Interferon-induced HERC5 is a novel and potent inhibitor of Ebola virus-like particle production

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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Abstract

Ebola’s severe pathogenesis can be attributed to its suppression of the Type I interferon (IFN) response, suggesting this pathway plays a role in restricting viral replication. One IFN-induced protein, HERC5, warrants further investigation as it inhibits the replication of evolutionarily diverse viruses. We showed that HERC5 drastically reduces the expression of structural protein VP40 at the protein and mRNA level. Mutagenesis of HERC5 demonstrated that the RCC-1-like domain is necessary and sufficient for restriction. This domain is also responsible for HERC5’s interaction with ZAP, a protein required for VP40 mRNA degradation. Finally, we showed that Ebola GP antagonizes HERC5 activity. Overall, we have identified a novel antiviral mechanism targeting Ebola RNA. Moreover, depletion of viral RNA via the RCC-1 like domain identifies a previously unknown function for HERC5. Moving forward, this information is crucial in building a solid foundation of knowledge regarding EBOV’s interaction with the IFN response during infection.

Keywords

Type I interferon response, HERC5, Ebola virus, restriction, antiviral, innate immunity, zinc finger antiviral protein, viral antagonism
Acknowledgments

I would first like to thank my supervisor Dr. Stephen Barr for the immeasurable guidance and knowledge he offered throughout my studies. I am forever grateful for having been given the opportunity to work in his laboratory and most of all, for the fact that he never shed an inch of doubt in me or my research.

I would also like to thank my advisory committee, Dr. Ilka Heinemann and Dr. Joseph Mymryk for giving me the constructive criticism and great ideas for the furtherance of my project. Committee meetings were always a positive environment where I felt encouraged to learn.

I would like to thank my colleagues, faculty and staff, who were always willing to offer a helping hand and provided a warm and welcoming atmosphere both in and out of the lab. Finally, I am incredibly grateful for my family and friends for their constant support and encouragement. You were crucial in getting me through graduate school (mostly) unscathed.
Table of Contents

Abstract ................................................................................................................................. i

Acknowledgments .............................................................................................................. ii

Table of Contents .............................................................................................................. iii

List of Tables ....................................................................................................................... vi

List of Figures ..................................................................................................................... vii

List of Appendices ............................................................................................................. viii

List of Common Abbreviations .......................................................................................... ix

CHAPTER 1: INTRODUCTION .......................................................................................... 1

1.1 Ebola virus disease .................................................................................................... 1

  1.1.1 Ebola virus genome ............................................................................................... 2

  1.1.2 Ebola life cycle ....................................................................................................... 2

1.2 Type I interferon response ......................................................................................... 5

  1.2.1 Interferon stimulated genes .................................................................................. 5

  1.2.2 Antiviral activity towards viral protein ................................................................. 6

  1.2.3 Antiviral activity towards viral RNA ................................................................. 8

1.3 Ebola restriction factors .......................................................................................... 9

  1.3.1 ISG15 ................................................................................................................... 9

  1.3.2 Interferon induced transmembrane proteins ...................................................... 10

  1.3.3 Tetherin ............................................................................................................... 10

1.4 HECT and RCC-1 like domain containing protein 5 (HERC5) ............................ 11

1.5 Ebola antagonism towards host .............................................................................. 14

  1.5.1 Viral protein 35 .................................................................................................. 15

  1.5.2 Viral protein 24 .................................................................................................. 15

  1.5.3 Glycoprotein ...................................................................................................... 16
1.6 Project rationale .................................................................................................................. 16
1.7 Hypothesis and specific aims .......................................................................................... 17

CHAPTER 2: MATERIALS AND METHODS .................................................................................. 18
2.1 Cells and cell lines ............................................................................................................. 18
2.2 Plasmids, transfections and antibodies .......................................................................... 18
2.3 Western blotting ............................................................................................................... 23
2.4 RNA isolation and reverse transcription polymerase chain reaction ......................... 23
2.5 Confocal immunofluorescence microscopy ................................................................. 25
2.6 Transmission electron microscopy .................................................................................. 25
   2.6.1 Immunogold transmission electron microscopy .................................................... 26
2.7 Co-immunoprecipitation ................................................................................................. 26
2.9 Statistical analyses and bioinformatics .......................................................................... 27
   2.10.1 Immunogold labelling quantification ...................................................................... 27
   2.10.2 CG dinucleotide content ....................................................................................... 27

CHAPTER 3: RESULTS ................................................................................................................... 29
3.1 Construction and verification of EBOV VP40 VLP assay .............................................. 29
3.2 HERC5 potently blocks the release of EBOV VP40 VLPs at the plasma membrane .......... 32
3.3 HERC5 potently restricts EBOV VP40 protein .............................................................. 35
3.4 Protein degradation is not involved in HERC5-mediated restriction of VP40 ............. 35
3.5 HERC5 restricts VP40 mRNA ......................................................................................... 39
3.6 HERC5 does not affect nuclear export of VP40 mRNA .............................................. 46
3.7 HERC5 is dependent on ZAP for restriction at the RNA level ..................................... 46
3.8 ZAP and HERC5 are interaction partners and require the RLD domain for interaction ................................................................. 48
3.9 HERC5’s specificity for VP40 is not related to CG dinucleotide content .................... 51
3.10 Ebola GP can antagonize HERC5 mediated restriction of VP40 protein and mRNA ................................................................. 51

3.11 GP antagonism is specific to filoviral GPs ................................................. 55

CHAPTER 4 – DISCUSSION ...................................................................... 61

4.1 Limitations of the study ........................................................................ 66

4.2 Future directions ...................................................................................... 67

4.3 Conclusions .............................................................................................. 68

References ................................................................................................. 70

Appendices ................................................................................................. 77

Curriculum Vitae ......................................................................................... 83
List of Tables

Table 1. Summary of plasmids and RNAs used in this study ........................................... 20

Table 2. Oligonucleotide primers used for cloning ...................................................... 22

Table 3. Oligonucleotide primers for quantitative PCR .................................................. 24

Table 4. Quantification of 5nm gold particle labelled anti-GFP in cells expressing HERC5 and VP40 .............................................................. 36

Table 5. Quantification of 5nm gold particle labelled anti-GFP in cells expressing empty vector and VP40 .............................................................. 36
List of Figures

Figure 1. Schematic of Ebola virus genome and lifecycle........................................ 4

Figure 2. Schematic of Type I interferon response to EBOV infection....................... 7

Figure 3. Schematic of HERC5. ............................................................................ 13

Figure 4. Plasmid containing GFP tagged VP40 at the N-terminus caused budding similar to wild type EBOV............................................................... 31

Figure 5. HERC5 blocks VP40 VLP formation along the plasma membrane.............. 34

Figure 6. HERC5 restricts EBOV VP40 protein ................................................... 38

Figure 7. MG132 treatment did not affect HERC5 mediated restriction............... 40

Figure 8. HERC5 restricts VP40 mRNA ............................................................ 42

Figure 9. The RLD is necessary and sufficient for restriction of VP40. .................. 45

Figure 10. HERC5 does not affect nuclear export of VP40 mRNA..................... 47

Figure 11. HERC5 necessitates ZAP for restriction of VP40 mRNA....................... 50

Figure 12. HERC5 interacts with ZAP through the RLD domain......................... 53

Figure 13. CG dinucleotide content within the EBOV genome .............................. 54

Figure 14. EBOV L and GP antagonize HERC5 mediated restriction of VP40 ....... 57

Figure 15. HERC5 antagonism is specific to filoviral GPs. ................................. 60
List of Appendices

Appendix A. Ebola protein sequences .................................................................................. 77

Appendix B. Knockdown efficiencies for shRNAs and siRNAs used within this study. 81

Appendix C. Copyright permissions for figures 1-2................................................................. 82
**List of Common Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBOV</td>
<td>Ebola virus (Zaire)</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HERC</td>
<td>HECT and RCC-1 like domain containing protein</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to E6-AP Carboxyl Terminus</td>
</tr>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
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<tr>
<td>IFITM</td>
<td>Interferon induced transmembrane protein</td>
</tr>
<tr>
<td>IFN</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>Jak/STAT</td>
<td>Janus Kinases/Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>L</td>
<td>L protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>NFκB</td>
<td>Nuclear factor κ B</td>
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<td>Nucleoprotein</td>
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<tr>
<td>OAS1</td>
<td>2´,5´-oligoadenylatesynthetase 1</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RCC</td>
<td>Regulator of chromatin condensation</td>
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<tr>
<td>RNA</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
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<td>Tripartate motif protein</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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<tr>
<td>VLP</td>
<td>Virus-like particle</td>
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</tr>
<tr>
<td>VSV</td>
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<tr>
<td>ZAP</td>
<td>Zinc finger antiviral protein</td>
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<tr>
<td>ZRE</td>
<td>Zap response element</td>
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CHAPTER 1: INTRODUCTION

1.1 Ebola virus disease

The latest Ebola outbreak in West Africa in 2014 recorded a staggering 28,616 cases and 11,325 deaths, demonstrating the severe and often lethal nature of Ebola virus infection. Ebola virus is a member of the Filoviridae family of negative sense RNA viruses, with a filamentous morphology. Though its natural reservoir is believed to be in fruit bats, it is transmitted between humans through bodily fluid. Once in the body, Ebola virus first infects macrophages and dendritic cells and is accompanied by flu-like symptoms after 8-12 days. The migratory properties of macrophages and dendritic cells allows for the spread of virus to fibroblasts, epithelial cells, hepatocytes and adrenal cortical cells. Ebola virus rapidly replicates within these cells, causing apoptosis and the release of viral particles. Circulation of extracellular particles leads to viral replication within lymph nodes, and eventual ablation of this crucial defense line. The body’s immune defense is further deteriorated by infection and apoptosis of dendritic cells and suppression of a key component of the innate immune defense, the Type I interferon (IFN) response. Extensive cell death and tissue necrosis ensues, inducing the release of massive amounts of pro-inflammatory cytokines and a systemic inflammatory syndrome.

The presence of pro-inflammatory cytokines and infection of endothelial cells are responsible for vasodilation of blood vessels, downregulation of cell adhesion proteins, pooling blood into various areas/tissues, and subsequent multi-organ failure known as septic shock. These cytokines are also responsible for inappropriate activation of coagulation cascades which deplete circulatory platelets and cause an inability of blood to clot. The body is thus prone to excessive bleeding and hemorrhages, which characterizes the quintessential hemorrhagic properties of the infection. An overall failure to clear this infection typically results in death within 6-9 days after the onset of symptoms—making Ebola virus one of the most virulent pathogens to infect humans.

There are five species within the Ebola virus genus: Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Bundibugyo ebolavirus (BDBV), Tai Forest ebolavirus
(TAFV) and Reston ebolavirus (RESTV). Unlike TAFV, which has only been found to be responsible for single, non-fatal infections of ebolavirus, SUDV, BDBV, RESTV and EBOV have been responsible for past outbreaks of human infection. Most notably, EBOV is responsible for the 2014 outbreak in West Africa. This outbreak not only shed light onto the severe manifestations of the disease, but also emphasized the knowledge gap regarding EBOV infection. The ability of EBOV to trigger the host immune response and cause severe disease, without triggering an effective antiviral response, demands critical analysis of the virus-host interplay during the course of EBOV infection.

1.1.1 Ebola virus genome

EBOV possesses a single-stranded, negative sense RNA genome of ~19 kilobases in size. The genome encodes seven genes, each of which are individual transcriptional units that encode seven viral structural proteins: Viral Protein 40 (VP40), Viral Protein 35 (VP35), Viral Protein 30 (VP30), Viral Protein 24 (VP24), Nucleoprotein (NP), Glycoprotein (GP), and Large Protein (L) as shown in Figure 1A. Transcriptional editing of viral RNA results in the production of both a soluble form (sGP) and membrane-bound form of GP, as well as an unedited sGP. Each viral protein plays an important role during the EBOV life cycle.

1.1.2 Ebola life cycle

EBOV enters the human cell via attachment and fusion mediated by viral surface GP, which leads to viral internalization via macropinocytosis. Acidification of the endosome induces cleavage of GP by cellular cathepsins B and L, and subsequent binding of GP to Neimann-Pick C1 (NPC1) in the late endosome. This allows for endosomal fusion and the release of the viral genome into the cytoplasm, where viral transcription and translation occur. Viral RNA synthesis begins on the uncapped, non-polyadenylated viral template, which is converted to 5'-capped, polyadenylated mRNA by L protein. VP35 acts to deliver L protein to the NP-encapsidated template by interacting with both L and NP. VP30 is also required for viral transcription and may play a role in the regulation of the viral polymerase complex during transcription and replication. The full-length
complement of the RNA genome is used as a template to create new negative-sense RNA genomes during replication\(^8\).

Following replication of the viral genome and transcription and translation of EBOV proteins, viral particles are assembled along the plasma membrane. Virus release occurs by budding and is mediated by the interaction of the main structural protein, VP40, with members of the cellular Endosomal Sorting Complex for Transport (ESCRT) pathway\(^{17,18}\). The N-terminal domain of VP40 promotes its own oligomerization, while a hydrophobic patch within the C-terminal domain is responsible for penetration of oligomerized VP40 into the inner leaflet of the plasma membrane\(^{19,20}\). The electrostatic and hydrophobic properties of VP40’s carboxy-terminus induce plasma membrane attachment and eventual VLP formation\(^{19}\). VP40 recruits ESCRT proteins Tsg101 and Nedd4, an ubiquitin ligase, to the plasma membrane for budding. Tsg101 and Nedd4 act together to ubiquitinate VP40\(^{17,18,21}\). The ubiquitinated form of VP40 is speculated to promote more efficient self-assembly, maturation and scission of viral particles from the plasma membrane\(^{21}\). Filamentous virions emerge from the cell via two distinct modes; infectious particles primarily bud horizontally, while less pathogenic empty virions bud vertically from the cell\(^{22}\).

Each EBOV protein, other than sGP, is present in the fully formed enveloped viral particle, but GP is the only protein that is exposed on the surface of viral particles. VP40 is the most abundantly produced of the seven proteins, and is the main structural protein within viral particles. Specifically, VP40 forms the viral matrix between the genome-associated nucleocapsid and lipid envelope to stabilize the virion shape\(^{19,23}\). Studies have shown that VP40, in the absence of any other Ebola protein, can self-assemble into virus-like particles (VLPs)\(^{24-26}\). These particles are non-infectious and are morphologically indistinguishable from natural Ebola virions, allowing studies of VP40 alone to be representative of some aspects of EBOV replication\(^{26}\). A summary of the EBOV lifecycle is shown in Figure 1B.
Figure 1. Schematic of Ebola virus genome and lifecycle.

(A) The genome is shown in 3’ to 5’ orientation to indicate that it is negative-sense. 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). (B) EBOV attaches to the cell surface via GP, and is taken up by macropinocytosis. Upon acidification of the endosome, cathepsins B and L cleave GP to allow for its interaction with NPC1 and fusion of the virus with the endosomal membrane. The viral RNA is released into the cytoplasm where viral transcription and replication occurs. VP30 is required for initial transcription of viral mRNAs, and genome replication follows. RNA synthesis also requires NP, VP35 and L proteins. NP, VP35, VP40, GP, VP30, VP24, and L are translated from the viral mRNAs and viral particles are assembled at the plasma membrane. VP40, the viral matrix protein, directs budding and VP24, GP and nucleocapsids are incorporated into the budding particles. Image retrieved from Messaoudi et al. (2015) Nat Microbiol 13:663-768.
1.2 Type I interferon response

The Type I IFN response is triggered by the presence of foreign pathogens such as EBOV and is one of the body’s first innate defense mechanisms against viral infection\textsuperscript{27}. A summary of this response is shown in Figure 2. Recognition of foreign bodies induces a downstream pathway leading to the expression of potent antiviral cytokines, most notably IFNα and IFNβ\textsuperscript{28}. The expression of IFNs is induced immediately following viral recognition, and is essential in producing numerous cellular antiviral proteins that initiate an antiviral state within the cell and signal for an antiviral state within neighboring cells. IFNα and IFNβ are produced by almost every cell in the body, making them indispensable for defense against viral infection\textsuperscript{28,29}. Not surprisingly, mice deficient in the IFNα/β receptor were severely impaired in resistance against numerous viruses including vaccinia virus, vesicular stomatitis virus (VSV) and semiliki forest virus \textsuperscript{30}.

As a single-stranded RNA virus, EBOV is first recognized by the Type I IFN response through the RNA helicase RIG-1 and Protein Kinase RNA-activated (PKR)\textsuperscript{31}. The downstream IFN cascade is activated, leading to dimerized IFN Regulatory Factor 3 (IRF3) and NFκβ translocation into the nucleus, where they act as a transcription factors for Type I IFNs IFNα and IFNβ \textsuperscript{32} (Figure 2). The release and binding of these IFNs to their respective receptors (IFN Alpha Receptor; IFNAR) on neighboring cells causes an association of the receptor with Tyk1 and Jak1 kinases. Tyrosine phosphorylation mediated by these kinases activates STAT1 and STAT2 to interact with IRF9 and form the transcription factor complex IFN Stimulated Gene factor 3 (ISG3)\textsuperscript{29}. ISG3 translocates to the nucleus and regulates the transcription of IFN stimulated genes (ISGs) via an interaction with IFN stimulated response elements\textsuperscript{29}. Interestingly, IRF7/IRF3 heterodimers amplify this response by acting as a transcription factor for IFNα and IFNβ as shown in Figure 2\textsuperscript{33}.

1.2.1 Interferon stimulated genes

Many interferon-stimulated genes (ISGs) have been identified which target various points of the viral lifecycle upon infection and stimulation of the Type I IFN response\textsuperscript{34}. In fact, over half of all early upregulated genes following EBOV infection of circulating
immune cells were ISGs\textsuperscript{35}. Some of the most upregulated genes include ISG15, Hect and RCC-1-like domain containing protein 5 (HERC5), oligoadenylate synthase 1 (OAS1), Interferon Induced Protein with Tetratricopeptide Repeats 2 (IFIT2), Interferon-induced GTP-binding protein Mx1 (Mx1), and DExH-box helicase 58 (DHX58) – most of which will be discussed further throughout this text\textsuperscript{35}. Upon upregulation via stimulation of the IFN response, ISGs create an antiviral state within the cell by performing antiviral defense mechanisms that may target viral protein or viral RNA.

1.2.2 Antiviral activity towards viral protein

The antiviral defense mediated by ISGs are involved in viral protein modification, degradation, and inhibition of protein translation. Viral proteins can be modified by ubiquitin and sent to be degraded by protein degradation factors\textsuperscript{36,37}. Most proteins are targeted to the proteasome by covalent attachment of ubiquitin molecules. Proteasomes are protein complexes which degrade unneeded or damaged proteins via proteolysis, a chemical reaction in which the peptide bonds of the protein are broken\textsuperscript{38}. The TRIM family of proteins act as ubiquitin E3 ligases\textsuperscript{39}. The ubiquitin ligase activity of the TRIM22 protein in particular has been shown to be required for antiviral activity against viruses such as encephalomyocarditis virus\textsuperscript{39}.

PKR is also a highly upregulated ISG, as shown in Figure 2. In addition to being a viral sensor upon initiation of the Type I IFN response, activation of PKR results in the dimerization of inactive monomers which can then go on to inhibit the translation of new viral proteins\textsuperscript{40}. As a protein kinase, PKR phosphorylates the protein synthesis initiation factor eIF-2\textalpha{} to achieve this antiviral activity\textsuperscript{40}. This is important as viruses rely on the host cell translation apparatus for expression of their proteins.
Figure 2. Schematic of Type I interferon response to EBOV infection.

Left panel: recognition of the viral genome by PKR and RIG-I leads to the activation of transcription factors NFκβ and IRF3, which translocate to the nucleus and activate the IFNβ promoter. IRF-3 is phosphorylated to cause dimerization by IKKe and TBK-1, which are activated by RIG-I, MDA5 and IPS-1/MAVS. Right panel: IFNβ binds to its receptor IFNAR to cause the activation of the JAK/STAT pathway and eventual transcription of ISGs. Mx, ISG20, OAS and PKR are examples of ISGs with antiviral activity. IRF7 amplifies the Type I IFN response by inducing the transcription of IFNα and IFNβ. The various points by which EBOV proteins counteract the Type I IFN response are shown. VP35, VP24 and GP are viral antagonists of the IFN response. More details regarding this response and EBOV antagonism towards it can be found throughout the text. Image retrieved from Haller et al. (2006) J Virol. 344: 119-130\textsuperscript{29}. 

\textsuperscript{29}
1.2.3 Antiviral activity towards viral RNA

The majority of well-characterized ISGs belong to a subset of proteins that affect viral RNA; this may involve RNA modification or degradation. One RNA-editing enzyme that is highly upregulated during the IFN response is ADAR1, an RNA-specific adenosine deaminase\(^{41}\). Deamination of viral or cellular RNA makes it incompatible with cellular machinery for further translation\(^{41}\). Additionally, OAS1 monomers oligomerize after activation by the IFN response and produce 2',5'-oligoadenylates which go on to activate RNase L, a ribonuclease that degrades both cellular and viral RNA\(^{42-44}\). Similarly, ISG20 is an ISG that acts to degrade viral RNA. ISG20 is an exoribonuclease that primarily acts on single-stranded RNA viruses\(^{45}\).

An important ISG that drives selective destruction towards viral RNA, as opposed to the broad-spectrum activities of the aforementioned enzymes, is the zinc finger antiviral protein (ZAP). ZAP is known to recognize ZAP response elements (ZREs) within viral mRNA, and cause downstream degradation by recruiting factors involved in RNA degradation and inhibition of translation\(^{46-48}\). These include the deadenylase poly(A) specific ribonuclease PARN, the RNA 3'-5'-directed exonuclease complex called the RNA processing exosome, and the Dcp1/2 decapping enzymes through their p72 helicase co-factor\(^{46}\). Many viruses contain ZREs and are restricted by ZAP, including Human Immunodeficiency Virus-1 (HIV-1), Sindbis virus, Murine gammaherpesvirus and filoviruses\(^{47,49-51}\). In fact, ZAP has been shown to target and reduce mRNA levels of all of the EBOV proteins, with L protein being affected the most\(^{50}\). The specific sequence of ZREs remains elusive, though Takada et al (2017) found that high CG dinucleotide content may be involved in ZAP recruitment\(^{52}\).

ZAP has four CCCH-type zinc fingers within its N-terminus, which are present in both known human isoforms of ZAP: ZAP-long (ZAPL) and ZAP-short (ZAPS). These zinc fingers are responsible for RNA binding\(^{48}\). Studies examining ZAP’s activity towards EBOV utilize ZAP from rat cells (rZAP), which corresponds to human ZAPS (77 kDa) and does not contain the C-terminal PARP domain that is present in ZAPL (101 kDa)\(^{50,53}\). However, more recent research has found ZAPL to mediate stronger antiviral activity towards viruses in general\(^{55}\).
Interestingly, ZAP has been shown to act synergistically with other ISGs to mount an antiviral response against viruses such as alphaviruses. Although this study did not determine the IFN induced factor that was responsible for the synergistic antiviral activity, it was posited that ISG15 modifies ZAP or another member of the IFN response. ISG15 is the most upregulated protein overall during the IFN response, and is a ubiquitin-like molecule. Given that ZAP is known to require ubiquitination by TRIM25 for optimal binding to viral mRNA and antiviral activity, ISG15 may play a similar role in enhancing ZAP’s activity. In fact, ZAP is a known putative target for ISG15 conjugation by E3 ligases within the IFN response.

1.3 Ebola restriction factors

Although many ISGs have been shown to be upregulated during the Type I IFN response to EBOV infection, such as those outlined by Caballero et al. (2016), the mechanisms by which most of these proteins carry out their anti-viral roles remains unclear. The role the Type I IFN response as a whole plays towards EBOV infection is quite elusive. However, a few ISGs have been identified as restriction factors specifically towards EBOV in vitro. The fact that EBOV strongly inhibits the Type I IFN response suggests that these restriction factors could potently restrict EBOV infection in vivo in the absence of IFN antagonists encoded by the viral genome.

1.3.1 ISG15

ISG15 is one of the most upregulated genes during the Type I IFN response to most viruses including EBOV, demonstrating its importance in the innate defense against viral infection. ISG15 is a ubiquitin-like molecule, which acts to modify proteins via a mechanism called ISGylation. ISGylation occurs through a series of steps in which UbeL1 (E1) activates ISG15, and UbcH8 (E2) and an E3 ligase together conjugate the molecule to substrates within the cell. Common E3 ligases include the HECT and RCC-1-like domain containing (HERC) and tripartite motif (TRIM) protein families. ISGylation plays a crucial role in restricting the replication of viruses such as Influenza A, HIV-1 and EBOV. During EBOV infection specifically, ISG15 inhibits the budding of VP40 VLPs. It does this by interacting with the E3 ligase Nedd4, which is required for the
ubiquitination of VP40 prior to viral budding. Free ISG15 acts to block an interaction between the E3 ligase Nedd4 and its E2 counterpart, thereby preventing further ubiquitination by Nedd4. Ultimately, such an interaction prevents the necessary ubiquitination of VP40, and may suggest that ISGylation is also blocking the site of ubiquitination. ISGylation of viral structural proteins to prevent viral assembly and release is not unique to EBOV, and is seen with other viral structural proteins such as Influenza A NS-1, HIV-1 Gag or Human Papillomavirus (HPV) L1. ISG15 plays other more general antiviral roles including immune modulatory activity, prevention of viral defense against IFNβ and increased susceptibility to viral infection when deficient.

1.3.2 Interferon induced transmembrane proteins

IFN-induced transmembrane proteins, or IFITMs, inhibit entry of EBOV particles by interfering with GP-mediated entry during the late stages of endosomal trafficking. IFITMs are a family of proteins that are primarily known for their IFN-mediated control of Influenza A virus by targeting the entry process. Although there has been less research done on other viruses, EBOV is likely similarly targeted at the stage of viral fusion. This is suggested by the fact that IFITM mediated restriction can be circumvented by complementing cells with cathepsin L, which allows for the late stages of endosomal compartment formation and viral fusion to occur.

1.3.3 Tetherin

Tetherin is known to inhibit release of EBOV by preventing diffusion of viral particles after budding. It is a transmembrane protein known to bind viral and host membranes, thereby preventing the release of viral particles by physically linking the viral and cellular membranes. This proteinaceous covalent bonding results in the accumulation or anchoring of viral particles along the plasma membrane, similar to the well-characterized effect that tetherin has on HIV-1. In retroviruses, these particles accumulate in a chain-like fashion and are ultimately re-internalized and subsequently degraded within the cell. Although tetherin’s activity towards EBOV is not as well studied, it is predicted that degradation of particles occurs similar to retroviruses.
For the vast number of IFN-induced proteins known to play a role in the Type I IFN response, it is unfortunate that ISG15, IFITMs and tetherin are the only well-established proteins that target EBOV. Furthermore, all of these proteins are ultimately antagonized by EBOV during an infection, giving rise to the overall suppression of the IFN response and allowing severe pathogenesis to manifest. No antiviral restriction factors have been identified which successfully circumvent EBOV infection. Investigating other IFN-induced proteins and their effect on EBOV is therefore important for better understanding of the virus-host interplay during infection.

1.4 HECT and RCC-1 like domain containing protein 5 (HERC5)

One IFN-induced protein that warrants further investigation is HERC5. HERC5 is known to inhibit the replication of evolutionarily diverse viruses including HIV-1, influenza A, murine leukemia virus and HPV. HERC5 is 1024 amino acids in length, or ~117 kDa, and is ubiquitously expressed with highest expression levels in the testis. It is the main mammalian E3 ligase, meaning it conjugates the ubiquitin-like protein ISG15 to substrates within the cell.

HERC5 belongs to a family of six HERC proteins located on chromosome 4, each containing an amino terminal Regulator of Chromosome Condensation 1 (RCC-1) like domain (RLD), a spacer domain that does not share homology with any known proteins, and a carboxyl terminal Homologous to E6-AP Carboxyl Terminus (HECT) domain (Figure 3). The RLD domain and HECT domain are highly conserved through evolutionarily diverse mammals, suggesting these domains play a fundamental role in mammalian biology. The RLD domain’s predicted structure closely resembles the crystal structure of the human RCC-1 protein, with its seven-bladed β-propeller structure. This domain has been speculated to be responsible for HERC5’s localization to polyribosomes translating novel proteins, and partially responsible for the ISGylation of some proteins. The HECT domain’s predicted structure is bi-lobed and is predominantly responsible for the E3 ligase activity of the HERC family of proteins. HERC5 in particular contains the active site for ISGylation at the Cys 994 residue located within the HECT domain. The HECT domain of HERC5 operates in conjunction with an
activating E1 and conjugating E2 enzyme to transfer ISG15 to specific cellular substrates through the ISGylation mechanism that has been previously explained\textsuperscript{64}.

HERC5 broadly targets newly synthesized proteins as well as constitutively expressed proteins involved in various cellular pathways, including RNA splicing, chromatin remodeling, transcription, cytoskeletal organization, the stress response and translation\textsuperscript{58,67,68,83–85}. By associating with polyribosomes, HERC5 can target a wide variety of proteins during their translation\textsuperscript{68}. Several targets are also IFN induced proteins involved in the antiviral response to foreign pathogens\textsuperscript{58,85}. Regulation of HERC5 expression is hence necessary to avoid ubiquitous ISGylation of cellular components. HERC5 expression is up-regulated in response to Type I IFNs \textit{in vitro} and \textit{in vivo} virus infection, lipopolysaccharide, tumor necrosis factor α, and interleukin-1β\textsuperscript{78}.

HERC5 inhibits viral replication through E3 ligase-dependent and -independent mechanisms. Woods \textit{et al.} (2011) has shown that HERC5 utilizes E3 ligase activity to ISGylate the structural protein of HIV-1, Gag, in order to cause the restriction of viral assembly and release\textsuperscript{64}. HERC5 directly interacts with Gag and post-translationally modifies it with ISG15, thereby causing arrest of an early stage of viral assembly at the plasma membrane. This restriction was distinct from expression of ISG15 alone. HERC5 has also been shown to ISGylate the structural protein of Influenza, NS-1, to prevent homodimer formation and corresponding host antagonism mediated by NS-1\textsuperscript{63,67}. HERC5 also ISGylates HPV L1 capsid protein to reduce viral infectivity of HPV pseudoviruses\textsuperscript{68}.

On the other hand, Woods \textit{et al.} (2014) has revealed an E3 ligase-independent mechanism by which HERC5 restricts HIV-1\textsuperscript{78}. Quantitative PCR (qPCR) and immunoprecipitation showed that HERC5 prevented nuclear export of viral RNA by interrupting the Rev/RRE pathway in an RLD dependent manner\textsuperscript{78}. Given the diverse viral targets and various ways by which HERC5 can restrict viral replication, HERC5 may restrict other viruses that have not been investigated, such as EBOV. Previous findings showing that E3 ligases such as Nedd4 regulate EBOV egress lends support to this idea\textsuperscript{21}. As previously mentioned, HERC5 is also one of the most upregulated genes during EBOV infection\textsuperscript{35}. 
Despite upregulation of various IFN-induced antiviral proteins such as HERC5, it should be noted that EBOV is still able to defeat the body’s defense mechanisms and cause debilitating disease. Similar to the other identified IFN-induced restriction factors of EBOV, any inhibitory activity mediated by HERC5 would likely be ablated in vivo due to the potent suppression of the Type I IFN response mediated by EBOV.

**Figure 3. Schematic of HERC5.**

HERC5 is an E3 ligase of ~117 kDa in size. It contains an amino-terminal RLD domain responsible for polyribosomal localization and restriction of some viruses by preventing nuclear export of viral mRNA. The predicted β-propellor structure is shown below the genomic representation of this domain. The carboxyl terminal HECT domain is responsible for E3 ligase activity, with its active site at Cys 994 indicated by a yellow box. The ISGylation capabilities of HERC5 are involved in restriction of HIV-1, Influenza A and HPV pseudoviruses through ISGylation of their structural proteins. This domain has a predicted bi-lobed structure shown below the genomic model. These two domains are connected by a spacer domain that contains many α-helices but does not share structural homology with any known proteins, and has no known function in HERC5’s activity.
1.5 Ebola antagonism towards host

The severe pathophysiology of EBOV infection can be attributed to its potent suppression of the IFN response. EBOV has been well documented to inhibit Type I IFN production within a wide array of cell types including human umbilical vein endothelial cells (HUVECs), peripheral blood mononuclear cells (PBMCs) and human monocyte-derived macrophages\textsuperscript{86,87}. Studies in HUVECs showed that there was rapid expression of pro-inflammatory cytokines during infection, but expression of IFN α and β was delayed until 3 days post-infection\textsuperscript{86}. This study also found that the formation of IFN-induced transcription factor complexes, as well as the expression of important proteins within the IFN pathway were reduced\textsuperscript{15}. The absence of these IFN-induced genes is responsible for depleting overall antiviral activity, and allows the severe pathogenesis of EBOV infection to take form. Not surprisingly, antagonism of the IFN response by EBOV is associated with increased virulence\textsuperscript{88}. Genomic analysis by Kash \textit{et al.} (2006) demonstrated that infection of human liver cells with an Ebola strain with a lower replication rate (Reston) resulted in increased activation of ISGs as compared to a highly replicating strain (Zaire)\textsuperscript{88}. This suggests that the Zaire strain is better able to evade the host immune response due to Type I IFN suppression, resulting in decreased antiviral proteins and increased virulence.

The ability of EBOV to selectively inhibit the Type I IFN response suggests that the IFN response harbors immune mechanisms that could ablate EBOV infection in the absence of Ebola antagonists\textsuperscript{89}. In fact, the difference between those who survived EBOV infection and those who did not was found to be in their innate immune responses, including the IFN response\textsuperscript{90,91}. Moreover, the viral antigen load did not vary between survivors and non-survivors, though the viral clearance by the innate immune system was significantly higher for survivors\textsuperscript{90}. Baize \textit{et al.} (1999) showed that survivors of the 1966 outbreak in Gabon, Africa had increased levels of IFN mRNA as well as other innate immune molecules such as IgG antibodies\textsuperscript{91}. Ultimately however, EBOV outbreaks hold up to 90% fatality rates due to its multifaceted ability to suppress the Type I IFN response. The viral genome encodes seven accessory proteins, three of which play a known role in this process.
1.5.1 Viral protein 35

The VP35 protein functions as a powerful virulence factor, and is a major contributor in shutting down the production of IFN-α/β. It does this by interacting with members of the RIG-1 pathway, one of the cascades depicted in Figure 2 that causes IFN-α/β expression and is triggered after the RNA helicase RIG-1 senses the EBOV genome. Particularly, VP35 interacts with two IFN regulatory factor (IRF) 3 kinases, IKKe and TBK-1, to prevent phosphorylation and activation of IRF3. IRF3 is a transcription factor that is responsible for transcription of IFN-α/β. Another way by which VP35 shuts down the IFN response is by interacting with an E2 and E3 ligase, Ubc9 and PIAS1 respectively, in order to cause the conjugation of a small ubiquitin-like modifier (SUMO) to IRF3 and IRF7. As functionally homologous transcription factors, SUMOylation of IRF3/7 represses transcription of ISGs. Other mechanisms of IFN suppression by VP35 include suppression of RNA silencing, and inhibition of dsRNA-activated protein kinase (PKR), an IFN-induced dsRNA-activated cellular serine/threonine kinase responsible for inhibiting viral replication. VP35 alone provides a significant virulence factor to the virus, though EBOV does contain other proteins to evade the Type I IFN response.

1.5.2 Viral protein 24

VP24 is considered the secondary matrix protein involved in capsid formation, but is also involved in virulence. Its primary role as a virulence factor is to prevent nuclear accumulation of STAT1, which, when dimerized, is allowed to enter the nucleus and act as a transcription factor for IFN-α/β and various ISGs. Western blotting and co-immunoprecipitation techniques show that VP24 interacts with a member of the NP-1 family of proteins responsible for the import of dimerized STAT1 into the nucleus. This protein, karyopherin α1 (KPNA), is a nuclear localization signal receptor for dimerized STAT1, which is inhibited by the interaction with VP24. Interestingly, VP24 uses mimicry of STAT1 to bind to KPNA, rather than ablating the receptor’s activity, and is itself imported into the nucleus.
1.5.3 Glycoprotein

GP potently prevents the activity of IFN-induced tetherin, through a mechanism that has yet to be elucidated\textsuperscript{74}. Using FACS analysis, Lopez et al. (2010) demonstrated that, unlike other viral mechanisms to counter tetherin such as HIV-1 Vpu, GP does not remove tetherin from the surface\textsuperscript{75}. GP also did not require specific sequences within the tetherin protein to cause restriction, suggesting that EBOV GP inhibits tetherin activity through a novel mechanism\textsuperscript{75}. Though studies have been inconclusive, it is possible that GP relocates tetherin within the plasma membrane or interrupts the structural integrity of the protein at filoviral budding sites. Radoshitzky et al (2010) observed that tetherin is excluded from the plasma membrane in sites that contained EBOV GP during infection, lending support to the idea that tetherin is relocalized in order to evade its tethering mechanisms\textsuperscript{100}.

1.6 Project rationale

In summary, EBOV is one of the most virulent pathogens that infects humans. Its extreme pathogenesis can be largely attributed to evasion of the innate immune response\textsuperscript{6,8}. Specifically, EBOV is known to harbor various mechanisms to evade the Type I IFN response, a response that triggers the production of antiviral proteins such as ISG15, IFITMs and tetherin that create an abrasive environment for the virus within the cell\textsuperscript{6}. For example, accessory proteins VP35 and VP24 are known to interrupt ISG transcription at numerous points\textsuperscript{92–94,96,98}. GP is also known to prevent the activity of IFN-induced tetherin, thereby allowing the budding and release of EBOV particles\textsuperscript{74,75}. The potent suppression of the IFN response by EBOV suggests that this defense mechanism would be effective in preventing viral replication in the absence of appropriate antagonists.

It is important for future studies to investigate the interplay between EBOV and the human host to further characterize the Type I IFN response and Ebola’s activity against it. It is likely that additional IFN induced proteins such as HERC5 act as antiviral proteins against EBOV infection, and that EBOV encodes appropriate antagonists. As aforementioned, HERC5 restricts various viruses using mechanisms that may or may not involve its E3 ligase activity\textsuperscript{64,67,68,78}. It is possible that HERC5 uses its E3 ligase activity
to ISGylate the structural protein of EBOV, VP40, similar to HIV-1, Influenza A or HPV\(^6^4\). Previous research showing that ISGylation of VP40 prevents viral release suggests that an E3 ligase is involved in the process\(^6^5,6^6\); HERC5 may be conjugating ISG15 to prevent VP40 VLP release. Alternatively, HERC5 may use an entirely novel defense mechanism to restrict EBOV, as the Spacer domain remains uncharacterized and the RLD domain has been recently discovered to be involved in a unique E3-ligase independent antiviral mechanism against HIV-1\(^7^8\).

HERC5 may cause downstream antiviral events against EBOV, such as degradation of viral protein, inhibition of translation or degradation of viral RNA. A major factor already known to be involved in reducing EBOV RNA levels within an IFN stimulated cell is ZAP\(^5^0\). ZAP is known to act synergistically with other IFN induced proteins, and is known to be activated by ubiquitination – a molecule resembling ISG15\(^5^4,5^6,5^7\). Considering ZAP is a putative target for HERC5 ISGylation\(^5^8\), HERC5 may utilize the ZAP pathway to mediate its restriction. The potential for HERC5’s involvement in the IFN response against EBOV infection has driven the initiation of this project, and lead to my hypothesis.

### 1.7 Hypothesis and specific aims

The overall objective of this project was to determine if HERC5 mediates antiviral activity towards EBOV. I hypothesized that, given the evolutionarily diverse set of viruses that HERC5 restricts, it would also restrict EBOV and that one or more of the genes encoded by EBOV would antagonize HERC5’s restriction capabilities.

To address this hypothesis I have four major specific aims: 1) Develop a model system of EBOV in order to study its replication. 2) Determine if HERC5 can mediate antiviral activity towards EBOV. 3) Characterize the mechanism by which HERC5 mediates restriction. 4) Determine if one (or more) of the EBOV proteins antagonize HERC5-mediated activity. The goal of this research is to further the understanding of the virus-host interplay between EBOV and their human host counterparts, and advance the current understanding of HERC5’s role in the innate immune response to viral infection.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cells and cell lines

Adherent 293T human fibroblast and HeLa cells were maintained in complete Dulbecco’s Modified Eagle’s Medium (cDMEM) (10% FBS, 1% Pen Strep (Sigma) in DMEM) at 37°C with 5% CO_2. Cell lines were obtained from American Type Culture Collection.

2.2 Plasmids, transfections and antibodies

A summary of the plasmids and RNAs used in this study and their sources can be found in Table 1. Note that pCR2.1-GP was restriction digested using HindIII and XbaI, and GP was inserted into pCMV3xFLAG using T4 Ligase according to manufacturer’s instructions (New England BioLabs). VP24 was unable to be cloned into a mammalian expression vector (ie. pCMV3xFLAG). pEGFP-VP40 and pFLAG-VP40 were cloned using the Gibson Assembly Master Mix kit (New England BioLabs) and the primers listed in Table 2. HERC5 mutants (HERC5-RLD-only and HERC5-ΔHECT) were constructed using site-directed mutagenesis (primers listed in Table 2), and cloned according to instructions in the Fast Cloning protocol described by Li et al. (2011)\textsuperscript{12}.

Unless otherwise stated, plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) or LipoD (Invitrogen) according to manufacturer’s instructions at a ratio of 1:10 for VP40:GL3/HERC5/ HERC5-ΔRLD/ HERC5-RLD-only/HERC5-ΔSpacer/HERC5-ΔHECT/HERC5-C994A (restriction assays) or 1:10:10 for VP40:GL3/HERC5:GL3/VP30/VP35/L/NP/GP (antagonist assays).

Knockdown of HERC5 using small interfering RNA (siRNA) was performed using Lipofectamine RNAiMAX (Life Technologies) transfection at a final concentration of 40 pM siRNA/sample as per the manufacturer’s instructions. Knockdown of HERC5 using short hairpin RNA (shRNA) was performed by transfecting HERC5\textsubscript{shRNA} (or Scram\textsubscript{shRNA}) with Lipofectamine 2000 at a ratio of 10:1 with VP40 and IFNβ (PBL Assay Science) inducing at 1000U/mL for 24h before harvesting. Knockdown of ZAP using shRNA was performed by transfecting ZAP\textsubscript{shRNA} (or Scram\textsubscript{shRNA}) 48h before transfection with HERC5.
and VP40 (ZAPshRNA/ScramshRNA:GL3/HERC5:VP40 10:10:1). 24h later, IFNβ induction at 1000U/mL was performed and harvested after another 24h. For the MG132 assay determining the effect of proteasome inhibition, 20 µM MG132 (Sigma) was added to culture supernatant at 32 hours post-transfection, and cells were treated for 16 hours before harvesting.

Antibodies: anti-FLAG was obtained from Sigma, anti-GFP from Clontech, anti-GAPDH from EMD Millipore Corp., and anti-ZAP from Abcam.
**Table 1.** Summary of plasmids and RNAs used in this study

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* University of Wisconsin, USA
† Genome Institute of Singapore, SG
‡ Western University, CA
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* All oligonucleotides are in 5’ to 3’ orientation
2.3 Western blotting

Clarified cell supernatants containing VLPs were pelleted over a 20% sucrose cushion for 2 hours at 21,000 × g. For cell lysates, cells were detached and centrifuged at 1500 × g for 5 mins. Pellets were lysed with 1× RIPA buffer (50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Protease Inhibitor Cocktail (Sigma), 1% 100mM phenylmethanesulfonyl fluoride in isopropanol, 1% TritonX-100, 0.1% SDS). For quantitative Western blotting, samples were mixed with 4× loading buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, and 5% beta-mercaptoethanol) to a final 1× concentration and separated on a 10% SDS-PAGE gel. Protein was transferred onto 0.2µm Low Fluorescence Amersham Hybond Polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences) by semi-dry transfer. Western blotting was carried out by blocking the membrane for 1 hour in Li-cor Blocking Buffer (Li-cor Biosciences) followed by a ~16 hour incubation with 1:1000 dilution of primary antibody. Detection was carried out using IRdye-labeled secondary antibody (1:20,000 for 1 hour) and the Li-cor Odyssey Clx Detection System (Li-cor Biosciences). Densitometric analysis was performed using ImageJ 1.48 u 64-bit version software (NIH, USA).

2.4 RNA isolation and reverse transcription polymerase chain reaction

Total RNA was isolated from cell lysates using the Purelink RNA Mini Kit and reverse transcribed using M-MLV reverse transcriptase according to manufacturer's instructions (Life Technologies). 40ng of cDNA was used as template for quantitative real-time polymerase chain reaction (qRT-PCR) reactions using gene specific primers listed in Table 2 and the SYBR Probe Master Mix (Qiagen). qRT-PCR was carried out using QuantiStudio5 Applied Biosystems with the following specifications: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 60 sec at 60°C (Thermo Fisher Scientific). Threshold cycle (C\text{T}) values for RNA were normalized to the C\text{T} values of GAPDH and relative fold changes were calculated using the Pfaffl Equation.
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*All oligonucleotides are in 5’ to 3’ orientation*
2.5 Confocal immunofluorescence microscopy

HeLa cells were cultured in 12-well plates on 18 mm coverslips and co-transfected with either pHERC5, pGL3 and pEGFP-VP40 (10:10:1 ratio) or pHERC5, pZAP and pEGFP-VP40 (10:10:1 ratio). Twenty-four hours after transfection, coverslips containing cells were washed twice with PF buffer (1× PBS + 1% FBS), fixed for 10 minutes in 1× PBS containing 5% formaldehyde and 2% sucrose, permeabilized in 1× PBS containing 5% NP-40 and then washed twice more with PF buffer. Coverslips were incubated with either primary antibody mouse anti-FLAG (1:500 dilution) or rabbit anti-ZAP (1:500) for 1 hour, washed 3x with PF buffer and incubated with either secondary antibody anti-rabbit 488 (1:10000 dilution) or anti-mouse 594 (1:1000) for 1 hour. Coverslips were washed 3x, incubated in Hoechst 33342 (1:10000 dilution) (Life Technologies) for 5 mins and washed 6x with PF buffer. Coverslips were then mounted on glass slides with 10 µL Vectashield mounting media (Vector Laboratories Inc.) and sealed with nail polish. Confocal micrographs were obtained using a Leica TCS SP8 (Leica Microsystems) microscope, and Leica Application Software X was used for image acquisition, Pearson’s coefficient and Mean Fluorescence Intensity calculations.

2.6 Transmission electron microscopy

293T cells were co-transfected with pGL3 or pHERC5 and pEGFP-VP40 at a 10:1 ratio. After 48 hours, cells were resuspended in media, fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) for 2 hours, and washed 3x in 0.1M sodium cacodylate. Cells were pelleted and fixed with 2% osmium tetroxide in sodium cacodylate. After ~1 hour in the dark, cells were washed 3x in ddH₂O. Cells were pelleted again, water was discarded and samples were left at 4°C overnight. Dehydration of samples was carried out by adding 1 mL 20% acetone in ddH₂O, mixing and incubating 10 min at RT. Cells were pelleted, acetone was removed and procedure was repeated with 50%, 70%, 90%, 100%, 100% and 100% acetone. Cells were embedded in resin by adding 1 mL of a 2:1 mix of acetone:resin (Epon) and incubating for ~4hrs at RT in a rotating tube shaker. Cells were pelleted, acetone:resin mix was discarded and this was repeated with a 1:1 mix overnight, 1:2 mix overnight and finally only resin overnight. Samples were cut in 70 nm slices using a Sorval Ultracut ultramicrotome, and placed onto 400 mesh nickel grids (Embra). Grids
were placed on drops of 2% uranyl acetate in ddH2O to stain for 20 mins in the dark, and washed 5-6x in ddH2O for 1 min. Samples were then stained in drops of Sato’s lead citrate (5 mM calcined lead citrate, 11 mM lead nitrate, 11 mM lead acetate, 95 mM sodium citrate) for 1 min and washed using ddH2O. NaOH pellets were placed beside each sample during lead staining to eliminate lead citrate exposure to atmospheric CO2 and therefore to prevent its precipitation. Samples were then imaged using a Phillips CM10 Transmission Electron Microscope. AMT Advantage digital imaging system was used for image acquisition.

2.6.1 Immunogold transmission electron microscopy

293T cells were fixed, embedded in resin, cut and placed onto grids as previously explained. Grids were blocked with a 0.2 uM filtered 1% BSA PBS Buffer (10.4 mM Na2HPO4, 3.2 mM KH2PO4, 20 mM Na3, 150 mM NaCl, 1% BSA, pH 7.4) and incubated at room temperature for 2 hours with 0.25 mg/ml mouse anti-GFP (1:20 dilution). Samples were washed and incubated at room temperature for 2 hours with secondary goat anti-mouse IgG colloidal 5 nm gold-conjugated antibody (Frogga cat# GA1003) at a dilution of 1:30. Samples were washed with BSA-PBS buffer and then with dH2O. Samples were stained with 2% uranyl acetate for 5 minutes and then washed with dH2O. Samples were imaged using a Phillips CM10 Transmission Electron Microscope. AMT Advantage digital imaging system was used for image acquisition.

2.7 Co-immunoprecipitation

Co-immunoprecipitation of pFLAG was performed using the μMACs anti-DYKDDDDK kit according to manufacturer’s instructions. Briefly, 293T cells were lysed using Lysis Buffer (150 mM NaCl, 1% Triton® X-100, 50 mM Tris HCl pH 8.0, 1% Protease Inhibitor Cocktail (Sigma)) for 30 min on ice. 50µL (per 10cm dish of 293T cells) anti-DYKDDDDK microbeads were mixed with lysed 293T cells to magnetically label FLAG tagged proteins. Lysate was run through a magnetic column and washed 4x with Wash Buffer 1 (150 mM NaCl, 1% Igepal CA-630 (formerly NP-40), 0.5% sodiumdeoxycholate, 0.1% SDS, 50 mM Tris HCl pH 8.0) and 1x with Wash Buffer 2 (20 mM Tris HCl pH 7.5). Protein was eluted with pre-heated (95ºC) 50µL Elution Buffer (50
mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol).

2.9 Statistical analyses and bioinformatics

GraphPad Prism v4.03 was used for all statistical analyses. P values and statistical tests were stated where appropriate. P values less than 0.05 were deemed significant.

2.10.1 Immunogold labelling quantification

Quantification of immunogold particle localization was performed as previously described [1]. Gold particles were counted for four fields of view per condition and scored as one of three regions: 1) Ebola VLPs and plasma membrane; 2) cytoplasm and nucleus; and 3) not containing particles or cells. The plasma membrane was defined as the outermost edge of the cell to a distance 100 nm inside the cell (approximately the diameter of an Ebola VLP [2]). This numerical frequency distribution (G₀) represents the 'observed' distribution [102]. The 'expected' or 'predicted' distribution was determined using a randomly positioned lattice of test points (P) superimposed on each field of view using the ‘grid’ feature in Adobe Photoshop CS3. The resulting distribution of test (or 'grid') points shows what would be expected if gold particles were scattered randomly across the cell [102]. The number of points that fell on each of the three regions of interest were scored. The expected number of gold particles (Gₑ) for each region was calculated using the formula: Gₑ = P*(total G₀)/(total P). The corresponding partial $X^2$ for each region was calculated from the observed and expected gold counts as: $X^2 = (G₀-Gₑ)^2/Gₑ$. If the total $X^2$ value for the given degrees of freedom (df) (given by 2-1 columns × 3-1 regions) indicated that the observed and expected distributions were significantly different, the null hypothesis of no difference from random labeling was rejected. Preferentially labeled regions were identified on the basis of satisfying two criteria. First, the G₀/Gₑ was > 1 and, secondly, the corresponding partial $X^2$ value accounted for a substantial proportion (≥ 10%) of the total $X^2$ value [102].

2.10.2 CG dinucleotide content

CG content of EBOV sequences was calculated by counting the number of CG dinucleotides in a 200 base pair sliding window of each viral cDNA sequence and a random
sequence, as shown by Muller et al. (2007)\textsuperscript{50}. Coding sequences for each Ebola protein can be found in Appendix A.
CHAPTER 3: RESULTS

3.1 Construction and verification of EBOV VP40 VLP assay

In order to measure and visualize HERC5’s effect on EBOV VLP production, we aimed to generate a GFP fusion protein with structural protein VP40. 293T cells were transfected with constructs encoding VP40 with N-terminal GFP (pEGFP-VP40-C1), or C-terminal GFP (pEGFP-VP40-N1) (Figure 4A). Western blot analysis of VP40 VLPs showed that VLPs were released into the supernatant of EGFP-VP40-C1 expressing cells but not EGFP-VP40-N1 expressing cells (Figure 4B). The band showing GFP-VP40 immunoreactivity appeared at ~65kDa, consistent with the fusion of the 40kDa VP40 protein with the 29kDa EGFP protein. To further investigate assembly and budding of the GFP-VP40 VLPs we performed confocal fluorescence microscopy and transmission electron microscopy (TEM). Confocal micrographs of HeLa cells expressing EGFP-VP40-C1 displayed punctate fluorescent GFP-VP40 bodies localized at the plasma membrane consistent with budding of VLPs (Figure 4C), whereas EGFP-VP40-N1 expressing cells showed GFP-VP40 evenly distributed throughout the cytoplasm (Figure 4C).

Transmission electron micrographs of 293T cells expressing EGFP-VP40-C1 displayed efficient VLP release when compared to an unperturbed membrane of a “mock” transfected cell (Figure 4D). VLPs appeared as both spherical electron dense cross sections ~ 100 nm in diameter, and as elongated filamentous cross-sections ~300 nm in length. Filamentous VLPs were protruding vertically from the membrane, as depicted in the schematic representation of vertical budding in Figure 4E. Successful budding of VLPs at the plasma membrane by EGFP-VP40-C1 allowed us to utilize this plasmid for further experiments involving EBOV VLPs.
Figure 4. Plasmid containing GFP tagged VP40 at the N-terminus caused budding similar to wild type EBOV.

(A) Plasmid constructs expressing N-terminus or C-terminus GFP-tagged VP40. (B) Detection of GFP-VP40 VLPs in cell supernatant by Western blot using anti-GFP for EGFP-VP40-C1 or EGFP-VP40-N1 transfected 293T cells. (C) Confocal micrographs showed GFP-VP40 fluorescent bodies localized at the plasma membrane in HeLa cells 48 hours after transfection with EGFP-VP40-C1. GFP-VP40 was spread evenly throughout the cytoplasm of HeLa cells 48 hours after transfection with EGFP-VP40-N1. Scale bars = 10µm (D) Transmission electron micrographs showed unperturbed membrane of 293T cells “mock” transfected, and VLP release in 293T cells transfected with EGFP-VP40-C1. Bottom panels of micrographs are zoomed images of the black boxes in the upper images. Scale bars = 500 nm. (E) Schematic representation of vertical (left) and horizontal (right) budding of EBOV (Takada 2012)\textsuperscript{15}. 
3.2 HERC5 potently blocks the release of EBOV VP40 VLPs at the plasma membrane

To determine the phenotypic effect of HERC5 expression on VP40 production we used both confocal and transmission electron microscopy (TEM) (Figure 5). In Figure 5A, HeLa cells co-expressing GFP-VP40 and empty vector depicted punctate fluorescence at the plasma membrane, consistent with the location of budding GFP-VP40 VLPs. However, cells co-transfected with GFP-VP40 and HERC5 lacked VP40 punctate fluorescence at the plasma membrane, suggesting an inhibition of VLP production and release (Figure 5A).

Transmission electron micrographs of 293T cells co-transfected with GFP-VP40 and empty vector also had a perturbed plasma membrane and spherical electron dense cross-sections of VLPs ~100 nm in diameter (Figure 5B). Cells co-transfected with GFP-VP40 and HERC5 showed substantially less VLPs compared to the control and displayed a smooth plasma membrane similar to mock transfected cells (Figure 5B). VLP production was quantified using immunogold TEM in which 293T cells were transfected, prepared for TEM and stained using a gold-tagged antibody targeting GFP (Figure 5C). HERC5 transfected cells exhibited significantly fewer immunogold particle counts as compared to control (Figure 5D) (P<0.01, student’s T test). It is interesting to note that some VLPs were produced in HERC5-transfected cells, however these particles appeared to be trapped at the plasma membrane as indicated with arrows shown in Figure 5B and Figure 5C.

To confirm that VP40 protein accumulated in EBOV VP40 particles and electron dense regions at the plasma membrane, we performed localization studies of immunogold particles. Quantification of immunogold localization on the electron microscopic thin sections from cells expressing HERC5 and VP40 showed that the gold labeling distribution was significantly different from a random distribution (Chi square analysis, $X^2 = 314.8$, df = 2, $P < 0.0001$) (Table 4) (see Methods for details). The VP40 was primarily localized along the plasma membrane, though VP40 was also found within VLPs that were formed
Figure 5. HERC5 blocks VP40 VLP formation along the plasma membrane

(A) Confocal micrographs of HeLa cells that were co-transfected with GFP-VP40 and either empty vector (control) or HERC5. Anti-GFP was used to detect GFP-VP40, and anti-Flag was used to detect HERC5. Scale bar = 10µm (B) Transmission electron micrographs of 293T cells that were “mock” transfected, transfected with empty vector (control) and VP40, or transfected with HERC5 and VP40. Bottom panels of micrographs are zoomed images of the black boxes in the upper images. Scale bar = 500nm (C) Micrographs of cells that were transfected with empty vector and immunogold labelled VP40, or HERC5 and immunogold labelled VP40. Gold particles are 5 ± 2 nm in size. Scale bars = 500 nm. (D) Quantification of immunogold particles. *** P<0.001, **P<0.01, *P<0.05. Student’s paired T-test.
in the presence of HERC5 (Table 4). The localization of VP40 along the plasma membrane and within VLPs was also seen in control cells, as expected (Table 5).

3.3 HERC5 potently restricts EBOV VP40 protein

Given the drastic effect HERC5 showed towards VP40 VLP production using microscopy, we next wanted to look at the protein levels of VP40. We co-transfected 293T cells with 0.15 µg EBOV VP40 and increasing amounts of HERC5 (0 µg, 0.38 µg, 0.75 µg and 1.50 µg), followed by Western blot analysis of VP40 levels. In Figure 6A, cells co-transfected with HERC5 showed a dose-dependent reduction in VP40 protein with increasing HERC5. The highest restriction was seen when HERC5 was transfected at a 10:1 ratio to VP40, with a 2.51-fold reduction in VP40 protein. (P<0.01, one-way ANOVA with post-hoc Dunnet’s square). Similarly, a dose-dependent pattern of HERC5-mediated restriction occurred with VP40 VLPs released into the supernatant (Figure 6B), with a potent 6.67-fold reduction when HERC5 was transfected at a 10:1 ratio with VP40. This is consistent with the reduction in VP40 VLPs as shown through immunogold TEM quantification. To confirm HERC5’s responsibility for the reduction in VP40 protein levels, we used HERC5_{shRNA} to knockdown endogenous levels of HERC5 within 293T cells (knockdown efficiency found in Appendix B). 1.5µg of HERC5_{shRNA} was co-transfected with 0.15µg of FLAG-VP40. Western blot analysis showed that intracellular VP40 levels were increased 1.52-fold, while VP40 VLP levels were increased 1.69-fold when HERC5 was absent (Figure 6C).

3.4 Protein degradation is not involved in HERC5-mediated restriction of VP40

To better understand the mechanism of how HERC5 reduces intracellular levels of VP40, we asked if HERC5 induces degradation of VP40 via the 26S proteasome complex, the major complex involved in protein degradation within the cell. We co-transfected 293T cells with EGFP-VP40 and empty vector or HERC5, and 20 µM of 26S proteasome inhibitor MG132 was used to treat cells for 16 hours before VLP purification. Without MG132, significantly decreased VP40 levels were shown in the supernatant (P<0.0001, student’s paired t test) and cell lysate (P<0.0001, paired t test) in the presence of HERC5.
### Table 4. Quantification of 5nm gold particle labelled anti-GFP in cells expressing HERC5 and VP40

<table>
<thead>
<tr>
<th>Region</th>
<th>Observed gold count, ( G_0 )</th>
<th>Point count, ( P )</th>
<th>Expected gold count, ( G_e )</th>
<th>( G_0/G_e )</th>
<th>( X^2 )</th>
<th>( X^2 ) as %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles + plasma membrane</td>
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<td>15</td>
<td>4</td>
<td>7.75</td>
<td>176.43</td>
<td>66.94</td>
</tr>
<tr>
<td>Cytoplasm + nucleus</td>
<td>35</td>
<td>49</td>
<td>13</td>
<td>0.71</td>
<td>39.62</td>
<td>15.03</td>
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<tr>
<td>Non-particle + non-cell</td>
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<td>192</td>
<td>53</td>
<td>0.02</td>
<td>47.52</td>
<td>18.02</td>
</tr>
<tr>
<td>TOTAL</td>
<td>70</td>
<td>256</td>
<td>70</td>
<td>263.57</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

For \( X^2 = 176.43 \) and \( df=2 \), \( P <0.0001 \) (\( X^2 \) analysis). The gold labeling distribution is significantly different from random. Only the particles/plasma membrane region (\( G_0/G_e = 7.75, X^2 = 66.9\% \) of total) meets the two criteria for being preferentially labeled (\( (G_0/G_e) \geq 1 \) and \( X^2 > 10\% \) of total).

### Table 5. Quantification of 5nm gold particle labelled anti-GFP in cells expressing empty vector and VP40

<table>
<thead>
<tr>
<th>Region</th>
<th>Observed gold count, ( G_0 )</th>
<th>Point count, ( P )</th>
<th>Expected gold count, ( G_e )</th>
<th>( G_0/G_e )</th>
<th>( X^2 )</th>
<th>( X^2 ) as %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles + plasma membrane</td>
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<td>17</td>
<td>14</td>
<td>7.73</td>
<td>656.78</td>
<td>79.28</td>
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<tr>
<td>Cytoplasm + nucleus</td>
<td>99</td>
<td>64</td>
<td>55</td>
<td>1.82</td>
<td>36.33</td>
<td>4.39</td>
</tr>
<tr>
<td>Non-particle + non-cell</td>
<td>7</td>
<td>175</td>
<td>149</td>
<td>0.05</td>
<td>135.35</td>
<td>16.34</td>
</tr>
<tr>
<td>TOTAL</td>
<td>218</td>
<td>256</td>
<td>218</td>
<td>828.46</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

For \( X^2 = 656.78 \) and \( df=2 \), \( P <0.0001 \) (\( X^2 \) analysis). The gold labeling distribution is significantly different from random. Only the particles/plasma membrane region (\( G_0/G_e = 7.73, X^2 = 79.3\% \) of total) meets the two criteria for being preferentially labeled (\( (G_0/G_e) \geq 1 \) and \( X^2 > 10\% \) of total).
Figure 6. HERC5 restricts EBOV VP40 protein

(A) HERC5 reduces intracellular VP40 protein levels in a dose-dependent manner. Representative Western blot of three independent trials is shown. Western blots were densitometrically analyzed using anti-FLAG and anti-GAPDH. N=3. (B) HERC5 reduces VP40 VLP release into supernatant in a dose-dependent manner. Representative Western blot of three trials is shown. Western blots analyzed using anti-FLAG and anti-GAPDH. N=3. (C) HERC5 knockdown rescues VP40 protein. 293T cells were co-transfected with ScramshRNA or HERC5shRNA and FLAG-VP40. Forty-eight hours after transfection, EBOV VP40 VLPs released into the supernatant and intracellular protein were analyzed via Western blot using anti-FLAG and anti-GAPDH. N=3. *** P<0.001, **P<0.01, *P<0.05. One way ANOVA with post-hoc Dunnet’s square for A-B, paired T-test for C. All data are representative of at least three independent experiments (N).
HERC5 also significantly decreased VP40 levels within the supernatant (P<0.0001, student’s paired t test) and cell lysate (P<0.05, student’s paired t test) in the presence of MG132 (Figure 7A-C). There was no significant difference in VP40 levels between cells expressing HERC5 alone and cells expressing HERC5 with MG132 treatment.

3.5 HERC5 restricts VP40 mRNA

HERC5-mediated restriction at the protein level corresponded with a drastic reduction of VP40 at the mRNA level as measured using qPCR. Cells expressing HERC5 showed an 8.93-fold reduction in EBOV VP40 mRNA (Figure 8A)(P<0.0001, student’s paired T test). VP40 mRNA levels following HERC5 depletion using HERC5siRNA (knockdown efficiency shown in Appendix B) exhibited a 2.04-fold increase (Figure 8B)(P<0.05, student’s paired T test). We showed that HERC5-mediated restriction is specific to VP40 mRNA by investigating mRNA levels of a control gene (GFP) after HERC5 co-transfection. GFP mRNA levels were not significantly different from control when HERC5 was present (Figure 8C). HERC5 was also specific to VP40 independent of its plasmid backbone. HERC5 mediated significant restriction towards VP40 mRNA when co-transfected with either FLAG-VP40 or EGFP-VP40 (Figure 8C)( P<0.001, one-way ANOVA with Dunnet’s square post-hoc) This allowed us to use EGFP-VP40 for microscopy, and FLAG-VP40 for most other experiments.

Given HERC5’s potent restriction of EBOV VP40, we also wanted to see if this restriction was specific to VP40, or whether HERC5 can also restrict the expression of other EBOV genes. We co-transfected cells with either empty vector and one of VP40, VP30, VP35, L, NP or GP at a 10:1 ratio, or with HERC5 and one of the aforementioned proteins at the same ratio. Interestingly, HERC5 was able to significantly reduce all of the EBOV mRNAs, but VP40 was reduced to the greatest extent (P<0.0001, One-way ANOVA with Dunnet’s square post-hoc) (Figure 8D).
Figure 7. MG132 treatment did not affect HERC5 mediated restriction

(A) 293T cells were co-transfected with empty vector or HERC5 and EGFP-VP40. 20μM of 26S proteasome inhibitor MG132 was added to cells 32 hours after transfection and left to treat cells for 16 hours. GFP-VP40 levels in VLPs released into supernatant (A) and cell lysate (B) were analyzed by Western blot using anti-GFP and anti-β-actin as a loading control, and quantified densitometrically (normalized to actin). (C) Representative Western blot of three independent trials. ***, P<0.0005. **, P<0.005. Student’s paired t test.
Figure 8. HERC5 restricts VP40 mRNA

(A) HERC5 reduces intracellular VP40 mRNAs. 293T cells were co-transfected with either empty vector (control) or HERC5 at a 10:1 ratio. 48 hours after transfection mRNA was isolated and quantified using qRT-PCR. N=4. (B) HERC5 knockdown rescues VP40 mRNA. 293T cells were co-transfected with ControlsiRNA or HERC5siRNA and FLAG-VP40. Forty-eight hours after transfection, cellular mRNA was isolated and quantified using qRT-PCR. N=3. (C) HERC5 reduces VP40 independent of expression vector backbone. 293T cells were co-transfected with either empty vector and one of VP40-EGFP or VP40-pFLAG (Control), or HERC5 and one of VP40-EGFP or VP40-pFLAG. GFP was used as a negative control. Forty-eight hours after transfection mRNA was isolated and quantified using qRT-PCR. N=3. (D) HERC5 reduces intracellular EBOV mRNA of other EBOV proteins. 293T cells were co-transfected with either empty vector and one of VP40, VP30, VP35, L, NP or GP (Control), or with HERC5 and one of the aforementioned proteins. Forty-eight hours after transfection, cellular mRNA was isolated and quantified using qRT-PCR. N=3. *** P<0.001, **P<0.01, *P<0.05. Paired T-test for A-B, one way ANOVA with post-hoc Dunnet’s square for C-D.
3.4 The RLD domain of HERC5 is necessary and sufficient to cause restriction

To assess HERC5’s ability to reduce VP40 mRNA levels and corresponding protein and VLP production, we utilized an assay involving HERC5 mutants to determine which domain is responsible for HERC5-mediated restriction of VP40. 293T cells were co-transfected with FLAG-VP40 and either empty vector (control), wild-type HERC5 or a HERC5 mutant lacking one of the three HERC5 domains. Two other mutants, HERC5-C994A and a HERC5 mutant only containing the RLD domain (HERC5-RLD-only) were also used. The HERC5-C994 residue is the active site for E3 ligase activity, and is known to be involved in HERC5-mediated restriction of HIV-1, influenza and HPV 16 pseudoviruses. E3 ligase activity is ablated by the C994A mutation.

Cells transfected with HERC5-ΔRLD showed a near complete rescue of intracellular VP40 protein as analyzed by Western blot, compared to the 1.92-fold reduction mediated by wild-type HERC5 (Figure 9A-B) (P<0.001, one-way ANOVA with post-hoc Dunnet’s square). This corresponded with a rescue in VP40 VLP release; an 8.17-fold reduction in VP40 VLPs mediated by HERC5 was reduced to a 1.92 fold reduction with the HERC5-ΔRLD mutant (Figure 9A&C) (P<0.001, one-way ANOVA with post-hoc Dunnet’s square). The other domain mutants showed restriction similar to wild-type HERC5, including HERC5-ΔHECT, HERC5-C994A and HERC5-ΔSpacer (Figure 9B). Since VP40 was transfected in a FLAG backbone, the VP40 and HERC-RLD-only mutants were too similar in size to be distinguished by Western blot analysis. Cells were instead co-transfected with GFP-VP40 and either empty vector (control), HERC5 or HERC-RLD-only and analyzed via Western blot. HERC5-RLD-only was able to reduce VP40 intracellular protein and VLP release similar to wild-type HERC5 (Figure 9D-E) (P>0.05, one-way ANOVA with post-hoc Dunnet’s square).

Similar findings suggesting that the RLD domain is both necessary and sufficient for HERC5-mediated restriction were seen at the RNA level. qPCR analysis revealed an 8.14-fold reduction in VP40 mRNA in the presence of wild-type HERC5, as opposed to a 1.72-fold reduction in the presence of HERC5-ΔRLD (Figure 9F) (P<0.01, one-way
Figure 9. The RLD is necessary and sufficient for restriction of VP40.

(A) Representative Western blot of three trials. 293T cells were co-transfected with FLAG-VP40 and empty vector, HERC5, HERC5-ΔRLD, HERC5-RLD-only, HERC5-ΔSpacer, HERC5-ΔHECT or HERC5-C994A. 48 hours after transfection VP40 levels in the cell lysate and VLPs released into the supernatant were analyzed by Western blot using anti-FLAG and anti-GAPDH. (B) Intracellular HERC5 restriction of VP40 protein was rescued by HERC5-ΔRLD, (C) as was the release of VP40 VLPs into the supernatant. (D) Representative Western blot of three trials. 293T cells were transfected with EGFP-VP40, and empty vector, HERC5 or HERC5-RLD only and analyzed via Western blot using anti-GFP and anti-GAPDH. Intracellular (E) and VLP release (F) of VP40 was similar between the WT and RLD-only HERC5. (G) HERC5 mediated restriction of VP40 mRNA was partially rescued by HERC5-ΔRLD, while HERC5-RLD-only mediated restriction similar to WT HERC5. 293T cells were co-transfected with FLAG-VP40 and empty vector, HERC5, HERC5-ΔRLD, HERC5-RLD-only, HERC5-ΔSpacer, HERC5-ΔHECT or HERC5-C994A. 48 hours after transfection mRNA was isolated and qRT-PCR was used to detect intracellular VP40 mRNA levels. N=3. *** P<0.001, **P<0.01, *P<0.05. One way ANOVA with post-hoc Dunnet’s square. All data are representative of at least three independent experiments (N).
ANOVA with post-hoc Dunnet’s square). The other domain mutants (HERC5-ΔSpacer, HERC5-ΔHECT and HERC5-C994A) did not show a significant rescue in VP40 mRNA levels (Figure 9F). Agreeing with the notion that the RLD domain is necessary for HERC5-mediated restriction, the RLD-only mutant mediated a reduction in VP40 mRNA similar to wild-type HERC5 (Figure 9F) (a 10.77-fold decrease in VP40 mRNA) (P<0.01, one-way ANOVA with post-hoc Dunnet’s square). This data also shows that the E3 ligase activity of HERC5 is not required for restriction.

3.6 HERC5 does not affect nuclear export of VP40 mRNA

Since we eliminated VP40 protein degradation as a major player in HERC5’s activity, we thought that mRNA was instead being affected. Although EBOV does not enter the nucleus during its regular life cycle, our VP40 assay is plasmid based and therefore enters the nucleus to be transcribed and translated. Since HERC5 is known to inhibit mRNA nuclear export of HIV-1’s structural protein, we tested if HERC5 induced the nuclear accumulation of VP40 mRNA. We performed a cytoplasmic extraction of cells co-transfected with VP40 and either empty vector or HERC5, and compared mRNA levels to the whole cell extracts. VP40 mRNA levels were not significantly different between the cytoplasmic extract and whole cell extract (P>0.05, one-way ANOVA with Dunn’s square post-hoc) (Figure 10). This indicates that HERC5 likely does not cause nuclear accumulation of VP40 mRNA, however we cannot rule out the possibility of degradation of accumulated mRNA. This is important in establishing the biological relevance of our findings.

3.7 HERC5 is dependent on ZAP for restriction at the RNA level

To further elucidate the mechanism that HERC5 utilizes to reduce EBOV VP40 mRNA levels, we investigated the involvement of ZAP, a protein known to recruit RNA degradation factors and has previously been shown to target EBOV proteins for degradation. We transfected 293T cells with FLAG-VP40 and either empty vector (control), HERC5, ZAP or HERC5 and ZAP together, followed by qRT-PCR to examine VP40 mRNA levels. We were able to show that ZAP reduces VP40 mRNA by 7.54-fold, which was not significantly different from HERC5 (P < 0.001, One-way ANOVA with
Figure 10. HERC5 does not affect nuclear export of VP40 mRNA

293T cells were co-transfected with VP40 and either empty vector or HERC5. mRNA was isolated from cytoplasmic and whole cell extracts, and subjected to qRT-PCR 48 hours after transfection to determine VP40 mRNA levels. N = 3 (independent trials). One-way ANOVA with Bonferroni post-hoc.
post-hoc Dunnet’s square) (Figure 11A). When co-transfected, HERC5 and ZAP trend towards increased restriction, with a 9.55-fold reduction in VP40 mRNA levels ($P < 0.001$, One-way ANOVA with post-hoc Dunnet’s square) (Figure 11A). To determine if HERC5 requires ZAP to restrict VP40, we used a shRNA targeting ZAP to deplete endogenous levels of ZAP (knockdown efficiency shown in Appendix B). 293T cells which basally express ZAP were co-transfected with either scrambledshRNA or ZAPshRNA. As a positive control showing HERC5-mediated restriction of VP40, we transfected scrambledshRNA expressing 293T cells with VP40 and either empty vector (control) or HERC5 after 48 hours. HERC5 transfected cells showed a 3.33-fold reduction in VP40 mRNA ($P < 0.01$, student’s paired T test) (Figure 11B). Contrastingly, in ZAPshRNA expressing cells, HERC5 did not mediate VP40 restriction ($P > 0.05$, student’s paired T test) (Figure 11C).

Conversely, we asked if ZAP requires HERC5 for restriction of VP40 mRNA. We co-transfected 293T cells with either scrambledshRNA or HERC5shRNA, followed by VP40 and either empty vector or ZAP (HERC5 knockdown efficiency can be found in Appendix B). ZAP was able to reduce VP40 mRNA 9.80-fold in scrambledshRNA expressing cells, agreeing with the restriction seen in Figure 11A ($P<0.0001$, student’s paired T test)(Figure 11D). ZAP was also able to reduce VP40 mRNA 8.77-fold in the absence of HERC5 (HERC5shRNA expressing cells) ($P<0.0001$, student’s paired T test)(Figure 11E), indicating that ZAP is able to restrict VP40 independent of HERC5 recruitment.

3.8 ZAP and HERC5 are interaction partners and require the RLD domain for interaction

Given ZAP’s involvement in HERC5-mediated restriction of VP40 mRNA, we asked whether HERC5 was recruiting ZAP to degrade viral RNA. To answer this question we used confocal microscopy of HeLA cells. Cells were transfected with empty vector, ZAP, HERC5, or ZAP and HERC5 and visualized using anti-ZAP and anti-pFLAG (Figure 12A). Interestingly, the mean fluorescence intensity of ZAP was significantly increased in the presence of HERC5 compared to ZAP alone ($P<0.0001$, student’s paired T test)(Figure 12B). Pearson’s correlation coefficient indicated that HERC5 and ZAP significantly colocalized ( $r = 0.458$, $P<0.0001$, One-way ANOVA with post-hoc Dunnet’s square)
Figure 11. HERC5 necessitates ZAP for restriction of VP40 mRNA

(A) HERC5 and ZAP mediate restriction of VP40 mRNA. Restriction increases (albeit not significantly different from H5 or ZAP alone) when HERC5 and ZAP are combined. 293T cells were transfected with VP40, and either an empty vector, HERC5, ZAP, or HERC5 + ZAP. RNA was isolated after 48 hours and quantified using qRT-PCR. N=3. (B-C) 293T cells were transfected with either Scrambled_shRNA or ZAP_shRNA. 48 hours after knockdown cells were co-transfected with VP40 and either empty vector or HERC5. mRNA was isolated and qPCR was used to detect VP40 levels 48 hours post-transfection. N=3. (B) HERC5-mediated restriction in Scrambled_shRNA transfected cells. (C) HERC5 no longer mediates potent restriction in ZAP_shRNA expressing cells. (D-E) 293T cells were transfected with either Scrambled_shRNA or HERC5_shRNA. 48 hours after transfection cells were co-transfected with VP40 and either empty vector or ZAP, and harvested for RNA isolation and qPCR 48 hours later. (D) ZAP mediated restriction in Scrambled_shRNA expressing cells and in (E) HERC5_shRNA expressing cells. **** P<0.0001, *** P<0.001, **P<0.01, *P<0.05. One way ANOVA with post-hoc Dunnet’s square in A and student’s paired T test in B-E. All data are representative of at least three independent experiments (N).
(Figure 12C). These data show that HERC5 may be upregulating ZAP expression, and supports HERC5’s recruitment of ZAP to VP40 mRNA.

To investigate this further, we determined if HERC5 and ZAP were interaction partners through co-immunoprecipitation. 293T cells were transfected with either empty vector, wild-type HERC5 or one of the HERC5 mutants. 48 hours after transfection cells were lysed under non-denaturing conditions and magnetic beads targeting FLAG tagged HERC5 were used to pull down HERC5 and interaction partners. Precipitated proteins were resolved using SDS-PAGE and subjected to Western blotting using anti-ZAP. ZAP was co-immunoprecipitated in the presence of wild-type HERC5 and HERC-RLD-only, but not in the presence of HERC5-ΔRLD (Figure 12D).

3.9 HERC5’s specificity for VP40 is not related to CG dinucleotide content

Recent research has shown that high CG dinucleotide content within viral genomes increases ZAP antiviral activity52. We thought that ZAP may be targeting VP40 mRNA for degradation by recognizing a high CG dinucleotide content within its RNA. We used a 200 bp sliding window to analyze CG dinucleotide content across the EBOV genome (sequences found in Appendix A). There was no significant difference between the CG dinucleotide content of each viral mRNA (P>0.05, One-way ANOVA with Bonferroni post-hoc) (Figure 13).

As a control we also analyzed HIV-1 coding sequences for CG dinucleotide content. This virus is known to exhibit CG dinucleotide suppression to avoid recognition by ZAP52. Consistent with this study, mRNA within the EBOV genome contained significantly higher levels of CG dinucleotides than HIV-1 (P<0.01, One-way ANOVA with Bonferroni post-hoc test) (Figure 13).

3.10 Ebola GP can antagonize HERC5 mediated restriction of VP40 protein and mRNA

EBOV is able to mount a potent suppression towards the Type I IFN response during infection, lending support towards the notion that EBOV harbours mechanisms to antagonize IFN induced HERC574,87,88,93,95. We tested a panel of EBOV proteins for potential antagonism of HERC5-mediated restriction of EBOV VP40. 293T cells were
**Figure 12. HERC5 interacts with ZAP through the RLD domain**

(A) Representative confocal micrographs of HERC5 and ZAP co-expression. HeLa cells were co-transfected with either empty vector, ZAP, HERC5, or ZAP and HERC5 and probed with anti-FLAG and anti-ZAP. Scale bars = 10 µm. (B) Mean fluorescence intensity of ZAP. Cells were outlined with the nucleus excluded, and analyzed for mean fluorescence intensity of the region of interest (ROI). (C) Pearson’s correlation coefficient showed significant colocalization between ZAP and HERC5. (D) 293T cells were transfected with either empty vector, HERC5, HERC5-ΔRLD, HERC5-RLD-only, HERC5-ΔSpacer, or HERC5-ΔHECT. HERC5 was immunoprecipitated under non-denaturing conditions using anti-FLAG magnetic beads. Precipitated proteins were resolved using SDS-PAGE and subjected to Western blotting using anti-ZAP. 30% of the input lysate from each condition was subjected to Western blotting using anti-ZAP and anti-GAPDH on the same blot. ZAP was pulled down in the presence of HERC and HERC5-RLD-only, but not HERC5-ΔRLD. *** P<0.001, **P<0.01, *P<0.05. Student’s paired T test in C. One-way ANOVA with post-hoc Dunnet’s square in D.
A 200 base-pair sliding window was used to count CG dinucleotide content within the EBOV (19kb) and HIV-1 genome (9kb) (nucleotide sequences shown in Appendix A). A model of the EBOV genome is shown along the top to indicate the regions of each viral protein. CG dinucleotide content for each EBOV protein sequence is significantly higher than HIV-1 (P<0.01, One-way ANOVA with Bonferroni post-hoc). No significant differences in CG dinucleotide content between EBOV protein sequences.

Figure 13. CG dinucleotide content within the EBOV genome
transfected with either empty vector or HERC5, plus VP40 and one of EBOV VP30, VP35, NP, L or GP. 48 hours after transfection, cells were harvested and analyzed using western blot or qPCR. The presence of VP35, VP30 or NP showed similar restriction of VP40 compared to cells transfected with HERC5 and VP40 alone (Figure 14A-B). Interestingly, the presence of L protein or GP showed a rescue in intracellular VP40 protein and mRNA that was not significantly different from control (P>0.05, student’s T test) (Figure 14A-B). Additionally, HERC5 was no longer able to mediate restriction of VLPs being released into the supernatant in the presence of GP (Figure 14A).

To determine if HERC5 levels were altered in the presence of GP, we measured HERC5 protein levels via quantitative Western blotting. HERC5 protein levels were not significantly altered when co-transfected with VP40 and any of the EBOV proteins (Figure 14C).

3.11 GP antagonism is specific to filoviral GPs

Since EBOV GP was able to potently antagonize HERC5 activity at the protein and RNA level, we decided to test whether this antagonism was specific to EBOV GP or if other viral envelope GPs are able to similarly antagonize HERC5. We transfected 293T cells with VP40, either empty vector or HERC5 and one of either EBOV GP, Marburg Virus GP or an irrelevant envelope protein – Vesicular Stomatitis Virus G (VSV-G). We examined intracellular VP40 protein levels, VP40 VLPs released into the supernatant and VP40 mRNA. Interestingly, the presence of VSV G did not affect HERC5’s ability to restrict intracellular VP40 protein (P<0.01, One-way ANOVA with Dunnett’s square post-hoc) (Figure 15A-B & C-D respectively). HERC5 was also able to significantly restrict VP40 mRNA in the presence of VSV G (P< 0.01, One-way ANOVA with Dunnett’s square post-hoc) (Figure 15E). In contrast, HERC5 expressing cells in the presence of EBOV GP or Marburg GP showed VP40 protein and mRNA levels similar to control (Figure 15A-E). This is interesting since EBOV GP only shares 34.40% identity with Marburg GP when the protein sequences were aligned using Clustal Omega (Figure 15F).
Figure 14. EBOV L and GP antagonize HERC5 mediated restriction of VP40

293T cells were co-transfected with VP40, either empty vector or HERC5 and one plasmid containing an EBOV protein. (A) VP40 levels in cell lysate and VLPs released into supernatant were analyzed by Western blot using anti-GFP and anti-GAPDH as a loading control, and quantified densitometrically (normalized to GAPDH). N=6. Includes representative Western blots analyzed using anti-GFP, and anti-GAPDH. (B) VP40 mRNA levels were quantified using qRT-PCR after co-transfection as explained previously. N=3. (C) HERC5 protein levels remained consistent in the presence of VP40 and each of the other EBOV proteins. 293T cells were co-transfected with VP40, HERC5 and one of the EBOV proteins followed by densitometric analysis.*** P<0.001, **P<0.01, *P<0.05. Student’s paired T test in A and one way ANOVA with post-hoc Dunnet’s square in B & C. All data are representative of at least three independent experiments (N).
Figure 15. HERC5 antagonism is specific to filoviral GPs.

293T cells were co-transfected with VP40, either empty vector or HERC5 and either EBOV GP, Marburg GP or VSV G. (A) VP40 levels in cell lysate were analyzed by Western blot using anti-FLAG and anti-GAPDH as a loading control, and quantified densitometrically (normalized to GAPDH). N=3. (B) Shows representative Western blots analyzed using anti-FLAG, and anti-GAPDH. (C) Densitometric analysis of VP40 VLP levels in supernatant. (D) Representative Western blots analyzed using anti-FLAG. Note that these samples were run on separate Western blots, therefore blots shown in B and D are a combination of 3 different blots. (E) VP40 mRNA levels were quantified using qRT-PCR after co-transfection as explained previously. N=3. (F) Clustal Omega multiple alignment of VSVG, EBOV GP and Marburg GP protein sequences. EBOV GP shares 34.40% identity with Marburg GP and 16.50% with VSV G. GenBank Accession EBOV GP: AAG40168.1; Marburg GP: APQ46224.1, VSV G: AAA48389.1. ***P<0.001, **P<0.01, *P<0.05. One way ANOVA with post-hoc Dunnet’s square. All data are representative of at least three independent experiments (N).
CHAPTER 4 – DISCUSSION

We have identified HERC5 as a novel IFN-induced protein that is able to restrict EBOV VP40 intracellular mRNA and restrict the release of assembled VLPs from the plasma membrane. The RLD domain located at the amino terminus of HERC5 was necessary and sufficient for HERC5-mediated restriction of EBOV VP40, and mediated an interaction between HERC5 and ZAP. ZAP is a protein involved in RNA degradation that we have demonstrated to be required for HERC5 restriction of VP40 mRNA. We also showed that GP antagonizes HERC5-mediated restriction of VP40 mRNA, protein and VLP release.

The mammalian Type I IFN response is strongly upregulated during EBOV infection, however few IFN-induced proteins have been identified which act to inhibit EBOV replication. The identification of HERC5 as an inhibitor of EBOV VP40 VLP production deduces a novel innate defense mechanism against EBOV. Data presented by Johnson et al. (2016) and Noda et al. (2002) show the formation of VP40 VLPs through confocal and transmission electron microscopy that are consistent with the VLPs produced in our assay. The particles in our transmission electron micrographs were ~100nm in diameter, agreeing with the aforementioned studies and with the size of wild-type virus (80nm). Most of our particles were spherical in shape, which are likely cross sections of filamentous particles. These particles also appeared to be budding vertically from the cell; vertical particles are known to be less infectious, which agrees with the nature of our assay – it does not contain the nucleocapsid hypothesized to be responsible for horizontal budding. Our confocal micrographs were also in agreement with prior literature showing GFP-VP40 bodies along the plasma membrane indicative of budding. However, this was only seen with the VP40 construct that has the GFP tag at the amino-terminus. The absence of immunoreactivity in Western blots shown in Figure 5B and diffuse fluorescence throughout the cytoplasm in the confocal micrographs of carboxyl-tagged GFP-VP40 suggests that this construct fails to bud and resembles what is seen with truncated VP40. Hence, GFP likely interferes with the function of the C-terminus of VP40, including electrostatic and hydrophobic properties inducing plasma membrane attachment and eventual VLP formation. Membrane attachment induces negative curvature changes in the plasma
membrane consistent with VP40 pushing the inner leaflet of the plasma membrane out of the cell to form a VLP\textsuperscript{19}. Despite the inefficient formation of VLPs in cells expressing GFP at the carboxy terminus of VP40, cells expressing VP40 with GFP at the amino terminus represented an efficient model of VLP release. We have therefore demonstrated a morphologically and biologically relevant assay that depicts HERC5-mediated restriction of VP40. It should also be noted that our studies were largely performed in 293T cells, which are human kidney fibroblasts – a cell type known to be infected during the onset of EBOV infection\textsuperscript{5}.

HERC5 showed its largest capacity to restrict VP40 mRNA, though other EBOV mRNAs were also reduced. It is known that HERC5 is recruited to polyribosomes concurrent with translation of new proteins, though it remains unclear why VP40 may be targeted more favorably than other EBOV RNAs\textsuperscript{68}. It is possible that the RLD domain is involved in the recruitment of HERC5 to particular viral mRNAs, as this domain has been shown to be important for co-localization of HERC5 and polyribosomes\textsuperscript{64,68}. The polyribosome is a likely site for HERC5 interaction with viral mRNA, as proteins are being newly synthesized from mRNA\textsuperscript{64,68}. This agrees with our findings that the RLD domain is both necessary and sufficient for HERC5-mediated restriction of EBOV VP40 mRNA and protein. We have revealed a novel antiviral mechanism for HERC5, which is independent of E3 ligase activity performed by the active site (Cys994) within the HECT domain\textsuperscript{64,104}. HERC5’s E3 ligase activity has been shown to block the function of many viral proteins including HIV-1 Gag and influenza A NS1, and to interfere with the infectivity of HPV pseudoviruses\textsuperscript{64,67,79}. It is therefore interesting to identify a novel mechanism not only independent of E3 ligase activity, but independent of any other known function of HERC5. Woods et al. (2011) were able to show that the RLD domain blocks the nuclear export of unspliced HIV-1 RNA, but this exhausts the known functions of the RLD domain within HERC5\textsuperscript{78}. Given that the amount of mRNA within the cytoplasmic and whole cell extracts did not significantly differ (Figure 10), we eliminated blocking of VP40 nuclear export as a possible HERC5 mediated mechanism for EBOV restriction. This lends support to the idea that HERC5 plays a role in the hosts innate defense against EBOV, as EBOV mRNA is translated in the cytoplasm and does not enter the nucleus. HERC5 blocking nuclear export of our plasmid-based VP40 construct would be biologically irrelevant; eliminating this possibility was important in supporting a biologically relevant mechanism for VP40
restriction. Delving further into the underlying mechanisms of HERC5 and the spectrum of pathogens that it targets is especially important given that HERC5 is evolutionarily conserved among mammalian vertebrate hosts\textsuperscript{78}. This research is therefore relevant to various hosts outside of humans.

We showed that HERC5’s mechanism does not involve targeting VP40 protein to the 26S proteasome, though increased VP40 levels may be seen with MG132 treatment due to normal protein turnover\textsuperscript{105}. MG132 acts by covalently binding the active site of the beta subunits in the 20S proteasome, blocking the proteolytic activity of the 26S proteasome complex\textsuperscript{106}. Since this complex is the major degradation site for proteins in eukaryotic cells\textsuperscript{106}, an increase in total protein would be expected when it is inhibited. Importantly, HERC5 was still able to restrict VP40 protein when cells were treated with MG132. Our findings differ from the mechanistic action of other HERC family members, namely HERC3. HERC3 directly interacts with the proteasome to lead to degradation of RelA\textsuperscript{37}. HERC5 on the other hand, reduces VP40 protein levels both without and with proteasome inhibition, suggesting that HERC5 does not interact with the 26S proteasome complex and that protein degradation is not involved in HERC5’s activity towards EBOV.

This study instead identified a mechanism of HERC5-mediated restriction requiring ZAP activity. ZAP is known to recognize ZREs within viral mRNA, and cause downstream RNA degradation by the RNA processing exosome\textsuperscript{46}. ZAP has also been shown to act synergistically with other Type I IFN induced proteins to mount an antiviral response against alphaviruses, making plausible its involvement and direct interaction with HERC5 to reduce EBOV VP40 mRNA\textsuperscript{54}. In fact, a study by Muller \textit{et al.} (2007) has shown that ZAP acts to decrease mRNA levels of EBOV proteins including VP40, although the RNA dependent RNA polymerase (L protein) was affected the most\textsuperscript{50}. Given that we have identified ZAP to be involved in HERC5-mediated restriction of VP40, it is logical that the reduction in VP40 is amplified greater than that seen by Muller \textit{et al.} (2007) when HERC5 is overexpressed\textsuperscript{50}.

We were also able to show that ZAP restricts VP40 independent of HERC5. When ZAP was overexpressed alone, VP40 mRNA levels were reduced similar to that seen in
the overexpression of HERC5. This suggests that HERC5 is not required for ZAP-mediated restriction at highly elevated levels of ZAP expression, although HERC5 and ZAP may have an additive effect when overexpressed together. This is supported by knockdown assays of HERC5, in which ZAP is still able to cause a reduction in VP40 mRNA when HERC5 is absent. We hypothesize that this is due to the nature of HERC5 acting upstream from ZAP; HERC5 activates or recruits ZAP to degrade VP40. ZAP activity is therefore enhanced in the presence of HERC5, but HERC5 is not absolutely required for degradation.

Co-immunoprecipitation assays determined that there is an interaction between HERC5 and ZAP that may promote activity towards VP40. Agreeing with our previous findings identifying the RLD domain as the active domain, the RLD mediated an interaction with ZAP. Given the RLD’s role in mediating HERC5’s recruitment to the polyribosome, we speculate that the RLD is recruiting ZAP to the complex for viral mRNA degradation. This was further investigated through confocal fluorescence microscopy, in which there was a strong colocalization between HERC5 and ZAP. Additionally, ZAP’s intensity was significantly higher in the presence of HERC5. Together, these findings suggest that HERC5 and ZAP form a complex, in which there could be a direct interaction between the two, or indirect interaction where HERC5 may perform ISGylation. Although ISGylation was proven to not be required for restriction, close proximity of the two proteins may incidentally ISGylate and stabilize ZAP. This would explain the increase in intensity of ZAP in the presence of HERC5, especially since ZAP is already known to be stabilized by an ISG15-like molecule, ubiquitin, through TRIM25. Stabilization by TRIM25 has been shown to be important in ZAP’s restriction of Sindbis virus. Furthermore, HERC5 is known to use ISGylation to stabilize other IFN-induced proteins such as IRF3.

Identification of ZAP as an interaction partner in HERC5-mediated restriction led us to wonder whether ZAP is also responsible for HERC5’s specificity towards VP40 mRNA. As previously mentioned, HERC5 can restrict all EBOV proteins, but VP40 was reduced to the highest extent. Although ZAP does not have a known target sequence, it is known to target and bind regions of foreign RNA that are rich in CG dinucleotides. After investigation of the CG dinucleotide content of each viral protein, we concluded that there
was not a significantly higher CG dinucleotide content within VP40 compared to the other EBOV mRNAs. However, the entire EBOV genome contained a significantly higher relative amount of CG dinucleotides compared to HIV-1. This agrees with previous findings showing CG dinucleotide suppression in HIV-1 and gives one explanation as to how HERC5 and ZAP coordinate to target EBOV but not HIV-1 RNA.

It is important to note that although we have identified ZAP as a downstream partner involved in HERC5 mediated restriction, there are likely other proteins or mechanisms at play given the complexity of the IFN response. Specifically, we believe there is a second blockade mediated by HERC5 in which fully formed VLPs are trapped at the plasma membrane. This is supported by our TEM images displaying VLPs beneath the plasma membrane, and confocal images showing GFP-VP40 VLPs localized along the membrane. A secondary mechanism is also supported by our Western blots. What we have found in this report is that, contingent on the RLD domain, HERC5 initiates mRNA degradation through an interaction with ZAP. A downstream reduction in VP40 protein and subsequent VLP release is thus caused. When the RLD is deleted, the intracellular VP40 protein is fully rescued, but VLPs being released into the supernatant are still modestly reduced – suggesting a mechanism exists between the production of protein and release of VLPs that is independent of the RLD domain. HERC5 has been shown to prevent an early stage of viral assembly in HIV-1, where it prevents the release of VLPs along the plasma membrane. Although this would not be the primary mechanism used by HERC5 towards EBOV, a mechanism similar to that seen in HIV-1 could contribute to the overall reduction in VP40 VLP release at the plasma membrane.

In HIV-1, ISGylation acts to interfere with an early stage of assembly of viral particles. In this study we have identified a mechanism of restriction that is independent of E3 ligase activity, which may seem contrary to our proposed secondary mechanism. However, we hypothesize that the primary mechanism involving ZAP saturates HERC5’s restriction capabilities when the RLD domain is present. This is supported by our transmission electron micrographs in which very few fully formed particles were measured; the minimal particles that were assembled could then be restricted by the secondary mechanism. This secondary mechanism may therefore only contribute
significantly when the RLD domain is deleted. It is thus important for future experiments to study whether HERC5 is able to mediate restriction in a HERC5-ΔRLD-ΔHECT or HERC5-ΔRLD-C994A mutant form.

EBOV’s potent ability to suppress the Type I IFN response is consistent with our finding that EBOV GP and L protein antagonize HERC5-mediated restriction of VP40 mRNA and protein. GP has already been shown to be involved in the suppression of the Type I IFN response by interrupting the antiviral activity of the IFN-induced protein tetherin. We have now identified a second mechanism by which EBOV GP defends itself from the host immune response. Although we began investigating the mechanism by which GP may act to antagonize HERC5, it remains elusive how this protein suppresses HERC5 function. It is likely that GP interrupts the restriction complex, given its ability to fully rescue VP40 mRNA, protein and VLP release. Since GP is a transmembrane protein, it is possible that GP also functions at the plasma membrane, as it is speculated to do during antagonism of tetherin. GP antagonism may therefore be involved in antagonizing HERC5’s ability to trap particles at the plasma membrane – a mechanism we have yet to investigate. Furthermore, our study showed that this antagonism is specifically mediated by filoviral GPs; our research may elude to a novel mechanism whereby filoviral GPs target the IFN response and HERC5 in particular.

Interestingly, we have identified a second antagonist, L protein, which prevents HERC5’s activity towards VP40 mRNA and modestly towards intracellular protein, but fails to antagonize HERC5’s restriction of VP40 VLP release into the supernatant. It is possible therefore that L protein antagonizes HERC5-mediated degradation of VP40 mRNA, but is unable to prevent the restriction of VLPs at the plasma membrane. Similar to GP, this mechanism remains to be elucidated.

4.1 Limitations of the study

As aforementioned, we were unable to clone VP24 into a mammalian expression vector. We were hence unable to test whether HERC5 reduces VP24 mRNA levels as it does the other EBOV proteins. We were also unable to test potential antagonism mediated
by VP24 towards HERC5. Given VP24’s known ability to counter the IFN response, it may potentially also target HERC5\textsuperscript{98}.

The main limitation of this study was the absence of a more biologically relevant system, involving all of the Ebola proteins in an infectious pseudovirus system. Investigating HERC5’s role in an infectious system is important in establishing the relevance of HERC5 in an actual EBOV infection. This will allow us to determine whether HERC5 plays a role in multi-round infections and to further study EBOV’s antagonism of HERC5 in the presence of all of the viral proteins. Using a biologically relevant system allows for a more thorough understanding of the virus-host interplay and how HERC5 is involved in the human defense against EBOV infection as a whole. This data is crucial moving forward and may lead to the study of HERC5 within a fully replication competent system modeling wild type infection.

4.2 Future directions

We aim to further elucidate the HERC5-ZAP pathway using mass spectrometry to examine other potential interaction partners such as the RNA processing exosome or PARN\textsuperscript{46,109}. These are downstream mRNA degradation factors known to be recruited by ZAP and would further confirm this pathway. We also plan to perform the co-immunoprecipitation studies in the presence of RNAse, to determine if viral VP40 mRNA is required for the HERC5-ZAP interaction to take place. Furthermore, a sequencing technology called CLIP-seq will allow us to identify binding sites for ZAP or HERC5 within viral mRNA. In brief, CLIP-seq couples proteins to their RNA targets, degrades RNA outside of the binding region and sequences this region. Although analysis of the EBOV genome showed that CG dinucleotide content is an unlikely cause for VP40 targeting, a specific target sequence may still be responsible. This would also potentially reveal a sequence specific target region for ZAP or ZREs, which has not been identified before.

Future directions also include exploring other possible mechanisms used by HERC5 to restrict VP40. Specifically, we plan to utilize TEM and confocal microscopy to study viral restriction that was seen at the plasma membrane but was not further
investigated in this study. Using our domain mutants will be important in determining which domain is responsible for virus trapping at the plasma membrane, and will be our first step in elucidating the potential for this secondary mechanism. Visualizing the effect of the GP antagonist on VLP release will also be important in beginning to study its method of antagonism.

Most importantly, we aim to test HERC5’s effect on the release of infectious EBOV pseudovirions using the system outlined by Mühlberger et al. (2017)\textsuperscript{110}. This a transcription and replication competent VLP system (trVLP) and is based on a tetracistronic minigenome that encodes a luciferase reporter, as well as VP40, VP24, and GP. Co-expression of this minigenome together with NP, VP35, VP30, and L drive genome replication and transcription, synthesis of the minigenome-encoded proteins, and formation of trVLPs. These trVLPs can incorporate minigenomes and infect target cells for multiple passages. Propagation of these trVLPs can be quantified over time (every three days) by measuring the luciferase reporter activity within cells at different passages. This system can be used within a wide range of cell types, can be utilized in a Biosafety Level 2 laboratory, and has been identified as a tool for antiviral drug screens for EBOV infection\textsuperscript{110}. This system will be important in our study of HERC5’s restriction capabilities during EBOV infection and research into the virus-host interplay by looking at the effect of HERC5 on multi-round infections. Additionally, we have initiated a collaboration with Dr. Gary Kobinger, the developer of ZMapp and chief of special pathogens at the Public Health Agency of Canada’s (PHAC) Biosafety Level 4 microbiology laboratory in Winnipeg, to study HERC5 in wild-type EBOV infection. Together these studies may lead to the long-term goal of studying novel small molecule drugs that exploit HERC5’s restriction capabilities while preventing viral antagonism towards the IFN response.

4.3 Conclusions

EBOV is one of the most virulent pathogens to infect humans. EBOV’s extreme pathogenesis can be largely attributed to its ability to globally and selectively suppress the Type I IFN response, yet the effect that the IFN response plays in defending the host during infection remains largely uncharacterized. Gaining a broad sense of the virus-host interplay during infection, especially with respect to the Type I IFN response, plays a large part in
understanding EBOV disease. Filling the gaps in knowledge regarding the interaction between EBOV and the innate immune response is critical in building a strong foundation of knowledge in EBOV pathology, immunological research, and the development of future therapeutic strategies.

Overall, by showing that the IFN-induced protein HERC5 targets EBOV VP40 mRNA, we have identified a novel antiviral mechanism targeting EBOV. Moreover, the ability of HERC5 to deplete viral RNA via its RLD domain and ZAP identifies a novel E3 ligase-independent antiviral mechanism for HERC5. Importantly, we have identified EBOV GP as an antagonist towards HERC5 activity – identifying a novel point at which EBOV can suppress the innate immune response and mitigate the host’s antiviral defense. Moving forward, this information not only furthers knowledge regarding EBOV and the human immune response towards it, but is also translatable to therapeutic strategies that can mimic the effects of HERC5 while inhibiting antagonistic activity mediated by Ebola virus GP.
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Appendices

Appendix A. Ebola protein sequences

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**Gene: Sequence**

### VP40:
ATGacatggatccatgatgtagtgaAGGCCGTTTATATTGCTACTGCTCTCCTCTCTGAATATATTGGAGCGCATATAATCACTACAGAGGAACCATGGCTCCTGCTGCTGCTGAGA

### VP35:
ATGACACTAAGAACAAAGGCCAGGGCCAGATACGCTGGCCAGCAGCTACTAAGAATAACGAGAGGAAGCTTGCACTACCTGGACCATCACTTTATGCAAGAAGTG

### VP30:
ATGGTCTAAAGCCAGTTTATGATATGGCAAAAACAATCTCCTCATTGAACAGGGTTTGTGCTGAGATGGTTTTGGCTACTGTTGCAACAACAAACCAT

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### VP40:

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### VP35:

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### VP30:

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### VP24:

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CAGTCCTCAATTTCATGGCCCAATCGGATAGTAAATTCAAGCAACGCGATTGATTGTTCTTCTAACAACACTTT
AGGTGAGTTTTCAGGAGGTGGCCAGTCTGCA

* CG dinucleotides are underlined
Appendix B. Knockdown efficiencies for shRNAs and siRNAs used within this study.

293T cells were transfected with either an shRNA targeting a scrambled sequence of RNA (Scrambled\textsubscript{shRNA} in A and C) or an siRNA targeting a scrambled sequence (Scrambled\textsubscript{siRNA} in B) as a control. To measure knockdown efficiency, cells were transfected with either (A) HERC5\textsubscript{shRNA} (shHERC5), (B) HERC5\textsubscript{siRNA} (siHERC5) or (C) ZAP\textsubscript{shRNA} (shZAP) and RNA levels were measured using qPCR. Knockdown efficiencies were 44\%, 60\% and 58\% respectively. **P<0.01, *P<0.05. Student’s paired T test.
Appendix C. Copyright permissions for figures 1-2.
Curriculum Vitae

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Western University, MSc 2016-2018
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Honours and Awards:
CIHR – Canada Graduate Scholarship Master’s 2017
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