

Review Article

Descriptive analysis of Ebolavirus proteins: towards development of effective therapies

Abstract

Ebolavirus (EBOV) is one of the deadliest human pathogens with case fatality rates of upto 90%. Ebolaviruses have seven structural proteins (Nucleoprotein, glycoprotein (GP), RNA polymerase L, VP24,VP30, VP35 and V40) and three non-structural protein (sGP, ssGP, and Δ-peptide). Functions of non-structural proteins are unknown but sGP is thought to act as a decoy for antibodies. Polymerase L,VP30,VP35, and Nucleoprotein form the ribonucleocapsid complex which is responsible for transcription and viral replication. VP40 represents about 40% of the molecular weight of EBOVs and plays an essential role in viral replication, assembly and budding.GP is a type II transmembrane envelope protein which is responsible for receptor binding and viral entry. It is a crucial component of vaccines and a target for neutralising antibodies. All EBOV proteins play varying roles in suppressing host immune responses. However, VP35 and VP24, are the major inhibitors of the host interferon signalling pathways and are capable of blocking maturation of dendritic cells. Currently, overexpression of genes that encode EBOV proteins is the primary approach to vaccine development. A number of potential virus vector and virus-like particle based Ebola vaccines are currently under research. Furthermore, synthetic antisense drugs such as phosphorodiamidate morpholino oligomers that inhibit gene expression of VP24, RNA polymerase L, and VP35 are currently under clinical trials. Together with siRNAs, they offer the best options for treating Ebola. However, there is still concern about the safety of these therapies and issues of pre-existing immunity against virus vector based vaccines still exist. Additionally, most of these vaccines and Antibody (Ab) concoctions only target GP, which unlike VP24 and VP35, is not the main protein that controls host IFN mediated immune responses. Thus, immunity stimulated by these GP specific vaccines may not be sufficient to control Ebolaviruses. Studies have shown that EBOV IgG positive sera reacts strongly to VP40 than to GP or NP. Other studies have also demonstrated that Abs from Ebola survivors have a preference for sGP than GP. Thus, vaccines or antibody concoctions must be designed to induce potent immunity against all major Ebola proteins if they are to be effective. Interferons play critical roles in stimulating antiviral immunity. Ebolaviruses use VP24 and VP35 to suppress IFN signalling pathways. Therefore, the use of exogenous interferons in combination with other therapies may offset the impact of VP24 and VP35, stimulate maturation of dendritic cells which are blocked by VP35 and promote interferon mediated immune responses. Tetherin, an interferon induced protein, may be important in the control of Ebola although its activities are blocked by EBOV GP. Hence, exogenous Tetherin may help to undermine the effects of GP on tetherin. In this article, we review the structure of Ebolavirus proteins, their mechanism of actions and regions within these proteins and mechanistic pathways that can be targeted by potential anti-Ebola therapies. We also review the weaknesses and strengths of Ebola therapies that are currently under research and suggest use of combinational treatment options. The mechanisms devised by bats to fight Ebola are also discussed.

44 **Key Words:** Ebolavirus proteins, Interferons, Tetherin, Phosphorodiamidate
45 **morpholino oligomers, siRNAs, Antibodies, vaccines.**

46

47 **Introduction**

48 The current outbreak of Ebola virus disease (EVD) in West Africa has led to a surge in public
49 interest and concern regarding the virus.¹ Humans initially contract the virus either through
50 contact with infected reservoir, which is currently thought to be fruit bats, or by contact with
51 infected wildlife, particularly Chimpanzees.² The outbreak is then driven by human to human
52 transmission through infected body fluids. Ebolaviruses, classified as biosafety level 4
53 viruses, have caused disease outbreaks in the equatorial regions of central Africa and Uganda
54 since the first simultaneous outbreaks in Zaire (now the Democratic Republic of the Congo)
55 and Sudan in 1976.³⁻¹⁴ However, the recent emergence of Zaire Ebolavirus in West Africa,
56 resulting in what is the largest outbreak to date shows that these viruses are more widely
57 distributed than previously thought.^{4,9,13} The Ebolavirus subtype currently causing outbreaks
58 in West Africa (Guinea, Liberia and Sierra Leone) is 97% homologous with the Zaire Ebola
59 virus.⁸ There are no licensed drugs and vaccines for the treatment of Ebola.^{5,6} The
60 epidemiology and ecology of Ebolaviruses are poorly understood but Rodents and fruitbats
61 (*Epomops franqueti*, *Hypsignathus monstrosus*, and *Myonycteris torquata*) have been
62 recognised as potential reservoir species and humans are end hosts.¹⁵⁻²⁶ Since the West
63 African Ebolavirus strain is an outlier within EBOVs, it is probably not an introduction of a
64 central African strain into that region but may have been present in bat populations
65 previously without infecting humans.²⁴ Ebolavirus disease is characterised by initial non-
66 specific symptoms such as sudden onset of fever, chills, and malaise, accompanied by other
67 signs such as myalgia, headache, nausea, abdominal pain, vomiting, and diarrhoea.^{1,27} The
68 incubation period varies from 3-21 days.²⁷ Approximately 30-50% of Ebola Zaire patients
69 develop a purplish-red maculopapular rash and more characteristic haemorrhagic symptoms
70 such as melena, haematochezia, epistaxis, haematemesis, and bleeding from injection sites
71 appear later in the course of the disease.^{2,8,21,27}
72 Persistence vomiting and development of multi-organ dysfunction leads to irreversible shock
73 and eventually death.^{6, 8, 27} Natural recovery from Ebola virus disease is associated with the
74 appearance of antibodies specific for Ebolavirus proteins.^{2,8}

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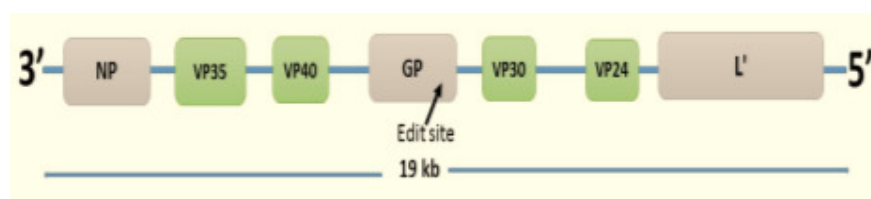
76 Much of the pathology seen in Ebolavirus disease is associated with the immunosuppressive
77 nature of Ebolavirus proteins. Ebolaviruses have seven structural proteins (Nucleoprotein,
78 glycoprotein (GP), RNA polymerase L, VP24, VP30, VP35 and VP40) and three non-
79 structural proteins (sGP, ssGP and Δ-peptide). This review attempts to synthesise data from
80 all of the published sources into a coherent explanation of the current knowledge about the
81 structure and mechanism(s) of action of Ebolavirus proteins. It will also review the
82 weaknesses and strengths of current potential anti-Ebola drugs/Ab concoctions and vaccines
83 that are aimed at these proteins, for treatment and stimulation of potent anti-viral immunity,
84 respectively. Suggestions of how to improve the efficacy of these therapies are also clearly
85 outlined. Furthermore, the article discusses the use of combinational therapy approaches to
86 treat Ebola in the light of the potential failure of some of these drugs and vaccines to control

the disease. Critical analysis is also extended to the potential role of IFNs and tetherin in the treatment of Ebola.

Classification and structure of Ebola viruses

Ebolavirus (EBOV) is a filamentous and pleomorphic enveloped negative sense RNA virus. Together with Cuevaviruses and Marburgviruses (MARV), Ebolaviruses belong to the family *filoviridae* and the order *Mononegavirales*.^{2,17} The family name is derived from a Latin word, *filum*, which means ‘thread’ reflecting the Ebola virus’s unique filamentous morphology.² There are five Ebolavirus subtypes: Zaire Ebolavirus (ZEBOV), Sudan Ebolavirus (SEBOV), Reston Ebolavirus (REBOV), Tai Forest Ebolavirus (TAFV), and Bundibugyo Ebolavirus (BEBOV).²⁸ All Ebolavirus subtypes are pathogenic for humans except REBOV, which is pathogenic for non-human primates.²⁸ Ebolavirus has a single non-segmented 19kb RNA genome (**Fig. 1**).¹⁰ Ebolavirus particles have a varying length of 800-1000nm but can reach upto 14,000nm long with uniform diameter of 80 nm and are covered in a lipid bilayer^{10, 29-30} The central core of each Ebolavirus is made up of a single RNA genome and its adherent nucleocapsid (composed of nucleoproteins NP and VP30).² They are linked by matrix proteins VP24 and VP40 to the inner surface of the lipid bilayer of the viral envelope, which is derived from the host cell.^{2, 10} Trimeric spikes of the surface envelope glycoprotein are anchored in the bilayer. Two other proteins, VP35 and large protein (RNA-dependent RNA polymerase L), are also present within the virion.^{2, 10} The genomic ends of the Ebola virus known as ‘trailer’ (5’end) and leader (3’end) are non-transcribed and possess cis-acting signals vital for transcription, replication and encapsidation of the virus.² Transcription begins at the 3’ end and the Ebola viral genome encodes 7 structural proteins: Nucleoprotein (NP), virion proteins [phosphoprotein (VP35), matrix proteins (VP40 and VP24), replication transcription protein (VP30), glycoprotein (GP), and the RNA polymerase protein (L)] and one major non-structural protein known as soluble glycoprotein (sGP) produced from an edited GP mRNA.^{2,10} Two other non-structural proteins (ssGP and a peptide) are secreted after manipulation of sGP. The seven genes that encode these proteins are arranged in a linear order 3-NP-VP35-VP40-GP-VP30-VP24-L-5 on the Ebola virus genome.^{6, 31} All the Ebola viral genes except the glycoprotein gene are monocistronic, they encode for one structural protein.¹⁰ The linear arrangement of the seven Ebola viral genes and the replication mechanisms or strategy in infected cells resemble those of the rhabdoviruses and paramyxoviruses.²

A.



B.

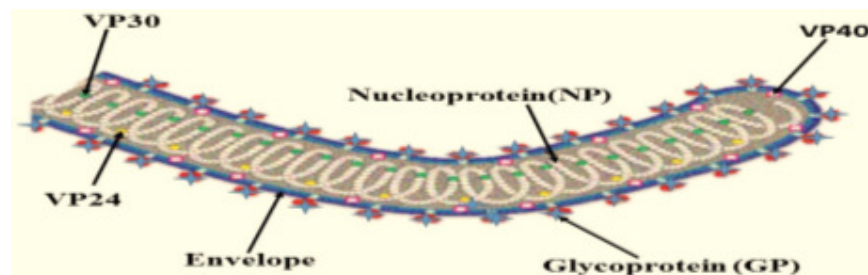


Figure 1: Structure of the single stranded negative sense Ebolavirus (EBOV) RNA genome. (A) Arrangement of Ebolavirus genes; GP=glycoprotein; NP=nucleoprotein; VP=viral protein; L=large protein (RNA-dependent RNA polymerase). **(B)** Configuration of the EBOV virion. The central core is composed of the RNA genomic molecule and its adherent nucleoproteins, NP and VP30. They are linked by VP24 and VP40 to the inner surface of the lipid bilayer of the viral envelop. Glycoprotein forms trimeric spike that are anchored in the lipid bilayer. VP35 and L-protein although not depicted are also present within the virion

Nucleoprotein and RNA polymerase L protein

The EBOV nucleoprotein (NP) is a principal constituent of the ribonucleocapsid complex together with the RNA-dependent RNA polymerase (L) and other viral proteins.³² The ribonucleocapsid complex encloses the viral RNA and is phosphorylated.³³⁻³⁴ The complex is responsible for viral transcription and replication.³⁵⁻³⁶ The NP of EBOV is the largest nucleoprotein of all non-segmented negative-stranded RNA viruses with 739 amino acid residues.³⁶ It has a hydrophobic N-terminal half composed of approximately 350 amino acids and a hydrophilic C-terminal half (≈ 400 amino acids).^{33, 37} The C-terminal half is responsible for interaction with matrix protein VP40 and the incorporation of NP into virions.³⁸⁻⁴⁰ On the other hand, the N-terminal half is believed to be essential for RNA binding through interaction with the phosphodiester backbone that is free of the nucleotide sequences, similar to paramyxoviruses and rhabdoviruses.³⁶ A study by Huang et al, 2002, demonstrated that EBOV NP is O-glycosylated and sialylated. They also showed that these modifications are necessary for NP's interaction with VP35 to support viral genome replication.⁴¹ However, the type of O-glycosylated and sialylation has not been confirmed.⁴² Huang et al, 2002, used transmission electron microscopy to confirm that co-expression of Ebolavirus NP, VP35, and VP24 results in the formation of nucleocapsid-like structures (≈ 50 nm in diameter).⁴¹ These findings were confirmed in experimental studies by Noda et al, 2006, who demonstrated that EBOV NP self-assembles (NP-NP interaction) to form helical tubes of approximately 20 nm in diameter whose morphological characteristics are different from authentic nucleocapsids.^{38, 43} These helical tubes presumably act as the core of nucleocapsids. The NP region from amino acids 1 to 450 was determined to be responsible for NP's self-assembly.³⁶ In addition, these amino-terminal 450 residues and the following 150 amino acid residues are essential for the formation of nucleocapsid-like structures and for viral genome replication.³⁶ NP's interaction with VP40 was found to be critical for the transportation of nucleocapsids to the host cell surface and for their incorporation into virions resulting in the formation of mature virus particles.⁴³

Glycoprotein

Ebolaviruses express a type I transmembrane viral envelope glycoprotein (GP) which is heavily glycosylated with N-linked and O-linked glycans accounting for nearly half of its molecular weight.^{17, 44} GP is a structural glycoprotein which is expressed by two open reading frames (ORFs) through transcriptional RNA editing (**Fig. 2**). The Ebola virus glycoprotein forms 7 nm spikes on the surface of virions at intervals of 5-10 nm.^{40, 45} This protein is responsible for receptor binding, viral entry into target cells, and is a principal immunogenic target.⁴⁵ EBOV exhibits a wider cell tropism but the cellular receptors used by GP for viral entry are unknown.⁴⁶ GP also augments budding of virus like particles which is driven by the viral matrix protein, VP40.^{39-40, 47-48} Maturation of EBOV GP involves posttranslational cleavage of a precursor (GP0) in the Golgi by a subtilisin-like endoprotease furin to yield two fragments, GP1 and GP2, which are covalently linked via a disulphide bond between the membrane anchored GP2 and the N-terminus of the extracellular GP1 subunit.^{17, 44-45, 49-50} The mature structural GP is a trimer of approximately 450 kDa made up of heterodimers between GP1 (~130 kDa) linked to GP2 (~24 kDa) by disulfide bonding.⁵¹ The proteins are expressed on the surface of infected cells and virions.⁵² Further studies using reverse genetics, which allows the artificial generation of Ebola virus from cloned cDNA, have also demonstrated that mutant ZEBOV in which the multi-basic sequence motif for furin cleavage (RRTRR at amino acids 497 to 501 of GP) is replaced with non-basic amino acids (AGTAA) prevents the generation of GP1 and GP2, but the mutant virus is able to grow in cell culture to titres similar to those of wild-type virus.⁵³⁻⁵⁴ In contrast, the glycoprotein of Reston Ebolavirus, the species that is non-pathogenic for humans, lacks an optimal furin cleavage site (contains lysine, but not arginine, at position 4).^{17, 44} This has led to the suggestion that Furin cleavage is a vital determinant of Ebola virus virulence. However, Neumann et al, found no appreciable differences in disease progression, serum biochemistry, haematology, , virus titers, or lethality in nonhuman primates infected with an Ebola virus mutant lacking the furin recognition sequence compared to those infected with wild-type virus.⁵⁰ Moreover, other studies of pseudotyped viruses expressing Ebola virus glycoprotein with an altered furin recognition sequence showed that GP cleavage by furin is expendable for in vitro infectivity of the pseudotyped viruses indicating that furin-mediated cleavage of GP is not essential for Ebola virus replication in cell culture.^{47, 55-56} Additionally, GP has also been recognised as a key determinant of viral pathogenesis and virus-like particles (VLPs) containing GP are able to activate human endothelial cells and macrophages.⁵⁷⁻⁵⁸ GP interacts with VP40 and NP to form viral particles.⁵⁹

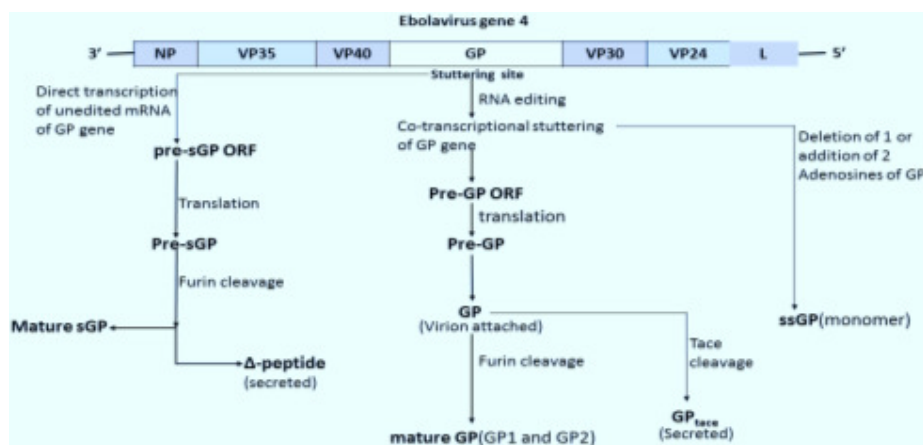


Figure 2. Transcription and processing of EBOV glycoproteins. Direct transcription of the unedited mRNA of EBOV gene leads to mRNA encoding a secreted non-structural soluble glycoprotein (sGP). Proteolytic processing of sGP at its C-terminus by furin yields a small secreted protein termed Δ -peptide and a mature sGP. GP is a structural glycoprotein which is expressed by two open reading frames (ORFs) through co-transcriptional RNA editing. Proteolytic processing of GP by furin yields two, disulphide linked proteins, GP1 and GP2. TNF- α -converting enzyme (tace) cleaves GP resulting in the production of GP_{tace}. Deletion of 1 or addition of 2 adenosines of GP during transcriptional stuttering yields a small secret glycoprotein (ssGP).

Of the two highly glycosylated trimer of GP1/GP2 heterodimers on the surface of Ebola virus virions, the GP1 subunit is required for interaction with receptors whereas the transmembrane associated GP2 is required for host cell membrane fusion.²⁸ GP1 subunit contains four different domains: serine-threonine rich mucin-like domain (MLD), base, glycan cap, and receptor-binding domain (RBD).²⁸ The base, glycan cap, and RBD domains make up the core of GP1 and are required for the expression and function of the prefusion glycoprotein.²⁸ The mucin domain encompasses approximately 150 amino acids and contains 4 N- and 13 O-linked glycosylation sites, and is a distinctive feature of filoviral glycoproteins.^{49, 60} The MLD appears to be essential for efficient interaction of GP with cell-surface C-type lectins but deletion of this domain does not adversely affect the infectivity of pseudotyped virions in vitro.⁶¹⁻⁶² The observed EBOV GP-mediated effects upon surface protein expression and cellular adhesion is sufficiently due to the MLD of GP1.⁶²⁻⁶³ Ebola virus GP lacking the MLD (GP1-muc) has increased protein expression and provides higher viral titres when pseudotyped onto retroviral or vesicular stomatitis virus (VSV) particle.⁶¹ The GP1 base subdomain has four interrupted sections (amino acid residues 33–69, 95–104, 158–167 and 176–189).⁶⁰ It forms a semi-circular hydrophobic surface that interacts with the internal fusion loop and heptad repeat region of GP2. GP1 base presumably forms a clamp that stabilises the GP2 prefusion conformation to prevent it from springing prematurely.⁶⁰ Additionally, within the GP1 β 2– β 3 loop, proximal to the viral membrane end of the GP1 base resides Cys53, which forms a disulfide bond to Cys609 of the GP2 subunit.⁶⁰ In contrast to GP1 base subdomain, the GP1 glycan cap consists of a non-interrupted polypeptide chain (from residue 227–310) that forms an α/β -dome/roof over the GP1 head subdomain.⁶⁰ Four predicted N-linked oligosaccharides are clustered within the GP1 glycan. Additionally, GP1 glycan cap subdomain is completely exposed on the outer surface of GP Δ muc Δ tm but is not involved in any contacts at the trimer interface.⁶⁰ A study

by Ou et al, 2010 found that two phenylalanines located at positions 88 and 159 of GP1 are critical for EBOV entry into cells.⁶⁴ Based on the GP crystal structure, F88 is partially exposed on the GP1 surface next to the putative receptor binding site and the GP2 internal fusion loop, while F159 is deeply buried within a hydrophobic pocket.⁶⁴⁻⁶⁵ Both of these residues are surrounded by a group of hydrophobic amino acids. The conserved GP1 receptor binding domain (RBD; also known as GP1 head subdomain) is located between the base and glycan cap subdomains.⁶⁴ It is made up of residues from four interrupted sections (residues 70–94, 105–157, 168–175 and 214–226).⁶⁴ RBD is surrounded by a heavy shield of glycans and disruption of this glycan shield via targeted mutagenesis results in an increase in cell entry, antiserum/antibody sensitivity, and protease sensitivity but is insufficient to allow virion binding to the intracellular receptor, Niemann-Pick C1 (NPC1).²⁸ Therefore, these studies provide evidence that Ebola viruses and other filoviruses maintain the heavy glycoprotein glycosylation to protect against antibody neutralization and proteases at the expense of efficient viral entry into host cells.²⁸ A significant amount of GP1 is also shed in a soluble form from the surface of infected cells after release from the GP2 subunit and may contribute to the complex pathogenesis of Ebola virus.⁶⁶ GP1,2 is a target for neutralizing antibodies, which have been shown to be an important element of immunity to EBOV and vaccination with GP1,2 offers protection from lethal EBOV challenge in rodent models.⁴⁴

The transmembrane associated GP2 subunit is responsible for fusion of the viral and host cell membranes. It contains a hydrophobic internal fusion loop (residues 511–556) and two heptad repeats (HR1 and HR2) which interact with the base subdomain of GP1.⁶⁰ Additionally, GP2 also contains a membrane-proximal external region, CX6CC disulfide bond motif, and a transmembrane anchor.⁶⁷ The internal fusion loop has a series of hydrophobic amino acid residues (Leu529, Trp531, Ile532, Pro533, Tyr534, Phe535 and Pro537) that are bounded by an antiparallel, disulfide-linked β -scaffold.⁶⁰ In the prefusion state, the internal fusion loop of GP2 wraps around the outside of the GP trimer. EBOV GP is a class I glycoprotein but the structure of its internal fusion loop is different from other class I viral GPs such as the haemagglutinin (HA) of influenza viruses and 5 F of Parainfluenza viruses.⁶⁸⁻⁶⁹ The internal fusion loop sequences of influenza virus and Parainfluenza virus GPs form helices and reside at the free N-terminus of GP2 in contrast to that of EBOV which resembles the β -sheet structured internal fusion loops of class II and III GPs, such as flavivirus E and vesicular stomatitis virus protein G. The heptad repeat of GP2 is known to interact with the semi-circular hydrophobic surface of GP1.⁷⁰⁻⁷⁴ However, only the first heptad repeat is ordered in the crystal structure of ZEBOV GP. This repeat region is divided into four segments: HR1A, HR1B, HR1C and HR1D. HR1A and HR1B form an α -helix with a 40° bend at Thr565 and within this tight bend occurs an unusual 3–4–4–3 stutter rather than the normal 3–4 periodicity of heptad repeats.⁶⁰ It has been suggested that this stutter may serve as a conformational switch.⁷⁵

EBOV, like many other viruses, affects the expression and/or function of several cell surface proteins, including adhesion factors and protein complexes that allow the immune system to recognise infected cells.¹⁷ Expression of high levels of main Ebola viral GP in cultured cells causes cell detachment leading to loss of cell-cell contacts, and cell rounding as well as loss

of attachment to the culture substrate.^{62, 76-77} This has been observed in a variety of cell lines and primary cell types.⁷⁷ However, transient GP expression does not cause death in human embryonic kidney 293T cells, but does so in primary human cardiac microvascular endothelial cells.⁷⁷⁻⁷⁸ Using flow cytometry, researchers have also found that cells expressing GP display lowered levels of a variety of surface proteins, including several members of the integrin family and Major histocompatibility complex class I (MHC1) although the exact complement of surface proteins affected by GP differs by cell type.⁷⁶⁻⁷⁷ In addition, infection of 293T cells with EBOV was observed to cause similar reduction of β 1 integrin and MHC1 staining by flow cytometry, confirming that observations from transient GP expression are not simply artifacts of overexpression.⁷⁹ Additionally, the GP of all Ebola virus subtypes except the Reston subtype has been shown to cause severe cell rounding and detachment supposedly due to down-regulation of MHC class I and cell surface adhesion proteins.³² Francica et al, 2010, found that the mechanism by which Ebola virus GP disrupts cell adhesion and other related effects mentioned above involves the heavily glycosylated domains of the EBOV glycoprotein which form a steric shield over proteins at the cell surface.⁴⁹ This steric interference blocks the detection of affected surface proteins using antibody reagents, but also has the functional effect of disrupting cell adhesion and preventing interactions with CD8 T cells.⁴⁹ Interestingly, other studies report that the GP of ZEBOV is not cytotoxic when expressed in isolation at similar levels to that seen during early virus infection.⁸⁰

The GP1,2 of ZEBOV counteracts the interferon-induced antiviral factor known as tetherin.⁴⁶ Tetherin and VP40 (important for EBOV budding), colocalise at the plasma membrane. Tetherin inhibits the release of viral matrix protein VP40-based virus-like particles (VLPs) and it is therefore assumed that tetherin plays a significant role to inhibit the spread of Ebola virus in the host.^{46, 81} Since GP 1,2 counteracts tetherin, it is thus possible that the EBOV-GP1,2 rescues the tetherin driven inhibition of VP40 based VLP release by interfering with the integrity of tetherin at filoviral budding sites.⁸² Additionally, it has also been suggested that the heavily glycosylated EBOV-GP1 subunit interferes with the formation of the 'tetherin-clamp' between the viral and cellular membrane.⁸³ Finally, it is now known that EBOV GP1,2 relocates tetherin within the cellular plasma membrane, thereby excluding it from membrane domains used by EBOV for budding.⁸⁴ This finding lends support to the theory that EBOV-GP blocks the antiviral action of tetherin by inducing its mislocalisation within the plasma membrane. However, it remains to be determined whether endogenous tetherin can reduce the release of EBOV from infected cells, as modest effects were observed in one study but not in another where a significantly higher multiplicity of infection (MOI) was applied, and may consequently restrict viral spread in the infected host.⁸³⁻⁸⁴ It would be interesting to determine whether African fruit bats, proposed as natural reservoirs of EBOV encode a tetherin-like protein and whether this tetherin homologue restricts EBOV spread in the light of the fact that bats are believed not to develop disease upon EBOV infection and that GP displays similar biological properties in human and bat cells.⁴⁶

Non-structural glycoproteins (sGP, ssGP and Δ-peptides)

Direct transcription of the Ebola virus GP gene leads to mRNA encoding a 364-residue secreted non-structural soluble glycoprotein (sGP) (**Fig. 2**).^{17, 60} The mRNA of sGP is the exact copy of the viral genome unlike the mRNA of the mature GP which is generated by transcriptional editing resulting in the addition of a non-templated adenosine residue to a run of seven adenosine residues.¹⁷ Post-translational proteolytic cleavage of pre-sGP by furin yields the mature sGP which has six predicted N-linked glycosylation sites, but only five sites are consistently glycosylated.^{60, 85} The two forms of Ebola virus glycoprotein, GP and sGP, are expressed from a single glycoprotein gene and have identical 295 amino acid residues at their NH₂-terminal ends but differ at the COOH termini by 69 amino acids.⁸⁶ Unlike GP1,2 which is heavily N- and O glycosylated, sGP carries solely N-linked carbohydrates.^{85, 87} The sGP is directly synthesised from the unedited mRNA of GP and is secreted from infected cells as a disulfide-linked homodimer.⁸⁸⁻⁹⁰ Volchkova et al, 1998, showed that the oligomeric structure of sGP is stabilized by a disulfide covalent linkage of monomers in antiparallel orientation through cysteine at positions 53 and 306 to form a 110 kDa homodimer that is secreted.⁹¹ However a study by Falzarano, 2006, proved that sGP is infact a parallel homodimer and is not in antiparallel orientation as first suggested by Volchikov et al, 1998.⁸⁵ The Ebola sGP lacks a transmembrane anchor and specifically shares the amino terminal 295 amino acids with GP1 and not GP2.⁴⁵ In addition, using mass spectrometry analysis of the Ebola virus sGP, researchers identified a rare post-translation modification, C-mannosylation, which was found on tryptophan (W) 288.⁸⁷ However, the functional consequence of modification is unknown because a mutant sGP lacking this modification was unaltered in its biosynthesis, processing, structure and potential anti-inflammatory function.⁸⁷ It is believed that C-mannosylation may be a common post-translational modification of viral transmembrane glycoproteins where it plays a role in particle maturation and/or entry by stabilizing the structure of these proteins.⁸⁷ Although the function of the sGP remains unclear, a study using a ZEBOV infectious clone, showed that in the absence of sGP, majority of full length GP synthesis is arrested in the endoplasmic reticulum(ER) or Golgi.⁵⁴ This protein also has anti-inflammatory properties which are observed in its ability to induce the recovery of the endothelial barrier function and it has been suggested that blockade of the inflammatory response by sGP, which is secreted in high amounts and detectable in patient sera, is a vital patho-mechanism in Ebola virus disease.^{32, 92} Since sGP is the predominantly transcribed glycoprotein, it has been postulated that the balance between sGP and GP serves to regulate the cellular effects of GP.⁵⁴ Other studies have also shown that GP is not detected in cells infected with ZEBOV.⁷⁶ This failure to detect GP during infection is thought to arise because GP is released from infected cells either as sGP) or a soluble form of GP1.⁵⁴ Taken together, these data suggest that high-level expression of GP overwhelms the transport and glycosylation machinery of the host cell resulting in increased cell rounding and cytopathology. On the other hand, the presence of sGP may modulate the expression of GP and cytopathology, allowing the virus to replicate for longer periods of time.⁵⁴ It is also understood that sGP plays a vital role in the pathogenesis of Ebola virus disease by inactivating neutrophils as well as acting as a decoy for anti-EBOV antibodies.^{93, 94}

During natural infection with EBOV, large amounts of sGP have been detected in the blood of Ebola virus disease patients.⁸⁹ Antibodies from sera of survivors appear to preferentially recognise sGP over GP.⁹⁵ Therefore, sGP could play an essential role in the evasion of host humoral immune response by absorbing elicited antibodies.⁶⁰ Ebola virus sGP has been reported to bind to human neutrophils through the IgG Fc receptor IIIb (FcγRIIIb, CD16b) and to inhibit early activation of neutrophils.^{96, 97} This study provided novel evidence that sGP may be involved in Ebola virus disease pathogenesis by diminishing innate immunity to the virus through its specific binding to neutrophils. Other researchers challenged the results of this report because sGP binding to neutrophils could not be detected with the use of anti-sGP Fab or F(ab')₂ fragments.⁸⁶ These findings suggested that the binding signal generated with the rabbit antibody against sGP occurred through its Fc portion to FcγRIIIb as an immune complex with sGP.⁹⁸ The authors of the initial report provided additional information by demonstrating that the binding of sGP to neutrophils as detected by the F(ab')₂ fragment could be restored by pre-incubating the neutrophils with purified nonspecific rabbit IgG.⁹⁷ Consequently, the reported binding of sGP to neutrophils remains unclear. Sui and Marasco, 2002, performed additional studies with purified sGP and human neutrophils to clarify the role of sGP in the pathogenesis of Ebola virus disease.⁸⁶ Unlike the previous findings, they did not find a specific interaction between sGP and CD16b but nonspecific formation of immune complexes on the neutrophil surface. Using an sGP absorption assay, they further demonstrated that sGP is not specifically bound but is non-specifically proteolysed by proteases released from neutrophils.⁸⁶ They concluded that neutrophils do not express a specific receptor for Ebola virus sGP and that sGP may not play a role in the pathogenesis of Ebola virus disease through interference with the innate immunity by targeting neutrophils.⁸⁶

Proteolytic processing of sGP at its C-terminus by furin yields a soluble heavily *O*-glycosylated, small, nonstructural, secreted protein termed Δ-peptide (**Fig. 2**).⁹⁹ Δ-peptide is released from infected cells just like sGP.⁸³ In contrast to a mature sGP, Δ-peptide is retained in producer cells for longer periods of time prior to its release into the cell culture supernatant, and the overall amount of secreted peptide is less than that of sGP.⁹⁹ Δ-Peptide has a predicted mass of ≈4.7 kDa and actual mass of ≈10 to 14 kDa.¹⁰⁰ The actual function of Δ-peptide is still obscure. Radoshitzky and co-workers, 2011, examined the cell surface binding and filovirus entry-inhibitory properties of Fc-tagged sGP, ssGP, and Δ-peptide. They found that sGP-Fc and ssGP-Fc do not influence the entry of filoviruses into host cells but Fc-tagged Δ-peptides do.¹⁰⁰ However, the phenotypic results were not obvious for the Fc-tagged Δ-peptide of an REBOV (non-pathogenic to humans) and that of BEBOV (has lower lethality in humans). In addition, the findings of this study suggested that functional peptides bind to filovirus-permissive cells, but not to filovirus-resistant cells.¹⁰⁰ The ability of these peptides to inhibit entry of both MARVs and EBOVs even though Δ-peptides are not produced by MARVs suggests that they probably interfere with a pathway that is used by all filoviruses to gain entry into target cells. Based on this information, it is possible that these peptides, like cathepsins and integrins, are modulators of filoviral cell entry and that they may play a role in the pathogenesis of filovirus diseases.¹⁰⁰ As stated above, the mRNA of sGP is the exact copy of the viral genome unlike the mRNA of the mature GP which is generated by

transcriptional editing resulting in the addition of a non-templated adenosine residue to a run of seven adenosine residues. However, Volchkov et al, 1995, discovered that deletion of one adenosine or addition of two adenines during transcriptional stuttering results in the expression of a third nonstructural, small sGP(ssGP) whose function is unknown (**Fig. 2**).⁸⁸ The ssGP shares 295 amino-terminal residues with sGP and GP and is secreted from cells in a monomeric form due to the lack of the carboxyl-terminal part, including cysteine at position 306.⁹¹ It is not known whether ssGP plays a role in Ebolavirus pathogenesis.

Role of glycoproteins in the uptake of Ebola virus into host cells

The mechanisms by which the Ebola virus enters the host cell are not well understood but significant progress has been made on the possible mechanisms. It has previously been proposed that clathrin- or caveolae-mediated endocytosis plays a significant role in EBOV entry, however, this idea is doubtful because Ebolavirus virions are larger than the plasma membrane invaginations that characterise this type of endocytosis.¹⁰¹⁻¹⁰³ Caveolae are small vesicles of about 50–80 nanometres (nm) in diameter, are rich in caveolin, sphingolipid, and cholesterol. Other studies have gone further to shed more light by suggesting that EBOV is internalised by an endocytic pathway, seeing that its entry is dependent upon low pH and the endocytic enzymes cathepsin B and L.¹⁰³⁻¹⁰⁶ Several other studies have also shown that EBOV entry depends on cholesterol, a major constituent of caveolae and lipid-rafts.⁶⁵ These disparities in the findings might be a reflection of the varying experimental systems and/or conditions used. Many of these studies have been carried out using retroviruses or vesicular stomatitis virus (VSV) pseudotyped with GP of Ebolaviruses.¹⁰³ It is well known that pseudotyped systems have limitations because the morphology of the virions (spherical for retrovirus or VSV-pseudotyped virions) differs outrightly from that of wild type Ebola virions.

It is currently clear that the spikes of the Ebola virus glycoprotein bind to host cells and mediate fusion between the viral envelope and the host cell membrane, enabling the virus to release its contents into the host-cell cytoplasm.⁸³ It is now thought that Ebolavirus is internalized and sequestered within an endosome where it is exposed to a low-pH environment and crosses the endosomal membrane to reach the cytoplasm. It has been established that conformational changes must occur in the viral GP spikes to enable its hydrophobic fusion domain to be introduced into the host cell membrane. The conformational changes act as a switch that ignites the fusion of the virus to the host cell.⁸³ Chandran et al discovered that the conformational changes are caused by proteolysis in the surface glycoprotein by two endocytic cysteine proteases, cathepsin B and L.¹⁰⁷ Further evidence suggests that host cells lacking these enzymes show a reduction in GP-mediated infection. Cathepsin B and cathepsin L are capable of individually cleaving Ebola virus GP1 to yield an approximately 18-kD N-terminal fragment, which is further digested by cathepsin B leaving only GP2.^{83,107} The efficiency of the GP mediated viral infection relies on the production of N-terminal fragment. Chandran et al's model predicts that internalisation of Ebola virus into the endosomes of cells leads to the removal of the C terminus of the viral GP1 by either of the protease enzymes or both in the endosome. This leaves the N-terminal fragment which is subsequently digested by cathepsin B to initiate membrane fusion by GP2,

the still-intact fusion domain of the glycoprotein molecule.⁸³⁻¹⁰⁷ It is therefore assumed that GP2 initiates fusion between the viral envelope and the endosomal membrane resulting in the release of the viral genome into the cytoplasm.⁸³ Inhibition of cathepsin B by a selective inhibitor, CA074, inhibits GP1 proteolysis, thereby preventing fusion and thus infection.⁸³ A study by Ou et al, 2010 found that a GP1 residue termed F88 plays an essential role in viral entry in a step after cathepsin processing but not in viral binding.⁶⁴ F88 is partially exposed on the GP1 surface next to the putative receptor binding site and the GP2 internal fusion loop, while another residue F159 is deeply buried within a hydrophobic pocket.^{64,65}

Other studies have added to the debate on the role of GP in the internalisation of Ebola virus by suggesting that Ebolaviruses are taken up into host cells via micropinocytosis in a glycoprotein dependant manner. Nanbo et al examined the mechanism of Ebolavirus internalization by real-time analysis of fluorescently labelled EBOV and Ebolavirus like particles and found that their internalization was independent of clathrinor caveolae-mediated endocytosis, but that they co-localised with sorting nexin (SNX5).¹⁰³ Sorting nexin (SNX) 5 comprises a family of peripheral membrane proteins and serve as markers of macropinocytosis specific endosomes known as macropinosomes.¹⁰³ Additionally, it was found that the internalisation of EBOV virions accelerates the uptake of a macropinocytosis-specific cargo, and was associated with plasma membrane ruffling.¹⁰³ The mechanism was reliant on cellular GTPases and kinases that are involved in macropinocytosis. Taken together, their data suggest that Ebolavirus is internalised into cells by stimulating macropinocytosis in a GP-dependent manner.¹⁰³ There is more work that needs to be done to unite these studies together to find the most appropriate role of the Ebola GP in the internalisation of Ebolaviruses into host cells.

Viral protein 30 (VP30)

The viral protein 30 (VP30) of Ebola viruses (EBOV) together with the L protein, VP35, and the N protein (NP) form the ribonucleocapsid complex, along with the genomic RNA.¹⁰⁸ Although VP30 is present in nucleocapsids, its inclusion is not a requirement for nucleocapsid to act as a template for transcription, because it can be provided in trans.¹⁰⁹ However, VP30's presence in the nucleocapsid is essential for initial transcription in newly infected target cells.¹⁰⁹ VP30 is a strongly acidophilic nucleocapsid protein and transcription of the EBOV genome strongly relies on the presence of this protein.^{35,54,108,110} It is thought that VP30 plays an essential role in Ebola virus replication probably by stabilizing nascent mRNA.¹¹¹ VP30 is vital for the initiation and re-initiation of viral transcription but is inessential for EBOV genome replication.^{35, 112-113} It has been established that VP30 acts as a transcription anti-termination factor immediately after initiation of transcription but does not affect transcription elongation.¹¹¹ Using the minigenome system, other studies failed to prove the proposed transcription anti-termination activity of EBOV VP30.¹¹⁴ Similar proteins of other viruses such as M2-1 of human respiratory syncytial virus support transcriptional elongation and formation of readthrough mRNAs.¹¹⁵⁻¹¹⁶ Formation of an RNA secondary structure (located within nucleotides 54-80 of the leader region) upstream of the transcriptional start site of the first gene of EBOV is the driving force behind the function of the heavily

phosphorylated VP30 as a transcription activator.^{108, 114, 117} This posttranslational modification has been shown to regulate the activity of VP30 during transcription.¹¹⁸ On the other hand, the molecular mechanisms by which VP30 activates viral transcription are not clearly understood. Sequence analysis of filoviral VP30 protein revealed that it contains a motif resembling that of an unconventional zinc-binding Cys3-His motif that was initially described for Nup475, a mammalian nuclear protein.¹¹⁹ This motif comprises amino acids 68 to 95 and is highly conserved among filoviruses.¹¹¹ In the proposed structure of the Nup475 metal-binding domain, the zinc ion is coordinated by the conserved cysteines and histidine.¹²⁰ A similar motif is present in M2-1 protein of pneumoviruses such as the human respiratory syncytial virus.¹²¹ Direct binding of zinc to M2-1 has not been demonstrated but the Cys3-His motif has been shown to have functional relevance for the M2-1 protein and for viral growth.¹²¹ However, by utilising a colorimetric zinc-binding assay, Modrof et al, 2003, found that the VP30-specific Cys3-His motif stoichiometrically binds zinc ions in a one-to-one relationship.^{111, 122} They further substituted the conserved cysteines and histidine within the motif leading to a complete loss of the capacity for zinc binding.^{111, 122} This confirmed the findings by Worthington et al, 1996, that the zinc ion is coordinated by the conserved cysteines and histidine.^{111, 120, 122} Additionally, using a bioinformatics approach, John et al, 2007, found that VP30 directly interacts with RNA and promotes transcription: they identified a region within the N terminus of VP30 with sequence features that characterise intrinsically disordered regions and a putative RNA binding site.^{108, 121, 123} The key amino acids that support RNA binding activity were mapped to a short arginine rich amino acid stretch (residues 26 to 40) of VP30.^{108, 121, 123} Like other past studies, they also recognised a zinc-finger motif downstream of this binding motif.^{108, 121, 123}

The VP30 of Ebolaviruses is phosphorylated at 2 serine clusters (amino acids 29–31 and 42–46), each containing 3 serine residues.¹¹⁸ Transcription activation by VP30 strongly relies on the phosphorylation state of VP30. It is now known that only none or weakly phosphorylated VP30 activates transcription while fully phosphorylated VP30 acts as an efficient inhibitor.¹¹⁸ In other words, phosphorylation of VP30 positively regulates its binding to NP and negatively regulates its transcriptional activity.¹¹⁸ The effect of phosphorylation on the function of VP30 has been studied further using alanine and aspartate substitutions, which mimic non-phosphorylated and constantly phosphorylated serines, respectively.¹¹³ Researchers have shown that a VP30 with all serine clusters replaced by alanine residues (VP30-AA) is active in the transcription of an EBOV minigenome and is also capable of supporting the rescue of a recombinant EBOV as observed in reverse genetics experiments.^{112-111, 122} On the other hand, replacement of serine residues by aspartate residues (VP30-DD) results in a VP30 protein that is defective in the transcription of the minigenome and is unable to support the recovery of recombinant EBOV.¹¹³ It has therefore been suggested that VP30 phosphorylation is essential for the function of the protein in viral replication and may be vital for either an early or a late step in EBOV life cycle. Martinez et al, 2011, concluded that substitution of serines by alanine or aspartate residues alters the replication of recombinant EBOVs by affecting transcription and replication of viral RNAs.¹¹³ The overall data from this study suggests that phosphorylation of EBOV VP30, plays an essential role in regulating viral RNA synthesis.

The function of VP30 as a transcription activator is exerted by a homooligomer of VP30, which requires a hydrophobic stretch of leucine residues in the N terminus.¹²⁴ VP30 forms hexamers in solution and infectious EBOV particles by an N-terminal oligomerisation domain housed within a stretch of hydrophobic amino acids (residues 94–112).¹²⁴ These studies suggest a hexameric stoichiometry of VP30 present in EBOV virions. Hartlieb et al, 2006, provided evidence that VP30 contains a second homooligomerization domain which is composed of the C-terminal half of VP30 (VP30_{CTD}).¹⁰⁹ Further structural analyses showed that VP30 C-terminal Domain (VP30_{CTD}) folds independently into a dimeric helical assembly with no relevant structural resemblance to known protein structures.¹⁰⁹ The dimerisation interface is sizeable and the majority of interactions are between the loop region connecting helices 6 and 7 and helix 1.¹⁰⁹ It is possible that the displacement of helix 7 could be enough to disrupt the dimerisation observed in VP30_{CTD} and allow other VP30 conformers. The two conserved regions of VP30_{CTD}, a basic cluster around Lys-180, and Glu-197, are essential for nucleocapsid interaction.¹⁰⁹ However, only mutagenesis of the basic cluster was shown to impair VP30's transcription activation, whereas exchange of Glu-197 left transcription activation function intact, suggesting that both processes are regulated independently.¹⁰⁹ Based on these experimental results, VP30 is a potential target for antiviral therapy because its essential homooligomerisation could be inhibited by synthetic peptides resulting in inhibition of EBOV propagation in cell culture.¹²⁴

Viral protein 35 (VP35)

The EBOV encoded VP35 protein fulfils several functions that are essential for viral amplification. As an important polymerase cofactor, VP35 is one of the proteins that are involved in the assembly of the EBOV ribonucleocapsid complex.⁸³ It is crucial for transcription and viral replication.³⁵ Consequently, interference with the expression of VP35 impairs the growth and virulence of Ebolaviruses.¹²⁵⁻¹²⁶ In all filoviruses, VP35 is the protein that is responsible for inhibition of the host interferon (IFN) responses by binding double stranded RNA (dsRNA).¹²⁷ This function is embedded in its 130 amino acid C-terminal dsRNA binding domain (RBD).¹²⁸ Crystal structures of the VP35 RBDs from EBOV, REBOV, and Marburg viruses (MARV) have been determined separately and in complex with dsRNA. These structures have revealed that the N-terminal half of RBD contains an α -helical subdomain whereas the C-terminal half contains a three-stranded mixed β sheet with a conserved basic patch.¹²⁹⁻¹³³ In addition, the N-terminal residues of both the unbound and bound VP35 RBD structures are disordered. Based on the structures of the complexes, previous researchers established that VP35s from Ebolaviruses bind the ends of dsRNA while those from MARV bind along dsRNA, primarily in the middle.¹³⁴ Using VP35 structures from REBOV, Kimberlin et al 2010, reported that EBOV VP35 uses a bimodal strategy to bind dsRNA for innate immune suppression.¹²⁹ They showed that VP35 RBD forms an asymmetric dimer with two distinctive modes of RNA binding: one monomer, termed the “endcapping molecule”, binds the terminal bases of dsRNA while another monomer termed the “backbone-binding molecule”, dimerises with the end-capping molecule and binds along the neighbouring sugar-phosphate backbone.¹²⁹ Kimberlin et al's REBOV VP35 crystal structure also illustrated that the third arginine, R301 in Reston (equivalent to R312 in ZEBOV) that was previously shown to be critical for RNA binding, does not contact dsRNA

but instead is an essential component of the VP35 dimeric interface between the two VP35 monomers.¹²⁹ Previously, only the central dsRNA backbone of MARV VP35 RBD dsRNA complexes was known to be bound by VP35 RBDs resulting in shielding of the backbone from PRRs while the end terminals remained free.¹³⁴⁻¹³⁵ Building on the findings by Kimberlin et al 2010, Bale et al, 2013 has demonstrated that Ebolavirus VP35 like MARV, also coats the dsRNA central backbone in solution and that binding to the dsRNA ends probably constitutes the initial binding event.¹²⁸ Upon the recognition of viral dsRNA, host pattern recognition receptors (PRRs) such as Melanoma Differentiation-Associated protein 5 (MDA-5), Retinoic acid-inducible gene-1(RIG-1), Toll like receptor-3 (TLR-3), and LGP2 activate a signalling cascade that leads to the phosphorylation and nuclear translocation of transcription factor IRF3/7(Interferon regulatory factor), and NFκB which in turn stimulate the production of type I interferons (IFNα/β) in infected and non-infected surrounding cells.¹²⁸ IFNα/β and other cytokines act in an autocrine and paracrine manner to activate the Janus kinases (JAK)/STAT pathway and IFN-stimulated response elements (ISRE s) or antiviral genes, such as RNA-dependent protein kinase (PKR), MHC class I and 2'5' Oligoadenylate synthetase (2'5' OAS). Activation of these antiviral molecules and the involvement of the adaptive immune responses limits viral replication. In response to this mechanism, filoviruses and other viruses have evolved to encode specific proteins that counter innate immune detection by binding to and shielding their dsRNA from the PRRs.¹²⁹⁻¹³¹ Shielding or coating of the dsRNA backbone prevents the recognition of dsRNA by PRRs.

The host innate immune system is the first line of defense against viral infections, which are mediated primarily through macrophages and dendritic cells. Production of type I IFNs is a fundamental component that is necessary to generate a potent innate immune response. Ebolavirus disease is marked by early suppression of the host innate immune signalling cascades involving silencing and sequestration of dsRNA by the viral protein VP35.¹²⁹⁻¹³¹ The ability of VP35 to antagonise IFN α/β response was first recognised by who demonstrated that VP35 can functionally replace the influenza A virus non-structural protein 1 (NS1), a viral type I IFN antagonist.¹³⁶ Basler and co-workers found that VP35 inhibited the virus-induced activation of type I IFN promoters by targeting a transcription factor known as IRF-3. On the other hand, activation of IFN promoters through exogenous interferons was not regulated by VP35.¹³⁷ VP35 has been shown to prevent phosphorylation and nuclear translocation of IRF-3 resulting in inhibition of IFN α/β expression.¹³⁶⁻¹³⁷ Consequently, IFNs are not available to activate the JAK/STAT pathway and IFN-stimulated response elements (ISRE s) such as PKR, MHC class I and 2'5' OAS thereby allowing the virus to replicate freely.¹²⁹ VP35 also serve as a suppressor of RNA silencing. VP35's ability to block the expression of IFN α/β has been traced back to its C-terminal dsRNA-binding domain, also known as the IFN inhibitory domain(IID).^{127,138} Researchers have found three basic residues, R305, K309, and R312 (as numbered in the ZEBOV) that are crucial for binding dsRNA and blocking the expression of IFN.¹³⁸ Kimberlin et al 2010, and Bale et al, 2013 demonstrated that Ebolavirus mask the central backbone of their dsRNA to avoid recognition by host pattern recognition receptors. By masking their dsRNA, these viruses prevent the activation of signalling cascades that would eventually stimulate the production of type I interferons (IFN).¹²⁸⁻¹²⁹ A study by Cardenas et al, 2006 and Hartman et al, 2008, shed more light to the

role of VP35 as a suppressor of early innate immunity by demonstrating that VP35 mutated viruses are incapable of blocking the IFN-dependent induction of antiviral factors.^{127, 139} Other mutational studies have also shown that the N-terminal coiled domain in VP35 mediates homo-oligomerisation, which is essential for IFN antagonism exhibited by the C-terminal half of VP35.¹⁴⁰

Retinoic acid-inducible gene-1 (RIG-1), one of the cytoplasmic pattern recognition receptors (PRRs) is activated by viral RNA and plays a role to induce type I IFN responses against viral replication. RIG-1 is also activated by Protein kinase, interferon-inducible double stranded RNA dependent activator (PACT). Filoviruses have developed specific mechanisms to counter innate immune detection by binding to and shielding their dsRNA from the PRRs. Luthra and co-workers, 2013, found another mechanism used by EBOV VP35 protein to suppress the immune system.¹⁴¹ They demonstrated that EBOV VP35 inhibits PACT-induced RIG-I ATPase activity in a dose-dependent manner. Wild-type VP35 disrupts the interaction of PACT with RIG-I in contrast to VP35 mutants that are unable to bind PACT.¹⁴¹ They also found that interactions between PACT-VP35 impair the association between VP35 and the viral polymerase, thereby diminishing viral RNA synthesis and modulating EBOV replication.¹⁴¹ Cells that are PACT-deficient are defective in IFN induction and are insensitive to VP35 function. Luthra and co-workers concluded that VP35-PACT interaction is mutually antagonistic and plays a significant role in determining the outcome of EBOV infections.¹⁴¹ Other studies suggest that EBOV VP35 probably inhibits the RIG-I pathway at several stages, including by acting as a decoy substrate for the cellular kinases IKK ϵ and TBK-1 and through interaction with the SUMOylation machinery.^{142, 143} The VP35 of Ebolaviruses has also been shown to inhibit the antiviral response induced by IFN- α by interfering with the pathway regulated by IFN-inducible dsRNA-dependent protein kinase (PKR). PKR recognises double stranded RNA produced by viruses during infection. Feng et al., 2007, showed that autophosphorylation of PKR is disrupted in cells expressing VP35 resulting in the inhibition of PKR activation.¹⁴⁴ Additionally, other researchers have demonstrated that ZEBOV VP35 not only blocks PKR activation but is also able to reverse the activation.¹⁴⁵ Furthermore, work by Schumann et al, 2009, indicates that mutations in the C-terminal dsRNA-binding domain of VP35 are sufficient to rescue the blockade to PKR activation imposed by VP35 and that the PKR-VP35 antagonism is functionally separate from dsRNA binding and inhibition of IRF-3.¹⁴⁵ The immunosuppressive role of EBOV VP35 does not end with RIG-1 inhibition. Recent studies indicate that VP35 suppresses RNA interference (RNAi). RNAi is a potent cellular defence mechanism that allows host cells to detect and destroy viral RNAs in order to combat viral infection.¹⁴⁶ To counter this mechanism, a number of viruses encode RNAi silencing suppressors (RSS) such as the NS1 protein of influenza viruses and the Tat protein of HIV-1. Haasnoot et al, 2007, demonstrated that VP35 of Ebolaviruses is an RSS whose activities rely on its dsRNA-binding capability indicating that VP35 shields dsRNA from recognition by a central molecule of the RNAi pathway known as dicer.¹⁴⁷ Other studies have refuted this suggestion by demonstrating that VP35 interacts with components of the RNA-induced silencing complex (RISC) independent of the presence of small interference RNA (siRNA).¹⁴⁸ Fabozzi et al., 2011, found that VP35 does not interact directly with dicer, but

interacts with two dsRNA-binding proteins (HIV-1 trans-activation response RNA-binding protein and PACT) which are part of the RISC complex.¹⁴⁸

The ability of Ebola viruses to control the host innate system is key to its virulence and pathogenicity. The innate immune system against viral infections is primarily mediated through macrophages and dendritic cells (DCs) which link innate and adaptive immunity.¹⁴⁹⁻¹⁵¹ DCs play an essential role in cytokine production, antigen presentation, antibody production and interaction with natural killer cells.¹⁵²⁻¹⁵³ Immature DCs reside in almost all peripheral tissues and are capable of capturing and processing viral antigens.¹⁵³ In response to viral infections, immature DCs migrate to lymph nodes where they undergo maturation. Mature DCs have relatively higher levels of co-stimulatory molecules and they release pro-inflammatory cytokines, which eventually activate the adaptive immune responses.¹⁵⁴⁻¹⁵⁶ To counter the immune mediated mechanisms by DCs, EBOVs replicate efficiently in DCs to inhibit the maturation of DCs without inducing the expression of cytokines resulting in impaired T-cell activation.¹⁵¹⁻¹⁵² However, the EBOV viral component that impairs DC function was unknown until a study by Jin et al, 2010, reported that EBOV VP35 is responsible for the inhibition of maturation of DCs induced by virus infection and toll like receptor-4 (TLR-4) signalling, leading to impaired T-cell proliferation.¹⁵⁴ In their study, Jin and co-workers revealed that expression of EBOV VP35 in mouse immature DCs prevented virus-stimulated expression of CD40, CD80, CD86 and MHC class II.¹⁵⁴ VP35 also suppressed the induction of cytokines such as interleukin (IL)-6, IL-12, tumour necrosis factor- α (TNF- α) and type 1 IFNs (IFN- α/β). Furthermore, Ebola VP35 attenuates the ability of DCs to stimulate the activation of CD4+ T cells and alters the functions of mouse DC induced by lipopolysaccharide, an agonist of TLR-4.¹⁵⁴⁻¹⁵⁶ The work of Jin and co-workers, 2010 highlighted an essential role of EBOV VP35 in viral interference of DC function and the resultant impairment of T-cell activation and function.¹⁵⁴ The activity of VP35 to block DC maturation and function contributes to the virulent nature of Ebola viruses. IFN type 1 is an activator of DCs and it is therefore possible that VP35 may modulate the function of DCs by interfering with IFN-mediated effects on DCs.¹⁵⁵⁻¹⁵⁶

Viral protein 24 (VP24)

VP24 is the smallest EBOV structural protein. It is a secondary matrix protein and a minor component of mature virions relative to a major EBOV matrix protein known as VP40.^{110, 157, 158} Together with VP30, L protein, VP35, and the N protein (NP), VP24 is required for the assembly of a fully functional nucleocapsid complex and contributes to the budding of virus-like particles (VLPs).^{39, 83, 157} As a component of the nucleocapsid complex, VP24 also plays a key role in the transcription and replication of EBOV viral genome even though Watanabe et al, 2007, reported that VP24 reduces transcription and replication of the EBOV genome by direct association with the ribonucleoprotein complex in virus-infected cells.¹⁵⁹ VP24 has a single domain, α/β structure whose crystal shape resembles a triangular pyramidal fold upon which sites required for virulence and for STAT1 binding have been mapped.¹⁶⁰ The three sides of the VP24 pyramid are termed Faces 1, 2 and 3. Located at the base of faces 1 and 3 are two pockets that are highly conserved in filoviruses.¹⁶⁰ The pocket at the base of Face 1 is

narrow and lined with a stretch of hydrophobic amino acid residues whereas that below Face 3 is shallow in depth and lined with polar residues. The function of these pockets remains unknown. Using Deuterium exchange mass spectrometry (DXMS), Zhang et al, 2012 identified a putative area of possible protein-protein interaction between VP24 and STAT1.¹⁶⁰ The putative STAT1-binding site on VP24 lies in the conserved region of Face 3 and is distinct from the site proposed to interact with karyopherin $\alpha 1$.¹⁶⁰ The identified three-dimensional templates of VP24 provides a platform to understanding the multiple roles of VP24 in the life cycle of Ebolaviruses and to design antiviral compounds against them.¹⁶⁰

VP24 is capable of inhibiting the host's IFN- α/β and IFN- γ response to viral infection. VP24 inhibits IFN responses by blocking the nuclear translocation of the transcription factor STAT1.¹⁶¹ STAT1 is a key component of the IFN-induced signalling pathway that controls the expression of IFN stimulating genes (ISGs). STAT1 predominately exists in an unphosphorylated state (U-STAT1) in healthy cells.¹⁶² During viral infection, production of interferons (IFN- α/β or IFN- γ) and signalling from other cytokines such as TNF α , growth factor, angiotensin, IL-6 and IL-10 causes STAT1 to be phosphorylated (PY-STAT1) by receptor associated Janus tyrosine kinases (JAKs).^{160, 163} Thereafter, tyrosine PY-STAT1 forms complexes with STAT2 and IRF-9 or it homodimerises.^{160, 163} Signalling from the IFN- α/β receptor results in the formation of PY-STAT1:STAT2 heterodimer complexes which additionally interact with IRF-9 whereas IFN- γ signalling leads to the formation of STAT1:STAT1 homodimers.¹⁶² Formation of PY-STAT1 heterodimers and homodimers exposes a nuclear localisation signal (NLS) in STAT1, which is subsequently recognised by nuclear import adaptor proteins known as karyopherin α (specifically $\alpha 1$, $\alpha 5$ and $\alpha 6$).⁸³ Karyopherin α is eventually bound by a nuclear import factor karyopherin β , which facilitates the translocation of dimerised STAT1-complexes into the nucleus of host cells where they regulate genes involved in the immune responses.¹⁶⁴ VP24 inhibits signalling downstream of IFN- α/β and IFN- γ by sequestering karyopherin α proteins.¹²⁷ Using VP24 protein, Ebolaviruses have been shown to indirectly affect the translocation mechanism of STAT1 by binding to karyopherin $\alpha 1$ and blocking it from transporting activated PY-STAT1 complexes into the nucleus of host cells.^{161, 165} Additionally, EBOV VP24 disrupts the recognition of the PY-STAT1 NLS by karyopherin α thereby blocking the translocation of PY-STAT1 into the nucleus.⁸³ Mutational studies showed that VP24 binds to amino acids 458–504 in karyopherin $\alpha 1$. This stretch of amino acids constitutes the armadillo repeat 10 (ARM 10) and encompasses part of the binding site for PY-STAT1.^{161, 165–166} VP24's binding to karyopherin α mimics that of exportin which also targets the ARM repeat 10.¹⁶⁷ Consequently, VP24 may inhibit the release of auto inhibitory NLS of karyopherin α , and this could prevent other proteins from binding to the NLS binding site of karyopherin- α including PY-STAT1.^{165–167} In other ways, targeting the ARM10 repeat of karyopherin- α by VP24 may block binding of PY-STAT1 because of competitive inhibition.¹⁶⁷ Furthermore, Mateo et al., 2010, reported that amino acid residues 42 and 142–146 in VP24 are critical for the inhibition of IFN- β -induced signalling and accumulation of PY-STAT in the nucleus.¹⁶⁸ VP24 mutants with amino acid exchanges at these positions neither bind karyopherin- α nor inhibit IFN- β -induced signalling confirming that binding of VP24 to karyopherin- α is essential for IFN antagonism.¹⁶⁸ Type I IFN signaling is mediated via a number of signaling pathways that

include the Janus kinase and signal transducer and activator (JAK-STAT), and p38 mitogen-activated protein (MAP) kinase pathways.¹⁶⁹ The EBOV VP24 protein is a distinct IFN antagonist and its antagonistic activities through the JAK-STAT pathway are well characterised. Halfmann and co-workers, 2011, demonstrated that VP24 also interferes with the p38 MAP kinase pathway by blocking IFN- β -stimulated phosphorylation of p38- α .¹⁶⁹ Although the JAK-STAT pathway is a well characterised IFN-induced signal transduction mechanism, the p38 mitogen-activated protein (MAP) kinase pathway is also crucial for type I IFN response.¹⁷⁰ Engagement of the type I IFN receptor by type I IFN activates a series of MAP kinases that eventually lead to the phosphorylation of the α -isoform of p38 (p38- α) at threonine 180 and tyrosine 182 positions.¹⁷¹ Phosphorylated p38 then initiates the phosphorylation of downstream transcription factors that play key roles in IFN responses.¹⁶⁹ As earlier described, VP24 inhibits the cellular response to type I IFN by blocking the translocation of phosphorylated STAT-1 into the nucleus of host cells.¹⁶⁵⁻¹⁶⁶ According to Halfmann and co-workers, VP24 also inhibits the p38 MAP kinase pathway by preventing the phosphorylation of p38- α .¹⁶⁹ Data from these studies suggest EBOV VP24 uses at least two mechanisms to counter the antiviral effects of type I IFNs: VP24-mediated blockade of IFN-induced signaling cascades, including the JAK-STAT and p38 MAP kinase pathways.

Viral protein 40 (VP40)

The viral protein 40 (VP40) is the major matrix protein of Ebola viruses and can drive the formation of filamentous virus like particles (VLPs) that resemble the morphology of wild type Ebola viruses.³² It is therefore used to study protein-protein and lipid-protein interactions as it serves as a representative model of budding and can be used in none BSL-4 laboratories.¹⁷² VP40 is the most abundant protein in EBOV particles, representing about 40% of EBOV molecular weight.¹⁷³ It is located beneath the viral lipid membrane, where it probably helps to maintain the structural integrity of the virus, and plays an essential role in viral replication, assembly and budding.^{40,48-47,174} VP40 and VP24 are linked to the ribonucleoprotein complex at the inner surface of the lipid bilayer of the viral envelope to maintain the structural integrity of the Ebola virus particles.¹⁷³ The nucleocapsid of EBOVs composed of the nucleoprotein (NP), VP24, VP30, VP35 and L protein, is crucial for transcription and replication of viruses. However, in the absence of VP40, the nucleoprotein is inefficiently carried to the plasma membrane and virus particles do not form.¹⁷² VP40 VLP budding is driven by viral L domains located in the N-terminus of VP40.^{39-40, 48} Through these domains, VP40 interacts with cellular factors such as the Nedd4, ubiquitin ligase, and TSG101 leading to the release of VLPs.¹⁷⁵⁻¹⁷⁷ Furthermore, VP40 has RNA binding and oligomerisation properties and is capable of producing VLPs from cells in the absence of other Ebola virus proteins when expressed in human cells.^{173, 178} However, recent studies have shown that co-expression of VP40 with other EBOV proteins enhance the budding of VP40 VLPs.^{39, 179} For example, when VP40 was co-expressed with GP and NP proteins, VLP release was enhanced almost 40-fold over what was observed when VP40 was expressed alone.³⁹ However, the mechanism by which EBOV proteins enhance VP40 budding remains unclear, as is their effect on VLP morphology.

Johnson and co-workers, 2006, found that VP40 is the major determinant of VLP density, and co-expression of NP, GP, and VP35 had little effects on morphology of VLPs.¹⁸² The crystal structure of VP40, first described by Dessen et al, 2000, consists of two distinct domains, N-terminal domain(NTD) and C-terminal domain(CTD), connected by a flexible linker.¹⁸⁰⁻¹⁸¹ NTD regulates dimerisation and CTD has been shown to mediate membrane binding and oligomerisation thus, inhibition of VP40 oligomerisation halts VLP budding.¹⁸⁰⁻¹⁸¹ Mutations (K127A, T129A, and N130A) in the loop region of NTD reduce oligomerisation and plasma membrane localisation of VP40 and consequently reduces VLP release from cells.^{173, 180-181} The loop region of NTD in VP40 that may be essential to viral egress.¹⁷² Further crystal structural studies of VP40 have revealed that VP40 is a dimer that assembles end-to-end into filaments through homologous interactions between their C-terminal domains.¹⁸³ Three immuno-dominant regions of EBOV VP40 located in CTD (amino acid residues 189–207, 213–235 and 289–319) reportedly play a critical role in virus assembly and budding and are a target of host humoral responses.¹⁷³ Becquart et al, 2014, demonstrated that EBOV IgG+ sera obtained from asymptomatic individuals during the Gabon outbreaks of Ebola virus disease reacted strongly to VP40 immuno-dominant regions than to GP, NP or VP35.¹⁷³ Sera from most survivors reacted mainly with GP peptides. The first domain that was targeted by IgG from asymptomatic individuals is found in the flexible linker (residues 195–200) joining CTD to NTD.¹⁸⁰ The second region is located in a conserved basic patch vital for electrostatic membrane interaction and for igniting assembly of VP40 into the viral matrix to bud nascent virions.¹⁸³⁻¹⁸⁴ The third region is found in the hydrophobic dimeric VP40 CTD-CTD interface, which has been described to be crucial for matrix assembly and budding as well as penetration of the plasma membrane.¹⁸³ Taken together, the study by Becquart et al, 2014, strongly suggests that humoral responses directed against the three key epitopes/domains of VP40 may contribute to protecting humans against EBOV infections.¹⁷³

Beside the established fact that VP40 assembles on the inner leaflet of the plasma membrane and a few studies that have highlighted possible mechanistic details of VP40 membrane binding that are vital for viral release, the actual mechanisms of VP40 membrane binding are still obscure. Phosphatidylserine, an anionic lipid present in the inner leaflet of the plasma membrane of human cells has been shown to be essential for VP40 CTD plasma membrane interactions.¹⁸⁴⁻¹⁸⁶ A cationic patch in the CTD is believed to mediate association with anionic membranes while a hydrophobic loop drives the penetration of CTD into the hydrocarbon core of the lipid bilayer.^{183, 185-186} Using fluorescence quenching of a tryptophan on the membrane-binding interface with brominated lipids along with mutagenesis of VP40, Soni et al, 2013, found that VP40 penetrates 8.1 Å into the hydrocarbon core of the plasma membrane bilayer and that it causes distinct changes to plasma membrane curvature as it tubulates liposomes and induces vesiculation into giant unilamellar vesicles.¹⁸⁶ This was found to be an essential step in viral egress as cellular assays demonstrated that hydrophobic residues that penetrate deeply into the PM are essential for plasma membrane localisation and formation and release of VLPs from host cells. However, mutations in the hydrophobic region of VP40 abrogated the observed effects of VP40 membrane penetration.¹⁸⁶ Furthermore, VP40's ability to mediate viral transcription is thought to be via the formation of an RNA binding

octameric ring.^{183,187,188} However, this ring has not been observed in infectious virions or VLPs and its absence does not affect VLP formation.¹⁸⁹ In other studies, VP40 has been shown to interact with actin, microtubules, and Ras GTPase-activating-like protein (IQGAP1).¹⁹⁰⁻¹⁹³ Actin has been implicated in the movement and assembly of VP40.¹⁹¹ Additionally, VP40 interacts with the COPII protein and Sec24C.¹⁷⁷ Thus, it is important to understand the nature of VP40 membrane interactions, which may be a prerequisite to VLP formation and release and may serve as platforms to develop vaccines and drugs against Ebola viruses.

Ebolavirus proteins in current therapeutics and vaccine development

Earlier attempts to develop vaccines against Ebolaviruses involved the use of formalin-, heat- or gamma-irradiation-inactivated whole virions.¹⁹⁴⁻¹⁹⁵ Unfortunately, these vaccines were largely ineffective in both Non-human primates (NHPs) and rodents. Currently, overexpression of genes that encode EBOV proteins is the primary approach to vaccine development. This approach aims at producing sufficient proteins that elicit potent T and B cell-mediated immunity against EBOVs. Initial vaccine strategies were centred on the overexpression of EBOV GP alone or in combination with NP and other viral proteins because of its well characterised roles in viral entry and ability to facilitate cell death and vascular permeability in Ebolavirus disease.¹⁹⁶ A number of viral and non-viral vectors are utilised to deliver genes for these antigens (viral proteins) in order to stimulate strong innate and adaptive anti-viral immune responses.¹⁹⁶

The first vaccine platform utilising a vector was a recombinant adenovirus type 5 (rAd5) vector expressing EBOV GP.¹⁹⁷ A single intramuscular (IM) injection of this vaccine after three priming doses of plasmid DNA encoding EBOV GP and NP, SEBOVGP, and Tai Ebola virus GP fully protected cynomolgus macaques against lethal EBOV challenge.¹⁹⁷ This approach boosted the levels of circulating anti-GP antibodies and T cell immune responses. Subsequent studies have shown that a single IM injection of the adenovirus vector expressing GP could protect animals from EBOV infections.¹⁹⁸ Furthermore, rAd5-based vaccine has been refined by optimising the GP expression cassette resulting in a 100 fold reduction in dosage and further evidence of protecting mice against EBOV, thirty minutes after exposure to a lethal dose of Ebola.¹⁹⁹ Despite this progress, concerns about rAd5-based vaccines still remain because significant populations across the globe have anti-adenovirus type 5 neutralising anti-bodies in their circulation hence the clinical utility of these vaccines is reduced.²⁰⁰⁻²⁰¹ Other vaccine platforms utilise live attenuated recombinant viruses bearing the EBOV GP. For example, a recombinant vesicular stomatitis virus (rVSV) in which the wild-type surface GP was replaced with EBOV GP showed reduced growth kinetics and tropism of Ebola viruses in vitro.¹⁹⁶ A single dose of this virus fully protected guinea pigs, mice and NHPs from a lethal dose of EBOV and prevented the appearance of clinical symptoms.¹⁹⁵ Recombinant VSV vector is a promising option for post-exposure treatment because a single intraperitoneal injection given 24 h after infection completely protected mice from a lethal EBOV challenge.¹⁹⁶ On the other hand, an inactivated form of VSV (gamma-irradiated) did not offer protection to animals against an EBOV lethal challenge, indicating that replication is key to the potency of this vaccine.¹⁹⁶ However, there are safety concerns

about this virus vector. Another live-attenuated vaccine platform that has been developed and confers full protection in NHPs and guinea pigs following EBOV lethal challenge is the recombinant human parainfluenza virus 3 expressing Ebolavirus GP alone or together with NP.²⁰²⁻²⁰⁴ Like the rAd5-based vaccines, the need for boost immunisation and the issue of pre-existing immunity to this vector is a major limitation of this vaccine platform because human parainfluenza viruses are common respiratory viruses. Chimeric human parainfluenza virus 3 vectors expressing EBOV GP as the lone surface protein have since been developed to avoid the effects of pre-existing immunity on vaccine potency.²⁰⁵ Other vaccine platforms that target the EBOV GP include Venezuelan Equine Encephalitis virus replicon, rabies virus based vaccines and the rare recombinant adenovirus serotypes (rAd26 prime /rAd35 boost).¹⁹⁶ These platforms have been found to confer protection to NHPs after a lethal EBOV challenge even though they all need boost immunisation.

Virus-like particle (VLP) vaccines are also promising vaccine platforms. VLPs depend on the properties of EBOV matrix protein, VP40, to drive budding of filamentous particles after co-expression in mammalian and insect cells.^{40, 206-208} VLPs can also be engineered to incorporate other EBOV proteins such as GP, VP24, and NP.^{40, 206-208} These spherical to filamentous structures are 400–600 nm with a diameter of 70–100 nm and they resemble authentic filoviruses.^{40, 206-208} VLP vaccines use particles containing two proteins (GP and VP40) or three proteins (GP and VP40 plus NP). Previous studies showed the efficacy of bivalent VLP vaccines in rodents, but more recent research has demonstrated that VLP vaccines containing GP, VP40 and NP confers full homologous protection against EBOV in a prophylactic setting in NHPs.²⁰⁹ As alternatives to vector-based vaccine platforms, VLP vaccines have emerged as potential vaccine candidates for several viruses and the recent approval of a VLP based human papillomavirus vaccine is a vital milestone.²⁰⁹ Currently, the VLP based vaccine candidate against filoviruses is an enveloped VLP with GP spikes on the surface of the lipid bilayer holding VP40.²⁰⁹ When NP is included, it is localised in the core beneath VP40. VLPs induce protective anti-filovirus immunity in NHPs unlike inactivated virions that do not induce reliable immunity.^{194-195,209} On the basis of several studies, VLP vaccines appear to be safe and may be effective prophylactic countermeasures for Ebola virus disease.

Virus entry is an important step in the life cycle of Ebola viruses. Inhibition of this process blocks viral replication and significantly reduces the chance of the virus causing severe pathological damage. Compounds that inhibit viral transcription and replication such as small interfering RNAs (siRNAs) bind to sequences within the EBOV polymerase L, VP24, and VP35 regions.²¹⁰ Intravenous administration of these compounds in NHPs and rodents through a seven-dose regimen effectively halted virus replication and protected these animals from a lethal challenge of EBOV. The only concern with these compounds is that their efficacy depends on multiple doses.¹⁹⁶ In a similar approach, recent silencing studies showed that phosphorylation of EBOV VP40 protein by c-Abl1 enzyme is essential for transport of the nucleocapsid complex to the host cell membrane and release of mature virions from the cell.²¹¹ Blocking this process with compounds (imatinib and nilotinib) that target the c-Abl1 enzyme, significantly reduced the amount of complete Ebola virions released in culture

medium.²¹¹The use of phosphorodiamidate morpholino oligomers, third generation synthetic antisense oligonucleotides, is also a very promising therapeutic platform against Ebola virus disease. Post exposure (30-60 minutes) administration of positively charged Ebolavirus-specific phosphorodiamidate morpholino oligomers (AVI-6002) targeting mRNA sequences within EBOV polymerase L, VP24 and VP35 regions fully protected rodents and five out of eight macaques infected with a lethal EBOV by blocking viral replication and subsequent inflammatory responses.²¹²The use of these oligomers is currently under phase I clinical trials but more research needs to be carried out to determine the exact timeframes within which post-exposure treatment with these compounds is effective.¹⁹⁵There are also significant developments and improvements of the antibody (Ab)-based approaches against EBOVs. In a recent study, a cocktail of monoclonal Abs conferred post exposure protection to 43% of monkeys when given 5 days after an EBOV lethal challenge.²¹³This cocktail of monoclonal Abs targeted the GP of EBOVs. Another therapy that combines monoclonal Abs with IFN- α provided almost complete protection of macaque monkeys when given 3 days post exposure.²¹⁴ Furthermore, Qiu et al, 2013, reported that an Ab therapy known as ZMapp that does not require IFN- α provided 100% protection to 3 groups of animals post exposure to a lethal dose of the Kikwit variant of EBOV. ZMapp contains a combination of chimerichumanised monoclonal Abs (in which fragments of human Abs are attached to Ab fragments from mice). ZMapp is produced in *Nicotiana benthamiana*, a close relative of the tobacco plant. According to Geisbert, 2014, the optimal formulation of ZMapp contains two Abs from a previously reported mix and a third from a different Ab cocktail.²¹³⁻²¹⁴ So far, ZMapp has been used to treat several Ebola virus disease patients on compassionate grounds in Spain, and the United states. ZMapp is currently undergoing clinical trials to determine its safety and efficacy in humans.

Towards development of effective therapies against Ebolaviruses

Each Ebolavirus protein has its own unique mechanism(s) of contributing to the ability of this virus to suppress or evade the host immune system. Nucleoprotein (NP), L protein, VP30 and VP 40 are mainly responsible for transcription and replication of Ebolaviruses. VP40 has an additional functions of maintaining structural integrity of the virus and also serves to drive the formation of virus like particles. These four proteins do not have known or welldefined suppressive mechanisms on the host immune system unlike VP24 and VP35. The two viral proteins, VP35 and VP24, are well characterised Ebola proteins that directly and indirectly inhibit the host IFN signalling pathways and subsequent adaptive T-cell immune responses. Therefore any anti-Ebola drugs or vaccines that target these immunosuppressive proteins hold the key to effective treatment of Ebolavirus disease.

a). Vaccines and antibody based therapies targeting GP

A number of virus vector based vaccines such as recombinant human parainfluenza virus 3, rAd5, rVSV, Venezuelan Equine Encephalitis virus replicon, and rabies virus based vaccines have been developed with the goal of stimulating Abs and T-cell immune responses against GP. GP is a cell surface glycoprotein responsible for receptor binding and fusion of the virus with host cell membranes. The major weakness with many of the current vaccines, Ab based therapies, and drugs being developed to treat Ebola or prevent infection and spread of the

disease is their sole focus on the Ebola Glycoprotein (GP) as the principal immunogenic target. They leave out other major proteins that make the Ebolavirus more virulent. In addition, the weaknesses of these vaccines does not only lie in their failure to induce innate and adaptive immune responses against other Ebola proteins rather GP but also in their safety, efficacy that depends on multiple doses, and issues of pre-existing immunity against the virus vectors used to carry the potential vaccines. Overall, vaccines that solely focus on the GP to induce anti-viral immunity are weakened further by the ability of Ebolaviruses to secrete a non-structural soluble GP (sGP) that serves as a decoy to Abs. Consequently, the virus is able to escape the wrath of Ab immunity. Moreover, it has been suggested that infection of host cells by Zaire Ebolavirus is enhanced by the complement protein (C1q) and antibodies to the viral glycoprotein.²¹⁵ Other studies have also demonstrated that Abs from Ebola survivors have a preference for sGP than GP probably because sGP is released in large amounts as a decoy.⁹⁵ In another study, EBOV IgG positive sera obtained from asymptomatic individuals during outbreaks of Ebolavirus disease in Gabon reacted strongly to VP40 than to GP, NP while Sera from most survivors reacted mainly with GP peptides.¹⁷³ This study suggested that Ab immunity against GP may help one to survive the disease but preventing the virus from causing disease may require a strong Ab immunity against VP40. To prove this theory, similar experiments must be carried out in survivors and asymptomatic individuals from the current Ebola outbreak in West Africa. This will assist with developing Ab concoctions that may be more effective by preventing the virus from causing disease and increasing the survival rate in infected people. It will also help researchers to verify whether the use of serum from Ebola survivors to treat the disease may be more effective if used in combination with serum from asymptomatic individuals. In that way, the concoction will contain a variety of Abs that will target the majority of Ebolavirus proteins.

Two Ab based therapies, one that combines monoclonal Abs with IFN- α , and another Ab therapy without IFNs, targeting the Ebola GP, have been shown to provide full or almost full protection to non-human primates.²¹³⁻²¹⁴ These therapies are still under human clinical trials and they look promising even though not all the people treated with these Ab therapies on compassionate ground have survived the disease. In the light of the fact that more than 50% of Ebola infected individuals in the current Ebola outbreak survive the disease, it is not yet confirmed that these Abs based therapies really work until after the clinical trials. We could be wrong by suggesting that any Ab based therapies must be broad based concoctions, targeting all the major immunosuppressive proteins of Ebolaviruses. However, studies on IgG positive sera from Gabon Ebola survivors and the ability of the virus to deceive Abs through the sGP protein convict us that this is the surest way to developing effective anti-Ebola therapies. This emphasis is not aimed at undermining the excellent work that other researchers have done but it is simply a suggestion towards making Ab based therapies more potent. Overall, the role of Ab responses in the control of Ebola cannot be overemphasised hence several routes and innovations must be explored in order to develop effective concoctions of Abs. Fatal cases of Ebola are associated with impaired humoral responses while recovery is associated with early and long lasting Ebolavirus specific IgG, followed by viral antigen clearance.¹⁷³

b). VLP based Ebola vaccines

Unlike viral vector based vaccines, virus-like particle (VLP) vaccines are one of the most promising vaccine platforms. They are safe and they are not affected by pre-existing immunity which is associated with other vaccines. VLPs depend on the properties of EBOV matrix protein, VP40, to drive budding of filamentous particles after co-expression in mammalian and insect cells.^{40, 206-208} Currently, potential VLP vaccines incorporate GP, VP40, and NP.^{40, 206-208} On the basis of the findings that Abs from asymptomatic individuals react strongly to Ebola VP40 than other proteins, we expect VLP to be more effective. Probably it is the reason why VLP based vaccines are the most promising therapy. However, the potency of these vaccines may be increased if VLPs are engineered to incorporate VP24 and VP35, the two most immunosuppressive Ebola proteins. In that way, the expected humoral immunity will have a broader but more specific targets leaving no chance for the virus to escape. The potency of VLP vaccines containing GP, VP24, VP35, VP40 and NP has not been studied at all. We believe that such a vaccine could provide the most potent therapy ever because innate and adaptive immune responses arising from such a vaccine would target all the major proteins of Ebolaviruses. This suggestion is based on the theory that such a VLP vaccine will stimulate the production of Abs and adaptive immune responses that have a wider target thereby increasing the chance of controlling Ebolaviruses and subsequent elimination of the virus.

c). Phosphorodiamidate morpholino oligomers and small interfering RNAs (siRNAs)

Analysis of Ebolavirus proteins and the mechanisms they employ to cause disease by suppressing the immune system has revealed that VP24 and VP35 are the key Ebola proteins responsible for much of the innate and adaptive immune suppression/evasion activities associated with Ebolavirus disease. Therefore, development of effective anti-Ebola therapies lies in the development of drugs and vaccines that target VP24 and VP35. It is therefore not surprising that Ebolavirus-specific phosphorodiamidate morpholino oligomers and small interfering RNAs (siRNAs) that target sequences within the regions of these two EBOV proteins, have been found to fully protect rodents and NHPs from lethal challenges of Ebola.^{196, 212} The success behind the use of siRNAs and oligomers in NHPs is their ability to prevent VP24 and VP35 from inhibiting the maturation of dendritic cells, mitogen activated pathways, IFN signalling pathways and inhibition of subsequent adaptive immune responses. This indicates that in order to overcome Ebolaviruses, all potential drugs must be aimed at these two proteins. The weakness of phosphorodiamidate morpholino oligomers and small interfering RNAs (siRNAs) is that they should be administered approximately 30-60 minutes after infection with Ebolaviruses and they require multiple doses (in the case of siRNAs). In the natural world, it is not possible to know when a person gets infected with Ebola. Moreover, Ebola has an incubation period of 21 days. The main challenge is to improve the timings of these drugs so that they can become effective several days after infection with Ebola otherwise, their potential efficacy will be limited to laboratory conditions. Therefore, we suggest that these drugs should be used in combination with other therapies in order to be more effective and reliable. Safety concerns about these potential therapies should also be addressed.

d). Interferons and Tetherin

IFNs play critical roles in direct antiviral immunity as well as linking innate and adaptive immune responses, thus, their role in controlling Ebola cannot underestimated. Ebolaviruses have devised mechanisms through VP35 and VP24 to inhibit all the IFN signalling pathways. It is therefore essential that exogenous IFNs should be part of any treatment against these viruses. This is suggestion to use exogenous IFNs in combination with other potential therapies is based on the evidence that VP35, the major IFN inhibitor, only inhibits virus-induced activation of type I IFN promoters but does not regulate the activation of IFN promoters through exogenous interferons.¹³⁷ The use of exogenous IFNs may also curtail the ability of VP24 and VP35 to prevent phosphorylation and nuclear translocation of transcription factors and the resultant inhibition of IFN expression.¹³⁶⁻¹³⁷ It is well established that IFNs play a role in the maturation and immunogenicity of dendritic cells (DCs).²¹⁶ However, the maturation of these cells is blocked by Ebola VP35 resulting in the inability of DCs to release pro-inflammatory cytokines that are needed to activate T-cell adaptive immune responses.¹⁵⁴ It is hoped that the suggested use of exogenous IFNs in combination with other therapies may help activate the suppressed maturation of DCs through a positive feedback mechanism and induce the expression of another antiviral protein known as tetherin. This theory is supported by Blanchet et al (2013) who demonstrated that expression of tetherin in immature myeloid and monocyte derived DCs is upregulated by IFN- α treatment and TLR-4 engagement with liposaccharides (LPS).²¹⁷

Tetherin, also known as BST-2 or CD317, is a type I IFN induced, transmembrane protein. It is a restriction factor that has been demonstrated to block the release of vesicular stomatitis virus, hepatitis C, Lassa viruses, HIV-1 and other retroviruses from infected cells by trapping virions at the plasma membrane of certain producer cells.²¹⁸ Kaletsky and other showed that tetherin functions as a broadly acting antiviral factor.⁸¹ They demonstrated that tetherin also potently inhibits the release of Ebolaviruses from the surface of infected cells. However, expression of the Ebolavirus GP antagonises the antiviral effect of tetherin and facilitated budding of Ebola particles.⁸¹ Clearly, tetherin is a critical anti-viral immune response protein that may serve as one of the potential agents to treat Ebola in combination with other therapies such as IFNs, phosphorodiamidate morpholino oligomers and small interfering RNAs (siRNAs).

e). Proposed treatment combinations for Ebolavirus disease

Based on analysis of the structure and mechanisms of action of Ebolavirus proteins and the various anti-Ebola therapies that are currently being developed or researched upon, we have realised that the fight against Ebola cannot be won using single therapies but combination of therapies. Each of the therapies that are being developed have their own weakness and strengths. The strengths and weaknesses of each of these therapies have been clearly outlined above. Therefore, the use of combined therapies may help deal with the inadequacies of some of these vaccine or drugs. The following combinations of therapies are proposed subject to in vitro experiments, testing in NHPs and subsequent human clinical trials:

- i. Phosphorodiamidate morpholino oligomers or siRNAs + monoclonal Abs
- ii. Phosphorodiamidate morpholino oligomers or siRNAs + IFNs

- iii. Phosphorodiamidate morpholino oligomers or siRNAs + concoction of monoclonal Abs +IFNs
- iv. Phosphorodiamidate morpholino oligomers or siRNAs + concoction of monoclonal Abs +IFNs +Tetherin
- v. Concoction of monoclonal Abs +IFNs +Tetherin

These proposed combinations can only work successfully if people living in endemic areas are vaccinated during the absence of an Ebola outbreak. Potential VLP based vaccines, carrying VP40, NP, VP35, VP24 and GP, offer the best alternative to the current vaccines that either under clinical trials or being researched upon in the Laboratory. It is possible that the combinations listed above may have adverse side effects thus we recommend that significant amount of work must be done in order to ascertain their safety. Furthermore, these combinations may already have been tried by other researchers but this review offers a significant platform to debate these ideas and provide other researchers a chance to conduct further research on these proposed therapies.

f). Bats in Ebola therapy research

Humans initially contract the Ebolavirus either through contact with infected reservoir, which is currently thought to be fruit bats, or by contact with infected wildlife, particularly Chimpanzees.² Detection of Ebola viral RNA nucleotide sequences and antibodies specific for Ebolavirus in three tree-roosting fruit bat species [Epomops franqueti, Hypsignathus monstrosus (the hammer-headed bat), and Myonycteris torquata (the little-collared bat)] near the areas where Ebola virus disease outbreaks occurred provided the first evidence for the potential presence of Zaire Ebolavirus in naturally infected fruit bats.²¹⁻²³ Much research has been focused on the prevalence of Ebola Abs in fruit bats. However, such research should be extended to determining the type of proteins that are specifically prioritised by bat antibodies to prevent the occurrence of clinical Ebolavirus disease after exposure of Ebolaviruses. This can be achieved by testing all the Ebolavirus proteins isolated from humans against the antibodies isolated from fruit bats. With such information, we can then design more specific Ab concoctions that can be used for treating Ebola in humans. If there is a cross reaction between bat Abs and human specific Zaire Ebolavirus, it could provide further information that the Ebolaviruses that infect both humans and bats may be the same in the light of the fact that no Ebolavirus has ever been isolated from bats. Bats may also hold the key to understanding how tetherin is utilised to control Ebola. As indicated earlier, it would be interesting to determine whether African fruit bats also encode a tetherin-like protein and whether this tetherin homologue restricts EBOV spread in bats to prevent occurrence of clinical disease. This thought is based on the established fact that EBOV GP displays similar biological properties in both human and bat cells.⁴⁶ Other interesting experiments can be carried out to compare how bat specific IFNs and human specific IFNs act to control Ebolaviruses in vitro. This could establish the missing links in the way human specific IFNs are easily suppressed by Ebolavirus proteins.

Conclusion

The fight against EBOVs through the development of potent vaccines and drugs relies on adequate understanding of the structure, function and mechanism of action of EBOV proteins. The current anti-Ebola therapies that are incapable of fighting the disease if used alone without combining with other therapies. Therefore, combining these therapies may be the only way to get the best out of these drugs, Ab based therapies, and vaccines. Furthermore, the search for a cure for Ebola must be extended to fruits bats in order to understand how they deal with Ebolaviruses to prevent occurrence of clinical Ebola.

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