PLOS ONE

RESEARCH ARTICLE

The Hemagglutinin of Bat-Associated Influenza Viruses Is Activated by TMPRSS2 for pH-Dependent Entry into Bat but Not Human Cells

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Citation: Hoffmann M, Krüger N, Zmora P, Wrensch F, Herrler G, Pöhlmann S (2016) The Hemagglutinin of Bat-Associated Influenza Viruses Is Activated by TMPRSS2 for pH-Dependent Entry into Bat but Not Human Cells. PLoS ONE 11(3): e0152134. doi:10.1371/journal.pone.0152134

Editor: Michael CW Chan, Centre of Influenza Research, The University of Hong Kong, HONG KONG

Received: December 1, 2015

Accepted: March 9, 2016

Published: March 30, 2016

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (DFG) grant PO 716/6-1, Göttingen Graduate School for Neurosciences, Biophysics, and Molecular Biosciences, Deutsche Forschungsgemeinschaft (DFG) grants GSC 226/1 and GSC 226/2 (www.dfg.de). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

New World bats have recently been discovered to harbor influenza A virus (FLUAV)-related viruses, termed bat-associated influenza A-like viruses (batFLUAV). The internal proteins of batFLUAV are functional in mammalian cells. In contrast, no biological functionality could be demonstrated for the surface proteins, hemagglutinin (HA)-like (HAL) and neuraminidase (NA)-like (NAL), and these proteins need to be replaced by their human counterparts to allow spread of batFLUAV in human cells. Here, we employed rhabdoviral vectors to study the role of HAL and NAL in viral entry. Vectors pseudotyped with batFLUAV-HAL and -NAL were able to enter bat cells but not cells from other mammalian species. Host cell entry was mediated by HAL and was dependent on prior proteolytic activation of HAL and endosomal low pH. In contrast, sialic acids were dispensable for HAL-driven entry. Finally, the type II transmembrane serine protease TMPRSS2 was able to activate HAL for cell entry indicating that batFLUAV can utilize human proteases for HAL activation. Collectively, these results identify viral and cellular factors governing host cell entry driven by batFLUAV surface proteins. They suggest that the absence of a functional receptor precludes entry of batFLUAV into human cells while other prerequisites for entry, HAL activation and protonation, are met in target cells of human origin.

Introduction

Influenza A viruses (FLUAV) are enveloped, negative stranded RNA viruses that pose a major threat to public health [1]. The ability of FLUAV to constantly adapt to immune pressure allows these viruses to continuously circulate in the human population, resulting in annual influenza epidemics (seasonal influenza [2, 3]). Infants, children and the elderly are at particular risk of developing severe disease upon infection with seasonal FLUAV and it has been estimated that world-wide 250,000 to 500,000 people die each year of seasonal influenza [1].

Competing Interests: The authors have read the journal's policy and the authors of this manuscript have the following competing interests: Stefan Pöhlmann currently serves as an academic editor for PLoS One. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Waterfowl has been shown to constitute the natural reservoir of FLUAV [4, 5] from which viruses with pandemic potential can be directly transmitted to humans or can emerge upon reassortment of avian and human FLUAV [2, 4, 6]. Influenza pandemics might have dramatic consequences, as highlighted by the 30–50 million deaths attributed to the influenza pandemic of the years 1918/1919 (Spanish influenza [7, 8]).

The viral surface proteins hemagglutinin (HA) and neuraminidase (NA) facilitate FLUAV entry and release from target cells, respectively. HA facilitates viral attachment to cells by binding to sialic acids on cell surface proteins or lipids [9–11] and, upon proteolytic activation by a host cell protease and exposure to endosomal low pH, mediates fusion of the viral membrane with the endosomal membrane [12–14]. In contrast, NA promotes release of progeny particles from infected cells by removing sialic acids from cell surface factors. Based on sequence and antigenic properties, sixteen HA (H1-16) and nine NA (N1-9) subtypes have been identified, and viruses representing all HA and NA subtypes are circulating in waterfowl [4, 15]. However, FLUAV-related viruses were recently discovered in New World bats [16, 17], provisionally termed bat-associated influenza A-like viruses (batFLUAV), and were shown to harbor HA- and NA-like proteins (termed HAL and NAL), which constitute new subtypes, H17/H18 (HL17/HL18) and N10/N11 (NL10/NL11), respectively. The question whether these viruses have the potential to infect and spread in humans is the focus of current research efforts.

Attempts to isolate batFLUAV were unsuccessful [16, 17] but, employing reverse genetics, it was demonstrated that the viral replication machinery and interferon antagonists are functional in mammalian cells [16, 18–21]. In contrast, the HAL and NAL proteins of batFLUAV were incompatible with viral spread in the cell culture systems examined so far [18, 19] for at present unknown reasons. Biochemical and structural studies imply that batFLUAV-HAL, unlike FLUAV-HA, does not engage sialic acids (SA) for host cell entry [17, 22–24], and that batFLUAV-NAL, unlike FLUAV-NA, neither shows neuraminidase activity nor possesses an active site that would allow interaction with sialic acids [17, 25–27]. However, it is currently unclear whether HAL and NAL can facilitate viral entry into certain target cells and it is unknown which determinants control the entry process.

Here, we utilize rhabdoviral vectors to analyze host cell entry driven by batFLUAV-HAL and -NAL. We show that HAL facilitates entry into certain bat but not human cell lines and that entry is independent of sialic acids. In contrast, HAL-driven entry was dependent on prior proteolytic activation of HAL and endosomal acidification. Moreover, we provide evidence that HAL can utilize the cellular protease TMPRSS2 for its activation, suggesting that batFLUAV entry into human cells is mainly restricted at the stage of receptor engagement while proteolytic activation and triggering of HAL are not limiting the entry process.

Materials and Methods

Cell culture

The following cell lines were used as targets for transduction and expression experiments and were maintained in Dulbecco's modified Eagle's medium (PAA Laboratories), supplemented with 10% fetal bovine serum (Biochrom) and antibiotics (penicillin/streptomycin, PAA Laboratories): HEK-293T, Huh7, Vero, MDCK, BHK-21, as well as chiropteran cell lines from five different bat species, RoNi/7, HypNi/1.1, EidNi/41, EpoNi/22.1 and CpKd (Table 1). All non-bat-derived cell lines were obtained from collaborators. The fruit bat cell lines (RoNi/7, HypNi/1.1, EidNi/41, EpoNi/22.1) were a kind gift of C. Drosten and M. A. Müller and have been described previously [28–31]. The CpKd cell line was described elsewhere [28]. All cell lines were grown in a humidified atmosphere at 37°C and 5% CO_2. For passaging and seeding, cells

Table 1. Cell lines used to study batFLUAV tropism.

Name	Species	Organ
HEK-293T	Human (*Homo sapiens*)	Kidney
Huh7	Human (*Homo sapiens*)	Liver
Vero	African green monkey (*Chlorocebus aethiops*)	Kidney
MDCK	Dog (*Canis lupus familaris*)	Kidney
RoNi/7	Egyptian fruit bat (*Rousettus aegyptiacus*)	Kidney
HypNi/1.1	Hammer-headed fruit bat (*Hypsignathus monstrosus*)	Kidney
EidNi/41	Straw-colored fruit bat (*Eidolon helvum*)	Kidney
EpoNi/22.1	Buettikofer's epauletted fruit bat (*Epomops buettikoferi*)	Kidney
CpKd	Seba's short-tailed bat (*Carollia perspicillata*)	Kidney

doi:10.1371/journal.pone.0152134.t001

were detached by either resuspension in fresh culture medium (HEK-293T cells) or by the use of trypsin/EDTA (PAA Laboratories).

Plasmids

Shuttle vectors harboring codon-optimized (for expression in human cells) open reading frames coding for the published amino acid (aa) sequences of the HAL of the two batFLUAV, A/little yellow-shouldered bat/Guatemala/153/2009 (H17/N10) (GenBank: CY103876.1, HAL17) and A/flat-faced bat/Peru/033/2010 (H18/N11) (GenBank: CY125945.1, HAL18), were purchased from a commercial service (Eurofins MWG Operon) and cloned into the pCG1 expression vector, that was kindly provided by R. Cattaneo, via *BamH*I and *Xba*I restriction sites. The pCAGGS-based expression plasmid for NAL of batFLUAV A/little yellow-shouldered bat/Guatemala/153/2009 (H17/N10) (GenBank: CY103878.1, NAL10) was provided by M. Schwemmle. HAL equipped with a C-terminal FLAG epitope (DYKDDDDK, HAL17-FLAG and HAL18-FLAG) were constructed by PCR and controlled for sequence integrity by automated sequence analysis. In addition, we used pCAGGS-based expression plasmids for the HA and NA of A/WSN/33 (H1N1) and A/Singapore/1/57 (H2N2) (GenBank: AY209895.1) [32, 33]. Furthermore, we employed pCAGGS-based expression plasmids for the HA of A/South Carolina/1/18 (H1N1) (GenBank: AF117241.1) and the NA of A/Brevig Mission/1/1918 (H1N1) (GenBank: AF250356.2) that were generated from previously used constructs [32]. The expression plasmid for the glycoprotein (G) of vesicular stomatitis virus (VSV, Indiana strain, VSV-G; GenBank: AJ318514.1) was generated by inserting the VSV-G ORF into the pCG1 expression vector and has been used in previous studies [28, 31, 34, 35]. Furthermore, expression plasmids for Nipah virus fusion protein (F, GenBank: AF212302) and glycoprotein (G, synthetic, GenBank: AF212302; derived from NiV/MY/HU/1999/CDC) were used [36]. For experiments analyzing proteolytic activation of HAL17 and HAL18 by human type-II transmembrane serine proteases (TTSPs), we employed expression plasmids for TMPRSS2, TMPRSS11E (DESC-1) and TMPRSS13 (MSPL), which have been described previously [32, 33].

Production of rhabdoviral pseudotypes

We employed a replication-deficient VSV vector for pseudotyping that contains two separate open reading frames, coding for enhanced green fluorescent protein (EGFP) and firefly luciferase (fLuc), instead of the genetic information for VSV-G, VSV*ΔG-fLuc [28, 31, 35]. Propagation of VSV*ΔG-fLuc was performed in a previously described VSV-G-expressing, transgenic cell line [37]. Generation of VSV pseudotypes (VSVpp) was performed as follows: HEK-293T

cells were transfected by calcium-phosphate precipitation with expression plasmids encoding viral surface proteins, VSV-G (positive control), NiV-F/G, FLUAV-HA and/or NA and bat-FLUAV-HAL and/or NAL, or empty plasmid (pCAGGS) as negative control. In order to investigate the potential of human TTSPs to proteolytically activate batFLUAV-HAL for host cell entry, we additionally cotransfected the cells with expression plasmids for TMPRSS2, DESC-1 or MSPL. At 16 h post transfection, the cells were inoculated with VSV*ΔG-fLuc at a multiplicity of infection of 3 for 1 h at 37°C and 5% CO_2. Subsequently, the cells were washed and incubated with an anti-VSV serum to neutralize residual input virus. Finally, the cells received fresh culture medium and were further incubated for 16–20 h, before the VSVpp-containing supernatants were collected, clarified from cell debris by centrifugation and aliquoted. Aliquots were stored at 4°C for a maximum of 7 days.

For proteolytic activation of HA/HAL by trypsin, pseudotypes were incubated with bovine trypsin (Sigma-Aldrich; final concentration: 50 μg/ml) for 20 min at 37°C. Subsequently, trypsin was inactivated by addition of soybean trypsin inhibitor (Sigma-Aldrich; final concentration: 50 μg/ml).

Treatment of cell lines with neuraminidase and ammonium chloride

To investigate the roles of sialic acids and endosomal acidification in batFLUAV entry, we used recombinantly-produced, bacterial sialidase (*Clostridium welchii*, Sigma-Aldrich) and ammonium chloride (Sigma-Aldrich). For treatment, the cell culture supernatant was removed and the cells were washed with phosphate buffered saline (PBS) before culture medium containing water (negative control), ammonium chloride (50 mM) or different concentrations of bacterial sialidase (1.5, 15 or 150 mU) was added. After 2 h of incubation at 37°C and 5% CO_2, the supernatant was removed, the cells washed and then inoculated with pseudotypes, as described below.

Transduction of cell lines with rhabdoviral pseudotypes and quantification of fLuc activity

All transduction experiments were performed in 96-well plates using quadruplicate samples. At 24 h post seeding, the cell culture medium was removed and the cells were washed with PBS. The cells were either directly inoculated with VSVpp or treated as specified above and then inoculated. VSVpp inoculation was performed for 1 h at 37°C and 5% CO_2. Afterwards, the inoculum was removed and the cells were again washed and incubated with fresh culture medium for 16–18 h at 37°C and 5% CO_2. For the quantification of the fLuc activity as an indicator of transduction efficiency, the cell culture supernatant was removed and the cells were washed with PBS. Next, 50 μl of 1x Luciferase Cell Culture Lysis Reagent (Promega) in PBS was added to each well and incubated for 30 min at room temperature, before the cell lysate was transferred to a white, opaque-walled 96-well plate (Thermo Scientific). The measurement of the fLuc activity was carried out in a microplate reader, Plate CHAMELEON (Hidex), using the MicroWin2000 software (version 4.44, Mikrotek Laborsysteme GmbH) and fLuc substrates from the Luciferase Assay System (Promega) or Beetle-Juice (PJK) kits. Transduction efficiency, represented by fLuc activity, was either displayed in counts per second (cps) or as normalized values.

Immunofluorescence analysis of HAL expression

To assess expression of HAL proteins, we transfected BHK-21 cells that were grown on coverslips with expression plasmids for HAL17-FLAG or HAL18-FLAG using the Lipofectamine2000 reagent (ThermoFisher Scientific) according to manufacturers' protocol. Cells

transfected with an empty expression vector served as negative control. At 24 h post transfection, cells were fixed with 4% paraformaldehyde/PBS, permeabilized by incubation with 0.2 M Triton X-100/PBS (10 min at room temperature) and subsequently incubated with anti-FLAG (mouse, 1:1,000, Sigma-Aldrich) and Cy3-labeled anti-mouse (1:750, Sigma-Aldrich) antibodies. After each antibody incubation, the cells were washed three times with PBS and finally incubated with DAPI (Roth, 5 min/37°C) to stain the nuclei before the coverslips were mounted on glass slides using Mowiol (AppliChem) supplemented with anti-bleaching reagent (DABCO, Roth). Representative pictures were taken at a 10x magnification using a Nikon Eclipse Ti fluorescence microscope and the NIS Elements AR software (Nikon).

Preparation of cell lysates and rhabdoviral pseudotypes for Western blot analysis

BatFLUAV-HAL cleavage was investigated by cotransfection of HEK-293T cells, which were grown in 6-well plates, with expression plasmids for batFLUAV-HAL and different TTSPs (TMPRSS2, DESC-1 or MSPL) or by incubation of HAL-expressing cells with trypsin (1, 5, 10, 50 μg/ml; 20 min/37°C) directly before cell lysates were produced. Cells cotransfected with empty plasmid and not subjected to trypsin treatment served as negative control. The 1918-HA served as positive control, since cleavage by TTSPs and trypsin has been previously shown [33]. At 24 h post transfection, the cells were washed with PBS, resuspended in 100 μl 2x 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 20 min at 96°C. To assess incorporation of HAL into VSVpp, 1 ml of the respective pseudotypes was loaded onto a 20% sucrose cushion in PBS and centrifuged at 17,000x g for 2 h at 4°C. After discarding the supernatant, the pelleted pseudotypes were mixed with 30 μl 2x SDS-containing lysis buffer and boiled for 20 min at 96°C. Finally, all samples were subjected to immunoblot analysis. For this, anti-FLAG (mouse, 1:1,000, Sigma-Aldrich), anti-FLUAV (goat, 1:1,000, Millipore), anti-VSV-M (mouse, 1:1,000, Kerafast), anti-VSV-G (I1, mouse hybridoma supernatant from CRL-2700, ATCC, 1:200) and anti-ß-actin (mouse, 1:1,000, Sigma-Aldrich) served as primary antibodies. Peroxidase-coupled anti-mouse (1:10,000, Dianova) and anti-goat (1:5,000, Dianova) antibodies served as secondary antibodies. Signal detection was carried out in a ChemoCam imager together with the ChemoStar professional software (both Intas) using a self-made chemiluminescence substrate (recipe available upon request).

Statistical analysis

In order to assess statistical significance, two-tailed student's t-tests were performed.

Results

Both HAL17 and HAL18 are comparably expressed in mammalian cells and incorporated into rhabdoviral pseudotypes

To test whether HAL17 and HAL18 are comparably expressed and incorporated into rhabdoviral pseudotypes, both proteins were equipped with a C-terminal FLAG epitope (HAL17-FLAG, HAL18-FLAG), since no batFLUAV-HAL-specific antibody was available. Upon transfection of BHK-21 cells similar numbers of HAL-expressing cells were detected by fluorescence microscopy (Fig 1A) and the intensity of the fluorescence signals emitted by the cells was comparable, indicating robust expression of both batFLUAV-HAL proteins. In order to assess HAL incorporation into rhabdoviral pseudotypes, we pelleted pseudotype preparations through a sucrose cushion and subjected the samples to SDS-PAGE and immunoblotting.

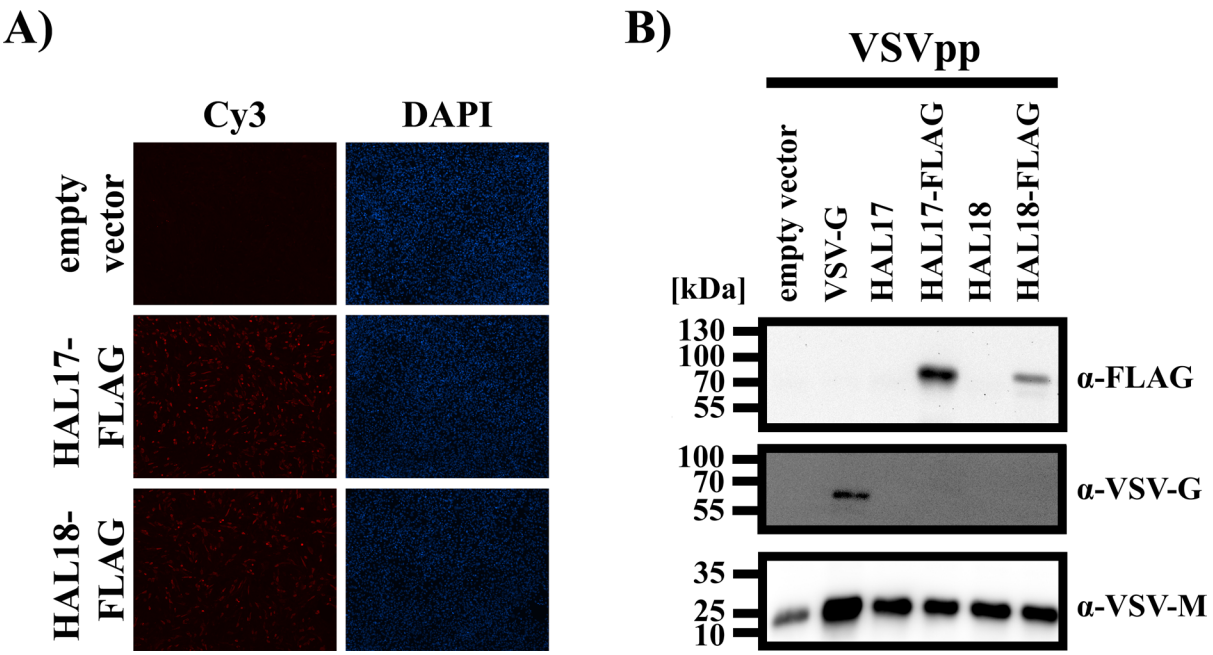

Fig 1. HAL of batFLUAV are robustly expressed and incorporated into rhabdoviral particles. (A) BHK-21 cells were transfected with the indicated HAL proteins harboring a C-terminal FLAG antigenic tag and protein expression was analyzed by immunofluorescence microscopy of permeabilized cells (magnification, 10x). Cells transfected with an empty expression vector served as negative control. Nuclei were visualized by DAPI staining. Similar results were obtained in a separate experiment. (B) For analysis of HAL incorporation into rhabdoviral particles, vesicular stomatitis virus (VSV)-based pseudotypes were pelleted though a 20% sucrose cushion and analyzed by SDS-PAGE and Western blotting with antibodies against the FLAG tag (α-FLAG), VSV glycoprotein (α-VSV-G) and matrix protein (α-VSV-M). The numbers on the left side of the blots indicate the molecular weight in kilo Daltons (kDa). The results were confirmed in an independent experiment.

doi:10.1371/journal.pone.0152134.g001

Using antibodies specific for the FLAG epitope, VSV-G and VSV matrix protein (VSV-M), we found that VSV-G, as expected, as well as both HAL17 and HAL18 proteins were incorporated into particles, with incorporation of HAL17 being more efficient than that of HAL18. (Fig 1B). Thus, both HAL17 and HAL18 were robustly expressed and incorporated into VSVpp, allowing their functional characterization.

HAL of batFLUAV mediates cellular entry into bat cell lines and entry depends on proteolytic activation

It has been previously reported that HAL and NAL of batFLUAV are not compatible with viral spread in the cell lines tested so far [18, 19], suggesting that these proteins mediate entry into a restricted set of target cells or are even inactive. In order to gain insights into the functional activity of batFLUAV-HAL and NAL, we employed rhabdoviral vectors pseudotyped with these proteins. We inoculated cell lines from different host species, including those standardly used for FLUAV research, as well as cell lines derived from different bat species (Table 1), as they are the natural reservoir for batFLUAV. We chose bat cell lines known to be susceptible to infection by viruses of different families [28, 29, 31] and previously used to functionally characterize surface glycoproteins of bat-borne viruses [28, 36, 38]. It is well established that FLUAV-HA depends on proteolytic cleavage by host cell proteases to transit into an active form [12] and it has been previously reported that batFLUAV can be activated by exogenous trypsin [39–41]. Therefore, we assessed whether trypsin treatment of the pseudotypes impacts transduction efficiency. As controls, we included the surface glycoprotein(s) of well-characterized FLUAV strains, A/WSN/33 (H1N1) (WSN-HA, WSN-NA), A/South Carolina/1/18

(H1N1) (1918-HA), A/Brevig Mission/1/1918 (H1N1) (1918-NA), and A/Singapore/1/57 (H2N2) (H2N2-HA, H2N2-NA), as well as the glycoproteins of Nipah virus (NiV-G, NiV-F) and vesicular stomatitis virus (VSV-G).

We found that none of the human, simian and canine cell lines was susceptible to entry driven by batFLUAV surface proteins (Fig 2A). In contrast, pseudotypes bearing VSV-G, NiV-F/G or WSN-HA/NA could readily enter these cells, whereas pseudotypes that harbored 1918- or H2N2-HA/NA required activation by exogenous trypsin for efficient transduction (Fig 2A). These results are in agreement with expectations, since activation of WSN-HA is known to be independent of trypsin [42–44], although viral infectivity can be enhanced by trypsin treatment.

When we focused on bat-derived cell lines, VSV-G, NiV-F/G and WSN-HA/NA again permitted pseudotype entry without prior trypsin treatment, while pseudotypes harboring 1918-HA/NA or H2N2-HA/NA were only able to transduce some of the bat cell lines after incubation with trypsin (Fig 2B). CpKd cells remained refractory to entry mediated by 1918-HA/NA or H2N2-HA/NA but also showed the lowest susceptibility to all other tested pseudotypes. Notably, three bat cell lines (EidNi/41, HypNi/1.1 and EpoNi/22.1) were susceptible to entry of pseudotypes bearing HAL and NAL of batFLUAV (Fig 2B), demonstrating that surface glycoproteins of batFLUAV can mediate cellular entry. Entry into the three bat cell lines was robust (1–3 log units above the threshold) and required prior treatment of pseudotypes with trypsin, which presumably resulted in the proteolytic activation of HAL. Furthermore, pseudotypes bearing HAL17/NAL10 or HAL18/NAL10 were both able to enter HypNi/1.1 and EpoNi/22.1 cells while EidNi/41 cells could only be transduced by pseudotypes bearing HAL18/NAL10 (Fig 2B), suggesting that batFLUAV of the HL17NL10 and HL18NL11 subtype might exhibit subtle differences in entry efficiency or cell tropism. Finally, HAL-proteins with a C-terminal FLAG tag facilitated host cell entry, although with somewhat reduced efficiency as compared to their untagged counterparts, indicating that the proteins used to study HAL expression and virion incorporation (Fig 1) were functional (data not shown). Taken together, we showed that batFLUAV surface proteins can mediate entry into certain bat cell lines. For further studies on the entry process, we focused on EpoNi/22.1 cells since they showed the highest susceptibility to entry driven by batFLUAV surface proteins.

Expression of batFLUAV-NAL in pseudotype producing cells does not impact particle infectivity

The NA proteins of human FLUAV facilitate release of progeny particles from infected cells by removing sialic acids from the cell surface. To study the impact of the batFLUAV-NAL on transduction efficiency, we produced pseudotypes bearing batFLUAV-HAL, -NAL or both proteins. For comparison, we generated pseudotypes harboring WSN-HA, WSN-NA or both proteins. These pseudotypes were then used for inoculation of MDCK (inoculated with pseudotypes bearing WSN proteins) and EpoNi/22.1 (inoculated with pseudotypes bearing WSN or batFLUAV proteins) cells. Pseudotypes harboring only WSN-NA were not able to transduce target cells (Fig 3) while pseudotypes bearing either WSN-HA alone or in combination with WSN-NA transduced both MDCK and EpoNi/22.1, as expected. Transduction efficiency was ~500–1,500-fold higher when WSN-NA was expressed in cells used for pseudotype production, in keeping with the findings that the presence of NA is required for efficient release of HA-bearing vectors and infectious FLUAV [32, 45, 46]. Pseudotypes harboring NAL were not infectious while pseudotypes bearing HAL robustly transduced EpoNi/22.1 cells, indicating that batFLUAV-HAL, like WSN-HA, is sufficient to mediate host cell entry. However, unlike WSN-NA, the expression of NAL in pseudotype producer cells did not increase transduction

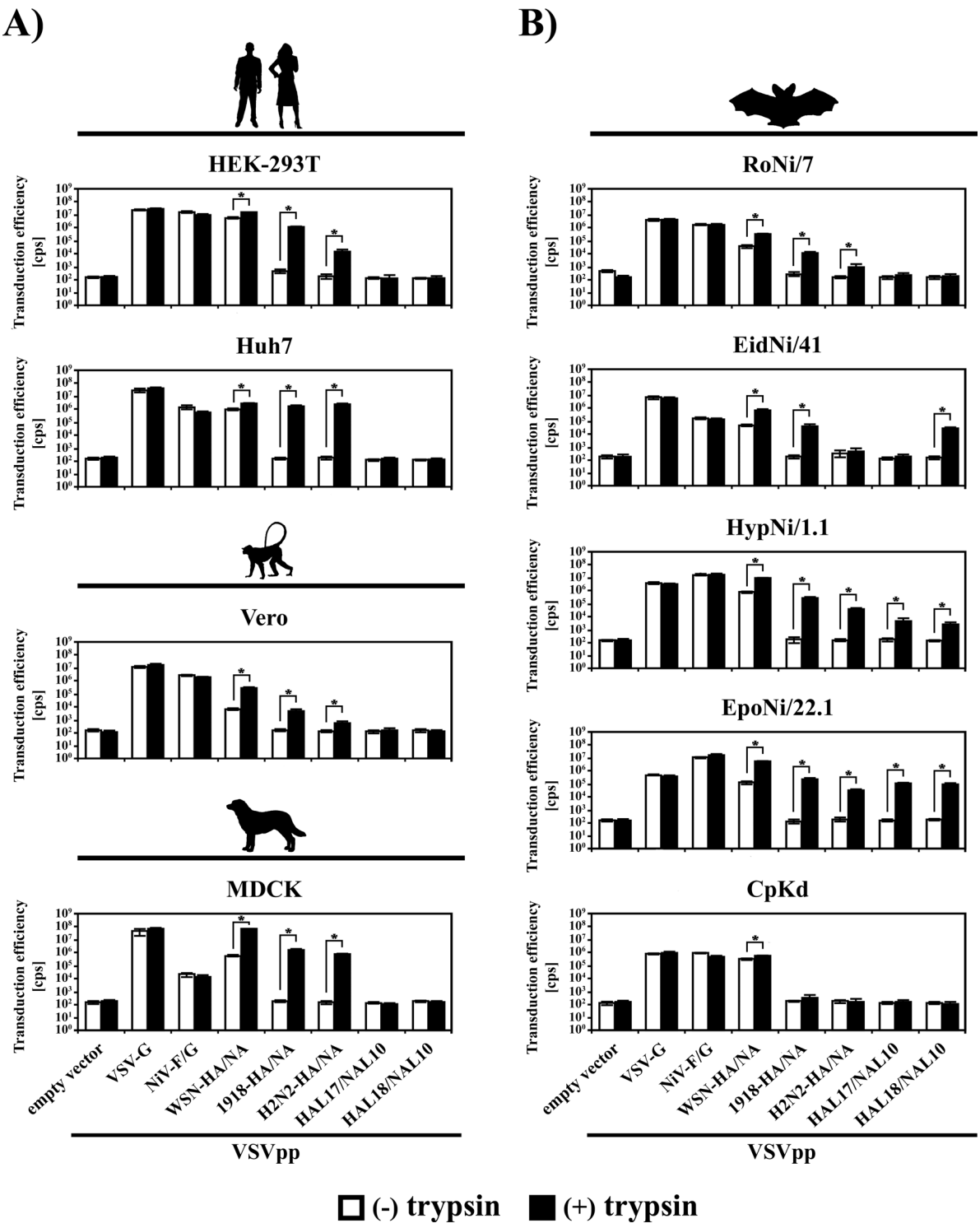

Fig 2. Surface glycoproteins of batFLUAV enable pseudotype entry into different bat but not human, simian and canine cell lines. Vesicular stomatitis virus-based pseudotypes (VSVpp) harboring no or the indicated viral glycoproteins were either left untreated (white bars) or treated with trypsin (black bars), before they were inoculated onto mammalian cell lines of human (HEK-293T, Huh7), simian (Vero) and canine (MDCK) origin (A) or bat cell lines (RoNi/7, EidNi/41, HypNi/1.1, EpoNi/22.1, CpKd) representing five different bat species (B). At 18–20 h post inoculation, the transduction efficiency was measured by quantification of the activity of the VSVpp-encoded luciferase (given as counts per second, cps, on a logarithmic scale). The result of a single representative experiment carried out with quadruplicate samples is shown. Similar results were obtained in four independent experiments carried out with separate pseudotype preparations. Error bars indicate standard deviations. A two-tailed, unpaired student's t-test was used to test statistical significance (* = $p < 0.05$).

doi:10.1371/journal.pone.0152134.g002

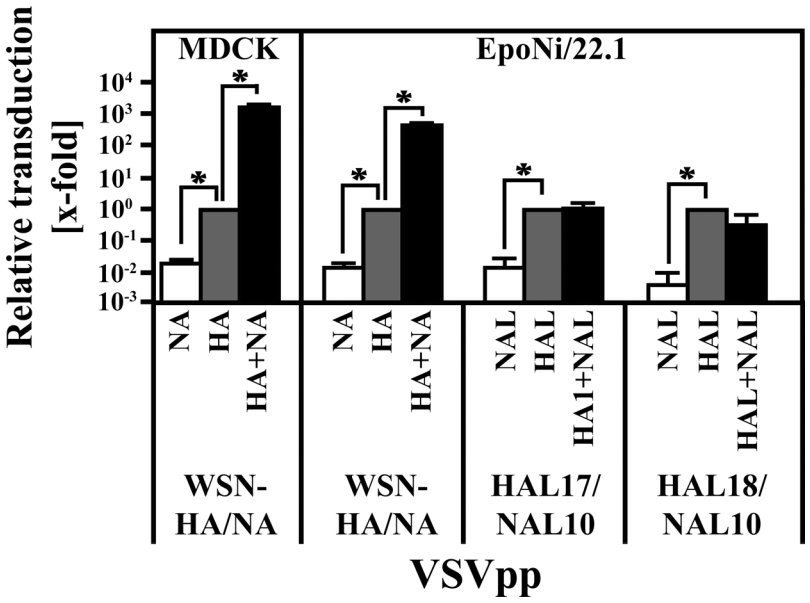

Fig 3. NAL of batFLUAV has no impact on the transduction efficiency of vectors bearing HAL. Vesicular stomatitis virus-based pseudotypes (VSVpp) harboring the indicated surface proteins of FLUAV or batFLUAV were treated with trypsin before inoculation of MDCK and EpoNi/22.1 cells. At 18–20 h post inoculation, the transduction efficiency was measured by quantification of the activity of the VSVpp-encoded luciferase. The combined data from three independent experiments (quadruplicate samples) with separate pseudotype preparations are shown. Transduction efficiencies were normalized against pseudotypes harboring only HA or HAL (set as 1) and are given as x-fold changes on a logarithmic scale. Error bars indicate standard error of the mean. A two-tailed, paired student's t-test was used to test statistical significance (* = $p < 0.05$).

doi:10.1371/journal.pone.0152134.g003

efficiency of HAL-harboring pseudotypes (Fig 3), suggesting that NAL is not required for release and/or infectivity of HAL containing particles, at least in the experimental system chosen.

BatFLUAV-HAL does not use sialic acids for host cell entry

FLUAV employ alpha-2,3- (avian viruses) and alpha-2,6-linked (human viruses) sialic acids as receptors for host cell entry [47–51]. In order to assess the potential role of sialic acids in HAL-driven entry, we pre-treated the cells with escalating doses of bacterial neuraminidase before transduction. Neuraminidase treatment of EpoNi/22.1 cells reduced transduction mediated by the FLUAV-HA proteins, as expected (Fig 4). In contrast, sialidase treatment had no effect on pseudotype entry mediated by batFLUAV-HAL, NiV-F/G or VSV-G. Moreover, pre-treatment of EpoNi/22.1 cells at the highest dose (150 mU) even enhanced transduction driven by HAL and VSV-G (Fig 4). These results indicate that HAL does not use sialic acids for host cell entry and suggest that removal of sialic acids might even increase batFLUAV infectivity, potentially by increasing accessibility of a cellular receptor.

HAL-driven entry of batFLUAV relies on endosomal acidification

Endosomal low pH triggers FLUAV-HA for membrane fusion. Therefore, we investigated whether increasing the endosomal pH in EpoNi/22.1 cells by ammonium chloride treatment impacts HAL-driven entry. As expected, ammonium chloride treatment led to a decrease in transduction efficiency mediated by pseudotypes bearing the HA-proteins of FLUAV of the H1N1 and H2N2 subtype and VSV-G (Fig 5). In contrast, pseudotype entry orchestrated by

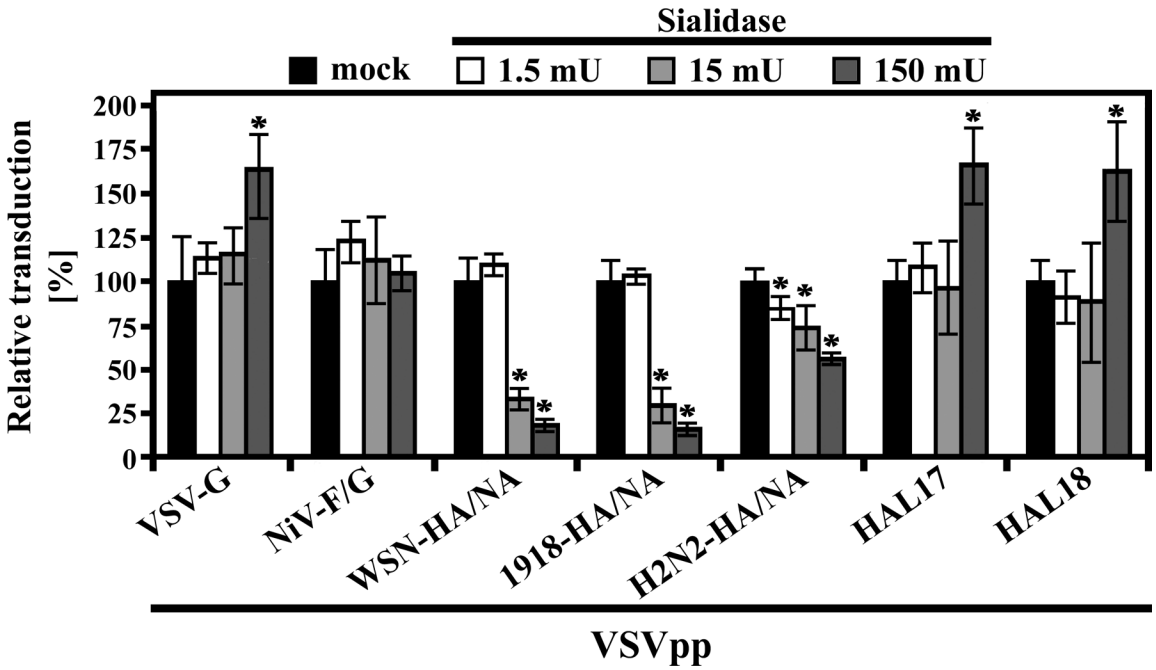

Fig 4. HAL of batFLUAV does not require sialic acids for host cell entry. EpoNi/22.1 cells were incubated for 1.5 h in the absence (black bars) or presence (white and grey bars) of increasing concentrations of exogenous sialidase and were subsequently inoculated with trypsin-treated vesicular stomatitis virus-based pseudotypes (VSVpp) harboring the indicated viral glycoproteins. At 1 h post inoculation, the inoculum was removed, the cells were washed and further incubated for 18–20 h with fresh medium before transduction efficiency was measured by quantification of the activity of VSVpp-encoded luciferase. Transduction of sialidase-treated cells is shown relative to that measured for mock-treated cells (on a linear scale), which was set at 100%. The result of a single representative experiment carried out with quadruplicate samples is shown. Similar results were obtained in two independent experiments carried out with separate pseudotype preparations. Error bars indicate standard deviations. A two-tailed, unpaired student's t-test was used to test statistical significance of differences measured for sialidase- versus mock-treated samples (* = $p < 0.05$).

doi:10.1371/journal.pone.0152134.g004

NiV-F and -G was unaffected, again in keeping with published data [52, 53]. Finally, HAL-driven entry was markedly reduced by ammonium chloride, demonstrating that the membrane fusion activity of batFLUAV-HAL is triggered by acidification (Fig 5).

TMPRSS2 activates batFLUAV-HAL

FLUAV-HA is synthesized as an inactive precursor and requires activation by host cell proteases to be responsive to low pH, the trigger for HA-driven membrane fusion [12–14]. Members of the TTSP family activate FLUAV-HA in cell culture [33, 54–58] and TMPRSS2 was previously shown to be essential for FLUAV-HA activation and viral spread in mice [59]. Therefore, we asked whether TTSPs able to activate FLUAV-HA can also activate batFLUAV-HAL. For this, we first investigated batFLUAV-HAL cleavage by TMPRSS2, DESC-1 and MSPL, and compared it to cleavage by trypsin. Cleavage of the 1918-HA served as positive control. We found that 1918-HA was efficiently processed by all proteases tested, as expected. Moreover, we could show that coexpression of TMPRSS2, DESC-1 and MSPL, and trypsin treatment resulted in cleavage of the HAL precursor (HAL_0) determined by the appearance of bands corresponding to the HAL_2 subunit (Fig 6A). While HAL18 was comparably cleaved by all tested TTSPs, HAL17 cleavage by TMPRSS2 was more pronounced than proteolysis by DESC-1 and MSPL (Fig 6A). Moreover, HAL18 was generally more sensitive to cleavage by TTSPs than HAL17 (Fig 6A). In order to assess whether batFLUAV-HAL cleavage by TTSPs also leads to HAL activation for host cell entry, we produced pseudotypes harboring batFLUAV-HAL (HAL17 or HAL18) in the presence of TMPRSS2, DESC-1 and MSPL. As a control,

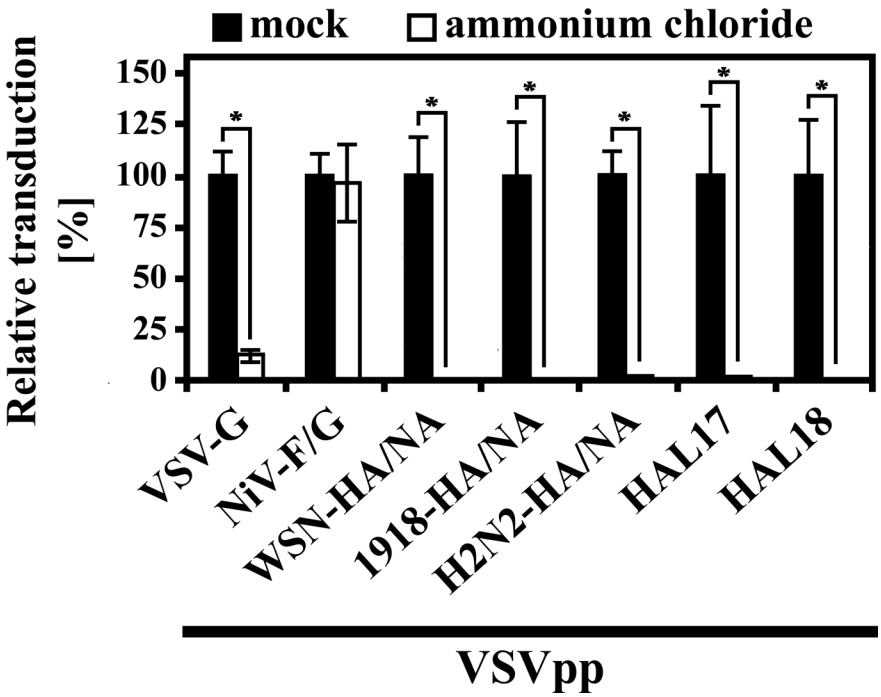

Fig 5. Entry driven by batFLUAV-HAL relies on an acidic pH. EpoNi/22.1 cells incubated for 3 h in the absence (black bars) or presence (white bars) of ammonium chloride (50 mM) were subsequently inoculated with trypsin-treated vesicular stomatitis virus-based pseudotypes (VSVpp) harboring the indicated viral glycoproteins. At 1 h post inoculation, the inoculum was removed and the cells were further incubated for 18–20 h in the presence or absence of ammonium chloride before transduction efficiency was measured by quantification of the activity of VSVpp-encoded luciferase. For each of the different pseudotypes, transduction efficiency (given as percentage on a linear scale) was normalized against the respective control (water). The result of a single representative experiment carried out with quadruplicate samples is shown. Similar results were obtained in two independent experiments carried out with separate pseudotype preparations. Error bars indicate standard deviations. A two-tailed, unpaired student's t-test was used to test statistical significance (* = $p < 0.05$).

doi:10.1371/journal.pone.0152134.g005

pseudotypes bearing 1918-HA and -NA were included in this experiment. The pseudotypes were treated with trypsin to activate HA/HAL or were mock-treated before addition to EpoNi/22.1 cells. Pseudotypes bearing 1918-HA and -NA and produced in the presence of TMPRSS2, DESC-1 and MSPL or treated with trypsin robustly transduced target cells (Fig 6B). In contrast, infectivity of FLUAV-HA pseudotypes produced in the absence of TTSPs or not treated with trypsin was in the background range (Fig 6B). Similarly, batFLUAV-HAL-bearing pseudotypes were activated by trypsin or TTSPs, including TMPRSS2 (Fig 6B). However, differences in the activation of HAL17 and HAL18 were observed and correlated with the efficiency of HAL protein cleavage, as determined above (Fig 6A). Thus, expression of TMPRSS2 but not DESC-1 and MSPL conferred robust infectivity to HAL17-bearing pseudotypes while all proteases were able to efficiently activate HAL18. Moreover, transfection of escalating amounts of TMPRSS2-encoding plasmids increased infectivity of HAL17-bearing pseudotypes in a concentration-dependent manner. In contrast, transfection of even the lowest amount of TMPRSS2 plasmid was sufficient to confer maximal infectivity of HAL18-bearing pseudotypes, confirming that the efficiency of TMPRSS2-mediated activation of HAL is subtype specific (Fig 6C). In sum, proteolytic activation of batFLUAV-HAL is critical for HAL-driven cell entry and proteases able to activate HA can also activate HAL.

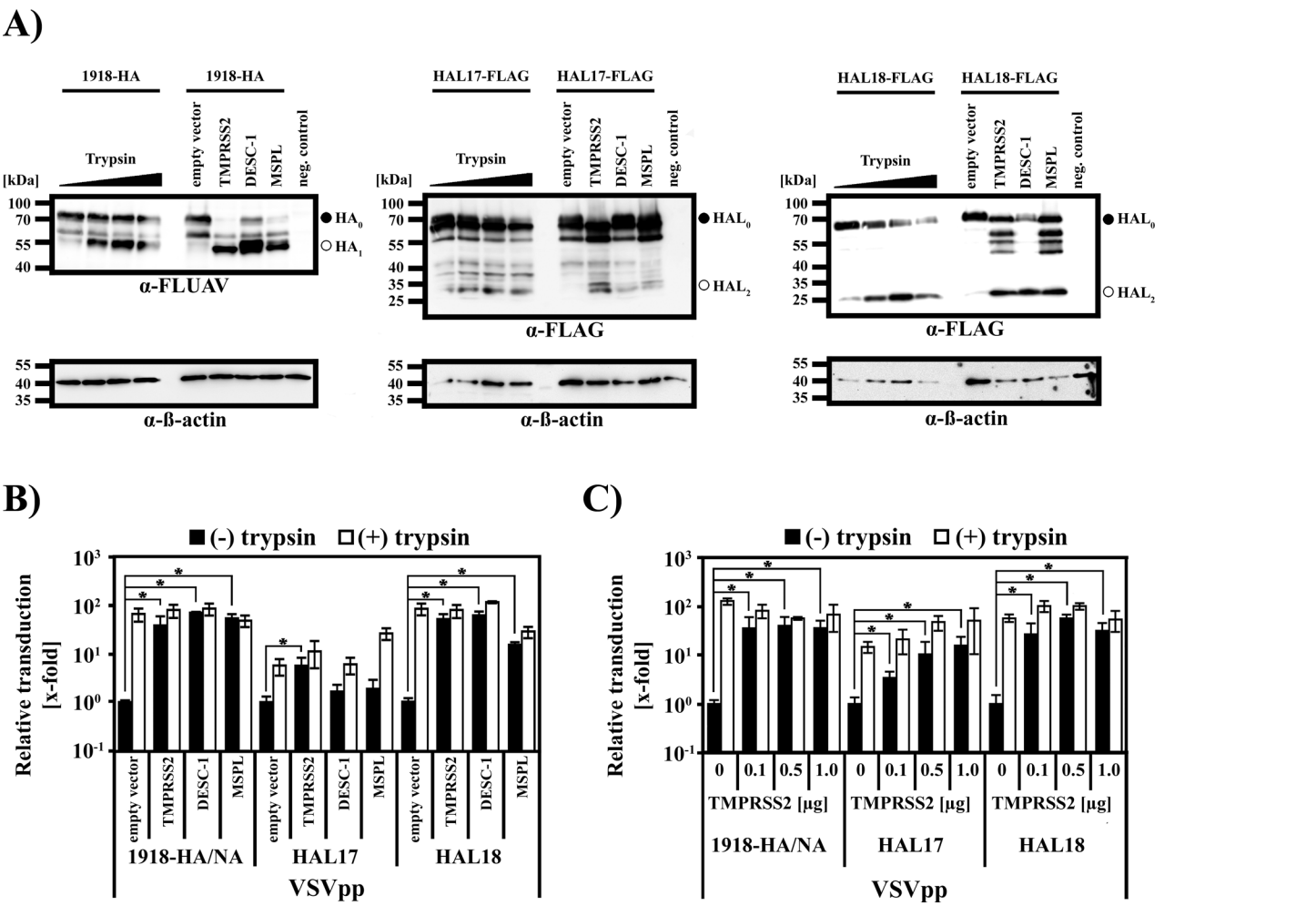

Fig 6. Human proteases that activate FLUAV-HA for cell entry also activate batFLUAV-HAL. (A) HEK-293T cells were transfected with plasmids encoding HA or HAL proteins and either trypsin treated or cotransfected with plasmids encoding type II transmembrane serine proteases. Transfection of empty vector served as negative control. Cleavage of HA/HAL proteins was analyzed by SDS-PAGE and Western blotting, employing antibodies against FLUAV-HA (α-FLUAV) and the FLAG epitope (α-FLAG). Detection of ß-actin served as loading control. Signals corresponding to uncleaved precursor proteins are marked by black circles, while products of proteolytic cleavage are indicated by white circles. The results were confirmed in a separate experiment. To assess proteolytic activation of HA/HAL proteins, vesicular stomatitis virus-based pseudotypes (VSVpp) were produced in cells transfected to express the indicated type II transmembrane serine proteases (B) or different amounts of TMPRSS2 (C). Pseudotypes were either directly used for transduction of EpoNi/22.1 cells (black bars) or previously treated with trypsin (white bars). At 24 h post inoculation, transduction efficiency was measured by quantification of the activity of VSVpp-encoded luciferase in cell lysates. For normalization, transduction by HA- or HAL-bearing pseudotypes that were produced in the absence of type II transmembrane serine protease expression (empty vector) and not treated with trypsin was set as 1. The result of a single representative experiment carried out with quadruplicate samples is presented. Similar results were obtained in three independent experiments carried out with separate pseudotype preparations. Error bars indicate standard deviations. A two-tailed, unpaired student's t-test was used to test statistical significance (* = $p < 0.05$).

doi:10.1371/journal.pone.0152134.g006

Discussion

The identification of two new FLUAV, subtypes H17N10 (HL17NL10) and H18N11 (HL18NL11), in new-world bats [16, 17] suggests that bats could serve as a natural reservoir of FLUAV [16, 17]. Unraveling the zoonotic potential of batFLUAV is of great importance since FLUAV are major human pathogens, responsible for influenza epidemics and pandemics. While the batFLUAV replication machinery appears to be functional in different mammalian (including human) cells [16, 18–21, 60], reassortment with FLUAV and FLUBV is unlikely [18, 19]. Regarding batFLUAV tropism of the viral surface proteins, HAL and NAL, only

limited information is available, which indicate that batFLUAV do not engage with canonical FLUAV receptors [17, 22–24]. However, until very recently no proof of functional activity of batFLUAV surface proteins was available [17, 22–27]. Here, we employed a vector system to analyze batFLUAV-HAL and -NAL. We show that HAL mediates entry into certain bat cell lines and that entry does not depend on the presence of sialic acids on the cell surface. Moreover, we demonstrate that NAL is not required for production of infectious HAL-bearing particles, at least under the conditions examined. Finally, our studies revealed that trypsin and TTSPs activate HAL for host cell entry.

We used a VSV-based vector system to study cellular entry of HAL and NAL-bearing particles. VSV pseudotypes allow convenient analysis of entry driven by diverse glycoproteins [28, 31, 61], although one should keep in mind that due to differences in particle shape and efficiency of glycoprotein incorporation pseudotypes might not adequately mirror all aspects of cellular entry of authentic viruses [62, 63]. We found that cell lines frequently used for FLUAV propagation were not susceptible to transduction by HAL and NAL bearing particles, which is in agreement with the finding that replacement of batFLUAV-HAL and -NAL by their FLUAV counterparts is required for spread of batFLUAV in the cell lines studied so far [18, 19]. In contrast, inoculation of bat cell lines originating from five different species of micro- and megachiropteran bats revealed that three cell lines, EidNi/41, HypNi/1.1, EpoNi/22.1, were susceptible to entry mediated by batFLUAV surface proteins. EpoNi/22.1 cells showed the highest susceptibility and were thus used for further studies, while EidNi/41 cells were found to be robustly susceptible only to transduction by pseudotypes harboring the HAL18. Collectively, these findings suggest that batFLUAV surface proteins can mediate entry into certain bat cells and that entry efficiency might differ between batFLUAV subtypes. Our finding that certain bat cell lines are susceptible to pseudotypes harboring HAL of batFLUAV are in line with observations very recently documented by Maruyama *et al.* who found that out of a diverse panel of bat cell lines tested, cells from *Miniopterus fuliginosus*, *Miniopterus schreibersii* and *Pteropus giganteus* were susceptible to pH-dependent, HAL-driven entry [64]. A cell line derived from *Eidolon helvum* spleen cells was found to be non-susceptible in contrast to our findings with a kidney cell line established from the same species, suggesting that receptor expression might differ between organs. Somewhat more surprising, Maruyama and colleagues also observed HAL-driven entry into MDCK cells [64], which was not detected in the present study, and these discrepant results might be attributed to use of MDCK cells from different sources or to differences in the method used to quantify pseudotype entry. Finally, it is noteworthy that cell lines from bats inhabiting different geographical locations (Africa, Asia, Europe) were found to be susceptible to HAL-driven entry, suggesting that entry is not a bottleneck for spread of batFLUAV between bat species.

The finding that batFLUAV surface proteins can facilitate entry into bat-derived target cells allowed us to investigate which viral and cellular components contribute to the entry process. We first focused on NAL. The expression of this protein, unlike expression of NA, in pseudotype-producing cells did not increase the titers of vectors harboring the corresponding batFLUAV-HAL protein. However, this finding does not exclude that NAL, like the NA of FLUAV, acts as a receptor-destroying enzyme. Thus, HEK-293T cells used for pseudotype preparation were not susceptible to HAL-driven entry, most likely because they do not express the appropriate receptor. It thus remains to be analyzed whether batFLUAV-NAL increases release of HAL-bearing vectors and authentic batFLUAV from susceptible bat cell lines. These endeavors might be challenging since transfection of bat cell lines by calcium-phosphate precipitation and liposome-based reagents was inefficient (not shown).

The FLUAV-HA is sufficient to mediate viral binding and entry into target cells and our findings indicate that the same applies to batFLUAV-HAL. In contrast to FLUAV-HA, however, HAL

does not depend on the presence of sialic acids for entry. Thus, treatment of EpoNi/22.1 cells with sialidase did not decrease HAL-mediated pseudotype entry. These findings are in keeping with the work by Maruyama *et al.* [64] and with structural data indicating that HAL does possess a distorted putative sialic acid binding site [17, 24]. Contrarily, high amounts of sialidase increased entry efficiency, potentially by increasing access to the elusive receptor. In addition, removal of sialic acids might increase electrostatic interactions of batFLUAV-HAL with cell surface factors, since sialic acids are negatively charged. Although HAL-driven entry was independent of sialic acids, it did require endosomal acidification (in accordance with Maruyama *et al.* [64]), which is known to trigger the membrane fusion activity of HA. Most likely, protonation also triggers batFLUAV-HAL for membrane fusion. However, it cannot be disregarded that the inhibitory effect of ammonium chloride was due to blockade of pH-dependent endosomal cysteine proteases, which activate the surface proteins of several coronaviruses and ebolaviruses [65–68].

Cleavage-activation of FLUAV-HA by host cell proteases is essential for FLUAV infectivity. Several TTSPs can cleave and activate HA in cell culture and recent studies demonstrated that TMPRSS2 is essential for FLUAV spread in mice [32, 33, 58, 59]. Moreover, polymorphisms in TMPRSS2 were shown to impact severity of influenza in humans [69]. Treatment of batFLUAV-HAL-expressing cells with trypsin led to proteolytic cleavage of HAL and exposure of HAL-bearing pseudotypes to trypsin was required for efficient transduction of target cells, indicating that proteolytic processing is also required for HAL function. Moreover, coexpression of batFLUAV-HAL with TMPRSS2, DESC-1 or MSPL resulted in proteolytic cleavage of HAL and rendered the particles infectious in the absence of trypsin treatment, suggesting that batFLUAV-HAL can utilize human proteases for their activation. Finally, titration of the amounts of TMPRSS2 had differential effects on the proteolytic cleavage of HAL17 and HAL18 and on infectivity of pseudotypes bearing these proteins, hinting towards subtle differences in the efficiency of TMPRSS2 use by these subtypes. Whether bat TMPRSS2 is also able to cleave and activate batFLUAV-HAL remains to be investigated.

Collectively, our results are most compatible with a scenario in which human cells allow for batFLUAV-HAL activation and triggering but fail to express a receptor, which can be employed by HAL for host cell entry. These results, jointly with the documented observation that the batFLUAV replication machinery is functional in human cells [16, 18, 19] suggest that HAL adaptation to a human receptor might be the major hurdle batFLUAV need to overcome to spread in humans. It will thus be highly interesting to identify the nature of this receptor and its interface with batFLUAV-HAL.

Of note, during the preparation of this manuscript, Maruyama and colleagues published a manuscript reporting batFLUAV-HAL-driven entry into bat cell lines different from those used in the present study (Maruyama *et al.*, 2015, doi: 10.1016/j.virol.2015.11.002.). Both studies show that HAL-driven entry requires prior proteolytic HAL-activation by trypsin and endosomal acidification but is independent of sialic acids. The present work extends these findings by demonstrating that HAL can utilize the human enzyme TMPRSS2 for its activation.

Acknowledgments

We would like to thank C. Drosten, M. A. Müller and M. Schwemmle for cell lines and expression plasmids. Furthermore, we thank E. Berger and I. Nehlmeier for excellent technical support.

Author Contributions

Conceived and designed the experiments: MH NK GH SP. Performed the experiments: MH NK PZ FW. Analyzed the data: MH NK PZ FW GH SP. Wrote the paper: MH SP.

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