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Filovirus pathogenesis and immune evasion: insights from Ebola virus and Marburg virus

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Abstract

Ebola viruses and Marburg viruses, members of the filovirus family, are zoonotic pathogens that cause severe disease in people. The Ebola virus epidemic in West Africa, which was first recognized in early 2014, highlights the threat posed by these deadly viruses. Filovirus disease is characterized by uncontrolled virus replication and the activation of damaging host pathways. Underlying these phenomena is the potent suppression of host innate antiviral responses, particularly the type I interferon (IFN) response, which allows high levels of replication. Here we review the mechanisms deployed by filoviruses to block host innate immunity and discuss aspects of virus replication that promote disease.

Introduction

In March 2014, cases of a severe, frequently lethal illness with symptoms that included fever, severe diarrhea and vomiting were reported to be occurring in the West African country of Guinea[1]. The causative pathogen was determined to be Ebola virus (EBOV), a member of the filovirus family of enveloped, negative-strand RNA viruses noted for their ability to cause outbreaks of severe, often fatal, viral hemorrhagic fevers[2,3]. Analysis of viral genome sequences and epidemiological investigation of the viruses from Guinea suggested an index case in a 2-year old child in December of 2013 with subsequent transmission of the virus to the prefectures of Guéckédou, Macenta, and Kissidougou[1]. The outbreak subsequently expanded into neighbouring Sierra Leone and Liberia. Consequent in-depth analysis of 78 EBOV sequences from Sierra Leone, obtained by using deep sequencing approaches, helped trace the spread of EBOV from Guinea and shed further light on transmission dynamics and genetic variation as the virus spread in the human population through mid-June 2014[4]. After a period of exponential spread in West Africa, the transmission of Ebola infections has generally decreased, but has not ended. As of May 10, 2015 the WHO reported a total of 26,759 confirmed, probable and suspected cases of EBOV

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disease and 11,080 deaths across 9 countries: Guinea, Liberia, Sierra Leone, United States, Mali, Nigeria, Senegal, Spain, and the United Kingdom, although the vast majority of cases have been in West Africa[5]. This outbreak of Ebola virus disease (EVD) is unique in that it is by far the largest outbreak on record and the first outbreak of EVD in West Africa (see Box 1 for description of past filovirus outbreaks).

The virus family *Filoviridae* is divided into three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*[6]. Within the ebolavirus genus are five species: Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Bundibugyo ebolavirus (BDBV), Tai Forrest ebolavirus (TAFV) and Reston ebolavirus (RESTV). The marburgvirus genus consists of a single species, Marburg marburgvirus (MARV). The genome of another filovirus, Lloviu virus, which is the only member of the Cuevavirus genus, was demonstrated to be present in bats in Northern Spain, but this virus has not yet been cultured[7]. EBOV is responsible for the current West Africa outbreak and along with SUDV, BDBV and MARV has caused past outbreaks of human disease; TAFV has only been associated with a single, non-fatal human infection[8]. RESTV appears to originate from the Philippines and has been isolated from non-human primates and pigs; infected non-human primates have been exported from the Philippines to other countries[8]. Humans exposed to RESTV have not become ill, suggesting that RESTV is significantly attenuated in humans compared to other filoviruses[9]. Whether Lloviu virus is hazardous to humans is not yet known. As is discussed below, filovirus pathogenesis is best characterized for the EBOV species, particularly in non-human primates. Current models suggest that potent suppression of host innate immune responses by filoviruses promotes high levels of systemic virus replication and that this triggers potent inflammatory responses that lead to severe manifestations of disease such as high fever, vascular leakage and bleeding.

The filoviruses are suspected to use bats as reservoir hosts, although current evidence is stronger for MARV than for EBOV (Box 2). As might be expected for a reservoir host, filoviruses do not appear to be overtly pathogenic in bats, despite the high virulence of these viruses in humans and non-human primates[10,11]. The absence of fatal disease following filovirus infection is not a property unique to bats. For example, experimentally-infected guinea pigs and mice do not die of filovirus infection, although serial passage of EBOV or MARV in these species can select for viruses that are lethal to these animals[12–15]. The factors that determine whether or not a filovirus is pathogenic in a particular host are not well-defined, although the type I interferon (IFN) response plays a role in containing infections in mice[16].

An overview of the filovirus genome and replication cycle is provided in Figure 1[3]. Filoviruses possess single-stranded, negative-sense RNA genomes of approximately 19 kilobases. They have seven distinct genes, each of which functions as a separate transcriptional unit (F). These encode seven viral structural proteins known as nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and the large protein (L). For the ebolaviruses, the GP gene encodes multiple different proteins due to transcriptional editing by the viral RNA-dependent RNA polymerase (RDRP) where non-template encoded “As” are added co-transcriptionally to the nascent mRNA. The unedited transcript encodes sGP, while editing results in translational frameshifting such that the

membrane-bound GP and a second secreted GP are produced from edited RNAs[3,17,18]. Additional editing events recently described for both EBOV and MARV mRNAs may further diversify the proteins translated from filovirus mRNAs[19].

With the exception of EBOV sGP, each viral protein is present in the enveloped viral particle. GP is a type I transmembrane protein and is the only viral protein exposed on the surface of viral particles. GP mediates entry into host cells, acting as the attachment and fusion protein. Within the cytoplasm, viral RNA synthesis occurs on the uncapped, non-polyadenylated template viral RNA, which is associated with the NP protein. NP, VP35, VP30 and L proteins are all required for viral RNA synthesis. L is the enzymatic component of the RDRP complex and in addition to RDRP activity possesses methyltransferase and guanyltransferase activities needed to 5′ cap viral mRNAs. VP35 acts as a viral polymerase cofactor, interacting with both L and NP, to deliver L to the NP-encapsidated template. VP30 is required for viral transcription (mRNA synthesis). The phosphorylation state of VP30 may also regulate the transcriptase and replicase functions of the viral polymerase complex. Tubular nucleocapsids form in the infected cell cytoplasm, and transfection-based studies indicate that these consist of RNA, NP, VP35 and VP24. The nucleocapsids are delivered to sites of viral assembly and release at the plasma membrane. Virus release occurs by budding and involves interactions of VP40 with components of the cellular ESCRT pathway. The VP40 protein is sufficient to bud on its own but budding efficiency is enhanced by other viral proteins, including GP, which reaches the plasma membrane. As is discussed in detail below, features that contribute to the severity of filovirus infections in humans and non-human primates include robust systemic virus replication and excessive inflammatory responses. This leads to the manifestations of filoviral disease, including high fever, vascular leakage and coagulopathy[2]. Underlying the uncontrolled replication is a potent suppression of innate antiviral defenses by filovirus gene products[20]. Below we discuss how several filoviral proteins counteract host defenses. Examples include the EBOV and MARV virus VP35s (eVP35 and mVP35, respectively), EBOV VP24 (eVP24) and MARV VP40 (mVP40) that disable specific innate immune signaling pathways and the GP protein that counteracts the antiviral effects of the cellular protein tetherin[21–25]. We also describe how these viruses have evolved mechanisms to take advantage of host pathways to increase the life of infected cells and promote optimal levels of viral protein translation.

Filovirus pathogenesis

Human infections are acquired by direct contact with infected bodily fluid and likely enter the human body via breaks in the skin or through mucosal surfaces[26]. Studies using nonhuman primates, the gold standard animal model for studying EBOV disease, have provided significant insight into the pathogenesis of filovirus infections. Immunohistochemistry and in-situ hybridization demonstrate that filoviruses can infect many cell types including macrophages, monocytes, dendritic cells (DCs), Kuppfer cells in the liver, fibroblasts, hepatocytes, adrenal gland tissue, endothelial cells and epithelial cells[27,28]. Time-course studies of infection with EBOV show that it preferentially and initially replicates in macrophages and DCs[28]. This conclusion is consistent with histopathology studies performed on human tissues[29]. A definitive basis for the preferential infection of these cells is lacking. However, Ebola virus has been demonstrated to effectively

infect macrophages and DCs following their differentiation from monocytes[30–32]. Differentiation correlates with the upregulation of cellular cathepsins and the protein Niemann-Pick C1 (NPC1), factors critical for GP-mediated membrane fusion[32–36]. Further, specificity for macrophages and DCs may be promoted through the interaction of virus with cell surface attachment receptors including dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and phosphatidylserine binding proteins[27,28,37–40]. Infection of these mobile cells allows the transport of the virus via lymphatics to lymph nodes and via blood to the liver and spleen, where the virus can infect resident tissue macrophages, including Kuppfer cells and DCs[27,28]. *In vivo*, filoviruses trigger the release of proinflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-10, MCP-1, MIP-1α, MIP-1β and TNFα, in addition to reactive oxygen species and nitric oxide[41–46]. This likely reflects production of these factors by infected macrophages[43,47–49]. Cytokines MIP-1α and MCP-1 can recruit additional macrophages to areas of infection, allowing EBOV to infect more target cells[28]. Infection of DCs inhibits their maturation and prevents antigen presentation to T cells. *In vitro* studies have shown that EBOV infection inhibits upregulation of co-stimulatory molecules CD40, CD80, CD86 and MHC class II[50–52]. These DC-suppressing functions have been linked to suppression of innate immune signaling pathways by viral proteins (see below)[50,52–54]. Exactly to what extent inhibition of DCs influences adaptive immune responses remains to be seen as EBOV-infected mice and people who survive infection have been demonstrated to develop adaptive immune responses to viral antigens[55,56].

In addition to disrupting the innate immune branch, EBOV infection has consequences for B and T cell responses. Lymphopenia has been detected following infection with EBOV and MARV[57]. For EBOV this occurs most notably among the CD4 T cells, CD8 T cells and NK cell populations in mouse models (2–3 dpi) and in nonhuman primates (4 dpi)[58,59]. Similarly, EBOV infection results in the loss of human CD4+ and CD8+ T cells *in vitro* 4 days post infection[60]. Apoptosis likely contributes to loss of lymphocytes, and several mechanisms have been proposed to explain this phenomenon[43,46,57,61–65]. The consequences of lymphopenia for pathogenesis are not fully elucidated, and in a mouse model, prevention of lymphocyte apoptosis did not prevent death in EBOV-infected mice[62]. However, loss of CD4+ T cells, which are required for isotype class switching, could result in the observed absence of EBOV specific IgM and IgG antibodies in case fatalities[61]. Another way in which EBOV may modulate B cell responses is via the sGP protein, which may redirect GP-specific antibody responses to non-neutralizing epitopes[66].

Hemorrhage and intravascular coagulation are features of severe EBOV infection. EBOV-induced paralysis of an effective host response may facilitate viral dissemination to hepatocytes, adrenal cortical cells and endothelial cells of connective tissue[28]. Hepatocellular necrosis may result in the decreased synthesis of coagulation proteins while adrenocortical infection and necrosis may negatively affect blood pressure homeostasis, potentially leading to hemorrhaging[28]. The increased vascular permeability and vascular leakage that typically lead to hemorrhagic fever could be attributed to the large release of TNFα from infected macrophages, which can increase endothelial permeability[43]. Similarly, the release of nitric oxide, which is an important effector molecule in the homeostasis of the

cardiovascular system, may result in loss of vascular smooth-muscle tone and hypotension[44,67].

More directly, studies in nonhuman primates show that coagulation abnormalities are initiated early during EBOV infection. A dramatic decrease in plasma levels of anticoagulant protein C occurred early in the coagulation cascade (~2 dpi). This was followed by an increase of both tissue plasminogen activator (tPA), which is involved in dissolving blood clots, and fibrin-degradation products (D-dimers) at day 5 post-infection[68]. In addition, EBOV infection of macrophages leads to the upregulation of tissue factor (TF) on the surface of monocytes and macrophages and the release of membrane microparticles that express TF. These likely promote the over-activation of the extrinsic pathway of coagulation and may promote disseminated intravascular coagulation[68]. It is postulated that expression levels of TF may be further upregulated by proinflammatory cytokines such as IL-6 that are abundant during acute EBOV, thereby exacerbating the intravascular coagulation phenotype[69]. Thrombocytopenia and prolonged pro-thrombin time are also indicators of dysregulation of blood coagulation. Fibrinolysis during filovirus infection may manifest as petechiae, ecchymoses, mucosal hemorrhages, and congestion[68,70]. The compounding effects of the increase of pro-coagulation factors and decrease of anti-coagulant factor Protein C could plausibly explain the activation of coagulation and hemorrhagic features characteristic of filovirus infection. Towards the terminal stage of infection and after the onset of hemorrhagic abnormalities, EBOV replicates in endothelial cells[27]. However, while endothelial cells are thought to play a role in pathogenesis, the molecular mechanisms that affect endothelial function are not yet fully understood[27,71–74].

The lethality of filovirus infection is likely due, in part, to the ability to replicate largely unimpeded by the host innate antiviral responses. This innate immune evasion probably facilitates the excessive virus replication that activates the damaging host responses outlined above. Underlying this robust growth are multiple mechanisms that allow filoviruses to counteract the host innate antiviral defenses, particularly IFN responses[20]. The type I IFNs (IFNβ and multiple IFNαs) constitute the major components of the innate immune response to viral infections[75]. IFNα/β expression can be triggered via signaling from pattern recognition receptors, including the RIG-I-like receptors (RLRs), which include RIG-I, MDA5 and LGP2, and select toll-like receptors (TLRs). The RLRs are cytoplasmic RNA helicases that detect viral RNAs and promote IFN-α/β responses. Of these, the RLRs RIG-I and MDA5 appear most relevant to filovirus infection. Activated by “immunostimulatory RNAs,” such as RNAs with 5′-triphosphates and double-stranded RNAs that may be produced during filovirus replication, RIG-I and MDA5 activate the Ser/Thr kinases TBK-1 and IKKε that phosphorylate IRF-3 and IRF-7. This promotes their nuclear accumulation and stimulates IFN-α/β gene expression. When expressed, IFNαβ is secreted from cells and signals via a heterodimeric receptor, the IFN-α/β receptor. This activates a JAK-STAT signaling pathway and induces expression of numerous genes, triggering an antiviral state[76].

EBOV and MARV infections effectively counter this critical antiviral defense system, blocking both the production of and the cellular responses to exogenously added IFNs[25,47,77–79] (Figure 2). That this inhibition is important is suggested by the facts that EBOV genomic RNA can activate RIG-I, activation of RIG-I signaling before EBOV

infection substantially reduced virus growth and recombinant viruses unable to block IFN responses are attenuated both in cell culture and in experimentally-infected animals (discussed further below)[16,80–83]. The mechanisms by which IFNα may exert its anti-filoviral effects have not been systematically studied, but several proteins encoded by IFN-stimulated genes (ISGs) exert anti-filoviral effects in cell culture. For example, interferon-induced transmembrane (IFITM) proteins inhibit filovirus entry into host cells and ISG15 is reported to impair budding by VP40[84,85]. Nonetheless, while IFNs can control infection in rodent models, filoviral infections are not controlled by host defenses in non-human primates and death of the host frequently ensues[14,16,86,87].

VP35 has multiple roles in suppressing cytosolic sensing

EBOVs and MARVs very effectively impair production and this is largely mediated by the VP35 protein. VP35 is multifunctional, serving not only as an antagonist of the innate immune response, but also functioning as a non-enzymatic cofactor for the viral RNA-dependent RNA polymerase and as a viral structural protein. The VP35 proteins of EBOV and MARV block signaling through RIG-I-like receptors and prevent the phosphorylation of IRF-3 and IRF-7, thereby short-circuiting the IFN response[88]. These inhibitory activities likely reflect inhibition at both upstream and downstream steps in the RLR signaling cascades. Downstream targets include the kinases IKKε and TBK1; VP35 has been demonstrated to interact with and act as a decoy substrate for these kinases such that IRF-3 interaction with the kinase is blocked and VP35 is phosphorylated instead[89]. VP35 is also reported to interact with IRF-7, SUMO conjugating enzyme Ubc9 and SUMO E3 protein ligase PIAS1 to promote SUMOylation of IRF-7, a modification that impairs IRF-7-dependent transcriptional responses[90,91]. Inhibitory activities upstream of RIG-I are connected to the dsRNA binding capacity of VP35, which resides in a carboxy-terminal domain referred to as the interferon inhibitory domain (IID)[92]. While wild-type VP35 (wtVP35) can bind dsRNA and suppress RLR-mediated activation of IFN responses induced by Sendai virus infection or by transfected dsRNAs, VP35 point mutants that lack dsRNA binding activity are greatly reduced in suppressive activity[53,93–95]. These data suggest a model where VP35 sequesters RLR-activating dsRNAs. Supporting this view, purified wild-type VP35, but not VP35 dsRNA binding mutants, can prevent dsRNA activation of RIG-I ATPase activity *in vitro*[96].

VP35 was also demonstrated to interact with the cellular protein PACT, a multifunctional protein that was originally described as a protein activator of the IFN-induced antiviral kinase, PKR[97]. PACT has also been shown to interact with and facilitate the activation of RIG-I[98]; VP35 blocks this interaction, thereby preventing PACT activation of RIG-I both in cells and *in vitro*. Interestingly, the interaction of VP35 with PACT can occur in the absence of dsRNA, but requires the same basic patch that mediates interaction with dsRNA[96]. These basic amino acid residues are capable of participating in either protein-dsRNA or protein-protein interactions. In the X-ray crystal structure of VP35 complexed with dsRNA, these residues were found to contact the RNA phosphodiester backbone and to facilitate VP35-VP35 contacts[95,99]. VP35 point mutants lacking dsRNA binding activity failed to interact with PACT and failed to block PACT-mediated activation of RIG-I. Demonstrating the relevance of PACT for EBOV infection, PACT over-expression overcomes the inhibitory

effects of VP35 and triggers an IFN-response in wild-type EBOV infected cells; on the other hand, the IFN response induced by EBOV carrying a mutated VP35 is decreased in PACT knockdown cells[96]. In sum, current models suggest that VP35 can inhibit RLR responses both by the sequestration of immunostimulatory dsRNAs and through interaction with PACT. These inhibitory mechanisms rely on the VP35 IID and contribute to the potent suppression of the IFNα/β response in filovirus-infected cells.

In addition to suppressing the production of IFNα/β, VP35 inhibition of RLR signaling suppresses maturation and function of DCs. As noted above, DCs are important targets of filovirus infection *in vivo* and are productively infected[28,50–52]. The role of DCs is to stimulate adaptive immune responses upon encountering pathogen[100], which requires that the DCs undergo a "maturation" process triggered by innate immune signaling pathways. However, *in vitro* infection of DCs with filoviruses results in aberrant DC maturation[50–52]. In contrast, EBOVs with mutant VP35s do induce DC maturation. Further, the expression of eVP35 in DCs recapitulates the suppressive effects of wild-type EBOV infection, with the inhibitory effects being mediated in large part by VP35 RLR inhibitory activity[50,52–54,101]. Therefore, the impact of VP35 RLR suppression likely extends beyond IFNα/β production and also impairs activation of adaptive immune responses.

Structural determinants of VP35 binding to dsRNA

The dsRNA binding activity of both EBOV and MARV VP35 has been explained by structural and biophysical methods. Overall, VP35 contains two identifiable domains, an N-terminal oligomerization domain and a C-terminal "interferon inhibitory domain (IID)." VP35 residues 220–340 were defined as the minimal region important for dsRNA binding and IFN inhibition using a combination of limited proteolysis and nuclear magnetic resonance (NMR)-based studies[92]. Consistent with this finding, the C-terminal IID is sufficient for suppression of IFN production, but the addition of the oligomerization domain enhances inhibition[102]. The structure of the EBOV VP35 IID, which has an alpha-helical subdomain and a beta-sheet subdomain, contains two basic regions. These regions, termed the first basic patch (FBP) and the central basic patch (CBP), are functionally important, with the latter playing a role in dsRNA binding and IFN inhibition[92,95,103]. Comparison of EBOV, RESTV and MARV VP35 IIDs reveal remarkable similarities in the overall structure as well as the highly conserved molecular surfaces[23,24,95,103]. Moreover, evaluation of the RESTV and MARV IIDs confirms that the CBP is conserved, whereas EBOV has the most extensive charge on the CBP[95,104]. In contrast, the FBP residues, which are conserved among ebolaviruses, appear to be important for virus replication, but not for dsRNA binding or IFN inhibition[105].

Despite the structural and functional similarities described above, the EBOV and MARV IIDs also show differences in their biochemical and structural characteristics. For example, *in vitro* EBOV VP35 (eVP35) can bind dsRNA with >8 bp (Figure 2B, left) and RESTV VP35 (rVP35) can bind dsRNA with >12 bp (Figure 2B, middle), whereas MARV VP35 (mVP35) binds to dsRNA with at least 12 bp[23] (Figure 2B, right); for dsRNAs >12 bps, eVP35 binds with greater affinity than mVP35. Data also suggests that eVP35 and mVP35 show significant differences in how they bind dsRNA: eVP35 displays two different binding

modes, recognizing both blunt ends of dsRNA and the dsRNA backbone, whereas mVP35 IID can only recognize the dsRNA backbone (Figure 2B). Because the 5′-triphosphate plays a critical role in RIG-I activation, the ability of eVP35 to mask dsRNA ends may provide eVP35 with an increased capacity to block IFN production.

Subversion of IFN-induced signaling

In addition to preventing IFNα/β induction, EBOVs and MARV block the cellular response to IFNs. As a consequence, IFN-induced gene expression is substantially impaired in EBOV- and MARV-infected cells[78,79]. In uninfected cells, IFNα/β addition activates a Jak-STAT signaling pathway in which receptor-associated Jak family kinases Jak1 and Tyk2 are activated. These kinases phosphorylate STAT1 and STAT2 (PY-STAT), which dimerize via phosphotyrosine-SH2 interactions[106]. The phosphorylation/dimerization reveals a non-conventional nuclear localization signal (ncNLS) on STAT1 that can interact with any of the three members of the NPI-1 subfamily of karyopherin alpha (KPNA) proteins, which comprises KPNA1, 5 and 6[107]. The interaction of PY-STAT1 with KPNAs mediates the nuclear import of the STAT protein dimers[107].

EBOV VP24 interferes with STAT nuclear entry

In EBOV-infected cells, IFNα/β trigger STAT phosphorylation; however, PY-STAT1 nuclear entry is impaired by eVP24[22]. eVP24 interacts with the same NPI-1 subfamily of KPNAs that are bound by PY-STAT1 and mapping studies suggest that eVP24 occupies the PY-STAT1 binding site on KPNA, thereby acting as a competitive inhibitor of PY-STAT1[108]. Structural and biochemical studies support this competition model of eVP24 binding to KPNA. eVP24 forms a pyramid-shaped tertiary structure with a series of α-helices resting on a β-sheet pedestal[109,110] (Figure 3AB, left). The KPNA proteins possess an N-terminal importin beta binding domain, ten armadillo (ARM) repeats and a C-terminal domain. Whereas proteins with classical, basic nuclear localization signals (cNLSs) bind to the central ARM repeats of KPNAs, PY-STAT interacts via its ncNLS with C-terminal regions of the NPI-1 KPNAs including ARMs 8–10. A recent study generated a stable KPNA construct (KPNA5C), which contains ARMs 7–10[111]. The structure of KPNA5C in complex with eVP24 (Figure 3B, right) identified three regions within eVP24, termed clusters 1, 2, and 3, that interact with KPNA5C ARMs 8, 9, and 10, respectively. Moreover, residues within KPNA5C identified as critical for eVP24 binding were also important for STAT1-KPNA binding, directly correlating binding with inhibitory activity. Thus, eVP24 targets well-conserved, functionally significant interactions to prevent the host response[111]. This same study also revealed that the KPNA binding site of monopartite cNLSs is largely unperturbed by eVP24 binding. Therefore, eVP24 may preserve nuclear import of such cargo. However, bipartite cNLSs as well as non-classical NLSs may be affected by eVP24-KPNA binding[111,112]. Further work will be required to determine if eVP24 mainly affects STAT1 or if import of other cargo is also affected.

mVP40 blocks JAK1 signaling

In contrast to eVP24, MARV VP24 (mVP24) does not bind to KPNAs or significantly alter IFN-induced gene expression[25]. The inhibition of IFN-signaling in MARV infected cells is

instead mediated by mVP40, which blocks the activation and function of Jak1[25] (Figure 3A). This recapitulates the phenotype of Jak1 knockout cells in that Jak family kinase and STAT protein tyrosine phosphorylation events were absent after addition of either IFNα/β or IFNγ[113]. Over-expression studies also suggest that mVP40 acts by specifically inhibiting Jak1; whereas over-expression of JAK1 triggers JAK1 autophosphorylation and STAT1 and STAT2 phosphorylation, mVP40 coexpression prevents these phosphorylation events. In contrast, when Tyk2 was over-expressed, phosphorylation of Tyk2, STAT1 and STAT2 was induced and expression of mVP40 had little to no impact on these phosphorylation events[25]. Although Jak1 function is inhibited by mVP40, the underlying mechanism remains to be defined and direct interaction of mVP40 with Jak1 has not been demonstrated. Thus, in contrast to EBOV, MARV infection abrogates IFN signaling by blocking the IFNα/β induced tyrosine phosphorylation Jak1, STAT1 and STAT2.

Inhibition of IFN-induced antiviral proteins

In addition to blocking cellular signaling pathways, mechanisms that counteract select IFN-induced antiviral proteins, including PKR and tetherin, have been described. PKR is a well-characterized IFN-induced, dsRNA-activated kinase with antiviral activity and VP35 has been shown to block its activity[114,115]. Although PKR is dsRNA-activated, some VP35 mutants unable to bind to dsRNA retain the capacity to inhibit PKR, suggesting a dsRNA-independent mechanism of action. Although the mechanism of inhibition remains to be elucidated, it is intriguing that VP35 interactor PACT is an activator of PKR[97,116]. The impact of PKR inhibition on EBOV replication also remains to be defined. That this function is biologically-significant is suggested by the facts that PKR phosphorylation and phosphorylation of the PKR substrate eIF-2α were not detected in EBOV-infected 293 cells and by the observation that the status of eIF-2α phosphorylation regulates replication in cells that are persistently infected with EBOV *in vitro*[115,117].

Tetherin, also known as BST-2 and CD317, is an IFN-inducible protein that restricts the budding and release of some enveloped viruses by tethering the particles to the cell surface[118,119]. The release of virus-like particles (VLPs), which occurs upon over-expression of either the EBOV or MARV VP40 matrix proteins, is likewise blocked by tetherin[120,121] (Figure 3C). In contrast to the inhibition of budding seen when VP40 was expressed outside the context of virus infection, particle release from EBOV-infected cells was relatively unaffected by tetherin because filoviral GPs counteract the anti-budding effects of tetherin[122–124] (Figure 3C). The molecular basis for GP antagonism of tetherin remains ambiguous, but GP does not alter tetherin levels, decrease tetherin levels at the cell surface or prevent tetherin association with lipid rafts[124–126].

Subversion of other host pathways

There is accumulating evidence that filoviruses not only block innate immune responses, but that they also adapt to their host to sustain replication and subvert translational regulation mechanisms of host cells. Filoviruses are unusual compared to many other non-segmented, negative-strand RNA viruses in that they produce mRNAs with long 5′ and 3′ untranslated regions (UTRs). The 5′UTRs of the EBOV VP35, VP30, VP24 and L mRNAs contain upstream AUGs (uAUGs), and the uAUGs of EBOV VP30, VP24 and L mRNAs mark the

beginning of short upstream open reading frames (uORFs)[127]. When assessed in reporter gene assays under normal conditions, the uORF in the L mRNA was found to suppress expression of the primary L ORF by approximately 10-fold. This suppression required the uAUG and reflects decreased translation initiation at the primary ORF. During cellular stress, translation initiation factor eIF-2α is phosphorylated, leading to a general decrease in translation (Figure 4). This suppression can serve as a mechanism to allow cells to recover from stresses or, in the case of virus infections, serve as a means to impair virus replication. However, translation of some cellular mRNAs that contain uORFs is increased under these conditions[128]. Therefore, the impact of the L 5′UTR and L uORF were compared in cells that were either not stressed or that were stressed by addition of thapsigargin, which triggers eIF-2α phosphorylation. The results demonstrated that the L mRNA uORF can sustain translation at the primary ORF when eIF-2α is phosphorylated, such as in the presence of thapsigargin, by increasing translation initiation efficiency at the primary AUG. This suggests a model whereby an uORF helps EBOV maintain expression of its polymerase L under conditions of stress and eIF-2α phosphorylation, such as might occur when the innate immune response leads to PKR activation (Figure 4). Furthermore, a virus mutant lacking the L uAUG site was impaired in replication by about 10-fold relative to wild-type virus and showed increased sensitivity to stress induced by thapsigargin, demonstrating that the L uORF is important for EBOV replication[127].

In another example, mVP24 has been demonstrated to interact with and modulate the function of cellular protein kelch-like ech-associated protein 1 (Keap1)[110,129,130]. Keap1 normally directs Cul3 ubiquitin ligase to target proteins such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that binds to and activates promoters possessing antioxidant response element (ARE) sequences. Such genes encode proteins that allow cells to recover from oxidative stress and other stresses. Under homeostatic conditions, Nrf2 is constitutively targeted for degradation due to ubiquitination directed by Keap1. However, when a cell experiences stress, Keap1-Nrf2 interaction can be disrupted, stabilizing Nrf2 and promoting ARE gene expression[131]. mVP24 interacts with the Kelch domain of Keap1 via an acidic motif present within a loop (called the “K-loop”). This loop possesses the amino acid residues “GE” preceded by acidic residues, which is reminiscent of interacting motifs on other Keap1 binding partners, including Nrf2. The specificity of mVP24 for the Keap1 KELCH domain is derived, in large part, from the extended beta-strands that can protrude from the body of mVP24 and presumably can reach into the deep binding pocket within the KELCH structure[110,132]. Functionally, mVP24 interaction with Keap1 disrupts Keap1-Nrf2 interaction and activates ARE gene expression[110]. Consistent with the binding data, eVP24 did not upregulate ARE genes, but chimeric VP24s possessing the mVP24 K-loop did induce upregulation. A comparison of MARV- and EBOV-infected cells demonstrated an upregulation of ARE gene expression MARV infection of macrophage-like THP1 cells and the absence of a response in EBOV-infected cells, consistent with the observation that mVP24 interacts with Keap1 but eVP24 does not. We hypothesize that turning on antioxidant responses could prolong the life of virus-infected cells.

Impact of immune evasion on pathogenesis

Among the described filoviral immune evasion functions, those most directly linked to pathogenesis are those that directly block innate immune signaling pathways. Recombinant EBOVs possessing mutated VP35s that are unable to bind dsRNA or PACT trigger nuclear accumulation of IRF-3 and induce robust IFNα/β responses, whereas wild-type EBOV does not. *In vivo*, these mutant viruses exhibited reduced replication and were non-lethal at the doses tested, providing compelling evidence that VP35 IFN-antagonist function is a critical determinant of EBOV disease *in vivo*[81,82].

Studies also implicate the IFN signaling inhibitors eVP24 and mVP40 as virulence determinants in rodent models. Although EBOV does not display virulence in mice or guinea pigs, the virus can be made lethal by serial passage in these animals[12,133]. The adapted viruses acquire amino acid changes in eVP24 and several other genes[12,133]. In the guinea pig-adapted EBOV, changes in VP24 were sufficient to confer lethality[134] and for mouse-adapted EBOV, a mutation in VP24 and a mutation in NP together make the virus lethal to mice[135]. It is presently unclear to what extent the adaptive changes are related to eVP24 IFN-antagonist activities, although the mouse-adapted EBOV was more resistant to the antiviral effects of IFNs in murine macrophages than was the parental, non-adapted virus[135].

Like EBOV, MARV does not cause lethal illness in guinea pigs or mice unless experimentally-adapted to these species[15,136]. Adaptation of the Ravn strain of MARV (RAVV) to mice led to changes across the viral genome; among these were several amino acid changes in VP40[137]. When the inhibitory activities of the adapted and parental mVP40 variants toward IFN signaling were compared, the parental mVP40 was impaired in inhibiting IFN responses in mouse cell lines relative to human cell lines. In contrast, the mouse-adapted mVP40 effectively inhibited IFN responses in both human and mouse cell lines[138,139]. Based on these data, it appears that the inhibitory function of VP40 is connected to virulence in mice[138,139]. Interestingly, the mouse-adapted mVP40 virus exhibits budding defects in human cells, and the defect was mapped to the same residues in mVP40 implicated in enhanced inhibition of the mouse IFN response. This correlates with an increased sensitivity to restriction by human, but not mouse, tetherin[140]. Therefore, there appear to be functional costs associated with increased IFN-antagonism in mice.

Conclusions and Future Directions

As discussed above, the mechanisms by which EBOV and MARV counteract host defenses are becoming increasingly clear. The VP35 protein antagonizes several aspects of host defense by blocking the cytoplasmic sensors RIG-I and MDA5, by inhibiting the antiviral kinase PKR and by impairing DC maturation. The eVP24 protein blocks cellular responses to IFNs by selectively blocking the nuclear import of phospho-STAT1; the mVP40 protein counters IFN signaling through inhibition of Jak1 and the filovirus GPs counter the antiviral effects of tetherin. In addition, mechanisms whereby EBOV and MARVs capitalize on specific cellular functions, such as stress-regulated translation or antioxidant responses, to

promote virus replication are also coming into focus. There is also strong evidence that these functions contribute to the severe infections that make filoviruses notable.

Nonetheless, fundamental questions remain to be asked. For example, it is unclear how these functions relate to specific mechanisms that drive disease, such as inflammation, vascular leakage and coagulopathy and how they relate to the relative virulence of different filovirus species. Differences in the efficiencies of innate immune antagonism, which might arise due to differences in how viral proteins function and/or in expression levels, could contribute to different degrees of virulence seen between various filoviruses, but such studies are yet to be performed. Because non-human primate models of filovirus infection are considered the gold-standard for pathogenesis studies, it will be important to define the contribution of innate immune antagonism and other proposed virulence determinants in these models. Given that filoviruses are zoonotic pathogens that are apparently not pathogenic in their presumed reservoir hosts, it will also be important to understand how filoviral immune evasion functions differ in bats versus humans. If the responses of different hosts diverge, it will be important to understand the mechanistic basis for such differences. Finally, it remains to be determined whether these functions can be exploited for therapeutic development. The West Africa Ebola epidemic has reinforced the need for effective medical interventions, yet approved therapeutics are lacking. Given the critical role of VP35 for virulence, drugs that either disrupt or bypass this function to restore RLR signaling in infected cells would be expected to have therapeutic benefit. Similarly, drugs that counteract eVP24 or mVP40 IFN-antagonist functions might enhance the benefit of naturally-produced IFNs or synergize with IFN administered therapeutically. Studies to identify inhibitors of other functions, such as the mechanisms regulating L translation and antioxidant responses are also warranted, as these would be useful as molecular probes for these functions and as possible leads for antiviral development. The need for better therapeutics and for improved understanding of filoviral disease should prompt continued delineation of filovirus innate immune evasion functions.

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Glossary

Cathepsins B and L
endosomal cysteine proteases that cleave EBOV GP during viral entry

humoral responses
antibody responses

isotype class switching
a recombination process that alters the isotype of an antibody (e.g. from IgM to IgG)

lymphopenia
low levels of lymphocytes in blood

micropinocytosis
an actin-dependent form of endocytosis that results in the formation of large fluid-filled vacuoles

necrosis
a non-apoptotic form of cell death

negative-sense RNA viruses
viruses possessing single-stranded RNA genomes that are negative-sense (i.e. complementary to the viral mRNAs)

phosphatidylserine
a phospholipid found on the surface of apoptotic bodies and on viral membranes that can promote intracellular uptake via interaction with specific cell surface receptors

pro-thrombin time
a blood test that measures clotting time

thrombocytopenia
low levels of platelets in the blood

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Box 1

Filovirus outbreaks – a historical perspective

The first documented outbreak caused by filoviruses occurred in 1967 in Marburg, Germany when animal staff and lab workers were infected while harvesting tissues from *Cercopithecus aethips* monkeys. These animals were imported from Uganda to produce kidney cell cultures needed for the production of live poliomyelitis vaccine[141,142]. In efforts to identify and characterize the infectious agent, plasma from infected guinea pigs were formalinized and analyzed by electron microscopy. The causative agent was identified as a novel virus and named Marburg virus (MARV)[142]. This pathogen is now classified in the *Marburgvirus* genus of the filovirus family. In 1976, another outbreak of hemorrhagic fever occurred in northern Zaire, now the Democratic Republic of the Congo (DRC), resulting in 318 cases with an 88% lethality rate. The causative agent of this outbreak was a second filovirus, Ebola virus (EBOV)[143]. *Ebolavirus* represents a second genus of the filovirus family. In the same year, a second outbreak of EBOV hemorrhagic fever also occurred in Sudan causing 284 cases with a 53% lethality rate[144]. This second outbreak was caused by a distinct virus, Sudan ebolavirus (SUDV). In the years since these early outbreaks, small sporadic outbreaks of MARV infection have occurred in Africa with the largest epidemics occurring in 1998 in the DRC, with 141 cases and an 82% lethality rate, and in 2004 in Angola, with 252 cases and a 90% lethality rate[145,146]. Subsequent outbreaks of EBOV revealed the presence of three additional species with varying pathogenicity in humans. In 1989 Reston ebolavirus (RESTV) was isolated from cynomolgus macaques in the Philippines and was found to be non-pathogenic in humans[147]. In 1994, the Ivory Coast ebolavirus (now called Tai Forrest ebolavirus; TAFV) was discovered from an infected patient in the Tai Forest reserve in Cote d'Ivoire[148]. Bundibugyo ebolavirus (BDBV) was discovered in 2007 in Uganda where it caused 149 cases and a 25% lethality rate. Most human cases of Ebola virus disease (EVD) are due to the emergence and re-emergence of EBOV in rural areas of Gabon, Republic of Congo, and DRC and of SUDV in Sudan and Uganda. Until 2014, there have been 1,383 EBOV and 779 SUDV cases, with fatality rates ranging from 50–90% and 40–65%, respectively[8]. The 2014 West Africa outbreak is unique in that it is the largest outbreak on record to date. The most recently identified filovirus, Lloviu virus (LLOV), which is classified in a distinct filovirus genus *Cuevavirus*, was identified in bats in Spain[7]. LLOV has yet to be cultured, but a near full-length viral genomic sequence has been determined from RNA in bat tissue. The pathogenic potential of LLOV is uncertain.

Box 2

Bats as filovirus reservoir hosts

Elucidating the reservoir host that harbors filoviruses has long been of interest. The identification of the reservoir might suggest mechanisms that drive the emergence of the viruses into human populations and strategies to avoid human infections. Because it is presumed that the reservoir host does not suffer the extreme mortality seen in humans and non-human primates, studies of virus-reservoir interaction could also provide unique insight into filovirus pathogenesis. However, it is only recently that conclusive evidence has been developed implicating select bat species as natural hosts of Marburg virus.

Circumstances surrounding several Marburg virus outbreaks suggested bats as a possible source of the virus for human infection. For example, bats were one of several animals to which the presumed index case of a small Marburg virus outbreak in 1975 had been exposed[149]. In several cases, human infections occurred in individuals, including miners and tourists, who had entered caves containing significant bat populations[146,150–152]. *Rousettus aegyptiacus* were repeatedly found in these caves[153]. More direct evidence of Marburg virus infection of bats was the detection of Marburg virus nucleic acid and anti-Marburg virus antibodies in *R. aegyptiacus*[153]. However, the conclusive evidence was the isolation of live virus from *R. aegyptiacus*[151]. Follow up studies, focused on Python Cave in Uganda where two tourists had become infected previously, found evidence of a seasonal pattern of infections within the bat population[154]. Consistent with the expected outcome, where infection of a reservoir host should not be consistently lethal, experimental inoculations of filoviruses into bats has not yielded signs of overt disease[10,11]. Interestingly, current data suggests that Marburg virus infections of *R. aegyptiacus* are not persistent, but instead are cleared after a period of replication. The animals develop a transient viremia, but mount a detectable antibody response to the infection and the virus is cleared. However, oral shedding as well as replication in multiple bat tissues has been detected. These data suggest potential routes that might lead to spread of infections among bats or to other species, including humans, although transmission between experimentally-infected bats has not been demonstrated[10,155].

The evidence for bats as Ebola virus reservoirs is currently based on serology and the presence of Ebola virus-derived RNAs in tissues and sera from bats in both Africa and Asia (see[10]). The near complete genome of Lloviu virus was characterized from bats in Northern Spain[7]. The isolation of infectious virus from bats, particularly from healthy bats, would provide stronger evidence that bats are reservoir hosts for Ebola virus and Lloviu virus. Nonetheless, the available data suggest that filoviruses may have a broader geographic distribution than is generally appreciated.

With the identification of bats as being susceptible to Marburg virus infection, but resistant to disease, an understanding of the mechanisms of disease resistance is needed. It will be of significant interest to determine how interactions of filoviruses with the bat innate or adaptive immune responses somehow contribute to disease outcome.

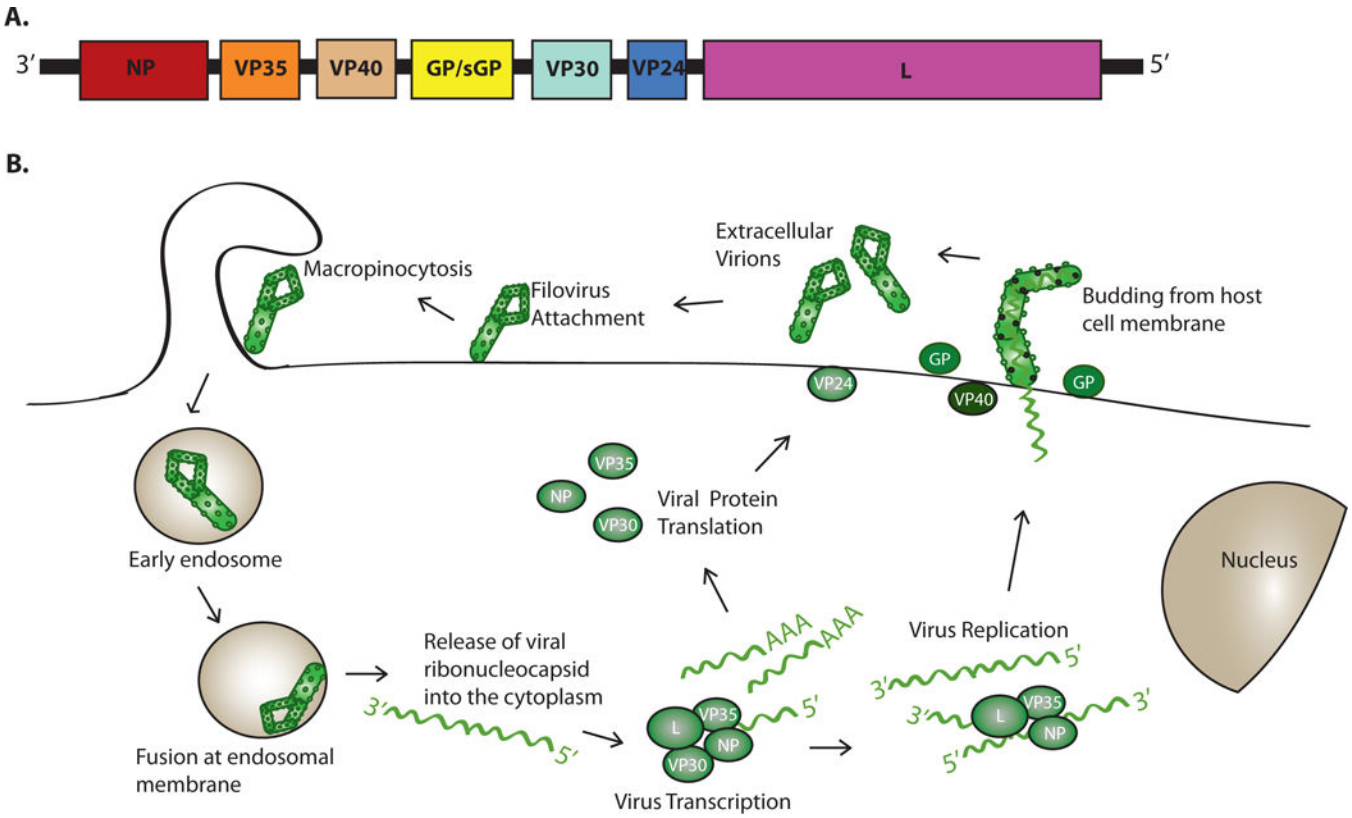

Figure 1. Filovirus genome structure and life cycle

A. The genome of EBOV is depicted. The genome is depicted in the 3′ to 5′ orientation to indicate that the genomic RNA is negative-sense. The genes are named after the proteins encoded by each. They are NP, nucleoprotein; VP35, viral protein 35; VP40, viral protein 40; GP/sGP, glycoprotein/soluble glycoprotein; VP30, viral protein 30; VP24, viral protein 24; L, Large protein (the viral polymerase). Note that Marburg virus does not encode a sGP protein. Filoviruses have unusually long non-coding regions at the 5′ and 3′ end of their mRNAs. The genome regions corresponding to these non-protein coding sequences are not drawn to scale. B. The general life cycle of a filovirus is displayed. First, the filovirus attaches to the cell membrane via its surface GP protein. Virus is taken up by a process known as macropinocytosis[38,156–158]. Upon acidification of the endosome, cathepsins B and L (cellular proteases) cleave GP[33]. This allows GP to interact with host protein NPC-1, an interaction which is a prerequisite for fusion of viral and endosomal membranes[34,35]. Host endosomal calcium channels called two-pore channels (TPCs) play a critical role in the endosomal trafficking of incoming viral particles to the site of fusion[159]. GP mediates fusion of the viral and the endosomal membrane, releasing the viral ribonucleocapsid into the cytoplasm where the negative strand RNA genome undergoes transcription and replication[160]. Production of 5′-capped, 3′-polyadenylated mRNAs from individual viral genes occurs and genome replication follows, in which the genomic RNA template is copied into a full-length complement. The full-length complement serves as template for synthesis of additional negative-sense genome. The RNA synthesis reactions require the NP, VP35 and L proteins. Initiation of transcription for Ebola virus also requires the VP30 protein, whereas the replication reactions do not require VP30. Viral proteins, including NP, VP35, VP40, GP,

VP30, VP24, and L are translated from the viral mRNAs. New viral particles are assembled at the plasma membrane. The VP40 protein functions as the viral matrix protein and directs budding of particles from the cell surface[161–163]. GP, a type I transmembrane protein, is incorporated into the budding particles, as are viral nucleocapsids containing the viral genome[164].

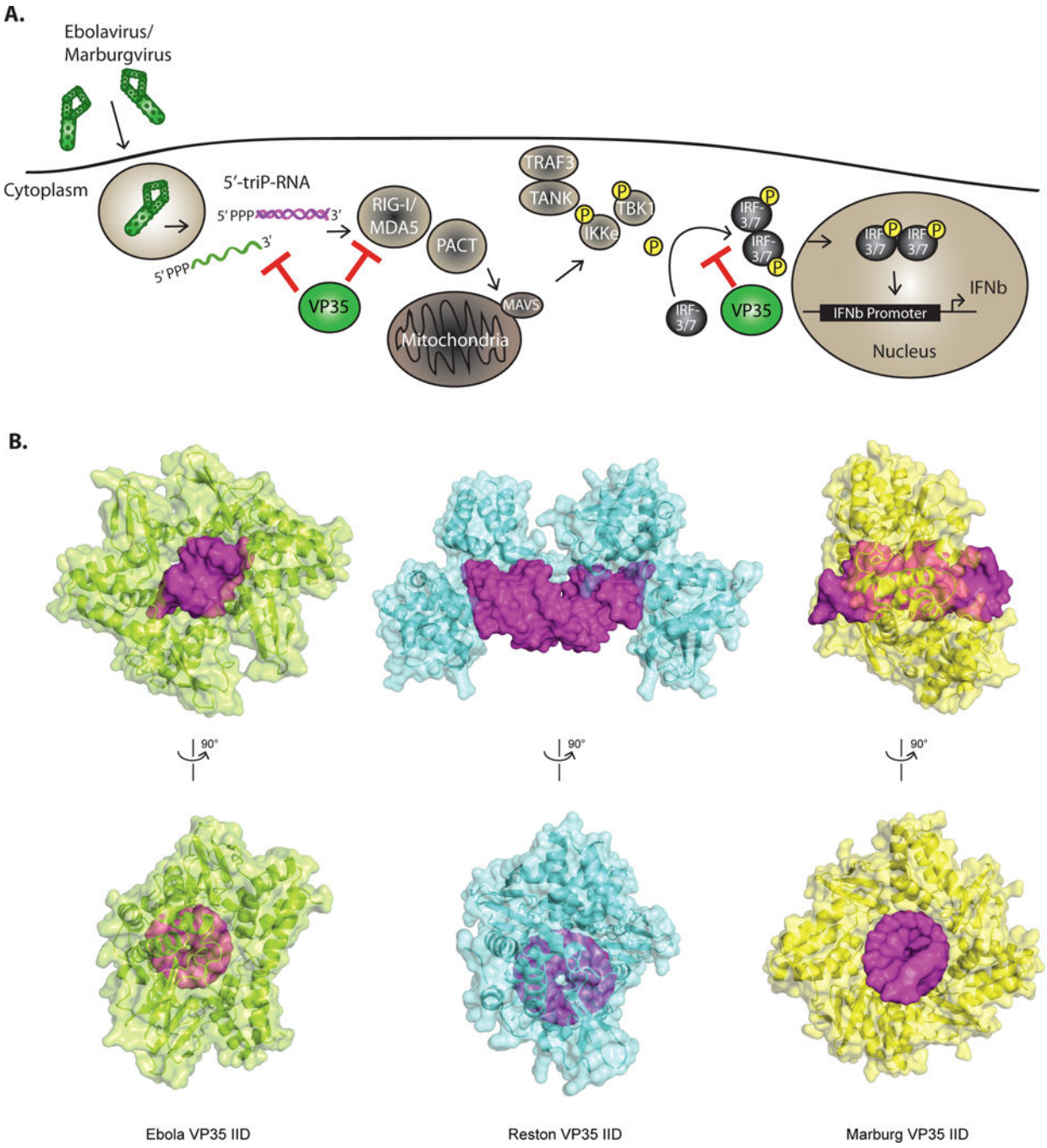

Figure 2. Subversion of IFN induction

A. Schematic of the IFN production pathway and mechanisms of filoviral evasion. The image depicts how EBOV VP35 (eVP35) and MARV VP35 (mVP35) proteins antagonize signaling pathways that lead to IFN-α/β gene expression. RIG-I-like receptors (RLRs) which include RIG-I and MDA5, detect products of viral replication, RNAs such as cytoplasmic dsRNAs or RNAs with 5′-triphosphates. Activation of RLRs is facilitated by the protein PACT. Upon activation, RLRs signal through the mitochondria-associated protein MAVS to activate kinases IKKε and TBK1. These phosphorylate interferon regulatory factor

(IRF)-3 or 7 then accumulate in the nucleus and promote IFN-α/β gene expression. VP35 proteins can bind to dsRNAs and to PACT, preventing RLR activation. In addition, VP35s interact with and act as decoy substrates for the kinases IKKε and TBK1. B. Structural analysis of filoviral VP35 protein interferon inhibitory domain (IID) binding to dsRNAs reveals differences among the dsRNA recognition mode for EBOV, RESTV, and MARV VP35 proteins. (Left) The structure of eVP35 bound to 8 bp dsRNA (PDB 3L25). (Middle) rVP35 bound to 12bp (PDB 4LG2). (Right) mVP35 IID bound to 12 bp dsRNA (PDB 4GHL). dsRNA shown in magenta.

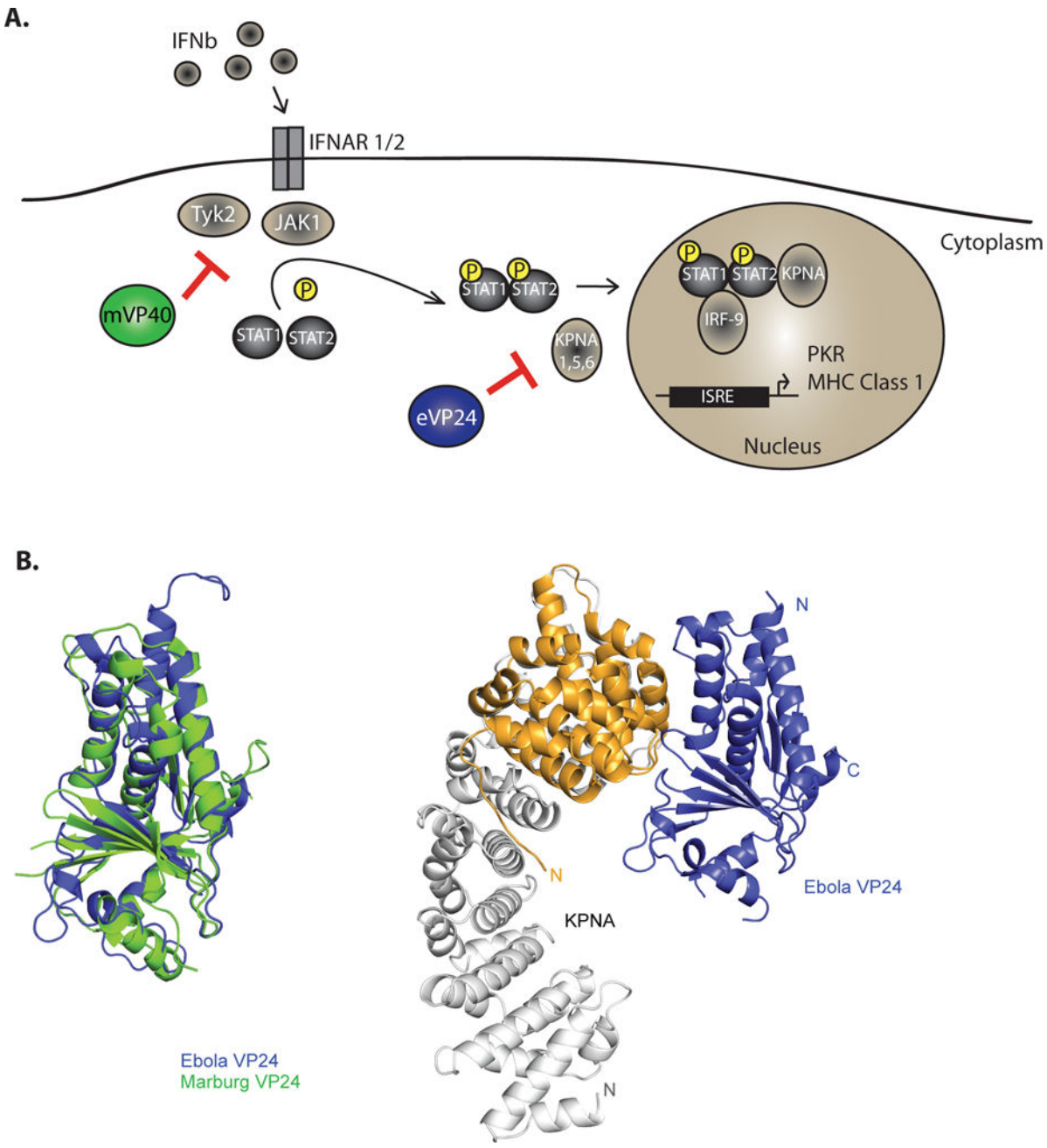

Figure 3. Subversion of IFN-induced signaling and of IFN-induced protein tetherin

A. Schematic of the IFN response pathway and mechanisms of filoviral evasion. IFN-α/β binds the extracellular domains of the heterodimeric IFN alpha receptor composed of subunits IFNAR1 and IFNAR2. This activates receptor-associated tyrosine kinases Jak1 and Tyk2. These phosphorylate STAT1 and STAT2 causing them to heterodimerize, associate with karyopherin alpha (KPNA)-1, 5 and 6 and be transported into the nucleus to induce interferon stimulating genes, including the antiviral kinase PKR and major histocompatibility complex 1 MARV VP40 (mVP40) inhibits the IFN induced activation of tyrosine kinase JAK1. EBOV VP24 (eVP24) binds KPNA1, 5 and 6 to block KPNA-STAT1

interactions. Both mechanisms inhibit IFN-induced gene expression. B. EBOV VP24 uses a unique non-classical nuclear localization site to interact with KPNA-1, 5 or 6. (Left) EBOV VP24 (PDB 4M0Q) and SUDV VP24 (PDB 3VNF) show a similar pyramidal structure[110]. (Right) eVP24 proteins bind the KPNA C-terminus (PDB 4U2X). C. The IFN-inducible protein tetherin can restrict budding of the filovirus VP40 protein (left). However, filovirus GP counteracts the antiviral effects of tetherin, allowing Ebola and Marburg viruses to be released in the present of tetherin (right).

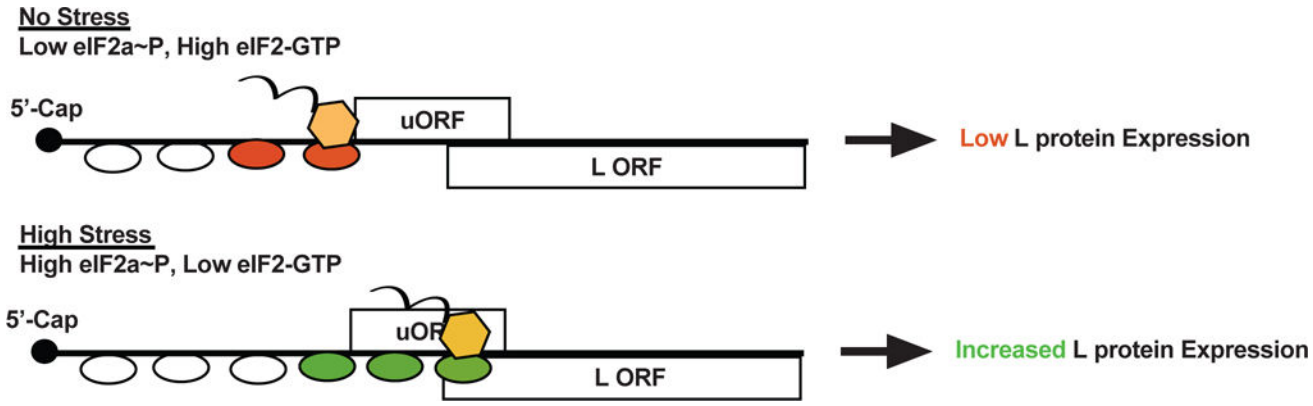

Figure 4. Subversion of host translation mechanisms to support virus replication

It has been observed that several of the 5′-capped viral EBOV mRNAs possess upstream open reading frames (uORFs) in their 5′ untranslated regions (UTRs). Under lowstress conditions, the uORF present in the mRNA encoding the L protein attenuates L translation initiation. This is because scanning ribosomes (depicted as ovals) initiate efficiently at the uORF AUG instead. However, under stress, such as occurs due to activation of innate immune responses, eIF2α phosphorylation (eIF2α~P) increases and translation initiation at the uORF becomes less efficient. This allows the scanning ribosome to bypass the uORF AUG and increases initiation at the L AUG, helping to sustain L expression despite the increase in eIF2α phosphorylation.