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Molecular Characterization of HERC5: A Novel Multifunctional Antiviral Protein

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Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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MOLECULAR CHARACTERIZATION OF HERC5: A NOVEL
MULTIFUNCTIONAL ANTIVIRAL PROTEIN

(Thesis format: Integrated Article)

by

Matthew W. Woods

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Interferon (IFN)-induced proteins serve as one of the first lines of defense against viral pathogens such as Human Immunodeficiency Virus (HIV)-1. IFN treatment has been shown to restrict multiple stages of HIV-1 replication. The identities and functions of IFN-induced proteins involved in the inhibition of HIV-1 and other viruses are not fully understood. Homologous to the E6-AP C-terminus (HECT) and regulator of chromosome condensation 1 (RCC1)-like domain (RLD) containing protein 5 (HERC5) is strongly upregulated by the Type I IFN response. HERC5, a member of the small HERC family, is composed of an N-terminus RLD domain and a C-terminus HECT domain separated by a spacer region. With the discovery that HERC5 is the main E3 ligase for ISG15, and given the well-known antiviral properties of ISG15, I hypothesized that HERC5 is an evolutionarily conserved restriction factor that interferes with HIV-1 particle production.

Using an HIV-1 particle release assay, I showed HERC5 inhibits HIV-1 assembly and budding using an ISG15 E3 ligase-dependent mechanism which modifies HIV-1 Gag with ISG15. Next, I determined that HERC5 restricts HIV-1 replication by inhibiting unspliced HIV-1 mRNA export, which is dependent on its RLD domain. I then assessed the antiviral activity of the other small HERC family members. HERC3 and HERC4 like HERC5 restricted HIV-1 replication by inhibiting the nuclear export of unspliced HIV-1 mRNA. HERC6 did not restrict HIV-1 replication. When the RLD of HERC6 was swapped with the HERC5 RLD, H6:H5-RLD-HERC6 was able to restrict HIV-1 particle production, further supporting that the HERC5 RLD contributes to HIV-1 restriction. Evolutionary analysis showed that strong positive selection is operating on HERC5 and HERC6, but not on HERC3 or HERC4.

My research characterizes HERC5 as an antiviral, cellular restriction factor that has the ability to potently restrict HIV-1 replication by two novel mechanisms. I provided new insight into how the innate immune system combats viral pathogens such as retroviruses. My research also lays the groundwork for future research into exploiting the antiviral

activity of HERC5 by developing drugs that mimic or enhance the antiviral activity of HERC5.

Key Words

HIV/AIDS, Cellular restriction factors, Interferon, Innate immunity, HERC5, ISG15, RCC1, Ran, Positive selection

Co-Authorship Statement

In chapter 1 the section 1.5.8 (ISG15) was partially published in: Jenna N. Kelly, Jessica G.K. Tong, Clayton J. Hattlmann, **Matthew W. Woods**, and Stephen D. Barr. (2011). ISBN 978-953-307-665-2. Book Title: HIV and AIDS: Updates on Biology, Immunology, Epidemiology and Treatment Strategies. Chapter Title: Cellular Restriction Factors: Can We Exploit the Body's Natural Antiviral proteins to Combat HIV/AIDS? (Permission **Appendix 7**)

Chapter 2 of this thesis was published in: *Retrovirology* **8**: 95 (2011). (Permission **Appendix 8**). C. Hattlmann was responsible for Figure 2.3A. M. Coleman was responsible for figure 2.4B. J. Kelly was responsible for cloning the HIS-tagged ISG15 construct. Figure 7 was completed by S. Barr and analyzed by S. Barr and M. Woods.

Chapter 3 of this thesis was published in: *Retrovirology* **11**:27 (2014). (Permission **Appendix 8**). Domain deletions were created by J. Tong. Monocyte purification was completed by P. Szabo. Figure 3.5B was completed by S. Barr. Figure 3.6 (positive selection analysis and 3D modeling) was done in collaboration with S. Barr and M. Woods.

In chapter 4 table 4.1 was completed by H. Kohio. Table 4.2 was completed by S. Barr and analyzed by M. Woods and S. Barr. Figure 4.7 (3D modeling) and 4.8 (positive selection analysis) were completed and analyzed in collaboration with S. Barr and M. Woods.

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List of Abbreviations

APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G
ARF	ADP Ribosylation Factors
BST-2	Bone marrow stromal antigen 2
CA	Capsid
CHMP-5	Chromatin-modifying protein/charged multivesicular body protein 5
CTE	Constitutive transport element
CypA	Cyclophilin A
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligating enzyme
EBOV	Ebola virus
ESCRT	Endosomal sorting complexes required for transport
EJC	Exon junction complex
GEF	Guanine nucleotide exchange
GPI	Glycophosphatidylinositol
HECT	Homologous to the E6-AP carboxyl terminus
HERC	HECT and RLD containing protein
HIV	Human immunodeficiency virus

HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HPV-16	Human papilloma virus 16
IAV	Influenza A virus
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFNAR	Interferon alpha/beta receptor
IRF-3	Interferon regulatory factor 3
IRF-7	Interferon regulatory factor 7
ISG	Interferon stimulated gene
ISG15	Interferon stimulated gene 15
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK/STAT	Janus kinases/signal transducers and activators of transcription
LTR	Long-terminal repeat
MA	Matrix
MLV	Murine leukemia virus
Mx	Myxovirus resistant protein
NC	Nucleocapsid
NES	Nuclear export signal

NF- κ B	Nuclear factor-kappa B
NLS	Nuclear localization signal
NXF1	Nuclear RNA export factor 1
Nup	Nucleoporin
OAS	2'-5'-oligoadenylate synthetase
PAMP	Pathogen-associated molecular pattern
PIC	Preintegration complex
PIP ₂	Phosphatidylinositol 4, 5-bisphosphate
PKR	Double-stranded RNA (dsRNA)-activated protein kinase
PRR	Pattern recognition receptor
Ran	Ras-related nuclear protein
RanGAP1	Ran GTPase activating protein 1
RanBP1	Ran binding protein
RCC1	Regulator of chromosome condensation
RING	Really interesting new gene
RLD	RCC1-like domain
RRE	Rev-response element
RLR	RIG-I-like receptor
SAMHD1	Sterile alpha motif and histidine/aspartic acid domain-containing protein

SIV	Simian immunodeficiency virus
STAT	Signal transduced and activator of transcription
TLR	Toll-like receptor
TRIM	Tripartite motif protein
TSG101	Tumor Susceptibility Gene 101
VPS4	Vacuolar protein sorting-associated protein 4

Chapter 1

1 Introduction

In the late 1970s and early 1980s people began to present with a previously undescribed and unexplained immune dysfunction. These patients had a significant depletion of the CD4⁺ T-lymphocyte subset in the peripheral organs and lymphoid tissues. They began to show symptoms of opportunistic infections and unusual cancers due to their immunocompromised state, which is now known as Acquired Immunodeficiency Syndrome (AIDS). In 1983 Montagnier and colleagues reported the isolation of Human Immunodeficiency Virus (HIV-1), which was named ‘lymphadenopathy-associated virus’ at the time [1, 2]. This virus was isolated from the lymph nodes of patients with an unknown cause of lymphadenopathy. HIV-1 was determined to be the sole causative agent responsible for the loss of CD4⁺ T cells and the progression to AIDS [1, 2].

HIV-1 exhibits a high degree of sequence heterogeneity due to the error-prone Reverse Transcriptase, the high levels of virus produced and the recombination of the diploid HIV-1 RNA genomes during HIV-1 reverse transcription. The heterogeneity of HIV-1 is a major obstacle for vaccine development and HIV-1 drug resistance, stressing the importance of novel strategies to combat HIV-1 [3, 4].

One prospect that is currently being explored is the anti-HIV-1 activity of the innate immune system. Cellular restriction factors are innate, intrinsic proteins that have the ability to restrict virus replication, some of which restrict HIV-1 [5]. Herein, I have identified and characterized a novel, innate cellular restriction factor called HERC5 which potently restricts HIV-1 replication by two novel mechanisms.

1.1 Introduction to HIV-1

HIV is a member of the viral family *Retroviridae* of the *Lentivirus* genus. *Lentivirus* comes from the Latin term “lenti” which means slow due to the fact that they cause slow, chronic disease. *Retroviridae* are a group of viruses that infect multiple animal species and have characteristic reverse transcription activity that creates a single strand of

complementary DNA from the positive sense RNA genome and then converts it into a double stranded form. The double stranded viral DNA is incorporated into the host genome as a provirus [2]. There are two types of HIV, HIV-1 and HIV-2. HIV-2 has been associated with lower infectivity and pathogenicity [6]. This thesis will primarily focus on the more pathogenic HIV-1.

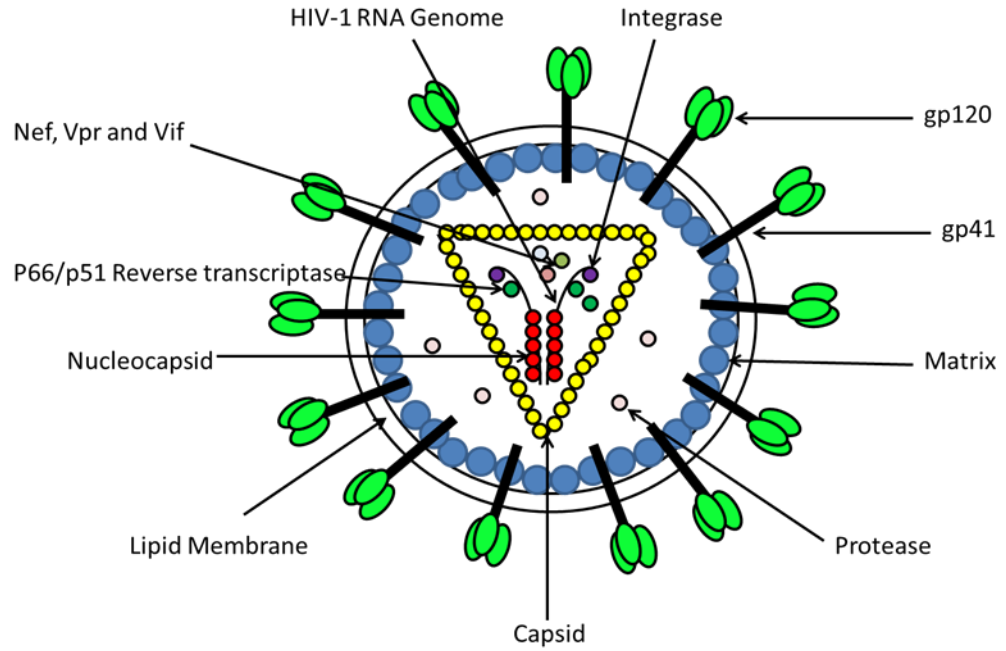
The HIV-1 genome contains multiple elements that are essential for efficient HIV-1 replication (**Figure 1.1**). The HIV-1 virion has two copies of the ~9-kb RNA genome. The RNA genome is reverse transcribed into double stranded cDNA followed by Integrase-mediated integration into the host genome forming the provirus. The HIV-1 provirus is flanked by two long terminal repeats (LTRs) [7]. The 5' LTR possesses the promoter for HIV-1 transcription. The TAR element region forms a hairpin stem loop which binds the essential HIV-1 Tat protein [7, 8]. This interaction prevents premature termination of elongation of viral mRNA during transcription of the HIV-1 LTR promoter. The packaging signal or ψ signal and the dimerization “kissing loop” site allows both copies of the HIV-1 RNA genome to be successfully incorporated into the budding HIV-1 virion [7]. HIV-1 also encodes a secondary stem loop structure which binds to the HIV-1 accessory Rev protein. Rev regulates the export of unspliced or partially spliced HIV-1 mRNA. This will be described in detail below (1.3.1) [9].

HIV-1 encodes nine open reading frames, which encode 15 HIV-1 viral proteins. Three of these open reading frames encode the Gag, Pol and Env polyproteins which are found in all retroviruses. The *gag* gene contains all the structural components of HIV-1 (MA, CA and NC) and the p6 region, which is essential for HIV-1 budding. The *pol* gene encodes the HIV-1 Reverse Transcriptase, composed of the p66 and truncated p51 subunits which are both required for reverse transcription [2, 7]. The *pol* gene also encodes the Protease which is required to cleave the polyproteins into their functional units. Lastly, the *pol* gene encodes the Integrase which is responsible for integrating the reverse transcribed double stranded DNA into the host genome [2, 7]. The *env* gene encodes a protein called

Figure 1.1: The HIV-1 virion and genome

A, HIV-1 is a 100-120µm enveloped virion. The gp120 and gp41 envelope complexes are bound to the surface of the HIV-1 virion. The inner membrane of the enveloped virion is lined with the HIV-1 Matrix (MA) protein. The Capsid (CA) protein forms the outer portion of the viral cone core. Within the CA cone core the two genomic RNA strands are present and are bound to Nucleocapsid (NC) proteins forming a ribonucleoprotein complex. The HIV-1 virion also contains functional integrase proteins, reverse transcriptase proteins, Vpr, Nef, Vif, proteases as well as multiple host proteins. **B**, HIV-1 encodes nine open reading frames which create the 15 HIV-1 viral proteins. The HIV-1 virion contains two copies of the ~9-kb RNA genome.

A



B

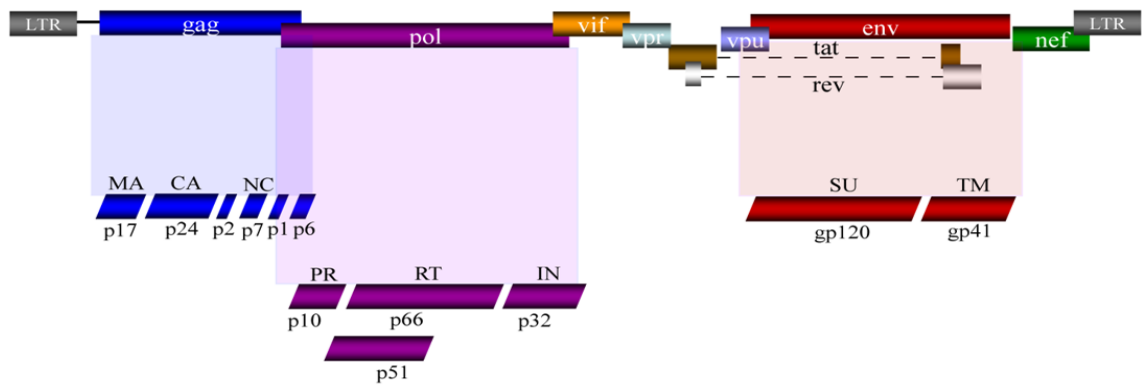


Figure: 1.1: The HIV-1 virion and provirus

gp160 which is targeted to the endoplasmic reticulum to be glycosylated and trafficked to the plasma membrane. The gp160 protein is cleaved into the functional gp120 and gp41 Env subunits by the cellular protease Furin [10]. These proteins are required to gain entry into the host cell (**Figure 1.1**) [7].

Unlike simple retroviruses, such as murine leukemia virus (MLV), HIV-1, a complex retrovirus, encodes several accessory proteins that aid in HIV-1 replication [7]. Tat as mentioned before is essential for the complete elongation of HIV-1 transcripts. Rev controls viral mRNA nuclear export [9] and HIV-1 Nef has the ability to down regulate surface molecules essential for immunologic communication. Two well defined examples are the CD4 and MHC-I surface proteins [11]. Nef can also modulate T cell activation by interacting with multiple Src family kinases known to increase CD4+ T cell activation. Hence, Nef modifies the infected T cell environment to enhance the replicative capacity of HIV-1 [11, 12]. Vpr is an accessory protein that enhances the LTR-driven expression of HIV-1 mRNA, promotes the nuclear import of the double stranded cDNA and has been shown to initiate a cell cycle arrest at the G2 stage which increases LTR transcription. Vpr also plays a part in HIV-1-mediated apoptosis [13].

Vpu is essential for efficient viral release, and has been shown to downregulate surface CD4 by binding the cytoplasmic tail of CD4 within the ER. This results in the degradation of CD4. If CD4 is not degraded during HIV-1 replication, the Env protein will not traffic to the plasma due to the fact that CD4 sequesters Env within the endoplasmic reticulum [14]. Vif is another accessory protein that allows HIV-1 to efficiently infect target cells. Both Vpu and Vif have been shown to antagonize antiviral host proteins (**Figure 1.1**) [15].

1.2 Early stage of HIV-1 life cycle

The HIV-1 lifecycle can be divided into two stages: an early stage and a late stage. The *early stage* of the HIV-1 lifecycle begins when the HIV-1 virion binds to the host cell via its CD4 receptor and CXCR4 or CCR5 co-receptor using the gp120 envelope protein [16]. Receptor binding initiates the gp41-mediated fusion between the envelope of the

HIV-1 virion and the plasma membrane of the host cell. Following fusion, the CA core of the virus is released into the cytoplasm. The single-stranded genomic RNA is reverse transcribed into double stranded DNA by the virion associated Reverse Transcriptase while the CA core uncoats. The dsDNA then associates in a complex, composed of both viral and host proteins, called the pre-integration (PIC) complex, and moves into the nucleus via microtubules [17]. The HIV-1 integrase protein then incorporates the viral cDNA into the genome of the host cell forming the provirus [18, 19].

1.3 Late stage of HIV-1 life cycle

The *late stage* of the HIV-1 lifecycle begins with LTR-mediated transcription of the integrated provirus in the nucleus. Both spliced and unspliced viral RNA are exported into the cytoplasm, where translation of viral proteins occurs [19]. During virus replication, early transcripts of HIV-1 are fully spliced viral mRNA and encode the early expressed regulatory proteins (Rev, Nef and Tat). The late transcripts are singly spliced or unspliced, and encode Gag, Pol, Env as well the accessory proteins Vpr, Vif, Vpu [9, 20] (**Figure 1.2**).

1.3.1 HIV-1 mRNA export

The transition of export from early to late transcripts is mediated by the HIV-1 Rev protein [21]. Rev is a 19kDa protein that is able to recognize and bind the secondary Rev-response element (RRE) RNA structure located within the HIV-1 *envelope* gene [22, 23]. Rev contains an arginine rich RNA binding domain that acts as a nuclear localization signal (NLS). The NLS is flanked by two protein segments that mediate Rev oligomerization [24]. Rev also contains a leucine rich nuclear export signal (NES). Rev is predominately located in the nucleus/nucleolus, but is able to shuttle between the nucleus and cytoplasm due to the NES and NLS [9].

During HIV-1 infection the Rev protein binds RRE of the viral mRNA within the nucleus. This triggers Rev oligomerization exposing the NES of the Rev. Rev oligomerization allows Rev to bind to the exportin protein called CRM1. The ras-related-nuclear protein (Ran)-GTP binds CRM1, which associates with the Rev/viral RNA

complex. This entire complex then docks to the large (>100Mda) nuclear pore complex (NPC) composed of up to 100 nucleoporin (Nup) proteins [9]. The Rev/viral RNA/Ran-GTP/CRM1 complex proceeds to translocate to the cytoplasm due to the interaction between CRM1 and Nup98 and Nup214 [25]. The cytoplasmic Ran GTPase Activating Protein 1 (RanGAP1) and Ran binding protein 1 (RanBP1) proceed to bind to Ran, resulting in the activation of Ran's GTPase activity. Ran-GTP is converted to Ran-GDP causing the Rev/viral RNA/Ran-GTP/CRM1 complex to dissociate. Rev is returned to the nucleus when Rev interacts with Importin- β and Ran-GDP forming a Ran-GDP/Importin- β /Rev complex. With the help of B23 [26], a chaperone protein, the Rev/Importin- β /Ran-GDP complex moves back into the nucleus. The nuclear regulator of chromosome condensation 1 (RCC1), which is a guanine nucleotide exchange factor (GEF) for Ran, changes the Ran-GDP to Ran-GTP liberating Rev from Importin- β , which allows the process to start over again (**Figure 1.2**) [9].

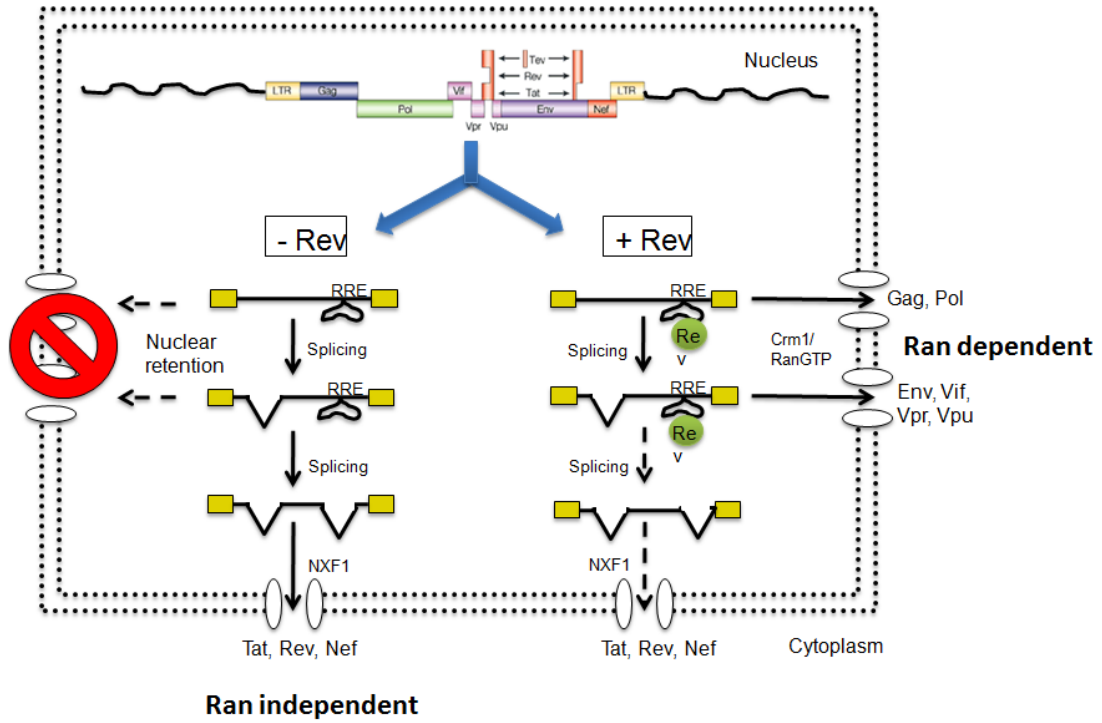
The early transcripts are exported in a Rev- and Ran-independent manner and are exported using the same mechanism as most cellular mRNAs. Generally, mRNA is exported out of the nucleus using the NXF1 (Tap) and the NXT1 (p15) proteins [27, 28]. Once cellular pre-mRNA is processed and fully spliced, a protein complex between the exons, called the exon junction complex (EJC) is formed. EJCs can recruit NXF1/NXT1 to the processed mRNA. NXF1/NFT1 bind to Nups in the NPC allowing the processed mRNA to move through the nuclear pore (**Figure 1.2**) [29].

1.3.2 HIV-1 assembly and release

The HIV-1 Gag polyprotein is translated from unspliced genomic RNA. HIV Gag is the main driving force for the assembly and budding of the HIV-1 virion. Gag contains three main structural components of HIV-1; MA, CA and NC. Gag also contains the p6 region that is required for budding and two spacer peptides P1 and P2. The MA portion of Gag contains basic residues and a hydrophobic groove, which interacts with phosphatidylinositol 4, 5-bisphosphate (PIP₂) located in the plasma membrane. The N-terminus of the MA^{Gag} is post-translationally modified with a hydrophobic myristic acid which is able to insert itself into the plasma membrane upon interaction with PIP₂ [30].

Figure 1.2: Role of Rev in the HIV-1 life cycle.

HIV-1 particle production requires cytoplasmic expression of unspliced, singly-spliced and fully-spliced viral mRNAs. In the absence of Rev, only fully spliced viral mRNAs encoding the regulatory proteins Tat, Nef and Rev, are exported from the nucleus via the NXF1-dependent pathway. In the presence of Rev unspliced or incompletely spliced viral mRNAs, encoding Gag, GagPol, Vif, Vpu and Vpr are expressed due to the recruitment of Rev and its associated cellular co-factors (e.g. Crm1 and Ran-GTP) to the RRE.



1.2: Role of Rev in the HIV-1 life cycle.

NC^{Gag} interacts with either host or viral RNA through basic residues within the domain and uses this RNA as a scaffold to promote Gag oligomerization, which is essential for Gag assembly and virion release [31, 32]. The C-terminus of CA^{Gag} is also involved in Gag-Gag interaction [33]. The Gag-Pro-Pol polyprotein which contains the Protease, the Reverse Transcriptase and the Integrase is created by a -1 frameshift event during translation due to the SLIP element, which is composed of a UUUUUA sequence, followed by a stem loop within the HIV-1 mRNA. The Gag-Pro-Pol polyprotein traffics to the membrane in a similar fashion as Gag [34].

Finally, two late (L) motifs, P(T/S)AP and YPXL, located in p6^{Gag} recruit the endosomal sorting complex required for transport (ESCRT) machinery to the plasma membrane [35]. The ESCRT machinery is responsible for the late stages of HIV-1 Gag particle budding. Normally, the ESCRT machinery selects proteins that are mono-ubiquitinated and sorts them into specific endosomal membrane compartments. The ESCRT machinery creates membrane invaginations away from the cytoplasm into the endosomal lumen. This results in the formation of vesicles that are released into the lumen of endosomes, creating multivesicular bodies. ESCRT machinery is recruited to the endosomal membrane by hepatocyte growth factor-regulated tyrosine kinase substrate [36]. HIV-1 has found a way to hijack this machinery by using the Gag P(T/S)AP L motif. P(T/S)AP motif mimics the ESCRT-I promoting HRS protein [37, 38]. P(T/S)AP binds to Tumor Susceptibility Gene 101 (TSG101), a component of the ESCRT-I complex. ESCRT-I sequentially recruits ESCRT-II then ESCRT-III complexes along with the AAA ATPase vacuolar protein sorting 4 (VPS4), resulting in the disassembly of the ESCRT machinery. This disassembly of the ESCRT machinery enables the initiation of viral budding. This mechanism, however, is currently not fully understood [36–42].

The other L motif is YPXL. This domain binds a protein called ALIX which recruits the ESCRT-III complex along with VPS4. This domain contributes to HIV-1 budding and release [38, 43, 44].

Multiple post-translational modifications of Gag with ubiquitin have been shown to take place near NC^{Gag} and p6^{Gag}. These Gag modifications are required for the normal budding

and release of HIV-1. Though not proven, it is speculated that the ubiquitination of Gag may assist with the recruitment of the ESCRT machinery complex [40, 45, 46]. As the virus buds out of the infected cell, the Protease from Gag-Pro-Pol is activated. The Protease is activated when it forms a homodimer allowing for the cleavage of the HIV-1 polyproteins into functional protein units resulting in the formation of mature HIV-1 virions [34, 47].

1.4 The IFN-I response and HIV-1 replication

The emergence of drug-resistant HIV-1 strains, and the lack of an effective HIV-1 vaccine, have both emphasized the importance of developing novel, non-toxic HIV-1 treatments. One exciting prospect that is currently being explored is the anti-HIV-1 activity of the innate immune system. The innate immune system is one of the immediate lines of defense against invading pathogens and is crucial for the onset of the delayed adaptive immune response [48]. The innate immune response is triggered after the detection of PAMP (pathogen-molecular patterns), which are often crucial components of the invading pathogen, not found in the host. PAMPs are recognized by cellular PRRs (pattern recognition receptors). The most widely studied PRRs are Toll-like receptors (TLRs). TLR 1, 2, 4, 5 and 6 are PRRs that are expressed on the cell surface and are able to recognize PAMPs from various bacteria fungi and protozoa whereas TLR 3, 7, 8, and 9 are almost exclusively localized on endosomes and are able to recognize nucleic acids from viruses [49–53]. RIG-I and melanoma-associated gene 5 are cytoplasmic PRRs that are able to detect the presence of viruses by sensing dsRNA or 5'-triphosphate RNA [54–56]. Once the PRR binds to the substrate PAMP, a complex signaling cascade occurs that activates cytoplasmic transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1) causing the upregulation of inflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF- α) and IL-6 [49]. Interferon regulatory factor (IRF) 3 and/or 7 are phosphorylated and activated upon viral PAMP-PRR stimulation. This initiates the homodimerization and nuclear translocation of these IRFs. Activated IRF3 and/or IRF7 are essential to produce IFN-I [52, 57].

The ssRNA genome of HIV-1 has been shown to act as a PAMP for the endosomal TLR7/8 (ssRNA PAMP) resulting in the production of IFN-I. Double stranded secondary structures in the HIV-1 genome act as a PAMP for TLR3 which also results in the production of IFN-I [53]. The cytoplasmic RIG-I and MDA5 PRRs (dsRNA) have also been shown to recognize double stranded regions in the genomic HIV-1 RNA resulting in the production of IFN-I [56].

HIV-1 has evolved a counter measure which inhibits the production of IFN-I in infected macrophages, DCs, and CD4+ T cells. In activated CD4+ T cells HIV-1 causes the degradation of IRF3 using the HIV-1 accessory proteins Vif and Vpr. These accessory proteins independently link IRF3 to an E3 ubiquitin ligase complex resulting in the proteasomal degradation of IRF3 [58]. In monocyte-derived macrophages as well as monocyte-derived dendritic cells, HIV-1 inhibits the translocation of IRF3 in a Vpr dependent manner [59, 60]. Plasmacytoid dendritic cells, the major producers of IFN- α , however, are still able to produce IFN-I despite the fact they can be infected with HIV-1 [61–65].

1.4.1 IFN family

IFN-I is a polypeptide cytokine, which is secreted from virally infected cells. IFN-I allows virally infected cells and neighboring cells to enter an antiviral state and promotes antigen presentation in innate cells [66]. IFN-I are one of the three families of IFNs and are composed of 13 human subtypes of IFN- α , and only one subtype of IFN- β [66]. Other less studied human IFN-I have also been identified such as IFN (ϵ , κ , ω) [67]. IFN-II only contains one type of IFN called IFN- γ . IFN- γ is produced primarily by lymphocytes and natural killer cells [68]. IFN- γ is not structurally similar to IFN-I however, it was classified as an IFN due to its ability to inhibit viral infection. Lastly, the IFN-III family is composed of IFN- λ 1, λ 2, λ 3. IFN-IIIs have a similar antiviral response compared to IFN-I and can be produced by many cell types directly upon viral infection. IFN-III, however, use receptors with limited tissue expression [69].

1.4.2 IFN-I signaling

All IFN-I bind to a common receptor called the IFN- α receptor (IFNAR) composed of two subunits called IFNAR1 and IFNAR2. When extracellular IFN-I binds the IFNAR, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) bound to the cytoplasmic side of the IFNAR, are activated and subsequently phosphorylated. This results in the phosphorylation of the cytoplasmic transcription factors signal transduced and activator of transcription 1 (STAT1) and STAT2 [66]. After STAT1 and STAT2 are phosphorylated they dimerize and subsequently interact with IFN-regulatory factor 9 to create a complex called IFN-stimulated gene factor 3 (ISGF3). ISGF3 then translocates to the nucleus where it binds to a consensus DNA sequence TTTCNNTTTC called the IFN-stimulated response element (ISRE) (**Figure 1.3**). Translocation results in the upregulation of hundreds of interferon-induced genes (ISGs). Among ISGs are cellular restriction factors which are defined as cellular proteins that inhibit viral replication. Interestingly, it has been shown that IFN-I restricts various stages of the early and late stages of HIV-1 replication in vitro [70–78]. IFN-I has been shown to reduce HIV-1 levels within a patient's blood, however, this treatment has undesirable and toxic side effects [73].

1.5 Cellular restriction factors

Cellular restriction factors were first identified in the late 1960 and early 1970s when Frank Lilly and colleagues observed that certain strains of mice were resistant to Friend Murine Leukemia Virus (MLV)-induced leukemia [79]. The resistance to the sensitive isolates of MLV was determined to be caused by the Friend virus susceptibility genes *Fv1* and *Fv4*. The *Fv1* gene encodes a cellular protein which is homologous to an endogenous retroviral Gag protein. This protein was found to restrict viral replication after cellular entry, but before integration [80, 81]. The *Fv4* gene encodes a protein that is

Figure 1.3: The IFN-I response.

Type I IFNs bind to the IFNAR1/2 receptor which is located on the cellular surface. After IFNAR1/2 bind IFN-I the JAK/STAT the JAK1 and TYK2 kinases are phosphorylated. This results in the recruitment and phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 form a heterodimer which initiates IRF-9 binding forming the ISGF3 complex. ISGF3 translocates to the nucleus where it binds to IFN-stimulated response elements (ISRE) inducing hundreds of IFN stimulated genes (ISGs).

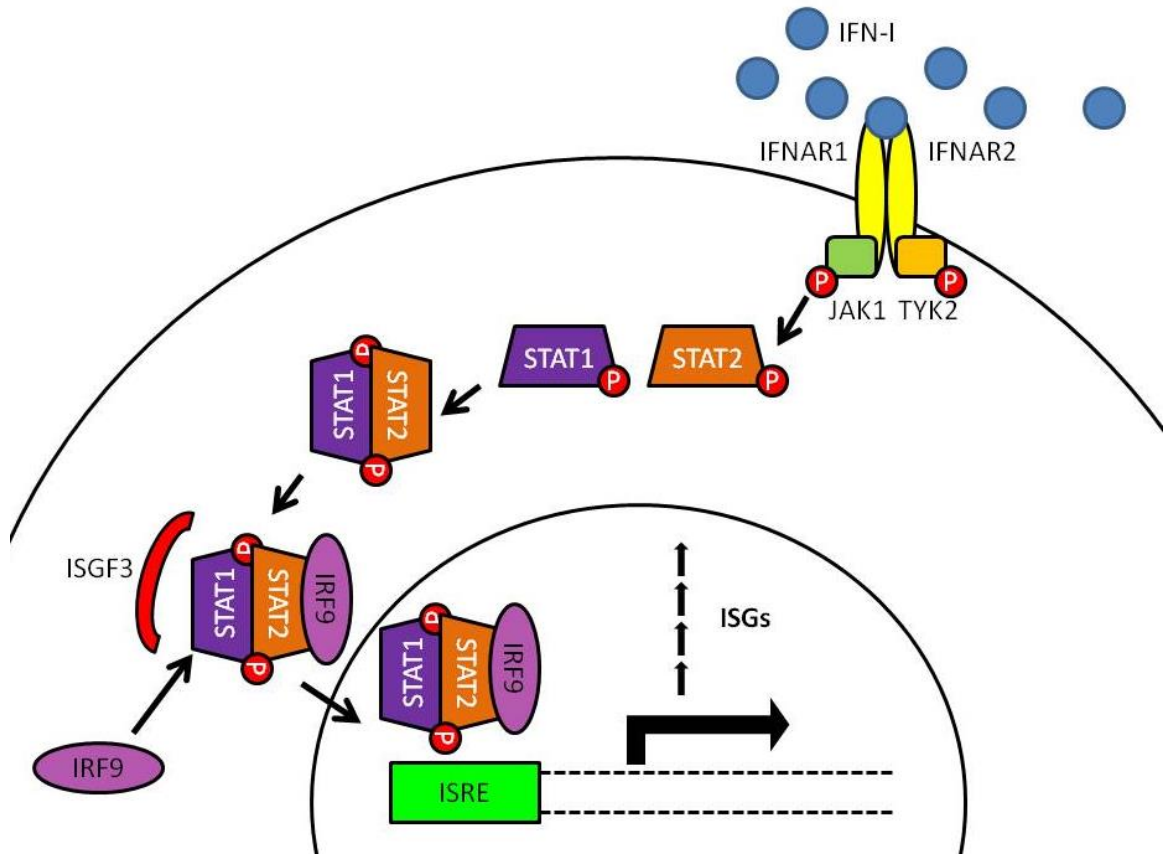


Figure 1.3: The IFN-I response.

similar to the *envelope* gene found in a MLV strain that infects Asian mice. This Envelope protein was shown to inhibit receptor binding [82].

Since the discovery of *Fv1* and *Fv4*, numerous restriction factors have been identified. There is now a better understanding of the hallmarks of a restriction factor. First, a cellular restriction factor's main biological function is antiviral in nature. Second, a cellular restriction factor is most likely induced by IFN or by virus infection. Third, there is a viral antagonist or a viral escape mutation that can counteract or escape the antiviral activity of the cellular restriction factor. Finally, the gene encoding the cellular restriction factor must evolve under positive selection [83, 84].

Most cellular proteins in the genome are under purifying selection, where non-synonymous mutations are removed from the population to preserve the biological function of the protein. Positive selection is a rare event where an antiviral host protein shows signs of genetic conflict throughout evolution due to an ongoing battle between the host and the invading pathogen or pathogens such as viruses. Non-synonymous mutations are selected in the host population with increased evolutionary fitness against the virus. This new selection pressure on the virus forces the virus to select for non-synonymous mutations that increases the fitness of the virus against the host. This is known as the “Red Queen” hypothesis described by Leigh Van Valen to describe the continual evolutionary conflict between the host and pathogen to maintain the *status quo* [83, 84]. Many cellular restriction factors that have been shown to restrict HIV-1 encompass most if not all of these hallmarks of a true cellular restriction factor.

To date, a small handful of IFN-I stimulated cellular restriction factors have been identified that restrict various parts of the HIV-1 lifecycle (**Table 1.1**), some of which inhibit steps in the HIV-1 lifecycle that are not targeted by any of the current United States Food and Drug Administration approved drugs (**Figure 1.4**). Further characterization of ISGs and HIV-1 cellular restriction factors may lead to the development of novel HIV-1 drugs that mimic or enhance their effects without the toxicity of IFN treatment.

Table 1.1: Cellular restriction factors that restrict HIV-1 replication.

Restriction factor	IFN/viral induced	Stage of life cycle inhibited	HIV/SIV viral antagonists or Escape mutations	Positive selection
MxB	Yes	Nuclear import or integration [85–91]	CA mutations evade the antiviral activity of MxB	Yes [83]
IFITM1	Yes	Gag accumulation [92–94]	Unknown	Yes [95]
IFITM2 and IFITM3	Yes	Endosomal fusion or uncoating [92–94]	Unknown	Yes [95]
TRIM5 α	Yes	CA uncoating [96–104]	CA mutations evade the antiviral activity of TRIM5 α	Yes [105]
APOBEC3G	Yes	Reverse transcription [15, 106–114]	Vif (HIV-1)	Yes [83]
SAMHD1	Yes	Reverse transcription [84, 115–118]	Vpx (HIV-2) Vpr (SIV)	Yes [83]
BST-2/Tetherin	Yes	Budding [119–126]	Vpu (HIV-1) Nef (SIV) Env (HIV-2)	Yes [83]
ISG15	Yes	Budding and release [75, 127]	Unknown	Unknown
TRIM22	Yes	Gag trafficking and transcription [128–130]	Unknown	Yes [105]
HERC5	Yes	mRNA export and assembly [77, 131]	Unknown	Yes [77]

Abbreviations: Apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3) G, Sterile alpha motif and histidine/aspartic acid domain-containing protein (SAMHD1), Tripartite motif 5 (TRIM5) α , Myxovirus resistant (Mx) protein, Interferon-induced transmembrane proteins (IFITMs), Tripartite motif 22 (TRIM22), Interferon Stimulated Gene 15 (ISG15) and HECT and RLD (RCC1-like domain) containing protein 5 (HERC5).

Figure 1.4: HIV-1 replication and cellular restriction factors.

The mature HIV-1 virion binds and enters the host cell via the CD4 receptor and CXCR4 or CCR5 co-receptor using the gp120 and gp41 envelope proteins. Following attachment the CA core of the virus is released into the cytoplasm where it uncoats. Next the single-stranded genomic RNA is reverse transcribed into double stranded DNA. The HIV-1 Integrase protein then incorporates the viral dsDNA into the genome of the host cell where it is known as a provirus. Transcription of the provirus later takes place using host and virus machinery. Viral RNA is exported into the cytoplasm where translation of the viral proteins occurs. The HIV-1 Gag polyprotein moves to the plasma membrane and interacts with the HIV-1 genome and other viral and host proteins. HIV-1 assembly occurs at the plasma membrane. An immature virion then buds out of the cell where Gag is cleaved by HIV-1 protease into its individual structural units to form a mature virion. MxB, TRIM5 α , APOBEC3G, SAMHD1 and IFITM2 and 3 are cellular restriction factors that have been shown to inhibit the early stages of HIV-1 replication prior to integration while Tetherin, TRIM22, IFITM1, ISG15 and HERC5 interfere with late post-integration stages of HIV-1 replication HIV-1. Modified from: Woods MW, Barr SD: Human HERC5 is a Novel E3 Ligase that Restricts an Early Stage of HIV-1 Assembly. In *Advances in Virus Research I*. Edited by Press iConcept. iConcept Press Ltd.; 2013 [19].

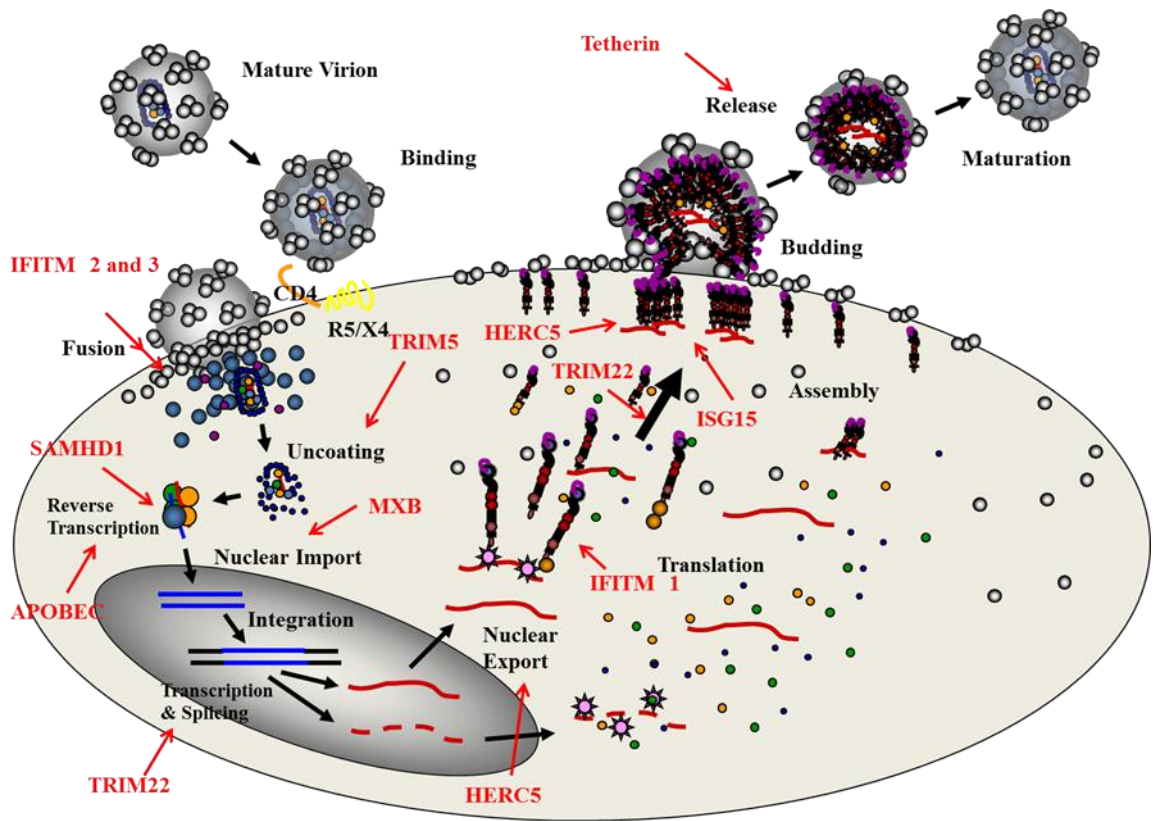


Figure 1.4: HIV-1 replication and cellular restriction factors.

1.5.1 Apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3) G

APOBEC3G was discovered to be a restriction factor responsible for the restriction of Vif-deficient HIV-1 replication [132]. APOBEC3G is a member of the APOBEC3 proteins that have the ability to act as a cytidine deaminases. APOBEC3G has the ability to catalyze deoxycytidine to deoxyuridine (C-U). This discovery hinted at the anti-viral mechanism of APOBEC3G and could explain the G-A hypermutation seen in HIV-1 from clinical samples [133, 134]. In Vif-deficient HIV-1 replication APOBEC3G becomes packaged into the HIV-1 virion by interacting with the HIV-1 genomic RNA and NC protein [107, 108, 110, 113, 135, 136]. During reverse transcription APOBEC3G deaminates cytidine to uridine in the newly synthesized minus strand. This results in G-A mutations in the second-strand synthesis during reverse transcription. This hypermutated DNA is either degraded or is integrated into the host genome where nonsense and missense mutations inhibit successful replication [137, 138]. APOBEC3D, F and H have also been shown to be incorporated into Vif deficient HIV-1 virions and inhibit HIV-1 [84]. APOBEC3A, B and C do not to inhibit HIV-1 replication [139].

The HIV-1 protein Vif acts as an antagonist against APOBEC proteins and initiates the polyubiquitination and subsequent degradation of the four APOBEC3 members that can inhibit Vif-deficient HIV-1 replication [139]. Vif has been evolutionarily conserved in all Lentivirus' with the exception of equine infectious anemia virus (EIAV) and can neutralize host antiviral APOBEC3 proteins. This highlights the evolutionary significance of Vif [140]. Vif mediates polyubiquitination of the antiviral APOBEC3 proteins by recruiting an E3 ubiquitin ligase complex called ELONGINB/C-CULLIN5-RBX1 which targets APOBEC3G for proteasomal degradation [106, 111, 114, 141].

1.5.2 Sterile alpha motif and histidine/aspartic acid domain-containing protein (SAMHD1)

SAMHD1 is composed of a sterile alpha motif (SAM) which is involved in protein interaction and a C-terminal dNTP phosphohydrolase domain that contains a conserved histidine and aspartate residue [78, 142]. SAMHD1 functions as a deoxynucleoside triphosphate hydrolase which cleaves dNTPs into deoxynucleosides and triphosphates. This effectively inhibits the reverse transcriptase due to the diminished concentration of dNTPs, which is essential for HIV-1 cDNA synthesis. It was also shown that SAMHD1 requires dGTP for its activation [78, 142, 143]. The HIV-2 and SIV accessory protein, Vpx, counteracts SAMHD-mediated restriction by acting as a scaffold for the formation of the E3 ubiquitin ligase complex consisting of DCAF1, DD81, and CUL4. This complex polyubiquitinates SAMHD1, which is subsequently degraded by the 26S proteasome [115, 118, 144].

1.5.3 Tripartite motif 5 (TRIM5) α and TRIM-Cyp Proteins

TRIM5 α is a member of the large TRIM family that contains over 100 TRIM proteins [145]. TRIM5 α is composed of an N-terminus really interesting (RING) domain which contains ubiquitin E3 ligase activity, a B-box 2 domain, a coiled-coil domain responsible for forming TRIM5 α trimers [146], and a B30.2 domain or PRYSPRY domain which has been shown to be involved in protein targeting. In owl monkeys and some macaques, retrotransposon events have replaced the PRYSPRY domain with CypA allowing TRIM5-Cyp to interact with the CA core of incoming retroviruses [97, 147].

TRIM5 α and TRIM5-CypA proteins restrict retrovirus events post-entry by interacting with the retrovirus CA protein and cause the CA uncoating process to occur prematurely. This causes the inhibition of the Reverse Transcriptase-mediated reverse transcription. The RING and B-box 2 domain domains of this anti-viral protein are involved in the inhibition of reverse transcription [15]. The B-box 2 domain domain forms high-order multimers that create cytoplasmic bodies. These multimers increase TRIM5 α 's and TRIM5-CypA's ability to interact with the CA lattice [98]. TRIM5 α and TRIM5-CypA

contain a functional ubiquitin E3-ligase RING domain. Importantly, proteasome inhibition has shown to prevent premature capsid disassembly and restore reverse transcription [15]. This, however, does not seem to be required for TRIM5's antiviral activity. This suggests that TRIM5 α and TRIM5-CypA have the ability to restrict retrovirus replication by multiple redundant mechanisms [100, 148].

1.5.4 Myxovirus resistant (Mx) proteins

IFN-induced Mx genes which encode Dynamin-like large GTPase proteins are present in all invertebrates and exhibit antiviral activities against multiple RNA viruses. The Human *Mx1* gene encodes the MxA protein which is cytoplasmic and possesses a wide range of antiviral activity. The human MxA protein has been shown to restrict viruses that replicate in the nucleus and the cytoplasm [149–160].

Humans also possess the *Mx2* gene which expresses the other Dynamin-like GTPase protein called MxB which has 63% sequence identity to MxA [160]. MxB is an IFN-induced protein that has only recently been demonstrated to have antiviral activity. MxB was thought to only be involved in normal biological nuclear import as well as cell cycle progression [161]. Unlike the cytoplasmic MxA protein, MxB is localized to the nuclear rim [162]. Recently, MxB was shown to restrict HIV-1 [163]. In particular, MxB restricts the early stages of the HIV-1 life cycle by inhibiting the nuclear import of the PIC thereby preventing viral DNA integration into the host cell genome [89]. MxB also restricts SIV as well as HIV-2 [164]. Certain strains of HIV-1 have developed mutations in the Cyclophilin A (CypA) binding domain within CA protein that allows HIV-1 to escape MxB-mediated restriction. CypA has been shown to promote the early stages of HIV-1 replication by increasing reverse transcription and altering the stability of the CA core of HIV-1. CypA has been shown to be essential for MxB-mediated restriction [88, 90, 164].

1.5.5 Interferon-induced transmembrane proteins (IFITMs)

Humans possess four functional IFITM proteins, IFITM 1, 2, 3 and 5. IFITM 1, 2 and 3 have been shown to possess antiviral activity and are expressed in the majority of cell

types. IFITM5 however, is only expressed in osteoblasts and is involved in the mineralization of bones [165]. The IFN-induced IFITM1, 2 and 3 proteins restrict early events of the replication of evolutionary diverse viruses such as West Nile virus, Influenza A and B viruses (IAV, IBV), SARS Coronavirus, dengue virus, hepatitis C virus the Ebola virus [75, 165–168]. IFITM proteins contain two hydrophobic membrane associating domains that are separated by a conserved intracellular loop [169]. IFITM proteins also restrict HIV-1 replication. IFITM 2 and 3 restrict HIV-1 entry; however, IFITM1 does not impede viral entry. Although IFITM1 does not impede viral entry, it can significantly reduce the amount of intracellular Gag produced by a mechanism that remains to be defined [93, 170]. More recently it was determined that IFITM2 and 3 co-localize with Env and Gag and are incorporated into the newly synthesized virions, which significantly decreases their infectivity by inhibiting fusion and entry into the host cell [171].

1.5.6 Tetherin (bone marrow stromal cell antigen 2 (BST2)/CD317)

HIV-1 encodes an accessory protein called Vpu which is required for HIV-1 to be released from cells [172]. In the absence of Vpu, HIV-1 can efficiently bud and mature normally from the cell using the ESCRT machinery, however, the virions are trapped on the cell surface by an IFN-induced protein called Tetherin [123, 172–175]. Tetherin is a type II single-pass transmembrane protein with a transmembrane anchor on the N-terminus and a glycosylphosphatidylinositol (GPI) lipid anchor at the C-terminus. The extracellular portion of Tetherin is composed of a long α -helix. It is not yet completely understood how Tetherin tethers HIV-1 to the cell surface. One theory suggests that the transmembrane domain of Tetherin incorporates into the lipid envelope of the assembling virus while the GPI lipid anchor remains incorporated in the cellular membrane. Also, Tetherin has been shown to tether multiple virions together, which suggests that both the N and C-terminal anchor can be incorporated into the viral envelope [122, 176].

Vpu decreases the expression of Tetherin on the cellular surface. Vpu initiates the internalization of Tetherin from the cell surface and interferes with the migration of

Tetherin to the plasma membrane [122, 123, 175–177]. Most strains of SIVs do not encode Vpu. SIV uses the accessory protein Nef to counteract the antiviral mechanism of Tetherin. Nef is post translationally myristoylated at the N-terminus and is localized at the plasma membrane. Nef interacts with multiple cellular proteins involved in endocytosis, however, it is not understood how SIV Nef is able to counteract the antiviral mechanism of Tetherin [178, 179]. SIV and HIV-2 have also been shown to counteract Tetherin by using their Env proteins, which sequester Tetherin into intracellular compartments [124, 126].

1.5.7 TRIM22

Like TRIM5 α , TRIM22 is a member of the tripartite motif family and is located directly next to the human TRIM5 α gene on chromosome 11 [105, 180]. TRIM22 contains a RING domain that possesses E3 ubiquitin ligase activity. TRIM22's E3 ligase activity has been shown to be essential to restrict hepatitis B virus, influenza A virus (IAV) and encephalomyocarditis virus [181–183]. Restriction of HIV-1 replication occurs both in the presence and absence of TRIM22's E3 ligase activity [128, 184].

TRIM22 restricts HIV-1 by at least two mechanisms. First, TRIM22 inhibits Gag trafficking to the plasma membrane in human cervical cancer cells (HeLa), as well as the human osteosarcoma cell line (HOS-CD4/CXCR4 cell line modified to expressing CD4 and CXCR4). This mechanism of TRIM22-mediated HIV-1 inhibition requires TRIM22's functional RING E3 ubiquitin ligase activity. TRIM22 inhibits the accumulation of intracellular HIV-1 Gag in U2OS and 143B (osteosarcoma cell lines) as well [128]. TRIM22 has also been shown to restrict HIV-1 replication by inhibiting LTR mediated transcription of HIV-1 [70, 130].

1.5.8 Interferon Stimulated Gene 15 (ISG15)

Interferon-stimulated gene 15 (ISG15) is an ubiquitin-like protein (Ubl) that was first discovered in 1979 and is induced in the presence of IFN- α/β [185–188]. ISG15 is composed of two Ubl domains [189]. The C-terminus of ISG15 contains a Gly-Gly motif which is required for ISG15 conjugation to target proteins. ISG15 is initially translated as

a precursor protein that cannot be conjugated to a substrate protein due to the additional amino acid residues on the C-terminus of the ISG15 protein [189, 190]. ISGylation requires an E1 activating protein, an E2 conjugating protein, and an E3 ligase protein to successfully ISGylate substrate proteins. The ISG15-specific IFN-induced E1 activating protein, Ube1L, uses ATP to adenylate the Gly-Gly motif of ISG15. Ube1L then forms a thioester bond between its catalytic cysteine residue and the C-terminal Gly residue of ISG15. With the help of the IFN-induced E2 conjugating protein, UbcH8, and a substrate-specific E3 ligase, ISG15 forms a covalent bond with the ϵ -NH₂ on the substrate lysine residue [19, 188, 191]. Ubp43 can cleave ISG15 from ISGylated proteins. Importantly, ISG15 is conjugated to both viral and host proteins, and can have an antiviral effect by altering the activity of substrate proteins or protein-protein interactions [127, 192, 193].

ISG15 can also act as a cytokine-like molecule. T-cells, B-cells, monocytes and epithelial cells have all been shown to release ISG15 after IFN-I treatment [194]. The release of ISG15 causes the proliferation of the innate immune natural killer cells and cytotoxic T cells. The secreted free form of ISG15 increases the production of the antiviral protein IFN- γ from both NK cells and other lymphocytes [195]. Free form ISG15 also has the ability to recruit neutrophils to the site of infection as well as promoting DC maturation [196].

ISG15 has the ability to restrict a wide range of evolutionary diverse viruses including; IAV [197], IBV [198–200], Sindbis Virus [200–202], herpes simplex type-1 [200], murine γ -herpesvirus [200], VSV [203], Dengue fever virus [204], ebola virus (EBOV) [127, 205], West Nile virus [204], lymphocytic choriomeningitis virus [203], Japanese encephalitis virus [206], as well as vaccinia virus [207]. To date only a few ISG15-mediated antiviral mechanisms have been characterized.

EBOV-mediated viral budding and release, is driven by the EBOV matrix protein called VP40. When VP40 is expressed on its own in the absence of the rest of the EBOV genome it can form virus-like particles [208]. VP40 contains two overlapping L domains, which are PTAP and PPEY. Both of these domains are required for EBOV to efficiently

bud and release [209]. These L domains are essential to recruit the ESCRT machinery through the recruitment of TSG101, a member of the ESCRT complex. Like HIV-1, the ESCRT complex is essential for EBOV to efficiently bud out of the host cell [127]. The PTAP motif of VP40 has been shown to directly recruit the ESCRT machinery by recruiting TSG101. The L domain PPEY of VP40 mediates the interaction between VP40 and the E3 ubiquitin ligase called Nedd4. Nedd4 ubiquitinates VP40 resulting in the increased recruitment of the ESCRT machinery required for budding [210]. Expression of ISG15 inhibits the interaction between Nedd4 and its E2 conjugating enzyme which is essential for Nedd4 to successfully modify VP40 with ubiquitin. This is essential for Nedd4 to sufficiently modify substrate proteins including VP40 [211]. The loss of VP40 ubiquitination results in the restriction of VP40 VLP release [212].

Recently, it has been determined that during the IFN response the ESCRT-I subunit, TSG101 is post-translationally modified with ISG15 during IAV infection. ISGylated TSG101 inhibits the trafficking of the essential influenza hemagglutinin (HA) protein to the plasma membrane which is essential for the proper assembly and release of infectious IAV virions [42].

ISG15 also modifies a well-known cellular restriction factor called the double-stranded RNA (dsRNA)-activated protein kinase (PKR), which inhibits a wide range of viruses including IAV, IAB, HCV, hepatitis D virus, WNV, sindbis virus, foot-and-mouth disease virus, and even DNA viruses such as HSV-1. PKR is activated when it binds to double stranded RNA introduced into the cell from viral infection. Once PKR detects the double stranded RNA, PKR dimerizes and autophosphorylates itself. This leads to the phosphorylation of the eukaryotic initiation factor 2 (eIF2 α), which is required for protein translation [213]. The phosphorylation of eIF2 α inhibits the translation of both viral and host proteins [214]. During the IFN response PKR is ISGylated at lysine residues K69 and K159 which activates PKR in the absence of viral RNA [189, 215].

ISGylation has been implicated in restriction of HIV-1 replication at the budding stage of the HIV-1 lifecycle [75, 127, 216]. The HIV-1 Gag protein contains a late-budding PTAP motif, which interacts with TSG101. TSG101 is a component of ESCRT-I complex,

which subsequently recruits ESCRT-II, then ESCRT-III [39]. ESCRT-III promotes viral budding and the recruitment of VPS4 which is an ATPase. VPS4 releases ESCRT factors from the membrane [37, 39]. ISG15 has been shown to interrupt the interaction between TSG101 and the HIV-1 Gag protein; however, in this study neither TSG101 nor HIV-1 Gag were modified with ISG15. This study also determined that the expression of ISG15 inhibited Gag ubiquitination which has been shown to be essential for HIV-1 to efficiently bud out of the cell [40, 75].

As well as inhibiting Gag ubiquitination, ISG15 also interferes with the recruitment of VPS4 to the HIV-1 budding complex. It was determined that the charged multi-vesicular body protein (CHMP)-5, a component of ESCRT-III, is ISGylated which prevents the recruitment of VPS4 to the ESCRT-III complex [127]. CHMP5 is essential to establish the interaction between LIP5, a co-activator of VPS4, and VPS4 which is crucial for HIV-1 release [127]. Further characterization of ISG15-mediated HIV-1 restriction is required to understand the antiviral effects of ISG15 on HIV-1 budding.

Many viruses have developed mechanisms to counteract the antiviral effect of ISG15. IAB inhibits ISG15 conjugation because it encodes a protein called NS1. NS1 binds ISG15 inhibiting the interaction between ISG15 and the E1 activation protein Ube1L, which ultimately inhibits cellular ISGylation [217]. The E3L protein of vaccinia virus can bind and sequester ISG15 ultimately inhibiting successful ISGylation as well. Mutated Δ EL3-vaccinia virus is no longer able to sufficiently replicate itself and is correlated to an increase in cellular ISGylation [218].

Multiple viruses have evolved proteins that have the ability to cleave ubiquitin as well as ISG15 from the substrate proteins. The cleavage of ISG15 from the substrate protein counteracts the antiviral activity of ISGylation. Crimean-Congo hemorrhagic fever virus encodes the L protein. The L protein decreases the total number of ubiquitinated and ISGylated conjugates, which results in an increase in viral replication [219]. SARS-CoV also encodes a protease that can cleave ISG15 from substrate proteins [220–222]. It is currently unknown if HIV-1 possesses any antiviral proteins that can counteract the antiviral effect of ISG15.

ISG15 encompasses many of the hallmarks of a true restriction factor. ISG15's main biological role is antiviral in nature and is one of the most highly upregulated proteins during IFN-I treatment and viral infection. A number of viruses have developed antagonists to counteract the antiviral effect of ISG15 [192]; however, it is currently unknown if ISG15 is undergoing positive selection. To successfully conjugate ISG15 to a substrate protein, an E3 ligase must be present. It has been determined that the main cellular ISG15 E3 ligase is called HERC5, which is a member of the HERC protein family [223, 224].

1.6 HERC family

The RCC1 superfamily is a family of proteins that contain at least one 350-500aa RLD. These domains fold into a seven bladed β -propeller tertiary structure. The first RLD that was discovered was the RCC1 itself [225–227]. Mutated alleles of RCC1 in the ts-BS2 hamster cell line induced G1 cell cycle arrest and premature chromosome condensation. This was reversed when wild type RCC1 was expressed. The RCC1 protein is a nuclear protein that has a guanine nucleotide exchange (GEF) activity for the Ras-related GTP binding protein (Ran). RCC1 has been shown to directly bind chromatin through the interaction between RCC1 and Histones 2A and 2B. RCC1, through its GEF activity for Ran, regulates multiple cellular processes that include nucleocytoplasmic transport, mitotic spindle assembly and nuclear envelope formation. To date, 18 members of the RCC1 superfamily have been identified with a wide range of cellular functions. One of the subgroups of the RCC1 superfamily proteins is called the HERC protein family which contain RCC1-like domains (RLD) [226–228].

HERC proteins contain at least one RLD that is predicted to fold into a seven bladed β -propeller tertiary structure. HERC proteins also possess an E3 ubiquitin ligase HECT domain. HERC proteins are further divided into large and small HERCs. The large HERC subgroup encompasses HERC1 and HERC2. The large HERCs possess at least two RLD domains in addition to a HECT domain. The small HERCs, HERC3, HERC4, HERC5 and HERC6 are composed of only one RLD and the E3 ligase HECT domain which is separated by the spacer region (**Figure 1.5**) [228].

1.6.1 HERC1

HERC1 is a 532kDa protein that contains multiple domains that are conserved in other proteins. It contains two RLD domains as well as a WD40 domain, which is also predicted to fold into a β -propeller. WD40 domains in other proteins have been shown to be involved in protein-protein interactions. HERC1 also contains a SPRY motif. SPRY domains in other characterized proteins are involved in protein-protein interactions as well as RNA-protein interactions. HERC1 contains other smaller motifs such as a SH3 binding sequence, a potential leucine zipper motif as well as multiple regions with polar and acidic side chains [227].

HERC1 stimulates the dissociation of guanine nucleotides from the ADP Ribosylation Factors (ARF) and Rab GTPases (ARF1, Rab3a and Rab5), which are proteins essential for normal membrane trafficking [227]. The RLD1 of HERC1 acts as a guanine release factor (GRF) and releases GDP from these ARF and Rab GTPases. RLD1 requires PIP₂ to activate its GRF activity [229]. The RLD2 domain of HERC1 does not possess GRF activity. It has been shown that RLD2 interacts with the heavy chain of clathrin and with the ARF1 GTPase. These observations provide evidence that HERC1 is involved in membrane trafficking as well as endocytosis [227].

Figure 1.5: Schematic comparing the protein domain organization of the different human HERC family members.

HERC proteins which are divided into the large HERC proteins HERC1 and HERC2 and the small HERC family HERC3, HERC4, HERC5 and HERC6. RLD, RCC1-like domain. SPRY, Spia andryanodine receptor domain; WD40, WD40 domain; cyt-b5, cytochrome b5-like heme/steroid binding domain; MIB, mind bomb herc2 domain; ZZ, zinc finger, ZZ type.

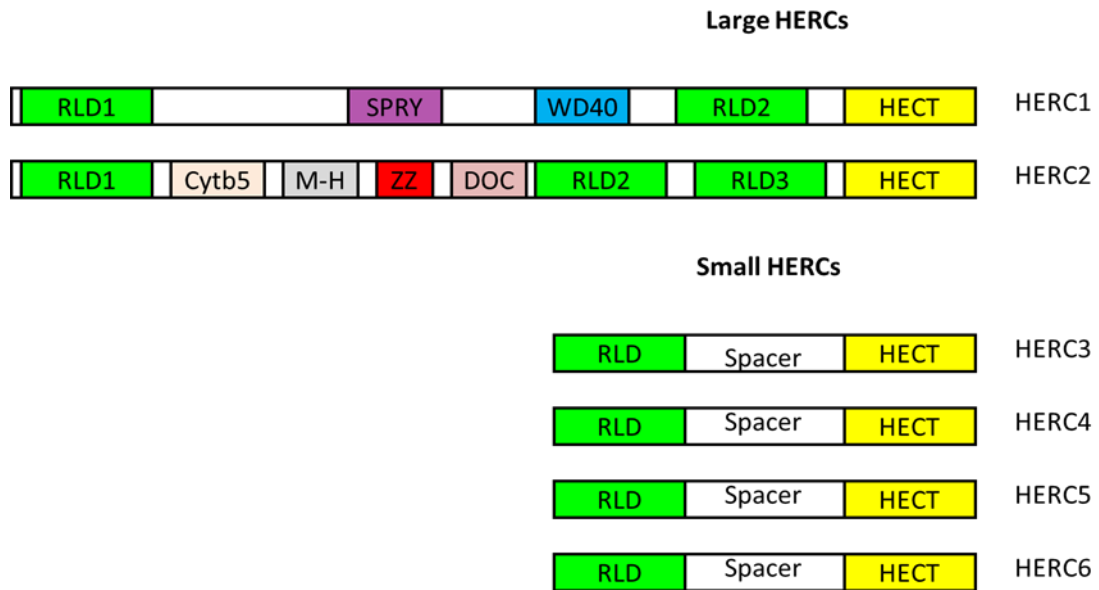


Figure 1.5: Schematic comparing the protein domain organization of the different human HERC family members.

With the help of the E2 conjugating enzyme UbcH5, HERC1 can form a thioester bond with the cysteine active site within the HECT domain of HERC1. This indicates that HERC1 most likely acts as a functional ubiquitin E3 ligase. No substrate proteins have yet been identified [227]. HERC1 is recruited to membrane protrusions where actin polymerization takes place. It has been hypothesized that HERC1 may also be involved in actin remodeling [229, 230].

1.6.2 HERC2

The HERC2 protein was first identified during mutational analysis of genetically modified mouse strains. When HERC2 was deleted in mice they displayed signs of hypopigmentation in their eyes as well as their coat [231–239]. These mice also showed signs of jerky gait, reduced size, and reduced spermatogenesis [225]. HERC2 is a giant 528kDa protein, which contains three RLD domains as well as a HECT domain. HERC2 possesses a predicted DOC domain which adopts a β -sandwich fold. DOC domains are crucial for substrate targeting for some E3 ubiquitin ligases [241]. HERC2 contains a domain that is predicted to be a cytochrome b5-like heme/steroid binding domain (cyt-b5), however, the function of this domain in the context of HERC2 is not known [227]. HERC2 also contains a Mib-HERC2 domain as well. Mib-HERC2 domains have been found in RING E3 ubiquitin ligases, suggesting that this domain may be involved in E3 ligase activity or substrate protein targeting [242]. Lastly, HERC2 contains a ZZ-type zinc finger motif composed of six conserved cysteine residues as well as two conserved histidine residues which may coordinate Zn^{2+} . The function of this domain is not currently understood [227].

Human HERC2 has been shown to be involved in eye pigmentation. HERC2 expression is correlated to blue eyes. Single nucleotide polymorphisms have been correlated to autism in humans as well [240]. Murine and human HERC2 expression appears to be elevated within the brain and testes, however, the function of HERC2 in these tissues have not been studied [226]. Overall, the cellular activities of HERC2 are understudied.

1.6.3 HERC3

HERC3 is an 117kDa protein that is a member of the small HERC proteins and contains only one RLD, a Spacer region as well as a functional HECT domain. HERC3 colocalizes with multiple membrane trafficking proteins such as β -COP, Rab5 and ARF1. The colocalization of HERC3 with these membrane trafficking proteins suggests that HERC3 is involved in membrane trafficking. HERC3 does not have GRF or GEF activity for the small GTPases Ran, Rab3a, Rab5, Rab8, ARF1, ARF6, Ras, Rac and RhoA [243]. It was also determined that the HECT domain of HERC3 is a functional E3 ligase. In the presence of the E2 conjugation enzymes UbcH7 or UbcH5 the cysteine active site of HERC3 in the HECT domain forms a thioester bond with ubiquitin. No substrate proteins have yet been identified. HERC3 has also been shown to be polyubiquitinated and sent for proteasomal degradation [243]. It has not yet been confirmed if HERC3-mediated autoubiquitination occurs or if an unknown E3 ubiquitin ligase is responsible for the polyubiquitination of HERC3.

1.6.4 HERC4

Two major splice forms of the cytoplasmic HERC4 protein are present in cells. The *herc4* gene expresses 1057 and 1049 residue HERC4 isoforms [228]. HERC4 is highly expressed in the brain and testes and is significantly involved in normal spermatogenesis in mice. The genetic deletion of HERC4 in mice resulted in the production of abnormal sperm. These sperm possessed an abnormal tail structure with an angular bend. The angular tail contained cytoplasmic components resulting in decreased fertility [244]. Studies to date have failed to demonstrate that HERC4's HECT domain exhibits any ubiquitination activity.

1.7 HERC5

HERC5 is an 117kDa protein that is ubiquitously expressed and is up-regulated in a variety of primary cells and immortalized cell lines by IFN- α/β , lipopolysaccharide, TNF α , and IL- β [224, 245–247]. HERC5 was first identified during a yeast-two hybrid

experiment to look for proteins that interact with the Cyclin E-p21 complex [19]. Cyclin E binds to the cyclin dependent kinase 2, which allows the transition from G₁ (G₁) to synthesis (S) phase during the cell cycle. The p21 protein inhibits cyclin dependent kinase 2 mediated cell cycle progression. This study also determined that HERC5 interacts with multiple other cyclin proteins, which are involved in regulating the multiple steps of the cell cycle. It is currently unknown, however, if HERC5 plays an essential part in cell cycle regulation [246].

HERC5 levels are highly expressed in both the testes and ovaries [244]. HERC5 contains an N-terminal RLD, a spacer region, and a C-terminal HECT domain [246]. The RLD is predicted to assume a seven-bladed β -propeller tertiary structure. Each blade is predicted to consist of a four-stranded antiparallel β -sheets and contains several highly conserved glycine residues required for the sharp turns of within the four-stranded anti-parallel β -sheets. HERC5 is predicted to contain a conserved cis-proline in the last strand of each blade as well. The RLD also contains several conserved hydrophobic amino acids that stabilize the seven blades [19, 226]. The tertiary structure of the RLD is predicted to be homologous to the human RCC1 protein which has a role in the cell cycle regulation as well as nuclear export [226]. The RLD mediates the interaction between ribosomes and HERC5, which is required for HERC5 to ISGylate newly synthesized proteins [248]. The function of the spacer region is unknown and is not homologous to any known protein. The spacer region is predicted to contain multiple alpha helices with no predicted coiled-coils (PSIPREDv2.5 and Jpred3 protein structure prediction servers) [19].

1.7.1 HERC5 mediated ubiquitination

E3 ubiquitin ligase proteins require an E1 activating protein and an E2 conjugating protein to successfully conjugate ubiquitin to a substrate proteins [191]. Like ISGylation, ubiquitination requires an E1 enzyme, which uses ATP to activate ubiquitin proteins. Ubiquitin is next conjugated to the respective E2 conjugating enzyme through a thioester bond between the ubiquitin C-terminal glycine residue and the E2 active site cysteine residue. RING E3 ligases function by initiating the interaction between the E2 conjugating protein and the substrate protein (**Figure 1.6**). This allows ubiquitin to pass

directly from the E2 conjugating protein to substrate protein. HECT E3 ligases form a thioester bond at the cysteine active site before modifying the substrate protein (**Figure 1.6**) [191]. E3 ligase-mediated ubiquitination usually modifies Lys residues on substrate proteins through isopeptide linkages, although cysteine residues can also be modified [249].

HERC5 has shown to be a functional E3 ubiquitin ligase and uses UbcH5a as its E2 conjugating enzyme [245]. Only one target has been identified for HERC5-mediated ubiquitination. This protein is called non-metastatic cells 23B (Nm23B), which is a nucleoside diphosphate kinase. The functional consequence of HERC5-mediated ubiquitination of Nm23B still remains to be determined [250].

1.7.2 HERC5-mediated ISGylation

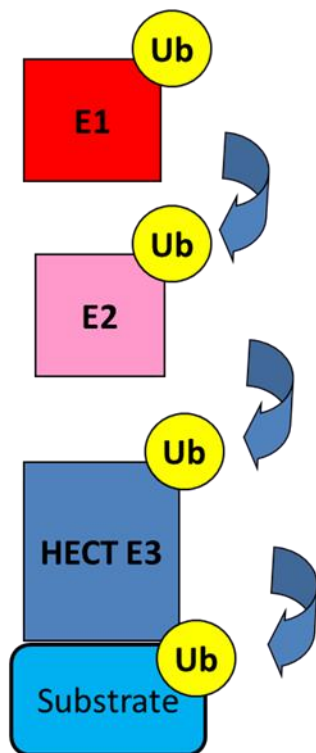
HERC5 is the main E3 ligase for ISG15 and uses the E1 activating enzyme Ube1L and the E2 conjugating enzyme UbcH8 to conjugate ISG15 to target proteins [223, 224]. The HERC5 E3 ligase active site is the cysteine amino acid at position 994 within the HECT domain. When this residue is mutated to alanine (HERC5-C994A), HERC5 loses its E3 ligase activity [224, 251]. Currently there is no evidence that HERC5-mediated ISGylation forms poly-ISG15 chains like ubiquitin. HERC5 conjugates ISG15 to several hundred endogenous proteins, some of which are involved in RNA splicing, chromatin remodeling/poIII transcription, cytoskeleton organization and regulation, stress responses, translation and glycolysis (**Figure 1.7**) [19, 223, 224, 247]. It has been demonstrated that HERC5 conjugates ISG15 to newly synthesized proteins while interacting with ribosomes. The first 100aa of the RLD are responsible for the interaction with ribosomes. The conjugation of ISG15 to proteins within a cell is greatly reduced when the RLD domain is deleted from HERC5 [223, 248].

As previously mentioned, it is well-known that ISG15 possesses potent antiviral activities towards divergent viruses [42, 75, 127, 206, 215, 218, 252, 253]. Although likely, it remains to be established whether HERC5 is the key E3 ligase responsible for these earlier observations. It has been shown that HERC5-mediated ISGylation reduces the

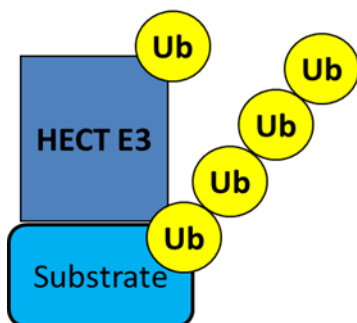
Figure 1.6: The HECT and RING E3 ligase-mediated ubiquitination.

Ubiquitin is activated by the E1 activating enzyme to and forms a thioester linkage with the active-site cysteine of E1. Ubiquitin is next passed to the active-site cysteine of the E2 conjugating enzyme. HECT E3 ligases first form a thioester bond between the C-terminal Gly residue of ubiquitin and the cysteine active site before modifying the substrate protein. RING E3 ligases mediate the interaction between the E2 and the substrate protein allowing the E2 to transfer of ubiquitin to a lysine residue on the substrate. The substrate can undergo monoubiquitination at one or more lysine residues or polyubiquitination. Chains that are linked together by the K48 residue of ubiquitin typically target substrates for 26S proteasomal degradation whereas ubiquitin chains linked by ubiquitin K63 are known to impact signal transduction or DNA repair.

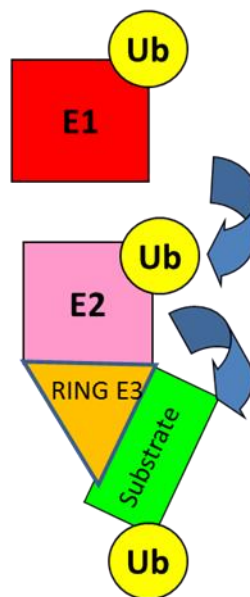
HECT E3 ligase ubiquitination



or



RING E3 ligase ubiquitination



or

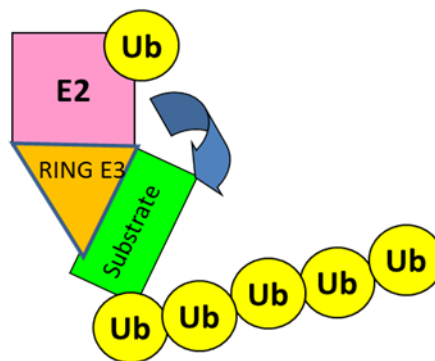


Figure 1.6: The HECT and RING E3 ligase-mediated ubiquitination.

infectivity of a Human Papillomavirus 16 pseudovirus by ISGylating the L1 capsid protein [248].

HERC5 also inhibits IAV replication via modification of the viral NS1A protein with ISG15 [254, 255]. The NS1A protein is crucial for IAV replication as it promotes the nuclear export and expression of viral mRNA and inhibits the export and translation of cellular mRNA. NS1A inhibits the activation of two potent cellular restriction factors PKR and 2'-5'-oligoadenylate synthetase (OAS) which are both activated by double stranded RNA. PKR inhibits cellular translation as mentioned previously where activated OAS results in the production of 2'-5'-oligoadenylate chains which subsequently activates RNase L, resulting in the degradation of cellular and viral RNA. NS1A inhibits the activation of PKR and OAS by binding and shielding the viral double stranded RNA [256]. NS1A also directly interacts with PKR causing its inhibition [257].

For NS1A to be functional it must form homodimers. HERC5-mediated ISGylation of NS1A inhibits NS1A homodimerization as well as its interaction with PKR [254]. HERC5 modifies NS1A at K41 so as to disrupt the interaction between NS1A and Importin- α which is essential for NS1A to localize to the nucleus. Nuclear localization is essential for NS1A to perform its pro-viral function [254–256]. Interestingly, NS1B from IAB binds to human ISG15 and inhibits HERC5-mediated cellular ISGylation [258].

1.7.3 HERC5-mediated regulation of IFN-I production

HERC5 is a key mediator and effector of the cellular IFN response. HERC5 up-regulates the IFN-I signaling cascade by modifying IRF3 with ISG15. IRF3 is a crucial transcription factor involved in the production IFN-I. It has been determined that once IRF3 is phosphorylated and activated after viral detection, the peptidyl-prolyl isomerase, Pin1 interacts with IRF3 to initiate ubiquitin-mediated proteasomal degradation of IRF3 [259]. HERC5 modifies IRF3 with ISG15, which interrupts the interaction between IRF3 and Pin1. This ultimately increases the production of IFN-I [193].

Figure 1.7: HERC5 mediated ISGylation.

ISGylation requires an E1 activating protein, an E2 conjugating protein, and an E3 ligase protein to successfully ISGylate substrate proteins. The ISG15-specific IFN-induced E1 activating protein, Ube1L, uses ATP to adenylate the Gly-Gly motif of ISG15. Ube1L then forms a thioester bond between its catalytic cysteine residue and the C-terminal Gly residue of ISG15. With the help of the IFN-induced E2 conjugating protein, UbcH8 HERC5 can modify substrate proteins with ISG15. ISG15 forms a covalent bond with the ϵ -NH₂ on the substrate lysine residue.

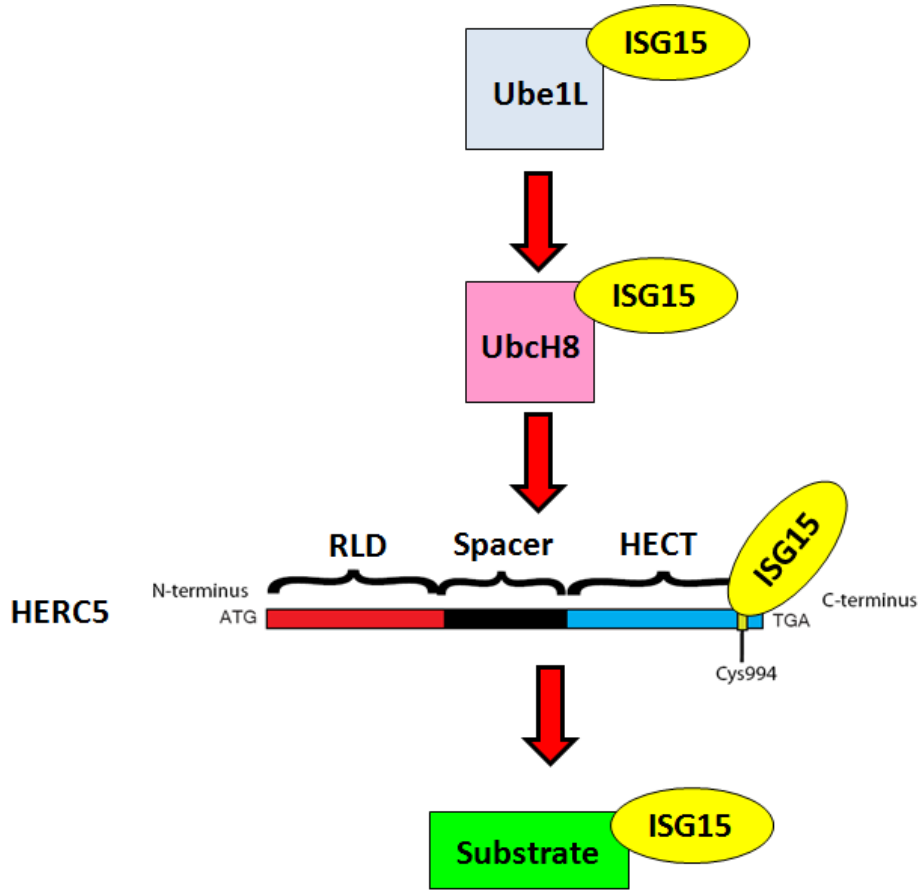


Figure 1.7: HERC5 mediated ISGylation.

1.8 HERC6

HERC6 is a 115kDa protein located adjacent to HERC5 on chromosome 4 [260]. Human HERC6 is IFN-induced and contains a HECT domain; however HERC6 does not function as the main cellular ISG15 E3 ligase in humans [223, 224]. In contrast, the murine HERC6 does act as the main ISG15 E3 ligase in murine cell lines and in mice *in vivo*. Mice do not express HERC5 [260, 261]. This suggests that either HERC5 or HERC6 must be functional as an ISG15 E3 ligase during mammalian evolution [261]. Murine HERC6 is also suspected to be involved in male fertility, as *herc6* gene knockout mice displayed enlarged seminal vesicles [261].

1.9 Hypothesis

With the discovery that the IFN-I-induced HERC5 protein is the main E3 ligase for ISG15, and given the well-known antiviral properties of ISG15, I hypothesized that HERC5 is an evolutionarily conserved anti-viral protein that interferes with HIV-1 particle production.

1.10 Thesis overview

Interferon (IFN)-induced proteins serve as one of the first lines of defense against viral pathogens such as HIV-1. The identities and functions of the critical proteins involved in the IFN-I response are not fully understood. Here, I have further characterized the anti-HIV-1 effect of IFN by identifying and characterizing a novel IFN-I-induced protein called HERC5. The work contained in this thesis presents the molecular characterization of the role that HERC5 plays during HIV-1 infection.

Chapter 2 summarizes my findings that HERC5 can restrict HIV-1 assembly, which is correlated with HERC5-mediated ISGylation of the HIV-1 Gag polyprotein. HERC5 does not inhibit Gag trafficking to the plasma membrane but restricts HIV-1 assembly at an early stage. HERC5's ISG15 E3 ligase activity results in the inhibition of MLV Gag particle budding as well. HERC5 is significantly up-regulated in biologically relevant cell types within patients infected with HIV-1.

Chapter 3 summarizes my findings that show that HERC5 expression results in the loss of Gag accumulation within cells expressing the HIV-1 provirus. This mechanism of HIV-1 restriction functions independently from HERC5's ISG15 E3 ligase activity. The RLD of HERC5 is responsible for the loss of Gag accumulation within the cell pellet by inhibiting late gene mRNA export required for Gag expression. HERC5 binds the crucial Ran protein and decreases the level of Ran-GTP within the cell. I determined that HERC5 has been evolving under strong positive selection indicating that HERC5 has been competing with invading pathogens throughout evolution.

In chapter 4, I characterized the antiviral activity of the small HERC family. HERC3, HERC4 and HERC5 expression results in the loss of Gag accumulation. HERC6 does not restrict HIV-1 replication. HERC3 and HERC4 inhibit the export of unspliced RNA similarly to HERC5. HERC5 and HERC6 are both IFN-I induced and have both evolved under strong positive selection indicating that HERC6 is, or once was, an antiviral protein. When the HERC6 RLD was swapped with the HERC5 RLD, HERC6 gained antiviral activity. In summary, I have identified members of the small HERC family as proteins with antiviral capabilities. HERC5, in particular, contains many hallmarks of a true restriction factor. First HERC5 is induced upon viral infection and is also IFN-I induced. HERC5 shows signs of genetic conflict throughout evolution. This indicates that HERC5 has a long history of competing with invading pathogens such as viruses. Lastly, HERC5's main biological activity is antiviral and contains two different domains that are antiviral that function independently of each other.

The potent antiviral nature of HERC5 makes it an attractive target for small drug design that can manipulate or increase the antiviral effect of HERC5. Further characterizing the antiviral effect of the HERC5 and the other small HERC proteins will provide insight into HIV-1 replication, and may be the ground-work for the creation of antiretroviral drugs that mimic the antiviral effect of these proteins. Currently, there are no antiretroviral drugs that inhibit HIV-1 RNA export or HIV-1 assembly. These drugs could provide people living with HIV-1 with alternative non-toxic options to inhibit HIV-1 replication and viral spread. The characterization of HERC5 has also provided further insight into how the innate immune system fights incoming pathogens such as viruses.

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Chapter 2

2 Human HERC5 restricts an early stage of HIV-1 assembly by a mechanism correlating with the ISGylation of Gag.

The identification and characterization of several interferon (IFN)-induced cellular HIV-1 restriction factors, defined as host cellular proteins or factors that restrict or inhibit the HIV-1 life cycle, has provided insight into the IFN response towards HIV-1 infection and identified new therapeutic targets for HIV-1 infection. To further characterize the mechanism underlying restriction of the late stages of HIV-1 replication, we assessed the ability of IFN β -induced genes to restrict HIV-1 Gag particle production and have identified a potentially novel host factor called HERC5 that blocks a unique late stage of the HIV-1 life cycle.

HERC5 inhibited the replication of HIV-1 over multiple rounds of infection and was found to target a late stage of HIV-1 particle production. The E3 ligase activity of HERC5 was required for blocking HIV-1 Gag particle production and correlated with the post-translational modification of Gag with ISG15. HERC5 interacted with HIV-1 Gag and did not alter trafficking of HIV-1 Gag to the plasma membrane. Electron microscopy revealed that the assembly of HIV-1 Gag particles was arrested at the plasma membrane, at an early stage of assembly. The mechanism of HERC5-induced restriction of HIV-1 particle production is distinct from the mechanism underlying HIV-1 restriction by the expression of ISG15 alone, which acts at a later step in particle release. Moreover, HERC5 restricted murine leukemia virus (MLV) Gag particle production, showing that HERC5 is effective in restricting Gag particle production of an evolutionarily divergent retrovirus.

HERC5 represents a potential new host factor that blocks an early stage of retroviral Gag particle assembly. With no apparent HIV-1 protein that directly counteracts it, HERC5 may represent a new candidate for HIV/AIDS therapy.

2.1 Introduction

Several IFN-induced proteins have been identified as correlates of HIV-1 infection and disease progression [1-4]. A few of these factors have already been shown to exhibit antiviral activity towards HIV-1, such as APOBEC3G, Tetherin and TRIM22 (reviewed in [5]). To identify and characterize additional potential host factors, we surveyed IFN β -induced genes from two previous studies [6, 7] for potential anti-HIV-1 activity and identified HERC5 as a new host factor that may block a late stage of the HIV-1 life cycle.

The *herc5* gene is one of six human *herc* family genes located on chromosome 4 and flanked by *herc6* and *herc3*. The HERC5 protein is 1024 amino acids in length and contains a RLD at its amino-terminus, followed by a unique Spacer region that does not share homology with any known protein, and a HECT domain at the carboxyl-terminus [8-10]. The function of the RLD domain is unknown; however it appears to be important, but not essential, for the conjugation of ISG15 to cellular proteins [6]. Also, the predicted tertiary structure of the RLD closely resembles the crystal structure of the human RCC1 protein (reviewed in [11, 12]). The Spacer region contains numerous α -helices and its function is unknown. The HECT domain is typically found in E3 ligases, which operate in conjunction with unique E1 “activating” and E2 “conjugating” enzymes to transfer ubiquitin or ubiquitin-like proteins, such as ISG15, to specific cellular substrates.

HERC5 is the main E3 ligase for the conjugation of ISG15 to proteins in human cells and works together with the E1 activating protein Ube1L as well as the E2 conjugating protein UbcH8 to conjugate ISG15 onto target proteins [6, 13, 14]. Cysteine 994 of the HERC5 HECT domain is essential for this E3 ligase activity of HERC5 [13]. Two reports have also identified HERC5 as an E3 ubiquitin ligase [15, 16]. HERC5 is ubiquitously expressed with the highest levels of expression observed in the testis. HERC5 is up-regulated in a variety of primary cells and immortalized cell lines by IFN, LPS, TNF α , and IL-1 β [6, 13, 16]. Moreover, HERC5 broadly targets newly synthesized proteins for ISG15 conjugation and many endogenous targets of HERC5 have been identified that function in a variety of cellular pathways including RNA splicing, chromatin remodeling/polIII transcription, cytoskeleton organization and regulation, stress responses,

translation, glycolysis, IFN signaling and antiviral responses [17-22]. Here we show that HERC5 inhibits HIV-1 replication by targeting a unique step of HIV-1 particle assembly at the plasma membrane.

2.2 Results

2.2.1 HERC5 inhibits HIV-1 particle production.

HERC5 is the main cellular E3 ligase that conjugates ISG15 to proteins and can target newly synthesized proteins, including foreign proteins [6, 13, 21]. Therefore, we tested the ability of HERC5 to restrict the release of newly made infectious HIV-1 particles. We co-transfected 293T cells with empty vector or plasmids encoding replication-competent HIV-1 (pR9) and HERC5. Cells expressing HERC5 released 4.0-fold less infectious virus than the control cells after a single round of replication ($P=0.008$, paired t test) (**Figure 2.1A**). Similar results were also obtained in HOS-CD4/CXCR4 cells, which support robust HIV-1 replication. HOS-CD4/CXCR4 cells expressing HERC5 released 10.8-fold less infectious HIV-1 particles into the supernatant after multiple rounds of replication ($P=0.001$, paired t test) (**Figure 2.1B**). Similar levels of inhibition of HIV-1 particle release after single and multiple rounds of replication were observed by Western blot analysis (**Figure 2.1C**). There was no significant difference in the viability of cells expressing HERC5 compared to the control cells ($90.2\% \pm 4.4\%$ SD versus $92.0\% \pm 4.3\%$ SD respectively; $P>0.05$, paired t test).

To assess the impact of reduced levels of HERC5 on HIV-1 particle production, we depleted HERC5 using short hairpin ribonucleic acid (shRNA) and measured the amount of HIV-1 particles released into the supernatant. 293T cells, which express basal levels of HERC5, were transiently co-transfected for 24 hours with either pLKO.1/scrambled_{shRNA} or pLKO.1/HERC5_{shRNA} and pR9. Cells expressing HERC5 shRNA exhibited 2.3-fold

Figure 2.1: HERC5 restricts a late stage of HIV-1 replication.

A, Inhibition of HIV-1 particle production. 293T cells were co-transfected with pR9 and either empty plasmid or pHERC5. Twenty-four hours after transfection, infectious HIV-1 virions released into the supernatant were quantified using GHOST(3) indicator cells. **B**, Inhibition of HIV-1 replication. HOS-CD4/CXCR4 cells were co-transfected with plasmids encoding a replication-competent HIV-1 provirus (R9) and HERC5 or an empty vector control. HIV-1 virions released into the supernatant were pelleted 72 hours after transfection and analyzed by Western blotting using anti-p24CA antibody. p24CA levels were quantified densitometrically. **C**, representative Western blot using anti-p24CA of HIV-1 particles released into the supernatant. Data shown is the average of at least two independent experiments performed in triplicate. **D-G**, 293T cells were co-transfected with pLKO.1/scrambled_{shRNA} or pLKO.1/HERC5_{shRNA} and pR9. Twenty-four hours after transfection, HIV-1 particles released into the supernatant, cellular RNA and cellular protein were isolated. HERC5, HERC3 and β -actin RNA were detected by reverse transcription polymerase chain reaction (RT-PCR) (**D**) and quantified densitometrically (**E**). HIV-1 particles released into the supernatant and intracellular Pr55Gag protein were detected by Western blotting using anti-p24CA (**F**) and quantified densitometrically (**G**). Data shown is representative of at least two independent experiments.

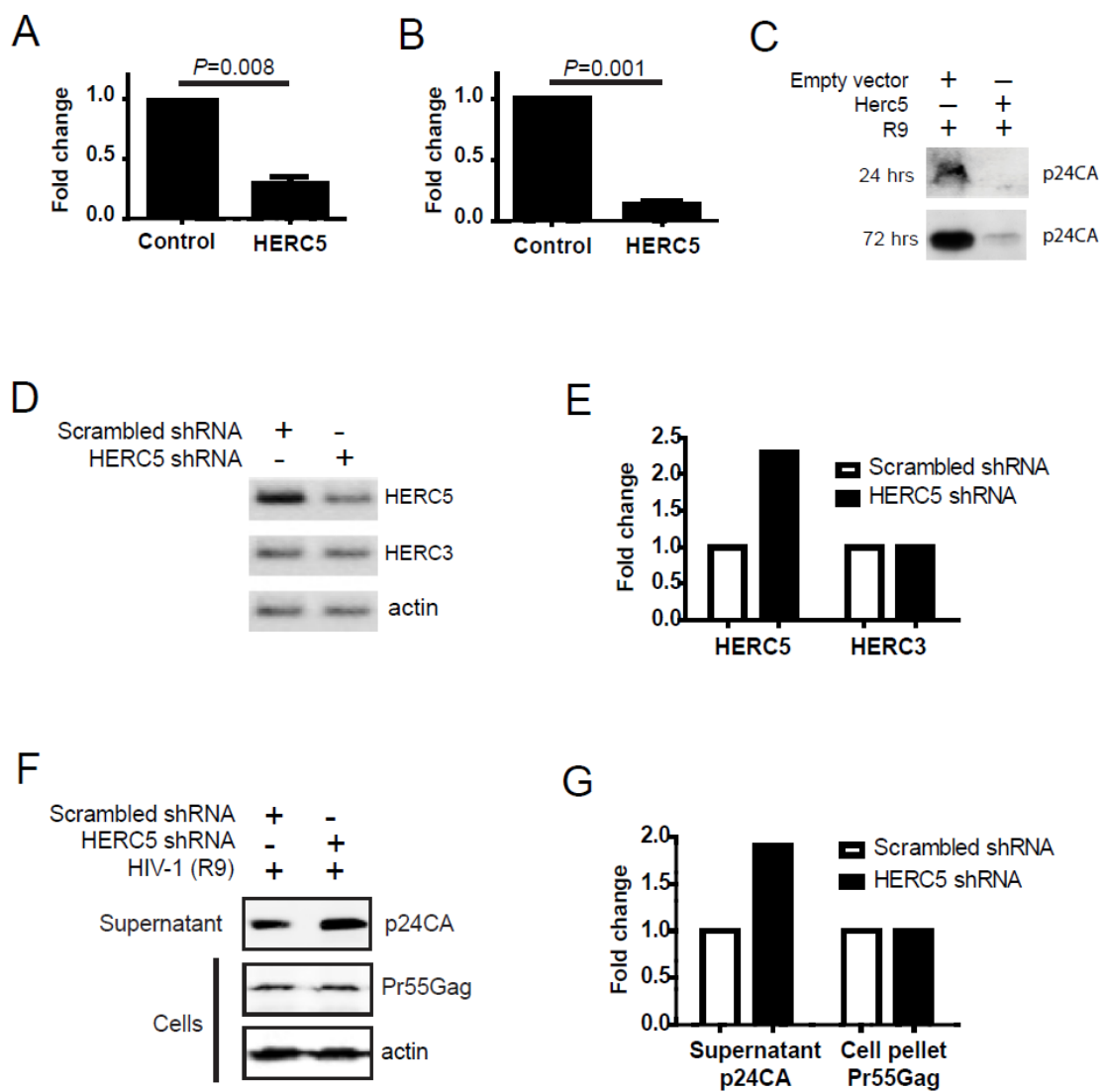


Figure 2.1: HERC5 restricts a late stage of HIV-1 replication.

less HERC5 RNA than the control cells expressing scrambled shRNA (**Figure 2.1D and 1E**). As a control for specificity, RNA levels of the related *herc3* gene was tested and found to be unaffected by the scrambled or HERC5 shRNA (**Figure 2.1D and 2.1E**). Accumulation of HIV-1 particles in the supernatant and cell pellets was monitored by Western blot using anti-p24CA (**Figure 2.1F**). Cells knocked down for HERC5 expression released 1.9-fold more HIV-1 particles into the supernatant compared to the control cells (**Figure 2.1F and 2.1G**). No substantial change in the intracellular Pr55Gag protein level was detected. Similar results were observed in HeLa cells, which also express basal levels of HERC5 (data not shown).

2.2.2 HERC5 blocks HIV-1 Gag particle production and this block correlates with the ISGylation of Gag.

Gag is the major structural polyprotein of HIV-1 that drives virion formation and can assemble and bud from cells in the absence of all other HIV-1 proteins as Gag-only particles [23]. Therefore, we asked if HERC5 could conjugate ISG15 to Gag, and in so doing, interfere with Gag particle production. To test this, we used a single-cycle HIV-1 Gag-only particle release assay in U2OS cells, which do not express HERC5 mRNA basally or after IFN β treatment, thereby providing a HERC5 null background (reference [14] and data not shown). The Gag-only particle release assay involved the co-transfection of plasmids encoding codon-optimized HIV-1 Gag with or without HERC5 and the measurement of the Gag-only particles released into the supernatant by quantitative Western blotting. To enhance detection of Gag modified with ISG15, plasmids encoding histidine-tagged ISG15, Ube1L and UbcH8 (referred to hereafter as the conjugation system (CS)), were included in the transfections.

As shown in **Figure 2.2A**, Western blot analysis revealed that cells expressing HERC5 + CS released substantially less Gag-only particles into the supernatant than the control cells. In contrast, cells expressing HERC5-C994A (defective for its E3 ligase activity) + CS failed to inhibit the release of Gag-only particles. Western blot analysis of Gag protein expression in cell lysates revealed similar levels of Gag (55kDa) in all samples. Notably, more slowly migrating Gag species (~85kDa and ~110kDa) were detected in

cells expressing HERC5 + CS. These slowly migrating Gag bands were not present in the HERC5-C994A + CS samples, suggesting that the E3 ligase activity of HERC5 was required for generating the more slowly migrating Gag species (**Figure 2.2A**). Western blot analysis of the cell lysates revealed the presence of ISG15 conjugates in cells transfected with HERC5 + CS, but not cells transfected with empty vector or HERC5-C994A, as previously shown [13]. The sizes of these slowly migrating Gag species are consistent with the conjugation of two or more ISG15 proteins (one ISG15= ~15kDa), although non-linear decreases in the electrophoretic mobility of proteins conjugated with ISG15 in SDS-PAGE gels has been previously reported [24, 25]. The identities of the slowly migrating Gag species were confirmed to be Gag after immunoprecipitation and protein identification using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). There was no significant difference in the viability of cells expressing HERC5 + CS compared to the control cells (92.0% \pm 4.3% SD versus 90.2% \pm 4.4% SD respectively; $P>0.05$, paired t test).

To determine if the more slowly migrating bands of Gag contained conjugates of ISG15, we co-expressed Gag and HERC5 with histidine-tagged ISG15 and the CS for 72 hours and then incubated the cell lysate with nickel agarose under non-denaturing conditions. Western blot analysis of the purified proteins using anti-p24CA revealed the presence of several species of Gag ranging from ~55 kDa to ~200 kDa in size (**Figure 2.2B**), including the ~85 kDa and ~110 kDa bands detected in the whole cell lysate (**Figure 2.2A**). Notably, when the ISG15ylated Gag species was purified under non-denaturing conditions, a substantial amount of 55 kDa Gag species was co-purified, suggesting that Gag species conjugated with ISG15 can interact with the 55 kDa unmodified Gag (**Figure 2.2B**). It is unknown why the ~85 kDa Gag species was more abundant than the ~110 kDa species after purification with the nickel agarose (compared to the levels observed in the cell lysate (**Figure 2.2A**)). We have noticed that the ~110 kDa species is more abundant if Gag is purified 48 hours after transfection instead of after 72 hours, possibly indicating that some of the modifications are labile.

To assess the importance of ISG15ylation in restricting Gag particle release, we co-expressed HERC5 + CS with the ISG15-specific deconjugating enzyme Ubp43 and

Figure 2.2: HERC5 restricts HIV-1 Gag particle production by a mechanism dependent on its E3 ligase activity.

A, U2OS cells were co-transfected with pGag, pUbe1L, pUbcH8, pMyc-ISG15 and either empty plasmid, pHERC5 or pHERC5-C994A. Gag particles released into the supernatant and intracellular Gag protein expression were analyzed after 24 hours by quantitative Western blotting using anti-p24CA and anti- β -actin as a loading control. The same blot was stripped and tested for ISG15 conjugates using anti-myc (bottom blot). **B**, Cells were co-transfected with empty plasmid or pHERC5 and pUbe1L, pUbcH8, pHis-ISG15 and pGag. Cells were lysed 72 hours later and histidine-tagged proteins were purified using nickel agarose. Purified proteins were resolved using SDS-PAGE and subjected to Western blotting using anti-p24CA. **C**, Cells were co-transfected with empty vector or pHERC5, pUbe1L, pUbcH8, pHis-ISG15 and pGag with or without pUbp43. Gag particles released into the supernatant and intracellular Gag protein expression were analyzed after 72 hours by quantitative Western blotting using anti-p24CA or anti- β -actin. **D**, In a similar transfection as in C, histidine-tagged proteins were purified from cell extracts using nickel agarose and subjected to Western blotting using anti-p24CA. The two lanes were digitally separated from the same image. Numerical values on the blots display the densitometric quantification of the specified bands. All Western blots shown are representative of at least two independent experiments.

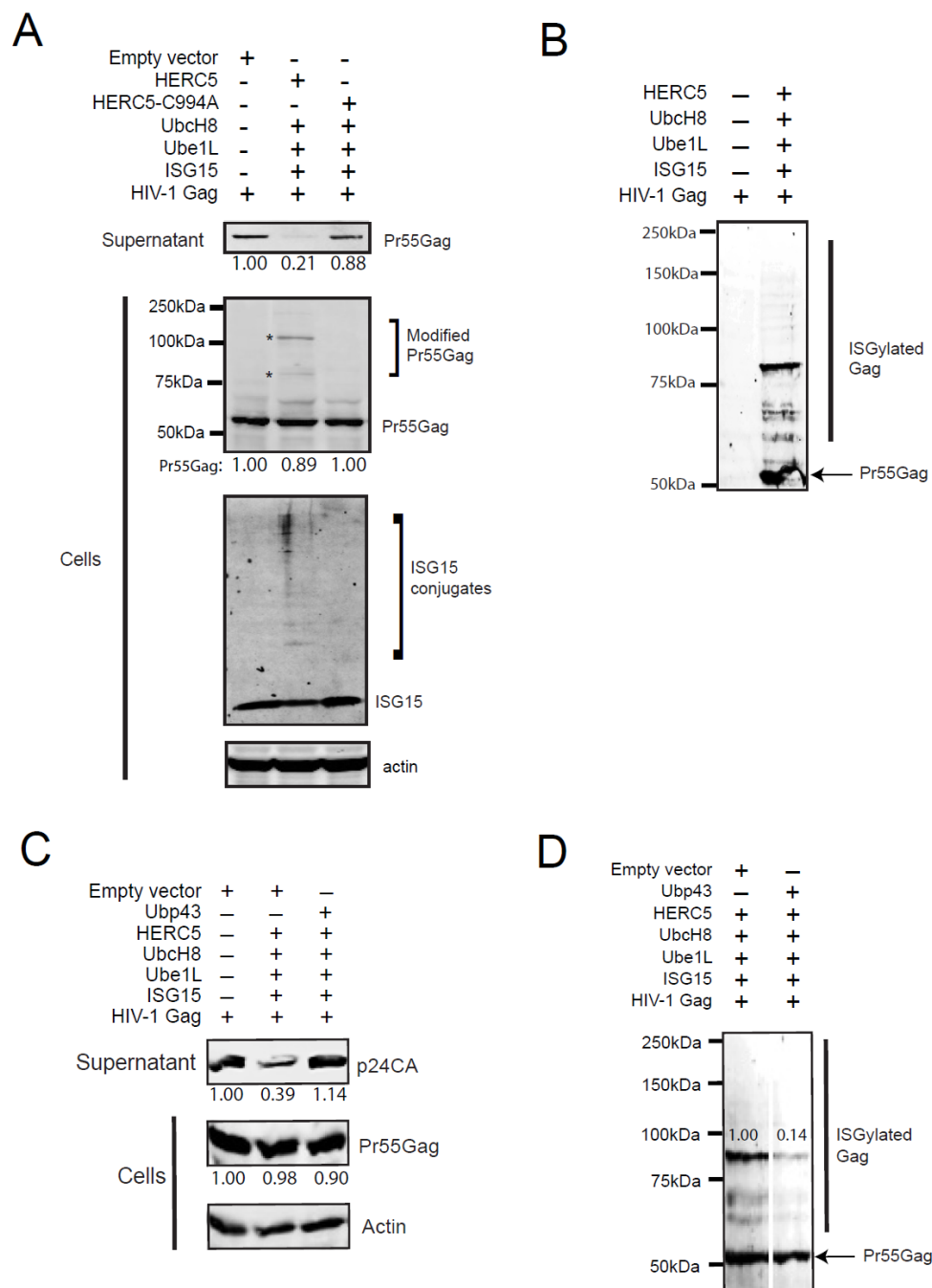


Figure 2.2: HERC5 restricts HIV-1 Gag particle production by a mechanism dependent on its E3 ligase activity.

assessed whether Ubp43 could rescue restriction imposed by HERC5 [26]. Quantitative Western blot analysis of Gag particles released into the supernatant showed that Ubp43 abolished HERC5-induced restriction, despite the presence of similar levels of intracellular Gag (**Figure 2.2C**). The rescue of Gag particle release by Ubp43 correlated with a 7.2-fold reduction in intracellular ISG15ylated Gag (**Figure 2.2D**).

2.2.3 Intracellular localization of HERC5 and HIV-1 Gag.

Confocal immunofluorescence microscopy of IFN β -treated PBMCs, human Jurkat cells, and immortalized murine mature dendritic cells (DC2.4) revealed that HERC5 localized to the cytoplasm in punctate bodies in each of the cell types (**Figure 2.3A**), consistent with the pattern of localization observed for HERC5 in human mammary epithelial cells [15]. Recently, it was shown biochemically in HeLa cells, but not microscopically, that HERC5 co-fractionated with polysomes due to an association with the 60S ribosomal subunit [21]. Consistent with this report, we observed that 59.9% \pm 0.12% SD of HERC5 co-localized with polyribosomes in IFN β -treated Jurkat cells using confocal immunofluorescence microscopy (mean Pearson's coefficient= 0.855; $n=10$) (**Figure 2.3B**). Similar observations were made in HOS-CD4/CXCR4 cells where 61.7% \pm 0.13% SD of IFN β -induced HERC5 co-localized with ribosomes (mean Pearson's coefficient= 0.863; $n=10$).

To determine if HERC5 and HIV-1 Gag co-localize in cells, we co-transfected plasmids encoding Gag-only (pGag) or replication-competent HIV-1 (pR9) with and without HERC5 + CS. Forty-eight hours after transfection, the localization of Gag and HERC5 protein was assessed in U2OS cells by examining optical slices through the center of cells. Gag expressed in the absence of HERC5 exhibited predominantly punctate fluorescence at the plasma membrane, consistent with the location of assembly and budding of Gag particles (**Figure 2.3C**). HERC5 expressed in the presence or absence of the conjugation system localized predominantly in the cytoplasm in punctate bodies (**Figure 2.3C**). When Gag was co-expressed with HERC5 + CS, Gag localized predominantly at the plasma membrane (**Figure 2.3D**). Interestingly, a substantial amount of HERC5 accumulated near the plasma membrane where 65.2% \pm 0.21% SD of

Figure 2.3: Intracellular localization of IFN β -treated HERC5 and HIV-1 Gag.

A, Localization of endogenous HERC5 (red) in IFN β -treated (500 units/ml for 16 hours) PBMCs (scale bar= 5 μ m), Jurkat cells (scale bar= 5 μ m), and murine dendritic cells (scale bar= 10 μ m). Images shown are representative of at least 2 independent experiments. **B**, Localization of endogenous ribosomes (green) and HERC5 (red) in control Jurkat cells or IFN β -treated Jurkat cells (scale bar= 5 μ m). Localization of Gag alone (red) or HERC5 (green) +/- CS (**C**), or co-expression of Gag and HERC5 + CS (**D**) in U2OS cells 48 hours after transfection. Scale bars= 20 μ m. Images shown are representative of at least 3 independent experiments. **E**, HeLa cells were co-transfected with pR9, pUbe1L, pUbcH8, and pMyc-ISG15 with or without pFlag-HERC5 or pFlag-HERC5-C994A. Gag was immunoprecipitated under non-denaturing conditions using anti-p24CA. Precipitated proteins were resolved using SDS-PAGE and subjected to Western blotting using anti-Flag or anti-p24CA. 10% of the input lysates was subjected to Western blotting using anti-p24CA and anti- β -actin on the same blot. HERC5 protein was undetectable in the 10% input lysates.

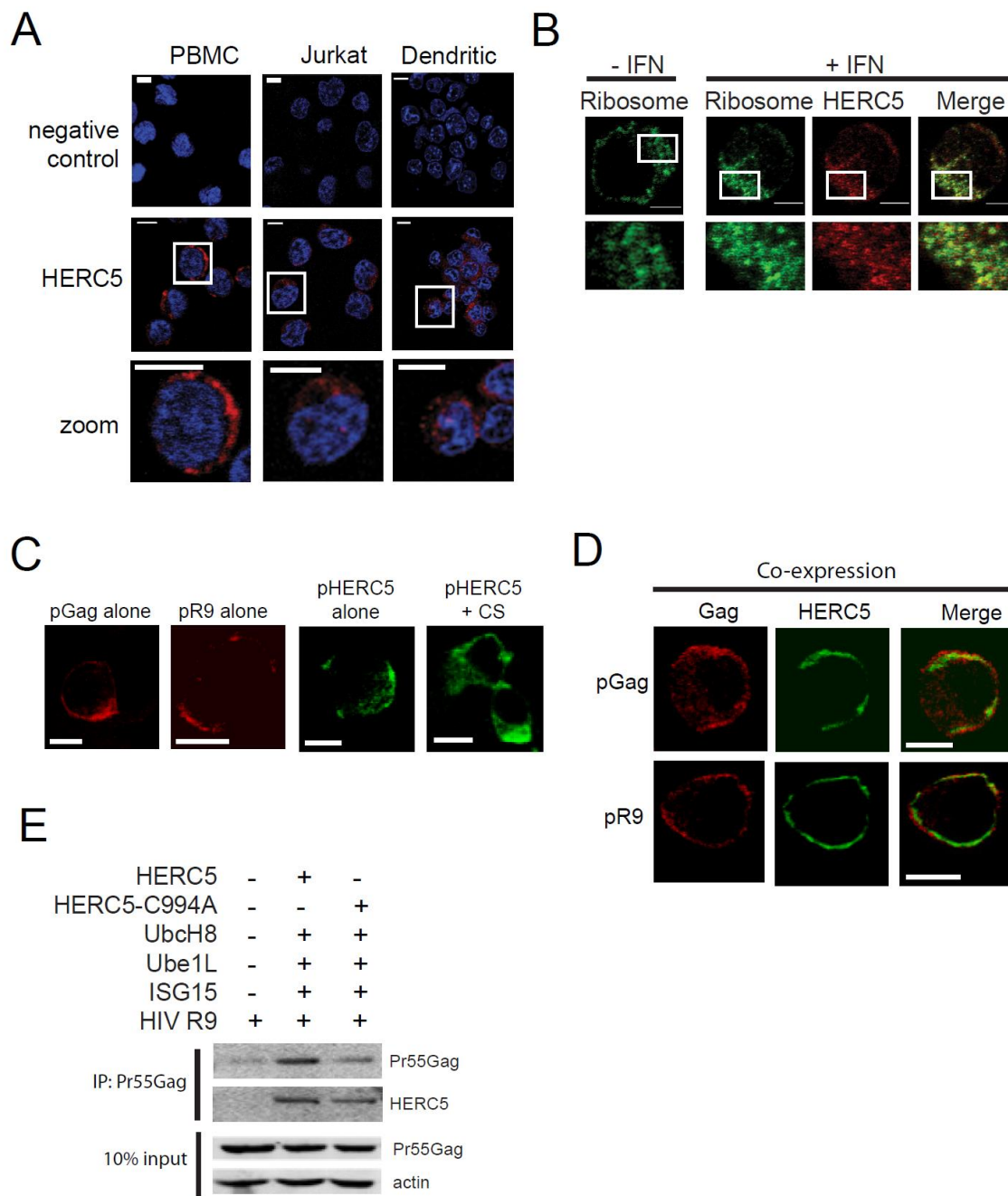


Figure 2.3: Intracellular localization of IFN β -treated HERC5 and HIV-1 Gag.

Gag expressed from pR9 co-localized with HERC5 (mean Pearson's coefficient= 0.324; n=21) and 53.1% \pm 0.16 SD of Gag expressed from pGag co-localized with HERC5 (mean Pearson's coefficient= 0.336; n= 15).

Since it was recently shown that HERC5 can conjugate ISG15 to exogenously expressed foreign proteins [21], we asked whether we could detect an interaction between flag-tagged HERC5 and HIV-1 Gag biochemically. Flag-tagged HERC5 + CS or flag-tagged HERC5-C994A + CS were co-expressed with replication-competent HIV-1 (R9) in HeLa cells. Forty-eight hours post-transfection, cells were lysed under non-denaturing conditions and subjected to co-immunoprecipitation using anti-p24CA. Western blot analysis of the precipitated proteins revealed that both flag-tagged HERC5 and HERC5-C994A co-precipitated with HIV-1 Gag (**Figure 2.3E**). Despite similar input levels of intracellular Gag, more Gag was consistently immunoprecipitated in the presence of HERC5 compared to the control and HERC5-C994A. Similar results were obtained with 293T cells.

2.2.4 HERC5 inhibits an early stage of Gag assembly.

We then utilized transmission electron microscopy (TEM) to examine HIV-1 Gag particles assembling at the plasma membrane in the presence or absence of HERC5. As normally seen phenotypically with TEM micrographs of negatively-stained samples, HIV-1 Gag particle assembly at the plasma membrane begins with the accumulation of Gag protein at the plasma membrane, which is visualized as small electron-dense regions at the plasma membrane often seen as small buds with the diameter of an immature Gag shell ~150-200nm (step I). This is followed by the formation of more pronounced half-spherical particles protruding from the plasma membrane (step II), more extensive budding with nearly complete spherical particles that are still contiguous with the cytoplasm via a stalk-like ("lollipop") structure (step III), scission of viral and cellular membranes (step IV), and finally release and distancing of the HIV-1 Gag particle from the plasma membrane (step V) (**Figure 2.4A**).

To differentiate between the various stages of assembly, budding and release, we examined TEM images of mock transfected U2OS cells and cells transfected with plasmids encoding codon-optimized HIV-1 Gag with or without HERC5 + CS. U2OS cells were chosen because of their HERC5 null background and that HIV-1 particles assemble and bud at the plasma membrane normally. As shown in **Figure 2.4B**, the mock control cells exhibited a relatively unperturbed plasma membrane, the absence of electron-dense accumulations/patches and budding structures at the plasma membrane. In contrast, cells expressing Gag in the absence of HERC5 + CS exhibited substantial perturbations of the plasma membrane and budding Gag-only particles. The Gag-only particles appeared as enveloped, spherical particles that resembled immature HIV virions but more heterogeneous in size (up to ~500nm in diameter). This observation is in agreement with previously published results [27]. Strikingly, cells expressing Gag in the presence of HERC5 + CS yielded substantially less Gag-only particles and exhibited pronounced electron-dense staining at the plasma membrane appearing in a polarized region of the plasma membrane or at regions of cell-cell-contact (**Figure 2.4B**).

To determine if the pronounced accumulation of Gag at the plasma membrane was due to gross over-expression from the cytomegalovirus (CMV) promoter-driven codon-optimized Gag (pGag), we performed a similar TEM experiment except that we transfected a plasmid encoding replication-competent proviral HIV-1 (pR9). This plasmid expresses biologically relevant levels of wild type Gag (in addition to all other HIV-1 proteins) from the HIV-1 long terminal repeat promoter. As seen in **Figure 2.4C**, the plasma membrane of mock transfected cells exhibited a relatively unperturbed profile with no viral particles released from the plasma membrane, whereas cells expressing HIV-1 alone produced numerous predominantly homogeneous particles (~100-150nm in diameter). Cells transfected with pR9 and pHERC5 + CS released substantially less HIV-1 particles compared to cells expressing R9 only. Notably, numerous cells exhibited electron-dense regions resembling early HIV-1 Gag assembly structures at the plasma membrane, with the absence of budded “lollipop” structures, extracellular virions, and intracellular virions. The electron dense patches accumulated in a polarized region of the plasma membrane or at regions of cell-cell contact. The electron-dense regions of the early budding structures are ~150-200nm in diameter, which is consistent with the

Figure 2.4: HERC5 inhibits an early stage of Gag assembly at the plasma membrane.

A, Schematic diagram of HIV-1 Gag assembly, budding and release at the plasma membrane. step I, Gag accumulates at the plasma membrane; step II, distortion of plasma membrane curvature; step III, increased distortion of the plasma membrane and formation of the “lollipop structure;” step IV, virion detaches its membrane from the host cell, undergoes proteolytic processing of Gag and remains closely associated with the host cell; step V virion distances itself from the host cell. **B**, Transmission electron micrographs of U2OS cells that were mock transfected (“mock”), transfected with pGag and empty vector (“-HERC5”), or co-transfected with pGag and pHERC5 + CS (“HERC5 + CS”). Scale bars= 500nm. **C**, Transmission electron micrographs of U2OS cells that were mock transfected (“mock”), transfected with pR9 and empty vector (“-HERC5”), or co-transfected with pR9 and pHERC5 + CS (“HERC5 + CS”). Scale bars= 300 nm. **D**, Transmission electron micrographs of U2OS cells that were transfected as described in parts B and C showing immunogold labeling of p24CA (Gag). Gold particles are 10 nm in size. Bottom panels of the micrographs are zoomed images of the white boxes in the upper panels. Scale bars= 100 nm. N, nucleus; C, cytoplasm; O, outside of cell.

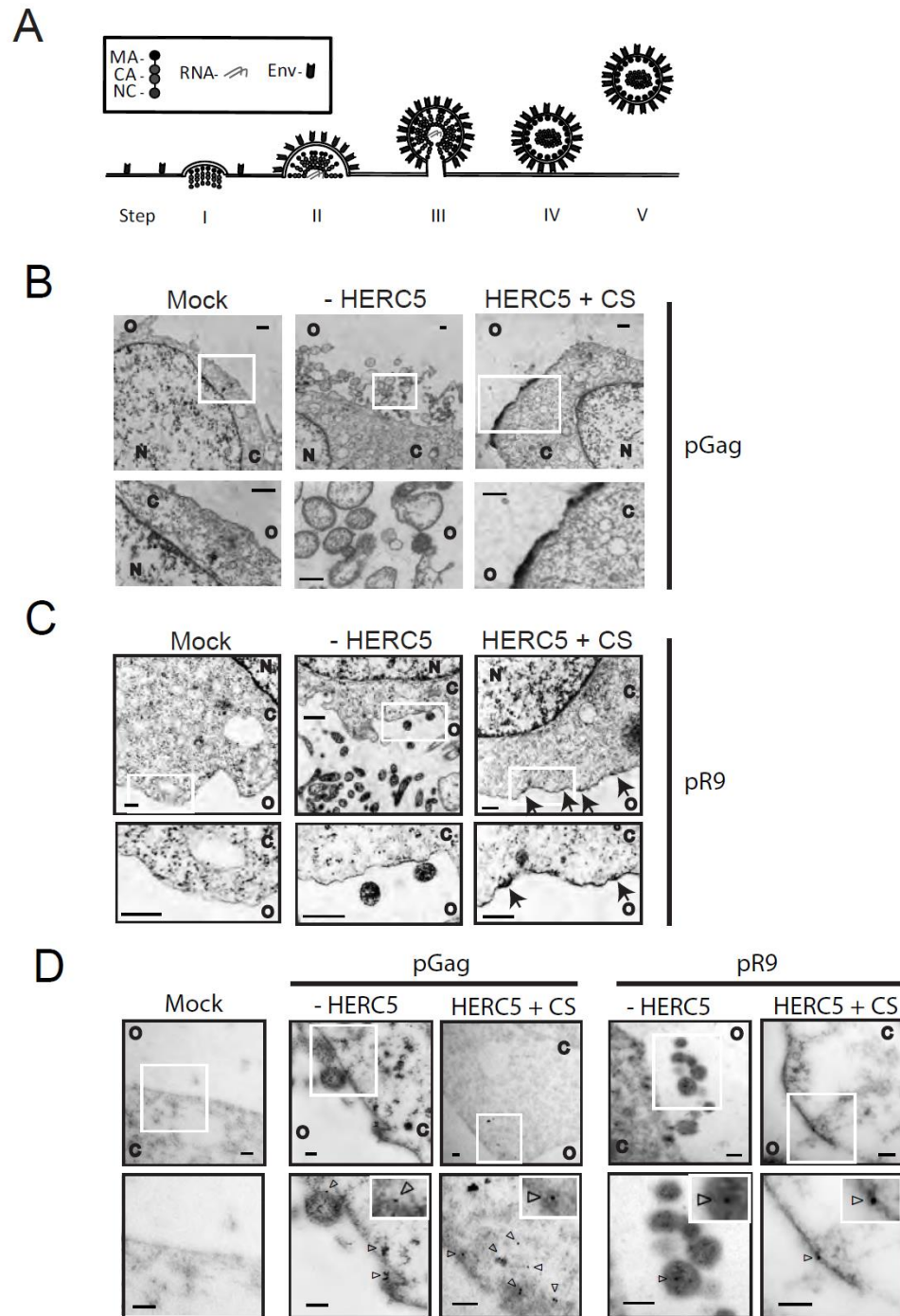


Figure 2.4: HERC5 inhibits an early stage of Gag assembly at the plasma membrane.

Table 2.1: Quantification of 10 nm gold particle-labeled anti-p24CA in cells expressing HERC5+CS and HIV-1 Gag.

Region	Observed gold count, Go	Point count, P	Expected gold count, Ge	Go/Ge	χ^2	χ^2 as %
Particles+ Plasma membrane	118	189	29	4.0	270.3	85.9
Cytoplasm+ Nucleus	80	875	135	0.6	22.5	7.1
Non-particle+ non-cell	18	335	52	0.3	22.0	7.0
TOTAL	216	1399	216		314.8	100.0

For $\chi^2=314.8$ and $df=2$, $P<0.0001$ (χ^2 analysis). The gold labeling distribution is significantly different from random. Only the particles/plasma membrane region ($Go/Ge=4.0$, $\chi^2=85.9\%$ of total) meets the two criteria for being preferentially labeled ($(Go/Ge) > 1$ and χ^2 value $\geq 10\%$ of total).

diameter of a budding immature Gag shell [28]. To confirm that Gag protein accumulated in HIV-1 Gag particles and electron dense regions at the plasma membrane, we performed immunogold-labeling using anti-p24CA. As shown in **Figure 2.4D**, Gag was observed to specifically localize in each of these regions. Quantification of immunogold localization on the electron microscopic thin sections from cells expressing HERC5 + CS and Gag-only or R9 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 2.1**) (see Methods for details).

2.2.5 HERC5-induced restriction of Gag particle production is distinct from ISG15-only restriction.

Three reports suggest that ectopic expression of the CS only (minus HERC5) restricts HIV-1 Gag particle budding and/or release [29-31]. Since HERC5 is the main enzyme responsible for the conjugation of ISG15 to proteins, we asked if HERC5 was the E3 ligase responsible for the restriction of Gag-only particle release induced by ISG15 expression. To address this question, we performed a single-cycle Gag-only release assay by co-expressing Gag + CS only in U2OS cells (which lack HERC5 expression). Consistent with the three previous published reports [29-31], we observed that the CS blocked Gag particle production without affecting the levels of intracellular Gag protein.

To determine if the inhibition of Gag particle production by the CS alone resembled the inhibition of Gag particle production induced by CS + HERC5, we examined TEM micrographs of cells expressing replication-competent HIV-1 (R9) + CS only. Analysis of cells expressing R9 + CS revealed an accumulation of apparently fully enveloped immature virions on the surface of the cell in addition to virions within intracellular compartments (**Figure 2.5A**). Virions found on the cell surface were closely associated with each other as doublet particles or in long chains. This phenotype is in contrast with that observed when HIV-1 is expressed in the presence of HERC5 + CS (**Figure 2.4C**).

Figure 2.5: ISG15 expressed in the absence of HERC5 causes retention of virions on cell surfaces and in intracellular compartments.

A, Transmission electron micrographs showing the plasma membrane and intracellular regions of U2OS cells that were mock transfected (“mock”), co-transfected with empty vector and pR9 (“R9 only”), or co-transfected with pR9 and CS only (“R9 + CS”). Insets show zoomed images of the dotted white boxes. Scale bars= 200 nm. **B**, Confocal immunofluorescence microscopy of U2OS cells mock transfected or co-transfected with pGag with or without HERC5 and the CS. Gag localization was detected using anti-p24CA (red) 48 hours post-transfection. Scale bars= 20 μ m. **C**, Quantitative analysis of cells from part B containing punctate Gag at the plasma membrane and large intracellular clusters of Gag or intracellular diffuse/punctate Gag. Data represents the average of two independent experiments +/- SD (n= 400). ns, non-significant. ***, $P < 0.0001$, Fisher’s exact test.

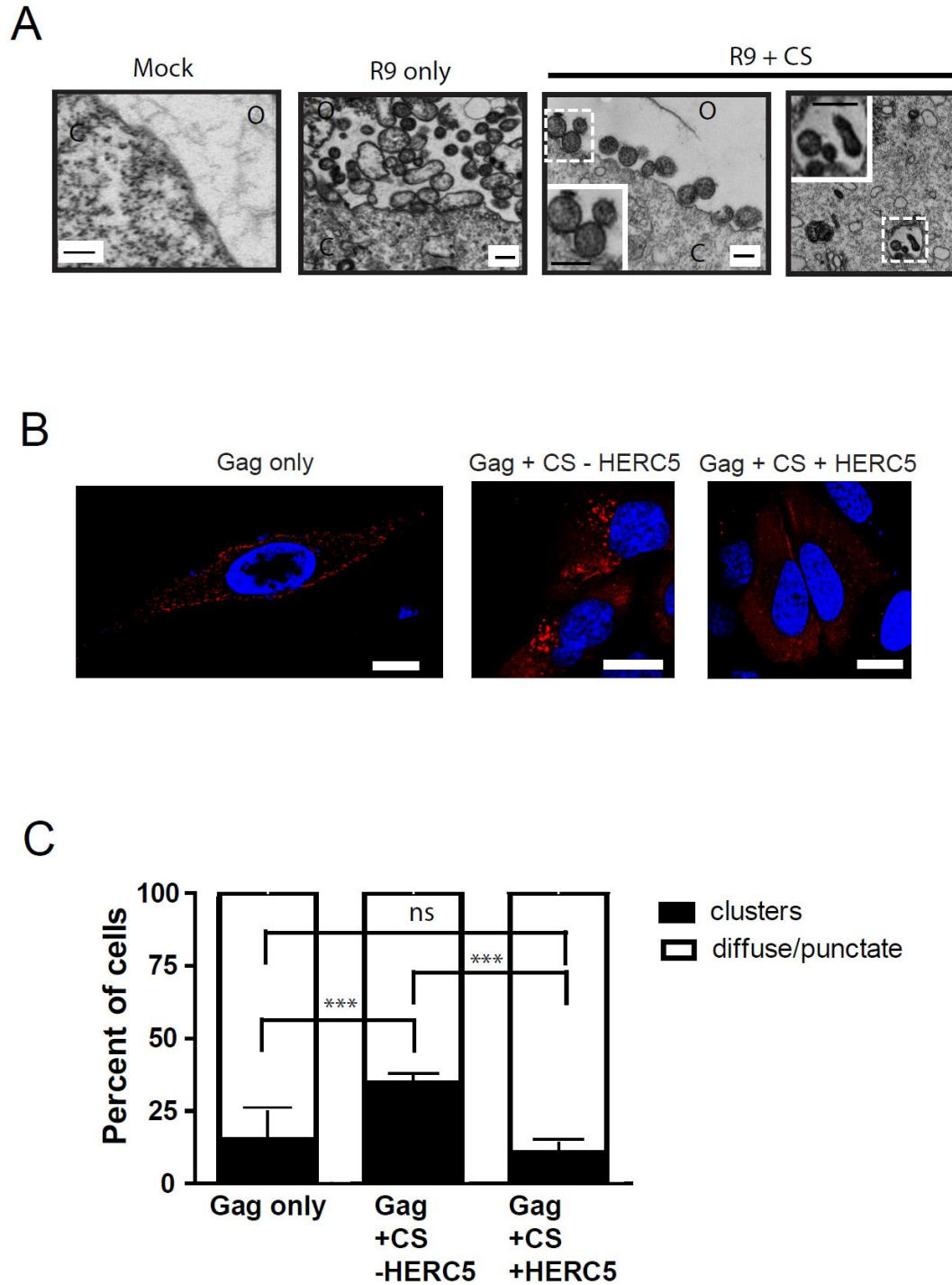


Figure 2.5: ISG15 expressed in the absence of HERC5 causes retention of virions on cell surfaces and in intracellular compartments.

We also assessed the intracellular localization of Gag in these cells by confocal immunofluorescence microscopy analysis. As shown in **Figure 2.5B**, control cells transfected with pGag + empty vector exhibited numerous punctate signals predominantly at the plasma membrane and regions of cell-cell contact, consistent with the site of Gag particle budding. Similar to the control cells, cells transfected with pGag + CS + pHERC5 exhibited an accumulation of Gag predominantly at the plasma membrane and regions of cell-cell contact. Interestingly, in cells transfected with pGag + CS, Gag accumulated at the plasma membrane and in the cytoplasm as large clusters (**Figure 2.5B**).

Quantitative analysis of cells co-expressing Gag + CS -HERC5 revealed that $34.5\% \pm 3.5\%$ SD of the cells contained Gag localized in the cytoplasm as large clusters. This is significantly more than cells expressing Gag only or Gag + CS + HERC5 where $15.0\% \pm 9.8\%$ SD and $10.5\% \pm 3.5\%$ SD of the cells contained large clusters of intracellular Gag, respectively ($P < 0.0001$, Fisher's exact test) (**Figure 2.5C**). Taken together, these data suggest that the mechanism of inhibition of HIV-1 Gag particle production is different in cells expressing CS + HERC5 than in cells expressing CS only.

2.2.6 HERC5 restricts MLV Gag-containing particle production

To assess the specificity of HERC5 for retroviruses, we tested the ability of HERC5 to restrict Gag particle production of the simple retrovirus MLV. Using a similar Gag particle release assay as that used above for HIV-1 Gag, we found that HERC5 + CS markedly restricted the release of MLV Gag-containing particles into the supernatant (**Figure 2.6**). Similar to that with HIV-1, Ubp43 co-expression rescued HERC5-induced restriction of MLV Gag particle production. HERC5-C994A + CS did not restrict MLV Gag particle production suggesting that the E3 ligase activity is also required for MLV restriction as it is for HIV-1. In contrast with that of HIV-1, expression of HERC5 + CS caused a substantial accumulation of intracellular MLV Gag protein. This accumulation

Figure 2.6: HERC5 restricts MLV Gag particle production.

U2OS cells were co-transfected with pMLV-Gag and either empty plasmid, pHERC5 + CS, or pHERC5-C994A + CS with or without pUbp43. Gag particles released into the supernatant and intracellular Gag protein expression were analyzed by quantitative Western blotting using anti-MLV antisera or anti- β -actin 48 hours post-transfection. Numerical values on the blots display the densitometric quantification of the specified bands after normalization with β -actin levels.

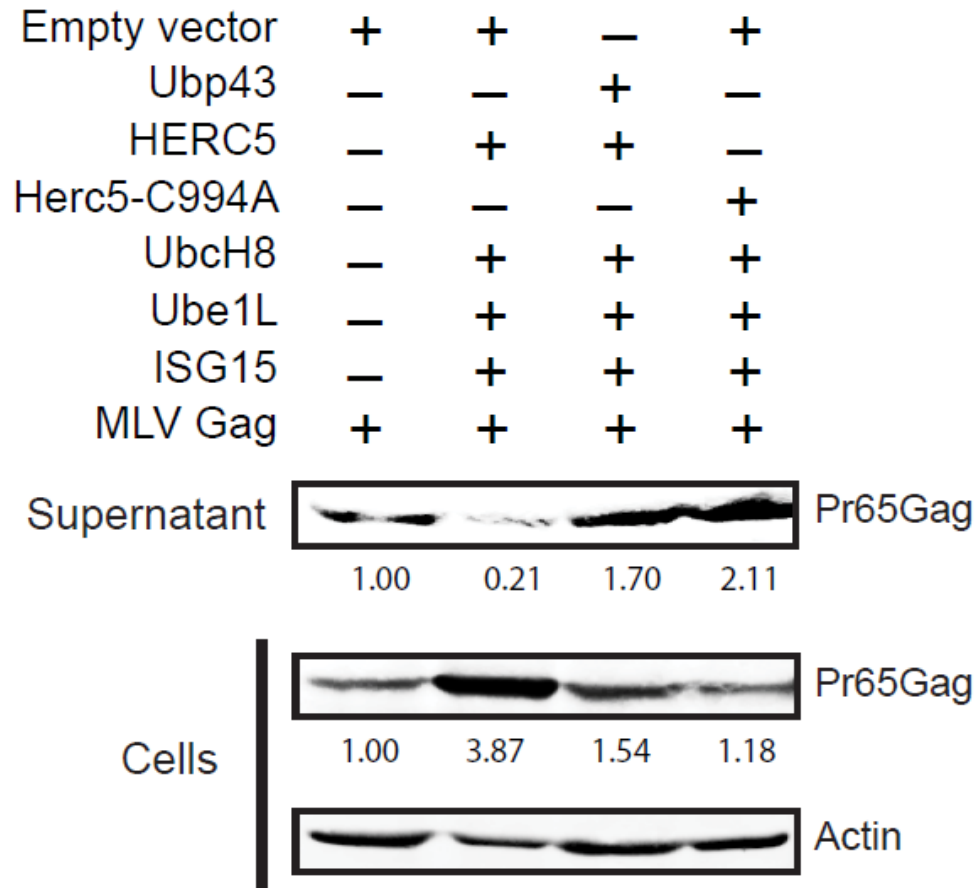


Figure 2.6: HERC5 restricts MLV Gag particle production.

was prevented by co-expression with Ubp43 (**Figure 2.6**). Taken together, these data suggest that HERC5 restricts Gag particle production from an evolutionarily diverse retrovirus and that the mechanism involves the E3 ligase activity of HERC5 for ISG15 conjugation.

2.2.7 HERC5 expression in HIV1-infected patients

To assess the expression of *herc5* in HIV-1-infected patients, we mined the Gene Expression Omnibus database repository (<http://www.ncbi.nlm.nih.gov/gds>) for published datasets containing gene expression profiles in HIV-1-infected individuals at various stages of disease progression. Transcriptional profiling data revealed that HERC5 and ISG15 RNA expression are significantly increased in lymphatic tissue during acute HIV-1 infection in patients with asymptomatic and acute stages of AIDS and patients with AIDS (**Figure 2.7A**) [2]. Significant increases in HERC5 and ISG15 RNA expression were observed in monocytes from viremic individuals after cessation of highly active antiretroviral therapy (HAART) compared to aviremic patients during HAART (**Figure 2.7B**) [3]. HERC5 and ISG15 expression are significantly increased in primary human PBMCs from seropositive patients compared to seronegative patients (**Figure 2.7C**) [4]. HERC5 and ISG15 expression is significantly increased in CD4+ T cells from acutely and chronically infected patients, but not in non-progressors (**Figure 2.7D**) [32]. HERC5, but not ISG15, is significantly increased in elite controllers and patients treated with HAART compared to HIV-1 negative patients (**Figure 2.7E**) [33]. Furthermore, HERC5 and ISG15 were identified as correlates of protection from HIV-1 infection in controllers compared to non-controllers (see reference [1]). In all datasets, there was no significant increase in the expression of beta actin, except in patients in the acute stages of AIDS, where a modest increase was observed (**Figure 2.7A**). Taken together, these data show that HERC5 expression is significantly increased in patients in the acute and chronic stages of infection and in patients in stages of long-term control of infection.

Figure 2.7: HERC5 and ISG15 expression in HIV-1-infected patients.

The Gene Expression Omnibus database repository was searched to identify published transcriptional profiling datasets of HIV-1- infected patient samples. The average relative RNA expression levels of HERC5, ISG15 and beta actin were compared for each dataset. **A**, GSE16363 dataset from Li et al. (2009) comparing patients with asymptomatic and acute stages of AIDS and patients with AIDS. **B**, GDS2168 dataset from Tilton et al. (2006) comparing monocytes from viremic individuals after cessation of HAART and aviremic patients during HAART. **C**, GDS1449 dataset from Ockenhouse et al. (2005) comparing primary human PBMCs from seropositive patients compared to seronegative patients. **D**, GDS2649 dataset from Hycza et al. (2007) comparing CD4+ T cells from non-progressors, and acutely and chronically infected patients. **E**, GSE23879 dataset from Vigneault et al. (2011) comparing elite controllers and patients treated with HAART. Significance was calculated using student's unpaired t test. *P* values <0.05 were considered significant.

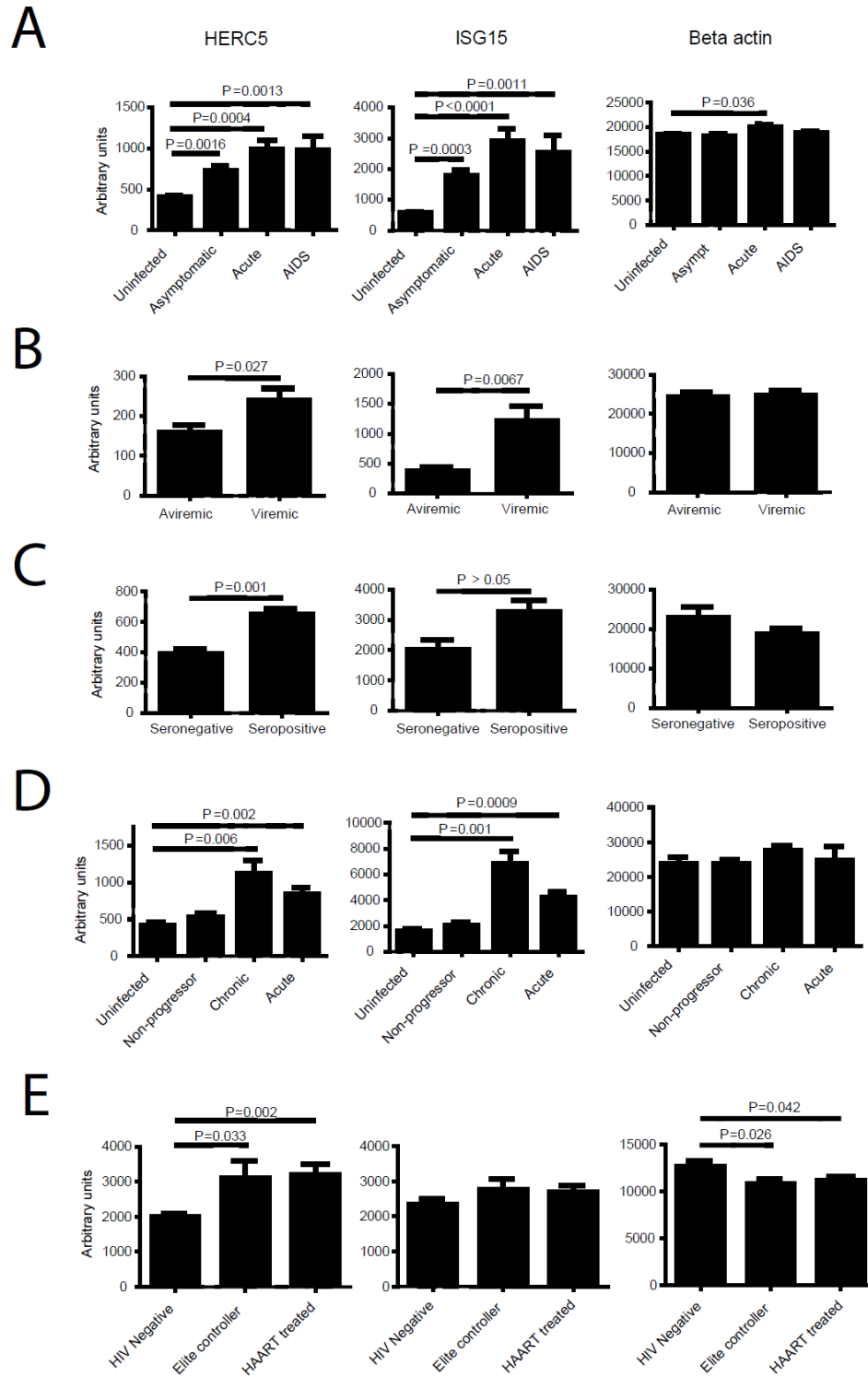


Figure 2.7: HERC5 and ISG15 expression in HIV-1-infected patients.

2.3 Discussion

Here we have identified HERC5 as an IFN-induced protein that is able to block an early step of HIV-1 particle assembly at the plasma membrane. HERC5 and Gag proteins were found to associate with each other *in vitro* and to co-localize within cells. These interactions correlated with the modification of Gag protein with ISG15. HERC5 did not alter the trafficking of Gag to the plasma membrane, however microscopic analysis revealed that Gag assembly was blocked at an early stage. HERC5 was also able to restrict the release of MLV Gag, showing that HERC5 can restrict an evolutionarily diverse retrovirus.

The mammalian Type I IFN response induces several host factors capable of restricting specific steps of HIV-1 replication at late stages of its life cycle in the absence of viral countermeasures; portraying a sophisticated and redundant defense mechanism (reviewed in [5]). Recently, TRIM22 has been shown to be a correlate of HIV-1 infection [34] and can block HIV-1 Gag particle production by altering intracellular trafficking of Gag to the plasma membrane and by inhibiting transcription from the HIV-1 long terminal repeat [7, 35]. Rhesus TRIM5 α has also been shown to degrade intracellular HIV-1 Gag polyproteins [36, 37]. ISG15 has been shown to interfere with late stages of HIV-1 Gag budding by disrupting interactions between Gag and Tsg101 [31] and between VPS4 and LIP5 [29, 30], interactions that are necessary for membrane scission and virus release. In the absence of HIV-1 Vpu, the host factor BST-2 (Tetherin) retains mature virions on the cell surface that are then subsequently endocytosed [38, 39]. The HERC5-induced post-translational modification of Gag is a potentially novel innate defense mechanism against HIV-1. It is unknown whether the modification of Gag is a prerequisite or consequence of restriction. Data presented by Durfee and colleagues (2010) show that HERC5 co-translationally modifies newly synthesized proteins with ISG15. Although we showed here that HERC5 interacts with Gag and co-localizes with Gag in cells, additional experiments are needed to determine if HERC5 post-translationally modifies newly made Gag polyproteins and if this modification directly interferes with Gag assembly at the plasma membrane. It is currently unclear how or why HERC5 accumulates more in a region near the plasma membrane in the presence of Gag and the conjugation system. It is

possible that some HERC5 associates with newly synthesized Gag on the way to the plasma membrane. However, it is also possible that HERC5 is recruited to regions of Gag assembly by other mechanisms. Further experiments are required to determine how and why the localization of some HERC5 protein changes in the presence of Gag.

The assembly and budding processes of HIV-1 Gag particles are relatively well understood (reviewed in [23, 40-45]), however much remains to be known about how the initial HIV-1 Gag bud forms and how the bud morphs into the “lollipop” structure. It has been suggested that energetic requirements for membrane distortion, which is a prerequisite for budding, occurs through the higher-order oligomerization of Gag molecules and is mediated through interactions between the interaction-domain of Gag. It is possible that HERC5 reduces Gag-Gag interactions below a threshold required to complete budding. HERC5-induced modification of a small fraction of Gag monomers may inhibit higher-order oligomerization and/or the recruitment of cellular factors due to steric hindrance of an attached ISG15 molecule. Alternatively, HERC5 could disrupt interactions with factors that manipulate membrane curvature and initiate or propagate formation of the bud. Such interactions have been observed between endophilin-2 and MLV Gag, and HIV-1 Gag and actin [46, 47]. Further experiments will be needed to address these and other possibilities.

Consistent with three previous studies [29-31], we were able to show that expression of ISG15 together with its E1 and E2 enzymes (in the absence of HERC5 expression) restricted Gag particle production without a reduction in intracellular Gag protein. Our data, together with data obtained by Okumura A., et al. (2006), Pincetic A., et al. (2010) and Kuang Z. et al. (2011), suggest that HERC5 restriction is distinct from the anti-HIV-1 activities of ISG15-only expression in that HERC5 induces the modification of Gag, whereas ISG15 alone does not. Moreover, HERC5 induces an arrest at an early step of assembly, whereas the expression of ISG15 alone causes an arrest at a late stage of budding/release where predominantly immature virions accumulate on the cell surface in doublet pairs or in chains. Interestingly, the phenotype resulting from the expression of ISG15 alone resembles that observed with HIV-1 p6 mutants where the authors reported that these virions accumulated on the surface in doublet pairs or in chains and were

largely immature [48]. The HIV-1 p6 protein regulates the final abscission step of nascent virions from the cell surface and accomplishes this primarily through the recruitment of Tsg101 and other members of the ESCRT complexes. The expression of ISG15 alone has been shown to interfere both with the recruitment of Tsg101 to p6 [31] and the interaction of Vps4 and LIP5, which are needed to promote the formation of the ESCRT-III-Vps4 double-hexamer complex required for membrane scission and virion release [29]. It is possible that in the absence of HERC5, another restrictive E3 ligase (yet to be identified) can be utilized, or that the E2-ISG15 complex can ISGylate substrate proteins in an E3 ligase-independent manner.

The ability of HERC5 to target viral proteins is not limited to HIV-1. We showed here that HERC5 blocked Gag particle production of an evolutionarily divergent retrovirus MLV. Interestingly, we found that HERC5 did not modify MLV Gag with ISG15 to appreciable levels compared to HIV-1 Gag and that HERC5 expression led to a substantial accumulation of intracellular MLV Gag protein. The reason for this difference is unclear, but could be due to differences in the rates of de-ISGylation and/or protein turnover. HERC5 E3 ligase activity has also been shown to block the antiviral function of the influenza A NS1 protein and interfere with the infectivity of human papillomavirus 16 pseudoviruses [20, 21]. Given that HERC5 is present in several vertebrates, it will be interesting to learn more about the spectrum of pathogens that can be targeted by HERC5 and the mechanisms underlying its restriction.

Our analysis of transcriptional profiling data of patient samples from various stages of infection and disease progression revealed that HERC5 expression is significantly increased in viremic patients (eg. acute, chronic, and AIDS). Given our data presented here that HERC5 inhibits HIV-1 particle production, one standing question is why HERC5 is not sufficient to suppress HIV-1 infection in patients who succumb to AIDS? One possibility is that these patients express non-functional HERC5 variants. Since we showed that the HERC5-C994A mutant failed to inhibit HIV-1 particle production, it is plausible that polymorphisms in the *herc5* gene that perturb the E3 ligase activity, or other polymorphisms affecting activity of the HERC5 protein, impact disease progression. For example, an insertion/deletion polymorphism (rs34457268) has been

identified in humans that leads to a translational frameshift and the production of a truncated HERC5 protein lacking the Cys994 active site residue.

A small percentage of HIV-1 infected patients remain asymptomatic for more than 10 years and maintain high CD4 cell counts without antiretroviral therapy. There are now several lines of evidence to suggest that these HIV-1 controllers are still infected with pathogenic virus, indicating that host factors of the innate and adaptive immune responses may play an important role in limiting HIV-1 replication and disease progression. Intriguingly, our analysis revealed that HERC5 expression is significantly increased in the elite controllers of the Vigneault et al. (2011) dataset, patients who have stable CD4⁺ T cell counts for >10 years without any clinical signs of disease progression. Notably, gene transcripts known to be involved in intrinsic cellular defense against retroviruses, such as members of the *TRIM* and *APOBEC* gene families, BST2/tetherin, and cyclophilin A, were not expressed differently between the elite controllers and the reference patients [33]. This begs the question of whether HERC5 is one factor, in addition to others, that can impact disease progression in these patients. Future work is needed to determine if HERC5 fits with the classical description of a cellular restriction factor, whether clinical isolates of HIV-1 possess HERC5 countermeasures, and whether *herc5* gene polymorphisms impact disease progression.

2.4 Methods

2.4.1 Ethics Statement

Informed consent was obtained from all subjects according to the ethics protocol #16682E, approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB).

2.4.2 Cells and Cell Lines

Cells were maintained in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM) for adherent cells and RPMI-1640 for suspension cells), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 µg/ml

Streptomycin) at 37 °C with 5% CO₂. Cell lines were obtained from American Type Culture Collection unless otherwise stated. The murine dendritic cell line DC2.4 was described previously [49]. HOS-CD4/CXCR4 was provided by Dr. F. Bushman (University of Pennsylvania, USA). The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (GHOST (3) R3/X4/R5; Cat. 3943) from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman [50]. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood from healthy volunteers using a Ficoll Hypaque (Sigma) gradient according to the manufacturer's instructions.

2.4.3 Plasmids, transfections and antibodies

Plasmids pUbe1L, pUbcH8, myc-tagged ISG15 (pMyc-ISG15), flag-tagged HERC5 (pHERC5) and flag-tagged HERC5-C994A (pHERC5-C994A) were kindly provided by Dr. K. Chin (Genome Institute of Singapore). The promoterless empty vector plasmid pGL3 was purchased from Promega, p3xFLAG from Sigma, and pUbp43 from Open Biosystems. pLKO.1/scrambled_{shRNA} was obtained from Addgene plasmid 1864; sequence 5' cctaa ggta agtcg cctc gctct agcga gggcg actta acctt agg 3') [51]. pLKO.1/HERC5_{shRNA} (cat. #RHS4533-NM_016323, [TRCN000004169](#)) was obtained from Open Biosystems. The plasmid encoding histidine-tagged ISG15 (pHis-ISG15) was created from the pMyc-ISG15 template by amplifying ISG15 using PCR and the primers: forward- 5' ACG TAA GCT TAC CAT GCA TCA TCA CCA TCA CCA TGG TGA TCA AAT GGG CTG GGA CCT GAC GG 3' and reverse 5' ACG TCT CGA GTC TAG ATT AGC TCC GCC CGC CAG G 3'. The forward primer encoded a 6x His tag (underlined). The amplified product was cloned into pcDNA3.1(+) (Invitrogen) using HindIII and XbaI. The plasmid encoding codon-optimized Gag (pGag) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Yingying Li, Feng Gao and Beatrice H. Hahn (p96ZM651gag-opt) [52]. Plasmid encoding the replication-competent provirus HIV-1 R9 was obtained from Dr. F. Bushman (University of Pennsylvania, USA). Plasmid transfections were performed using standard calcium phosphate transfection for co-transfections of 3 or more plasmids or Lipofectamine 2000 (Invitrogen). Plasmids were co-transfected at a

ratio of 10:7.5:7.5:7.5:1 for pHERC5 or pHERC5-C994A, pUbcH8, pUbe1L, pMyc-
ISG15 and pGag or pR9 respectively, unless otherwise noted. The following reagents
were obtained through the NIH AIDS Research and Reference Reagent Program,
Division of AIDS, NIAID, NIH: HIV-1 p24 Monoclonal Antibody (183-H12-5C) from
Dr. Bruce Chesebro and Kathy Wehrly [53-55]); HIV-1SF2 p24 antiserum. MLV
antiserum was a generous gift from Dr. S. Ross (University of Pennsylvania, USA).
Antibodies: anti-HERC5 was obtained from Abnova, anti-FLAG from Sigma, anti-ISG15
and anti- β -actin from Rockland, anti-HA from Roche, anti-myc from Santa Cruz, and
anti-ribosomal protein S6 from Cell Signaling Technology.

2.4.4 Quantification of infectious virus

Clarified supernatants containing virus particles were pelleted over a 20% sucrose
cushion for 2 hours at 21,000 x g. Pellets were lysed for quantitative Western blot
analysis or resuspended in fresh medium and used to infect GHOST(3) indicator cells.
Quantification of infectious virus release using GHOST(3) indicator cells has been
described previously [50].

2.4.5 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). The cDNA was amplified by PCR using the following primers:
HERC5 forward- 5' CTG GCA CTG TTT AAG AAA C 3'; HERC5 reverse- 5' TCA
GCC AAA TCC TCT G 3'; HERC3 forward- 5' ATG TTA TGT TGG GGA TAT TGG
3'; HERC3 reverse- 5' TCA GGC CAA ACT AAA CCC TTC ATA G 3'; β -actin
forward- 5' GGT CAT CAC CAT TGG CAA TGA GCG G 3'; β -actin reverse- 5' GGA
CTC GTC ATA CTC CTG CTT GCT G 3'. Amplified DNA were separated on a 1%
agarose and quantified densitometrically using ImageJ 1.43u 64-bit version software
(NIH, USA).

2.4.6 Immunoprecipitation

For immunoprecipitation, cells were lysed with cold non-denaturing lysis buffer (1% (w/v) SDS, 50 mM Tris-Cl pH 7.4, 5 mM EDTA pH 8.0, 300 mM NaCl, 0.02% (w/v) sodium azide with Roche protease inhibitor) for 20 minutes. Cyanogen bromide-activated Sepharose beads (GE Health Care) were swollen in 1 mM HCl for 10 minutes followed by antibody coupling using either 5 μ l of rabbit anti-p24CA or 1 μ l of mouse anti-FLAG (Sigma) per 75 μ l of beads in coupling buffer (0.1 M NaHCO₃ pH 8.3 with 0.5 M NaCl) overnight at 4°C. The beads were then washed three times with coupling buffer to remove excess antibody. The beads were blocked with blocking buffer (0.1 M Tris-HCl buffer pH 8.0) for 2 hours at 4°C. The beads were then washed with 3 cycles of alternating pH (0.1 M sodium acetate pH 4 with 0.5 M NaCl; 0.1 M Tris-HCl pH 8.0 with 0.5 M NaCl). The cell lysates were added to the beads for 1 hour at 4°C. The beads were washed 8 times with non-denaturing lysis buffer and the protein was eluted with 0.5 mM NaCl.

2.4.7 Western blotting

Clarified supernatants containing virus or Gag-only particles were pelleted over a 20% sucrose cushion for 2 hours at 21,000 x g. For cell lysates, cells were detached, centrifuged at 350 x g for 5 mins, washed twice with phosphate-buffered saline (PBS). Pellets were lysed with 1x RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1x Complete Protease Inhibitor (Roche), 1% Triton X-100, 0.1% SDS). For quantitative Western blotting, samples were mixed with 4x loading buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, and 5% beta-mercaptoethanol) to a final 1x concentration and separated on a 10% SDS-PAGE gel. Protein was transferred to FluorTransW (Pall) membrane 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 an ~16 hour incubation with 1:1000 dilution of primary antibody. Detection was carried out using IRdye-labeled secondary antibody (1:20,000 for 30 mins) and the Li-cor Odyssey Detection System (Li-cor Biosciences). Densitometric analysis was performed using ImageJ 1.43u 64-bit version software (NIH, USA).

2.4.8 Protein identification by mass spectrometry

Gel slices were excised and protein subjected to trypsin digestion followed by peptide identification by MALDI-MS using the Applied Biosystems® 4700 Proteomics Discovery System. A search of the NCBI nr viral database using Mascot identified HIV-1 Gag protein with >5 matching peptides and scores >50.

2.4.9 Purification of histidine-ISG15 tagged proteins

Cells were cultured in 10 cm dishes and co-transfected with empty vector plasmid or pHERC5, pUbcH8, pUbe1L, pISG15 and pGag (10:7.5:7.5:7.5:2 ratio respectively) with or without pUbp43. Nickel pull down of histidine-tagged ISG15ylated proteins was completed as previously described [20]. Briefly, cells were lysed with His-lysis buffer (50 mM Tris-Cl [pH 7.4], 300 mM NaCl, 1% Triton-X 100, 10 mM imidazole, 10 mM 2- β -mercaptoethanol) with 5 mM *N*-ethylmaleimide (Sigma), 1 mM PMSF (Sigma), and a protease inhibitor mixture (Roche). Ni-NTA agarose (Qiagen) was added to clarified cell extracts and incubated overnight at 4°C. The Ni-agarose was washed 3 times in His-lysis buffer and the protein was eluted with 0.5 M imidazole.

2.4.10 Confocal Immunofluorescence Microscopy

Cells were cultured in 12-well plates on 18 mm coverslips and co-transfected with pHERC5 (or pHERC5-C994A or empty vector), pUbcH8, pUbe1L, pISG15 and pGag (10:5:5.5:1 ratio respectively). Twenty-four hours post-transfection, the coverslips containing the cells were washed twice with PF buffer (1x PBS + 1% FBS), fixed for 10 minutes in 1x PBS containing 5% formaldehyde and 2% sucrose, permeabilized in 1x PBS containing 5% NP-40 and then washed twice more with PF buffer. The coverslips were incubated with primary antibodies for one hour, washed 6x with PF buffer, incubated with secondary antibodies (Alexa Fluor 546 anti-mouse or AlexaFluor 488 anti-rabbit, Invitrogen) for one hour and then washed 6x with PF buffer. Coverslips were mounted onto glass slides with ~10 μ l of Vectashield mounting media with DAPI (Vector Laboratories) and then sealed with nail polish. Slides were examined using a Zeiss LSM 510 confocal fluorescence microscope and images were obtained with sequential

imaging. Spatially-calibrated images were analyzed using the “Co-localization Threshold” plugin of the ImageJ 1.43u 64-bit version software (NIH, USA) for co-localization using the method of Costes et al. (2004) for spatial intensity correlation analysis, automatic thresholding and statistical significance testing [56]. The Pearson’s correlation coefficient of co-localized volumes measures the correlation between the intensities of the two labels in the co-localized voxels and is used to express the extent of co-localization where a value of 1.0 represents perfect correlation.

2.4.11 Transmission Electron Microscopy

Cells were transfected with pHERC5 or pHERC5-C994A, pUbe1L, pUbcH8, pMyc-*ISG15* and pR9 (10:7.5:7.5:7.5:1 ratio) using calcium phosphate. Forty-eight hours post-transfection cells were fixed in 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer. Cells were washed 3 times in 0.1 M sodium cacodylate buffer. The cells were fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour and then washed 3 times in 0.1 M cacodylate buffer and enrobed in 5% noble agar. Agar was washed 5 times with distilled water then stained with 2% uranyl acetate for two hours. Agar was then dehydrated with 50% ethanol for 15 minutes then dehydrated in 70% ethanol overnight at 4°C. The next day the agar was dehydrated with 85% then 95% ethanol for 15 minutes each. The agar was dehydrated in propylene oxide for 15 minutes and added to a 1:1 ratio of epon resin/propylene oxide for 3 hours followed by incubation in a 3:1 ratio of epon resin/propylene oxide overnight at 4°C. The following day, the agar was incubated in pure epon resin for 6 hours. Lastly, the samples were kept at 60°C for 2 days. Slices were cut and visualized using a Philips EM 410 equipped with high resolution 35mm film output. Developed photos were digitized using an Epson Expression 1680 scanner.

For immunogold staining, U2OS cells were fixed in 3% paraformaldehyde, 0.025% glutaraldehyde, in 0.1 M cacodylate (CAC) buffer adjusted to pH 7.4 for two hours. Cells were then washed with 0.1 CAC buffer. Cells were later enrobed in 5% noble agar. Solidified agar and cell mixture was dehydrated in 50, 70, 85, 95, 2x 100% ethanol respectively. Samples were rotated in a 1:1 LR white resin:100% ethanol then in pure LR white. The sample was placed in a gelatine capsule and placed in an oven at 50 °C for 24

hours. Samples were cut and placed on nickel grids. Grids were blocked with a 0.2 μ M filtered 1% BSA PBS Buffer (10.4 mM Na₂HPO₄, 3.2mM KH₂PO₄, 20mM NaN₃, 150mM NaCl, 1% BSA, pH 7.4) and incubated at room temperature for 2 hours with 0.25 mg/ml anti-p24CA (1:20 dilution). Samples were washed and incubated at room temperature for 2 hours with secondary goat anti-mouse IgG colloidal 10nm gold-conjugated antibody (Invitrogen cat# A31561) 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.

2.4.12 Statistical Analyses

GraphPad Prism v5.03 was used for all statistical analyses stated in the text. *P* values and statistical tests were stated in the text where appropriate. *P* values less than 0.05 were deemed to be significant. *Immunogold-labeling quantification*: Quantification of immunogold localization on the electron microscopic thin sections was performed as described previously [57]. Gold particles were counted for each field of view and scored as falling on one of three regions of interest: 1) HIV-1 Gag particles and plasma membrane; 2) cytoplasm and nucleus; and 3) not containing particles or cells. The plasma membrane was defined as the outer most edge of the cell to a distance 100 nm inside the cell. 100 nm was chosen since this is approximately the diameter of a mature HIV-1 virion. The resulting numerical frequency distribution (G_0) represents the ‘observed’ distribution [58]. The ‘expected’ or ‘predicted’ distribution of gold particles was determined using a randomly positioned lattice of test points (*P*) superimposed on each field of view. This was done by viewing the ‘grid’ feature in Adobe Photoshop CS3 for each image. The resulting distribution of test (or ‘grid’) points represents that which would be expected if gold particles were scattered randomly across the cell [58]. The number of points that fell on each of the three regions of interest were scored. The expected number of gold particles (G_e) for each region was calculated using the formula: $G_e = P \cdot (\text{total } G_0) / (\text{total } P)$. The corresponding partial X^2 for each region was calculated from the observed and expected gold counts as: $X^2 = (G_o - G_e)^2 / G_e$. If the total X^2 value for the given degrees of freedom (*df*) (given by 2-1 columns x 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 G_o/G_e was >1 and, secondly, the corresponding partial X^2 value accounted for a substantial proportion ($\geq 10\%$) of the total X^2 value [58].

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Chapter 3

3 Interferon-induced HERC5 is evolving under positive selection and inhibits HIV-1 particle production by a novel mechanism targeting Rev/RRE-dependent RNA nuclear export.

Type I interferon (IFN) inhibits virus replication by activating multiple antiviral mechanisms and pathways. It has long been recognized that type I IFNs can potently block HIV-1 replication *in vitro*; as such, HIV-1 has been used as a system to identify and characterize IFN-induced antiviral proteins responsible for this block. IFN-induced HERC5 contains an amino-terminal Regulator of Chromosome Condensation 1 (RCC1)-like domain and a carboxyl-terminal HECT domain. HERC5 is the main cellular E3 ligase that conjugates the IFN-induced protein ISG15 to proteins. This E3 ligase activity was previously shown to inhibit the replication of evolutionarily diverse viruses, including HIV-1. The contribution of the RLD to the antiviral activity of HERC5 was previously unknown.

In this study, we showed that HERC5 inhibits HIV-1 particle production by a second distinct mechanism that targets the nuclear export of Rev/RRE-dependent RNA. Unexpectedly, the E3 ligase activity of HERC5 was not required for this inhibition. Instead, this activity required the amino-terminal RCC1-like domain of HERC5. Inhibition correlated with a reduction in intracellular RanGTP protein levels and/or the ability of RanGTP to interact with RanBP1. Inhibition also correlated with altered subcellular localization of HIV-1 Rev. In addition, we demonstrated that positive evolutionary selection is operating on HERC5. We identified a region in the RCC1-like domain that exhibits an exceptionally high probability of having evolved under positive selection and showed that this region is required for HERC5-mediated inhibition of nuclear export.

We have identified a second distinct mechanism by which HERC5 inhibits HIV-1 replication and demonstrate that HERC5 is evolving under strong positive selection. Together, our findings contribute to a growing body of evidence suggesting that HERC5 is a novel host restriction factor.

3.1 Introduction

The cellular HERC5 protein was recently identified as an antiviral protein that inhibits replication of evolutionarily diverse viruses [1-3]. HERC5 belongs to a family of 6 HERC proteins containing an amino-terminal RCC1-like domain, a spacer region that does not share homology with any known protein, and a carboxyl-terminal HECT domain. Phylogenetic analysis of the *HERC* family revealed that the *HERC4* ancestor emerged in nematodes and that the *HERC* family expanded to six members during animal evolution, with *HERC5* being the most recently emerged family member [4]. HERC5 is ubiquitously expressed in many cell types and tissues including, but not limited to, effector and central memory T cells, dendritic cells, CD14⁺ monocytes, monocyte-derived macrophages, embryonic and induced pluripotent stem cells, hematopoietic and granulopoietic stem cells, testis (germ and leydig cells), ovary, liver and lung [5-17]. HERC5 expression is up-regulated in response to IFN [18, 19], *in vitro* and *in vivo* virus infection [1, 20-25], lipopolysaccharide, tumor necrosis factor α , and interleukin-1 β [26].

HERC5 is the main cellular E3 ligase that conjugates the ubiquitin-like protein ISG15 to proteins in human cells via a hierarchical enzymatic cascade involving E1 activating enzyme Ube1L and E2 conjugating enzyme UbcH8 [18, 19]. The conjugation of ISG15 to proteins is commonly referred to as ISGylation. We previously showed that HERC5 inhibits HIV-1 Gag particle assembly by a mechanism correlating with the post-translational modification of Pr55Gag with ISG15 [1]. HERC5 inhibits influenza A virus replication by catalyzing the conjugation of ISG15 to the viral NS1 protein, thereby preventing NS1 from forming homodimers and inhibiting corresponding antiviral processes [2]. Furthermore, HERC5 conjugates ISG15 to the human papillomavirus (HPV) L1 capsid protein, conferring a dominant-inhibitory effect on the infectivity of HPV16 pseudoviruses [3].

Although much is known about the HECT domain of HERC5 and its critical role in E3 ligase activity, little is known about the contribution of the RLD region to the antiviral activity of HERC5. Proteins such as HERC5 that contain RCC1-like domains belong to a phylogenetically widespread RCC1 superfamily of proteins [27, 28]. The prototypical member of this superfamily is human RCC1. RCC1 is characterized by the presence of 7 repeats of ~60 amino acids in length that assume a 7-bladed β -propeller structure. RCC1 is localized in the nuclei of eukaryotic cells where it binds and activates the GTPase Ras-related nuclear (Ran) protein [29, 30]. Subsequent hydrolysis of GTP to guanosine diphosphate (GDP) by its intrinsic GTPase activity returns Ran to an inactive state. RCC1 maintains a higher level of RanGTP in the nucleus compared to the cytoplasm (>1000-fold), which is critical for Crm1-dependent nuclear export of macromolecules [31].

Here we present the molecular characterization of a second distinct and novel antiviral function of HERC5. This function targets HIV-1 Rev/RRE function and involves the RCC1-like domain of HERC5. We also demonstrate that HERC5 is evolving under strong positive evolutionary selection. Together, these observations provide new insight into the innate immune response towards HIV-1.

3.2 Results

3.2.1 HERC5 inhibits HIV-1 particle production by an E3 ligase-independent mechanism

The E3 ligase activity of HERC5 was previously shown to contribute to the inhibition of Pr55Gag particle production [1]. To examine the contribution of the RLD to inhibition of infectious HIV-1 particle production, we generated flag-tagged HERC5 constructs lacking the RLD (HERC5- Δ RLD) or lacking E3 ligase activity (HERC5-C994A) (**Figure 3.1A**). Cysteine 994 is essential for HERC5-induced ISGylation [18]. Quantitative Western blot analysis of cell lysates showed that all HERC5 constructs exhibited similar levels of intracellular protein expression (**Figure 3.1B**). The HERC5 constructs were also analyzed by confocal immunofluorescence microscopy and found to be localized in the

cytoplasm of U2OS cells, similar to wild type HERC5 (**Figure 3.1C**). Similar localization was observed in 293T and HeLa cells (data not shown).

We then tested the ability of these HERC5 constructs to inhibit HIV-1 replication. 293T cells were co-transfected with plasmids encoding full-length, replication-competent HIV-1 (pR9) and either empty vector, wild type HERC5, HERC5- Δ RLD or HERC5-C994A. Forty-eight hours after transfection, infectious virus released into the supernatant was measured using an infectious HIV-1 release assay (**Figure 3.1D**). The expression of HERC5 and HERC5-C994A significantly reduced the amount of infectious virus released into the supernatant compared to the control cells ($P < 0.0001$, student's paired t test). In contrast, HERC5- Δ RLD failed to inhibit infectious virus release compared to the control cells ($P > 0.05$, student's paired t test). Quantitative Western blot analysis of viral particles released into the supernatant and of producer cell lysates revealed that inhibition of HIV-1 particle production by HERC5 and HERC5-C994A resulted in a substantial reduction in total intracellular Gag protein and in HIV-1 particles released into the supernatant (**Figure 3.1E and 3.1F**). Cells expressing HERC5- Δ RLD produced similar levels of intracellular Gag protein as the empty vector control cells; however, these cells did not exhibit a full rescue of released HIV-1 particles into the supernatant.

Notably, HERC5-C994A inhibited infectious HIV-1 particle production significantly better than wild type HERC5 and yielded substantially less intracellular Gag protein compared to wild type HERC5 and the control cells ($P < 0.01$, student's unpaired t test) (**Figure 3.1E and 3.1F**). This enhanced activity correlated with the loss of slowly migrating species of HERC5 protein (**Figure 3.1G**). We used a standard ISGylation assay to determine if these slowly migrating forms of HERC5 represented ISGylated HERC5 protein [1]. To enhance detection of ISGylated species, wildtype HERC5 was expressed in the presence of the ISG15 conjugation system consisting of E1 activating enzyme Ube1L, E2 conjugating enzyme Ubch8 and ISG15. As shown in **Figure 3.1H**, slowly migrating forms of HERC5 were observed. When wildtype HERC5 was expressed in the presence of the ISG15-specific deconjugating enzyme Ubp43 [32], substantially less

Figure 3.1: HERC5 inhibits HIV-1 particle production via an E3 ligase-independent mechanism.

A, Schematic representation of the HERC5 protein domains and the various mutant constructs generated (not to scale). **B**, Western blot analysis of 293T cell lysates transfected with either empty plasmid or a plasmid encoding HERC5, HERC5-C994A or HERC5- Δ RLD. Anti-flag was used to detect the flag-tagged HERC5 constructs and anti- β -actin was used as a loading control. Numbers above the bands represent densitometric quantification relative to wildtype HERC5 after normalization to β -actin levels. **C**, U2OS cells were transfected with plasmid encoding flag-tagged HERC5, HERC5-C994A, or HERC5- Δ RLD. Forty-eight hours post-transfection, the localization of the HERC5 construct was analyzed by confocal immunofluorescence microscopy using anti-flag. Images shown are representative confocal optical slices taken through the center of cells from z-stacks. Scale bars= 10 μ m. **D**, 293T cells were co-transfected with pR9 and either empty plasmid, pHERC5, pHERC5-C994A, or pHERC5- Δ RLD. Forty-eight hours after transfection, infectious HIV-1 virions released into the supernatant were quantified using GHOST(3) indicator cells. The averages \pm SD from at least three independent experiments are shown. **E**, Western blot analysis of 293T cell lysates co-transfected with pR9 and either empty plasmid or a plasmid encoding HERC5, HERC5-C994A or HERC5- Δ RLD. Forty-eight hours after transfection, HIV-1 virions released into the supernatant and Gag levels within the cell lysates were analyzed by quantitative Western blotting using anti-p24CA or anti- β -actin as a loading control. **F**, Densitometric analysis of the indicated bands (virion p24CA, cell Pr55Gag or cell p24CA) relative to the empty vector control from at least three independently generated Western blot experiments similar to that shown in panel E. Values were normalized to β -actin levels. **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$; n.s. $P > 0.05$ (student's paired t test). **G**, Western blot analysis of 293T cell lysates co-transfected with pR9 and either empty plasmid, flag-tagged pHERC5 or flag-tagged pHERC5-C994A. Forty-eight hours after transfection, cell lysates were analyzed by quantitative Western blotting using anti-p24CA, anti-flag or anti- β -actin as a loading control. **H**, Cells were co-transfected with pUbe1L, pUbcH8 and myc-tagged ISG15 and either empty vector, pUbp43, flag-tagged pHERC5 or flag-tagged pHERC5-C994A. Cell lysates were subjected to Western blotting using anti-flag or anti-

β -actin as a loading control. Western blot data shown is representative of at least three independent experiments.

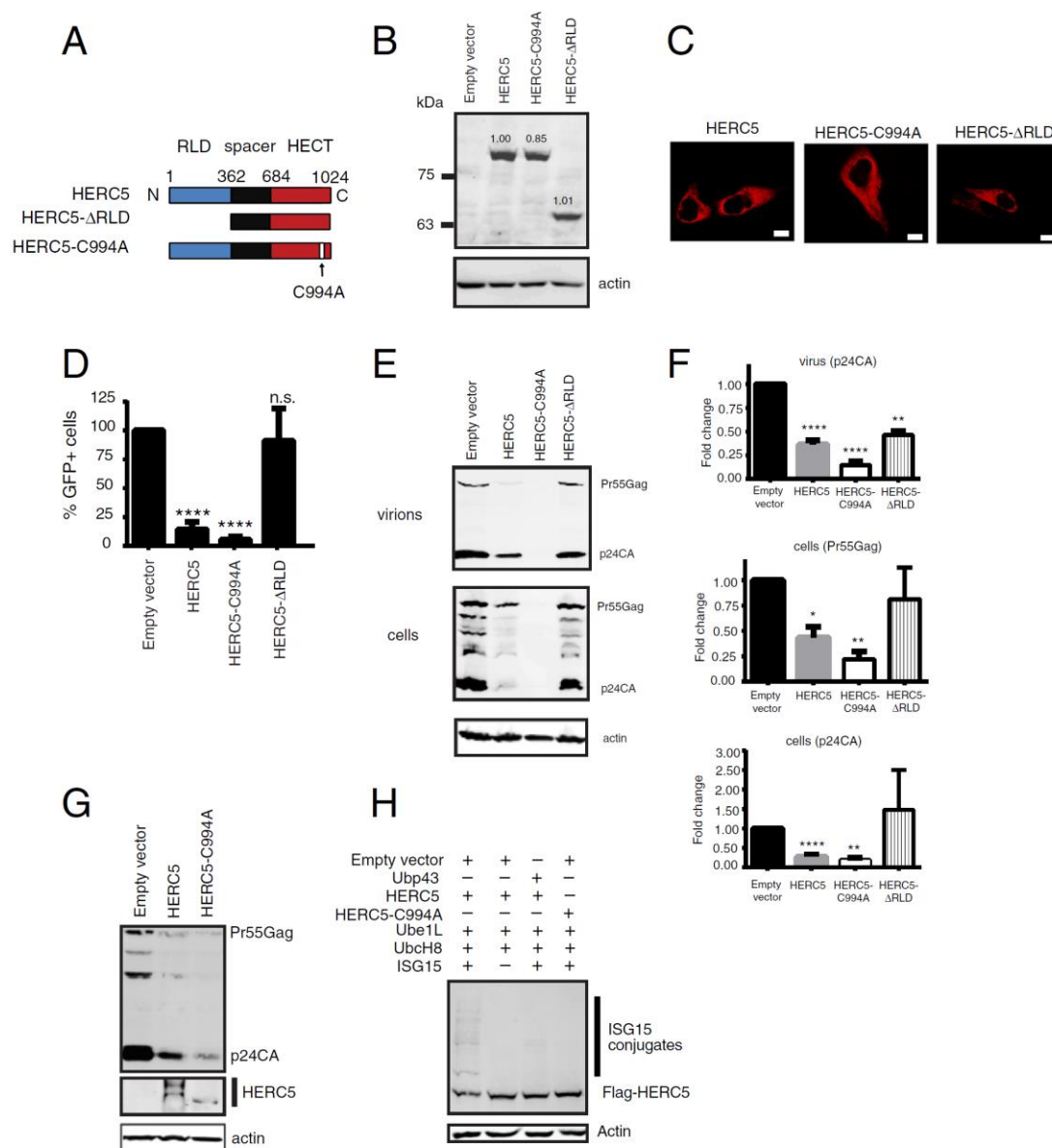


Figure 3.1: HERC5 inhibits HIV-1 particle production via an E3 ligase-independent mechanism.

slowly migrating forms of HERC5 were observed. Cells expressing HERC5-C994A, which is defective for ISGylation, also did not yield slowly migrating forms of ISGylated HERC5-C994A protein. These data indicate that the slowly migrating forms of HERC5 are modified with ISG15.

3.2.2 Endogenously expressed HERC5 inhibits intracellular Gag protein production

To assess whether endogenously-expressed HERC5 inhibits intracellular Gag protein expression from infectious HIV-1, we knocked down endogenous HERC5 RNA using short hairpin RNA (shRNA) and examined the influence of reduced HERC5 expression on intracellular Gag protein production, compared with cells expressing scrambled shRNA. Western blot analysis of cell lysates from transfected 293T cells, before or after treatment with IFN- β , revealed that HERC5 shRNA-expressing cells exhibited substantially more intracellular Gag protein than cells expressing scrambled shRNA (**Figure 3.2A and 3.2B**). A similar effect was observed in primary human macrophages from two different donors (**Figure 3.2C**). Cells expressing HERC5 shRNA exhibited an average of 3.2-fold less HERC5 RNA than control cells expressing scrambled shRNA, as determined by quantitative reverse transcription polymerase chain reaction (qPCR). As a control for specificity, we similarly measured the effect of the scrambled and HERC5 shRNA on HERC3 RNA levels and found them to be equivalent. Together, these data suggest that endogenously expressed HERC5 provides a significant barrier to intracellular Gag protein production with and without IFN pre-treatment.

3.2.3 HERC5 inhibits nuclear export of HIV-1 genomic RNA

To investigate the mechanism by which HERC5 inhibits intracellular Gag protein production, we asked if HERC5 induced the degradation of intracellular Gag protein. 293T cells were co-transfected with plasmids encoding replication-competent HIV-1 and

Figure 3.2: Endogenous HERC5 inhibits intracellular Gag protein production.

A, 293T cells were co-transfected with pR9 and either pScram or pHERC5_{shRNA}. Forty-eight hours after transfection, cell lysates were subjected to Western blotting using anti-p24CA or anti- β -actin as a loading control. **B**, Cells were transfected as in part A for 24 hours and then treated with 500 units/ml of IFN- β . Sixteen hours after IFN- β treatment, cell lysates were subjected to Western blotting using anti-p24CA or anti- β -actin as a loading control. Data shown in panels A and B are representative of at least three independent experiments. **C**, Primary human macrophages from two different donors were transfected as in panel A. Seventy-two hours after transfection, cell lysates were subjected to Western blotting using anti-p24CA or anti- β -actin as a loading control.

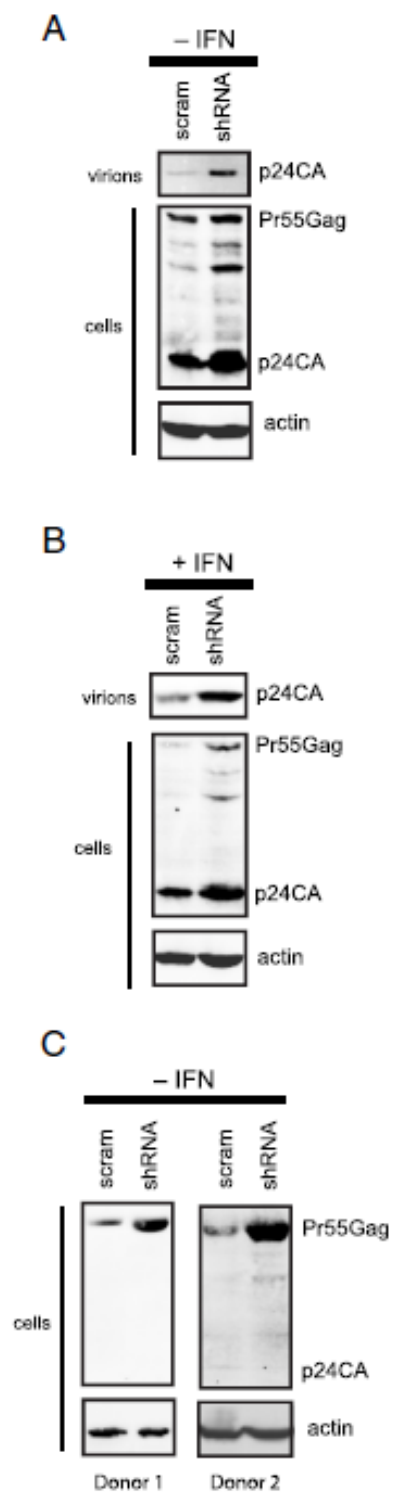


Figure 3.2: Endogenous HERC5 inhibits intracellular Gag protein production.

HERC5 and then treated with the proteasome inhibitor MG132 or the lysosome enzyme inhibitor amantidine. Western blot analysis of cell lysates revealed that MG132 or amantidine treatment did not rescue levels of intracellular Gag protein (**Figure 3.3A**). This finding suggests that HERC5 does not induce Gag protein degradation. MG132 and amantidine treatment also did not rescue the release of extracellular HIV-1 particles into the cell supernatant. MG132-treated control cells exhibited a reduction in the production of extracellular virus, as previously reported [33]. Similar results were obtained using U2OS cells (data not shown).

Since Gag protein is expressed from unspliced HIV-1 genomic RNA in the cytoplasm, we asked if HERC5 interfered with the nuclear export of unspliced HIV-1 RNA. 293T cells were co-transfected with plasmids encoding full-length replication-competent HIV-1 (pR9) and either empty vector or HERC5. A plasmid encoding green fluorescent protein (GFP) was also co-transfected to serve as a transfection control. Total RNA was harvested from total cell extract or cytoplasmic extract only and subjected to qPCR with primers specific to either unspliced HIV-1 genomic RNA (e.g. Gag), fully spliced RNA (e.g. Rev), β -actin (loading control) or GFP (transfection control). Cells expressing HERC5 exhibited a 2.7 to 4.2-fold reduction in the amount of HIV-1 genomic RNA exported to the cytoplasm compared to the control cells ($P = 0.0003$, student's paired t test). In contrast, no significant difference was observed in the export of fully-spliced HIV-1 Rev transcripts (**Figure 3.3B**).

To further investigate the effect of HERC5 on the localization of unspliced HIV-1 RNA, we utilized an established assay involving the bacteriophage MS2 coat protein to determine the localization of HIV-1 genomic RNA. HIV-1 NL4-3 genomic RNA was tagged with 24 copies of the MS2 binding RNA stem loops (NL4-3-SL). These stem loop structures bind with high affinity and specificity to a fusion protein consisting of the bacteriophage MS2 coat protein and GFP (MS2-GFP). The MS2 RNA stem loops were inserted such that unspliced full-length genomic RNA would be labelled with MS2-GFP, as previously described [34]. MS2-GFP contains a nuclear localization signal sequence that targets the fusion protein to the nucleus; however, MS2-GFP can shuttle to the cytoplasm when bound to cargo destined for the cytoplasm.

Figure 3.3: HERC5 inhibits nuclear export of HIV-1 genomic RNA.

A, 293T cells were co-transfected with pR9 and either empty vector or pHERC5. Forty hours post-transfection, cells were treated with the proteasomal inhibitor MG132 (20 μ M) or the lysosomal inhibitor amantidine (1.5 mM) for 16 hours. Virus released into the supernatant or total cell lysates were then subjected to quantitative Western blot analysis using anti-p24CA and anti- β actin as a loading control. **B**, Cells were transfected as in part A. Forty-eight hours after transfection, total RNA was extracted and reverse transcribed into cDNA from whole cell lysates or from the cytoplasmic fraction only. Quantitative PCR was performed on each fraction using primers specific to either unspliced HIV-1 genomic RNA (e.g. Gag), fully spliced RNA (e.g. Rev), β -actin (loading control) or GFP (transfection control). The proportion of unspliced or fully-spliced HIV-1 RNA in the cytoplasmic fraction compared to the total amount of HIV-1 RNA (nuclear plus cytoplasmic) was determined for the control cells and cells expressing HERC5. The fold-change in copy number relative to the control cells is shown. Data shown represents the average (\pm SEM) from six independent experiments. *** $P=0.0003$; n.s. $P>0.05$ (student's paired t test). **C and D**, HeLa cells were co-transfected with plasmids encoding MS2-GFP alone, MS2-GFP and NL4-3-SL, or MS2-GFP + NL4-3-SL and either flag-tagged HERC5, HERC5-C994A or HERC5- Δ RLD. Forty-eight hours post-transfection, cells were fixed, stained with anti-flag and DAPI and imaged using fluorescence confocal microscopy. MS2-GFP localization was assessed in each cell and categorized according to localization in the nucleus only or both the nucleus and cytoplasm (C). Results shown are from at least three independent experiments (n=331). Representative images of the predominant phenotypes are shown (D). Blue, nucleus; green, MS2-GFP; red, flag-tagged HERC5. Scale bars = 10 μ m.

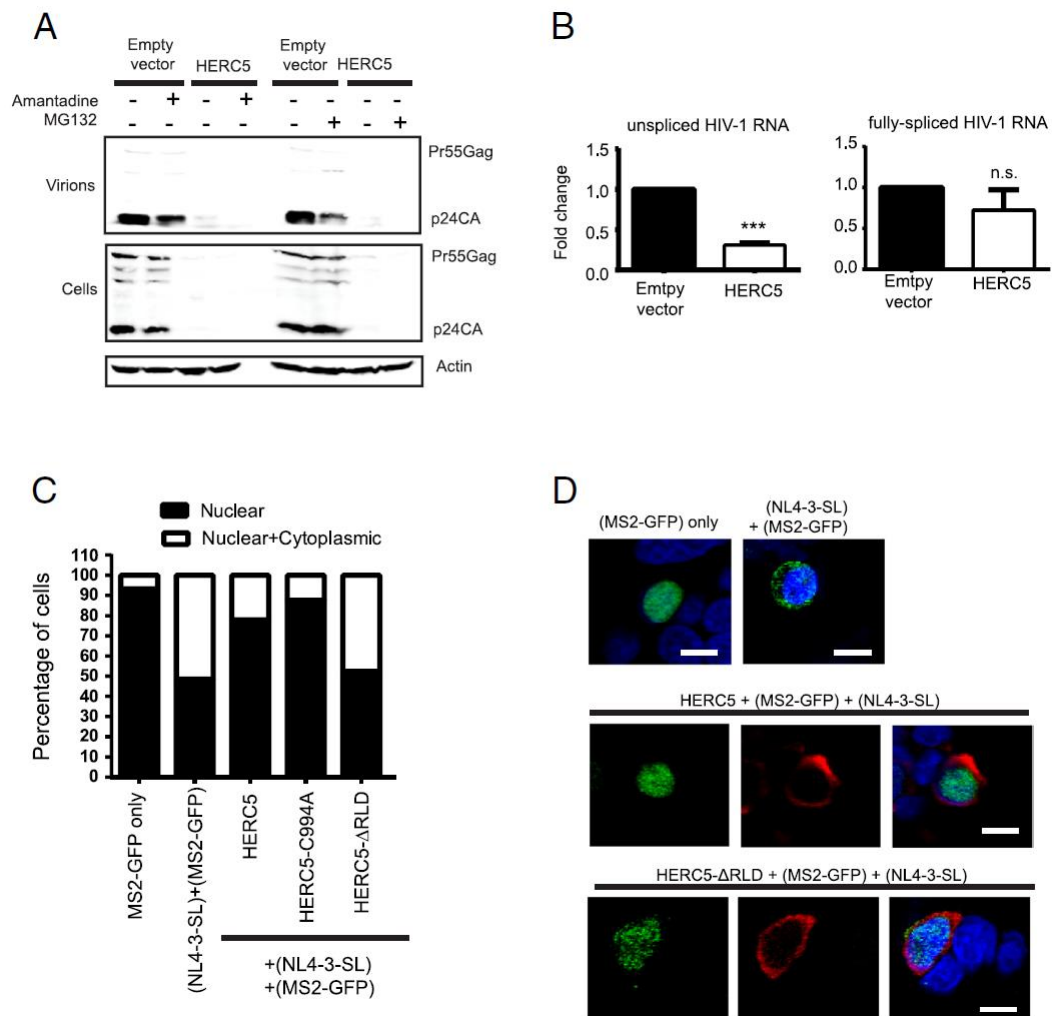


Figure 3.3: HERC5 inhibits nuclear export of HIV-1 genomic RNA.

MS2-GFP expressed alone localized exclusively in the nucleus as expected (**Figures 3.3C, 3.3D and Appendix 1**). When the MS2-GFP signal intensity was increased, no MS2-GFP signal was observed in the cytoplasm (**Appendix 1**). When MS2-GFP was co-expressed with NL4-3-SL, MS2-GFP localized in both the nucleus and cytoplasm as expected. However, in the presence of HERC5 or HERC5-C994A, MS2-GFP localized predominantly in the nucleus (**Figure 3.3C and 3.3D**). Similar to the MS2-GFP only control, no MS2-GFP signal was observed in the cytoplasm after the signal intensity was increased (**Appendix 1**). In contrast, MS2-GFP localized in both the nucleus and cytoplasm in the presence of HERC5- Δ RLD, indicating that the HERC5 RCC1-like domain is required for inhibiting nuclear export of HIV-1 genomic RNA (**Figure 3.3C and 3.3D**). Taken together, these data demonstrate that HERC5 inhibits nuclear export of unspliced HIV-1 RNA.

3.2.4 HERC5 inhibits nuclear export of Rev/RRE-dependent HIV-1 RNA

In eukaryotic cells, intron-containing messages are normally retained in the nucleus, whereas completely spliced messages are allowed to exit into the cytoplasm. HIV-1 overcomes this checkpoint in cells through expression of the HIV-1 regulatory protein Rev. Rev promotes nuclear export of intron-containing HIV-1 mRNAs by binding to a specific *cis*-acting element called the rev-response element (RRE), located within the HIV-1 intron [35-39]. Rev binds to the CRM1/RanGTP complex and translocates through the nuclear pore complex to the cytoplasm via the CRM1-dependent nuclear export pathway. The constitutive transport element (CTE) from Mason-pfizer monkey virus (MPMV) is a structured RNA element that also functions in *cis*, but it does not require co-expression of a viral Rev-like protein for the nuclear export of intron-containing RNA. Instead, CTE-containing RNA recruits the NXF1/NXT1 proteins, which direct nuclear export of the RNA via the NXF1-dependent pathway and is independent of RanGTP [40-42].

To determine if HERC5-mediated inhibition of HIV-1 RNA nuclear export was Rev/RRE-dependent, we tested the ability of HERC5 to inhibit Gag expression from

Figure 3.4: HERC5 targets Rev/RRE function.

A, Schematic depicting the different Gag constructs used in the experiment. 293T cells were co-transfected with increasing concentrations of plasmids encoding HERC5 and either Rev-dependent GagPol (containing RRE) +/- Rev (**B**), Rev-independent GagPol (containing 4xCTE in place of the RRE) (**C**), or Rev-independent Gag-only (codon-optimized) construct (**D**). Total DNA transfected was kept equal with empty vector plasmid. Gag levels within the cell lysates were analyzed by quantitative Western blotting using anti-p24CA and anti- β -actin as a loading control. Numbers below the bands represent densitometric quantification of the indicated bands relative to the empty vector control after normalization to β -actin levels.

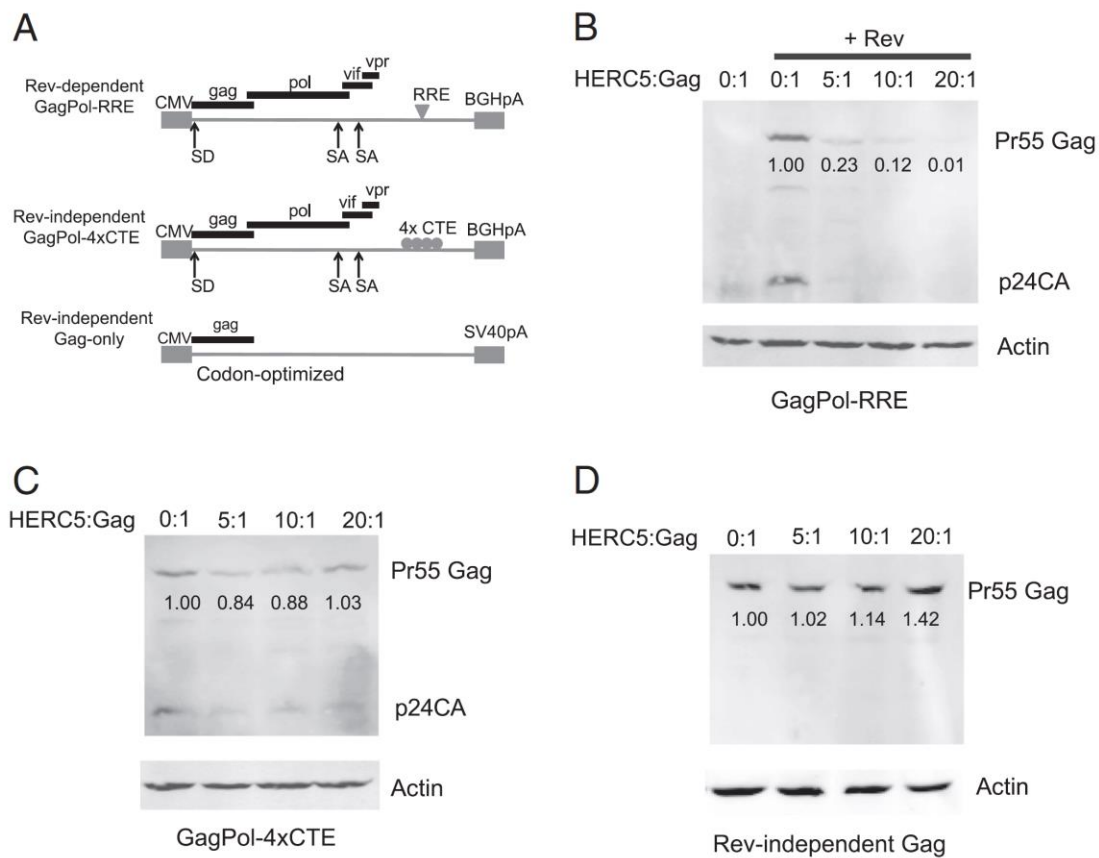


Figure 3.4: HERC5 targets Rev/RRE function.

Rev-dependent (e.g. GagPol-RRE) and Rev-independent (e.g. GagPol-4xCTE and codon-optimized Gag-only) constructs, as previously described (**Figure 3.4A**) [43]. 293T cells were co-transfected with increasing concentrations of plasmids encoding HERC5, with or without pGagPol-RRE, pGagPol-4xCTE or pGag. As shown in **Figure 3.4B**, HERC5 potently inhibited Gag expression from the GagPol-RRE construct. In contrast, a modest reduction in Gag expression was observed from the GagPol-4xCTE construct (**Figure 3.4C**). No reduction in Gag expression was observed from the RRE/CTE-independent Gag-only construct (**Figure 3.4D**).

3.2.5 HERC5 targets RanGTP and alters Rev localization.

RCC1 is localized in the nucleus and stimulates the conversion of RanGDP into RanGTP. A steep gradient of RanGTP between the nucleus and cytoplasm is essential for Crm1-dependent nuclear export, but not the NXF1-dependent export [31]. To determine if HERC5 interacts with Ran, cells expressing or not expressing flag-tagged HERC5 were lysed under non-denaturing conditions and subjected to co-immunoprecipitation using anti-Ran or anti-flag. Western blot analysis of the precipitated proteins revealed that Ran and HERC5 co-precipitated (**Figure 3.5A**). We then asked if HERC5 expression affected intracellular RanGTP levels. Due to the lack of a specific antibody that distinguishes RanGTP from RanGDP, we utilized a RanGTP pull-down assay involving Ran binding protein 1 (RanBP1)-coated agarose beads. RanBP1 binds specifically to RanGTP and not RanGDP. Control cells treated with non-hydrolyzable GTP γ S, transfected with empty vector, or transfected with pHERC5 were lysed and mixed with RanBP1-coated beads. RanGTP protein eluent was measured using quantitative Western blotting with anti-Ran. Control cells treated with GTP γ S and empty vector control cells readily pulled down RanGTP (**Figure 3.5B**). In stark contrast, substantially less RanGTP was pulled down in cells expressing HERC5, indicating that HERC5 reduced intracellular levels of RanGTP or interfered with the interaction between RanGTP and RanBP1.

Since nuclear-cytoplasmic shuttling of HIV-1 Rev is critically dependent on RanGTP, we asked if the effect of HERC5 on RanGTP correlated with aberrant Rev localization.

Figure 3.5: HERC5 interacts with Ran and reduces RanGTP levels and/or its binding with RanBP1.

A, 293T cells were co-transfected with either empty vector or a plasmid encoding flag-tagged HERC5. Forty-eight hours after transfection, cells were lysed under non-denaturing conditions and subjected to immunoprecipitation using anti-Ran or anti-flag. Precipitated proteins were separated by SDS-PAGE and subjected to Western blotting using anti-HERC5 or anti-Ran. Numbers below the lower blot represent the densitometric quantification of the non-specific band in the empty vector control and Ran in the HERC5-expressing cells. **B**, U2OS cells were transiently-transfected with empty vector or a plasmid encoding HERC5. Forty-eight hours after transfection, total cell lysate was mixed with RanBP1-bound agarose beads to selectively isolate and pull-down RanGTP. As a control, cell lysate was incubated with non-hydrolyzable GTP γ S prior to incubation with the agarose beads. The eluate, including input lysate (3%), were separated by SDS-PAGE gel and subjected to quantitative Western blotting using anti-Ran. Note, bands shown in the input samples represents total Ran protein and does not distinguish RanGTP or RanGDP. The blot shown is representative of three independent experiments. Numbers represent the densitometric quantification of RanGTP after normalization to Ran levels. **C**, U2OS cells were co-transfected with plasmids encoding HIV-1 Rev and either flag-tagged HERC5 or HERC5-C994A. Forty-eight hours post-transfection, cells were fixed, stained with anti-flag and/or anti-Rev and DAPI and imaged using fluorescence confocal microscopy. Rev localization was assessed in each cell and categorized as either: near the nuclear membrane, in both the nucleus and cytoplasm, in the cytoplasm only, or nucleolar. Results shown are from at least three independent experiments (n=500). **D**, Representative images of the predominant phenotypes. Blue, nucleus; green, flag-tagged HERC5; red, Rev. Scale bars = 10 μ m.

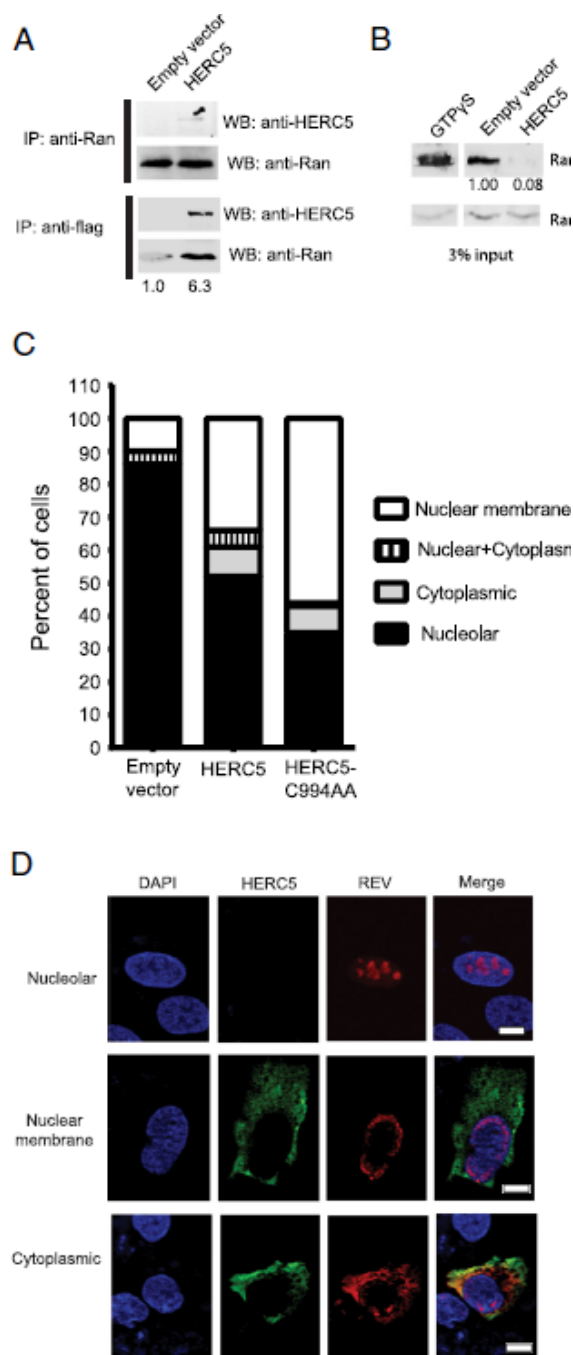


Figure 3.5: HERC5 interacts with Ran and reduces RanGTP levels and/or its binding with RanBP1.

U2OS cells, which do not express endogenous HERC5, were co-transfected with plasmids encoding flag-tagged HERC5 and HIV-1 Rev and imaged by confocal immunofluorescence microscopy. Quantitative analysis of cells co-expressing HERC5 and Rev revealed that 34% of cells expressing HERC5 and 56% of cells expressing HERC5-C994A exhibited Rev localization at or near the nuclear membrane compared to 10% in the control cells ($P=0.0004$ and $P<0.0001$ respectively, Fisher's exact test) (**Figure 3.5C and 3.5D**). Nine percent of cells expressing HERC5 and 8% of cells expressing HERC5-C994A exhibited Rev localization in the cytoplasm only compared to 0% in the control cells ($P=0.0032$ and $P=0.0032$ respectively, Fisher's exact test) (**Figure 3.5C and 3.5D**). These data indicate that HERC5 expression significantly alters the subcellular localization of Rev.

3.2.6 Positive selection is operating on HERC5

The antiviral activities of HERC5 towards evolutionarily diverse viruses, together with the realization that HERC5 orthologs exist in evolutionarily diverse mammals, identifies HERC5 as a candidate host factor that has likely experienced genetic conflict with viruses during mammalian evolution. Therefore, we asked if positive evolutionary selection is operating on HERC5. The computer software Selecton (Server for the Identification of Site-Specific Positive Selection & Purifying Selection) combines the implementation of state-of-the-art methods for detecting positive evolutionary selection [44, 45]. Selecton has been shown to successfully detect site-specific selection forces on the retroviral restriction factor TRIM5 α , a protein that has recently been shown to have undergone positive selection during the course of primate evolution [44, 46, 47]. Selecton analysis also enabled the detection of positively selected regions that correlated with the previously identified species-specificity determinants of TRIM5 α [44].

We used a similar Selecton analysis to test for positive selection on HERC5 using 13 evolutionarily diverse HERC5 sequences as input sequences (**Figure 3.6A**)

Figure 3.6: Positive evolutionary selection analysis of HERC5.

A, Neighbor-joining phylogenetic tree for progressive alignment of 13 different HERC5 species using constraint-based alignment tool (COBALT) for multiple protein sequences. Branch lengths are proportional to the amount of inferred evolutionary changes. **B and C**, Selecton analysis for positive selection was performed using HERC5 sequences from human, chimpanzee, gorilla, marmoset, baboon, squirrel monkey, gibbon, horse, panda, sheep, cow, dog and cat. Evolutionary analysis for positive selection in HERC5 using various models of evolution where M8 and MEC allow for sites to evolve under positive selection and M7 and M8a models do not. **B** A plot of the Ka/Ks ratio at each codon in an alignment of HERC5 coding sequences is shown. Codons with Ka/Ks ratios >1 indicate positive selection, $=1$ neutral selection and <1 purifying selection. **C**, A plot showing the results of a Bayesian analysis approach to identify sites where Ka/Ks >1 , mapped to the different HERC5 domains. Shown are the sites where Ka/Ks >1.5 and the 95% confidence interval is larger than 1; hence considered statistically significant. **D**, The HERC5 RLD was modeled using SWISS-MODEL (Swiss Institute of Bioinformatics: <http://swissmodel.expasy.org/>) and visualized and colored using DeepView/Swiss-PDBViewer, v4.0.1. The region corresponding to amino acids 2-103 is colored red. All other colors are arbitrary and used to highlight the different blades of the β -propeller structure. The inset numbers identify the different blades. “N” and “C” denote the amino- and carboxyl-termini respectively.

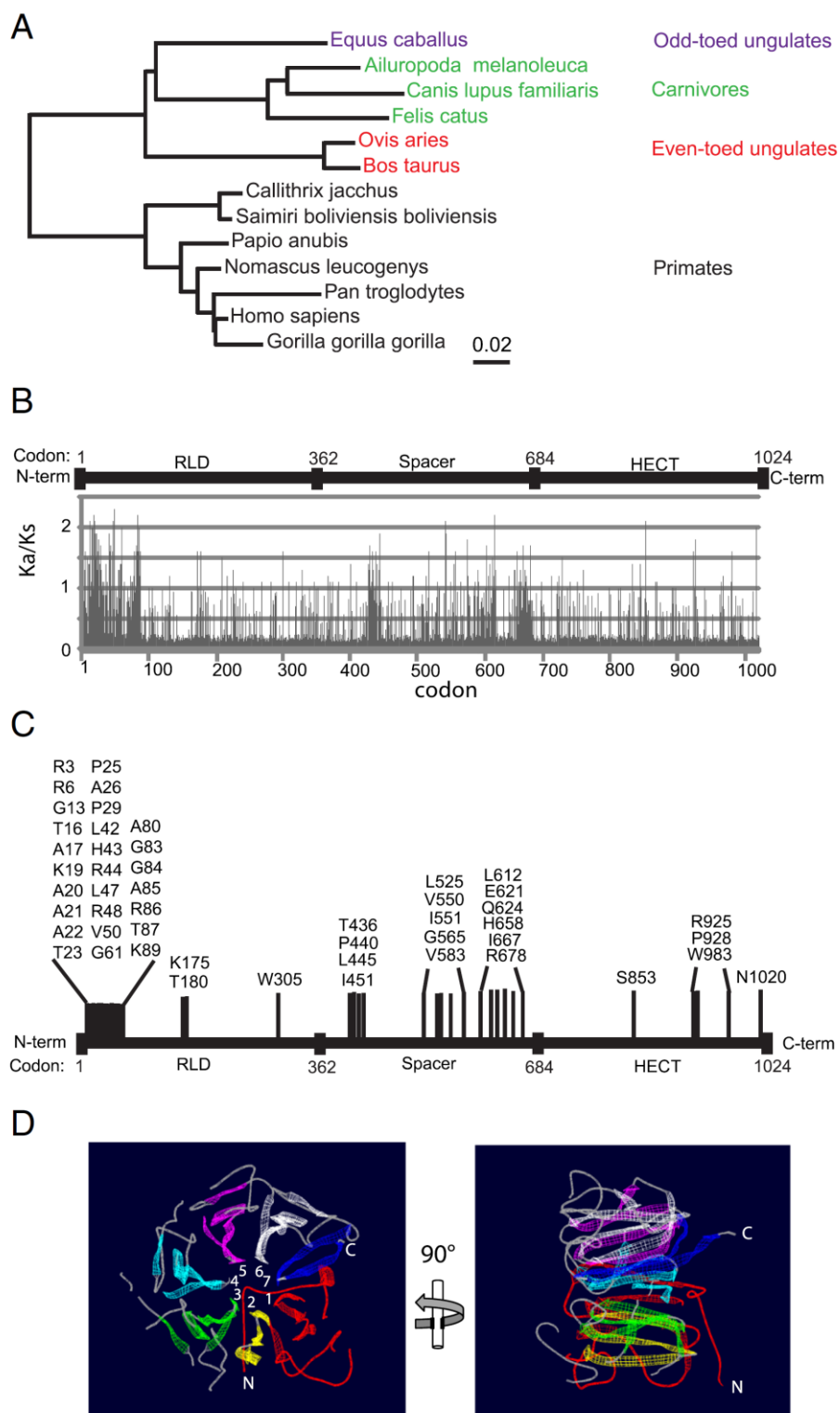


Figure 3.6: Positive evolutionary selection analysis of HERC5.

HERC5 evolution in mammals was evaluated under several standard models of sequence evolution as implemented in the Selecton program. This comprised two nested pairs of models (M8a and M8; and M7 and M8), in which the first model of each pair is nested in the second model. The M8 model, but not the M8a or M7 models, allows sites to evolve under positive selection. A non-nested pair (M8a and MEC) model comparison was also performed. The MEC model differs from the other models in that it takes into account the differences between amino acid replacement rates [44]. The nested models were compared using the likelihood ratio test. In each case, allowing sites to evolve under positive selection (M8) gave a significantly better fit to the HERC5 sequence data than the corresponding model without positive selection (M8a and M7) (**Appendix 2**). The MEC model, which allows for positive selection, was compared with the M8a null model, which does not allow for positive selection. Comparison of the AIC_c scores (M8a: 28432; MEC: 28016) revealed that the MEC model fits the HERC5 data better than the M8a model (**Appendix 2**). The results of the MEC analysis were projected by Selecton onto the primary sequence of human HERC5 (**Figures 3.6B, 3.6C and Appendix 3**). The results show that positive selection is operating on HERC5 and that several codons situated in the RCC1-like domain, the spacer region and the HECT domain exhibit exceptionally high probabilities of having evolved under positive selection. Notably, 27 out of 50 of these codons cluster within the first 100 amino acids of the amino terminus of the RCC1-like domain, encompassing blade 1 and part of blade 2 of its predicted 7-bladed β -propeller structure (**Figure 3.6D**).

Since there was a marked enrichment in positively selected sites within blades 1 and 2, we generated a HERC5 mutant lacking amino acids 2-103 (HERC5-RLD Δ 2-103) and tested whether these blades contributed to the HERC5-mediated inhibition of HIV-1 particle production. Similar to our observations with HERC5- Δ RLD, HERC5-RLD Δ 2-103 produced similar levels of intracellular Gag protein as the empty vector control cells and did not exhibit a full rescue of released HIV-1 particles into the supernatant (**Figure 3.7A**). Furthermore, HERC5-RLD Δ 2-103 failed to inhibit nuclear export of HIV-1 genomic RNA compared to wild type HERC5 (**Figure 3.7B and 3.7C**). These findings indicate that blades 1 and 2 are required for the HERC5-mediated inhibition of nuclear export.

Figure 3.7: Amino acids 2-103 of HERC5 are required for inhibiting nuclear export of HIV-1 genomic RNA.

A, Western blot analysis of 293T cells co-transfected with pR9 and either empty plasmid or a plasmid encoding HERC5, HERC5-C994A, HERC5- Δ RLD and HERC5-RLD Δ 2-103. Forty-eight hours after transfection, HIV-1 virions released into the supernatant and Gag levels within the cell lysates were analyzed by quantitative Western blotting using anti-p24CA or anti- β -actin as a loading control. **B**, HeLa cells were co-transfected with plasmids encoding MS2-GFP alone, MS2-GFP and NL4-3-SL, or MS2-GFP + NL4-3-SL and either flag-tagged HERC5, HERC5-C994A, HERC5- Δ RLD or HERC5-RLD Δ 2-103. Forty-eight hours post-transfection, cells were fixed, stained with anti-flag and DAPI and imaged using fluorescence confocal microscopy. Representative images of the predominant phenotypes are shown. Blue, nucleus; green MS2-GFP; red, flag-tagged HERC5. Scale bars = 10 μ m. **C**, MS2-GFP localization was assessed in each cell and categorized according to localization in the nucleus only or both the nucleus and cytoplasm. The results of the phenotypic quantification are shown and were obtained from at least three independent experiments (n=235).

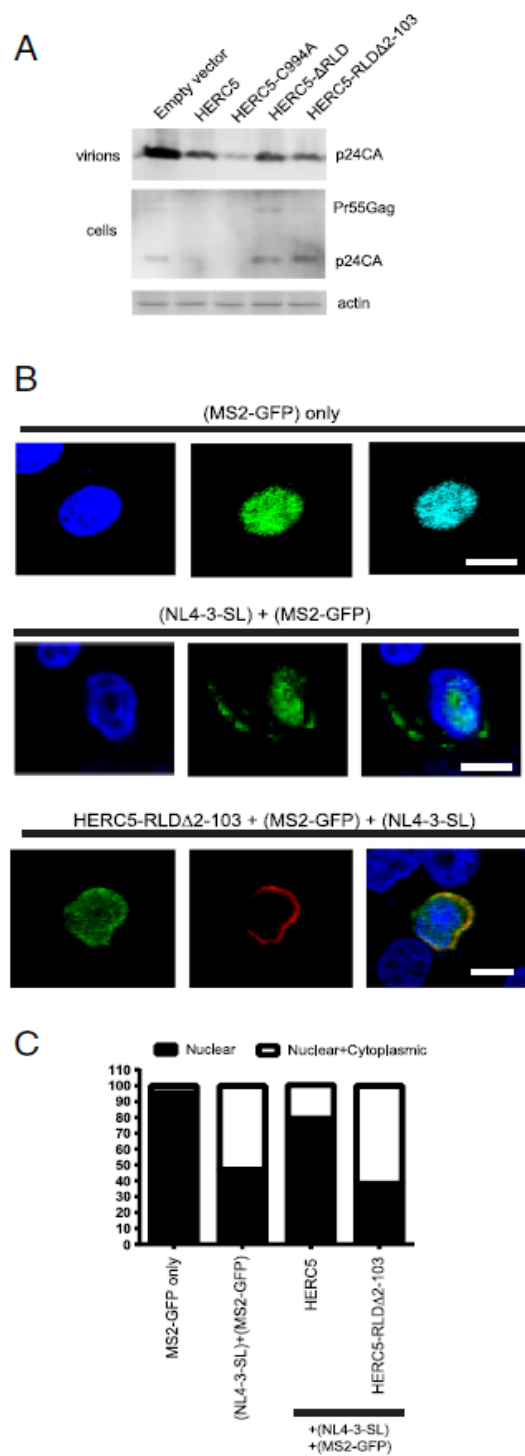


Figure 3.7: Amino acids 2-103 of HERC5 are required for inhibiting nuclear export of HIV-1 genomic RNA.

3.3 Discussion

Previously, HERC5 was shown to inhibit an early stage of HIV-1 Gag assembly at the plasma membrane by a mechanism correlating with the modification of Pr55Gag with ISG15 [1]. Here we have shown that HERC5 inhibits HIV-1 particle production by a second distinct mechanism targeting Rev/RRE function. A region of the RCC1-like domain of HERC5 was required for this inhibition, which is also evolving under strong positive selection. Although deleting the RCC1-like domain of HERC5 rescued inhibition of Rev/RRE function, HERC5- Δ RLD inhibited HIV-1 particle release at levels comparable to wild type HERC5. HERC5- Δ RLD has been shown to possess some E3 ligase activity for ISG15 conjugation; therefore, it is likely that this E3 ligase activity contributed to inhibition of particle release via ISGylation of HIV-1 Gag [19, 49]. However, the infectivity of HIV-1 particles released from cells expressing HERC5- Δ RLD did not differ from those released from the control cells. By inhibiting the nuclear export of HIV-1 genomic RNA, the RCC1-like domain may also promote the release of non-infectious HIV-1 particles (i.e. particles lacking genomic RNA). We also showed that HERC5-C994A was able to inhibit HIV-1 particle production better than wild type HERC5. This potent inhibition correlated with the loss of ISGylated forms of HERC5. Given that HERC5 modifies itself with ISG15, it is possible that auto-ISGylation negatively regulates HERC5 antiviral activity. This auto-regulation may represent a mechanism by which HERC5 maintains tight control over its E3 ligase-independent activity, particularly during periods of high-expression such as after induction by IFN. A similar finding was previously observed for the E3 ligase TRIM25 where auto-ISGylation negatively regulated its activity for conjugating ISG15 to 14-3-3sigma [48].

HERC5 expression is significantly up-regulated in cells after exposure to IFN [18, 19]. We showed here that knocking down HERC5 in a background of IFN exposure substantially increased intracellular HIV-1 Gag production after 48 hours, indicating that HERC5 is an important mediator of the IFN response towards HIV-1. Knocking down endogenous levels of HERC5 in primary macrophages, in the absence of exogenous IFN, also resulted in a substantial increase in HIV-1 particle production. This finding suggests that endogenous levels of HERC5 may serve to limit, but not fully restrict, HIV-1 particle

production in the absence of IFN. As HERC5 levels increase, such as after exposure to IFN, HERC5 may be more able to restrict HIV-1 particle production. This finding contrasts our previous finding that knockdown of HERC5 did not exhibit a substantial effect on the intracellular production of Pr55Gag from full-length replication-competent HIV-1 [1]. A likely explanation for the difference is that the data in the present study was obtained 48 hours post-transfection, compared to 24 hours in our previous study. Another contributing factor could be that the level of HERC5 knockdown achieved in the present study was higher than our previous study (3.2-fold versus 2.3-fold respectively). In both studies, HERC5 had no substantial effect on intracellular Pr55Gag levels when expressed from a Rev-independent Gag-only construct.

Most eukaryote messenger RNAs undergo splicing to remove introns before they are exported to the cytoplasm via the NXF1/NXT1-dependent nuclear export pathway [50-53]. However, the expression of HIV-1 genes is a notable exception. Unspliced and singly-spliced HIV-1 RNA must be exported to the cytoplasm before they are fully-spliced by host machinery in the nucleus [54-57]. These incompletely-spliced RNAs are essential for steps such as incorporation of full-length genomes into new virions and for expression of Gag, Gag-Pol, Env, Vif, Vpr and Vpu proteins. Nuclear export of incompletely-spliced HIV-1 RNAs occurs when the Rev/RRE complex recruits the dimeric complex of Crm1/RanGTP before translocating through the nuclear pore to the cytoplasm via the Crm1/RanGTP-dependent nuclear export pathway. Once in the cytoplasm, the complex dissociates and RanGTP is converted to RanGDP with the help of RanBP1 and RanGAP1, which then shuttles back into the nucleus for another round of export [35-39, 58].

We showed that HERC5 interacts with Ran and substantially reduces intracellular levels of RanGTP and/or inhibits the association of RanGTP with RanBP1. Cells require a high concentration (>1000-fold) of RanGTP in the nucleus, which is believed to provide directionality for nuclear export to the cytoplasm [31]. Perturbing this nuclear:cytoplasmic RanGTP gradient by either reducing total RanGTP levels or increasing the cytoplasmic RanGTP level by interfering with the interaction between RanGTP and RanBP1, halts nuclear export of Crm1/RanGTP-dependent cargo.

Consistent with this idea, we observed altered localization of Rev protein and Rev/RRE-dependent RNA in the presence of HERC5. With the ability of HERC5 to interact with Ran, it is possible that HERC5 binds and sequesters Ran in the cytoplasm. This activity would interfere with the shuttling of RanGDP into the nucleus, thereby interfering with the production of RanGTP in the nucleus. Another possibility is that HERC5 stimulates guanine nucleotide release from Ran in the cytoplasm. It was previously shown that the related RCC1-like domain 1 of human HERC1 stimulates GDP release from the small GTPase proteins ARF1/6 and Rab, but not from Ran [59, 60]. Therefore, it is plausible that the HERC5 RCC1-like domain performs a similar function on Ran, with which it interacts. Further experiments are needed to further dissect this mechanism.

HERC5 orthologs have been identified in a variety of evolutionarily diverse mammals spanning >75 million years of evolution. Genetic conflict arising from the co-evolution of hosts and pathogens can lead to rapid selection of amino acid substitutions that alter amino acid composition of the host factors and their pathogen antagonists, thereby conferring an evolutionary advantage to the host or the pathogen [61, 62]. This process of positive selection is not a common phenomenon and is typically not apparent in most examined datasets [63, 64]. However, recent evolutionary studies on host antiviral factors have shown that they are rapidly evolving genes due to genetic conflict between hosts and pathogens [65]. Several of these host factors such as APOBEC3G [66]; TRIM5 α [46]; (BST2)/Tetherin [67, 68]; SAMHD1 [69]; MxA [70] contain genetic ‘signatures’ of positive selection. Positively selected residues have been shown to play key functional roles in the antiviral activities of these proteins.

We demonstrate here that *HERC5* also contains genetic signatures of strong positive selection. Twenty-seven of 50 codons predicted to be evolving under strong positive selection in *HERC5* map to blades 1 and 2 of the predicted β -propeller structure of the RCC1-like domain. This finding identifies blades 1 and 2 as a functionally important region of *HERC5* and may represent a highly dynamic interface with viral antagonists. Fifteen of 50 codons predicted to be evolving under strong positive selection map to the spacer region of *HERC5*.

The high proportion of amino acids predicted to be evolving under purifying selection in blades 3-7 of the RCC1-like domain and the majority of the HECT domain indicates that purifying selection is playing an important role in maintaining the long-term stability of these domains. These two domains are highly conserved in evolutionarily diverse mammals, suggesting they play fundamental roles in biology. Indeed, the HECT domain of HERC5 confers its E3 ligase activity and HERC5 has been shown to be the main cellular E3 ligase for host ISGylation.

Here we demonstrate that HERC5 possesses a second distinct mechanism by which it blocks HIV-1 particle production. By being able to inhibit both nuclear export of incompletely-spliced HIV-1 RNA and an early step in HIV-1 Gag assembly at the plasma membrane, HERC5 represents a significant challenge for HIV-1 replication. The work presented here contributes to the growing body of evidence that HERC5 is a novel host restriction factor. Currently, HERC5 satisfies 3 of the 4 hallmarks of restriction factors: HERC5 exhibits strong 'signatures' of positive selection, is up-regulated by IFN β and virus infection, and has antiviral activity as its major biological function. A direct viral antagonist of HERC5 is yet to be identified; although antagonists to HERC5 function (e.g. ISGylation) have been identified from several evolutionarily diverse viruses [77-84]. It will be interesting to discover the evolutionary pressures that drive positive selection in HERC5 and how HIV-1 and/or other viruses circumvent HERC5 activity *in vivo*.

3.4 Methods

3.4.1 Ethics statement

Informed consent was obtained from all subjects according to an ethics protocol approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB).

3.4.2 Cells and cell lines

All cell lines were obtained from American Type Culture Collection unless otherwise stated. Cells were maintained in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM)), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 µg/ml Streptomycin) at 37 °C with 5% CO₂. Human primary monocyte-derived macrophages were generated from peripheral blood mononuclear cells (PBMCs) of healthy volunteer donors. PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma). Monocytes were purified using a CD14+ cell isolation kit from Miltenyi Biotec. The purity of CD14+ cells was >90% as determined by flow cytometric analysis using anti-human CD14 PE-Cyanine7 (eBiosciences). Monocytes were cultured for 6 days in RPMI-1640 supplemented with 10% FBS and 50 ng/ml recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech) as previously described [85]. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (GHOST (3) R3/X4/R5; Cat. 3943) from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman [86].

3.4.3 Plasmids, transfections and antibodies

Plasmids: pR9, pUbe1L, pUbcH8, myc-tagged ISG15 (pMyc-ISG15), flag-tagged HERC5 (pHERC5) and flag-tagged HERC5-C994A (pHERC5-C994A) were described previously [1]. The promoterless empty vector plasmid pGL3 was purchased from Promega, p3xFLAG from Sigma, and pUbp43 from Thermo Scientific. Plasmids encoding HERC5- Δ RLD, and HERC5-RLD Δ 2-103 were generated using standard domain deletion mutagenesis using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Primer pairs used in the reactions are as follows: HERC5- Δ RLD= forward- 5' GAC ATG GAG CGC CGC AGC ATG ATT GCT GGA GGG AAT CAA AGC ATT TTG CTC TGG 3' and reverse 5' GCT TTG ATT CCC TCC AGC AAT CAT GCT GCG GCG CTC CAT GTC GTC 3'; HERC5-RLD Δ 2-103= forward 5' GAC ATG GAG CGC CGC AGC CAG GGA GCC GAA CAC ATG CTG 3' and reverse 5' GTG TTC GGC TCC CTG GCT GCG

GCG CTC CAT GTC GT 3'. pMS2-GFP was obtained from Addgene (cat.# 27121). pNL4-3-SL was kindly provided by Dr. Hu (National Cancer Institute, Frederick, Maryland, USA). pGagPol-RRE and pGagPol-4xCTE were provided by Dr. M. Malim (King's College London). pScram was described previously [1]. pHERC5_{shRNA} (cat. #RHS4533-NM_016323, TRCN0000004171) was obtained from Thermo Scientific (Open Biosystems). Transfections: plasmid transfections were performed using standard calcium phosphate transfection or Lipofectamine 2000 (Invitrogen). Transfection of primary human macrophages was performed using GenJetTM In Vitro DNA Transfection Reagent for Primary Macrophages (FroggaBio). Co-transfections were performed at a ratio of 10:1 (pHERC5 construct:pR9) unless otherwise stated. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly [87-89]). Antibodies: anti-HERC5 was obtained from Abnova, anti-FLAG from Sigma, and anti-myc and anti- β -actin from Rockland.

3.4.4 Quantification of infectious virus

Clarified supernatants containing virus particles were pelleted over a 20% sucrose cushion for 2 hours at 21,000 x g and lysed for quantitative Western blot analysis. Alternatively, the clarified supernatants were used to infect GHOST(3(3)R3/X4/R5 indicator cells. Quantification of infectious virus release using GHOST(3)R3/X4/R5 indicator cells has been described previously [86].

3.5.5 Quantitative real-time PCR

Total RNA was extracted from total cell lysate or the cytoplasmic fraction only using the R&A-BLUE Total RNA Extraction kit (Frogga Bio). 3 μ g of RNA was reverse transcribed to cDNA using the M-MLV reverse transcriptase and Oligo(dT) primers (Life Technologies). Prior to real-time PCR, cDNA samples were diluted 1:10 with water. Each PCR reaction consisted of 10 μ l of SYBR Green Master Mix, 2 μ l of Gag or Rev-specific primers (1 μ l of 10 μ M forward primer and 1 μ l of 10 μ M reverse primer), 1 μ l of diluted cDNA, and water to a total volume of 20 μ l. Real-time PCR was run on the Rotor-Gene 6000 real-time PCR machine (Corbett Life Science) under the following cycling

conditions: 10 min at 95°C and 40 cycles of 10 sec at 95°C, 15 sec at 60°C, and 20 sec at 72°C. The Rotor-Gene 6000 series software (version 1.7) was used to determine the C_T for each PCR reaction.

3.4.6 Western blotting and confocal microscopy

Cells were cultured in 12-well plates on 18 mm coverslips and co-transfected with pHERC5 (or pHERC5-C994A or empty vector), pUbcH8, pUbe1L, pISG15 and pGag (10:5:5.5:1 ratio respectively). Twenty-four hours post-transfection, the coverslips containing the 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. The coverslips were incubated with primary antibodies for one hour, washed 6× with PF buffer, incubated with secondary antibodies (Alexa Fluor 546 anti-mouse or AlexaFluor 488 anti-rabbit, Invitrogen) for one hour and then washed 6× with PF buffer. Coverslips were mounted onto glass slides with ~10 µl of Vectashield mounting media with DAPI (Vector Laboratories) and then sealed with nail polish. Slides were examined using a Zeiss LSM 510 confocal fluorescence microscope and images were obtained with sequential imaging.

3.4.7 Immunoprecipitation

For immunoprecipitation, cells were lysed with cold non-denaturing lysis buffer (1% (w/v) SDS, 50 mM Tris-Cl pH 7.4, 5 mM EDTA pH 8.0, 300 mM NaCl, 0.02% (w/v) sodium azide with Roche protease inhibitor) for 20 minutes. Cyanogen bromide-activated Sepharose beads (GE Health Care) were swollen in 1 mM HCl for 10 minutes followed by antibody coupling using either 5 µl of rabbit anti-p24CA or 1 µl of mouse anti-FLAG (Sigma) per 75 µl of beads in coupling buffer (0.1 M NaHCO₃ pH 8.3 with 0.5 M NaCl) overnight at 4°C. The beads were then washed three times with coupling buffer to remove excess antibody. The beads were blocked with blocking buffer (0.1 M Tris-HCl buffer pH 8.0) for 2 hours at 4°C. The beads were then washed with 3 cycles of alternating pH (0.1 M sodium acetate pH 4 with 0.5 M NaCl; 0.1 M Tris-HCl pH 8.0 with 0.5 M NaCl).

The cell lysates were added to the beads for 1 hour at 4°C. The beads were washed 8 times with non-denaturing lysis buffer and the protein was eluted with 0.5 mM NaCl.

3.4.8 RanGTP pull-down assay

Forty-eight hours after transfection, the media was aspirated from a confluent 10cm dish of 293T cells. Cells were washed twice with ice-cold 1x PBS, scraped from the dish and placed into an appropriately sized tube and kept on ice at all times. Cells were processed using the Ran Activation Assay Kit (Cell BioLabs, Inc.) according to the manufacturer's protocol.

3.4.9 Positive selection analysis

HERC5 sequences were aligned and a phylogenetic tree generated using COBALT (<http://www.ncbi.nlm.nih.gov/tools/cobalt/>) [90]. *HERC5* sequences were obtained from Genbank: *Homo sapiens* ("Human") (NP_057407.2), *Pan troglodytes* ("Chimpanzee")(XP_003310459.1), *Gorilla gorilla gorilla* ("Gorilla") (XP_004039179.1), *Callithrix jacchus* ("Marmoset") (XP_002745648.1), *Papio anubis* ("Baboon") (XP_003898997.1), *Saimiri boliviensis boliviensis* ("Squirrel monkey") (XP_003924055.1), *Nomascus leucogenys* ("Gibbon") (XP_003265940.1), *Equus caballus* ("Horse") (XP_001915115.2), *Ailuropoda melanoleuca* (Giant Panda") (XP_002913645.1), *Ovis aries* ("Sheep") (XP_004009762.1), *Bos taurus* ("Cow") (NP_001095465.1), *Canis lupus familiaris* ("Dog") (XP_535652.3), *Felis catus* ("Cat") (XP_003985249.1). At least 2 independent sequences were available for human, sheep, baboon, marmoset, gibbon, squirrel monkey. The following sequences were not independently validated: cat, dog, cow, horse, sheep and giant panda. The identification of site-specific positive selection and purifying selection was generated using the Selecton Server (<http://selecton.tau.ac.il/index.html>) as previously described [44, 45]. The *HERC5* phylogenetic tree was used in the Selecton analysis. Nested pairs of models (M8a and M8; and M7 and M8) and a non-nested pair (M8a and MEC) were compared using the likelihood ratio test implemented in the Selecton program.

3.4.10 Statistical Analyses

GraphPad Prism v5.03 was used for all statistical analyses stated in the text. *P* values and statistical tests used are stated in the text where appropriate. *P* values less than 0.05 were deemed significant.

3.5 References

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Chapter 4

4 Evolution-guided structural and functional analyses of the anti-HIV-1 activity of the small HERC family

Host restriction factors are rapidly evolving genes due to genetic conflict between hosts and viruses. Genetic conflict arising from the co-evolution of hosts and viruses can lead to rapid selection of amino acid substitutions that confers an evolutionary advantage to the host or the virus. HERC5, a cellular restriction factor that we have shown potently restricts HIV-1 replication, is evolving under positive selection which suggests that HERC5 is an important effector molecule for our cells in the fight against viruses such as HIV-1. HERC5 inhibits HIV-1 assembly and budding using an ISG15 E3 ligase-dependent mechanism. HERC5 also restricts HIV-1 replication by inhibiting unspliced HIV-1 mRNA export which is dependent on its RLD. Here we compare the anti-HIV-1 activity of the HERC family members HERC3, HERC4, HERC5 and HERC6.

Using an HIV-1 particle release assay, we showed that HERC3, HERC4, HERC5 but not HERC6 significantly inhibited the amount of virus released into the supernatant as well as the amount of intracellular Gag protein produced. We found HERC3, HERC4 and HERC5 inhibit the nuclear export of unspliced HIV-1 RNA. Using a nuclear export assay, we demonstrated that the mechanism underlying this inhibition specifically targets the HIV-1 Rev/RRE-mediated nuclear export of HIV-1 RNA. Evolutionary analysis showed that strong positive selection is operating on HERC5 and HERC6, but not HERC3 and HERC4. Structural analyses and domain-swapping mutagenesis between HERC5 and HERC6 has identified the HERC5 RLD as a determinant of antiviral activity.

Our research characterizes the HERC family as an antiviral protein family that has the ability to restrict HIV-1 replication. Our research provides a better understanding of how our cells fight viruses and lays the groundwork for future research into exploiting the critical interactions between HIV-1 proteins and HERC proteins for novel drug and/or gene therapy design.

4.1 Introduction

IFN-induced HERC5 is a novel multi-functional host restriction factor that inhibits the replication of viruses such as HIV-1, influenza A virus, and human papillomavirus [1–4]. By virtue of its amino-terminal RCC1-like domain, HERC5 belongs to a phylogenetically widespread RCC1 superfamily of proteins. The prototypical member of this superfamily is RCC1, and is characterized by the presence of seven repeats of 51-68 amino acids that assume a seven-bladed β -propeller structure. The primary sequences of mammalian RCC1-like domains are poorly conserved; however, their predicted tertiary structures are highly conserved [5]. We previously showed that HERC5 inhibits nuclear export of incompletely-spliced HIV-1 RNA, resulting in a severe reduction in the level of intracellular Gag protein and production of viral particles. The region encompassing blade 1 (amino acids 1-103aa) of the RCC1-like domain of HERC5 is required for inhibiting nuclear export of incompletely spliced HIV-1 RNA, which is essential for HIV-1 particle production. Interestingly, blade 1 of the RCC1-like domain is evolving under strong positive evolutionary selection, potentially identifying this region as a key interface between HERC5 and pathogens such as viruses [2].

By virtue of its C-terminal HECT domain, HERC5 is the main cellular E3 ligase that conjugates ISG15 to proteins via a hierarchical enzymatic cascade involving the E1 activating enzyme Ube1L and the E2 conjugating enzyme UbcH8 [6, 7]. The conjugation of ISG15 to proteins is referred to as ISGylation and can interfere with key protein-protein interactions or disrupt protein function [8]. Several groups have shown that HERC5 ISGylation activity exhibits potent antiviral activity against evolutionarily diverse viruses such as IAV, HPV-16 and HIV-1 [1–4]. For example, HERC5 inhibits an early stage of HIV-1 Gag assembly at the plasma membrane by modifying Gag with the ubiquitin-like protein ISG15 [3]. HERC5 knockdown studies in the presence and absence of IFN- β have also demonstrated that HERC5 is an important contributor to the type I IFN response towards HIV-1 and IAV [1, 3].

HERC5 is one of six HERC family members (numbered 1 through 6) that are present in evolutionarily diverse mammals. The family can be divided into large HERCs (HERC1

and HERC2) and small HERCs (HERCs 3-6) [9, 10]. The small HERCs are the most homologous members, containing a single N-terminal RCC1-like domain and a carboxyl-terminal HECT domain. *HERC5* is the most recently emerged member of the family, having arisen from a *HERC6* gene-duplication event [9]. Gene-duplication events are believed to be an effective evolutionary strategy of host restriction factors for accelerating host adaptation to viruses. Several examples where gene duplication has given rise to restriction factor families in mammals include Mx1, IFITM, TRIM5 and, most notably, APOBEC3 [11–18]. Increasing the copy number of a restriction factor is believed to allow the host to rapidly evolve in response to several different viruses (reviewed in [19]). For example, several APOBEC3 genes show evidence of positive selection in primates; however, the residues under positive selection differ, suggesting that these *APOBEC3* genes evolved in response to different viruses. Alternatively, restriction factor families may have evolved to target the same virus in different ways. For example, APOBEC3H and APOBEC3G both deaminate cytosines during first strand cDNA synthesis, however their target sequences differ [20]. It is also important to note that restriction factor families can also 'contract' such as in the absence of selective forces. For example, APOBEC3H has antiviral activity against lentiviruses; however, two independent loss-of-function mutations in APOBEC3H are present at high frequencies in humans [21]. As such, valuable evolutionary and functional information can be gained from characterizing the antiviral activities of restriction factor families.

We recently showed that *HERC5* is highly conserved in mammals and has been evolving under strong positive evolutionary selection. This suggests that *HERC5* plays a fundamental role at the host-pathogen interface [2]. HERCs 3-6 are predicted to be structurally homologous, begging the question of whether the antiviral activity of *HERC5* is conserved among these family members. We performed evolution-guided structural and functional analyses of HERCs 3-6 and show here that *HERC3*, *HERC4* and *HERC5*, but not *HERC6*, exhibit antiviral activity towards HIV-1. Evolutionary analysis revealed that *HERC3* and *HERC4* are not evolving under positive selection; however, the non-functional human *HERC6* exhibits strong signatures of positive selection, more so than its more recently evolved functional counterpart *HERC5*. Our predicted structural

analyses of the β -propeller in the RLD identified blade 1 as a potential key structural determinant of small HERC antiviral specificity.

4.2 Results

4.2.1 HERC3, HERC4, HERC5, but not HERC6, inhibit HIV-1 particle production

We previously showed that human HERC5 potently inhibits HIV-1 particle production [2, 3]. We asked whether human HERC3, HERC4 and HERC6 were also capable of inhibiting HIV-1 particle production. Human 293T cells were co-transfected with plasmids encoding full-length, replication-competent HIV-1 (pR9) and either empty vector, HERC3, HERC4, HERC5 or HERC6. Forty-eight hours after transfection, intracellular viral protein production and viral particles released into the supernatant were analyzed by Western blotting using antibodies to HIV-1 capsid protein (anti-p24CA). As expected, HERC5 potently inhibited intracellular Gag protein production and HIV-1 particle release compared to the empty vector control cells (**Figure 4.1**). Cells over-expressing HERC3 or HERC4 also exhibited a substantial reduction in intracellular Gag protein production and HIV-1 release (**Figure 4.1**). In stark contrast, cells expressing HERC6 failed to inhibit intracellular Gag protein production and HIV-1 release (**Figure 4.1**). Next, we assessed the localization of the small HERC family. As previously shown for HERC5 [3], HERC3, HERC4 and HERC6, are all diffusely localized in the cytoplasm in punctate bodies (**Figure 4.2**). HERC6 appears to exhibit more pronounced localization at the peri-nuclear region (**Figure 4.2**).

Figure 4.1. HERC3, HERC4, HERC5 but not HERC6 restrict HIV-1 replication.

293T cells were co-transfected with pR9 and peGFP and either empty vector, pHERC3, pHERC4, pHERC5 and pHERC6 in increasing concentrations (R9:HERC-1:3, 1:6 and 1:12). Forty hours post-transfection, virus released into the supernatant and total cell lysates were subjected to Western blot analysis using anti-p24CA, anti- β actin as a loading control and anti-eGFP as a transfection control.

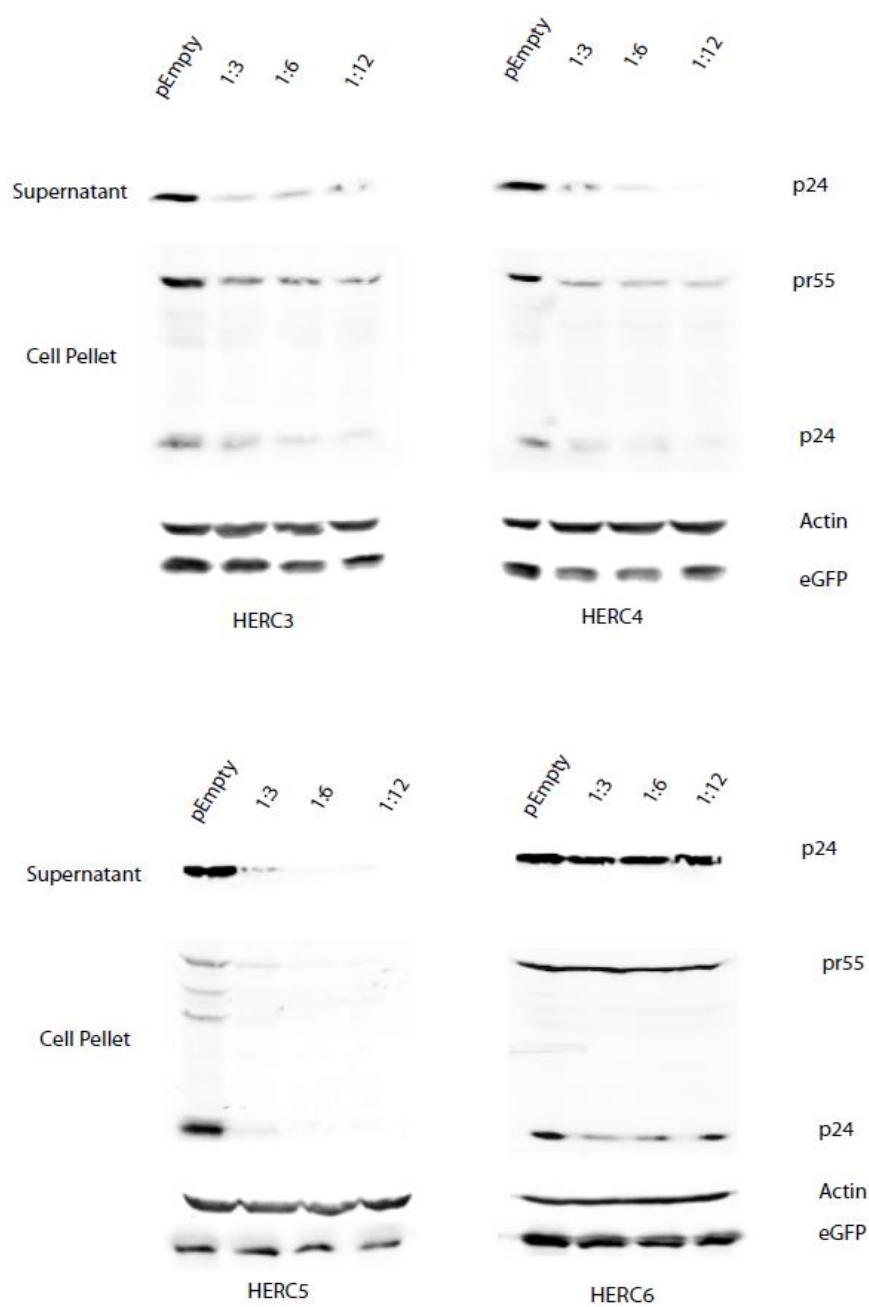


Figure 4.1: HERC3, HERC4, HERC5 but not HERC6 restrict HIV-1 replication.

Figure 4.2. HERC localization

HeLa cells were transfected with either pFlag-HERC3, pFlag-HERC4, pFlag-HERC5 or pFlag-HERC6. The nucleus (Blue) was stained with Dapi and the HERC proteins (Red) was imaged using a mouse anti-Flag antibody with the secondary antibody alexa fluor anti-mouse 546. Scale bar=10 μ m.

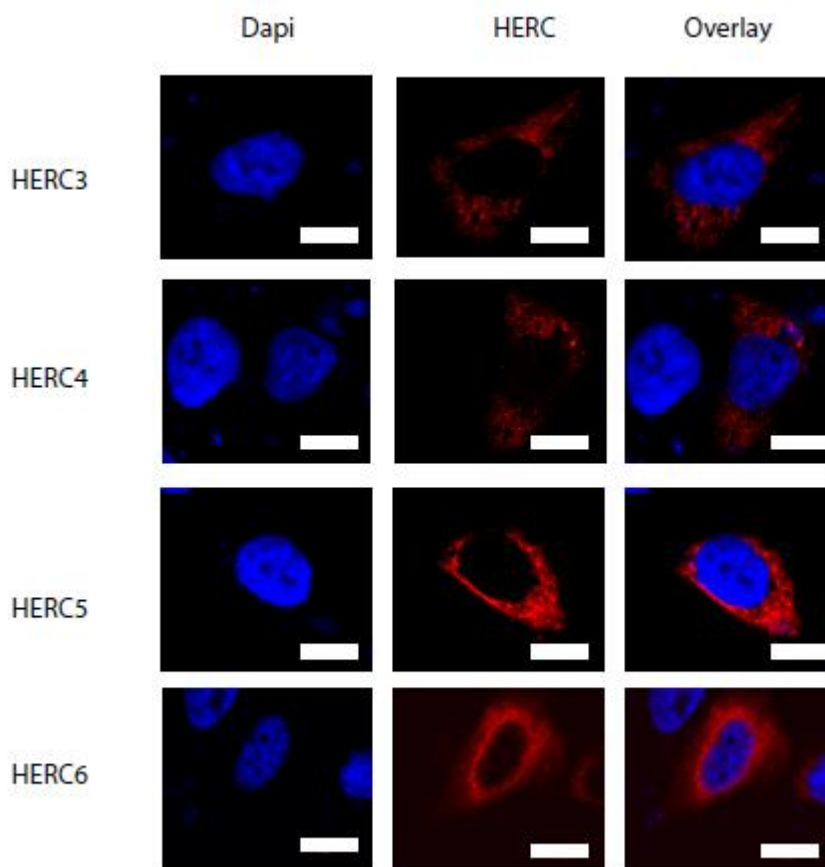


Figure 4.2: HERC localization.

4.2.2 The antiviral mechanism of HERC3 and HERC4 is similar to HERC5

We have previously determined that HERC5 restricts HIV-1 replication by two independent mechanisms. First we determined that HERC5 modifies HIV-1 Gag with ISG15, which correlated to the inhibition of an early stage of Gag assembly [3]. This mechanism is dependent on the ISG15 E3 ligase activity of the HECT domain within HERC5. We next determined that HERC5 inhibits the export of unspliced HIV-1 mRNA which resulted in the loss of Gag accumulation. This mechanism is dependent on the HERC5 RLD domain [2]. Given that HERC3 and HERC4 expression resulted in a substantial reduction in intracellular Gag protein, we asked if they inhibited intracellular Gag protein production by a mechanism similar to HERC5, which inhibits nuclear export of incompletely-spliced HIV-1 mRNA [2].

First, 293T cells were transfected with both R9 and either HERC3, HERC4, HERC5 or HERC6 and treated with proteasome inhibitor MG132 to determine if the loss of Gag accumulation was due to proteasomal degradation. As expected, MG132-treated control cells exhibited a slight reduction in the production of extracellular virus [2]. Treatment of cells transfected with either HERC3, HERC4, HERC5 or HERC6 with the proteasome inhibitor MG132 did not rescue levels of intracellular Gag protein or HIV-1 release from cells (**Figure 4.3**).

MG132 treatment increased intracellular levels of both HERC3 and HERC6. MG132 treatment resulted in an increase of slowly migrating forms of HERC3 and HERC6, possibly suggesting that HERC3 and HERC6 levels are controlled by ubiquitination and degradation by the proteasome. HERC3 and HERC4 had lower expression compared to HERC5 and HERC6 (**Figure 4.4**).

Figure 4.3. HERC3, HERC4 and HERC5 mediated restriction is not mediated by the proteasome.

293T cells were co-transfected with pR9 and either empty vector pHERC3, pHERC4, pHERC5 or pHERC6. Twenty-four hours post-transfection, cells were treated with the proteasomal inhibitor MG132 (20 μ M) for 16 hours. Virus released into the supernatant or total cell lysates were subjected to Western blot analysis using anti-p24CA and anti- β actin as a loading control.

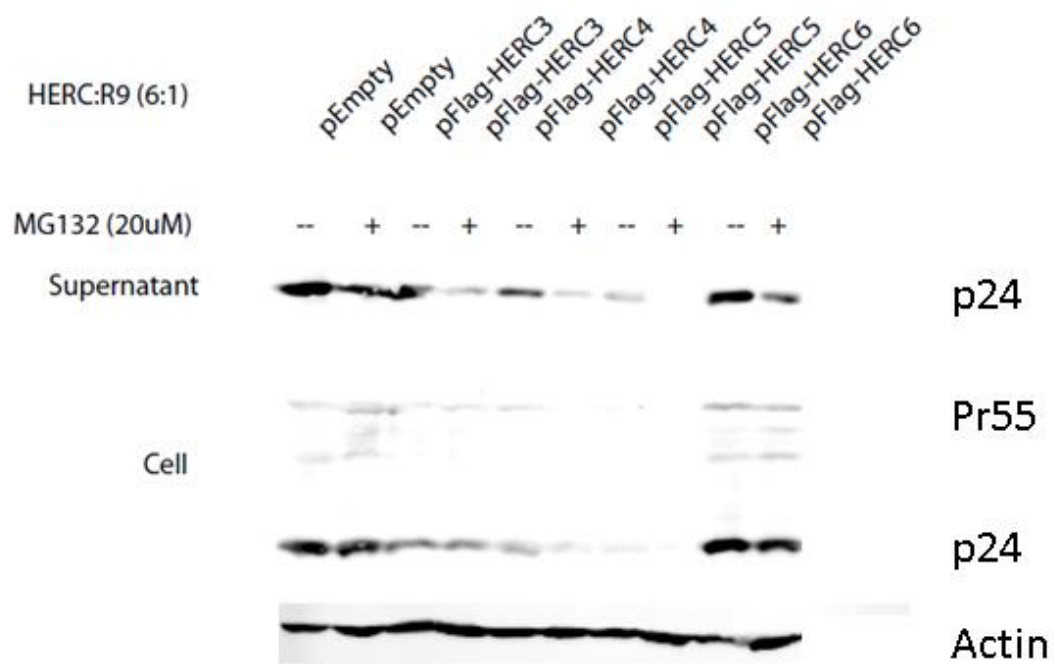


Figure 4.3: HERC3, HERC4 and HERC5 mediated restriction is not mediated by the proteasome.

Figure 4.4. HERC3 and HERC6 protein levels are controlled by the proteasome.

293T cells were co-transfected with either empty vector pHERC3, pHERC4, pHERC5 or pHERC6. Twenty-four hours post-transfection, cells were treated with the proteasomal inhibitor MG132 (20 μ M) for 16 hours. Western blot analysis was completed using anti-pFlag to detect HERC levels and anti- β actin to detect actin as a loading control.

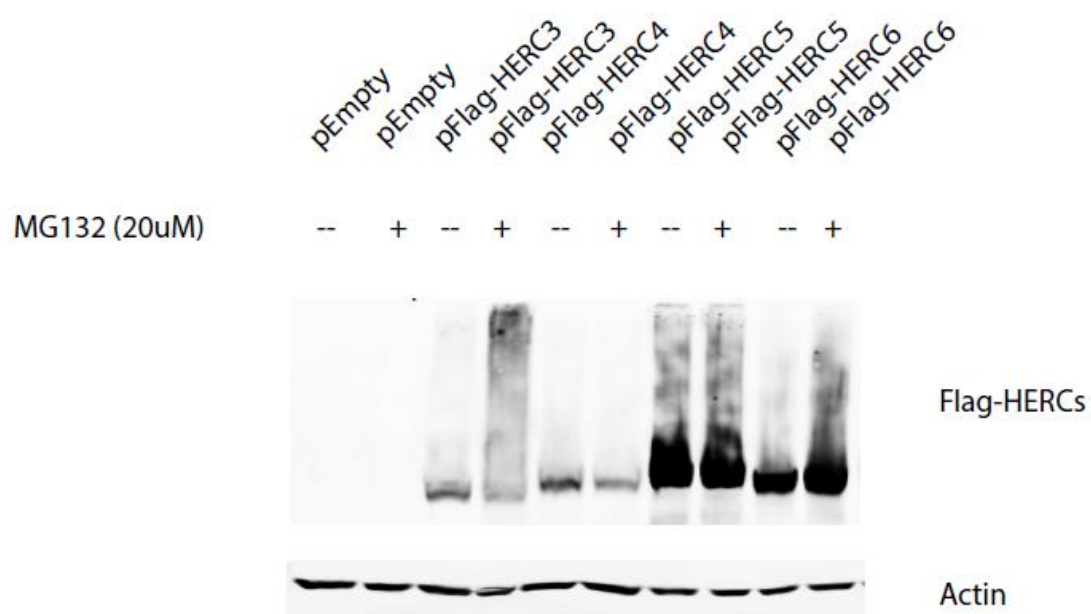


Figure 4.4: HERC3 and HERC6 protein levels are controlled by the proteasome.

To determine if HERC3 and HERC4 restricted the export of unspliced HIV-1 mRNA similarly to HERC5, the cytoplasmic RNA was subjected to qPCR with primers specific to either unspliced HIV-1 genomic RNA (e.g. Gag) and fully spliced HIV-1 genomic RNA (e.g. Rev) or β -actin (loading control). Cells expressing HERC5 exhibited a 3.6-fold reduction in the amount of HIV-1 genomic RNA exported to the cytoplasm compared to the control cells (**Table 4.1**). HERC3 also caused a 1.9-fold reduction in the export of HIV-1 genomic RNA. Similarly, HERC4 caused a 1.9-fold reduction in unspliced genomic RNA. In contrast, HERC3, HERC4 and HERC5 did not significantly decrease the export of fully-spliced HIV-1 Rev transcripts (**Table 4.1**).

We asked if HERC3 or HERC4-mediated inhibition of HIV-1 RNA nuclear export was Rev-dependent, as previously described for HERC5 [2]. Rev promotes nuclear export of incompletely-spliced HIV-1 mRNAs by binding to a specific cis-acting element called the rev-response element (RRE). Successful export of incompletely-spliced RNA can be assessed by Western blotting for Gag protein expression. We tested the ability of HERC3 and HERC4 to inhibit Gag expression from Rev-dependent (GagPol-RRE) and Rev-independent (GagPol-4xCTE and codon-optimized Gag-only) constructs as previously shown with HERC5 [2]. 293T cells were co-transfected with increasing concentrations of plasmids encoding the HERC proteins, with either (pGagPol-RRE + pRev) or pGagPol-4xCTE. As shown in **Figure 4.5**, HERC3, HERC4 and HERC5 inhibited Gag expression from the GagPol-RRE construct but no substantial decrease of Gag was observed from the GagPol-CTE construct (**Figure 4.5**). Also, no reduction in Gag expression was observed from the Gag-only construct (**Figure 4.5**).

4.2.3 ISG15 E3 ligase activity of the small HERC family

It was previously determined that HERC5 is the main cellular E3 ligase [6, 7], however, it is unknown if the other small HERCs possess ISG15 E3 ligase activity, which may also contribute to the antiviral activity towards HIV-1 as observed with HERC5. After transfecting the various HERC proteins together with the conjugation system Ube1L, UbcH8 and myc-ISG15, it was evident that HERC5, as expected, exhibited substantial E3 ISG15 ligase activity. In contrast, HERC6 exhibited minor ISG15 E3 ligase activity and

Table 4.1: HERC3, HERC4 and HERC5 restrict export of unspliced HIV-1 mRNA

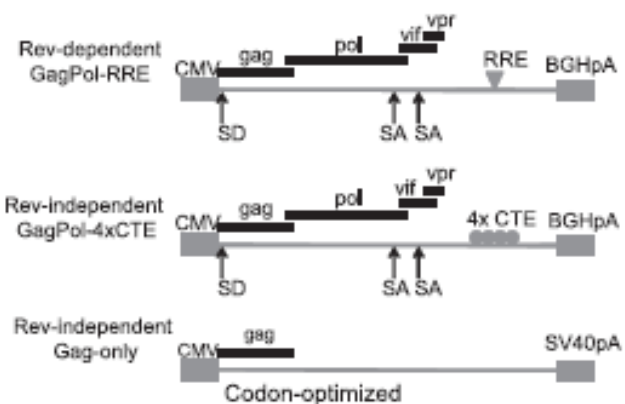
	Unspliced	Fully Spliced
pEmpty	1.00	1.00 ns
HERC3	0.52 **	1.55 ns
HERC4	0.52 *	1.10 ns
HERC5	0.28 *****	0.58 ns

293T cells were co-transfected with R9 and either HERC3, HERC4 or HERC5. Forty-eight hours after transfection, total RNA was extracted and reverse transcribed into cDNA from the cytoplasmic fraction only. Quantitative PCR was performed on each fraction using primers specific to unspliced HIV-1 genomic RNA (e.g. Gag), fully spliced RNA (e.g. Rev) and β -actin (loading control). The fold change of unspliced or fully-spliced HIV-1 RNA in the cytoplasmic fraction was determined for control cells and cells expressing the HERC proteins. N=4 Significance was calculated using student's paired t test. Not significant (ns) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, ***** $P < 0.0001$)

Figure 4.5. HERC3, HERC4 and HERC5 target Rev/RRE Function

(A) Schematic depicting the different Gag constructs used in the experiment. (B) 293T cells were co-transfected with increasing concentrations (R9:HERC at 1:3, 1:6 and 1:12) of plasmids encoding the small HERC3-5 and either Rev-dependent GagPol (containing RRE) + Rev or the Rev-independent GagPol (containing 4xCTE in place of the RRE). A Gag-only (codon-optimized) construct was also transfected with either HERC3, HERC4 or HERC5 in a 1:6 ratio. Total DNA transfected was kept equal with empty vector plasmid. Gag levels within the cell lysates were analyzed by Western blotting using anti-p24CA and anti- β -actin as a loading control.

A



B

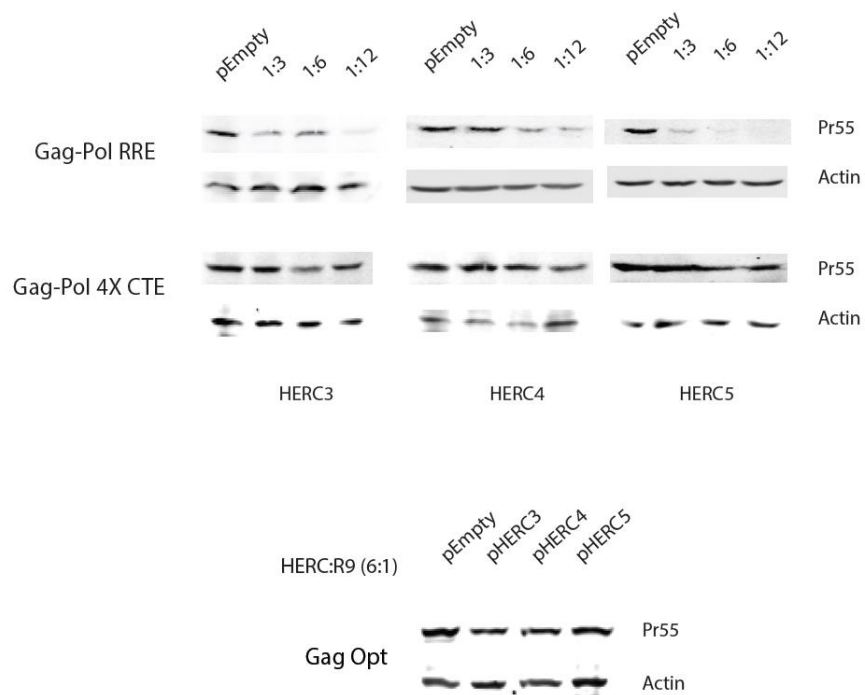


Figure 4.5: HERC3, HERC4 and HERC5 target Rev/RRE function

HERC3 and HERC4 did not exhibit detectable E3 ligase activity (**Figure 4.6**). The minor E3 ligase activity of HERC6 was lost when the HERC6 RLD was replaced with the HERC5 RLD (H6:H5-RLD-HERC6), suggesting that the HERC6 RLD is involved in protein targeting during HERC6-mediated ISGylation. It is also possible that the HERC5 RLD inactivates the HERC6-mediated E3 ligase activity.

4.2.4 Predicted structure of RLD domains of the small HERC family

HERC3 and HERC4 cause the loss of Gag accumulation and inhibit the export of genomic RNA. It is possible that the RLD of HERC3 and HERC4 are responsible for the loss of Gag accumulation similarly to the HERC5 RLD [2]. We assessed the predicted structures of the RLD from HERC3-6 to look for clues as to why HERC3, HERC4 and HERC5 restrict HIV-1 replication and why HERC6 does not (**Figure 4.7**). The HERC RLDs were modeled using 3D-Jigsaw and visualized and colored using Visual Molecular Dynamics (VMD), v1.9.2. Structural variation is most evident in blades 1 and 7 of the β -propeller. Blade 7 directly connects to the spacer region of the HERC proteins, and therefore the predicted structure of this region may vary depending on the spacer region, for which there is no predicted homology to any other protein. The predicted structures also varied in the loop regions connecting each blade of the propeller (**Figure 4.7**). The first blade of HERC3, HERC4 and HERC5 protrude away from the RLD domain. In contrast, blade 1 of HERC6 is predicted to fold back and interact with the RLD. Amino acids 1-103 of HERC5 which encompass blade 1 have been shown to be responsible for RLD-mediated restriction [2]. This suggests that the structure and orientation of blade 1 may correlate with the restriction of Gag accumulation (**Figure 4.7**).

4.2.5 Positive evolutionary selection of the small HERCs

We previously showed that HERC5 has been evolving under strong positive selection for over 75 million years [2]. The majority of the residues that are evolving under positive selection are located in the first 103 amino acids of the RCC1-like domain. We

Figure 4.6. Characterization of the small HERC ISG15 E3 ligase activity

293T cells were co-transfected with either pEmpty, pHERC3, pHERC4, pHERC5, pHERC6 or pH5:H6-RLD-HERC6. The pHERCs or pEmpty were transfected with pMyc-ISG15, pUbe1L and pUbcH8 in a 10:7.5:7.5:7.5 respectively. All cells were transfected with eGFP as well. Forty hours post-transfection cell lysates were subjected to Western blot analysis using anti-myc, anti- β actin as a loading control and anti-eGFP as a transfection control.

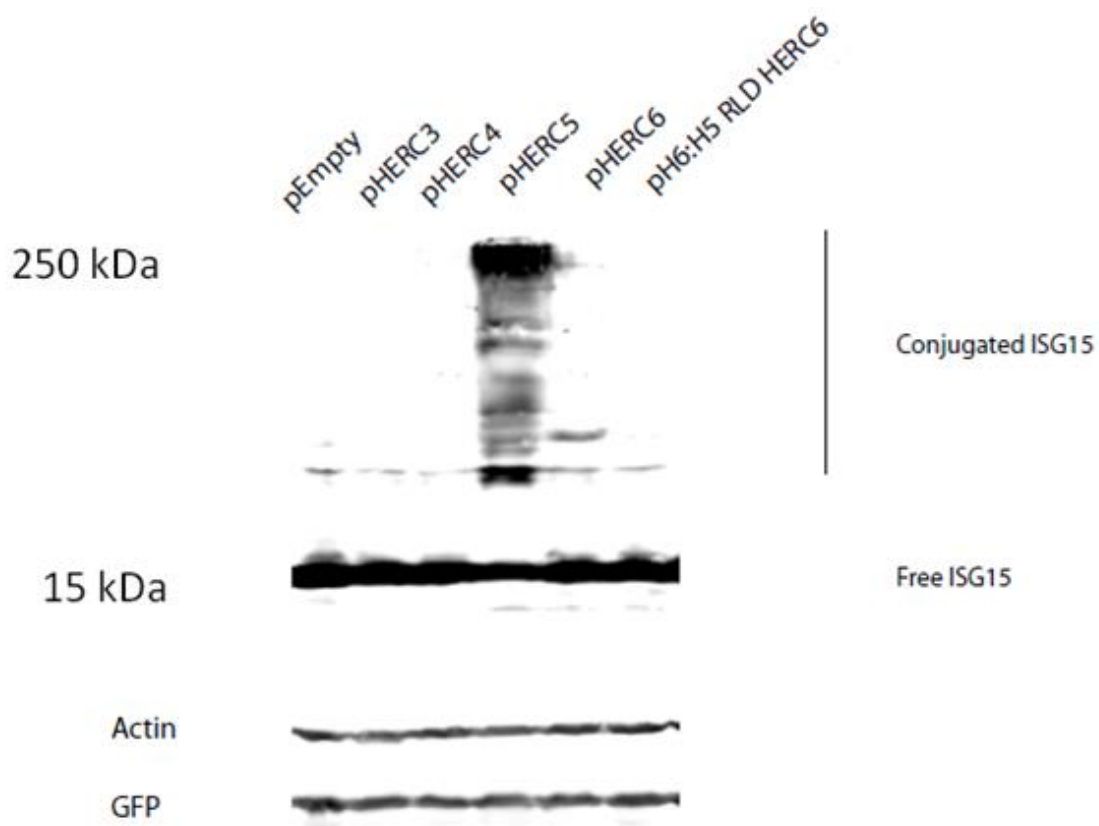


Figure 4.6: Characterization of the small HERC ISG15 E3 ligase activity

Figure 4.7: Predicted RLD structures of the small HERC family

The HERC RLDs were modeled using 3D-Jigsaw and visualized and colored using Visual Molecular Dynamics (VMD), v1.9.2. Each amino acid is colored according to the degree of conservation within the alignment: blue means highly conserved, white means somewhat conserved, and red means very low or no conservation.

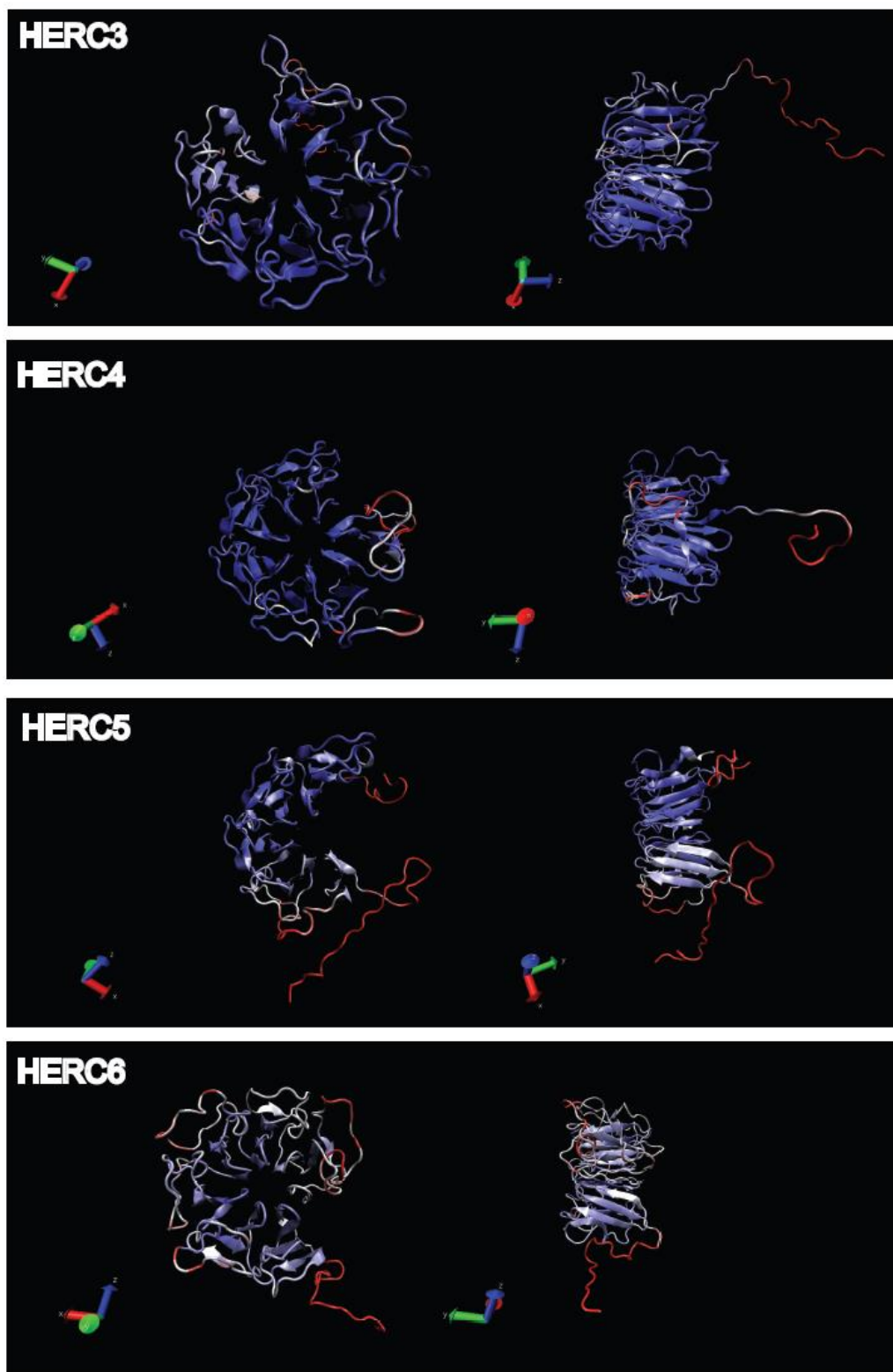


Figure 4.7: Predicted RLD structures of the small HERC family

performed a similar evolutionary analysis for positive selection for each of the small HERC members to determine if the entire small HERC family has been evolving under positive selection, possibly indicating that multiple HERC proteins have experienced genetic conflict with pathogens such as viruses during mammalian evolution. HERC evolution in mammals was evaluated under several standard models of sequence evolution using the Server for the Identification of Site-Specific Positive Selection & Purifying Selection (Selection) program [22–25]. This comprised two nested pairs of models (M8a and M8; and M7 and M8), in which the first model of each pair is nested in the second model. The M8 model, but not the M8a or M7 models, allows sites to evolve under positive selection. A non-nested pair (M8a and MEC) model comparison was also performed. The MEC model differs from the other models in that it takes into account the differences between amino acid replacement rates [22]. The nested models were compared using the likelihood ratio test. Analysis of 13 evolutionarily diverse HERC sequences revealed that HERC3 and HERC4 are evolving under strong purifying selection and are not evolving under positive selection.

Allowing sites to evolve under positive selection (M8) gave a significantly better fit to the HERC6 sequence data than the corresponding model without positive selection (M8a and M7) (**Appendix 4**). The MEC model, which allows for positive selection, was compared with the M8a null model, which does not allow for positive selection. Comparison of the AICc scores (M8a: 25806; MEC: 25557) revealed that the MEC model fits the HERC6 data better than the M8a model (**Appendix 4**). The results of the MEC analysis were projected onto the primary sequence of human HERC6 (**Figures 4.8** and **Appendix 5**). The results show that strong positive selection is operating on HERC6 and that several codons situated in the RCC1-like domain, the spacer region and the HECT domain exhibit exceptionally high probabilities of having evolved under positive selection. Notably, ~23% (23 of 102) of the codons cluster within the first 80 amino acids of the amino terminus of the RLD (**Figure 4.8**). Another ~30% (30 of 102) of positively selected amino acids cluster at the carboxyl-terminus of the spacer region. The level of positive selection operating on HERC6 was considerably higher than that predicted for HERC5, with twice as many sites evolving under positive selection [2].

Figure 4.8: Positive evolutionary selection analysis of HERC6.

(A) Neighbor-joining phylogenetic tree for progressive alignment of 12 different HERC6 species using constraint-based alignment tool (COBALT) for multiple protein sequences. Branch lengths are proportional to the amount of inferred evolutionary changes. (B) Selecton analysis for positive selection was performed using HERC6 sequences from human, chimpanzee, gorilla, marmoset, baboon, squirrel monkey, gibbon, horse, sheep, cow, dog and cat. Evolutionary analysis for positive selection in HERC6 using various models of evolution where M8 and MEC allow for sites to evolve under positive selection and M7 and M8a models do not. A plot of the Ka/Ks ratio at each codon in an alignment of HERC6 coding sequences is shown. Codons with Ka/Ks ratios >1 indicate positive selection, $=1$ neutral selection and <1 purifying selection. (C) A plot showing the results of a Bayesian analysis approach identifying sites where the Ka/Ks >1.5 and the 95% confidence interval is larger than 1; hence considered statistically significant.

4.2.6 Swapping the RLD of HERC6 with the RLD of HERC5 results in the anti-HIV-1 activity of HERC6

We have previously shown that the loss of Gag accumulation and inhibition of genomic HIV-1 RNA nuclear export is mediated by the HERC5 RLD. We have also discovered that both the HERC6 and HERC5 RLDs have evolved under positive selection demonstrating the potential importance of these domains [2]. We asked if replacing the HERC6 RLD with the HERC5 RLD (H6:H5-RLD-HERC6) confers antiviral activity to HERC6 and whether it results in the loss of Gag accumulation as observed with HERC5. Swapping out the HERC6 RLD with the HERC5 RLD converted the HERC6 protein from one that normally exhibits no antiviral activity against HIV-1 to one that now exhibits potent inhibitory activity against HIV-1 replication (**Figure 4.9**). H6:H5-RLD-HERC6 restricted HIV-1 replication to the same degree as HERC5 indicating that the HERC5 RLD is necessary and sufficient to confer antiviral activity to HERC6.

4.2.7 Small HERC expression in HIV1-infected patients

To determine if the small HERC family proteins have biological significance during the various stages of HIV-1, we mined the Gene Expression Omnibus database repository (<http://www.ncbi.nlm.nih.gov/gds>) for published datasets containing gene expression profiles in HIV-1-infected individuals at various stages of disease progression. Transcriptional profiling data revealed that HERC5 and HERC6 expression are significantly increased in lymphatic tissue during acute and asymptomatic stages of HIV-1 infection. HERC5 and HERC6 are also significantly upregulated in patients with AIDS [26]. Significant increases in HERC5 but not HERC6 expression were observed in monocytes from viremic individuals after cessation of highly active antiretroviral therapy (HAART) compared to aviremic patients during HAART [27]. HERC5 and HERC6 expression is significantly increased in CD4⁺ T cells from acutely and chronically infected patients, but not in non-progressors [28]. HERC5, but not HERC6, is significantly increased in

Figure 4.9. Swapping the RLD of HERC6 with the RLD of HERC5 results in the anti-HIV-1 activity of HERC6

293T cells were co-transfected with pR9 and peGFP and either empty vector pHERC3, pHERC4, pHERC5, pHERC6 or pH6:H5-RLD-HERC6. Transfections were done in a 1:6 ratio (R9:HERC). Forty-eight hours post-transfection, virus released into the supernatant or total cell lysates were subjected to Western blot analysis using anti-p24CA, anti- β actin as a loading control and anti-eGFP as a transfection control.

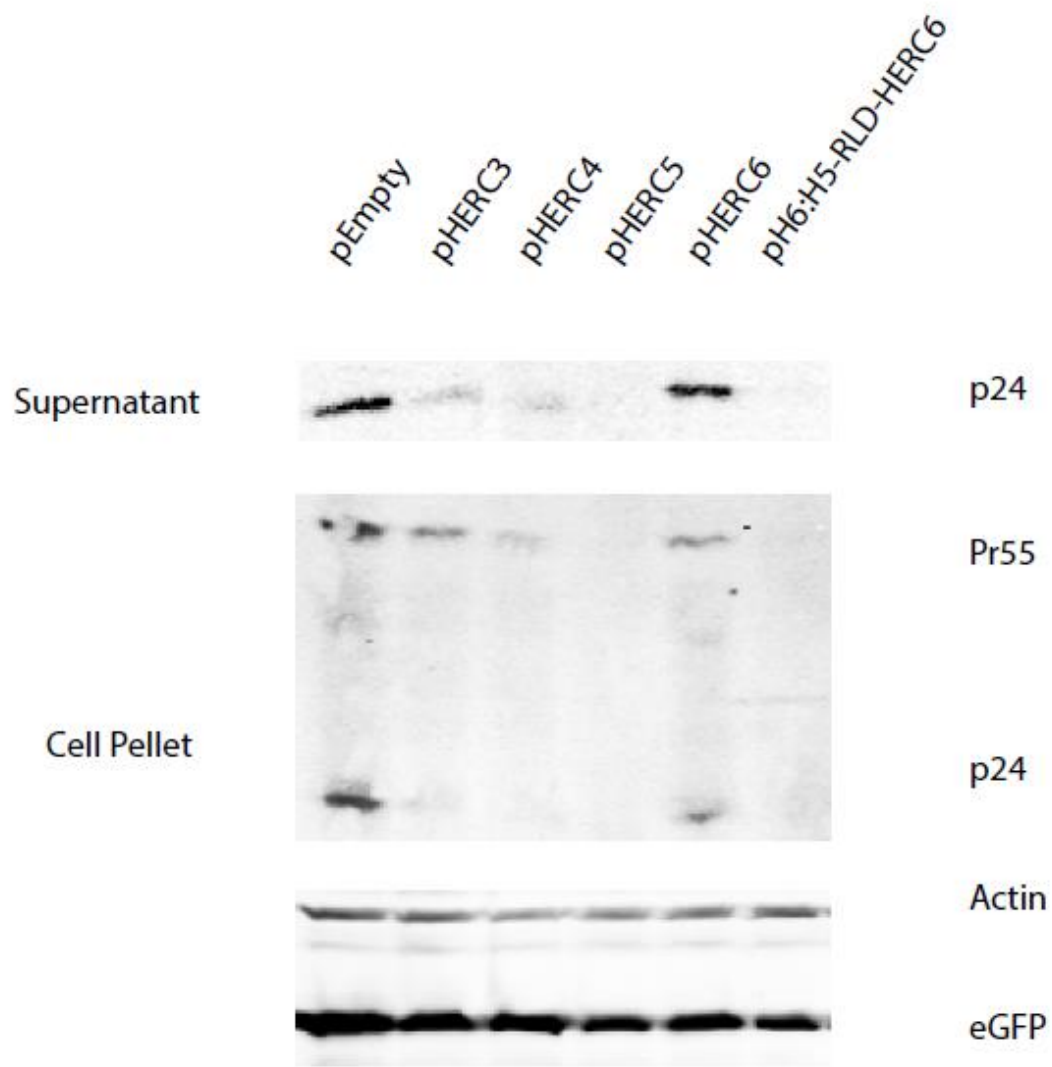


Figure 4.9: Swapping the RLD of HERC6 with the RLD of HERC5 results in the anti-HIV-1 activity of HERC6.

elite controllers and patients treated with HAART compared to HIV-1 negative patients [29]. Both HERC5 and HERC6, however, were identified as correlates of protection from HIV-1 infection in controllers compared to non-controllers (see reference [30]). There was no increase in levels of HERC3 and HERC4 transcript levels during HIV-1 infection in all of the data sets. Taken together, these data show that HERC5 and HERC6, but not HERC3 and HERC4, expression is significantly increased in patients in the acute and chronic stages of infection and in patients in stages of long-term control of infection (**Table 4.2**).

4.3 Discussion

In this study we have further characterized the structure, function and evolutionary signatures of the small HERC family. We have determined that HERC3, HERC4, HERC5 but not HERC6 restrict HIV-1 replication by decreasing the level of Gag accumulation. HERC3 and HERC4 were expressed at lower levels than HERC5 indicating that HERC3 and HERC4 are potent inhibitors of HIV-1 replication despite their relatively low levels of expression. HERC3, HERC4 and HERC5 inhibit the export of unspliced, genomic mRNA in a Rev/RRE dependent manner, which results in the loss of Gag accumulation. The predicted tertiary structure of the HERC RLDs from HERCs that restricted HIV-1 replication revealed that blade 1 protruded away from the RLD domain while blade 1 of HERC6 appeared to fold back towards and interact with, the RLD. Next, evolutionary analysis determined that only the IFN-induced HERC5 and HERC6 have been evolving under strong positive selection while HERC3 and HERC4 are evolving under purifying selection. Next, by analyzing previously published transcriptional profiling data we determined that HERC5 and HERC6 mRNA levels generally increase during the various stages of HIV-1 replication [26–29]. It was found that both HERC5 and HERC6 are upregulated in controllers of HIV-1 progression compared to non-controllers within T-cells [30].

Table 4.2: HERC expression in HIV-1-infected patients.

	HERC3	HERC4	HERC5	HERC6	Actin	Dataset
Uninfected	1.0	1.0	1.0	1.0	1.0	Li
Asymptomatic	1.0	1.0	1.8 **	1.6 *	1.0	
Acute	1.1	1.0	2.5 ***	2.4 **	1.1	
AIDS	1.0	0.9	2.5 **	2.3 **	1.0	
HIV Negative	1.0	1.0	1.0	1.0	1.0	Vigneault
Elite Controllers	1.0	0.8 ****	1.6 *	1.0	0.8 *	
HAART Treated	1.0	0.6 ****	1.6 **	0.8 ****	0.9 *	
Uninfected	1.0	1.0	1.0	1.0	1.0	
Acute	1.0	1.2	1.7 ***	2.3 ***	1.0	Hyrzca
Chronic	1.0	1.0	2.8 ***	3.3 ****	1.1	
Non-progressor	1.1	1.1	1.1	1.1	0.9	
Aviremic (on HAART)	1.0	1.0	1.0	1.0	1.0	Tilton
Viremic (off HAART)	1.6	1.4	1.5 *	1.3	1.0	

Table 4.2. HERC expression in HIV-1-infected patients. The Gene Expression Omnibus database repository was searched to identify published transcriptional profiling datasets of HIV-1- infected patient samples. The average relative RNA expression levels of HERC3, HERC4, HERC5, HERC6 and beta actin were compared for each dataset. GSE16363 dataset from Li et al. (2009) comparing patients with asymptomatic and acute stages of AIDS and patients with AIDS. GDS2168 dataset from Tilton et al. (2006) comparing monocytes from viremic individuals after cessation of HAART and aviremic patients during HAART. GDS2649 dataset from Hyrcza et al. (2007) comparing CD4+ T cells from non-progressors, and acutely and chronically infected patients. GSE23879 dataset from Vigneault et al. (2011) comparing elite controllers and patients treated with HAART. Significance was calculated using student's unpaired t test. (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, **** $P < 0.0001$)

We have previously determined that amino acids 1-103 of the HERC5 RLD are responsible for inhibiting Gag accumulation [2]. This region encompasses blade 1 of the HERC5 RLD. It is possible that blade 1 from HERC3, HERC4, HERC5 but not HERC6 are responsible for inhibiting the unspliced HIV-1 mRNA export. Blade 1 of both the HERC5 and HERC6 have evolved under strong positive selection. It is possible that blade 1 of the HERC6 RLD but not HERC5 interacts with a viral antagonist that inhibits the function or stability of HERC6. Importantly, accessory proteins such as Vif and Vpu encoded by HIV-1, have been shown to antagonize cellular restriction factors [31]. It would be important to determine if swapping the positively selected blade 1 of HERC6 with the positively selected blade 1 of HERC5 causes HERC6 to gain antiviral activity. If restriction is conferred to HERC6, alanine mutagenesis could then be performed to try and determine the specific amino acids that are important for conferring restriction.

It is also possible that the large amount of non-synonymous substitutions that occurred in blade 1 throughout evolution due to viral pressure has resulted in a defective HERC6 RLD. When the HERC6 RLD was replaced with the HERC5 RLD, HERC6 gained antiviral activity demonstrating that HERC6 is antiviral, provided it contains a functional RLD domain. We have also found that HERC6 is very polymorphic within the human population. It is possible that certain *herc6* SNPs within blade 1 confer anti-HIV-1 activity and could explain why HERC6 has been found upregulated in HIV-1 controllers [30].

We have previously shown that HERC5 interacts with Ran. HERC5 also causes a reduction in the levels of Ran-GTP and/or the interaction of Ran-GTP with RanBP1 within the cell [2]. Ran-GTP levels and the interaction between Ran-GTP and RanBP1 are essential for normal export of unspliced HIV-1 RNA. It would be interesting to determine if HERC3 and HERC4 also bind Ran and whether they too can reduce intracellular levels of Ran-GTP. It is possible that the protruding blade 1 of the RLD from HERC3, HERC4, HERC5 but not HERC6 bind and sequester Ran perturbing the essential Ran gradient.

In summary, we have characterized the antiviral activity of the small HERC family. We have determined that HERC3, HERC4, HERC5, but not HERC6 restrict HIV-1 replication by inhibiting nuclear export of unspliced mRNA. The work completed may offer further insight into the innate immune system and how it combats incoming viruses. This research may be the groundwork for drug designs that mimic the antiviral effect of HERC3, HERC4 and HERC5, which could provide a further line of defense against HIV-1 replication.

4.4 Materials and Methods

4.4.1 Cell Lines

293T and HeLa cell lines were obtained from American Type Culture Collection unless otherwise stated. Cells were maintained in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM)), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 µg/ml Streptomycin) at 37°C with 5% CO₂.

4.4.2 Plasmids, transfections and antibodies

Plasmids: The promoterless empty vector plasmid pGL3 and p3xFLAG were obtained from Promega and Sigma respectively. Plasmids pFLAG-HERC3, pFLAG-HERC4, pFLAG-HERC5, and pFLAG-HERC6 were created by amplifying inserts from each respective template using PCR and primers: HERC3— forward 5' ACG TGA ATT CCA TGT TAT GTT GGG GAT ATT GG 3' and reverse 5' ACG TGG TAC CTC AGG CCA AAC TAA ACC CTT CAT AGT TGT C 3', HERC4— forward 5'ACG TGA ATT CTA TGT TGT GCT GGG GAA ATG C 3' and reverse 5' ACG TTC TAG ATT ATA TTA AAC TGA AGC CTT CAT TGT G 3', HERC5— forward 5' AAT CGA GAT CTT ATG GAG CGC CGC AGC 3' and reverse 5' TAT GCG GAT CCT CAG CCA AAT CCT CTG 3', and HERC6— forward 5' AGA TAA GAT CTT ATG TAC TTC TGT TGG GGC 3' and reverse 5' TAG GAG ATA TCT TAT GAC TGT GTG AGC ATG 3'. Amplified products of each HERC insert were cloned into pFLAG using restriction

enzymes: HERC3— EcoRI and KpnI, HERC4— EcoRI and XbaI, HERC5— BglIII and BamHI, and HERC6— BglIII and EcoRV. The H6:H5-RLD-HERC6 construct was created using the forward 5' GGATGACGATGACAAG ATGGAGCGCCGCAGCC 3' and the reverse 5' TATGTTCCAGCAAAA TTATTAACCTCTTTTCTGAGGTATGGCTTTCAAG 3' for the HERC5 RLD insert and forward 5' TTTTGTGCTGGAACATATGCCAACTTTG 3' and reverse 5' CTTGTCATCGTCATCCTTGTAATCGATG 3' for HERC6 vector back bone. The H6:H5-RLD-HERC6 was created using the fast cloning technique [32]. Transfection: Transfections: plasmid transfections were performed using standard calcium phosphate transfection or Lipofectamine 2000 (Invitrogen). Co-transfections were performed at a ratio of 6:1 (pHERC constructs:pR9) unless otherwise stated. Antibodies: The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly. Anti-FLAG was purchased from Sigma, anti-myc and anti- β -actin was purchased from Rockland, and anti-eGFP was purchased from ClonTech.

4.4.3 Quantitative real-time PCR

Total RNA was extracted from the cytoplasmic fraction only using the R&A-BLUE Total RNA Extraction kit (Frogga Bio). 3 μ g of RNA was reverse transcribed to cDNA using the M-MLV reverse transcriptase and Oligo (dT) primers (Life Technologies). Prior to real-time PCR, cDNA samples were diluted 1:10 with water. Each PCR reaction consisted of 10 μ l of SYBR Green Master Mix, 2 μ l of Gag or Rev-specific primers (1 μ l of 10 μ M forward primer and 1 μ l of 10 μ M reverse primer), 1 μ l of diluted cDNA, and water to a total volume of 20 μ l. Real-time PCR was run on the Rotor-Gene 6000 real-time PCR machine (Corbett Life Science) under the following cycling conditions: 10 min at 95°C and 40 cycles of 10 sec at 95°C, 15 sec at 60°C, and 20 sec at 72°C. The Rotor-Gene 6000 series software (version 1.7) was used to determine the C_T for each PCR reaction.

4.4.4 Confocal microscopy

HeLa cells were cultured in 12-well plates on 18 mm coverslips and co-transfected with either HERC3, HERC4, HERC5 or HERC5. Twenty-four hours post-transfection, the coverslips containing the 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. The coverslips were incubated with primary antibodies for one hour, washed 6× with PF buffer, incubated with secondary antibodies (Alexa Fluor 546 anti-mouse) for one hour and then washed 6× with PF buffer. Coverslips were mounted onto glass slides with ~10 µl of Vectashield mounting media with DAPI (Vector Laboratories) and then sealed with nail polish. Slides were examined using a Zeiss LSM 510 confocal fluorescence microscope and images were obtained with sequential imaging.

4.4.5 Western Blotting

Clarified supernatants containing virus particles were pelleted over a 20% sucrose cushion for 2 hours at 21,000 × g. For cell lysates, cells were detached, centrifuged at 350 × g for 5 mins. Pellets were lysed with 1× RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1× Complete Protease Inhibitor (Roche), 1% Triton X-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 to FluorTransW (Pall) membrane 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 an ~16 hour incubation with 1:1000 dilution of primary antibody. Detection was carried out using IRdye-labeled secondary antibody (1:20,000 for 30 mins) and the Li-cor Odyssey Detection System (Li-cor Biosciences). Densitometric analysis was performed using ImageJ 1.43 u 64-bit version software (NIH, USA).

4.4.6 Positive selection analysis

HERC5 sequences were aligned and a phylogenetic tree generated using COBALT (<http://www.ncbi.nlm.nih.gov/tools/cobalt>) [33]. *HERC5* sequences were obtained from Genbank: *Homo sapiens* (“Human”) (NP_057407.2), *Pan troglodytes* (“Chimpanzee”) (XP_003310459.1), *Gorilla gorilla gorilla* (“Gorilla”) (XP_004039179.1), *Callithrix jacchus* (“Marmoset”) (XP_002745648.1), *Papio anubis* (“Baboon”) (XP_003898997.1), *Saimiri boliviensis boliviensis* (“Squirrel monkey”) (XP_003924055.1), *Nomascus leucogenys* (“Gibbon”) (XP_003265940.1), *Equus caballus* (“Horse”) (XP_001915115.2), *Ailuropoda melanoleuca* (Giant Panda”) (XP_002913645.1), *Ovis aries* (“Sheep”) (XP_004009762.1), *Bos taurus* (“Cow”) (NP_001095465.1), *Canis lupus familiaris* (“Dog”) (XP_535652.3), *Felis catus* (“Cat”) (XP_003985249.1). At least 2 independent sequences were available for human, sheep, baboon, marmoset, gibbon, squirrel monkey. The following sequences were not independently validated: cat, dog, cow, horse, sheep and giant panda. The identification of site-specific positive selection and purifying selection was generated using the Selecton Server (<http://selecton.tau.ac.il/index.html>) as previously described [22, 23]. The *HERC5* phylogenetic tree was used in the Selecton analysis. Nested pairs of models (M8a and M8; and M7 and M8) and a non-nested pair (M8a and MEC) were compared using the likelihood ratio test implemented in the Selecton program.

4.4.7 Statistical analyses

GraphPad Prism v5.03 was used for all statistical analyses stated in the text. *P* values and statistical tests used are stated in the text where appropriate. *P* values less than 0.05 were deemed significant.

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Chapter 5

5 Overall discussion

5.1 Summary of results

The IFN-I response potently restricts HIV-1 replication of many evolutionary diverse viruses [1–9]. Although a handful of antiviral IFN-induced proteins have been identified, further investigation is essential to completely understand the complexity of this potent anti-viral response. I have further characterized the anti-viral response of IFN-I. I have identified and characterized an IFN-induced protein called HERC5 which potently restricts HIV-1 replication by two novel and independent mechanisms [10, 11]. I have also characterized the antiviral function of the small HERC family.

In chapter 2, I characterized HERC5 as an IFN-induced protein which is able to restrict HIV-1 Gag particle production. HERC5 expressed with the Ube1L, UbcH8 and ISG15 conjugation system (CS), substantially reduced HIV-1 Gag particle production. This was correlated with the ISGylation of the HIV-1 Gag polyprotein. HIV-1 Gag particle production was restored when the de-ISGylation protease Ubp43 was added to the system, resulting in the loss of Gag ISGylation. The HERC5 E3 ligase active site at position 994 was required for the modification of Gag and did not restrict HIV-1 Gag particle production. HIV-1 Gag interacted and co-localized with HERC5. HERC5, which is normally cytoplasmic and diffuse, migrated to the plasma membrane when HIV-1 Gag was present. HERC5 did not disrupt Gag trafficking to the plasma membrane but arrested HIV-1 Gag at the plasma membrane, resulting in its accumulation at the plasma membrane. In contrast, when the CS was transfected without HERC5, fully enveloped immature HIV-1 virions were bound to the plasma membrane. These immature virions were also found in internalized compartments. In addition to HIV-1 Gag particle budding, the ability of HERC5 + CS to restrict the evolutionary diverse simple retrovirus MLV Gag particle production was assessed. Again, I saw that HERC5 + CS restricted the release of MLV Gag only particles. Unlike HIV-1 Gag, however, no ISGylated forms of MLV Gag were detected and there was an increased accumulation of MLV Gag within the cell pellet.

Lastly, I examined *herc5* gene expression in HIV-1 infected patients by analyzing transcriptional profiling data published by others and revealed that HERC5 is expressed during the acute and chronic stages of HIV-1 infection as well in patients with AIDS [12–15]. Importantly, elite controllers had significantly higher levels of HERC5 RNA in two data sets published by others [16, 17].

In chapter 3, I identified a second mechanism of HIV-1 restriction independent of the E3 ligase activity of HERC5. In cells expressing replication competent HIV-1, an E3 ligase independent mechanism of restriction, decreased Gag accumulation within the cell pellet, resulting in restricted HIV-1 particle release. The RLD domain of HERC5 was found to be essential for the loss of Gag accumulation within the cell pellet. The HERC5-C994A mutant that does not contain E3 ligase activity resulted in a greater loss in Gag accumulation compared to that of wild type HERC5. This correlated with the loss of auto-ISGylation within the HERC5-C994A mutant.

HERC5 expression resulted in the inhibition of late viral mRNA nuclear export, but not early viral mRNA transcripts. Rev/RRE-dependent expression of Gag was inhibited by HERC5; however, CTE-dependent expression of Gag was not substantially affected in the presence of HERC5. HERC5 interacted with the small GTPase Ran and reduced the level of Ran-GTP and/or the interaction with RanBP1 within the cell. Expression of HERC5 also altered HIV-1 Rev localization, causing an increase of Rev around the nuclear border as well as an increase in cytoplasmic Rev localization.

I also examined signatures of evolutionary conflict within HERC5 by identifying HERC5 residues undergoing positive selection. HERC5 was shown to evolve under strong positive selection. Twenty-seven out of the 50 positively selected residues clustered within the first two blades of the RLD. When these two blades were deleted, HERC5 no longer was able to reduce the levels of intracellular level of Gag.

In chapter 4, I explored the anti-HIV-1 activity of the entire small HERC family. HERC3, HERC4 and HERC5 restricted HIV-1 particle production and caused the loss of Gag accumulation within the cell pellet. HERC6, however, had no effect on HIV-1 particle production. I determined that both HERC5 and HERC6 have evolved under strong

positive selection. Furthermore, after analysis of transcriptional profiling data published from others, it was revealed that HERC6 expression in HIV-1 infected patients was strikingly similar to the expression of HERC5 [12–16]. HERC3 and HERC4 have not evolved under positive selection and generally the expression levels of HERC3 and HERC4 did not change in HIV-1 infected patients [12–16]. When the HERC6 RLD was replaced with HERC5 RLD (H6:H5-RLD-HERC6), HERC6 was able to potently inhibit Gag accumulation and HIV-1 particle production. I also determined that HERC5 was the only small HERC that had the ability to act as a major cellular ISG15 E3 ligase capable of modifying many cellular proteins. HERC6, however, had modest ISGylation activity and mediated the ISGylation of a protein or proteins with that were/are approximately 60kDa in size.

In summary, I have identified an IFN-induced protein called HERC5 with a novel activity capable of restricting HIV-1 replication by two independent mechanisms and have characterized the antiviral functions of the entire small HERC family.

5.2 HERC5 mediated ISGylation of HIV-1 Gag is a potent inhibitor of HIV-1 assembly

HERC5 + CS modifies HIV-1 Gag with ISG15, which correlates with the restriction of an early stage of HIV-1 Gag assembly. HERC5-mediated ISGylation of Gag may inhibit an early step of Gag budding by inhibiting or reducing Gag ubiquitination by modifying Lys residues with ISG15 that are normally ubiquitinated. It has been shown that Gag ubiquitination is crucial for normal assembly and release of HIV-1 [18–20]. It is also possible that the ISGylation of Gag may inhibit normal Gag-Gag interaction, which is essential for normal assembly and release [21–23]. ISG15 is a relatively bulky protein composed of two ubiquitin-like domains [24], which may result in steric hindrance disrupting Gag-Gag interactions. Multiple examples of ISG15-mediated inhibition of normal protein-protein interactions have been identified and characterized [25–27, 24]. Gag may also be ISGylated in the basic region of NC^{Gag}, which is essential for Gag oligomerization. NC^{Gag} binds RNA which acts as a scaffold between multiple Gag proteins [22].

ISGylation of HIV-1 Gag could inhibit the interaction between host factors essential for driving the formation of fully enveloped immature virions. For example, the ATP-binding cellular protein (ABCE1) binds Gag and is involved in HIV-1 assembly [28]. It is possible that the ISGylation of Gag inhibits the interaction between Gag and ABCE1 interfering with normal assembly. It has also been shown that HIV-1 Gag can remodel the actin cytoskeleton [29]. Actin polymerization has been thought needed to drive the membrane curvature and physical force to allow HIV-1 and Gag virus-like particles to bud. The NC region of Gag has been shown to bind actin [30, 31] and when the NC region is mutated, HIV-1 Gag can no longer initiate cytoskeleton rearrangement [29]. If Gag is ISGylated in the NC region by HERC5, HIV-1 Gag may not initiate actin polymerization at the budding site resulting in the sequestration of Gag at the plasma membrane.

To further characterize the anti-HIV-1 effect of HERC5-mediated ISGylation of HIV-1 Gag, the specific modified Lys residues must be identified. HIV-1 is modified with ISG15 on multiple residues. To determine where Gag is modified with ISG15, HIS-tag purification then immunoprecipitation (IP) followed by MALDI-TOFF mass spectrometry could be performed. Identification of HIV-1 Gag peptide mass increases will indicate which peptides of HIV-1 Gag are modified with ISG15. Once the modified peptides are identified, the Lys residues within the peptide will be sequentially mutated to Ala to determine which mutation causes the loss of Gag modification. Identifying the sites of ISGylation could provide clues on how Gag assembly is interrupted.

I determined that the expression of HIV-1 Gag only (no other HIV-1 proteins expressed) with HERC5 + CS resulted in the modification of Gag multiple times with ISG15. It would be interesting to determine if the ISGylation of HIV-1 Gag remains present during the expression of replication competent HIV-1. It is possible that HIV-1 possesses an antagonist that cleaves ISG15 from Gag. Another important function of the HIV-1 protease, may be to cleave ISG15 from HIV-1 Gag. Other viruses such as the SARS-CoV encode a protease that can cleave ISG15 from substrate proteins [32–34]. HIV-1 may also have the ability to recruit the specific ISG15 protease, Ubp43, to the site of assembly to promote viral budding. HIV-1 could also encode an antagonist that inactivates the E3

ISG15 ligase activity of HERC5. Other viruses have been shown to possess similar mechanisms. For example the NS1B protein from IBV has been shown to inhibit HERC5 mediated ISGylation [35].

5.3 HERC5 is a potent inhibitor of REV/RRE mediated export of late HIV-1 transcripts

The RLD of HERC5 was determined to be responsible for the loss of Gag accumulation by inhibiting the export of unspliced HIV-1 mRNA. The RLD domain is homologous to the crucial nuclear RCC1 protein which is a GEF for the GTPase Ran [37–40]. Like RCC1, HERC5 interacts with Ran. The RLD domain may be responsible for this interaction. It was determined that HERC5 dramatically reduces levels of Ran-GTP and/or inhibits the interaction between the crucial RanBP1 and Ran-GTP protein. The cytoplasmic HERC5 protein may bind and sequester Ran in the cytoplasm, not allowing Ran-GDP to move to the nucleus where it is converted to Ran-GTP. The RLD1 of HERC1 has also been shown to act as a GRF for ARF and Rab proteins, which are proteins involved in membrane trafficking [41, 42]. It would be interesting to know if the RLD of HERC5 acts as a GRF, which results in the release of GDP from Ran which could explain the loss of cellular Ran-GTP.

To test if the RLD of HERC5 acts as a GRF, the HERC5 RLD could be purified by cloning it into a bacterial expression vector including a HIS tag. RCC1 would also be cloned into this vector to act as a positive control. I have successfully purified the recombinant His-tagged Ran-GTPase using a nickel HPLC column (**Appendix 6**). GRF activity of the RLD of HERC5 would be measured using fluorescent GDP analogue. The purified Ran would be pre-loaded with fluorescent GDP. GDP will only fluoresce when bound to Ran but will lose its fluorescence if it dissociates. A decrease of fluorescence in the presence of the HERC5 RLD would indicate GRF activity.

Most cellular mRNA does not require the Ran-GTP nuclear energy gradient and uses an entirely different export pathway. Generally, mRNA is exported out of the nucleus using the NXF1 (Tap) and the NXT1 (p15) proteins after it is fully spliced. This process is Ran-

GTP independent as discussed in chapter 1 [43, 44]. To determine what nuclear export pathways are affected by HERC5 HIV-1 Gag constructs that either expressed Gag using the RRE/Rev/Ran dependent pathway, or expressed Gag using the CTE/NXF1/NXT1 Ran independent pathway, were transfected with HERC5. HERC5 potently inhibited the Rev/RRE Ran-dependent expression of Gag, while it did not substantially affect the CTE/NXF1/NXT1 Ran-independent expression of Gag. This indicates that HERC5 shuts down Ran-dependent export while maintaining the expression of Ran-independent mRNA and therefore may act as a nuclear regulator during viral infection. HERC5 expression could allow the expression of cellular mRNA which includes mRNAs that encode antiviral proteins while inhibiting viruses that harness the Ran-GTP gradient to replicate.

5.3.1 HERC5 auto-ISGylation and HIV-1 restriction

The E3 ligase mutant (C994A-HERC5) inhibited the nuclear export of incompletely-spliced HIV-1 RNA and the intracellular production of HIV-1 Gag protein, more potently than the E3 ligase active HERC5. This raised the intriguing possibility that auto-ISGylation impedes the RLD-mediated mechanism of restriction. E3 ligase active HERC5 was found to auto-ISGylate itself with ISG15, while C994A-HERC5 did not. Auto-ISGylation of HERC5 may act as a “switch” to turn off or reduce the RLD-mediated mechanism of restriction. Auto-ISGylation may be required for HERC5 to tightly regulate nuclear import and export. Similarly, the E3 ISG15 ligase TRIM25 undergoes auto-ISGylation which impedes its ability to target and ISGylate its substrate 14-3-3 sigma, a protein involved in many cellular signaling pathways [45].

The sites of HERC5-mediated auto-ISGylation will be crucial to identify in order to fully understand the antiviral function of HERC5. Identification of these sites will give us insight into why HERC5-C994A causes a greater reduction in accumulated intracellular Gag compared to the E3 ligase active HERC5. It is possible that the auto-modification of HERC5 inhibits its interaction with Ran not allowing HERC5 to perturb the Ran-GTP gradient. This could be tested by performing a co-IP for Ran with either HERC5 or HERC5-C994A to determine if HERC5-C994A interacts with Ran to a greater degree.

5.3.2 HERC5 has evolved under positive selection

HERC5 is a multifunctional protein shown to be a potent antiviral factor that inhibits IAV, HPV and HIV-1 replication [10, 11, 46, 47]. Multiple viruses most likely have co-evolved with HERC5 resulting in signatures of genetic conflict. The first 103 amino acid region of the RLD domain is composed of multiple residues that have undergone strong positive selection. This region is essential for the RLD-mediated mechanism of restriction. It is possible that a virus, or multiple viruses, have developed viral antagonists throughout evolution that interact with this region resulting in the inhibition of the RLD-dependent anti-viral activity. Interestingly, it has been determined that the first 100 amino acids of HERC5 are crucial for its ribosomal binding allowing for HERC5-mediated ISGylation of newly synthesized proteins [46]. Multiple viruses may have developed antagonists that bind to the first 100 amino acids of the RLD inhibiting the interaction between HERC5 and the ribosome. This could severely effect HERC5's global ISG15 E3 ligase activity which has been shown to interfere with the replication of multiple viruses [40, 46, 47]. There are also strongly positive selected residues within the spacer region; however, the function of this region is unknown. It is possible that this domain is involved in viral targeting or aiding in the interaction between Ran, the ribosome, Ube1L and/or UbcH8.

5.4 HERC3, HERC4 and HERC5 but not HERC6 inhibit cellular Gag accumulation and restrict HIV-1 particle production

HERC3, HERC4 and HERC5 restricted HIV-1 particle production and caused the loss of Gag accumulation while HERC6 did not. HERC3 and HERC4 restrict the export of unspliced HIV-1 mRNA in a similar manner to HERC5. HERC6 may not restrict HIV-1 replication due to an antagonist HIV-1 contains that may inhibit the function of HERC6 but not HERC5. To determine if HERC6 is antagonized by proteins encoded by HIV-1, HIV-1 mutants that have accessory proteins deleted, such as Nef, Vpr, Vpu and Vif could be tested. All of these accessory proteins have been shown to antagonize other antiviral

proteins [48–53]. It is also possible that HERC6 has lost its antiviral activity against HIV-1 throughout millions of years of evolution.

Both HERC5 and HERC6 display signs of genetic conflict and have evolved under strong positive selection. The IFN-induced *herc5* and *herc6* genes are located right next to each other on chromosome 4, and were most likely created from a gene duplication event [39]. Similarly, the positively selected IFN-induced *mxα* and *mxβ* genes are also located right next to each other and have both been shown to have antiviral properties. MXA has been shown to potently inhibit a wide range of evolutionary diverse RNA and DNA viruses, but cannot restrict HIV-1 replication [54–61]. Until recently, it was thought that MXB did not contain antiviral activity, until it was discovered that MXB restricts HIV-1 replication [62–68]. It is therefore possible that HERC5 can restrict HIV-1 while HERC6 can target and restrict multiple other viruses.

It will be essential to determine the range of antiviral activities of both HERC5 and HERC6 due to the fact that these proteins have evolved under strong positive selection. Replication of evolutionary diverse viruses could be tested in the presence of HERC5 and HERC6 to determine if these proteins have antiviral activity. Interestingly, it was shown that HERC6 has limited ISG15 E3 ligase activity, which is dependent on its RLD domain. It is possible that HERC6's RLD domain targets certain viral proteins for ISGylation, which could restrict viral replication.

In humans, it has been shown that HERC5 is the main ISG15 E3 ligase, however mice do not possess HERC5. Instead the mouse HERC6 protein is the main ISG15 E3 ligase [69, 70]. This suggests that either HERC5 or HERC6 must be a functional ISG15 E3 ligase and antiviral protein. Testing HERC5 and HERC6 orthologs from evolutionary diverse mammals could assess the evolutionary conservation of the antiviral effect of HERC5 and HERC6 as well as cellular ISGylation. ISG15 E3 ligase assays, as well as virus release assays, could be conducted to determine if either HERC5 or HERC6 act as the main cellular ISG15 E3 ligase within these mammals, and whether these orthologs restrict viral replication. Feline Immunodeficiency Virus and Equine Infectious Anemia Virus are both complex retroviruses, found in cats and horses (respectively), and use

Rev-mediated mRNA export in order to replicate efficiently [71, 72]. It will be interesting to determine if HERC5 or HERC6 from cats and horses restrict export of FIV and EIAV late mRNAs and/or Gag assembly. These experiments would provide us with information that will help us better understand the antiviral role of HERC5 and HERC6 throughout evolution.

5.5 HERC polymorphisms

Although the mutation rate in viruses is much higher than in the host's, the host has additional advantages that viruses do not to maintain the *status quo* during evolutionary conflict. For example, humans are diploid and can contain two forms of antiviral proteins due to polymorphisms within the population. HERC5 is highly polymorphic with multiple alleles within the population. This may increase the breadth and alter the potency of HERC5's antiviral function. We have identified polymorphisms in HERC5 which cause a frame shift mutation resulting in a truncated HERC5 protein with no HECT domain [11]. Without the HECT domain, HERC5 would not be able to modify cellular and viral proteins with ISG15 possibly making these individuals more susceptible to viral infection. I have shown however, that HERC5's RLD-mediated mechanism of restriction is more potent when HERC5's E3 ligase activity is not functional. These individuals may control HIV-1 replication more efficiently via increased RLD activity. As such, it would be interesting to determine if there is a correlation between HERC5 polymorphisms and HIV-1 disease progression. HERC5 polymorphism could potentially be used as a marker to predict disease progression.

It is also possible that polymorphisms within the positively selected *herc6* gene may activate an anti-HIV-1 function within HERC6. These polymorphisms could be generated using site-directed mutagenesis followed by a virus release assay to determine if certain HERC6 polymorphisms have the ability to restrict HIV-1.

5.6 Concluding remarks

It is well known that the IFN-I response can restrict the replication of multiple evolutionary diverse viruses due to the upregulation of hundreds of ISGs, many

demonstrating antiviral activity. While the IFN-I response has been extensively studied, only a small fraction of the IFN-induced cellular restriction factors have been identified and characterized. I have further characterized the anti-HIV-1 effect of the IFN-I response by identifying a novel IFN-induced protein called HERC5.

HERC5 is a potent antiviral protein that can restrict HIV-1 replication by two novel mechanisms. First, HERC5 restricts the export of unspliced HIV-1 RNA, which is required for the production of HIV-1 Gag and is mediated by the RLD domain [10]. The small amount of Gag that is produced in the presence of HERC5 is stopped by the second E3-ligase dependent mechanism of HERC5, which modifies Gag with ISG15 to inhibit assembly of HIV-1 particles [11] (**Figure 5.1**). This makes HERC5 a very potent restriction factor that has two lines of defence against HIV-1 replication.

It was also determined that overexpression of the HERC family members, HERC3 and HERC4, have the ability to restrict HIV-1 replication by inhibiting late HIV-1 protein production. This may indicate that the HERC family may be a family of restriction factors similarly to the APOBEC3, IFITM, TRIM and Mx families [68, 73–76].

HERC5 is significantly upregulated during all stages of HIV-1 infection yet some individuals still progress to AIDS. Although HERC5 is upregulated, it is possible that polymorphism within HERC5 alter the antiviral function not allowing HERC5 to control HIV-1 in certain individuals. This could explain differences in disease progression between infected individuals. It is also possible that the large frequency of mutations and heterogeneity of HIV-1 allow for escape mutations or the creation of a viral antagonist against HERC5. Of importance, however, HERC5 has been identified as a protein that is expressed significantly higher in HIV-1 controllers compared to typical progressors [17].

In summary, I have identified HERC5 as a restriction factor that encompassed three of the hallmarks of a *bonafide* restriction factor. First, HERC5 is induced by viral infection and is IFN induced. Second, HERC5's main biological function is antiviral in nature and lastly HERC5 shows sign of genetic conflict and has evolved under strong positive selection. Further characterization of HERC5 and the HERC family may provide us with further understanding of HIV-1 replication and alternative methods to combat this

Figure 5.1: HERC5 is a multifunctional antiviral protein

HERC5 is a potent antiviral protein that can restrict HIV-1 replication by two novel mechanisms. First, HERC5 restricts the export of unspliced HIV-1 RNA. Gag that is produced in the presence of HERC5 is stopped by the second E3-ligase dependent mechanism of HERC5, which modifies Gag with ISG15 to inhibit budding of HIV-1 particles.

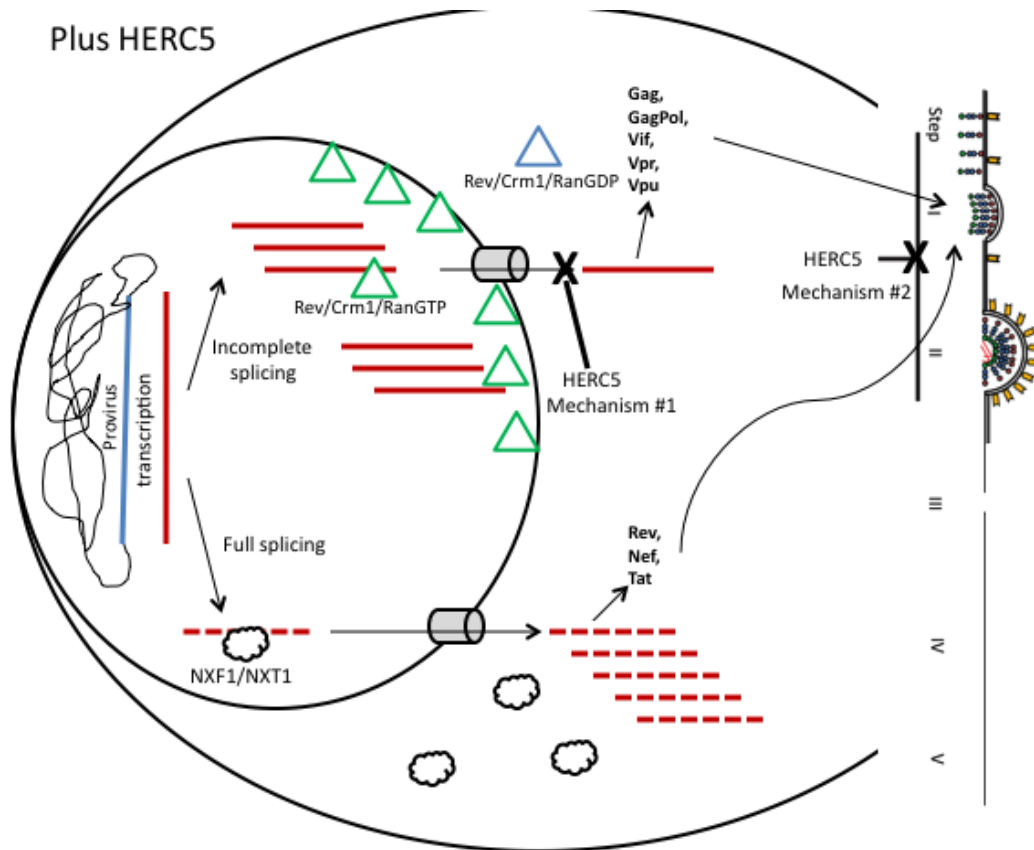


Figure 5.10: HERC5 is a multifunctional antiviral protein

disease. To date there are no drugs that inhibit the export of unspliced HIV-1 RNA or drugs that inhibit assembly. This research may be the ground work for drug development that mimic or enhance the effect of HERC5.

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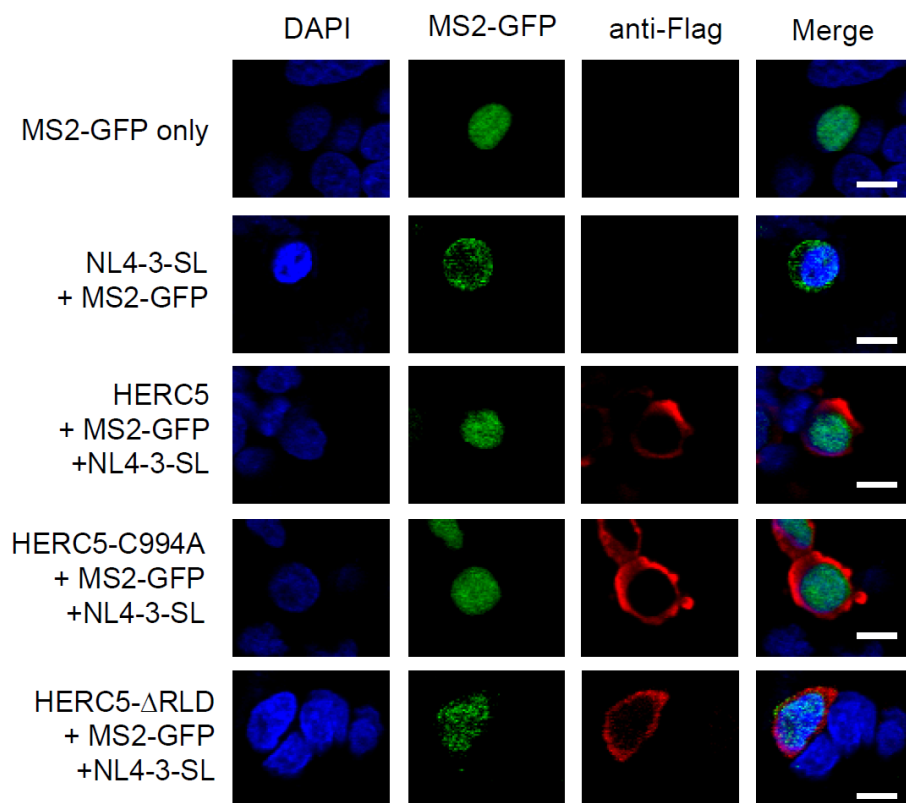
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Appendix

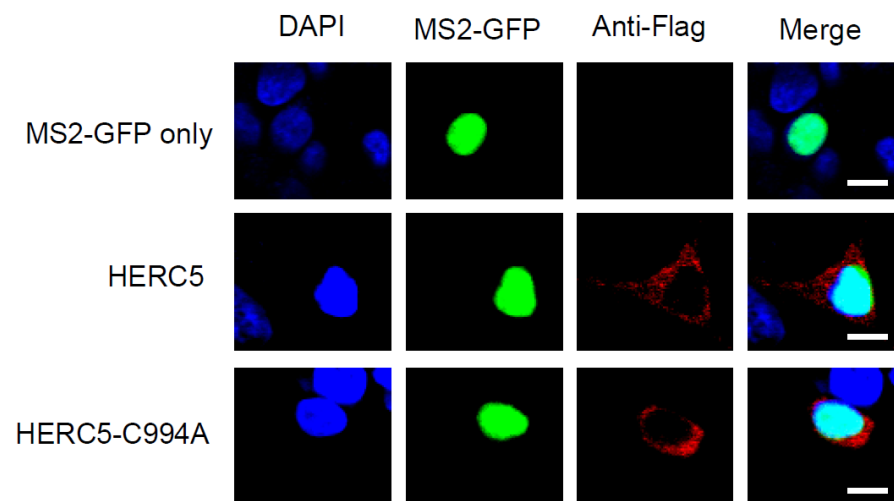
Appendix 1: HERC5 inhibits nuclear export of HIV-1 genomic RNA.

A, HeLa cells were co-transfected with plasmids encoding MS2-GFP alone, MS2-GFP and NL4-3-SL, or MS2-GFP, NL4-3-SL and either flag-tagged HERC5, HERC5-C994A or HERC5- Δ RLD. Forty-eight hours post-transfection, cells were fixed, stained with anti-flag and DAPI and imaged using fluorescence confocal microscopy. **B**, Over-exposure of cells exhibiting nuclear localization of MS2-GFP. HeLa cells were co-transfected with plasmids encoding MS2-GFP and NL4-3-SL and either flag-tagged HERC5, or HERC5-C994A. Forty-eight hours post-transfection, cells were fixed, stained with anti-flag and DAPI and imaged using fluorescence confocal microscopy. Cells exhibiting nuclear localization were imaged after increased laser intensity.

A



B



Appendix 1: HERC5 inhibits nuclear export of HIV-1 genomic RNA.

Appendix 2: Analysis for positive selection in HERC5

Nested models	Log-likelihood	$2(\ln L_1 - \ln L_0)$	Degrees of Freedom	χ^2
M8a, M8	-14212, -14193	38	1	<0.001
M7, M8	-14224, -14193	62	2	<0.001

Non-nested model	Log-likelihood	AIC _c Score ^Δ : $-2 \cdot \log L + 2p \cdot (N/N - p - 1)$	Parameters
M8a, MEC	-14212, -14003	28432, 28016	M8a: 4 free parameters MEC: 5 free parameters

^Δ L represents the likelihood of the model given the data, p represents the number of free parameters and N represents the sequence length. The lower the AIC_c score, the better the fit of the model to the data, and hence the model is considered more justified.

Appendix 3: Selection results for HERC5 run on 13 HERC5 sequences with the MEC model.

Positive selection is colored in shades of yellow, neutral selection in white, and purifying selection in shades of magenta. Shades of yellow (colors 1 and 2) indicate a Ka/Ks ratio > 1 , white indicates a Ka/Ks ratio $= 1$, and shades of magenta (colors 3 through 7) indicate a Ka/Ks ratio < 1 .



Legend:
 The selection scale:
 1 2 3 4 5 6 7
 Positive selection Purifying selection

Box 1 Sequences queried	
NP_057407.2	= Human
XP_003310459.1	= Chimpanzee
XP_004039179.1	= Gorilla
XP_002745648.1	= Marmoset
XP_003898997.1	= Baboon
XP_003924055.1	= Squirrel monkey
XP_003265940.1	= Gibbon
XP_001915115.2	= Horse
XP_002913645.1	= Panda
XP_004009762.1	= Sheep
NP_001095465.1	= Cow
XP_535652.3	= Dog
XP_003985249.1	= Cat

Appendix 3: Selection results for HERC5 run on 13 HERC5 sequences with the MEC model.

Appendix 4: Analysis for positive selection in HERC6.

Nested models	Log-likelihood	$2(Ln_1 - Ln_0)$	Degrees of Freedom	χ^2
M8a, M8	-12899, -12871	56	1	<0.001
M7, M8	-12912, -12871	82	2	<0.001

Non-nested model	Log-likelihood	AIC_c Score^Δ: $-2 \cdot \log L + 2p \cdot (N/N - p - 1)$	Parameters
M8a, MEC	-12899, -12773	25806, 25557	M8a: 4 free parameters MEC: 5 free parameters

^Δ L represents the likelihood of the model given the data, p represents the number of free parameters and N represents the sequence length. The lower the AIC_c score, the better the fit of the model to the data, and hence the model is considered more justified.

Appendix 5: Selection results for HERC6 run on 13 HERC6 sequences with the MEC model.

Positive selection is colored in shades of yellow, neutral selection in white, and purifying selection in shades of magenta. Shades of yellow (colors 1 and 2) indicate a Ka/Ks ratio > 1 , white indicates a Ka/Ks ratio $= 1$, and shades of magenta (colors 3 through 7) indicate a Ka/Ks ratio < 1 .

```

1           11           21           31           41
MYFCWGADSR ELQRRRTAGS PGAE LLQAAS GERHSL LLLT NHRVLS CGDN
51          61          71          81          91
SRGQLGRRGA QRGELPEPIQ ALE LIVDLV SCGKEHSLVY CHKGRVFWG
101         111        121        131        141
AGSGQLGIG FKEISFTK KIM LND IYSCGHYHS LLSKDSQVF
151        161        171        181        191
SWGKNSHGQL GLGKEFPSQA SPQVRSLG IPLQVNGG AHSPALS LCG
201        211        221        231        241
SEGWGNSA GQLALSGRV PVQSNKPLSV GALKNLGVVY ISCGDAHAV
251        261        271        281        291
LTQDG VFF GD RSGQLGY SPTPEKRGPO LVER DGLS QICGSYHTL
301        311        321        331        341
YVHTGQVY SFGHGPSDTS KPT PEALTE NFDISCLIS EDFVDVQVNH
351        361        371        381        391
FAGTYA FV HHQDTSSTR APG TLPE S RISQSMAEW IAVKRRSTH
401        411        421        431        441
EMAKS IIRM FSSPCLLAL FLK LGTGT TSIQVDLEM R TFKKLKK
451        461        471        481        491
EWSSMI TC LEDLLR LFP CHSPHQALS V FLLLP CPV HSKWKNL
501        511        521        531        541
VFPK VCE LSKQSLQVLK KCWAPLQSS LNPLIQ LLA AIISQLLHQT
551        561        571        581        591
KTEQDHCNV ALLGMM ELH K KANCRLP NTFN LLS NLLFYIDG
601        611        621        631        641
RQLFRDNHL PAETPSPVIF HPPFIENSL S KLLQDS HIKMQMSEKK
651        661        671        681        691
AYMLMHETIL QKKDEFPSPS R F I LRVRRS L K L RQL QA ADFCV
701        711        721        731        741
LVV F IN C PESGGV SSEF F HCM F ETK PEYG FMYF MGSC WFFAK
751        761        771        781        791
FPEKRVFL TGMLCGLSLF L VAN L PFP L LYK LLDQ KPSLE L L L
801        811        821        831        841
PRIGKSLQ VLDDAADDIG DALCIRTS H WDQ D D L F NGISIPVDQT
851        861        871        881        891
KRDYYSKYI YIFVSVKA YEFQGFY RVCEKEILRH FYPE L L T I
901        911        921        931        941
IGT YDWKQ TQSKVEQG YQKS P IQL F W F H K L L D K K F L F L
951        961        971        981        991
TGR RLHARG IQKME VERC PE F R RHP TSITCN LLS LKYS I IERM
1001       1011       1021
EEALQVAIIN RGEVSEMIT QS

```

Legend:

The selection scale:

1 2 3 4 5 6 7

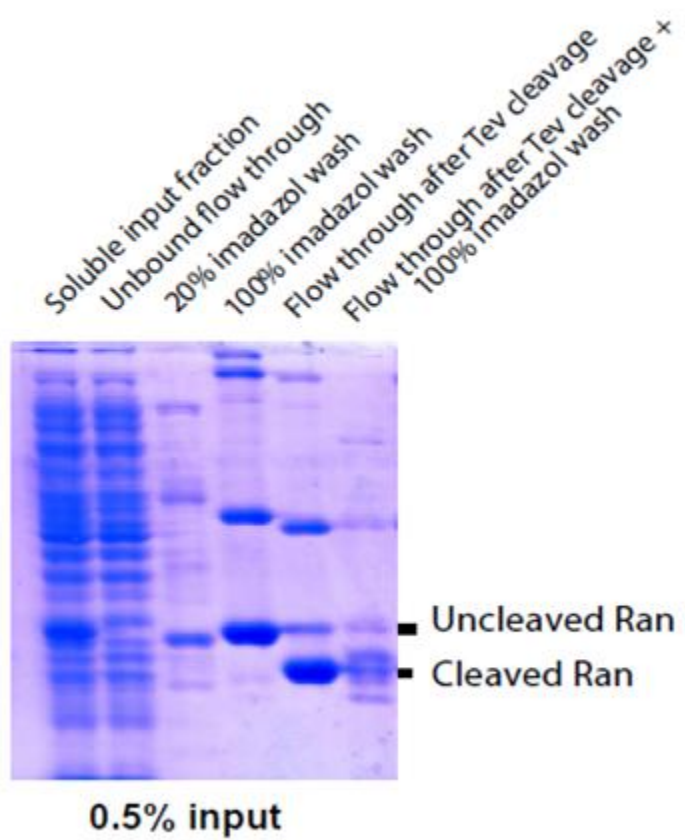
Positive selection Purifying selection

Box 1 Sequences queried	
NP_057407.2	= Human
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XP_003265940.1	= Gibbon
XP_001915115.2	= Horse
XP_002913645.1	= Panda
XP_004009762.1	= Sheep
NP_001095465.1	= Cow
XP_535652.3	= Dog
XP_003985249.1	= Cat

Appendix 5: Selection results for HERC6 run on 13 HERC6 sequences with the MEC model.

Appendix 6: Purification of recombinant Ran

Ran was cloned into pET28a with an amino-terminal histidine tag and a TEV cleavage site separating the tag and Ran. *E. coli* BL21(DE3) cells were transformed with the constructs and grown to an O.D.=0.8 at 37°C. Cells were induced with IPTG (1 mM) for 16 hours at room temperature, lysed, and subjected to standard purification over nickel agarose. Beads were treated with a 20% imidazole stringency wash, followed by a 100% imidazole wash to measure the efficiency of binding and assess the degree of non-specific protein binding. Recombinant proteins bound to the nickel agarose were washed with 20% imidazole, followed by cleavage with TEV to release the target protein from the histidine tag, which remained bound to the column. The identity of the target proteins was confirmed by Western blotting with anti-histidine. 0.5% Ran fractions were loaded on the SDS-PAGE gel.



Appendix 6: Purification of Ran.

Appendix 7: Permission to publish partial 1.5.8 (ISG15) section

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Appendix 8: Permission to publish chapters 2 and 3

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Curriculum Vitae

Name Matthew William Woods

Education

2009	BSc	Western University, London, Ontario, Canada Honors Specialization in Microbiology and Immunology (Distinction)
2009-2015	PhD	Western University, London, Ontario, Canada Department of Microbiology and Immunology Supervisor: Dr. Stephen Barr

Honors and Awards

2014		International AIDS Society/ French National Agency for Research on AIDS Young Investigator Award (International)
2014		AIDS 2014: 20th International AIDS Conference Travel Scholarship (International)
2013-2016		Canadian Institutes of Health Research (CIHR) Doctoral Research Award: HIV/AIDS (National)
2013		Ontario Graduate Scholarship (OGS) (Declined to accept National award) (Provincial)
2013		Ontario HIV Treatment Network (OHTN) travel Award (Provincial)
2012		Ontario Graduate Scholarship (OGS) (Provincial)
2012		Queen Elizabeth II Graduate Scholarships in Science and Technology (QEII GSST) (Declined to accept more prestigious Provincial award) (Provincial)
2012		Ontario HIV Treatment Network (OHTN) travel Award (Provincial)

2012	Graduate Thesis Research Award Fund recipient. (Internal)
2011	Microbiology and Immunology Graduate Entrance Award. (Internal)
2011	Graduate Thesis Research Award Fund recipient (Internal)
2010	Cedarlane Infection and Immunity Research Forum (IIRF) Best oral presentation

Research Publications

1. **Woods MW**, Tong JG, Tom SK, Szabo PA, Cavanagh PC, Dikeakos JD, Haeryfar SMM, Barr SD: Interferon-induced HERC5 is evolving under positive selection and inhibits HIV-1 particle production by a novel mechanism targeting Rev/RRE-dependent RNA nuclear export. *Retrovirology* 2014, 11:27. (Impact Factor at time of publication: 5.66) Highly accessed*
2. **Woods MW**, Kelly JN, Hattlmann CJ, Tong JGK, Xu LS, Coleman MD, Quest GR, Smiley JR, Barr SD: Human HERC5 restricts an early stage of HIV-1 assembly by a mechanism correlating with the ISGylation of Gag. *Retrovirology* 2011, 8:95. (Impact Factor at time of publication 6.47) Highly accessed*
3. **Woods MW**, Barr SD: Human HERC5 is a Novel E3 Ligase that Restricts an Early Stage of HIV-1 Assembly. In *Advances in Virus Research I*. Edited by Press iConcept. iConcept Press Ltd.; 2013.
4. Kelly JN, **Woods MW**, Xhiku S, Barr SD: Ancient and Recent Adaptive Evolution in the Antiviral TRIM22 Gene: Identification of a Single-Nucleotide Polymorphism That Impacts TRIM22 Function. *Hum Mutat* 2014, 35:1072–81.
5. McAllister RG, Liu J, **Woods MW**, Tom SK, Rugar CA, Barr SD: Lentivector integration sites in ependymal cells from a model of metachromatic leukodystrophy: non-B DNA as a new factor influencing integration. *Mol Ther Nucleic Acids* 2014, 3:e187.
6. Jenna N. Kelly, Jessica G.K. Tong, Clayton J. Hattlmann, **Matthew W. Woods**, and Stephen D. Barr. (2011). ISBN 978-953-307-665-2. Book Title: HIV and AIDS: Updates on Biology, Immunology, Epidemiology and Treatment Strategies. Chapter Title: Cellular Restriction Factors: Can We Exploit the Body's Natural Antiviral proteins to Combat HIV/AIDS?

Related Work Experience

2010-2013 Teaching Assistant
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Committees and Activities

2011-Present Virtual Researcher on Call (VROC)

2009-Present Ambassador at the Regional HIV/AIDS Connection
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2012 Global Health Research Sub-Committee member
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