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Investigation and Characterisation of Viral and Host Factors that Impact Intraspecies EBOV Infectivity using a Viral Pseudotyping Assay

Jayasree Dey
BSc, MSc

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Abstract

Zaire Ebola Virus (EBOV) is the etiological agent of Ebola Virus Disease, which is characterised by inflammation and damage to endothelial cell linings that can rapidly advance to internal haemorrhaging and multi-organ failure, with mortality rates observed as high as 90%. As a zoonotic virus, current evidence suggests that bats may be the natural reservoir for EBOV. With outbreaks becoming more frequent, EBOV remains an ongoing threat to public health. Further work investigating the intraspecies variation present within EBOV and the viral and host genetic factors that impact viral fitness, is needed to provide insight into potential genetic drivers of virus spillover and pathogenesis.

This project developed the first EBOV GP pseudotyping panel that is representative of the intraspecies variation the virus displayed in outbreaks between 1976 and 2016. The panel was then tested in HuH-7 and Hyp/Lu 45.1 cells to determine the differences in infectivity profiles between the EBOV variants. The project demonstrated that EBOV outbreak strains display differential infectivity in both cell lines, with the Mbandza 2003 strain exhibiting the lowest infectivity in multiple cell lines. This characteristic was deemed a product of the T527 residue that is present in the internal fusion loop of the Mbandza 2003 GP. The assay also confirmed that the tissue culture adaptation, T544I, significantly impacted the infectivity of the Mayinga 1976 strain, increasing it relative to wild-type strains in both cell lines. The panel was further tested alongside sequences representative of theoretical ancestral GPs to determine the impact of the genetic differences between these sequences on viral fitness and also provide more insight into the theory that bats are the reservoir for the virus. Investigations showed that ancestral sequences are more infectious than outbreak sequences in both HuH-7 and Hyp/Lu 45.1 cells, suggesting that the *H. monstrosus* bat species that the Hyp/Lu 45.1 cells were derived from, may not be a natural reservoir for EBOV and that other selective pressures may be driving evolution.

Finally, the project investigated the impact of single nucleotide polymorphisms (SNPs) that were present in the NPC1 receptor of African populations to determine their impact on the susceptibility to EBOV. It was determined that the NPC1 containing the P434S and M642I SNPs was more permissive to EBOV infection relative to its wild-type homologue. In conclusion, this project established that variation in both the viral GP and host receptor impacts the entry fitness of EBOV.

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Abbreviations

6HB	Six-helical bundle
ADE	Antibody-dependent enhancement
Amp	Ampicillin
AAV	Adeno-associated virus
APC	Antigen-presenting cell
ANOVA	Analysis of variance
BDBV	Bundibugyo virus
BOMV	Bombali virus
bp	Base pairs
bGH	Bovine growth hormone polyadenylation signal
BSA	Bovine serum albumin
bsd	Blasticidin
BSL	Biosafety level
CAT B/L	Cathepsin B/L
CDC	Centre of Disease Control and Prevention
cDNA	Complementary DNA
CFR	Case fatality rate
ChAd	Chimp Adenovirus
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CV	Coefficient of variation
DC	Dendritic cell
DENV	Dengue virus
Delta E	Delta envelope protein
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EBOV	Zaire Ebola Virus
EDTA	Ethylenediaminetetraacetic acid

ESCRT	Endosomal sorting complexes required for transport
EtBr	Ethidium Bromide
ER	Endoplasmic reticulum
ERVEBO	Recombinant vesicular stomatitis virus–Zaire Ebola virus (rVSV-ZEBOV) vaccine
EVD	Ebola virus disease
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GE	Gene end
GP	Glycoprotein
GFP	Green fluorescent protein
GS	Gene start
HCV	Hepatitis C
HIV	Human immunodeficiency virus
HEK293T	Human embryonic kidney-293T cells
HR	Heptad repeats
HuH-7	Human hepatocarcinoma cells
Hyp/Lu 45.1	Hypsignathus monstrosus lung
IRES	Internal ribosomal entry site
IFL	Internal fusion loop
IFN	Interferon
IL	Interleukin
IgG/M	Immunoglobulin G/M
JAK	Janus kinase
kb	Kilobase
kDa	Kilodalton
KLD	Kinase, ligase and Dpn1
KPN-α1	Karyopherin α 1
L	Polymerase
LB	Luria-Bertani
LDL	Low-density lipoprotein
LLOV	Lloviu virus
MARV	Marburg virus
MCP-1	Monocyte chemoattractant protein 1
MDA-5	Melanoma differentiation-associated gene 5
MIP1-α	Macrophage inflammatory protein 1 α

MHC	Major histocompatibility complex
MLD	Mucin-like domain
MLAV	Mengla virus
MLV	Murine leukaemia virus
MME	Multiple mass extinctions
MRCA	Most recent common ancestors
mRNA	Messenger RNA
NEAA	Non-essential amino acids
NHP	Non-human primate
NK	Natural killer
NP	Nucleoprotein
NPC1/2	Niemann-pick C1/2
nt	Nucleotide
ORF	Open reading frame
PALM	Pamoja tulinde maisha
PAMPs	Pathogen-associated molecular pattern molecules
PPR	Pattern recognition receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PP1/2A	Protein phosphatases 1/2A
PREVAC	Partnership for Research on Ebola VACCination
PREVAIL II	Partnership for Research on EbolaVirus in Liberia II
PtdSer	Phosphatidylserine
Puro	Puromycin
PV	Pseudovirus
RAVV	Ravn virus
RBD	Receptor binding domain
RC	Republic of Congo
RdRp	RNA-dependent RNA polymerase
RESTV	Reston virus
REGN-EB3	REGN3470, REGN3479 and REGN3471 antibody cocktail
RIG-1	Retinoic inducible gene
RLU	Relative light units
RNA	Ribonucleic acid
RNP	Ribonucleoprotein

rMFI	Relative median fluorescence units
SD	Standard deviation
SDM	Site-directed mutagenesis
sGP	Soluble GP
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SSD	Sterol sensing domain
ssGP	Small soluble GP
SRPK-1	Serine-arginine protein kinase 1
STAT1/2	Signal transducer and activator of transcription 1/2
SUDV	Sudan virus
TAE	Tris –acetate-EDTA buffer
TAFV	Tai Forest virus
TIM-1	T-cell immunoglobulin and mucin domain 1
TLR	Toll-like receptor
TMD	Transmembrane domain
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
U2OS	Human bone osteosarcoma epithelial cells
Vero E6	African green monkey epithelial cells
VLP	Virus-like particles
VSV	Vesicular stomatitis Indiana virus
WHO	World Health Organisation
YFV	Yellow fever virus
ZMab	Cocktail of mouse monoclonal antibodies: c1H3, c2G4, and c4G7
ZMapp	c2G4, c4G7 (from ZMab) and c13C6 antibody cocktail

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1. Introduction

1.1 Introduction to the Viral Order, *Mononegavirales*

1.1.1 Characteristics of the Order, *Mononegavirales*

Mononegavirales is the taxonomic home to numerous families of viruses that can cause a variety of respiratory, neurological and haemorrhagic diseases within human and/or animal populations (Amarasinghe *et al.*, 2019). The classification of viral families belonging to this order has undergone numerous iterations in previous years. The order is currently comprised of eleven viral families, which include *Filoviridae*, *Paramyxoviridae*, *Bornaviridae*, *Pneumoviridae* and *Rhabdoviridae*, to name a handful that have had a considerable impact on public healthcare as well as the global economy (Lamb, 2007; Amarasinghe *et al.*, 2019). Although each viral family is distinguishable by many factors, including their morphologies, pathogenic potential and distinctive host ranges (Table 1.1), they are all primarily characterised by their linear non-segmented, negative-sense, single-stranded RNA genomes, which can extend up to 19 kb in length and occasionally contain limited overlapping genes (Pringle, 1991; Muhlberger *et al.*, 1999; Whelan, Barr and Wertz, 2004).

Table 1.1 Characteristics of families of viruses within the order, *Mononegavirales*

These viruses are some that are significant to human or animal health. Adapted from (Easton and Ling, 2010)

	<i>Filoviridae</i>	<i>Paramyxoviridae</i>	<i>Pneumoviridae</i>	<i>Rhabdoviridae</i>	<i>Bornaviridae</i>
Genome Size (~nt)	15-19,000	13–18,000	13-15,000	11-15,000	8900
Virion Morphology	Filamentous, pleomorphic	Pleomorphic, spherical, filamentous	Spherical, filamentous	Bullet-shaped or bacilliform	Spherical
Replication Site	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm except for nucleorhabdoviruses	Nucleus
Host Range	Primates, bats, swine, fish	Vertebrates, including mammals, birds, reptiles	Mammals, Birds	Vertebrates, arthropods, plants	Mammals, birds, reptiles
Pathogenic Potential	Viral haemorrhagic fever	Respiratory or neurological diseases	Respiratory syndromes	Mild febrile illnesses to fatal neurological diseases	Disturbances in behaviour to encephalomyelitis

The negative-sense genomes are considered to be non-infectious, lacking a capped structure and polyadenylation, however, they are flanked by a 3' leader (containing the promoter region) and a 5' trailer sequence, which regulates both transcription and replication of the genome (Latorre, Matternberger and Geller, 2018). These processes rely on the activity of a large, multi-domain RNA-dependent RNA polymerase (RdRP) alongside essential polymerase cofactors, which are crucial in bridging the interactions between the nucleoprotein and the RdRP, thus resulting in the formation of the ribonucleoprotein complex (RNP) (Mühlberger *et al.*, 1992, 1998; Lamb and Parks, 2007; Morin *et al.*, 2013). The necessity of the RNP to drive viral genome transcription and replication within a host cell is conserved in all members of the order (Pfaller, Cattaneo and Schnell, 2015). These processes usually occur within the cytoplasm of the host cell, except for bornaviruses and plant rhabdoviruses, which follow an uncharacteristic model of transcription and replication that occur in the host cell nucleus (Richman *et al.*, 2016; Gaafar *et al.*, 2019).

1.1.2 Transcription and Replication to Form Viral Progeny

Mononegavirales share a homology regarding their genome organisation, typically following the order of a 3' untranslated region, genes that code the core proteins and envelope proteins, the polymerase gene and a 5' untranslated region (Latorre, Matternberger and Geller, 2018). Each gene acts as an individual transcription unit that is enclosed by transcription initiation and termination sequences, often showing similarities between members within the same family (Mühlberger *et al.*, 1992; Pringle and Easton, 1997). Transcription results in a capped and polyadenylated positive sense messenger RNA and whilst most genes code for a single protein product, some viral families contain genes that can produce up to three proteins (Whelan, Barr and Wertz, 2004). As transcription is a sequential process, initiating at the 3' end of the genome, a gradient is observed in terms of the different protein concentrations that are expressed (Pagan, Holmes and Simon-Loriere, 2012). This is due to polymerase dissociation, which occurs at the gene junctions. It is the concentration of these proteins that regulate the switch from transcription to genome replication, which is initiated through the formation of a positive sense intermediate anti-genome that acts as a replication template from which negative-stranded viral progeny genomes can be transcribed (Banerjee, Barik and De, 1991; Lvov *et al.*, 2015; Pfaller, Cattaneo and Schnell, 2015). The preservation of the elements essential for modes of gene expression and replication throughout the order, illustrates that members have originated from a common ancestor.

Following the expression of essential viral proteins and replication of the genome, virions can begin to assemble. Virus maturation occurs at membrane sites, where virions are encompassed by a membrane-derived envelope, as they typically bud away from the cell. Most viruses bud from the plasma membrane, although the rhabdovirus, *Rabies lyssavirus*, can bud into intracellular membranes and some plant rhabdoviruses bud from the nuclear membrane (Goodin *et al.*, 2007; Lamb, 2007). The envelope composition is reflective of the exploited host cell, however, typically consists of phospholipids and glycolipids, which represent approximately 55-60% and 35-40% of the envelope's total lipid composition, respectively. Additionally, carbohydrates present as N and O-linked glycan chains attached to various surface glycoproteins, constitute approximately 3% of a virion's weight (Fauquet *et al.*, 2005; Lamb, 2007).

1.1.3 Impact of the Viral Order on Global Health and Economy

The diversity of these viruses in terms of their tropism and disease presentation illustrates the relevance of this viral order to human and animal health. Many families within the order encompass viruses that are well-established pathogens, which can pose a severe and often lethal risk to individual and public health, whilst also contributing to a major economic burden on worldwide healthcare systems. For example, reoccurring outbreaks of viral haemorrhagic fever in human and non-human primate populations, for which filoviruses are etiological agents, have some of the highest mortalities observed within the realm of infectious diseases (Nyakarahuka *et al.*, 2016; Wing *et al.*, 2018; Languon and Quaye, 2019). Even vaccine-preventable diseases such as the neurological disease, rabies, continue to result in over 59,000 annual worldwide fatalities, with the estimated yearly cost of disease diagnostics, prevention and control in the USA ranging between \$245 and \$510 million, alone (Hampson *et al.*, 2015; Pieracci *et al.*, 2019).

Some viruses belonging to the order, including pneumoviruses and paramyxoviruses, display a broad tropism encompassing humans, birds and other mammals, although individual viruses have a more restricted host range. Several members of these families are responsible for many common infectious childhood respiratory syndromes, including respiratory syncytial syndromes, measles, mumps, and parainfluenza (Virtue, Marsh and Wang, 2009; Griffiths, Drews and Marchant, 2017; Maykowski *et al.*, 2018; Shafagati and Williams, 2018; Mina *et al.*, 2019). However, the families also contain viruses that harm a variety of animals, including canines, birds and domestic cattle, with associated diseases resulting in an economic burden for the commercial farming and agricultural industries

(Easton, Domachowske and Rosenberg, 2004; van Boheemen *et al.*, 2012; Piewbang and Techangamsuwan, 2019; Suarez *et al.*, 2020). Additionally, paramyxoviruses are inclusive of zoonotic pathogens of emerging prevalence, such as Nipah and Hendra viruses. These viruses not only result in acute respiratory illness and fatal encephalitis in humans but can also largely affect animal populations, causing overwhelming epidemics that leave devastating financial consequences for the farming communities that are involved, once again demonstrating the high medical and economical relevance of viruses from this order (van Boheemen *et al.*, 2012; Zeltina, Bowden and Lee, 2016; Thibault *et al.*, 2017).

1.2 Introduction to the *Filoviridae* Family

1.2.1 General Properties of Filoviruses

Filoviruses are unique members of the order in that they share a characteristic filamentous morphology that is typically 790 to 970 nm in length when purified and 80 nm in width (Geisbert and Jahrling, 1990, 1995). The viral genome is encapsidated by nucleoprotein (NP), which when combined with the polymerase (L) the polymerase cofactor, VP35, and the transcription activator, VP30, forms the RNP, a protein structure that encompasses a transcriptase and replicase function. RNP interacts with the nucleocapsid-associated VP24 and is enclosed by the matrix protein VP40, which is vital in particle formation and filovirus morphology (Emanuel, Marzi and Feldmann, 2018; Kuhn *et al.*, 2019; Feldmann, Sprecher and Geisbert, 2020). Filoviruses express a single surface glycoprotein (GP), a trimeric transmembrane GP that mediates viral entry through attachment to host cells, internalisation via macropinocytosis and entry into the host cell cytoplasm, the site of filovirus replication, through membrane fusion with an endosomal receptor (Hoffmann *et al.*, 2016).

Filoviruses are differentiated by their antigenic cross-reactivity, the similarity of their genomes, the number of overlapping genes and whether they can implement the phenomenon of transcriptional editing of the GP that leads to its multiple forms as shown in Figure 1.1 (Feldmann *et al.*, 1994; Carroll *et al.*, 2013; Kuhn *et al.*, 2019). The classification of the family and the associated diseases has recently been revised following the accumulation of data surrounding the 2013-2016 West African Ebola virus outbreak, as well as the discovery of new viruses. The family currently houses six different genera of viruses with varying host ranges (Amarasinghe *et al.*, 2019; Kuhn *et al.*, 2019).

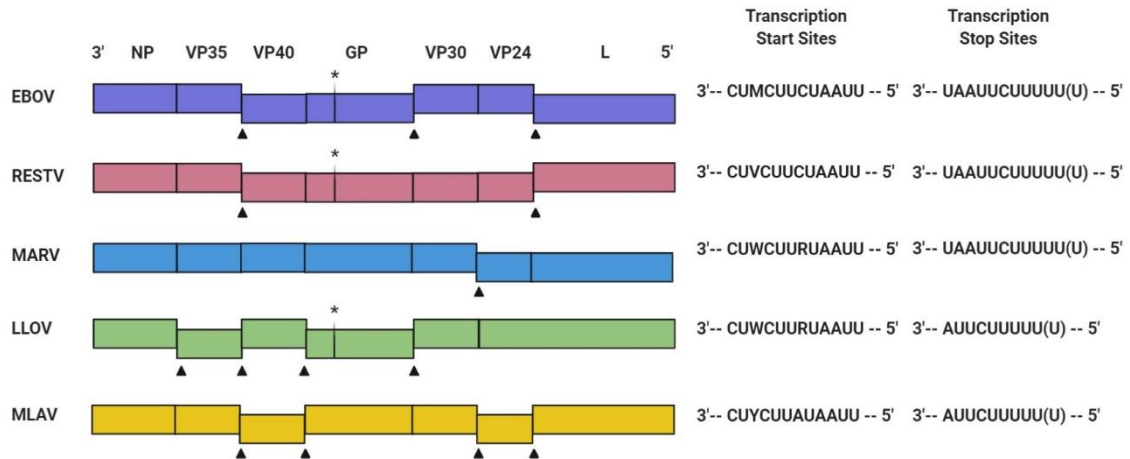


Figure 1.1 Genome organisation of filoviruses

Genes are shown as boxes, gene overlapping sites are indicated by triangles and sites of transcriptional editing within the glycoprotein gene are highlighted by an asterisk. TAFV and BDBV share the same overlapping gene sites as EBOV and SUDV, the same as RESTV. LLOV only contains 6 genes, with a bicistronic mRNA resulting in the expression of both the polymerase and VP24. Adapted from (Yang *et al.*, 2019).

1.2.2 Emergence of Filoviruses and their Current Classification

Arguably, the most widely recognised of these are *Marburgvirus* and *Ebolavirus*. The *Marburgvirus* genus comprises a single species that has two members, Marburg virus (MARV) and Ravn virus (RAVV), whereas *Ebolavirus* now consists of six species. Members of both these families have been considered a severe public health risk since their initial identification during outbreaks occurring in 1967 and 1976, respectively (Siegert *et al.*, 1967; WHO, 1978; Kuhn *et al.*, 2019). This is due to their ability to initiate unpredictable and sporadic outbreaks of lethal haemorrhagic fever in human and non-human primate populations (Feldmann *et al.*, 2003; Messaoudi, Amarasinghe and Basler, 2015).

Filoviruses are thought to be regionally epizootic rather than endemic, supported by the frequent re-emergence of the viruses throughout the years (Feldmann, Sprecher and Geisbert, 2020). For decades, the origins, geographic prevalence and ecology of filoviruses remained elusive. However, it is now clear that outbreaks of disease or zoonotic origin, occur when viruses spillover from their animal reservoir to human or mammalian populations, resulting in spread via human-to-human transmission (Messaoudi, Amarasinghe and Basler, 2015). Identifying risk factors that lead to the occurrence of spillover events is crucial in preventing disease outbreaks and therefore, identifying the natural reservoir species of the virus is of paramount importance. Bats (*Rosettus aegyptiacus*) are well-established reservoirs of MARV and RAVV, whereas ambiguity still exists regarding the *Ebolavirus* animal reservoir (Carroll *et al.*, 2013; Olival and Hayman,

2014). Nevertheless, growing evidence points towards fruit bats also being the primary suspect implicated in the transmission of ebolaviruses to hosts susceptible to disease, based on studies relating to serology and viral genome detection (Leroy *et al.*, 2005).

Similarly, the viral RNA of Lloviu virus (LLOV), belonging to the genus *Cuevavirus*, was also first identified in insectivorous bats (*Miniopterus schreibersi*) in Europe (Negredo *et al.*, 2011; Kemenesi *et al.*, 2018). Interestingly, all dead bats found at the location were positive for LLOV RNA, whereas healthy bats sampled from the same location remained viral RNA-free. Histopathological studies detected signs of viral pneumonia in carcasses, suggesting an alternative reservoir for LLOV may exist, rather than *M. schreibersi*. There have not been any reported cases of an associated disease in humans and therefore, the virus has not yet been biologically characterised with regard to its pathogenicity within humans (Negredo *et al.*, 2011).

This also applies to Mengla virus (MLAV), the single-member species of the genus *Dianlovirus*, which was recently identified in *Rousettus* bat species originating from China. Although the transmissibility of LLOV and MLAV in human populations is a possibility, due to their use of the identical host cell receptor as Marburg and ebolaviruses to mediate cell entry and instigate membrane fusion, this has not yet been observed within a human population (Negredo *et al.*, 2011), (Yang *et al.*, 2019). Additionally, species tropism is not solely determined by receptor usage, but by additional factors such as the ability of the virus to replicate within a host, which may also explain why no human infections have thus far been noted. MLAV is discernably evolutionarily related to the Ebola virus and Marburg virus, demonstrated by some shared functional similarities. However, it is genetically distinct, sharing a similarity between 32 and 54% at a nucleotide base level, relative to other known filovirus genomes (Yang *et al.*, 2019).

Moreover, two additional RNA virus genomes were recently discovered in striated frogfish and filefish. Although there was no attempt to isolate these viruses, phylogenetic analysis of the RdRP placed both these viruses, termed *Striavirus* and *Thamnovirus* respectively, as genera within the filovirus family, further demonstrating its geographic diversity and broad host range (Shi *et al.*, 2018).

Although members of the *Filoviridae* family are biologically similar with limited differences in their genome structures, in how they edit their RNA and their interferon antagonistic properties, the vast amount of available knowledge surrounding filoviruses has been obtained from studies of mostly EBOV and MARV (Emanuel, Marzi and Feldmann, 2018).

As biosafety level (BSL) 4 pathogens, there has been a necessity for the establishment of modelling systems, to allow for the elucidation of the filovirus life cycle, within BSL1 and 2 laboratories (Hoenen, Groseth and Feldmann, 2019). These systems, which will be discussed in further detail in future chapters have been crucial in gaining insight into viral entry pathways, viral replication and infection and potential therapies and as such, should be applied to cultivate a greater knowledge and understanding of the more newly discovered filoviruses with unknown pathogenic potential.

1.2.3 The *Ebolavirus* Genus

There are five well-acknowledged species of *Ebolavirus*; Zaire Ebola Virus (EBOV), Bundibugyo Virus (BDBV), Tai Forest Virus (TAFV), Sudan Virus (SUDV) and Reston Virus (RESTV), the latter of which was, until recently, the only known species that seemed to be non-virulent in humans, with individuals testing as seropositive for antibodies against RESTV, yet remaining asymptomatic with regards to disease presentation (CDC, 1990; Barrette *et al.*, 2009; Feldmann and Geisbert, 2011).

However, in 2018, a new species of *Ebolavirus*, Bombali Virus (BOMV), was discovered in free-tailed, insectivorous bats from Sierra Leone. Since its discovery, the virus has also been identified in the same species of bat in Kenya, highlighting its potential geographical prevalence (Goldstein *et al.*, 2018; Forbes *et al.*, 2019). In this instance, viral RNA was also isolated from the spleen, liver, heart, intestine and mouth and faecal swabs taken from the bats, indicating that the presence of the BOMV genome was not simply an artefact of the host species' diet, as might have previously been argued with its original discovery, during which only oral and rectal swabs were positive for the virus (Forbes *et al.*, 2019). Although a viral pseudotype expressing BOMV GP was shown to be able to initiate entry into human cells via the same receptor as other filoviruses, similarly to LLOV and MLAV, there is currently no evidence that this virus causes disease in humans, however, this does highlight its zoonotic potential and provides more evidence to the growing argument that bats are the natural reservoir for Ebolaviruses (Goldstein *et al.*, 2018).

1.2.4 Geographic Prevalence of Ebolaviruses

Ebolaviruses are predominantly of African origin, with reoccurring outbreaks of viral haemorrhagic disease characteristically arising in regions within proximity to the African

equator. EBOV outbreaks have typically occurred in central and western African countries. Contrastingly, SUDV and BDBV have been geographically observed in eastern African countries. RESTV is the only exception to this. The virus was initially detected in the USA with several outbreaks occurring in 1989-1990, after the import of cynomolgus macaques (*Macaca fascicularis*), which originated from a facility in the Philippines. This facility became the source of future outbreaks in Italy (1992) and Texas (1996). The infected animals presented with symptoms including a cough, swollen eyelids, weight loss, and an enlarged spleen and kidneys, although no symptomatic human cases were reported (Jahrling *et al.*, 1990).

Since then, it has been found in domestic pigs throughout the Philippines and China, raising fear around food safety since the zoonotic potential of the virus remains unclear (Barrette *et al.*, 2009; Pan *et al.*, 2014). *Rosettus amplexicaudatus* bats from the Philippines have been seropositive for RESTV, implicating them as a possible reservoir for the virus in Asia (Taniguchi *et al.*, 2011). Recently, separate studies showed that bat species in Bangladesh and China have also been shown to be seropositive for antibodies against EBOV and RESTV, the first evidence of EBOV infecting wildlife in mainland Asia (Yuan *et al.*, 2012; Olival *et al.*, 2013; Jayme *et al.*, 2015). Although no live virus was detectable in these cases, it once again highlights the potential geographic range of *Ebolavirus* and corroborates the notion that filoviruses, in general, may be harboured across a broader range than was originally assumed.

1.2.5 Ebola Virus Disease

EBOV is one of the etiological agents of Ebola Virus Disease, with major outbreaks described in West Africa, between 2013 and 2016, as well as the outbreak that commenced in 2018, in the Democratic Republic of Congo (DRC). Outbreaks of EBOV have been occurring since 1976 and genetic analysis of virus genomes shows a 2.7% nucleotide variation between isolated sequences. Genetic diversity within a single outbreak was found to be even lower. For example, sequence divergence for samples taken from patients during the outbreak that occurred in Luebo between 2007 and 2008, was less than 0.07% (Carroll *et al.*, 2013). Outbreaks have a zoonotic origin and are then sustained via human-to-human transmission. This often occurs from direct contact with bodily fluids/ secretions of infectious individuals or the deceased, for example, during traditional burial practices (Nkoghe *et al.*, 2011).

Those unfortunate enough to contract the deadly disease experience fever, systemic inflammation and damage to their endothelial cell lining. These symptoms rapidly advance to internal haemorrhaging, shock and multi-organ failure, leading to observed mortality rates of up to 90% (Falasca *et al.*, 2015). In more recent outbreaks, however, The case fatality rates (CFR) have been markedly lower, often between 50 and 60% (Hemingway-Foday *et al.*, 2020; Rohan and McKay, 2020; Keita *et al.*, 2021; WHO, 2021).

Case fatalities associated with other *Ebolaviruses* seem to be considerably lower, regardless of them having similar disease presentation and routes of transmission. These range from between 53 to 66% for SUDV, the most common species to cause outbreaks after EBOV and 40% for BDBV based on the 2007 outbreak in Uganda (Baron, McCormick and Zubeir, 1983; Towner *et al.*, 2004; MacNeil *et al.*, 2010). There has only ever been one recorded case of TAFV; the virus was isolated from an ethnologist completing an autopsy on a chimpanzee found dead in Tai National Park and this incidence was not fatal (Guenno *et al.*, 1995).

EBOV is a threat to global public health, particularly in areas often lacking the appropriate infrastructure to support the containment of the disease and provide the best responses in disease management. With the limited widespread availability of therapeutic options to combat the virus, a continued understanding of EBOV, its interactions and how it may evolve is needed at a molecular level to aid in infection control, outbreak management and the development of drugs and other available therapies.

1.3 Zaire Ebola Virus

1.3.1 EBOV Structure and Morphology

As mentioned previously, EBOV is a negative-strand RNA virus which comprises of filamentous, pleomorphic particles that are typically 1 μm in length and 80 nm in diameter (Figure 1.2) (Ellis *et al.*, 1979; Geisbert and Jahrling, 1995). Morphologically, these thread-like particles can be found in 'U', '6' or circular forms (torus), where the nucleocapsid is bent back on itself within the envelope, however, most frequently, they are observed as long filaments via scanning electron microscopy (SEM) (Watanabe *et al.*, 2004; Bharat *et al.*, 2012; Golding *et al.*, 2016).

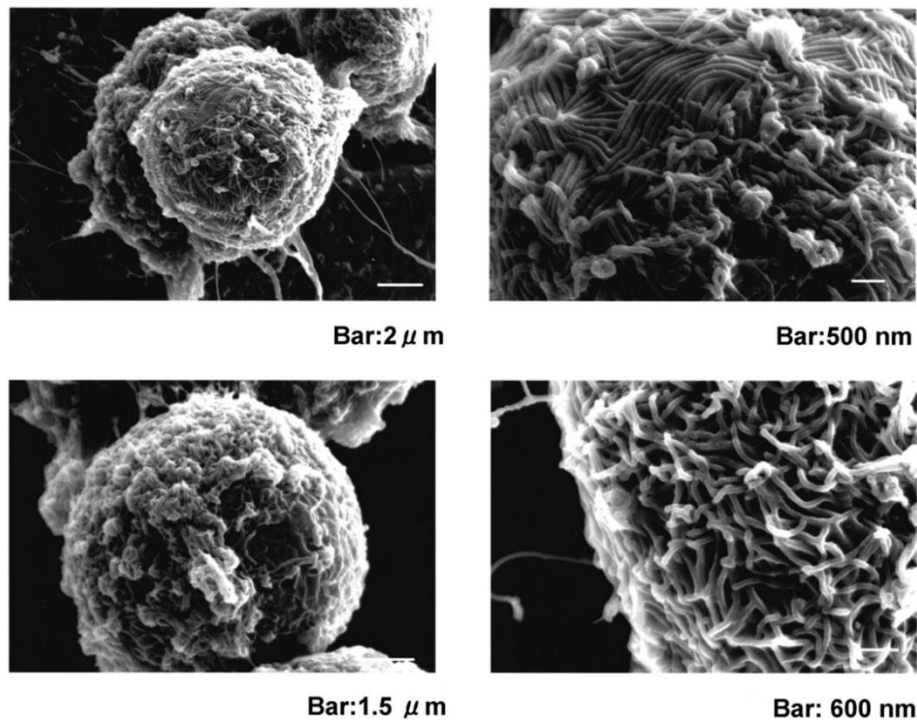


Figure 1.2 SEM of Vero E6 cells infected with Ebola virus
Image taken from Watanabe *et al.*, (2004).

As a pleomorphic, enveloped virus, EBOV is less ordered than its non-enveloped counterparts. Accordingly, it is theorised that the virus can display a level of flexibility in genome size and copy number. EBOV is unusual as it can package several copies of its RNA genome, resulting in the production of polyploid viral particles that are of variable length (Beniac *et al.*, 2012). Approximately 50% of EBOV particles exhibit this characteristic with as many as 22 copies of a genome being observed within one viral particle (Beniac *et al.*, 2012). This phenomenon has also been identified in some *Paramyxoviridae* and *Birnaviridae* families of non-enveloped RNA viruses (Loney *et al.*, 2009; Luque *et al.*, 2009).

As true for all filoviruses, the EBOV genome is encompassed by the nucleoprotein, with which it forms tight interactions (Figure 1.3). It also has direct interactions with the transcriptional activator, VP30, the RdRP (L) and the polymerase co-factor, VP35. Together, these viral components form the RNP complex, a structure that as described before, all *Mononegavirales* depend upon for rounds of transcription and replication. However, only pneumoviruses seem to contain a homologue of VP30 in their genomes. The respiratory syncytial virus M2-1 protein has been shown to act as a transcription elongation factor, and similarly to VP30, has been shown to contain a zinc-binding motif that is critical to the protein function (Collins *et al.*, 1996; Fearn and Collins, 1999; Hardy and Wertz, 2000; Modrof, Becker and Mühlberger, 2003).

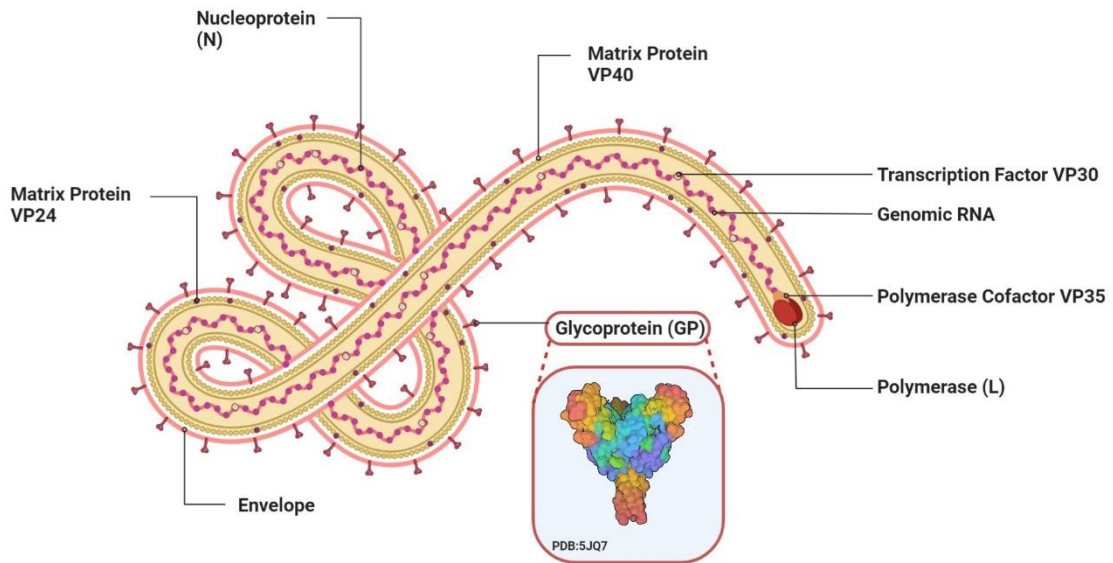


Figure 1.3 EBOV structure and morphology

EBOV is an enveloped filovirus with a -ssRNA genome. N is the key component of the nucleocapsid, encompassing the viral genome. VP30 is a phosphoprotein that is crucial in transcription. L is a viral polymerase, catalyzing replication, with VP35 acting as a polymerase cofactor. VP40 and VP24 are the matrix proteins that regulate virus morphogenesis and they are paramount in viral assembly and budding. GP, located on the viral envelope, aids in viral attachment and entry into host cells.

VP24 protein is unique to filoviruses and is traditionally described as a minor matrix protein, nonetheless, there is mounting evidence to suggest that VP24 directly interacts with the RNP, to play a central role in nucleocapsid formation and the modulation of transcription and replication (Watanabe, Noda and Kawaoka, 2006; Lamb, 2007; Watanabe *et al.*, 2007; Banadyga *et al.*, 2017; Cantoni and Rossman, 2018). The filovirus nucleocapsid is perhaps the most complicated in the order, however, of the five proteins described above, studies have shown that only NP, VP35 and VP24 are needed to form nucleocapsid structures that are morphologically comparable to those observed during EBOV infection, and thus are recognised as the minimal components of the nucleocapsid (Watanabe, Noda and Kawaoka, 2006).

The nucleocapsid is surrounded by the matrix space, consisting of one of the most abundant proteins in filoviruses, VP40, which acts as an important stimulus for particle morphogenesis and budding of the virus (Noda *et al.*, 2002). Expression alone of VP40 can result in the expression and release of EBOV-like viral particles (VLPs), analogous to a wild-type viral morphology (Jasenovsky *et al.*, 2001). The final component, the trimeric GP_{1,2} is found embedded in the host-derived envelope of the virus and functions crucially

in the viral entry pathways, with roles in both viral attachment and membrane fusion (Wang *et al.*, 2016).

1.3.2 EBOV Genome Structure and Organisation

The EBOV genome consists of seven genes; 3' *NP-VP35-VP40-GP-VP30-VP24-L* 5' (Figure 1.1) (Mühlberger *et al.*, 1999). This is typical for all filoviral genomes, except for the LLOV genome, which contains six genes, the final being bicistronic and therefore resulting in both the expression of VP24 and RdRP (Negredo *et al.*, 2011). The genome is flanked by two regulatory regions that contain the cis-acting signals required for transcription, replication and encapsulation of the genome, the 3' leader sequence (60 nt) and a 5' trailer sequence (676 nt) (Mühlberger *et al.*, 1999; Volchkov *et al.*, 1999). The trailer consists of a sequence that complements the replication promoter sequence and can be utilised by the RdRP to synthesise negative-stranded viral RNA, from the replication intermediary anti-genomes produced (Volchkov *et al.*, 1999).

Replication of the genome relies on a bipartite promotor (Mühlberger, 2007). The first element is located in the leader sequence (nt 1-55) and the second promotor element (nt 81-128) is found in the non-translated region of the *NP* gene, comprising a unique structure of eight UN₅ hexamers, where N is any nucleotide. The elements are separated by a spacer region that includes the start signal driving the transcription of the *NP* gene. Studies have identified that replication requires appropriate spacing of the two promoter elements, although the actual sequence of the spacer appears to be unimportant (Weik *et al.*, 2005). Furthermore, deletions of six or multiples of six nucleotides within the spacer region were tolerated, with replication being inhibited by any other changes, indicating that the promotor follows the rule of six, although this is not observed with regards to the total genome length, as is seen with many paramyxoviruses (Kolakofsky *et al.*, 1998; Weik *et al.*, 2005).

Genes are flanked by conserved initiation and termination sequences and are usually divided by small intergenic regions that are between four to seven nucleotide bases. However, a long intergenic sequence between VP30 and VP24 has been observed in ebolaviruses and one between the GP and VP30, in marburgviruses (Sanchez *et al.*, 1993; Neumann, Watanabe and Kawaoka, 2009). Each gene typically contains a single open reading frame. Nonetheless in the case of ebolaviruses and LLOV, the *GP* gene contains overlapping open reading frames (ORFs). These viruses display the phenomenon of co-

transcriptional editing of the *GP* gene, leading to the formation of multiple *GP* products that further undergo post-translational processing (Volchkov *et al.*, 1995; Sanchez *et al.*, 1996; Mehedi *et al.*, 2011; Negredo *et al.*, 2011).

1.3.3 Gene Order as a Transcriptional Regulator

Like other filoviruses, EBOV is suspected to follow the stop-start model of viral transcription, as briefly described previously, because a gradient is observed concerning the proteins that are expressed (Mühlberger *et al.*, 1996; Shabman *et al.*, 2013). According to this model, the viral polymerase binds to a promoter at the 3' end of the genome and synthesises mRNA in a 5' direction (Whelan, Barr and Wertz, 2004). At gene borders, a term which encompasses the region from a gene end (GE) signal of an upstream gene and a gene start (GS) signal of a downstream gene, the polymerase can either continue to reinitiate downstream transcription or dissociate from the template. Re-entry to the genome can only be achieved at the 3' promoter, resulting in the more frequent synthesis of transcripts located near the promoter, for example, *NP* and low levels of expression of genes closer to the 5' end, such as *L* (Lamb and Parks, 2007). As well as guiding the polymerase to mRNA synthesis, GS signals direct the co-transcriptional capping and methylation of the mRNA's 5' end, whilst GE signals facilitate polyadenylation as well as the termination of the transcripts (Mühlberger, 2007). Both are highly conserved in EBOV (Brauburger *et al.*, 2014, 2016).

1.3.4 Transcriptional Editing of the *GP* Gene

The primary product of an unedited mRNA transcript is a 364 residue, non-structural, soluble glycoprotein (sGP), however, transcriptional editing leads to the expression of the full-length 676-residue transmembrane glycoprotein precursor (GP₀), which gets post-translationally processed to produce GP_{1,2} (Figure 1.4) (de La Vega *et al.*, 2015; Ning *et al.*, 2017; Zhu *et al.*, 2019). This occurs due to a 'slippage' phenomenon displayed by the RdRP at the editing site of the *GP* gene. The template gene sequence contains seven successive uridines, however, during transcription, an eighth untemplated adenosine can be incorporated into the transcript, resulting in the amalgamation of two different ORFs, thus superseding the premature termination of *GP* transcription that would otherwise result in the sole production of sGP. The addition of two adenosines leads to the production of a small secreted glycoprotein (ssGP), a 298 residue protein, the first 295 of which are the same as those that sGP and GP_{1,2} are comprised of, however, each protein has a different

carboxyl-terminal region (Sanchez *et al.*, 1996; Mehedi *et al.*, 2011). Recent experimental data shows that transcripts for sGP, GP and ssGP are present at a ratio of 71:24:5, in Vero E6 cells, however, it remains unclear if this is observed at a protein level (Mehedi *et al.*, 2011).

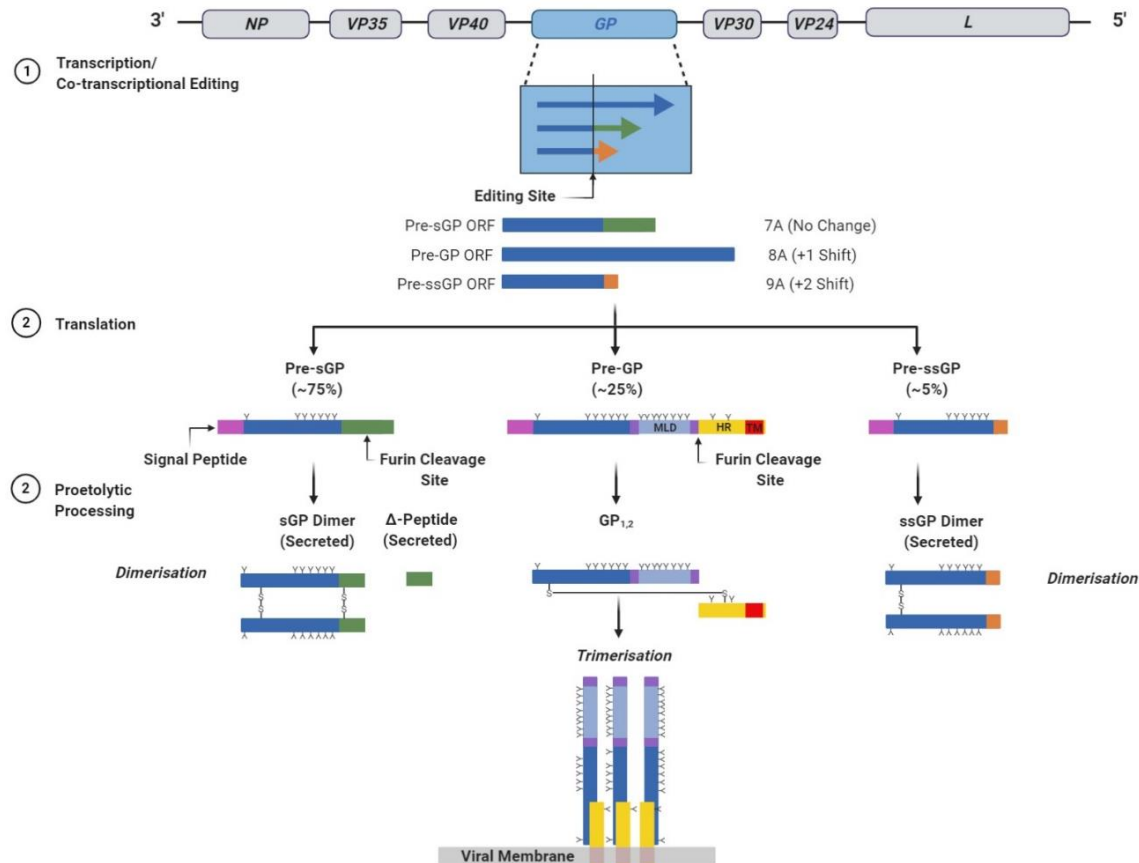


Figure 1.4 Co-transcriptional editing of the GP gene

The primary ORF of the EBOV GP gene determines a truncated, sGP, which undergoes, proteolytic cleavage by furin, resulting in the mature form of sGP. Co-transcriptional editing at the stuttering site can also form GP and ssGP. GP also undergoes furin cleavage to reach a mature form comprising of the GP1 and GP2 subunits, which are linked by a disulfide bond. Image adapted from Lee and Saphire (2009).

1.3.5 EBOV Protein Function within the Viral Life Cycle

1.3.5.1 Transcription and Replication

The RNP is the only viral component that is released into the host cell cytoplasm during an initial EBOV infection. There, it facilitates a primary round of transcription, resulting in the formation of the first viral mRNA, the synthesis of which has been detected 6-7 hours post-infection (Sanchez and Kiley, 1987; Mühlberger *et al.*, 1999). The mRNA is translated into additional viral proteins through the activity of host cell ribosomes. These

newly synthesised proteins supplement the activity of the RNP by facilitating secondary rounds of transcription and subsequent replication, which occurs in inclusion bodies within the cytoplasm that form with the production of NP (Hoenen *et al.*, 2012). Replication is dependent on the transcription of an anti-genomic intermediate (cRNA), which can be copied to form viral genomic RNA (Hoenen, Groseth and Feldmann, 2019).

The principal components of the RNP are the major and minor nucleoproteins NP and VP30. Primarily, the NP functions critically in protecting the viral RNA against exogenous nucleases and encapsidates the RNA during viral assembly. It is thought that the C-terminus of NP is essential in RNA binding, whilst the N-terminus is important during the incorporation of NP into VLPs generated by VP40 (Noda *et al.*, 2007). NP-NP interactions that self-oligomerise to form the typical helical morphology, require the N-terminal 450 amino acid residues (Watanabe, Noda and Kawaoka, 2006). Studies have shown that without a helical morphology, nucleocapsid assembly cannot occur (Watanabe, Noda and Kawaoka, 2006). The RdRP does not directly interact with N, relying on its cofactor VP35 to bridge the interaction (Becker *et al.*, 1998). As the enzymatic component of the RNP, large parts of the gene are highly conserved to maintain the polymerase function, with both enzymatic and template binding domains having been identified (Trunschke *et al.*, 2013; Schmidt and Hoenen, 2017).

Typically, transcription in *Mononegavirales* is regulated by the NP, P and L, which are homologous to the filovirus proteins NP, VP35 and L (Whelan, Barr and Wertz, 2004). Although these proteins have been shown to adequately support the transcription of MARV mini-genomes, EBOV transcription and regulation towards replication strongly rely on the transcription factor VP30 (Mühlberger *et al.*, 1998; Weik *et al.*, 2002; Boehmann *et al.*, 2005). VP30 binds genomic RNA at a stem-loop structure that is present in the 3' leader sequence of the genome and promotes polymerase read-through to enhance viral transcription (Weik *et al.*, 2002; Hartlieb *et al.*, 2003). VP30 also has a role in transcription re-initiation at gene junctions, promoting the transcription of downstream genes (Martínez *et al.*, 2008).

VP30 function is dependent on phosphorylation (Biedenkopf, Lier and Becker, 2016). Consequently, host cell phosphatases such as protein phosphatase 1 and 2A (PP1/PP2A) are recruited to the RNP complex by EBOV NP, within NP-induced inclusion bodies, to regulate the VP30 phosphorylation status (Modrof *et al.*, 2002; Lier, Becker and Biedenkopf, 2017; Kruse *et al.*, 2018). Additionally, it has been elucidated that serine-arginine protein kinase 1 (SRPK1) and SRPK2 are recruited to EBOV-induced inclusion

bodies and act to phosphorylate VP30 (Takamatsu, Krähling, *et al.*, 2020). Phosphorylation of VP30 blocks transcription and favours replication, which is accomplished by VP35 and L alone (Figure 1.5) (Martínez *et al.*, 2008; Martinez *et al.*, 2011).

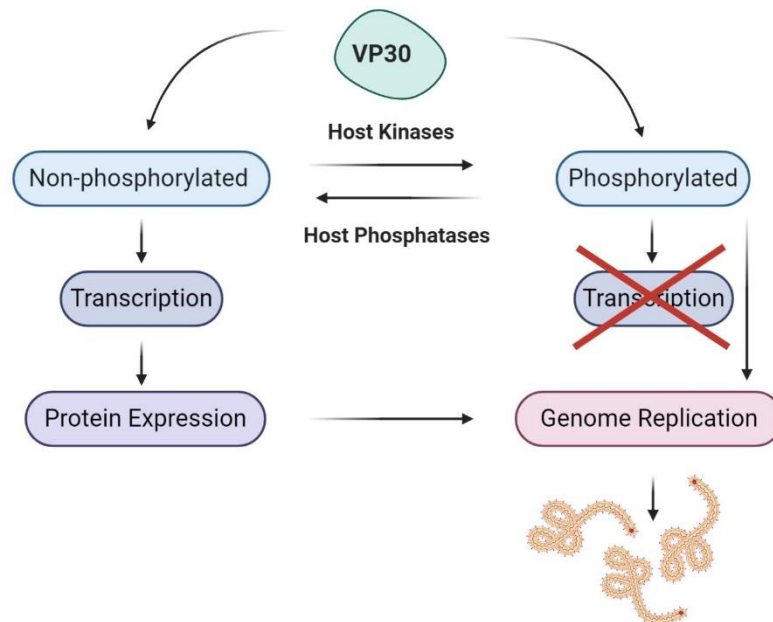


Figure 1.5 The regulatory role of VP30 phosphorylation in the EBOV replication cycle
Changes in the phosphorylation status of VP30 are driven by host kinases and phosphatases. Non/weakly phosphorylated VP30 favours transcription and the production of viral proteins. Phosphorylated VP30 favours replication of the viral genome, which is independent of VP30. Image adapted from Martinez *et al.*, (2011).

The switch is perhaps due to a weakened association with the polymerase complex, specifically with VP35, although it is unclear if a lack of VP30 results in the transcriptase complex converting to a replicase complex, or if transcriptase complexes are unavailable, replicase complexes display preferential access the NP-RNA templates (Biedenkopf, Lier and Becker, 2016). Inhibitors that bind PP2A, thus preventing recruitment of the phosphatase by NP, have been shown to suppress viral RNA synthesis (Kruse *et al.*, 2018).

1.3.5.2 Virus Assembly and Budding

As a minor matrix protein, VP24 plays a role in the viral release. It is suspected that the association of VP24 with the surface of the RNP complexes formed after replication, causes the RNP to condense, thus changing its form to transcription and replication

inactive but packaging competent, ready to be transported to viral budding sites located at the plasma membrane, primarily via interaction with the actin cytoskeleton of host cells (Watt *et al.*, 2014; Schudt *et al.*, 2015; Banadyga *et al.*, 2017). VP24 is reliant on amino acids V170 and N171, to interact with the NP, resulting in its condensation (Banadyga *et al.*, 2017). Knockdown of VP24 has been shown to obstruct nucleocapsid assembly, the primary step in viral particle formation (Mateo *et al.*, 2011). VP24 has also been shown to have a regulatory role in RNA synthesis, with overexpression of the protein resulting in RNA synthesis inhibition (Watanabe *et al.*, 2007; Takamatsu, Kolesnikova, *et al.*, 2020).

Nucleocapsids are co-transported to sites of viral assembly with VP40. VP40 has also been shown to utilise host cellular trafficking components such as actin and microtubules and has specifically been shown to interact with Sec24C, an element of the COPII transport system (Ruthel *et al.*, 2005; Noda *et al.*, 2006; Yamayoshi *et al.*, 2008; Adu-Gyamfi *et al.*, 2012). Contrastingly, GP_{1,2} the final component of the assembled particle utilises the classical secretory pathway for transport and incorporation into the membrane which envelopes the virus as it buds (Ning *et al.*, 2017).

Both the processes of viral morphogenesis and assembly are mediated by VP40 (Madara *et al.*, 2015). As the main structural component of the viral matrix, VP40 enables RNP envelopment by the cellular membrane, instigating viral budding from the cell (Jasenosky and Kawaoka, 2004). VP40 consists of two protein domains, the N-terminal oligomerisation domain, through which it forms hexameric and octameric ring structures *in-vitro* and the C-terminal membrane-binding domain, which contains a cationic patch that can interact with plasma membrane lipids such as phosphatidylserine, an association that is critical to viral budding (Dessen *et al.*, 2000; Jasenosky *et al.*, 2001; Del Vecchio *et al.*, 2018).

Octameric structures exhibit perinuclear localisation, where they play a role in transcriptional regulation, whereas hexameric structures are membrane localised and are important in processes such as viral budding, alongside the interactions of VP40 with additional recruited host factors such as elements of the endosomal sorting complex required for transport (ESCRT) and various ubiquitin ligases (Hoenen *et al.*, 2010; Bornholdt *et al.*, 2013; Madara *et al.*, 2015; Han *et al.*, 2016). Budding efficiency is enhanced by the EBOV GP_{1,2}, which can inhibit tetherin activity, a type IFN I inducible cellular factor that can prevent the budding of enveloped viruses from plasma membranes (Neil *et al.*, 2007; Kaletsky *et al.*, 2009).

1.3.5.3 GP_{1,2} Processing and Structure

The GP_{1,2} is the virion's only surface protein and the best characterised of all filovirus proteins due to its high functional importance and immunogenic potential (Miller and Chandran, 2012). Via this protein, which is incorporated into the lipid envelope, all filoviruses, including EBOV, mediate their entry into host cells. GP is further involved in subsequent steps of pathogenesis including the recognition of viral receptors and membrane fusion of the virus and host cell to allow for the release of viral particles into the cytoplasm (Ito *et al.*, 2001).

As a class I fusion protein, GP_{1,2} is proteolytically processed from a distinct uncleaved precursor protein, GP₀, which is otherwise incapable of membrane fusion. GP₀ consists of an N-terminal signal peptide that directs its passage through the cellular endoplasmic reticulum, (ER) (Sapir *et al.*, 2008; Podbilewicz, 2014). Within the ER, the signal peptide is cleaved by a signalase and disulphide bonds are formed at key cysteine residues (Figure 1.4) (Sanchez *et al.*, 1998). Mutagenesis of cysteine residues to glycine residues in GP₁ determined that the cys53 mutation was the only one that resulted in the efficient secretion of GP₁ and is, therefore, most likely to participate in the only disulphide bridge that links the two subunits together, alongside cys609 present in GP₂ (Jeffers, Sanders and Sanchez, 2002).

At the trans-Golgi, GP₀ is cleaved by the cellular protease furin at residue 501, leading to the formation of two subunits, GP₁ (130 kDa) and GP₂ (24 kDa) as shown in Figure 1.6, which are connected through the disulphide bond (Volchkov *et al.*, 1998). Studies have shown that furin cleavage is dispensable for viral entry and replication, suggesting the need for an additional cleavage event which results in the primed form of the GP (Neumann *et al.*, 2007; Ströher *et al.*, 2007). GP₁ consists of an N-terminal base that interacts closely with GP₂ and locks it in its pre-fusion form, as well as the receptor-binding domain (RBD) (Figure 1.6). A flexible β 13- β 14 loop joins these structures to a C-terminal glycan cap and the mucin-like domain (MLD). GP₂ consists of a fusion peptide, two canonical heptad repeats (HR) and a transmembrane domain (TMD) (Lee *et al.*, 2008). Atypical when compared to many class I fusion proteins, the fusion peptide is located in a loop near the N-terminal of GP₂, instead of at the N-terminus. Within this loop, lie hydrophobic residues, which when mutated to alanine and arginine residues, have been shown to inhibit the infectivity of pseudoviruses expressing GP_{1,2}. (Ito *et al.*, 1999).

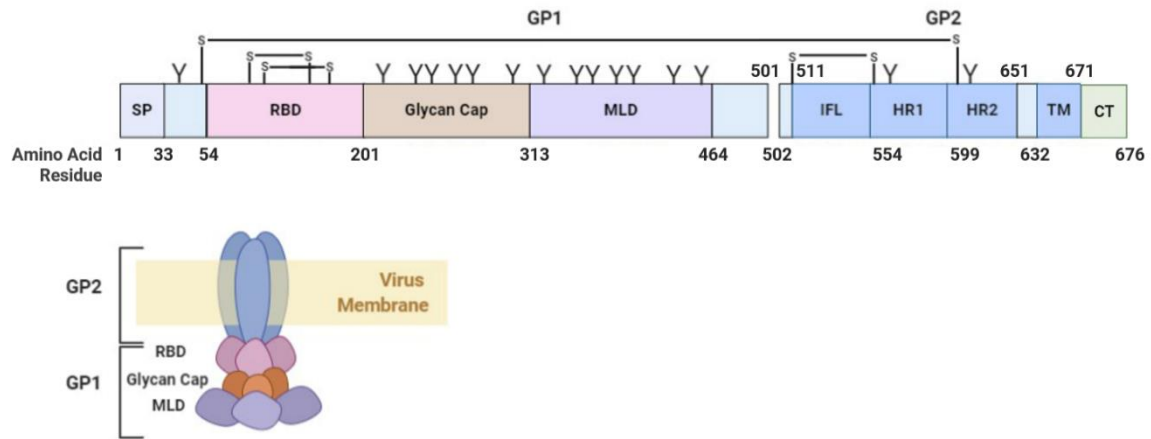


Figure 1.6 Structure of the EBOV GP

GP₁ consists of the RBD, glycan cap and the MLD and shares a disulphide bond with GP₂, which consists of the IFL, HR1, HR2 and TM domain. Image adapted from Lee *et al.*, (2008).

Structural modelling has shown that this results in the formation of GP₁-GP₂ heterodimers, which are arranged in trimers that are approximately 150 kDa in size and adopt a chalice-like structure that are the spiked protrusions observed on the virion surface. GP₁ is recognised as having a role in cell attachment through mediating viral adhesion to host cells, whereas GP₂ has been described as catalysing membrane fusion (Lee *et al.*, 2008). Trimerisation is thought to be instigated by coils of heptad repeat sequences that are found in GP₂. Although the majority of GP_{1,2} is set into the envelope through the transmembrane domain in GP₂, shed forms of GP do exist. GP₁ can be shed as a result of the instability of the disulphide bond linking it to GP₂, additionally, a cleaved form of GP_{1,2} trimers are also shed, due to excision of the GP₂ transmembrane anchor, by the metalloprotease, tumour necrosis factor α -converting enzyme (TACE) (Dolnik *et al.*, 2004). Shed GP has been shown to initiate the Toll-like receptor (TLR) 4 pathway, leading to pro-inflammatory cytokine expression, which impacts endothelial permeability (Dolnik *et al.*, 2004; Okumura *et al.*, 2010).

1.3.5.4 N and O-linked Glycosylation

More than half the molecular weight of GP_{1,2} is attributed to the N and O-linked glycans that are present in its structure (Feldmann *et al.*, 1994). N-linked glycosylation occurs primarily in the ER, whilst the Golgi is the site of N-glycan maturation and O-linked glycosylation. It is thought that this glycosylation helps to mask antigenic epitopes that exist, in particular, within the MLD (Reynard *et al.*, 2009). Studies have demonstrated that

N-linked glycans are a heterogeneous assortment of high mannose, hybrid and complex oligosaccharides, whilst *O*-linked glycans are smaller structures that contain variable quantities of sialic acids (Ritchie *et al.*, 2010).

The similarity in patterns and types of *N*-linked glycosylation can be observed between different *Ebolavirus* GPs, however *O*-linked glycosylation widely varies both inter and intraspecies, with GP variants taken from the original outbreak (Yambuku) and the 2013-2016 west African outbreak, presenting with significant dissimilarities (Collar *et al.*, 2017). GP₁ is predicted to have up to 15 *N*-linked glycosylation sites, with its MLD having up to 80 *O*-linked glycosylation sites (Figure 1.6) (Jeffers, Sanders and Sanchez, 2002; Lennemann *et al.*, 2014). Contrastingly, GP₂ does not appear to contain any *O*-linked glycans, however, it does have two predicted *N*-linked glycosylation sites (Jeffers, Sanders and Sanchez, 2002). These two residues, N563 and N618, are conserved for all filoviruses, suggesting a functional significance and are located at the HR1 and HR2 regions of GP₂. These glycans have been identified as having a role in GP processing, oligomerisation and conformation and subsequently, impact GP incorporation into EBOV VLPs and retroviral pseudoviruses (Wang *et al.*, 2017). Glycans at N618 have been shown to improve viral entry and deglycosylation of both residues interrupts viral entry altogether (Wang *et al.*, 2017).

The density of glycans present on the GP is thought to provoke an unfavourable environment for neutralising antibody function (Lennemann *et al.*, 2014). Heavy glycosylation masks critical regions of the GP such as the RBD (Jeffers, Sanders and Sanchez, 2002). It is thought that the glycans trigger the host immune system to produce antibodies against parts of the virus that are considered dispensable or are variable, for example, the MLD, the variable site of *O*-linked glycosylation (Lee *et al.*, 2008). It is also suggested that the glycans can shield surface epitopes thereby inhibiting surface protein recognition by host cell factors such as major histocompatibility complex (MHC) class one proteins (Reynard *et al.*, 2009; Francica *et al.*, 2010). Additionally, glycans have been shown to bind to cell surface lectins to enhance EBOV attachment and entry (Lennemann *et al.*, 2014).

Deletion of the MLD has been shown to enhance GP_{1,2} processing and its assimilation into pseudotyped retroviral particles and therefore results in higher transduction. However, it is unclear if this is because deletion of MLD, permits a more rapid transit of GP precursors through the ER and trans-Golgi, meaning high levels of GP_{1,2} processing and subsequent expression on the virion surface, or because of the reduced toxicity observed

with MLD deletion (Jeffers, Sanders and Sanchez, 2002). Deletion of the O-linked glycosylation region has also been shown to reduce the cytopathic effects of EBOV GP expression (Yang *et al.*, 2000).

1.3.5.5 Structure and Function of the Secreted GP Products

As observed with GP₀, soluble GP (sGP) is originally synthesised as a Golgi-specific precursor protein (pre-sGP), which undergoes intracellular processing (Figure 1.4). The precursor is cleaved by the enzyme furin, at a cleavage site that is located on its C-terminus (residue 324 at the R-X-R-R site) (Volchkova, Klenk and Volchkov, 1999). As a result, mature sGP and a secondary, heavily O-glycosylated product called delta peptide are produced (Volchkova, Klenk and Volchkov, 1999). Furin cleavage is not observed in the production of small soluble GP (ssGP), however, both sGP and ssGP form parallel homodimers for secretion through disulphide bonding which occurs at specific cysteine residues within the proteins. Disulphide bonds form between paired Cys53-Cys53 residues in ssGP monomers, whilst sGP requires an additional disulphide bond between Cys306-Cys306 residues to form a 110 kDa homodimer (Volchkova *et al.*, 1998; Barrientos *et al.*, 2004; Mehedi *et al.*, 2011). Both sGP and ssGP have been shown to have similar patterns of N-glycosylation, with six predicted sites being described (Mehedi *et al.*, 2011; de La Vega *et al.*, 2015).

Although the role of sGP remains unclear, it is theorised to have a function in EBOV pathogenesis through several different mechanisms. sGP has anti-inflammatory properties, including the inactivation of neutrophils and partial restoration of the endothelial barrier function, which is antagonised by GP_{1,2} and VP40 (Wahl-Jensen *et al.*, 2005). The induced recovery of barrier function following incubation with the pro-inflammatory cytokine, tumour necrosis factor α (TNF α), may function to support viral replication in these cells (Wahl-Jensen *et al.*, 2005). sGP has also been shown to reduce the production of TNF α and Interleukin 6 (IL-6) by macrophages, whilst having no impact on anti-inflammatory cytokine production, including IL-10 (Bradley *et al.*, 2018).

Additionally, both sGP and GP share common epitopes that competitively bind host antibodies, thus leading to evasion of the host immune system. This may contribute to disease severity and explain why patients with acute infection have increased levels of sGP present in their blood (Mohan *et al.*, 2012). Animal models have shown that most derived antibodies are directed towards sGP rather than GP_{1,2} (Druar *et al.*, 2005). Unlike

sGP, ssGP does not show anti-inflammatory function in endothelial cells treated with TNF α and its role remains largely unknown (Mehedi *et al.*, 2011).

Perhaps most importantly, sGP production functions to limit GP_{1,2} derived cytotoxicity with cytotoxic effects observed both *in-vivo* and *in-vitro* (Yang *et al.*, 2000). Studies have shown that the expression of GP can be lethal for host cells, instigating cell rounding and cell death, which is preceded by the downregulation of cell surface molecules including integrins (Simmons *et al.*, 2002).

This GP_{1,2} mediated outcome is suspected to be one of the causes of the typical haemorrhaging which is often observed following endothelial cell infection, although the resulting cytokine storm that follows EBOV infection also has a role in exacerbating vascular leakage, leading to widespread haemorrhaging (Reynard *et al.*, 2019). However, as the virus is reliant on the host cellular replicative machinery for its pathogenesis, premature cell death is an unfavourable consequence and thus, it has been proposed that co-transcriptional editing is a mechanism by which the virus regulates its membrane GP_{1,2} production in order to limit the cytotoxic impacts of GP overexpression (Simmons *et al.*, 2016). Indeed, when expressed at a moderate level, GP_{1,2} does not result in cell rounding as is not cytotoxic (Alazard-Dany, 2006).

Studies have shown that mutational deletion of the editing site in an EBOV recombinant to ensure the sole production of GP_{1,2} leads to high cytopathogenicity in *in-vitro* infections, with the virus also growing to lower titres relative to wild-type counterparts (Volchkov, 2001). Other studies have shown that the co-expression of sGP and GP, in ratios that reflect what is observed in natural infection, is accountable for a three-fold higher level of infectivity compared to the expression of GP alone and that the enhancement of infectivity was due more to the absence of GP_{1,2} than to the co-expression of sGP (Mohan *et al.*, 2015).

Interestingly, it has been demonstrated that the serial passaging of the virus in Vero E6 cells, leads to it rapidly mutating its editing site to assume a phenotype that predominantly formed GP_{1,2}. However, when reintroduced back into an animal model (guinea pigs), a reversion to the wild-type editing site of the virus resulted in normal sGP expression levels and the restoration of a highly pathogenic phenotype was observed. This suggests that environmental restrictions may impact the selection of a high or low GP_{1,2} phenotype (Volchkova *et al.*, 2011). Additionally, although the passage of EBOV in Vero E6 cells leads to a seven-uridine phenotype favouring GP_{1,2} production, passaging in Huh-7 cells

leads to an eight-uridine phenotype favouring sGP production, suggesting that cell type may also play a role (Tsuda *et al.*, 2015).

The secondary product of sGP cleavage, delta peptide, is retained by producer cells for longer periods before being secreted and overall, less delta peptide is secreted compared to sGP (Volchkova, Klenk and Volchkov, 1999). Recent studies reveal that delta peptide contains an amphipathic α -helical structural motif that is similar to that of pore-forming peptides and a conserved pair of cysteine residues (Cys29 and Cys38) that are present in some antimicrobial peptides and have a role in membrane permeation and pore formation. (Yount and Yeaman, 2004; Nieva, Madan and Carrasco, 2012; Gallaher and Garry, 2015). Experimental studies have confirmed that delta peptide exhibits membrane-permeabilising, viroporin-like activity and thus, may be involved in the pathology of EBOV. The protein can homo-oligomerise in the cell membrane and form amphipathic pores, as well as permeabilise plasma membranes of variable origin, whilst also increasing ion permeability across confluent cell monolayers (He *et al.*, 2017; Pokhrel *et al.*, 2019). Delta peptide can also inhibit the interactions of retroviruses pseudotyped with EBOV GP_{1,2} with cellular attachment factors, although the significance of this is unknown (Radoshitzky *et al.*, 2011).

1.4 The Viral Entry Pathway

1.4.1 The Role of Cell Surface Attachment Factors

The primary step in infection is the entry of the virus into a susceptible host cell (Figure 1.7). This frequently involves interaction between the surfaces of the virus and the host cell, leading to a fusion event in the case of enveloped viruses such as EBOV. This is the first barrier encountered by a virus when it jumps from its reservoir to a host that is vulnerable to infection. EBOV has a very broad species as well as cellular tropism. Monocytes, macrophages and dendritic cells, all integral components of the innate immune system, have been associated with the support of early viral replication and dissemination (Feldmann *et al.*, 1996; Schnittler and Feldmann, 1999; Bosio *et al.*, 2003).

EBOV can bind to differential cellular attachment factors depending on the targeted permissive cell, thus enhancing viral entry and infectivity. Examples of such attachment factors include the C-type lectins, DC-SIGN and L-SIGN, calcium-dependent carbohydrate-binding proteins that have been shown to stimulate viral internalisation,

through interactions with the glycan cap and MLD of GP₁ (Alvarez *et al.*, 2002; Takada *et al.*, 2004; Matsuno, Kishida, *et al.*, 2010; Collar *et al.*, 2017). However, not all susceptible cells express these lectins and research has shown that both these components of GP₁ can be successfully deleted, without any observed losses of viral transduction efficiency. This suggests that the interactions between the cell surface proteins and the GP_{1,2} partake in enhancing attachment rather than being an essential requirement for the internalisation of the virus (Matsuno, Nakayama, *et al.*, 2010).

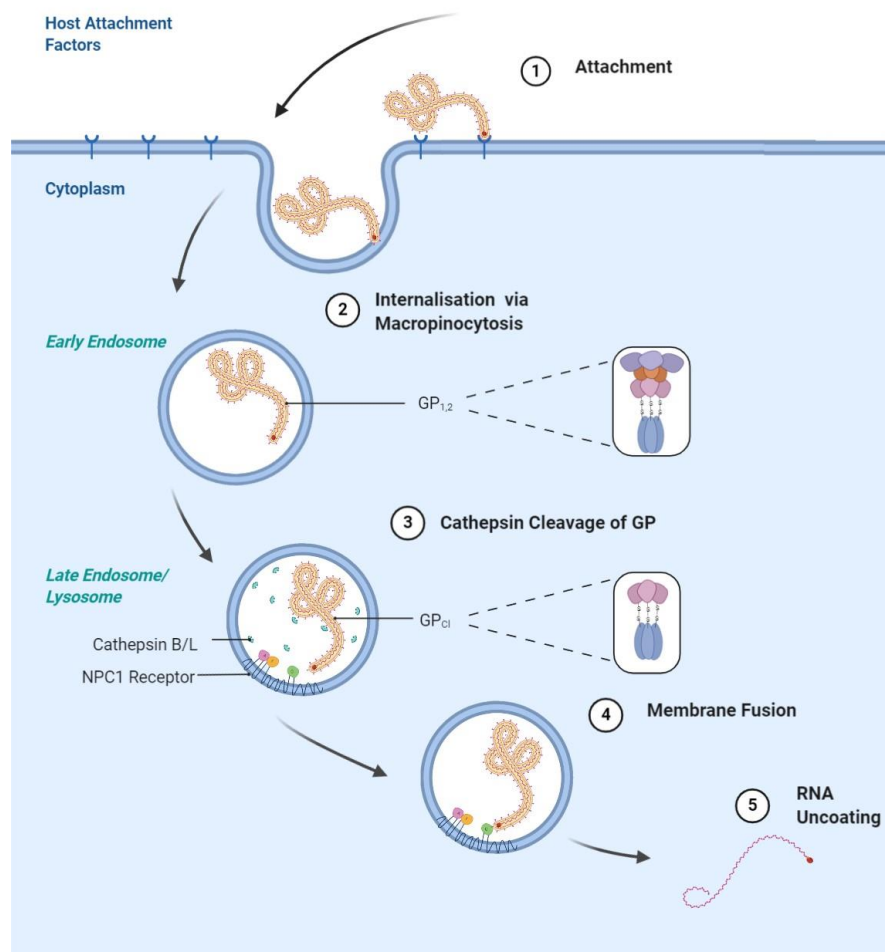


Figure 1.7 EBOV entry pathway

1) The virus interacts with varying attachment factors that are dependent on the target cell. 2) Endocytosis is achieved through macropinocytosis. The endosome is trafficked further into the cytoplasm. 3) Late-endosomal, cathepsin cleavage of GP1 occurs, thus exposing the RBD and allowing for the interaction with the host cell receptor, NPC1. 4) This instigates membrane fusion, resulting in the release of the viral RNP into the host cell cytoplasm. Image adapted from Bhattacharyya, Mulherkar and Chandran, (2012).

Several other surface proteins have been implicated in the attachment process, including β -integrins, molecules that are important for cellular attachment to the matrix, with antibodies directed against β -integrins reducing the entry of pseudoviruses (PVs)

expressing EBOV GP GP_{1,2} (Takada *et al.*, 2000; Simmons *et al.*, 2002). T cell immunoglobulin and mucin domain (TIM) expression in poorly permissive cells has been demonstrated to enhance EBOV entry, whilst loss of expression in permissive cells reduced viral infection/transduction (Kondratowicz *et al.*, 2011). A commonly expressed factor on epithelia, TIM-1 proteins have been shown to interact with phosphatidylserine, (PtdSer) present on the viral membrane, independent of GP_{1,2}, the downregulation of which negatively impacts viral entry in epithelial cells (Moller-Tank *et al.*, 2013; Younan *et al.*, 2018). TAM receptor tyrosine kinase (Tyro3, Axl, Mer) family members, also seem to utilise interactions with viral membrane PtdSer, via the soluble serum protein, Gas6, to enhance viral internalisation by macropinocytosis in some cell types (Shimojima *et al.*, 2006; Hunt *et al.*, 2011; Morizono *et al.*, 2011). Since EBOV has such a broad cellular tropism, with different cells expressing different attachment factors, it is thought that multiple factors are needed for enhanced entry and a single surface receptor has not yet been identified (Miller and Chandran, 2012).

Studies have also shown that EBOV can also exploit antibodies to enhance entry into specific cells. Known as antibody-dependent enhancement (ADE), EBOV-antibody complexes interact with Fc receptors that are expressed specifically on cells of the immune system and are internalised (Takada *et al.*, 2003, 2007). An additional mechanism that can mediate this enhancement involves the C1q complement protein. C1q can also bind EBOV-antibody complexes via the Fc portion of the antibodies and its receptor, C1qR, which is expressed on the surface of most mammalian cells (Takada *et al.*, 2003, 2007).

1.4.2 Mechanism of Viral Internalisation

The most common endocytic pathways, caveolin-dependent endocytosis, clathrin-dependent endocytosis and macropinocytosis, have all been associated with EBOV internalisation. However, EBOV particles can often be larger than the plasma membrane invaginations that typically characterise clathrin or caveolin-mediated pathways (Sanchez, 2007; Traub, 2009; Bhattacharyya *et al.*, 2010). Additionally, the overexpression of caveolin 1 in a T cell line that is poorly permissive to the virus, does not increase EBOV transduction. This implies that EBOV relies on other pathways for internalisation (Simmons *et al.*, 2003).

It has since been determined that EBOV can utilise multiple pathways although, it is primarily dependent on macropinocytosis for internalisation into permissive cells,

occurring due to the formation of outward extensions of the plasma membrane, known as ruffles, driven by localised actin polymerisation (Nanbo *et al.*, 2010; Saeed *et al.*, 2010; Aleksandrowicz *et al.*, 2011). Ruffles can fold back in on themselves, forming vesicles containing the internalised virus, which then follow an atypical endocytic pathway (Saeed *et al.*, 2010).

1.4.3 Proteolytic Processing of GP_{1,2}

For some viral fusion proteins, receptor engagement and the acidification of endocytic vesicles are adequate to instigate the conformational changes of the GP, that lead to membrane fusion (Harrison, 2015). However, EBOV GP_{1,2} must undergo a multistep priming process as well as additional events, which result in the final fusion-ready form of the GP that can participate in membrane fusion.

Priming is reliant on the low, internal endocytic pH, which is essential for the function of cysteine proteases that catalyse the enzymatic proteolysis of the GP_{1,2}. Studies that have manipulated the use of selective protease inhibitors and protease-deficient cell lines have established the impact of both cathepsin B and L (Cat B and L) in VSV pseudotyped with EBOV GP_{1,2}-mediated entry into Vero E6 cells (Chandran *et al.*, 2005). The process consists of two steps, whereby GP₁ is primarily cleaved by Cat L at the unstructured β 13- β 14 loop of GP₁, causing the formation of a 20 kDa product. This is further digested by Cat B, to form a final 19 kDa product, a primed GP (GP_{Cl}) which is thought to be able to contribute to the initiation of membrane fusion (Figure 1.8) (Chandran *et al.*, 2005; Dube *et al.*, 2009). Specifically, the cysteine protease inhibitor E64d, reduced VSV-GP infection in Vero E6 cells by greater than 99% (Chandran *et al.*, 2005). Integrins have been implicated as regulators for endosomal cathepsins. Chinese hamster ovary (CHO) cells deficient in $\alpha_5\beta_1$ -Integrin, lacked double-chain forms of Cat B and L, which correlated with a decrease in Cat L activity (Schornberg *et al.*, 2009). Additionally, cells devoid of cathepsin activity are refractory towards EBOV infection, highlighting the virus' dependence on these enzymes during entry (Schornberg *et al.*, 2006).

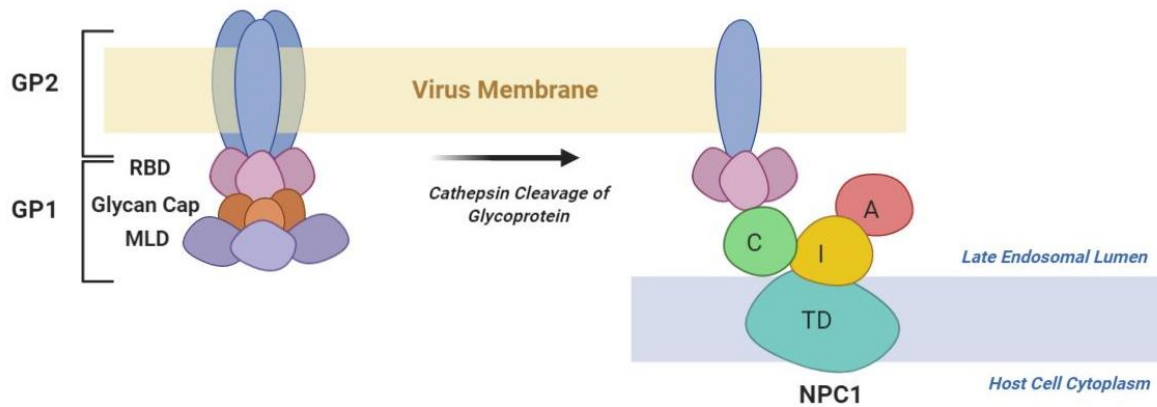


Figure 1.8 Cathepsin cleavage of GP_{1,2} and interactions with NPC1

The GP1 subunit is necessary for the initial adhesion steps and receptor interactions whereas GP₂ has a major role in membrane fusion. GP₁ consists of the glycan cap and MLD, which are later cleaved by cathepsin to expose the RBD. This structure can then interact with the endosomal/lysosomal, host cell receptor NPC1, permitting the cytoplasmic entry of the virus. Image adapted from Urbanowicz *et al.* (2016).

Cathepsin cleavage of GP₁ removes its glycan cap and MLD, thereby exposing its RBD, however during these events, GP₂ remains fully protected and linked to the GP₁ remnants via the disulphide bond (Brecher *et al.*, 2012). The roles of Cat B and L, in the viral entry pathway, have been recapitulated *in-vitro*. Incubation of VSV pseudoviruses expressing EBOV GP with purified human Cat B and Cat L, or with the bacterial protease thermolysin, leads to cleavage and the removal of MLD and glycan cap sequences (Schornberg *et al.*, 2006; Dube *et al.*, 2009). Cat B and not Cat L activity has shown to be a requisite for infection in dendritic cells, with studies demonstrating that EBOV can circumvent this requirement via mutations in the GP_{1,2} subunit interface that prompt conformational changes in the pre-fusion GP_{1,2} trimer, resulting in a heightened sensitivity to proteolysis (Martinez *et al.*, 2010; Wong *et al.*, 2010).

The direct impact of cleavage remains ambiguous. It had previously been argued that priming releases a structural constraint on the GP₂ fusion loop, permitting its insertion into the lipid bilayer of host cells during membrane fusion (Wong *et al.*, 2010). However, it has since been determined that cathepsin cleavage alone is insufficient to trigger the insertion (Bale *et al.*, 2011; Brecher *et al.*, 2012). Cleavage of GP_{1,2} does, however, result in the unmasking of its binding site thus allowing it to interact with the fusion receptor Niemann-Pick C1 (NPC1), which provides a physical link between viral particles and the host membrane; unlike full-length GP_{1,2}, GP_{C1} can directly interact with NPC1 via the receptor's second luminal domain (Lee *et al.*, 2008; Miller *et al.*, 2012; Wang *et al.*, 2016). Studies

have also suggested that aside from endosomal receptor engagement, GP_{CI} requires an additional trigger for membrane fusion, which cathepsin cleavage may allow GP_{CI} to respond to (Brecher *et al.*, 2012).

1.4.4 NPC1, the Endosomal Receptor

NPC1 consists of 1,278 residues and comprises thirteen transmembrane helices and three distinct luminal domains, also known as domains A, C and I. It is expressed ubiquitously in lysosomes and endosomes within different cell types and plays a critical role in the lysosomal efflux of cholesterol (Gong *et al.*, 2016). NPC1 works concurrently with the small soluble glycoprotein, NPC2, to mediate intracellular cholesterol trafficking (Infante *et al.*, 2008). The process begins with the entry of low-density lipoproteins (LDLs) into endosomes/lysosomes, through endocytosis, from the surrounding extracellular fluid. LDLs then undergo lipolysis to release unesterified cholesterol that binds to NPC2, which delivers it to a cholesterol-binding pocket in the first luminal domain of NPC1. (Friedland *et al.*, 2003; Ko *et al.*, 2003; Xu *et al.*, 2007; Infante *et al.*, 2008; Kwon *et al.*, 2009; Deffieu and Pfeffer, 2011).

Crystal and cryo-EM structures have elucidated that the transmembrane domains three to seven of NPC1 constitute the sterol sensing domain (SSD). The SSD acts by monitoring the sterol concentrations in surrounding membranes and facilitating the egress of cholesterol from the late endosomes and lysosomes, to other destinations within the cell (Davies and Ioannou, 2000; Millard *et al.*, 2005; Li, Saha, *et al.*, 2016; Wang *et al.*, 2016).

In concordance with the fundamental roles of NPC1 and NPC2 proteins in cellular homeostasis, individuals with homozygous or compound heterozygous mutations in either of their genes, suffer from Niemann-Pick diseases (type C1 or C2, respectively). These are rare, autosomal-recessive lipid storage disorders, which can be lethal (Millat *et al.*, 2001; Verot *et al.*, 2007; Runz *et al.*, 2008; Bountouvi *et al.*, 2017). Specifically, mutations in these proteins have been linked to the toxic accumulation of unesterified cholesterol and other fatty acids within cells, which in turn, reduces the availability of these compounds for essential biological processes, such as in the maintenance of membrane integrity and the synthesis of steroid hormones and bile acids. (Tabas, 2002; Rosenbaum and Maxfield, 2011; Trunschke *et al.*, 2013). Since both proteins are vital in cholesterol trafficking, types C1 and C2 diseases have a similar clinical presentation, with symptoms including liver damage, progressive neurological degeneration and impairment of intellectual function

from childhood, ultimately resulting in untimely death (Kelly *et al.*, 1993; Imrie *et al.*, 2007; Tang, Li and Liu, 2010).

1.4.5 NPC1, a Filovirus Receptor

As mentioned previously, the late remodelling of the EBOV GP_{1,2} has now been well described, whereby the heavily glycosylated GP1 subunit is enzymatically trimmed by pH-dependent cathepsin proteases, post-endocytosis (Chandran *et al.*, 2005). However, further work utilising antibody binding studies demonstrated that several factors, including incubation at low pH and cleavage of the glycan cap and mucin domain, were inadequate in eliciting the conformational changes in the GP that are paramount in membrane fusion (Bale *et al.*, 2011). This led to speculation that the GP must encounter an additional receptor that facilitates its entry into the host cell cytoplasm.

A breakthrough finally occurred when two independent studies implicated the transmembrane protein, NPC1, in the role of EBOV entry into cells, through the use of chemical and genetic screening, respectively (Carette *et al.*, 2011; Côté *et al.*, 2011). Carette *et al.* (2011) exploited haploid screening to identify various genes required in lysosomal function. The most significant hit was NPC1 and they showed that it was necessary at a late entry step and that was essential for filovirus infection and pathogenesis *in-vivo*. Additionally, this finding was reinforced through the use of small-molecule screening, with the specific aim of identifying any molecules which inhibited PV entry into Vero E6 cells (Côté *et al.*, 2011). It was concluded that a small molecule that exclusively recognised NPC1, the critical host factor for viral entry, also inhibited EBOV entry in cell culture (Côté *et al.*, 2011).

Both groups were able to determine that cleaved GP can interact with membrane-bound NPC1 and that the disruption of NPC1 could negatively impact EBOV entry in a range of cell lines. Interestingly, both studies also demonstrated that NPC1-null cell lines or cells in which it had been disrupted either biochemically or via mutagenesis, displayed an intrinsic resistance to EBOV infection (Carette *et al.*, 2011; Côté *et al.*, 2011). Additionally, in both studies, NPC1-deficient CHO cells that were fully resistant to viral infection were successfully infected once the expression of NPC1 was re-established.

In-vivo studies confirmed the importance of the receptor for viral infection with a mouse-adapted virus to challenge homozygous, NPC1-null mice, which remained free of infection

and viral replication (Herbert *et al.*, 2015). Interestingly, NPC2-deficient mice were still found to be susceptible to GP-mediated filovirus entry, suggesting that resistance to infection specifically depends on the NPC1 receptor and is independent of the accumulation of cholesterol in the late endosome/lysosomes, a trait usually displayed in individuals with compromised NPC1/NPC2 (Herbert *et al.*, 2015).

Another milestone in evidencing the role of NPC1 in EBOV entry came with the determination of the crystal structure of domain C, which is responsible for binding GP_{Cl}. These structures revealed that domain C is composed of a helical core that is encompassed by numerous beta strands with two projecting loops. The second loop specifically interacts with a hydrophobic cavity that is present on the primed GP_{Cl} (Wang *et al.*, 2016). This increased insight into the molecular foundation of EBOV entry has important therapeutic implications as drug candidates can be specifically developed to inhibit this surface interaction, thus preventing infection.

The interactions that occur between viral molecules and their receptors are expected to develop into what is commonly termed a 'genetic arms race.' As hosts are under pressure to develop resistance to pathogens, whilst pathogens endeavour to acquire corresponding counter-measures for evasion, both partners are under a constant and mutual selective pressure resulting in a cyclical adaptation and counter-adaptation (Sironi *et al.*, 2015). This results in positive selection in which amino-acid replacements are favoured over synonymous base substitutions. Such observed changes are stronger at codons that form part of the interacting surfaces.

Three such codons have been identified either within or close to the second loop of domain C, however, the study was primarily looking into different polymorphisms in NPC1 and their association with type-2 diabetes, rather than the function of NPC1 as the receptor for filovirus entry into cells. Nonetheless, the authors acknowledged that positive selection in this gene may occur to avoid the binding of specific viral components rather than dietary changes (Al-Daghri *et al.*, 2012).

More recently, positive selection has been detected in loop 2 through a study that was specifically designed to investigate how NPC1 contributes to EBOV susceptibility in bat cells (Ng *et al.*, 2015). The group identified a single viral mutation in GP_{Cl} (V141A) that permitted escape from receptor control. Thus, they hypothesised that this codon may have been targeted by positive selection, in response to a mutation (D502F) observed in the NPC1 of African straw-coloured fruit bats, which reduces receptor binding. In doing so,

the virus has been able to broaden its host tropism by circumventing the barrier that was imposed by NPC1, which otherwise was able to greatly diminish the susceptibility of cells sourced from different tissues to EBOV (Ng *et al.*, 2015). These theories were taken a step further by the pursuit of alternative residues in a range of mammalian orders and with the conclusion that filovirus GP_{CI} evolved by adaptive changes that occur in the RBD, but at the position S142Q, although no evidence of selection was detected at codon 502 of NPC1, suggesting that it is a bat-specific selection target only (Pontremoli *et al.*, 2016). Additionally, analysis of EBOV GP_{CI} bound with domain C, indicates that there is no direct contact between residue 141 in GP_{CI} and 502 in NPC1, however, the substitution D502F does restore GP binding to NPC1 (Pontremoli *et al.*, 2016).

Neither V141A nor S142Q are likely to have a large impact on EBOV virulence in humans however, they may have a role in determining the large host range of EBOV in the wild and give evidence that different species of *Ebolavirus* may not share the same reservoir. Additionally, the ability of the virus to battle for binding affinity and the concept that some mammalian NPC1 may be more liable to EBOV entry has severe implications in the context of an outbreak. GP_{1,2} could mutate so that it can preferentially bind to human NPC1 rather than that of the reservoir or an intermediate host species.

1.4.6 Membrane Fusion

Until fairly recently, the intricacies regarding the cellular entry of EBOV remained elusive. Crystal structures of GP₂ have shown that it undergoes extensive conformational changes from a stable pre-fusion state to a lower energy state, post-fusion six-helix bundle (6HB), following the collapse of the heptad repeats into its coil structure (Weissenhorn *et al.*, 1998; Malashkevich *et al.*, 1999; Lee *et al.*, 2008; Miller and Chandran, 2012; Harrison, 2015). 6HB formation is common to class I viral fusion proteins that are thought to facilitate viral and cellular bilayer mixing by drawing both into proximity with each other and by fusion pore formation, thus permitting the delivery of the RNP to the cytoplasm (Skehel and Wiley, 2000; White *et al.*, 2008; Harrison, 2015).

This process requires GP₂ to be released from its pre-fusion complex with GP₁, which serves to restrain the internal fusion loop, preventing premature fusion events (Lee *et al.*, 2008; Gregory *et al.*, 2011). With GP₂ release, a core sequence in the internal fusion loop is inserted into the host endosomal membrane, instigating membrane fusion. Crystal structures of NPC1 bound to GP_{CI} have elucidated that following CatB/L cleavage and

NPC1 binding, GP_{Cl} undergoes conformational changes whereby the upward movement of a 3¹⁰ helix in the $\beta 3 - \alpha 1$ loop is thought to aid the release of the N-terminal portion of the IFL within the GP₂ subunit, thus resulting in membrane fusion (Wang *et al.*, 2016). Loop insertion into the host membrane then leads to a change in the GP₂ trimers to an energetically unfavourable state, exposing the heptad repeats to the physiological conditions of the endosome, possibly resulting in their collapse (Brecher *et al.*, 2012).

As mentioned previously, it remains unclear if enzymatic cleavage and binding alone, although indispensable to function, are sufficient to stimulate conformational changes or if they are reliant on an alternative fusion trigger (Spence *et al.*, 2016). Early work showed that GP_{Cl} remained sensitive to lysosomotropic agents that raise pH and E64d, suggesting further cleavage steps and an additional role for low pH (Schornberg *et al.*, 2006). Recently, live-cell imaging studies utilising VSV pseudotyped with EBOV GP_{Cl} showed that an E64d-sensitive step did indeed follow hemifusion, where cathepsins are thought to play a key role in the formation of the fusion pore that allows RNP release, possibly by mediating 6HB formation (Spence *et al.*, 2016).

The role of low pH as well as other cofactors, including Ca²⁺ ions and reduction, remains ambiguous (Hunt, Lennemann and Maury, 2012; Sakurai *et al.*, 2015; Markosyan *et al.*, 2016; Fénéant *et al.*, 2019; Das *et al.*, 2020). Interestingly, the co-localisation of intracellular TIM-1 and NPC1, in vesicles where EBOV GP-mediated membrane fusion occurred, has been demonstrated. With the use of EBOV VLPs the study established that a monoclonal antibody directed against TIM-1 inhibited GP-mediated membrane fusion and prevented the interactions between TIM-1 and NPC1 in Vero E6 cells (Kuroda *et al.*, 2015). This suggests a model where TIM-1, as well as acting as a surface attachment factor, may facilitate the binding of GP_{Cl} to NPC1, perhaps by acting as a bridge between the viral and endosomal membranes, thus being an important step in triggering the membrane fusion that occurs due to the conformational change in GP_{Cl}, or that TIM-1/NPC1 interactions act as a direct trigger for the change in conformation. However, it is important to note that EBOV has a broad cellular tropism and not all cells susceptible to infection, express TIM-1.

1.5 Pathogenesis

The virulence that is associated with EBOV is largely due to its ability to dysregulate the host immune response (Dowell *et al.*, 1999). EBOV enters the human body through

breakages in the skin or the mucosa and after 2-21 days of incubation, early, non-specific symptoms include fever, chills and malaise (Nicastri *et al.*, 2019). As the infection becomes systemic, gastrointestinal symptoms, such as nausea and vomiting become frequent, leading to a loss of bodily fluids, and respiratory symptoms including chest pain and shortness of breath and vascular hypotension and oedema can all be observed (Leligdowicz *et al.*, 2016; Baseler *et al.*, 2017). Subsequent increases in vascular permeability, tissue injury, coagulation cascade dysfunction and haemorrhaging lead to shock, multi-system organ failure and death (Figure 1.9) (Feldmann and Geisbert, 2011; Rivera and Messaoudi, 2016). Some survivors have subsequently developed chronic manifestations of the disease, which resemble inflammatory or autoimmune conditions, suggesting that the virus remains replicative at low levels (Forrester, 2018). Additionally, the virus has been shown to remain in bodily fluids such as semen, for prolonged lengths of time in survivors (Deen *et al.*, 2017). This persistence increases the risk of further transmission of the virus.

The clinical manifestations of EVD occur due to the direct cytopathogenic effects of viral replication, intracellular inclusion body formation and cell lysis, in addition to more indirect effects that impair the crucial functions of the innate and adaptive immune responses and endothelium (Muñoz-Fontela and McElroy, 2017). More specifically, innate response disruption involves inhibition of type-I interferon (IFN) responses, dysregulation of cytokine/chemokine networks leading to 'storms,' and compromised dendritic cell (DC) function as antigen-presenting cells (APCs) that govern cell activation and cell-mediated immune responses (Falasca *et al.*, 2015).

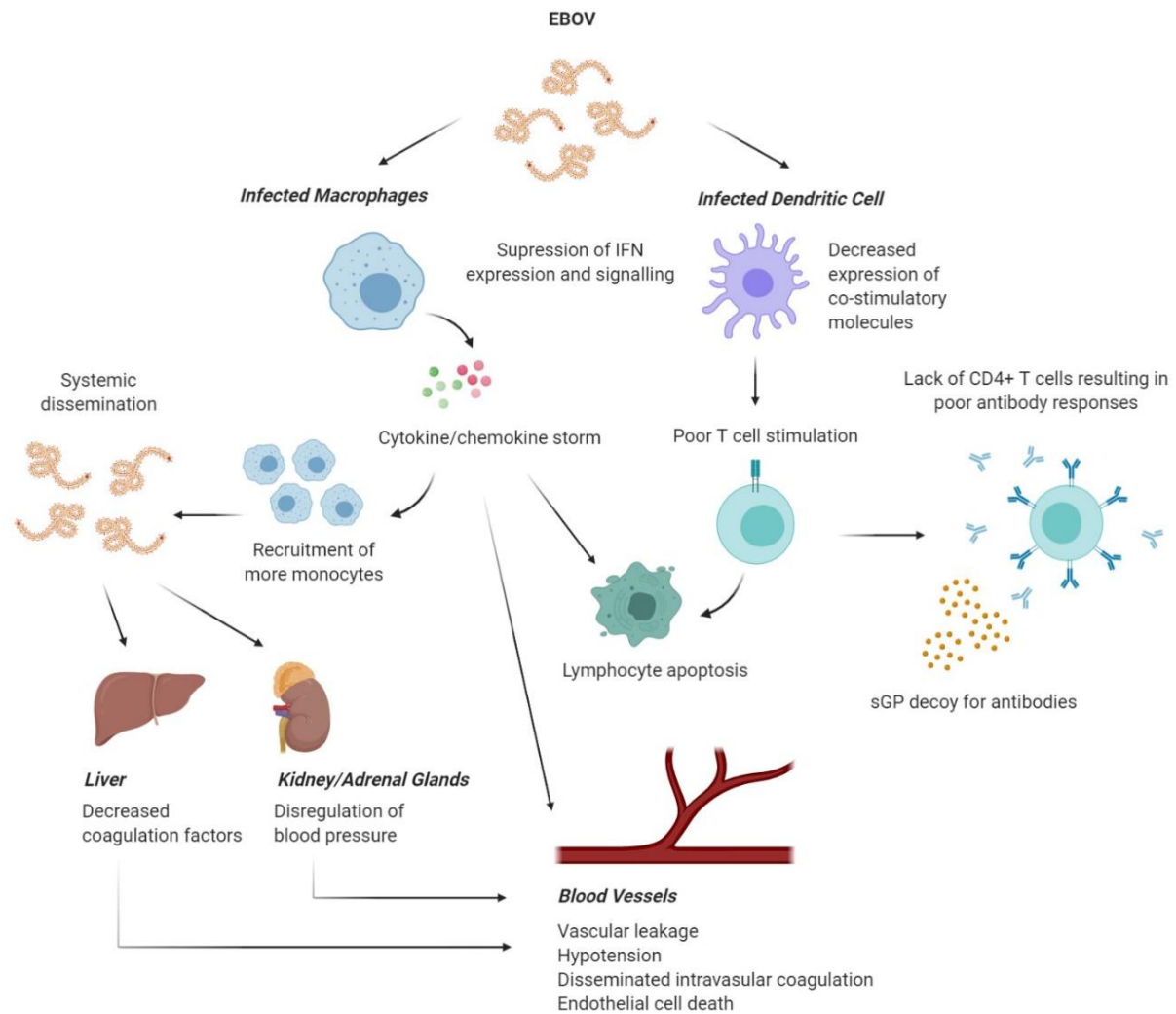


Figure 1.9 EBOV pathogenesis

EBOV initially favours infection of monocytes, macrophages and DCs, impairing their maturation and suppressing type 1 IFN signalling and expression, the host's primary defence against viral infections. This prevents T-cell activation. Infected macrophages overexpress inflammatory mediators, which recruit further cells of the immune system to sites of infection. Inflammatory mediators, reactive oxygen species and nitric oxide instigate T lymphocyte apoptosis. Lower levels of CD4+ cells result in an inhibition of the production of an antibody response. Infected cells disseminate the virus through the lymphatic system, to vital organs and endothelial cells, altering their functions, which results in the symptoms of EVD such as haemorrhaging and DIC, eventually causing shock and multisystem organ failure. Image adapted from (Rivera and Messaoudi, 2016).

1.5.1 Functional Impairment of Dendritic Cells

Studies have shown that APCs in lymphoid tissues are the preferred targets for EBOV, which serve as primary sites of viral replication but also may have a role in the systemic dissemination of the virus as once infected, the virus can use these cells as a vehicle for transportation throughout the lymphatic system and blood to sites including lymph nodes, the liver and spleen. At these sites, EBOV can attack fixed macrophages, with infection then spreading to surrounding cells, including hepatocytes, fibroblasts and endothelial

cells (Geisbert, Hensley, *et al.*, 2003; Geisbert, Young, Jahrling, Davis, Larsen, *et al.*, 2003; Martinez *et al.*, 2015).

This has broad implications, as APCs, in particular, DCs bridge the innate and adaptive arms of the immune system due to their role in processing pathogenic materials into defined epitopes and presenting them to T-cells, thus inducing a cell-mediated response (Rossi and Young, 2005). The disruption of DC maturation by EBOV infection reduces the stimulation of adaptive immune responses needed to clear infections. Considering that most human populations are immunologically naive to EBOV, proper APC function is crucial for the initiation of any virus-specific adaptive immune responses (Van der Groen and Pattyn, 1979; Bouree and Bergmann, 1983).

In-vitro infections of DCs have resulted in their inability to upregulate vital co-stimulatory molecules, including CD40, CD80 and CD86, as well as MHC class I and II, or produce cytokines, interfering with cell-mediated T-cell responses and B-cell activation (Bosio *et al.*, 2003; Mahanty *et al.*, 2003). NK cell reduction has also been observed in the peripheral blood, following infection in non-human primate models, likely contributing to the impairment of DC maturation, as well as resulting in a reduced clearance of infected cells (Reed *et al.*, 2004).

1.5.2 'The Cytokine Storm'

Contrastingly, EBOV infections of monocytes and macrophages result in overexpression of inflammatory cytokines and chemokines including interleukin 1 β (IL-1 β), IL-6, IL-8, macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , MCP-1, and TNF- α , *in-vitro* (Gupta *et al.*, 2001; Ströher *et al.*, 2001; Hensley, 2002). EBOV exposure has been shown to induce inflammatory mediators within the first hour of infection, prior any viral gene expression, suggesting a direct role for EBOV GP_{1,2} in inducing initial inflammatory responses (Wahl-Jensen *et al.*, 2011). As mentioned previously, shed GP can bind and activate macrophages and DCs via TLR-4, contributing to the secretion of cytokines, which can cause endothelial cell dysregulation that can result in increased vascular permeability and fluid extravasation (Dolnik *et al.*, 2004; Escudero-Pérez *et al.*, 2014). In particular, Increased TNF- α levels have been shown to instigate increased endothelial permeability that results in the characteristic vascular leakage that is observed as the disease progresses (Hensley, 2002).

Chemokines such as MIP1- α and MCP-1 can recruit further monocytes and macrophages to sites of infection, thus leading to the infection of more target cells, as well as an amplification of the production of inflammatory mediators by these cells, which likely facilitates the deterioration of the vascular system that is typical to EBOV infections (Gupta *et al.*, 2001; Osvaldo Martinez *et al.*, 2013). Infected macrophages produce tissue factor, which is also upregulated by elevated concentrations of IL-6, causing the over-activation of the intrinsic pathway of coagulation, leading to the formation of fibrin and thrombin deposits. Subsequent disseminated intravascular coagulation is observed, causing microthrombi that hinder adequate blood supply, eventually leading to tissue necrosis, commonly observed in the liver (Neumann *et al.*, 1997; Geisbert *et al.*, 2003). Excessive amounts of reactive oxygen species such as nitric oxide have also been detected during EBOV infections. As fundamental molecules in cardiovascular homeostasis, the increase in nitric acid might likely lead to the loss of vascular smooth muscle and an increase in vasodilation (Blatter and Wier, 1994; Geisbert *et al.*, 2000; Sanchez *et al.*, 2004). The impact of these factors simultaneously contributes to vascular hypotension, eventually resulting in shock and multisystem organ failure.

1.5.3 Immune Response Subversion by Interferon Antagonisers VP24 and VP35

Type-I IFN responses are a vital antiviral mechanism that the innate immune system employs to perturb infection. Acting as a master regulator, IFNs drive an antiviral state in non-immune cells, whilst simultaneously preventing further viral replication in already infected cells. They also enhance antigen presentation that instigates the adaptive immune response (Kühl and Pöhlmann, 2012).

Type-I IFN expression is induced by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including retinoic inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) (Rehwinkel and Gack, 2020). Upon the activation of these pathways, IFN-promoter stimulator 1 induces kinases (TBK-1 and IKK ϵ) to phosphorylate the transcriptional regulators, IFN regulatory factors (IRFs) 3 and 7, instigating their dimerisation and thus, subsequent translocation to the nucleus, resulting in the transcription of IFN- α/β (Prins, Cárdenas and Basler, 2009). When IFN- α/β binds to its cellular receptors, the JAK and STAT pathways are activated, whereby the phosphorylation of JAK1 results in the phosphorylation of STAT1/2, also dimerise and translocate to the nucleus, where they trigger the transcription of IFN

stimulated genes that can limit viral replication (Fleming, 2016). EBOV has developed mechanisms by which it can block the production of type I IFN, as well as the cellular responses to IFN production. VP24 can block the nuclear translocation of phosphorylated STAT1 by binding to the nuclear importer protein karyopherin $\alpha 1$ (KPN- $\alpha 1$), thus inhibiting the induction of IFN-stimulated gene expression (Reid *et al.*, 2007; Zhang *et al.*, 2012). Moreover, VP24 can bind to STAT1 directly, inhibiting its phosphorylation and translocation (Zhang *et al.*, 2012).

VP35, another IFN antagonist, has an IFN inhibitory domain (IID), which can bind dsRNA formed as a replication intermediary, thus preventing RIG-1 and MDA-5 recognition. It can also bind PACT, a dsRNA binding protein that can also be recognised by RIG-1, to activate downstream signalling. Subsequently, IRF3 and 7 phosphorylation are impaired by the inhibition of kinases, thereby inhibiting type I IFN expression (Basler *et al.*, 2000; Prins, Cárdenas and Basler, 2009; Schümann, Gantke and Mühlberger, 2009; Luthra *et al.*, 2013). Additionally, VP35 can increase PIAS1-mediated SUMOylation of IRF7, resulting in transcriptional repression of IFN genes. (Chang *et al.*, 2009). Furthermore, VP35 has been shown to also prevent DC maturation by interfering with the RIG-1 signalling pathways. This inhibits the upregulation of co-stimulatory molecules (CD40, CD80 and CD86) as well as MHC 1 and 2, resulting in impaired antigen presentation to T cells and their activation (Yen *et al.*, 2014).

1.5.4 Lymphopenia

Although lymphocytes are refractory to infection, lymphopenia, a reduction in T-cells in peripheral blood, is also a common condition associated with fatal EBOV infections (Younan, Iampietro and Bukreyev, 2018). Although further investigation is needed into the contribution of cellular and viral factors and molecular mechanisms of lymphopenia, studies show that prolonged exposure to a cytokine storm is toxic to T-cells (Sanchez *et al.*, 2004). T-cell apoptosis has also been shown to be induced by the release of exosomes containing VP40 from DCs, formed when VP40 hijacks ESCRT machinery (Pleet *et al.*, 2016). Early *in-vitro* studies have shown that infected macrophages can upregulate TNF-related apoptosis ligand (TRAIL) expression which can induce apoptosis in lymphocytes, although more recently, TRAIL expression was not observed in macrophages, rather an upregulation in TRAIL mRNA was seen in T cells, upon the infection of PMBCs (Hensley, 2002) with EBOV, causing non-activated CD4⁺ and CD8⁺ T-cells to undergo apoptosis (Gupta, Spiropoulou and Rollin, 2007). EBOV has recently been shown to bind and

activate CD4+ T-cells via the GP interactions with TLR4 and by PtdSer on the viral membrane interactions with TIM-1, further stimulating the cytokine and chemokine cascade and increasing cell death as observed in *in-vitro* experiments (Iampietro *et al.*, 2017; Younan *et al.*, 2017). Although the implications are unclear, CD4+ T-cells are crucial in isotype class switching in B-cells, and thus, cell death could result in reduced EBOV-specific immunoglobulin M (IgM) and IgG antibodies in fatal cases (Baize *et al.*, 1999).

1.6 Vaccines and Therapies

1.6.1 Therapeutic Interventions

Without the availability of licenced vaccines and therapies, the traditional management of patients with EVD relied primarily on the provision of supportive and critical care to manage the symptoms of the disease, with the replacement of fluid losses and the treatment of concurrent diseases being standard practice (Lamontagne *et al.*, 2019). In recent years, the advent of investigational therapies and vaccines has reached clinical trials and many were tested for efficacy during the 2013-2016 West African outbreak (Table 1.2) (Cross *et al.*, 2018; Dodd *et al.*, 2019). Such therapies for EVD aim to limit viral replication to reduce the exacerbated inflammatory storm that is triggered by the viral infection spreading systemically (Malvy *et al.*, 2019). Although none of these treatment options showed unequivocal efficacy, promising therapies were identified and their administration to critical patients during these trials formed an important part of the global outbreak response. During the outbreak in the DRC (2018-2020), experimental therapies including the monoclonal antibody cocktails ZMapp and REGN-EB3, the single monoclonal antibody MAb114 and the small molecule antiviral drug Remdesivir were administered to patients in a randomised clinical trial known as the Pamoja Tulinde Maisha (PALM) trial (Mulangu *et al.*, 2019).

ZMapp, a drug consisting of three recombinant monoclonal antibodies that were generated from mice that were exposed to EBOV proteins and chimerised with human constant regions, has demonstrated anti-EBOV activity *in-vitro*, and in non-human primate models (Davidson *et al.*, 2015; Davey *et al.*, 2016). The cocktail was first used in humans during the West African outbreak within the Partnership for Research on Ebola Virus in Liberia II (PREVAIL II), a randomised clinical trial, however, although the drug resulted in favourable outcomes in patients, the efficacy of the drug could not be determined as the

sample size of patients was insufficient to provide statistically significant results (Davey *et al.*, 2016).

The provisional results of the PALM trial, in which the patients receiving ZMapp served as the control group based on the results of the PREVAIL II trial, determined that patients receiving MAb144 or REGN-EB3 showed improved survival rates than those treated with ZMapp or Remdesivir (Mulangu *et al.*, 2019). REGN-EB3 contains three antibodies that were generated by immunising mice that expressed human antibody variable gene segments, meaning that the antibodies that are produced are fully human. The antibodies bind to specific-non overlapping epitopes on the EBOV GP, potentially improving the cocktail's efficacy and reducing the frequency of escape mutants (Sivapalasingam *et al.*, 2018).

Contrastingly, Mab114 is derived from the memory B-cells produced by a survivor of the Kikwit 1995 EBOV outbreak and binds to a conserved epitope within the RBD of GP₁, thus preventing the GP from interacting with NPC1 (Gaudinski *et al.*, 2019). As the antibody targets the RBD, the chance of escape mutants is once again reduced as mutations in this area would be detrimental to the virus causing infection, thus the virus would be less fit (Levine, 2019). Intravenously delivered Remdesivir was shown to display good efficacy against EBOV in non-human primates, having a positive safety profile in phase 1 studies (Warren *et al.*, 2016). As an adenosine nucleotide triphosphate analogue, the drug can interfere with the action of the RdRP and cause premature chain termination, thus interfering with viral replication (Jordheim *et al.*, 2013). Although the trial has found that the drug is less effective than the antibody treatments tested, the drug may provide the option of a broader, synergistic treatment if administered alongside antibodies (Mulangu *et al.*, 2019). These trials represent a promising step forwards toward attaining a licensed therapy that can be provided to manage EVD outbreaks.

Additionally, outside the remit of clinical trials, the WHO permitted the administration of further drugs, including nucleoside/nucleotide analogues, nucleic acid-based drugs and immunosuppressants, for emergency and compassionate use, although their application under these circumstances was determined to be unsubstantial in providing evidence towards the safety or efficacy (Gignoux *et al.*, 2016; Uyeki *et al.*, 2016; Dörnemann *et al.*, 2017). Thus, even drugs that appeared to be effective on the front line, require further testing to determine safety profiles, effective dosages and treatment combinations.

Table 1.2 Overview of therapeutics assessed in clinical trials for EVD

Trials were undertaken during the 2013-2016 West African outbreak. Adapted from (Malvy *et al.*, 2019).

<i>Drug/Intervention</i>	<i>Description</i>	<i>Details of Clinical Trial</i>
Favipiravir (T-705)	• Small-molecule, direct-acting antiviral • Inhibitor of viral RdRP • Repurposed influenza drug (Avigan) • Established anti-EBOV activity <i>in vivo</i>	• JIKI started in December 2014, in Guinea, for acute Ebola virus disease • 126 participants enrolled – no significant survival benefit in patients with a high viral load (Sissoko <i>et al.</i>, 2016)
Convalescent plasma	• Antibody-based treatment • ABO-compatible plasma taken from convalescent donors • Treatment is dependent on unverified levels of neutralising antibodies present in the plasma of donors	• Ebola-Tx started in February 2015, in Guinea, for acute Ebola virus disease • 84 participants enrolled – no significant survival benefit (van Griensven <i>et al.</i>, 2016)
ZMapp	• Monoclonal antibody cocktail • Three chimerised antibodies (c13C6, 2G4 and 4G7) • Target distinct epitopes on the viral glycoprotein • Antibodies are selected from previously established antibody cocktails (MB-003 and ZMab)	• PREVAIL II started in February 2015, in multiple locations, for acute Ebola virus disease • 72 enrolled participants (target was 200). Suggestive positive outcomes but not statistically conclusive. • Raised concerns about side effects, staffing resource commitment and the requirement of a cold chain (Davey <i>et al.</i>, 2016).
TKM-130803 (TKM)	• Lipid nanoparticle formulation of small interfering RNAs (siRNAs) • Directed against <i>L</i> and <i>VP35</i>	• RAPIDE-TKM started in February 2015, in Sierra Leone, for acute Ebola virus disease • 12 participants enrolled – no significant survival benefit • Concerns of side effects - acute cytokine release syndrome was observed in healthy volunteers (Dunning <i>et al.</i>, 2016)

1.6.2 Vaccine Development

Although in past outbreaks, the World Health Organisation (WHO) had approved the implementation of experimental vaccines, monoclonal antibodies and antivirals that were in clinical development. on the grounds of compassionate use, it was not until 2019 that

the first vaccine was licenced for use against EBOV, having shown success when employed as part of experimental ring vaccination strategies (WHO, 2015a; Gsell *et al.*, 2017; Keusch *et al.*, 2017; WHO, 2018; Levine, 2019). Named commercially as 'ERVEBO,' the vaccine is a live and attenuated recombinant vesicular stomatitis virus (VSV) vaccine. In this vaccine, the VSV G surface protein has been substituted with the EBOV glycoprotein (rVSV Δ G-ZEBOV-GP_{1,2}) (Khan, 2019).

Vaccine development against EBOV started in the 1970s and progress was limited for many decades. It was not until the West African outbreak that the clinical development of vaccines was drastically accelerated; before this outbreak, only EBOV DNA and adenovirus-based vaccines had surpassed the preclinical testing stages (Feldmann, Feldmann and Marzi, 2018; Gross *et al.*, 2018). ERVEBO was evaluated via a phase 3, randomised, placebo-controlled clinical trial within the remits of PREVAIL I (Henao-Restrepo *et al.*, 2017). Preliminary results indicated that under a protocol of ring vaccination (the vaccination of confirmed patients, direct contacts and contacts of direct contacts). The vaccine demonstrated high efficacy and was well tolerated among patients, inducing an immune response in them (Henao-Restrepo *et al.*, 2017; Kennedy *et al.*, 2017).

A similar protocol was utilised in the 2018-2020 outbreak in the DRC in which over 60,000 people were vaccinated, including members of the population that were more likely to come into contact with the sick, such as religious leaders and traditional healers (Malvy *et al.*, 2019). ERVEBO was approved by the European Medicines Agency and the American Food and Drugs Administration in 2019, becoming the first licensed vaccine/treatment for EVD, although unlikely the last (Khan, 2019).

The accelerated development of several alternative vaccine candidates, including the chimpanzee adenovirus serotype 3 vaccine (ChAd3-EB0-Z), a vaccine that was assessed simultaneously with ERBEVO (PREVAIL I), showing safety and immunogenicity for up to 1 year, means that the gap between investigational use and clinical use is swiftly diminishing (Kennedy *et al.*, 2017). A further vaccine candidate that combines a human adenovirus vaccine (Ad26.ZEBOV), boosted with a modified vaccinia Ankara virus (MVA), aiming to improve long-term immunity, was also tested, with and without the booster in the Partnership for Research on Ebola Vaccinations (PREVAC) trial within the 2018 outbreak in the DRC (Callendret *et al.*, 2018; Badio *et al.*, 2021). The trial also re-evaluated ERBEVO within the context of single-shot versus boosted regimens, aiming to generate data on the long-term safety and immunity associated with both vaccines. Although much

progress has been made, questions surrounding the storage, transport and administration of these vaccines remain and require addressing.

1.7 Outbreaks

1.7.1 The Impact of EBOV Outbreaks on Primate Populations

As a zoonotic disease, EBOV is spread via direct contact with animals or carcasses housing an infectious viral load. However, the ecology of EBOV and the role that wildlife plays in EBOV disease epidemiology remains to be completely elucidated. Wildlife acts as a reservoir for numerous diseases impacting humans and it is frequently postulated that an increase in urbanisation, deforestation and the general encroachment of human populations into the wilderness has caused greater contact with wildlife and consequently, any pathogens that use these animals as a reservoir for infection or as a host for disease, thus encouraging a spillover into human populations (Walsh *et al.*, 2003; Neiderud, 2015). Specifically, remote sensing techniques have been used to determine a positive correlation between EBOV outbreaks in central and western Africa and deforestation (Olivero *et al.*, 2017).

The first outbreak of EBOV was described in 1976, occurring in DRC, formerly known as Zaire (Table 1.3). The outbreak occurred near the Yambuku village, with 318 known cases, leading to a fatality rate of 88%. Since then, sporadic outbreaks have occurred throughout the decades. However often, the index case and under what circumstances they may have contracted the disease has not been identified (Languon and Quaye, 2019). This is more applicable to the earlier outbreaks (Figure 1.10). Nonetheless, several outbreaks have also been linked to contact with animal carcasses, in particular, the 1996 outbreak in Mayibout (Gabon), in which the index cases were children who had come into contact with and butchered a chimpanzee carcass (Figure 1.11), although direct contact with other hosts of the virus has been reported (Georges *et al.*, 1999; Leroy *et al.*, 2009). Subsequent human-to-human transmission follows within local communities as well as health facilities.

Chronology of outbreaks and some molecular studies have shown that there is a significant correlation between EBOV outbreaks in human populations and the rapid decline of wildlife in parts of central Africa, which particularly impacted non-human primate populations. Between August 2001 and June 2003, 50 gorilla, 15 chimpanzee and 14 duiker carcasses were discovered in an outbreak region that bordered Gabon and the RC

(Leroy *et al.*, 2004; Rouquet *et al.*, 2005). During this timeframe, two notable increases in animal deaths were recorded, the first being in the Ekata region of Gabon between November and December 2001 (51 animal carcasses, including 30 great apes and 8 duikers) followed by deaths in the Lossi animal sanctuary in the Republic of Congo (RC) between December 2002 and February 2003 (20 animal carcasses, including 17 great apes, 2 duikers, and 1 *Cercopithecus cephus*.) (Rouquet *et al.*, 2005). A population census undertaken within this sanctuary before and after the 2003 EBOV outbreak in Kelle outbreak, determined that the populations of gorillas and duikers were estimated to have declined by 50%, whilst the chimpanzee population by 88% (Leroy *et al.*, 2004). Such high mortality is not indicative of a host that maintains the virus but does emphasise the importance of these animals in further transmission events

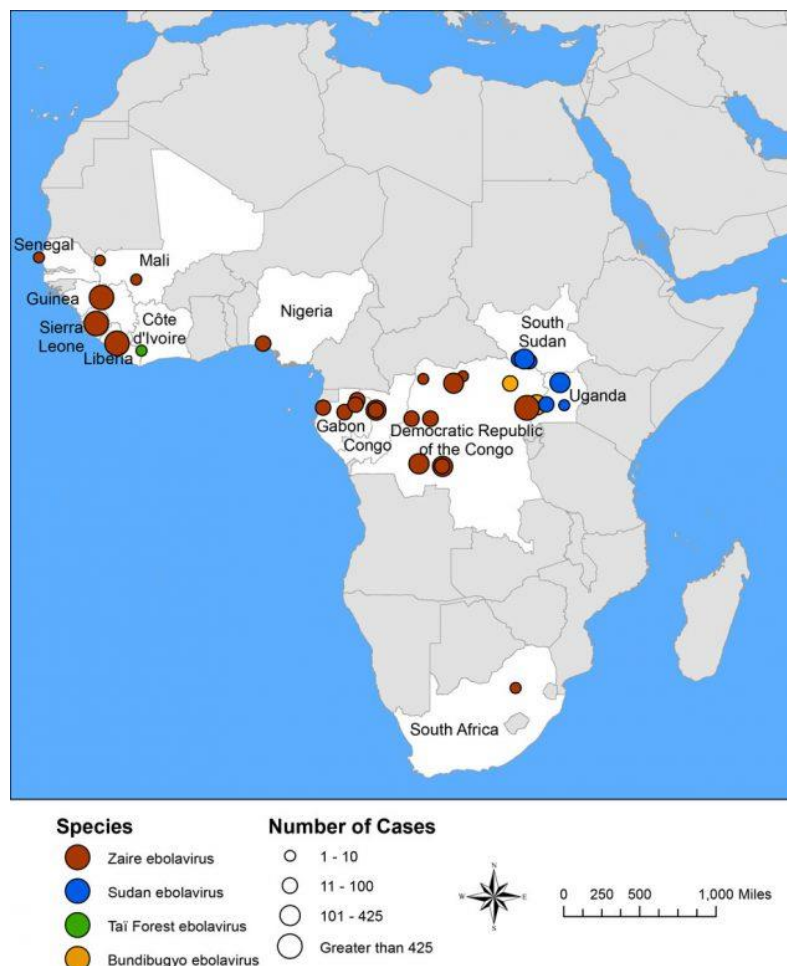


Figure 1.10 Distribution map of outbreaks caused by *Ebolavirus*

The map is limited to depicting outbreaks in Africa. Image sourced from the CDC (2020).

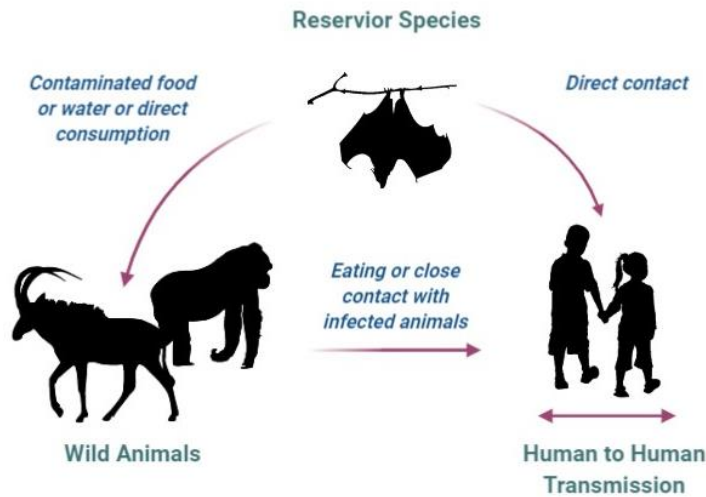


Figure 1.11 Possible routes of transmission of EBOV during outbreaks

EBOV is transmitted from a suspected reservoir species to intermediate animal hosts. Human infection can occur from either direct contact with a reservoir species, or with an intermediate host.

Sequencing of the EBOV *GP* gene available from all human samples that were taken during the 2001-2003 outbreaks identified sequence variation at a nucleotide level, between viruses that were isolated from people part of different epidemic chains and samples that were taken from individuals from the same chain. In the five outbreaks that occurred during this period, eight viral strains and ten epidemic chains were identified. Consequently, it was concluded that the outbreaks started due to the introduction of separate viral strains into the population rather than the introduction of a single strain. The index cases were reported to have been handling carcasses of animals including gorillas, chimpanzees and duikers (Leroy *et al.*, 2004). Subsequent testing of carcasses of 34 animals in Gabon between 2001 and 2003, showed that 14 tested positive (10 gorillas, 3 chimpanzees and 1 duiker) for EBOV infection. EBOV-specific IgG, as well as viral antigens, were detected in the soft tissue taken from the carcasses. Viral proteins were also detected via immunohistochemistry (IHC) (Rouquet *et al.*, 2005; Wittmann *et al.*, 2007). Interestingly, all muscle tissues tested for EBOV-specific IgG, indicating that the animals had died without developing humoral responses, similar to that seen in fatal human cases.

These observations suggest that non-human primates and other animals susceptible to infection are taking an intermediary role in the transmission of EBOV between its reservoir and human populations. Transmission of EBOV in these populations is enough to cause substantial outbreaks, particularly in great ape populations, which can pose a large threat to animal conservation. Given that the recovery of these populations will take many years,

non-human primates are not a likely contender for the role of the EBOV reservoir however, they do have a part in further transmission events into human populations.

Table 1.3 List of all EBOV outbreaks which have occurred since 1976 to date

The content of the table is adapted from the CDC website (CDC, 2022).

Date of Outbreak	Region Affected	Case Numbers	Deaths (%)	Details	References
1976	Zaire (DRC)	318	280 (88%)	Occurred in Yambuku . Disease was spread by close personal contact and contaminated needles and syringes in healthcare settings. This was the first recorded incidence of EVD.	(WHO, 1978)
1977	Zaire	1	1 (100%)	Noted retrospectively in the village of Tandala .	(Heymann <i>et al.</i> , 1980)
1994	Gabon	52	31(60%)	Occurred in Mékouka and other areas in nearby rainforests. Initially misdiagnosed as yellow fever but recognised as EVD in 1995.	(Georges <i>et al.</i> , 1999)
1995	DRC (formerly Zaire)	315	250 (81%)	Occurred in Kikwit . The index case was a patient who worked in the surrounding forest. This was the second largest outbreak to have occurred at the time.	(Khan <i>et al.</i> , 1999)
1996 (January-April)	Gabon	37	21(57%)	Occurred in Mayibout area. An infected chimpanzee was eaten by people hunting for food. Children also came into contact with the carcass. Nineteen people who were involved in the butchery of the animal became ill; other cases occurred in family members.	(Georges <i>et al.</i> , 1999)
1996-1997 (July-January)	Gabon	60	45 (74%)	Occurred in Booué . Index case patient was a hunter who lived in a forest camp. The virus was spread to those who came into close contact with infected persons.	(Georges <i>et al.</i> , 1999)
1996	South Africa	2	1 (50%)	A medical professional travelled from Gabon to Johannesburg , South Africa, after having treated Ebola-infected patients. He was hospitalized, and a nurse who took care of him became infected and died.	(WHO, 1996)

1996	Russia	1	1(100%)	Laboratory contamination.	(Borisevich <i>et al.</i> , 2006)
October 2001-March 2002	Republic of the Congo	59	44 (75%)	Outbreak occurred over the border of Gabon and the Republic of the Congo (RC) . The first reported incidence of EVD in the RC.	(WHO, 2003)
2001	Gabon	65	53 (82%)	Again, occurred along the border of RC and Gabon. An abnormal decline in wildlife was observed in this region.	(WHO, 2003)
December 2002-April 2003	Republic of the Congo	143	128 (89%)	Outbreak occurred in the districts of Mbomo and Kéllé .	(Formenty <i>et al.</i> , 2003)
November-December 2003	Republic of the Congo	35	29 (83%)	Outbreak occurred in Mbomo and Mbandza villages located in Mbomo district.	(WHO, 2015b)
2004	Russia	1	1(100%)	Laboratory contamination.	(Akinfeyeva <i>et al.</i> , 2005)
2005	DRC	12	10 (83%)	Two hunters (index patients) died in Etoumbi Medical Centre in April 2005.	(Nkoghe <i>et al.</i> , 2011)
2007	DRC	264	187 (71%)	Outbreak occurred in Kasai Occidental Province. The outbreak was declared over November 20. Last confirmed case on October 4 and last death on October 10.	(WHO, 2007)
December 2008-February 2009	DRC	32	15 (47%)	Outbreak occurred in the Mweka and Luebo health zones of the Province of Kasai Occidental.	(Leroy <i>et al.</i> , 2009)
2013-2016	Multiple countries	28,616	11,310 (39%)	Outbreak primarily in Guinea, Liberia and Sierra Leone . The first incidence of the virus being exported internationally. 36 confirmed cases were reported from Italy, Mali, Nigeria, Senegal, Spain, the United Kingdom, and the United States 32. A further 24 Ebola-confirmed patients were transported to France, Germany, Italy, the Netherlands, the United Kingdom, the United States, Norway, Spain, and Switzerland. The largest recoded EBOV outbreak.	(Bell <i>et al.</i> , 2016)
August-November 2014	DRC	69	49 (71%)	Outbreak occurred in multiple villages in the DRC. The outbreak was unrelated to the outbreak of Ebola in West Africa .	(Maganga <i>et al.</i> , 2014)

May – July 2017	DRC	8	4 (50%)	Outbreak occurred in the Likati health zone of the province of Bas Uélé . Containing the outbreak was challenging due to the remoteness of the area and limited available services. Mobile diagnostic laboratories provided testing of samples in the affected areas.	(Hemingway-Foday <i>et al.</i> , 2020)
May – July 2018	DRC	54	33 (61%)	Outbreak occurred Bikoro region but infections quickly spread to other regions such as Mbandaka .	(Mbala-Kingebeni, Pratt, <i>et al.</i> , 2019)
August 2018 - June 2020	DRC	3,470	2,287 (66%)	The DRC government declared the outbreak on August 1 in North Kivu province. Confirmed cases were also reported in Ituri provinces. The second-largest outbreak of EBOV.	(Rohan and McKay, 2020)
June- November 2020	DRC	130	55 (42.3%)	Confirmation of a new outbreak in Mbandaka .	(WHO, 2020)
February- June 2021	Guinea	23	12 (52.2%)	The first confirmed cases in Guinea since the West African outbreak (2013-2016). First cases were detected in N'Zérékoré Prefecture .	(Keita <i>et al.</i> , 2021)
February- May 2021	DRC	12	6 (50%)	Outbreak occurred in Biena Health Zone, North Kivu Province .	(Khan <i>et al.</i> , 2021)
October- December 2021	DRC	11	6 (55%)	Outbreak occurred in Beni Health Zone, North Kivu Province . Sequencing determined a link to the 2018-2020 outbreak in the same region. Likely caused by persistent infection in an EVD survivor.	(WHO, 2021)
April- July 2022	DRC	5	5 (100%)	Outbreak occurred in Mbandaka health zone, Equateur Province. Sequencing data indicates that this is a new spillover event.	(WHO, 2022)

Future rapid genomic characterisation of human and non-human primate outbreaks of EBOV will remain imperative to continued understanding of the zoonotic and epidemiological origins of EBOV, and whether outbreaks are due to a single introduction event from the reservoir, or intermediate host with ensuing human-to-human transmissions, or due to multiple introductions with fewer transmissions within human populations, but more genetic diversity (Carroll *et al.*, 2013).

1.7.2 Bats as the Reservoir for EBOV between Outbreaks

The reservoir species for EBOV was elusive for years. In the past, studies have shown that arthropods are unable to support EBOV viral replication, an interesting observation considering that arthropods are traditional vectors for other haemorrhagic fever-causing viruses including Yellow Fever Virus (YFV) and Dengue Virus (DENV) (Turell, Bressler and Rossi, 1996; Carrington and Simmons, 2014; Klitting *et al.*, 2018). Following the Kikwit 2005 EBOV outbreak, over 3000 animals consisting of 78 mammalian, 51 bird and 22 reptile and amphibian species, were sampled from areas surrounding the home of the index case in attempts to identify evidence of EBOV infection, although none was found through serological studies. Of these animals, approximately 20% were bats, however, the sample size per species was low, with only four bat species having sample sizes of more than 20 individuals (Leirs *et al.*, 1999).

Experimental inoculation of multiple different plant species as well as reptiles, invertebrates and some vertebrates failed to yield any virus replication and were therefore also determined to be unlikely reservoirs for infection (Swanepoel, 1996). However, in this study, two species of insectivorous bats, *Tadarida condylura* and *Tadarida pumila* and one species of fruit bat, *Epomophorous wahlbergi* were also inoculated with EBOV. These bats were able to support EBOV replication and display an adaptive immune response. Additionally, the virus could be isolated from the bats for up to three weeks (Swanepoel, 1996). Notably, faecal shedding was only observed in one species, indicating that this may not be a favoured mechanism for transmission amongst bat populations (Swanepoel, 1996).

Fruit bats have since been identified as the reservoir species for both *Marburgvirus* and *Cuevavirus*, (Towner *et al.*, 2009; Negrodo *et al.*, 2011). During an outbreak of MARV in 2007, virus-specific antibodies and Marburg virus RNA were detected in *Rousettus aegyptiacus*. However, the evidence that implicated the bat as the reservoir species was that for the first time, isolation of the live virus was achieved directly from the wild animals, with it present in approximately 5% of the bats that were sampled (Towner *et al.*, 2009). Additionally, it was found that the genome sequences that were circulating in this bat population, were highly divergent (21%). Such diversity could be a consequence of a long-term association with a reservoir host of a large population size.

Following this, studies have demonstrated MARV replication in multiple tissues and the blood of *Rousettus*, oral viral shedding and antigen presence in the liver and spleen, all

without evidence of the virus causing any clinical symptoms or any observed gross pathology (Paweska *et al.*, 2012; Amman *et al.*, 2015). Paweska *et al.* (2012), demonstrated that the virus could be detected in several tissues for up to 9 days post-infection and that IgG could be detected between 9 and 21 days post-infection, outcomes that when combined with a lack of clinical symptoms, are consistent with describing the bats as a reservoir rather than an intermediary to human infection. However, the virus that was used in this study was passaged almost 40 times in Vero E6 cells before the bats were exposed and this could have a significant impact on the infectivity of the virus, due to it gaining tissue culture adaptations. Recently, studies of the *R. aegyptiacus* genome have identified an expanded family of NK cell receptors, MHC class I genes and type 1 IFNs, which are different from mammalian counterparts, suggesting that this bat species employs novel modes of antiviral defence that permit it to tolerate viral infection and asymptotically host the virus (Pavlovich *et al.*, 2018).

Evidence that implicates other species of fruit bat, as the reservoir species for *Ebolavirus* has also been uncovered. EBOV-specific antibodies and EBOV RNA have been detected in *Hypsignathus monstrosus*, *Myonictoris torquata* and *Epomops franqueti* (Leroy *et al.*, 2005). In a trapping expedition led by Leroy *et al.* (2005), 1030 animals were captured. This included 679 bats. IgG specific for EBOV was detected in the three species of bat that are listed above. Viral nucleotide sequences were also isolated from the organs of other bats within this population, via PCR. Interestingly, the PCR-positive bats were not IgG-positive, and vice versa. This is likely because the PCR-positive bats may have only been recently infected and therefore, had not had the appropriate time required to develop a humoral response (Leroy *et al.*, 2005).

Notably, no live virus was isolated from these bats or any other species of bats which have since been implicated as a potential reservoir, in which infection occurs without clinical symptoms, and only parts of the viral genome were detected using molecular techniques. Additionally, in Leroy's study (2005) the only PCR-positive organs were the liver and the spleen, and even then, the levels of viral RNA were low. No RNA was detected in several blood-filled organs such as the heart, liver and kidneys. Once again, this raises questions about how the virus would be transmitted within bat populations and whether it has a limited ability to shed in bat bodily fluids. However, persistent infection and horizontal transmission between colonies have been suggested as a mechanism which has allowed for the preservation of the virus, in the wild (Wittmann *et al.*, 2007).

1.7.3 Origins of the West African Outbreak and the Sustained Transmission of EBOV

EBOV outbreaks have been categorised into those that occur because of a single introduction of a virus into a human population, resulting in person-to-person transmission or those that occur because of multiple introductions of viruses and subsequent short chains of transmission, as determined for the outbreaks in Gabon between 2001 and 2003 (Leroy *et al.*, 2004; Wittmann *et al.*, 2007). In previous outbreaks, once the virus had entered a human population, there seemed to be little molecular evolution of the virus, with genomes remaining relatively stable and exhibiting little diversity (Leroy *et al.*, 2009; Carroll *et al.*, 2013). When diversity was observed it was within hypervariable regions of specific genes, particularly *GP*, *N* and *L* (Jun *et al.*, 2015). Clinical samples taken from the 1995 Kikwit EBOV outbreak, in particular, demonstrated a limited amount of genetic variability was displayed by the virus, throughout the outbreak, suggesting the outbreak was due to a single zoonotic event (Muyembe-Tamfum *et al.*, 1999; Rodriguez *et al.*, 1999). This genetic stability was based on sequencing of a 249-nucleotide region in *GP* and may be explained by the relatively short durations of earlier outbreaks; the Kikwit outbreak only lasted six months and limited sequencing of genomes took place, whilst the sequence availability of outbreak strains has only improved in recent years (Rodriguez *et al.*, 1999; Leroy *et al.*, 2002; Carroll *et al.*, 2013; Jun *et al.*, 2015).

The outbreak in West Africa which occurred between 2013 and 2016 was unprecedented in terms of its geographical location, scale and its duration. Comparatively, all previous outbreaks occurred on a much smaller scale (Table 1.3) and were restricted to central Africa. However, the duration of the outbreak also permitted the evolution of the viral genome over its course and for the first time, via advancements in deep sequencing techniques that were utilised directly on samples that were taken from EBOV-positive patients, these evolutionary events were tracked (Baize *et al.*, 2014; Gire *et al.*, 2014; Kugelman *et al.*, 2015; Park *et al.*, 2015; Simon-Loriere *et al.*, 2015; Tong *et al.*, 2015). In doing so, this allowed for an improved understanding of viral transmission chains throughout the outbreak, as well as provided insight into the genomic sequences under positive selection, within the EBOV genome (Ueda *et al.*, 2017).

The strain of EBOV that circulated throughout the outbreak was termed Makona and it caused over 28,000 cases and 11,000 deaths. Throughout the outbreak, various teams of researchers sequenced the viral genome attained different afflicted individuals that permitted the cataloguing of a multitude of outbreak-specific, non-synonymous mutations

occurring throughout the EBOV genome (Baize *et al.*, 2014; Gire *et al.*, 2014; Kugelman *et al.*, 2015; Park *et al.*, 2015; Simon-Loriere *et al.*, 2015; Tong *et al.*, 2015). As a result, a concern was raised regarding the ability of EBOV to evolve concerning its pathogenicity and its transmissibility, by improving its viral fitness. There was also a concern regarding the emergence of less pathogenic strains that may be able to establish a more endemic presence (Sofonea *et al.*, 2018). However, the identification of these circulating strain variants has also provided the ability to predict whether they would be able to escape current vaccine candidates and available alternative therapies (Holmes *et al.*, 2016).

The index case was concluded to be a two-year-old boy from Gueckedou, Guinea, who is said to have contracted EBOV after contact with a bat in December 2013 (Baize *et al.*, 2014). EBOV was then sustained in the human population by subsequent transmission events. Specifically, studies combining analysis of epidemiological information and data generated by parallel sequencing of EBOV genomes, determined that two attendees at a funeral in Guinea became infected and introduced variants of the virus to Sierra Leone, where the virus further spread and a new lineage emerged (Gire *et al.*, 2014).

Two genetically distinct lineages are considered to have been introduced from Guinea to Sierra Leone. One of these (SL1) was more closely related to Guinean EBOV genome sequences, whereas SL2 was characterised by four further mutations, suggesting that it had evolved from SL1 (Gire *et al.*, 2014). A third lineage, SL3 evolved from SL2 and became the most prevalent lineage present in Sierra Leone, with SL1 disappearing soon after its emergence (Park *et al.*, 2015). These lineages circulated locally and rarely crossed borders, except for SL2, which had more widespread transmission (Park *et al.*, 2015). These datasets provided details about how EBOV spread across different populations and how the virus genetically changed over time as it transmitted from person to person.

1.7.4 The Impact of Significant Mutations Altering EBOV Phenotype

One significant mutation that originated near the beginning of the outbreak (lineage SL2) was A82V. This was the first substitution that was identified in the GP that could alter how it interacted with NPC1. The importance of this general region to EBOV infection is demonstrated by a study in which F at residue 88 was mutated to an A, which resulted in the inhibition of viral particles in human cell lines (Martinez *et al.*, 2013). The A82V mutation was present in all sequences sampled after March 2014. Recently, two

independent but complementary papers characterised the infectivity of the GP mutation, as well as other significant mutations, in different cell lines (Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016). Both groups demonstrated that this mutation resulted in an increase in infectivity in human cells, showing enhanced viral fitness of 82V over wild-type 82A strains, using pseudotyping assays.

Diehl *et al.* (2016) concluded that this single mutation was able to increase infectivity in cell lines including human dendritic cells and that this was directly due to virus entry into the cytoplasm. Additionally, rodent and carnivorous cell lines were also tested, however, there was no infectivity in these cases. This supports the theory that this was likely, a primate, and thus human-specific adaption that occurred due to positive selection, during the outbreak and then remained fixed in the population as it provided the virus with a selective advantage (Diehl *et al.*, 2016). Urbanowicz *et al.* (2016) took a slightly different approach when studying the mutation, as the group were specifically testing for differences in infectivity in human cell lines and the presumed reservoir species, fruit bats. This allowed them to also identify several other mutations that were associated with primate-specific adaptations, namely, R29V, T206M, and T230A. Perhaps most interestingly, the group demonstrated that some mutations that were associated with a primate-specific adaption, also conferred a loss of infectivity in bat cells, providing further evidence to the theory that EBOV relies upon species of fruit bats as a reservoir (Urbanowicz *et al.*, 2016). This is because the specific nature of these mutations would mean that they would not be tolerated during the infection of a reservoir species.

Additional epidemiological analysis highlighted that there was a rise in viremia and mortality, post-March 2014, which corresponded with the appearance of the mutation (Faye *et al.*, 2015). However, both teams emphasised that it is still difficult to connect the strain variant to transmission patterns that were observed in the outbreak because it is unclear whether it was the mutation itself, or the epidemiological circumstances that were present during the arrival of this viral strain into Sierra Leone, that propelled this variant into dominance during the outbreak (Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016). Both studies are limited in that the mutations were not studied in the context of live EBOV Makona and no *in-vivo* work was completed to determine the impact of the mutations on disease progression.

A further study showed that as well as A82V occurring in the GP, R111C observed in the nucleoprotein (NP) and D795L in the polymerase (L), also emerged early on in the outbreak and were found in approximately 90% of isolates that were sequenced (Dietzel

et al., 2017). The three mutations were consecutively obtained by the virus, in the order L, GP, and finally NP. D795L was first detected in March 2014 and was then supplemented with A82V, which was then replaced by the triple mutant that was identified in Sierra Leone in May 2014. The group found that recombinant EBOV VLPs with a combination of these mutations displayed a growth advantage compared to a prototype isolate (Makona C7) which was representative of the virus strain in circulation before this mutant became dominant. This again shows that strains with improved viral fitness emerged during this outbreak (Dietzel *et al.*, 2017).

Biochemical assays analysing the effect of A82V showed that the mutation decreases the stability of the pre-fusion conformation of the GP, which, therefore, reduces the threshold for activation of the membrane fusion activity and conformational changes in GP2, which is triggered by host factors, cathepsins and NPC1 (Wang *et al.*, 2017). This is in direct contradiction to the claims that were made by both Diehl *et al.* (2016) and Urbanowicz *et al.* (2016) that the mutation alters the affinity that the GP has for the receptor.

Further to this, an analysis of low-passage Makona isolates taken from different points of the outbreak, containing these mutations, was completed to determine if there was any difference in pathogenicity in two animal models; mice and macaques. It was found that there was no significant difference in the behaviour of the clinical Makona variants tested; they all caused severe or lethal disease in the animal models and had similar patterns of viral shedding. This suggested that perhaps the unique combination of mutations found in the clinical strain was not accounted for in the PV models used in previous studies and in an *in-vivo* model, these combinations of mutations could have had an inhibitory effect (Marzi *et al.*, 2018).

T455I has also been identified as an additional mutation in Makona, in the alpha-helix of the GP, which comprises the NPC1 binding pocket. Although A82V had only been found in the Makona strain that had been fixed in the population, T455I has been reported to have frequently occurred in past outbreaks. Additional pseudotyping assays showed that although both mutations increased EBOV infectivity in HuH-7 cells, A82V only caused small increases in infectivity in contrast to T455I (Wang *et al.*, 2017).

This high infectivity of T455I is also supported by a separate study that demonstrated that T455I, found in the fusion loop of GP, enhances GP2-mediated cell entry, with these mutants displaying three times higher entry than wild-type. However, perhaps most significantly, the study concluded that the mutation plays no role in pathogenesis and

disease transmission and that in fact, this mutation is a tissue culture adaptation that spontaneously appears after multiple passages of the viral GP within Vero E6 cells (Ruedas *et al.*, 2017).

1.8 The Use of Pseudoviruses as a Model for Cell Entry

Research into viruses such as EBOV poses several challenges. As a BSL-4 pathogen that is a serious risk to human health, there are a limited number of facilities equipped worldwide that permit the handling of the live virus. Typically to obtain stocks of the live virus, EBOV must be isolated from clinical samples and serially passaged in cell lines (Kugelman *et al.*, 2012; Beitzel *et al.*, 2021). As mentioned previously, this may result in tissue-cultured adapted genome mutations that lead to the virus behaving atypically in *in-vitro* and *in-vivo* experiments (Hoffmann *et al.*, 2017; Ruedas *et al.*, 2017, 2018).

Surrogate models are often used to combat some of the issues faced in handling live-replicating high-containment level viruses (Temperton and Page, 2015; King *et al.*, 2016; Saito *et al.*, 2020). PVs are often used as safe substitutes for enveloped viruses that would require a high level of bio-containment (King *et al.*, 2016). It is a method that is used to study aspects of viral entry and tropism and assess vaccine efficacy and serological studies. In basic terms, PVs are chimeric viral particles in which the envelope protein is substituted with one originating from a different enveloped virus, thus creating a recombinant hybrid (Sanders, 2002). The PV will consist of a core, encompassed by a cell-derived membrane that expresses the foreign viral envelope protein or GP. Importantly, the core does not contain any genetic material that would allow for the expression of a viral envelope protein, therefore the PV can only enter and transduce a target cell, thus they are capable of initiating nucleic acid replication, however, they do not produce infectious viral particles, making them safe to use (Li *et al.*, 2018).

Pseudotype platforms have great utility in outbreak management because as soon as the genomic sequences for the virus are available, PVs can be quickly and efficiently generated to instigate sero-surveillance and determine antigenic epitopes studies at BSL-2, therefore aiding in the development of therapeutics and targeted vaccines. However, optimisation of the system is also required to ensure that the levels of expressed GP on the surface of a PV are not toxic to the cells that it will be infecting (Volchkova *et al.*, 2015).

1.9 Aims

The first barrier EBOV must overcome to successfully infect a host is cell entry. Since this step is facilitated by the GP, the primary aim of this project was to determine the intraspecies GP variation among EBOV strains that circulated between the first outbreak ever recorded (1976) and the West African outbreak that ended in 2016. Such an approach was used to test the hypothesis that genetic variations may impact the GP's primary function in cell entry and therefore, the infectivity of EBOV strains in different cell lines. To this end, a panel of replication-deficient lentiviral PVs representative of specific outbreak strains were generated and used to establish the impact of genetic variation on the infectivity of each strain. This was accomplished through a series of *in-vitro* experiments where PVs were used to challenge cell lines representing different EBOV hosts and reservoirs. In doing so, the aim was to identify specific genetic signatures, which might impact the infectivity of the virus. As well as determining the effect on viral infectivity, this work could be further expanded to investigate whether intraspecies genetic variation may impact the sensitivity of the virus to existing therapeutics and to inform future improved designs, ensuring they account for the variation that EBOV displays.

In addition to factors such as environment and public health, viral evolution is known to significantly impact epidemic spread and disease severity. Therefore, this project's additional aim was to define the impact of the long-term evolution of EBOV on its infectivity and tropism. This was achieved by determining the evolutionary relationship between ancestral node GP sequences (inferred by Professor Edward Holmes, University of Sydney) and the outbreak GP sequences described above. Subsequently, the impact of genetic differences between ancestral and outbreak sequences on EBOV fitness was also determined in a series of infectivity assays.

Moreover, it is known that EBOV can circulate in the environment within a reservoir species between outbreaks. There is also evidence to suggest that fruit bats are this reservoir, although this has not been definitively proven. Therefore, it was also hypothesised that, compared to their outbreak-derived counterparts, ancestral node GP sequences could contain genetic signatures that favoured infection of cells derived from a potential reservoir species (Hyp/Lu 45.1), with the opposite being true for cells from a human host (HuH-7). To prove this, PV assays were used to determine the difference in infectivity between outbreak GP sequences and ancestral node sequences in the cell lines mentioned above. In doing so, this work aimed to provide more insight into the current theory that fruit bats are the reservoir species for EBOV.

The final aim of this project was to determine if any naturally-occurring polymorphisms within NPC1 genes of humans living in different geographic areas could impact their function as EBOV receptors. This enabled the identification of genetic signatures intrinsically present in some populations that may confer increased susceptibility or resistance to zoonosis and any subsequent human transmission events. Consequently, public health policies could be informed to improve future outbreak management and responses. Notably, since EBOV outbreaks are traditionally restricted to Africa, it was hypothesised that some NPC1 mutations that may be enriched in African populations (due to the selective pressure resulting from small and restricted outbreaks) may not be observed in other populations at similar frequencies and that these mutations may confer increased resistance to infection relative to the genetic signatures observed in wild-type NPC1. This was assessed by generating cell lines that could stably express NPC1 receptor variants and challenging them with EBOV PVs. Analysis of the NPC1 sequence of different animal hosts and their expression in a similar model may also help to define species determinants that shape EBOV entry, thus providing an improved understanding of spillover events that have occurred in the past.

2. Materials and Methods

2.1 Bacterial Cells

The *E. coli* cells, Stellar competent cells (TakaraBio) and STBL3 competent cells (Thermo Fisher) were used in the maintenance of plasmids containing the EBOV *GP* gene and the human *NPC1* gene variants, respectively. Cells were grown in Luria-Bertani (LB) medium or broth with Ampicillin (100 µg/mL). Stellar cells were grown at 37 °C, whereas STBL3 cells were grown at 30 °C. Glycerol stocks of all cultures were made by adding equal volumes of culture and 50% glycerol. Cultures were vortexed before storage at -80 °C freezer, for future use.

2.2 Mammalian Cell Lines

Human Embryonic Kidney 293T (HEK293T), HuH-7 human hepatoma, A549 human lung carcinoma and Vero E6 African green monkey kidney cell lines were obtained from the European Collection of Authenticated Cell Cultures. These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, 41965), containing high glucose (4.5g/L), without HEPES and with pyruvate, which was supplemented with 10% fetal bovine serum (FBS; Gibco) and 0.1 mM nonessential amino acids (NEAA; Gibco), at 37 °C and 5% CO₂. Although the absence of mycoplasma was verified, no steps were further taken to verify them following their receipt.

Hyp/Lu 45.1 bat cells were obtained from Dr Marcel Müller (Kühl *et al.*, 2011) and were grown in DMEM supplemented with 10% FBS, 0.1 mM NEAA and 0.1 mM sodium pyruvate (Gibco), at 37 °C and 5% CO₂.

NPC1 Null U2OS cells were kindly gifted by Dr W. E. Diehl (University of Massachusetts). These cell lines had been generated by CRISPR knockout. Positive cells were selected with blasticidin. A control cell line, the U2OS BST2 stuffer cell line, was also provided. These cells contained the *BST2* gene within the gRNA, preventing successful CRISPR knockout. The cells were grown in DMEM supplemented with 10% FBS and 0.1 mM

NEAA, at 37°C and 5% CO₂. Transduced U2OS cells were maintained in the same media, with an additional supplement of either 0.5 µg/ mL or 1.25 µg/ mL of puromycin.

All cell lines were passaged every 72 and 96 hours after confluency of approximately 80% was obtained. After the removal of excess media from the flasks, cells were washed with phosphate-buffered saline (PBS, Sigma Aldrich). Cells were then incubated with 0.05% Trypsin-EDTA (Gibco), for 5 minutes at 37 °C. An equal volume of supplemented DMEM was then added to the cells to neutralize the trypsin, after which the cells were centrifuged at 300 x *g* for 7 minutes in a swinging bucket rotor (Beckman Coulter). 10 µL of cell suspension was removed before centrifugation, to obtain an accurate cell count via haemocytometry. Cell pellets were resuspended in supplemented DMEM and seeded into either a 75 cm² or a 175 cm² Corning flask, between 1.5 x10⁶ and 3 x 10⁶ cells per flask. For longer-term storage in liquid nitrogen, cells were resuspended in a freezing medium containing 90% FBS and 10% Dimethyl sulfoxide (DMSO) at a density of approximately 6 x 10⁶ cells per mL. Cells were aliquotted into cryovials, which were placed into a receptacle containing 100% isopropanol. This was frozen at -80 °C for a minimum of 48 hours before the cryovials were transferred into liquid nitrogen.

2.3 Determining EBOV Ancestral Node Sequences

The previous reconstruction of EBOV ancestral node sequences, representative of the most common recent ancestors (MRCAs), was completed by Professor E. Holmes' laboratory (The University of Sydney). Briefly, a bootstrap phylogenetic tree based on amino acid sequences was estimated using PhyML using WAG and JTT substitution models. Node sequences were estimated using the joint and marginal approaches. Posterior probabilities for the marginal reconstruction revealed five amino acid positions with low support (310, 377, 378, 422, and 443). Amino acids with the highest probability were chosen for inclusion in the MRCAs.

Node constructs were then synthesised in-house by colleagues, via site-directed mutagenesis (SDM) of a plasmid construct containing the Zaire EBOV Mayinga strain GP gene [GenBank: U23187.1], kindly provided by Dr F. Rey (Pasteur Institute), or a construct obtained from Professor G. Kobinger (Laval University) containing the Zaire EBOV Makona C07 GP gene [GenBank: MG572233.1]. Both these constructs contained a pcDNA3.1+ backbone. In instances where SDM on these two constructs was not possible,

pcDNA3.1+ constructs containing the desired node sequences were commercially synthesised via the GeneArt gene synthesis service (Thermo Fisher Scientific).

2.4 Determining EBOV Outbreak GP Panel Candidates

GP constructs within the mammalian expression vector pcDNA3.1+ (Addgene) that were representative of the first EBOV outbreak (DRC; 1976; Mayinga strain) and the West African outbreak (2013-2016; Makona C15 variant) had previously been obtained or synthesised in-house by colleagues. An exhaustive search of the GenBank (NCBI) database was completed to determine which further EBOV GP sequences were to be included in a mutant panel that was representative of all other outbreak strains up to the West African outbreak.

Briefly, all outbreak-specific sequences were filtered by date (up until the West African Outbreak) and sequence length (2000-20,000 base pairs). Glycoprotein and full genome sequences that had been isolated from samples collected from non-human primates were excluded. This led to the collation of both GP and whole-genome sequences for which, a multiple genome alignment was completed using the MUSCLE algorithm available within the MEGA7 software package. Whole-genome sequences were edited to isolate the GP sequence specifically and all sequences were further modified at base 877 to include an extra adenosine base, thus ensuring the correct open reading frame and preventing the early truncation of the GP sequence with a premature stop codon.

Ancestral node sequences and sequences from the first and West African outbreaks were also included in the alignment. Sequences were translated and the MEGA7 software was used to complete a model test to determine the recommended amino acid substitution model that should be applied to the alignment of sequences. As a result, the final maximum likelihood tree was generated with a JTT+G model of substitution and a bootstrap value of 1000, based on translated amino acid sequences. Eight outbreak sequences, representative of each prominent clade of the phylogenetic tree, were selected to be tested alongside the already generated clones of Mayinga and Makona strain variants, leaving a final panel of ten outbreak EBOV GP mutants. Alignments and trees have since been updated with sequences that have become available for more recent outbreaks, although sequences have been limited to those that emerged early within the outbreaks.

2.5 Generation of the EBOV GP Panel Candidates via SDM

Phylogenetic analysis permitted the identification of the nearest ancestral node sequence, for each chosen outbreak sequence. Alignments for each outbreak sequence and the closest ancestral node sequence were completed to serve as a template for SDM (New England Biolabs; NEB). For each outbreak strain, all non-synonymous mutations were identified, relative to the chosen template node sequence.

2.5.1 Primer Design to Insert Point Mutations to Template Sequences to Generate Outbreak Sequences

Primers (Table 2.1) to insert each desired mutation were designed and annealing temperatures were determined using the online tool, NEBaseChanger (NEB), synthesised by Eurofins and consequently used as part of the Q5 SDM kit (NEB), as per manufacturer's instructions. The desired single-base mutation was incorporated into the forward primer in all instances.

2.5.2 Exponential Amplification of GP Template to Generate Outbreak Variants

The constructs pcDNA3.1_N21 and pcDNA3.1_N26 served as templates for all mutagenesis pathways. Amplification of the template to include the desired mutation was completed through polymerase chain reaction (PCR) using the Q5 polymerase, as per the manufacturer's instructions. Briefly, plasmids were diluted in nuclease-free water (1:100) to achieve a recommended concentration between 1 and 25 ng/ μL . 1 μL of diluted plasmid was used as a template for mutagenesis in each reaction, in addition to 6.25 μL of Q5 Hot Start High Fidelity 2 x Master Mix, 0.625 μL of both forward and reverse primer (10 μM) and 4.5 μL of nuclease-free water to obtain a total reaction volume of 12.5 μL . Recommended cycling conditions (Table 2.2) were used with an extension time of 4 min and 35 cycles of amplification. Successful amplification was visualised via gel electrophoresis utilising a 2% gel, as described in Section 2.5.3. In instances where amplification was not observed, the PCR reaction was optimised by varying the concentrations of the template and by implementing a range of annealing temperatures (55-72 $^{\circ}\text{C}$) using a gradient PCR thermal cycler.

This methodology was also used to insert the I527T mutation into the N21 construct and revert the mutation (T527I) in the Mbandza 2003 construct (Table 2.1) when investigating the impact of these singular, internal fusion loop mutations on the infectivity of the GP.

Table 2.1 Templates and primers used to insert mutations into template GP sequences

These were used to generate the desired outbreak sequences. Mutations in bold were present in multiple sequences. Mutation F676L, present in DRC Etoumbi 2005, was inserted via InFusion cloning after SDM was unsuccessful. InFusion cloning was also used to ligate amplified Mbandza 2003 GP sequence into Eco-RI digested pcDNA3.1.

Outbreak Strain	Accession Number	Template Used	Mutations to be inserted	Primers, Forward and Reverse (5'-3')
Gabon 1994	KC242792.1	pcDNA3.1_N26	Q376R – CAA ->CGA	CCCATACCAAACCAGGTCCGGA
			T379I – ACA ->ATA	GGGTCGGAGACTCGTGGAGATTGTG
Gabon 1996	AY058898.1	pcDNA3.1_N26	R219K – AGA ->AAA	CCCATACCAAACCAGGTCCGGAC
			L368P – CTT ->CTT	AGAATAGTAGCCACTAGAC
			A474T – GCC ->ACC	CTAACAACCCCTGCCACAATC
				AGAATAGTAGCCACTAGAC
Gabon 2001	KY471124.1	pcDNA3.1_N21		AGAAGAGAGTACCAGCAGCGG
				CCGGTATCTTGGTGATGAG
			H455Y – CAC ->TAC	CCCCCAAACCTACAGCGAGACTG
			T483A - ACC ->GCC	CTTGTCGTGGTGGCGGGG
DRC Mbandza 2003	EU051635.1	pcDNA3.1_N21	A349T – GCA ->ACA	AGGCTTAATTGCCAATACTATTGC
			T373A – ACG ->GCG	TGGAGTAGCAGG
			H455Y – CAC ->TAC	AGCTTCCCGCTGCCGGCA
				AAATTCCTCTACAATGGTTCAAG
			I527T – ATC ->ACC	TCTGAAGCCATGATTTTG
DRC Kelle 2003	EU051635.1	pcDNA3.1_N21		CACAATCTCCGCGAGTCCTCA
				GCAAGGGTTATCAGATGC
				CCCCCAAACCTACAGCGAGACTG
				CTTGTCGTGGTGGCGGGG
				GGTGCTGCAACCGGATTGGCC
DRC Kelle 2003	EU051635.1	pcDNA3.1_N21	E337G - GAA ->GGA	TTCATCCTGAGTAGTCCAGTAATG
			N346D – AAT ->GAT	TAAATTG
			L368F – CTT ->TTT	ACAACAACCTGGAGACCACAAAATC
				GTAGGTCTCTGGGTCGGA
			L481S – TTA ->TCA	GGCTTCAGAAGATTCTCTGC
DRC Kelle 2003	EU051635.1	pcDNA3.1_N21		ATGATTTTGTGGTCTTCAG
				TCTGATAACCTTTGCCACAATCTC
				C
				TGCGACACTGCAGCTTCC
				AAGCTAGGCTCAATTACCAATACT
DRC Kelle 2003	EU051635.1	pcDNA3.1_N21		ATTGCTGGAGTAG
				CCCGCTGCCGGCACTCTC

			V661G – GTT -> GGT	GTTACAGGTGGTATAATTGCAG TCCAATACCTGCCGGTAT
			S319G – AGT -> GGT	CAAAGACATCGGTGGTCAGAG GCTCCGTTTGATACAGCTG
			A349T - GCA -> ACA	AAATTCCTCTACAATGGTTCAAG TCTGAAGCCATGATTTTG
			H455Y – CAC -> TAC	CCCCCAAACCTACAGCGAGACTG CTTGTCGTGGTGGCGGGG
			F676L - TTT -> CTT	GCAAATTTGTCCTTAGATGGG ATATACAGAATAAAGCGATAAC
DRC Etoumbi 2005	EU051634.1	pcDNA3.1_N21		
DRC Luebo 2007	KC242790.1	Commercially synthesised	N/A	N/A
DRC Lomela 2014	KP271018.1	Commercially synthesised	N/A	N/A
DRC Mbandza 2003 sub-clones	N/A	DRC Mbandza 2003 (pcDNA3.1_N21_H4 55Y_A349T_T373A_I527T)	N/A	CAGTGTGGTGGGAATTCACCATGG GTATTACAGG GATATCTGCAGAATTCATAAAGAC GGTGCTGCAACCGGATTGGCC
N21_I527 T	N/A	pcDNA3.1_N21	I527T – ATC -> ACC	TTCATCCTGAGTAGTCCAGTAATG TAAATTG
Mbandza_T527I	N/A	DRC Mbandza 2003 (pcDNA3.1_N21_H4 55Y_A349T_T373A_I527T)	T527I - ACC ->ATC	GGTGCTGCAATCGGATTGGCC TTCATCCTGAGTAGTCCAGTAATG TAAATTAGGGTTGC

Table 2.2 Cycling conditions used for SDM reactions

These were the basic perimeters used to ensure the mutagenesis of different plasmid templates.

Step	Temperature (°C)	Time (sec)
Initial denaturation	98	30
35 cycles	98	10
	50-72	10-30
	72	20-30/kb
Final Extension	72	120
Hold	8	-

2.5.3 Gel Electrophoresis

All PCR products were visualized under UV light after running 2% (w/v) agarose-gel electrophoresis with ethidium bromide (EtBr). 2 g of agarose was heated in 100 mL of 1 x TAE buffer. After cooling, 5 µL of EtBr was introduced to obtain a final concentration of 10 mg/ mL and the gel was subsequently cast. 2.5 µL 10 x FastDigest Green Buffer (Thermo Fisher) was added to PCR products. From this mix, 5 µL of the product was loaded into each well. A GeneRuler 1 Kb Ladder Mix (Thermofisher) was also loaded to aid in

determining the size of any bands. Gels were run in 1 x TAE buffer containing 30 μL EtBr, again, at a final concentration of 10 mg/mL, under a voltage of 90 V for 36-42 minutes. Bands were visualised under a UV light and desired products were confirmed via Sanger sequencing (Source Bioscience), as described in Section 2.5.6.

2.5.4 Kinase, Ligase and Dpn1 (KLD) Treatment of PCR Products and Transformation of Stellar Cells

KLD ligation of PCR products was completed as per the manufacturer's instructions (NEB), except for the use of half-size reaction volumes. Briefly, 0.5 μL of PCR product was incubated with 2.5 μL of 2 x KLD reaction buffer, 0.5 μL of 10 x KLD enzyme mix and 1.5 μL of nuclease-free water, for 5 minutes at room temperature. 2.5 μL of ligation product was added to 25 μL of Stellar competent cells, which were subsequently incubated on ice for 30 minutes. Cells were then heat shocked at 42 °C for 45 seconds and immediately re-incubated on ice for a further 5 minutes. 250 μL of SOC media was added to allow for the recovery of cells during a 1-hour incubation at 37 °C in a shaking incubator (250 RPM; C24 incubator shaker, New Brunswick). The cells were then plated onto an LB-ampicillin (100 $\mu\text{g/mL}$) agar plate and grown overnight at 37 °C.

2.5.5 Colony Screening for Mutants by PCR

Successful mutagenesis, ligation and transformation of plasmid were identified via colony screen PCR. 0.075 μL Hotstart Taq Polymerase (5 units/ μL , Qiagen) was used as per the manufacturer's instructions in a 15 μL reaction volume, which also included 1.5 μL PCR Buffer, 0.3 μL of both the vector-specific forward (T7F; 5'-TAGAAGGCACAGTCGAGG-3') and reverse (bGHR; 5'-TAATACGACTCACTATAGGG-3') screening primers (10 μM), 0.6 μL of dNTPs (10 mM of each), and 12.225 μL of DEPC-treated water. Single colonies were selected from the LB-ampicillin plate and added to the reaction mix. Colonies were also stabbed onto fresh LB-ampicillin to permit identification after PCR confirmation. Recommended cycling conditions (Table 2.3) were used, with a 2-minute extension time, and an annealing temperature of 55 °C and 35 cycles. Positive colony-PCR products were confirmed via Sanger Sequencing (Source Bioscience), once again, using the T7F and bGHR primers. Positive colonies were then inoculated into LB broth with ampicillin and incubated overnight at 37 °C.

Table 2.3 Cycling conditions used for colony screening PCR reactions

Colony screening via PCR was essential in determining which colonies had been correctly transformed with plasmids containing the desired mutation.

<i>Step</i>	<i>Temperature (°C)</i>	<i>Time (min/sec)</i>
Initial denaturation	95	15 min
35 cycles	95	20 sec
	55	20 sec
	72	1 min/kb
Final Extension	72	2 min
Hold	8	-

2.5.6 Plasmid Extraction, Quantification and Confirmation of Product

Plasmid extraction was completed by either GenElute Miniprep or Midiprep kits (Sigma-Aldrich), depending on the desired volume of the plasmid. The kits utilise a spin column and vacuum method for plasmid recovery, respectively. The manufacturer's protocols were followed with no alterations with all centrifugation steps undertaken at a maximum force of 14,000 x *g*. Briefly, bacterial pellets were resuspended and lysed for no longer than 5 minutes after which a neutralisation solution was added. The suspension was centrifuged for 10 minutes to separate the cell debris and the lysate was then loaded onto a binding column. The column was once again centrifuged and the flow-through was discarded before the columns were washed with solutions containing ethanol. The columns were dried for 2 minutes before the plasmid was eluted into a volume of 100 µL.

Subsequently, plasmids were quantified by spectrophotometry (NanoDrop One; Thermofisher). Plasmid concentrations were read at a wavelength of 260 nm and reported in ng/ µL. Purity was reported as 260/280 and 260/230 ratios. Plasmids were verified by Sanger sequencing (Source Biosciences), using the T7F and bGHR primers. The electropherograms generated were visualized by Finch TV software and the Nucleotide BLAST tool (NCBI) was used to confirm the sequences' identities.

2.6 Generation EBOV GP Panel Candidates via Infusion Cloning

For outbreak sequences that required generation by more than six non-synonymous mutations within a pathway (Table 2.1) the GP sequence construct was commercially synthesised via GeneArt (Thermo Fisher). These constructs also include vector-specific In-Fusion tags for the EcoRI site that is present in the vector, pcDNA3.1+, which would permit cloning of the GP gene into a linearized form of the vector.

In-Fusion cloning was also used to insert the final mutation required to achieve the outbreak GP sequence DRC Etoumbi 2005, for which SDM was unsuccessful (Table 2.1). To complete this, the primer design for InFusion cloning was completed using the Primer3 Web tool. Briefly, the first and last 20 bases of the desired gene sequence were determined and increased or decreased in length as necessary to obtain an annealing temperature between 55 and 60 °C. A Kozak sequence (CACC) was then added in the 5' region of the forward primer to ensure subsequent expression in mammalian cell lines. Finally, 15 base pair overlaps, homologous to the desired site of insertion in the linearized vector (EcoRI restriction site), were included in both the forward and reverse primers. Insertion of the specific mutation at the end of the *GP* gene sequence was ensured by designing the reverse primer to include the desired base mutation in the region of sequence homologous to the end of the gene. These primers were used in a PCR reaction to amplify the *GP* gene present in a pcDNA3.1+ template produced as part of SDM pathways, as described in Section 2.5.2 and the resulting products, were visualised via gel electrophoresis as described in 2.5.3.

This cloning method was also used to ligate the amplified Mbandza 2003 PCR products generated with EcoRI In-Fusion tags (Table 2.1) with linearised pcDNA3.1+ to generate the Mbandza 2003 sub-clones.

2.6.1 Digestion of Plasmid Vector

The chosen plasmid for both ancestral and outbreak GP expression was pcDNA3.1+, a high copy number plasmid, containing a human CMV promoter, multiple cloning sites, the bovine growth hormone (BGH) polyadenylation signal for termination and polyadenylation of mRNA, and Ampicillin-resistant gene (Invitrogen). The vector was digested with the EcoRI enzyme (New England Biolabs) for use in InFusion cloning. Approximately 10 µg of plasmid was used in a 50 µL restriction digest.

Briefly, 5 µL of EcoRI enzyme, 5 µL 10 x CutSmart Buffer (New England Biolabs), 30 µL of plasmid and 10 µL of water were incubated overnight at 37 °C. The enzyme was then heat-inactivated following incubation at 65 °C for 20 minutes. The enzyme was removed using a NucleoSpin Gel and PCR Clean-up (Machery-Nagel) as per the manufacturer's instructions with all centrifugation steps undertaken at 11,000 x *g*. Briefly, the plasmid was added to two volumes of buffer NT1 and transferred to a NucleoSpin binding column. The binding column was centrifuged and the flow-through was discarded. The silica membrane

was then washed with buffer NT3 and the membrane, dried via a further centrifugation step. The digested plasmid was then eluted into 15 µL of elution buffer and quantified using spectrophotometry, as described in Section 2.5.6.

2.6.2 Ligation Reaction

Optimal concentrations of tagged inserts were between 50 and 100 ng/µL for use in the ligation reaction. Briefly, 1 µL of digested plasmid, 1 µL of insert and 0.5 µL of InFusion enzyme mix (Takarabio) were incubated at 50 °C for 15 minutes. Following this, the transformation of Stellar competent cells, colony screening and plasmid extraction were completed, as described in Sections 2.5.4 to 2.5.6.

Table 2.4 Ancestral and outbreak GP sequences that were tested in cell entry assays

All GP sequences were cloned into the pcDNA3.1 expression vector.

<i>Node or Outbreak Strain</i>	<i>Plasmid Name</i>
Node 1	pcDNA3.1_N1
Node 3	pcDNA3.1_N3
Node 21	pcDNA3.1_N21
Node 22	pcDNA3.1_N22
Node 23	pcDNA3.1_N23
Node 26	pcDNA3.1_N26
Mayinga 1976	pcDNA3.1_May1976
Gabon 1994	pcDNA3.1_N26_QT376+9RI
Gabon 1996 Bouee	pcDNA3.1_N26_R219K_L368P_A474T
Gabon 2001	pcDNA3.1_N21_H455Y_T483A4
DRC 2003 Mbandza	pcDNA3.1_N21_H455Y_A349T_T373A_I527T
DRC 2003 Kelle	pcDNA3.1_N21_H455Y_A349T_S319G_F676L
DRC 2005 Etoumbi	pcDNA3.1_N21_N346D_E337G_L368F_L481S_V661G
Luebo 2007	pcDNA3.1_Lue07
Lomela 2014	pcDNA3.1_Lom14
Makona C15	pcDNA3.1_MakC15
Mbandza_2003_Subclone_4/5	pcDNA3.1_Mbandza_2003_Subclone_4/5
N21_I527T	pcDNA3.1_N21_I527T
Mbandza_T527I	pcDNA3.1__H455Y_A349T_T373A_T527I

2.7 Pseudotype Production via HEK293T Cells

A three-plasmid lentiviral gene expression system was used for the production of EBOV PVs (Figure 2.1). This included the packaging plasmid phCMV5349, a Murine-Leukaemia Virus (MLV) based construct containing Gag/Pol genes. Additionally, a transfer plasmid

pTG126, containing a ψ packaging signal and flanking MLV long terminal repeats, as well as a luciferase reporter gene under the control of a Cytomegalovirus (CMV) immediate early promoter, was used. Both the packaging and transfer plasmids were a gift from Francois-Loic Cosset (Bartosch, Dubuisson and Cosset, 2003). The final component of the system was the pcDNA3.1+ based GP constructs as discussed throughout Sections 2.5 and 2.6. The protocol followed was previously described by Urbanowicz *et al.* (2016).

Briefly, HEK293T cells were seeded into 10 cm Primaria-surface coated tissue culture dishes (Corning) at a density of 1.5×10^6 cells per dish. 10 mL of supplemented DMEM (Section 2.2) was added and cells were incubated overnight at 37 °C and 5% CO₂. After reaching a confluency of approximately 50%, cells were transfected with 2 µg of both the backbone and reporter plasmids and 200 ng of the GP construct alongside 24 µL of the transfection reagent 25 kDa, linear polyethylenimine (PEI; Polysciences, Germany; diluted to 1 mg/ mL), and OptiMem (GIBCO) resulting in a total reaction volume of 600 µL. The cells were then further incubated with 7 mL of OptiMem for 6 hours at 37 °C. At this point, the OptiMem was replaced with 10 mL of supplemented DMEM and incubated for a further 72 hours at 37 °C. PVs were harvested by passing the supernatant through a 0.45 µm filter (Sartorius). If not immediately used, PVs were stored at 4 °C. Positive controls were made using phCMV5349, pTG126 and the pMD2.G construct, which encodes the Vesicular Stomatitis Virus (VSV) G protein. pMD2.G was a gift from Didier Trono (unpublished; available on Addgene, plasmid number 12259). For negative controls, only the packaging and transfer vectors were transfected.

2.8 Infectivity Assays

Infection assays were performed using HuH7, Vero E6 and Hyp/Lu 45.1 cells as well as U2OS variant cell lines described in Section 1.2. Solid-bottom 96-well corning plates (Costar) were seeded with 2×10^4 cells per well and incubated overnight at 37 °C and 5% CO₂. Triplicate wells were then infected with 100 µL of each PV and incubated for four hours, after which, an additional 150 µL of DMEM was added to the wells. The plates were incubated for 72 hours. 50 µL of 1:5 diluted luciferase lysis buffer (Promega, E1501) was added to each well and the plates then were placed for 15 minutes on a shaker at a speed of 20 RPM. Luminescence was then detected using a luminometer equipped with direct injection (BMG Labtech FluoStar Omega Plate Reader). For this machine, the PMT gain was set to 3600. The machine was programmed to inject 50 µL of luciferase substrate

(Promega), shake the plate for two seconds and read luminescence immediately afterwards for one second as relative light units (RLU values).

All data was analysed using the software, GraphPad Prism. Normality tests were completed on datasets using the software, to determine a normal distribution of data. The type of normality test completed was dependent on the number of pooled experimental repeats obtained for an experiment. A Kolmogorov-Smirnov test or a Shapiro-Wilk test were primarily used as these could be completed in cases where the number of experimental repeats were fewer. In cases where data failed these tests, the results of an Anderson-Darling test and a D'Agostino & Pearson test were also taken into consideration to determine the distribution of data. Normalised, pooled data were presented as either box plots showing minimum and maximum values, or bar graphs showing standard deviation (SD).

Statistical analysis was completed on normally distributed data using a parametric analysis of variance (ANOVA) test with additional multiple comparisons testing. A one-way ANOVA was used when only one variable was measured within an experiment i.e. the GP clone and a two-way ANOVA was used when one variable was measured against a second variable in an experiment i.e. comparing the difference in values of a GP clone in multiple cell lines. Based on software recommendations, either a Dunnett's multiple comparisons test (for a one-way ANOVA in which the mean of all variables was compared to that of a control), a Tukey's multiple comparisons (for a one-way ANOVA where the mean of each variable was compared with each other) or a Sidak's multiple comparisons (for a two-way ANOVA in which the mean of each variable was compared with each other), was completed.

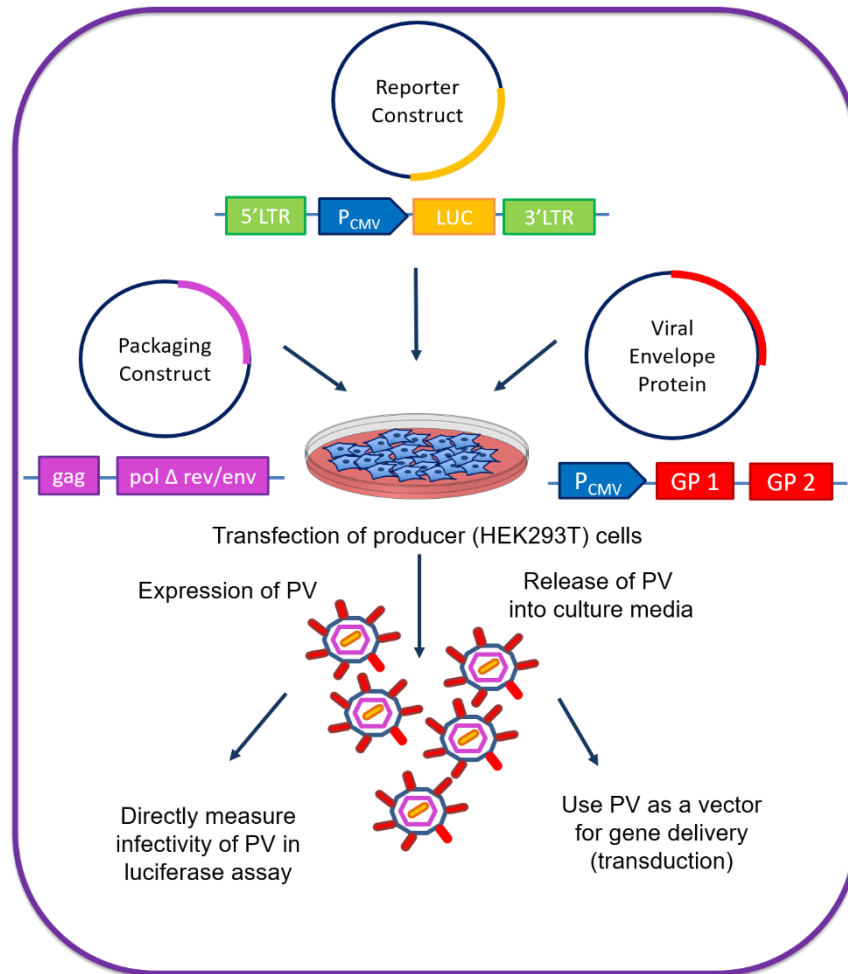


Figure 2.1 A three-plasmid lentiviral packaging/ gene delivery system

This system was used in the following: A) Production of PVs containing a Murine-Leukemia Virus (MLV) backbone, a luciferase reporter gene and the variant EBOV GPs. B) Production of a virion containing a Human Immunodeficiency Virus (HIV) backbone, the variant NPC1 genes tagged with GFP or a selection marker and the Vesicular stomatitis Virus (VSV) GP, as a transduction unit to deliver NPC1 variants to null cell lines.

2.9 Measuring GP Expression in PVs

2.9.1 Concentration of PVs via a 20% Sucrose Cushion

PVs were concentrated by a 20% sucrose cushion. Briefly, a 20% sucrose solution was prepared using distilled water and filtered through a 0.45 μ M filter. 1 mL of the solution was pipetted into a 10.4 mL polycarbonate ultracentrifuge tube (Beckman Coulter). Approximately 9 mL of culture supernatant containing PVs were slowly layered on top of the sucrose solution using a fine-tip Pasteur pipette, with extra care taken to ensure that the interface between the two solutions was left undisrupted. Tubes were prepared this

way in pairs to act as balances for each other in the centrifuge and were marked to ensure the position of the site of the pelleted PVs. PBS was used to adjust any differences in mass between the tubes. A 70 Ti fixed angle rotor was chilled to 4 °C in the ultracentrifuge (Beckman Coulter). Samples were centrifuged at 40,000 RPM, for 2.5 hours. Following the centrifugation cycle, the supernatant was carefully removed from each tube with a fine-tip Pasteur pipette, without disrupting the pellet. The pellet was then resuspended overnight in 30 µL of sterile PBS (Sigma Aldrich).

2.9.2 Western Blot to Determine GP Expression

Western blot was completed to determine GP and core protein expression in the PV preparations. All antibody dilutions were made in a blocking solution, consisting of PBS containing 0.01% tween 20 (PSB-T), supplemented with 10% milk powder. Further, all antibody incubation steps were completed at room temperature, on a plate rocker, for 1 hour and all membrane wash steps following an antibody incubation comprised of three, 15-minute washes in PBS-T.

Firstly, PV resuspensions were mixed with 4 x Laemmli buffer (Bio-Rad) and heated at 95°C for 10 minutes. Samples then were loaded in duplicate onto pre-cast, 10% SDS-PAGE gels (Bio-Rad), alongside a spectra multi-coloured ladder (Abcam). The gels were placed in a gel tank containing running buffer (25 mM tris base, 190 mM glycine, 0.1% sodium dodecyl sulphate) and an electric field of 90 volts was applied for 40 minutes. Following this, the gels were placed on top of methanol-activated, PVDF membranes and were sandwiched between three layers of filter paper, which had been pre-soaked in transfer buffer (25 mM tris-base, 192 mM glycine, 20% methanol). The sandwiches of filter paper, gel, membrane and additional filter paper were placed in a TransBlot SD semi-dry transfer cell (Bio-Rad), where a constant current of 100 mA was applied for 1 hour.

Ponceau S (Sigma Aldrich) staining was subsequently completed on the membranes to ensure successful protein transfer. The membranes were then washed with PBS-T and then blocked overnight at 4 °C in blocking solution. Following this, membranes were incubated with 10 mL of the primary antibodies; a pan-filovirus α-GP mouse monoclonal antibody (21D10; IBT) diluted to a concentration of 4 µg/mL (1:250) and an α-MLV p30 mouse antibody (ab130757, Abcam), diluted to a concentration of 0.67 µg/mL (1:1500). The membranes were then washed before subsequent incubation with 10 mL of the secondary antibody, a rabbit, α-mouse HRP (P0399; Dako) diluted to a concentration of

0.65 µg/mL (1:2000), after which, the membranes were washed again. Finally, they were incubated with Super Signal West Pico Plus Chemiluminescent Substrate (Thermoscientific), as per the manufacturer's instructions. Bands were visualised by a G-Box (SynGene) with images taken at different exposure times.

2.9.3 Densitometry to Quantify GP and p30 Protein Expression

Quantification of ancestral and outbreak GP expression was completed using the images of the western blots that were produced by the G-Box (SynGene). The images were uploaded as TIFF files onto the software ImageJ and converted to an 8-bit format. Bands in each lane were highlighted using the selection tool. Plot profiles were produced for each lane, representing the relative density of contents of each band that was selected. On each plot profile, peaks were closed off using the straight-line tool to remove the background from each peak of interest. Each peak was then highlighted and a results table was generated to determine the area of each peak and the percentage of the total size of all of the highlighted peaks. These values were then used to work out relative densities for each peak, corresponding to GP or core expression for each PV. Raw RLU values obtained through infectivity assays were then normalised to these results, with data analysed in GraphPad Prism 9.

2.10 Generation of the NPC1 Variants in a pHIV-eGFP Lentiviral Vector

2.10.1 Determining Relevant SNPs in NPC1

A search of the ENSEMBL Genome browser led to the identification of 21 missense allelic variants of human NPC1 that existed in a frequency of greater than 1% in specific populations. Of these, two specific SNPs of interest were highlighted as existing as a haplotype, only within NPC1 of African populations: P434S and M642I.

A low-resolution, partial structure for NPC1 complexed with cleaved GP was obtained from Wang *et al.* (2016). The deposited PDB (5F1B) was retrieved and visualized using the molecular visualisation software, PyMOL. In this structure, the cleaved glycoprotein was highlighted in orange, and domain C was highlighted in blue. The Phyre2 homology modelling software was then utilised in the generation of the NPC1 haplotype identified in African populations. The amino acid sequence for the haplotype, containing the specific

SNPs, was inputted into the software web server and modelling was completed using 'normal' mode. A model with a confidence that was closest to 100% was selected and also visualised on PyMOL. In this ribbon model, domain C was highlighted in magenta, and the remainder of NPC1 was highlighted in yellow. The P434S and M642I mutations were visualised as cyan and red sticks, respectively. An overlay was then generated by aligning the Phyre2-generated haplotype model of NPC1, to the partial NPC1 structure, complexed with cleaved GP (5F1B).

Alternatively, protein-protein docking was also performed by colleagues using ClusPro2.0 to generate the complex between the NPC1 haplotype model and cleaved GP1 structure (PDB 5F1B). To generate the complex, restraints needed to be applied in ClusPro2.0, based on published interactions cited by Wang *et al.* (2016). A restraint group file was created to limit docking confirmations by specifying pairs of interacting residues, one from NPC1 and (Y423, P424, F503, F504, and Y506) and one residue from the cleaved GP. The specific contacts with an acceptable distance range were listed by Wang *et al.* (2016) and the group was set to 50% as the required percentage of restraints. The generated file was subsequently added to the ClusPro2.0 docking job with the "restraints" option. The resulting complex with the highest score was selected based on the largest group member with the lowest energy and the selected model with the top score was analysed for detailed structural characterization. PyMOL was used to overlay this structure, with 5F1B and visualised to determine any further differences in interaction between NPC1 haplotype and the cleaved GP.

2.10.2 RNA Extraction and cDNA to obtain Template for NPC1 Amplification

RNA was extracted from A549 cells by colleagues through the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Briefly, approximately 6×10^6 cells were harvested and pelleted. Cells were lysed on ice and mixed with one volume of 70% ethanol. Lysed cells were transferred to a spin column and washed with buffers RWI and RPE. The membranes were then dried by centrifugation of the column at a maximum speed at $14,000 \times g$ for 1 minute and RNA was then eluted into 50 μ L RNase-free water. RNA was then quantified via spectrometry, as described in Section 2.5.6 and samples were stored in a -80°C , freezer for long-term usage.

cDNA was synthesised using the RNA to cDNA EcoDry Random Hexamer Premix kit (Takara Clontech), as per the manufacturer's instructions. Briefly, 3 μ g of RNA was added

to RNase-free water, to achieve a total volume of 20 μ L. This was added to lyophilised random hexamers and the reaction mixture was then incubated at 42 °C for 1 hour. The reaction was terminated by heating the sample to 70 °C for 10 minutes. After synthesis, cDNA was stored in a -20°C freezer.

2.10.3 Design of pHIV-eGFP containing an NPC1 Cassette

The Lentiviral mammalian expression vector pHIV_eGFP (Addgene), as referenced by Welm *et al.* (2008) was digested by BamHI restriction enzyme (NEB) as described in Section 2.6.1. The introduction of a cassette fragment containing a Kozak sequence, the NPC1 signal peptide (bases 1 to 81) and an NsiI restriction site was completed via Infusion cloning. The inclusion of the cassette was to permit the universal expression of NPC1, whilst the addition of the NsiI restriction site would ensure the remainder of the NPC1 gene could be inserted in-frame. Primers pHIV-NsilcasF and pHIV-NsilcasR (Table 2.5) were used to amplify the NPC1 signal peptide with BamHI tags, as described in Section 2.5.2, from cDNA synthesised from A549 RNA extracts. The resulting cassette was then cloned into the linearised pHIV-eGFP vector at the BamHI site, as described in Sections 2.6.1 and 2.6.2. Finally, plasmid preparation from transformed colonies was completed as described between Sections 2.5.4 and 2.5.6. Plasmids were quantified via spectrophotometry and confirmed via Sanger sequencing using the pHIV_F and pHIV_R primers (Table 2.5).

2.10.4 Amplification of the Remainder of the NPC1 Gene

The resulting pHIV_eGFP_Nsil_Casette construct was linearised as described in Section 2.6.1 with the NsiI restriction enzyme (NEB) and it was then used as a vector backbone for the in-frame insertion of the remainder of the NPC1 gene. Bases 82 to 3834 were amplified with primers PanNPCspF and huNPC1>pHIVR (Table 2.5) from a cDNA template synthesised from the A549 RNA extracts.

Briefly, amplification was completed using CloneAmp HiFi PCR Premix (Takara Clontech), with a final reaction volume of 25 μ L. 12.5 μ L of the premix was added to 0.5 μ L of both the forward and reverse primer (5 μ M), 1 μ L of cDNA template and 10.5 μ L of nuclease-free water. Recommended cycling conditions (98 °C for 10 seconds, 62.9 °C for 15 seconds and 72 °C for 3 minutes) were used for 30 cycles of amplification. Tagged PCR products were then purified using the Monarch® PCR & DNA Clean-up Kit (NEB) as per the

manufacturer's instructions. Briefly, PCR products were diluted with a binding buffer containing isopropanol and were loaded onto a spin column. Wash buffer was then added to the membrane, which was dried by centrifugation of the column at a maximum speed of 14,000 x *g* for 1 minute. The purified PCR product was then eluted into 20 µL of nuclease-free water.

InFusion cloning of the ligated vector and purified PCR product containing the NsiI tags was completed as described in Section 2.6.2. Plasmid preparation from transformed colonies was completed as described between Sections 2.5.4 and 2.5.6. Plasmids containing the full NPC1 construct were quantified via spectrophotometry and confirmed via Sanger sequencing using the primers pHIV_F pHIV_R, R215H_F and N931K_R (Table 2.5).

Table 2.5 Primers used in the generation of the NPC1 variant constructs by SDM

Some primers that were used for SDM or screening were also used for sequencing of the final plasmid construct.

Primer	Purpose	5'-3' Sequence
pHIV-NsilcasF	Amplification for cloning	ACCCGGGCTAGGATCCACCATGACCGCTCGCGGCC TGGCCCTTGGCCTCCTCCTGCTGCTACTGTGTCCAG C
pHIV-NsilcasR	Amplification for cloning	GGAGAGGGGCGGATCCTATGCATACCAAACACAGG ACTGTGAAAACACCTGCGCTGGACACAGTAGCAGCA G
PanNPCspF	Amplification for cloning	CAGTCCTGTGTTTGGTATGGAGA
huNPC1>pHIVr	Amplification for cloning	GAGGGGCGGATCCTAGAAATTTAGAAGCCGTTT
pHIV_Scr_F	Screening/sequencing	TGGAATTTGCCCTTTTGTAG
pHIV_Scr_R	Screening/sequencing	AGGAACTGCTTCCTTCACGA
R215H_F	SDM R215H CGT>CAT	TTTCCAGTCCATGGGATGGAG
R215H_R		ATCTGAAAACACAGGAGTGATG
I642M_R	SDM I642M ATC>ATG	TGGGGCACATGAAAAGCTGTC
I642M_F		AGGCTAGGGAAATATATAGAAAC
P434S_F	SDM P434S CCG>TCG	CTTTGGACCTTCGCTTGACATAC
P434S_R		GGTACATCAGCTCCCGAA
V858I_F	SDM V858I GTT>ATT	CAAAGTAGATATTGGATTGGATCAGTC
V858I_R		TTCAGGACTGCATGCTG
N931K_R	Sequencing	TTTGTCCCACCAGAAATATCGCGCC

2.10.5 Insertion of P434S and M642I Mutations into NPC1

An alignment with the wild-type NPC1 reference sequence [Genbank: NM_000271.5] indicated that the A549 NPC1 contained two SNPs that needed reversion; R215H and V858I. Additionally, one of the desired SNPs, M642I, was already present. P434S also needed to be inserted. SDM primers were designed (Table 2.5) and were used to amplify the NPC1 whilst inserting mutations at the desired site, as described in Section 2.5, with the only difference being that a six-minute extension time was used during amplification, due to the size of the template. Additionally, STBL3 cells were used during transformation, to reduce the chance of unwanted recombination of the long terminal repeats (LTRs) that are present in lentiviral expression vectors. These cells were grown at 30 °C, as described in Section 2.1. Multiple primers (Table 2.5) were used for colony screening, due to the size of the insert, depending on the site of the desired mutation. Plasmid preparation from transformed colonies was completed as described between Sections 2.5.4 and 2.5.6. Plasmids containing the NPC1 construct with the desired SNPs were quantified via spectrophotometry and were confirmed via Sanger sequencing using the primers pHIV_F pHIV_R, R215H_F and N931K_R (Table 2.5). The final constructs that were produced are described in Table 2.6.

Table 2.6 Summary of pHIV_eGFP NPC1 constructs used for transduction of U2OS cells
Constructs were made via site-directed mutagenesis of the A549 cell line NPC1 sequence.

<i>NPC1 Variant</i>	<i>Plasmid Name</i>
Wild-type NPC1	pHIV_eGFP_NPC1_R215H_V858I_I642M
NPC1 Haplotype (P434S/M642I)	pHIV_eGFP_NPC1_R215H_P434S_V858I
NPC1 P434S	pHIV_eGFP_NPC1_R215H_P434S_V858I_I642M
NPC1 M642I	pHIV_eGFP_NPC1_R215H_V858I

2.11 Production of NPC1 Transducing Units using a GFP Reporter

The production of viral transducing units to deliver the NPC1 receptor variants was completed in a very similar method to that described in Section 2.7. In this instance, the plasmid pCMVR87.4 acted as the packaging construct and provided a backbone to the transducing units based on the Human Immunodeficiency Virus (HIV). The pHIV_eGFP constructs containing the NPC1 variant genes to be delivered also contained green fluorescent protein (GFP) and thus, also acted as the reporter construct. Finally, the construct pMD2.G (unpublished; available on Addgene, plasmid number 12259), was

used to express the VSV G protein on the surface of the transducing viral particle to allow efficient delivery of the gene into a range of cells. 2 µg of each plasmid, alongside PEI was used to transfect HEK293T cells (Section 2.7). For the positive control, pHIV_eGFP plasmid was used without a receptor insert. For the negative control, no reporter plasmid was used.

Transducing units were harvested by passing the supernatant through a 0.45 µm filter. GFP-positive HEK293T cells were visualised using an iRiS™ Digital Cell Imaging System (Logos Biosystems). The efficiency of these units was then tested on HuH-7 cells and U2OS NPC1 knockout cells, which had undergone an overnight incubation at 37 °C after being seeded onto a 24-well clear bottom plate (Costar) at a density of 8×10^4 cells per well. A 72-hour incubation at 37 °C was completed after the transducing units were added and GFP-positive cells were visualised via the iRiS imaging system. All images were taken at 10 x magnification and a scale bar representative of 50 µm was added to each image. Images were saved in the TIFF file format and displayed using ImageJ Software.

2.12 Optimisation of Transducing Unit Production

2.12.1 Improving Transduction Efficiency

To improve the efficiency of transduction, the transducing units were concentrated via a sucrose cushion, exactly as described in Section 2.9.1. The resulting pellet was then resuspended overnight in 120 µL of sterile PBS (Sigma Aldrich). Resuspended transducing units were then added to HuH-7 cells seeded on a 96-well clear flat bottom plate (Corning) at a density of 2×10^4 cells per well. Plates were incubated at 37 °C for 4 hours before the addition of 150 µL of supplemented DMEM. After incubation at 37 °C for 72 hours, GFP-positive cells were visualised via the iRiS imaging system.

To ensure that transducing units were being formed correctly by the HEK293T producer cells, the resuspended pellet was also tested by western blot, as described in Section 2.9.2. A 10% gel was used to run 15 µL of the resuspended pellet for each transducing unit. The primary antibody used was α-HIV p24 rabbit antibody (ab9044, Abcam) at a 1:1000 dilution. The secondary antibody used was swine, α-rabbit HRP; (P0260, Dako), diluted in a blocking solution to a concentration of 0.17 µg/mL (1:2000).

2.12.2 Production of pHIV_Puromycin NPC1 Variant Constructs

After struggling to observe successful receptor transduction via a GFP reporter in both HuH-7 and the U2OS NPC1 knockout cell lines, an alternative methodology was determined whereby the GFP reporter gene was replaced in the original receptor constructs with the gene for puromycin-N-acetyltransferase. The presence of this gene confers resistance to puromycin. Therefore, successful transduction of the receptor constructs into the U2OS NPC1 knockout cell lines would also ensure the resistance of these cells to puromycin, thus, transduced cells could be selected for using this antibiotic, resulting in the creation of a stable cell line able to continuously express the *NPC1* transgene.

To achieve this, all four pHIV_eGFP NPC1 variant constructs were used as templates for PCR amplification of the vector backbone. The forward primer, Delta_GFP_F: (5'-AGCGGCCGCGATCGATACC-3'), was designed to anneal directly downstream of the *GFP* gene stop codon and the reverse primer, Delta_GFP_R (5'-GGTTGTGGCCATATTA TCATCGTGTTC-3'), was designed to anneal directly upstream of the *GFP* gene start codon. This ensured amplification of the remainder of the plasmid construct, including the *NPC1* gene variants, during a reaction, utilising the Q5 high fidelity polymerase (NEB). The reaction set-up and cycling conditions are as described in Section 2.5.2, with the exception that a six-minute extension time was used to account for the size of the plasmid template. Products were then purified using the Monarch PCR & DNA Clean-up Kit (NEB), as described in Section 2.10.4.

The gene for puromycin-N-acetyltransferase was codon optimised for mammalian expression and commercially synthesised (GeneArt; Thermo Fisher) to include tags that were homologous to the site of insertion in the PCR amplified plasmid backbones. The lyophilised construct was then reconstituted to a concentration of 50 ng/μL. A ligation reaction was completed via InFusion, as described in Section 2.6.2. Transformation of STBL3 cells (NEB) with ligated vectors was completed as described in Section 2.5.4, following which, the successful transformation was determined by colony screening using the primer, Puro_dwnstrm_Scr_R (5'-GCATTGGTAGCTGCTGTATT-3'), as described in Section 2.5.5. Plasmids were then extracted and quantified via spectrophotometry and their sequences were confirmed by Sanger sequencing using pHIV_F pHIV_R, R215H_F, N931K_R and Puro_dwnstrm_Scr_R primers, as described previously in Section 2.5.6.

Table 2.7 Summary of pHIV_PuroR NPC1 constructs used for transduction and creation of U2OS stable cell lines

The puromycin resistance gene was cloned into each construct, replacing the *GFP* gene downstream of the IRES.

<i>NPC1 Variant</i>	<i>Plasmid Name</i>
Wild-type NPC1	pHIV_PuroR_WTNPC1
NPC1 Haplotype (P434S/M642I)	pHIV_PuroR_PS_MI_NPC1
NPC1 P434S	pHIV_PuroR_P434S
NPC1 M642I	pHIV_PuroR_M642I

2.13 Antibiotic Sensitivity Assays on U2OS NPC1 Knockout Cells

To determine the concentration of puromycin that would be required to ensure the death of any untransduced U2OS NPC1 knockout cells, a puromycin kill curve was completed. U2OS NPC1 knockout cells were seeded onto a clear, flat bottom, 96-well plate (Corning) at a density of 2×10^4 cells per well. The cells were then incubated overnight at 37 °C after which, images were taken of the cells using a 10X objective lens, at 100X magnification, via the iRiS imaging system. 10 µg/mL and 12 µg/mL preparations of puromycin in supplemented DMEM were then made and used in two simultaneous serial dilutions to provide a range of concentrations between 0 and 12 µg/mL of puromycin to test on the U2OS NPC1 knockout cells. Spent media was removed from the wells and 100 µL of the various antibiotic concentrations were added to the wells. Eight replicates were prepared for each concentration tested. The cells were then incubated at 37 °C for 72 hours, with images of the cells taken at 24, 48 and 72-hour time points. This experiment was also repeated with different concentrations of blasticidin to characterise the cells on receipt from Dr W. E. Diehl. During the generation of the original NPC1 knockout cell line by Dr Diehl's team via CRISPR-Cas9, blasticidin was used to select knockout mutants.

2.14 Transduction of U2OS NPC1 Knockout Cells with NPC1 Receptor Variants and Testing Permissibility to EBOV

To produce the lentiviral transducing units capable of delivery of the *NPC1* variant transgenes to the U2OS NPC1 knockout cells, a three-plasmid gene expression system was used, as described in Section 2.11. The plasmid pCMVR87.4 acted as the packaging construct and provided a backbone to the transducing units based on the Human Immunodeficiency Virus (HIV). The pHIV_PuroR constructs containing the NPC1 variant genes as well as the puromycin resistance gene under the control of a bicistronic promoter

acted as transfer plasmids and finally, the construct pMD2.G was used to express the VSV G protein on the surface of the transducing viral particle to allow efficient delivery of the gene into a range of cells. Briefly, HEK923T cells were seeded into 10 cm Primaria-coated dishes at a density of 1.5×10^6 cells per dish. 10 mL of supplemented DMEM (Section 2.2) was added and cells were incubated overnight at 37 °C and 5% CO₂. After reaching a confluency of approximately 50%, for each reaction, cells were transfected with 8 µg of both the backbone and VSV G plasmids and 2 µg of the pHIV_PuroR NPC1 variant constructs alongside PEI (1 mg/mL) and OptiMem (GIBCO) resulting in final reaction volumes of 600 µL. The cells were then further incubated with 7 mL of OptiMem for 6 hours at 37 °C. The OptiMem was replaced with 10 mL of supplemented DMEM and incubated for a further 72 hours at 37 °C. Transducing units were harvested by passing the supernatant through a 0.45 µm filter (Sartorius).

1 mL of transducing units were then added to U2OS NPC1 knockout cells, which had been seeded onto a clear, flat bottom 12-well plate (Corning) 24 hours previously at a density of 16×10^4 cells per well. A cells-only control lacking any transducing units was used to compare the impact of the antibiotic treatment. The transducing units were incubated with the cells for 5 hours before they were carefully removed without disruption of the cellular sheath. 1 mL of fresh supplemented DMEM was added to each well before incubation at 37 °C for a further 24 hours. Following this spent media was replaced with 1 mL of fresh DMEM containing puromycin at a concentration of either 0.5 or 1.25 µg/mL, as determined by the puromycin kill curve. Cells were monitored for the next 72 hours, with images taken at 24, 48 and 72 hours post-treatment using the iRIS imaging system.

Cells that were still alive 72 hours after the addition of both transducing units and antibiotics were confirmed to have undergone successful transduction. This was relative to cells where transducing units had not been added but had subsequently been treated with antibiotics, where 100% cell death was observed after 72 hours. Transduced cells were harvested as described in Section 2.2. All transduced cells were seeded into a 25 cm³ flask, in media containing either 0.5 or 1.25 µg/mL of puromycin. Cells were split every 72 and 96 hours, with the size of the flasks being seeded increasing as the transduced cell population expanded. Once the cell population had expanded enough to be confluent in a 175 cm³ flask, approximately 7×10^6 cells for each transduced line were frozen and stored in liquid nitrogen. The remainder of the cells were maintained in a 75 cm³ flask at the correct concentration of antibiotic for the duration of their use.

To determine if the transduced cell lines showed any difference in their permissibility to EBOV, infectivity assays as described in Section 2.8 were completed. Briefly, PVs expressing EBOV GP variants that had previously been observed to be either highly or lowly infectious in HuH-7 cells were produced via transfection of HEK293T cells, as described in Section 2.7. PVs were harvested and added to U2OS cell lines expressing NPC1 variants as described in Section 2.8, with the only exception being that cells were incubated for 72 hours in media containing either 0.5 or 1.25 µg/mL of puromycin before they were lysed.

2.15 NPC1 Gene Expression Analysis by qPCR

NPC1 gene expression analysis was completed in a variety of cell lines to determine any differences in transcript level. To achieve this, RNA extraction was completed from multiple cell lines and was then synthesised to cDNA as described in Section 2.10.2. It was ensured that 3 µg of RNA was inputted into each cDNA synthesis reaction. qPCR reactions for each cell line were completed in triplicate. Each reaction had a total volume of 20 µL and consisted of 10 µL 2x SYBR Green PCR Master Mix (Applied Biosystems) 0.8 µL of both the forward and reverse primers (10 µM), 1 µL cDNA and 7.4 µL nuclease-free water. Primer pairs to target *NPC1* and *GAPDH* (Table 2.8), which was used as a housekeeping gene, were designed by colleagues to be exon spanning, to ensure amplification of cDNA. Cycling conditions included an initial annealing temperature of 95 °C for 2 minutes, 40 cycles at 95 °C for 5 seconds and 60 °C for 20 seconds with a final extension at 95 °C for 10 seconds. Melt curve analysis was also completed from 65 °C to 95 °C incrementing in 0.5 °C for 5 seconds. All analysis was completed using the CFX Maestro Software (Bio-Rad). Data was normalised to the housekeeping gene or the RNA input.

Table 2.8 Primers used for qPCR gene expression analysis of *NPC1*

Multiple primers targeting bat *GAPDH* were tested.

<i>Primer</i>	<i>Target</i>	<i>5'-3' Sequence</i>
qNPC1_F	Universal <i>NPC1</i>	TGCACCAGGTTCTTGACTTACA
qNPC1_R	Universal <i>NPC1</i>	CTTGTATCATTCAGAGAGGCAGG
GAPDH_F	<i>GAPDH</i> mammalian	GACTTCAACAGTGACTCCAC
GAPDH_R	<i>GAPDH</i> mammalian	CCTGTTGCTGTAGCCAAATTC

2.16 NPC1 Expression Analysis by Flow Cytometry

NPC1 endosomal protein expression was measured across a variety of cell lines using flow cytometry. In each cell line, antibodies specific to NPC1 and an isotype control were tested and an unstained sample served as a negative control. All centrifugation steps used to pellet cells were completed in a swinging bucket rotor (Beckman Coulter) at $300 \times g$ for 5 minutes. For each cell line, three FACS tubes were prepared with 2.5×10^5 cells. Cells were then pelleted by centrifugation at $300 \times g$ for 5 minutes and washed twice with 1 mL of sterile PBS (Sigma Aldrich). After washing, cells were resuspended in 0.5 mL of 4% paraformaldehyde and incubated at room temperature for 5 minutes to instigate fixation. Cells were then pelleted and washed with PBS a further two times before incubation with 0.5 mL of 90% methanol at room temperature for 15 minutes to permeabilise them. Following this, cells were pelleted and washed twice with FACS buffer, consisting of PBS plus 2% bovine serum albumin (BSA) and 2 mM of ethylenediaminetetraacetic acid (EDTA).

Cells were again pelleted and one tube from each cell line was incubated with 10 μ L of the primary antibody (1:150 dilution in FACS buffer of NPC1 rabbit IgG, Novus Biologicals, NB400-148, 6.67 μ g/mL) or 10 μ L the isotype control, (0.6:150 dilution in FACS buffer of recombinant Rabbit IgG, ab172730; 6.67 μ g/mL) for 1 hour at room temperature, in the dark. One tube of cells was left as an unstained negative control. The cells were then pelleted and washed twice with 2 mL of FACS buffer before another incubation step at room temperature for a further hour in the dark, with 10 μ L of 1:1000 (2 μ g/mL) dilution in FACS buffer of the secondary antibody, goat α -rabbit IgG H&L (Alexa Fluor® 488; ab150077). Cells were washed and pelleted for a final time and resuspended in 500 μ L of FACS buffer before overnight storage at 4 °C until flow cytometry. The samples were run on an FC500 flow cytometry analyser (Beckman Coulter) with 10,000 events recorded for each sample. All analysis was completed using the Kaluza analysis software (Beckman Coulter). Unstained samples were used for gating and calculating rMFI, completed by dividing the median fluorescence intensity of stained cells in each sample, by that of the unstained sample.

2.17 NPC1 Expression Analysis by Western Blot

NPC1 expression in cell lines was determined by western blot. To detect protein in cell lines, cells were lysed in RIPA buffer (50 mM tris-base, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% igePAL 650),

containing a 1:25 dilution of protease inhibitor (9 mg/mL), on ice, for 20 minutes. The lysed cells were then centrifuged at a maximum force of 14,000 x *g* for 10 minutes. The cell lysate was separated from the pellet of debris and stored at -80 °C until needed. Total protein quantification was completed on cell lysates using the Pierce BCA Protein Assay Kit (ThermoFisher) as per the manufacturer's instructions, against a known standard of bovine serum albumin (BSA). Briefly, preparations of diluted albumin standards were made at concentrations ranging between 0 and 2000 µg/mL. 10 µL of each unknown lysate and standard was added to a well on a clear flat bottom 96 well plate. 200 µL of reagent WR was added to each well and the plate was incubated at 37 °C for 30 minutes. The plate was cooled to room temperature and the absorbance was then measured on a BMG Labtech FluoStar Omega Plate Reader at an absorbance of 562 nm. A standard curve was completed by plotting a 562 nm measurement for each BSA standard against its concentration in µg/mL. This curve was then used to determine the protein concentration of each unknown sample.

To determine protein expression from cell lysates, 30 µg of total protein was mixed with 4 x Laemmli protein sample buffer (Bio-Rad) and heated at 95 °C for 10 minutes to denature the protein. Samples were loaded alongside a spectra multi-coloured ladder (Abcam), onto a pre-cast 4-15% SDS-PAGE gel (Bio-Rad). The gel was placed in a gel tank containing running buffer (25 mM tris base, 190 mM glycine, 0.1% sodium dodecyl sulfate) and an electric field of 120 volts was applied for 40 minutes. Following this, the gel was placed on top of a nitrocellulose membrane and both gel and membrane were placed in a sandwich of filter paper that had been soaked in transfer buffer (25 mM tris-base, 192 mM glycine, 20% methanol) and together, they were placed in a TransBlot SD semi-dry transfer cell (Bio-Rad), where a constant current of 100 mA was applied for 1 hour. All subsequent antibody and incubation, wash and visualisation steps were completed as described in Section 2.9.2. The primary antibody used was α-NPC1 rabbit IgG (Novus Biologicals; NB400-148), diluted in a blocking solution to a concentration of 1 µg/mL (1:1000). The secondary antibody used was swine, α-rabbit HRP; (P0260, Dako), diluted in a blocking solution to a concentration of 0.17 µg/mL (1:2000). GAPDH was used as a loading control. The primary antibody was α-GAPDH goat IgG (1:4000) (A00191) and the secondary antibody used was α-goat HRP (Sigma; A8919), diluted in a blocking solution to a concentration of 0.002 mg/mL (1:5000).

3. Creation of a Panel of EBOV GP Expression Plasmids and Characterisation of Mbandza 2003 GP

3.1 Introduction

EBOV (formerly known as EBOV-Z or ZEBOV) causes zoonotic disease through a spillover following contact by a susceptible host species, with the virus in its natural reservoir. Among the four known species of *Ebolavirus* that are responsible for haemorrhagic disease in humans, EBOV is the most severe with mortality rates observed in outbreaks reaching as high as 90% (Feldmann, Sprecher and Geisbert, 2020). To date, there have been 24 outbreaks recorded in Africa. Historically, these were sporadic, geographically confined and typically self-limiting. However, recent outbreaks have become more frequent and of a larger scale, with the two largest outbreaks both lasting several years (Claude, Underschultz and Hawkes, 2018; Languon and Quaye, 2019; Keita *et al.*, 2021).

While the natural reservoir for EBOV remains unknown, the isolation of fragmented viral RNA and antibodies against EBOV from several bat species, suggests that they could be the most likely candidate. However, no live virus has been isolated from any bat species to date (Leroy *et al.*, 2005; Pourrut *et al.*, 2007, 2009; Yuan *et al.*, 2012; Olival *et al.*, 2013; Olival and Hayman, 2014). Given its high mortality rate, understanding how the virus interacts with the human host and how behaves in the environment, remain crucial prerequisites for managing outbreak preparedness, thus limiting the number of deaths caused by EBOV, as well as guiding the continued design of effective vaccines and therapeutics.

3.1.1 The Role of the EBOV GP

As mentioned in Chapter 1, GP_{1,2} mediates membrane fusion by undergoing several conformational changes, with GP₁ playing an important role in the attachment to the host

cell surface, whereas GP₂ directs the fusion of the viral and host membranes, thus allowing the egress of the virus (Lee *et al.*, 2008).

Once the virus is internalised into an endosome, a low pH allows the cysteine proteases cathepsins B and L, to catalyse GP_{1,2} proteolysis. This process involves two cleavage steps that lead to the formation of a final 19 kDa product known as primed GP (GP_{Cl}) (Chandran *et al.*, 2005; Harrison, 2015). Furthermore, cathepsins act on GP₁, to remove the glycan cap, thereby exposing the receptor binding domain (RBD) which plays an essential role in membrane fusion interacting with the second luminal domain of the host cell receptor, NPC1 (Schornberg *et al.*, 2006; Dube *et al.*, 2009). Studies have demonstrated that changes in the EBOV GP can impact the efficiency of viral entry into cells of different mammalian species, thus highlighting the importance of the GP in determining both host tropism and viral fitness (Martinez *et al.*, 2013). Consequently, research into vaccine development is typically based on the GP and many antibody-based therapies are designed to target GP epitopes (Wolf *et al.*, 2021; Crunkhorn, 2022; Milligan *et al.*, 2022).

3.1.2 Limitations of Live Virus Studies

Whilst essential, research into viruses such as EBOV poses several challenges. Primarily, as a BSL-4 pathogen presenting a serious risk to human health, there are a limited number of facilities equipped worldwide that permit the handling of live EBOV. Additionally, obtaining live stocks typically requires the virus to be isolated from clinical samples and serially passaged in cell lines (Kugelman *et al.*, 2012; Beitzel *et al.*, 2021). However, several studies have shown that this practice may potentiate tissue-cultured adapted genome mutations that may lead to the virus behaving atypically *in-vitro* and *in-vivo* experiments, as well as leading to inaccurate phylogenetic analyses (Volchkova *et al.*, 2011; Hoffmann *et al.*, 2017; Ruedas *et al.*, 2017, 2018).

This adaptive property known as sequence plasticity was first observed when it was reported that the GP gene could acquire an additional uracil nucleotide in a stretch of seven uracil residues, resulting in an open reading frame shift that favours the expression of GP_{1,2} rather than sGP (Volchkova *et al.*, 2011; Kugelman *et al.*, 2012). sGP is secreted by cells and interacts with the host immune system (Wahl-Jensen *et al.*, 2005). *In-vivo*, the secretion of sGP is expected to contribute to viral fitness by limiting the expression of otherwise cytopathic GP_{1,2} (Yang *et al.*, 2000).

Another well-established sequence adaptation is T544I, which spontaneously occurs in the internal fusion loop of the viral GP. Ruedas *et al.*, (2017) demonstrated that after two passages of recombinant VSV expressing Makona GP in Vero E6 cells, T544I became detectable and increased viral entry was observed in these cells. This was an extension of work that first drew the connection between the mutation and tissue culture, but could not determine if it was a spontaneous mutation or an outgrowth of a strain that was present in patients, that became dominant in tissue culture (Hoffmann *et al.*, 2017). Ruedas *et al.* (2017) used pseudotyping to confirm that the mutation was a spontaneous occurrence. Interestingly, the adaptation occurred only during passing using Vero E6 cells and not HuH-7 cells, implying the latter can sustain the replication of clinical isolates of EBOV, whilst providing a lesser selective pressure. They also determined that the mutation was not found to occur in other species of *Ebolavirus*, including TAFV, BDBV and SUDV. Subsequent analysis of sequence data collected from the West African outbreak confirmed that the mutation was not found in the original clinical samples from which the Makona sequence was extracted.

Further work completed by the group that pseudotyped EBOV Makona GP into replicating VSV and passaged it in Vero E6 cells confirmed the presence of T544I in two isolates as well as the mutations H549R in seven isolates and D552N in two isolates (Ruedas *et al.*, 2018). Three extra mutations, D522E, R574H, and I584L were also identified, each in one isolate. In total 19 isolates were tested. Notably, no isolates consisted of two or more changes to the GP and wild-type GP did not contain any substitutions before the passaging of the pseudotypes (Ruedas *et al.*, 2018). The study also observed the detection of the R574H mutation after the passage of the pseudotyped virus in HuH-7 cells, contradicting previous reverse genetics studies, which suggested that EBOV GP showed greater stability in these cells (Tsuda *et al.*, 2015).

Analysis of the sequences of clinical isolates and lab-grown stocks deposited on GenBank showed that aside from the T544I mutation, the D552N mutation was the only other that was found in five laboratory-passaged EBOV samples, two of which were from lab-passaged stocks of the Makona C07 clinical isolate, reported to encode D552 in its wild-type strain (Ruedas *et al.*, 2018).

These potentially critical findings highlight the need for researchers who are depositing data onto NCBI to specify exactly how samples have been collected and whether sequencing of the GP gene was completed directly from clinical samples, or after passaging isolates to create lab-grown virus stocks. If the latter is the case, it would leave

room for mutations to occur in the genome that may affect the virulence of the strain in a way that might never be observed in strains circulating during outbreaks. Thus, any subsequent work regarding the subtle differences in infectivity between strains or variants may involve an element of bias. More importantly, efficacy testing of antivirals and therapeutics that rely on the same data may provide inaccurate results. For example, a study has recently demonstrated that T544I can alter the susceptibility of EBOV to 3.47, which is a small molecule inhibitor of the virus (Beitzel *et al.*, 2021).

Nonetheless, with the advent of deep sequencing, more clinical isolates are directly being sequenced (Gire *et al.*, 2014). This was not the case in the past, which could complicate data interpretation. For instance, it has previously been mentioned that the T544I variant has been observed in past outbreak strains (Ueda *et al.*, 2017). However, with a lack of knowledge surrounding the sample collection and passaging of these isolates, it is impossible to determine whether the observed mutation was due to tissue culture or whether it was naturally occurring (Beitzel *et al.*, 2021).

3.1.3 Surrogate Models for EBOV

To overcome some of the issues in handling live-replicating, high-level bio-containment viruses, surrogate models are often used to study aspects of viral entry and tropism, assess vaccine efficacy and perform serological studies (Temperton and Page, 2015; King *et al.*, 2016; Saito *et al.*, 2020). Pseudoviruses (PVs) are an example of safe surrogates for enveloped viruses. In general terms, PVs are recombinant, hybrid viral particles consisting of a core encompassed by a cell-derived membrane in which the original envelope protein has been substituted with a heterologous protein obtained from a different enveloped virus (Sanders, 2002; Li *et al.*, 2018). Importantly, the core does not contain the genetic material that would allow for the expression of a viral envelope protein, therefore, PVs can enter a target cell and initiate nucleic acid replication, however, they are not capable of producing infectious viral particles, making them safe to use (King *et al.*, 2016).

A well-established model is one based on VSV, which was originally designed for the functional analysis of the EBOV GP (Takada *et al.*, 1997). The strength of this system is that the genome is transcribed into five separate mRNAs, meaning that the GP gene can be easily substituted (Farzani, Chov and Herschhorn, 2020). Typically, the gene is replaced with a reporter gene such as *GFP*, whereas a separate plasmid contains the

gene for the GP of interest (King *et al.*, 2016). The downside of this system, however, is the complication added by the numerous steps involved comprising the recovery of VSV from plasmid, the generation of G-complemented VSV, and finally the pseudotyping of VSV with the envelope protein from an alternative virus (Whitt, 2010). The process requires producer cells that are infected with modified Vaccinia Ankara T7, followed by co-transfection of plasmids containing rVSVΔG genome, the desired heterologous GP and VSV accessory plasmids that encode N P L and G proteins under the control of a T7 promoter. The expression plasmid containing the native G protein is also included to support the entry of the recovered virus during the initial steps (Yahalom-Ronen *et al.*, 2020). This method supports a single round of infection with pseudotyped VSV (Whitt, 2010; Farzani, Chov and Herschhorn, 2020).

Reverse genetics protocols have also allowed the development of a recombinant viral system, that directly encodes the heterologous envelope gene in the VSV genome, replacing the G protein (Tani, Morikawa and Matsuura, 2012; Farzani, Chov and Herschhorn, 2020). This results in a recombinant virus that is replication-competent both *in-vitro* and *in-vivo* providing an improved model for infection compared to PVs, which are the favoured model for viral entry (King *et al.*, 2016). Both VSV systems have been used widely in filovirus research, with the latter forming the basis of the recently licenced ERVEBO vaccine which is based on a replication-competent recombinant VSVΔG expressing the EBOV GP to elicit a targeted immune response (Wolf *et al.*, 2021).

The gold standards for many labs, however, are lentiviral or retroviral-based systems that utilise the HIV-1 or MLV pseudotyping systems, respectively (Cronin, Zhang and Reiser, 2005). These systems rely on the ability of the virus to integrate its genomes into that of the host, thus providing an efficient mechanism of reporter gene delivery into the desired cell line (Dautzenberg, Rabelink and Hoebe, 2020). Lentivirus-based pseudotypes can broadly infect both dividing and non-dividing cells, whereas retrovirus-based systems are limited to infecting dividing cell types. Studies have shown that both Mayinga 1976 and Makona C07 (isolated from Guinea, early in the West African outbreak) strains of EBOV PVs show an increased signal for infectivity based on a luciferase reporter when using an HIV-1 backbone compared to an MLV backbone, in HuH-7 cells (Urbanowicz *et al.*, 2016). However, MLV backbones offer advantages to HIV-1 backbones in work utilising cells from multiple species as certain cell lines have been shown to have restriction factors against HIV-1, impacting the ability of PVs to infect cells (Stremlau *et al.*, 2004; Yap *et al.*, 2014). These findings demonstrate the importance of optimising transfection protocols depending on which cells that relative infectivity shall be assessed in.

EBOV GP PVs based on an MLV core were first described in 1998 and have since been broadly utilised (Wool-Lewis and Bates, 1998). The system used by Urbanowicz *et al.*, (2016) that was considered for this study is based on a three-plasmid MLV system, typically employing producer cells, such as HEK293T cells, that are transfected. One plasmid produces the viral ‘backbone,’ which in this instance, were MLV backbones carrying the *gag* and *pol* genes that encode the viral core structure and the reverse transcriptase, respectively. An additional plasmid contains a quantifiable reporter gene (typically luciferase), flanked by retroviral long-terminal repeat sequences and a packaging signal. The final plasmid contains the gene for the envelope protein of interest, which in the case of Urbanowicz *et al.*, (2016) were variants of the Makona GP that evolved during the lengthy 2013-2016 West African Outbreak. The MLV system was favoured by the authors as the bat cells they were testing their PVs in contained restriction factors against HIV (Urbanowicz *et al.*, 2016).

Pseudotyping has been invaluable in the field of EBOV research. PVs have been used in studies investigating the efficacy of therapeutic mAbs and antiviral drugs, elucidating the interactions between the virus and the host cell receptor and determining viral tropism (Côté *et al.*, 2011; Ng *et al.*, 2015; Ndungo *et al.*, 2016; Zhang *et al.*, 2016; Steeds *et al.*, 2020; Takadate *et al.*, 2020; Tian *et al.*, 2020). By 2015 Edward Wright’s and Nigel Temperton’s groups established a panel of PVs that included all five *Ebolavirus* species known at the time, with both Mayinga and Makona strains representative of EBOV, and isolates of MARV and LLOV (Bentley, Mather and Temperton, 2015; Temperton and Page, 2015). The utility of these pseudotypes was highlighted in immunogenicity studies of the Chimpanzee-derived adenovirus-vectored (ChAd) vaccine, ChAd3 EBOZ at the Jenner institute (Ewer *et al.*, 2016). In these studies, the pseudotyped GPs acted as substitute antigens in virus neutralisation assays. As described in a more recent study looking at the cross-neutralisation of BOMV by EBOV antibodies, the group was also successful in producing lentiviral pseudotypes expressing the BOMV GP at high enough titres for downstream assays, further adding to their existing filovirus GP panel (Bentley *et al.*, 2021).

Although the utility of the systems previously discussed is undeniable, one limitation is that studies relying on PVs expressing EBOV GP or reverse genetics systems to simulate replicating viruses, typically utilise sequences from well-characterised strains such as Mayinga 1976, Kikwit 1995 or Makona strains (Beitzel *et al.*, 2021). Studies that

incorporate a comprehensive panel of different outbreak strains to look at intraspecies variation remain a novel concept.

3.1.4 The Impact of Intraspecies Variation

The overall genomic diversity between the EBOV strains is low, with nucleotide sequences varying by a maximum of approximately 3% (Carroll *et al.*, 2013). Despite this, variation does exist in intergenic regions and in genes of key proteins such as the GP, a target for many existing medical countermeasures (Jun *et al.*, 2015; Dudas *et al.*, 2017). Therefore, when it comes to the design of effective vaccines, antivirals and antibody-based therapeutics, it is important to have a good understanding of the genomic variation within the species, particularly, the heterogeneity of GP epitopes across multiple strains. Failure to do so could lead to conclusions surrounding the efficacy of vaccines and therapeutics that are based on a single strain, which may not be reflective of the diversity that the virus has displayed since first being discovered in 1976. In fact, sequence variations between strains could alter the structure of an epitope, reducing the efficiency of treatments that were developed based on a specific strain (Ghosh *et al.*, 2021).

This concept was emphasised by Kugelman *et al.*, (2015) in a study evaluating the impact of EBOV genetic drift on therapeutic candidates, during the midst of the West African outbreak. The group identified that all monoclonal antibodies used for passive immunotherapies at the time were generated against the GP of the Kikwit 1995 strain. They then demonstrated that there were 21 non-synonymous mutations between the Kikwit 1995 GP and the Makona variant from the early West African outbreak. The ZMapp cocktail of antibodies tolerated 18 of these mutations, but not all mutations that appeared through the course of the outbreak were tested for resistance (Kugelman, Sanchez-Lockhart, *et al.*, 2015). Although therapies such as ZMapp target several GP epitopes, reducing the likelihood that an EBOV variant is completely tolerant, this work highlighted the need to take into account the diversity that the EBOV species has displayed since first appearing in 1976. Similarly, they identified that all siRNA treatments at the time had been generated against the Mayinga GP, raising the same concerns (Kugelman, Sanchez-Lockhart, *et al.*, 2015). Therefore, evaluating the genetic differences of EBOV through historic outbreaks should be a priority in determining the efficacy of current therapies, as well as in providing greater insight into how different strains may enter host cells, thus aiding in managing future outbreak preparedness.

Understanding the genetic diversity of the EBOV GP may have wider implications because it can help to determine MRCA sequences through modelling that can predict viral sequences by going backwards in theoretical time. Such ancestral/node sequences have been proposed for use in vaccine design in the past, particularly for RNA viruses that display a high level of genetic diversity, in response to the inability of existing vaccines to induce sufficiently broad immunity against different infectious strains (Doria-Rose *et al.*, 2005; Nickle *et al.*, 2007). A similar approach was more recently employed in the design of an antigen for a ChAd-based Rabies vaccine. In the work, the chosen antigen was described as a 'medoid' G sequence consisting of the amino acids with the highest average percentage identity to all other available online G protein sequences (Napolitano *et al.*, 2020).

3.1.5 Aims

One of the main aims of this study is to generate a panel of EBOV GP pseudotypes that are representative of all outbreaks that occurred between 1976 and 2016. This will allow the determination of the subtle difference in infectivity between all outbreak strains as well as the efficiency of viral entry across them, which can be supplemented with *in-vivo* models to determine if any differences correlate to a difference in pathogenicity of the viral strains. The construction of a comprehensive library of EBOV GP pseudotypes would be also invaluable in determining the effect of these genetic differences against existing therapies, particularly, in elucidating if existing antibody therapies display comparable neutralisation patterns between strains. Such a tool would also be useful in the development of vaccines and other medical countermeasures, thus helping the prompt response to future outbreaks. The proposed EBOV GP panel will be used in a three-plasmid MLV lentiviral system to generate PVs. PVs will be tested to determine the efficiency of cell entry in multiple cell lines. Finally, the system will also be used to generate a panel of reconstructed ancestral/MRCA GP sequences, as determined by Professor E. Holmes (University of Sydney), which will also be tested alongside outbreak sequences in different cell lines. This aims to determine if mutations observed between MRCAs (presumed to represent the virus as it existed in the reservoir host) and viral strains circulating in humans, increase fitness in the latter and provide more insight into what the natural reservoir for the virus may be and if multiple hosts may exist.

3.2 Results

3.2.1 Selection of Representative Outbreak EBOV GP Sequences for Panel

The first recorded outbreak of EBOV occurred in 1976 with the circulating strain during the outbreak named Mayinga. EBOV GP sequences representative of this wild-type strain, its tissue-cultured variant, the Makona-Kissidougou C15 GP sequence taken from the strain circulating during the early stages of the 2013-2016 West African outbreak and six theorised ancestral EBOV GP sequences previously inferred by Professor Holmes, were already available for use in pcDNA3.1 mammalian expression vectors. The first step was to identify which other EBOV variant sequences should be included in a panel of GP constructs that aimed to be representative of strains circulating in outbreaks between 1976 and 2016. To achieve this, a phylogenetic tree was generated.

This involved creating nucleotide alignments of all GP sequences sourced from a human host, for each individual outbreak. These alignments allowed the identification of any duplicate nucleotide sequences, which were excluded from the final tree. As many of the earlier outbreaks were time-limited and geographically contained, for some outbreaks limited or no sequence data was available. The final tree was generated through an amino acid alignment of outbreak sequences alongside the inferred ancestral node sequences. The only exceptions to this were sequences from the 2013-2016 West African and 2018 DRC outbreaks. The sequence for Makona-Kissidougou C15 GP, which was already in pcDNA3.1, was included in the alignment as the representative sequence for this outbreak. Furthermore, only sequences from early-outbreak strains circulating in the 2018 DRC outbreak were included. Figure 3.1 shows the final maximum-likelihood phylogenetic tree with a JTT + G model of substitution and bootstrap value of 1000 that was generated to include all intermediate outbreak sequences available on GenBank (Figure 3.1).

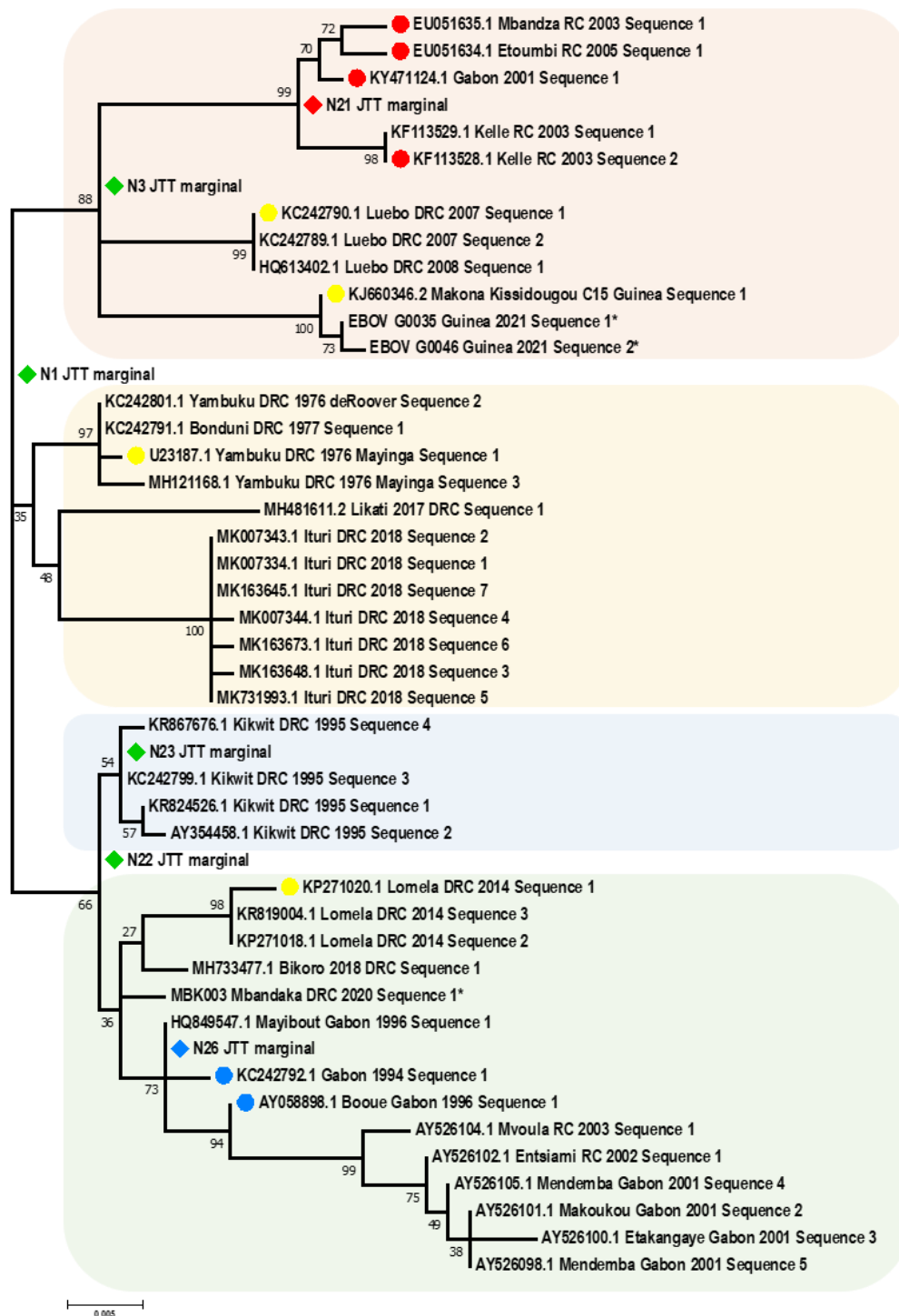


Figure 3.1 Maximum-likelihood tree of EBOV GP sequences from outbreaks since 1976

Tree contains 46 amino acid sequences including ancestral node sequences. A JTT+G model of substitution was applied and the tree topology was assessed using bootstrap analysis of 1000 iterations. Values on branches represent percent support based on this analysis. All outbreak sequences were obtained from GenBank, excluding asterisked sequences which were obtained from Virological. The tree was rooted to N1, the first ancestral node sequence generated. Coloured sequences are those generated for this investigation, with diamonds highlighting the ancestral nodes and circles signifying the outbreak sequences generated. Red sequences are derived from the N21 sequence, whilst blue sequences are from the N26 sequence. The tree is drawn to scale, with branch lengths measured in the number of amino acid substitutions per site and was generated using the MEGA7 software.

All theoretical ancestral EBOV GP sequences appear at the nodes of the tree topology whereas all outbreak/extant sequences appear on branch tips following analysis. As discernible, although all the GP sequences present in the tree belong to viruses from the *Zaire ebolavirus* species, there is a substantial amount of variation between the different sequences at an amino acid level, which may impact the viruses' abilities to enter cells and cause pathogenesis, as well as limit the efficacy of existing vaccines and therapies targeting any regions of variation. Outbreak sequences are clustered into four main clades, with ancestral node sequences visible at branch nodes.

Generally, sequences from outbreaks that occurred more closely together in time, are clustered in the same sub-clade. This can be seen with the 1976 Yambuku and 1977 Bonduni outbreaks in the DRC, the 1994 and 1996 outbreaks in Gabon, the 2007 and 2008 outbreaks in Luebo, DRC and the 2017 Likati and 2018 Ituri outbreaks in DRC. Other clades contain sequences from outbreaks several years apart, for example, the Guinea 2021 sequences cluster closely to Makona C15 and the 2018 Bikoro and 2020 Mbandaka outbreaks in the DRC also cluster together. Both these patterns may indicate a potential latency of the virus in the population or environment between outbreaks. Additionally, sequences that were associated with outbreaks that resulted in heavy loss of local wildlife in the beginning to mid-2000s have also clustered primarily in the first clade. The remainder of the outbreaks from this period have clustered together in the final clade. Finally, in outbreaks for which multiple sequences of circulating strains were available, there is little amino acid variation observed limited to one or two residues, for example, as seen in the 1995 Kikwit and 2003 Kelle and 2007 Luebo outbreaks.

From the topology displayed in Figure 3.1, an outbreak strain from each major outbreak between 1976 and the 2013-2016 West African outbreak was selected at random to be included in the panel. The only exception to this was the sequence selected for the 1976 outbreak, as this was already available in a construct, and the sequences for the 1995 Kikwit DRC outbreak. As N3 was identical to one of the three Kikwit strains present in the tree, no further sequence was selected for the panel.

Following this, the chosen sequences were analysed further because many of the GP sequences deposited on GenBank had been obtained following the tissue culture of the virus. Consequently, it was important to determine whether the outbreak sequences chosen for generation in the panel contained tissue culture-adapted mutations, which may impact their infectivity. Table 3.1 shows tissue culture adaptations in the EBOV GP that have been identified in the literature and the corresponding residues present at these sites in

the outbreak strains chosen. All outbreak strains contain wild-type residues at each site of potential adaptation and were thus, deemed suitable for inclusion in the panel. Figure 3.2 shows the final panel of ancestral node sequences and outbreak strains that were selected to test in infectivity assays and all sites of amino acid variation present in their GPs that may impact their ability to enter different cell lines. The figure shows that a large number of these mutations are found in the mucin-like domain of the GP (amino acid residues 313-464).

Table 3.1 Residues in outbreak sequences at sites of tissue culture adapted mutations
All chosen sequences contained wild-type residues at these specific sites.

Outbreak Strain	GenBank Reference	522	544	549	552	574	584
Mayinga 1976	U23187.1	D	T	H	D	R	I
Gabon 1994	KC242792.1	D	T	H	D	R	I
Gabon 1996	AY058898.1	D	T	H	D	R	I
Gabon 2001	KY471124.1	D	T	H	D	R	I
Mbandza 2003	EU051635.1	D	T	H	D	R	I
Kelle 2003	KF113528.1	D	T	H	D	R	I
Etoumbi 2005	EU051634.1	D	T	H	D	R	I
Luebo 2007	KC242790.1	D	T	H	D	R	I
Lomela 2014	KP271020.1	D	T	H	D	R	I
Makona C15 2013-2016	KJ660346.2	D	T	H	D	R	I

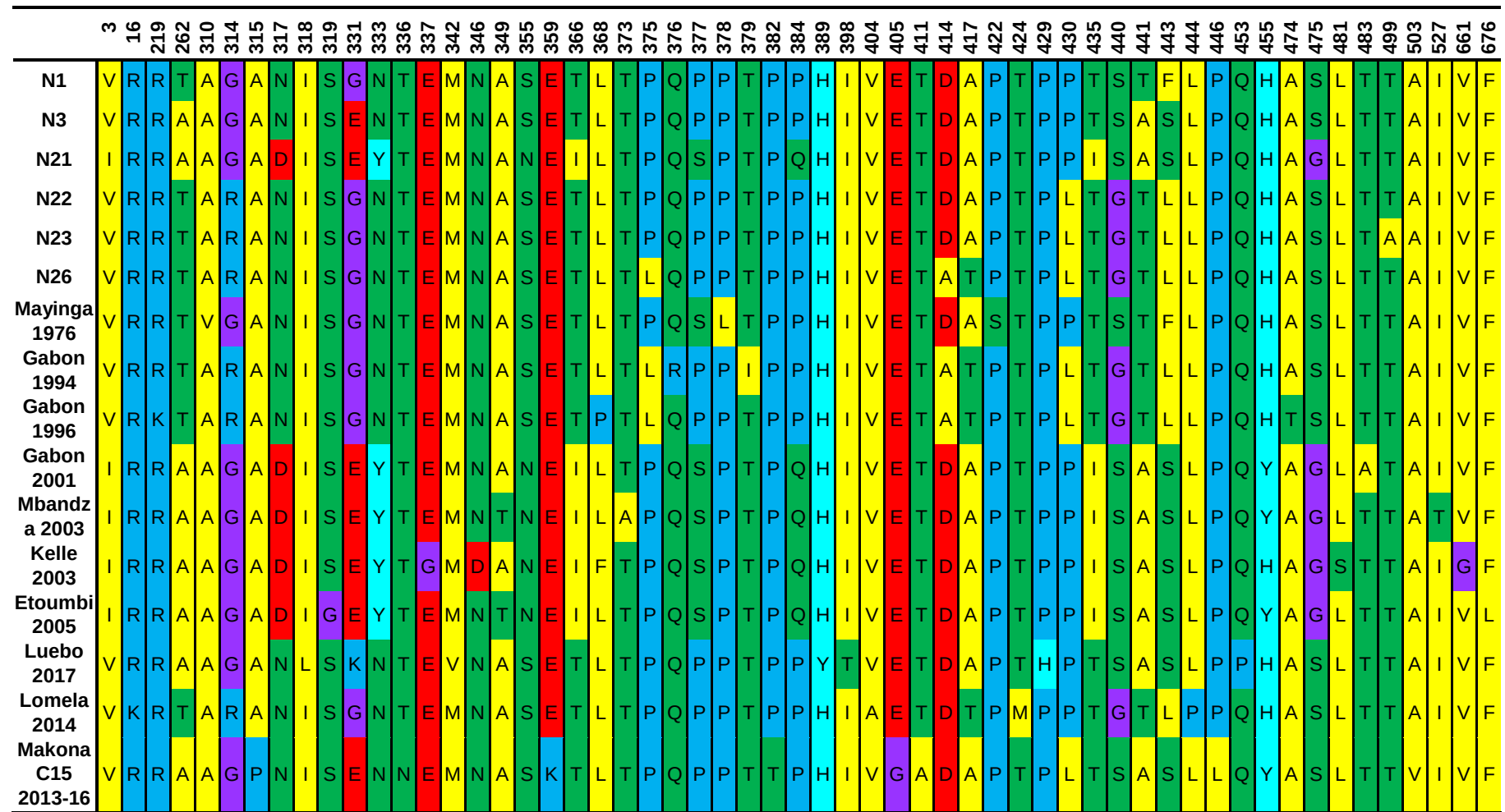


Figure 3.2 Sites of variation in selected ancestral and outbreak EBOV GP sequences

Yellow shows hydrophobic side chains, blue shows aliphatic amino acids, green shows polar amino acids, purple shows glycines, red shows negatively charged amino acids and cyan shows aromatic amino acids. Colour scheme based on the MUSCLE alignment algorithm in the MEGA7 software.

3.2.2 Generation of EBOV GP Constructs Representative of Outbreak Sequences

Of the outbreak sequences, plasmid constructs containing both wild-type and tissue-cultured Mayinga 1976, Makona C15 and the ancestral node GP sequences, had previously been generated by colleagues. To generate the remaining constructs, Figure 3.1 was used to determine the closest ancestral node sequence, to each of the remaining outbreak GP sequences. Alignments of each outbreak sequence to the closest ancestral node sequence allowed identification of the mutational pathway required to generate each outbreak sequence, from the closest ancestral node sequence.

Figure 3.3 shows that six out of the eight chosen outbreak sequences could be derived from either N21 or N26, with five or fewer mutagenesis steps. The Luebo 2007 and Lomela 2014 sequences differed greatly from their nearest ancestral node and were, therefore, synthesised commercially. For sequences to be generated from N21, three of the four outbreak sequences shared the common mutation, H455Y. Therefore, this was the first mutation inserted into the N21 GP gene present in a pcDNA3.1 vector construct (Figure 3.4) via an SDM reaction. On a different N21 template, E337G was also inserted as the first mutation to generate Kelle 2003 and QT376/379TI and R219K were inserted into N26 as the first mutations to generate Gabon 1994 and Gabon 1996, respectively.

Figure 3.5 demonstrates this process. Of the primer pairs used for the reaction, the forward contained the desired mutations. Successful PCR amplification of the template was confirmed by gel electrophoresis, with bands visible at approximately 7.5 Kb. The products were then ligated in a KLD reaction before transformation into Stellar bacterial cells. PCR screening of selected bacterial colonies confirmed the successful ligation and uptake of the plasmid. These colonies were then grown in LB broth for subsequent plasmid extraction. Successful mutagenesis of the template plasmid was confirmed with Sanger sequencing. Purity was determined by spectrophotometry and the generated plasmids were then used as templates for the remaining mutagenesis steps in the pathway to generate each variant, as shown in Figure 3.3. The final EBOV panel of pcDNA3.1 constructs consisted of six ancestral nodes and ten outbreak GP sequences (Figure 3.2).

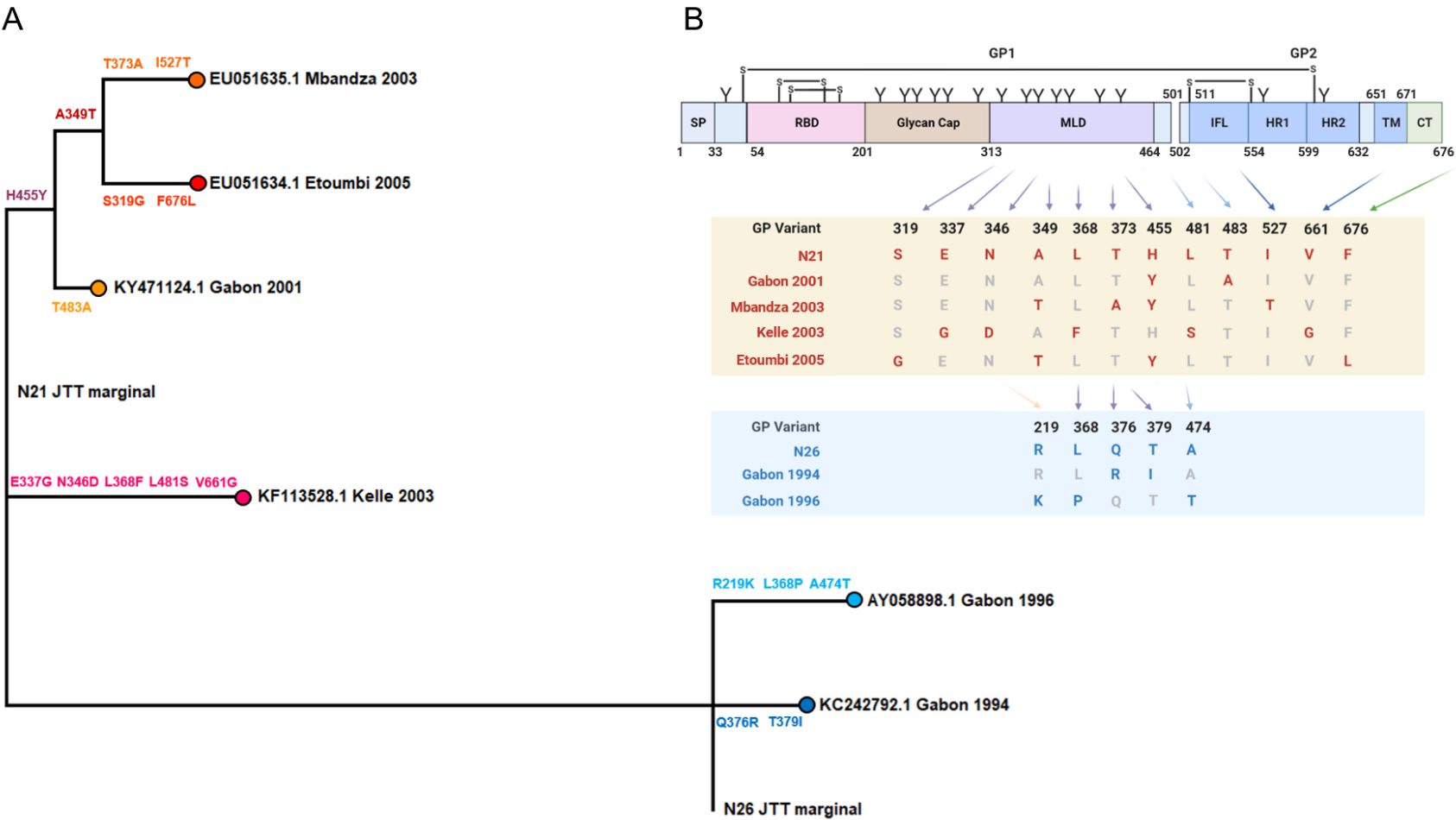


Figure 3.3 Relationship between ancestral EBOV GP sequences and outbreak derivatives
A) Schematic of a maximum-likelihood tree depicting the relationship between the nodes and their derivative outbreak sequences. The mutations needed to generate outbreak sequences have been highlighted. B) Schematic of the sites of the variations present in the ancestral nodes and outbreak GP sequences and their relation to the GP structure.

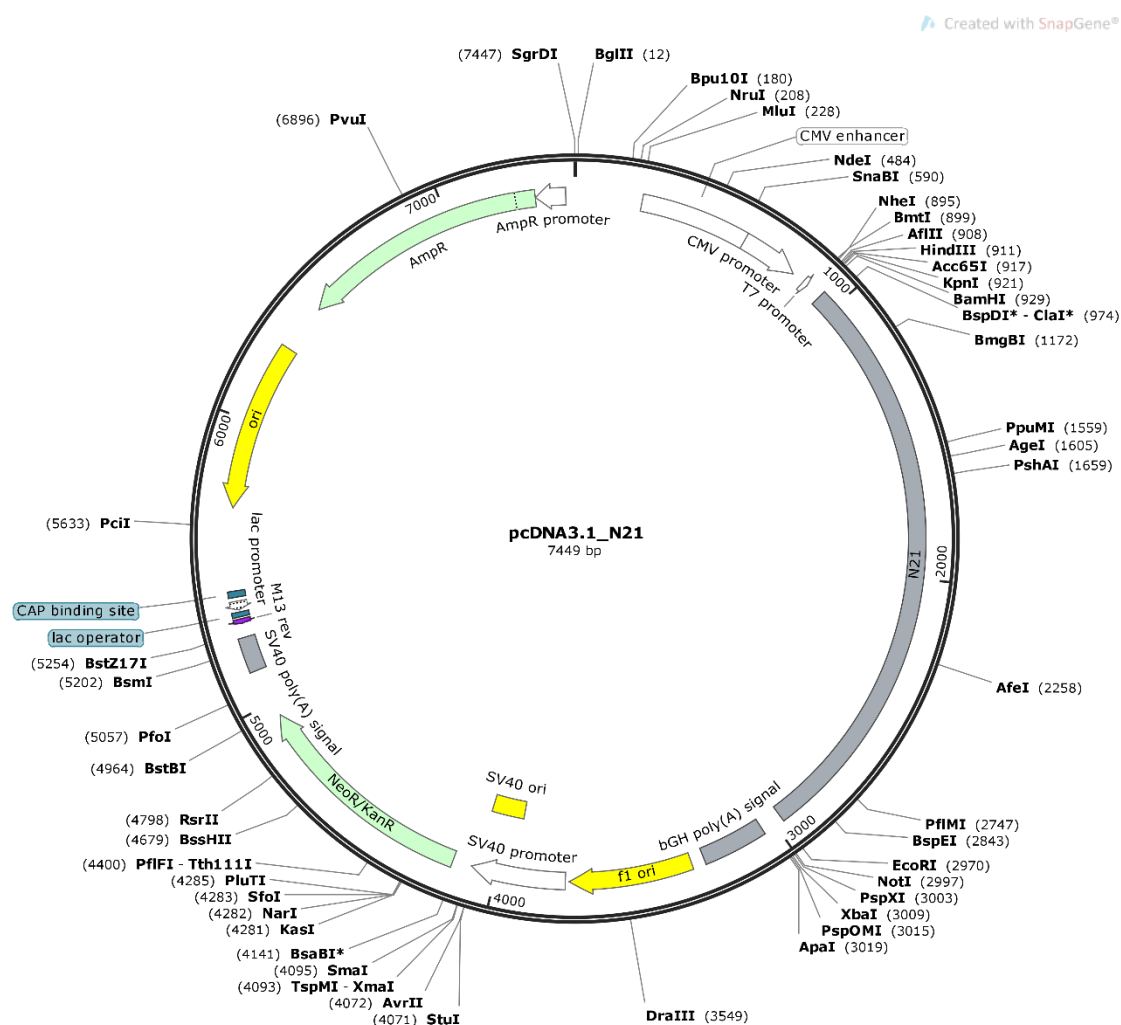


Figure 3.4 Plasmid map of pcDNA3.1_N21

The pcDNA3.1 mammalian expression vector was used to express the EBOV GP variants. Ancestral node sequences (N21 and N26) were used as templates to generate several outbreak sequences via SDM, with the exception of Luebo 2007 and Lomela 2014, which were codon optimised and commercially synthesised into this same vector.

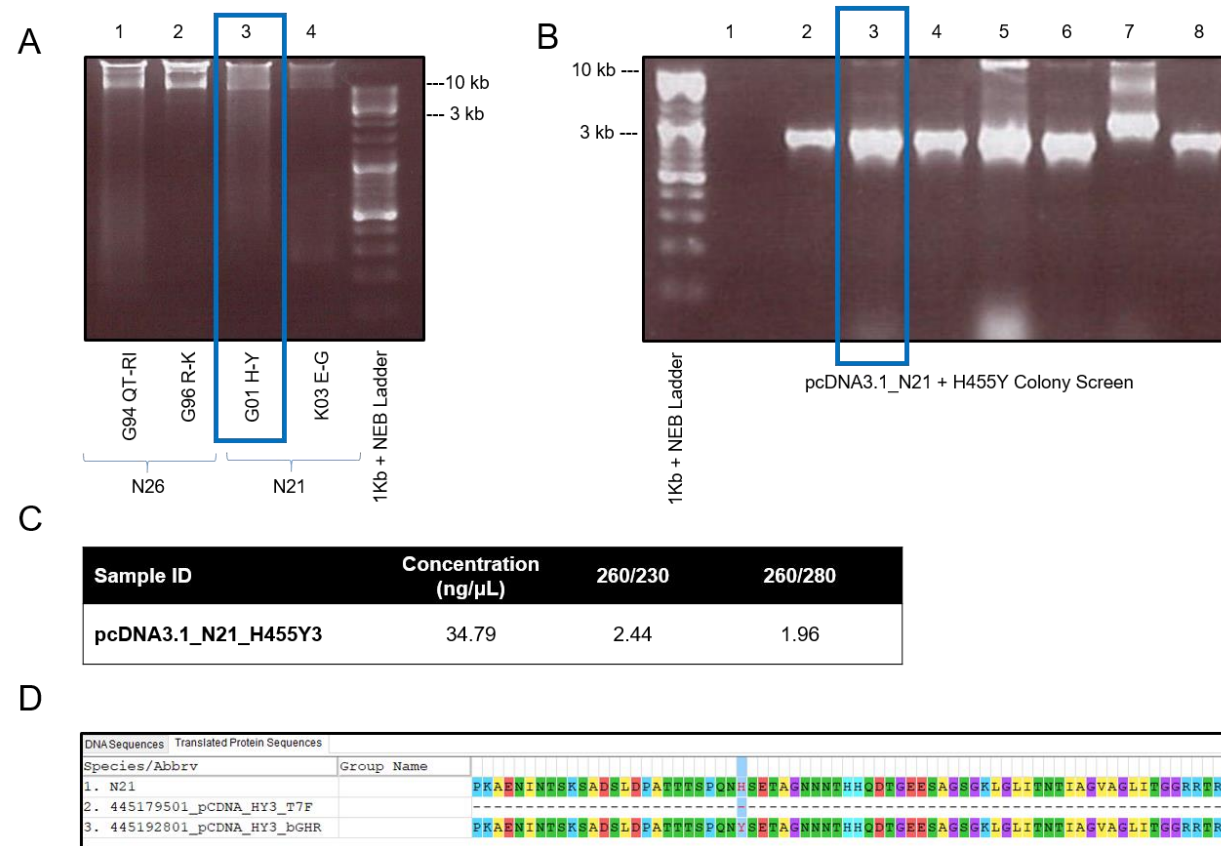


Figure 3.5 Generation of outbreak GP sequences from pcDNA3.1_N21/N26 templates

A) Site-directed mutagenesis was used to insert individual mutations into vectors containing N21 and N26. H455Y was a mutation common to three sequences so was inserted into N21 first. Successful amplification was determined by a band at approximately 7.5 Kb. The PCR product was then used in a kinase, ligase, DpnI (KLD) reaction after which Stellar cells were heat-shocked and transformed. B) Colony screen PCR to determine which colonies contained the desired plasmid. Sequencing of PCR products helped to determine if the GP gene contained the correct mutation. Positive colonies were then grown in a culture and plasmid was then extracted. C) NanoDrop results for the extracted plasmid to determine concentration and purity. D) Sequencing results of plasmid, analysed on MEGA7, showing the desired mutation present at the correct site.

3.2.3 Testing of Ancestral and Outbreak GP Panels for the Production of Infectious Pseudoviruses

As mentioned, constructs containing the Mayinga GP, both wild-type (544I) and the tissue-cultured (544T) sequences as well as the Makona-Kissidougou C15 sequence, had been previously generated by colleagues. The Makona C15 sequence GP construct was demonstrated to produce infectious PVs by colleagues in published work (Urbanowicz *et al.*, 2016). It was therefore used as a positive control for cell culture experiments, alongside VSV G.

To determine the ability of the Mayinga 1976 constructs to produce PVs that were correctly expressing functional glycoprotein, as well as the impact of the tissue culture mutation in the GP, HEK-293T producers cells were transfected in a three-plasmid system (Chapter 2.7 and 2.8). Generated PVs were then used to infect HuH-7 cells and Hyp/Lu 45.5 cells. In doing so, a reporter plasmid containing the luciferase gene was delivered to these cells, so that once lysed and challenged with the substrate luciferin, the resulting bioluminescence emitted from lysed cells could be used as a measure of GP infectivity.

Figure 3.6 shows the infectivity of these EBOV variants, relative to Makona C15 in HuH-7 cells and Hyp/Lu 45.5 cells. In both cell lines, tissue-cultured Mayinga 1976 displays significantly higher infectivity compared to its wild-type counterpart and Makona C15 ($P < 0.0001$). In HuH-7 cells, there is approximately a six-fold difference in infectivity between wild-type and cultured Mayinga 1976. In Hyp/Lu 45.1 cells, this difference is approximately two-fold. As this increase is not representative of the virus as it circulates in the wild, the cultured Mayinga 1976 GP was discarded from the panel and wild-type GP was used in further experiments as representative of the first EBOV outbreak.

Subsequently, all ancestral node and outbreak constructs were also tested in the same cell culture system, to determine if these could also produce functional PVs expressing the desired GP. PVs were used to infect HuH-7 cells as shown in Figure 3.7. The data has been normalised to Mayinga 1976 as the earliest outbreak sequence. All ancestral node sequences are shown to be significantly more infectious than Mayinga 1976 and Makona C15 in HuH-7 cells ($P < 0.0001$). In contrast, the general infectivity of the outbreak sequences was either similar to or markedly higher than these two sequences. The only exceptions to this are Mbandza 2003 and Luebo 2007, which are significantly lower in infectivity relative to both Mayinga 1976 and Makona C15 ($P < 0.0001$). The average raw

RLU values for Mbandza 2003 was 37,073 across three independent experiments and 45,945 for Luebo 2007.

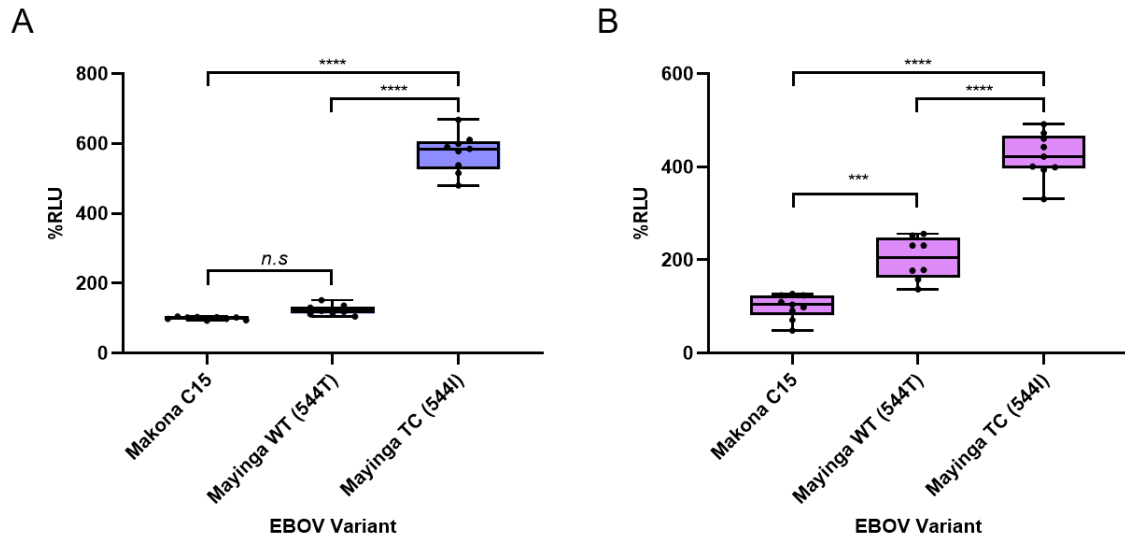


Figure 3.6 Infectivity of wild-type and tissue-culture adapted Mayinga GP in A) HuH-7 cells and B) Hyp/Lu 45.1 cells

In both cell lines, there was a significant difference in infectivity between Makona C15 and wild-type Mayinga 1976, compared to the tissue-cultured variant ($P < 0.0001$). In HuH-7 cells, there was no significant difference in the infectivity of Makona C15 and wild-type Mayinga 1976. Experiments were completed in triplicate and normalised to Makona C15 (100%) as the positive control and the delta envelope protein negative control (0%; not shown on graph). Normalised triplicate data from three independent experiments were pooled. Normal distribution of pooled data presented in both A) and B) was confirmed through a Kolmogorov-Smirnov normality test. Bars on box plots show minimum and maximum values of pooled data. Statistical significance was determined by a one-way ANOVA and a Tukey's multiple comparisons test. A) All GP clones displayed a coefficient of variation (CV) of $<10.0\%$ with the exception of Mayinga WT (544T). B) All GP clones displayed a CV of $>10.0\%$. CV was calculated from data pooled from three independent experiments.

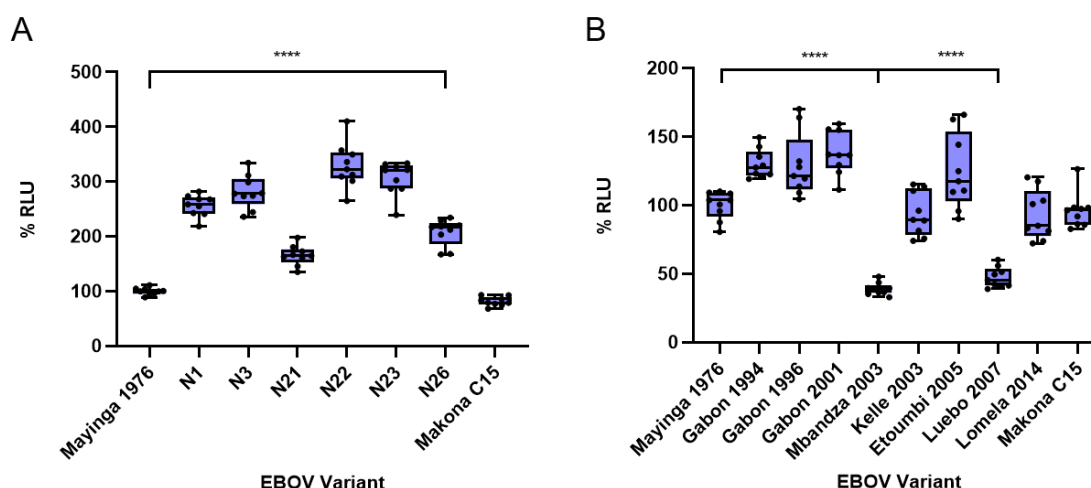


Figure 3.7 Infectivity of A) ancestral and B) outbreak GP sequences in HuH-7 cells

All ancestral node sequences were significantly more infectious than wild-type Mayinga and Makona C15 ($P < 0.0001$). All outbreak GPs had similar or increased infectivity compared to wild-type Mayinga, except for Mbandza 2003 and Luebo 2007, which were significantly less infectious. Experiments were completed in triplicate and normalised to Mayinga 1976 (100%) as the first outbreak strain and the delta envelope protein negative control (0%; not shown on graph). Normalised triplicate data from three independent experiments was pooled. Normal distribution of pooled data presented in A) and B) was confirmed through a Kolmogorov-Smirnov normality test, with the exception of Makona C15 in dataset B). Bars on box plots show minimum and maximum values of pooled data. Statistical significance was determined by a one-way ANOVA and a Dunnett's multiple comparisons test. A) Mayinga 1976, N1, N23 and Makona C15 displayed a coefficient of variation (CV) of $<10.0\%$; all remaining clones, $>10.0\%$. B) Gabon 1994 displayed a CV of $<10.0\%$ all remaining clones; $>10.0\%$. CV was calculated from data pooled from three independent experiments.

These experiments show all constructs produce infectious PVs that can be successfully used in future experiments. Although the Luebo 2007 GP displayed significantly lower infectivity in HuH-7 cells compared to Mayinga 1976 and Makona C15, it was hypothesised that this was unlikely due to an issue with the pcDNA3.1 construct containing the GP gene as it was commercially synthesised. However, the low infectivity of the Mbandza 2003 GP required further investigation in experiments using multiple cell lines, to determine whether it was a property of the GP, only observable in HuH-7 cells or an issue with the construct used to generate the PVs, resulting in poor infectivity in all cell lines. This was because the construct was generated by SDM which relies on PCR to amplify the entire plasmid to include the desired mutation. Consequently, this method of amplification may have resulted in a mutation in a fragment of the plasmid that is important in GP expression, such as the promoter. Therefore, the infectivity of both constructs was also tested in the Hyp/Lu 45.1 and Vero E6 cell lines to determine if the low infectivity observed in HuH-7 cells could be observed in other cell lines, which could potentially indicate a construct issue. This data is shown in Figure 3.8.

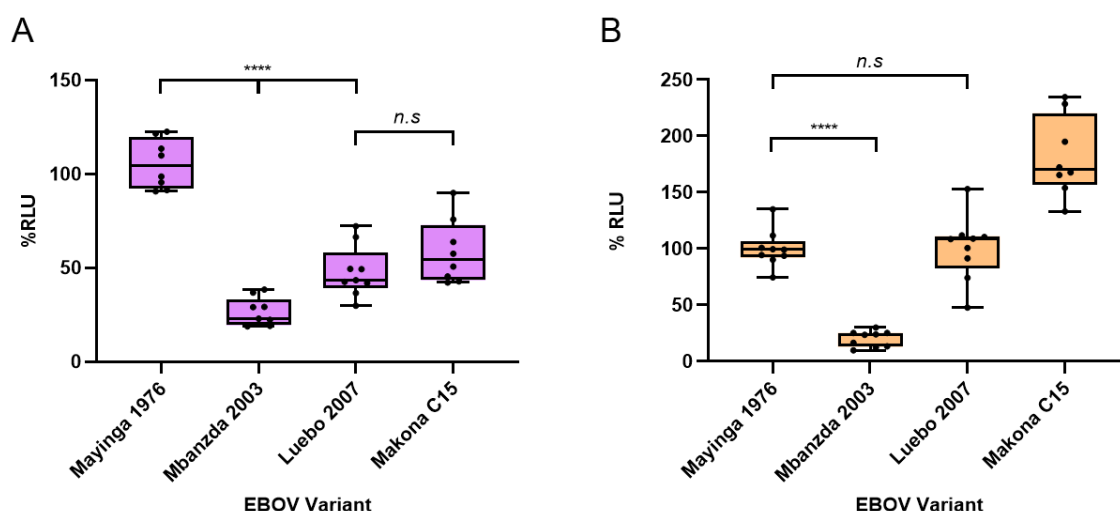


Figure 3.8 Infectivity of Mbandza 2003 and Luebo 2003 GP in A) Hyp/Lu 45.1 cells and B) Vero E6 cells

In both cell lines, the Mbandza 2003 GP is significantly less infectious relative to the Mayinga 1976 and Makona C15 GPs. Although the Luebo 2007 GP is significantly less infectious relative to Mayinga 1976 in Hyp/Lu 45.1 cells, there was no significant difference in infectivity between Luebo 2007 and Makona C15 in this cell line. Furthermore, there is no significant difference in infectivity between it and Mayinga 1976 in Vero E6 cells. Experiments were completed in triplicate and data was normalised to Mayinga 1976 (100%) as the first outbreak sequence and the delta E negative control (0%; not shown on graph). Normalised triplicate data from three independent experiments was pooled. Normal distribution of pooled data presented in both A) and B) was confirmed through a Kolmogorov-Smirnov normality test. Bars on box plots show minimum and maximum values of pooled data. Statistical significance was determined by a one-way ANOVA and Tukey's multiple comparisons test. A) and B) All clones displayed a coefficient of variation (CV) of >10.0%. CV was calculated from data pooled from three independent experiments.

Results showed that in both cell lines, the Mbandza 2003 construct displayed significantly low infectivity relative to Mayinga 1976 and Makona C15 ($P < 0.0001$). The average raw RLU value for Mbandza 2003 was 22,651 in Vero E6 cells and 21,457 in HypLu45.1 cells. Contrastingly, the Luebo 2007 construct displayed infectivity comparable to Makona C15 in Hyp/Lu 45.1 cells, and Mayinga 1976 in Vero E6 cells. The results suggested that further investigation into the viability of the pcDNA3.1 construct of the Mbandza 2003 GP to produce infectious PVs was required. However, as expected, the Luebo 2007 GP displayed differential infectivity that was cell-line dependent and required no further investigation.

3.2.4 Characterisation of the Mbandza 2003 GP Construct

Due to the repeated low infectivity observed in the Mbandza 2003 GP construct across multiple cell lines, investigations were completed to determine if the pcDNA3.1 plasmid construct was correctly forming functional PVs. Figure 3.9 shows a western blot of the GP

and MLV p30 core protein from Mbandza 2003 PVs that had been concentrated through ultracentrifugation by a 20% sucrose cushion and resuspended in PBS. The expression of both proteins was compared to that of Mayinga 1976 and Makona C15. As can be observed, Mbandza 2003 displayed intermediate GP expression relative to the Makona C15 GP, which showed the highest expression. Mayinga 1976 GP expression was the lowest (bands observed at ~130 kDa) and VSV G was used as a negative control and showed no GP expression. However, the accuracy of quantification in this experiment is limited as serial dilutions of the GP products were not completed with corresponding levels of protein expression determined by western blot. As a result, it is difficult to determine if the bands show true expression or an oversaturation of the blot. If time had permitted, an additional western blot comparing two-fold dilutions of the GP products would have been completed, so that the intensity of GP expression could be compared at different dilutions to provide a more accurate quantification of expression.

The expression pattern seen did not correlate with the patterns of infectivity observed in the PVs. For example, Figure 3.7 showed that Mbandza 2003 exhibited the least infectivity in HuH-7 cells. However, it consistently displayed levels of GP expression that were higher than the infectious Mayinga 1976 strain in western blot. p30 expression, however, was comparable between all PVs tested although some MLV gag polyprotein precursors were observable, particularly in Makona C15 for which a fairly strong band can be seen at ~80 kDa.

Following this, experiments were completed to determine if there was an issue with the plasmid construct containing the GP itself. The Mbandza 2003 GP sequence was PCR amplified from the original pcDNA3.1 construct, to contain EcoRI overhangs and then the amplified product was sub-cloned into an EcoRI digested version of the plasmid, which had previously been sequenced. After Sanger sequencing confirmed the sequence of the promoter, the insertion site and that of the GP to be correct, the Mbandza 2003 sub-clones were used to produce PVs and their infectivity was tested alongside the original construct that was made through SDM and therefore, could have had undesirable mutations in regions of the plasmid associated with GP expression.

Figure 3.10 shows the results of these experiments in HuH-7, Hyp/Lu 45.1 and Vero E6 cells, relative to the Makona C15 construct which was used as a positive control. In all three cell lines, there was no difference in infectivity between the newly generated Mbandza 2003 sub-clones, and the SDM-generated original Mbandza 2003 construct. This confirmed that the low infectivity observed with the Mbandza 2003 GP, across

multiple cell lines, was a true characteristic of this GP and not due to the way the original plasmid construct was generated.

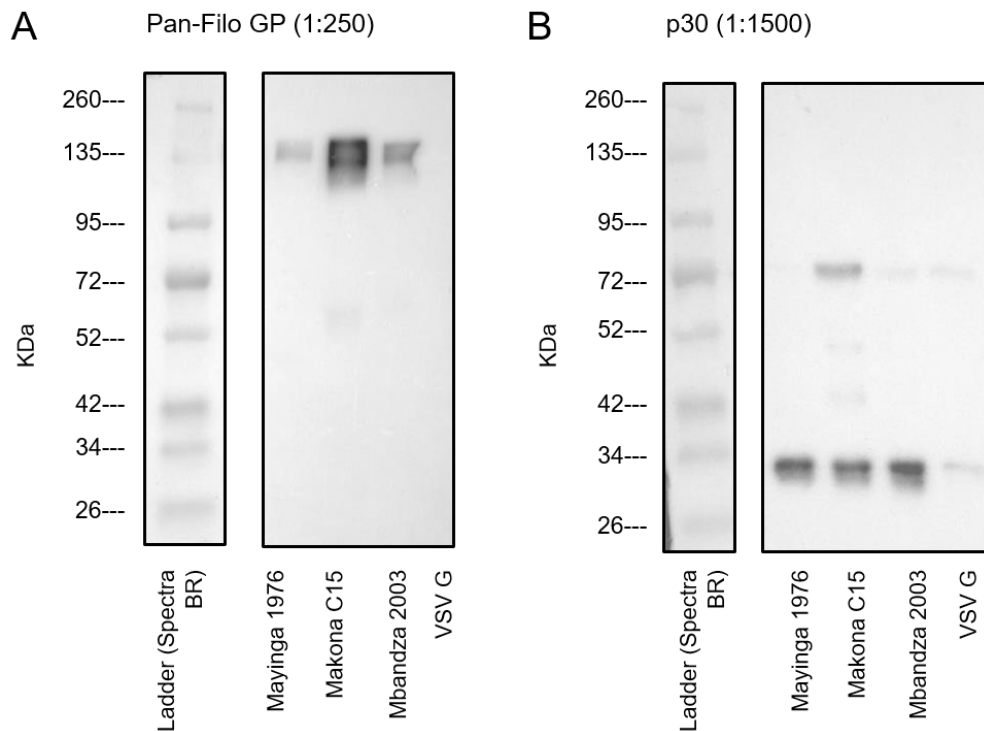


Figure 3.9 Western Blot of Mbandza 2003 GP and p30 core protein

A) A 1:250 dilution of a Pan-filo α -GP antibody (IBT) was used to detect the GP at approximately 130 kDa. There was a differential expression of GP between the three EBOV variants, with the highest observed in Makona C15. VSV G protein was used as a negative control. B) MLV p30 core protein was detected at 30 kDa with a 1:1500 dilution of MLV α -p30 antibody (Abcam). Consistent expression of p30 is observable between the three variants, however, p30 precursors are also observable, particularly for Makona C15. A 1:2000 dilution of a rabbit, α -mouse HRP (Dako) was used as a secondary antibody for both blots. Bands were visualised by a G-Box (SynGene) with an exposure time of 15 minutes. The image was cropped to remove irrelevant wells. Data representative of three independent experiments.

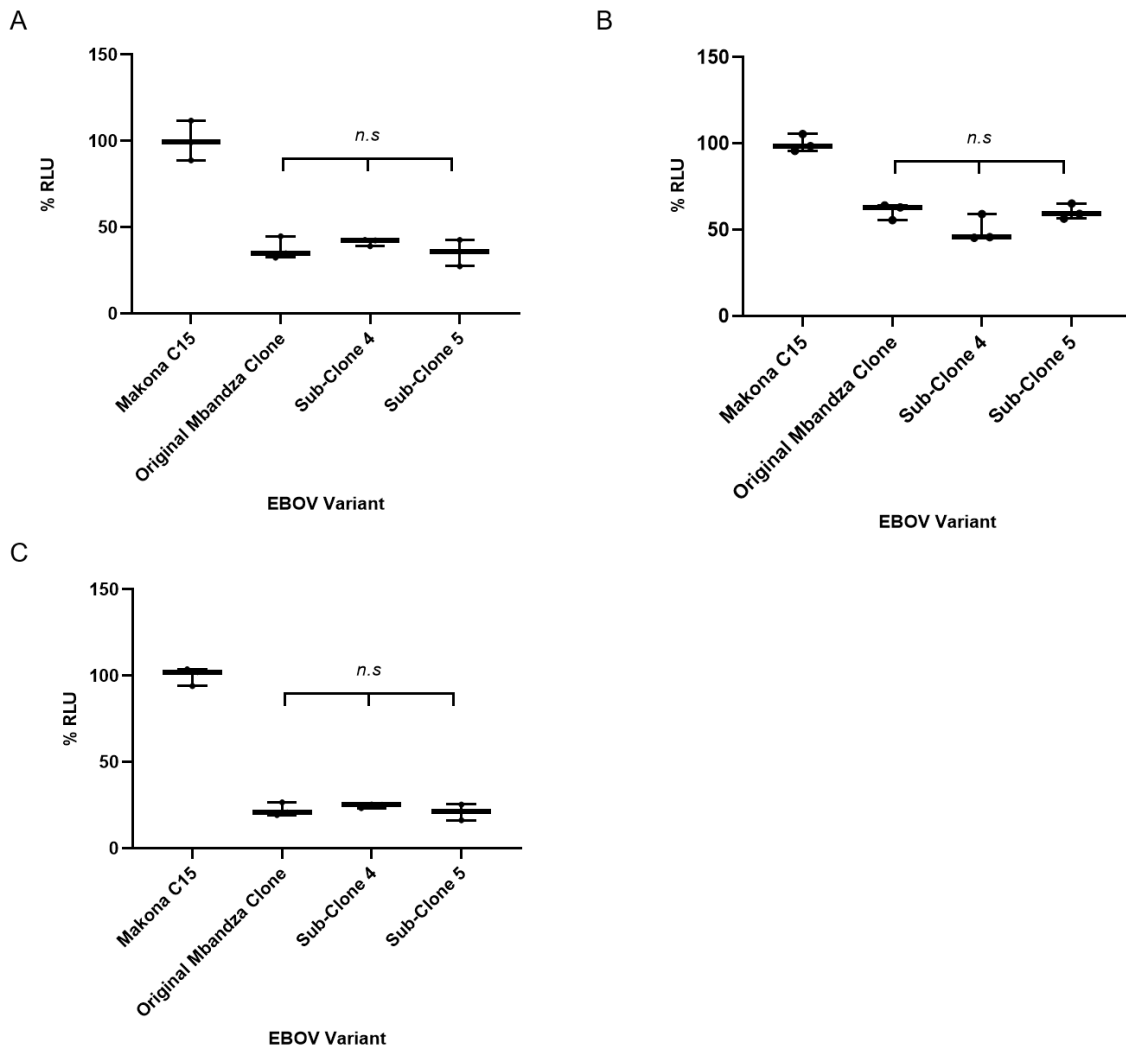


Figure 3.10 Infectivity of Mbandza 2003 GP sub-clones in multiple cell lines

Mbandza GP was sub-cloned into linearized pcDNA3.1 and the corresponding infectivity was compared to that of the original construct. There is no difference in infectivity between the original construct and the two sub-clones tested in A) HuH-7, B) Hyp/Lu 45.1 or C) Vero E6 cells. Data was obtained in triplicate and normalised to the positive control, Makona C15 (100%) and Delta E (0%, excluded from the graph). Normal distribution of data presented in A), B) and C) was confirmed through a Shapiro-Wilk normality test, with the exception of Sub-clone 4 in B). Bars on box plots show minimum and maximum values of triplicate data. Statistical significance was determined by one-way ANOVA and a Tukey's multiple comparisons test. A) Data for all clones displayed a coefficient of variation (CV) of >10.0%, with the exception of Sub-clone 4 (4.9%). B) All clones displayed a CV of <10.0%, with the exception of Sub-clone 4. C) Makona C15 and Sub-clone 4 displayed a CV of <10.0%. CV was calculated from an individual experiment.

3.2.5 The I527T Mutation Causes Low infectivity of Mbandza 2003 GP

Having concluded that the low infectivity of Mbandza 2003 across multiple cell lines was a true characteristic of the GP, investigations were made into which specific residues could be responsible for this observation. As Figure 3.3 showed, Mbandza 2003 was generated from the N21 plasmid after four consecutive rounds of SDM to successively insert the

mutations H554Y, A349T, T373A and I527T. Consequently, alongside the N21 template, intermediate pcDNA3.1 constructs containing the N21 GP + H455Y, N21 + H445Y and A349T and N21 + H455Y, A349T and T373A mutations were all available to test to determine if the accumulation of these mutations sequentially in N21 impacted its infectivity. Figure 3.11 shows the impact of these sequential mutations on the infectivity of N21 relative to Makona C15, in HuH-7, Hyp/Lu 45.1 and Vero E6 cell lines. As can be seen, in all cell lines, there is no significant difference in infectivity between N21 and these intermediate GP constructs. This suggested that the reduction in infectivity observed is due to the final mutation, I527T, inserted in N21 GP to produce Mbandza 2003.

To test this theory, SDM was used to insert I527T as a single mutation into the N21 GP template. This mutation was also reversed (T527I) in the Mbandza 2003 construct. These constructs were used to produce PVs, the infectivity of which was tested alongside N21 and Mbandza 2003. Figure 3.12 shows the result of this experiment, relative to Makona C15, once again in the HuH-7, Hyp/Lu 45.1 and Vero E6 cell lines.

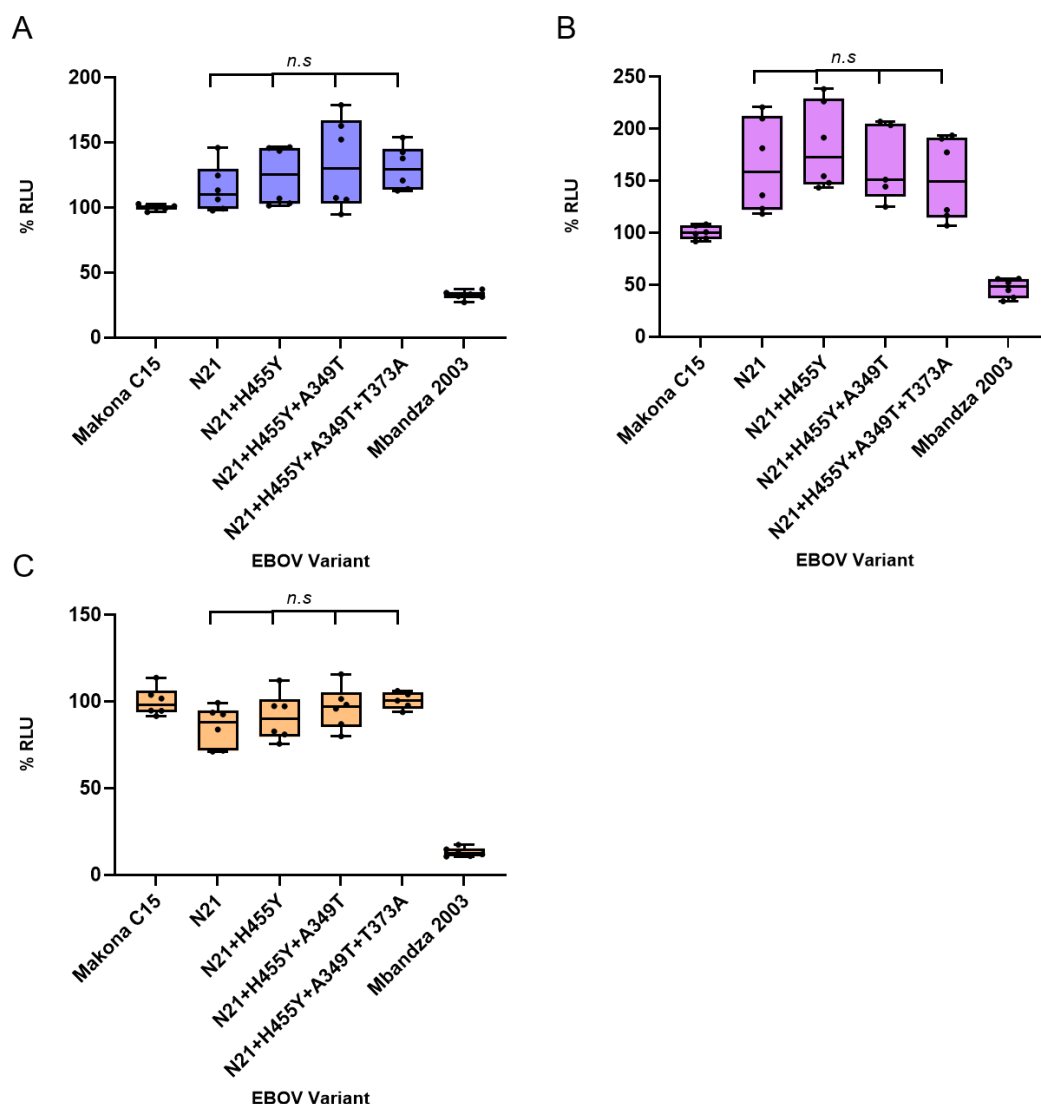


Figure 3.11 Infectivity of N21, Mbandza 2003 and all intermediate GP constructs in multiple cell lines

There is no difference in infectivity between N21 and any of the intermediate constructs tested in A) HuH-7, B) Hyp/Lu 45.1 and C) Vero E6 cells. Data was obtained in triplicate and normalised to the positive control, Makona C15 (100%) and Delta E (0%, excluded from graph). Normalised triplicate data from two independent experiments were combined. Normal distribution of combined data presented in A), B) and C) was confirmed through a Kolmogorov-Smirnov normality test. Bars on box plots show minimum and maximum values of combined data. Statistical significance was determined by a one-way ANOVA and a Tukey's multiple comparisons test. A) Makona C15 displayed a coefficient of variation (CV) of 2.2%; all remaining clones, >10.0%. B) Makona C15 displayed a CV of 6.5%; all other clones, >10.0%. C) Makona C15 and N21+H455Y+A349T+T373A displayed a CV of <10.0%; all other clones, >10.0%. CV was calculated from combined data from two independent experiments.

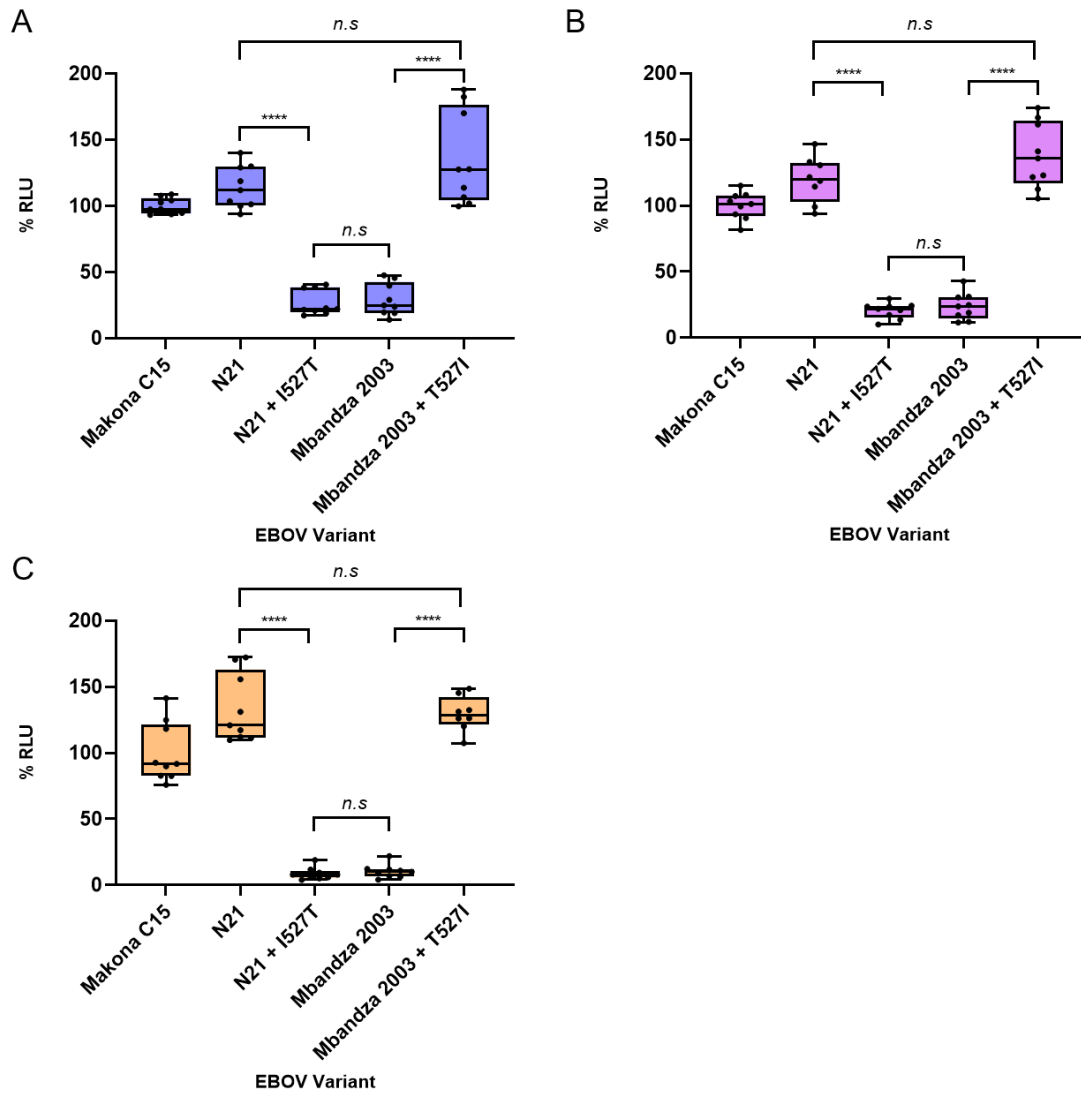


Figure 3.12 Impact of the I527T mutation on N21 infectivity in multiple cell lines

The addition of I527T to N21 causes a decrease in infectivity ($P < 0.0001$) so that there is no significant difference between N21+ I527T and the Mbandza 2003 constructs. Conversely, reverting this mutation in Mbandza 2003, causes its infectivity to increase so that there is no significant difference between Mbandza 2003+ T527I and N21. This was observed in A) HuH-7, B) Hyp/Lu 45.1 and C) Vero E6 cells. Data was obtained in triplicate and normalised to the positive control, Makona C15 (100%) and Delta E (0%, excluded from the graph). Normalised triplicate data from three independent experiments was pooled. Normal distribution of data was determined using a Kolmogorov-Smirnov normality test, with the exception of N21 + I527T in dataset A) and Makona C15 in dataset C). Bars on box plots show minimum and maximum values of pooled data. Statistical significance was determined by a one-way ANOVA and a Tukey's multiple comparisons test. A) Makona C15 displayed a coefficient of variation (CV) of 6.0; all others clones $>10.0\%$. B) and C) All clones displayed a CV of $>10.0\%$. CV was calculated from data pooled from three independent experiments.

Interestingly, the data shows that in all three cell lines, adding I527T to the N21 GP reduces its infectivity dramatically so that there is no significant difference between this and the original Mbandza 2003 construct. Furthermore, reverting this mutation in the Mbandza 2003 GP increases its infectivity so that there is no significant difference

between the revertant and N21. It can therefore be concluded that the low infectivity observed for the Mbandza 2003 GP in multiple cell lines, is due to the I527T mutation.

3.3 Discussion

The first challenge that zoonotic viruses such as filoviruses encounter when jumping from their natural reservoir to a susceptible host species is attachment and entry into the host's cells. For EBOV and many other enveloped viruses, the key determinant involved in these steps is the GP (Hofmann-Winkler, Kaup and Pöhlmann, 2012; Hunt, Lennemann and Maury, 2012; Hoffmann *et al.*, 2016). Natural variation within the structure of the GP could, therefore, alter the efficiency of these steps, as was previously witnessed during the West African outbreak (2013-2016). Subsequent analysis of over 1500 genome sequences that had been clinically sampled and deposited online, demonstrated that the virus had been evolving during the outbreak and additional phenotypic studies demonstrated that the observed mutations resulted in the virus adapting to the human host (Baize *et al.*, 2014; Gire *et al.*, 2014; Park *et al.*, 2015; Kugelman, Sanchez-Lockhart, *et al.*, 2015; Kugelman, Wiley, *et al.*, 2015; Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016).

In particular, two groups published complementary studies that investigated the effects of A82V, a mutation located on the receptor-binding domain of the GP (Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016). This mutation appeared early on in the outbreak and subsequently rose to a high frequency, appearing in over 90% of the sampled genomes (Holmes *et al.*, 2016; Dietzel *et al.*, 2017). Both groups demonstrated that A82V increased the infectivity of the virus in human cell lines (Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016). If a single variation within an outbreak can lead to phenotypic changes, it is apparent that addressing the variation that might exist between EBOV strains that circulated in different outbreaks, should also be a priority. To this end, pseudotyping represents a simple and efficient model to investigate the variation in the GP that may have an impact on factors such as cell entry and host cell tropism, as well as providing a substitute antigen to test the efficiency of existing vaccines and therapeutics (Gutierrez-Guerrero, Cosset and Verhoeyen, 2020).

In line with the above, this work provided the first comprehensive panel of EBOV GP pseudotypes that were representative of outbreak sequences in Figure 3.1 taken from the early stages of outbreaks occurring between 1976 and 2016. Interestingly, the comparison between the tissue-cultured Mayinga 1976 strain and its wild-type counterpart

confirmed the findings of other studies, specifically, that the T544I adaptation increased the infectivity of the virus in different cell lines, including bat cells ($P < 0.0001$) (Hoffmann *et al.*, 2017; Ruedas *et al.*, 2017, 2018). Consequently, the wild-type strain was included in the panel of outbreak constructs.

Alongside ancestral node constructs that were previously modelled by Professor Holmes, this panel was fully operational in a three-plasmid MLV lentiviral system and was shown to produce functional PVs capable of infecting HuH-7 cells. Most importantly, the study indicated that both the ancestral node sequences and the outbreak strains possess differential patterns of efficiency of cell entry within a HuH-7 cell line, meaning that within this *in-vitro* model, some outbreak strains are more infectious than others (Figure 3.7). This finding will be investigated further in the next chapter, whereby the differential patterns of infectivity of the outbreak sequences will be compared to that of the ancestral nodes in HuH-7 and Hyp/Lu 45.1 cells, to gain more insight into how EBOV may be evolving and to provide more evidence into what the reservoir species may be.

This study also demonstrated that the Mbandza 2003 strain, consistently displayed low infectivity in HuH-7 Vero E6 and Hyp/Lu 45.1 cells, each representative of a theoretical bat reservoir, an intermediate primate host and a human end host, respectively (Figures 3.7 and 3.8). This result suggests that there may have been an atypical loss of viral fitness when Mbandza 2003 jumped to a new host species (Leroy *et al.*, 2005; Pourrut *et al.*, 2007, 2009).

Following the observation that Mbandza 2003 displayed poor infectivity in all cell lines tested, western blots were used to confirm GP and p30 core protein expression in the preparations containing the Mbandza 2003 PVs. However, this method is not sufficient to determine whether the PVs were correctly forming and expressing GP. Therefore, the Mbandza 2003 GP was sub-cloned into linearized pcDNA3.1 to exclude any issues with the vector sequences that could lead to sub-optimal GP expression.

The original construct was generated by SDM of the N21 plasmid template, to insert amino acid substitutions at four different positions, H455Y, A349T, T373A and I527T. The intermediate constructs generated in the SDM pathway (containing the first three mutations above) were tested for their impact on cell entry. Although they were only tested sequentially, they were not found to contribute to the low infectivity observed by Mbandza 2003. The insertion of the I527T mutation in N21 caused its infectivity to drop to levels comparable to Mbandza 2003. Similarly, reverting this mutation (T527I) in Mbandza 2003

caused its infectivity to increase to levels comparable to N21 in the three cell lines tested. Therefore, it can be concluded that it is the I527T mutation that is responsible for the low infectivity of Mbandza 2003 in HuH-7, Vero E6 and Hyp/Lu45./1 cells, suggesting that it is not well adapted to either the potential reservoir or a different host species. If time had permitted, a western blot of the N21+ I527T and the Mbandza 2003 + T527I GP variant proteins would have been completed alongside the N21 and Mbandza 2003 GPs, to determine if there was any impact of the I527T mutation on protein expression.

One challenge faced throughout this work was the broad range in the raw infectivity values displayed by clones, between experiments. Although triplicate results for a clone within an experiment displayed a very small range, the range of data between experiments varied and as a result, when calculating the coefficient of variation (CV) of pooled data, the values obtained for different clones often exceeded the typical thresholds that indicate a high or moderate precision of data (<5.0% and <10.0%, respectively). Although a limitation to this work, the patterns of infectivity that different clones displayed in different experimental repeats, followed the same trend post-normalisation of the data and thus, were deemed acceptable in drawing conclusions. The differences observed in the raw data may be due to inconsistencies in the handling of producer cells between experiments and if time had permitted, further experimental repeats would have been completed to obtain more precise data. Additionally, once data were normalised, normality tests were completed to determine whether the data displayed a normal distribution. In most cases, pooled data for clones displayed normal distribution according to test results, however in some instances, a clone failed the chosen test. Again in these instances, as data should be conceptually normally distributed, data not fitting a normal distribution is likely due to under-sampling and insufficient repeats rather than the data displaying an alternative distribution and therefore, once again, further experimental repeats would have likely solved this problem.

The I527T mutation is located on the internal fusion loop (IFL) of the GP (Lee *et al.*, 2008). Previous studies investigating the impact of the T544I mutation, also present in this region, showed the dramatic implications that mutations of the IFL can have on viral entry (Hoffmann *et al.*, 2017; Ruedas *et al.*, 2017). This is because IFL is fundamental for membrane fusion between the virus and the host cell receptor (Gregory *et al.*, 2011). Following the cleaved GP binding to the NPC1 receptor, an unknown fusion trigger results in a conformational change in GP₂ that exposes the IFL, which in turn, is inserted into the target cell membrane (Gregory *et al.*, 2011; Brecher *et al.*, 2012; Hoffmann *et al.*, 2017). Studies have shown that the apex of the IFL is structured into a hydrophobic 'fist,'

supported by a scaffold that includes residues 529, 535 and 544 (Gregory *et al.*, 2014). Mutations in this region, particularly at positions 529 and 544, can severely disrupt the hydrophobic surface associated with the first structure (Gregory *et al.*, 2014). It is therefore postulated that the substitution from I to T at position 527 may result in a change in structure that poorly supports the fusion of the viral membrane with the host cells, likely due to the disruption of the IFL scaffold or the hydrophobic surface of the first structure. Further modelling of the structure of the Mbandza 2003 IFL and the NPC1 residues it interacts with would be required to determine exactly how I527T causes the low infectivity phenotype.

The outbreak caused by this variant was relatively short, occurring between October and December of 2003 and resulting in 35 cases and 29 deaths (Nkoghe *et al.*, 2011; Pigott *et al.*, 2014). With very limited sequencing data available, it is unclear if I527T persisted in the GP throughout the outbreak. Given its negative impact on viral fitness, it is also unclear whether it could have been subject to purifying selection, although this phenomenon has not been observed in larger outbreaks albeit with the absence of supporting phenotypic studies (Olabode *et al.*, 2015). Regardless of its poor infectivity in different cell lines, the case fatality rate for Mbandza 2003 outbreak was high at 88%. This shows that poor infectivity in an *in-vitro* model may not always translate to poor disease progression or virulence of EBOV strains in an outbreak and that any correlation between poor infectivity and other factors impacting viral fitness and pathogenesis would need to be confirmed with *in-vivo* models.

The Mbandza 2003 outbreak was one of several that occurred between 2001 and 2005 on the border between Gabon and RC, including in Kelle (2003) and Etoumbi (2005) (Leroy *et al.*, 2004; Pourrut *et al.*, 2005; Rouquet *et al.*, 2005; Grard *et al.*, 2011; Nkoghe *et al.*, 2011). These outbreaks were shown to coincide with large losses of wildlife in the surrounding area, particularly within gorilla and chimpanzee populations (Walsh *et al.*, 2003; Leroy *et al.*, 2004, 2005). The identification of multiple epidemic chains during some of these outbreaks caused by different strains of the virus led to the conclusion that EBOV was repeatedly and independently spilling over from an unknown reservoir species (Leroy *et al.*, 2004).

Subsequent phylogenetic analysis of the viral GP and NP genes found in sequences obtained from great ape carcasses as well as the human outbreaks between 2001 and 2005, revealed the existence of two new genetic EBOV lineages; Group B viruses, which were mainly gorilla-derived sequences that showed phylogenetic differences compared to

the sequences from viruses obtained from outbreak between 1976 and 1996 (denoted Group A) and Group R viruses, which were mainly outbreak sequences obtained between 2001 and 2003 (Wittmann *et al.*, 2007). The study theorised that Group R viruses occurred from a recombination event between Group A and Group B viruses, based on their phylogenetic proximity to the GP gene of Group A viruses and the NP gene of Group B viruses (Wittmann *et al.*, 2007). Interestingly, analysis of GP and NP genes taken from sequences obtained from the Mbandza 2003 and Etoumbi 2005 outbreaks showed phylogenetic similarity to the gorilla-derived Group B viruses rather than to previous human outbreak strains that formed Group A viruses (Wittmann *et al.*, 2007).

Since phylogenetically, Mbandza 2003 clusters with Group B viruses, comparing phenotypic characteristics between Group B gorilla-derived sequences and Mbandza 2003 in further pseudotyping assays, may provide additional insight into whether the strain could be behaving more like its gorilla-derived counterparts rather than the outbreak strains it was compared to in this study. Interestingly, Etoumbi 2005 also clustered with Group B viruses, however, this strain displayed slightly higher infectivity than Mayinga 1976 ($P=0.0126$) in HuH-7 cells.

Lastly, Group R viruses were included in the phylogenetic analysis performed in this study as shown in the final clade in Figure 3.1. However, a representative sequence was not included in the final panel as the respective alignment of these recombinant GP sequences demonstrated that they all contained a stop codon at position 481, resulting in a truncated GP product that was predicted to be 29% shorter. Therefore, the Group R viruses were deemed incomparable to other outbreak GP sequences, even though they were involved in a series of outbreaks between 2001 and 2002 (Leroy *et al.*, 2004; Wittmann *et al.*, 2007).

In conclusion, this work resulted in the successful creation of a panel of EBOV GPs representative of outbreaks between 1976 and 2016. This panel will be tested in PV assays alongside ancestral node GPs to determine how the strains involved may behave in different cell lines and to provide insight into how the virus may evolve. Additionally, the Mbandza 2003 strain was shown to display low infectivity in three different cell lines. This difference was attributed to the I527T mutation in the IFL of the GP and may be due to structural changes that interfere with the hydrophobic fist of the IFL that is required to instigate membrane fusion but further studies modelling the GP IFL and its interacting partners would be required to confirm this.

4. Determining Infectivity of EBOV Ancestral and Outbreak GPs in Multiple Cell Lines

4.1 Introduction

Zoonotic diseases are caused by organisms whose natural reservoirs are typically wild or domesticated animals (Pigott *et al.*, 2014). Statistics estimate that up to 60% of known infectious diseases and up to 75% of emerging infectious diseases are caused by zoonotic transmission events (Salzer *et al.*, 2017). With any zoonotic virus, the pathogen must be able to enter a host cell, replicate, infect more cells and then successfully be sustained in a chain of transmission within the new host species (Warren and Sawyer, 2019). As urbanisation, human encroachment into once rural areas and globalisation increase, the frequency of zoonotic transmission events is bound to rise, a pattern that is becoming more and more apparent with EBOV outbreaks, in which CFR can range between 50 and 90% (Feldmann and Geisbert, 2011; Pigott *et al.*, 2014; Neiderud, 2015).

4.1.1 Evolution of EBOV in Outbreaks

Since it was first recorded in 1976 in the DRC (formerly known as Zaire), EBOV has caused the most human outbreaks among all incidents of EVD (Pourrut *et al.*, 2005; de La Vega, Stein and Kobinger, 2015; Languon and Quaye, 2019). Early outbreaks occurred sporadically and were confined to regions of central Africa, particularly the DRC and the border between RC and Gabon. These outbreaks consisted of a moderately small number of cases, often occurring in rural areas and were successfully controlled by basic public health and quarantine measures due to the lack of vaccines and therapeutics available at the time (Muyembe-Tamfum *et al.*, 2012).

However, in the last decade, outbreaks have become more frequent, impacting larger populations and extending into urban areas. Before 2013, the largest documented EBOV outbreaks were the first one occurring in 1976, consisting of 318 cases with a CFR of 88%, and the 1995 Kikwit outbreak in which 315 cases and a CFR of 81% were recorded (WHO, 1978; Muyembe-Tamfum *et al.*, 1999). Whilst subsequent smaller outbreaks occurred on

the border between the RC and Gabon in the beginning to mid-2000s it was the West African outbreak (2013-2016) that marked a major shift in EBOV epidemiology (Leroy *et al.*, 2004; Wittmann *et al.*, 2007; Nkoghe *et al.*, 2011; Simon-Loriere *et al.*, 2015). The West African outbreak saw not only the introduction of the disease into a region that was inexperienced with disease management but also the exportation of the virus internationally. This led to the largest outbreak to date, causing approximately 11,000 deaths (de La Vega, Stein and Kobinger, 2015).

In contrast to previous outbreaks which relied on the retrospective study of the viral strains in circulation (often through stocks of cultured virus), the NGS techniques utilised during the West African outbreak permitted the identification of viral sequences directly from patient samples (Ghosh *et al.*, 2021). During its earlier stages, any efforts in genome characterisation relied on exporting positive samples to specialist centres abroad but as the outbreak progressed, the use of benchtop sequencing systems such as MiSeq (Illumina) and Ion Torrent (ThermoFisher Scientific) became routine and nanopore sequencing technologies such as MinION (Oxford Nanopore Technologies) allowed efficient processing of patient samples to extract genomic sequences directly on site (Baize *et al.*, 2014; Gire *et al.*, 2014; Kugelman *et al.*, 2015; Arias *et al.*, 2016; Quick *et al.*, 2016; Ghosh *et al.*, 2021).

As a result, the West African outbreak was comprehensively sampled, with over 1500 full-length sequences of the circulating Makona strain being generated which formed the basis of extensive epidemiological studies (Baize *et al.*, 2014; Gire *et al.*, 2014; Carroll *et al.*, 2015; Park *et al.*, 2015; Holmes *et al.*, 2016). The sequencing data obtained at the time identified a strain of EBOV that was different from the strains circulating in earlier outbreaks. The genome sequences had a high degree of genetic similarity suggesting a single transmission of the virus from its natural reservoir (Simon-Loriere *et al.*, 2015; Holmes *et al.*, 2016). Whilst this finding contrasted with the outbreaks in Gabon and RC (2001-2003), where multiple viral spillovers occurred concurrently or over a short period of time, it drew parallels to the 1995 Kikwit outbreak, which is also believed to be caused by a single transmission event (Rodriguez *et al.*, 1999; Leroy *et al.*, 2004).

However, a study by Rodriguez *et al.*, (1999) determined that there was no sequence variation in the *GP* gene of sampled virus from the Kikwit 1995 outbreak, which was explained by a reduced number of human transmissions in what was a relatively small outbreak. This differs from what was observed in the West African outbreak, where once EBOV entered the human host species, several sites in the viral genome were thought to

be subject to positive selection. This resulted in mutations that were functionally selected for during the recurrent human-human transmission chains, although their impact on pathogenesis or transmission remains largely unclear (Simon-Loriere *et al.*, 2015; Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016; Dietzel *et al.*, 2017; Ueda *et al.*, 2017).

The difference in GP stability observed between the Kikwit 1995 and the West African outbreak might be explained by the fact that the primers used to amplify and sequence the GP gene in the study by Rodriguez *et al.*, (1999) only targeted a pre-determined variable region rather than the entire gene, meaning that variations present in other regions could have been missed. As Figure 3.1 demonstrated, sequence data from the outbreak available on GenBank showed variation at positions 47 (E/D) and 544(T/I), although the latter may be attributed to the fact that the sequence was obtained from a tissue-cultured sample.

The widely available sequence for the West African outbreak also meant that it was the first in which the genetic evolution of EBOV could be tracked. Early predictions were estimated by Gire *et al.*, (2014) at approximately 2.0×10^{-3} substitutions/site/year. However, this study used sequences that were sampled during a short interval between March and June, which may be insufficient time to account for the action of purifying selection and thus, inflated predicted rates.

The short time frame over which viral sequences are sampled during an outbreak means that typically, estimations of evolutionary rates are predicted to be higher within outbreaks than between them. This is because early sampled sequences are more likely to contain a surplus of deleterious variants which would otherwise be eradicated by purifying selection over a longer period, leading to exaggerated estimations of rates of viral evolution during outbreaks (Holmes, 2009; Holmes *et al.*, 2016). However, it is suggested that the length and increased sampling seen during the West African outbreak meant that analysis estimations of evolution conducted in several studies eventually converged into a reliable estimate. Early estimates based on sequences sampled between March and June suggested that Makona was evolving at approximately twice the rate relative to previous outbreak strains that had been analysed (Gire *et al.*, 2014).

Subsequent studies have predicted varied rates, all lower than the original predicted by Gire *et al.*, (2014) showing that the virus was not evolving at a higher rate compared to previous outbreak strains. This highlights the challenging nature of predicting rates of viral evolution using different predictive models and how factors such as sampling periods,

whether whole genomes or specific genes are included and what type of molecular clock model is used, may all impact the outcome and thus, should be interpreted cautiously (Azarian *et al.*, 2015; Carroll *et al.*, 2015; Tong *et al.*, 2015).

A study by Holmes *et al.*, (2016) analysed publicly available sequencing data from the outbreak as a whole and estimated that the mean evolutionary rate of EBOV during the course of the outbreak was 1.2×10^{-3} substitutions/site/year (Holmes *et al.*, 2016). A subsequent study used a Bayesian approach to determine that the evolutionary rate of EBOV between different strains across all outbreaks occurring from 1976 to 2018, was approximately 0.47×10^{-3} substitutions/site/year (Mbala-Kingebeni *et al.*, 2019). These estimations follow the expected trend of evolutionary rates, which are typically higher within outbreaks rather than between them. (Holmes *et al.*, 2016).

Furthermore, these rates are much lower than those of many other RNA viruses, such as HIV-1 and Hepatitis C (HCV), suggesting that EBOV is relatively genetically stable. This may explain why there is not more phenotypic variation in EBOV, which is unusual for an RNA virus subject to error-prone replication (Saphire, 2015). Such behaviour might be a consequence of EBOV not being subjected to high selective pressure or immune surveillance in its natural reservoir or could be due to a selective pressure that is driving purifying selection, thus leading to little change.

4.1.2 The EBOV Reservoir

As discussed in Chapter 1, the natural reservoir for EBOV is definitively known. Evidence shows animals such as primates succumb to the virus similarly to humans, with human outbreaks correlating with losses of wildlife in surrounding areas; numbers of gorillas, chimpanzees and duikers have significantly declined during EBOV outbreaks, with many reported cases originating from well-documented human outbreaks that occurred between the border of Gabon and the RC between 2001 and 2003, around the Lossi Animal Sanctuary (Walsh *et al.*, 2003; Leroy *et al.*, 2004; Rouquet *et al.*, 2005; Bermejo *et al.*, 2006).

In 2005, in a large survey of mammals in the area around Gabon that included 679 bats, EBOV-specific IgG antibodies were identified in three species; *H. monstrosus*, *M. torquata* and *E. franqueti*, none of which displayed any clinical symptoms (Leroy *et al.*, 2005). Viral RNA was also identified from different tissues via PCR (Leroy *et al.*, 2005). To date, this

provides some of the strongest evidence of bats being the reservoir for EBOV. However, notably, no live virus was isolated from these bats and only parts of the viral genome were detected using molecular techniques.

This work complements a later study whereby 2147 bats from different species were sampled over five years from EBOV hotspots in Gabon and North Congo, in search of IgG antibodies specific to EBOV and MARV (Pourrut *et al.*, 2009). Antibodies for EBOV were isolated from six bat species, including the three species of fruit bat identified in the study by Leroy *et al.* (2005). The other species of bat that were found to be seropositive were *Micropteropus pusillus*, *Mops condylurus* and *R. aegyptiacus*. Additionally, an *R. aegyptiacus*-derived cell line was supportive of EBOV replication *in-vitro* (Krähling *et al.*, 2010).

Interestingly, Jones *et al.* (2015) provided insight as to whether *R. aegyptiacus* could also be a reservoir species for EBOV. After the inoculation of four groups of bats with either one of the Ebolaviruses or MARV, limited RNA distribution in tissues was observed for Ebolaviruses, and no viremia or viral shedding was detected. Contrastingly, MARV RNA was found with viremia and oral and rectal shedding was observed (Jones *et al.*, 2015). Viral antigen was also found in the spleen and liver (Jones *et al.*, 2015). The study suggests that *R. aegyptiacus* is an unlikely source for EBOV in nature as it seemed generally refractory to infection, however, experimental inoculations may not have been enough to induce infection and no serological studies were completed for EBOV infections. Additionally, the authors stated that the bat species breeds particularly well in captivity and flourishes in a laboratory environment and therefore, may not be a suitable experimental model to investigate EBOV relationships with a reservoir host.

There has been one incident of EVD where the index case came into direct contact with fruit bats. Through the collection of qualitative data via interviews with the local villagers who were present during the 2007 outbreak in Luebo (DRC), it was found that migrating fruit bats established colonies in the region for several weeks before the outbreak and were hunted by locals as a source of protein (Leroy *et al.*, 2009). It was theorised that the index case had purchased fruit bat meat from the local market and transmitted the virus to his four-year-old daughter, the first to contract EVD (Leroy *et al.*, 2009). This evidence supports the suspected role that fruit bats have in the natural cycle of EBOV and also highlights the importance of tracking the seasonal migration of bat populations in limiting the risk of EVD. However, EBOV was not isolated from any wildlife in the region (Leroy *et al.*, 2009). The re-emergence of the virus just a year later, with very little genetic

divergence does suggest that the virus had persisted locally in the environment or the wildlife, between outbreaks.

It is undeniable that there is accumulating evidence that both fruit bats and insectivorous bats could be exposed to filovirus in the wild and are a possible reservoir to harbour the virus. As mentioned in Chapter 1.2.1, several other species of bat have since been shown to be seropositive for EBOV and RESTV in the Philippines, China and Bangladesh (Taniguchi *et al.*, 2011; Yuan *et al.*, 2012; Olival *et al.*, 2013). The identification of antibody-positive bats suggests these species commonly survive filovirus infection. This long-term survival was noted in a study in which a seropositive bat was shown to be alive thirteen months following its release with a radio collar (Hayman *et al.*, 2010). The presence of bat species that are seropositive for EBOV in areas where there have been no recorded EBOV outbreaks suggests that sampling of bat populations may not be reflective of original outbreak regions. Seropositive samples may be obtained in areas that are geographically distinct from those in which they may have been originally exposed to EBOV due to the long life span of bats and their ability to fly over great distances. (Yuan *et al.*, 2012; Olival *et al.*, 2013). Furthermore, it is also unknown whether these viruses are in regular circulation within the aforementioned bat populations.

However, bats are a known reservoir for other filoviruses including BOMV, MARV and MLAV, adding to the evidence suggesting that they could also be an EBOV reservoir (Towner *et al.*, 2009; Goldstein *et al.*, 2018; Yang *et al.*, 2019). Additionally, a study has recently analyzed 1180 reported multiple mortality events (MMEs) in bat populations between 1790 and 2015, by their cause of death. The study covered 152 species of bat, attributing death in only 25 MMEs, which encompassed 14 species of bat, to disease. The presence of LLOV found in *M. schreibersii* in Spain in 2011, was the only MME relating to filoviruses, with most being caused by rabies and many remaining without confirmatory evidence of a specific agent. The very few numbers of MMEs as a result of viral infection combined with the fact that bats can support many viruses in the wild is once again suggestive that bats can act as a reservoir for not only filoviruses but multiple viruses, without falling victim to their pathogenicity (O'Shea *et al.*, 2016).

It also remains a possibility that bats are intermediate hosts that are exposed to the virus through an unknown reservoir and that the emergence of the virus amongst populations might also be due to environmental or ecological factors. Nonetheless, the fact that to date, EBOV has not been able to be isolated from any bat species, must not be overlooked. Unfortunately, without the certainty of the natural reservoir, it is difficult to

predict which populations are at risk and manage outbreak preparedness (Negredo *et al.*, 2011; Goldstein *et al.*, 2018; Kemenesi *et al.*, 2018; Yang *et al.*, 2019).

4.1.3 Aims

The presence of EBOV RNA fragments in bats and the existence of seropositive species have implicated them as a likely reservoir for the virus. However, the absence of live viruses in these species leaves ambiguity regarding the true reservoir. It is hypothesised that reconstructed MRCA sequences will be representative of different points in the evolutionary history of EBOV in the reservoir host and therefore will contain natural host-specific determinants. This study aims to use a PV system to test the infectivity of these MRCA/node sequences (kindly modelled by Professor Holmes at The University of Sydney) in a variety of mammalian cells and compare them to the infectivity of outbreak sequences. This aims to determine if mutations observed between MRCAs (presumed to represent the virus as it existed in the reservoir host) and viral strains circulating in humans, increase fitness in the latter and provide more insight into what the natural reservoir for the virus may be and if multiple hosts may exist. MRCA sequences may also have wider implications in vaccine development, as a tool to combat the impact of inter-strain variability of rapidly evolving viruses.

4.2 Results

4.2.1 NPC1 Expression is higher in Hyp/Lu 45.1 than Vero E6 or HuH-7 Cells

Having established that all ancestral and outbreak EBOV GPs produce infectious PVs (see Chapter 3), the infectivity of these constructs was to be tested on multiple cell lines representative of a theorised EBOV reservoir (Hyp/Lu 45.1), an intermediate host (Vero E6) and human end hosts (HuH-7). Initially, the cell lines were characterised for NPC1 mRNA expression and NPC1 receptor expression. Figure 4.1 shows average Ct values from triplicate results in qPCR analysis of mRNA expression in two different experiments. For each cell line, the same quantity of RNA was used to synthesise cDNA. Additionally, for NPC1 cDNA amplification, primers were designed across the three species in a region where there was no variability in sequence to ensure primer binding efficiency would be the same in all cell lines tested. Therefore, any variability in Ct values was restricted to the natural variation in the levels of NPC1 mRNA and thus, Ct values were directly compared.

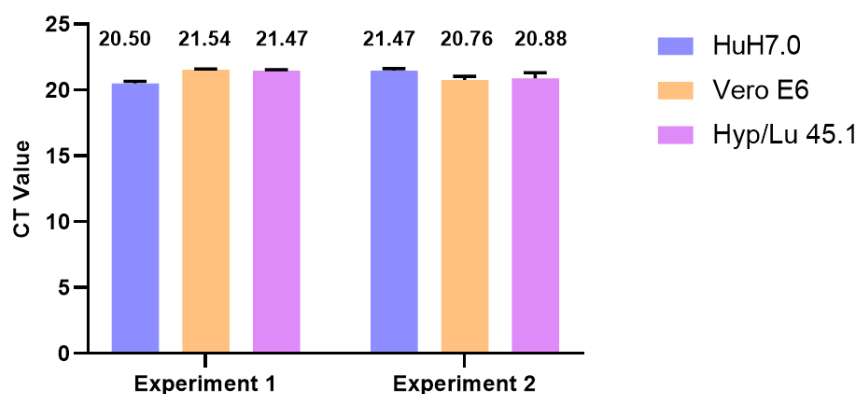


Figure 4.1 NPC1 mRNA expression in multiple cell lines

3 µg of RNA extracted from each cell line was used to synthesise cDNA, of which 1 µL was inputted into each SYBR Green qPCR reaction mix. Experiments were completed in triplicate. Data has been normalised by RNA input. Error bars are representative of +/- SD.

Results from the first experiment show that there is a difference of approximately 1 Ct between the HuH-7 and Hyp/Lu 45.1 cells suggesting a two-fold difference in the levels of NPC1 mRNA between these cell lines. However, in the second experiment, the Ct value for HuH-7 cells was higher than that of Hyp/Lu 45.1. In both experiments Ct values for Vero E6 cells were intermediary. RNA extractions from these cell lines were completed using cells from different passage numbers for each independent experiment. The levels of NPC1 mRNA may have differed depending on the passage number of the cells, accounting for the different trends between the two experiments.

As mRNA expression does not always correlate to protein expression, to gain further insight into NPC1 receptor expression in the cell lines, analysis by flow cytometry was completed. Briefly, for each cell line, approximately 250,000 cells were either left unstained (as an indicator of background fluorescence and to set negative populations/gates), stained with anti-NPC1 antibody (to determine NPC1 expression in the cell line) or stained with an isotype control antibody (to determine that staining is due to specific antibody binding to NPC1 rather than any other artefacts). Figure 4.2 shows the output from the analysis of NPC1 expression in HuH-7 cells using an FC500 flow cytometer. The analysis was completed on single cells only.

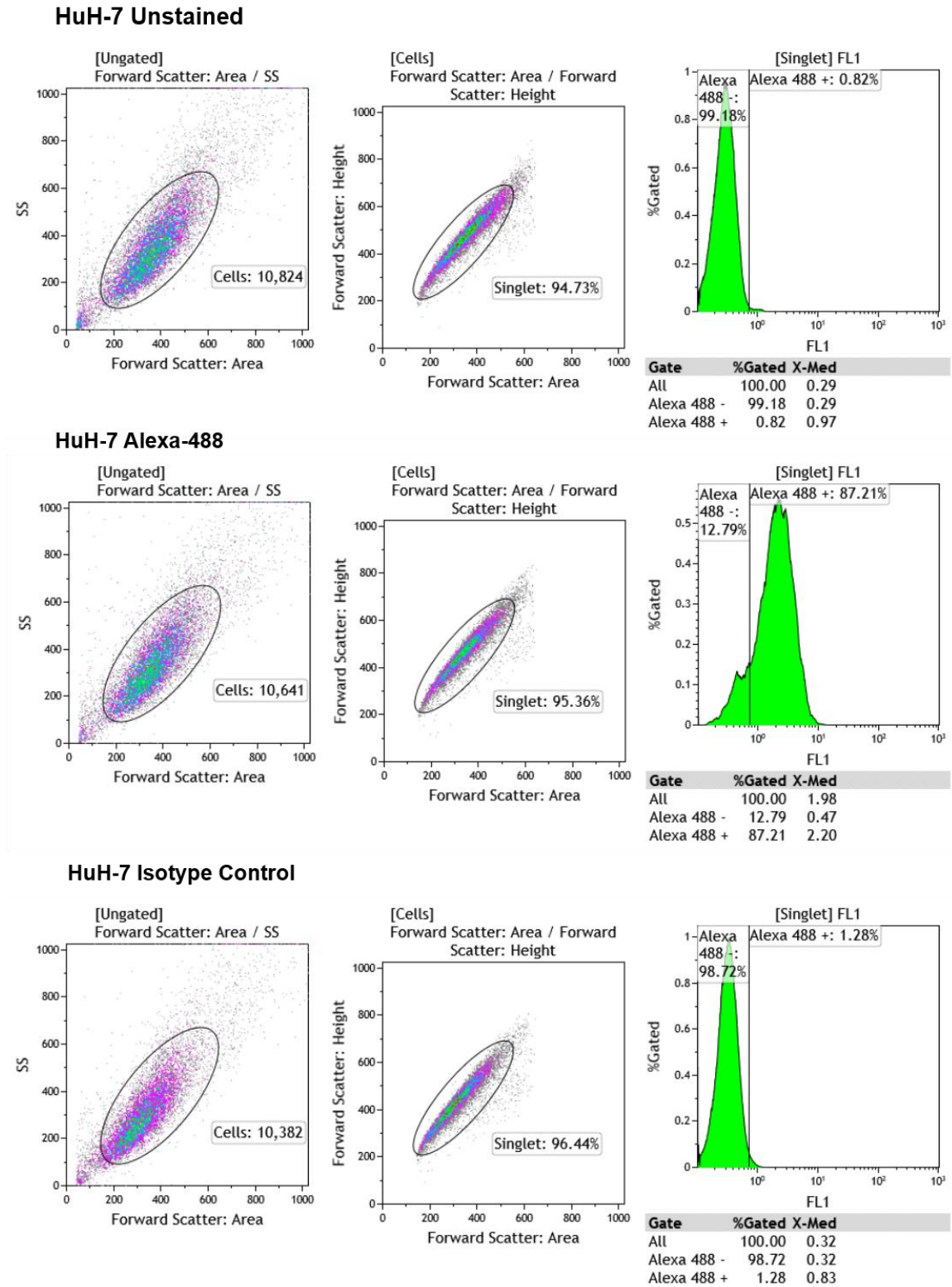


Figure 4.2 Characterisation of NPC1 receptor expression in HuH-7 cells
Expression was determined using an unstained control, an α -NPC1 Alexa-488 antibody and an isotype control in HuH-7. Cells of the correct population were gated. Of these cells, singlet cells were identified and the fluorescence intensity was determined for the selected population. The unstained control was used for each cell line to set gates and background parameters. This was completed for Vero E6 cells and Hyp/Lu 45.1 cells (data not shown). Data is representative of two independent experiments

The unstained population was initially used to set the background parameters including all gating. A similar analysis was completed for Vero E6 and Hyp/Lu 45.1 cells (data not shown). Figure 4.3 shows overlaid fluorescence intensity in unstained, Alexa-88 and isotype control-treated HuH-7, Vero E6 and Hyp/Lu 45.1 cells. Median values for fluorescence intensities were used to determine the rMFI values that are also depicted in Figure 4.3. The data shows that in two independent experiments, rMFI values for Hyp/Lu 45.1 cells were consistently higher compared to HuH-7 and Vero E6 cells, suggesting higher levels of NPC1 expression in Hyp/Lu 45.1 cells, despite the difference in mRNA expression between both cell lines being more ambiguous (Figure 4.1).

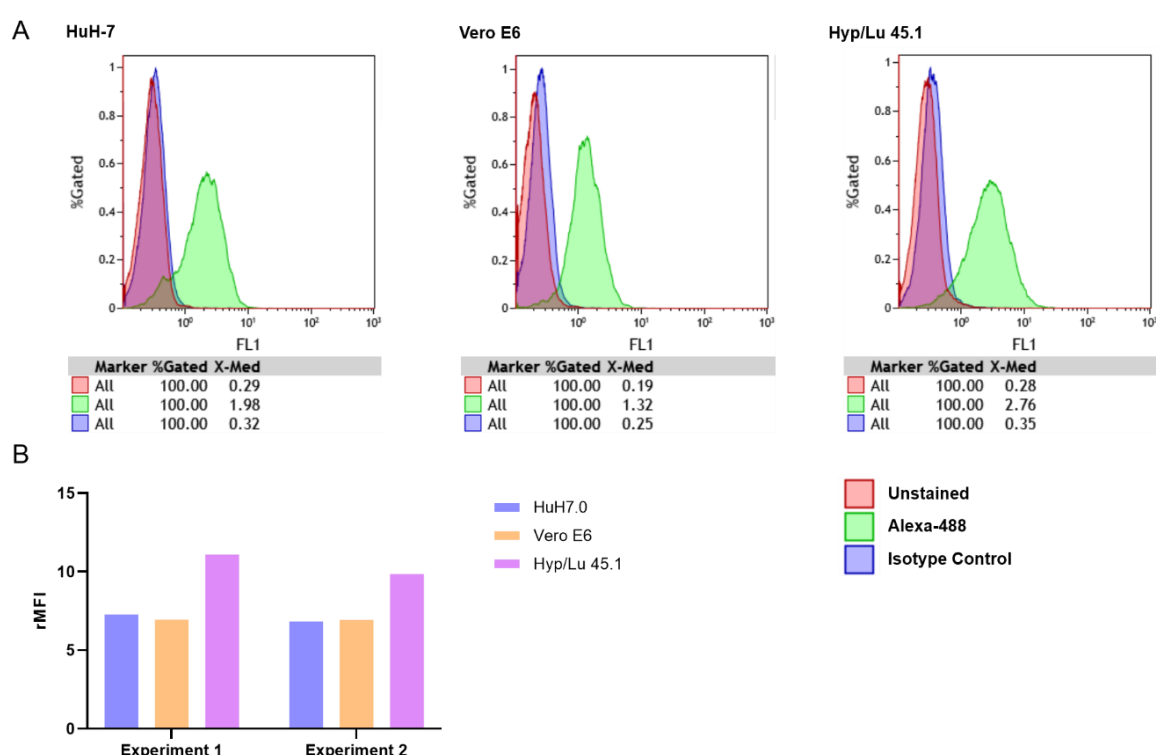


Figure 4.3 Overlay images showing NPC1 protein expression in multiple cell lines

Data for each cell line was generated with the method described in Figure 4.2. A) Overlay images for each cell line showing the fluorescence intensities of unstained cells and those treated with the NPC1-Alexa 488 or isotype control antibodies. This data was used to determine the rMFI values for each sample, in each cell line. The analysis was completed using the Kaluza software (Beckman Coulter). Data is representative of two independent experiments. B) rMFI values for NPC1 expression in HuH-7, Vero E6 and Hyp/Lu 45.1 cells as determined in two independent experiments.

4.2.2 EBOV Ancestral GP Sequences are more Infectious than Outbreak Sequences in HuH-7 and Hyp/Lu 45.1 Cells

Following the results shown above, PVs expressing ancestral and outbreak GPs were generated using the three-plasmid system described in Chapter 3. The PVs were tested for their infectivity in HuH-7 and Hyp/Lu 45.1 cells. Due to time limitations, the infectivity of the PVs could not be tested in Vero E6 cells as was originally planned. Figure 4.4 shows the results of three independent experiments after the normalization of the data to Mayinga 1976. In the HuH-7 cell line, several node sequences, including N22, N23 and N26 displayed a marked increase in infectivity relative to Mayinga 1976. Early outbreak sequences including Gabon 1994, 1996 and 2001 displayed levels of infectivity that were comparable to Mayinga 1976. As discussed in Chapter 3, Mbandza 2003 displayed significantly lower relative infectivity. Contrastingly, the Etoumbi 2005 and Lomela 2014 constructs displayed infectivity levels that were significantly higher than Mayinga 1976 and comparable to some of the node sequences.

In Hyp/Lu 45.1 cells, N1, N23 and N26 displayed a slight increase in infectivity relative to Mayinga 1976, whereas early outbreak sequences and Etoumbi 2005 displayed comparable levels of infectivity. In contrast, Mbandza 2003 and Luebo 2003 displayed infectivity that was significantly lower than in Mayinga 1976. Figure 4.4 also shows the correlation of normalised PV infectivity between the two cell lines. An R^2 value of 0.8080 demonstrates a strong correlation in patterns of infectivity in HuH-7 and Hyp/Lu 45.1 cells, indicating that the relative level of infectivity of the EBOV PVs will be similar in both cell lines and that both the panel of nodes and outbreak sequences show differential infectivity in both cell lines.

As mentioned in Chapter 3, N21 was used to generate the Gabon 2001, Mbandza 2003, Kelle 2003 and Etoumbi 2005 GP constructs, whilst N26 was used to generate Gabon 1994 and 1996 GP constructs. Consequently, the difference in amino acid sequence between the ancestral sequences and their derivatives is limited to no more than five residues. To determine the impact of these residue changes on the phenotype of outbreak sequences, the infectivity of the outbreak sequences displayed in Figure 4.4 was normalised to the corresponding ancestral sequence for both HuH-7 and Hyp/Lu 45.1 cell lines. A one-way ANOVA and Dunnet's multiple comparisons test were subsequently used to determine if there was a significant difference in infectivity between the outbreak strain and the ancestral sequence it was generated from. Figure 4.5 displays the averages in infectivity data for both cell lines. This data shows that, with the exception of Etoumbi 2005,

the amino acid changes that occurred between the ancestral sequences and their outbreak derivatives, lead to a loss of infectivity in both cell lines. This difference is of significance in Gabon 1994, Gabon 1996, Kelle 2003 and Mbandza 2003 in both HuH-7 and Hyp/Lu 45.1 cell lines, albeit to different degrees.

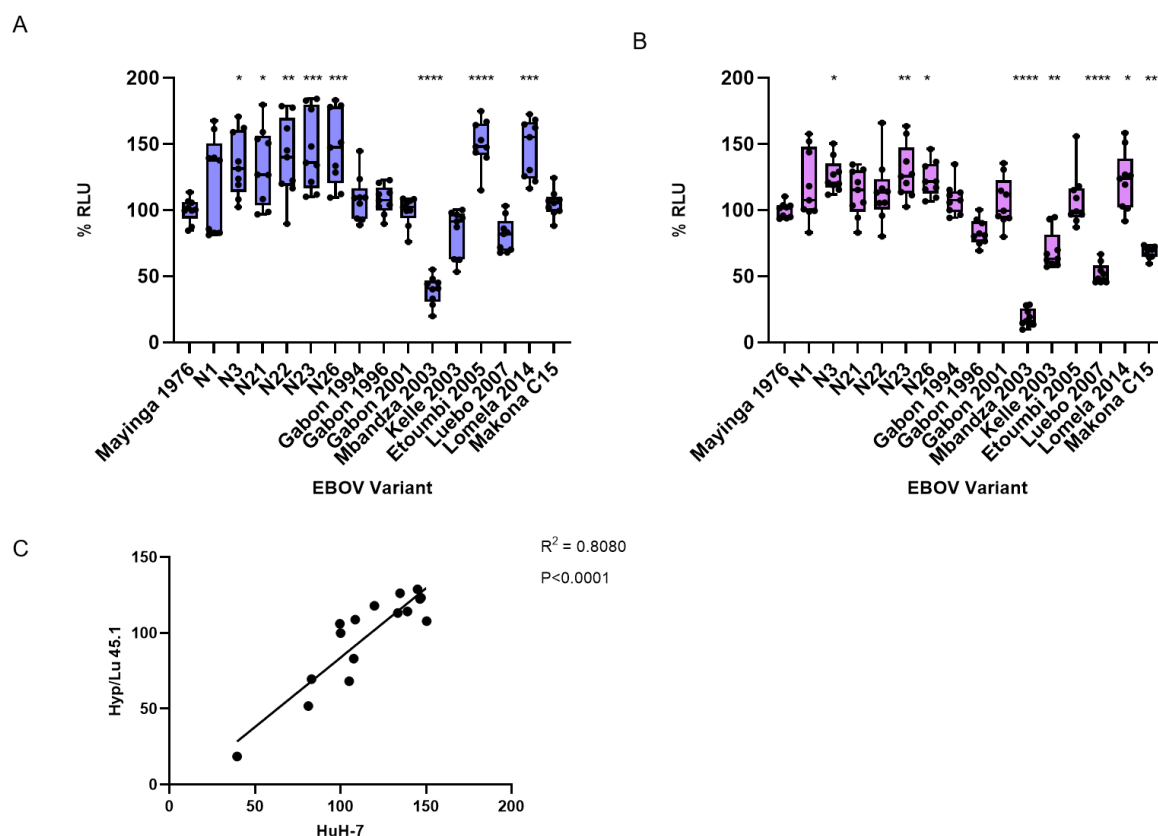


Figure 4.4 Infectivity of EBOV ancestral and outbreak GPs in A) HuH-7 and B) Hyp/Lu 45.1 cells

PVs were generated using a three-plasmid system and used to infect cell lines. Infections with each variant were completed in triplicate within an experiment. Data has been normalised to Mayinga 1976 (100%) as the first outbreak sequence and delta E (0%, not shown on graph). Triplicates for three independent experiments were pooled following normalisation. Normal distribution of data was determined using a Kolmogorov-Smirnov normality test, with the exception of Gabon 2001 in dataset A). Bars on box plots show minimum and maximum values of pooled data. Differences in the infectivity of PVs relative to Mayinga 1976 were assessed using a one-way ANOVA with Dunnet's multiple comparisons test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; values that were not significant ($p > 0.05$) have not been highlighted in the figure. A) and B) Mayinga 1976 and Makona C15 display a coefficient of variation (CV) of $<10.0\%$; all other clones, $>10.0\%$. CV was calculated from data pooled from three independent experiments. C) Plots of normalized infectivity used to calculate Pearson's correlation test.

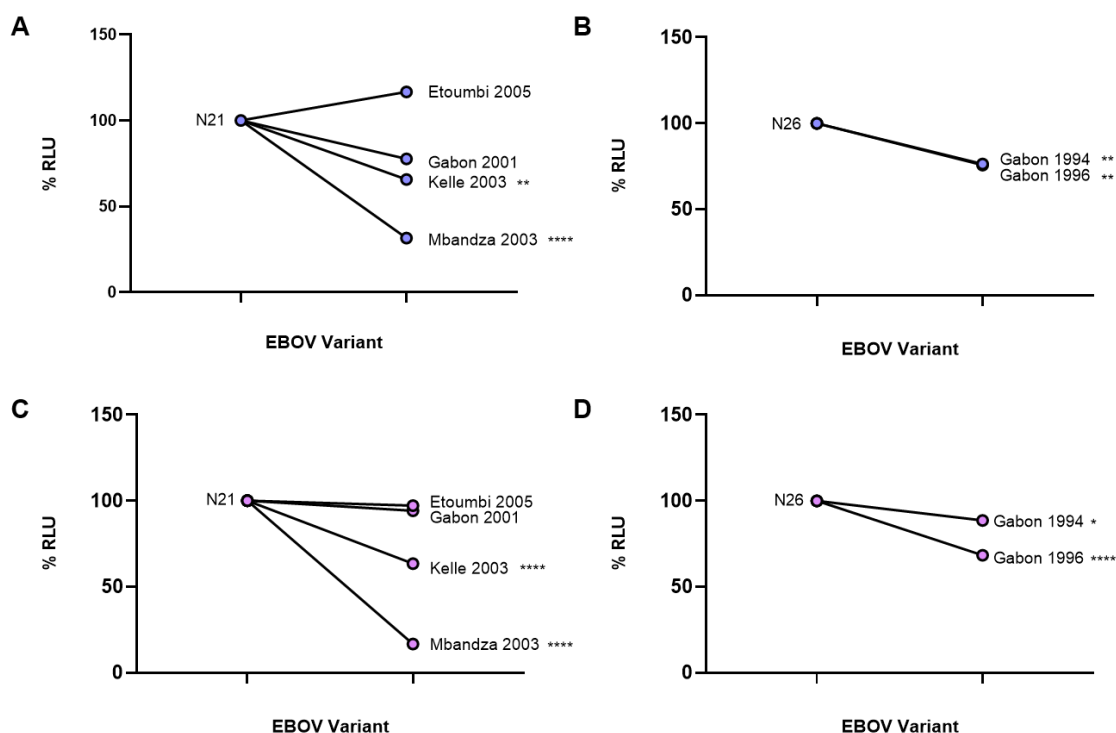


Figure 4.5 Infectivity of outbreak GPs relative to the ancestral GPs they were generated from A) N21 derivatives in HuH-7 cells B) N26 derivatives in HuH-7 cells C) N21 derivatives in Hyp/Lu 45.1 cells. D) N26 derivatives in Hyp/Lu 45.1 cells. Triplicates from the same three independent experiments displayed in Figure 4.5 were normalised to either N21 or N26 and the normalised data was pooled. Statistical analysis comparing infectivity to either N21 or N26 was completed with a one-way ANOVA with Dunnet's multiple comparisons test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Values that were not significant ($p > 0.05$) have not been highlighted in the figure. The plots show average values from pooled data from three independent experiments.

4.2.3 GP Expression Varies Across Ancestral and Outbreak EBOV PVs

To confirm the correct incorporation of the PVs, western blot analysis was carried out to assess GP and p30 expression, thus ensuring the robustness of the infectivity data obtained for each PV. Figure 4.6 shows the differential expression of the ancestral GPs, with Makona C15 used as the positive control and delta E as the negative control. The data is representative of three independent experiments, with Makona C15 repeatedly showing the highest GP expression in a western blot. This added to observations made in the previous Chapter in Figure 3.9, where Makona C15 expression was notably higher than Mayinga 1976 and Mbandza 2003 expression. p30 expression was relatively similar across the PVs however, as Figure 4.6 shows with the band for N21, some PVs show stronger p30 expression throughout experimental repeats. Additionally, some PVs also displayed expression for p30 precursor proteins, with bands for p70 expression being particularly strong for Makona C15 in this instance. Figure 4.7 shows the same experiments repeated for outbreak GPs. Makona C15 and delta E were once again used

as positive and negative controls, respectively. Again, differential GP expression is observed between outbreak sequences, with Kelle 2003, Mbandza 2003, Luebo 2007 and Makona C15 showing the strongest GP expression. Interestingly, p30 expression across outbreak PVs was consistent, with no precursor expression observed.

Figures 4.4, 4.6 and 4.7 show that there does not seem to be any correlation between GP expression and infectivity. For example, Figure 4.4 shows that after normalisation to Mayinga 1976, the infectivity of all the nodes is generally higher than Makona C15 in both HuH-7 and Hyp/Lu 45.1 cells, however, Makona C15 GP expression was consistently higher than all the node GPs. Additionally, the infectivities of Mbandza 2003 and to a lesser degree, Luebo 2007, are amongst the lowest observed in both cell lines, however, the GP expression was consistently some of the highest observed relative to other outbreak sequences. Furthermore, there is no significant difference between the infectivity of Mayinga 1976 and Makona C15 in HuH-7 cells. However, the western blot data shows that Mayinga 1976 GP expression was amongst the lowest observed. This correlates with observations made by Urbanowicz *et al.*, (2016), who found that in the case of HCV PVs, GP incorporation does not effectively predict the level of viral entry as PVs that were expressing low GP levels, which were undetectable by western Blot, were still able to mediate infection. Additionally, there seems to be no pattern in EBOV p30 expression, which although generally similar amongst PVs between experimental repeats, did display occasional variability in the band intensity of some PVs.

The western blot images were uploaded onto the ImageJ software for densitometry analysis to generate quantitative values for GP and p30 expression in each PV, to which the corresponding infectivity data could be normalised. Prior to pelleting PVs for western blot, they were used to infect both HuH-7 and Hyp/Lu 45.1 cells. Figure 4.8 shows the raw infectivity data in RLU for the PVs expressing ancestral GPs, normalised to GP and p30 expression. Figure 4.9 shows the same for the PVs expressing outbreak GPs. For both ancestral and outbreak GPs, similar patterns of infectivity are displayed in both HuH-7 and Hyp/Lu 45.1 cells. Thus, once normalised to either GP expression or p30 expression, patterns are again, observably similar in both cell lines.

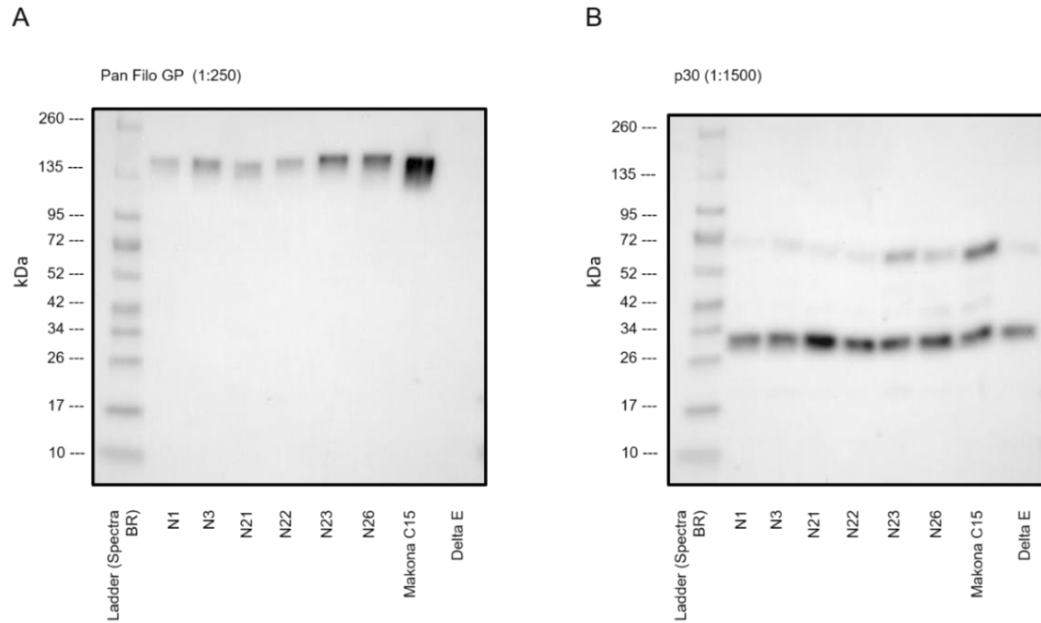


Figure 4.6 Western blot of EBOV ancestral GPs and p30 core protein
A) A 1:250 dilution of a Pan-filo α -GP antibody (IBT) was used to detect the GP at approximately 130 kDa. Makona C15 was used as a positive control and delta E as a negative control. B) MLV p30 core protein was detected at 30 kDa with a 1:1500 dilution of MLV α -p30 antibody (Abcam). p30 precursors can be observed, particularly p70. A 1:2000 dilution of a rabbit, α -mouse HRP (Dako) was used as a secondary antibody for both blots. Bands were visualised by a G-Box (SynGene) with an exposure time of 25 minutes. Data is representative of three independent experiments. No differences in the sizes of the bands were expected.

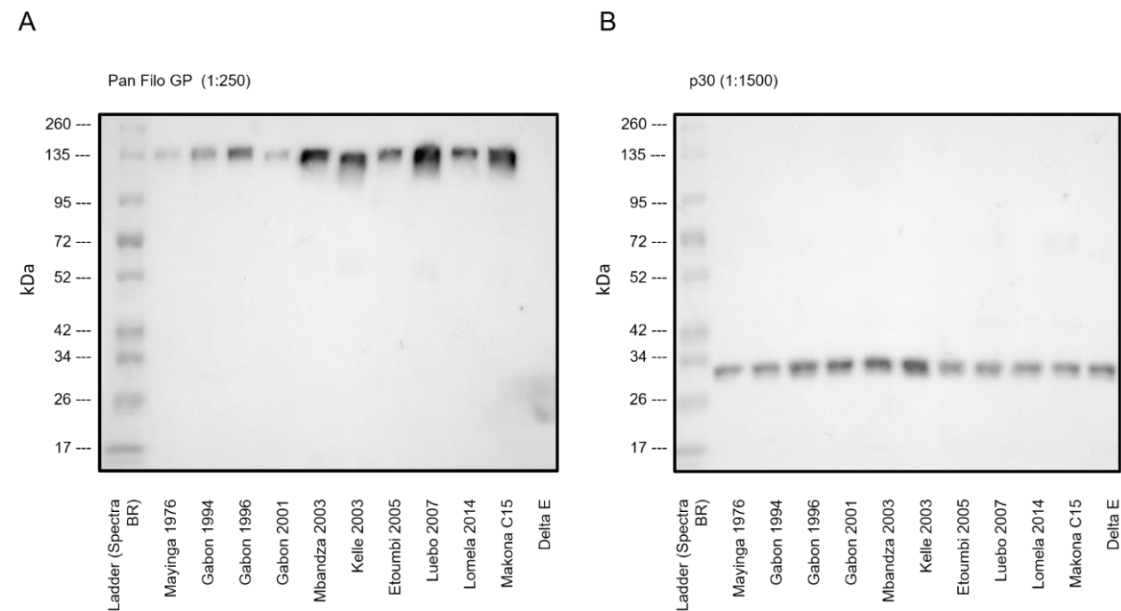


Figure 4.7 Western blot of EBOV outbreak GPs and p30 core protein
A) A 1:250 dilution of a Pan-filo α -GP antibody (IBT) was used to detect the GP at approximately 130 kDa. Makona C15 was used as a positive control and delta E as a negative control. B) MLV p30 core protein was detected at 30 kDa with a 1:1500 dilution of MLV α -p30 antibody (Abcam). A 1:2000 dilution of a rabbit, α -mouse HRP (Dako) was used as a secondary antibody for both blots. Bands were visualised by a G-Box (SynGene) with an exposure time of 25 minutes. Data is representative of three independent experiments. No differences in the sizes of the bands were expected.

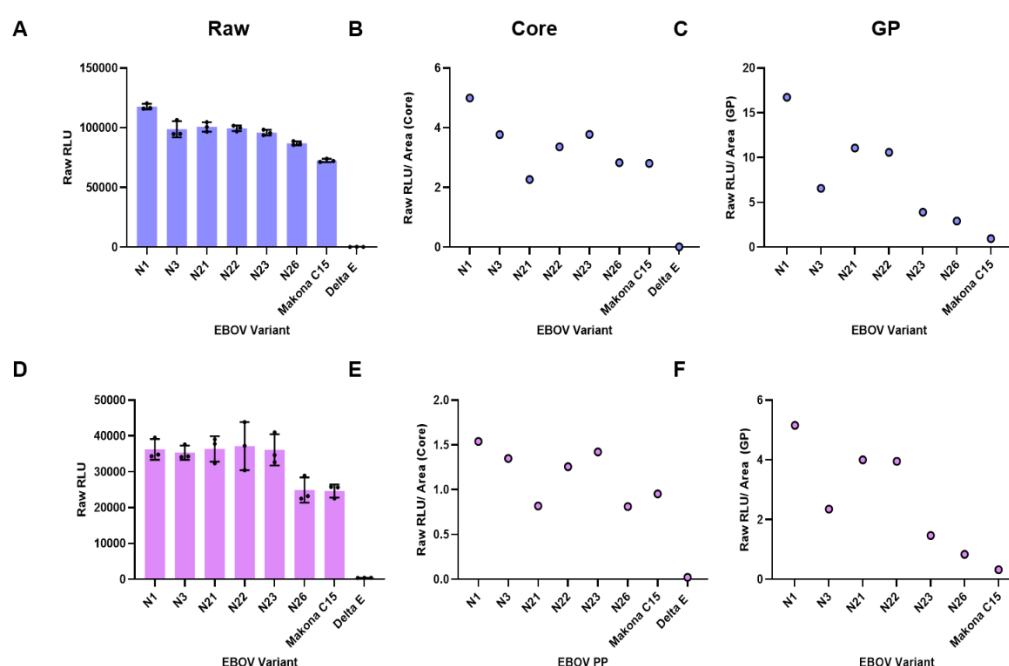


Figure 4.8 Infectivity of ancestral GPs normalised to GP and p30 expression

A-C) HuH-7 cells and D-F) Hyp/Lu 45.1 cells. Densitometry was completed on the western blots shown in Figure 4.6 using the ImageJ software. The relative density values for GP and p30 bands were determined and the raw RLU values from infections performed using the same PV preparations were normalised to these results. Data is representative of three independent experiments. Error bars represent $\pm$ SD.

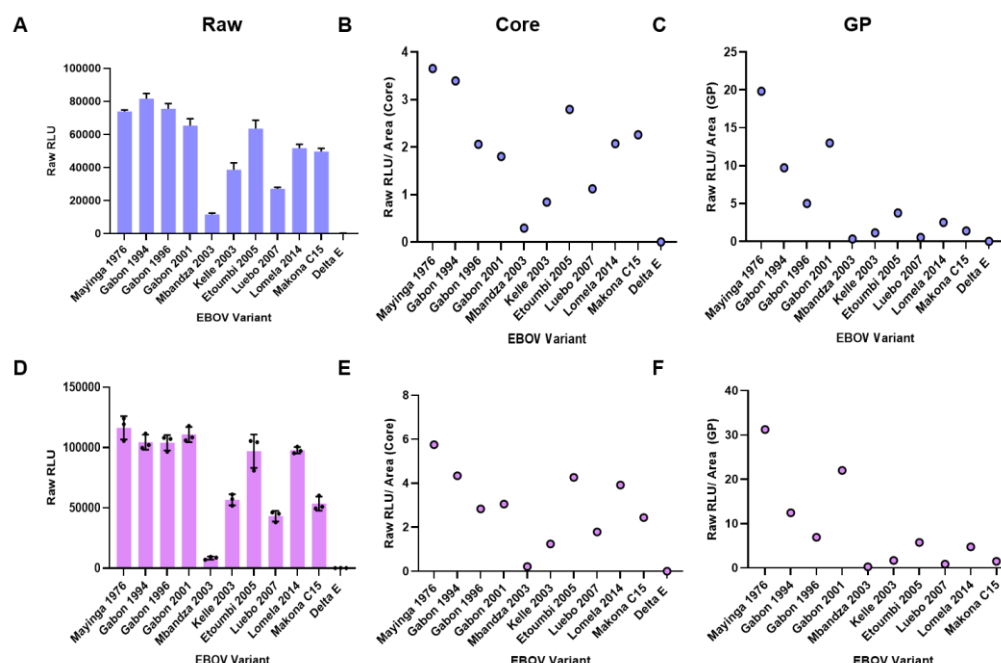


Figure 4.9 Infectivity of outbreak GPs normalised to GP and p30 expression

A-C) HuH-7 cells and D-F) Hyp/Lu 45.1 cells. Densitometry was completed in western blots shown in Figure 4.7 using the ImageJ software. The relative density values for GP and p30 bands were determined and the raw RLU values from infections performed using the same PV preparations were normalised to these results. Data is representative of three independent experiments. Error bars represent $\pm$ SD.

4.3 Discussion

The mechanisms by which EBOV persists in the environment and causes outbreaks have long been disputed. A 2005 study by Walsh *et al.*, challenged the common belief that EBOV was persistently present at different outbreak sites. They employed both Maximum likelihood and Bayesian estimations to generate phylogenetic analyses of the GP gene from strains circulating in outbreaks between 1976 and 1996 and between 2001 and 2004. Both methods highlighted one major distinguishable lineage originating from the first outbreak strain in 1976 (which was placed close to the root corresponding to an estimate of the MRCA), with all subsequent outbreak strains appearing as its direct, closely-related descendants, regardless of their locality of isolation. Based on this, they suggested that rather than the viral strains evolving independently *in situ*, EBOV may be spreading in a wave-like manner. Further, they showed that a similar ancestor-descendent relationship connected the outbreaks from the mid-90s and early 2000s, suggesting that the replacement of viral strains over time and space by related but consistently more divergent genotypes is also more typical of a continuous spread of the virus. However, the group could not provide further insight as to whether EBOV was present in central Africa before its discovery in 1976, or if it was brought to the region as an invader. It also remains unclear whether the wave-like spread may occur through a species that was endemic to the region or through the invasion of an infected reservoir.

A study published in the same year by Leroy *et al.*, (2005) provided compelling evidence for the argument that fruit bats could be a natural reservoir for EBOV. In particular, *H. monstrosus*, *E. franqueti*, and *M. torquata* were found to be seropositive for antibodies against EBOV in several cases and yielded viral sequences that were similar to those found in human outbreaks during the early 2000s. However, to date, no live replicating virus has been successfully isolated from bat species sampled for EBOV.

Subsequently, Biek *et al.*, (2006) completed a phylogenetic analysis of the L gene from outbreak sequences between 1976 and 2002, as well as the bat-derived sequences presented by Leroy *et al.*, (2005). Consistent with the previous findings of Walsh *et al.*, (2005), analysis of the L gene also showed that viruses from more recent outbreaks seemed to be direct descendants of viruses that were circulating in earlier outbreaks. In fact, they postulated that all the genetic variation observed in EBOV since its discovery, including the variation present in the viral RNA fragments amplified from fruit bats, is a product of mutations that have occurred since its discovery in the 1970s and that all viruses sampled from humans and bats in the early 2000s can be traced back to a recent

MRCA, thus suggesting a recent merging of genetic lineages. Further, they suggested that if the three fruit bats that Leroy *et al.*, (2005) investigated were a natural reservoir for EBOV, the recent common ancestry of all sequences analysed could be explained by the virus experiencing a genetic bottleneck, disrupting the bat population and causing the descendants of all but one of the previously existing viral lineages to be lost.

This study aimed to provide more evidence as to whether the virus gained a change in phenotype whilst crossing the species barrier as well as to provide more insight into what the reservoir for EBOV may be. To this end, the infectivity of modelled ancestral GP sequences representative of different time points in the evolutionary history of EBOV was compared with that of outbreak sequences isolated between 1976 and 2016, using cell lines representative of a reservoir and an endpoint host. Specifically, an immortalised cell line generated from lung cells of *H. monstrosus* was deemed the perfect candidate to represent a theorised reservoir species, while HuH-7 cells were selected as representative of an end-point host.

In both cell lines, it was observed that outbreak sequences that were derived from the nearest ancestral sequence resulted in a general loss of infectivity, except for Etoumbi 2003. In both cell lines, this difference was most significant in Kelle 2003 and Mbandza 2003, which were derived from N21 and Gabon 1994 and Gabon 1996, which were derived from N26. The low infectivity of Mbandza 2003 was discussed extensively in the previous chapter. Figure 3.3 in the previous chapter showed that Kelle 2003 does not share any common mutations with other derivative outbreak sequences. Therefore, a similar approach to that taken in the previous chapter to identify what residue caused the low infectivity of Mbandza 2003 could be taken for this outbreak sequence, by determining the individual or combined impact of E337G, N346D, L368F, L481S and V661G on the N21 GP. This would give further insight into what mutations are causing this observable change in phenotype when comparing the infectivity of an ancestral sequence that is presumed to represent the virus as it existed in the reservoir host and outbreak viral strains that have circulated in humans. The N26 derivatives had fewer mutations in relation to their closest ancestral sequence, with Gabon 1994 only differing by two mutations (Q376R and T379I) and Gabon 1996 by three (R219K, L368P and A474T). The relative losses of infectivity can be attributed to either one or a combination of these mutations, but once again, without completing further tests it is unclear which.

As it was suggested that ancestral node GPs may have species-specific determinants that favour tropism for a reservoir host, it was hypothesised that these GP sequences would

result in the more favourable infectivity of Hyp/Lu 45.1 cells compared to sequences from recent outbreaks. The converse was hypothesised to be observed in HuH-7 cells, with it postulated that outbreak GPs would display greater infectivity in these cells. However, this study showed that the ancestral node sequences were generally significantly more infectious than outbreak sequences in both cell lines. Further, based on the phylogenies observed in Figure 3.1, if the assumption is made that the outbreak sequences were descendants from the nearest hypothesised ancestral node sequences, the data obtained showed that outbreak sequences between 1994 and 2005 were generally less infective relative to the node sequence they may have descended from, as observed in both Hyp/Lu 45.1 and HuH-7 cell lines. Additionally, the general pattern of infectivity between nodes and outbreak sequences in both cell lines seems to be similar, as indicated by the R^2 values following a Pearson's correlation test (0.8080).

Attempts were made in this study to normalise the infectivity of the GPs to the NPC1 receptor expression (rMFI values listed in Figure 4.3) in HuH-7 and Hyp/Lu 45.1 cells, as shown in Appendix ii. This was to ensure that the infectivity of PVs between different cell lines could be compared to see if any were significantly more infectious in a particular cell line. This data suggested that generally, both ancestral and outbreak GP sequences were minimally more infectious in HuH-7 cells than Hyp/Lu 45.1 cells although, with the exception of N21, these results were not significant. Although interesting, the data was interpreted with caution as due to time restrictions, it was not possible to repeat the cell entry and flow cytometry assays using the same stock of cells at the same passage number, which would be the only way to draw robust conclusions regarding infectivity comparisons between cell lines.

Overall, the study concluded that the mutations that were gained by theoretical EBOV ancestral sequences to form outbreak sequences that have circulated in the last half-century, resulted in a loss of viral fitness that was observable in both a human cell line and a bat cell line generated from a hypothesised reservoir (Leroy *et al.*, 2005). The data obtained did not support the theory that *H. monstrosus* may be a possible reservoir of EBOV in the classical sense where the virus and the reservoir share a long-standing relationship allowing EBOV to be sustained persistently between outbreaks, but perhaps that the virus was more recently introduced to this bat population. The fact that the ancestral sequences are more infectious in both cell lines suggests that the bat species is similarly susceptible to infection as an end-point host. However, it is important to note several limitations of this study when considering such a conclusion.

Firstly, as an *in-vitro* model, the use of a single cell line from a species (particularly when the cells are derived from different organs) may not be reflective of the EBOV infectivity in reservoirs and endpoint hosts in nature. Additionally, these conclusions were made based on the use of PVs, which are morphologically very different from filoviruses such as EBOV, the structure of which is primarily determined by the expression of VP40 as discussed in Chapter 1 (Noda *et al.*, 2006). Once again, this is a factor that may skew the infectivity observed *in-vitro* when compared to what may be naturally observed.

Furthermore, although the GP is a key determinant of EBOV entry, viral fitness during an outbreak is a composite of many factors such as replication capacity, the ability to egress host cells as well as how it challenges the host immune system. An example of this is to analyse how the virus acts in response to the presence of tetherin, a viral restriction factor that is expressed when the IFN-dependent antiviral pathway is induced. Studies expressing VP40 to form EBOV-like VLPs have shown that the egress of EBOV VLPs can be restricted by the presence of tetherin. However, EBOV GP has been shown to antagonise tetherin activity, thus promoting the budding of VLPs in tetherin-expressing cells, in a dose-dependent manner, with complete restoration of budding observed when high concentrations of GP are expressed (Kaletsky *et al.*, 2009). Unfortunately, high GP expression has also been shown to increase cytotoxicity, impacting viral titres, with a moderate expression limiting such detrimental impact (Alazard-Dany, 2006; de La Vega *et al.*, 2015). This may explain why some of the most infectious EBOV PVs such as those expressing the ancestral GPs showed poor GP expression in western blot (Figure 4.7) and some of the least infectious PVs (Mbandza 2003 and Luebo 2007; Figure 4.8) showed some of the highest expression, although does not explain the behaviour of highly infectious strains showing high GP expression such as Makona C15.

These findings highlight that optimising GP expression for entry in such a system as that used in this project may impact other factors, for example, viral egress and pathogenicity and vice-versa and once again, may not be reflective of behaviour that is observed in nature. Furthermore, these processes require the use of many other EBOV proteins, as discussed in Chapter 1, which can also mutate during outbreaks and show variation between them, again impacting viral fitness (Albariño *et al.*, 2016). Therefore, patterns of infectivity observed in these assays may not correlate to the viral fitness observed for strains within the outbreaks that were tested for within the remit of this project, or the theoretical ancestors of EBOV that node sequences were modelling.

In addition, western blots were completed to ensure that GP and p30 core protein expression was being achieved as an indicator of the correct incorporation of these proteins in PVs and thus, their functionality. Differential GP expression was observed between isolates, although p30 expression remained consistent with the additional detection of p30 precursor proteins on occasion. Patterns of GP expression displayed by both ancestral and outbreak GPs may be explained by the commercially obtained antibody that was used to target the EBOV GPs. The epitope of the antibody was unknown and therefore it was unclear if it bound to a site that contained some variability in protein structure. If this was the case, the antibody may have displayed differing affinities for the panel of ancestral and outbreak GPs that were tested. Therefore, the normalisation of infectivity to core protein expression, as completed in this study, is more favourable when accounting for differences between PV preparations.

It is also unclear if this assay was only detecting correctly assimilated PVs, as studies have previously shown that supernatant containing PVs as generated by a producer cell line can also contain cell-derived microvesicles containing GPs that are non-infectious, as well as core particles, that can be difficult to separate by methods such as ultracentrifugation (Bess *et al.*, 1997; Herrera *et al.*, 2005). Consequently, the detection of either GP or p30 via western blot may result in an overestimation of expression levels, thus normalisation of infectivity to either protein may be inaccurate and difficult to standardise.

Finally, it is important to note that although the ancestral node sequences chosen were modelled with high confidence, in essence, they are still only theoretical sequences and as such, they may not be representative of the behaviour that a genuine MRCA of EBOV strains (circulating in the environment pre-1970s in a reservoir species) may display. Nonetheless, the results of the present study suggest that perhaps EBOV was introduced to fruit bats around the same time it infected other wildlife populations or human hosts. This implies that the primary reservoir for EBOV is yet to be found. More evidence to reinforce this theory may have been obtained if the original scope of this study had been met, which was to test the infectivity of the PVs in a cell line that was representative of an intermediate host such as Vero E6 cells. If the PVs behaved in the same way in this cell line, it would again suggest that EBOV was introduced to all three hosts at a similar point in history.

5. Identification and Characterisation of SNPs Present in NPC1 that are Enriched in African Populations

5.1 Introduction

Predominantly a cholesterol transporter, NPC1 is an endosomal/lysosomal protein consisting of thirteen transmembrane domains and three luminal domains (A, C and I) (Davies, Chen and Ioannou, 2000; Li, Wang, *et al.*, 2016). It works concurrently with the small soluble glycoprotein, NPC2, to mediate intracellular cholesterol trafficking, with mutations either resulting in Neimann-Pick C1 or C2 diseases. (Infante *et al.*, 2008). The protein is ubiquitously expressed in cells, with an increased tissue distribution of *NPC1* mRNA observable in the liver, lungs and adrenal glands (Zhang *et al.*, 2003; Ramirez *et al.*, 2011). The wide tissue distribution of NPC1, as well as the conservation it displays across a variety of species, including all mammals, demonstrates the importance of NPC1 to biological function.

In the last decade, the additional role of NPC1 as the filovirus receptor for cellular entry has been well characterised (Carette *et al.*, 2011; Côté *et al.*, 2011; Miller *et al.*, 2012; Spence *et al.*, 2016). The primary step of any viral infection is the entry of the virus into a susceptible cell. In the case of enveloped viruses such as filoviruses, this involves the interactions between a viral glycoprotein and a host receptor protein, initiating membrane fusion. In the case of EBOV, domain C in NPC1 is indispensable for this purpose, although the additional trigger that follows the binding of cleaved GP to NPC1 to instigate membrane fusion, is still unknown. Further studies have continued to show the importance of NPC1 in several other viral infections including HIV, and flaviviruses such as HCV and Dengue (DENV) (Tang *et al.*, 2009; Jupatanakul, Sim and Dimopoulos, 2014; Stoeck *et al.*, 2018).

5.1.1 Impact of *NPC1* Variation on its Biological Role as a Cholesterol Transporter

Over 400 mutations have been identified in *NPC1*, accounting for approximately 95% of the cases of Neimann-Pick diseases, with the remainder being caused by anomalies in *NPC2* (Ribeiro *et al.*, 2001; Park *et al.*, 2003; Runz *et al.*, 2008; Jahnova *et al.*, 2014; Bountouvi *et al.*, 2017). While many of these are private mutations, some are more globally occurring (Bountouvi *et al.*, 2017). One of the most prevalent mutations in *NPC1* is I1061T, which is commonly identified in patients of European ancestry (Millat *et al.*, 1999; Park *et al.*, 2003; Gelsthorpe *et al.*, 2008; Feng *et al.*, 2019). In a study completed by Millat *et al.*, (1999) examining a cohort of 115 unrelated patients presenting with C1 disease, I1061T constituted 14% of disease-causing alleles, whilst being absent in the control group. Furthermore, all homozygous patients displayed a juvenile-onset, neurological form of the disease (Millat *et al.*, 1999). A more recent study involving *in-vitro* models based on *NPC1*-null CHO cells transfected with vectors containing different *NPC1* variants has identified that I1061T causes partial clearance of cholesterol deposits in a time-dependent manner, suggesting that some functional retention may be attributed to this variant (Feng *et al.*, 2019).

SNPs in *NPC1* across different populations have also been associated with other chronic health conditions, including high body fat mass, adult-onset obesity and type 2 diabetes (Meyre *et al.*, 2009; Al-Daghri *et al.*, 2012; Liu *et al.*, 2017; Chiorean, Garver and Meyre, 2020). A genome-wide study completed in 2009 identified two SNPs, rs1805081/H215R and rs1805082/I858V, occurring in European populations. These SNPs displayed strong linkage and were associated with an increased risk of obesity in adults, as confirmed by further studies focussing on H215R and its association with increased BMI in addition to obesity, within these populations (Meyre *et al.*, 2009; Sandholt *et al.*, 2011). Furthermore, the rs1788799/I642M SNP, occurring in the SSD, has been found to modulate the risk of type 2 diabetes in Saudi Arabian populations (Al-Daghri *et al.*, 2012).

Unfortunately, although mutations in *NPC1* have been shown to cause C1 disease or be associated with other chronic conditions relating to its biological function, the molecular mechanisms underpinning this relationship remain vaguely understood and require further elucidation.

5.1.2 The Indispensable Role of Domain C in Filovirus Infection

Alongside its fundamental role in the physiological mechanism of intracellular cholesterol transport, NPC1 has also been shown to play a less beneficial role as the receptor used by filoviruses to enter host cells, as first evidenced by Carette *et al.*, (2011) and Cote *et al.*, (2011) (see Chapter 1.4.4). NPC1 as a viral receptor has been widely studied as well as the impact of its mutations on viral tropism and cell entry. However, some human cell types that express NPC1, including those of a lymphoid origin, are refractory to EBOV infection. In particular, *in-vitro* studies have demonstrated that T cells, which have been shown to have relatively high NPC1 expression are not permissive to infection, suggesting that NPC1 may not be the only protein of importance (Geisbert *et al.*, 2000; Bokelmann *et al.*, 2020). Regardless, EBOV can still bind directly to T cells and can cause cell death, without causing infection, (Iampietro *et al.*, 2017; Younan *et al.*, 2017; Younan, Iampietro and Bukreyev, 2018).

In efforts to clarify the involvement of NPC1 in EBOV infection, Miller *et al.*, (2012) demonstrated that while the full-length protein is not a requisite for filovirus entry, domain C (residues 372-620) is indispensable. In the study, NPC1-deficient CHO CT43 cells were engineered to express NPC1 variants lacking either domains A, C, or I and the ability of each variant to mediate viral infection was examined by challenging the cells with wild-type VSV or a recombinant version expressing filovirus GP. The results showed that the introduction of NPC1 deprived of domain C was unable to rescue viral entry, regardless of the expression level and intracellular distribution of the receptor, which were comparable amongst the variants tested.

A similar finding was obtained during a study involving chimaeras of NPC1 and NPC1L1, a protein with substantial structural homology to NPC1 that has been implicated in HCV cell entry (Krishnan *et al.*, 2012; Sainz *et al.*, 2012). When CT43 cells were engineered to express wild-type NPC1-L1, they were refractory to infection by VSV expressing filovirus GP. However, the same cell line expressing an NPC1L1 chimaera with domain C from NPC1 still showed susceptibility to viral infection. The opposite was observed in CT43 cells expressing an NPC1 chimaera with domain C from NPC1L1 (Krishnan *et al.*, 2012). This indicates that the divergent domain C in NPC1L1 cannot bind to EBOV GP.

Further insight into the interaction between EBOV GP and domain C of NPC1 was provided by solving the crystal structure of cleaved GP bound to NPC1. The resulting structure confirmed the implication of domain C in the binding of cleaved GP and thus, in

facilitating membrane fusion and viral entry into the cell (Miller *et al.*, 2012; Gong *et al.*, 2016; Wang *et al.*, 2016; Zhao *et al.*, 2016). Biochemical analysis of purified NPC1 and cleaved GP has also shown that amino acid substitutions in domain C can impact its binding to cleaved GP (Wang *et al.*, 2016). In particular, amino acid substitutions in two protruding loops that interact with the cleaved GP, such as P424A, F503G, and F504G, were shown to reduce the binding affinity between the domain and its ligand.

5.1.3 NPC1 Variation Impacting Filovirus Tropism

Residues within domain C of NPC1 have been demonstrated to be an important determinant of EBOV tropism. A study investigating the susceptibility of cell lines from different bat species to EBOV infection also found that a single amino acid change in domain C (residue 502), impacted susceptibility to GP-mediated infection (Ng *et al.*, 2015). In particular, the mutation D502F resulted in the reduced susceptibility of cells from the African straw-coloured fruit bat to EBOV. Contrastingly, cells from Buettikofer's epauletted fruit bat and Egyptian rousette fruit bat were both predisposed to infection. Additionally, studies investigating the molecular basis of the NPC1-dependent block to EBOV entry in reptilian cells, showed that amino acid changes in the adjacent domain C residue (residue 503) can affect the binding between the domain and cleaved GP in these cells, without impacting the folding, endosomal delivery or cholesterol clearance function of NPC1 (Ndungo *et al.*, 2016).

Although these studies focus on mutations in animal NPC1 orthologues, a more recent study identified ten naturally occurring missense SNPs that occur in human NPC1, specifically in the loops that form part of the interface with cleaved GP. They compared the susceptibility of Vero E6 cells expressing human NPC1 with the SNPs, to the *Ebolavirus* species infection. It was concluded that some SNPs reduced susceptibility to filoviruses. Specifically, P424A and D508N significantly altered EBOV, BDBV, TAFV, and SUDV PV entry, into cells (Kondoh *et al.*, 2018). However, there was no reference in this study as to what frequency the investigated SNPs occurred or in which populations. Investigation of SNPs occurring at a higher frequency in exposed populations, for example, African populations, could provide further insight into *Ebolavirus* epidemiology and why the virus seems to be geographically centralised to Africa.

Notably, all the sites of interest discussed in these studies are located in one of the two protruding loops of domain C that directly interact with the receptor-binding site of cleaved

GP. The mechanism of this interaction is analogous to how domain C of NPC1 binds to NPC2 to facilitate the bi-directional transfer of cholesterol to the N-terminal luminal domain (Li, Saha, *et al.*, 2016; Wang *et al.*, 2016). However, the binding affinity of domain C for cleaved GP is approximately eight-fold higher than that observed for NPC2, perhaps due to the extensive interface created as a result of the engagement of multiple residues in loops 1 and 2 with cleaved GP, compared to NPC2 (Gong *et al.*, 2016; Li, Saha, *et al.*, 2016).

5.1.4 Aims

Growing evidence suggests that in certain populations, mutations in NPC1 can be associated with both severe disease and chronic illnesses, prevalently due to the receptor's role as a cholesterol trafficker. Furthermore, several investigations have proved that key mutations in NPC1 can impact its effectiveness as a receptor for filoviruses in both animals and humans. In light of the above, we set up an *in-vitro* model to determine if the enrichment of SNPs in populations more frequently exposed to EBOV outbreaks, could impact the infectivity of EBOV. Specifically, we focused on African populations as we hypothesised that the outbreaks of endemic EBOV may constitute a selective pressure leading to the enrichment of SNPs that, once fixed in the population, may provide resistance to virus infections. To this end, we used a specialised database to identify a haplotype genotype for NPC1 that was specific to African populations and completed a structural analysis *in-silico* to inform subsequent *in-vitro* tests using NPC1 knockout U2OS cells. Finally, cells were transduced to stably express NPC1 variants carrying SNPs of interest and their susceptibility to EBOV infection was tested by challenging them with lentiviral pseudotypes expressing EBOV GPs.

5.2 Results

5.2.1 Determination of SNPs in NPC1 Deemed Suitable for Investigation

Investigations into polymorphisms within human *NPC1* began with the interrogation of the Ensembl genome browser to ascertain all minor allelic variations in *NPC1*. Of these, 21 missense allelic variants were identified occurring at a frequency that was greater than 1% within a particular population and thus, were determined to be SNPs (Table 5.1). These were either present close to or within the two-loop structures of domain C (amino acids 419-428 and 500-508), which constitute the binding partner of cleaved EBOV GP.

Table 5.1 21 missense allelic variants of human NPC1

Variants that existed at a frequency greater than 1% within specific populations were included. Data was generated using the ENSEMBL genome browser to identify SNPs of potential interest. Amino acids present at these variable sites, within the wild-type reference sequence NM_000271.5 and the sequence for NPC1 derived from the A549 cell line, have also been identified to determine which mutations (highlighted in red) would need to be inserted or reverted to generate the desired wild-type or haplotype sequences

Percentage (%) of Variant Present in Population											
Variant ID	Position	Allele	Amino acid	All	African	American	East Asian	European	South Asian	NCBI Ref. Sequence: NM_000271.5	A549 Sequence
rs143205855	149	C	G	99.8	100	100	100	100	99	G	G
		T	R	0.2	0	0	0	0	1		
rs1805081	215	T	H	78	98	74	74	62	75	H	R
		C	R	22	2	26	26	38	25		
rs55680026	222	T	N	>99	>99	>99	>99	>99	>99	N	N
		C	S	<1	<1	<1	<1	<1	<1		
rs80358251	237	G	P	>99	>99	>99	>99	98	>99	P	P
		A	S	<1	<1	<1	<1	2	<1		
rs138151007	291	C	W	>99	>99	>99	>99	>99	>99	W	W
		A	C	<1	<1	<1	<1	<1	<1		
rs77080672	411	C	R	>99	99	100	100	100	100	R	R
		T	Q	<1	1	0	0	0	0		
rs61731962	434	G	P	99	96	100	100	100	100	P	P
		A	S	1	4	0	0	0	0		
rs13381670	511	G	T	>99	99	>99	100	100	100	T	T
		A	M	<1	1	<1	0	0	0		
rs201791992	517	C	V	>99	100	100	>99	100	100	V	V
		T	I	<1	0	0	<1	0	0		

Chapter 5. Identification and Characterisation of SNPs Present in NPC1 that are Enriched in African Populations

rs1788799	642	C	M	17	4	22	8	35	22	M	I
		G	I	83	96	78	92	65	78		
rs145362908	810	C	V	>99	100	>99	100	100	100	V	V
		A/T/G	I	<1	0	<1	0	0	0		
rs190298665	842	A	F	>99	100	99	100	100	100	F	F
		G	L	<1	0	1	0	0	0		
rs1805082	858	T	I	51	64	39	39	54	54	I	V
		C	V	49	36	61	61	46	46		
rs34302553	911	C	G	>99	99	100	100	100	100	G	G
		T	S	<1	1	0	0	0	0		
rs1140458	931	G	N	52	64	39	39	54	55	N	N
		A	K	48	36	61	61	46	45		
rs34084984	961	T	N	>99	99	100	100	100	100	N	N
		C	S	<1	1	0	0	0	0		
rs141440861	1073	C	G	>99	100	100	100	>99	100	G	G
		T	S	<1	0	0	0	<1	0		
rs34226296	1115	C	V	>99	99	100	100	100	100	V	V
		A/T	I/F	<1	1	0	0	0	0		
rs61731969	1179	T	M	>99	99	>99	100	100	100	M	M
		C	V	<1	1	<1	0	0	0		
rs35248744	1200	T	S	>99	99	>99	100	100	100	S	S
		C	G	<1	1	<1	0	0	0		
rs1805084	1266	C	R	85	70	90	81	97	93	R	R
		T	Q	15	30	10	19	3	7		

This targeted approach also revealed that some SNPs were present in different frequencies in African populations, compared to other geographic regions. 411Q, 434S were of particular interest due to their position in the NPC1 protein structure and their low frequencies which are restricted to African populations, suggesting that their emergence may be due to a selective pressure provided by EBOV circulation in the population, during short-lived outbreaks. Further analysis of haplotypes showed that 434P>S,642M>I (Table 5.2) was only present in African populations. This informed the decision to investigate the impact of these two SNPs as both single variants and as a combination variant (from herein, referred to as the haplotype variant), within an *in-vitro* model.

Prior to this, an in-silico model was used to determine if the changes in the amino acid at these positions impacted the structure of NPC1 significantly, particularly at the binding site for the cleaved GP. Homology modelling was completed using the Phyre2 online software to compare the suspected structure of the haplotype variant (434P>S,642M>I), with that of the crystal structure of cleaved GP bound to domain C of NPC1 (PDB 5F1B), as determined by Wang *et al.*, (2016). The model shows 434S, displayed as a cyan stick, which is located within close proximity to the two loops that interact with cleaved GP, whereas 642I, located in the transmembrane SSD, is positioned in further proximity to the EBOV binding site. The model showed that relative to the domain C structure in 5F1B, which is shown in blue, the haplotype model predicts missing beta-sheets near the 434S mutation (Figure 5.1), suggesting a potential change in structure, and perhaps function. This was confirmed using the docking software ClusPro2.0, where missing beta sheets were again observed (Figure 5.2).

Table 5.2 Frequency of 434P>S, 642M>I haplotype

Further searches determined that the protein haplotype 434P>S, 642M>I variant is only present in African populations at a frequency of 0.0424% based on the 1000GENOMES database, with a combined frequency across all samples present in ENSEMBL, being 0.00931%. Numbers in parenthesis equal observed count.

<i>Protein haplotype</i>	<i>Frequency (count)</i>	<i>African</i>	<i>American</i>	<i>East Asian</i>	<i>European</i>	<i>South Asian</i>
434P>S,642M>I	0.00931 (56)	0.0424 (56)	0 (0)	0 (0)	0 (0)	0 (0)

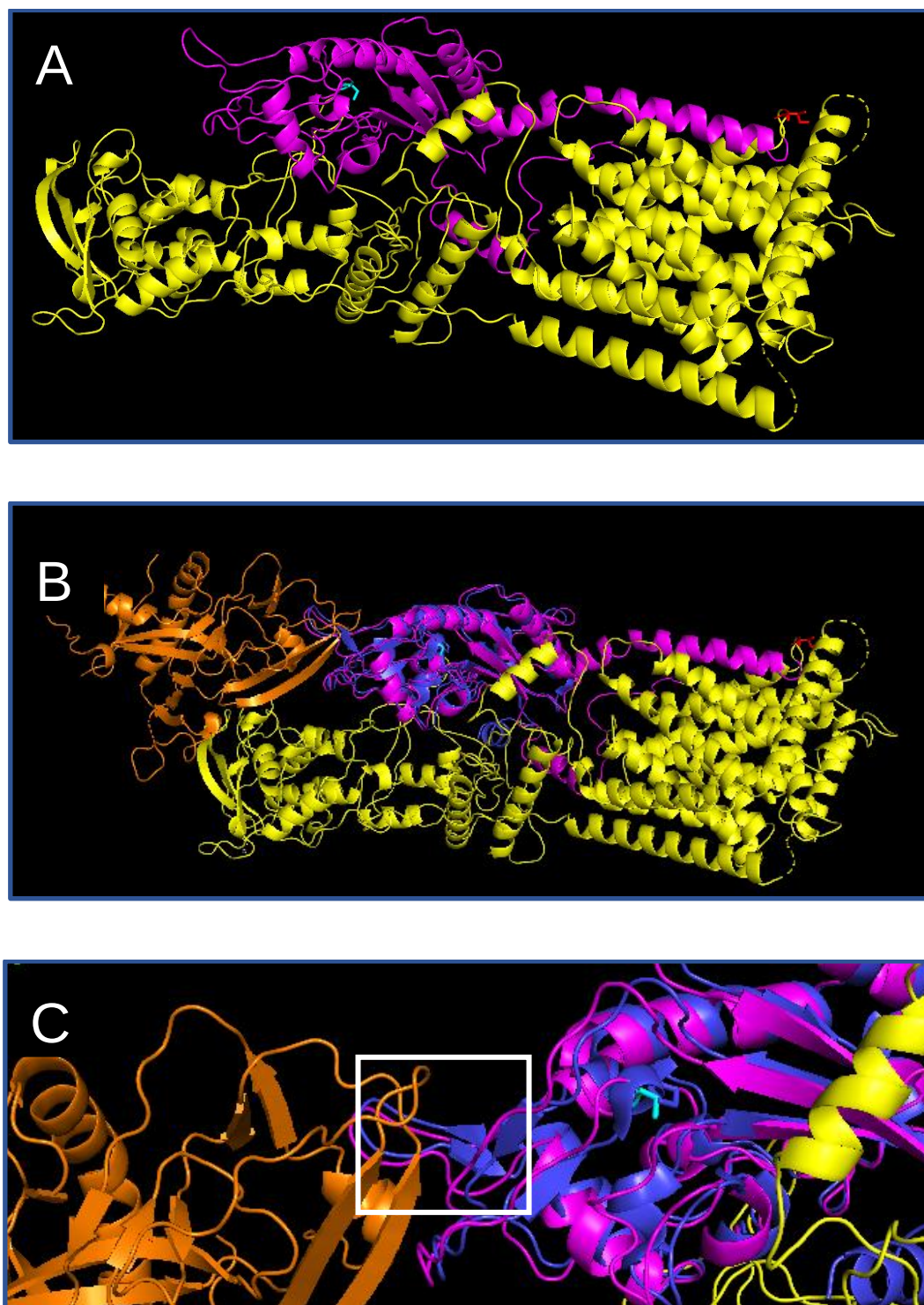


Figure 5.1 Homology modelling of NPC1

A) A homology model of the NPC1 haplotype variant in which flat arrows represent beta-sheets within the protein structure. Domain C (residues 327 to 640), as described by Wang *et al.*, (2016) has been highlighted in magenta and the remainder of the NPC1 structure in yellow. Both SNPs P434S and M642I, have been depicted as turquoise and red sticks, respectively. B) The haplotype variant model has been aligned with the structure of wild-type NPC1 domain C bound to the cleaved GP ligand (PDB 5F1B), depicted in orange. C) Zoom of the two protruding loops of domain C that interact with cleaved GP and the close vicinity of the P434S SNP to these loops. Missing beta-sheets near the site can be observed in the haplotype structure. The model was created using the Phyre2 web portal for protein modelling and visualised using PyMOL2.

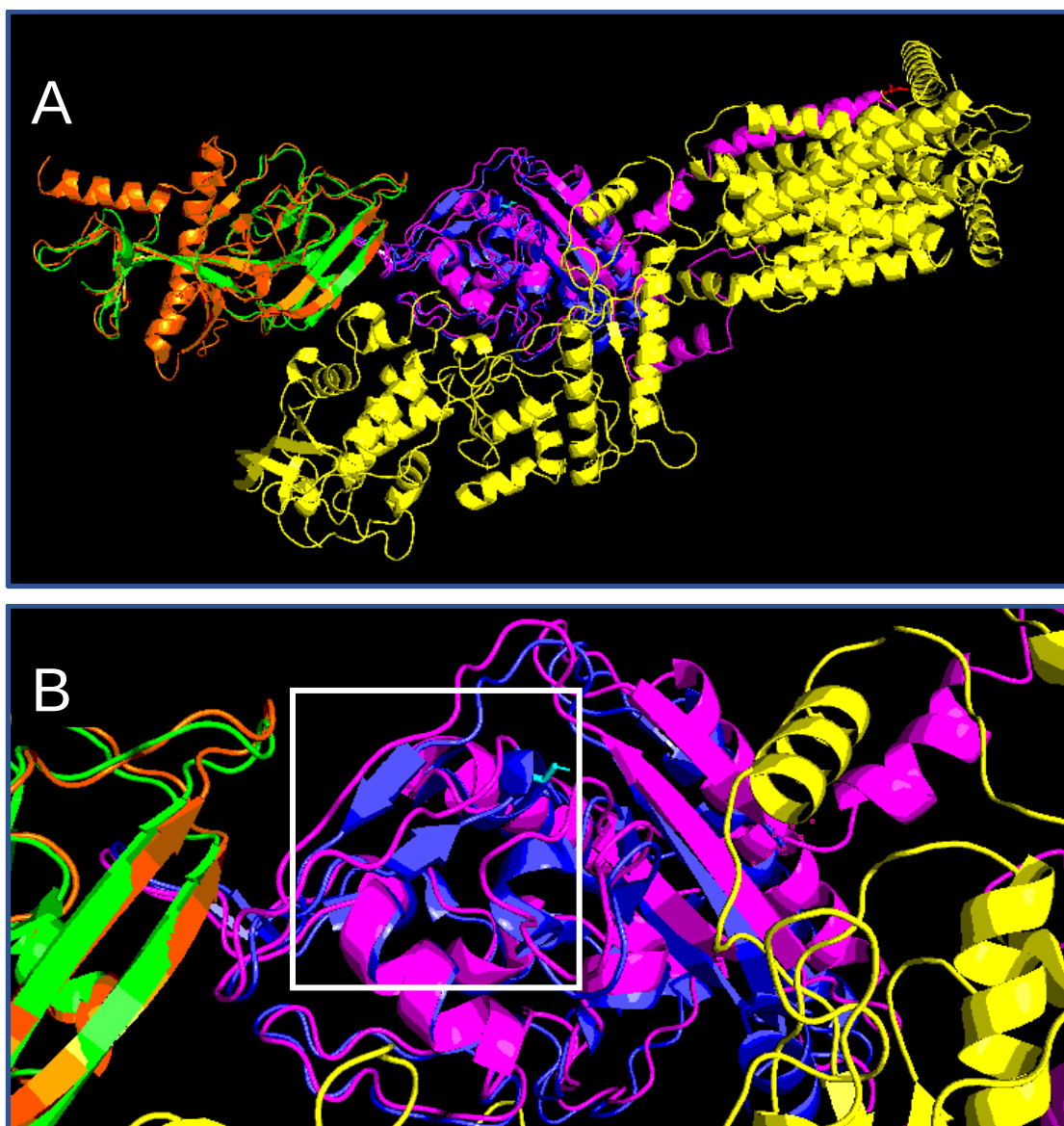


Figure 5.2 NPC1 haplotype variant docked against PDB 5F1B

The NPC1 haplotype variant structure was docked against the cleaved GP ligand chains A and B taken from PDB structure 5F1B, using the ClusPro 2.0 protein docking online software. This model was visualised on PyMOL2 and then aligned with 51FB. Beta-sheets close to site 434 can be observed in the haplotype variant but are not visible in the original PDB structure, suggesting a possible change in structure and perhaps in the resulting function of the receptor. Green represents cleaved GP, orange represents GP from 5F1B, blue represents domain C in 5F1B, magenta is domain C in the haplotype model and yellow is the remaining NPC1 structure in the haplotype model.

5.2.2 Mutagenesis to Generate NPC1 Variants

To obtain constructs that contained the SNPs 434S and 642I as both single variants and a combined haplotype variant, relative to the wild-type human NPC1 sequence (NM_000271.5), an alignment was completed using the MUSCLE programme available on the MEGA7 software, between the wild-type sequence and the sequence for NPC1

that had previously been isolated from RNA extracted from the A549 cell lines and synthesised into cDNA. The alignment determined three sites of variation between the wild-type sequence and A549 NPC1; H215R and I858V and M642I. To obtain the M642I mutation as a single variant in the NPC1 sequence, H215R and I858V were reverted using SDM. To this template, P434S was added to obtain the combined variant P434S/ M642I. M642I was then reverted to obtain P434S as a single variant. To obtain the original wild-type sequence. H215R and I858V and M642I were all reverted. Figure 5.3 provides an example of how the P434S single variant was generated.

The template used to generate the variant was the pHIV-eGFP vector that had the A549 *NPC1* cloned into the multiple cloning site and the mutations R215H, V858I and P434S already inserted via SDM. This template was used in a gradient PCR reaction, using primers that contained the desired mutation (I642M) in the reverse primer. From the amplified PCR products, gel electrophoresis identified the temperature at which the least non-specific products were present (55 °C). This product was processed further in a KLD ligation and during the transformation of stable cells. Colony screening of part of the *NPC1* gene containing the desired mutation was used to ensure that the PCR product had ligated correctly into the vector and that the gene contained the correct mutation, as confirmed via Sanger sequencing. A positive colony was then selected for growth in broth media and plasmid extraction. The final purified plasmid quality was characterised by spectrophotometry. Products with 260/280 ratios between 1.8 and 2.0 and 260/230 ratios between 2.0 and 2.2, were considered substantial for use in cell culture experiments. The plasmid sequence was confirmed to contain the I642M mutation by Sanger sequencing.

This process was used to obtain all four desired constructs (Figure 5.4). In instances where the initial templates were not sufficiently amplified for downstream processing, PCR reactions were optimised by using multiple primer annealing temperatures, as shown in Figure 5.3, as well as using varying template dilutions (data not shown)

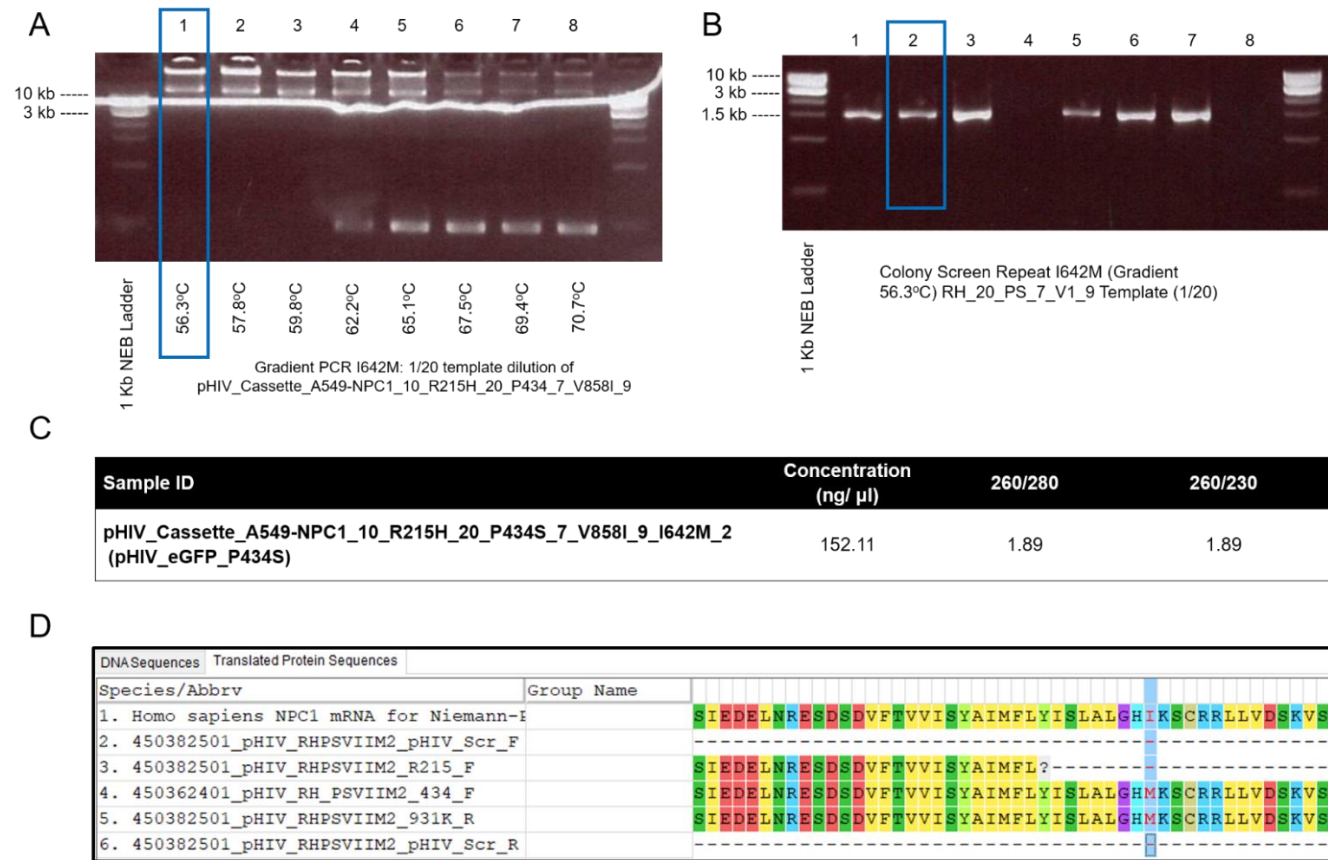


Figure 5.3 Reverting the final mutation to generate the NPC1 + P434S variant

A) A gradient PCR was completed to determine the best annealing temperature for the SDM primers used to revert the mutation 642I>M on a template plasmid containing NPC1 taken from the A549 cell line. 215R>H, 434P>S and 858V>I had already been generated. The PCR product was then used in a KLD reaction after which STBL3 cells were heat-shocked and transformed. B) Colony screen PCR to determine which colonies contained the desired plasmid. Sequencing of PCR products helped to determine if NPC1 contained the correct mutation. Positive colonies were then grown in a culture and plasmid was then extracted. C) NanoDrop results for the extracted plasmid to determine concentration and purity. D) Sequencing results of plasmid, analysed on MEGA7, showing the desired mutation present at the correct site.

There was a significant difference between the infectivity of C15, in the U2OS stuffer cell line and the U2OS delta NPC1 cell line ($P < 0.0001$), with the latter showing infectivity comparable to that of the negative control (delta E). Both cell lines were extremely permissive to the positive control, VSV G. These data confirmed that the delta NPC1 did not express NPC1 and was refractory to infection by C15 pseudotypes and thus, could be used in transduction experiments to express alternate NPC1 variants of interest.

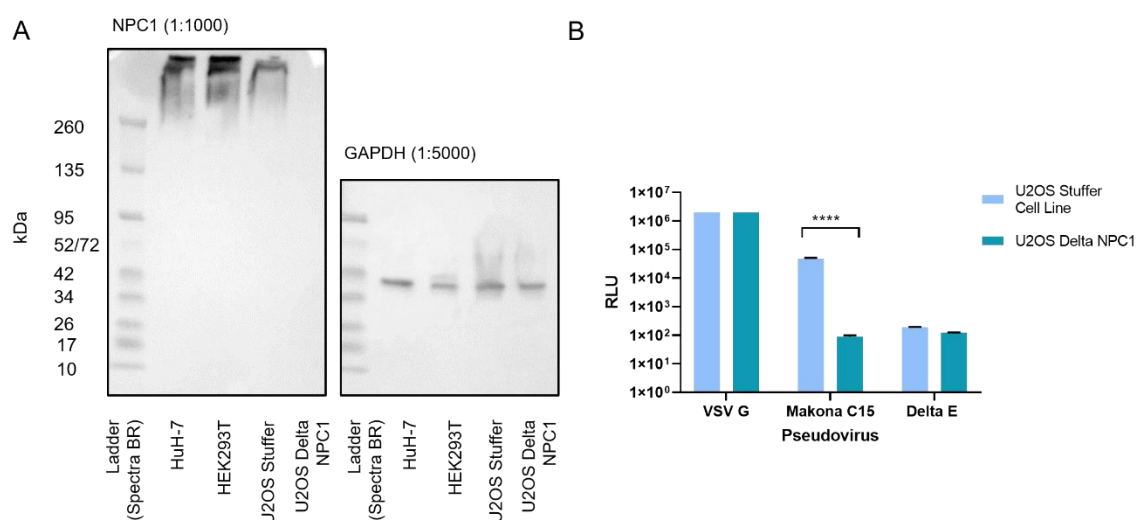
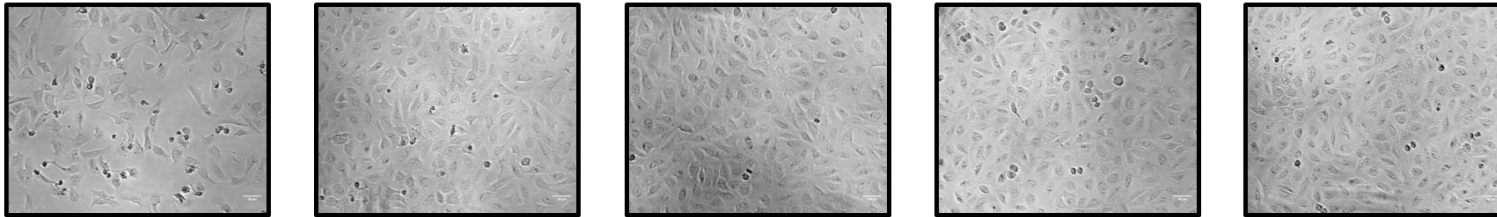


Figure 5.5 Characterising the U2OS Cell lines

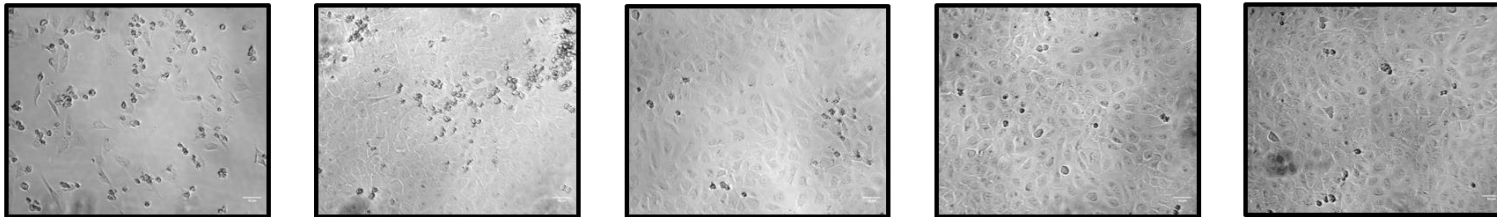
Western blot showing NPC1 expression in HuH-7 and HEK293T cells (positive controls), the U2OS stuffer cells and the U2OS delta NPC1 cell line. A 1:1000 dilution of an α -NPC1 antibody was used to detect protein expression slightly above 260 kDa for the stuffer cell line, although no expression is observed for the knockout cell line. A 1:2000 dilution of a swine, α -rabbit HRP (Dako) was used as the secondary antibody. GAPDH was used as a loading control. The primary antibody was a 1:4000 dilution of an α -GAPDH goat IgG (A00191) alongside a 1:5000 dilution of an α -goat HRP (Sigma) secondary antibody. B) Infectivity assay showing the permissibility of the two cell lines to Makona C15. The U2OS stuffer cell line has high permissibility to the virus whereas RLU values for U2OS delta NPC1 cells are comparable to the negative control, delta E, confirming a lack of receptor expression. VSV G was used as a positive control. $P < 0.0001$ as determined by a two-way ANOVA and a Sidak's multiple comparisons test. Error bars represent $\pm$ SD.

The U2OS delta NPC1 cell line had been generated by collaborators, via CRISPR and they had selected positive clones using blasticidin. Thus, the U2OS delta NPC1 cell line was expected to be resistant to the antibiotic, up to a concentration of 10 mg/ mL. To confirm this, a blasticidin kill curve was completed, in which the cells were challenged with concentrations of blasticidin that ranged between 0-30 mg/ mL (Figure 5.6). 72 hours post the addition of the antibiotic, a confluent sheet of cells was still observable in wells treated with 10 mg /mL of blasticidin. However, in cells that were treated with higher concentrations, cell rounding and death were seen. Again, this helped to verify the status of the cells before their transduction with NPC1 variant constructs.

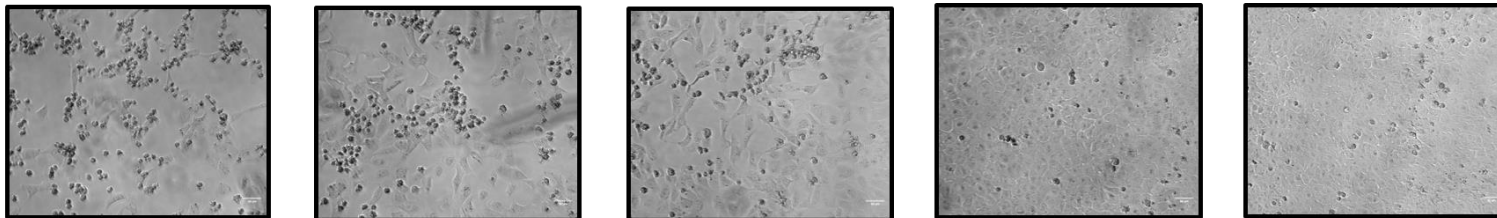
24 Hours Post Treatment



48 Hours Post Treatment



72 Hours Post Treatment



30 µg/ mL

20 µg/ mL

15 µg/ mL

10 µg/ mL

7.5 µg/ mL

Figure 5.6 Blasticidin sensitivity assay performed on U2OS delta NPC1 cells

On receipt, the U2OS delta NPC1 cell line was treated with multiple concentrations of blasticidin to confirm that they were resistant to concentrations up to 10 µg/ mL. A full cell sheet is observable 72 hours post-treatment with 10 µg/mL of blasticidin. At 15 µg/mL a disrupted sheet and cell rounding/death were observable. Images were taken at 10 x magnification with scale bar representative of 50 µM.

5.2.4 NPC1 Variant Transduction of U2OS Cells

A three-plasmid lentiviral transduction system was used to transduce the U2OS delta NPC1 cell line to establish four different cell lines, expressing NPC1 and GFP, through a bi-cistronic promoter. Initial experiments were additionally completed with HuH-7 cells because they had been successfully transduced by colleagues in the past and therefore, could be used to validate the method. This would ensure that all plasmids used to produce transducing units consisting of a lentiviral core, expressing VSV G and encasing the genomic material required for expression of the transgene in the desired cell lines, were functional and that the system was operational.

Figure 5.7 shows the HEK293T producer cells that were transiently transfected with the different plasmids, after 72 hours. Some cell death was observed due to the expression of VSV G, which is known to have high fusogenicity. GFP production can be observed in the HEK293T cells, suggesting that transducing units have been formed and are auto-transducing the producer cells. These units were then harvested and added to HuH-7 cells, which were observed over 72 hours for GFP expression that would indicate successful transduction of the transgene in each instance. Figure 5.8 shows there was little evidence for successful expression of the NPC1 variants in HuH-7 cells, even though a transduction efficiency of at least 30-40% was expected. The greatest indication of expression was observed within the positive control, which contained an empty GFP vector, suggesting that the addition of NPC1 variants to the vector, may impact either the efficiency of transducing unit production or transgene integration and expression.

The experiment was repeated under the same conditions, using the same plasmid constructs and reagents, substituting the HuH-7 cell line with the U2OS delta NPC1 cell line (Figure 5.9). Transfections were limited to the positive control and the wild-type variant to determine if the system was functional using U2OS delta NPC1 cells. The cellular morphology of the HEK293T cells 72 hours after transduction was unusual; much more cell rounding and cell death were observed. GFP expression was also observable but predominantly restricted to cells that were displaying or starting to display rounding. U2OS delta NPC1 cells that were transduced with these units displayed no GFP expression, indicating no NPC1 expression (Figure 5.9).

Subsequently, the transducing units were concentrated via ultracentrifugation so that p24 expression could be detected via western blot. As the major core protein, this was

completed to determine if the transducing units were integrating correctly and if the p24 core protein was being processed correctly from its precursors. Figure 5.10 showed that in these transducing units, although p24 expression was detectable, there were also strong bands at 51 and 44 kDa, indicating that many precursors of p24 were present, such as p51 and p44. This suggests compromised transducing unit production by the HEK293T cells, which explained why transduction of the U2OS cells was not observed.

Several attempts to optimise this experiment were made to produce transducing units that could be used as a positive control, and units that could transduce both HuH-7 cells and U2OS delta NPC1 cells with wild-type NPC1. Initially, fresh plasmid preps were completed, to exclude the possibility that the plasmids that were being used in the *in-vitro* system, had been contaminated and thus, a substance that was toxic to the cells, was causing higher HEK293T cell death than expected. This had no impact on the results, with HEK293T cells still showing significant cell death and the HuH-7 and U2OS delta NPC1 cells showing no GFP expression at all (data not shown).

Plasmid concentrations were then varied to determine if a sub-optimal concentration of plasmids was causing poor lentivirus production (Figure 5.11). Typically, 2000 ng of each plasmid is used in the system and this has been demonstrated to be successful (Figure 5.7). Varying concentrations of the VSV G plasmid, the wild-type NPC1 plasmid or the positive control plasmid also had no positive impact on the cells or transduction. In fact, the HEK293T cells displayed increased cell rounding and death with every combination of plasmid concentration tested (data not shown). Finally, transducing units were produced in triplicate by producer cells, combined and concentrated using a 20% sucrose cushion in an ultracentrifuge. In this manner, cells subsequently treated with pelleted transduction units would be exposed to a much higher quantity of units in a smaller volume. It was hoped that this may increase transduction efficiency by sufficient amounts so that cells expressing GFP could be sorted via flow cytometry, if not the actual desired amount. Again, no GFP expression was observed in either HuH-7 cells or U2OS delta NPC1 cells, with either the wild-type NPC1 or the positive control (data not shown).

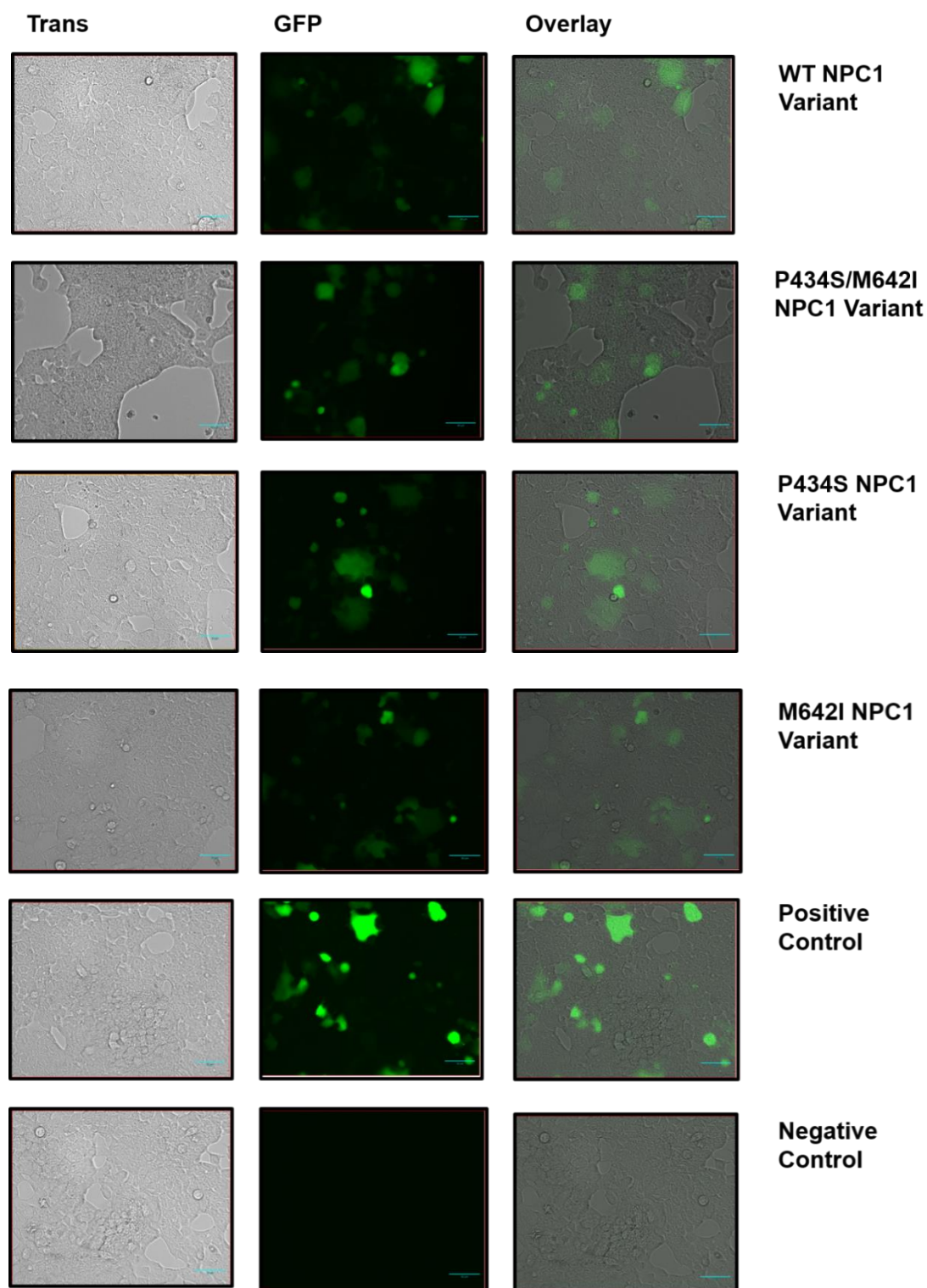


Figure 5.7 Transfection of HEK293T cells to produce transducing units

A three-plasmid system was used to produce transducing units containing pHIV-eGFP NPC1 variants. Images were taken using a white filter cube that permitted transmitted light imaging (trans) and a filter cube that allowed GFP visualisation. Images taken with the transmitted light and GFP lenses were superimposed to create overlay images. All images were taken at 10x magnification, with a scale bar representative of 50 μ M. Images were taken 72 hours post-transfection, on the day of the harvest of transducing units.

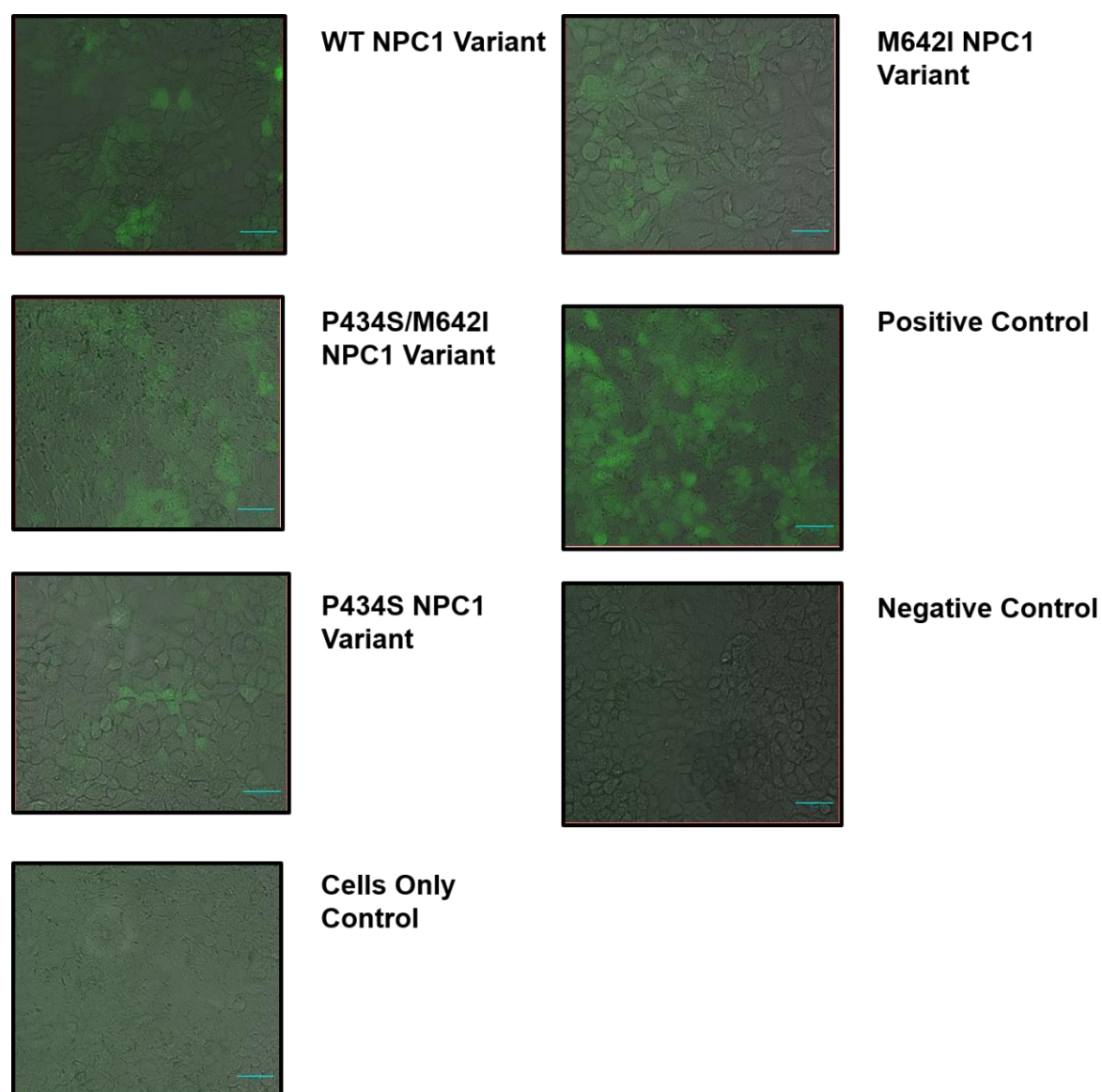


Figure 5.8 Transduction of HuH-7 cells with NPC1 variant receptors

Overlay images of HuH-7 cells 72 hours post-transduction with units containing pHIV_eGFP NPC1 variants. Images show successful transduction efficiency of approximately 40% for different variants, with discernibly higher efficiency for the positive control. No expression of GFP was observed in the negative control or the cells-only control. Images were taken at 10x magnification with the scale bar representing 50 μ M.

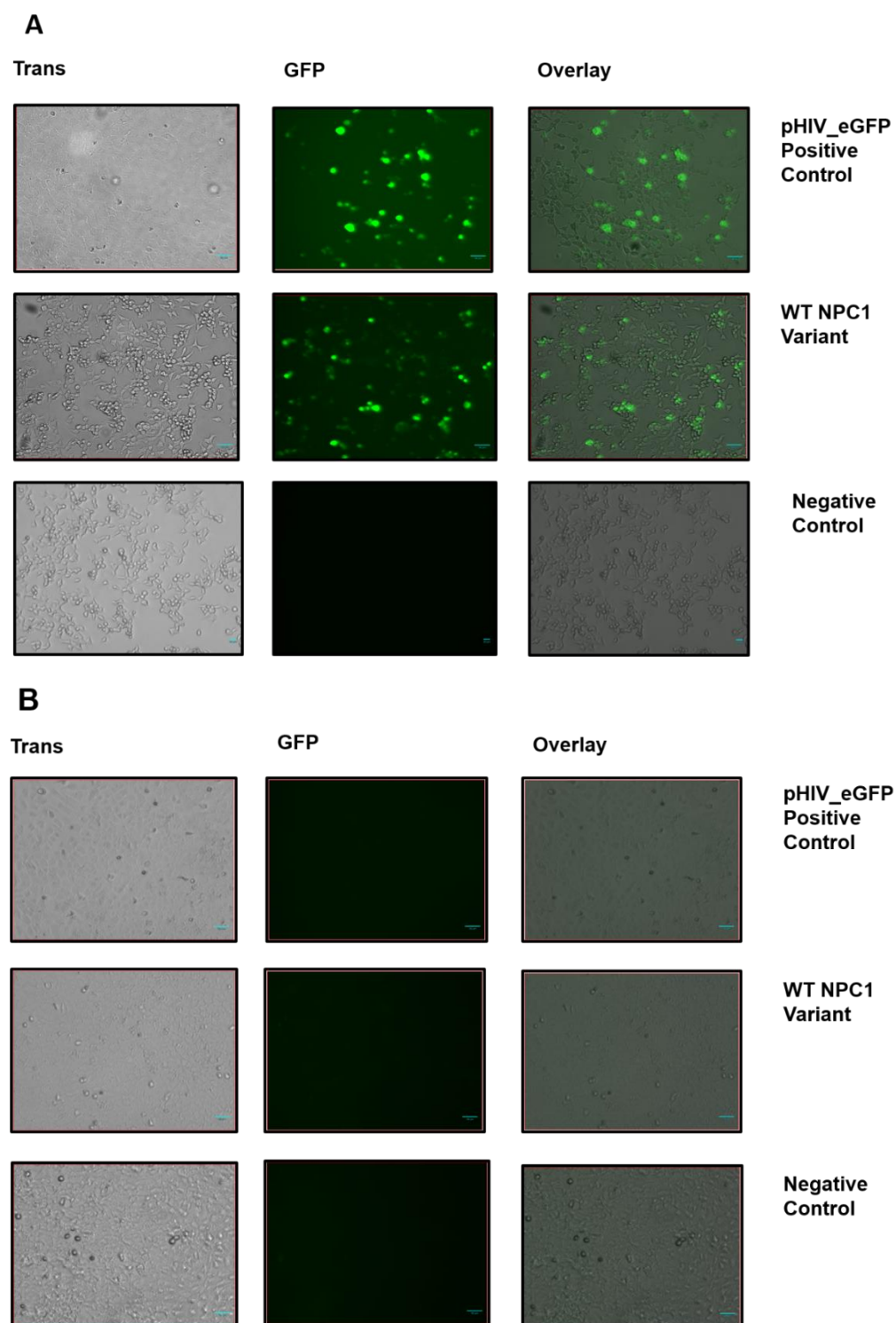


Figure 5.9 Transduction of U2OS delta NPC1 cells with wild-type NPC1

Images were taken using a white filter cube that permitted transmitted light imaging (trans) and a filter cube that allowed GFP visualisation. Images taken with the transmitted light and GFP lenses were superimposed to create overlay images. All images were taken at 10x magnification, with a scale bar representative of 50 μ M. A) Images of HEK293T cells 72 hours post-transfection showing GFP expression. Cells were transfected with the positive control and wild-type NPC1 variant. B) No GFP expression in U2OS delta NPC1 cells 72 hours post-transduction, showing unsuccessful receptor transduction.

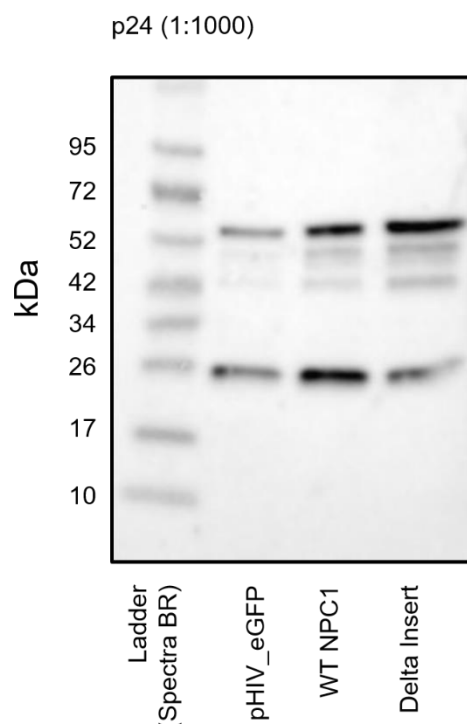


Figure 5.10 p24 detection in transducing units produced by HEK293T cells

Transducing units were pelleted via a 20% sucrose cushion in an ultracentrifuge. Pellets were resuspended in 15 μ L of PBS. Pellets were run on a 10% gel to determine if transducing units were forming correctly via the detection of the p24 core protein in western blot. A 1:1000 dilution of an α -HIV p24 (Abcam) primary antibody was used alongside a 1:2000 dilution of a swine, α -rabbit HRP (Dako) secondary antibody to detect expression. Data shows that although p24 is detectable, so are several of its precursors including p51 (51 kDa) and p44 (44 kDa) suggesting that transducing units may not be forming properly and thus, be less efficient in function.

A dummy transfection was then completed, in which cells were grown in DMEM, DMEM plus the transfection reagent, PEI and DMEM, PEI and VSV G plasmid, backbone plasmid and PEI, the plasmid containing wild-type NPC1 and PEI and the positive control plasmid and PEI. Cells were observed for morphological differences and death after 72 hours (Figure 5.11). Confluent sheets were observed for the cells grown in DMEM only and DMEM + PEI, although in the latter, fewer cells were observed. Patchy growth of cells was observed in the cells treated with PEI and VSV G plasmid. However, the majority of cells retained their typical star-shaped morphology. This was observed for the remaining cells treated with different plasmid combinations, suggesting that none of these components of the system in these given combinations was the problem. However, GFP expression in cells treated with the wild-type plasmid and PEI and the positive control and PEI, was still not as strong as expected or as prevalent as observed in earlier experiments.

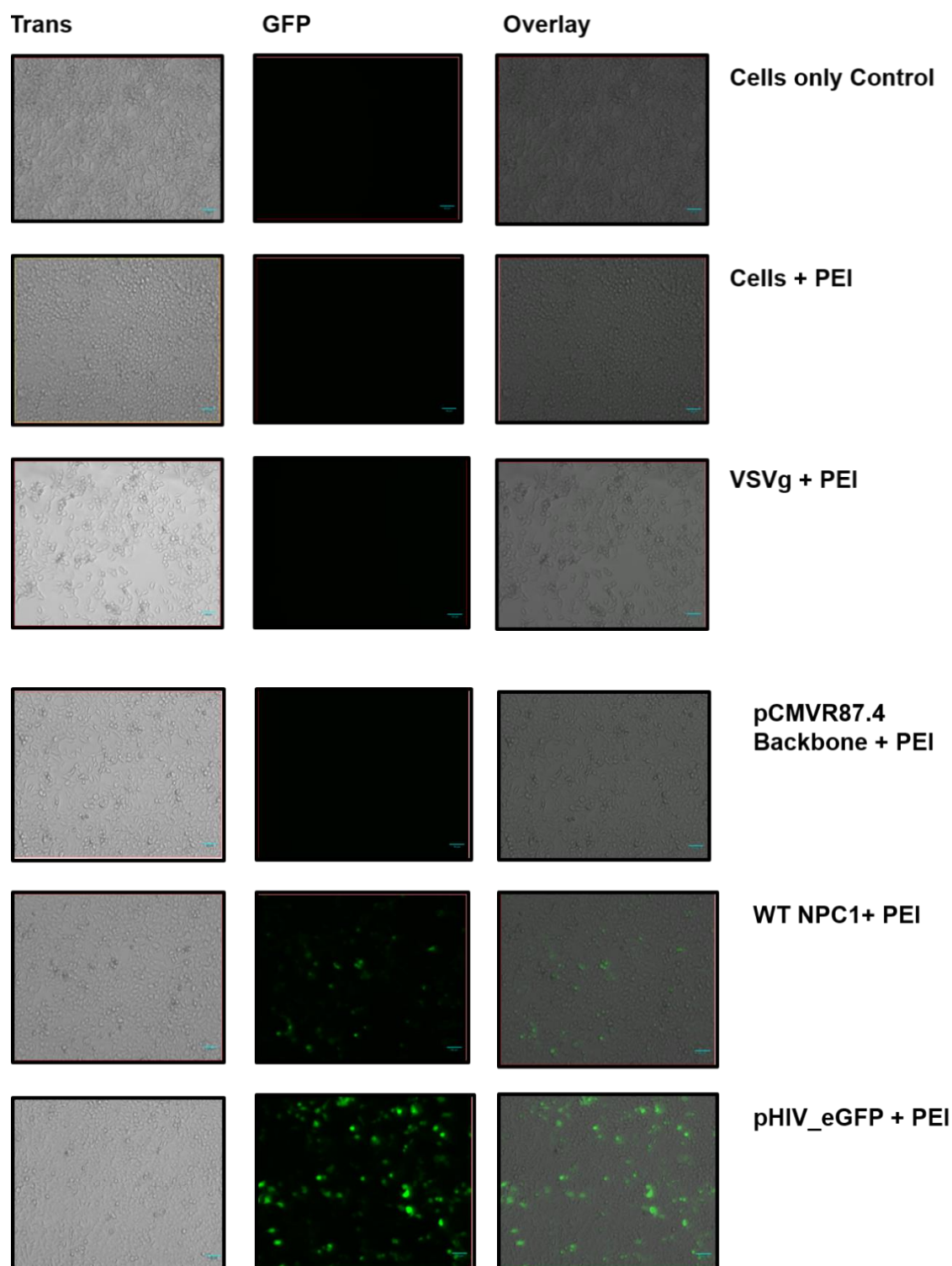


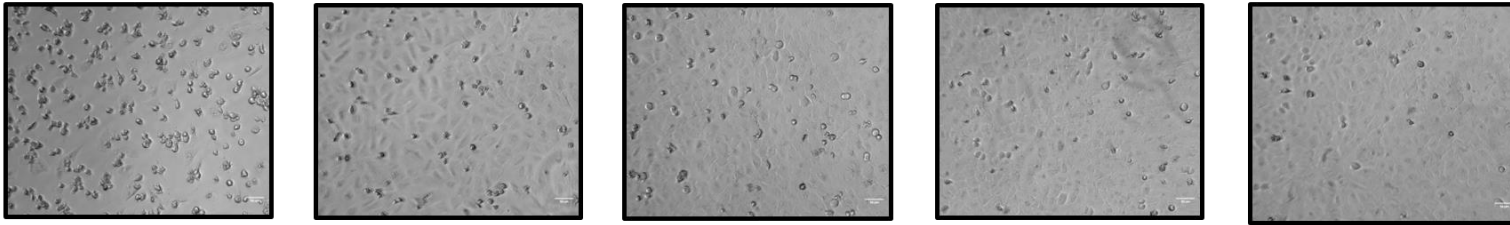
Figure 5.11 Dummy transfection of HEK239T cells

This was completed to determine if individual components of the system could be causing cell death. Each individual component was tested alongside the transfection reagent PEI, which was also tested by itself. No abnormal growth of HEK293T cells was observed after 72 hours. Images were taken using a white filter cube that permitted transmitted light imaging (trans) and a filter cube that allowed GFP visualisation. Images taken with the transmitted light and GFP lenses were superimposed to create overlay images. All images were taken at 10x magnification, with a scale bar representative of 50 μ M.

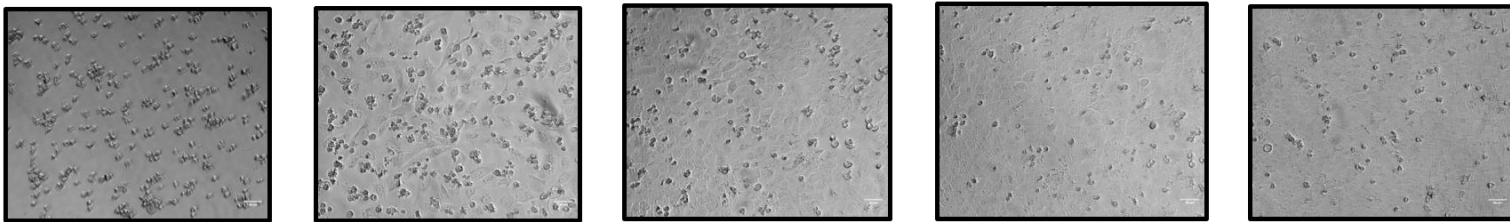
With no consistency observed with this methodology, it was decided that an alternative approach was to be taken, in which cells would be transduced to stably express the NPC1 variants, alongside a puromycin-resistant gene. Cells would then be treated with puromycin and surviving populations of transduced cells would be expanded to create stable cell lines. Similarly to the blasticidin kill curve, a puromycin kill curve was completed using the U2OS delta NPC1 cells, to determine an appropriate concentration of antibiotics to treat the cells with, post-transduction. Figure 5.12 shows the results of the puromycin kill curve. After 72 hours, some cell death was observed in cells treated with 0.3125 µg/ mL of puromycin and absolute cell death could be observed in those treated with 0.625 µg/ mL.

This helped to determine which narrow range of concentrations was to be tested during transduction experiments. Transduction experiments initially involved attempting to get stable cell line expression of wild-type NPC1 and the haplotype variant. The remainder were to be completed if time permitted. HEK293T cells were transfected with the backbone plasmid, VSV G and a plasmid containing either wild-type NPC1 or haplotype transgene, with a downstream puromycin resistance gene. The units were harvested and used to transduce U2OS delta NPC1 cells which were then treated with 0.5 µg/ mL and 1.25 µg/ mL puromycin (Figure 5.13). Both sets of cells survived 5 and 9 days post-treatment, relative to untransduced cells, which showed absolute cell death. These populations of cells were then expanded and used for infectivity assays.

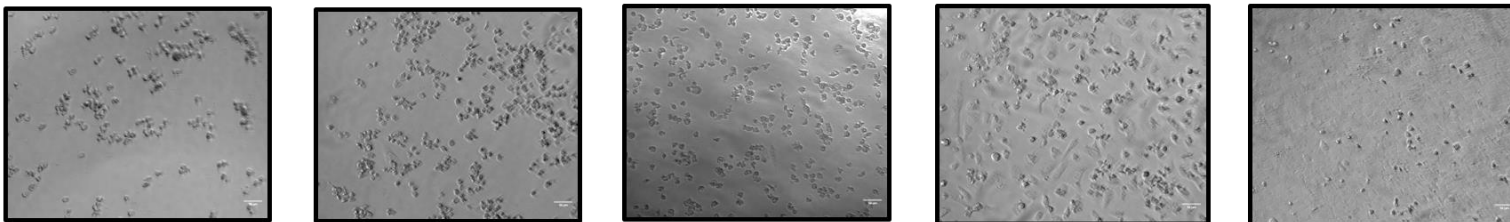
24 Hours Post Treatment



48 Hours Post Treatment



72 Hours Post Treatment



2.5 µg/ mL

1.25 µg/ mL

0.625 µg/ mL

0.3125 µg/ mL

0 µg/ mL

Figure 5.12 Puromycin sensitivity assay in U2OS delta NPC1 cells

Puromycin resistance was to be used as an alternative way to select successfully transduced cells. The sensitivity of U2OS delta NPC1 to puromycin had to be determined to elucidate the appropriate concentrations of antibiotic to use for selection. Cell death was observed 72 hours post-antibiotic treatment at a concentration of 0.625 µg/ mL. Images were taken at 10x magnification with the scale bar representing 50 µM.

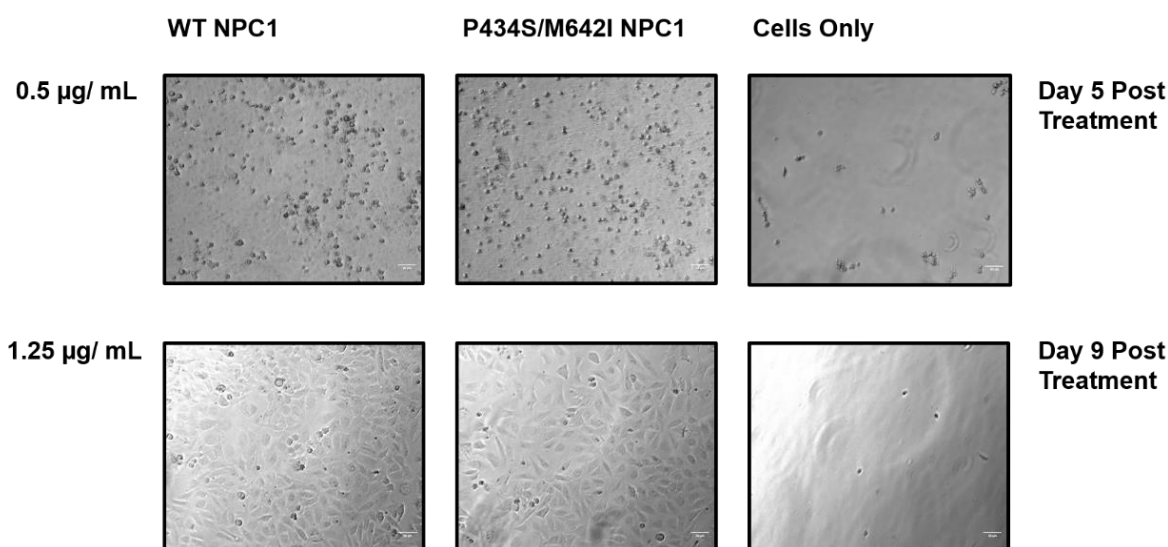


Figure 5.13 U2OS cell lines expressing different receptor variants

Cells that were successfully transduced with plasmids containing NPC1 variants and the puromycin-resistance genes reached confluency after being treated with 0.5 µg/ mL and 1.25 µg/ mL puromycin at days 5 and 9 post-treatment, respectively. The same concentration of antibiotics resulted in complete cell death in untransduced cells on days 5 and 9. Images were taken at 10x magnification with the scale bar representing 50 µM.

5.2.5 Characterising NPC1 Variant Expression in Transduced U2OS Cells

Once stable cell lines had been established expressing wild-type NPC1 and the haplotype variant genotype containing the P434S and M642I mutations, the next stage involved characterising the lines with regards to their NPC1 expression. This was investigated at both a genomic and phenotypic level, to determine if there was any correlation between mRNA levels within the cells and NPC1 expression in the late endosome/lysosome. qPCR was completed to determine mRNA expression of the NPC1 variants of the transduced cell lines, compared to that present in the U2OS stuffer and U2OS delta NPC1 cell lines and to determine if there was relative overexpression in the transduced cell lines. Primers to identify *NPC1* and *GAPDH* were obtained from colleagues. The primers had been designed to anneal onto different exons, to determine whether amplification products were obtained from mRNA or genomic DNA. RNA was extracted from approximately 6×10^6 cells and for each reaction, 3 µg was used to synthesise cDNA, which was then used in the qPCR reaction. To ensure that there were only single products amplified during the qPCR reaction, gel electrophoresis was used to identify the amplification of single products.

Figure 5.14 shows clean bands for an expected 262 base pair product in all cell lines, indicative of *NPC1* presence. Primers for *NPC1* targeted non variable regions, ensuring the same primer binding efficiency across the cell lines tested. The *GAPDH* amplification, however, gave multiple bands. This suggests a low primer specificity. Without time restrictions, alternative *GAPDH* primers would have been designed to ensure optimal efficiency specific to the U2OS cell line, compared to this primer set, which was designed to be universal to mammalian cell lines, thus, perhaps compromising efficiency. This would have been tested by creating a standard curve, with serial dilutions of cDNA. Additionally, the use of multiple housekeeping genes would have been considered. However, as this work involved only one cell line, with any variability limited to *NPC1*, the *GAPDH* gene sequence is the same across cell lines, thus primer binding efficiency would be consistent between cell lines. Consequently, any differences in *NPC1* expression between cell lines, analysed relative to *GAPDH* would remain consistent. *NPC1* expression was assessed relative to *GAPDH*, using the $2^{-\Delta\Delta C_q}$ method of analysis.

Figure 5.15 shows that mRNA expression of *NPC1* in the U2OS cells transduced with wild-type *NPC1* was 25-fold higher, relative to the U2OS stuffer cell line which acted as the control cell line, whereas U2OS cells transduced with the haplotype *NPC1* was 48-fold higher. Although the number of cells to be transduced in each instance was the same, these transduction units were not titred before addition to the *NPC1* deficient U2OS cells. Therefore, it is likely that the U2OS cells that were transduced by the *NPC1* haplotype had a higher multiplicity of infection, leading to increased levels of mRNA. However, levels of protein expression might not always correlate to levels of mRNA and thus it was important to characterise this using flow cytometry (Figures 5.16 and 5.17).

As discussed in Chapter 4, for each of the U2OS cell lines, approximately 250,000 cells were either left unstained, stained with anti-*NPC1* antibody to determine *NPC1* expression in the cell line or stained with an isotype control antibody. Figure 5.16 shows the output from the analysis of *NPC1* expression of the U2OS cells that were transduced with wild-type *NPC1* and maintained in 1.25 $\mu\text{g/mL}$ puromycin, using an FC500 flow cytometer. Analysis was completed on single cells only. This was repeated for cells transduced with the haplotype *NPC1* variant, *NPC1*-null U2OS cells and U2OS stuffer cells (data not shown). For all cell types, the unstained population was initially used to set the background parameters including all gating.

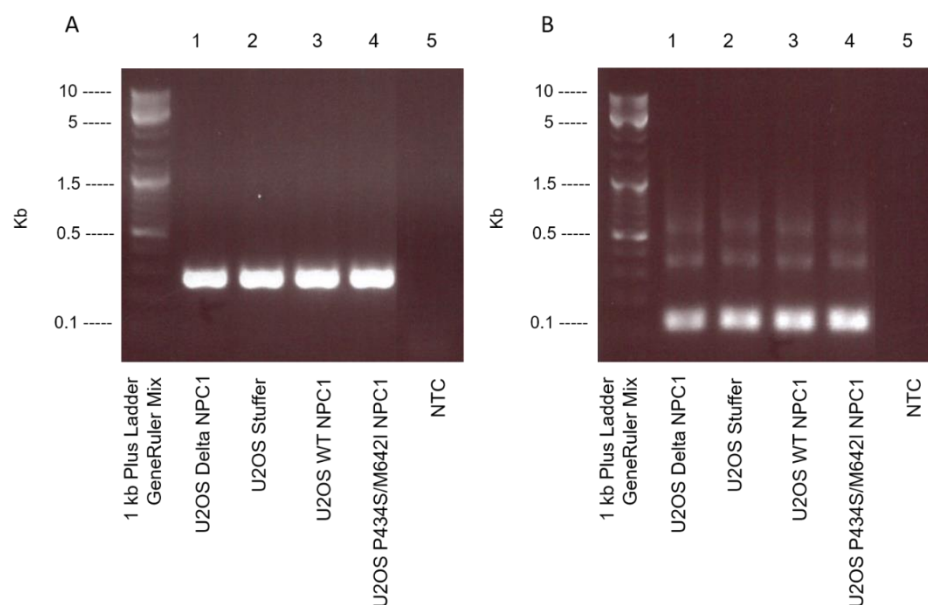


Figure 5.14 qPCR products from the amplification of *NPC1* and *GAPDH* in U2OS cell lines
Clean amplification of products was observed for *NPC1* (expected product size: 262 bp). Multiple products were observed for *GAPDH* amplification (expected product size 117 bp). NTC is representative of a no template control.

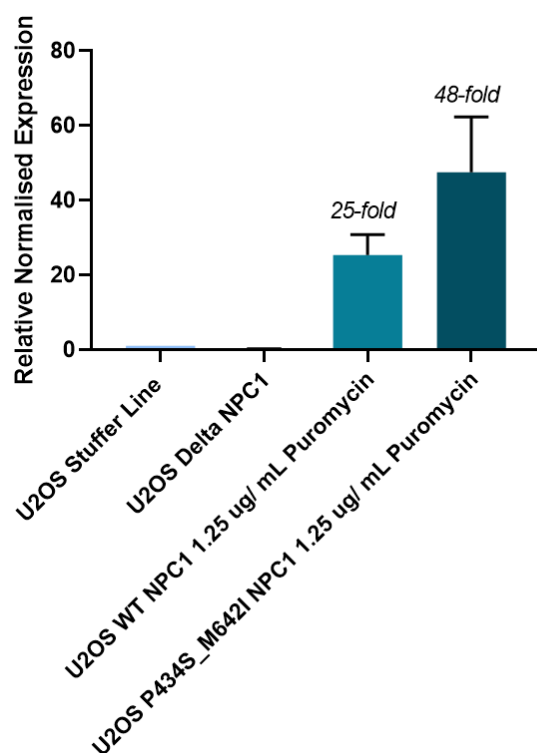
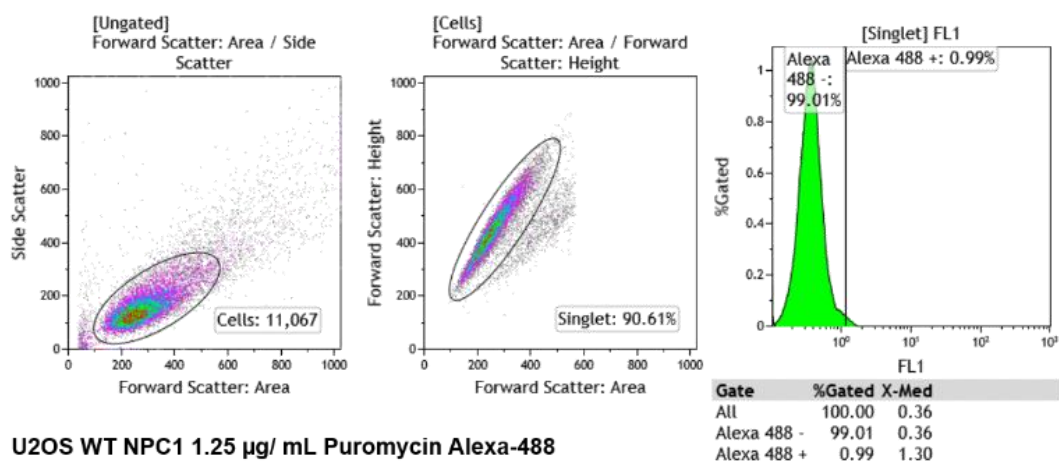
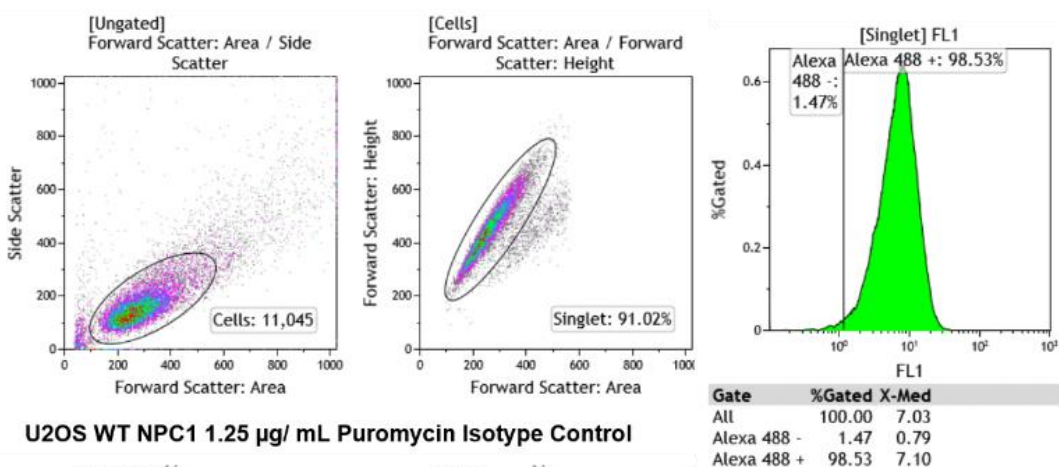


Figure 5.15 Relative gene expression of *NPC1* in U2OS cell lines
Gene expression was assessed in U2OS cell lines pre and post-transduction, with the U2OS stuffer line acting as the control cell line. Gene expression was normalised by RNA input and also to *GAPDH* expression using the $2^{-\Delta\Delta C_q}$ method. Data is the mean $\pm$ 1 SD of two independent experiments, each performed in triplicate.

U2OS WT NPC1 1.25 µg/ mL Puromycin Unstained



U2OS WT NPC1 1.25 µg/ mL Puromycin Alexa-488



U2OS WT NPC1 1.25 µg/ mL Puromycin Isotype Control

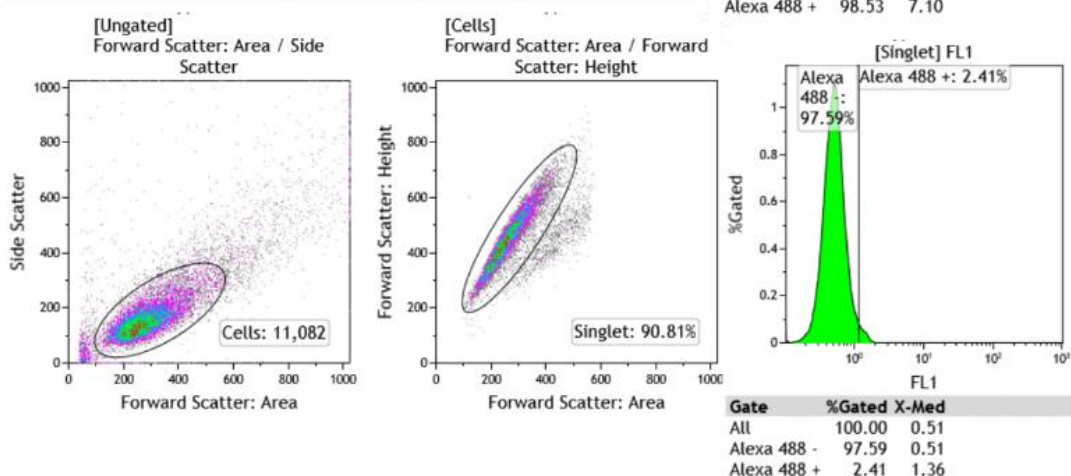


Figure 5.16 Fluorescence intensity of U2OS cells transduced with wild-type NPC1 receptor

NPC1 expression, shown in U2OS cells transduced with wild-type receptor and treated with 1.25 µg/mL of puromycin, was determined using an unstained control, anti-NPC1 Alexa-488 and an isotype control. Cells of the correct population were gated. Of these cells, singlet cells were identified and the fluorescence intensity was determined for the selected population. The isotype control was used for each cell line to set gates and background parameters. This was completed for the U2OS stuffer cell line, the U2OS delta NPC1 cell line and the U2OS P434S/M642I NPC1 variant grown in 1.25 µg/mL of puromycin (data not shown). Analysis was completed using the Kaluza software (Beckman Coulter). Data is representative of three independent experiments.

However, as Figure 5.17 shows, there was a slight shift in peak fluorescence intensity observed in all cells treated with the isotype control, implying very slight non-specific binding. This was observed in all experimental repeats (data not shown), albeit to a lesser degree by the final experiment. Therefore, the final gating was determined using the isotype control.

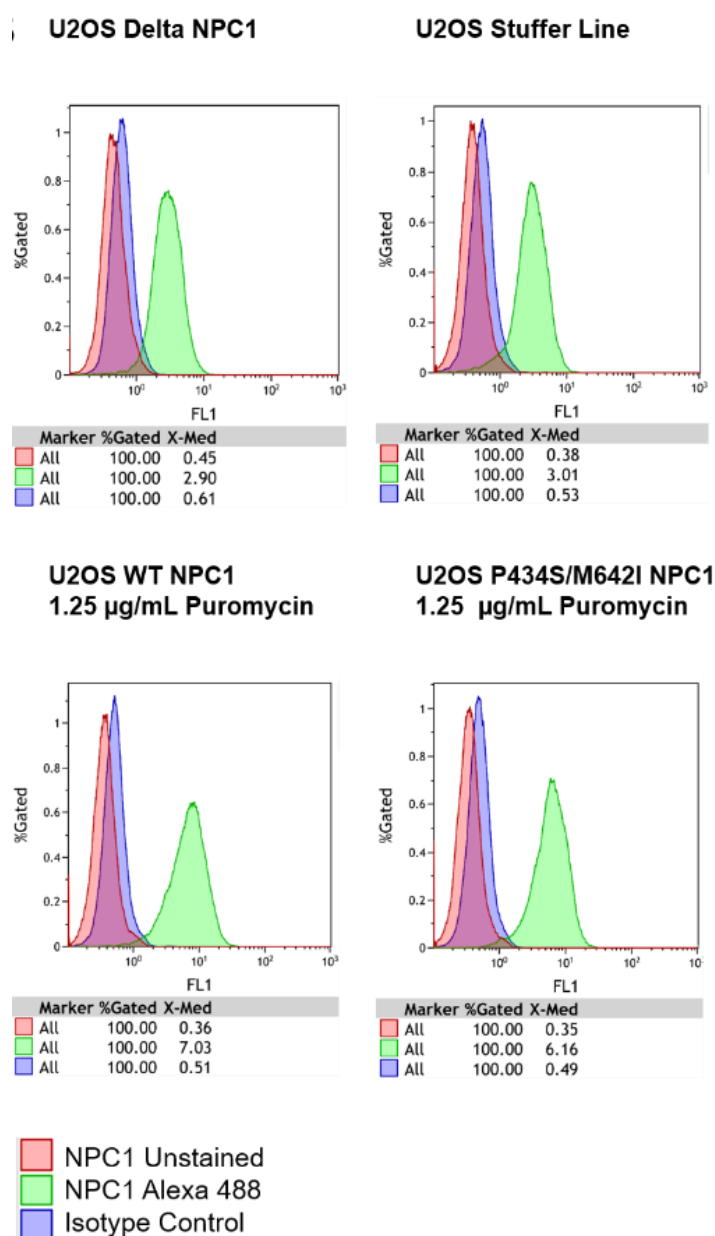


Figure 5.17 Overlay images showing fluorescence intensities of all U2OS cell lines expressing NPC1 variants

Overlay images for each U2OS cell line showing fluorescence intensities of unstained cells and those treated with the NPC1-Alexa 488 or isotype control antibodies. Analysis was completed using the Kaluza software (Beckman Coulter). Data is representative of three independent experiments.

As the experiments all involved one cellular population, rather than a true negative and positive population, which would typically result in two peaks in fluorescence intensity, negative cells in the stained sample were more likely to be displaying low expression/fluorescence intensity and therefore, it was determined that the most informative approach of analysis would be to measure the rMFI of the cells of stained cells, relative to the isotype control. Figure 5.18 shows the change in rMFI in the cell population over three independent experiments. Relative to the isotype control, the rMFI is highest in the U2OS cell line transduced with wild-type NPC1 in every experiment, and approximately five-fold higher relative to the stuffer cell line (6.42) by experiment 2 and approximately two-fold higher than the U2OS cell line transduced with haplotype NPC1. However, by the final experiment, the expression was relatively similar, although still much higher than observed in both the NPC1-null U2OS and stuffer cell lines.

Interestingly, there was some NPC1 expression observed in the U2OS delta NPC1 cell line. This may be due to how the cells were generated. Depending on the site of CRISPR editing, small amounts of a truncated, non-functional protein that still included the antibody epitope for anti-NPC1, may have been detectable through flow cytometry (Spence *et al.*, 2016). However, as previous experiments show, these cell lines are not permissive to EBOV PVs expressing C15 GP, which is the interaction that is under scrutiny in these experiments. Stabilisation of NPC1 expression in the presence of puromycin, in newly transduced cells, may also explain the variability of rMFI between experiments, observed in these cell lines.

All U2OS cell lines were then challenged with EBOV PVs that were known to be highly infectious (Makona C15) and poorly infectious (Mbandza 2003) in HuH-7 cell lines, to determine if there was a difference in the permissibility of cells transduced with wild-type NPC1 or the haplotype NPC1, attributing any observed differences to the 434P>S and 642M>I SNPs. Figure 5.18 shows that over two independent experiments, there is a significant difference in the infectivity of Makona C15 and Mbandza 2003 between the transduced cell lines, with it being higher for both variants in the haplotype ($P < 0.0001$ and $P = 0.0057$, respectively).

Infectivity data was then normalised to the expression data obtained from cells of the same passage number, as shown in Figure 5.18. Interestingly, normalisation showed that the ratio between infectivity and rMFI, was highest in the U2OS stuffer cell line, although there

was still a significant increase in haplotype cells compared to cells transduced with wild-type NPC1 ($P = 0.0004$).

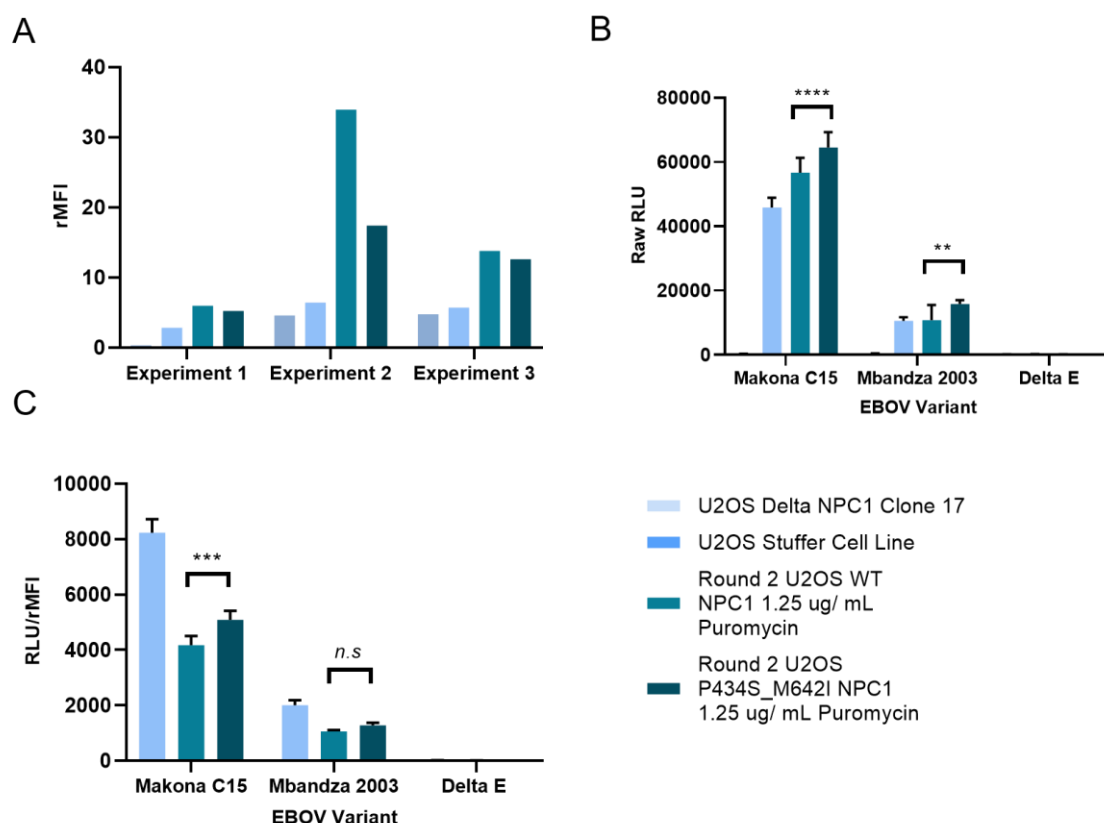


Figure 5.18 Characterisation of infectivity of EBOV PVs, relative to differential NPC1 expression in U2OS cell lines

A) Relative mean fluorescence intensity (rMFI) of NPC1 variants in U2OS cell lines across three independent experiments. To determine rMFI for each cell line, the mean fluorescence intensity was taken for cells treated with NPC1 Alexa-488 and divided by the mean fluorescence intensity for cells treated with the isotype control. B) Differential infectivity PVs expressing EBOV GP variants in U2OS cell lines. Data is the mean + 1 SD of two independent experiments, each performed in triplicate. Differences in the mean infectivity for each PV, between different cell lines, were assessed using a two-way ANOVA with Tukey's multiple comparison test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s, not significant ($p > 0.05$). C) Infectivity normalised to NPC1 expression in cells of the same passage number.

5.3 Discussion

Following the West African EBOV outbreak, several studies demonstrated the ability of the virus to rapidly evolve to become better adapted to human cell entry (Diehl *et al.*, 2016; Urbanowicz *et al.*, 2016; Dietzel *et al.*, 2017). The residues in the glycoprotein that had interacting partners within domain C of NPC1 and their importance in the viral entry process were also identified (Krishnan *et al.*, 2012; Gong *et al.*, 2016; Wang *et al.*, 2016).

However, less attention has been given to the variability of NPC1 both within and across species and how this may impact viral entry. Nevertheless, some studies have successfully identified residues in NPC1 that impact the tropism of the virus in different species. (Ng *et al.*, 2015; Ndungo *et al.*, 2016). Further studies that focussed on characterising the susceptibility of two different bat cell lines to both EBOV and MARV infection, revealed that three and two amino acid differences in the protruding loops of domain C resulted in the preferred susceptibility of the bat cell lines to either EBOV or MARV infection, respectively (Takadate, Kondoh, Feldmann, *et al.*, 2020).

The investigations into the variability in human NPC1 remain limited and rather than elucidating the impact of this variability on its role as a viral receptor, they focus predominantly on population-specific studies of mutations that disrupt its primary role as a cholesterol trafficker and the acute and chronic health conditions that consequently arise (Lamri *et al.*, 2018; Touma *et al.*, 2021). Given the limited knowledge available on host genetic factors involved in human susceptibility or resistance to EBOV, the aim of this study was to generate a uniform system to investigate population-specific NPC1 polymorphisms and their impact, with the hope to inform public health policy and determine what populations may be inherently more susceptible or at risk of infection.

The need for such investigations is supported by the cases of infection by some viruses such as macrophage-tropic (M-tropic) HIV-1 strains. These viruses utilise the CD4 receptor and the co-receptor CCR-5 (a chemokine receptor) to gain entry into susceptible cells. A mutant allele of CCR-5 (known as CCR5-delta 32) discovered in Caucasian populations at low allelic frequencies was shown to code for a non-functional receptor that does not support entry and infection in M-tropic HIV-1 viruses (Samson *et al.*, 1996). Thus, patients who are homozygous for the allele are resistant to infection. This mutation is either very rare or absent in African and Asian populations (Samson *et al.*, 1996; Novembre, Galvani and Slatkin, 2005). Although this mutation is a larger genetic change than the polymorphisms that were investigated in this chapter, this demonstrates how receptor mutations linked to specific populations can impact viral entry and infection.

In the case of NPC1, the identification of SNPs only present in African populations as a haplotype variant (434P>S and 642M>I) was of interest for several reasons. Firstly, given the geographic origin of all known EBOV outbreaks, it was interesting to determine if the virus could impose a selective pressure on African populations resulting in an enrichment of certain alleles that may cause increased resistance to EBOV disease.

Additionally, the P434S SNP was particularly interesting due to its proximity to sites of positive selection and other SNPs which studies have shown to impact tropism and infectivity. For example, Kondoh *et al.* (2018) demonstrated the impact of ten different SNPs in domain C of human NPC1 on filovirus infectivity. In particular, the P424A substitution resulted in the reduced entry of VSV pseudotyped with EBOV GP, but not MARV GP. Consequently, any structural changes in NPC1 resulting from the mutation of a neutral proline to a polar serine residue were also considered to be worth investigating in relation to their impact on the function of NPC1.

However, there were some limitations in the methods used to determine the specific *NPC1* sequences that were to be investigated in this study. Firstly, the sequence that was utilised as the 'wild-type' *NPC1* sequence and used throughout the study was the canonical reference sequence listed in ENSEMBL for human *NPC1* (NM_000271.5). However, the source of this receptor sequence and which population subset it was obtained from was undetermined. Therefore it is unknown if the receptor sequence was sourced from a population who are mainly infected with EBOV (African populations) or from other populations where the receptor has been studied in relation to its biological significance as a cholesterol transporter and the impact that mutations can have in Niemann-Pick C1 disease. Additionally, database searches using UniProt determined that isoforms of NPC1 can be generated by alternative splicing of the protein. The wild-type sequence used in this study is the isoform that has been determined as the canonical sequence but a shorter isoform of the spliced protein (UniProt ID: O15118-2) can be produced. This may have an impact on the expression of the protein, for example, nucleotide substitutions near the splice site may cause a different form of the protein to be expressed, even if the inferred protein sequence would be the same.

The present work determined that transduced U2OS cell lines expressing the NPC1 haplotype variant resulted in greater permissibility to infection by PVs containing the EBOV GP, compared to cells expressing the wild-type receptor. This outcome was in contrast to the originally proposed hypothesis. Although the mutations may not provide a relative level of resistance against the virus and in fact, makes it more susceptible to infection, the reasons for their increased frequency within the population may be explained through the protein's biological function within the cell. As discussed before, domain C of NPC1, where the P434S mutation lies, is also active in the transport of cholesterol and therefore, may have an impact on this mechanism and related health conditions. Evidence of a similar correlation already exists for M642I, which has been linked to diabetes in some populations, therefore, any impact on its function as a filovirus receptor may be secondary

(Al-Daghri *et al.*, 2012; Zhao *et al.*, 2016). This may provide a more likely explanation for the frequency of alleles as historically, EBOV outbreaks have been infrequently occurring, (although are becoming more commonplace in recent years), have been localised in nature and were relatively self-limiting. Previous studies have determined that a change from P424A results in reduced affinity to GP (Wang *et al.*, 2016). Perhaps P434S results in a stronger affinity to the GP, thereby explaining the increase in infectivity that was observed. Further investigations would be needed to determine which GP residue acts as the interacting partner for this amino acid and how binding could be impacted.

Another factor to consider is that although there was a statistically significant increase in the permissibility of the haplotype receptor variant relative to the wild-type variant, this may not have a biological significance. It has been well established that viral tropism is influenced by many additional factors aside from the host receptor engagement that the assays used in this study measured. Examples include a virus's ability to successfully replicate and egress from the cells without inhibition by restriction factors (Yap *et al.*, 2014; Vande Burgt, Kaletsky and Bates, 2015). Therefore, a significant increase in infectivity displayed by EBOV through a particular NPC1 variant may not result in any clinical significance. This can be observed in Chapter 3, where although the Mbandza 2003 EBOV variant consistently displayed the lowest infectivity in cell lines originating from multiple species, in a human outbreak setting, the variant had one of the highest CFRs observed in EBOV outbreak history, at 83% (Table 1.3), indicating that the virus may have displayed efficiency at other stages of its life cycle, relevant to its pathogenicity.

The transduction of a cell line that had no functional NPC1 expression was central to the model used during this investigation. Previous experiments confirmed that not only does NPC1 expression differ between tissues within a species, but also between different species (Urbanowicz *et al.*, 2016; Bokelmann *et al.*, 2020). Furthermore, other studies have revealed that the passage number of a cell line obtained from a given species can also have an impact on NPC1 expression, depending on the tissue that the cells are from and whether they are primary or immortalised (Bokelmann *et al.*, 2020). Additionally, some, such as bat and Vero E6 cell lines have been shown to contain restriction factors that can target PVs that use an HIV-1 backbone (Stremlau *et al.*, 2004; Yap *et al.*, 2014; Urbanowicz *et al.*, 2016). This suggests that together with polymorphisms, factors like variable NPC1 expression and abundance as well as the presence of unknown restriction factors may have an impact on viral entry and infection. Therefore, determining the role of specific SNPs in viral entry becomes challenging without taking into consideration these additional variables. The benefit of a model such as the one proposed in this work,

whereby different receptor variants, reflecting within and between species, could be generated by molecular techniques, is that theoretically, the variants could be expressed in the same NPC1- deficient cell line, maintained at the same passage number and display similar receptor expression levels, thus eliminating the background biological variability arising from the use of different cell lines. Consequently, the scope of this work is vastly increased by providing a model to complete comparative studies using any orthologue of NPC1.

For this investigation, several optimisation steps were required to generate the transduced cell lines. Originally transduction efficiency with each NPC1 variant was to be monitored through bicistronic GFP expression, through a vector (pHIV_eGFP) containing an IRES sequence that would enable the coordinated expression of both *NPC1* and *GFP* (Makrides, 2003). This way, NPC1 expression could be normalised to GFP expression, whilst also providing a quick and easy way to visibly determine if the transduction had worked. Positive cells were then to be sorted via flow cytometry and maintained to permit rounds of infection with EBOV PVs. As the results show, several steps to achieve this successfully to produce U2OS cells transduced with NPC1 variants, proved ineffective, although it is unclear why. Optimisation steps included using different plasmids, varying plasmid concentrations and testing individual components of the system for cellular toxicity.

U2OS cells are transiently transfectable (Ndungo *et al.*, 2016). Therefore, attempts were made to transfect the NPC1 null U2OS cells directly, using both the transfection reagents PEI and *TransIT* (Mirus Bio). Transfection efficiency was noticeably higher in cells treated with *TransIT* (data not shown). However, due to the limitations of reagent price and availability, the experiment could not be further optimised. Alternatively, the creation of stable cell lines was attempted to circumvent the long-term issues of using a GFP marker. Although GFP expression provides a quick way to visually determine successful transduction, there is no selective pressure forcing the cells to maintain the expression plasmid. As a result, the negative cells in the population continue to expand and cells typically have to be discarded after a few passages. This means that multiple transductions would have been required throughout the investigation. The generation of stable cell lines, although initially more time-consuming, meant that NPC1 could be continuously expressed, in the presence of the antibiotic puromycin, which ultimately proved to be the most successful method.

Once successful transduction with the receptor variants was completed, mRNA expression levels were measured using qPCR. U2OS cells transduced with the 434P>S 642M>I haplotype showed almost double the mRNA expression relative to cells transduced with wild-type *NPC1*. However, protein expression studies using flow cytometry showed that by the third experimental repeat, the receptor expression had stabilised in both lines, although wild-type *NPC1* expression was marginally higher than in the haplotype (1.08 times).

Due to the slight difference observed in *NPC1* expression level between cell lines, the infectivity data taken from cells of the same passage number was normalised to the expression level. In doing so, it became apparent that the U2OS stuffer cells had a higher infectivity/expression ratio than either of the two transduced cell lines. The stuffer cells were generated by CRISPR-Cas9 as a control cell line that would be analogous to the unmodified U2OS cell line. This was because the gRNA used to target the *NPC1* gene for CRISPR-Cas9 contained an additional BST2 ‘stuffer’ sequence to disrupt the process. The relationship between infectivity and receptor expression in the U2OS stuffer cells was not observable when looking at the infectivity data alone. This data, before normalisation, repeatedly showed that the lowest infectivity was observed in the U2OS stuffer cells and the highest in the cells expressing the haplotype receptor variant. It would have been interesting to determine the sequence of the *NPC1* present in the U2OS stuffer cell line and what polymorphisms it may have contained, relative to the wild-type *NPC1* sequence obtained from Ensembl (NM_000271.5), as well as the haplotype sequence.

If time had not been a limiting factor, several additional studies would have been undertaken to investigate the link between receptor and variant function. Firstly, RNA extractions of the transduced cell lines would have been completed. This would have allowed cDNA synthesis and the sequencing of both receptors to confirm their genotype. Further to this, co-localisation studies would have been completed to ensure that *NPC1* was being expressed correctly. Rab7 and Rab9 (GTPases associated with the early and late endosome, respectively) are often used as markers for *NPC1* and could have been employed in the visualization of receptor expression through confocal microscopy (Spence *et al.*, 2016; Podinovskaia *et al.*, 2021). The endogenous lysosome-associated membrane protein 2 (LAMP2) could also have been used to confirm that *NPC1* was correctly expressed in lysosomes (Li, Saha, *et al.*, 2016). Additionally, the expression data obtained via flow cytometry would have been confirmed through western Blot.

Throughout the iterations of flow cytometry, it was clear that there was a slight shift in fluorescence intensity, in the cells treated with the isotype control antibody, relative to those unstained, although by the final experiment, this effect had markedly reduced. rMFI was therefore calculated relative to the cells treated with isotype control antibody rather than the unstained cells. However, if time had permitted, the experiment would also have been repeated using further dilutions of the antibody to observe high-affinity interactions between the antibody and antigenic epitopes, thus reducing the non-specific binding. Alternatively, a new antibody would have been tested to determine if the cells treated with isotype control were providing comparatively similar results to the unstained cells, to confirm that the observed shift in fluorescence could be attributed to negligible unspecific binding.

Finally, the expression vectors utilised contained a CMV promoter. Because the CMV promoter is designed to over-express genes depending on the targeted cell line, subtle differences in the corresponding infectivity may not be observable with the system used here (Qin *et al.*, 2010; Kallunki *et al.*, 2019) Therefore, testing cloning vectors featuring a weaker promoter or an inducible system such as the Tet system may have allowed for a better appreciation of the differences in infectivity observed in cell lines expressing different receptor variants.

In conclusion, this work successfully identified and developed a methodology that permits investigation into NPC1 orthologues. It was used to determine the impact of the SNPs P434S and M642I, which are enriched in the genotypes of African populations on EBOV entry. Surprisingly, relative to the wild-type receptor, In combination, these SNPs showed increased permissibility of the receptor, to viral entry, contradicting the original hypothesis that the frequency of these SNPs within African populations may be due to a limited selective pressure that EBOV outbreaks provide, resulting in a low-level resistance to the virus. An alternative explanation for the presence of this haplotype NPC1 variant may be regarding the role of NPC1 a biologically essential cholesterol transfer protein, and thus any impact on EBOV entry is secondary. Constructs to test these mutations singularly were also made, however, time restrictions meant that these constructs were not used to produce stable cell lines. Assessing the impact of these mutations individually in NPC1 may provide further insight into their impact on EBOV entry.

6. Final Conclusions

Emerging viral diseases are a continuous threat to the global population and economy. The increasing interaction between humans and animal reservoirs that naturally harbour pathogenic viruses has amplified the frequency of zoonotic diseases in already susceptible areas (Salzer *et al.*, 2017). Additionally, the modern accessibility of international travel has transformed these localised episodes into worldwide challenges. The recent West African EBOV outbreak (2013-2016), the Zika virus epidemic (2016) and the ongoing SARS-CoV-2 pandemic are the most obvious evidence of the impact that such events can have on global health, welfare and economics. In each of these occasions, the scientific community has come together to rapidly develop therapeutic and preventative measures (Lelgowska *et al.*, 2016; Nicastri *et al.*, 2019; Tregoning *et al.*, 2021; Li *et al.*, 2022; Robinson *et al.*, 2022). However, to be effective, such interventions require a deep understanding of the molecular mechanisms that underpin the interaction between a virus and its host, particularly those involved in entry pathways. A deeper understanding of entry pathways is also crucial in determining the possible genetic drivers that may lead to spillover events and better adaptability of the virus to a specific host.

To this end, PVs provide an effective model to elucidate the entry mechanisms and receptor interactions of BSL-4 pathogens since their modular nature allows for the key molecular determinant of cell entry in an enveloped virus, the viral GP, to be expressed on a heterologous core protein (Temperton and Page, 2015; Urbanowicz *et al.*, 2016). For this reason, PV models have been used extensively in EBOV research, including in the present study. In our work, we used an MLV-PV system to provide more insight into the genetic variability of the EBOV GP and the human NPC1 receptor, as we hypothesised that different variants of both of these proteins could impact virus-host interaction, bearing effects on the infectivity and entry fitness of the virus.

As the focus of this investigation concerned the initial steps during viral infection, the use of viral pseudotypes provided a safe and efficient technique to probe the viral entry pathway (Takada *et al.*, 1997; Temperton *et al.*, 2007; King *et al.*, 2016; King and Tarr, 2017). One challenge that was faced throughout the project was the batch variability between PV preparations leading to a substantial range in raw RLU values between

different preparations of the same PV. Typically, this was due to either the quality of the transfection reagent, which is reduced with long-term storage or the passage number of both the producer cells and the cells to be infected. To combat this, efforts were made to consistently use fresh reagents and strictly maintain cell lines under optimal conditions for no more than 50 passages. Regardless, the CV of triplicate data that was then pooled from independent experiments was typically in excess of 10.0%, indicating limitations in the precision observed between independent experiments. However, normalisation of the infectivity data to that of a chosen EBOV variant limited the impact of the ranges in infectivity displayed by the same variant between experiments, without effecting the distribution of the data or patterns of infectivity.

As described in Chapter 3, the first step was the generation of a panel of pcDNA3.1 constructs carrying EBOV GP sequences representative of all outbreaks between 1976 and 2016. This novel panel was used together with a set of pre-existing constructs containing ancestral EBOV GP sequences (previously inferred through evolutionary modelling by Professor Edward Holmes, University of Sydney) to produce the corresponding PVs. When tested in a HuH-7 cell line, the PV panel displayed an appreciable range of infectivity, confirming the initial hypothesis that variability within the EBOV GP sequence correlates to variability in the infectivity of the virus. In particular, it became apparent that the Mbandza 2003 GP conferred the lowest infectivity across the panel, relative to the wild-type Mayinga 1976 GP. This observation was investigated in further tests using Vero E6 and Hyp/Lu 45.1 cells, which confirmed that the behaviour was not species-specific. After ensuring that this pattern was not a result of mutations in the pcDNA3.1 construct, the cause of the low infectivity was identified to be the result of an amino acid substitution within the internal fusion loop of the Mbandza 2003 GP (I527T).

However, as all the observations discussed above were based on data obtained *in-vitro*, it should be noted that an important limitation of this study is that it cannot determine if the GP variants tested would also have an impact on factors such as pathogenesis and the interaction between the virus and the host immune system, all important contributors to overall viral fitness in an outbreak setting. To elucidate such aspects would require *in-vivo* experiments on animal models (Prescott and Feldmann, 2016; Piorkowski *et al.*, 2017; Marzi *et al.*, 2018). Nevertheless, the present findings highlight the importance of continued investigations around the genetic and molecular variations that exist within a viral species and what these differences may mean (Kugelman *et al.*, 2015).

An example may be that the variation seen in EBOV GPs could also impact factors such as the efficacy of current therapies and thus, investigations could provide precious insight for the improvement of current vaccines and therapeutics, as well as the design of new ones. Assessing the impact of GP variants on the efficacy of current therapies that target the GP was in fact, one of the original aims of this project. To this end, a series of neutralisation assays were planned. Unfortunately, due to time restrictions, these experiments could not be completed and would therefore constitute important future work. For such assays, target PVs would be incubated with multiple dilutions of the antibody KZ52, a key component of antibody cocktails used therapeutically, which is thought to bind to the base of the EBOV GP (Davidson *et al.*, 2015). PV-antibody dilutions would then be applied to HuH-7 cells in triplicate and the difference in luminescence between the KZ52-treated PVs and uninhibited controls would be used to infer neutralisation patterns in order to determine if the efficacy of KZ52 is comparable between the outbreak strains tested (Urbanowicz *et al.*, 2016). The data obtained could provide insight into how effective the currently available therapies may be against strains originating from different outbreaks, which in turn may help to inform future outbreak management, especially if new variants were found to possess sequence similarity to past strains.

The work presented in Chapter 4 focused on comparing the infectivity conferred by ancestral and outbreak GP sequences in both the HuH-7 and Hyp/Lu 45.1 cell lines, the latter representing a potential reservoir species for EBOV. The results showed that both groups of GPs conferred variable degrees of infectivity in Hyp/Lu 45.1 cells, as was previously observed in HuH-7 cells in Chapter 3, with infectivity patterns that were consistent between both cell lines. Notably, in both cell lines, the pattern observed included the ancestral GPs conferring higher infectivity than the outbreak sequences, with the only exception being the Etoumbi 2005 sequence, which displayed infectivity comparable to that observed for the N21 sequence that it is thought to have evolved from.

Interestingly, the GP sequence for Etoumbi 2005 (an outbreak that occurred in close proximity to that of Mbandza 2003) evolved from N21 through the mutation of four residues (S319G, A349T, H455Y and F676L), two of which (A349T and H455Y) were among those included in the Mbandza 2003 sequence analysed in Chapter 3. Subsequent investigations would involve considering a similar approach to that taken for the Mbandza 2003 GP to identify the mutation(s) responsible for the contrasting behaviour of the Etoumbi 2005 GP in HuH-7 and Hyp/Lu 45.1 cells, which can be attributed to the S319G and/or F676L residues.

These results led to the conclusion that the mutations that evolved in the outbreak sequences (Gabon 1994, Gabon 1996, Gabon 2001, Mbandza 2003, Kelle 2003 and Etoumbi 2005) resulted in a phenotype generally characterised by lower viral infectivity relative to that of the ancestral sequences. These observations were unexpected and contradicted the initial hypothesis that the ancestral GP sequences (representative of the virus in its reservoir) would be better adapted to the Hyp/Lu 45.1 cells (generated from a predicted reservoir species), whereas the outbreak sequences would be better adapted to the HuH-7 cell line.

The most direct explanation for the observed trend is that the mutations that have led to the evolution of outbreak-causing EBOV strains in nature have also caused the virus to lose entry fitness. It is also possible that *H. monstrosus* may not be a long-established reservoir for EBOV since both ancestral and outbreak sequences behave similarly in both HuH-7 and Hyp/Lu 45.1 cell lines and that the virus may have been introduced into this bat population only recently by one or more other reservoir species, although this would require further investigation. Additionally, the impact of other selective pressures that may effect the GP entry, such as the presence of cell line-specific restriction factors, may account for the differences in infectivity observed between the panel of GPs tested (Wrensch *et al.*, 2015).

The aims of Chapter 4 draw some comparisons to a study completed by Hofmann-Winkler *et al.*, (2015), which attempted to compare the cell entry of Mayinga 1976 to that of a Makona variant taken from 2014 (Accession number: KM233105), in different cell lines including multiple cell lines of human, primate and bat origin. Using a VLP system, they found that both GPs transduce the same spectrum of cells and are susceptible to blockade by the same IFN-induced proteins and antibodies. The study showed that cell entry efficiency between both GPs in HuH-7 cells was comparable and that appreciable differences were not observed in Hyp/Lu 45.1 cells, a contrast to the findings presented in Chapter 4, which suggested that the Makona C15 variant is less infectious than Mayinga 1976 in Hyp/Lu 45.1 cells. This difference may be explained by the fact that the Makona variant used by Hofmann-Winkler *et al.*, (2015), was circulating later in the West African outbreak compared to Makona C15.

However, to expand the work presented in Chapter 4 to further elucidate the relationship between EBOV and its natural reservoir(s), the ancestral and outbreak GP variants could be tested on multiple cell lines of bat, primate and human origins, similarly to Hofmann-Winkler *et al.*, (2015). These could include Vero E6 cells (representative of an intermediate

host) and cell lines derived from other hypothesised reservoir bat species, such as the remaining species described by Leroy *et al.*, (2005).

Importantly, as the evolutionary relationship between ancestral and outbreak GP sequences was determined as part of this work, the implementation of molecular clock dating could be considered to measure the rapidity of EBOV evolution both within outbreaks (where extensive sampling was not completed) and between them (while the virus was circulating in the reservoir) (de La Vega *et al.*, 2015; Holmes *et al.*, 2016). This approach would also allow estimations of when the ancestral sequences may have diverged, thus providing more clues as to what the reservoir species may be and when spillover events may have occurred.

Lastly, in Chapter 5, this study shifted its focus to the NPC1 endosomal receptor, the binding partner of EBOV GP during viral cell entry and membrane fusion. It investigated whether host species variations in this receptor may impact viral infectivity. It was hypothesised that SNPs present in NPC1 may be enriched in populations that are more frequently exposed to EBOV outbreaks and that these SNPs may provide a low-level resistance to viral infections. To verify this, an NPC1-knockout U2OS cell line that was developed by a collaborator was transduced to stably express mutated NPC1, carrying amino acid substitutions corresponding to a haplotype only found in African populations (P434S and M642I). The permissibility of this receptor to EBOV was compared to the wild-type receptor. Surprisingly, susceptibility testing of the transfected cells to EBOV infection revealed that the NPC1 haplotype variant was more permissive to EBOV infection compared to the wild-type receptor. This finding called for further examination of the two NPC1 mutations individually but unfortunately, this was not possible due to time restrictions and therefore, it would constitute important future work.

Additional future experiments would include investigations into NPC1 orthologues from different species to identify important residues that contribute to viral tropism. Plasmid constructs that contained NPC1 from *E. beuttikoferi* and *H. monstrosus* (which only differ by their L405 and M405 residue, respectively) were generated as part of this work. This was to assess whether a single variation in the NPC1 of potential EBOV reservoirs may have an impact on viral entry and susceptibility to infection and thus, provide more evidence towards determining a true reservoir species. Furthermore, a primate-specific mutation (405T) was also inserted into the otherwise bat-like receptor to determine whether the change would confer a difference in phenotype. Unfortunately, due to time restrictions, the production of transduced cell lines expressing these NPC1 orthologues

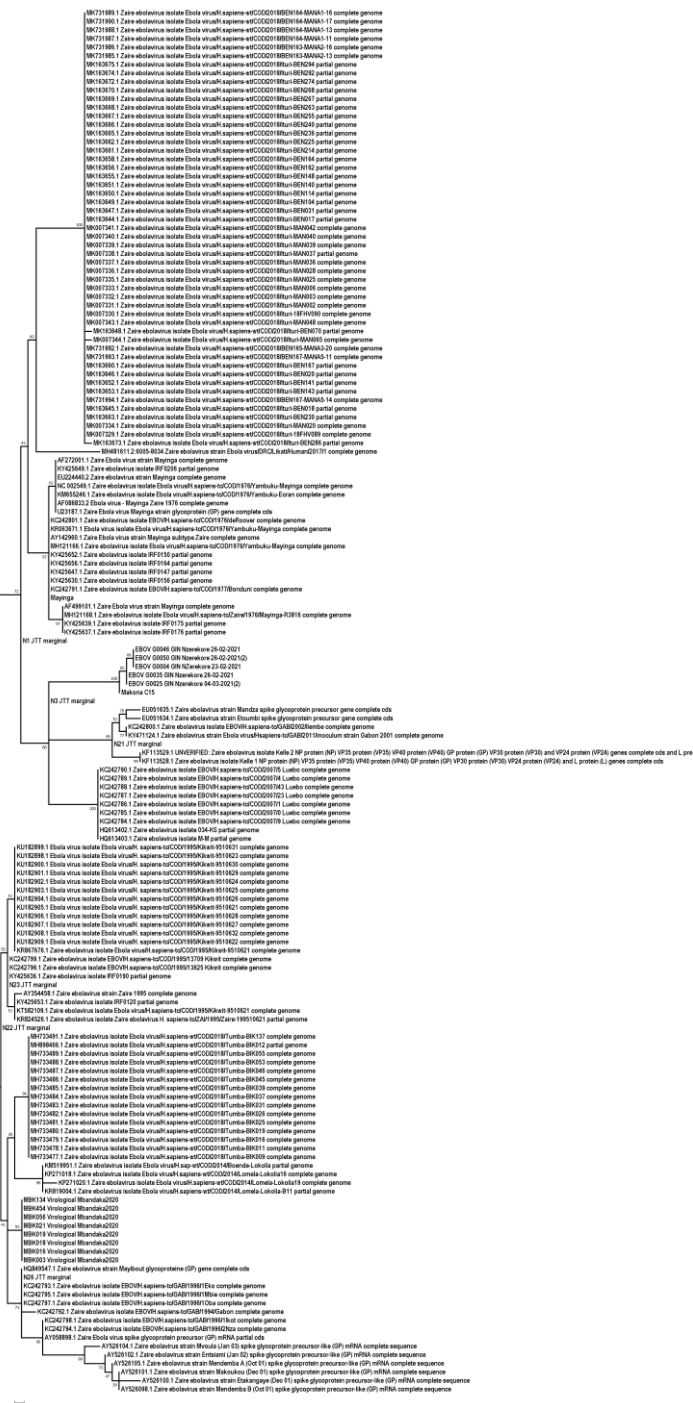
and the experiments required to determine any resulting differences in viral infectivity could not be completed. This line of work, however, remains a priority for the future of this study and could be used to identify key determinants of viral tropism in different species. Regardless, the broad applications of this model demonstrate its utility in the field of characterising viral and receptor interactions within and between different species.

Additionally, the scope of this work could be expanded further, by completing studies utilising fully replicating EBOV, to determine if the variation that is observed in human NPC1, results in a biological difference in relation to the virus's infectivity, particularly as replication is an important factor contributing to tropism. One method to complete this might be through the delivery of the human *NPC1* variants to *NPC1*-null mice, through viral gene therapy, for example through an AAV vector (Naldini, 1998). Observations in the mice regarding pathogenicity and disease progression, post-challenge, might provide insight into whether the virus responds differently to different NPC1 variants. However, in such experiments, it would be challenging to elucidate which aspect of the viral life cycle and how it is interacting with the host, and which other viral proteins aside from the GP, may be contributing to any observed differences. Furthermore, extraction of viral mRNA from sites of replication could allow determination as to whether the virus sequence further mutates and adapts to receptor usage (Watson *et al.*, 2021).

In conclusion, this project successfully used the flexible nature of a PV model to develop the first panel of EBOV GP variants to encompass the range of genetic variation that the EBOV GP displayed in outbreaks up until 2016. It also successfully developed a model utilising an NPC1 receptor knockout cell line in which different receptor variants could be transduced to permit the characterisation of NPC1 receptor variation both within a species and between species, as well as to determine the impact this has on viral infectivity. Together, these models form an invaluable tool for investigating virus and host receptor interactions that could be applied in the investigation of other enveloped viruses and their entry pathways. Importantly, these models could be used to inform animal studies, which remain a contentious issue, particularly in the field of EBOV research.

7. Appendix

i) Phylogenetic Analysis of All Sequences



ii) Infectivity of GPs Normalised to NPC1 Receptor Expression

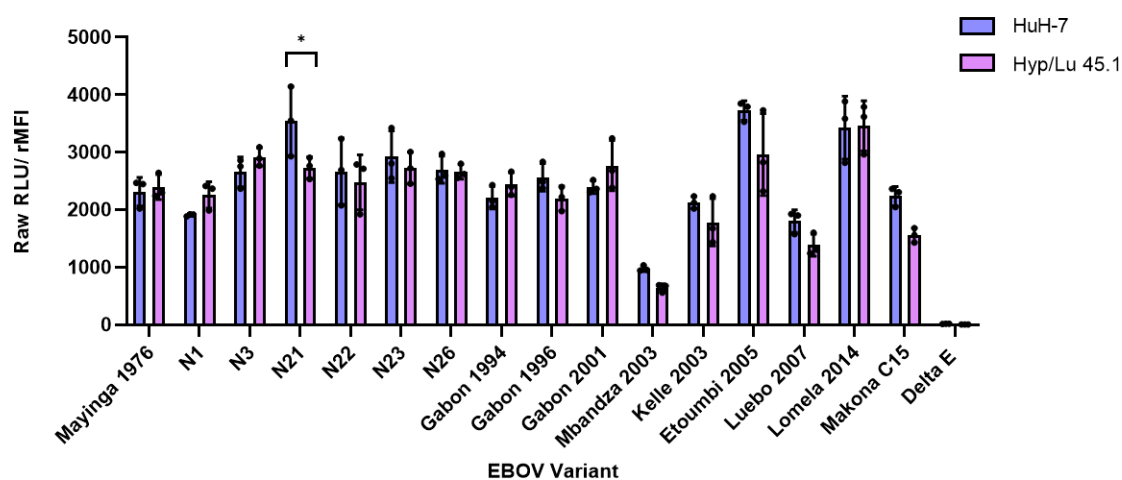


Figure 7.1 Infectivity of GPs normalised to NPC1 receptor expression

Raw Infectivity data from the final independent repeat that was used to obtain Figure 4.4 was normalised to the rMFI values obtained in the second experimental repeat shown in Figure 4.3. Graph shows mean values $\pm$ SD. Differences in the infectivity of PVs between HuH-7 and Hyp/Lu 45.1 cells were assessed using a two-way ANOVA with Sidak's multiple comparison test; $*p < 0.05$; values that were not significant ($p > 0.05$) have not been highlighted in the figure.

iii) Internship Reflective Statement

During my PhD, I completed a placement at the University of Nottingham Asymptomatic Testing Service, which provided asymptomatic testing for the SARS-CoV-2 virus to staff and students on campus. This ensured that all university campuses remained a safe environment throughout the height of the pandemic, by working towards limiting transmission chains within the university population.

The pandemic restricted many options that I was previously considering for placements as much of what was available at the time was distant placements. Therefore, I felt fortunate to be able to take this opportunity to gain some hands-on experience whilst working as part of a larger team in a laboratory environment who were tasked with meeting daily targets in delivering an essential service. This was a new experience that I had to learn to adapt to after years of managing my workload for my PhD project and working to my own timescales and deadlines.

As part of the role, I gained experience working in a CL2+ laboratory environment, where I was trained to process saliva samples collected from asymptomatic individuals across all campuses and accommodations at the university, ensure all samples were inputted correctly into a database, pool samples for COVID-19 testing, prepare samples and reagents and test them via qPCR and finally manage the storage of samples. Once samples were received, either a positive or negative result was expected to be confirmed within 24 hours using CDC-approved and UKAS-accredited techniques. This involved maintaining a consistent workflow, particularly during very busy periods when we were processing over 1000 samples a day. I became well versed in qPCR techniques, which were used to detect the *N1* and *E* genes present in SARS-CoV-2 and the *RNase P* control to detect human saliva. I learnt how to analyse this data using the Bio-Rad CFX Maestro software, which proved beneficial in experimental work completed during my PhD project.

Overall, I was grateful to work as part of a good team of motivated individuals and supportive management staff. The work completed as part of the service was published as three papers, two of which I was listed as an author. This will prove beneficial in future endeavours both in and outside of an academic environment. Mostly, I was appreciative of the chance to apply the knowledge and skills that I had gained in the field of molecular virology throughout my PhD, to aid in the pandemic response. This is something that I will continue to be proud of in years to come.

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