Understanding the bat as a host of zoonotic viruses: biosurveillance of bat populations in Southeast Asia and examination of the autophagy pathway as an antiviral mechanism in bats

by

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DEDICATION

For Robin, always inspirational

The love of complexity without reductionism makes art; the love of complexity with reductionism makes science.

E.O. Wilson, Consilience: The Unity of Knowledge

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ABSTRACT

Understanding the bat as a host of zoonotic viruses: biosurveillance of bat populations in Southeast Asia and examination of the autophagy pathway as an antiviral mechanism in bats

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Zoonotic infectious diseases account for the majority of all recently emerged infectious diseases, including Ebola virus, Marburg virus, Nipah virus, Hendra virus, and SARS-coronavirus. Bats, Order Chiroptera, are the natural host of these viruses. Outbreaks of Ebola virus disease have historically been confined to continental Africa. However, Reston virus, an *Ebolavirus* sp., has origins in the Philippines where it has been isolated from monkey, domestic pig, and bat populations, which suggests that the geographic distribution of ebolaviruses is more extensive than previously thought. In light of the recent outbreak of Ebola virus disease in West Africa, improved biosurveillance of bat populations is critical for understanding the geographic distribution

and potential for spillover events of ebolaviruses and other pathogenic zoonotic viruses. To investigate the geographic distribution of the ebolaviruses, marburgviruses, and henipaviruses, we developed a Luminex-based multiplex binding assay that can be used to detect antibodies specific to the soluble envelope glycoproteins of these viruses. We used this multiplex binding assay to screen bat sera from three Pteropodidae bat species that have vast geographic ranges within Asia for evidence of past exposure to ebolaviruses, marburgviruses, and henipaviruses. We discovered past exposure to viruses that were most antigenically similar to African ebolaviruses in all three Southeast Asian bat species. This discovery corroborates evidence for the geographic distribution of ebolaviruses within the Asian continent and extends this distribution into Southeast Asia.

Unlike most terrestrial mammals, infection of bats species with Ebola virus and Nipah virus does not result in the development of symptomatic disease. How zoonotic viruses persist within bats at the cellular level is incompletely understood. To explore antiviral defenses in bats, we focus on the autophagy pathway, a cellular homeostatic process that has intrinsic immune functions. We compared autophagy responses after infection with Australian bat lyssavirus (ABLV) in cells derived from the Black Flying Fox, *Pteropus alecto*, the natural bat host of ABLV, and human cell lines. We observed that ABLV induced autophagy in both bat and human cell lines. Together, pharmacological and genetic approaches suggested that autophagy has an antiviral role during ABLV infection of bats and humans. Finally, treatment with an autophagy-activating drug was determined to be a potentially protective therapeutic during ABLV infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Bats are reservoirs for emerging viruses

EMERGING VIRUSES

The majority of recently emerging infectious diseases are zoonotic, originating in wildlife and spilling over into human populations through direct contact or through intermediate amplifying wildlife or domestic animal hosts, or vectors (134; 275; 297). The growing human population and resulting land-use changes such as deforestation, urbanization, and agricultural intensification are known drivers of infectious disease spillover events (141; 220; 291). Ecological changes present new opportunities for disease emergence to occur, as spatial barriers of disease transmission between human and wildlife habitats are lifted. A survey of new emerging human pathogens discovered since 1980 found that two-thirds of the 87 new human pathogens are viruses, and that approximately 56% are RNA viruses, and of these RNA viruses 94% are zoonotic (151; 297). Importantly, RNA viruses have high rates of genome mutations, which is thought to contribute to the high degree of host-plasticity and broad species tropisms that more readily permits infection of new hosts, cross-species transmission, and emergence into human populations (151) (119; 298).

ZOONOTIC RNA VIRUSES

Emerging zoonotic RNA viruses from several families have caused epidemics in human communities with high mortalities and in some cases have caused pandemics that have threatened global health security. Notable epidemic zoonotic RNA viruses include influenza A virus subtype H1N1, responsible for 1918 and 2009 pandemics, human immunodeficiency virus (HIV), Ebola virus (EBOV), Hendra virus (HeV), Nipah virus

(NiV), influenza A virus subtype H5N1, severe-acute respiratory syndrome coronavirus (SARS-CoV), and Middle Eastern respiratory coronavirus (MERS-CoV) (Table 1). Not shown in Table 1 is HIV, which has origins as a zoonotic RNA virus and approaches a 100% case fatality rate in the absence of treatment.

Table 1. Select zoonotic RNA virus outbreaks of the 20th and 21st centuries

Year	Virus species	Virus family	Cases (% fatality)	Country of origin	Animal source
1918	Influenza A virus H1N1 ("Spanish flu)	*Orthomyxoviridae*	500 million (10-20%)	Inconclusive hypotheses	Inconclusive hypotheses
1957-1958	Influenza A virus H2N2 ("Asian flu")	*Orthomyxoviridae*	400 million (.1-.5%)	China	Avian, human
1968	Influenza A virus H3N2 ("Hong Kong flu")	*Orthomyxoviridae*	200 million (.1-.5%)	Hong Kong	Avian, swine, human
1976	Zaire ebolavirus (EBOV)	*Filoviridae*	318 (88%)	Democratic Republic of Congo	Bats†
1994	Hendra virus (HeV)	*Paramyxoviridae*	4 (25%)	Australia	Horses* (bats)
1997	Influenza A virus H5N1 ("Avian flu")	*Orthomyxoviridae*	1,000-2,650 (14-33%)	Hong Kong	Avian
1999	Nipah virus (NiV)	*Paramyxoviridae*	265 (40%)	Malaysia	Domestic pigs* (bats)
2001	Nipah virus (NiV)	*Paramyxoviridae*	280 (75%)	Bangladesh	Bats
2002-2003	SARS-coronavirus	*Coronaviridae*	8,098 (10%)	China	Palm civets* (bats)
2009	Influenza A virus H1N1 ("Swine flu")	*Orthomyxoviridae*	200-400 million (<.1%)	Mexico	Swine, human
2012-	MERS-coronavirus	*Coronaviridae*	1,841 (36%)	Saudi Arabia	Camels
2013-2016	Zaire ebolavirus (EBOV)	*Filoviridae*	28,652 (40%)	Guinea	Bats†

Cases and case fatality rates were estimated from various WHO and CDC reports, and the WHO Pandemic Index. Secondary, or amplifying animal host, are indicated with * and the natural host is indicated within parentheses (natural host). Suspected animal sources are indicated with †.

Filoviruses and henipaviruses

The filoviruses and henipaviruses are zoonotic RNA viruses that have emerged from animal reservoirs over the past 70 years (12; 211). The *Filoviridae* family and *Henipavirus* genus each contains virus species that require biosafety-level 4 (BSL-4) containment and are classified as select agents by the United States Department of Health and Human Services. These viral pathogens have been top priorities for biodefense research and ongoing efforts have been focused on improving prevention and detection of outbreaks to minimize the threat posed to global health security by these viruses.

Ebolaviruses

The ebolaviruses, marburgviruses, and cuevaviruses are three genera included in the virus family, *Filoviridae*. The prototypical *Ebolavirus* species, Ebola virus (EBOV), was first recognized as a pathogen in 1976 following an outbreak of viral hemorrhagic fever disease in the Democratic Republic of Congo (2). Severe disease presentation with pathogenic *Ebolavirus* spp. follows a non-specific prodrome of flu-like symptoms such as fever and myalgias however unlike seasonal flu; these symptoms are accompanied by a high fatality rate. Additionally, during EBOV infection patients experience vomiting, diarrhea, and abdominal pain, and death is frequently caused by septic shock, hypovolemia, and multi-organ system failure (162). Case fatality rates within outbreaks (1976-2013) have ranged from 40-70%.

EBOV has emerged into human populations 14 times since the first confirmed outbreak in 1976, primarily clustered in Republic of Congo, Democratic Republic of Congo, and Uganda (236). Other ebolaviruses include Sudan virus (SUDV), Bundibugyo

virus (BDBV), Tai forest virus (TAFV) and Reston virus (RESTV). SUDV and BDBV are pathogenic species that have caused lethal disease in humans (1; 236; 280). TAFV was isolated from one non-fatal human case in Côte-d'Ivoire (156). RESTV was first isolated in 1989 from non-human primates and traced back to the Philippines (3; 129; 184). In the Philippines, RESTV has shown to cause lethal disease in monkeys and pigs, and asymptomatic infection in humans (23; 185; 248). RESTV is the only confirmed *Ebolavirus* species found in Asia (211).

Marburgviruses

The *Marburgvirus* genus includes two closely related viruses, Marburg virus (MARV) and Ravn virus (RAVV) (133). MARV was first identified during a laboratory exposure outbreak of viral hemorrhagic fever in lab personnel that handled imported African green monkeys from Uganda (74; 235). Clinical symptoms of MARV infection are similar to those observed during EBOV infection. Natural infections of MARV have occurred in visitors to the Kitum Cave in Mount Elgon National Park, Kenya, and also in miners in the Democratic Republic of Congo and Uganda (25; 133; 264). Most persons naturally exposed to MARV have participated in spelunking or mining activities in caves with roosting bat colonies (7; 8; 277).

Cuevavirus

The third *Filoviridae* genus is *Cuevavirus,* which includes the single species Lloviu virus (LLOV), was identified following a mass die-off of cave bats in Spain in 2002 (199). Phylogenetically, LLOV is more closely related to the ebolaviruses than the marburgviruses (199). Further, LLOV is the first filovirus to be isolated in Western Europe from endemic wildlife.

2013-2016 Ebola virus disease outbreak

Recently, the largest outbreak of Ebola virus disease (EVD) occurred from 2013-2016 and resulted in 28,652 cases with a 40% case fatality rate (18; 234). This outbreak primary afflicted populations in Guinea, Liberia, and Sierra Leone. Unlike past outbreaks, this epidemic was sustained over three years and spread to neighboring countries such as Nigeria. Furthermore, this outbreak validated some ideas about the potential threat that EBOV posed to the larger global health community.

Human adaptation as a result of sustained EBOV transmission was a concern during the outbreak. One of the earliest articles to examine evolution of the EBOV Makona variant during the outbreak provided evidence of a high frequency of nonsynonymous mutations in the EBOV genome (92). A follow-up study that examined a seven-month collection of EBOV sequence data from Sierra Leone concluded that the majority of the mutations were deleterious and were selected against, but did note that follow-up experiments were needed to investigate some observed changes in the mucin-like domain of the virus envelope glycoprotein (Gp) (219). One subsequent viral lineage that appeared at high frequency as the outbreak progressed was defined by a single alanine to valine substitution (A82V) in the EBOV Gp (62; 282). This A82V Gp substitution became a dominant variation during the outbreak and was observed to increase infectivity of pseudotyped viruses in human cells compared to the pseudotyped viruses with the Gp of the ancestral EBOV variant (62). This was the first evidence of positive selection for human adaptation that might have enhanced virulence during the EVD outbreak.

Access to highly resourced supportive care was critical to manage EVD and to improve survivor success (162). No EBOV-specific anti-viral therapies exist at this time,

but a promising vaccine candidate, rVSV-EBOV, was used in a ring vaccination program in Guinea during the EVD outbreak and was demonstrated to be efficacious (118). The rVSV-EBOV vaccine is being further deployed in a phase II/III clinical trial through the STRIVE (Sierra Leone Trial to Introduce a Vaccine against Ebola) program (290).

Since this outbreak, the global health community recognized the potential threat to global health security posed by EBOV and has created the Global Health Security Agenda to address perceived inadequacies in detection, response, and prevention of emerging diseases. A large diversity of viruses remains to be identified, and one study has modeled an estimation of 320,000 unknown mammalian viruses (14). An undiscovered viral diversity of both pathogenic and non-pathogenic virus species most likely exists in the *Filoviridae* family. Increased virus surveillance and elucidation of patterns that can be used to predict virus spillover events will be necessary to control future outbreaks (15).

Henipaviruses

The *Henipavirus* genus, family *Paramyxoviridae*, is comprised of Hendra virus (HeV), Nipah virus (NiV), both of which are classified as BSL-4 pathogens and Cedar virus (CedPV) (287; 307). The first recorded HeV zoonosis occurred 1994 in Queensland, Australia following outbreaks of fatal respiratory illness in horses and humans (255). Hendra virus was first termed as an equine morbillivirus following fatal infection of horses with a previously unknown respiratory virus (255). Subsequently, this virus was identified as a new virus species and taxonomically placed in its own genus in the *Paramyxoviridae* family (287). Annual HeV spillover events have occurred since 2004, temporally occurring between April and October.

Nipah virus (NiV) was identified in 1999 after causing fatal encephalitic and respiratory illness in pigs and agricultural workers in Malaysia and Singapore (4; 5; 54; 161). This first outbreak was caused by spillover from flying foxes, the animal reservoir, via contaminated food waste that was consumed by domestic pigs. This resulted in an epizootic outbreak within the pig population and enzootic persistence of the NiV in the amplifying host population on the index farm (238). Movement of infected pigs from the index farms to surrounding farms contributed to the regional spread and reemergence of NiV Malaysia strain (NiV-M) (238).

In 2001, A NiV strain spilled over into human populations in Bangladesh (121). This NiV Bangladesh strain (NiV-B) causes annual outbreaks of disease in humans in Bangladesh and is capable of person to person as well as foodborne transmission (6; 27; 70; 105; 170). The main source of human infection is NiV-B contaminated, unpasteurized, date palm sap, which is a local drink in Bangladesh (170). Public health efforts have focused on encouraging the use of engineered modifications that provide a barrier between flying foxes and the date palm sap collection vessels (196). In addition, pasteurized date palm is another public health effort to reduce the transmission risk of NiV, however, may conflict with cultural norms of raw date palm sap consumption. Unlike NiV-M, NiV-B has a case fatality of 70% compared to 40% observed during NiV-M outbreaks.

Disease presentation following HeV and NiV differs between the terrestrial mammals infected (68). Horses and pigs infected with HeV and NiV, respectively, display signs of febrile and respiratory illness (22; 186). Humans infected with HeV and NiV have disease presentation similar to flu-like illness and can experience acute

encephalitic disease with or without respiratory illness (95; 296). There has been one noted case of fatal encephalitis in a human infected with HeV, which occurred one year after the patient recovered from meningitis caused by HeV (296). Relapsed encephalitis has been observed in many cases of NiV infection, which together with the aforementioned case of fatal encephalitis one year after HeV infection raises questions and concerns about the risk of HeV and NiV recrudescence in humans (213; 273).

CedPV is the most recently identified *Henipavirus* species and the only known non-pathogenic in species in the genus (177). As a non-pathogenic *Henipavirus* spp., CedPV can potentially be used in comparative experiments with HeV or NiV to elucidate the mechanisms responsible for severe pathogenesis of these viruses. Molecular virology experiments with HeV and NiV can be cumbersome at BSL-4 and CedPV would most likely provide an adequate model *Henipavirus* spp. to explore novel therapeutic strategies and to understand the host transcriptomic and proteomic response to *Henipaviruses.*

The expression of soluble HeV envelope attachment glycoprotein (G) facilitated the creation of a HeV G subunit vaccine that has been demonstrated to stop the progression of *Henipavirus* disease in ferret, feline, and non-human primate models of HeV and NiV infection (34; 192; 217). The Equivac® HeV (Zoetis; NJ, USA) vaccine is currently being used as part of a horse vaccination program in Australia, which breaks the chain of HeV transmission to humans through vaccination of the intermediate or virus-amplifying host. This HeV G subunit vaccine strategy fits well with current One Health efforts to simultaneously address animal and human health (182). Furthermore, soluble HeV G has been used to generate monoclonal antibodies such as m102.4, which are potent antibody therapeutics during both HeV and NiV infection (35; 40; 88).

Lyssaviruses

Lyssavirus is the genus of zoonotic RNA viruses that cause Rabies disease. Bats are animal reservoirs of all known *Lyssavirus* species with the exception of Mokola virus, which natural infects shrews (21). The prototypical virus species is Rabies virus (RABV). Presently, 14 genotypes of lyssaviruses have been identified. Australian bat lyssavirus (ABLV) was first identified in 1995 and has been isolated from Black Flying Foxes (*Pteropus alecto*), which are regarded as the natural host (101) (80; 266). ABLV has been transmitted with fatal results to humans who have been in contact with and scratched or bitten by bats (84; 246). One case of fatal human infection manifested 27-months after exposure (104). Additionally, two fatal cases of horses infected with ABLV have been reported. (261). All ABLV infections have resulted from exposure to bats, *Pteropus alecto* and an insectivorous bat, *Saccolaimus flaviventris,* which respectively are host to *Pteropus* and *Saccolaimus* strains of ABLV in Australia (98; 120).

BIOSURVEILLANCE

The multitude of data indicates that bats are the natural hosts, or animal reservoirs, of filoviruses and henipaviruses (112). Infectious MARV has been isolated from cave roosting *Rousettus aegyptiacus* fruit bats, and entry of bat roosting caves is an associated risk factor for natural infection with MARV (25; 278; 279). Serological and nucleic acid evidence, and experimental infection studies have identified other African bat species: *Eidolon helvum, Hypsignathus monstrosus, Epomops franqueti, E. gambianus, Myonycteris torquata,* and *Micropteropus pusillus* as putative animal reservoirs of ebolaviruses (113; 115; 164; 207; 211; 233).

Bats are also the confirmed animal reservoirs of HeV, NiV, and CedPV (55; 102; 177; 305). Surveillance studies identified all four *Pteropus* species of bats endemic in Australia as animal reservoirs of HeV, but the Black Flying Fox, *Pteropus alecto,* is considered to be the primary reservoir for the majority of transmission to domestic animal and human populations, and remains the species of most concern to public health (45; 79; 81; 96). Additionally, *Pteropus* bats sampled in India were discovered to have evidence of past infection with henipaviruses (71). Serological and RNA evidence of henipa-like viruses was discovered in West African fruit bats (64; 114; 223). Additional biosurveillance of African bat populations for evidence of infection with henipaviruses and paramyxoviruses has identified a new species (Ghana virus) and implicated bats as the potential ancestral source of all human paramyxoviruses (65; 173; 227). Henipaviruses have been detected from pteropodid bats in West Africa, India, and Madagascar, indicating that the geographic distribution of henipaviruses coincides with the distribution of the pteropdid bat reservoir host (71; 114; 123).

The tropics and subtropics are hotspots of both mammalian and pathogen diversity, of which currently only a small fraction of the potential viral richness and novel viruses have been identified (109; 195). Inadequate surveillance in the tropics and subtropics, and uneven geographic sampling has been implicated as the reason for the slow discovery of new viruses (112; 243.). The sustained EBOV 2013-2014 outbreak in the West African countries of Guinea, Liberia, and Sierra Leone resulted in 28,616 reported human cases and 11,310 deaths {Prevention, #214). This outbreak highlighted the potential global security risk of emerging viruses, such as EBOV, and the necessity for increased surveillance to proactively determine the geographic distribution of

ebolaviruses and other viruses. Several groups have published both serological and nucleic acid evidence of *Ebolavirus* species and ebola-like virus circulation in bat populations in Asia (116; 131; 212; 274). This research has supported predictive models that the geographic distribution of ebolaviruses extends beyond the African continent (211; 228).

VIRUS ECOLOGY

Henipavirus ecology and persistence

The biotic and abiotic factors that drive zoonotic spillover events are largely unknown. Understanding how zoonotic viruses persist in animal reservoirs is fundamental to addressing what drives spillovers and what intervention strategies can be developed to minimize zoonotic events. Of the bat-borne zoonotic viruses, the ecology of HeV has been one of the more extensively researched. Transmission of HeV spillover into human populations flows from bats (natural host) to horses (amplifying host) and lastly, to humans (dead-end or accidental hosts) (81). In 2011, there were eighteen HeV spillover events in a short span of twelve weeks, which surpassed the cumulative total of fourteen previous spillovers over the preceding sixteen years (77). Spatial surveillance discovered high HeV excretion from bat populations in southern Queensland and northern New South Wales and increased temporal excretion corresponding with winter seasonality (79). Seasonal variations that have been associated with increased HeV seroprevalence and excretion in *Pteropus* bats include nutritional status, birthing, and lactation (39; 230).

Persistence of HeV in *Pteropus* bat populations has been thought to rely on horizontal transmission, waning immunity, and metapopulation migration structures with

immigration of infected, shedding bats into naïve populations, or immigration of naïve individual bats into populations with shedding individuals, thus perpetuating infectious episodes (230; 231; 286). In contrast to metapopulation transmission dynamics, high seroprevalence of isolated and small bat populations supports evidence of episodic HeV recrudescence as a mechanism of HeV persistence at the population level (39; 223). Recrudescence of NiV in naturally infected *Pteropus* species has been observed, and late onsets of fatal encephalitis from NiV and HeV are suggestive of latent infections and potential recrudescence in human patients (205; 239; 265; 273). A model of HeV recrudescence has been explored to understand whether episodic shedding maintains HeV infection in endemic populations, and if episodic shedding explains the clustered transmission to susceptible horse populations (286).

Virus persistence occurs at community, population, individual, and cellular levels (229). To understand virus persistence and transmission dynamics in natural hosts, new models of bat-borne zoonotic virus persistence and transmission have proposed inclusive hypotheses of metapopulation and endemic population structures with migration, waning immunity, birth pulses, and episodic shedding (229; 232). For episodic shedding to occur, we reason that the bat host possesses antiviral mechanisms that inhibit the progression to clinical disease, without the complete abolishment of virus replication, facilitating recrudescence. These antiviral mechanisms constitute part of the immune response and contribute to virus persistence at the cellular level in bat reservoir species.

Lyssavirus ecology

The relationship between bats and rabies virus (RABV) was one of the earliest descriptions of the role of bats as animal reservoirs for zoonotic viruses, however, persistence of lyssaviruses in bat host populations in less well defined when compared to HeV (69; 221; 222). Torpor, hibernation, transmission of non-lethal infectious dose, and bat metapopulation structure have all been hypothesized to contribute to persistence of lyssaviruses in bat host populations (20; 21; 33). Potential mechanism of recrudescence could be facilitated through hibernation, as there is evidence of virus replication in brown fat tissue, which might contribute to persistence of the virus in the host during periods of torpor (267). Persistence in adipose tissue would permit the virus to overwinter and reactivate when metabolic activities resume, thus maintaining infection in the same individual and population through seasons and birth pulses (90). However, most recent infection modeling based on field studies in Peru suggest that RABV persistence in bat populations results from non-lethal infections, subsequent immunization of the population to clinical infection, waning immunity, then introduction of infected, or non-immunized bats through meta-population structures, followed by a wave of clinical and subclinical infections (33). Mexican Free-Tailed Bats are less susceptible to experimental infection with RABV when compared to Little Brown Bats, and infection susceptibility between species of bats supports the importance of meta-population structures in virus persistence (267).

BAT IMMUNITY

Experimental and natural infection of bats

Bats have been implicated as animal reservoirs for several highly pathogenic zoonotic viruses including HeV, NiV, EBOV, MARV, Severe Acute Respiratory Syndrome-like coronaviruses (SARS-CoV), and lyssaviruses such as Rabies virus (RABV) (20; 100; 102; 168; 278; 279). In contrast to most terrestrial mammals, bats do not develop symptomatic diseases following infection with these viruses.

Lyssaviruses

Rabies disease is often regarded as uniformly fatal in humans and other mammalian hosts. However, bats infected with RABV can survive infection and show evidence of seroconversion (281). Three out of ten Grey-Headed Flying Foxes inoculated with Australian bat lyssavirus (ABLV) developed clinical signs of infection after approximately two to three weeks, however, of the seven bats that survived ABLV challenged, five seroconverted and did not develop signs of clinical infection (179). Four of twenty Big Brown Bats seroconverted after RABV challenges and survived infection without signs of clinical disease, however sixteen Big Brown Bats challenged with RABV did develop clinical disease (127). Experimental *Lyssavirus* infection studies have demonstrated that bats are not uniformly asymptomatic hosts of lyssaviruses, however, the pathogenicity of lyssaviruses in the natural bat host remains somewhat contentious. Several factors that might affect disease outcome and rate of seroconversion in the bat host that necessitate further investigation include the route or site of infection, infectious dose, and host species and *Lyssavirus* species or strain. The development of disease following RABV infection of Vampire bats was dependent on intracranial, intramuscular,

and subcutaneous routes of infection and infectious dose (188). In contrast, to *Lyssavirus* infection, filoviruses and henipaviruses appear to uniformly cause only subclinical infections in their bat hosts.

Filoviruses

Experimental infection of bat species with EBOV resulted in the recovery of infectious virus titers from sera, recovery of live virus from blood and organs, and detection of virus antigen in lungs without histopathologic lesions (271). Similarly, MARV was isolated at high titers from naturally infected *R. aegypticus* bats, and antigen was detected in organs with scarcely observed virus induced lesions (278).

Henipaviruses

Grey-headed Flying Foxes experimentally infected with NiV developed subclinical infection with seroconversion and virus shedding in urine, and the same bat species inoculated with HeV also developed subclinical disease (183; 295). Malaysian and Australian *Pteropus* species inoculated with NiV and HeV, respectively, by natural routes of infection did not develop clinical disease or productive infection, and low-level virus titers were excreted by urine in a minority of experimental-infected bats (100). Combined, these studies demonstrated that the viruses replicated in bats, but that replication did not result in productive infection or the development of clinical disease adding further evidence that bats are the natural hosts of these viruses. These experimental infection studies have been important in the establishment and recognition of bats as the natural reservoir hosts for these viruses. As a natural host, or reservoir species, we assume that infection remains subclinical or non-pathogenic, thus, the host is able to carry on with normal activities that promote fitness and fecundity. The lack of

clinical disease in bats raises several questions about potential immune responses, mechanisms of intracellular virus control, and whether these viruses truly persist as latent infections in bat hosts (46).

Bats versus rodents: are bats 'special' virus reservoirs

The question then arose: are bats 'special' in their ability to control viral infection or is the lack of clinical disease the result of a co-evolutionary history between host and pathogen? To answer this question, we have to compare bats to other animal hosts of zoonotic viruses. Rodents and bats are respectively the first and second most diverse orders of the mammalian class, and both are the animal reservoirs of several emerging infectious diseases (171). Primarily rodents, but in some cases, shrews and bats, are the natural hosts of zoonotic viruses including the hantaviruses (e.g. Sin Nombre virus) and arenaviruses (e.g. Lassa virus), which cause pulmonary and hemorrhagic diseases in accidental human hosts (72; 76; 157; 159; 187) (16; 117). Like the relationship between bats and the viruses, hantaviruses are known to persist in rodent reservoirs causing little to no symptomatic disease. Regulatory T-cells and regulation of pro-inflammatory responses have been linked with persistence of hantaviruses in rodent hosts (66; 67; 251). So if rodents are also hosts to zoonotic viruses, and persistence of these viruses appears to be a co-evolutionary relationship involving dampened pro-inflammatory responses, what separates bats as 'special' reservoirs of zoonotic viruses?

In support of bats as 'special' virus reservoirs, a comparison of bats and rodents revealed that when the diversity difference between bat and rodent species is corrected for, bats, on a per species basis, are host to more zoonotic viruses compared to rodents (171). Behavioral factors that might contribute to this pathogen diversity in bats include

the semi-arboreal lifestyle, which through flight locomotion increases their exposure to novel pathogens in the ecosystem compared to terrestrial mammals.

The interferon response and bats

Recent research has focused on understanding the potential immune responses underlying the ability of bats to co-exist with pathogenic viruses. A lack of commercially available reagents, immune cells, and few published bat genomes present serious challenges when attempting to address questions about the bat immune response to viral infection or virus persistence. Despite these challenges, several bat immunity studies have focused on the interferon (IFN) pathway, as the IFN response is typically the first line of defense for the innate immune response during virus infection. As a result, many viruses encode proteins that target and antagonize interferon production and signaling. Henipaviruses express a non-structural phosphoprotein (P) with an alternate start site that leads to the expression of the accessory C protein, and a RNA editing site that results in transcriptional slippage and the expression of V and W proteins (106; 153; 287). P, V, and W proteins are potent antagonists of the IFN pathway through interactions with intracellular STAT proteins (56; 153; 256-258; 287). Ebolaviruses encode vp24 protein, which also antagonize the IFN response through STAT1 sequestration (309).

The conserved and ubiquitous nature of the IFN pathway was an early target of genomic and functional investigations into bat immunity (29; 57; 130; 167; 311-314). IFN-α levels are elevated in bats compared to other mammals, but infection of bat cells with henipaviruses inhibited the induction of a type-1 IFN response (283; 315). Upregulation of a type-III IFN response in bat cells following virus infection and no activation of type-I IFN demonstrated one potential antiviral response in bats (313). At

this time, conclusions about the protective antiviral role of the IFN response in bats remain unclear and additional studies are needed to better understand the role of the IFN response in controlling pathogenic viruses in bat reservoir species.

Enhanced longevity, oxidative stress, flight and life histories of bats

Genomic sequencing of the Black Flying Fox (*Pteropus alecto*), a confirmed reservoir of HeV and Australian bat lyssavirus (ABLV), and related insectivorous bats, *Myotis davidii* and *M. brandtii,* showed evidence of positive selection for genes involved in DNA-damage repair (254; 310). Further genome analysis suggested that bats might experience dampened inflammatory responses or decreased immunopathology as a result of the loss of the PYHIN gene family, which are genes involved in inflammasome activation (9).

Additionally, bats are considered 'special' within the mammalian class with the adaptation to flight. The evolution of flight and the effects on bat life history has been hypothesized to be responsible for the ability of bats to function as virus reservoirs (204; 310). For example, the metabolic costs and demands of flight for bats are higher than those experienced by other mammalian species during physical exertion (269). High levels of aerobic respiration are associated with increased reactive oxygen species and free radical generation. The process of cellular aging is theorized to be a result of an accumulation of free radicals and oxidative stress over time (107).

In contradiction to the 'free radical theory of aging', both birds and bats, live relatively longer than terrestrial counterparts (193). Furthermore, bats have longer life spans than is predicted for their body size and measured metabolic rate. (43; 75; 254). For most animals, the relationship between life span and body size follows a linear

relationship with long-lived animals being larger in size with slower metabolisms than smaller animals with faster metabolisms. However there are many examples of bat species that have life spans well beyond predicted longevities (294). The Brandt's myotis, with an adult body mass of 4-8 grams, has been documented to live up to 42 years (75; 254). The cellular and genetic mechanisms that facilitate longevity in bats are incompletely understood. However, a growing body of work has begun to explore antioxidant defenses in bats and discovered that mitochondria from Little Brown Bats generated lower levels of reactive oxygen species and hydrogen peroxide compared to shrew and mouse species (42). Additionally, two antioxidant defenses, superoxide dismutase and catalase, had higher activities in bat species (293). In summary, the cellular responses to flight and long life spans in bat species may also protect bats from viral pathogenesis while promoting their ability to serve as effective virus reservoirs.

AUTOPHAGY

In addition to reduced oxidative stress, elevated levels of protein homeostasis such as macroautophagy have been observed in long-lived bat species (237; 245). Macroautophagy, hereafter referred to as autophagy, is a cellular proteostatic mechanism that is activated in response to a variety of stressors. Dr. Yoshinori Ohsumi was awarded the 2016 Nobel Prize in Medicine for his seminal work in the autophagy field that established a system to accurately monitor the autophagy pathway in yeast (203). In addition to developing a novel approach to monitor the autophagy pathway in yeast, Dr. Ohsumi subsequently pioneered the research that identified autophagy-related genes and ubiquitin-like systems that regulate the pathway, which are discussed in greater detail below.

A catabolic process during nutrient deprived conditions, autophagy is characterized by the formation of double-membrane vesicles in the cytoplasm that engulfs cytoplasmic substrates and delivers them to lysosomes for degradation (93). Induced by a variety of stimuli including starvation, DNA damage and reactive oxygen species, and pathogens, autophagy functions in response to environmental nutrient and energy availability, and cellular stress (60; 63; 215). Autophagy is regulated by the interactions between nutrient and energy sensing serine-threonine kinases: AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), and unc-51-like kinase 1 (ULK1) (11). Under insulin, growth factor, and nutrient rich conditions mTOR phosphorylates ATG13, which inhibits ATG13-ULK1 complex association and autophagy induction (11; 139). Nutrient starvation, stress, or rapamycin all inhibit mTOR suppression of autophagy, which results in dephosphorylation of ATG13 and activation of ULK1 (139). In addition to nutrient availability, pathogen pattern receptors (PRRs) such as RIG-like receptors and toll-like receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs) and activate the autophagy pathway demonstrating the role of autophagy as an immune response (59; 208).

Steps of the autophagy pathway

The process of cellular recycling by the autophagy pathway is referred to as autophagic flux (Figure 1). In response to specific stimuli, an isolation membrane called a phagophore develops in the cytoplasm. The membrane of the phagophore is thought to originate from the mitochondria and the endoplasmic reticulum (260). The formation of the phagophore, or pre-autophagosomal structure (PAS), is the initiation step in the autophagy pathway (270). Elongation of the double-membrane phagophore marks the

next step in the autophagy pathway. During elongation cytoplasmic substrates are non-selectively and selectively engulfed by the developing autophagosome. The mature autophagosomal double-membrane vesicle traffics to and fuses with lysosomes. The acidic environment of the lysosome and the lysosomal proteases degrades the contents of the autolysosome. For further review and description of the autophagy pathway see Kaur J. and J. Debnath, 2015 (142).

Initiation of the phagophore and elongation of the autophagosome is a coordination of autophagy-related gene (ATGs) proteins, kinases, and ubiquitin-like conjugating enzymes (Figure 2). Inhibition of mTOR, results in the activation of ULK1, phosphorylation of Beclin-1 (becn1) and enhanced function of the class III phosphatidylinositol (PI) 3-kinase, Vps34, and ATG14 complexes (48; 144; 244). The class III PI 3-kinase, Vps34, is a critical regulator of autophagy initiation and phosphorylates PI, generating PI(3)P, which functions as scaffolds for the recruitment of proteins to the developing pre-autophagosome structure (44; 125; 126).

Phagophore initiation and elongation of autophagosomes requires activating, conjugating, and ligating enzymes similar to the ubiquitination system. Microtubule-associated proteins 1A/1B light chain 3B (LC3B) is the mammalian homolog of ATG8 in yeast, and along with ATG12 are two ubiquitin-like proteins in the autophagy pathway that are recruited to the elongating autophagosome membrane (89). The ATG12-ATG5-ATG16 complex is necessary for formation of autophagosomes in steps prior to the recruitment and lipidation of LC3B-phosphatidylethanolamine (PE), but is also required LC3B lipidation (285). ATG7 and ATG10 act as ubiquitin activating (E1)-like and ubiquitin conjugating (E2)-like enzymes, respectively, during ATG12-ATG5

conjugation, whereas ATG7 and ATG3 act as E1-like and E2-like proteins, respectively, during LC3B-PE conjugation (89; 122). Lipidated LC3B-PE is required for autophagosome membrane biogenesis, and is initiated by the cysteine protease, ATG4, which cleaves the c-terminus of LC3B facilitating attachment of PE (145). Lipidation of LC3B is reversible, and regulated by ATG4, which removes LC3B from mature autophagosomes and recycles LC3B from inappropriately lipidated organelles (non-autophagosome vesicles), thus maintaining a cytoplasmic pool of unconjugated LC3B that ensures biogenesis of autophagy when up-regulated (145; 198).

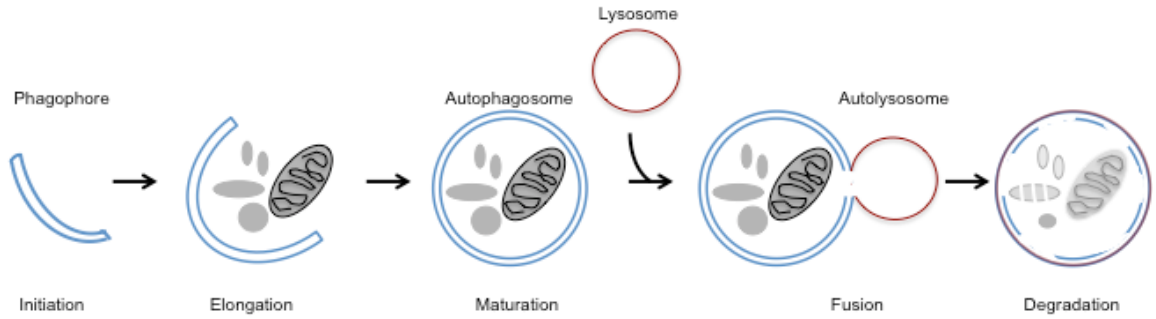


Figure 1. The autophagy pathway

Upon activation a double membrane forms in the cytoplasm known as a phagophore. During elongation of the double membrane, cytoplasmic substrates such as long-lived proteins and damaged organelles; e.g. mitochondria, are engulfed by the growing autophagosome. Once the double membrane is a fully formed vesicle it traffics towards lysosomes. Autophagosomes fuse with lysosomes forming the autolysosomes, delivering cytoplasmic substrates for degradation and recycling. The steps involved in cycling from initiation to fusion and degradation are commonly referred to as autophagic flux.

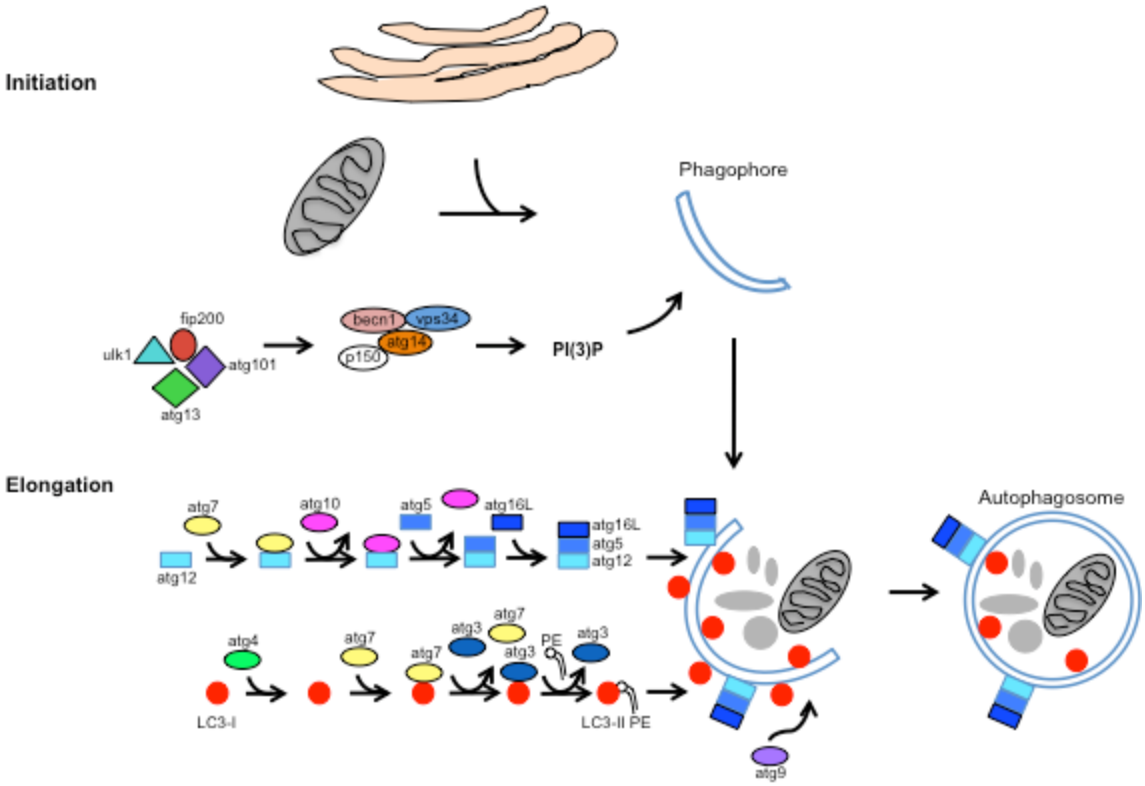


Figure 2. Autophagy-related genes (ATGs) involved in initiation and elongation of autophagosomes.

The autophagosome double membrane originates from the mitochondrial membrane and the endoplasmic reticulum. Various protein kinases are recruited and generate phosphatidylinositol 3-phosphate (PI(3)P), which are necessary for the initiation of the phagophore. Following suppression of mTOR, ulk1 phosphorylates beclin1 (becn1), which promotes ATG14/Vps34 complexes and the generation of PI(3)P. Two separate ubiquitin-like conjugation systems sequentially modify atg12 and LC3-I to generate atg16L-atg5-atg12 and LC3-II PE, respectively, which are inserted into the elongating autophagosomal membrane. Atg9 assists in additional lipid recruitment during elongation. Figure adapted from (241).

Selective autophagy

In addition to the canonical, non-selective nature of the autophagy pathway, research has also elucidated how autophagy selectively degrades substrates. Selective autophagy describes a process of autophagy that is mediated by cargo receptor proteins such as p62, neighbor of BRAC1 (NBR1), nuclear dot 52 kDa protein (NDP52), and optineurin that recognize specific cytoplasmic substrate for autophagosomal degradation (31; 146; 218; 284; 304). Selective autophagy is mediated by mono- or poly-ubiquitination, which targets cytoplasmic proteins and organelles for autophagosomal degradation (147). Autophagy cargo receptor proteins have both a LC3B-interacting region and an ubiquitin-interacting region that simultaneously recognize ubiquitinated substrates and autophagosomal LC3B (132; 201). In contrast to proteasomal degradation, which requires lysine residue 48 (K48)-ubiquitination, autophagosome-bound substrates are K63-ubiquitinated (147; 149). Cargo receptor proteins recognize this ubiquitination and serve as adaptors that transport the K63-ubiquitinated substrates into the autophagosomes. Selective autophagy has been a focus for research that examines the interactions between intracellular pathogens and the autophagy pathway (38; 268).

Autophagy as an intrinsic immune response

There are several mechanisms by which the autophagy pathway can also function as an immune response to invading pathogens: (1) directly degrade intracellular pathogens in a process termed xenophagy, (2) deliver foreign nucleic acid to endosomes containing toll-like receptors (TLRs), thereby activating the innate immune response, and (3) process intracellular antigens for MHC class II presentation, also contributing to the adaptive immune response (Figure 4) (124; 158; 165; 249; 262). In terms of virus

infection, autophagy has been shown to function as both an anti- and a pro-viral immune defense mechanism with roles differing based on the infectious agent (52). For some RNA viruses, such as poliovirus, the induction of autophagy provides intracellular scaffolds that support virus replication (155). Measles virus has been shown to induce autophagy through receptor engagement and virus C protein expression, which enhances infection in a pro-viral manner (242). During Chikungunya virus, autophagy has been observed to support virus replication and provide cytoprotective effects that limit cell death resulting from infection (136; 137; 150). As a conserved cellular process, viruses will encounter the autophagic response during the intracellular life cycle. To evade autophagy, several viruses encode virulence factors that inhibit the initiation of autophagy, or the maturation of autophagosomes and delivery of cytoplasmic substrates to autolysosomes, and in some cases, the virus life cycle is dependent on the formation of the double-membrane that is characteristic of autophagosomes (128; 152).

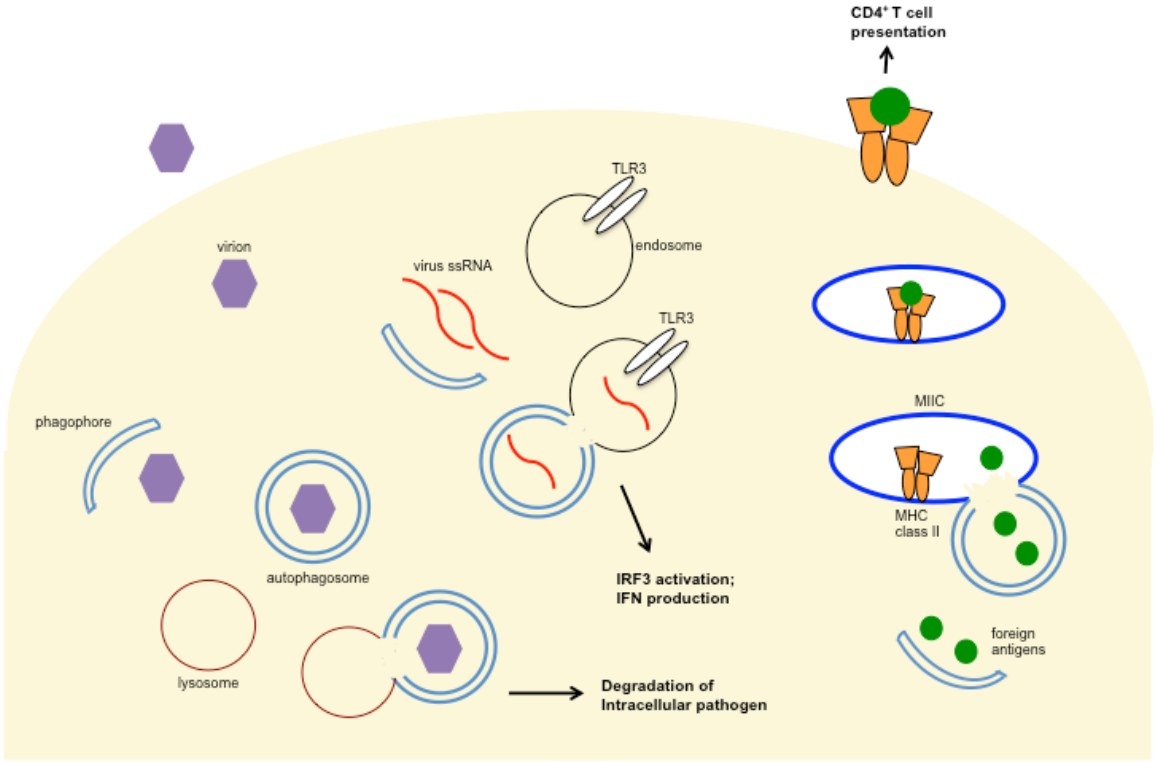


Figure 3. Intrinsic immune function of the autophagy pathway.

An intracellular pathogen, a virus is depicted here in purple, enters the cytoplasm where a phagophore, the initial autophagosomal isolation membrane, engulfs the virion and targets it for lysosomal degradation. This process has been termed 'xenophagy.' Alternatively, the viral nucleic acid, depicted in red, is released into the cytoplasm and engulfed by the autophagosome and delivered to mature toll-like receptor 3 (TLR3) containing endosomes, which results in interferon regulatory factor 3 (IRF3) activation, signaling and the production of interferons (IFNs). Lastly, autophagosomes can engulf foreign antigens, depicted in green, and deliver these antigens to the late endocytic antigen processing, MHC class II compartment (MIIC). This pathway results in the delivery of foreign antigens to MHC class II and presentation to $CD4^+$ T-cells.

The antiviral role of autophagy

Autophagy has been demonstrated to have direct antiviral effects on virus replication. In fruit flies, autophagy functions as an antiviral mechanism, which has been seen as evidence that autophagy, like RNAi, is an ancient antiviral mechanism (259). The promotion of autophagy was also observed to be an essential part of fruit fly immunity during Rift Valley fever virus infection (190). Selective autophagy during Sindbis virus infection has also been shown to remove the accumulation of virus protein aggregates, harmful to cellular homeostasis, without having any effect on virus replication, thereby conferring a cyto-protective effect (216; 268). Additionally, distinct species-specific interactions with Chikungunya virus and autophagic machinery have been observed (138). For an additional summary of interactions between animal viruses and the autophagy pathways see Table 2. The role of autophagy as an innate immune response that could potentially limit virus pathogenicity in reservoir species, such as bats, is largely unknown. Investigations into the role of autophagy as an antiviral mechanism in bat reservoir species could therefore begin to elucidate the cellular mechanisms of virus persistence in reservoir hosts.

Table 2. Selected examples of interactions between animal viruses and the autophagy pathway

Virus species	Virus family	Interaction with autophagy	Ref
Measles virus (MeV) (-)ssRNA	*Paramyxoviridae*	Activates autophagy through CD46 receptor engagement; MeV C protein induces autophagy; MeV-induced fusion activates autophagy; Promotion of autophagy is considered pro-viral	(135; 242)
Rabies virus (RABV) (-)ssRNA	*Rhabdoviridae*	Activated by RABV infection; RABV matrix protein induces autophagy	(226)
Vesicular stomatitis virus (VSV) (-)ssRNA	*Rhabdoviridae*	Activates autophagy through Toll-7 and VSV envelope glycoprotein interactions; in *Drosophila* autophagy is pro-survival, anti-viral	(197; 259)
Rift valley fever virus (-)ssRNA	*Bunyaviridae*	Activates autophagy in human and murine cell lines; Pharmacologic induction has anti-viral effects; anti-viral role in *Drosophila*	(190)
Influenza A virus (-)ssRNA	*Orthomyxoviridae*	Influenza A virus encodes a M2 protein that blocks fusion of autophagosomes with lysosomes	(85)
Chikungunya virus (CHKV) (+)ssRNA	*Togaviridae*	Induces autophagy to inhibit apoptosis; Pro-survival and pro-viral	(49)
Sindbis virus (+)ssRNA	*Togaviridae*	Activates autophagy; removes toxic aggregates of Sinbis virus nucleocapsid; pro-survival in mice	(216)
Dengue virus (DENV) (+)ssRNA	*Flaviviridae*	Induces autophagic degradation of lipids to produce ATP necessary for viral replication; pro-viral	(49)
Herpes simplex virus (HSV) dsDNA	*Herpesviridae*	HSV virulence factor γ34.5 inhibits initiation of autophagy through interaction with beclin-1	(94; 292; 306)
Human immunodeficiency virus (HiV) ssRNA-RT	*Retroviridae*	HIV Nef accessory protein blocks the fusion of autophagosomes with lysosomes	(154)
Poliovirus (+)ssRNA	*Picornaviridae*	Induction of autophagy and recruitment of double membrane lipid structures promotes virus replication; pro-viral	(155)

RESEARCH GOAL, RATIONALE, AND AIMS

Research Goal

The overall research goal of this dissertation is to understand the bat as a natural host, or animal reservoir, of emerging zoonotic viruses.

Aim 1: Understand the geographic distribution of filoviruses and henipaviruses in Southeast Asian bat populations

Rationale

The preponderance of evidence suggests that bats, Order Chiroptera, are the animal reservoir of the filoviruses and henipaviruses. The global distribution of these viruses is incompletely understood. The sustained outbreak of Ebola virus disease in Liberia, Sierra Leone, and Guinea demonstrated the global security and health risks posed by Ebola virus. What was once thought to be a disease confined to rural and forest regions of Central Africa emerged in urban areas of West African countries, highlighting the potential for zoonotic viruses such as Ebola virus to emerge in new geographies. Additionally, models have predicted that filoviruses are geographically distributed throughout the Asian continent and serological surveillance studies have discovered evidence of past infection of bats with Ebola virus and Reston virus in Bangladesh, China, and the Philippines. As part of efforts to prevent zoonotic disease emergence, biosurveillance is critical to elucidating the geographic distribution of emerging viruses and the potential wildlife sources. To undertake surveillance of emerging viruses, we developed a Luminex-based multiplex binding assay that can detect antibodies specific to antigens from all known ebolaviruses, marburgviruses, and henipaviruses

Hypothesis: E. spelaea, C. brachyotis, and P. lucasi bat populations sampled in Singapore will have serological evidence of past exposure to filoviruses.

Aim 1A. Express soluble glycoproteins from ebolaviruses and maburgviruses, and confirm specificity of sGps and activity of sGp-conjugated BioPlex microspheres

Aim 1B. Explore the geographic distribution of filoviruses across Southeast Asia by screening Pteropodidae bat species for serologic evidence of past exposure

Aim 2: Investigate whether the autophagy pathway functions as an antiviral defense in bats

Rationale

The unique life history of bats, which includes flight and longevity, suggests that bats possess cellular processes that maintain the cell body despite high levels of aerobic respiration and potential DNA damage. Autophagy is a cellular homeostatic process that is stimulated by DNA damage, reactive oxygen species, and viruses. With consideration to the unique life history of bats, we wanted to explore the role of autophagy in bats and elucidate its potential as an antiviral defense. Specifically, we wanted to address whether autophagy reduces virus replication or removes toxic virus protein aggregates to levels that maintain cellular homeostasis under normal conditions, thus creating a threshold between asymptomatic and symptomatic disease. Using Australian bat lyssavirus and *Pteropus alecto* derived cells presented us with an opportunity to explore potential differences between natural host (bat)-pathogen and accidental host (human)-pathogen interactions. Understanding, the antiviral defenses of animal reservoirs, allowed us to investigate potential mechanisms such as autophagy, contribute to virus persistence at the cellular level. Additionally, investigating the antiviral role of autophagy in the natural host would demonstrate whether the pathway could be therapeutically harnessed for treatment of human disease.

Hypothesis. We hypothesize that autophagy functions as an effective antiviral mechanism during ABLV infection of bat cells.

Aim 2A. Elucidate the antiviral role of autophagy during bat cell infection

Aim 2B. Explore the therapeutic potential of pharmacologic autophagy activation during neurotropic viral infection

CHAPTER 2: Application of a multiplex serology assay to detect evidence of filovirus circulation in bat populations

2.1. INTRODUCTION

Zoonotic events have accounted for nearly 50% of all recently emerged infectious diseases (134). Biosurveillance of wildlife is critical for the identification of emerging zoonoses that have the potential to threaten global health. Bats have been implicated as the natural reservoirs of several highly pathogenic, zoonotic viruses, including the ebolaviruses, marburgviruses, and henipaviruses (100; 102; 164; 271; 278). Ebola virus spillover events have historically occurred in Central African countries. However, the 2014 outbreak of Ebola virus disease (EVD) in the West African countries of Guinea, Liberia, and Sierra Leone, ~2000 miles away from past outbreaks in the Republic of Congo, the Democratic Republic of Congo, and Gabon, demonstrated that Ebola virus outbreaks are not geographically restricted to Central Africa, and may be distributed along the habitat ranges of the natural bat hosts (26). The ecological factors that lead up to the EVD outbreak in West Africa are unclear but an initial spillover event from a bat host has been hypothesized (83; 175). Virus transmission from bats has driven outbreaks of EBOV in the Democratic Republic of Congo, Nipah virus (NiV) in Malaysia and Bangladesh, and Hendra virus (HeV) in Australia (81; 163; 169; 238).

Five virus species make up the *Ebolavirus* genus: Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo virus (BDBV), Tai Forest virus (TAFV), and Reston virus (RESTV). Reston virus is the only *Ebolavirus* species presently known within the Asian continent. The origins of Reston virus were traced to the Philippines following an outbreak of viral hemorrhagic fever in cynomolgus macaques at a quarantine facility in

Reston, Virginia, USA (3; 129). Reston virus is mostly closely related to Sudan virus and these two species form a sister clade to the branch of EBOV, BDBV, and TAFV (280). Marburg virus (MARV) and Ravn virus (RAVV) are two related viruses in the *Marburgvirus* genus. NiV and HeV are classified as biological safety level-4 pathogens, and with the non-pathogenic related species Cedar virus (CedPV) form the *Henipavirus* genus. Ecological models and geographic ranges of putative bat hosts of ebolaviruses and marburgviruses have predicted that these viruses are potentially distributed throughout the Asian continent (211; 228).

Past surveillance work in Asia has demonstrated serological evidence of infection with a virus related to Ebola virus and Reston virus in *Rousettus leschenaultii* bats sampled in Bangladesh (212). A surveillance study of 843 bats in China discovered serological evidence of past infection with EBOV- and RESTV-like viruses with high seroprevalence in *R. leschenaultii* and *Pipistrellus pipistrellus* populations (308). Serology and nucleic acid based tests have demonstrated that RESTV circulates in bat populations in the Philippines (131; 274).

Our lab has developed a serological Bio-Plex multiplex binding assay that is able to detect antibodies specific to soluble versions of virus envelope glycoproteins (sGps) from all presently known *Ebolavirus, Marburgvirus,* and *Henipavirus* species. The research included in this chapter was part of an effort to initiate collaborative biosurveilance in Southeast Asia to investigate the geographic distribution of these viruses with partners at the Duke-NUS Graduate Medical School, Singapore. Bat sera samples from *Eonycteris spelaea* (Cave-Nectar Bat), *Cynopterus brachyotis* (Short-Nosed Fruit Bat), and *Penthetor lucasi* (Dusky Fruit Bat) were screened with the

multiplex assay. All three fruit bats are members of the family *Pteropodidae*, have habitat ranges that extend from India to Indonesia, and share ecological niches (180). Infectious disease surveillance of bats has heavily focused on *Rousettus* and *Pteropus* genera; confirmed reservoirs of MARV, and NiV and HeV, respectively. Parasite density models highlighted that *E. spelaea*, *C. brachyotis*, and *P. lucasi* are notable bat species less sampled by past surveillance studies, and predicted that all three species should have high parasite richness (86). A model of filovirus-bat hosts has ranked *E. spelaea* as the fifth most likely un-sampled putative host (103).

In our study, we focused on the *E. spelaea*, *C. brachyotis*, and *P. lucasi* fruit bats to investigate whether that these three species have roles as reservoirs of filoviruses or henipaviruses, and to understand whether filoviruses are geographically distributed in Southeast Asia. Individual *E. spelaea, C. brachyotis,* and *P. lucasi* sera samples screened by the multiplex assay were seropositive for antibodies specific to sGps from ebolaviruses. No bat sera samples from any of the three species screened were seropositive for marburgviruses. Analyses of the effects of age, sex, and year on seroprevalence for each species revealed a statistically significant increase in seroprevalence due to sex in *C. brachyotis* populations. No other significant associations were revealed by univariate analysis. Sera from all three species preferentially reacted with EBOV, BDBV, and SUDV sGps. In the absence of virus nucleic acid data, it remains a possibility that these bats have been previously exposed to each of these three *Ebolavirus* species. However, the more likely explanation is that one of these viruses (EBOV, BDBV, SUDV) or a novel, uncharacterized *Ebolavirus* species antigenically similar to these three viruses circulates in these bat populations and polyclonal sera is

cross-reactive because of shared conformational sGp epitopes. Surprisingly, we did not find any sera to be seropositive for RESTV sGps.

2.2. Aims and Hypotheses

Hypothesis. *E. spelaea, C. brachyotis,* and *P. lucasi* bat populations sampled in Singapore will have serological evidence of past exposure to filoviruses.

Aim 1A. Express soluble glycoproteins from ebolaviruses and maburgviruses, and confirm specificity of sGps and activity of sGp-conjugated BioPlex microspheres

Aim 1B. Explore the geographic distribution of filoviruses across Southeast Asia by screening Pteropodidae bat species for serologic evidence of past exposure

2.3. Materials and Methods

Soluble glycoprotein expression

Chan YP *et al.* have previously described the methodology for the preparation of soluble virus envelope glycoproteins (sGps) from mammalian cell-culture systems by our lab (50). This system was adapted for the expression of sGps from ebolaviruses and marburgviruses. Soluble versions of each *Ebolavirus* and *Marburgvirus* species were identified from the National Center for Biotechnology Information (NCBI) database (Table 3). The EBOV and SUDV Gps clones were gifts from Drs. Chad Mire and Tom Geisbert, and Dr. Katherine Bossart, respectively. The remaining Gp clones were synthesized as codon optimized genes and all sGps were designed with a C-terminal GCN domain, to assist proper folding, followed by factor Xa (fXa) sequence, and lastly a Twin-Strep-tag (TST; (IBA, Göttingen)). sGp-fXa-TST constructs were ordered from Genscript (Piscataway, NJ) (Figure 4). Gps from ebolaviruses and marburgviruses undergo conformational changes in low pH environments to facilitate cellular entry (99;

108; 160). The TST enabled the use of physiological buffers for protein elution during affinity and size exclusion chromatography, thereby retaining pre-entry, native, confirmations of sGp oligomers.

Table 3. Virus envelope glycoproteins and Bio-Plex beads used in multiplex assay

	Ebolaviruses	Soluble env. proteins	Bio-Plex bead #
1	Ebola virus (EBOV)	Gp	#33
2	Ebola virus (EBOVΔM)	GpΔmucin	#17
3	Sudan virus (SUDV)	Gp	#77
4	Reston virus (monkey strain) (RESTVm)	Gp	#85
5	Reston virus (pig strain) (RESTVp)	Gp	#72
6	Tai forest virus (TAFV)	Gp	#57
7	Bundibugyo virus (BDBV)	Gp	#64
	Marburgviruses		
8	Marburg virus (Musoke) (MARV(Mus))	Gp	#37
9	Marburg virus (Angola) (MARV(Ang))	Gp	#28
10	Ravn virus (RAVV)	Gp	#49
	Henipaviruses		
11	Hendra virus (HeV)	G	#46
12	Nipah virus (NiV)	G	#42
13	Cedar virus (CedPV)	G	#53

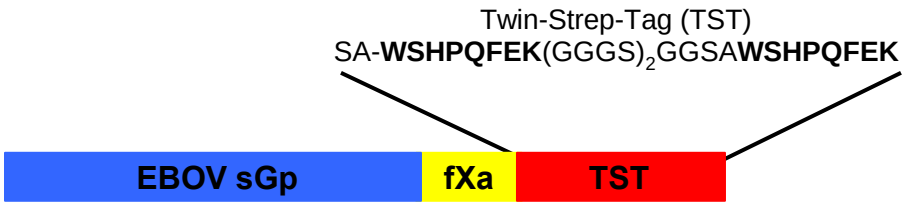


Figure 4. Representative schematic of soluble virus envelope glycoprotein (sGp) construct

Factor Xa (fXa) cleavage is used to remove the TST prior to conjugation with Bio-Plex COOH beads so that only the sGp remains in the binding assay.

sGp antigen coupling to Bio-Plex beads

fXa cleavage of eluted sGp-fXa-TST proteins was performed to remove the TST. Bio-Plex COOH beads with specific internally labeled fluorescent dyes were purchased from BioRad (Table 3). Amine coupling of sGps to Bio-Plex COOH beads was performed as per manufacturer's instructions (BioRad; Hercules, CA).

Control sera and antibodies

Several collaborators provided sterilized, gamma irradiated, polyclonal sera from African green monkeys (AGM) infected with Nipah virus (NiV), and Marburg virus (MARV) or Ebola virus (EBOV) from cynomologus monkeys (CM). Dr. Xiangguo Qui (National Microbiology Laboratory, Winnipeg, MD) kindly provided anti-EBOV CM sera, and monoclonal antibodies (mAb) specific to EBOV (m5D2) and MARV (m2B8). A human immunoglobulin DNA phage library was used for panning against specific virus sGps to produce fragment-antigen binding fragments (Fabs). A human Fc domain was fused to Fab specific for CedPV G and used to generate a CedPV G-specific monoclonal antibody (m14F). Anti-NiV, MARV, and EBOV sera, and monoclonal antibodies were used to test proper activation of sGp-coupled Bio-Plex COOH beads and assay signal specificity between the sGps of the virus species represented in this multiplex assay. Control monoclonal antibodies were diluted to starting concentrations of 1.0µg/mL. Polyclonal control sera were diluted to starting concentrations of 1:250 (anti-MARV and EBOV CM sera) or 1:200 (anti-NiV AGM sera). These sera and antibodies were tested with the multiplex binding assay.

Bio-Plex multiplex assay

An earlier publication by Bossart KN *et al*. detailed the protocol for the Luminex-based Bio-Plex assay (36). Earlier versions of this multiplex microsphere assay, which included soluble versions of *Henipavirus* envelope glycoproteins, have been validated as a method for *Henipavirus* serology studies in Australia and Bangladesh, and have been used to detect antibodies specific to henipa-like viruses in West African fruit bats (36; 53; 114).

Reagents and equipment

BioRad Bio-Plex 200 machines installed at the Uniformed Services University and Duke-NUS Medical School were used to test control sera and experimental field collected bat sera, respectively. Soluble virus envelope glycoprotein (sGp) antigens were coupled to Bio-Plex COOH (carboxylated) beads, specific internal dye regions indicated in Table 3.

Screening experimental bat sera

Sera collection and storage

Bat sera samples were collected from 2011-2016 from roosting and feeding sites within Singapore in accordance with Duke-NUS Graduate Medical School IACUC guidelines (IACUC Permit #B01/12). Bats were captured with mist nets, and a venipuncture was performed to collect blood that was field-diluted 1:10 with 1XPBS. Age (juvenile and adult), sex, and morphological measurements (ear length, forearm length, and weight) were also recorded. Sera supernatant was removed after centrifugation of the blood clot, heat inactivated for 30 minutes at 56°C, then stored at -80°C.

Preliminary screen

During June – July 2015, 238 bat sera samples from three bat species, Short-Nosed Fruit Bat (*Cynopterus brachyotis*), Cave-Nectar Fruit Bat (*Eonycteris spelaea*), and Dusky Fruit Bat (*Penthetor lucasi*) were screened for evidence of past infection with ebolaviruses, marburgviruses, and henipaviruses using the BioRad Bio-Plex 200 machine at Duke-NUS Graduate Medical School. A total of 104 *C. bracyhotis*, 94 *E. spelaea,* and 40 *P. lucasi* sera samples were screened with the multiplex assay, which at the time included sGps from EBOV, SUDV, MARV (Musoke variant), and an EBOV sGp that lacked the mucin domain (EBOVΔM). Sera samples were tested at dilutions of 1:50 and 1:100.

Screen with completed virus sGp panel

In April 2016, a second screening was performed with 409 bat sera samples (153 *C. brachyotis*, 186 *E. spelaea*, 70 *P. lucasi*), which included all previously screened samples, with the complete *Ebolavirus* and *Marburgvirus* sGp-bead multiplex assay (Table 1). Sera samples were diluted 1:100 and tested in duplicates.

Binding assay protocol

Figure 5 is a schematic of the multiplex binding assay. Bat sera samples were thawed on ice and diluted to final concentrations in Ca^{2+}/Mg^{2+} free 1X PBS (PBSA). 96-well filter plates (Millipore-Sigma; Darmstadt, Germany) were pre-wet with 100µL PBSA. Each sGp-conjugated bead was vortexed for one minute followed by an additional minute of sonication. For 96 wells, 100 µL of each sGp-coupled bead were diluted in a 10 mL master mix of PBSA so that 1 µL in 100 µL of PBSA of each bead is added per well. The 96-well filter plate was vacuumed to remove excess PBSA. The sGp-

conjugated beads remained in the wells. 100µL of each sera sample was added to each well. The plate was covered with aluminum foil to protect from light and shaken at room temperature for 45 minutes at 900 rpm. The 96-well filter plate was vacuumed to remove sera not bound to the sGp-conjugated beads. Biotinylated-proteins A and G (Thermo Fischer Scientific; Waltham, MA) were mixed 1:1 at a 1:500 dilution in PBSA, and then added to each well. Protein A and protein G bound to the sera immunoglobulin. Again, the plate was covered with aluminum foil and shaken on a microtiter plate shaker for 45 minutes at 900 rpm. At the conclusion of this incubation step, the plate was vacuumed to remove excess biotinylated-protein A/G and 100µL of streptavidin-phycoerythrin (PE) (Bio-Rad; Hercules, CA) was added to each well. The plate was incubated and shaken for a final 30 minutes. The plate was then loaded into the BioRad Bio-Plex 200 machine, internal dyes for each Bio-Plex COOH beads were detected and PE signal for each bead is recorded as median fluorescence intensity (MFI).

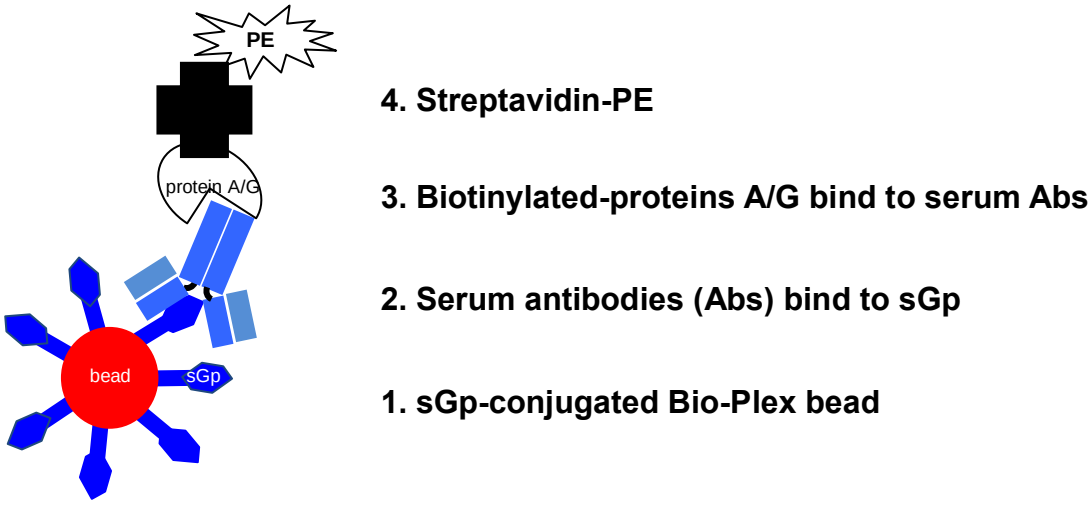


Figure 5. Schematic of Bio-Plex multiplex binding assay

Western blots

Preparative-well protein gels (8%) were loaded with 2μg of EBOV or BDBV sGp. Proteins were transferred to membranes and blotted with selected multiplex binding assay seropositive and seronegative sera (1:100) from *E. spelaea, C. brachyotis,* and *P. lucasi* samples. A 1:1 combination of biotinylated-Protein A/G was used to detect bat sera and streptavidin-HRP (Thermo Fischer Scientific; Waltham, MA) was used for signal detection.

2.4. RESULTS

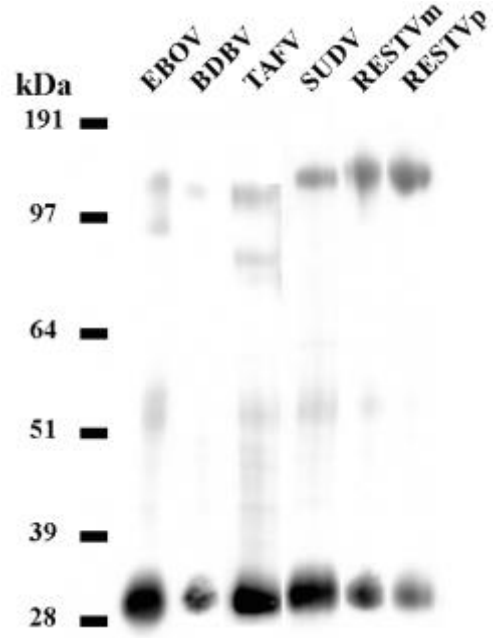
sGp-bead activation and signal specificity

Prior to binding assay testing, sGp-fXa-TST eluted proteins were checked by western blot (WB) for appropriate Gp sizes by SDS-page gels under reducing conditions (Figure 6). After confirming sGps by WB we tested several control monoclonal antibodies (mAb) and polyclonal sera that were generated against EBOV, MARV, NiV, and CedPV with the multiplex binding assay to examine whether the sGp-bead coupling was successful and to begin to elucidate sGp-bead signal specificity. As predicted, m5D2 (a mAb specific to EBOV) had the highest reactivity with EBOV sGp, and exhibited minimal reactivity with other virus species sGps (Figure 7). Also, the 5D2 mAb did not react with the EBOVΔM sGp, which showed that the antibody epitope is within the mucin domain. EBOV, BDBV, and TAFV sGp sequence similarities share between ~63-73% sequence identity. Sera from a cynomologus monkey (CM) inoculated with EBOV preferentially reacted with EBOV, BDBV, and TAFV sGps (Figure 7). This is in agreement with the cross-reactive nature of anti-EBOV IgG antibodies to antigens from heterologous ebolaviruses (172). Additionally, the intensity of anti-EBOV CM sera

reactivity with the sGps in the binding assay parallels the phylogenetic relatedness of these viruses; EBOV, BDBV, and TAFV cluster together while SUDV and RESTV form sister clade (Figure 8). Despite an absence of RESTV-positive sera or antibodies, anti-EBOV CM sera reacted with RESTV sGps at MFIs higher than background and MARV sGps, which demonstrated that the RESTVm and RESTVp sGps-beads were functional and activated (Figure 7 inset). The polyclonal anti-EBOV CM sera did not react with sGps-beads from *Marburgvirus* or *Henipavirus* species, which validated that the assay is able to differentiate polyclonal sera specific to ebolaviruses.

Anti-MARV(Mus) AGM sera preferentially reacted with MARV(Mus) and Mus(Ang) sGp-beads (Figure 7). The anti-MARV(Mus) AGM reacted with BDBV, EBOV, and TAFV sGp-beads at lower MFIs. This reactivity is most likely a reflection of some shared epitopes and cross-reactivity. An anti-MARV(Mus) mAb (2B8) specifically reacted with the MARV(Mus) sGp-bead (Figure 7). As expected anti-CedPV mAb (m14F) specifically reacted to CedPV (Figure 7) and anti-NiV AGM sera preferentially reacted with NiV sGp-beads (Figure 7). Combined, these mAbs and sera controls demonstrated that the sGps-beads were functionally active and can be used to differentiate IgGs with specificity between *Ebolaviruses, Marburgviruses,* and *Henipaviruses* in the multiplex binding assay.

A.



B.

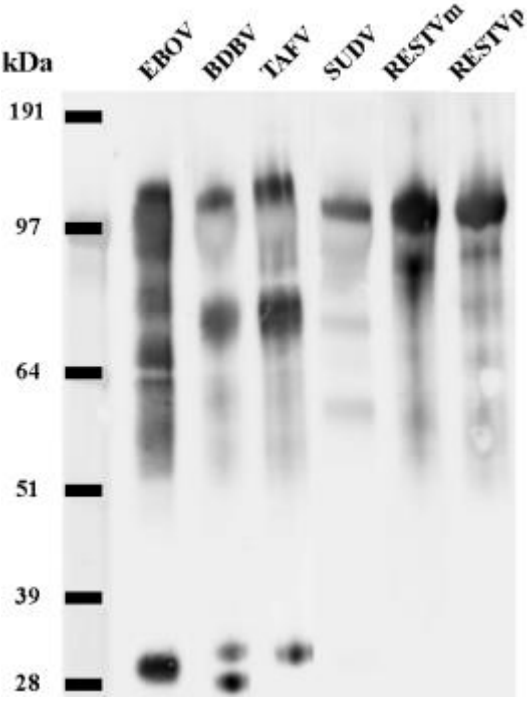


Figure 6. Western-blot analysis of *Ebolavirus* species sGps.

(A) Proteins were immunoblotted with anti-TST antibody.

(B) Proteins were immunoblotted with anti-EBOV CM sera. The denatured sGp monomers are detected at ~120kDa (Gp1) and ~28kDa (Gp2).

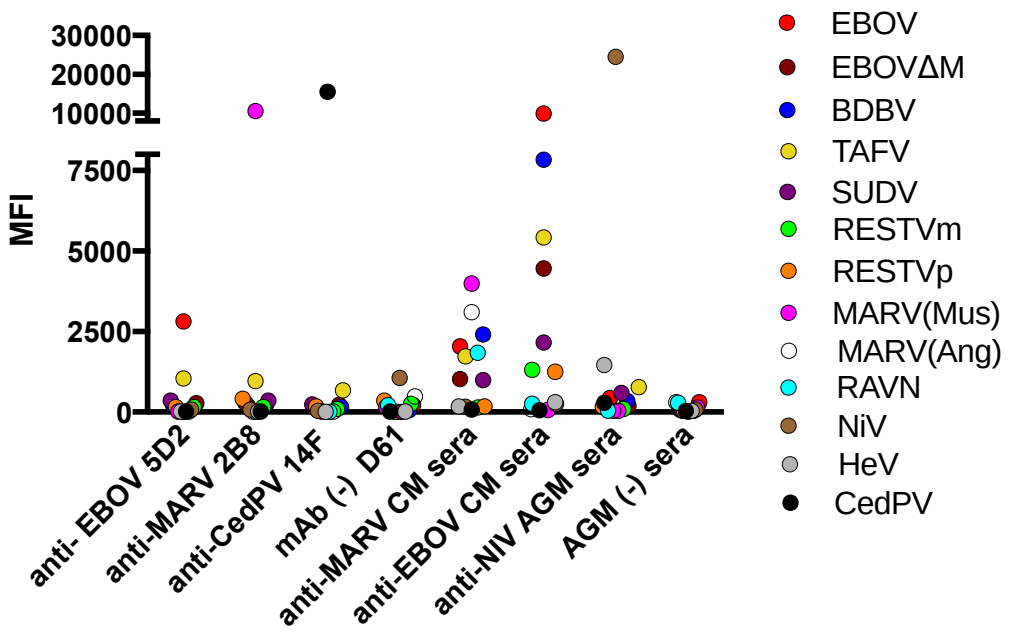


Figure 7. Bio-Plex multiplex binding assay tested control monoclonal antibodies and sera.

Monoclonal antibody was diluted to concentrations of 1.0 µg/mL, and sera from cynomolgus monkeys and African green monkeys were diluted to 1:200 and 1:250, respectively.

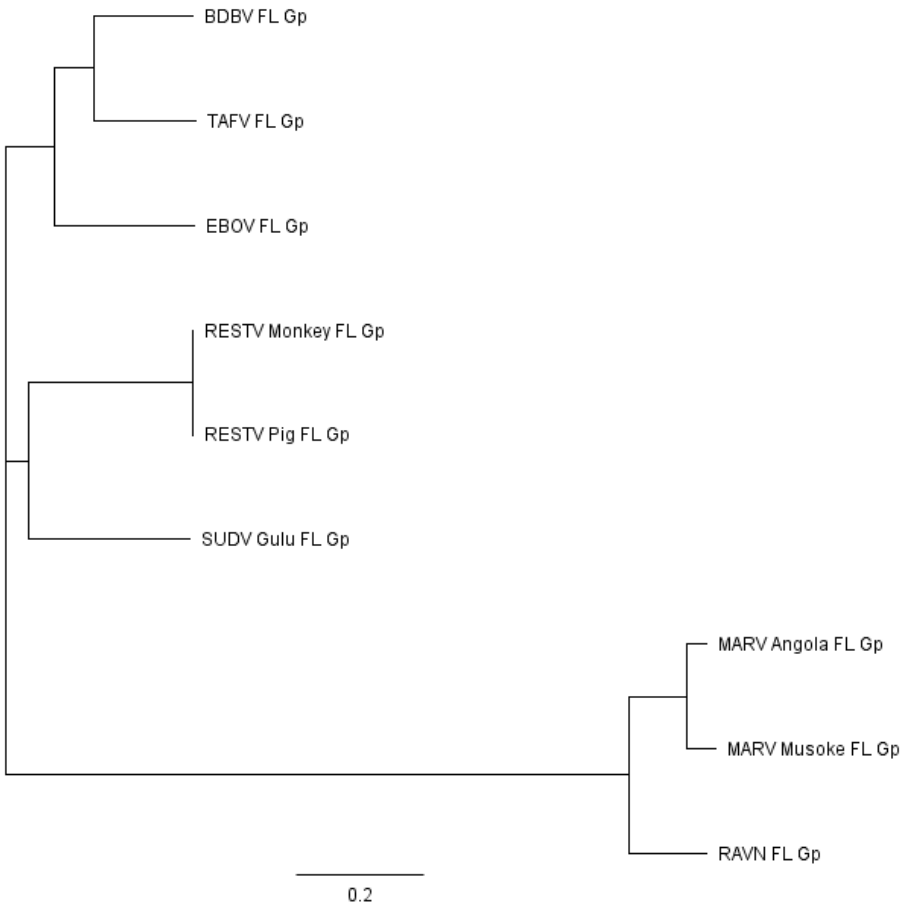


Figure 8. Phylogenetic tree relatedness of *Ebolavirus* and *Marburgvirus* species full-length envelope glycoprotein (Gp)

* FL (full-length); figure was generated with the Geneious® software package

Screening experimental bat sera

Preliminary screen

An initial screen of 238 sera samples (104, *C. bracyhotis*; 94, *E. spelaea*; 40, *P. lucasi*) diluted 1:50 and 1:100 was performed with sGps from EBOV, EBOVΔM (deleted mucin domain), SUDV, and MARV(Mus). In the absence of negative control sera for each bat species samples, we employed methods developed by Peel AJ *et al.* to establish a MFI cutoff value to discriminate between positive and negative sera samples (53; 224). Using this approach we established 200 median fluorescence intensity (MFI) as the cutoff for positive bat sera samples. A 200 MFI cutoff has also been used for *Eidolon helvum* bat sera screened with a Luminex-based binding assay (114). We identified (20, *C. bracyhotis*; 21, *E. spelaea*; 7, *P. lucasi*) samples that were cross-reactive with EBOV and SUDV sGps (Figure 9 and Table 4). No bat sera samples were seropositive with MARV(Mus) sGp (Figure 9 and Table 4). Five out of twenty, *E. spelaea* sera samples, and three out of 20 *C. brachyotis* were seroreactive with henipaviruses. The seroreactivity seen in *E. spelaea* and *C. brachyotis* samples with henipaviruses was in addition to cross-reactivity with EBOV and SUDV sGps. However, in *P. lucasi* samples, three sera samples were uniquely reactive with NiV sG (Table 4).

Looking at the MFIs for each seropositive sample, we observed that no EBOVΔM samples had MFIs higher than 600(+), whereas there were two samples that had SUDV and EBOV positive seroreactivity higher than 1000 (+++) (Table 5). This is consistent with the immunodominant nature of the mucin domain (209; 301). This data indicated that when the SUDV and EBOV sGps mucin-like domain is intact, sera immunoglobulins are strongly reactive with these antigens.

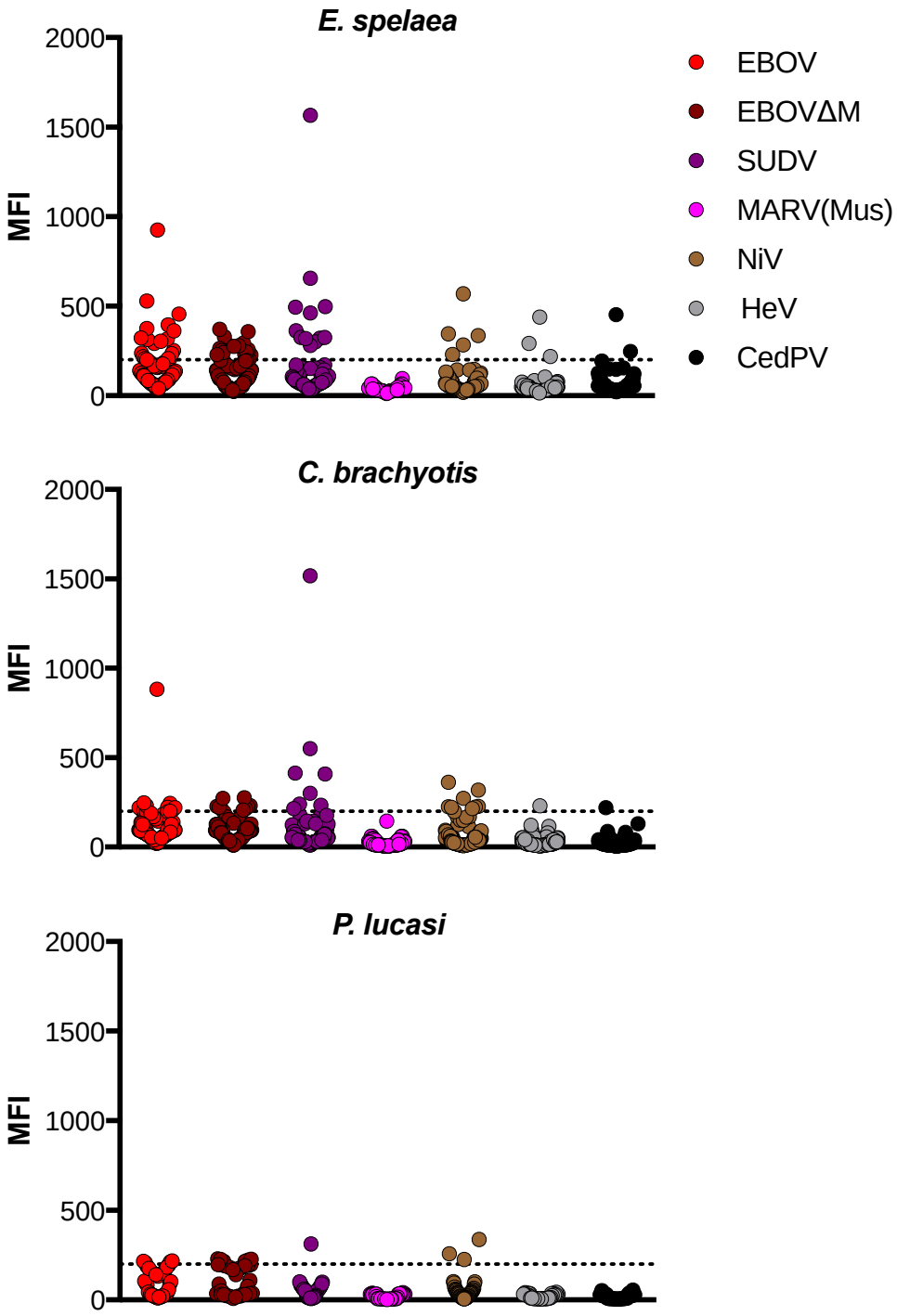


Figure 9. Serologic evidence of past exposure to ebolaviruses detected by preliminary Bio-Plex multiplex binding assay in three Pteropodidae bat species.

*Preliminary screen of bat sera samples diluted 1:100, dashed line indicates 200MFI

Table 4. Bio-Plex median fluorescence intensities (MFI) for bat sera samples positive in the preliminary screen

E. spelaea (n=94)							
Year;ID	E	EΔM	S	Mm	H	N	C
2011;0805149‡	**925**	**227**	58.5	-	34	36	23
2012;080810	**252**	**233**	**320**	33	32	39	87
2012;080814‡	**395**	**280**	**1565**	43	44	78	61
2012;080832	**208**	**257**	152	65	55	96	111
2012;080858	194	**202**	132	37	39	61	22
2012;080869	**317**	**294.5**	**655**	45.5	52	230	155
2012;082138	**455**	**329**	**494**	33	59	105	54
2012;082141	**292**	**264**	121.5	51	43	52	44
2013;013112	183	**221.5**	154.5	28	60	144	122
2013;013120	**234**	**358**	**301.5**	95.5	**292**	569	148
2013;030713	**208**	**252**	88	36.5	33	73	53
2013;030717	**314**	**244**	**363**	34.5	49	99	87
2013;052313‡	**375**	**279**	**462**	64	72	61	75
2013;110704	**237**	151	**281**	39.5	52	61	64
2013;110764	**218**	154	**326.5**	39	25	78	126
2014;052023	**303**	189.5	**326.5**	-	**439**	284	**453**
2014;052031	**208**	**240.5**	119.5	29	41	49	64
2014;111903‡	**323**	**229**	109	24.5	45	49	59
2014;111907‡	**529**	**371**	**497**	47	**219**	345	194
2015;012204	**201**	197	171	43.5	75	336	46
2015;012208	**362**	**275**	**318.5**	32	59	125	82
C. brachyotis (n=109)							
Year;ID	E	EΔM	S	Mm	H	N	C
2011;051258	129	66	**414**	44	49	52	32
2011;0516620	192	**210**	122	61	83	**226**	65
2011;0516629	194	**232**	173	60	100	159	88
2011;0516632‡	**244**	104	**550**	-	41	55	30
2011;0721110	**208**	171	170	30	56	85	67
2011;08181611	**220**	68	143	-	43	43	42
2011;08181615	**205**	196	96	43	34	54	37
2011;11032432	**208**	**226**	79	40	38	70	31
2011;11032449	183	**228**	64	29	31	44	26
2011;11032463	**223**	**222**	122	27	78	60	55
2011;1103248	197	**213**	130	41	58	87	82
2013;112805	**220**	118	**409**	14	19	63	16
2014;021303	**882**	**273**	**1517**	10	16	38	22
2014;021357‡	115	101	**300**	10	17	88	16
2014;032703	120	92	**240**	8	7	16	14
2014;050804	**201**	129	146	10	17	20	11
2014;050818‡	**232**	126	**234**	11	16	20	10
2015;040804‡	189	**275**	**215**	145	122	**363**	30
2015;072314	**248**	**207**	177	-	**230**	**216**	**220**
2011;051258	129	66	**414**	44	41	27	43
P. lucasi (n=40)							
Year;ID	E	EΔM	S	Mm	H	N	C
2012;062505	**207**	**228**	96	33	35	57	37
2012;062510	193	**222**	75	35	35	89	37
2012;062525	**212**	**216**	79	32	38	60	26
2012;062580	163	**216**	66	32	27	53	30
2012;062595	176	169	**312**	33	23	27	31
2012;0625106	**217**	**226**	88	34.5	38	30	23
2012;0625112	**216**	**227**	80	41	35	51	44
2013;091905	94	109	54	28	39	**226**	40
2013;091909	148	181	82	37	32	**337**	29
2013;091928	149	197	71	32	41	**258**	52

*E (EBOV), EΔM (EBOVΔM), S (SUDV), Mm (MARV(Mus)), H (HeV), N (NiV), C (CedPV); ID (Specimen identification).

†Values in boldface are positive results.

‡Indicates sera samples that repeated seropositive in the second round of screening

Table 5. Positive sera samples screened (1:100) in the preliminary screen had a range of median fluorescence intensities (MFI)

	MFI > 1000 (+++)	1000 > MFI > 600 (++)	600 > MFI > 200 (+)
C. brachyotis			
SUDV	1	0	8
EBOV	0	1	10
EBOVΔM	0	0	9
MARV	0	0	0
HeV	0	0	1
NiV	0	0	3
CedPV	0	0	1
E. spelaea			
SUDV	1	1	11
EBOV	0	1	18
EBOVΔM	0	0	17
MARV	0	0	0
HeV	0	0	3
NiV	0	0	6
CedPV	0	0	1
P. lucasi			
SUDV	0	0	1
EBOV	0	0	4
EBOVΔM	0	0	6
MARV	0	0	0
HeV	0	0	0
NiV	0	0	3
CedPV	0	0	0

Screen with completed virus sGp panel

The Bio-Plex multiplex assay, when originally created, only contained envelope glycoproteins from henipaviruses. This *Henipavirus* multiplex assay and the individual soluble HeV and NiV envelope glycoproteins have been used in previous bat and wildlife biosurveillance by collaborators (53; 114; 223; 224). Given our lab's extensive experience in generating conformational-dependent, oligomeric virus envelope glycoproteins, the panel of glycoproteins included in this binding assay was expanded to include sGps from all known species of ebolaviruses and marburgviruses. With this now complete multiplex panel, we rescreened the bat sera collection at Duke-NUS Medical School, Singapore.

RESTV is the only presently known filovirus in Asia, and there is serological and molecular evidence of RESTV infection in bats found in the Philippines (131; 274). RESTV is thought to have originated in Asia, but its close phylogenetic relationship with SUDV raises questions about its true geographic and evolutionary origins (228). Because of the close proximity of Singapore to the Philippines and the phylogenetic relationship between SUDV and RESTV, we hypothesized that all bat sera samples that were cross-reactive with EBOV and SUDV sGps would be seropositive with sGps from RESTV. Additionally, we included two strains of RESTV, one isolated from infected pigs and a second from infected monkeys to increase our chances of identifying RESTV seropositive samples. Other ebolaviruses included in the complete panel included TAFV, responsible for one non-fatal human infection; BDBV a closely related *Ebolavirus* sp.; a second variant of MARV, MARV Angola; and RAVV (Table 3).

A total of 409 sera samples collected from *C. brachyotis* (n=153), *E. spelaea* (n=186), and *P. lucasi* (n=70) bat species were screened with the complete multiplex

Ebolavirus, Marburgvirus, and *Henipavrus* sGp binding assay. All sera samples screened in the first round were included in the second round. The data demonstrated evidence of bat sera cross-reactivity with EBOV, BDBV, TAFV, and SUDV sGps (Figure 10 and Table 6). EBOV, BDBV, and TAFV cluster together as one clade based on phylogenetic analysis so it was not surprising that samples seropositive for one of these sGps would be cross reactive with other species. The highest MFIs were observed with the EBOV and BDBV sGps, which suggested that the ebolaviruses that circulate in these three bat species are most antigenically similar to EBOV and BDBV (Figure 10 and Table 6). Unexpectedly, there was no detection of sera reactivity with RESTV sGps. We identified zero seropositive samples with sGps from *Marburgvirus* species despite the addition of two more *Marburgvirus* sGps.

We identified 23 *E. spelaea* (12%), 11 *C. brachyotis* (8%), and 3 *P. lucasi* (4%) samples as seropositive (Table 6). Between the two independent experiments, five *E. spelaea* sera samples and four *C. brachyotis* positive samples repeated positive results (Table 4). All three bat species sampled exhibited sera reactivity to BDBV or EBOV sGps (Table 6). Positive *E. spelaea* sera samples were seroreactive with BDBV, EBOV, and SUDV sGps. Eleven out of fifteen EBOV seropositive *E. spelaea* sera samples were cross-reactive with BDBV sGps (Table 6). Two *E. spelaea* samples were only reactive with EBOV sGp and four samples were only reactive with BDBV sGp (Table 6). Positive *C. brachyotis* samples displayed a preferential reactivity with BDBV sGps (Figure 10 and Table 6). Of the eleven seropositive samples, six samples were seroreactive only with BDBV, five samples were cross-reactive with EBOV and BDBV, and one sample was only seropositive with SUDV sGp (Table 6). Similar to *C. brachyotis,* two out of three

seropositive *P. lucasi* samples were only seroreactive with BDBV, and the third positive sample was cross-reactive to BDBV and EBOV (Table 6). Ten *E. spelaea* and five *C. brachyotis* positive samples bound to SUDV (Table 6). The majority of seropositive bat sera samples were cross-reactive with BDBV, EBOV, and SUDV sGps. Examination of cross-reactivity for *C. brachyotis* seropositive samples revealed that five out of thirteen samples were preferentially reactive with BDBV only, one out of thirteen samples was only seropositive for SUDV sGp, three out of thirteen samples were seroreactive with BDBV, SUDV, and EBOV sGp, and two out of thirteen samples were EBOV and BDBV seropositive (Table 6). BDBV and TAFV Gp share ~81% sequence identity, yet despite this sequence similarity we did not observe high binding to, as measured by MFI values, TAFV Gp in the BDBV and EBOV cross-reactive seropositive samples (Table 6). Altogether, these results suggested that EBOV, BDBV, and SUDV sGps share conserved immunodominant epitopes. We also observed seroreactivity in one *E. spelaea* sample to CedPV and two *C. brachyotis* samples were seroreactive with CedPV and NiV (Figure 10 and Table 6).

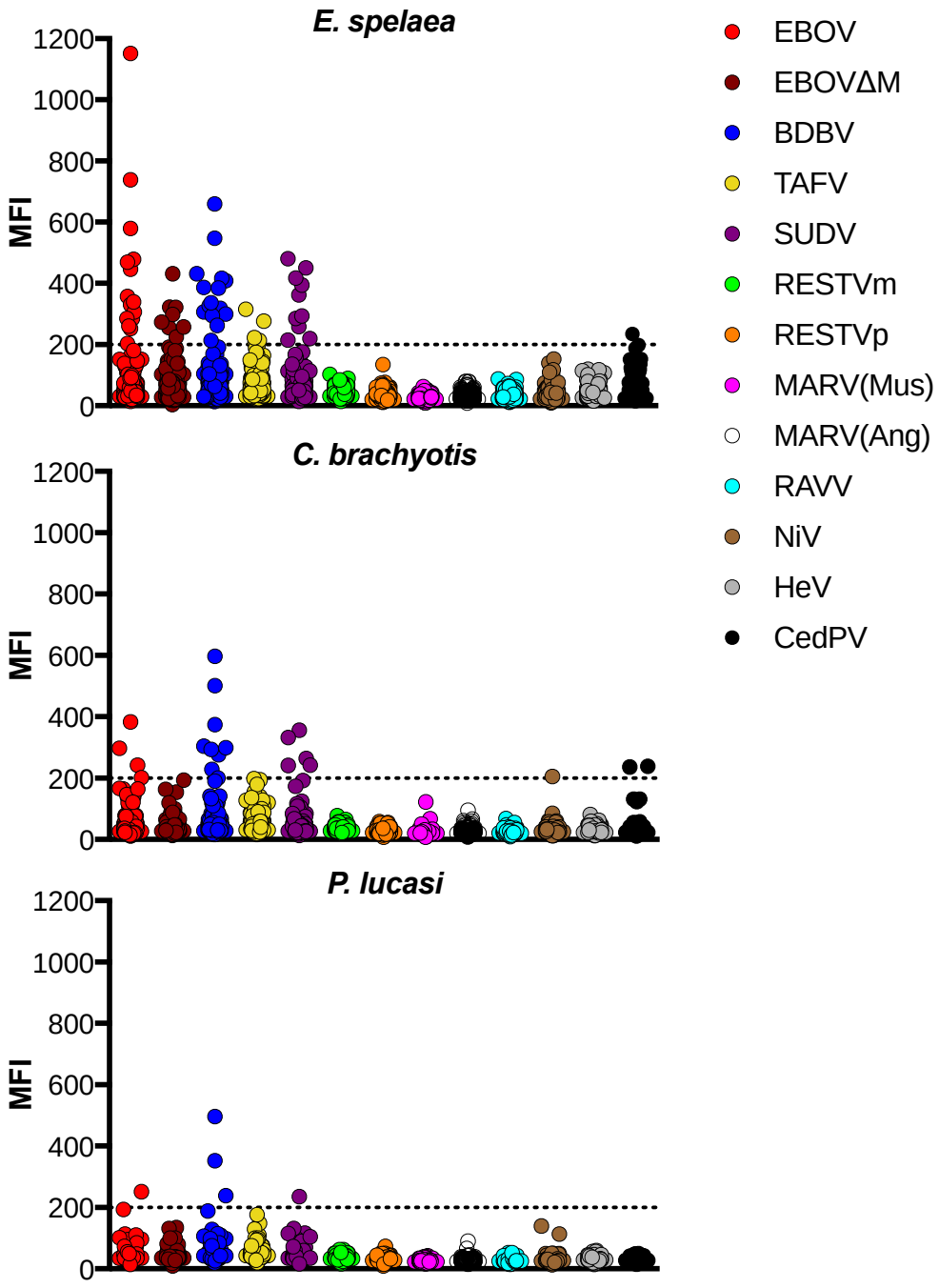


Figure 10. Serologic evidence of past exposure to ebolaviruses detected by complete Bio-Plex multiplex binding assay in three Pteropodidae bat species.

*Bat sera samples screened with the full sGp panel, dashed line indicates 200MFI

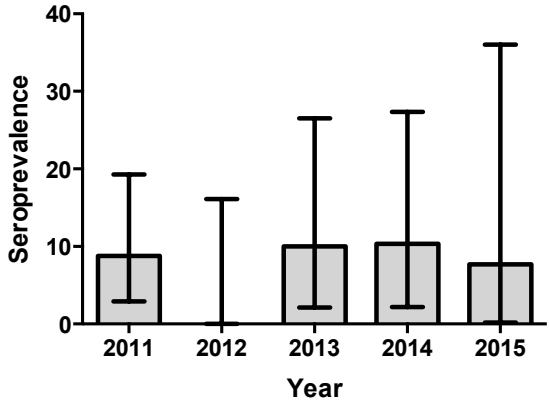
Table 6. Bio-Plex median fluorescence intensity (MFI) serology results for bats screened in the second round

E. spelaea (n=186)													
Year;ID	E	EΔM	S	B	T	Rm	Rp	Mm	Ma	R	H	N	C
2011;0805149	**738**	105	40	124	68	44	22	23	21	24	27	32	25
2012;080814	86	39	**258**	**318**	105	26	12	17	16	20	18	23	21
2012;082154	143	62	**214**	161	113	35	41	21	31	39	34	36	31
2013;030704	125	**255**	90	142	122	62	65	59	52	85	107	97	152
2013;052313	**284**	88	**285**	**408**	177	89	72	29	23	30	28	45	28
2013;052335	**203**	98	**219**	191	124	42	21	38	38	24	31	43	106
2013;052339	**357**	144	**293**	**306**	141	54	31	26	26	42	33	24	38
2013;041112	104	**322**	73	103	93	62	59	53	42	87	91	103	84
2013;071839	**330**	146	**480**	**299**	164	65	44	28	33	45	78	74	81
2013;071842	**446**	**321**	**362**	**327**	**202**	65	49	42	38	57	85	60	118
2013;110733	126	94	95	**416**	166	58	42	34	42	58	68	49	84
2014;011603	**1151**	**298**	69	130	91	36	32	51	35	39	56	41	151
2014;011616	**252**	184	175	294	168	32	49	47	29	50	43	152	37
2014;011656	**306**	115	**394**	**386**	**204**	89	73	18	39	37	59	71	53
2014;012309	**579**	**224**	69	**659**	**315**	35	31	27	33	35	51	48	65
2014;021303	**478**	**257**	**450**	**431**	188	52	37	24	30	47	27	22	30
2014;052008	25	50	30	33	27	42	33	23	21	25	48	35	**234**
2014;111903	**469**	102	113	**384**	**276**	52	57	37	69	54	45	71	123
2014;111907	**285**	85	158	**336**	**213**	39	36	29	50	30	37	44	85
2016;042701	**339**	138	**417**	**547**	**222**	60	78	54	25	62	40	109	45
2016;042709	146	**431**	118	169	139	77	60	78	49	64	79	84	68
2016;042722	**260**	179	**1**67	**262**	174	75	31	54	24	42	80	76	110
2016;042724	89	**273**	95	131	88	74	66	68	23	51	71	117	91
C. brachyotis (n= 153)													
Year;ID	E	EΔM	S	B	T	Rm	Rp	Mm	Ma	R	H	N	C
2011;051253	121	47	**242**	133	59	40	41	19	25	68	31	22	31
2011;0516613	146	69	73	**293**	127	47	36	25	29	22	34	40	27
2011;0516632	138	56	**356**	139	86	35	25	28	34	34	26	31	28
2011;0726122	119	-	60	**501**	100	40	46	25	19	29	37	-	27
2011;1103241	84	-	**241**	141	128	50	47	66	38	34	62	-	57
2013;100903	148	73	108	**201**	71	42	33	18	16	36	31	27	29
2013;100914	74	47	55	**228**	70	39	38	30	27	26	42	40	39
2013;100925	166	72	116	**304**	109	43	18	33	30	28	52	35	30
2014;021357	**201**	119	**264**	**299**	179	65	44	25	55	47	58	50	42
2014;050804	**242**	193	124	**276**	140	41	30	34	33	44	81	57	55
2014;050818	**383**	163	**332**	**374**	198	60	55	29	26	68	50	32	41
2014;050822	50	60	45	**33**	30	41	63	30	32	38	54	60	**236**
2015;040807	**297**	154	192	**597**	194	40	38	122	95	32	45	205	**238**
P. lucasi (n=70)													
Year;ID	E	EΔM	S	B	T	Rm	Rp	Mm	Ma	R	H	N	C
2012;062590	34	31	39	**496**	93	36	18	23	17	23	41	24	20
2013;070409	95	89	89	**238**	129	62	27	34	36	37	39	41	41
2013;112112	**251**	131	**235**	**352**	148	51	29	23	23	29	42	50	35

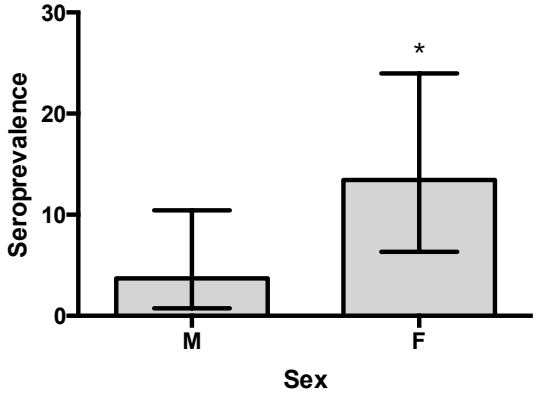
*E (EBOV), EΔM (EBOVΔM), S (SUDV), B (BDBV), T (TAFV), Rm (RESTVm), Rp (RESTVp), Mm (MARV(Mus)), Ma (MARV(Ang)), R (RAVV); ID (Specimen identification); A (adult), J (juvenile)
†Values in boldface are positive results

Univariate analyzes were performed to determine whether age, sex, and year sampled had any associations with seroprevalence (Figures 11-13). Seroprevalence in all three bat species was not significantly associated with age and or year sampled. *C. brachyotis* females had a significantly higher seroprevalence.

A.



B.



C.

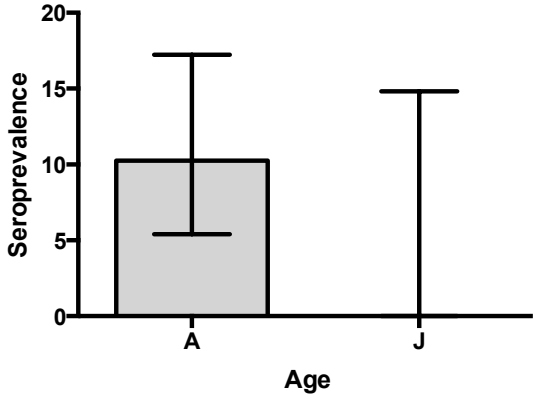
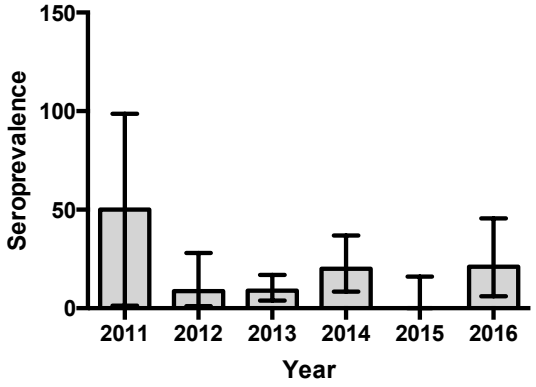
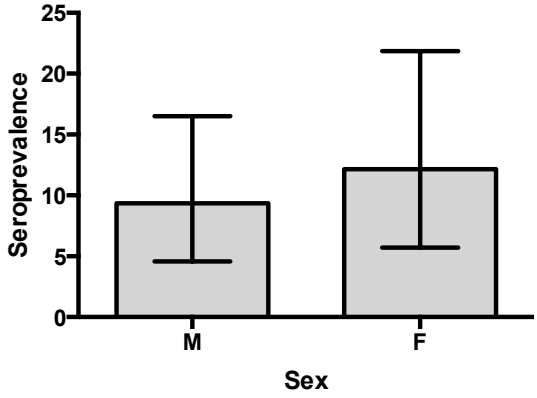


Figure 11. Univariate analysis of *C. brachyotis* seroprevalence.
95% confidence intervals were calculated for all three panels (A, B, C).
*Chi-square test $p<.05$
†A (adult), J (juvenile)

A.



B.



C.

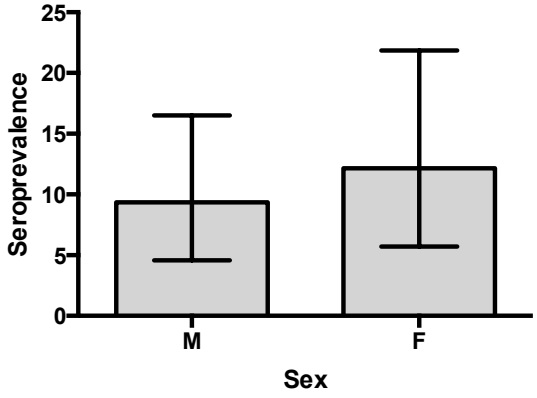
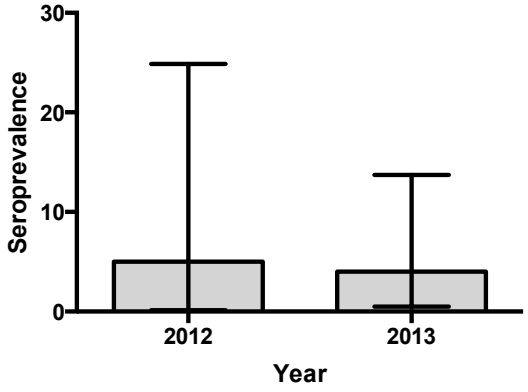
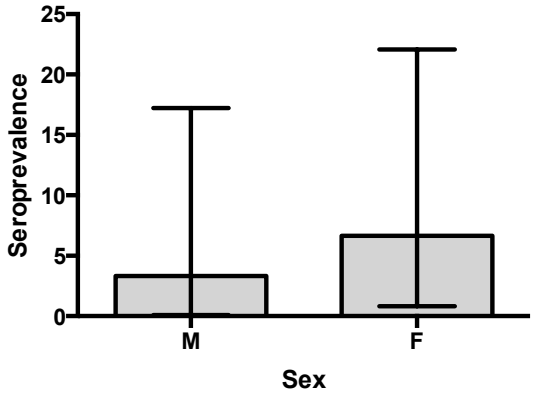


Figure 12. Univariate analysis of *E. spelaea* seroprevalence.
95% confidence intervals were calculated for all three panels (A, B, C).
†A (adult), J (juvenile)

A.



B.



C.

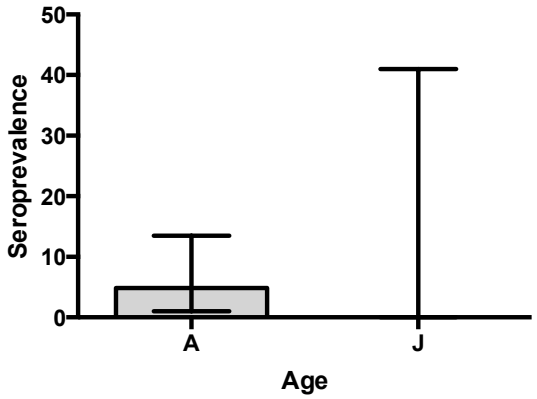
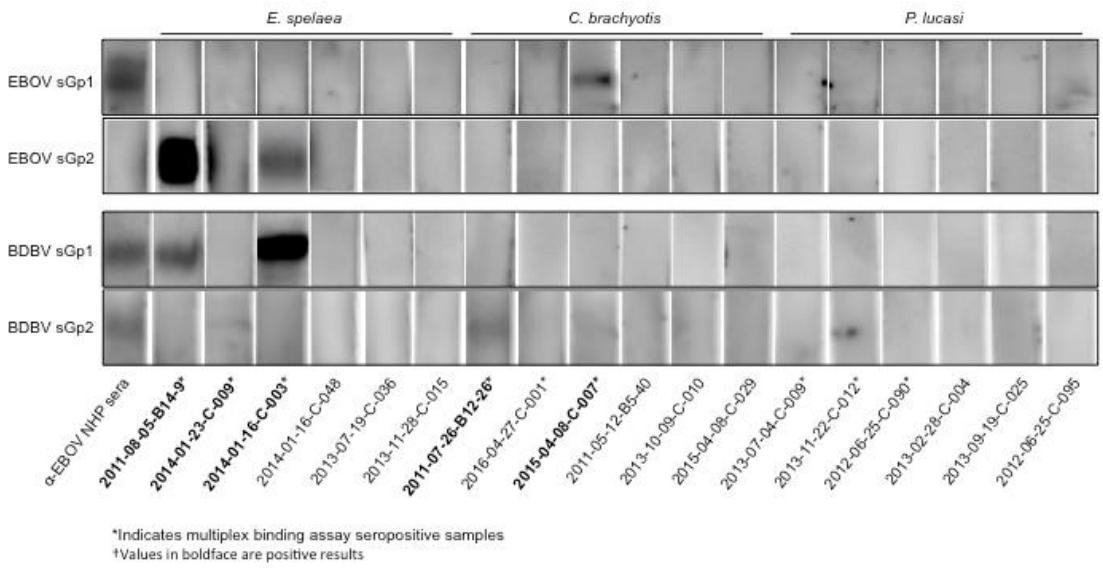


Figure 13. Univariate analysis of *P. lucasi* seroprevalence.
95% confidence intervals were calculated for all three panels (A, B, C).
†A (adult), J (juvenile)

Western blots were used to confirm seropositive Bio-Plex multiplex assay results for a select number of bat sera samples. We selected the three highest MFI binding assay seropositive samples for each species to test by Western blot, and three binding assay seronegative samples were tested as controls. Confirmation by Western blot was performed to first, determine whether the MFI cutoff at 200 was appropriate, and more importantly to explore the cross reactive nature of binding assay seropositive bat sera. Three out of three *E. spelaea* and two out of three *C. brachyotis* samples that were EBOV or BDBV seropositive were WB positive; no *P. lucasi* binding assay positive samples were WB positive (Figure 14).



*Indicates multiplex binding assay seropositive samples
†Values in boldface are positive results

Figure 14. Western blots of select seropositive and seronegative bat sera samples

2.5 Discussion and Future Directions

To our knowledge, this is the first bat serological surveillance study to comprehensively examine preferential reactivity to all known ebolaviruses and marburgviruses. The majority of *R. leschenaultii* sera collected in Bangladesh preferentially reacted to EBOV nucleoprotein (Np) compared to RESTV Np (212). Seropositive *R. leschenaultii, Pipistrellus pipistrellus and Myotis* species samples collected in China and screened by RESTV Np and EBOV Np ELISA reacted equally with both virus Nps (308). Inclusions of sGp antigens from all ebolaviruses allowed this multiplex assay to further discriminate preferential reactivity of bat immunoglobulins. The majority of *E. spelaea, C. brachytois,* and *P. lucasi* seropositive samples had the highest seroreactivity with BDBV and EBOV sGps and a majority of positive sera was cross-reactive.

Observed seroreactivity was specific to sGp antigens from ebolaviruses, and we saw no seroreactivity with marburgviruses. Surprisingly, we did not observe any seroreactivity with RESTV sGps. This was in contrast to previous surveillance projects that reported cross-reactivity of bat sera with RESTV Np (212; 274; 308). One explanation for the lack of RESTV seropositive samples in this study is that our binding assay is based on sGp antigens in contrast to virus Nps used in previous serological ELISA-based surveillance studies. The use of conformational sGp versus bacterially expressed Np has increased specificity in Luminex-based *Henipavirus* assays (personal communication, G Crameri). It is likely that use of sGps as antigens in our Bio-Plex multiplex binding assay increased specificity to detect bat IgGs specific for *Ebolavirus* species compared to binding assays that used virus Np. Furthermore, our lack of evidence of RESTV sGps seroreactivity with *Cynopterus* and *Eonycteris* sera samples is in line

with the seronegative findings of these bat species sampled in the Philippines. Of 13 confirmed bat species sampled in the Philippines, which included *C. brachyotis* (n=35) and *E. spelaea* (n=5), only *R. amplexicaudatus* sera samples had positive serology results with RESTV Gp and Np (274). *R. aegyptiacus* experimentally infected with two ebolaviruses and MARV, only developed viremia and virus shedding when infected with MARV, which was the first evidence to suggest host restriction within the filovirus family (Megan E.B, 2015). The observed MARV-*R. aegyptiacus* host restriction might extend to other members of the *Filoviridae* family such as RESTV and putatively *R. amplexicaudatus*, and may explain why we did not detect reactivity with *E. spelaea, C. brachyotis,* and *P. lucasi* sera and RESTV sGps.

One limitation of serological surveillance methods is the issue of antibody cross-reactivity with antigens included in the serology assays, and the degree of unknown cross-reactivity between experimental sera and the virus antigens included in the assay (91). This limitation becomes more important when serological surveillance is performed in geographies where virus distribution is unknown as was this study. In an effort to address cross-reactivity limitations we designed the multiplex binding assay to include sGps antigens from all presently known ebolaviruses, marburgviruses, and henipaviruses. Human survivors of EBOV, BDBV, and SUDV infection generated IgGs that were cross-reactive to antigens from heterologous ebolaviruses (172). The inclusion of Gp antigens from all known ebolaviruses, marburgviruses, and henipaviruses helped to address some limitations of a serology-based binding assay. Antigens from *Marburgvirus* and *Henipavirus* genera essentially functioned as negative controls within the multiplex, thus

permitting our interpretation that the bat sera screened had evidence of past infection with a virus mostly closely related to the *Ebolavirus* genus.

Previous serological surveillance studies of infectious diseases in wildlife have used antigens from one or two virus species to detect immunoglobulins from animal hosts (114; 212). In these studies, researchers reported finding evidence of antibodies specific to ebola-like viruses or henipa-like viruses. An absence of reactivity with antigens from one or two of the three virus genera (*Henipavirus* and *Marburgvirus*) increased our confidence that the cross-reactivity with BDBV, EBOV, and SUDV sGps was not an artifact of the binding assay. Additional research is needed to optimize final sera dilutions that might permit discrimination at a virus species level and decrease cross-reactivity with related virus sGp-beads. Whether this assay can be further developed to differentiate immunoglobulins specific for each ebola- or marburgvirus species remains to be determined. A final sera dilution of 1:250 has been used to discriminate between anti-HeV immunoglobulins and anti-NiV immunoglobulins in polyclonal sera with the same Luminex-based multiplex platform (36). Non-lethal sampling of non-*Pteropus* bats yields low volumes of sera, so a Luminex-based multiplex approach to screening is the ideal platform for such an analysis.

Between the two rounds of screening we discovered that sera diluted 1:100 provided a more discriminatory dilution to investigate preferential reactivity with sGps in the binding assay. Most of the 1:50 bat sera samples that had MFI > 1000 with the SUDV sGp in the initial round of screening were lost during the second round when tested only at 1:100 dilutions. One serum sample that was negative in the first screen became positive in the second screen because of seroreactivity with BDBV that was not included

during the initial screen. This highlights the need for multiplex assays to be designed to be as inclusive as possible so that differences in antigen sequences do not limit surveillance detection.

Comparing Western blot results with Bio-Plex binding assay MFI it appears that MFI of >500 are a good indication of whether a sera sample will test positive by Western blot. Conformational sGps were used as antigens in the Bio-Plex binding assay and denatured, linear sGp peptides were used as antigens in the WB assays making these two techniques fundamentally disparate. Seropositive samples with MFI >500 most likely reflects a high IgG titer and the majority of the IgGs in these sera samples are conformational-dependent. Thus, when the sGp antigen is linearized, only samples with high IgG titers have the minority populations of IgGs required to react with linearized sGp and be detected by Western blot.

There are two likely explanations for the observed preferential reactivity to BDBV, EBOV, and SUDV. One, or all of these viruses circulates in populations of these bats, and cross-reactivity is a reflection of shared epitopes that are the result of the Gp sequence similarity (EBOV and BDBV Gp sequences are 70% similar); or a novel, antigenically similar ebolavirus species is in circulation. Given the absence of Ebola-virus disease in Singapore and Southeast Asia, we think that the mostly likely interpretation based on the data presented, is that a novel *Ebolavirus* species, antigenically related to BDBV, EBOV, and SUDV is in circulation. The Western blot results provide additional support to this conclusion. The five out of nine binding assay seropositive samples that tested positive by WB displayed differential reactivity to EBOV and BDBV Gp1 or Gp2 antigens. We expected that bat sera preferentially reactive to

EBOV sGp by binding assay would be preferentially reactive to EBOV sGp by Western blot. Unexpectedly, we observed that the two *E. spelaea* samples that were seropositive by binding assay for EBOV and no other sGp reacted with both EBOV sGp2 and BDBV sGp1. We think reactivity with more than one *Ebolavirus* species denatured sGp supports our assertion that a novel *Ebolavrius* species, antigenically similar to EBOV and BDBV is in circulation in these bat species.

Bats are known to maintain a high diversity of viruses (171). Biosurveillance of global bat populations and sequence analysis of known and newly identified paramyxoviruses have suggested that bats might be the ancestral host of all paramyxoviruses, and that the current number of known paramyxoviruses is under represented (65). Furthermore, the bat coronaviruses include a large diversity of viruses with only a few known to cause human disease (87). The same diversity most likely exists for filoviruses and has yet to be characterized. Genomic sequencing identified a putative filovirus strain in *R. leschenaultii* bats collected in China (116). More recent, twenty-three novel filovirus sequences have been discovered in two species of bats endemic in the Yunnan province of Southern China (302). Further comprehensive surveillance approaches that include serology and nucleic acid sequencing will help to elucidate the geography of filovirus diversity distributed within Asia.

Cynopterus sphinx sampled in China were seropositive to RESTV Np and EBOV Np antigens (308). We have contributed additional serological evidence of antibodies specific to ebolaviruses found in a *Cynopterus* species (*C. brachyotis*), and have added *E. spelaea* and *P. lucasi* to the growing list of putative hosts of filoviruses in Asia. Sex, age, and year collected were analyzed for any effect on seroprevalence for each bat species.

There were no significant increases in seroprevalence due to years or age in any of the three species so no conclusions can be drawn about timing of introduction of the virus in the bat populations or whether birth pulses contribute to virus persistence in the populations as demonstrated for filoviruses in African bats (111). Female *C. brachyotis* bats had a significant association with seroprevalence. *Cynopterus* species are known to have a harem-type social organization, and this social structure might contribute to virus transmission dynamics (47).

Live MARV has been isolated from *R. aegypticus* bats, however, a definitive maintenance reservoir has not been established for any species of *Ebolavirus* (13; 278). Bats are gregarious, and sympatric bat species have been demonstrated to be host to more zoonotic viruses (171). A study of *Eidolon helvum* in Africa revealed panmictic population connectivity (225). All three bat species that we screened in this study have overlapping geographical ranges in Southeast Asia and share ecological niches and foraging sites in Singapore (180). A minority of *C. brachyotis* bats included in this study were captured at the main *E. spelaea* roosting colony in Singapore, and *E. spelaea* bats were captured at locations where the majority of bats collected were *C. brachyotis*. We observed that *E. spelaea* sera samples had the highest number of samples that were cross-reactive with three or more virus sGps. In addition, *E. spelaea* was the only species sampled that had positive cross-reactive sera with TAFV. We can examine HeV ecology studies to gain insight into observations about higher virus exposure in *E. spelaea* populations. HeV is known to infect all four *Pteropus* species in Australia yet the Black Flying Fox, *P. alecto* is regarded as the most competent reservoir species responsible for most transmission to target populations (45; 78). Drawing conclusions about the status of

E. spelaea as the primary animal reservoir of this putatively novel *Ebolavirus* and potential transmission of this virus to *C. brachyotis* and *P. lucasi* populations at this point must remain speculative. However, the roost size of the *E. spelaea* colony in Singapore is large in size and might be large enough to allow for persistent transmission.

Serologic and nucleic acid evidence of past infection in Asian bat species distributed from Bangladesh to the Philippines suggests the importance of bats in the maintenance of ebolaviruses in this geography. The metapopulation structure of *E. spelaea, C. brachyotis,* and *P. lucasi* in Singapore and Southeast Asia, and whether community structure facilitates virus transmission is unknown. It is easy to hypothesize that the *Ebolavirus* that we discovered serological evidence of in these bat populations is maintained through multi-bat host transmission. *Rousettus leschenaultii* and *E. spelaea* share roosting sites in India, and both have been identified as putative filovirus-hosts through serological surveillance ((212; 302)personal communication, IH Mendenhall). Comprehensive surveillance projects will be needed to understand the multi-host dynamics of *Ebolavirus* maintenance in Southeast Asia, and whether maintenance and non-maintenance bat species or populations can be identified (110).

2.6 ACKNOWLEDGEMENTS

sGps were prepared by Lianying Yan and Spencer L. Sterling at USU. Ian H. Mendenhall, Sophie A. Borthwick, Erica S. Neves, Dolyce Low Hong Wen at Duke-NUS, and Benjamin PY-H Lee at the National Parks Board comprised the bat surveillance team. Julia Lim Sze Lynn and Dolyce Low Hong Wen optimized and performed the experimental western blots. Yihui Chen, Martin Linster, and Ian H. Mendenhall provided cut-off analysis. Ian Mendenhall and Martin Linster provided

valuable input about data interpretation. This work was supported in part by a National Science Foundation (NSF) East Asia and Pacific Summer Institutes (EAPSI) Fellowship, and funding from the Bio-Defense Research Directorate.

CHAPTER 3: The role of autophagy during virus infection in bat (natural) and human (accidental) hosts

3.1 INTRODUCTION

Bats are the only volant mammals, have life spans that are longer than what is typically predicted in relation to their small body size, and are natural hosts for several highly pathogenic, zoonotic viruses (e.g. Nipah virus, Ebola virus, and SARS-like coronavirus) (75; 100; 168). Within the mammalian class, flight remains exclusive to bats and has been hypothesized to contribute to the ability of bats to be virus reservoirs (204; 310). In contrast to most terrestrial mammals, bats do not develop symptomatic diseases following infection with these zoonotic viruses.

Comparative genome sequencing of the Black Flying Fox (*Pteropus alecto*), the natural host of Hendra virus and Australian bat lyssavirus (ABLV) with *Myotis davidii*, an insectivorous bat species, revealed positive selection of genes involved in the DNA-damage repair pathway (97; 100; 310). The aerobic demands of flight in bats is higher than the aerobic demands of other terrestrial mammals experienced during exertion, and both bats and birds are longer lived than non-flying terrestrial counterparts, which is counterintuitive to oxidative stress theories of aging that propose senescence follows the accumulation of DNA and cellular damage caused by reactive oxygen species (24; 193; 269). Positive selection of genes involved in DNA-damage repair pathways in bats is suggestive of a response to deleterious accumulation of reactive oxygen species from the high aerobic demands of flight. Whether bats possess unique immune responses that suppress viral infection or limit disease pathogenesis has recently become an area of increased research focus.

Interestingly, long-lived mammalian species including bats have been demonstrated to possess enhanced mechanisms for maintaining protein homeostasis (237; 245). Improved cellular homeostatic mechanisms might contribute to longevity in animal species with high metabolic and oxidative stress. Further, macroautophagy, hereafter referred to as autophagy, is a catabolic process involved in cellular homeostasis and survival. Autophagy functions as a homeostatic mechanism in response to stressors and promotes cellular survival by removing damaged organelles, and misfolded or aggregated proteins from the cytoplasm (93). Autophagy is activated by a variety of cellular stressors including DNA-damage and reactive oxygen species (63; 82). In addition to removal of damaged organelles and protein aggregates, autophagy functions as an intrinsic immune response during infection with a variety of pathogens (52; 128; 189; 215).

Upon infection, viruses typically 'hijack' the host cell transcriptional and translational machinery to facilitate replication. The dysregulation of homeostatic mechanisms goes hand-in-hand with the viral exploitation of the host cell machinery (176). In essence, virus infection represents just one of many stressors that homeostatic mechanisms must contend with to avoid cell death. Therefore, if long-lived bats have improved proteostatic mechanisms, and have evolved mechanisms to reduce DNA and cellular damage from reactive oxygen species, then these same proteostatic mechanisms might function as antiviral defenses. Episodic shedding of latently infected natural hosts or virus recrudescence in natural hosts has been proposed as a model that maintains virus persistence in host populations and drives pulses of zoonotic virus spillover (229). However, it is not understood whether zoonotic viruses persist in natural hosts at a cellular level. For episodic shedding to occur, natural hosts cannot completely abolish

virus infections. Of note, autophagy has been demonstrated to function as a pro-survival defense during Sindbis virus infection by removing toxic virus protein aggregates, but does not effect virus replication (216; 268). Indeed, a pro-survival autophagic response might explain how natural hosts permit virus persistence at the cellular level, but still allow for viral replication and spillover events to occur. At the crossroads of cellular homeostasis, the autophagy pathway responds to DNA damage, reactive oxygen species, and intracellular pathogens, which are associated with three characteristics of bats: flight, longevity despite high metabolic costs, and being natural hosts of pathogenic RNA viruses.

We undertook a comparative study to examine the potential antiviral defenses of autophagy in cell lines derived from a natural bat host of zoonotic viruses. Brain, lung, kidney, and fetal tissue-derive cell lines have been established from *P. alecto* (58). *P. alecto* is a model bat species and derived cell lines have been used to investigate the interferon response, as well as transcriptomic and proteomic responses after HeV infection (300; 315). ABLV, a neurotropic, Rabies virus-related virus, is classified as a biosafety-level 2 (BSL-2) pathogen and naturally infects *P. alecto* bats (97). ABLV, like all lyssaviruses, has a 3'-5'(-)ssRNA genome with five protein-coding genes. The nucleoprotein (Np) encapsidates the genomic RNA and facilitates interactions with the phosphoprotein (P) and RNA polymerase (L) that are necessary for transcription and replication. The matrix protein (M) is responsible for virion maturation and budding. The envelope glycoprotein (G) facilitates attachment and entry in host cells.

Using ABLV and *P. alecto* as our model virus and natural host, respectively, we examined potential differences between natural (bat) host-pathogen interactions and

accidental (human) host-pathogen interactions with the autophagy pathway. We hypothesized that autophagy functions as an antiviral mechanism in cells derived from the natural bat host, *P. alecto,* during ABLV infection.

In this study, we rescued a modified ABLV virus that expresses a green fluorescent protein (ABLV-GFP). We observed that infection with ABLV-GFP resulted in the activation of the autophagy pathway in both bat and human derived cell lines. Wild-type ABLV (ABLV-WT) infection of primary *P. alecto* brain cells similarly activated autophagy. In *P. alecto* cells, the basal level of autophagy was significantly higher than the levels of autophagy quantified in the human cell line used for comparative purposes. Activation of autophagy decreased ABLV replication in both bat and human cells, and an incomplete genetic knockdown of autophagy-related-gene 5 (ATG5) resulted in slight decreases in autophagy levels and increases in ABLV replication. Combined these results suggested that autophagy functions as an antiviral defense in bat cells. To determine whether the induction of autophagy could be harnessed as a therapeutic strategy, a human neuroblastoma cell line was treated with NVP BEZ235 (BEZ), a pharmacologic autophagy activator, during ABLV infection. Pre- and post-infection treatment with BEZ reduced ABLV replication, demonstrating that activation of autophagy might be an effective treatment against neurotropic viruses.

3.2 AIMS AND HYPOTHESIS

Hypothesis. We hypothesize that autophagy functions as an effective antiviral mechanism during ABLV infection of bat cells.

Aim 2A. Elucidate the antiviral role of autophagy during bat cell infection

Aim 2B. Explore the therapeutic potential of pharmacologic autophagy activation during neurotropic viral infection

3.3 Materials and Methods

Cell Lines

PaBrH (hTERT immortalized) and PaKiT (SV40 T antigen immortalized) cell lines are brain and kidney tissue cells, respectively, derived from the Black Flying Fox, *P. alecto* (58). Immortalized *P. alecto* cells were grown in DMEM (GIBCO©, Thermo Fischer Scientific; Waltham, MA) supplemented with 10% cosmic calf serum (CCS) (HyClone, Fischer Scientific; Hampton NH) and 1% L-glutamine (GIBCO©). A human neuroblastoma cell line, NBF-L, was a kind gift from Dr. Aviva Symes (272). NBF-L cells are a fibroblast-like neuroblastoma cell line and were used in comparative experiments with PaBrH, which morphologically also appear fibroblast-like. NBF-L cells were grown in DMEM supplemented with 10% CCS, 5% horse serum (GIBCO©), and 1% GlutaMAX™ (GIBCO©). Mouse neuroblastoma Neuro 2a (N2A) cell line and human embryonic kidney cell line (HEK293T) were used in experiments to allow for additional comparisons. HEK293T was discovered to be a less suitable cell line for autophagy Western blot experiments, but was still chosen for some comparative experiments with the modified ABLV-GFP reporter virus because both the PaKiT and HEK293T cell lines have been immortalized via SV40 T antigen. Primary *P. alecto* brain cells were grown in DMEM:F12 with L-glutamine (Sigma-Aldrich; St. Louis, MO), 10% fetal bovine serum (GIBCO©), and 0.1% penicillin/streptomycin (GIBCO©).

Reagents

Antibodies used in this study that cross-reacted with proteins harvested from *P. alecto* whole cell line lysates included anti-β-actin polyclonal antibody (Abcam: Cambridge, UK), anti-LC3B polyclonal antibody (Sigma-Aldrich), anti-p62 polyclonal antibody (Sigma-Aldrich), anti-NDP52 polyclonal antibody (Aviva Systems Biology; San Diego, CA), and anti-ATG5 polyclonal antibody (Novus Biologicals LLC; Littleton, CO). Additional antibodies included: anti-GAPDH polyclonal antibody (Abcam) and anti-turboGFP polyclonal (Evrogen; Moscow, Russian Federation). Anti-Rabies virus nucleoprotein polyclonal rabbit sera, a kind gift from Dr. Ina Smith (CSIRO), anti-Rabies virus phosphoprotein polyclonal antibody (MyBioSource; San Diego, CA) and FITC anti-Rabies monoclonal globulin (Fujirebio Diagnostics, Inc.; Malvern, PA) were used for detection of ABLV proteins. Chemical modulators of autophagy included: NVP-BEZ235 (Selleck Chemicals; Houston, TX). bafilomycin A1 (InvivoGen; San Diego, CA), chloroquine (Sigma-Aldrich), rapamycin (Sigma-Aldrich), SMER28 (TOCRIS; Bristol, UK), 3-methyladenine (Sigma-Aldrich) and VPS34IN1 (Millipore-Sigma; Darmstadt, Germany). Polyinosinic:polycytidylic acid (poly(I:C)) (InvivoGen) was used as a TLR-3 ligand. Cells were stained with CYTO-ID® Autophagy detection kit (Enzo Life Sciences, Inc.; Famingdale, NY) to monitor levels of autophagy by flow cytometry. We utilized several pharmacological inhibitors of autophagy in this study; Figure 15 provides a schematic of the autophagy pathway stages inhibited by the drugs used in experiments.

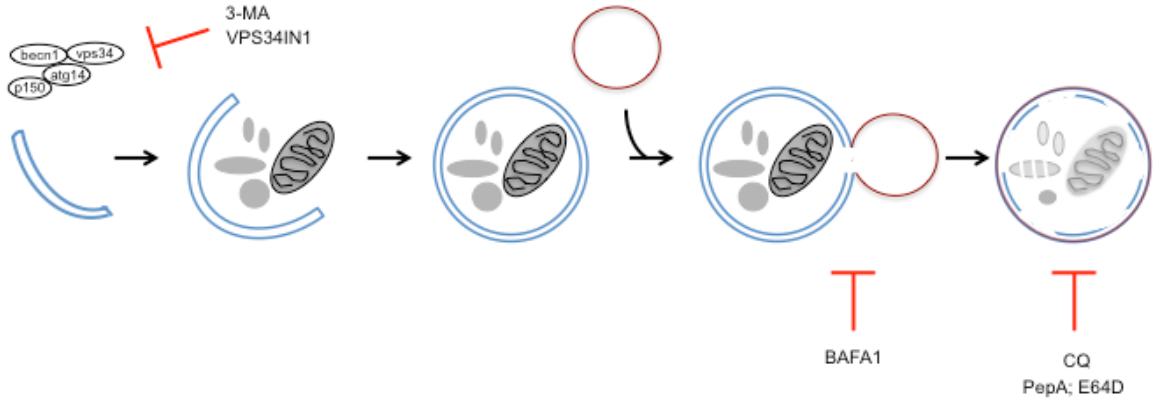


Figure 15. Pharmacological inhibitors of autophagy.
Autophagy inhibitors are shown in red. 3-Methyladenine (3MA) is a pan-inhibitor of class I and III PI 3-kinases, whereas, Vsp34IN1 is a selective inhibitor of the class III PI 3-kinase (Vsp34). Both 3-MA and Vsp34IN1 are considered to inhibit the activation of autophagy through inhibition of PI 3-kinases necessary for phagophore initiation. Bafilomycin A1 inhibits the fusion of autophagosomes with autolysosomes. Chloroquine (CQ) and pepstatin A function as inhibitor of lysosomal degradation. CQ treatment increases the pH of the lysosome, thereby abolishing the degradative nature of the organelle. Pepstain A (PepA) and E64D are inhibitors of lysosomal proteases such as cathepsins. Figure is adapted from (51)

Rescue of modified ABLV-GFP reporter virus

A recombinant Australian bat lyssavirus (ABLV) (*Pteropus* isolate) plasmid, provided by Dr. Ina Smith (CSIRO), was modified to contain a green fluorescent protein (GFP) open reading frame between the glycoprotein (G) and RNA polymerase (L) genes. To generate the modified ABLV green fluorescent protein (GFP) reporter virus (ABLV-GFP), 2 x 10^5 HEK293T cells were transfected with the full-length ABLV-GFP antigenome plasmid (2µg), and virus RNA polymerase complex helper plasmids: nucleoprotein (N; 1µg), phosphoprotein (P; 0.5µg), and RNA polymerase (L; 0.5µg). Virus plasmid DNA was mixed with Lipofectamine LTX® (Thermo Fischer Scientific) (4µg plasmid DNA:10µL Lipofectamine LTX®) in OPTI-MEM (GIBCO©). The HEK293T cell DMEM, 10%CCS, 1%L-gluatmine culture media was removed 24 hours after transfection and replaced with DMEM/6%CCS/1%L-glutamine. Transfected cells remained in culture for three days, at which time GFP expression was examined by microscopy. Transfected cells were then passaged and co-cultured with N2A cells. GFP expression was monitored by microscopy and cell culture supernatant containing infectious ABLV-GFP was passaged to fresh HEK293T cells. To generate large preparations of ABLV-GFP, supernatant from fifteen T-125 infected HEK293T cell culture flasks was harvested, overlaid on a 20% sucrose cushion, and ultracentrifuged at 27,500rpm for two hours.

ABLV infections and immunoblotting

Bat PaBrH, PaKiT, N2A, and HEK293T cells were seeded 1.25x10^5 cells per well in six-well cell culture containers and infected on the next day following seeding. NBF-L cells were seeded 2.5x10^5 cells/well. All cell lines were cultured such that the number of

cells per well at the time of infection was $2.5x10^5$ cells per well. Primary *P. alecto* brain cells were seeded $1.25x10^5$ cells per well in six-well cell culture containers and infected on the next day following seeding. Cells were infected with ABLV-GFP or ABLV-WT at multiplicities of infection (moi) indicated. Cell culture media was removed and replaced with one mL of fresh cell culture media containing ABLV. Infected cell cultures were rocked every fifteen minutes for one hour then a second mL was added to the cultures. Whole cell protein lysates at specific hours post-infection (hpi) were processed per protocol guidelines (181) and protein samples (15µg) were loaded into a 4-12% Bis-Tris protein gel (Invitrogen; Carlsbad, CA). Protein gels were transferred to PVDF membranes (Bio-Rad; Hercules, CA) at 350mA for 90 minutes. PVDF membranes were blocked in 5% milk PBS for 15 minutes then incubated with antibodies specific to LC3B, p62, NDP52, ABLV nucleoprotein (N), ABLV phosphoprotein (P), virus GFP (vGFP), ß-actin, or gapdh. Antibody incubation proceeded at 4°C overnight and two hours at room temperature the next day, with rocking. Protein levels were quantified using ImageJ software.

Basal autophagy experiments

To examine differences in the basal rates of autophagy, bat PaBrH and PaKiT cells were seeded $5x10^5$ cells per well and NBF-L cells were seeded $1x10^6$ cells per well. After 48 hours in culture, all three cell lines reached approximately equal densities in six-well culture plates. Cells were then treated with bafilomycin A1(BAFA1) for two hours before whole cell lysates were harvested. LC3B levels were determined by Western blot.

ABLV titers

Cell culture supernatant containing ABLV-GFP and ABLV-WT was collected and centrifuged at 2600rpm for ten minutes to remove cell debris. Virus supernatant was then serial diluted and incubated with HEK293T cells in quadruplicate in 96-well plates. Titers were counted 72hpi and averaged. For the ABLV-vGFP, the number of fluorescent foci was counted as plaques per mL. To count ABLV-WT titers, 72hpi, HEK293T cells were fixed for thirty minutes at 37°C with 4% paraformaldehyde. Cells were permeabilized, and then incubated with FITC anti-Rabies monoclonal globulin overnight at room temperature.

shRNA transductions

Autophagy related gene 5 homolog (ATG5) shRNA vectors were gifts from Dr. Chou-Zen Giam. Three ATG5 shRNA vectors with target sequences that were 100% identical to *P. alecto* ATG5 nucleotide sequences were transfected along with a lentiviral packaging plasmid, and a Vesicular Stomatitis Virus (VSV)-G envelope plasmid into HEK293T cells. Cell culture supernatant containing VSV-G pseudotyped lentiviruses were collected after three days. NBF-L, PaBrH, and PaKiT cells were infected with ATG5-shRNA lentiviruses and selected with puromycin (1µg/mL).

NVP BEZ235 treatments

The effects of pre-infection and post-infection treatment with NVP BEZ235 (BEZ) on ABLV replication were tested. Human NBF-L cells were pre-treated for four hours with BEZ, after which time cell culture media was removed, then cells were washed with DMEM, and fresh cell culture media containing ABLV-GFP was added to the cells. Human NBF-L cells were pre-treated for four hours with BEZ, and then

infected with ABLV-GFP without any washing to remove BEZ from the cell culture. In this treatment, BEZ remains in the cell culture for a total of 52 hours. Human NBF-L cells were infected with ABLV-GFP, and then 24hpi, BEZ was added to the culture. Whole cell lysates were harvested from all pre-infection and post-infection treated cells 48hpi. In ABLV-WT, BEZ treatment experiments, NBF-L cells were infected with ABLV-WT for 24 hours. At which time, the cell culture supernatant was removed and fresh cell culture media containing serial dilutions of BEZ was added. Supernatant and whole cell lysates were collected after an additional 24 hours, or 48hpi.

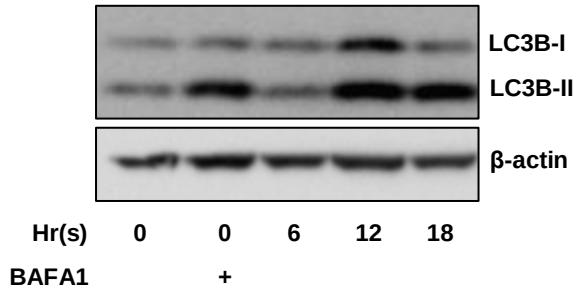
3.4 RESULTS

Infection with ABLV induced the autophagy pathway

Autophagy has been previously demonstrated to function as an antiviral mechanism during vesicular stomatitis virus (VSV) infection (197; 259). VSV is a member of the *Rhabdoviridae* family, which includes the *Lyssavirus* genus. Quite recently, a wild-type Rabies virus (RABV) was discovered to activate the autophagy pathway upon infection of a human neuroblastoma cell line (226). Following specific stimuli, the cytosolic, microtubule-associated protein 1A/1B light chain 3B (LC3B-I) is lipidated, and incorporated into the developing phagophore. Increased levels of the lipidated, autophagosomal-associated LC3B-phosphatidylethanolamine (LC3B-II) are used as a marker for the induction of autophagy (140). Human and bat LC3B protein sequences have high sequence identity, differing by one amino acid (C113Y), which is located in the portion of the LC3B C-terminus that is post-translationally cleaved, making human and bat cytosolic LC3B-I and autophagosomal LC3B-II identical. Stimulation of toll-like receptors (TLRs) with polyinosine–polycytidylic acid (poly(I:C)), a ligand of TLR-3, is

known to activate autophagy (60). We stimulated the bat brain cell line (PaBrH) with poly(I:C) and monitored LC3B-II to examine whether autophagy could be induced in bat cells by Western blot (Figure 16A). In bat PaBrH cells, poly(I:C) stimulation increased LC3B-II levels, consistent with autophagy induction (Figure 16B).

A.



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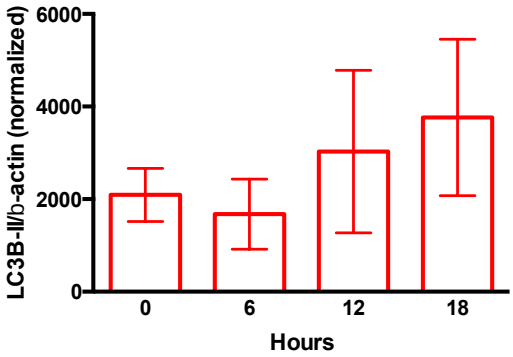


Figure 16. TLR-3 stimulation induces autophagy in bat cells.

(A) LC3B Western blot image. Bat PaBrH cells were treated with poly(I:C) (33µg/mL) for hours indicated. Positive control wells were treated with bafilomycin A1 (BAFA1; 400nM) for two hours.

(B) LC3B-II protein levels. Mean ± SEM. Data is a representation of two independent experiments.

To examine whether autophagy is induced during virus infection in bats, we rescued an ABLV reporter virus that expresses a green fluorescent protein (ABLV-GFP), which served as a model virus in initial experiments.

The full-length anti-genome recombinant ABLV plasmid was modified to express a GFP gene inserted as an open reading frame between the envelope glycoprotein (G) and RNA polymerase (L) genes (Figure 17). Quantification of vGFP was used to indirectly measure virus replication, and was favored for quantification of virus titers in place of staining with FITC anti-Rabies virus monoclonal globulin. The reduced strength of ABLV vGFP signal was the first indication that ABLV replication is more restricted in bat cells.

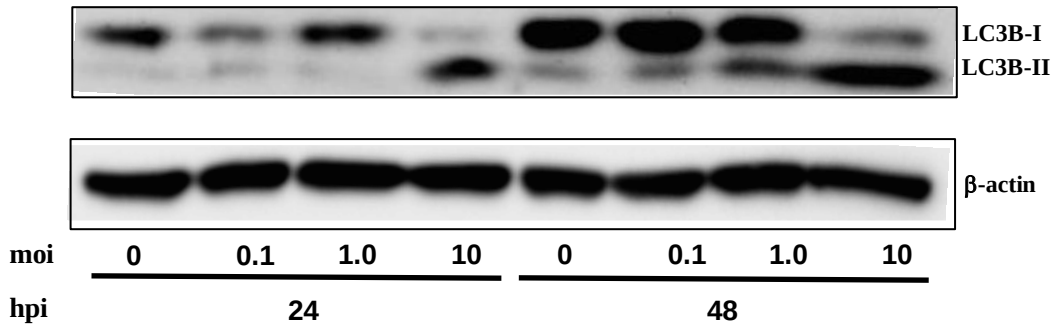
Figure 17. ABLV genome schematics.

* N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (envelope glycoprotein), L (RNA polymerase), le (leader sequence), tr (trailer sequence), cmv (cytomegalovirus promoter), rbz (Hepatitis delta virus ribozyme sequence), GFP (green fluorescent protein)

We monitored levels of LC3B-I and LC3B-II to determine whether ABLV-GFP infection activated autophagy. To compare quantities of LC3B-II between the bat and human cell lines, LC3B-II was expressed as a percentage of total LC3B. The bat brain cell line (PaBrH) had a significant percentage of LC3B-II when infected with a multiplicity of infection (moi) of ten at 24 and 48 hours post infection (hpi) (Figure 18). The human neuroblastoma cell line (NBF-L) had a significant percentage of LC3B-II when infected for 48hpi at moi(s) of one and ten (Figure 19). These results suggested that activation of autophagy in bat cells was dependent on a high virus dose.

Several viruses are known to inhibit autophagic flux, which describes the 'recycling' process of autophagosome fusion with lysosomes, delivery, and degradation of autophagosomal contents (128). Accumulation of LC3B-II after viral infection could potentially be misinterpreted as induction of autophagy when in actuality autophagic flux is being blocked by the virus. The HIV Nef protein and influenza M2 protein have been demonstrated to block the fusion of autophagosomes with autolysosomes, thereby inhibiting autophagic flux (85; 154). Blocking this fusion step leads to an accumulation of autophagosomes and thus an increase in the observed quantity of LC3B-II. To further investigate the induction of autophagy after ABLV infection, we treated cells with bafilomycin A1(BAFA1), a drug that inhibits the fusion of autophagosomes and autolysosomes, and examined levels of autophagy associated cargo receptor proteins (303). During unobstructed autophagic flux, these cargo receptor proteins will be degraded upon fusion of the autophagosomes with the autolysosomes. Any inhibition of autophagic flux at this step will lead to an increase in these proteins and increases or decreases of cargo receptor

A.



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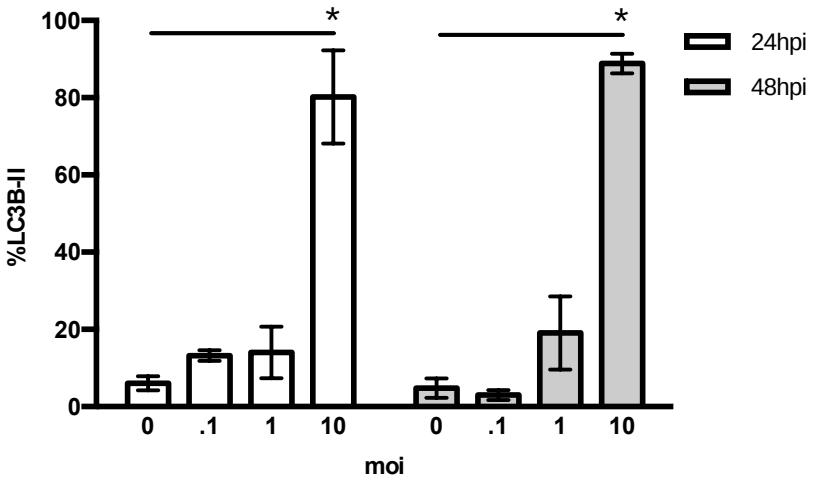
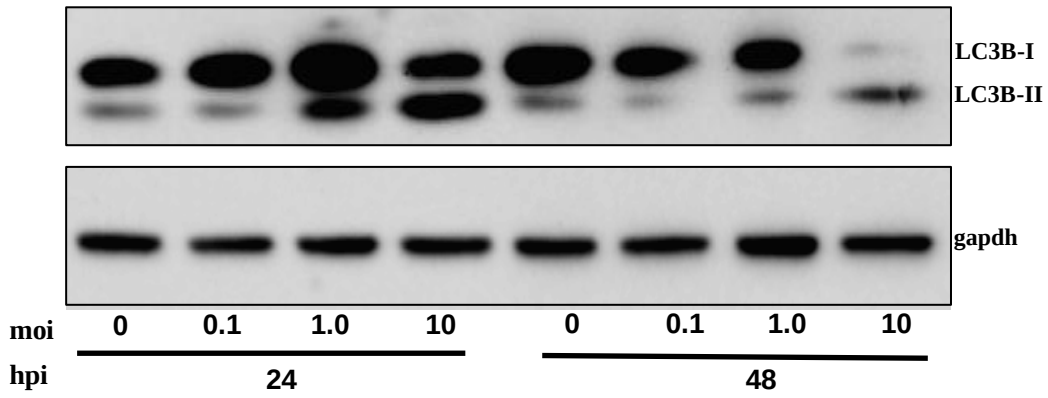


Figure 18. ABLV activates autophagy in bat brain cells.

(A) LC3B Western blot image. Bat PaBrH cells were infected with ABLV-GFP for 24 or 48 hours at moi 0, 0.1, 1.0, and 10.

(B) LC3B-II (ß-actin normalized) expressed as a percentage of total LC3B. Mean ± SEM *p<.05, student's t-test. All data is a representation of three independent experiments.

A.



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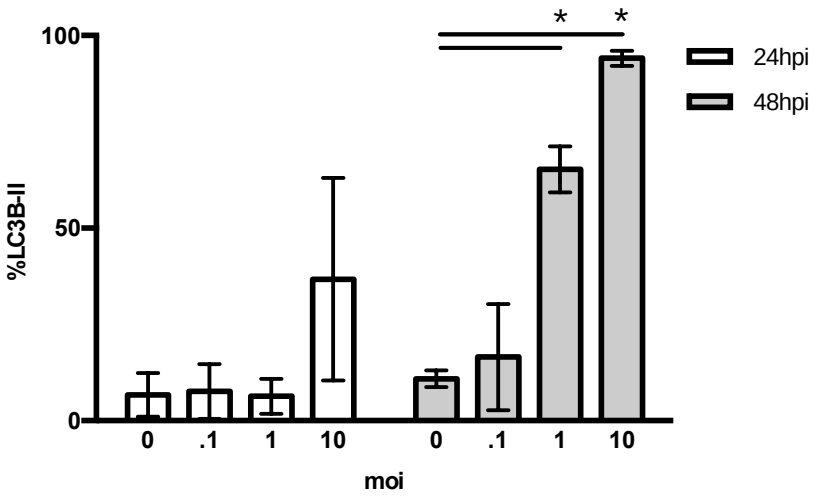


Figure 19. ABLV activates autophagy in human cells.

(A) LC3B Western blot image. Human NBF-L cells were infected with ABLV-GFP for 24 or 48 hours at moi 0, .1, 1, and 10.

(B) LC3B-II (ß-actin normalized) expressed as a percentage of total LC3B. Mean ± SEM *p<.05, student's t-test. All data is a representation of three independent experiments.

proteins can be used to monitor autophagic flux (32). p62 and NDP52 are two autophagy-associated cargo receptor proteins that have roles in selective autophagy and levels of these proteins after ABLV infection were monitored (31; 218; 276; 284). Western blots were used to examine levels of p62 and NDP52 in ABLV infected bat cells, and levels of p62 in ABLV infected human cells (Figure 20A; Figure 21A). ABLV-GFP infection, alone, caused a mean fold decrease of p62 and NDP52 at 48hpi in bat PaBrH cells, and a mean fold decrease of p62 at 48 and 72hpi in human NBF-L cells (Figures 20B and 16C; Figure 21B). Treatment with BAFA1 significantly increased p62 fold change compared to ABLV-GFP infection (Figure 20B; Figure 21B). There were no differences in p62 levels between the combination of ABLV-GFP infection and BAFA1 treatment, or BAFA1 treatment alone, and if ABLV infection was inhibiting the autophagic flux, we would expect that ABLV infection and BAFA1 treatment would have additive effects on p62 or NDP52 levels compared to BAFA1 treatment alone. Additionally, the mean fold decreases in p62 and NDP52 levels in bat PaBrH cells and p62 mean fold decreases NBF-L cells over ABLV-GFP infection time course indicated that both of these cargo receptor proteins were being degraded, and that autophagic flux was not being inhibited.

ABLV-WT infection induced autophagy in primary bat brain cells

To substantiate induction of autophagy in bat cell lines after ABLV-GFP infection, primary *P. alecto* brain cells were infected with ABLV-WT. Infection with ABLV-WT increased levels of LC3B-II at 48hpi with a moi 10, which was consistent with the infectious dose and infection time course that caused a significant LC3B-II level in the bat brain cell line (PaBrH) (Figure 22A and 22B). Though not significant, we observed a decreasing trend of p62 in primary bat brain cells that were infected with

ABLV-WT (Figure 22C). Collectively, these results indicated that ABLV infection activated autophagy in both bat and human cells (Figures 18-22).

Bat tissue derived cells lines have high basal autophagy

When monitoring p62 levels in bat cells, we observed that at 72hpi, treatment with BAFA1 resulted in a ~0.6 fold increase in p62 levels in bat PaBrH cells compared to human NBF-L cells. Though, not a statistically significant difference, this was still an interesting observation given the role of autophagy in nutrient recycling. The PaBrH cells at this time point had been in culture for 96hpi and were confluent, and we suspected whether the p62 fold increase at this time point indicated that autophagy had become active because of a potential nutrient deprivation in the cell culture media. Particularly, whether autophagy was more active in bat cells under nutrient stress. To compare whether basal autophagy levels were different between bat and human cells, we cultured bat PaBrH, PaKiT, and human NBF-L cells to equal confluences then treated all three cell lines with BAFA1 for two hours. Treatment for two hours with BAFA1 allowed us to assess the autophagic flux, and we observed a significantly higher percentage of LC3B existed as autophagosomal LC3B-II in bat PaBrH and compared to human NBF-L cells (Figure 23A and 23B). PaBrH cell lines express hTERT while PaKiT cells were immortalized thru SV40 T antigen transformation. These two bat cell lines exhibited similar autophagic flux results despite the differences in immortalization technique and tissue origin suggestive that the observed increases in autophagic flux are not the result of cell type, tissue source, or immortalization technique, but rather a cellular process that is unique to cells from this bat species. Indeed, when compared with the human cell line, the increased LC3B-II percentage demonstrated that the bat cells appear to possess an

elevated level of basal autophagy, and this is intriguing observation suggests that autophagy could be a factor in how bats might be regulating viral infection.

Figure 20. ABLV-GFP does not inhibit autophagic flux in a bat brain cell line.

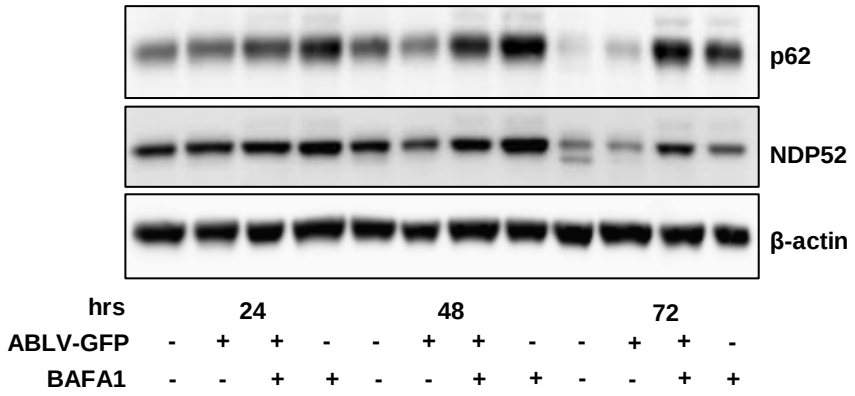
(A) p62 and NDP52 protein Western blot image, ABLV-GFP (moi 1) and BAFA1 (400nm; 2hrs)

(B) Log transformed fold change in p62 normalized ß-actin and compared to uninfected, DMSO-mock treated time point controls. Mean ± SEM. *ANOVA (two-way) Tukey's multiple comparisons test.

(C) Log transformed fold change in NDP52 normalized ß-actin and compared to uninfected, DMSO-mock treated time point controls. Mean ± SEM. *ANOVA (two-way) Tukey's multiple comparisons test. All data represent three independent experiments.

Figure 20. ABLV-GFP does not inhibit autophagic flux in a bat brain cell line (PaBrH)

A. Western blot



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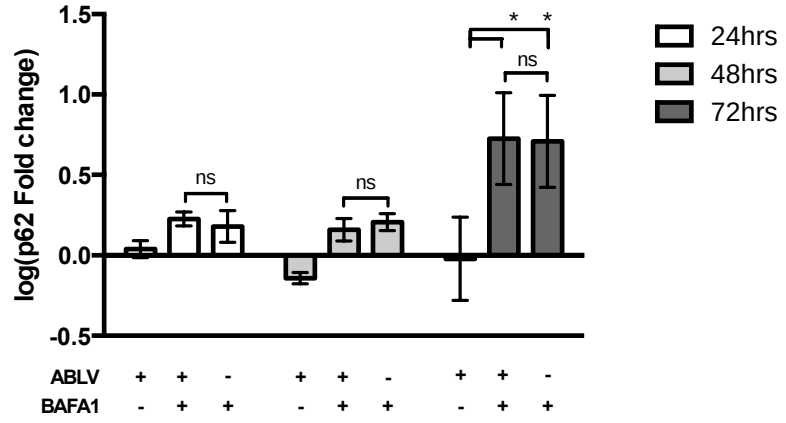


C.

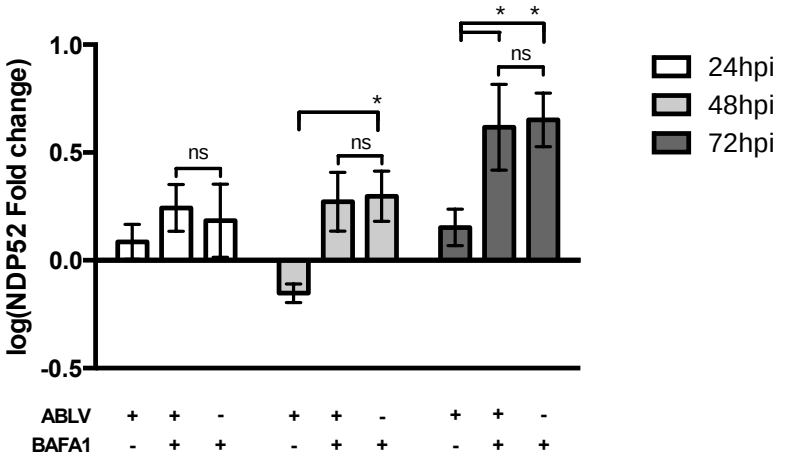


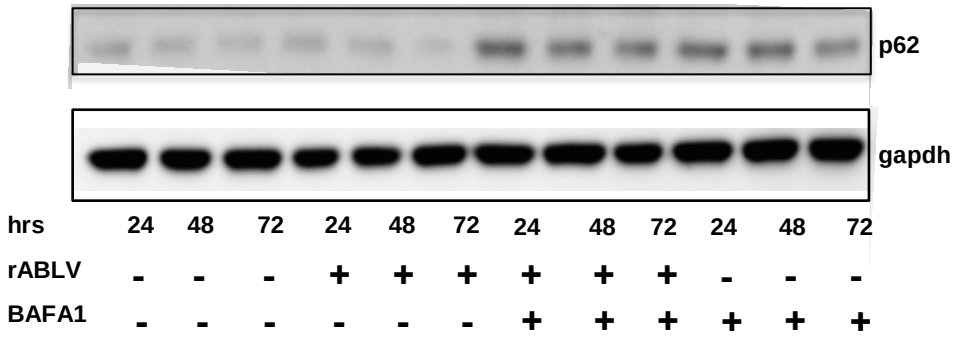
Figure 21. ABLV-GFP does not inhibit autophagic flux in a human cell line.

(A) p62 and NDP52 protein Western blot image, ABLV-GFP (moi 1) and BAFA1 (400nm; 2hrs)

(B) Log transformed fold change in p62 normalized ß-actin and compared to uninfected, DMSO-mock treated time point controls. Mean ± SEM. *ANOVA (two-way) Tukey's multiple comparisons test.

Figure 21. ABLV-GFP does not inhibit autophagic flux in a human cell line (NBF-L).

A. Western blot



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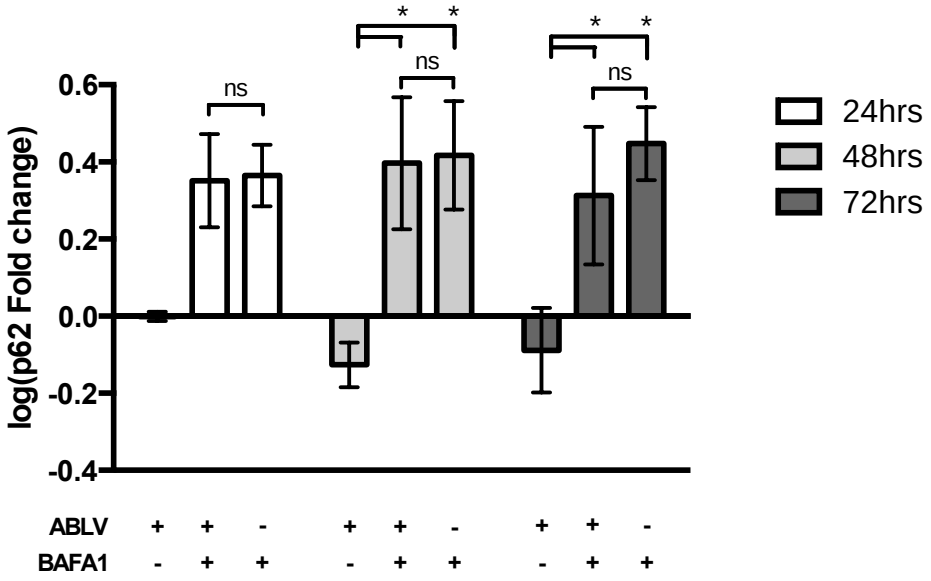


Figure 22. ABLV-WT infection induces autophagy in primary bat brain cells.

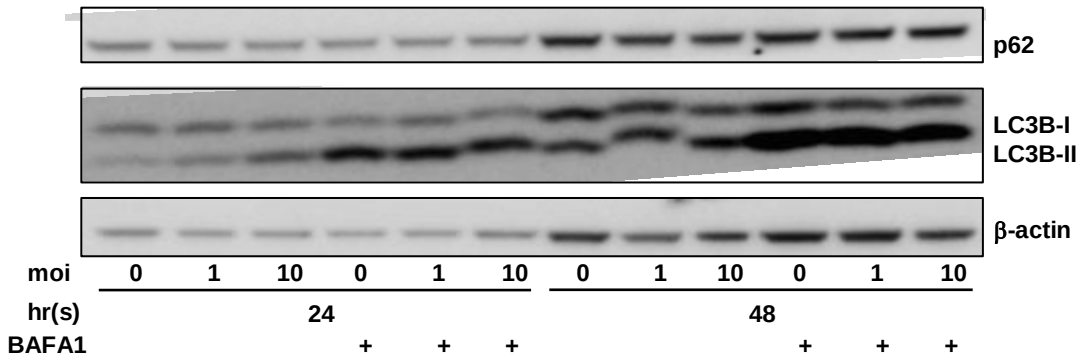
(A) LC3B and p62 Western blot image. Primary bat brain cells were infected with ABLV-WT for 24 or 48 hours at mois of 1 and 10. Bafilomycin A1 (BAFA1; 400nm) was added to the cell cultures for 2 hours prior to the end of the infection time course.

(B) LC3B-II protein levels, mean ±SEM, student's t test *p<05

(C) p62 protein level, mean ±SEM. No significant differences in p62 fold change during infection, although p62 levels exhibited a decreased trend in the infected samples. All data are representations of three independent experiments.

Figure 22. ABLV-WT infection induces autophagy in primary bat brain cells.

A. Western blot



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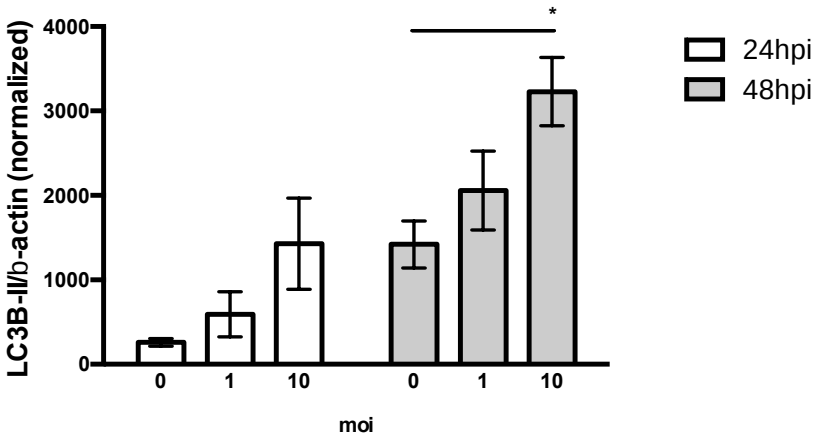

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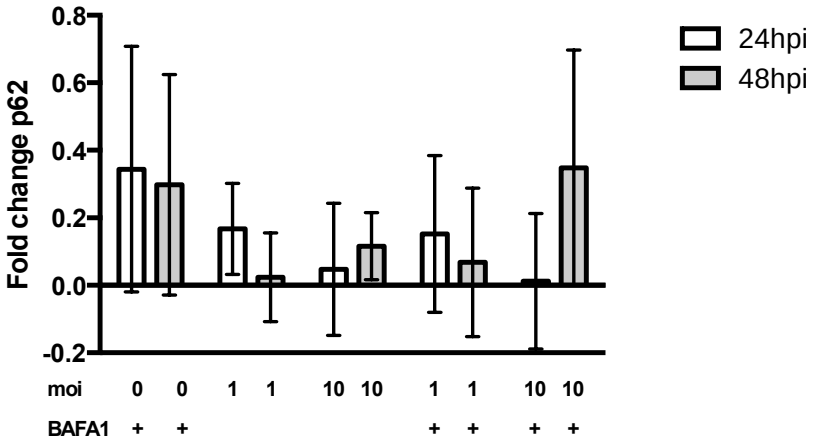
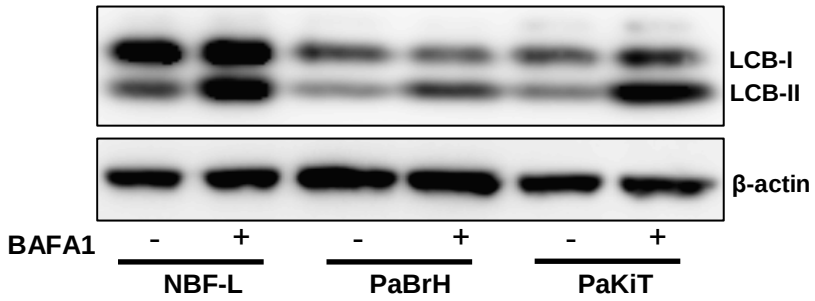


A.



B.

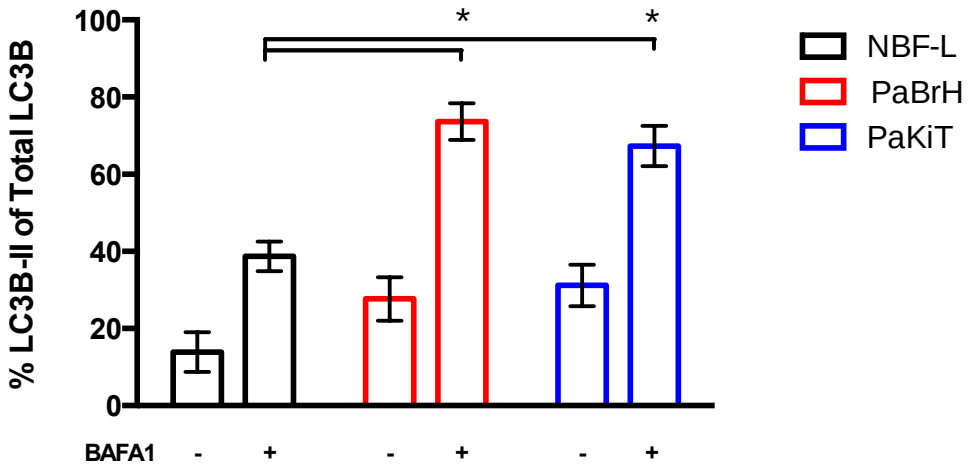


Figure 23. Bat cells have high basal autophagic flux

(A) LC3B Western blot image. Human NBF-L cells, bat PaBrH, and PaKiT cells were treated with bafilomycin A1 (BAFA1; 400nM) for two hours.

(B) LC3B-II (ß-actin normalized) expressed as a percentage of total LC3B. Mean ± SEM *p<.05, student's t-test. All data is a representation of three independent experiments.

Antiviral role of autophagy during ABLV infection

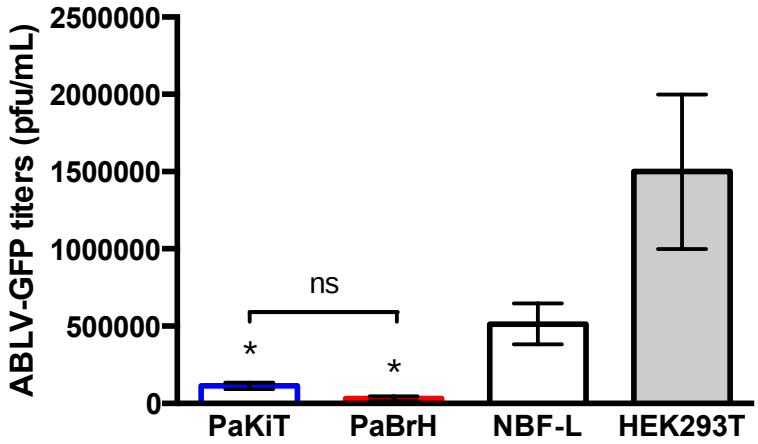
Pharmacological modulators of autophagy

We compared the ABLV-GFP titers and vGFP levels, from virus produced in bat verses human and mouse cells, and observed that both were consistently lower in bat cells compared to human cells (Figure 24A-B). Having observed that bat cells induced the autophagy pathway after ABLV infection, that bat cells had an elevated basal autophagic flux, and that ABLV titers were lower after infection of bat cells, we hypothesized that autophagy was restricting virus replication. To understand whether the autophagy pathway has an antiviral role during infection in bat cells the effects of several pharmacological modulators of autophagy on ABLV replication were tested.

Rapamycin (RAPA) and small-molecule enhance of autophagy-28 (SMER) are known to activate the autophagy pathway through mTOR-dependent and mTOR-independent mechanisms, respectively (240; 247). Bat PaBrH and PaKiT cells were treated with RAPA and SMER during ABLV infection to elucidate whether activation of autophagy had antiviral effects. Human NBF-L cells were treated in comparison. Bat PaKiT cells were treated with RAPA and SMER, and at the conclusion of the time course, autophagy levels were quantified by flow cytometry (Figure 25A). Chloroquine induces the accumulation of autophagosomes and was used as a positive control. Treatment with both drugs induced autophagy, but RAPA had a stronger effect than SMER. Bat and human cells were infected with ABLV, and RAPA or SMER was added to the cell cultures 24hpi. RAPA induced autophagy resulted in significant reductions in ABLV titers in both bat and human cells (Figure 25B). SMER induced autophagy resulted in a significant reduction in ABLV titers in human cells only, but even though not statistically significant, a decreasing titer trend is observed in SMER treated bat cells.

ABLV titer fold decreases between bat and human cells were not significantly different, with all three cell types exhibiting similar fold reductions.

A.



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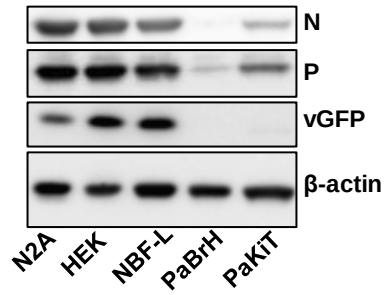
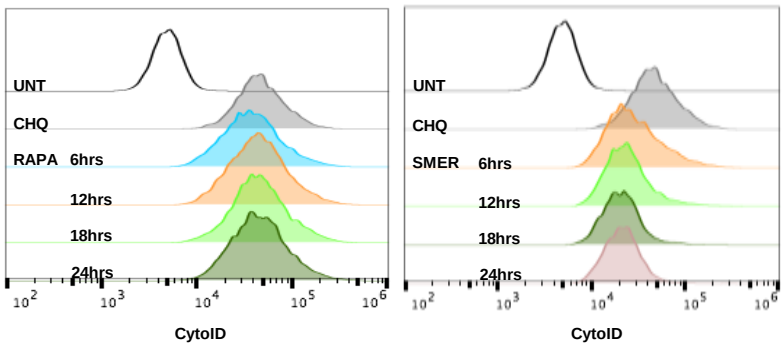


Figure 24. ABLV has lower replication in bat cells.

(A) ABLV-GFP titers 48hpi from bat PaKiT, PaBrH, human NBF-L, and HEK293T cells infected with moi 1. PaKiT, PaBrH, and NBF-L are a quantification from three independent experiments. *p<.05, ANOVA (one-way) compared to NBF-L cells. HEK293T is a quantification of two independent experiments.

(B) N, P, and vGFP Western blot image. Data is a representation from two independent experiments.

A.



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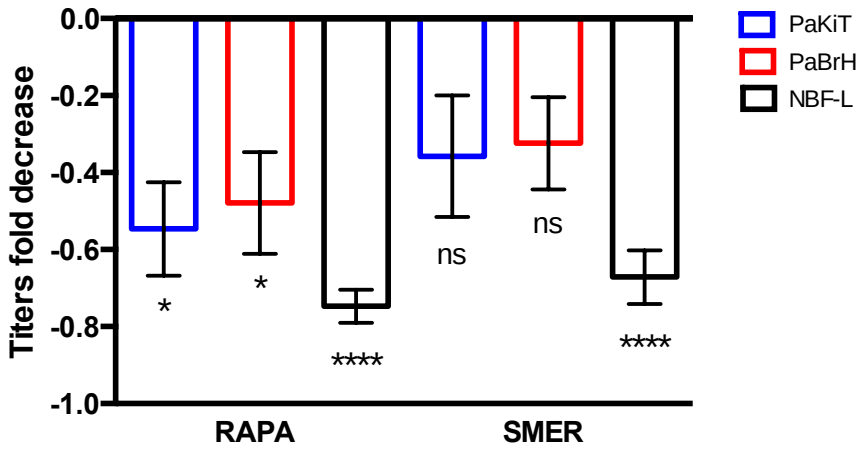


Figure 25. Pharmacological induction of autophagy reduces ABLV titers.

(A) Flow cytometry plots of bat kidney cells (PaKiT) stained with Cyto-ID© to monitor autophagy levels. PaKiT cells were treated with rapamycin (RAPA; 2µM) and smer28 (SMER; 50µM) four hours indicated or chloroquine (CHQ; 1µM) for 18 hours.

(B) ABLV-GFP titers (pfu/mL). Bat (PaKiT and PaBrH) and human (NBF-L) cells were infected with ABLV-GFP (moi 1) for 48 hours. RAPA (RAPA; 2µM) and smer28 (SMER; 50µM) were added to cell cultures 24hpi. *ANOVA (one-way), multiple comparisons to DMSO-mock treated 48hpi controls. Fold change data is a representation of four independent experiments

As a potential mechanism underlying reduction in ABLV, we examined whether treatment with RAPA and SMER resulted in degradation of virus proteins. Replication of ABLV is dependent on virus nucleoprotein (N), phosphoprotein (P), and RNA polymerase (L) which associate to form the virus polymerase complex (PC) (250). Because of slippage of the PC in the intergenic regions, (-) ssRNA viruses experience a transcriptional gradient where upstream genes are transcribed more frequently than genes further downstream from the leader sequence (Figure 26). If activation of autophagy results in the degradation of virus PC proteins, then even a minor reductions, may have amplifying negative effects on overall viral replication, as (-) ssRNA replication is highly dependent the levels of available virus protein.

ABLV protein levels after RAPA and SMER treatment of PaKiT cells were examined by Western blot (Figure 27A). RAPA and SMER treatment did not have any significant effects on N or P protein levels in bat PaKiT cells, however N protein fold change had a decreasing trend in RAPA treated cells (Figure 27B and 27C). Western blot quantification of vGFP can be difficult because of low replication and vGFP expression levels in bat cells. We were able to successfully quantify vGFP from two of the four independent experiments with PaKiT cells. The noticeable vGFP fold decrease caused by RAPA and SMER stands in contrast to fold changes in N and P proteins (Figure 27D). ABLV protein levels in bat PaBrH cells infected and treated with RAPA and SMER were examined by Western blot (Figure 28A). As seen in the PaKiT cells, N protein fold change had a decreasing trend in bat PaBrH cells when treated with RAPA, but not SMER (Figure 28B). P protein levels were quantified for two independent experiments, and also have a trending decease in both RAPA and SMER treated cells (Figure 28C).

Human NBF-L cells had a significant fold decrease of vGFP levels in both RAPA and SMER treated cells, but neither N nor P levels had any significant reductions (Figure 29A-D).

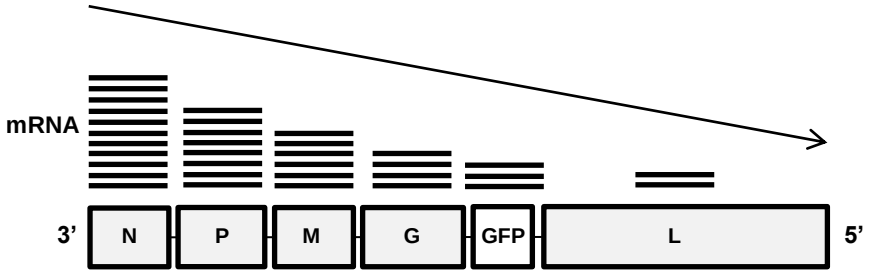


Figure 26. ABLV transcription results in a gene concentration gradient dependent on the distance from the 3' leader sequence before the nucleoprotein gene.

Figure 27. Pharmacological induction of autophagy reduces ABLV vGFP levels in bat cells (PaKiT).

(A) Western blot image of ABLV N, P, and virus GFP (vGFP). PaKiT cells were infected with ABLV-GFP (moi 1) for 48hrs. RAPA (RAPA; 2µM) and smer28 (SMER; 50µM) were added to cell cultures 24hpi.

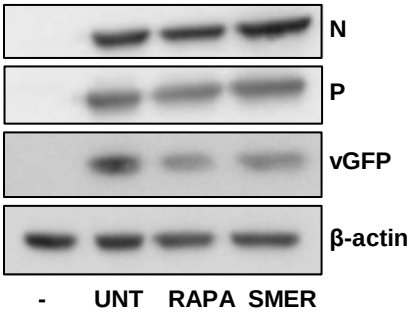
(B) ABLV N protein (ß-actin normalized) fold change. An ANOVA (one-way), multiple comparisons test was performed to compare RAPA and SMER treated cells to 48hpi ABLV-GFP (moi 1), DMSO mock-treated cells. Data is a representation of three independent experiments.

(C) ABLV P protein (ß-actin normalized)fold change. Data is a representation of three independent experiments.

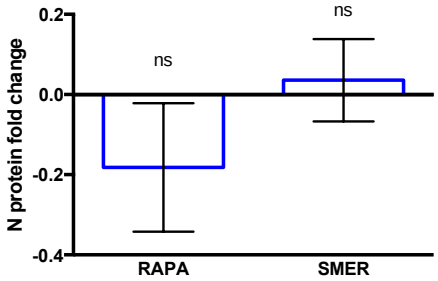
(D) ABLV vGFP (ß-actin normalized) fold decrease. Data is a representation of two independent experiments.

Figure 27. Pharmacological induction of autophagy reduces ABLV vGFP levels in bat cells (PaKiT).

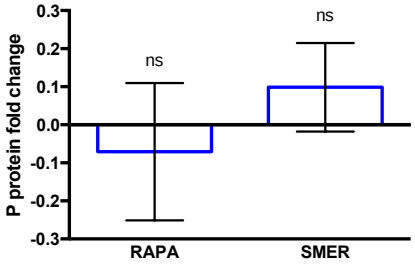
A. Western blot



B.



C.



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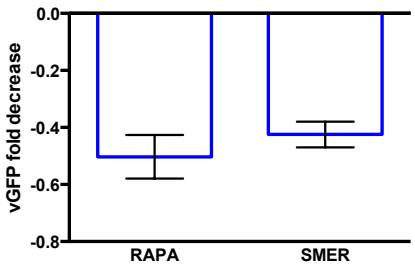
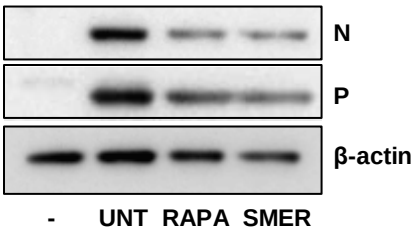
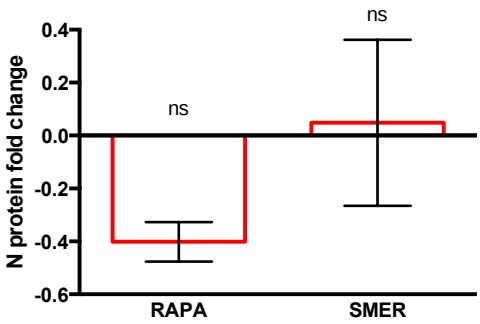


A. Western blot



B.



C.

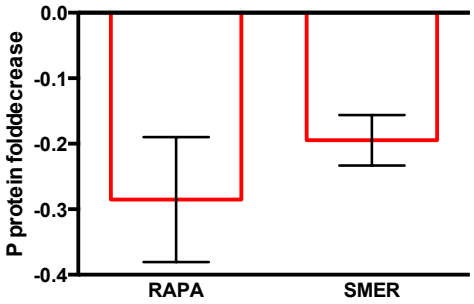


Figure 28. Pharmacological induction of autophagy reduces ABLV vGFP levels in bat cells (PaBrH).

(A) Western blot image of ABLV N, P, and virus GFP (vGFP). PaBrH cells were infected with ABLV-GFP (moi 1) for 48hrs. RAPA (RAPA; 2µM) and smer28 (SMER; 50µM) were added to cell cultures 24hpi.

(B) ABLV N protein (ß-actin normalized) fold change. An ANOVA (one-way), multiple comparisons test was performed to compare RAPA and SMER treated cells to 48hpi ABLV-GFP (moi 1), DMSO mock-treated cells. Data is a representation of three independent experiments.

(C) ABLV P protein (ß-actin normalized) fold decrease. Data is a representation of two independent experiments.

Figure 29. Pharmacological induction of autophagy reduces ABLV vGFP levels in human cells (NBF-L).

(A) Western blot image of ABLV N, P, and virus GFP (vGFP). NBF-L cells were infected with ABLV-GFP (moi 1) for 48hrs. RAPA (RAPA; 2µM) and smer28 (SMER; 50µM) were added to cell cultures 24hpi.

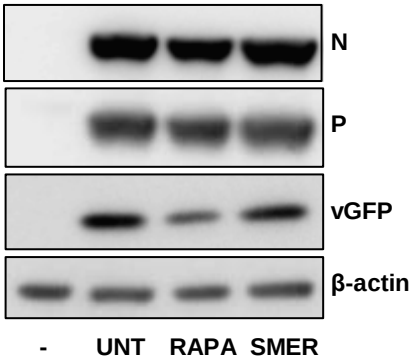
(B) ABLV N protein (ß-actin normalized) fold change.

(C) ABLV P protein (ß-actin normalized) fold change.

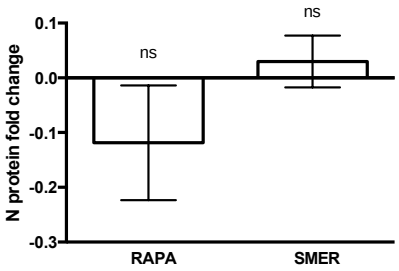
(D) ABLV vGFP fold decrease (ß-actin normalized). *An ANOVA (one-way), multiple comparisons test was performed to compare RAPA and SMER treated cells to 48hpi ABLV-GFP (moi 1), DMSO mock-treated cells. All data are a representation of three independent experiments.

Figure 29. Pharmacological induction of autophagy reduces ABLV vGFP levels in human cells (NBF-L).

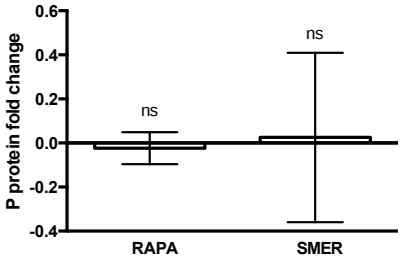
A. Western blot



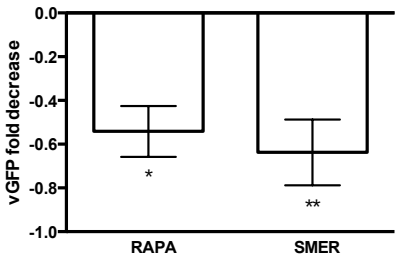
B.



C.



D.



Primary *P. alecto* brain cells were similarly treated with RAPA and SMER during ABLV infection. Here, we observed a reduction in ABLV titers following SMER treatment, but not with RAPA (Figure 30A). ABLV proteins were examined by Western blot (Figure 30B). However, RAPA treatment did decrease P protein and vGFP levels (Figure 30C). The results from autophagy activation experiments with bat and human cell lines indicated that autophagy could function as an antiviral defense during ABLV infection when induced. To further examine the antiviral role of autophagy we examined the effects of pharmacological inhibition of autophagy on ABLV replication.

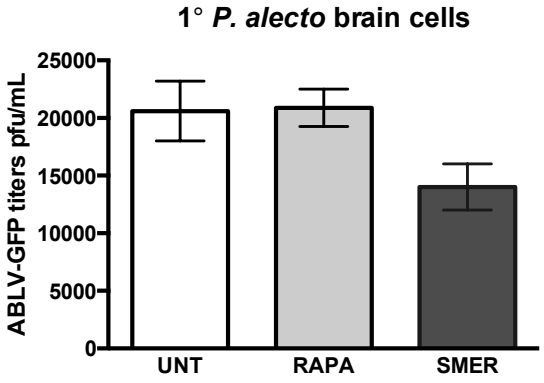
If autophagy is acting as an antiviral defense, we hypothesized that inhibition of autophagy would increase ABLV replication. We next tested the effects of 3-methyaldenine (3-MA), a pan-inhibitor of phosphoinositide 3-kinase (PI3K) activity, on ABLV replication. PI3Ks phosphorylate phosphatidylinositol, creating phosphatidylinositol 3-phosphate (PtdIns(3)P), which are necessary for the recruitment of proteins involved in the initiation of autophagy and the development of autophagosomes (202; 206; 263). Classically, 3-MA is considered an autophagy inhibitor, however, dual functions as both activator and inhibitor, dependent on nutrient conditions, have been described (299). Bat brain cells were treated with 3-MA under nutrient-rich and nutrient-deprived conditions to confirm the role of 3-MA as an inhibitor. We observed a decrease in LC3B-II in bat cells treated with 3-MA with 10% serum media, consistent with the role of 3-MA as an

Figure 30. Activation of autophagy reduces ABLV vGFP levels in primary bat brain cells.

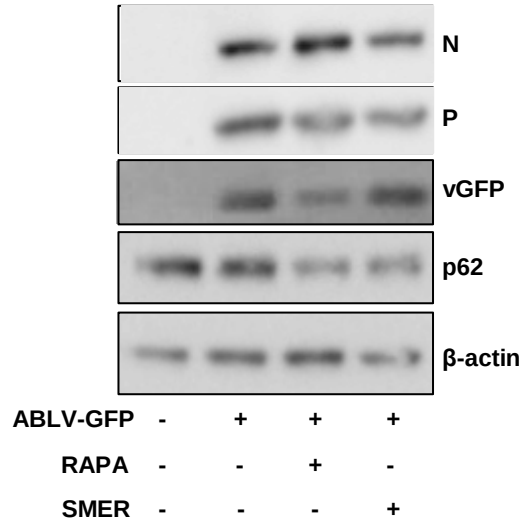
(A) ABLV-GFP titers, primary *P. alecto* cells were infected with ABLV-GFP (moi 1). Cells were DMSO-mock treated (UNT) or treated with RAPA (2µM) and SMER (50µM) for 24hrs. Virus supernatant and whole cell lysates were harvested 48hpi.
(B) ABLV N, P, vGFP, and p62 Western blot image.
(C) ABLV N, P, and vGFP levels expressed as a fold change compared to DMSO-mock treated, 48hpi cells. All data is a presentation of two experiments.

Figure 30. Activation of autophagy reduces ABLV vGFP levels in primary bat brain cells.

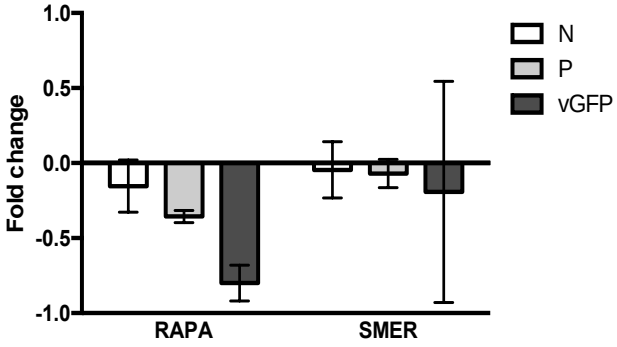
A. Virus titers



B. Western blot



C.

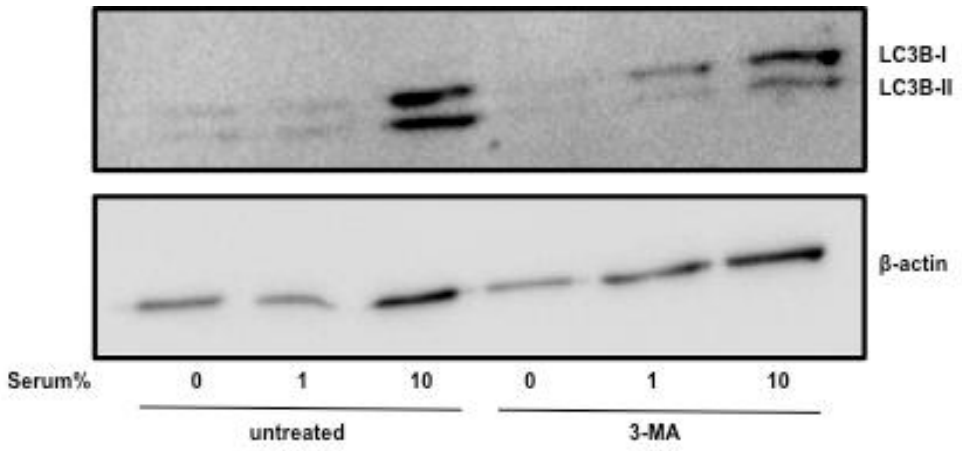


autophagy inhibitor (Figure 31A). If autophagy has an antiviral role during ABLV infection, we hypothesized that inhibition of autophagy through 3-MA treatment would result in increased levels of vGFP protein expression. Unexpectedly, in mouse and bat cells pre-treated with 3-MA, the amount of ABLV vGFP decreased (Figure 31B). The cellular receptor that facilitates RABV entry is unknown, however RABV is known to fuse with endosomes and specifically, ABLV G-mediated entry is dependent on Rab-5 endocytosis (166; 288; 289). Given the multiple roles of PI3Ks in membrane trafficking, we concluded that inhibition of PI3K activity by 3-MA most likely blocked ABLV entry.

To further examine pharmacological modulators of autophagy, we next tested VPS34IN1, a selective inhibitor of vacuolar protein sorting 34 (VPS34) type III PI3K (17; 178). VP34 is the only known type III PI3K and a main generator of PtdIns(3)Ps necessary for phagophore development and autophagy initiation (61; 126). Treatment of human (NBF-L) cells with VPS34IN1 decreased the amount of LC3B-II, but did not have defnitive effects on other cell lines (Figure 32A). p62 and LC3B-II levels increased in bat PaBrH cells treated with VPS34IN1 (Figure 32B). Given the recent discovery and application of VPS34IN1 as a type III PI3K inhibitor, its effect on the autophagy pathway remains less characterized than other established pharmacologic autophagy modulators and may have unexpected effects on autophagosomal, endosomal, and lysosomal pathways (194). The accumulation of p62 and LC3B-II following VPS34IN1 treatment of PaBrH (Figure 32B) suggested that VPS34IN1 treatment caused a potential block in turnover of these proteins. ABLV vGFP levels decreased in mouse cells pre-treated with VPS34IN1 (Figure 33A). The expression vGFP in bat PaBrH cells is so low that visualization and quantification by Western blot is difficult. Post-infection treatment with

VPS34IN1 appeared to have no effect on vGFP levels in mouse and bat cells (Figure 33B). As with 3-MA inhibition, we concluded that because of the reduction in vGFP in mouse cells pre-treated with VPS34IN1, but not post-infection treated, inhibition of VPS34 again interfered with ABLV entry.

A.



B.

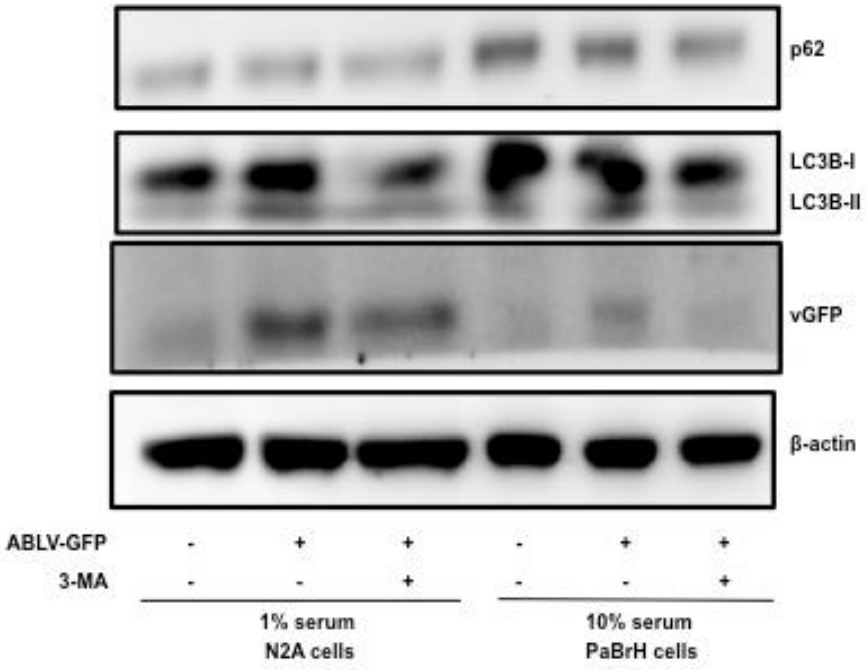
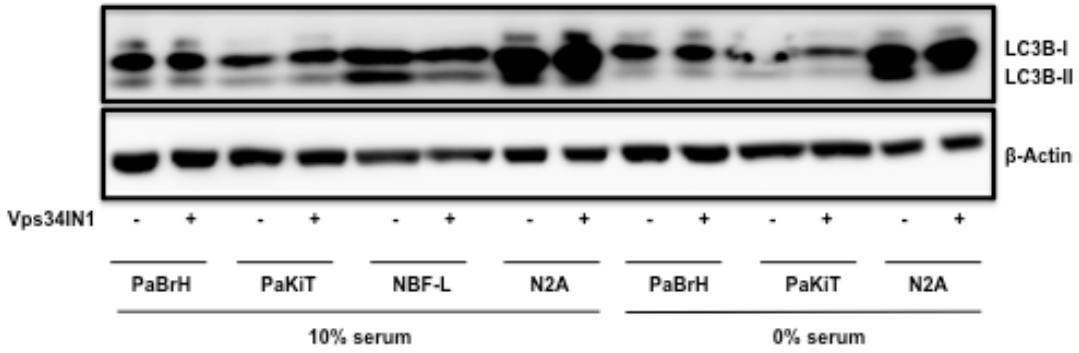


Figure 31. PI3kinase inhibition blocks ABLV-GFP entry.

(A) LC3B protein Western blot image. PaBrH were treated with 3-MA (1mM) for 16hrs.

(B) N2A and PaBrH cells were pre-treated with 3-MA (1mM) for 16hrs then infected with ABLV-GFP (moi 1). Whole cell protein lysates were harvested 48hpi.

A.



B.

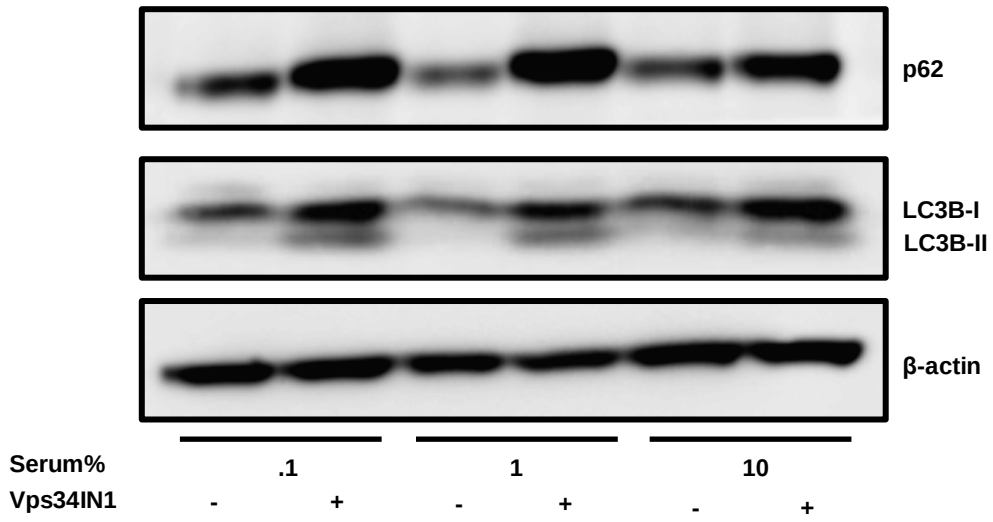
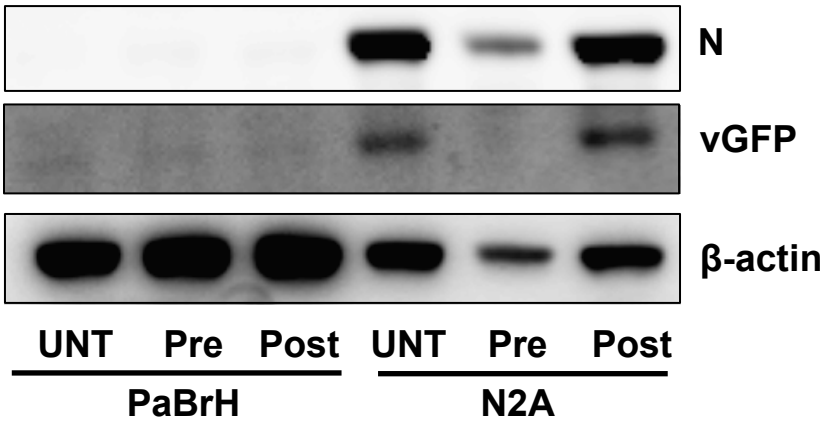


Figure 32. Vps34IN1 inhibition of autophagy.

(A) LC3B protein Western blot image. PaBrH, PaKiT, NBF-L, and N2A cells were treated with Vps34IN1 (18hrs; 1uM).

(B) p62 and LC3B protein Western blot image. PaBrH cells were treated with Vps34IN1 (18hrs; 1uM).

A.



B.

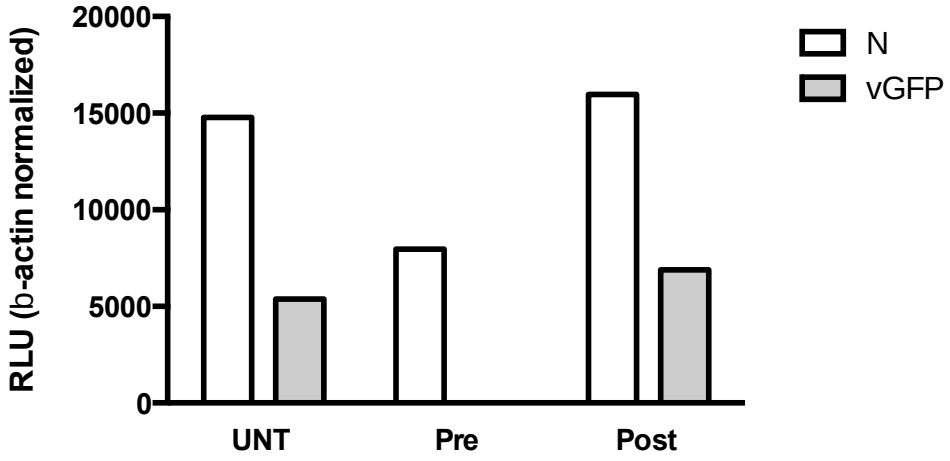


Figure 33. Vps34IN1 interferes with ABLV infection

(A) ABLV N protein and vGFP Western blot image. PaBrH and N2A cells were treated with Vps34IN1 (1µM; 16 hours) Pre- or Post-infection. Whole cel lysates were process 48hpi, ABLV-GFP (moi 1).

(B) ABLV N protein and vGFP levels (normalized to ß-actin).

Genetic knockdown of autophagy

Following our examination of pharmacological inhibitors of autophagy o investigate the antiviral role of autophagy during ABLV infection, we next tested a genetic approach for the inhibition of the autophagy. Here, bat PaBrH, PaKiT, and human NBF-L cells were transduced with ATG5 shRNA to knockdown autophagy and examine the effects of autophagy inhibition on ABLV replication. The knockdown of ATG5 was examined by Western blot (Figure 34A). Autophagy levels in NBF-L WT and ATG5 knockdown (ATG5KD) cells were monitored by flow cytometry to examine the functional effects of ATG5KD. The NBF-L ATG5KD cell line had a minor 0.32 fold decrease in autophagy levels (Figure 34B). We next examined the effects of this slight reduction in autophagy in the NBF-L ATG5KD cells on ABLV replication. We observed an ~0.8 fold increase in vGFP% NBF-L ATG5KD cells (Figure 34C). In bat and human ATG5KD cells we observed trending ABLV titer fold increases (Figure 35A and 35B). Although a quite interesting observation, further confirmation of the antiviral role for autophagy during virus infection will require additional genetic approaches to silence autophagy-related genes.

Figure 34. Minor autophagy inhibition increases ABLV replication.

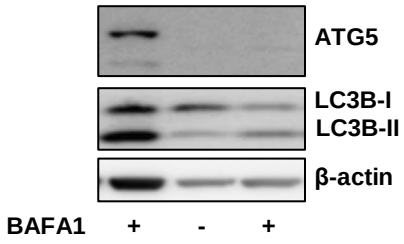
(A) ATG5 knockdown Western blot image. Bat PaBrH cells.

(B) Flow cytometry analysis of autophagy levels between human NBF-L WT and ATG5KD cells.

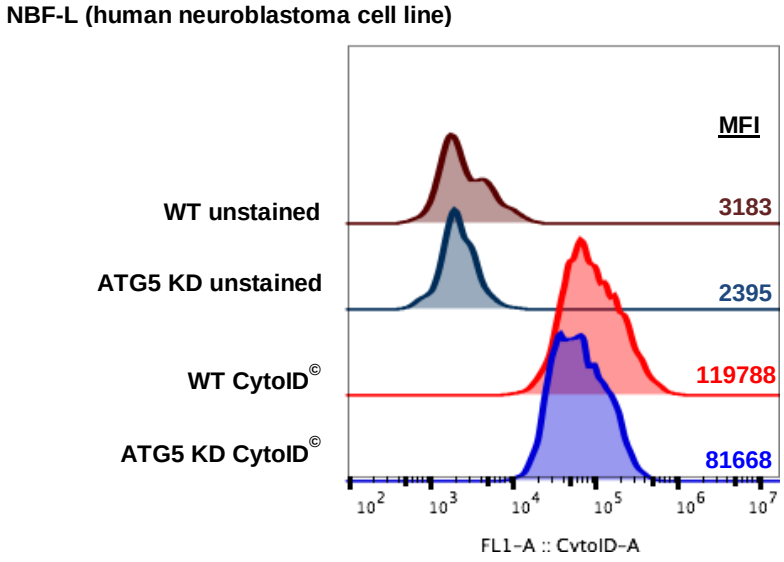
(C) Flow cytometry quantification of vGFP% positive NBF-L WT and ATG5KD cells 24hpi.

Figure 34. Minor autophagy inhibition increases ABLV replication.

A. PaBrH cells



B.



C. NBF-L cells

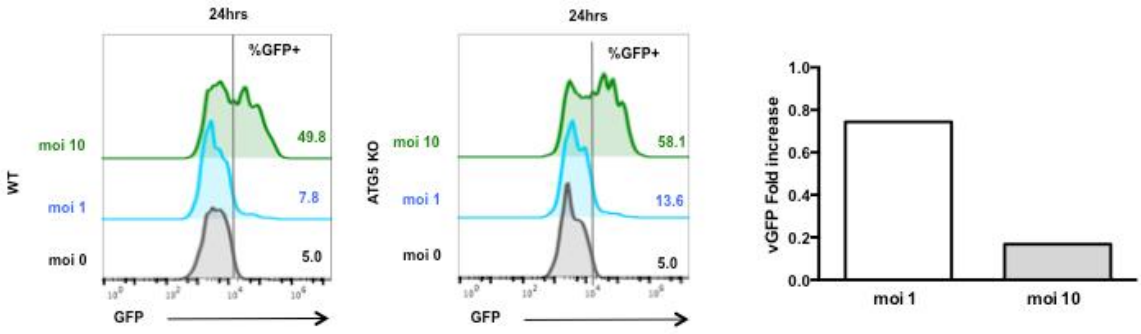
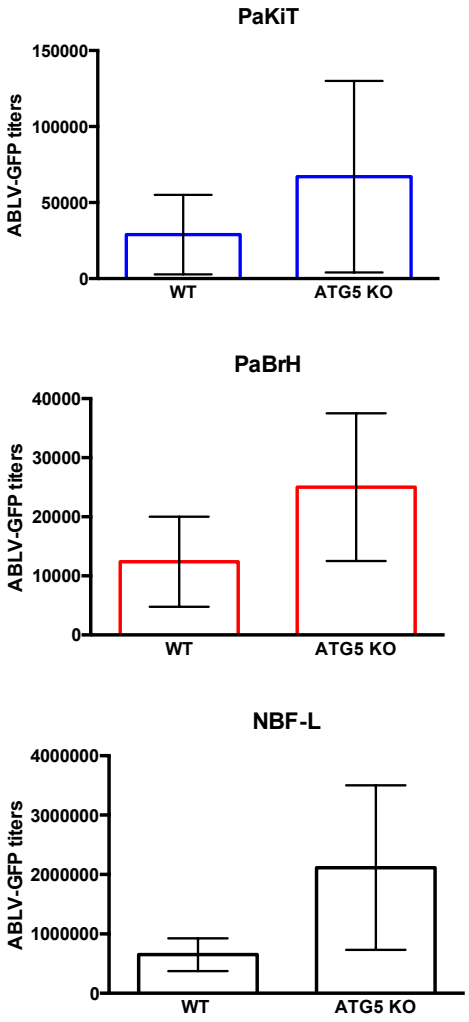
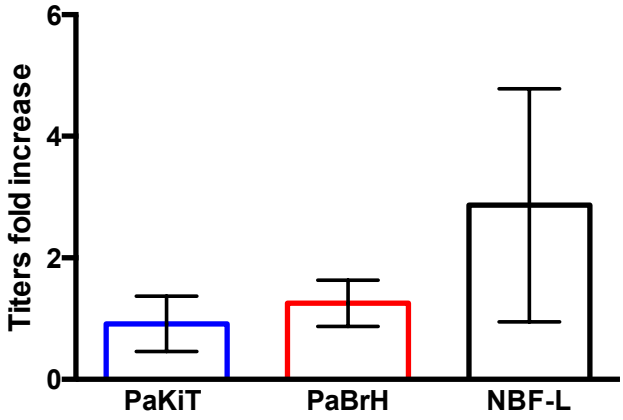


Figure 35. ATG5 knockdown increases ABLV replication in bat and human cells.
(A) ABLV-GFP titers 72hpi of bat PaKiT, PaBrH, and human NBF-L cells.
(B) ABLV-GFP titer fold increases in ATG5KD cell lines. All data are a representation two independent experiments.

Figure 35. ATG5 knockdown increases ABLV replication in bat and human cells.
A.



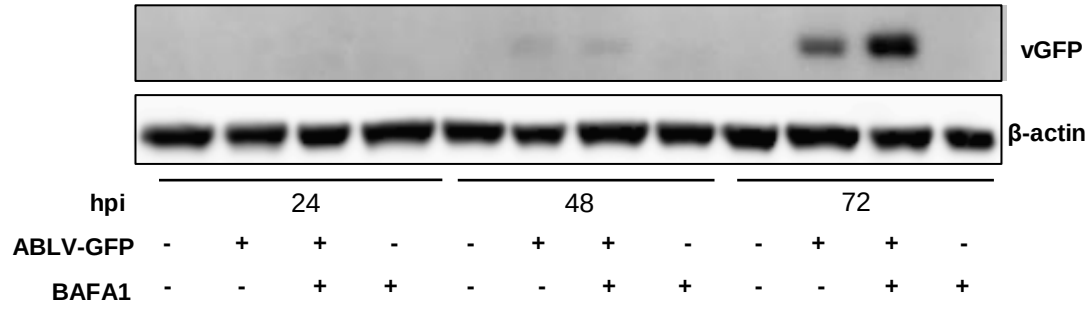
B.



Shuttling of ABLV proteins to the autophagosomal pathway

Another way to explore the impact of cellular autophagy on virus replication was to evaluate whether the amounts of viral proteins varied in response to manipulation of the autophagic pathway. When we inhibited autophagic flux in bat PaBrH cells with BAFA1, we noticed a significant increase in vGFP levels at 72hpi (Figure 36A and 36B). This was the first piece of evidence that ABLV proteins might be shuttled to autophagosomes for degradation in bat cells. Primary bat brain cells infected with ABLV-WT and similarly treated with BAFA1 had a significant increase in P protein levels at 48hpi (Figure 37A-C). If ABLV proteins were being shuttled to autophagosomal-autolysosomal pathway for degradation, we hypothesized that inhibition of lysosomal degradation would result in an increase in ABLV proteins. In contrast to our hypothesis, we did not observe increased levels of ABLV proteins when lysosomal proteases were inhibited (Figure 38A-D). As a follow-up to these experiments, we next used chloroquine to inhibit lysosomal acidification, and again, did not observe any increases in ABLV protein levels (Figure 39A-C). We concluded from these experiments that ABLV N and P proteins were not shuttled to the autophagosomal-autolysosomal pathway for degradation. The BAFA1-induced increase in vGFP and P protein levels might be the result of an off-target effects that remain to be determined.

A.



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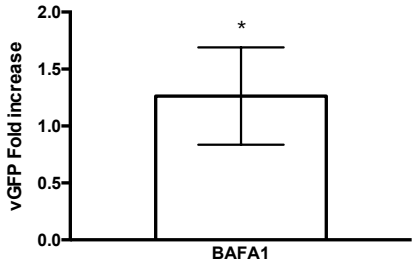


Figure 36. Short-term inhibition of autophagic flux increases vGFP levels.

(A) vGFP Western blot image. Bat PaBrH cells were infected with ABLV-GFP (moi 1) for times indicated. Cells were treated with BAFA1 (400nM) for two hours.

(B) vGFP fold increase (normalized to ß-actin), compared to vGFP levels in the absence of BAFA1, 72hpi. Mean ± SEM. *$p<.05$, student's t-test.

Figure 37. Short-term inhibition of autophagic flux increases ABLV P levels.

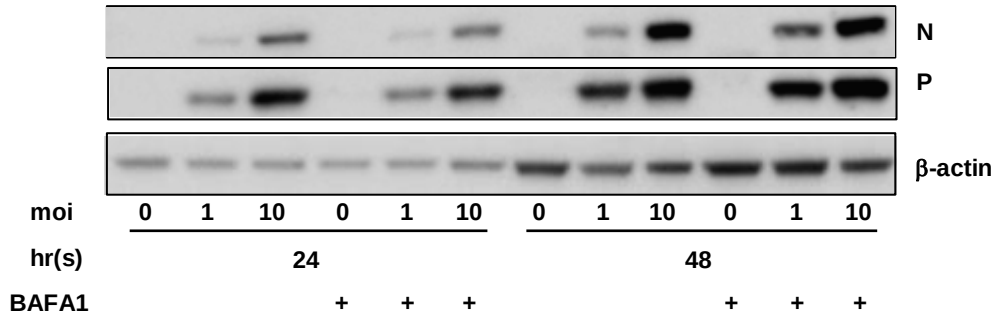
(A) ABLV N and P Western blot image.

(B) ABLV nucleoprotein (N) levels, mean ±SEM. Primary bat brain cells (PaBr) were infected with ABLV-WT for 24 or 48 hours at mois of 1 and 10. Bafilomycin A1 (BAFA1; 400nm) was added to the cell cultures for 2 hours prior to the end of the infection time course.

(C) ABLV phosphoprotein (P) levels, mean ±SEM, student's t test *$p<.05$.

Figure 37. Short-term inhibition of autophagic flux increases ABLV P levels.

A. Western blot (primary bat brain cells)



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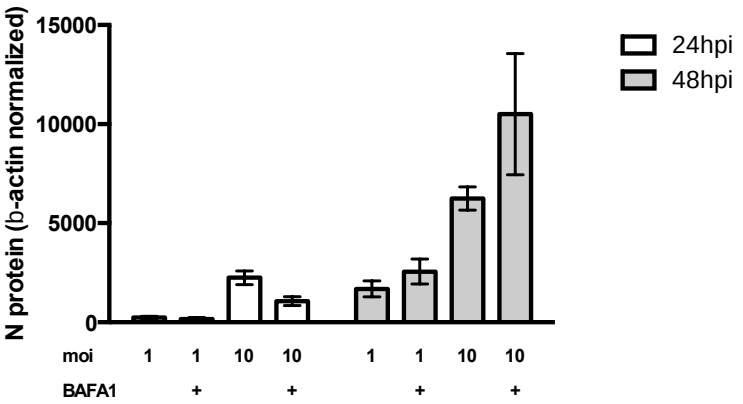


C.

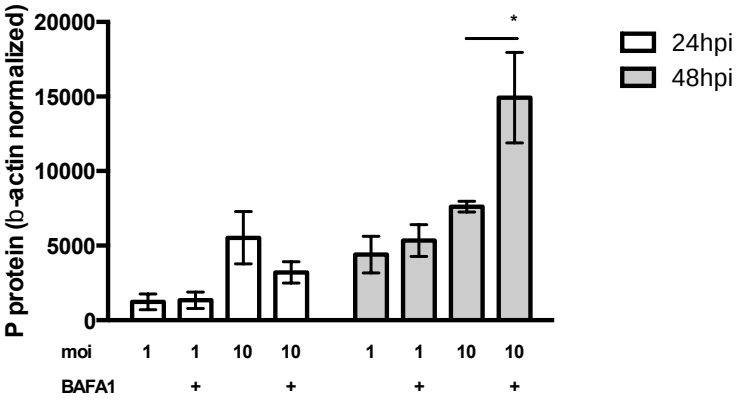


Figure 38. Inhibition of lysosomal proteases does not increase ABLV protein levels

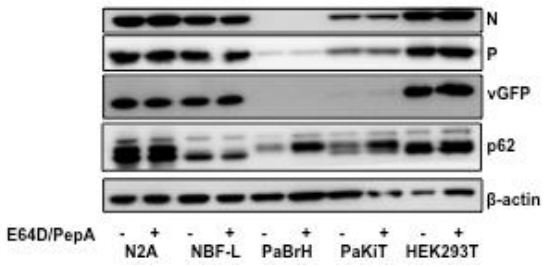
(A) ABLV N, P, and vGFP Western blot image. Whole cell protein lysates were harvested from cell lines infected with ABLV-GFP (moi 1) 48hpi. Cells were treated with E64D (10µg/mL) and pepstatinA (PepA; 10µg/mL) for 18 hours.

(B) ABLV N, P, and vGFP Western blot image. Primary bat brain cells (PaBr) were infected with ABLV-WT (48 hours). Cells were treated with E64D (10µg/mL) and PepA (10µg/mL) for 24 hours.

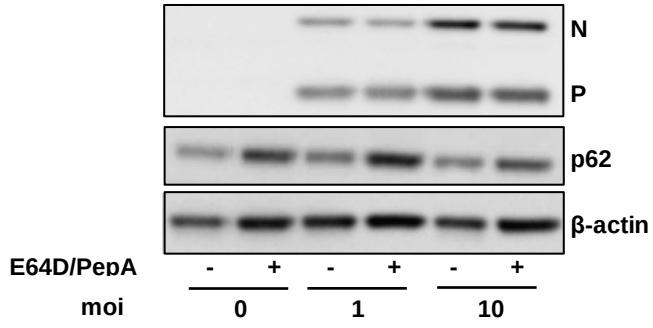
(C) p62 protein levels, mean ±SEM.

(D) ABLV N and P protein levels, mean ±SEM. Data (B-D) are a representation of two independent experiments.

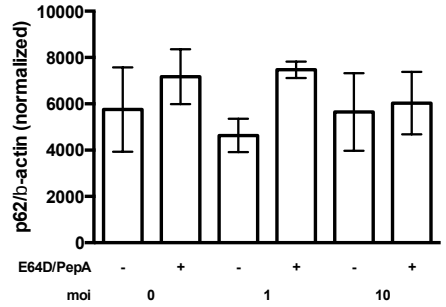
Figure 38. Inhibition of lysosomal proteases does not increase ABLV protein levels
A.



B. Western blot, primary bat brain cells (PaBr)



C.



D.

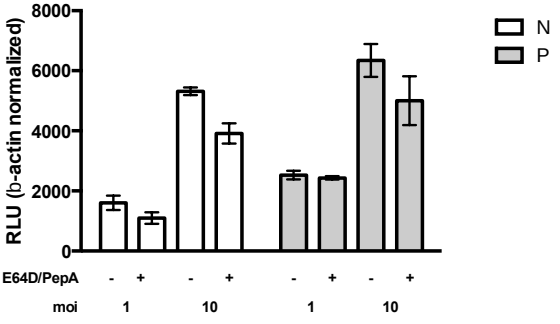
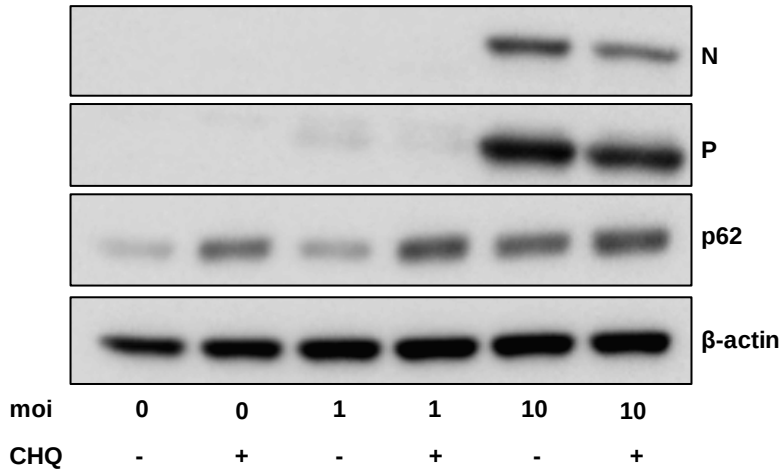


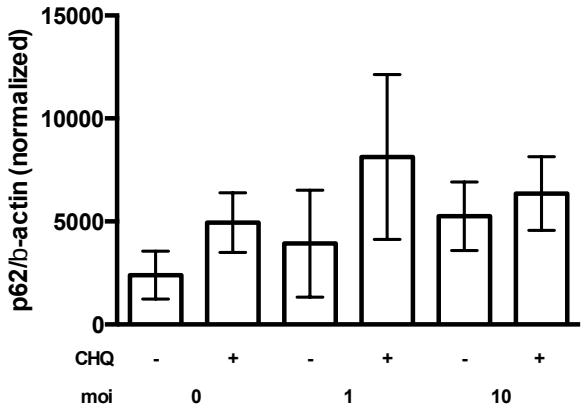
Figure 39. Inhibition of lysosomal acidification does not increase ABLV protein levels.
(A) ABLV N and P Western blot image. Whole cell protein lysates were harvested from bat brain cell lines (PaBrH) infected with ABLV-WT, 48hpi. Cells were treated with chloroquine for 24 hours.
(B) p62 protein levels, mean ±SEM.
(C) ABLV N and P protein levels, mean ±SEM. Data is a representation of two independent experiments.

Figure 39. Inhibition of lysosomal acidification does not increase ABLV protein levels in bat brain cell line (PaBrH)

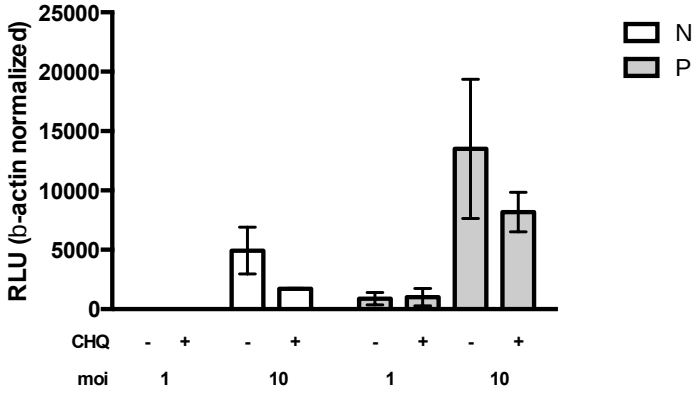
A.



B.



C.



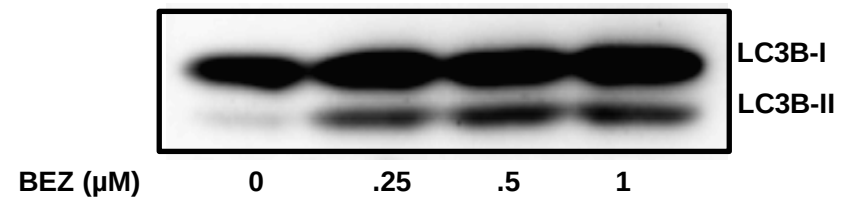
NVP BEZ235 (BEZ) restricts ABLV infection

An overall goal of my comparative studies between bat and human cells and the possibility of an antiviral role of cellular autophagy, was to elucidate whether the induction of autophagy would be a beneficial therapeutic modality in treating viral infection. Nearly half of all emerging viruses cause encephalitic disease (210). RABV infection and Rabies disease still causes significant mortality in developing countries in Africa and Asia where Rabies disease control efforts are insufficient and access to post-exposure prophylaxis is limited (37; 148). Autophagy plays a critical role in the maintenance of cellular homeostasis in post-mitotic cells such as neurons (214). Neurons cannot rely on the anti-viral effects of interferon (IFN)-induced apoptosis to control viral infection because of the potential damages to the central nervous system as a result of immune response induced cell death (306). Virulent strains of RABV are known to subvert or dampen intrinsic immune responses (252). NVP BEZ235 (BEZ) is a pharmacological drug that has advanced to clinical trials stages as a potential anti-cancer therapy (28; 73; 143). BEZ is a dual inhibitor of PI3Ks and mTOR, making it a potentially potent autophagy inducer. Given the neurotropic nature of lyssaviruses and ABLV we examined the protective effect of BEZ on physiologically relevant human NBF-L cells.

BEZ treatment activated autophagy as examined by an increase in the amount of LC3B-II in NBF-L cells (Figure 36A). Treatment of NBF-L cells with BEZ did not result in significant cytotoxic effects (Figure 36B). To examine the antiviral effect of BEZ treatment on ABLV replication, NBF-L cells were pre-infection and post-infection treated with BEZ. Treatment with BEZ resulted in decreased ABLV-GFP titers and ABLV N and vGFP levels (Figure 37A-C). The antiviral effect of BEZ treatment was

also examined with ABLV-WT. NBF-L cells were inoculated with ABLV-WT for 24hrs, the supernatant was removed and replaced with culture media containing BEZ. BEZ treatment resulted in a dose-dependent decreases in ABLV-WT titers, and N and P protein levels (Figure 38A-C).

A.



B.

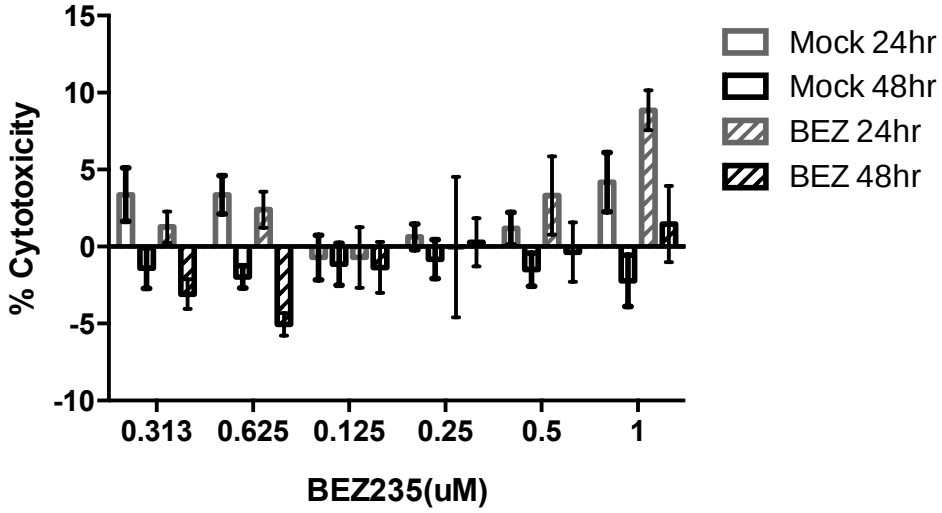


Figure 40. NVP BEZ235 (BEZ) induces autophagy and does not cause significant cytotoxicity.

(A) LC3B protein Western blot image. NBF-L cells were treated with BEZ for 4hrs

(B) Lactate dehydrogenase cytotoxicity assay of NBF-L cells treated with BEZ or mock-treated with DMSO. No significant differences between spontaneous cytotoxicity, DMSO or BEZ treatments.

Figure 41. BEZ treatment limits ABLV-GFP replication in human cells.

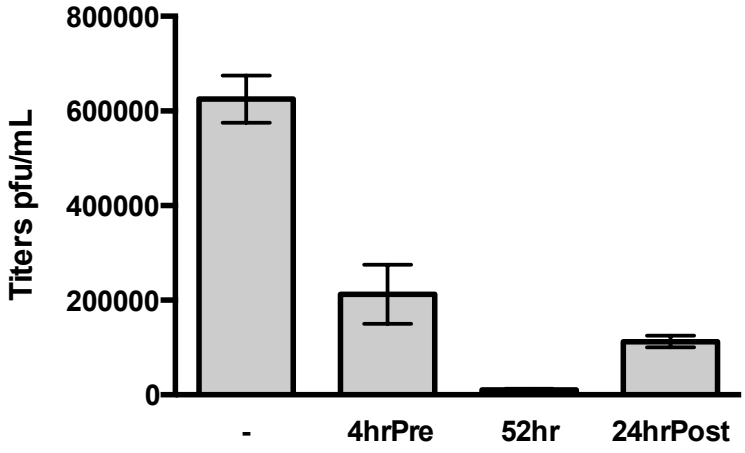
(A) NBF-L cells were infected with ABLV-GFP (moi 1) for 48 hours. NBF-L cells received three variations of BEZ (1um) treatment. 4hrPre: Cells were pre-treated with BEZ for 4hrs before infection. After 4hrPre treatment, culture media was removed, cells were washed, and fresh media without BEZ was inoculated with ABLV-GFP then added to the culture. 52hr: Cells were 4hrPre treated, then infected with ABLV-GFP so that the BEZ remained in cell culture during the entire infection. 24hrPost: BEZ was added to NBF-L culture 24hrs post-infection and remained in culture.

(B) ABLV-GFP nucleoprotein (N) and viral-GFP (vGFP) Western blot image.

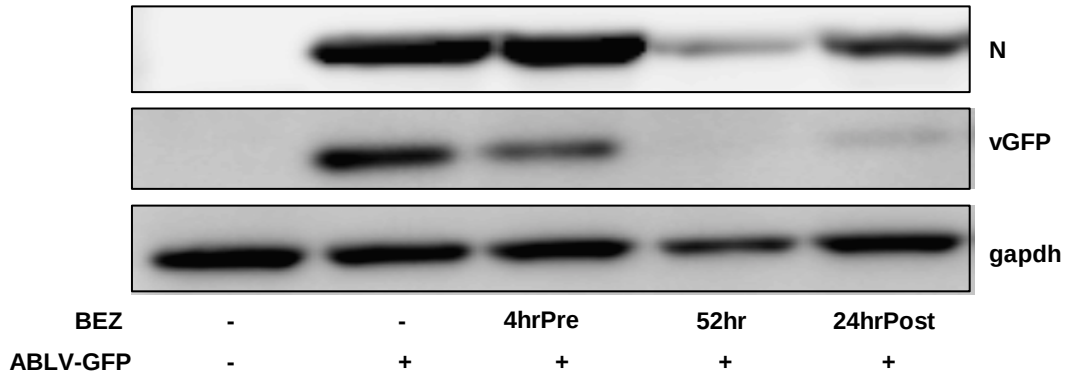
(C) ABLV-GFP nucleoprotein (N) and viral GFP (vGFP) were normalized to gapdh. All data represent two independent experiments.

Figure 41. BEZ treatment limits ABLV-GFP replication in human (NBF-L) cells

A.



B.



C.

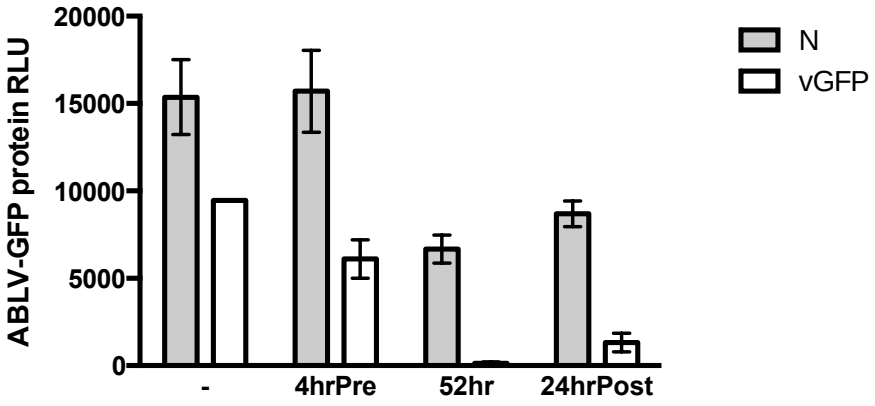
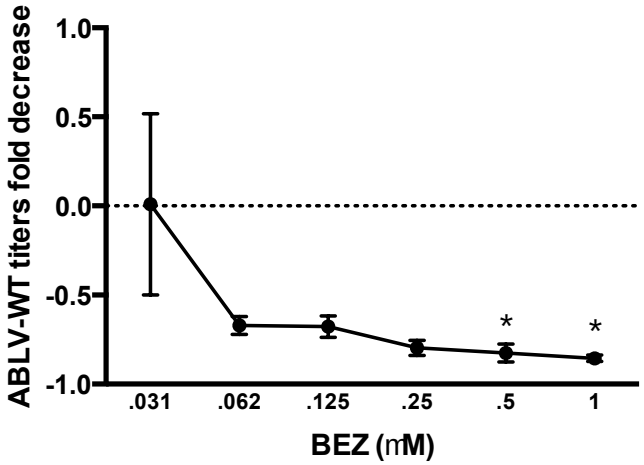


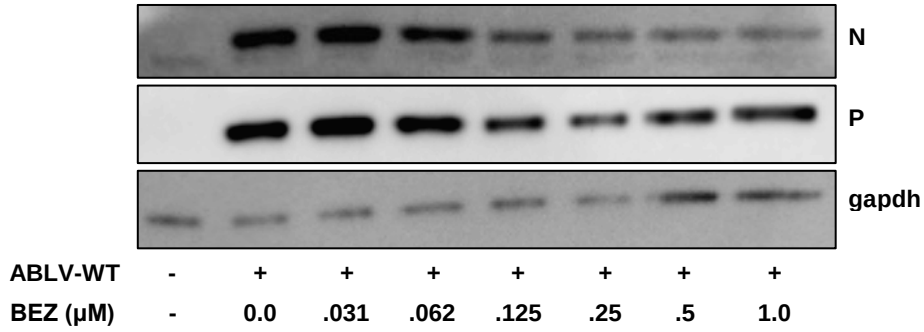
Figure 42. BEZ treatment limits ABLV-WT replication in human cells.
NBF-L cells were infected with ABLV-WT (moi 1) for 24 hours. NBF-L culture media was removed and replaced with cell culture media containing varying concentrations of BEZ. The infected and BEZ treated cells remained in culture for an additional 24 hours. (A) ABLV-WT titers (pfu/mL) fold decrease (B) Western blot image of ABLV-WT N and P protein (C) ABLV-WT N and P protein fold decreases. Data are a representation of three independent experiments. *Statistical significance determined by ANOVA (one-way) tests.

Figure 42. BEZ treatment limits ABLV-WT replication in human (NBF-L) cells

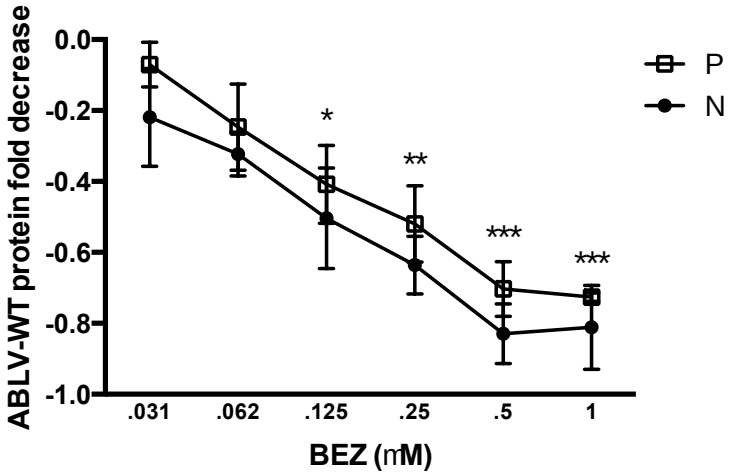
A.



B.



C.



3.5 Discussion and Future Directions

Autophagy is cellular homeostatic process that responds to cellular stressors, including starvation, DNA and organelle damage, and intracellular infection. In this study, we found that in bats and humans, ABLV infection induced autophagy. Furthermore, activation of autophagy restricted replication of ABLV, a neurotropic, Rabies virus-related virus. While this is the first study to examine whether autophagy is an antiviral defense in bats, recently another lab group independently hypothesized that the autophagy pathway was a critical antiviral defense in bats based on similar observations in regards to the enhanced longevity in bats, flight, autophagy, and control of viruses (41).

Infection with ABLV resulted in the induction of autophagy in bat and human cells, which aligns with previous observations that RABV infection induces autophagy and did not inhibit autophagic flux (226). Together, these results suggest that autophagy induction in response to virus infection appears to be conserved across the *Lyssavirus* genus. The specific pathogen associated molecular patterns (PAMPs) that were responsible for autophagy induction during ABLV infection have not yet been investigated. Pathogen recognition receptors (PRRs) are known to stimulate the autophagy pathway (241). We demonstrated that the TLR-3 ligand, poly(I:C), activated autophagy in bat cells. Similarly, TLR-3 has previously been shown to be a functional PRR in bat cells involved in the interferon pathway (58; 311; 313). As a synthetic analog of dsRNA, we have demonstrated the potential for virus dsRNA to stimulate autophagy in bats. In fruit flies, envelope glycoprotein engagement of Toll-7 by related (-)ssRNA viruses, VSV and Rift Valley fever virus has been demonstrated to activate the autophagy pathway (191; 197). Future autophagy induction experiments with UV-inactivated

ABLV-GFP will elucidate whether ABLV glycoprotein engagement at the cell surface is responsible for autophagy activation.

We found that treatment with two inducers of autophagy, rapamycin and small molecule enhancer of autophagy-28 (SMER), reduced ABLV replication in bat and 1human cells. In bat cells, rapamycin treatment had a greater effect on ABLV replication than SMER treatment. Treatment with rapamycin and SMER had little effect on ABLV N or P. The N and P proteins have the first and second highest transcriptional and translational expression, respectively (10). Moreover, given the high expression levels of these genes along the transcriptional gradient of the virus, it is possible that the reduction in ABLV replication might not cause observable effects on these two proteins.

Decreased vGFP was seen in both bat cells and human neuroblastoma cells. Expression of vGFP directly relates to ABLV replication, and since we observed a reduction in ABLV titers after rapamycin and SMER treatment, we expected to observe decreased levels of vGFP expression. The observed reduction in ABLV titers after rapamycin treatment makes it unlikely that activation of autophagy resulted in the selective degradation of vGFP since vGFP has no role in ABLV replication. At this time, we were not able to investigate the levels of ABLV matrix (M), envelope glycoprotein (G), or RNA polymerase (L) proteins, but we hypothesize that degradation of one or more of these proteins, which are involved in virus egress and replication, results in reduced ABLV replication when autophagy is induced. Experiments are currently being designed to quantify the transcript levels of each ABLV gene after autophagy induction to provide additional evidence that control of virus replication is occurring at the protein level.

Little is known about mTOR signaling in bats. Rapamycin induced higher levels of autophagy than SMER in bat kidney cells, and rapamycin had a stronger effect on reductions of ABLV replication compared to SMER in the both bat cells. Quantification of autophagy levels by flow cytometry after rapamycin and SMER treatment in human cells is needed to understand whether RAPA is a more potent activator of autophagy in bat cells and whether this observed difference can provide insight regarding mTOR signaling in bats. Inhibition of mTOR has been demonstrated to increase longevity in animal models, and as discussed, bats have unexpected longevities (30). The mTOR-signaling pathway might be an interesting avenue for future work targeted at understanding cellular mechanisms involved in the long life span of bats.

We experienced unexpected difficulties using classical and novel pharmacological modulators to examine the effects of autophagy inhibition on ABLV replication. Our preliminary data with 3-MA, an autophagy inhibitor, suggested that pre-infection treatment with 3-MA was inhibiting ABLV entry. Additionally, observations with inhibition of the class 3 PI 3-kinase, Vps34, through VPS34IN1 treatment suggested that inhibition of Vps34 was possibly blocking ABLV entry. Unlike 3-MA, VPS35IN1 specifically inhibits Vps34, which is required for formation of autophagosomes, and not other class 1 PI 3-kinases, however, since the drug is relatively new its effects on the autophagy pathway are not completely characterized (17; 178). Experiments that utilize live cell imaging of fluorescently labeled autophagosomes during VPS34IN1 treatment will help to elucidate whether VP34IN1 has downstream effects on autophagosome maturation and fusion with lysosomes. Furthermore, refinement of experiments with 3-MA or VPS34IN1 and ABLV will help to understand what step in the cellular life cycle

of ABLV these drugs alter, and might result in conclusions that PI 3-kinase inhibitors have potential roles as therapeutic strategies during ABLV infection, and more broadly, emerging neurotropic zoonotic viruses.

Despite multiple attempts, bat cells haven proven difficult to transfect and so siRNA knockdown of autophagy-related genes (ATGs) was never pursued as an option. Lentiviral delivery of shRNA to knockdown ATG5 levels did not decrease autophagy levels as expected, most likely due to redundancies in autophagy-related genes. However, despite this modest reduction in ATG5 expression and autophagy levels, we still observed that inhibition of ATGs resulted in slight increases in ABLV replication. Given the limited number of experiments with these ATG5 knockdown bat and human cells, we remain hopeful that additional attempts to silence ATGs will further support the notion that autophagy plays a role as an antiviral defense in bats.

Autophagy is regarded as a cell survival response that is the first check that cells make in response to potential cell damage, which can determine the fate of the cell and progression towards apoptotic cell death (174; 200). An experiment that is of particular interest, but dependent on efficient autophagy knockdown would be to monitor levels cell death during ABLV infection in wild-type and autophagy deficient cells. Chikungunya virus activates the autophagy pathway as a pro-viral strategy to maintain cellular homeostasis and down-regulate apoptosis and subsequently the antiviral effects of apoptotic cell death (137). Cross-talk between autophagy and apoptosis is of particular interest during ABLV infection because pathogenicity of RABV strains has an inverse relationship with apoptosis induction (19). Further characterization of autophagy

activation and cell death during ABLV infection in bat and humans cells will help to refine whether autophagy has a cytoprotective role in bat cells during virus infection.

We observed that short-term inhibition of autophagic flux resulted in significantly higher percentages of LC3B-II in bat cells during the time frame of inhibition. These results suggested that bats have an elevated level of basal autophagy compared to human cells. To understand whether elevated basal autophagy results in more rapid protein turnover, a lactate dehydrogenase autophagic sequestration assay should be performed (253). In such an experiment, autophagosomes would be isolated by gradient centrifugation, lysed, and lactate dehydrogenase levels in bat and humans would be quantified by color-metric assays to determine whether differences in autophagic flux exist between the cell lines. Before making far reaching conclusions about this observation, additional experiments focused on measuring basal autophagy in bat cell lines, primary bat cells, and other comparative mammalian cells are needed. Most promising from this work, has been the observed protective effects of BEZ treatment during both ABLV-GFP and ABLV-WT infection. However, additional experiments using genetic inhibition of autophagy are required to confidently conclude that reduction in ABLV replication during BEZ treatment is the dependent on autophagy. Future work will be aimed at inhibiting ATGs in human neuroblastoma cells and monitoring whether the effects of BEZ on ABLV replication are lost.

In conclusion, we have provided the first evidence that autophagy is activated during virus infection of a natural bat host. Additionally, the cumulative evidence suggests that autophagy acts as an antiviral defense in bats during ABLV infection. These findings helped to explore whether autophagy induction should be therapeutically

targeted during neurotropic virus infection and provide insights into innate immune responses that control viral replication in disease reservoirs.

3.6 ACKNOWLEDGEMENTS

Spencer L. Sterling performed all cytotoxicity experiments with BEZ and experiments with ABLV-WT and BEZ treatment. Dr. Dawn L. Weir successfully rescued the ABLV-GFP reporter virus. Dr. Sasha Atkins provided help with flow cytometry and Cyto-ID® staining.

CHAPTER 4: General discussion and future directions

DISSERTATION SUMMARY

The research aims of this dissertation were to 1) understand the geographic distribution of filoviruses and henipaviruses in Southeast Asian bat populations and 2) to investigate whether the autophagy pathway functions as an antiviral defense in bats. To address these research aims, we developed a Luminex-based multiplex binding assay that is capable of simultaneously detecting antibodies specific to all presently known ebolaviruses, marburgviruses, and henipaviruses, and we comparatively investigated the interaction of ABLV with autophagy in cells derived from the natural bat host and human cell lines. In collaboration with partners at the Duke-NUS Graduate Medical School, Singapore, we screened a library of endemic bat sera samples that were collected as part of a five-year longitudinal study with our multiplex binding assay. The principal goal that encompasses both of these aims is to understand persistence and maintenance of zoonotic viruses in animal reservoirs. Detection of zoonotic viruses in unknown geographies and wildlife populations, and examination of cellular mechanisms of antiviral defense in natural hosts are necessary to understand the persistence of emerging viruses in host populations, and the abiotic and biotic drivers of zoonotic events.

CHAPTER SUMMARIES

Chapter 2 Summary

In chapter 2, we hypothesized that *E. spelaea, C. brachyotis,* and *P. lucasi* bat populations sampled in Singapore will have serological evidence of past exposure to filoviruses. To test this hypothesis, we expanded an already established, and field-tested

Luminex-based multiplex assay for henipaviruses to include envelope glycoprotein antigens from all currently known marburgviruses and ebolaviruses. Our bat sera screening results demonstrated that all three bat species have been exposed to viruses most antigenically-related to ebolaviruses. Our results also suggested that all three bat species tested were exposed to a novel filovirus species, which is more antigenically similar to ebolaviruses than marburgviruses. Additionally, the putatively novel filovirus(es) detected in our multiplex assay are more antigenic similar to African ebolaviruses such as EBOV, BDBV, and SUDV than to RESTV, which naturally infects bat populations in the Philippines, and is the only presently characterized *Ebolavirus* species in Asia.

Limitations

Our bat sera screening results are limited by several factors. The long-lived nature of antibodies is advantageous for less invasive serological biosurveillance studies where animals can be captured, sampled, and released. However, interpretations of serology studies have inherent limitations. As observed in our study, without nucleic acid evidence to support serological evidence of past infection we are limited to draw conclusions based on polyclonal sera cross reactivity. The multiplex binding assay has been designed to be inclusive of known species of ebolaviruses, marburgviruses, and henipaviruses. Yet, when we are conducting biosurveillance for viruses where distribution is unknown, additional virus sequence evidence would be helpful for confidently narrowing down the viruses circulating in wildlife populations. Additional integration of techniques such as the collection of urine excretion, the "gold standard" for identification of novel viruses from bats, and improved guidelines for serology surveillance studies including repeated

sampling of individuals and age-stratified analysis will help to address several hypotheses about transmission dynamics and virus persistence within bat host populations (232). Moreover, an insufficient understanding of some bats species, such as *Eonycteris* spp., leaves gaps in our understanding about the migration between roosting colonies, the critical community size, and transmission dynamics of ebolaviruses in Southeast Asia within bat populations.

Future directions

Current and future biosurveillance studies in Southeast Asia and India are incorporating our Luminex-based multiplex binding assay. These biosurveillance studies will permit us to better understand the cross-reactive nature of bat sera from wild populations in this region. Additionally, the experimental protocol of the multiplex binding assay permits the screening of all domestic or wildlife species, not just bats. Biosurveillance at animal interfaces such as agricultural areas that are high-risk areas for zoonotic spillovers can be used to monitor virus chatter between domestic and wildlife populations as part of preventive measures. The binding assay protocol can be further modified to screen for evidence of recent infections by testing human sera for IgMs, which could potentially be useful for differential diagnosis of uncharacterized virus infection in future studies. To facilitate this modification, commercially available anti-human IgM sera would be substituted in the Bio-Plex assay in place of protein A/G as the secondary antibody.

Finally, additional studies using radio collar tracking of individual bat species are needed to assist in understanding migration patterns, endemic colonies, and metapopulation dynamics of the less studied *Pteropodidae* bat species in Southeast Asia.

Together these data have the potential to provide insight into the different bat species and populations that are responsible for virus maintenance in the environment.

Chapter 3 Summary

We investigated whether autophagy has an antiviral role in bats. We setup comparative experiments with brain and kidney cell lines derived from the Black Flying Fox, *P. alecto*, and a human neuroblastoma cell line. In these experiments, ABLV, a Rabies-virus related virus that has been isolated from *P. alecto* was used as a model bat-borne virus so that we could compare infection dynamics between a zoonotic virus and its natural host. When bat and human cells were grown to equal densities in culture, bat cells appeared to have an elevated basal autophagic flux. This observation fits our hypothesis that with an adaption for flight, bats required cellular proteostatic mechanisms to maintain cellular homeostasis in response to high oxidative stress and to support increased longevity.

In these experiments, we discovered that autophagy was induced during ABLV infection in both bat and human cell lines. Induction of autophagy in bats was only seen 48hpi with a MOI of 10 in both primary brain cells and a brain-tissue cell line. This observation suggests that either activation of autophagy required a pathogenic level of virus or that the elevated basal autophagic flux in bat cells turns over LC3B-II protein rapidly so that a low MOI infection does not result in observable LC3B-II increases. Treatment of bat and human cells with inducers of autophagy, rapamycin and SMER28, resulted in decreased ABLV replication. We further explored pharmacological autophagy to control ABLV replication with a FDA-approved therapeutic, NVP BEZ235, and

observed that treatment with NVP BEZ235 reduced replication of our ABLV reporter virus and a wild-type ABLV isolate.

Limitations

This aim has been limited by several factors. One limiting factor has been an overreliance on Western blots to monitor levels of autophagy and autophagic flux. Adherence of bat cell lines and growth on coverslips has been inconsistent, and has limited our ability to perform fluorescent microscopic imaging of autophagy during ABLV infection. A second method to monitor autophagy activation that does not rely on immunoblotting, such as flow cytometry, will be useful to strengthen conclusions about autophagic flux during ABLV infection and bafilomycin treatments. The interference of ABLV entry observed during treatment with pharmacological inhibitors of autophagy, limited our ability to make stronger conclusions about the antiviral role of autophagy in bats. Genetic approaches to inhibit autophagy are critical. A lack of reagents specific to ABLV matrix (M) and RNA polymerase (L) leave us with an incomplete idea of how autophagy induction is causing reductions in ABLV replication at the protein level.

We have used ABLV as our model bat-borne virus because of biosafety-level restrictions for other bat-borne viruses such as Hendra virus (HeV), which also naturally infects and is maintained by *P. alecto*. One limitation is whether these interactions will translate to other bat-borne viruses. Applying the observations of ABLV and autophagy interactions in *P. alecto* and human cells might not fit with how HeV and NiV interacts with autophagy in bat cells. The immunocompetence bat hosts infected with lyssaviruses is not completely understood. Additionally, the natural routes of infection of lyssaviruses

and the henipaviruses vary significantly and these viruses differ in clinical disease development in the natural bat host.

Future directions

The current annotated *P. alecto* genome, supply of *P. alecto* and ABLV cross reactive reagents, non-traditional methods for monitoring autophagy, wild-type ABLV, and successful culture of primary *P. alecto* cells, presents the laboratory with opportunities for several future studies that could build upon the work started by this research aim. A well-established technique to monitor autophagic flux is to dual-tag LC3B protein with a green fluorescent protein (GFP) and a red fluorescent protein (RFP). This GFP-RFP-LC3B construct can be used to monitor the maturation of autophagosomes and complete autophagic flux, as GFP is acid labile and upon autophagosomal fusion with lysosomes the GFP signal is lost. Autophagic flux can be monitored in stable cell lines expressing this GFP-RFP-LC3B construct can be infected with wild-type ABLV.

A truly comparative cell line study of ABLV replication in cell lines from rats and mice will help to further understand whether low titers of ABLV is the result of cell type differences or a host restriction specific to bats. Furthermore, a comparative study including the additional mouse and rat cell lines will be helpful to understand whether elevated basal autophagic flux observed in *P. alecto* cells is unique to bat physiology. The autophagy and the interferon pathways are both described as cell-autonomous intrinsic immune responses. The completed annotation of the *P. alecto* genome and advances in CRISPR-Cas9 technologies will enable us to explore any differential contributions of these two immune response to antiviral defense in bats. Lastly, the

Amnis® imaging flow cytometer installed at the Biomedical Instrumentations Center presents unique opportunities to monitor, quantify, and compare mitophagy in bat and human cells after ABLV infection, which will help to address hypothesizes about the physiological evolution of autophagy as a homeostatic mechanism to control damage from reactive oxygen species and mitochondrial dysfunction in bats.

Lastly, performing these experiments with other bat-derived cell lines will contribute to our understanding as to whether these observations about autophagy and ABLV are unique to *P. alecto* or can be more generally applied to *Pteropodid* bat hosts. Collaborators are in the process of establishing cell lines from *Eonycteris spelaea*, a fruit bat related to *P. alecto*. Additionally, this study can be used as a model for new BSL-4 experiments aimed as investigating whether HeV infection results in activation of autophagy in the natural *P. alecto* host cells. Lastly, as a non-pathogenic *Henipavirus* species, Cedar virus, once rescued using a reverse genetics approach at BSL-2 will also present new opportunities to understand virus-host interactions between natural and accidental cell lines.

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