (12) and that replacing the EBOV-GP TMD with that of the Lassa virus glycoprotein (LASV-GPC, which does not antagonize tetherin) abrogates tetherin antagonism (16). However, only limited conclusions could be drawn from these results since EBOV-GP harboring the LASV-GPC TMD was unable to mediate entry (16). Moreover, LASV-GPC equipped with the EBOV-GP TMD failed to counteract tetherin (16). Strong evidence for a role of the EBOV-GP TMD in tetherin counteraction was then provided by Vande Burgt and colleagues. They showed that soluble GP (sGP), which is the primary translation product of the GP open reading frame (ORF), fails to counteract tetherin and that addition of the EBOV-GP TMD to sGP is sufficient to bestow tetherin antagonism upon sGP (15). However, the TMD amino acids required for tetherin antagonism remained unknown.

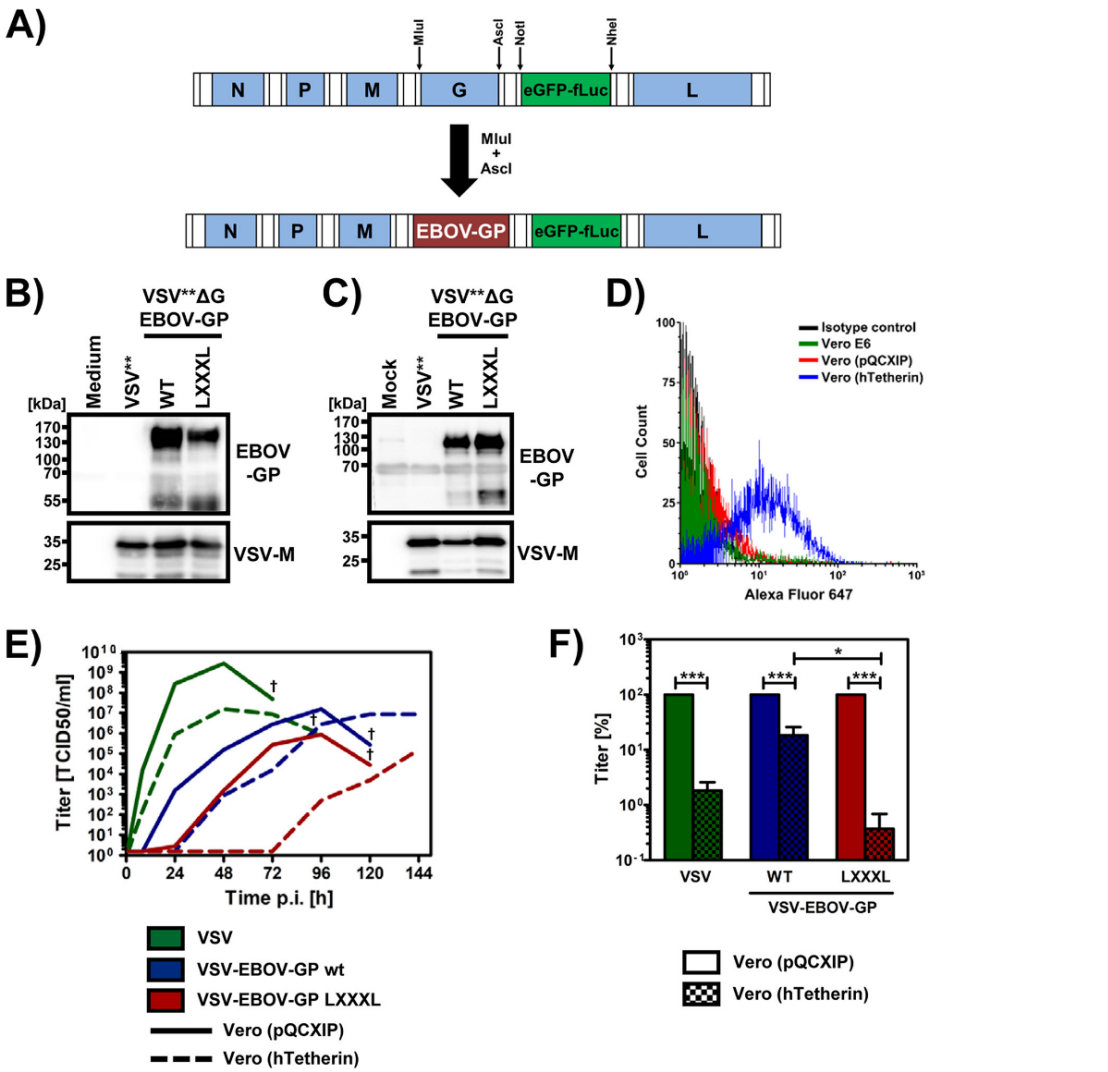


**FIG 4** Disruption of the GXXXA motif reduces EBOV-GP-dependent viral spread in tetherin-positive cells. (A) Schematic illustration of the VSV** genome and the derived chimeric genome harboring the open reading frame (ORF) for EBOV-GP (red) instead of VSV-G. Transcription units coding for structural proteins of VSV are highlighted in light blue, while nontranslated regions of the genome are displayed in white. All VSV genomes code for a dual reporter consisting of eGFP linked to firefly luciferase from an additional transcription unit downstream of the respective glycoprotein ORF (green). (B) Replication-competent VSV chimeras encoding the indicated glycoproteins instead of VSV-G were pelleted through a 20% sucrose cushion and evaluated for incorporation of EBOV-GP and VSV-M by Western blotting. Similar results were obtained in two independent experiments. (C) Vero E6 cells were infected with VSV wt or the indicated VSV chimeras at an MOI of 1. At 20 h postinfection, cell lysates were prepared and analyzed for the expression of EBOV-GP (wt and mutant LXXXL) and VSV-M by Western blotting. Mock-infected and VSV wt-infected cells served as controls. Similar results were obtained in two independent experiments. (D) Tetherin surface expression on Vero cell lines used for the experiment shown in panel E was analyzed by flow cytometry. Vero (pQCXIP) were incubated with an isotype control and secondary antibody (black) or Vero E6 (green), Vero (pQCXIP) (red), Vero (hTetherin) (blue) cells were incubated with anti-tetherin antibody and secondary antibody. Antibody staining was analyzed by flow cytometry. A single representative experiment is shown; similar results were obtained in a second independent experiment. (E) Control Vero cells transduced with plasmid pQCXIP and Vero cells transduced to express human tetherin (hTetherin) were infected with VSV wt (green) or a VSV chimera coding for either EBOV-GP wt or the LXXXL mutant (MOI of 0.0001) for 1 h. After removal of the inoculum and washing of the cells, samples of the cell culture supernatants were taken at different time points (1, 8, 24, 48, 72, 96, 120, and 144 h postinfection), and viral titers were quantified by $TCID_{50}$ assay. The results of a single representative experiment are shown. Similar results were obtained in two additional, independent experiments.

(Continued on next page)

Our results show that a GXXXA motif within the TMD of EBOV-GP is essential for tetherin counteraction. The underlying mechanism is at present unclear. However, inferences can be made based on the work by Hacke and colleagues. They showed that the well-known ability of EBOV-GP to mediate the detachment of cells from a culture flask depends on the integrity of a GXXXA motif within the TMD and on the availability of membrane cholesterol (17). On the basis of these findings, they postulated that the GXXXA motif is required for interactions between GP trimers in cholesterol-rich microdomains (lipid rafts), which in turn is a prerequisite for GP-mediated detachment of cells and viral budding. Our results confirm that alteration of the GXXXA motif reduces cellular detachment and suggest that this defect can be rescued by cholesterol. However, addition of cholesterol to cells expressing the LXXXL mutant did not restore tetherin antagonism, and a previous report suggested that GP is neither located in lipid rafts nor alters tetherin localization in such domains (14). Moreover, evidence was provided that targeting sGP to lipid rafts is not sufficient for tetherin antagonism (15). In the light of these findings, membrane cholesterol does not seem to play a major role in tetherin antagonism by GP, and alternative scenarios should be considered. For instance, the GXXXA motif might contribute to formation and/or stability of GP trimers, and reduced trimerization might be incompatible with tetherin antagonism. However, our initial analyses revealed that particles bearing EBOV-GP wt and mutant LXXXL were comparably sensitive to inactivation by elevated temperature (data not shown), suggesting that the stability of the respective GP trimers may not be markedly different. Alternatively, the GXXXA motif might promote protein-protein interactions essential for tetherin antagonism. Given that the sequence integrity of the tetherin TMD is dispensable for GP-mediated tetherin antagonism (13), one can speculate that the GXXXA motif might mediate interactions of GP with a cellular protein that is required for tetherin counteraction.

Most studies employed VLP systems to analyze tetherin counteraction by EBOV-GP. In contrast, it remains unknown whether GP counteracts tetherin in the context of EBOV infection. Two studies provided evidence that EBOV infection might not be appreciably inhibited by tetherin (12, 28), but whether lack of tetherin sensitivity was due to the action of GP was not addressed. In the present study, alteration of the GXXXA motif in the context of VSV-EBOV-GP did not impact particle incorporation of GP and GP expression levels in infected cells. Moreover, mutation of the GXXXA motif only modestly reduced replication kinetics and peak viral loads in tetherin-negative cells, in keeping with the modestly diminished viral entry observed for the LXXXL mutant in single-cycle assays. In contrast, replication kinetics of the LXXXL mutant in tetherin-positive cells were markedly delayed compared to the kinetics of control cells while a clearly less pronounced delay was observed for the virus bearing wt GP. These results, jointly with the observation that VSV (which does not encode a tetherin antagonist that is active in infected cells) is more robustly inhibited by tetherin than VSV-EBOV-GP, suggest that tetherin counteraction by GP can occur in infected cells. Altering the GXXXA motif in the context of authentic EBOV might thus allow determination of the impact of GP-mediated tetherin antagonism on viral spread.

Based on these collective observations, we identified a mutation that abrogates tetherin antagonism with reasonable selectivity and consider it possible that even higher selectivity might be attainable by mutating only the glycine or the alanine residue within the GXXXA motif. Moreover, we provide, for the first time, evidence that GP can counteract tetherin in the context of cells infected with an EBOV surrogate. Future studies are required to confirm our results with infectious EBOV and to further elucidate how the GXXXA motif contributes to tetherin counteraction.

**FIG 4** Legend (Continued)
Crosses indicate time points at which kinetics were stopped due to complete virus-induced cell lysis. (F) Mean viral peak titers (VSV wt, 48 h; VSV-EBOV chimeras, 96 h postinfection) resulting from inoculation of Vero control (pQCXIP) cells and Vero cells expressing hTetherin measured in three independent experiments conducted as described for panel E. Error bars indicate standard errors of the means, and statistical significance was analyzed using one-way ANOVA with Bonferroni posttest analysis (*, $P \leq 0.05$; ***, $P \leq 0.001$).

## MATERIALS AND METHODS

**Cell culture.** HEK-293T (human, kidney), HeLa (human, cervix carcinoma), Vero and their subcloned derivate Vero E6 (both African green monkey, kidney), BHK-21(G43) (hamster, kidney), and EpoNi/22.1 (Buettikofer's epauletted fruit bat, kidney) cell lines were used as targets for transfection, transduction, infection, and detachment experiments. HEK-293T and HeLa cells (29) were obtained from DSMZ (ACC-635; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) and the NIH AIDS reagent program (https://www.aidsreagent.org/), respectively, while Vero, Vero E6, BHK-21(G43) (30), and EpoNi/22.1 (12) cells were provided by collaborators. All cell lines were maintained in Dulbecco's modified Eagle medium (PAN-Biotech), supplemented with 10% fetal bovine serum (Biochrom) and 1% penicillin-streptomycin (PAN-Biotech). Medium for EpoNi/22.1 cells was additionally supplemented with 1 mM sodium pyruvate (PAA Laboratories) and 1× nonessential amino acids (from a 100× stock solution; PAN-Biotech). After being transfected with the retroviral pQCXIP vector, Vero cells stably expressing human tetherin [Vero (hTetherin)] and Vero control cells expressing pQCXIP [Vero (pQCXIP)] were selected and maintained using 10 and 0.5 $\mu$g/ml of puromycin (Cayman chemical), respectively. All cells were incubated in a humidified atmosphere at 37°C and 5% $CO_2$. For subcultivation and seeding, cells were detached by either resuspension in fresh culture medium (HEK-293T cells) or by the use of trypsin-EDTA (PAN-Biotech).

**Plasmids.** Expression plasmids encoding the proteins used in this study were described previously, as follows: EBOV and VSV glycoproteins EBOV-GP (GenBank accession number AF086833.2) and VSV-G, respectively (10, 31); human tetherin (32); HIV-1 Vpu (12); HIV-1 p55-Gag (12, 16); and EBOV-VP40 harboring an N-terminal c-Myc epitope, EQKLISEEDL (33). The GXXXA motif in the transmembrane of EBOV-GP was genetically modified to LXXXL by employing overlap extension PCR. Expression plasmids for the coding sequences of VSV nucleoprotein (VSV-N), phosphoprotein (VSV-P), and RNA-dependent RNA-polymerase (VSV-L) were generated by amplifying the respective open reading frames (ORF) from a plasmid-encoded VSV anti-genome (Indiana strain; kindly provided by G. Zimmer) and inserting them via standard cloning procedures into the pCAGGS expression vector.

In order to produce replication-competent, chimeric VSV expressing EBOV-GP wild-type (wt) or the LXXXL mutant instead of VSV-G, as well as a dual reporter consisting of an enhanced green fluorescent protein (eGFP) and firefly luciferase (fLuc) from an additional transcription unit, we generated a plasmid-encoded VSV anti-genome (GenBank accession number J02428.1) as follows. First, we introduced unique MluI and NheI restriction sites upstream and downstream of the VSV-G transcription unit, respectively. Next, a cassette was inserted that consisted of the coding sequences of VSV-G and the eGFP-fLuc dual reporter, separated by a minimal intergenic region (34). To obtain the eGFP-fLuc dual reporter, the stop codon of the eGFP open reading frame (ORF) was removed and then combined with the genetic information of fLuc, linked via a nonflexible amino acid linker sequence (GPDPPVAT). This strategy gave rise to VSV** (the two asterisks indicate the dual reporter, later referred to as VSV). As this construct also contained a unique AscI restriction site downstream of the VSV-G transcription unit, it allowed us to replace VSV-G with the EBOV-GP wt or mutant LXXXL, thus generating VSV**ΔG EBOV-GP wt, VSV-EBOV wt, VSV**ΔG EBOV-GP LXXXL, and VSV-EBOV LXXXL. The integrity of all PCR-amplified sequences was verified by automated sequence analysis.

**Detachment assay.** HeLa cells were transiently transfected following calcium-phosphate precipitation with plasmids encoding EBOV-GP wt, EBOV-GP LXXXL, or GFP as a control. At 6 h posttransfection, cells were treated with water-soluble cholesterol (Sigma) at a concentration of 100 $\mu$M or water alone for 30 min. Subsequently, cells were washed with phosphate-buffered saline (PBS), and fresh medium was added. Supernatants were collected at 48 h after transfection, and the number of detached cells was quantified using a Becton Dickinson LSR II flow cytometer and BD FACSDIVA software.

**Production of rhabdoviral pseudotypes.** The pseudotypes were generated and used for transduction as described previously (35). In brief, 293T cells were seeded in six-well plates and transfected by calcium phosphate precipitation with plasmids encoding VSV-G, EBOV-GP wt, or EBOV-GP LXXXL or with an empty plasmid (pCAGGS) as a negative control. At 18 h posttransfection, the cells were inoculated with a replication-deficient VSV, in which the ORF for VSV-G was replaced by two separate ORFs for eGFP and fLuc (36) (kindly provided by G. Zimmer), at a multiplicity of infection (MOI) of 3. At 1 h postinfection, cells were washed with PBS and incubated with a 1:1,000 dilution of I1 (an anti-VSV-G mouse hybridoma supernatant from CRL-2700; American Type Culture Collection) for 1 h at 37°C in order to neutralize residual input virus. Finally, fresh culture medium was added to the cells. At 18 to 20 h postransduction, supernatants were collected, clarified from cell debris by centrifugation, aliquoted, and stored at −80°C until use.

**Transduction of cell lines and quantification of fLuc activity.** To assess cell entry driven by EBOV-GP LXXXL, 293T, Vero E6, and EpoNi/22.1 cells were seeded in 96-well plates. At 24 h after seeding, medium was removed, and cells were transduced with equal volumes of VSV pseudotypes. At 16 to 18 h postransduction, intracellular fLuc activity was measured as an indicator of transduction efficiency. For this, the cell culture medium was aspirated, and cells were incubated for 30 min at room temperature with 50 $\mu$l of luciferase cell culture lysis reagent (Promega). Lysates were transferred to a white, opaque-walled 96-well plate (Thermo Scientific), and fLuc activity was measured in a microplate reader (Plate Chameleon; Hidex) using MicroWin2000 software (version 4.44; Mikrotek Laborsysteme, GmbH) and fLuc substrate from a Beetle-Juice kit (PJK GmbH).

**Rescue and quantification of replication-competent VSV chimeras encoding EBOV-GP wt and EBOV-GP LXXXL.** For the rescue of the replication-competent VSV-EBOV wt or LXXXL, we used a strategy developed by others (37) with some modifications. First, BHK-21(G43) cells were seeded in 12-well plates and treated with mifepristone (Sigma-Aldrich) at a final concentration of 1 nM [BHK-21(G43) cells

inducibly express VSV-G upon stimulation with mifepristone] (30). At 16 h posttreatment, cells were infected with recombinant modified vaccinia virus Ankara expressing T7 polymerase (vMVA-T7; kindly provided by G. Sutter) (38) at an MOI of 3. The inoculum was aspirated 1 h after infection, and cells were washed with PBS. Next, the cells were transfected with expression plasmids for VSV-N, -P, and -L (polymerase complex) as well as the respective plasmid-encoded VSV anti-genome using Lipofectamine 2000 (ThermoFisher Scientific) as a transfection reagent. In this anti-genome, a T7 promoter sequence that precedes the leader region of the VSV genome drives cytoplasmic synthesis for negative-sense, full-length VSV genomes with defined genome ends through the activity of the hepatitis delta virus ribozyme located directly downstream of the trailer region. The negative-sense, full-length VSV genomes serve then as templates for mRNA transcription and further genome replication (both driven by the VSV polymerase complex). The following DNA amounts per well were used: 0.4 $\mu$g of VSV-N, 0.35 $\mu$g of VSV-P, 0.25 $\mu$g of VSV-L, and 1 $\mu$g of plasmid-encoded VSV anti-genome. At 6 h posttransfection, cells were washed, and fresh medium containing 1 nM mifepristone was added. We used mifepristone-stimulated BHK-21(G43) cells for initial transcomplementation of newly produced VSV particles with VSV-G in order to boost rescue efficiency. At 18 h posttransfection, the culture medium was supplemented with 100 $\mu$g/ml rifampin and 40 $\mu$g/ml cytosine $\beta$-D-arabinofuranoside (both from Sigma-Aldrich) to limit rMVA-T7 replication. After an additional 48 h of incubation, the supernatant was collected, clarified from cellular debris by centrifugation (4,700 rpm, 10 min, 4°C), and twice filtered through 0.2-$\mu$m-pore-size filter membranes to exclude residual rMVA-T7 from the preparation. Next, Vero E6 cells grown in a T-25 flask were inoculated with a 1:3 dilution of the filtered supernatant and incubated for 72 h in the presence of anti-VSV-G mouse hybridoma supernatant to neutralize subsequent host cell entry by *in trans*-delivered VSV-G. Afterwards, culture medium was collected and filtered again. Finally, Vero E6 cells grown in a T-75 flask were inoculated with a 1:10 dilution of the supernatant for virus stock production.

Quantification of titers of virus stocks was carried out on confluent Vero E6 cells seeded in 96-well plates. First, cell culture medium was removed, and cells were inoculated with 10-fold serial dilutions of virus. At 1 h postinfection, inoculum was removed, and cell culture medium containing 2% methylcellulose (Sigma-Aldrich) was added. After 48 h of incubation, eGFP-positive foci were counted under a fluorescence microscope. Virus stock titers were determined as the number of focus forming units (FFU) per milliliter to calculate the MOI for subsequent infection experiments. To verify the integrity of VSV-EBOV wt and VSV-EBOV LXXXL, viral RNA was extracted from virus stocks using a QIAamp Viral RNA minikit (Qiagen) and reverse transcribed into cDNA using a SuperScript III first-strand synthesis system (ThermoFisher Scientific) according to the manufacturer's protocol (for random hexamers). A fragment of approximately 1,800 bp was then amplified with primers binding in the intergenic region upstream of EBOV-GP (forward) and near the 5′ end of the eGFP ORF (reverse) using Phusion polymerase (ThermoFisher Scientific), separated by agarose gel electrophoresis, and extracted from the gel by commercial kits (Macherey & Nagel), before being subjected to automated sequence analysis (SeqLab).

**Growth kinetics of VSV-EBOV-GP in control and tetherin-positive cells.** To evaluate the effect of the GXXXA motif on viral spread, Vero cells stably expressing human tetherin (hTetherin) or control Vero cells containing empty vector (pQCXIP) were infected with VSV-EBOV-GP wt or VSV-EBOV-GP LXXXL (MOI of 0.0001) for 1 h. Afterwards, cells were washed with PBS to remove residual inoculum, and fresh culture medium was added. Cells were further incubated, and samples were collected at 1, 8, 24, 48, 72, 96, 120, and 144 h postinfection. Viral titers were determined on Vero E6 cells by calculating the 50% tissue culture infective dose ($TCID_{50}$) according to the Spearman-Kärber method (39, 40).

**Analysis of tetherin surface expression by flow cytometry.** For analysis of tetherin surface expression, Vero E6, Vero control (pQCXIP), and Vero cells stably expressing human tetherin were detached and incubated for 30 min at 4°C with a 1:50 dilution of purified anti-human CD317 (BST2; tetherin) antibody (BioLegend) or with an isotype-matched control antibody (BioLegend). Afterwards, cells were washed twice with PBS and then incubated with Alexa Fluor 647-conjugated goat anti-mouse secondary antibody at a 1:100 dilution for 30 min at 4°C. Finally, cells were washed with PBS, fixed with 2% paraformaldehyde (PFA), and analyzed in a Becton Dickinson LSR II flow cytometer. Data were further analyzed using FCS Express, version 4, software.

**Immunoblotting.** Expression and incorporation of EBOV-GP wt and EBOV-GP LXXXL into VSV pseudotypes and replication-competent VSV-chimeras were determined by Western blotting. For expression analysis, HEK-293T cells were transiently transfected with plasmids encoding EBOV-GP wt, EBOV-GP LXXXL (both harboring an N-terminal V5 tag), or empty plasmid (pCAGGS) as a negative control. At 48 h posttransfection, culture medium was aspirated, and cells were lysed using 300 $\mu$l of 2× sodium dodecyl sulfate (SDS)-containing lysis buffer (50 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% $\beta$-mercaptoethanol, 0.1% bromophenol blue, 1 mM EDTA) and boiled for 30 min at 95°C. To assess EBOV-GP wt and EBOV-GP LXXXL incorporation into pseudotypes and VSV-EBOV chimeras, equal volumes of pseudotype preparations or supernatant of virus stocks were pelleted by centrifugation (17,000 × *g*, 2 h, 4°C) through a 20% sucrose cushion. Samples were resuspended in 20 $\mu$l of 2× SDS lysis buffer and boiled for 30 min at 95°C. Afterwards, samples were separated by SDS-PAGE using 12.5% polyacrylamide gels and transferred onto nitrocellulose membranes (0.2-$\mu$m pore size; GE Healthcare Life Sciences). Membranes were blocked for 1 h in 5% milk powder in PBS with 0.1% Tween 20. The expression of V5-tagged glycoproteins was detected using anti-V5 antibody (Invitrogen) at a 1:1,000 dilution; $\beta$-actin was detected after the membranes were stripped (Tris-HCl, SDS, $\beta$-mercaptoethanol; 50°C, 30 min) using an anti-$\beta$-actin antibody (Sigma) at a dilution of 1:1,000. Particle incorporation of EBOV-GPs was detected using a GP1-specific rabbit serum at a 1:1,000 dilution. To show that equal amounts of pseudotypes and replication-competent virus were used, a separate membrane with the same samples was incubated with an antibody against VSV-M (matrix protein, raised in mice [KeraFast];

1:1,000). Bound antibodies were detected using horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit secondary antibodies (Dianova) at a dilution of 1:10,000. Signals of bound secondary antibodies were detected using an in-house-made enhanced chemiluminescence (ECL) solution (0.1 M Tris-HCl, pH 8.6, 250 $\mu$g/ml luminol [Sigma], 1 mg/ml *para*-hydroxycoumaric acid [Sigma], 0.3% $H_2O_2$) and visualized using a ChemoCam imaging system along with the ChemoStar Professional software (Intas).

**Virus-like particle release assay.** Release of HIV-Gag or EBOV-VP40-based virus-like particles (VLPs) was analyzed as described before (10) to assess the importance of the GXXXA motif for EBOV-GP-dependent tetherin antagonism. In brief, 293T cells were seeded in 48-well plates and cotransfected following calcium-phosphate precipitation with plasmid encoding HIV-1 p55-Gag or EBOV-VP40, human tetherin, and a tetherin antagonist or empty plasmid as control. At 48 h posttransfection, supernatants were collected and clarified from cell debris, and VLPs were pelleted by centrifugation through a 20% sucrose cushion. Concentrated VLPs were resuspended in 20 $\mu$l of 2× SDS buffer. In parallel, cells were lysed with 50 $\mu$l of 2× SDS lysis buffer. Both whole-cell lysates (WCL) and VLPs were boiled at 95°C for 30 min. Presence of HIV-Gag in lysates and VLPs was analyzed by immunoblotting according to the same protocol as described in the previous paragraph ("Immunoblotting"). Gag protein was detected using a 1:100 diluted supernatant of hybridoma cells secreting a mouse anti-Gag antibody (183-H12-5C) (41), while EBOV-VP40 was detected using undiluted supernatants of 9E10 cells, a hybridoma cell line that secretes anti-Myc antibody (42). Finally, antibody binding was detected employing an HRP-linked anti-mouse secondary antibody (Dianova) at a dilution of 1:10,000.

**Statistical analyses.** To assess statistical significance, unpaired and paired Student *t* tests were conducted for comparison of two data groups, while one-way analysis of variance (ANOVA) with Bonferroni posttest analysis was conducted for comparison of multiple data groups. All statistical analyses were done employing the GraphPad Prism software (GraphPad Software).

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## 5. Discussion

Ebola virus constitutes a severe threat to human health. Understanding how EBOV enters cells and evades control by the immune system may pave the way to novel options for antiviral intervention.

EBOV must be handled in biosecurity level 4 (BSL-4) laboratories which slows down research progress. Therefore, surrogate systems have been developed to study EBOV infection. For instance, MLV- and VSV-vectors pseudotyped with EBOV-GP (MLVpp, VSVpp) have been used to study viral entry [248–250]. Moreover, EBOV-like particles containing VP30 linked to luciferase (VP30-luc) are used to study viral entry in the context of exclusively EBOV-derived proteins [128]. However, VP30-Luc EBOV-VLPs, VSVpp and MLVpp allow only one round of infection and are not able to replicate. In contrast, a novel transcription- and replication-competent virus-like particle (trVLP) minigenome system allows to study all steps of EBOV infection [417] in the context of authentic filamentous particles [418].

Similar to other viral glycoproteins, including influenza hemagglutinin [419], EBOV-GP depends on priming by host cell proteases to transit into a membrane-fusion competent form. Proteolytic priming of EBOV-GP can be facilitated by the endosomal cysteine proteases CatB/CatL [241,248]. However, CatB/CatL dependence was mainly documented with surrogate systems and a study conducted with authentic EBOV and CatB or CatL knockout mice found that expression of these proteases was dispensable for viral spread and pathogenesis [251]. Therefore, the first goal of this work was to identify determinants governing the CatB/L dependence of EBOV-GP-driven entry into host cells. The results of this research are reported in manuscript 1 and will be discussed below.

EBOV-GP post-translational modifications involve cleavage by furin into the subunits GP1 and GP2. However, this cleavage is dispensable for GP-driven entry [420–422]. In contrast, removal of the MLD and glycan cap in host cell endosomes exposes the RBD and is required for viral entry. These cleavage events are facilitated by CatB/CatL [241]. CatB/CatL are involved in apoptosis and have been targeted for treatment of diseases like traumatic brain injury [423], cancer [424] or viral infections [425]. Their

enzymatic activity can be inhibited by E-64d, an epoxysuccinate inhibitor, and MDL28170, an aldehyde inhibitor [426–429], and both compounds inhibit EBOV-GP-driven entry [243,248,250]. In the present study, these inhibitors and the above listed surrogate systems were used to determine whether the shape of the GP presenting particle impacts CatB/CatL dependence of GP-driven entry. These studies were motivated by the observation that many published studies examining CatB/CatL dependence of GP-driven entry used spherical retroviral or bullet shaped rhabdoviral particles [220,241,247,248,250], which might be taken up into cells via different mechanisms [220,221] as compared to filamentous particles [84,182,223] and might thus exhibit differential CatB/CatL dependence. I was able to show that GP-driven entry was CatB/CatL dependent irrespective of whether GP was presented in the context of spherical, bullet shaped or filamentous particles and irrespective of the target cell line.

My results suggested that host cell entry of filamentous particles bearing GP is CatB/CatL dependent and hence fails to provide an explanation for the striking finding that CatB or CatL are dispensable for EBOV spread in mice [251]. A second scenario that may account for the findings of Marzi and colleague is that other cathepsins can compensate for the loss of CatB or CatL expression. These compensatory enzymes might be responsible for GP processing and allow efficient spread in mice. In this context, it is important to note that although E-64d and MDL28170 inhibit CatB and CatL respectively, they can also interfere with the proteolytic activity of other cysteine proteases and may thus block the activity of compensatory proteases.

My studies revealed that the lung derived cell line Calu-3 is largely refractory to entry driven by diverse filovirus glycoproteins. In contrast, the cells were susceptible to entry driven by rhabdo-, paramyxo- and arenavirus glycoproteins. The absence of robust GP-driven entry was striking, since EBOV has a broad cell tropism and its GP can drive entry into a diverse range of cells, with only T and B cells being refractory for incompletely understood reasons [249,430–432]. A study by Park and colleagues revealed that Calu-3 cells express low levels of CatL. Therefore, uncleaved Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein is unable to mediate entry into Calu-3 cells [433]. This finding raised the question whether the low

expression levels of CatL could also account for the inefficient EBOV-GP-driven entry into Calu-3 cells. Using quantitative RT-PCR, I could confirm that Calu-3 cells express lower mRNA levels of CatL than 293T or Huh-7 cell lines, while CatB mRNA copies were only slightly lower than those measured for 293T and Huh-7 cells. In order to reveal if CatL expression limits filovirus GP-driven entry, I generated Calu-3 cell lines that stably express CatL. I could show that directed expression of CatL significantly increased MARV-GP-driven entry and moderately improved EBOV-GP-, SUDV-GP-, TAFV-GP-, RESTV-GP-driven entry but did not appreciably enhance BDBV- and LLOV-GP-driven entry and had no effect on VSV-G driven entry which is CatL independent. My observations are in keeping with previous studies which show that CatB/CatL dependence varies among filovirus GPs [247,250]. For instance, directed expression of CatB but not CatL in CatB/CatL knockout cells promotes EBOV-GP- and TAFV-GP-driven entry. In contrast, CatL but not CatB increased MARV-GP-driven entry while expression of both enzymes promoted RESTV-GP-driven entry [247].

Expression of attachment factors can greatly augment EBOV-GP-driven entry. Therefore, in parallel to CatL, I also evaluated whether filovirus attachment factors (DC-SIGN, Axl) or receptor (NPC1) could account for limited GP-driven entry into Calu-3 cells. To investigate this, I generated stable Calu-3 cell lines expressing either DC-SIGN, Axl, NPC1 or CatB. I could show that CatB, NPC1 and Axl do not increase entry into Calu-3 cells. The finding that directed expression of CatB and NPC1 did not rescue was not unexpected since it is well known that these proteins are ubiquitously expressed [229,252,253]. In contrast, expression of Axl is cell line dependent. However, its entry enhancing activity should not depend on the cellular context and it is unclear why directed expression of Axl failed to augment entry into Calu-3 cells. One can speculate that expression levels of Axl in Calu-3 cells might have been insufficient for entry augmentation. In contrast, directed expression of DC-SIGN which can boost GP-driven entry up to 50-fold [200,216] and renders Jurkat cells susceptible to GP-driven entry [216], rescued EBOV-GP, SUDV-GP and MARV-GP entry into Calu-3 cells. Moreover, DC-SIGN expression moderately increased entry driven by RESTV-GP and TAFV-GP. Finally, I provided evidence that increased entry into Calu-3 cells engineered to express DC-SIGN or CatL was due to direct effects of these

proteins on the entry reaction. In sum, my results indicate that entry driven by filovirus GPs in Calu-3 cells is limited at the stage of attachment and priming.

The IFN system is the first line of defense against viral infections. Upon infection, signaling cascades stimulate IFN production, and IFN then triggers the expression of approximately 500 ISGs, many of which exert antiviral activity [300,301,306]. Tetherin is an IFN-induced protein that prevents viral release by forming a physical bridge between viral and cellular membrane [347,359,360]. Several viruses encode proteins that antagonize tetherin, usually by interfering with tetherin expression [361,383]. Filovirus GPs can antagonize tetherin but the mechanism used by these proteins to counteract tetherin remains incompletely understood. Therefore, the second goal of my research was to identify domains and amino acid motifs in GP that are required for tetherin antagonism. The results of my research have been documented in manuscript 2 and will be discussed below.

It has been shown that the glycan cap, RBD and TMD in GP contribute to tetherin antagonism [413–415]. However, no specific amino acids within the TMD have been identified that are required for tetherin antagonism. However, a GXXXA motif within the TMD of EBOV-GP has been associated with GP-mediate cellular detachment [416], raising the question whether this motif could also contribute to EBOV-GP-mediated tetherin antagonism.

My results revealed that the GXXXA motif is not required for EBOV-GP expression, particle incorporation and viral entry, although it should be stated that mutation of the motif reduced entry by about 50% in Vero E6 and EpoNi/22.1 cells. Moreover, mutation of this motif did not affect the ability of EBOV-GP to promote release of HIV- and EBOV-based viral particles from tetherin-negative cells [373,413,415,434]. In contrast, mutation of this motif abrogated tetherin counteraction by EBOV-GP in the context of spherical HIV Gag- [373,413] and filamentous EBOV VP40-based particles [103,104].

Hacke and colleagues reported that the GXXXA motif and membrane cholesterol are required for GP-mediated cellular detachment. Based on these findings they suggested that the GXXXA motif drives GP-GP homotypic interactions within lipid rafts

and that this is required for cellular detachment [416]. In contrast to what Hacke and colleagues postulated, a previous study suggested that GP is not located in lipid rafts [435]. Moreover, it was suggested that GP does not alter tetherin localization in lipid rafts [435] and that targeting sGP, which does not counteract tetherin, to lipid rafts is not sufficient for it to mediate tetherin antagonism [414]. My results show that cellular detachment but not tetherin antagonism by a LXXXL mutant of GP (i.e. GXXXA motif mutated) can be rescued by addition of exogenous cholesterol, arguing against an important role of cholesterol in tetherin antagonism. It has been suggested that the interaction between the TMD of single-pass membrane proteins depends on GXXXG or (small amino acid)XXX(small amino acid) motifs, and the presence of these motifs could be used, in some cases, as indicator of TMD oligomerization [436]. This raises the possibility that the GXXXA motif might contribute to formation and/or stability of EBOV-GP trimers, which in turn might be required for tetherin antagonism. However, I obtained no evidence for an impact of the GXXXA motif on trimer stability. As an alternative explanation for the role of the GXXXA motif in tetherin counteraction one could speculate that this domain is required for GP interactions with a cellular factor required for tetherin antagonism and this possibility remains to be investigated.

Two studies demonstrated that EBOV replicates efficiently in tetherin-positive cells [365,373]. However, it was not elucidated whether this was due to GP-mediated tetherin antagonism. A replication-competent VSV-EBOV-GP chimera is an EVD vaccine candidate [42,43] and offers the possibility to study whether GP counteracts tetherin in infected cells without having to recur to BSL-4 experiments [437,438]. I could show that both, GP wt and the LXXXL mutant, were expressed at similar levels upon infection and were both efficiently incorporated into viral particles. Moreover, mutation of the GXXXA motif had little effect on VSV-EBOV-GP replication in tetherin-negative cells, and the small effect observed might be due to slightly reduced viral entry, as discussed above. Finally, replication of VSV was strongly inhibited by tetherin, in keeping with the finding that this virus does not encode for a tetherin antagonist [439]. Collectively, these results suggest that GP-mediated tetherin counteraction can occur in the context of infected cells, at least when surrogate systems are used. Therefore, mutation of the GXXXA motif in the context of authentic EBOV might allow to determine whether tetherin antagonism occurs in infected cells and contributes to viral spread.

## 6. Outlook

During my doctoral thesis I was able to determine that proteolytic priming of EBOV-GP by CatB and CatL is independent of viral particle shape and cell line. A remarkable finding was the identification of Calu-3 cells as one of the few cell lines that are largely resistant to filovirus GP-driven entry and I could provide evidence that entry is restricted at the stages of attachment and GP priming. Future studies need to determine whether Calu-3 cells are also resistant to entry of authentic EBOV and if entry is enhanced in Calu-3 cells expressing CatL or DC-SIGN. Furthermore, it needs to be elucidated whether co-expression of both proteins further augments entry efficiency. Finally, it will be interesting to determine if usage of certain attachment factors like DC-SIGN might impact CatB/CatL dependence of viral entry potentially by introducing viral particles into different uptake pathways.

My work on the antiviral host cell factor tetherin revealed that a mutation within the TMD domain of EBOV-GP abrogates tetherin antagonism while largely maintaining efficient host cell entry. This finding provided the first evidence that EBOV-GP can antagonize tetherin in the context of an infectious surrogate system. Further studies are required to identify fully selective mutations that have no effect on entry but completely abrogate tetherin antagonism. Moreover, it remains to be determined whether mutating the GXXXA motif in the context of infectious EBOV inhibits tetherin antagonism and whether this inhibition affects viral spread. Finally, it is essential determine how GP counteracts tetherin and how the GXXXA motif contributes to this process.

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# 8. Appendix

## 8.1. List of abbreviations

| | |
|---|---|
| ADAM17 | A disintegrin and metalloproteinase 17 |
| ASGPR | Asialoglycoprotein receptors |
| ASGPR-1 | Asialoglycoprotein receptor-1 |
| BDBV | Bundibugyo virus |
| BOMV | Bombali virus |
| BSL4 | Biosecurity level 4 |
| BST-2 | Bone marrow stromal antigen 2 |
| CARD | caspase recruitment domains |
| CatB | Cathepsin B |
| CatL | Cathepsin L |
| CD209 | Cluster of differentiation 209 |
| CD317 | Cluster of differentiation 317 |
| CD4+ | Cluster of differentiation 4 |
| Cdc42 | Cell division control protein 42 homolog |
| CHIKV | Chikungunya virus |
| CLR | C-type lectin receptors |
| cRNA | Complementary ribonucleic acid |
| CTL | C-type lectins |
| Cys | Cysteine |
| DC | Dendritic cells |
| DC-SIGN | Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| DENV | Dengue virus |
| DNA | Deoxyribonucleic acid |
| DRC | Democratic Republic of Congo |
| dsRNA | Double-stranded ribonuclec acid |
| EBOV | Ebola virus |
| EBOV-GP | Ebola virus glycoprotein |
| EGFR | Epidermal growth factor receptor |
| ER | Endoplasmic reticulum |
| EVD | Ebola virus disease |
| Gas6 | Growth arrest-specific 6 |
| GDP | Guanidine diphosphate |
| GED | GTPase effector domain |
| GP | Glycoprotein |
| GP0 | Glycoprotein 0 |
| GP1 | Glycoprotein 1 |
| GP2 | Glycoprotein 2 |
| GPI | Glycosylphosphatidylinositol |
| GTP | Guanidine triphosphate |
| GTPase | Guanosine triphosphatase |
| HCV | Hepatitis C virus |
| HIV-1 | Human immunodeficiency virus 1 |

| | |
|---|---|
| HIV-2 | Human immunodeficiency virus 2 |
| hPIV-2 | Human parainfluenza virus type 2 |
| HR | Heptad repeat |
| HSV | Herpes simplex virus |
| IAV | Influenza A virus |
| ICAM-1 | Intercellular cell adhesion molecule-1 |
| IFITM | Interferon-induced transmembrane protein |
| IFL | Internal fusion loop |
| IFN | Interferon |
| IFNR | Interferon receptor |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IgM | Immunoglobulin M |
| IgV | Immunoglobulin variable |
| IRF | Interferon-regulatory factors |
| ISG | Interferon stimulated gene |
| JAK | Janus-kinases |
| KPNA | Karyopherin α |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| LGP2 | Laboratory of Genetics and Physiology 2 |
| LLOV | Lloviu virus |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeats |
| LSECtin | Liver and lymph node sinusoidal endothelial cell C-type lectin |
| L-SIGN | Lymph node-specific intercellular adhesion molecule-3-grabbing integrin |
| MARV | Marburg virus |
| MAVS | Mitochondrial antiviral signaling protein |
| MD | Middle domain |
| MD5A | Melanoma differentiation-associated protein 5 |
| MERS | Middle east respiratory syndrome |
| MFG-E8 | Milk fat globule-epidermal growth factor-factor 8 |
| MGL | Macrophage galactose C-type lectin |
| MLD | Mucin-like domain |
| MLV | Murine leukemia viruses |
| mRNA | Messenger ribonucleic acid |
| Mx | Myxovirus resistance protein |
| MyD88 | Myeloid differentiation factor 88 |
| Nedd4 | Neural precursor cell expressed developmentally down-regulated protein 4 |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHP | Non-human primate |
| NK | Natural killer |
| NLR | NOD-like receptors |
| NOD | Nucleotide-binding oligomerization domain |
| NP | Nucleoprotein |

| | |
|---|---|
| NPC1 | Niemann-Pick C1 |
| NPC2 | Niemann-Pick C2 |
| NTD | N-terminal domain |
| OAS | 2’-5’-oligo-adenylate synthetase |
| OASL | OAS-like |
| ORF | Open reading frame |
| PACT | PKR activator |
| Pak1 | Serine/threonine-protein kinase |
| PAMP | Pathogen-associated molecular patterns |
| PECAM-1 | Platelet/endothelial cell adhesion molecule-1 |
| PHEIC | Public Health Emergency of International Concern |
| PI3K | Phosphatidylinositol 3-kinases |
| PKC | Protein kinase C |
| PKR | Protein kinase R |
| PRR | Pattern recognition receptors |
| PtdSer | Phosphatidylserine |
| PTK | Protein tyrosine kinase |
| PVEERs | Phosphatidylserine-mediated virus entry enhancing receptors |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RAVV | Ravn virus |
| RBD | Receptor binding domain |
| RESTV | Reston virus |
| RIG-I | Retinoic acid-inducible gene-I |
| RLR | RIG-I-like receptor |
| RNA | Ribonucleic acid |
| RNase L | Ribonuclease L |
| RNP | Ribonucleoprotein |
| Rsp5 | Reversion of Spt phenotype |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SARS-CoV | Severe acute respiratory syndrome coronavirus |
| sGP | Soluble Glycoprotein |
| siRNA | Small interfering ribonucleic acid |
| SIV | Simian immunodeficiency virus |
| SP | Signal peptide |
| SSD | Sterol sensing domain |
| ssGP | Small soluble Glycoprotein |
| ssRNA | Single-stranded ribonucleic acid |
| STAT | Signal transducer and activator of transcription |
| SUDV | Sudan virus |
| TACE | Tumor necrosis factor alpha-converting enzyme |
| TAFV | Taï Forest virus |
| TAM | Tyro-3, Axl, and Mer receptors |
| TBK1 | TANK-binding kinase 1 |
| TGN | Trans-Golgi network |
| TIM | T-cell immunoglobulin and mucin-domain containing protein |
| TIR | Toll-IL-1 receptor |

| | |
|---|---|
| TLR | Toll-like receptor |
| TMD | Transmembrane domain |
| TRAF | TNF receptor-associated factor |
| TRIF | TIR-domain-containing adaptor-inducing IFN- factor |
| TRIM | Tripartite motif protein |
| trVLP | Transcription- and replication-competent virus-like particle |
| Tsg101 | Tumor susceptibility gene 101 |
| TYK2 | Tyrosine kinase 2 |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VLP | Virus-like particle |
| VP24 | Viral protein with molecular mass of 24 kilodalton |
| VP30 | Viral protein with molecular mass of 30 kilodalton |
| VP35 | Viral protein with molecular mass of 35 kilodalton |
| VP40 | Viral protein with molecular mass of 40 kilodalton |
| Vpu | Viral protein U |
| VSV | Vesicular stomatitis virus |
| VSV-EBOV-GP | Vesicular stomatitis virus with Ebola virus glycoprotein |
| WHO | World Health Organization |

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### 8.3. Curriculum Vitae

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**Academic Education**

| | |
|---|---|
| 08/2015 - 03/2019 | PhD within the Graduate School "Emerging Infectious Diseases" (EIDIS)<br>Georg-August University School of Science (GAUSS)<br>Göttingen, Germany<br><br>Thesis "Interactions of the Ebola virus glycoprotein with host cell factors during viral entry and release"<br><br>Supervisor: Prof. Dr. Stefan Pöhlmann |
| 08/2012 – 08/2014 | M.Sc., Master in Health Science<br>Escuela Superior de Medicina - Instituto Politécnico Nacional<br>Mexico City, Mexico<br><br>Thesis "Identification and validation of epitopes of Dengue virus serotypes for serological diagnosis"<br><br>Supervisors: Dr. Leopoldo Aguilar Faisal<br>Dr. Adolfo Chávez Negrete<br><br>Graduated with Honors |
| 08/2007 – 12/2011 | Bachelor in Biochemistry Engineering<br>Escuela Nacional de Ciencias Biológicas- Instituto Politécnico Nacional<br>Mexico City, Mexico<br><br>Thesis "Activation and retrieval of the four serotypes of Dengue virus and its identification using RT-PCR"<br><br>Supervisors: M.Sc. Verónica Alcántara Farfán<br>M.Sc. Arturo Barbachano Guerrero |
| 08/2004 – 07/2007 | High School, Chemical Technician<br>Centro de Estudios Científicos y Tecnológicos No.6 Miguel Othón de Mendizábal-Instituto Politécnico Nacional<br>Mexico City, Mexico |

**Honors and Awards**

| | |
|---|---|
| 10/2015 – present | Scholarship holder within the DAAD country-related cooperation program with the National Council on Science and Technology of Mexico (CONACYT) |
| 08/2015 – 07/2018 | The mobility scholarship within the MayaNet Project-Erasmus Mundus Action 2 |
| 08/2012 – 08/2014 | Stipend recipient within the master's degree program division from the National Council on Science and Technology of Mexico (CONACYT) |
| 03/2013 – 09/2014 | The scholarship for health research within the Research Development Division from the "Instituto Mexicano del Seguro Social (IMSS)" |
| 02/2013 – 06/2014<br>02/2011 – 12/2011 | Scholarship holder within the Institutional Program for Training of Researchers (PIFI) from the "Instituto Politécnico Nacional (IPN)" |
| 02/2005 – 12/2011 | The Institutional scholarship from the "Instituto Politécnico Nacional (IPN)" |

### 8.4. Conference participations

26th Annual meeting of the Society for Virology (GfV), Münster, Germany, 2016

Poster presentation: Importance of the GXXXA motif in the Ebolavirus GP transmembrane domain for entry and sensitivity towards host-cell encoded antiviral factors

National Symposium on Zoonoses Research, Berlin, Germany, 2016

Oral presentation: GXXXA motif within the transmembrane domain of the Ebola virus glycoprotein is important for counteraction of the antiviral host factor tetherin

Keystone Symposia on Hemorrhagic Fever Viruses, Santa Fe, New Mexico, USA, 2016

Poster presentation: The GXXXA motif within the GP transmembrane domain is important for Ebola virus counteraction of the antiviral host cell factor tetherin

28th Annual meeting of the Society for Virology (GfV), Würzburg, Germany, 2018
Oral presentation: A GXXXA motif in the transmembrane domain of the Ebola virus glycoprotein is required for tetherin antagonism

National Symposium on Zoonoses Research, Berlin, Germany, 2018
Poster presentation: Ebola virus glycoprotein-driven entry requires cathepsin B/L activity irrespective of particle shape and target cell line

### 8.5. List of publications

**González-Hernández, M.**, Hoffmann, M., Brinkmann, C., Nehls, J., Winkler, M., Schindler, M., Pöhlmann, S. 2018. A GXXXA motif in the transmembrane domain of the Ebola virus glycoprotein is required for tetherin antagonism. *Journal of Virology* pii: JVI.00403-18. doi: 10.1128/JVI.00403-18.

**González-Hernández, M.**, Hoffmann, M., Pöhlmann, S. 2019. Calu-3 cells are largely resistant to entry driven by filovirus glycoproteins and the entry defect can be rescued by directed expression of DC-SIGN or cathepsin L. *Virology* (Submitted Manuscript)

Hoffmann, M., Crone, L., Dietzel, E., Paijo, J., **González-Hernández, M.**, Nehlmeier, I., Kalinke, U., Becker, S., Pöhlmann, S. 2017. A polymorphism within the internal fusion loop of the Ebola virus glycoprotein modulates host cell entry. *Journal of Virology* 91: e00177-17. https://doi.org/ 10.1128/JVI.00177-17

Brinkmann, C., Nehlmeier, I., Walendy-Gnirß, K., Nehls, J., **González-Hernández, M.**, Hoffmann, M., Qiu, X., Takada, A., Schindler, M., Pöhlmann, S. 2016. The tetherin antagonism of the Ebola virus glycoprotein requires an intact receptor-binding domain and can be blocked by GP1-specific antibodies. *Journal of Virology* 90:11075–11086. doi:10.1128/JVI.01563-16

Hoffmann, M., **González-Hernández, M.**, Berger, E., Marzi, A., Pöhlmann, S. 2016. The Glycoproteins of All Filovirus Species Use the Same Host Factors for Entry into Bat and Human Cells but Entry Efficiency Is Species Dependent. *PLoS ONE* 11(2): e0149651. doi:10.1371/journal.pone.0149651

Plegge, T.*, Spiegel, M.*, Krüger, N., Winkler, M., **González-Hernández, M.**, Pöhlmann, S. 2019. Inhibitors of signal peptide peptidase and subtilisin/kexin-isozyme 1 inhibit Ebola virus glycoprotein-driven cell entry by interfering with activity and cellular localization of endosomal cathepsins. *PLoS One* (Submitted manuscript) *shared first authorship