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Molecular Determinants of Trim5α Restriction and Recruitment of Autophagic Effectors

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MOLECULAR DETERMINANTS OF TRIM5α RESTRICTION AND RECRUITMENT OF AUTOPHAGIC EFFECTORS

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To Ma, Dad and Kaiser

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ABSTRACT

TRIM5α is one of the best characterized anti-viral restriction factors and works specifically to inhibit the lifecycle of retroviruses. Following fusion of a retrovirus with its target cell, TRIM5α binds directly to the retroviral capsid, a proteinaceous core that houses the viral genome. Upon capsid recognition, TRIM5α forms a hexameric lattice around the retroviral capsid and initiates its antiviral activities, which include: (1) inhibition of viral infection; (2) inhibition of viral reverse transcription; (3) disassembly of the capsid; and (4), activation of innate signaling pathways. Importantly, the formation of this assembly also activates the E3 ubiquitin ligase function of TRIM5α. Ubiquitin modification is associated with directing substrates to particular cellular pathways. We and others have previously shown that TRIM5α spontaneously forms assemblies known as cytoplasmic bodies in cells, and these bodies colocalize with proteins involved in the cellular degradative pathway of autophagy. The autophagy pathway and its association with other TRIM proteins has been implicated in serval models of pathogen clearance, and therefore we hypothesized that autophagy may play a critical role in the function of TRIM5α as a retroviral restriction factor. The goal of this dissertation is to define the molecular interactions required for the association of TRIM5 proteins with autophagy effectors and to delineate the roles of ubiquitination and autophagy in retroviral restriction by TRIM5α. We first assessed the contribution of the autophagy pathway to the restriction of retroviral infection or reverse transcription by TRIM5α. We demonstrated that, when the autophagy factors ATG5 or Beclin1 are depleted in human

cell lines, the restriction of N-MLV by human TRIM5α, and HIV-1 by Rhesus macaque TRIM5α and owl monkey TRIM-Cyp is preserved. These data indicate that autophagy machinery is not required for retroviral restriction by TRIM5 proteins. However, given TRIM5α's activity as a ubiquitin ligase, we wanted to further probe the ubiquitin-dependent steps during retroviral restriction. We generated fusion proteins in which the catalytic domain of different deubiquitinase (DUb) enzymes, with different specificities for polyubiquitated linkages, was fused to the N-terminal RING domain of Rhesus macaque TRIM5α. Using these fusion proteins as tools, we assessed the role of ubiquitination in restriction and the degree to which specific types of ubiquitination are required for the association of TRIM5α with autophagic proteins. We determined that K63-linked ubiquitination by TRIM5α is critical for its association with autophagosome membranes. In the absence of K63-specific ubiquitin ligase activity, TRIM5α forms a stable association with the capsid, allowing reverse transcription to proceed, however, infection is still blocked. These data favor a model whereby the formation of a TRIM5α assembly around a capsid is sufficient to inhibit infection. Further, while the ubiquitin ligase activity of TRIM5α is needed to inhibit reverse transcription, recruitment of autophagic effectors is not required for restriction of infection or reverse transcription.

CHAPTER ONE

REVIEW OF LITERATURE

HIV-1 Pathogenesis

Despite the advent of Highly Active Anti-Retroviral Therapy (HAART), infection with Human Immunodeficiency Virus-1 (HIV-1) continues to be a significant healthcare burden worldwide, as 40,000 Americans and ~2 million people worldwide become infected with this virus annually [1]. Furthermore, there are currently nearly 40 million people worldwide living with HIV-1 infection [2], and this prevalence continues to rise as people are living longer with HIV-1 infection due to the increasing availability of HAART [3, 4]. Encouragingly, the number of deaths associated with HIV and Acquired Immunodeficiency Syndrome (AIDS) has decreased in recent years [3].

HIV-1 was identified as the causative agent of AIDS in the 1980s [5-7]. The epidemic arose from the cross-species transmission of simian immunodeficiency viruses found in African primates to humans [8]. The lineage of HIV-1 can be traced to four different subgroups, based on their proposed species of origin: Groups M, N, O, which were transmitted from chimpanzees; and Group P, which was transmitted from gorillas [3]. Group M is responsible for 48% of infections worldwide and is considered the cause of the global epidemic [9]. A related virus, HIV-2, was transmitted to humans from sooty mangabey monkeys, and although this virus causes similar clinical symptoms as HIV-1, it is considered to be less pathogenic because it is less transmissible and disease progression is slower [3, 8].

HIV-1 primarily targets $CD4^+$ T lymphocytes, although other cells bearing the CD4 marker, including macrophages, monocytes, and dendritic cells, can also be targets of infection [3]. HIV-1 is predominantly transmitted via sexual or percutaneous routes, and mucosal transmission represents the major (80%) route of infection [10, 11]. A mixture of quasispecies of virus, representing defective viruses, less fit viruses, and competent viruses, are typically present in the blood or mucosal sites [12]. However, in most cases, a single virus is responsible for transmission and productive infection, and this virus is known as the Transmitted Founder virus [8, 12, 13]. Early replication of the Transmitted Founder virus is associated with an induction of cytokines and chemokines and the opportunity for the virus to infect more cells. $CD8^+$ T cells are critical during the early stages of infection, as they kill infected cells and facilitate other arms of the innate and adaptive immune response [14]. Unfortunately, the development of the adaptive immune response is sometimes associated with the mutation of epitopes, such as in the viral envelope glycoprotein, which drives immune escape [14]. Approximately 20% of infected individuals are able to produce broadly neutralizing antibodies that are able to neutralize a number of HIV-1 subtypes. In infected individuals, however, these antibodies do not lead to clearance, but rather act as drivers of escape mutations [15]. Furthermore, over time, many individuals experience a progressive loss of function of HIV-1 specific $CD8^+$ T cells, termed exhaustion [16]. Exhaustion is associated with a loss of effector function and the expression of one or several inhibitory receptors, particularly PD-1, on the cell surface [16].

Clinically, HIV-1 infection is associated with a loss of $CD4^+$ T cells that occurs as

a result of toxicity of infection or the immune response [17]. If infection is left untreated, the loss of $CD4^+$ T cells is associated with the development of opportunistic infections and significant mortality [3]. However, regardless of treatment status, HIV-1 infection is also associated with inflammation and general immune activation. This activation is thought to be driven by activation of plasmacytoid dendritic cells, which produce copious amounts of type I IFN [18]. Importantly, this heightened inflammation contributes to a number of HIV-related complications and comorbidities, including cardiovascular disease, cancer, neurological disease, and liver disease [3].

Latency

Despite the success of antiretroviral therapy in suppressing viral loads, the virus almost universally rebounds after treatment interruption [3]. As a result, HIV-1 infected individuals are generally required to remain under therapy for life in order to maintain low viral loads and avoid the adverse sequelae associated with continued viral replication. One of the perplexing questions in the field, and the major burden to a cure, is determining where and how the virus remains latent during the treatment phase. This latent reservoir is the subject of significant ongoing research. Latency in the HIV field refers to the integration of virus's DNA into a host's DNA without the production of virus [3]. The precise identity of cells forming the latent reservoir is still under debate, with studies implicating resting, memory, and naïve T cells as potentially composing the reservoir [19-21]. Several hypotheses have been suggested to explain the establishment of the latent reservoir, and these hypotheses largely focus on events or conditions that keep the virus in a transcriptionally silent state, such as integration of the

viral DNA into transcriptionally inactive chromatin; epigenetic control of the HIV-1 promoter; the absence of transcription factors, such as NFκB, which drive the expression of viral genes; or low expression of the viral protein Tat, which is needed for efficient transcription of the viral genome [3, 8, 22]. Furthermore, latently infected cells are known to persist long term and are thus a critical barrier to a cure.

Therefore, there is significant research in the field to identify methods of eradicating the latent reservoir. One of the prevailing strategies involves activating the cells to allow for transcriptionally silent viruses to become expressed (“Shock”) and subsequently be targeted by antiretroviral drugs or immune responses (“Kill”) [3, 8]. To date, many studies have investigated drugs that are able to reactivate the expression of latent viruses, including chromatin remodeling agents and NFκB activators [3]. However, there is limited data supporting the ability of these reactivation efforts to reverse latency *in vivo* [3, 8].

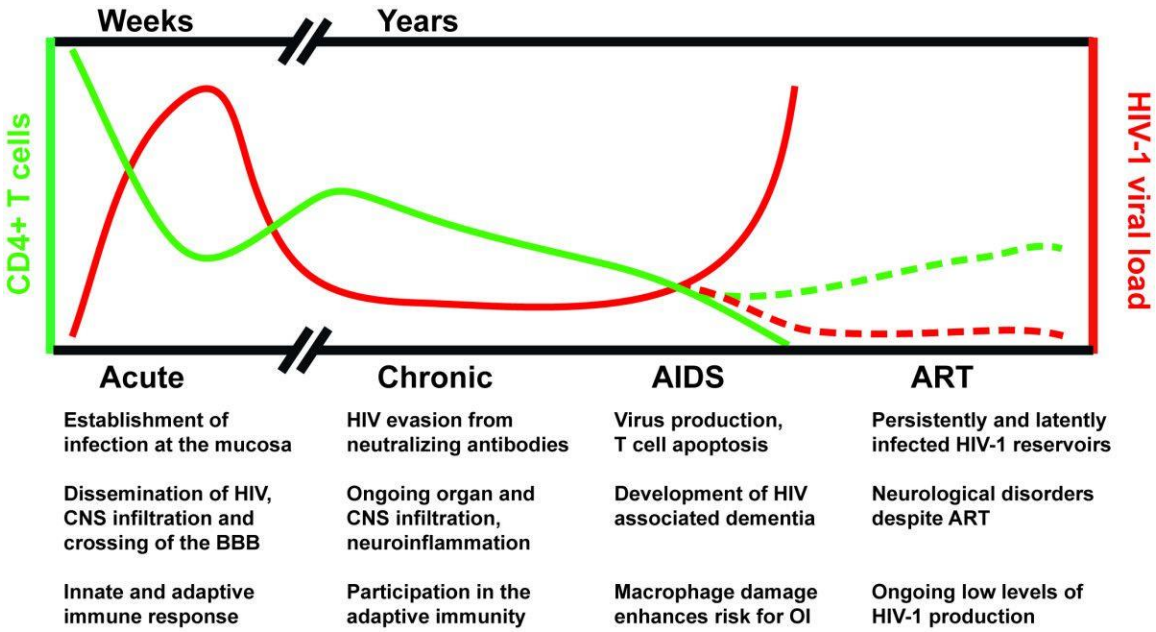


Figure 1. The Clinical Course of HIV-1 Infection. The course of HIV-1 infection can be divided into acute and chronic stages. The acute phase is associated with a high viral load in the blood and the dissemination of the virus to different sites in the body, as well as a decline in $CD4^+$ T cell numbers. Over time, the chronic phase of infection develops and is associated with the development of viral escape mutants and increased pathological sequelae affecting multiple organ systems. Over time, AIDS develops as the $CD4^+$ T cell count is further depleted. The introduction of HAART therapy can forestall many of the complications associated with late stages of disease, though the virus remains present in latently infected cells. Reprinted with permission from [23].

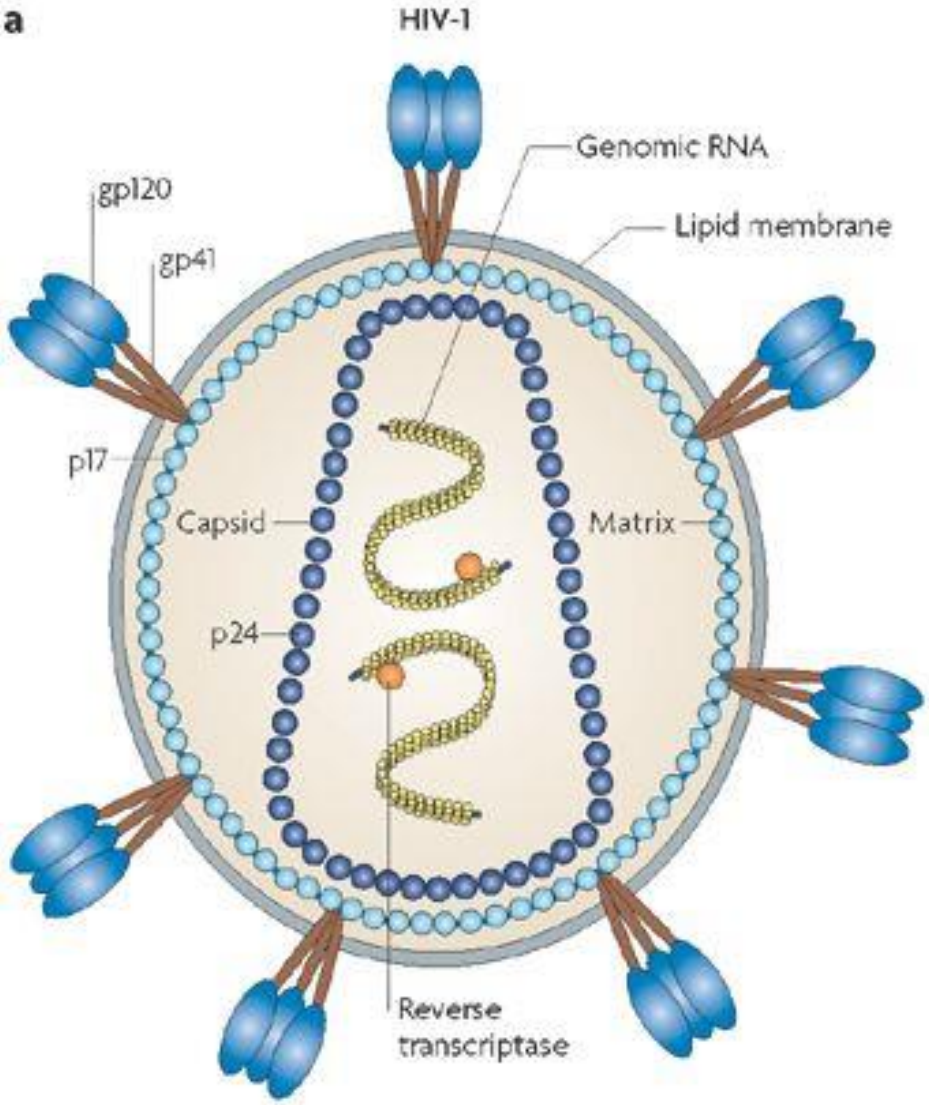


Figure 2. The Structure of HIV-1. Depicted here is the structure of the mature HIV-1 virion. HIV is an enveloped RNA virus. The viral envelope, derived from the host cells, is studded with viral Env glycoproteins, which facilitate fusion to the target cell. Encased within the envelope is the core. The core consists of the capsid, a proteinaceous conical structure housing the viral genomic RNA and viral proteins, such as integrase, reverse transcriptase, and viral accessory proteins. Reprinted with permission from [24].

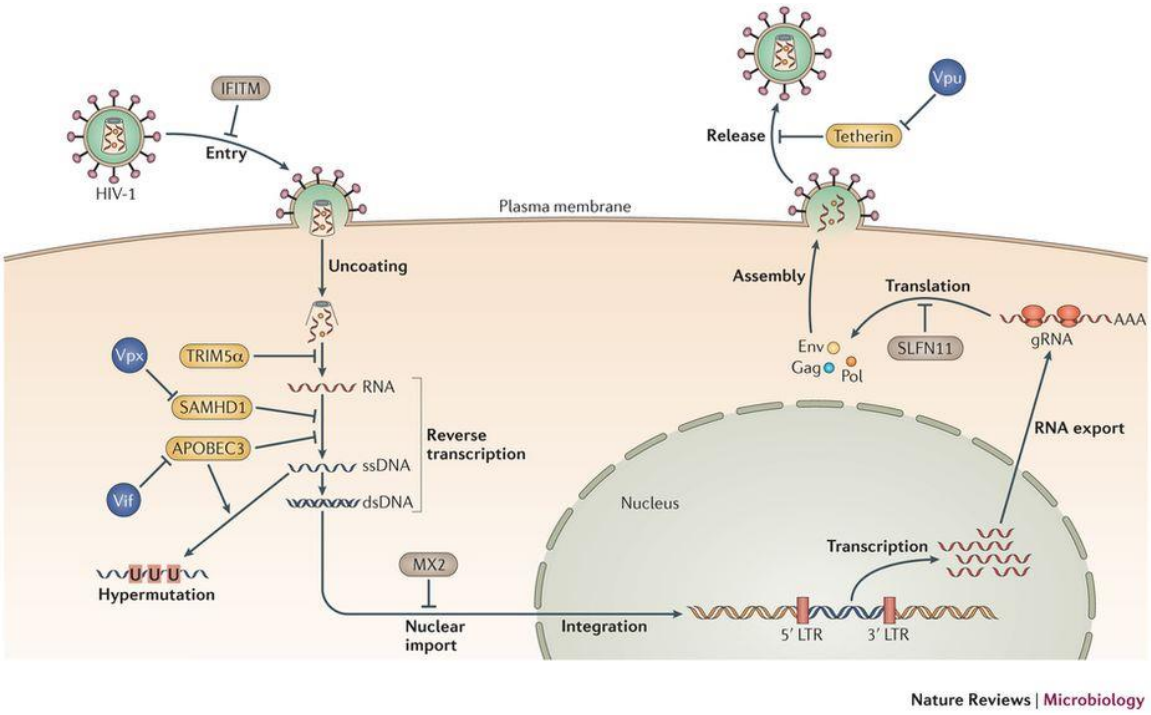


Figure 3. The Life Cycle of HIV-1. Upon fusion, HIV-1 undergoes reverse transcription of its RNA genome into DNA, uncoating of the capsid, and trafficking to the nucleus, where it integrates into the host genome. HIV-1 RNA, consisting of genomic RNA and messenger RNA, is transcribed and exported to the cytoplasm, where they can be utilized in the synthesis of viral proteins and the generation of the next generation of virions. Many of these steps can be inhibited by cellular proteins knowns as restriction factors (yellow boxes). However, these restriction factors in turn can be antagonized by viral proteins (blue circles). Reprinted with permission from [25].

HIV-1 Lifecycle

The lifecycle of HIV-1 (Figures 2 and 3) begins with the binding of the virus's envelope glycoprotein, gp120, to the CD4 receptor and coreceptors, such as the chemokine receptors CXCR4 or CCR5, on target cells [26]. Subsequently, the virus fuses with the host cell, and the viral capsid enters into the target cytoplasm. The capsid is a proteinaceous conical core containing the viral genome and viral proteins, such as integrase, reverse transcriptase, and viral accessory proteins (Figure 2). The term

capsid refers to the cone-shaped unit composed of individual CA monomers, while the core refers to the capsid structure along with its associated genome and viral proteins [27]. Upon entry, the capsid engages the cellular microtubule network to move through the cell and traffic toward the nucleus [27, 28].

During the core's movement through the cytoplasm, the single-stranded RNA genome of HIV-1 is converted into double-stranded DNA via the enzyme reverse transcriptase within the core [29]. Reverse transcription initiates a process known as uncoating, whereby the capsid undergoes a regulated process of disassembly or loss of integrity, though the details of this are under considerable debate [27, 30-32]. Importantly, some amount of CA remains associated with the reverse-transcribed genome (known at the Pre-Integration Complex, or PIC), as numerous studies have identified host cellular factors that bind to the capsid and that are critical for trafficking of the PIC across the Nuclear Pore Complex (NPC) and protection of the genome from cytoplasmic sensors [27, 33, 34]. However, the capsid itself can also function as a Pathogen Associated Molecular Pattern (PAMP) and be recognized and sensed by cellular factors, namely TRIM5α [35]. Given these interactions with cellular factors, it would be advantageous for the virus to mutate its capsid in order to avoid cellular recognition. However, the capsid is considered "genetically fragile", as mutations within it are associated with impaired reverse transcription, trafficking, and integration, and have been associated with innate sensing [27, 33, 34, 36, 37]. Therefore, the centrality of the capsid to HIV-1's life cycle and its genetic vulnerability make it an important target for antiviral approaches, and understanding factors that bind to and target the capsid

could give important insight into the next generation of treatment approaches to target HIV-1 infection.

Upon reaching the nucleus, the PIC engages cellular factors at the nuclear pore and enters into the nucleus. There are several models of how the PIC translocates into the nucleus, concerning whether an intact core or a partially disassembled core is the species that reaches the nuclear pore complex [27]. Nevertheless, several studies have identified CA proteins in the nucleus, which could be relevant for optimal nuclear import and integration site selection [27, 38, 39]. Integration of the HIV-1 reverse transcribed DNA into the host DNA is mediated by the viral enzyme integrase, which is associated with the PIC.

Once integrated, the virus's genome can be transcribed to produce both viral genomic RNA as well as mRNA [40]. Transcription relies on cellular transcription factors, namely NFκB and NFAT, and occurs relatively inefficiently until expression of the viral protein tat, which drives efficient viral transcription [22]. With Tat present, several different types of viral transcripts are produced (Figure 4), and these include: unspliced RNA, which comprises the genomic RNA and Gag or GagPol precursors (discussed more, below); singly spliced RNA, which encodes the messages for the Envelope glycoprotein (Env) and the accessory proteins Vif, Vpr, Vpu; or fully spliced RNA, which encodes messages for Tat, Rev, and Nef [22].

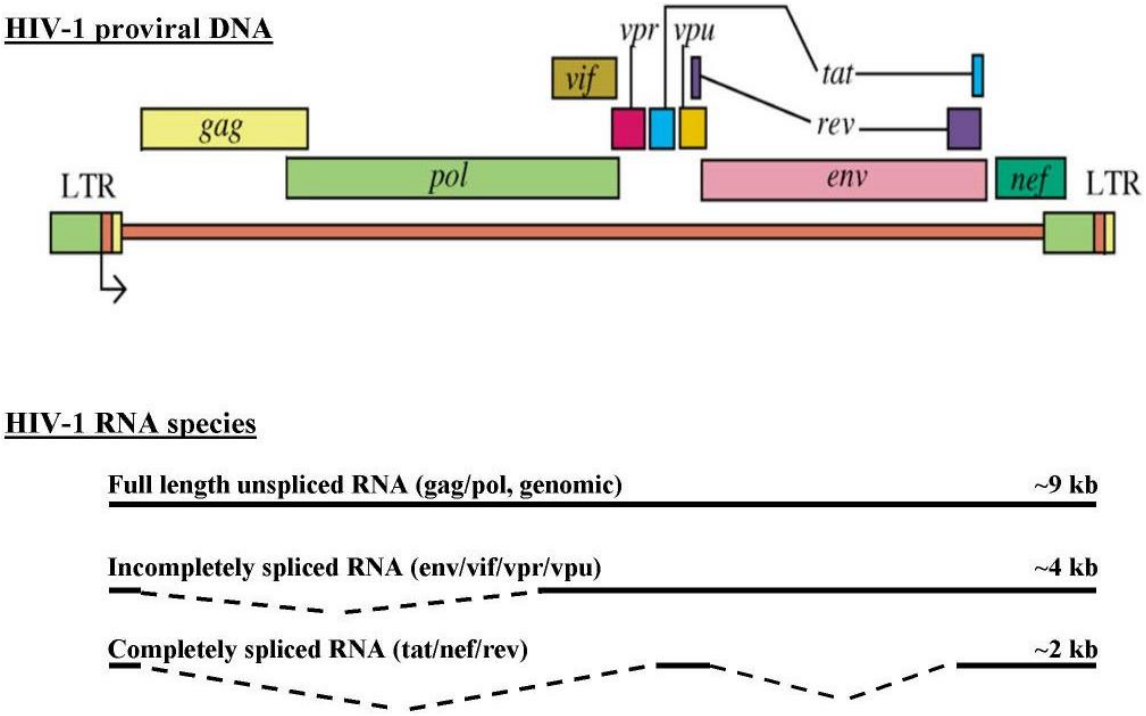


Figure 4. Transcription of HIV-1 RNA. Transcription of HIV-1 RNA produces several species, due to differential splicing. These include: full length unspliced RNA (~9 kb), which comprises the genomic RNA and Gag or GagPol precursors; singly spliced RNA (~4 kb), which encodes the messages for the Envelope glycoprotein (Env) and the accessory proteins Vif, Vpr, Vpu; or completely spliced RNA (~2 kb), which encodes messages for Tat, Rev, and Nef. Reprinted with permission from [41].

Subsequently the viral protein Rev facilitates the transport of viral RNAs to the cytoplasm where they can be used to produce viral proteins and the next generation of virions [22, 40]. Translation of viral RNA produces several products. The Gag polyprotein precursor, which encodes the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains, is produced by translation of the full-length viral RNA [40]. In addition, a frameshift during the translation of the Gag precursor produces GagPol, which in turn encodes MA, CA, NC, protease, reverse transcriptase, and integrase proteins [40]. Newly synthesized Gag binds to and recruits viral genomic RNA as it is exported from

the nucleus to the cytoplasm, and in turn this complex, along with newly synthesized envelope glycoprotein (Env), is concentrated at the plasma membrane [40]. Subsequently, the assembling virus engages members of the cellular endosomal sorting complex required for transport (ESCRT) pathway to facilitate budding and release of the viral particle [40]. Once the virus has been released, the protease is activated and can cleave the Gag precursor protein into its constituent proteins [40].

During this time, the individual CA monomers assemble into the fullerene cone structure of the capsid, in the process encapsidating the viral RNA, integrase, reverse transcriptase, and some accessory proteins [27]. The capsid is comprised of approximately 1,500 CA monomers that spontaneous assemble into hexamers (predominantly) and pentamers to make up the structure of the cone [27, 42]. It is this mature core that is released into the cytoplasm during the subsequent round of infection.

Antiretroviral Therapy

The development of combinatorial antiretroviral therapy regimens was essential for control of the HIV-1 epidemic and is responsible for the decreased mortality associated with infection [43]. Currently, over 25 drugs have been developed to target various aspects of the HIV-1 life cycle, and importantly, these drugs are utilized in combinations to limit the potential development of drug resistance [3]. Standard regimens include several reverse transcription inhibitors, a protease inhibitor, and an integrase inhibitor [3]. The reverse transcription inhibitors fall into one of two classes: nucleoside reverse transcription inhibitors (NRTI), which inhibit reverse transcription by

acting as a “chain terminator” of DNA synthesis; and non-nucleoside reverse transcription inhibitors (NNRTI), which bind to and inhibit the reverse transcriptase enzyme directly [44, 45]. Significantly, the onset of therapy produces a substantial decrease in plasma viral load, usually to levels below the limit of detection of common laboratory tests [3]. In contrast, the rebound of $CD4^+$ T cells is variable among patients, and the extent of $CD4^+$ T cell recovery, which is critical to forestall the onset of HIV/AIDS related complications, such as the development of opportunistic infections, could be related to the timing of initiation of treatment following acute infection [3].

Restriction Factors

Through the course of completing its life cycle, HIV-1 potentially interacts with many different host proteins, and over the course some of these host proteins evolved to specifically inhibit one or more steps in the viral life cycle. These factors can collectively be termed restriction factors (Figure 3). Restriction factors were first described in the context of defining the cellular factors that protected mice from infection by murine leukemia virus, and the identification of Fv1 in the 1960s (discussed in more detail, below) opened the door to look for cellular proteins that inhibit viral replication. Since then, a number of specifically-anti-HIV restriction factors have been characterized. Generally, restriction factors represent different classes of proteins and act through different mechanisms, although they share some common characteristics. Some common features include: inducibility by interferon; they can be antagonized by viral proteins or accessory proteins; and they may display evidence of positive selection, suggesting co-evolution with the targeted pathogen over time [46].

APOBEC3 Proteins.

Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like 3 (APOBEC3) proteins are widely expressed cellular proteins that utilize their cytidine deaminase activity to convert cytidine residues in RNA or DNA into uridines, which in turn introduces mutations in the resulting sequence [46]. APOBEC3 proteins are particularly deleterious to HIV-1 because the APOBEC3 proteins can be packaged into assembled virions. Upon fusion and release of the core into the cytoplasm, APOBEC3 proteins can promote the development of mutations in the newly reverse-transcribing DNA [47-50]. This hypermutated DNA often severely compromises viral fitness, and as such, the HIV-1 accessory protein Vif evolved to specifically counteract APOBEC3. Vif acts by binding to APOBEC3 proteins and promoting their ubiquitination by a cellular ubiquitin ligase, ultimately leading to the proteasomal degradation of APOBEC3 [51].

SAMHD1.

SAM Domain and HD Domain-Containing Protein 1 (SAMHD1) is a cytoplasmic dNTPase that acts to regulate the pool of free dNTPs in the cytoplasm, which in turn limits the ability of HIV-1 to reverse transcribe its genome [52-54]. Importantly, SAMHD1 can be antagonized by accessory proteins from HIV-2 or SIV, namely Vpr and Vpx [54]. However HIV-1 does not encode an antagonist to SAMHD1, and this thought to explain the relative inability of HIV-1 to infect macrophages compared to its HIV-2 and SIV counterparts [54], as macrophages are known to possess a much lower concentration of cytoplasmic dNTPs available for reverse transcription compared to that found in $CD4^+$

T cells [55].

MxB.

Myxovirus Resistance Protein B (MxB) is another interferon-inducible inhibitor of HIV-1 infection. It interacts with the assembled capsid structure, rather than CA monomers [56-59]. As Mx2 is a relatively newly discovered restriction factor, much is unknown about its mechanism of action. It is thought to act after viral reverse transcription but before integration of the viral DNA, and prevailing models propose that Mx2 interferes with nuclear import of the PIC [60].

Tetherin.

Tetherin was identified as an interferon-inducible cellular factor that inhibits the release of assembled virions from the producer cell [61, 62]. Tetherin is known to anchor into the plasma membrane, however the precise mechanisms by which it block viral release are still under debate [46]. Importantly, through the action of tetherin, viruses are thwarted from release and are instead internalized into endosomes for degradation [46, 62]. The HIV-1 protein Vpu antagonizes the effects of tetherin, though its mechanism of action is also still under debate [46, 61, 62].

Capsid Binding Restriction Factors: Fv1, Ref1, and Lv1

The discovery of factors that are able to bind to the retroviral capsid began in the 1960s, as researchers investigated the factors responsible for the susceptibility of mice to infection by Murine Leukemia Virus (MLV) [63]. Eventually, Friend virus 1 (Fv1) was identified as the cellular factor that protects mice from MLV infection [64-67]. Two alleles of Fv1, denoted $Fv1^{n}$ and $Fv1^{b}$, were found to be responsible for conferring resistance

to B-tropic MVL (B-MLV) and N-tropic MLV (N-MLV), respectively [64-67]. Sensitivity to Fv1-mediated restriction was mapped to the capsid protein of MLV [68, 69], as the capsids of B-MLV and N-MLV differ by a single amino acid [68]. Furthermore, restriction of infection was found to occur after the completion of viral reverse transcription but before integration into the host's DNA [70]. Pre-integration Complexes (PICs) isolated from cells expressing Fv1 were found to be competent for integration *in vitro*, suggesting that Fv1 arrests PICs that are on a productive path to infection [71]. In addition, researchers found that restriction by Fv1 could be abrogated by the addition of increasing amounts of restriction-sensitive virus, suggesting that the activity of this factor could be saturated [72, 73]. In 1996, the gene encoding Fv1 was identified [74, 75], and it was found that this gene was related to the Gag protein of an endogenous retrovirus present in the mouse genome [76].

Following the discovery and characterization of Fv1, researchers sought to identify similar cellular factors that conferred resistance to retroviral infection of non-murine cells. In 2000, a factor known as Ref1 was identified, and it was found to protect mammalian cells, including human cells, from infection by N-MLV but not B-MLV [77]. Intriguingly, Ref1 shared some properties with Fv1, namely, that its target mapped to the capsid of N-MLV, and its activity could be saturated by the addition of increasing amounts of restriction-sensitive virus [77]. However, in contrast to Fv1, Ref1 restricts retroviral infection prior to the completion of reverse transcription [77].

Several years after the discovery of Ref1, researchers identified a similar factor, termed Lv1, which is responsible for the restriction of lentiviral infection [78-81]. As

Ref1, restriction by Lv1 mapped to the lentiviral capsid, could be saturated, and occurred prior to the completion of reverse transcription [78-81]. Eventually, the discovery of TRIM5α, a restriction factor that inhibits HIV-1 infection in Old World Monkeys, was identified to be a species-specific variant of Lv1 and Ref1 [82-86].

TRIM Family of Proteins

The Tripartite Motif (TRIM) family is a diverse group of cellular proteins that function in a variety of cellular pathways (Figure 5). Over 80 TRIM proteins have been identified, and they have been implicated in functions as diverse as regulating cell cycle progression, autophagy, innate immunity, signaling, pathogen clearance, and the degradation of proteins [87]. The members of the TRIM family are distinguished by the presence of the RBCC motif, consisting of: a RING domain, which acts as an E3 ligase; one or two Bbox domains, and a Coiled-coil domain, which are important for self-association and higher-order assembly [87, 88]. The functions of these domains will be discussed below.

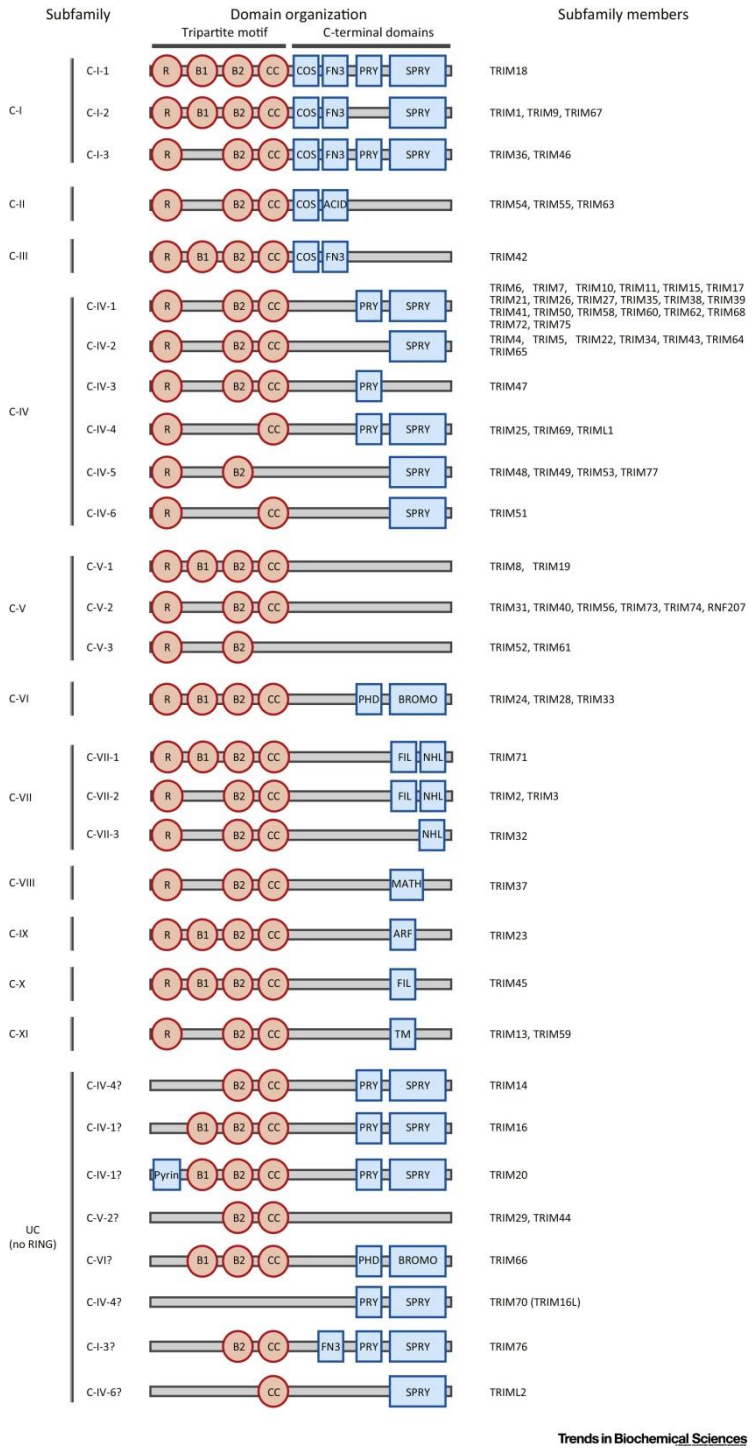


Figure 5. The TRIM Family of Proteins. TRIM proteins share the Tripartite Motif, consisting of a RING, one or two Bbox, and Coiled-coil domains. The different members of this family are distinguished by the presence of different C-terminal domains. Reprinted with permission from [88]**.**

Domains of TRIM Proteins

RING Domain.

Most TRIM proteins possess an N-terminal Really Interesting New Gene (RING) domain, which functions as an E3 ligase to catalyze the addition of ubiquitin (most commonly), small ubiquitin-like modifier (SUMO), or Interferon-stimulated proteins of 15 kDa (ISG15) to substrates [89]. The activity of the RING domain can be regulated by assembly mediated by the other domains, and this activity can influence the overall function of the particular TRIM protein [87, 90].

Bbox and Coiled-coil Domains.

Both the Bbox and Coiled-coil domains of TRIM proteins are critical for their self-association and the formation of higher-order assemblies [90]. There are two types of Bbox domains, type 1 and type 2, which differ in their ability to coordinate zinc ions [91]. Most TRIM proteins possess the type 2 Bbox (Bbox2), whereas others possess both type 1 and type 2 [90]. The Coiled-coil domain is critical for dimerization among TRIM proteins. This domain is generally helical in structure, and structural studies of several TRIM proteins, including TRIM5α, TRIM20, TRIM25, and TRIM69, demonstrate that the dimerization of these TRIM proteins involves the formation of an antiparallel dimer mediated by the CC domain [87, 90].

C-terminal Domain of TRIM Proteins.

The C-terminal domains of TRIM proteins are variable and differentiate the members of this family from one another. The C-terminal domains are important for the binding of TRIM proteins to other factors, and they can be involved with directing TRIM

proteins to different subcellular localizations [87, 90]. TRIM proteins are subdivided into 11 groups based on their C-terminal domains, the most common of which is the PRYSPRY domain (also known as B30.2), which can be found in over 30 TRIM proteins [90, 92].

Discovery of TRIM5α

For many years, scientists observed that certain primate species were naturally resistant to infection by HIV-1, and many groups sought to identify the determinant(s) responsible for this resistance[77, 79-81, 93]. In 2004, a screen from a cDNA library prepared from Rhesus macaque fibroblasts identified TRIM5α as the cellular factor that protects Old World monkey cells from infection by HIV-1 [85]. Similar to Ref1 and Lv1, TRIM5α targets the HIV-1 capsid, its activity can be saturated, and restriction occurs before reverse transcription [85]. Subsequent studies concluded that Ref1 and Lv1 are species-specific variants of TRIM5α [82, 83]. Importantly, TRIM5 proteins possess the ability to restrict retroviruses from different species (Figure 6), and different primate orthologues of TRIM5 were shown to have variable restriction efficiency of different retroviruses [94, 95].

While TRIM5α and Fv1 are both retroviral restriction factors, they have several important differences in their structure and function. First, Fv1 and TRIM5α proteins are very different at the primary sequence level, and Fv1 does not have a RING or BBox2 domain [63]. Nevertheless, both TRIM5α and Fv1 possess a Coiled-coil domain, which facilitates multimerization, as well as a C-terminal domain responsible for binding to the retroviral capsid [96-99]. Also, while restriction of retroviral infection is a conserved

property between TRIM5α proteins and Fv1, the timing of this restriction occurs either before or after reverse transcription [63].

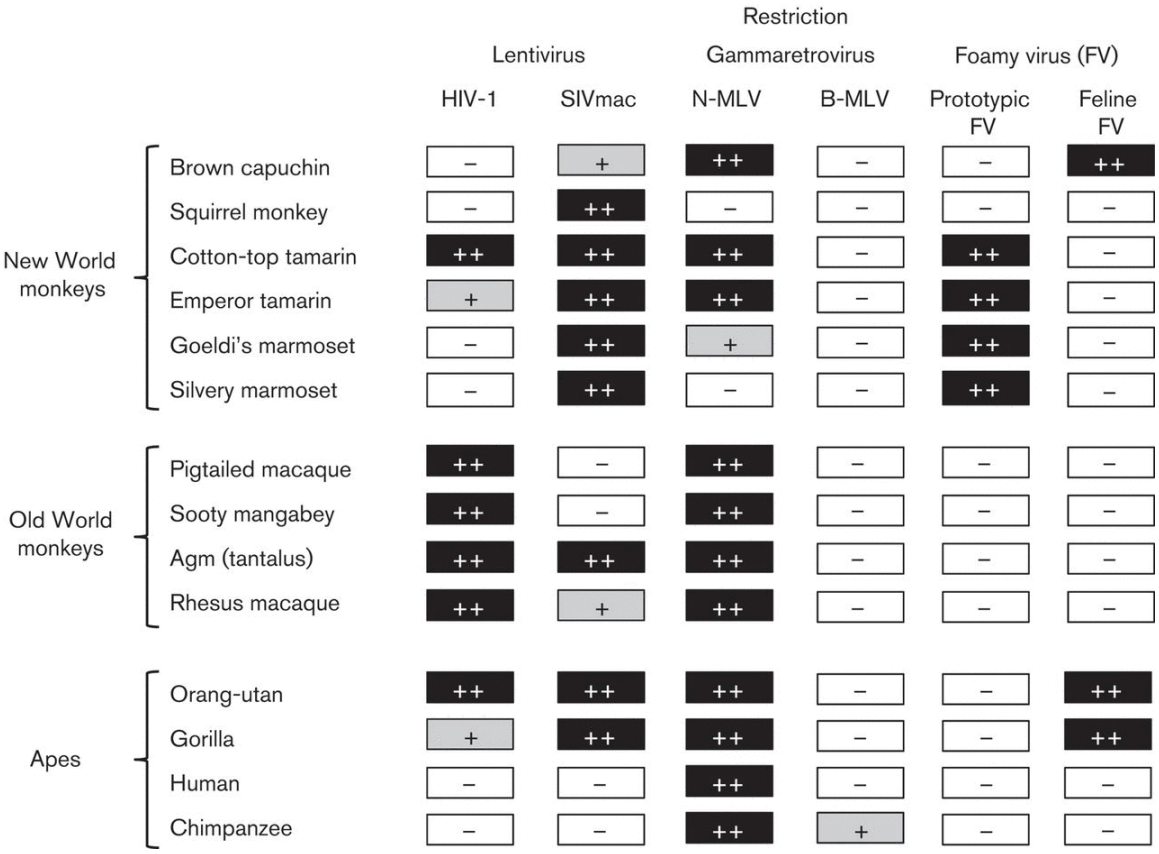


Figure 6. Specifies Specificity of Retroviral Restriction by Different TRIM5 Proteins. TRIM5 proteins demonstrate species-specific restriction of different retroviruses. Depicted here is the restriction efficacy of different TRIM5 proteins against retroviruses, classified as strong restriction (++), mildly restriction (+) or no restriction (-). Reprinted with permission from [63].

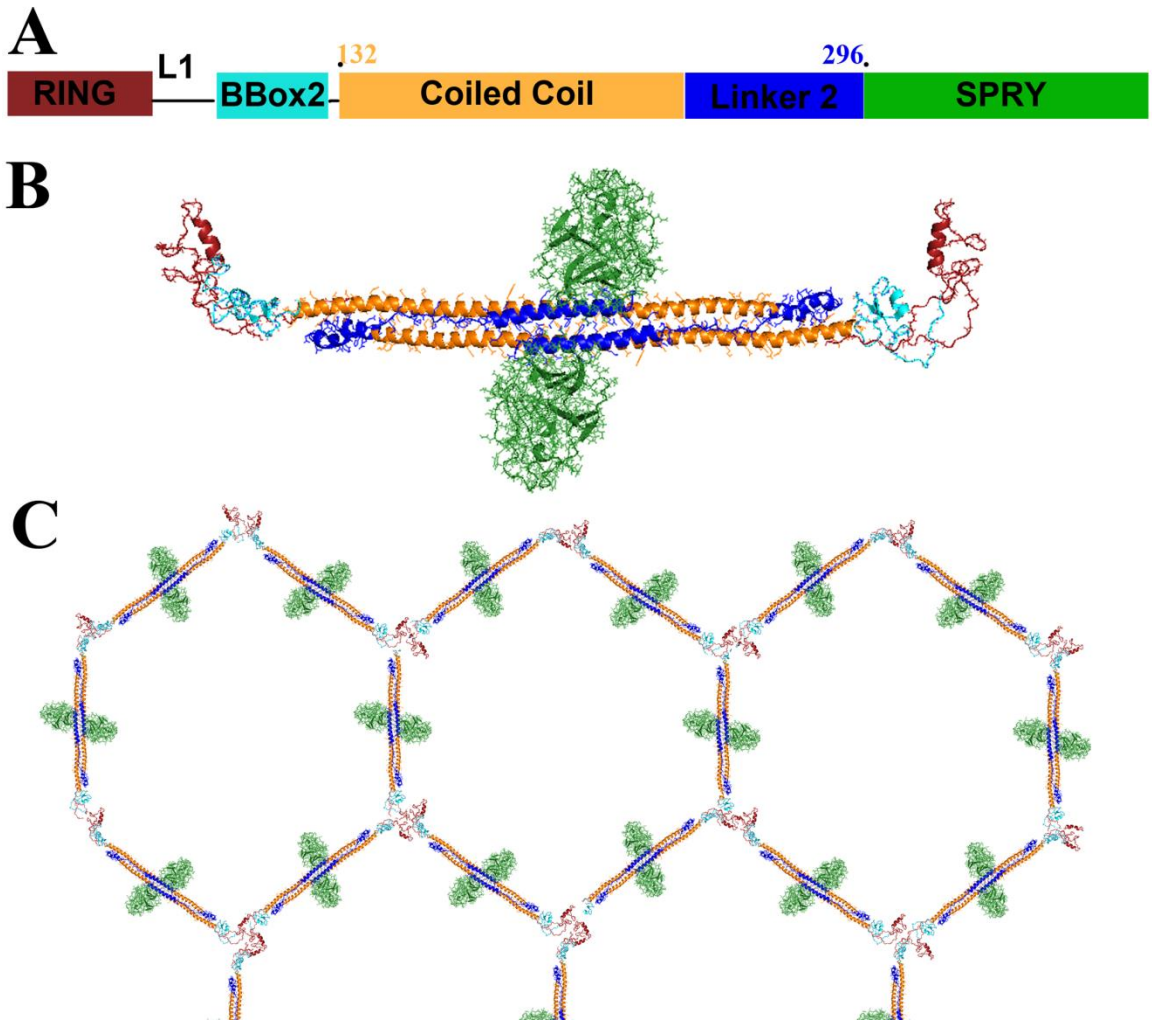


Figure 7. Domains and Assembly of TRIM5α. (A) The domain structure of TRIM5α, discussed below. TRIM5α forms an antiparallel dimer (putative structure in (B)). TRIM5α further assembles into a hexagonal lattice formation, a model of which is depicted in (C). Reprinted with permission from [100]**.**

TRIM5α: Domain Structure and Function

RING Domain.

TRIM5α possesses the RBCC motif characteristic of this family. The RING domain of TRIM5α proteins functions as an E3 ubiquitin ligase. Early investigations of the function of the RING domain were complicated by the observation that mutation of the catalytic cysteine residue in the RING domain produced unstable or large, non-functional aggregates of protein [98, 101]. Nevertheless, later studies found that the function of the RING domain is activated in the context of higher-order assembly of TRIM5α proteins [102], and that the RING domain catalyzes the autoubiquitination of TRIM5α itself [103], as well as the synthesis of free, unanchored polyubiquitin chains [35, 103].

Bbox and Coiled-coil Domains.

As with other TRIM proteins, the Bbox2 and Coiled-coil domains of TRIM5α are necessary for the formation of higher-order assemblies [104, 105]. Furthermore, dimerization of TRIM5α is mediated by the Coiled-coil domain, which forms an antiparallel dimer (Figure 7B) [106, 107], and is required for restriction [98, 108].

C-terminal PRYSPRY Domain of TRIM5α Proteins.

TRIM5α proteins possess a C-terminal PRYSPRY (hereafter referred to as SPRY) domain, which confers restriction specificity for different retroviruses [109-113]. Early experiments investigating the saturation of restriction factors identified that the retroviral capsid protein is the target of restriction factor binding. However, individual TRIM5α proteins demonstrate low affinity for binding to individual CA monomers [114].

In contrast, this low affinity binding can be overcome by the multimerization of TRIM5α proteins around the capsid, which thereby fosters a higher avidity interaction between TRIM5α and the capsid [108, 115-117]. Furthermore, this low affinity interaction between TRIM5α proteins and the capsid offers the advantage of allowing TRIM5α to restrict multiple retroviruses, as well as to readily accommodate changes in the retroviral capsid [115]. Owl monkeys express a variation of TRIM5α in which the SPRY domain is replaced by a Cyclophilin A (Cyp) domain [118, 119], producing a restriction factor known as TRIM-Cyp. As CypA is known to bind to the HIV-1 capsid [120], TRIM-Cyp remains a potent restrictor of HIV-1 infection.

The binding of TRIM5α proteins to the retroviral capsid is a critical determinant of restriction, and several groups have identified regions in the SPRY domain, particularly a 13-amino acid stretch in the variable loops of the SPRY domain, that are under strong positive selection [121, 122]. This observation supports the hypothesis that TRIM5α is constantly undergoing evolution in order to restrict retroviruses. Furthermore, although human TRIM5α is less potent at restricting HIV-1 infection compared to Rhesus macaque TRIM5α, it is likely that human TRIM5α evolved to restrict an ancient retrovirus for which its specificity was better suited [121, 123]. Notably, introduction of a single amino acid substitution at position 332 in human TRIM5α is sufficient to impart potent anti-HIV-1 restriction capabilities to human TRIM5α [124]. This observation supports the hypothesis that human TRIM5α is not grossly incapable of restriction of HIV-1, and that strategies may exist to enhance the anti-HIV-1 restriction activities of human TRIM5α.

Higher Order Assembly of TRIM5α

With the cooperation of its Bbox2 and Coiled-coil domains, TRIM5α readily forms higher-order assemblies structured as a hexagonal lattice (Figure 7C, 8) [115-117, 125]. This hexagonal lattice formation was observed to occur spontaneously *in vitro* (Figure 8), suggesting the formation of such an assembly is an “intrinsic property” of TRIM5α proteins [115]. However, lattice formation forms much more readily in the presence of *in vitro* assembled viral capsid assemblies, suggesting the capsid may act as a template for seeding the formation of the TRIM5α lattice [115]. Furthermore, the TRIM5α lattice matches the symmetry of the viral capsid lattice (Figure 8) [115]. Finally, formation of the TRIM5α lattice facilitates dimerization of RING domains on neighboring TRIM5α monomers within the lattice, and this arrangement thereby activates the E3 ligase function of the RING domains [102]. Activation of the RING domain has important implications for TRIM5α’s anti-retroviral functions, and these effects will be examined in more detail below. Importantly, it is clear that assembly of the TRIM5α lattice around the retroviral capsid is a critical aspect of downstream restriction functions.

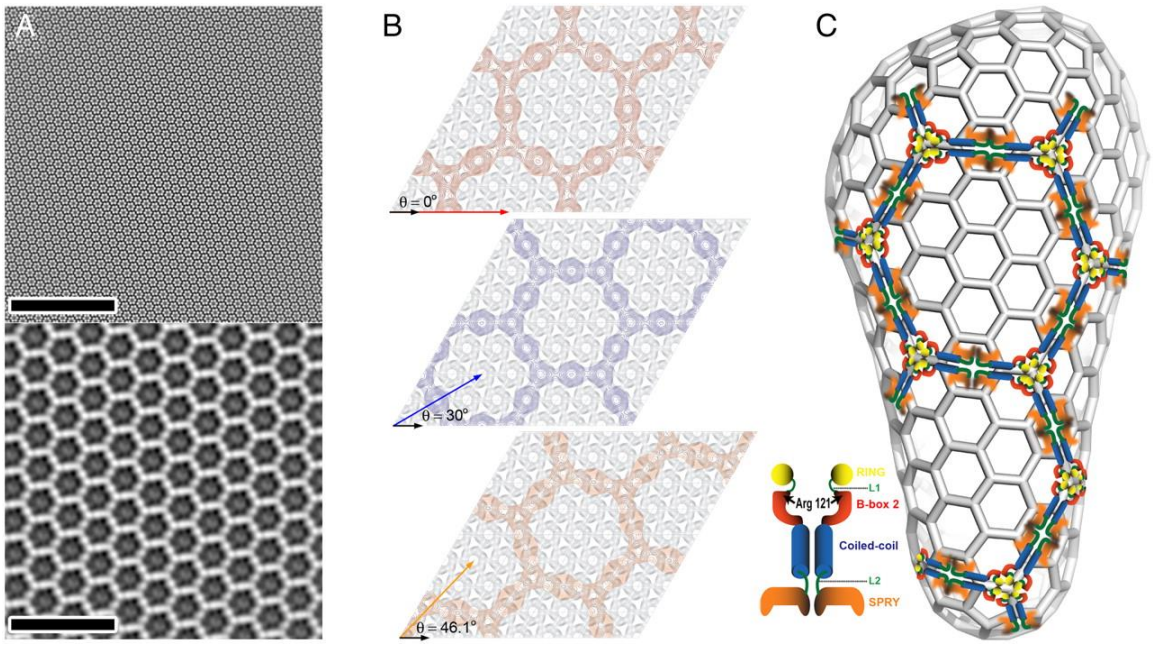


Figure 8. Assembly of TRIM5α around the Retroviral Capsid Lattice. (A) TRIM5α spontaneously forms a hexagonal lattice structure *in vitro* on assembled CA assemblies. (B) TRIM5α assembles a lattice around the viral capsid, matching the symmetry of the viral capsid. Reprinted with permission from [115].

Ubiquitin and Ubiquitin Ligases

Ubiquitin is a 76-amino acid protein that can be covalently or noncovalently attached to substrates, and this modification is often the signal to direct substrates to particular cellular pathways [126, 127]. The addition of ubiquitin to a substrate involves the coordinated action of several enzymes. A particular E1 enzyme, known as the ubiquitin activating enzyme, binds to ubiquitin that can be found freely in the cell [87]. Subsequently, E2 and E3 enzymes, which are known as ubiquitin conjugating and ligating enzymes respectively, catalyze the addition of the ubiquitin protein to a particular lysine residue in the substrate [87]. Ubiquitin has seven internal lysines (residues 6, 11, 27, 29, 33, 48, and 63), and these residues, along with the N-terminal

methionine of ubiquitin, can be targets of ubiquitination themselves, thereby producing polyubiquitin linkages [87, 128]. The polyubiquitin linkages can be anchored to a particular protein substrate, or they can be unanchored [129, 130]. Importantly, different polyubiquitin linkages are associated with different cellular fates. K48 and K63-linked polyubiquitin chains are the most prevalent, representing approximately 80% of all ubiquitin linkages observed in mammalian cells [131]. K48-linked polyubiquitin chains are canonically associated with directing substrates to the proteasome for degradation, while K63-linked polyubiquitin modification is associated with endosomal trafficking, intracellular signaling, and DNA repair [126-128, 132-134]. Intriguingly, substrates bearing K48 and K63-linked polyubiquitin chains have been shown to bind to proteasome components with similar affinities *in vitro* [135], and proteins modified by K63-linked polyubiquitin chains have been demonstrated to be proteasome targets *in vitro* [136]. Nevertheless, there is little evidence that K63-linked polyubiquitination is a signal for proteasomal degradation *in vivo*.

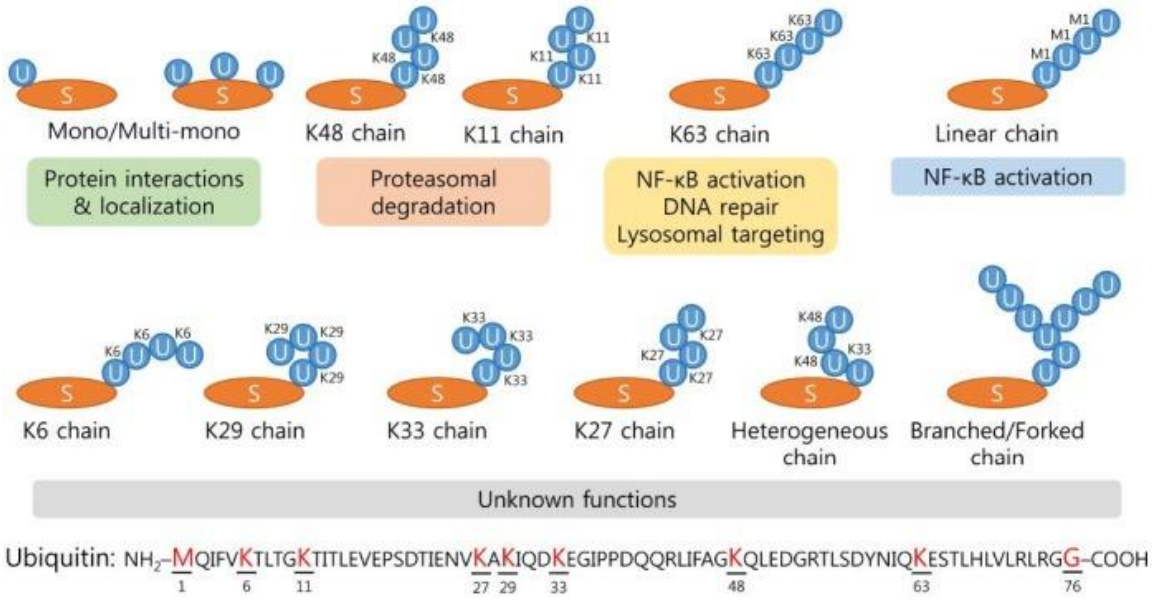


Figure 9. Overview of Ubiquitin and Polyubiuquitin Linkages. Ubiquitin contains seven internal lysine residues (K, shown in red in the bottom panel) that can be modified by ubiquitin themselves, producing polyubiquitin chains. The structure and most common function of these linkages is listed in the figure. Reprinted with permission from [137].

Proteasomes and Proteasomal Degradation

The proteasome is one of the major cellular degradative pathways (Figure 10). The eukaryotic proteasome is a 2.5 megadalton complex consisting of several important domains [138]. The central part of the proteasome complex is composed of a hollow 20S core, within which resides the proteolytic enzymes that are responsible for the degradation of protein substrates [138]. In addition, the central region of the proteasome also contains a 19S regulatory region, which houses ubiquitin-binding receptors that bind to ubiquitinated substrates and direct them into the degradative region of the proteasome [135, 138]. The ubiquitin-binding receptors within the proteasome bind to the ubiquitin tag, after which the proteasome machinery directs the substrate into the

core, processively unfolding the substrate as it moves through the core [139, 140]. From there, the unfolded protein is sensitive to degradation by the proteolytic enzymes present within the 20S core [139, 140]. The ubiquitin itself is cleaved away from the substrate via proteasome-associated deubiquitinase enzymes (DUBs) so that it can be recycled [141].

Degradation of substrates via the proteasome is classically associated with K48-linked polyubiquitination. Interestingly, several studies have reported that the ubiquitin-binding receptors present within the 19S region of the proteasome bind to both K48 and K63-linked polyubiquitin chains with equal affinity *in vitro* [135, 138]. However, another study found that the processivity of the proteasome varies based on the polyubitination modifications on a substrate, with K48 or mixed polyubiquitination linkages promoting greater processivity of the proteasome compared to K63-linked polyubiquitin chains [134]. One proposed model to explain the role of ubiquitin chains in influencing proteasomal processivity is that K48-linked polyubiquitin chains switches the proteasome to an activated state, which facilitates substrate degradation [134].

Generally, K63-linked polyubiquitin chains are not associated with directing substrates to the proteasome for degradation *in vivo* [142, 143]. Several models have been proposed to explain why this linkage may not be preferred for proteasomal degradation. First, cellular factors may occlude binding the of K63-linked polyubiquitin chains to the proteasome [138]. One study found that members of the ESCRT ((Endosomal Sorting Complex Required for Transport) family of proteins binds to proteins marked by K63-linked polyubiquitin chains and blocks their ability to bind to

proteasomal components [138]. Second, proteasomal deubiquitinase enzymes may cleave K63-linked polyubiquitin chains from substrates, thereby promoting their release from the 19S region prior to degradation [138, 141]. Finally, it is possible that cellular factors may selectively enhance the recruitment and binding of substrates marked with K48-linked polyubiquitin chains to components of the proteasome, thereby promoting their efficient degradation [138, 141]. One exception to this model is that K63-linked ubiquitin modification to a substrate has been shown to, in some cases, act as a seed for the generation of mixed-linkage K48- and K63- linked polyubiquitin chains, which in turn can be directed to the proteasome for degradation [144].

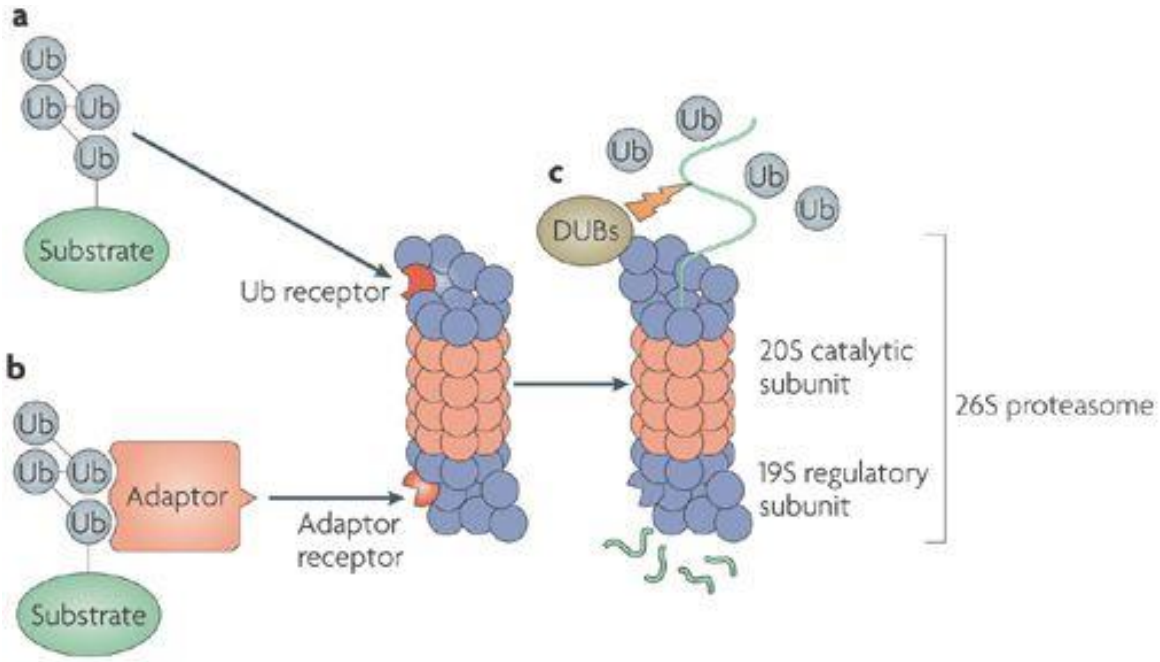


Figure 10. Schematic of the Eukaryotic Proteasome. Ubiquitinated substrates engage the proteasome either directly (a) or via adaptor proteins (b). Subsequently, the proteasome degrades these substrates, and ubiquitin is recycled via proteasome associated deubiquitinases (DUBs). Reprinted with permission from [145].

Autophagy and TRIM Proteins

Macroautophagy (hereafter autophagy) is a conserved mechanism of degradation of cytoplasmic substrates and can be induced by a number of stimuli, including starvation, stress, or invasion by pathogens [146, 147]. It initiates with the *de novo* formation of a double-membraned vesicle, termed the autophagosome, around a particular substrate, such as cellular proteins, organelles, or even pathogens, and this action, in turn, sequesters the contents from the cytoplasm [146, 147]. A number of cellular proteins are involved in the formation of the autophagosome. Studies of autophagy in yeast have identified at least thirty-one autophagy related proteins (Atg proteins) as being involved in autophagosome formation and maturation [147]. In the past, autophagy was considered to be a relatively nonselective degradative mechanism, with the proteasome being considered the more selective cellular degradative pathway [147]. However, emerging evidence identifies adaptor proteins, including members of the TRIM family, may be responsible for the selective targeting of substrates to the autophagosome for degradation [146]. One well-characterized adaptor protein is SQSTM1/p62. p62 is known to be able to bind to the autophagosome membrane marker LC3, and importantly, it is also able to bind to ubiquitinated proteins [147-149]. Once formed, the autophagosome is delivered to the lysosome, where degradative enzymes within the lysosome can degrade or recycle the contents of the autophagosome [147].

Recent studies have highlighted the importance of TRIM proteins in autophagic clearance [150]. The proposed role of TRIMs in autophagy first involves the recognition

of particular targets by TRIM proteins. Subsequently, the TRIM proteins may assemble and recruit autophagy machinery to promote the formation of the autophagosome and downstream clearance [150]. For example, TRIM20 and TRIM21 have been shown to bind to components of the inflammasome pathway and the type I interferon response, respectively, and subsequently, the TRIMs recruit autophagic machinery to these targets to promote their degradation [151].

Mechanisms of Restriction by TRIM5α

Similar to Fv1, TRIM5α is able to restrict retroviral infection. However, early studies of TRIM5α quickly identified important differences between the restriction mechanisms of these two factors. Upon fusion of the virus with the host cell, the capsid undergoes a regulated program of gradual or partial disassembly known as uncoating, although the precise mechanisms of this process are still under debate [27]. However, the consensus in the field is that reverse transcription of the viral genome is closely tied to uncoating [27, 30-32]. Capsids that are too stable or too labile demonstrate impaired reverse transcription and replication [36], and therefore, the precise timing of reverse transcription and uncoating is a critical aspect of retroviral infection. Binding of TRIM5α and its orthologues to the retroviral capsid occurs within minutes after viral fusion [152, 153]. TRIM5α readily forms a lattice structure around the retroviral capsid [115-117], and subsequently, TRIM5α is able to promote the premature disassembly of the capsid [154, 155]. This disassembly event was uncovered as researchers determined that upon infection, pelletable capsids, which represent the higher-order structure of the conical core, were unable to be recovered from cells expressing a restriction-competent

TRIM5α orthologue [155]. In cells expressing human TRIM5α, one observes a decrease in pelletable N-MLV capsid and a corresponding increase in soluble monomeric capsid (CA) protein [155]. In contrast, while cells expressing Rhesus macaque TRIM5α produced a similar decrease in pelletable HIV-1 capsid, an increase in soluble CA was difficult to detect due to the presence of pre-existing soluble CA in cytoplasm of cells [155]. Nevertheless, the loss of pelletable capsid was, in all cases, associated with a block to infection, and the implication was that disassembly of the capsid is a critical aspect of restriction by TRIM5α proteins [155].

Since then, a several groups have sought to determine the mechanism of how TRIM5α disrupts capsids, leading to their premature disassembly. One hypothesis was that following capsid binding, TRIM5α recruits the capsid and its associated viral components to cellular degradative machinery, such as the proteasome or autophagy pathways. Early investigations focused on the proteasome as having a central role in restriction because, in the presence of proteasome inhibitors such as MG132, the block to retroviral infection remains intact, but the block to reverse transcription is relieved [156, 157]. The reverse transcription products that accumulate in the presence of MG132 are competent for integration into DNA *in* vitro, indicating these products are on a productive path to infection [156]. Furthermore, in the presence of MG132, TRIM5α forms stabilized associations with capsids, and this has been observed by many groups in biochemical and imaging-based approaches [152, 155, 158]. These observations helped shape the hypothesis that TRIM5α could utilize the proteasome to promote the degradation of the capsid and its associated viral components; when the proteasome is

inhibited, the capsid is not destabilized, and reverse transcription can proceed, while the block to infectivity, measured as gene expression from an integrated provirus, remains [156, 157]. These data suggest that, similar to Fv1, restriction of reverse transcription is not critical for restriction of infection by TRIM5α [156-158]. Furthermore, these data suggest that TRIM5α has likely evolved multiple mechanisms to inhibit retroviral infection [77, 156-158].

Nevertheless, the observation that TRIM5α forms stabilized complexes with the capsid in the presence of proteasome inhibitors [152] suggested that the proteasome has some role in the destabilization and/or degradation of viral components. Two studies have observed colocalization between TRIM5α proteins and components of the proteasome [159, 160]. To determine if this association with proteasomes had any functional impact on TRIM5α's restriction mechanism, one study tracked the fates of the capsid, integrase enzyme, and viral RNA, throughout infection in the presence or absence of MG132 [158]. The study found that during restriction, Rhesus macaque and human TRIM5α solubilize the capsid and viral RNA of HIV-1 and N-MLV, respectively [158]. However, in the presence of MG132, the core complex, containing the capsid, integrase, and viral RNA, is retained, and these cores are indistinguishable from unrestricted viral cores [158]. These studies concluded that the proteasome is required for disruption of the core but not restriction of infection [158]. Collectively, these studies suggest a two-step model of restriction by TRIM5α. In the first step, TRIM5α or its orthologues binds to the retroviral capsid, and this is sufficient to block infection. In the second step, which is sensitive to proteasome inhibitors, TRIM5α induces the

premature disassembly of the viral capsid and prevents the accumulation of reverse transcription products.

However, these studies raised key questions about the role of proteasomes in capsid destabilization, as proteasomes are more associated with the degradation of linear peptides rather than the consumption of a large complex, such as TRIM5α bound to a capsid. As an E3 ubiquitin ligase, TRIM5α promotes both its own autoubiquitination as well as the synthesis of unanchored K63-linked polyubiquitin chains [35, 103]. To date, no study has identified the capsid as being a substrate for ubiquitination by TRIM5α proteins [155, 158], however, it is possible that only a small portion of CA monomers could be ubiquitinated, which would be difficult to resolve by Western blot [158, 161]. However, another study found that expression of TRIM5α itself decreased in the presence of a restriction-sensitive virus, and this reduction in expression could be reversed in the presence of MG132 [162]. Notably, the reduction in expression of TRIM5α proteins was associated specifically with being in the presence of a restriction-sensitive virus, as no reduction was observed in the context of an unrestricted virus [162]. These data suggested a possible connection between the degradation of TRIM5α proteins and their ability to restrict retroviruses. TRIM5α has a rapid turnover in cells (approximately 50-60 min) [163], and one hypothesis was that the degradation of the capsid is linked to TRIM5α's turnover. However, cells expressing a RING domain mutant of TRIM5α with a longer half-life still maintained the ability to restrict infection, indicating that the fast turnover of TRIM5α proteins is not driving restriction of retroviruses [163]. Therefore, although there appears to be a step in restriction (the

inhibition of reverse transcription) that is sensitive to proteasome inhibitors, it is unclear whether proteasomal degradation per se is necessary for capsid disassembly.

Importantly, several pieces of evidence argue against the proteasome having a central role in capsid disassembly. First, TRIM5α proteins are known to bind to CA monomers with low affinity [114], and therefore it is unclear how this low affinity binding would transmit enough force to drive both TRIM5α and its bound capsid to the proteasome for degradation. In addition, proteasomal degradation is classically associated with K48- rather than K63-linked polyubiquitin chains, and it is therefore unclear how TRIM5α, which has been associated with the production of K63-linked polyubiquitin chains *in vitro* [103], is recruited to the proteasome for degradation.

Previous studies have also interrogated autophagy and its role in TRIM5α-mediated restriction. We have previously observed that TRIM5α associates with the autophagic adaptor protein p62/sequestosome1, and depletion of p62 by siRNA caused a reduction in retroviral restriction in cells expressing human TRIM5α (huTRIM5α) or Rhesus macaque TRIM5α (RhTRIM5α) [164] ; however, because the depletion of p62 also reduced the expression level of TRIM5α, we could not conclude that p62 is directly required for the restriction of retroviruses by TRIM5α. However, two recent studies implicated autophagy in the clearance of HIV-1 by TRIM5α, proposing that after binding to the viral capsid, TRIM5α recruits autophagic machinery to degrade the virus within the lysosome [146, 165]. Although this observation appears to disagree with other studies suggesting a proteasome-dependent step in restriction, this apparent discordance might be explained by crosstalk between autophagic and proteasomal

pathways, which is known to occur in many contexts [166, 167], or by gross perturbation of ubiquitin homeostasis caused by pharmacological inhibition of the proteasome.

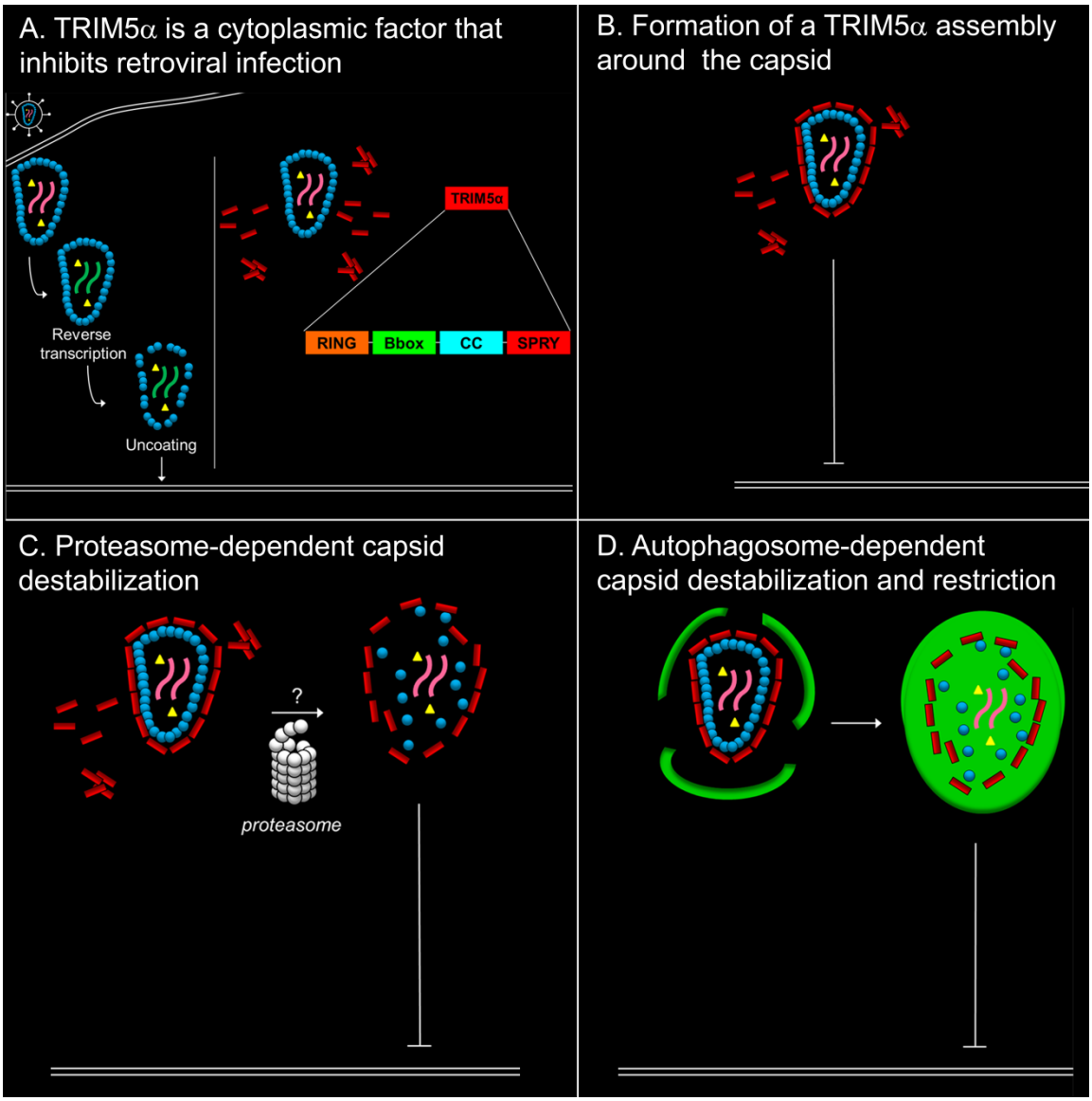


Figure 11. Proposed Models of Retroviral Restriction and Core Destabilization by TRIM5α. (A) (left side) During the HIV-1 lifecycle, the core is delivered to the cytoplasm of the target cell. The virus reverse transcribes is genome, uncoats its capsid, and traffics to the nucleus for integration. (right side) TRIM5α exists as diffuse proteins or aggregations called cytoplasmic bodies. During restriction, TRIM5α forms assemblies around the retroviral capsid (B), and in turn this assembly formation could be sufficient to inhibit infection. Alternatively, TRIM5α could engage the proteasome (C) or autophagosome (D) to destabilize capsids, which may or may not be important to block infection.

CHAPTER TWO

MATERIALS AND METHODS

Cells and Pharmaceuticals

Expression plasmids for yellow fluorescent protein-tagged Rhesus macaque TRIM5α (YFP- RhTRIM5α) and hemagluttinin (HA)-tagged RhTRIM5α or TRIM-Cyp have been described previously [168, 169]. To quantify TRIM5α accumulation, a lentiviral plasmid (pLVX, Clontech) expressing human TRIM5α containing a C-terminal Firefly luciferase reporter gene was created. HeLa and TE671 cell lines were obtained from the American Type Culture Collection. THP-1 cells were obtained from the AIDS reagent repository. Wt and ATG5-/- Mouse Embryo Fibroblasts (MEFs) were generously provided by Noboru Mizushima (University of Tokyo). HeLa, TE671 and wt and ATG5 -/- MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan) UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml ciprofloxacin. THP-1 cells were cultured in RPMI medium with identical FBS and antibiotics as above. Cells were maintained in the presence of 5% CO2 at 37°C. Bafilomycin A1 and MG132 (Cayman Chemical Company, Ann Arbor, Michigan, USA) were used at 100 nM and 1 µg/ml, respectively. Cyclohexamide was used at 20 µg/ml. Cyclosporine A (CsA; Sigma Aldrich) was used at a final concentration of 2.5 µM.

Cloning and Generation of Stable Cell Lines

Stable expression of YFP-LC3 was achieved by cloning YFP-LC3, described previously [170], into a retroviral vector [21]. To generate stable cell lines, retrovirus was prepared by transfecting equal amounts of VSV-G, pCigB packaging plasmid, EXN YFP-LC3 into HEK293T cells. Viral supernatant was harvested 48 hrs post-transfection, filtered through 0.45 μm filters (Milipore), and applied to A549 cells. 48 hrs after transduction, G418 was added to the cells, and following selection, cells were collected for phenotypic analysis.

Generation of deubiquitinase Rhesus-TRIM5α fusion proteins was performed as described previously [171]. Briefly, catalytically active and inactive deubiquitinase enzymes utilized in this study were: HSV-1 UL36 DUb (residues 15-260, [171, 172]); AMSH-LP (residues 265-436, a kind gift from the Fukai Lab, University of Tokyo, [173]); and OTUB1 (a kind gift from the lab of Wade Harper, (Addgene plasmid # 22551), mutation described in [174]). Each of these DUBs was cloned, in frame, into a pLVX Flag-Rhesus TRIM5α, in between the Flag-tag and a short linker sequence before the start codon of Rhesus TRIM5α, using SOEing PCR. To generate stable cell lines, lentivirus was prepared by transfecting equal amounts of VSV-G, psPAX2 (from Dr. Didier Trono, NIH AIDS Reagent Program, Cat. # 11348) [175], and the pLVX-DUB-Rhesus TRIM5α constructs into HEK293T cells. Viral supernatant was harvested 48 hrs post-transfection, filtered through 0.45 μm filters (Milipore), and applied to TE671, HeLa, or A549 cells. 48 hrs after transduction, puromycin was added to the cells, and following selection, cells were collected for phenotypic analysis.

Generation of Knockout Cells Using CRISPR/Cas9 Genome Editing

Indicated knockout TE671, HeLa, and A549 cell lines were generated using LentiCRISPRv2 (Addgene plasmid #52961), a gift from Dr. Feng Zhang [176]. Guide sequences were generated using the CRISPR design tool at http://www.crispr.mit.edu or were taken from available guide sequences from the Genome-scale CRISPR knockout (GeCKO2) library [176]. The following oligos were annealed and cloned into LentiCRISPRv2 (puromycin resistance) or LentiCRISPRv2-Hygromycin (which we designed): oligo targeting ATG5: 5'- CACCGGATGGACAGTTGCACACACT-3'; oligo targeting Beclin1: 5'- CACCGATCTGCGAGAGACACCATCC-3'; oligo targeting p62/SQSTM1: 5'- CACCGTGAAACACGGACACTTCGGG-3'; oligo targeting control sequence: 5'-CACCGGCACTACCAGAGCTAACTCA-3'. Lentivirus was prepared by transfecting equal amounts of VSV-G, psPAX2 (from Dr. Didier Trono, NIH AIDS Reagent Program, Cat. # 11348) [175, 177], and LentiCRISPRv2 (containing the guide RNA of interest) into HEK293T cells. Viral supernatant was harvested 48 hrs post-transfection, filtered through 0.45 μm filters (Milipore), and applied to TE671, HeLa, or A549 cells. 48 hrs after transduction, puromycin or hygromycin was added to the cells, and following selection, cells were collected for knockout assessment by Western blot and phenotypic analysis.

Flow Cytometry

For viral infectivity assessment by flow cytometry, equivalent numbers of indicated cell lines were plated in 24-well plates. Dilutions of viral supernatant were applied to the cells, after which the cells were subject to spinoculation (1200 x g, 2 hrs

at 13°C). For experiments involving cells expressing TRIM-Cyp, cyclosporine A (final concentration of 2.5 μM) or DMSO was added to the cells concurrently with viral supernatant. Following spinoculation, media was subsequently changed, and after 48 hrs, the cells were harvested and fixed in a 1% formaldehyde-PBS solution for flow cytometric analysis. Percent infectivity was determined by measuring the proportion of GFP-positive cells in the FITC channel for 10,000 events per sample, using a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

Immunofluorescence Microscopy

Cells were allowed to adhere to fibronectin-treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], pH 6.8. Cells were permeabilized with 0.1% saponin, 10% normal donkey serum, 0.01% sodium azide in PBS. We used the following primary antibodies: rabbit anti-LC3b (Sigma-Aldrich, St. Louis, MO, USA); mouse anti-LAMP2A (BD Pharmigen, San Diego, CA, USA); rabbit anti-Flag (Sigma-Aldrich, St. Louis, MO, USA), and mouse ant- HIV-1 p24 (Santa Cruz) . Primary antibodies were labeled with fluorophore-conjugated donkey anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Images were collected with a DeltaVision microscope (Applied Precision, Issaquah, WA, USA) equipped with a digital camera (CoolSNAP HQ; Photometrics, Tucson, AZ, USA), using a 1.4-numerical aperture (NA) 100x objective lens, and were deconvolved with SoftWoRx software (Applied Precision, Issaquah, WA, USA).

Image Analysis

20-30 Z-stack images were acquired using identical acquisition parameters. Deconvolved images were analyzed using Imaris software (Bitplane). For analysis of colocalization between YFP-RhTRIM5α and autophagy markers, surfaces were generated around YFP-RhTRIM5α. Then, the maximum fluorescence intensities of LC3b and LAMP2A within each surface were quantified. Background fluorescence intensities were calculated and used to set LC3b and LAMP2A intensity thresholds. For analysis of colocalization between Flag-RhTRIM5α or the deubiquitinase-fusion proteins and YFP-LC3b and/or p24, surfaces were generated around Flag (Figure 12). Maximum fluorescence intensities of YFP-LC3b and p24 within each surface were quantified. Both graphing and statistics calculations were performed in Prism (Graphpad Software, Inc). As indicated on each graph, data is presented as the mean and SEM, and significance was determined by Student's t-test or ANOVA, as indicated.

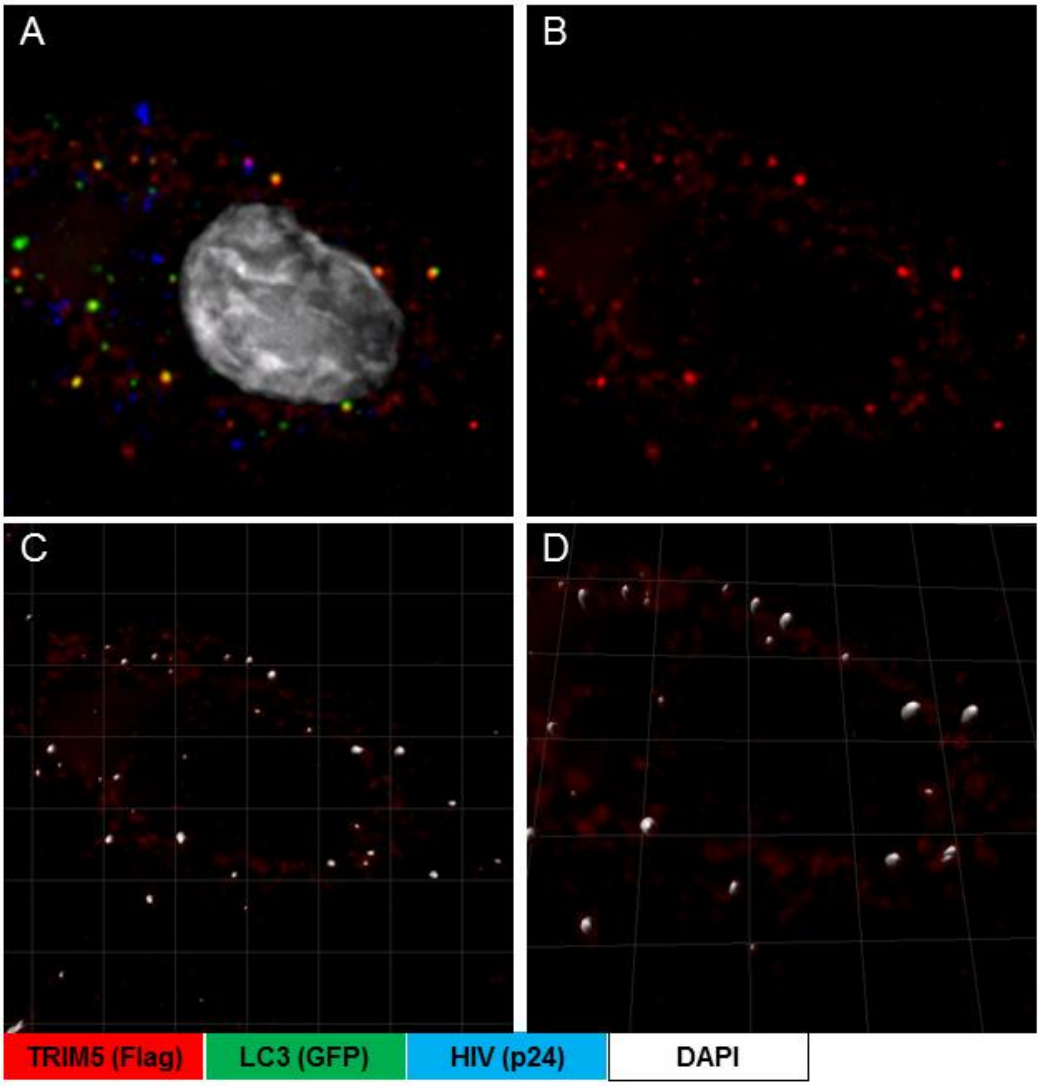


Figure 12. Image Analysis Schematic. To analyze images, a particular channel of interest is selected (B) and, and we design three dimension surfaces around the puncta within the given channel (C, D). Then, one can determine the intensity of the other channels within each of these surfaces in order to assess the degree of colocalization. The surfaces are designed with an algorithm and applied to all images.

siRNA Transfections

Transcripts for several macroautophagy factors were targeted using the following siRNAs: ATG5 (Santa Cruz Cat. No. sc-41445), Beclin1 (Santa Cruz Cat. No. sc-

29797), p62/SQSTM1 (Santa Cruz Cat. No. sc-29679), and Control siRNA (Santa Cruz Cat. No. sc-37007). 300,000 TE671 cells were plated in 6-well plates and were transfected with 30 nM of the indicated siRNAs twice over a 48-hour period. The siRNAs were transfected using Lipofectamine 2000 (Life Technologies, Cat. No. 11668027, Grand Island, NY, USA), according to manufacturer's instructions. Whole-cell lysates were prepared 72 hrs following the second transfection, as described above. Proteins were separated via SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with α-Atg5 (Novus, Cat. No NB110-53818), α-Beclin1 (Cell Signaling, Cat. No. 3738), α-p62/SQSTM1 (Cell Signaling, Cat. No. 7695S), and anti-β-actin and anti-β-tubulin antibodies. Secondary antibodies conjugated to HRP (Thermo Fisher Scientific, Waltham, MA, USA) were used where necessary, and antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescence was detected using a UVP EC3 imaging system (UVP LLC Upland, CA, USA).

Virus Generation and Titering

HIV and MLV reporter virus were prepared as described previously [164]. Briefly, HIV-1 reporter virus was produced by polyethylenimine [26] transfection of 293T cells with 10 μg of VSV-G and 15 μg of the proviral construct R7ΔEnvGFP, in which the Nef gene was replaced with GFP. MLV reporter virus was produced by PEI transfection of 293T cells with equal amounts of VSV-G, pCigN or pCigB packaging plasmid (for N-MLV and B-MLV generation, respectively), and GFP reporter vector. Virus was

harvested as previously described [178]. MLV was titered on CRFK cells to normalize viral input in infectivity studies, as described previously [156]. R7ΔEnvGFP was titered as described previously [177]

Quantitative Real-Time PCR for Viral RT Products

Quantitation of viral RT products was performed as previously described [156, 157]. Briefly, equivalent numbers of indicated cells were seeded in 12-well plates. Cells were infected with indicated virus, and they were subsequently incubated for 18 hrs at 37°C. Genomic DNA was harvested using a DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and digested with 1 unit/µl DpnI (New England Biolabs, Ipswich, MA, USA) for 4 hrs at 37°C to remove residual plasmid DNA. Real-time PCR was performed with SYBR green PCR reagent (Applied Biosystems, Carlsbad, California, USA) using primers for late RT, GFP and GAPDH. Dilutions of proviral plasmid and GAPDH (10-fold) were used to generate standard curves. Samples were normalized to 10 ng of total cellular DNA or GAPDH standards.

CHAPTER THREE

HYPOTHESIS AND SPECIFIC AIMS

Host cell restriction factors are a class of proteins that inhibit viral replication by blocking the ability of a virus to complete its life cycle. TRIM5α is one of the best characterized anti-viral restriction factors. Members of the TRIM family of proteins are defined by having a tripartite motif consisting of an N-terminal RING domain, which functions as an E3 ubiquitin ligase; one or two B-box domains; and a Coiled-coil domain. TRIM5α is distinguished from other members of this family by its C-terminal SPRY domain, which allows TRIM5 proteins to directly bind to the retroviral capsid, a proteinaceous core that houses the viral genome [85, 155]. Upon capsid recognition, the B-box and Coiled-coil domains, which are critical for self-assembly among many TRIM family members [97, 108, 117, 179], facilitate assembly of TRIM5 to form a multimeric lattice surrounding the viral core [115, 116, 180]. Furthermore, higher-order assembly of TRIM5 also activates the E3 ubiquitin ligase function of the RING domain [102]. Through the formation of this multimeric assembly, TRIM5 is able to initiate its antiviral activities, which include: (1) inhibition of viral infection; (2) inhibition of viral reverse transcription; disassembly of the capsid; and (4), synthesis of K63-linked ubiquitin chains that activate innate signaling pathways and induce an "antiviral state" for the cell [35, 103, 155, 157]. While the inhibition of viral infection by TRIM5α is well-characterized, the precise mechanism by which TRIM5α promotes the degradation of the retroviral capsid remains unknown.

Several groups, including our own, have determined that there is step in restriction that is sensitive to proteasome inhibitors, as treatment with proteasome inhibitors allows reverse transcription to proceed, though infection is still blocked, suggesting a proteasome dependent step in the restriction process [152, 156, 157, 160, 162]. However, several recent high profile studies have implicated autophagy as having a central role in retroviral restriction by TRIM5α [146, 165]. Thus the relationship between the autophagic and proteasomal degradation pathways and the functions of TRIM5α remains unresolved.

Autophagy is a conserved cellular process whereby cargoes such as proteins or organelles are sequestered into a double-membraned vesicle and transported to the lysosome for degradation or recycling. We and others have previously shown that TRIM5α spontaneously forms assemblies known as cytoplasmic bodies in cells, and these bodies colocalize with proteins involved in autophagy, including the adaptor molecule p62/SQSTM1 and the autophagosome membrane marker LC3b [164, 181]. Furthermore, we and others have also demonstrated that the turnover of TRIM5α is partially autophagy-dependent [146, 181]. Intriguingly, autophagy has also been implicated in pathogen clearance. For example, a recent study described how cells employ autophagy to clear *Salmonella* infection by tagging the invasive bacteria with ubiquitin [182, 183]. These ubiquitinated bacteria are then recognized by p62/SQSTM1 and directed to autophagosomes for degradation [182, 183]. Similarly, another recent study highlighted how infection with certain RNA viruses triggers the activation of TRIM23, which in turn activates p62/SQSTM1 and promotes the induction of autophagy

[160, 184]. Taken together, these observations suggest that the autophagic pathway may play a critical role in the function of TRIM5α as a restriction factor. The goal of this proposal is to define the molecular interactions required for the association of TRIM5 proteins with autophagy effectors and to delineate the roles of ubiquitination and autophagy in TRIM5α restriction.

Aim 1: Determine if TRIM5 proteins require autophagy effectors for the restriction of retroviral infection and reverse transcription. To test the requirement of autophagy in restriction, we examined the ability of TRIM5α to restrict retroviral infection in cells depleted of autophagic mediators ATG5, Beclin1, and p62. In all cases, restriction of retroviral infection and reverse transcription by human TRIM5α, Rhesus macaque TRIM5α, and owl monkey TRIM-Cyp remained potent in cells depleted of these autophagic effectors by siRNA knockdown or CRISPR/Cas9 genome editing [181].

Aim 2: Determine if the ubiquitin ligase activity of TRIM5α is required for its association with autophagy effectors and its ability to restrict retroviral infection and reverse transcription. We generated fusion proteins in which the catalytic domain of different deubiquitinase (DUb) enzymes, with different specificities for polyubiquitinated linkages, was fused to the N-terminal RING domain of Rhesus TRIM5α. Using these fusion proteins as tools, we sought to assess the role of ubiquitination in: (A) the restriction of infection and reverse transcription; (B) destabilization of the capsid core; and (C) the degree to which specific types of ubiquitination are required for the association of TRIM5α with autophagic proteins.

CHAPTER FOUR

RESULTS

Inhibition of Autophagy Alters the Cellular Localization of Rhesus TRIM5α

A novel characteristic of the TRIM family of proteins is their intrinsic ability to form higher order assemblies, and in the case of TRIM5α, this activity is essential for the ability of the protein to act as a retroviral restriction factor [169]. Normally, RhTRIM5α localizes to both small, discrete cytoplasmic puncta, termed cytoplasmic bodies [85] and a diffuse pool of cytoplasmic protein that is capable of forming cytoplasmic bodies de novo around individual virions [152, 168]. We previously observed that cytoplasmic bodies of RhTRIM5α colocalize with the autophagic adaptor protein p62 [164]. In contrast, previous studies have observed that treatment of cells stably expressing RhTRIM5α with MG132, a proteasome inhibitor, drives the protein to form cytoplasmic bodies that are larger in size than normal bodies [157]. Therefore, to study the effects of autophagic or proteasomal inhibition on the cellular localization of YFP-RhTRIM5α, HeLa cells stably expressing YFP-RhTRIM5α were treated with BafA1, which prevents hydrogen flux through the ATPase present on lysosomal and endosomal membranes [185], or MG132 for 18 hrs, after which the abundance of YFP-RhTRIM5α in cells was quantified. As shown in Figure 13, inhibition of autophagy by BafA1 altered the localization of TRIM5α, resulting in the accumulation of more numerous cytoplasmic bodies than observed in untreated cells (Fig. 13A and C), while treatment with MG132 recapitulated previously published findings (Fig. 13B). These observations were

validated by quantitative image analysis to characterize YFP-RhTRIM5α localization in data sets obtained from the individual treatment groups. BafA1 treatment produced a substantial increase in the number of cytoplasmic bodies per cell (Fig. 13D), compared to both untreated and MG132 treated cells. Therefore, BafA1 treatment alters the subcellular localization of RhTRIM5α, resulting in more numerous cytoplasmic bodies, consistent with these bodies being autophagosomal structures destined for clearance via lysosomal degradation pathways.

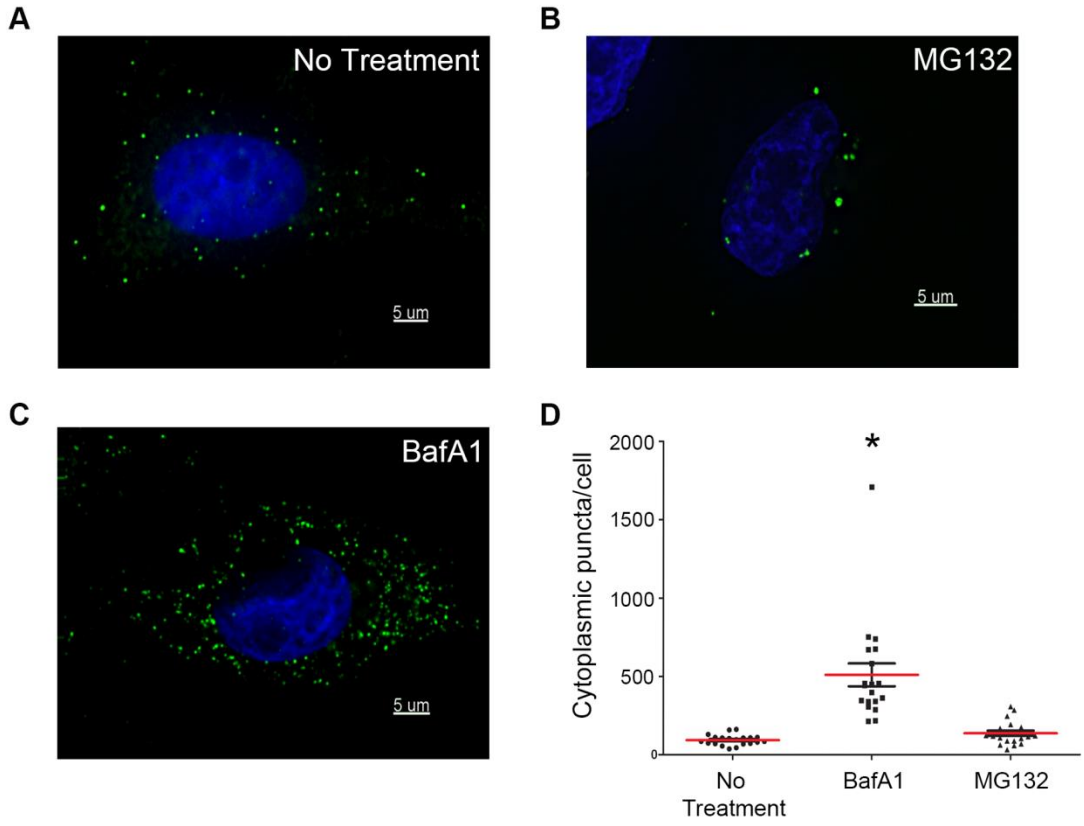


Figure 13. Subcellular Localization of YFP-RhTRIM5α Changes in the Presence of BafA1 and MG132. (A-C) HeLa cells stably expressing YFP-RhTRIM5α were seeded onto fibronectin treated coverslips for 18 hrs. Cells were left untreated or treated with BafA1 or MG132 during this time. Cells were subsequently fixed and stained with DAPI. Z-stack images were collected with a DeltaVision microscope equipped with a digital camera using a 1.4-numerical aperture (NA) 100× objective lens, and were deconvolved with SoftWoRx deconvolution software. Individual channel images were superimposed to create the merged panels. Images of cells left untreated (A), treated with MG132 (B), or treated with BafA1 (C) are presented. Images are representative of at least three experiments. (D) To quantify the number of RhTRIM5α cytoplasmic bodies in each treatment group, 20 images were taken per treatment under identical acquisition parameters. Each image was analyzed using Imaris imaging software. The mean and standard error of the mean [186] are highlighted in red. *, P<0.0001.

Rhesus TRIM5α Colocalizes with Autophagic Markers LC3b and LAMP2A following BafA1 Treatment

Our observation that treatment with BafA1 increases the accumulation of YFP-RhTRIM5α suggested that TRIM5α is degraded by an autophagic pathway. Accordingly, if YFP-RhTRIM5α is degraded by autophagy, then we would expect that the cytoplasmic bodies of YFP-rhTRIM5α, which accumulate upon BafA1 treatment, to colocalize with markers of autophagy. To test this hypothesis, we utilized immunofluorescence microscopy to quantify the degree of colocalization between YFP-RhTRIM5α and LC3b, a common marker of autophagosomes, and Lysosomal Associated Membrane Protein 2A (LAMP2A) in HeLa cells. In untreated cells, a subset of YFP-RhTRIM5α puncta was observed to colocalize with LC3b and LAMP2A (Fig. 14A and B), with approximately 30% of the YFP-rhTRIM5α cytoplasmic puncta being positive for at least one of these markers (Fig. 14C). However, after 6 hrs of treatment with BafA1, the colocalization of YFP-RhTRIM5α with both markers substantially increased (Fig 14A and B), such that ~68% of puncta were positive for one of the two markers, and approximately 35% were positive for both LC3b and LAMP2A (Fig. 14C). These data suggest that YFP-RhTRIM5α is rapidly turned over by autophagic degradation. When autophagy is inhibited by BafA1, YFP-RhTRIM5α that has been targeted for degradation accumulates in compartments containing LC3b and LAMP2A.

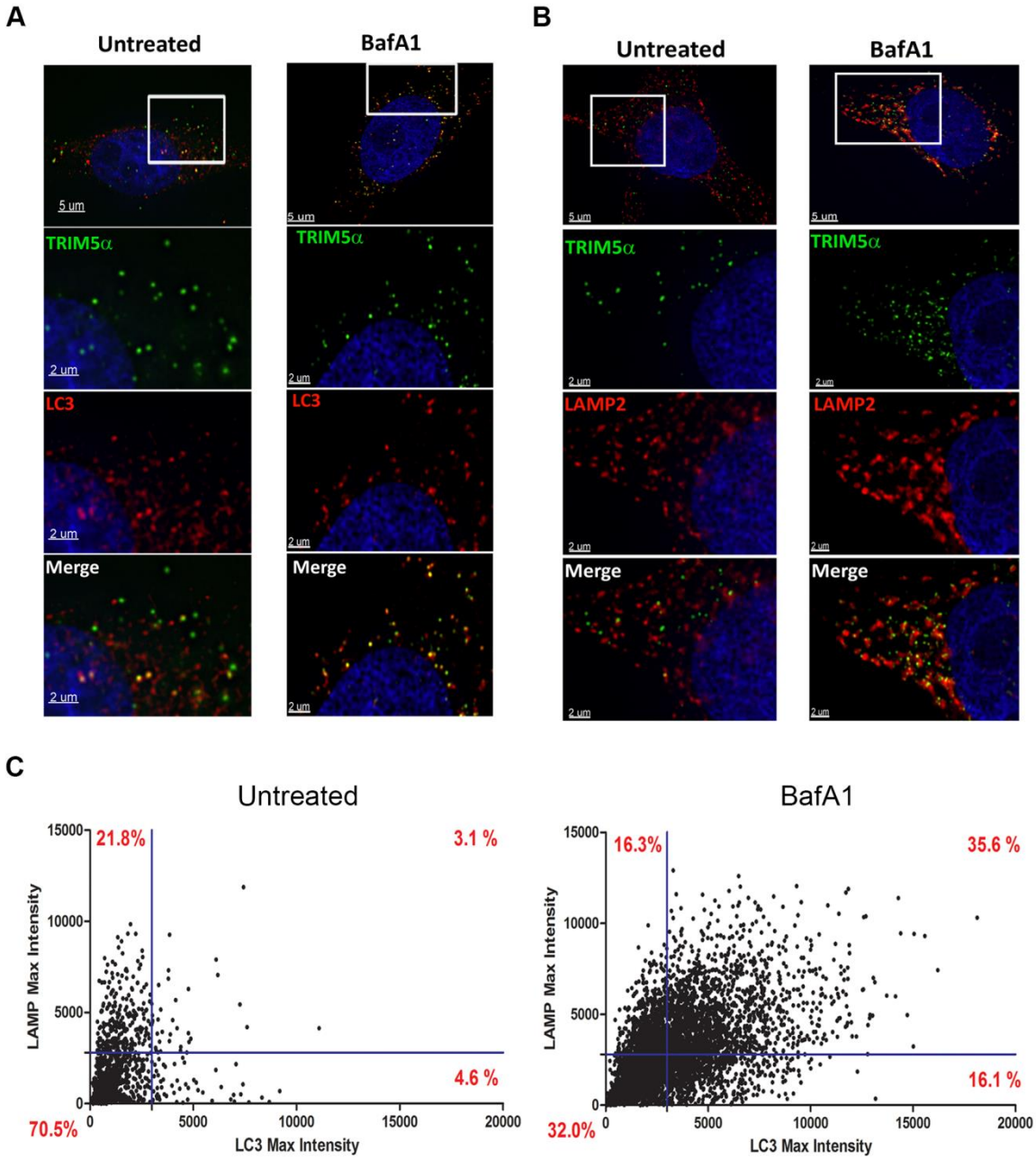


Figure 14. RhTRIM5α Colocalizes with the Autophagy Markers LC3b and LAMP2. (A and B) HeLa cells stably expressing YFP-RhTRIM5α were seeded onto fibronectin treated coverslips. Cells were left untreated or treated with BafA1 for 6 hrs. Cells were fixed, permeabilized and costained with rabbit anti-LC3b (A) and mouse anti-LAMP2A (B) and DAPI. Representative images of cells left untreated or treated with BafA1 are presented. (C) To quantify the number of RhTRIM5α cytoplasmic bodies that were positive for LC3b or LAMP2A following each treatment, 20 Z-stack images were taken

per treatment, under identical acquisition parameters. Imaris imaging software was used to identify YFP-RhTRIM5α puncta, and the maximum LC3b and LAMP2A staining intensity in each surface was calculated and plotted. Percentages indicate the number of RhTRIM5α cytoplasmic bodies that are positive for LAMP2A, LC3b, both, or neither. Images are representative of at least three independent experiments.

Depletion of Autophagic Effectors Does Not Relieve N-MLV Restriction by Human TRIM5α

The above studies provide evidence to suggest that YFP-RhTRIM5α is degraded by an autophagic pathway. We next asked if the depletion of key macroautophagy effector proteins was able to perturb TRIM5α-mediated retroviral restriction. To this end, we assessed retroviral restriction in human TE671 cells, which endogenously express human TRIM5α and therefore potently restrict N-tropic murine leukemia virus (N-MLV) but are permissive to infection by B-tropic MLV (B-MLV) [84]. TE671 cells were transfected with siRNAs targeting ATG5, Beclin1 or p62, and the infectivity of N-MLV and B-MLV was assessed. As expected, N-MLV infection was potently inhibited compared to B-MLV infection in TE671 cells subject to control siRNA transfection (Fig. 15B). Notably, knockdown of ATG5, Beclin1 or p62 did not relieve the restriction of N-MLV infection (Fig. 15B), suggesting that these effectors of macroautophagy are not required for the restriction of N-MLV by huTRIM5α.

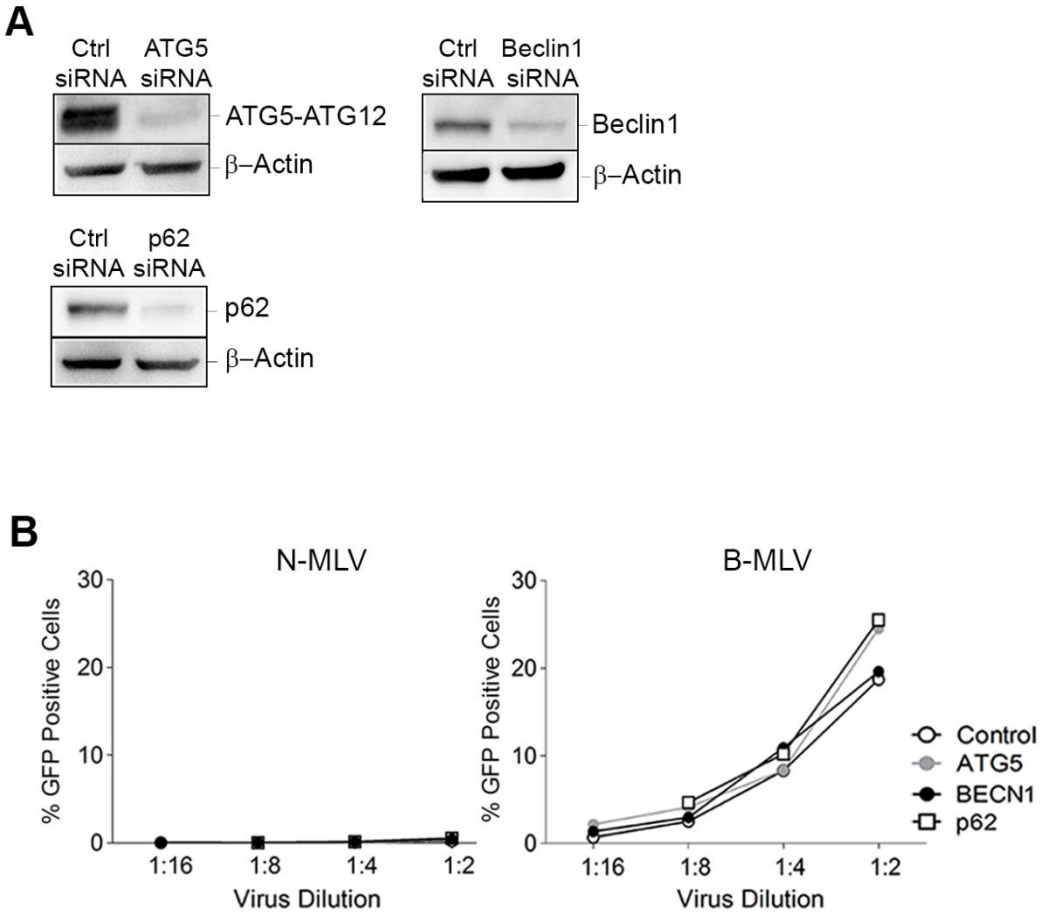


Figure 15. Depletion of Autophagic Mediators by siRNA Does Not Affect N-MLV Restriction by HuTRIM5α. (A) TE671 cells were transfected with siRNAs targeting ATG5, Beclin1, or p62, or a control siRNA. Expression of the indicated proteins was detected by Western blot 72 hrs post-transfection. (B) TE671 cells transfected with siRNAs targeting ATG5, Beclin1, or p62, or a control siRNA were collected at 72 hrs post-transfection. Equal numbers of siRNA-transfected cells were plated and infected with equivalent titers of VSV-G pseudotyped N-MLV or B-MLV. Cells were harvested 48 hrs after infection and infectivity, signified by the percentage of GFP-positive cells, was measured by flow cytometry. The data shown here is representative of three independent experiments.

To confirm and extend this observation, we generated TE671 cells in which the ATG5 gene or the Beclin1 gene were disrupted using CRISPR/Cas9 genome editing (Fig. 16C and E). Similar to our findings in cells depleted of ATG5 or Beclin1 by siRNA, we observed no relief of TRIM5α-mediated restriction of N-MLV in TE671 cells in which ATG5 or Beclin1 was knocked out (Fig. 16D and F).

To determine if ATG5 or Beclin1-dependent macroautophagy are required for the restriction of reverse transcription by TRIM5α, we also measured reverse transcription products generated by N-MLV and B-MLV in these cells. As we and others have previously observed, reverse transcription by N-MLV was reduced, relative to reverse transcription by B-MLV, in unmodified TE671 cells (Fig. 16G and H). Importantly, the restriction of N-MLV reverse transcription, relative to that of B-MLV, was preserved in cells depleted of ATG5 or Beclin1 (Fig. 16G and H). These data demonstrate that perturbation of macroautophagy does not abrogate restriction of retroviral infection or reverse transcription by endogenously expressed huTRIM5α.

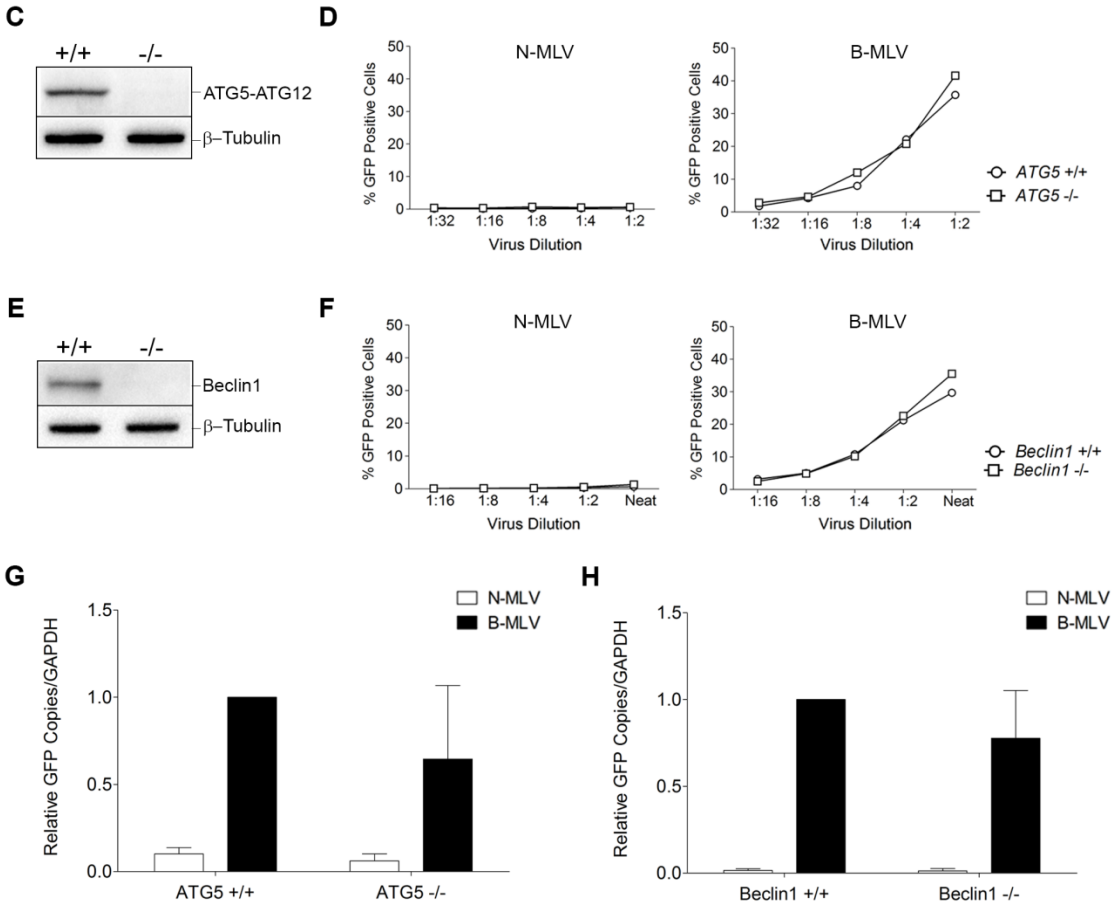


Figure 16. **Depletion of Autophagic Mediators by CRISPR/Cas9 Genome Editing Does Not Affect N-MLV Restriction by HuTRIM5α.** TE671 cells were depleted of ATG5 (C) or Beclin1 (E), using CRISPR/Cas9 genome editing, and protein expression of ATG5 in wild-type and knockout cells was confirmed by Western blot. Infectivity of VSV-G pseudotyped N-MLV or B-MLV in wild-type or ATG5 (D) or Beclin1 (F) knockout TE671 cells was assayed. The data shown here is representative of three independent experiments. Wild-type or ATG5 (G) or Beclin1 (H) knockout TE671 cells were infected with equivalent titers of VSV-G pseudotyped N-MLV or B-MLV, and viral reverse transcription products were measured. Three independent experiments were conducted, and the amount of viral DNA detected in each experiment was normalized to the wild-type untransduced sample infected with B-MLV of that experiment. Error bars represent the standard deviation of the relative number of viral DNA products detected across three independent experiments.

Depletion of Autophagic Effectors Does Not Relieve HIV-1 Restriction by Rhesus TRIM5α or Owl Monkey TRIM-Cyp

We next assessed if autophagic adaptors are also required for the restriction of HIV-1 by RhTRIM5α and owl monkey TRIM-Cyp. We generated HeLa cell lines in which ATG5 or Beclin1 were disrupted by CRISPR-Cas9 genome editing (Fig. 17A and C and 18A). When challenged with HIV-1, we observed extensive infection in wild-type HeLa cells and in HeLa cells depleted of ATG5 or Beclin1 (Fig. 17B and D). In contrast, when wild-type and ATG5 or Beclin1-depleted HeLa cells were transduced to stably express RhTRIM5α, these cells potently restricted HIV-1 infection relative to their untransduced counterparts (Fig. 17B and D). Analogous results were obtained in ATG5 knockout mouse embryonic fibroblasts (not shown).

We next examined the ability of RhTRIM5α to inhibit the formation of HIV-1 reverse transcription products in cells depleted of macroautophagy factors. In untransduced ATG5 and Beclin1 knockout HeLa cells, reverse transcription was reduced relative to unmodified HeLa cells, consistent with the reduction in infectivity observed in these cells (Fig. 17E and F). In each case, however, potent restriction of reverse transcription was observed in cells expressing rhTRIM5α, compared to their untransduced counterparts.

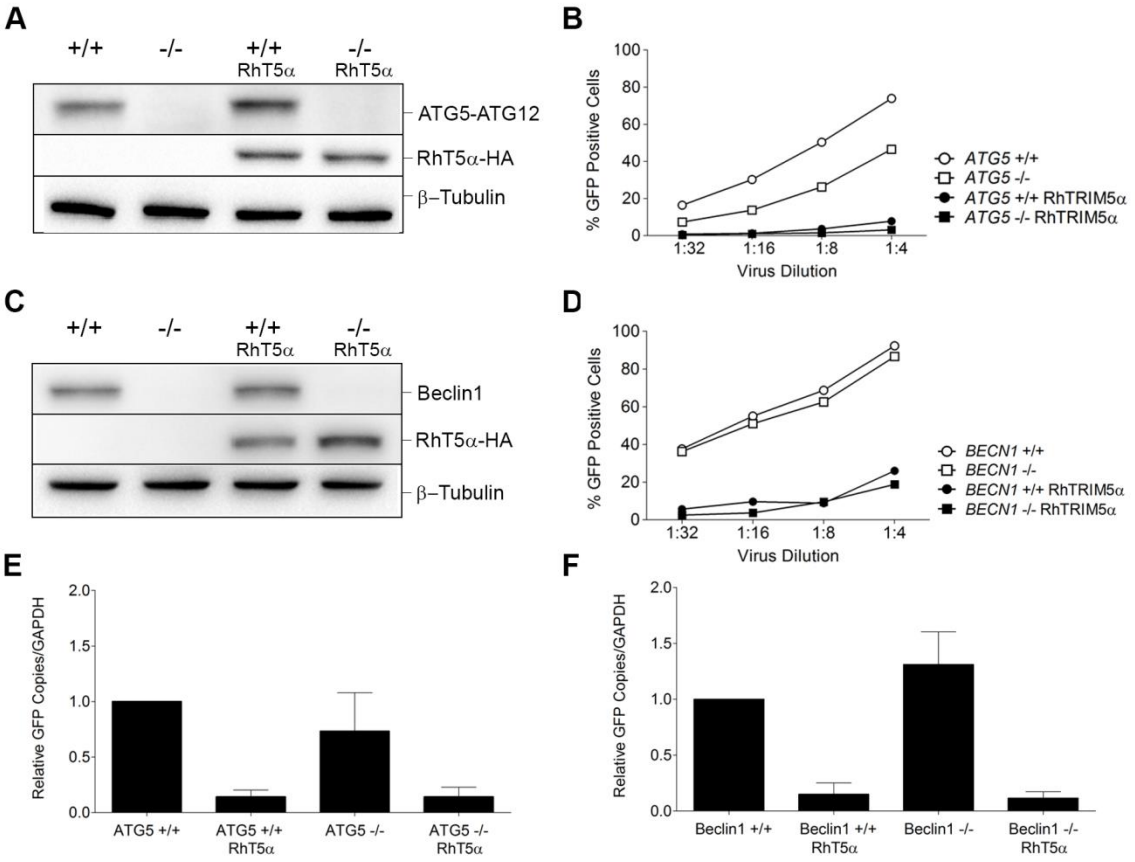


Figure 17. Depletion of Autophagic Mediators Does Not Affect the Restriction of HIV-1 by RhTRIM5α. HeLa cells depleted of ATG5 (A) or Beclin1 (C) by CRISPR/Cas9 genome editing were transduced to stably express HA-tagged rhTRIM5α (or left untransduced). Wild-type, ATG5 (B), or Beclin1 (D) knockout HeLa cells, either with or without exogenous RhTRIM5α expression, were infected with a VSV-G pseudotyped HIV-1 reporter virus, and infectivity was measured by flow cytometry. The data shown here is representative of three independent experiments. (E) Wild-type, ATG5 (E), or Beclin1 (F) knockout HeLa cells, either with or without exogenous RhTRIM5α expression, were infected with equal titers of VSV-G pseudotyped HIV-1, and viral reverse transcription products were measured by quantitative PCR. For each sample, viral DNA, as measured by the number of GFP reporter copies detected, was normalized to the amount of GAPDH observed in parallel samples. Three independent experiments were conducted, and the amount of viral DNA detected in each experiment was normalized to the wild-type untransduced sample of that experiment. Error bars represent the standard deviation of the relative number of viral DNA products detected across three independent experiments.

Furthermore, we observed similar restriction in wild-type and ATG5 or Beclin1 knockout HeLa cells stably expressing owl monkey TRIM-Cyp, compared to untransduced cells (Fig. 18B). Notably, relief of restriction by TRIM-Cyp was only observed when infection was carried out in the presence of cyclosporine A (CsA), which is known to inhibit the interaction of TRIM-Cyp with the capsid of HIV-1 (Fig. 18B) [119, 153]. Collectively these data reinforce that the restriction of HIV-1 infection by both RhTRIM5α and owl monkey TRIM-Cyp is independent of macroautophagy adaptors ATG5 and Beclin1. Furthermore, RhTRIM5α does not require ATG5 or Beclin1 to complete restriction of HIV-1 infection or reverse transcription.

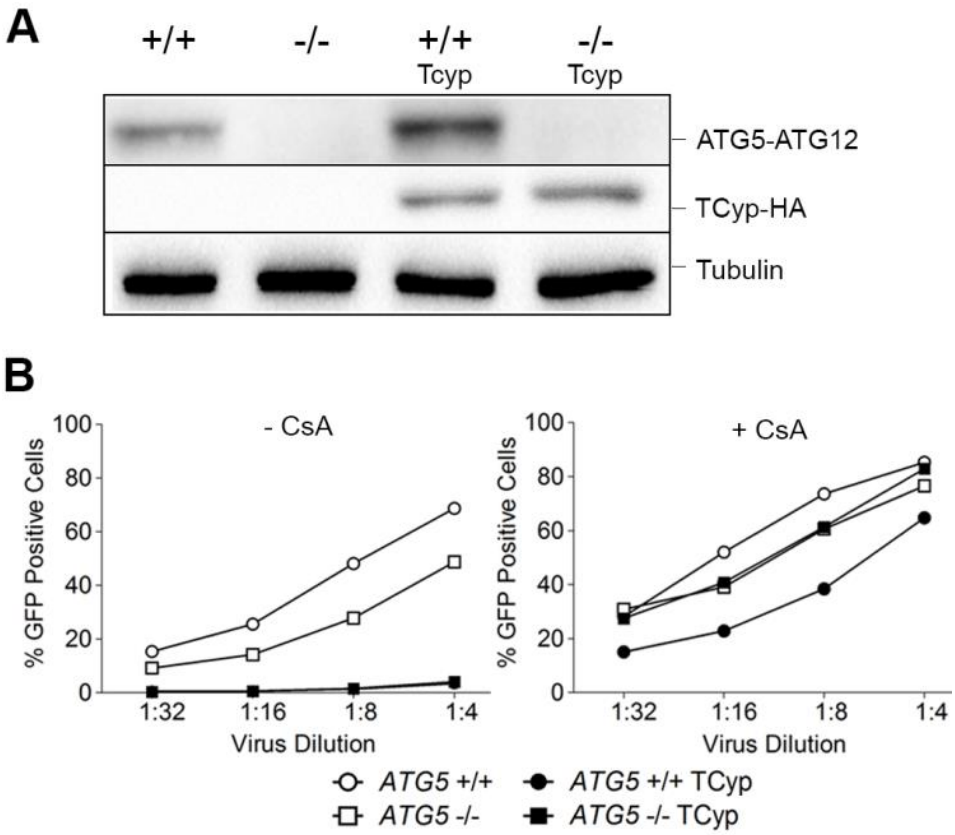


Figure 18. Depletion of ATG5 Does Not Affect the Restriction of HIV-1 by Owl Monkey TRIM-Cyp. (A) HeLa cells depleted of ATG5 by CRISPR/Cas9 genome editing were transduced to stably express HA-tagged owl monkey TRIM-Cyp (or left untransduced). (B) Wild-type or ATG5 knockout HeLa cells, either with or without exogenous owl monkey TRIM-Cyp expression (as depicted in (A)) were infected with a VSV-G pseudotyped HIV-1 reporter virus either in the absence or presence of cyclosporine A (- or + CsA, respectively). Cells were harvested 48 hrs after infection and infectivity, signified by the percentage of GFP-positive cells, was measured by flow cytometry. The data shown here is representative of three independent experiments.

Inhibition of Ubiquitination Produces Stable Association of TRIM5α and HIV-1 in THP-1 and A549 Cells

Initial studies investigating the mechanism of restriction of retroviral infection by TRIM5 proteins determined that TRIM5 causes a destabilization of the retroviral capsid [155, 187], and early models invoked cellular degradative machinery, such as the proteasome or autophagy pathways, as being critical mediators of this destabilization. Previous studies identified that in the presence of the proteasome inhibitor MG132, Rhesus TRIM5α is unable to restrict HIV-1 reverse transcription, although infection is still inhibited [156, 157]. In addition, Rhesus TRIM5α forms stabilized complexes with HIV-1 virions in the presence of MG132 [152, 155, 158]. Intriguingly, these complexes stain positively for ubiquitin [152], possibly indicating a role for ubiquitin or ubiquitination in TRIM5α's anti-retroviral functions. As an E3 ubiquitin ligase, TRIM5α has been shown to autoubiquitinate itself and produce unanchored K63-linked ubiquitin chains *in vitro* [103]. Ubiquitination is often a marker to direct substrates to particular cellular pathways, and we and others have observed that TRIM5α colocalizes with markers of the autophagy pathway [146, 164, 181]. These observations suggested a possible role for autophagy in TRIM5α's restriction functions. However, we previously established that restriction of retroviral infection or reverse transcription by TRIM5 proteins does not require the autophagy effector molecules ATG5 or Beclin1 [181]. Nevertheless, it is possible that ubiquitination contributes to other functions of TRIM5α. Therefore, our goal was to delineate the role of ubiquitination in the anti-retroviral functionsTRIM5α and its recruitment to autophagosomes.

A recent study from our group determined that the E3 ubiquitin ligase function of TRIM5α is required for its ability to destabilize retroviral capsids [171]. TRIM5α proteins in which the Herpes Simplex Virus UL36 deubiquitinating enzyme (hereafter referred to as DUb) fused to the N-terminal RING domain of Rhesus macaque TRIM5α (DUb-RhTRIM5α) were able to restrict HIV-1 infection, however, viral cores in complex with DUb-RhTRIM5α accumulated in the cytoplasm of infected cells, suggesting impaired destabilization of cores in the absence of competent ubiquitination [171]. Importantly, cells expressing a catalytically inactive version of the DUB, termed DUb*-RhTRIM5α, maintained the ability to both restrict infection and destabilize viral cores [171]. We previously observed the accumulation of DUb-RhTRIM5α-core complexes in the cytoplasm of infected HeLa cells [171]. These findings were recapitulated in both THP-1 cells differentiated into macrophages and A549 cells (Fig 19-21). Importantly, from these studies, it was unclear if the stabilized DUb-RhTRIM5α-capsid complexes were being sequestered into autophagosomes for subsequent degradation.

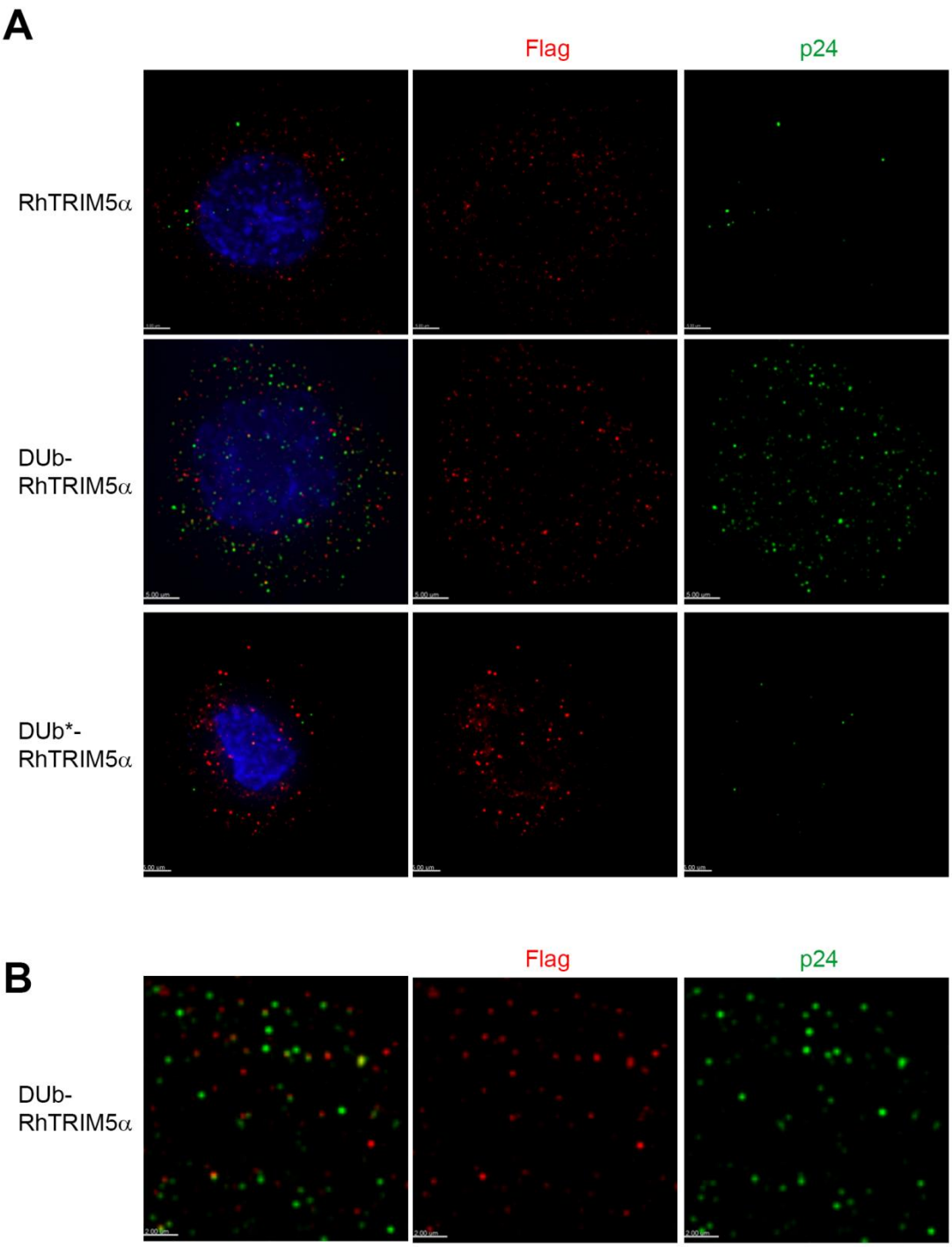


Figure 19. Co-localization of DUB-Fusion Proteins and HIV-1 p24 in THP-1 Cells. THP-1 cells expressing the indicated Flag-tagged RhTRIM5 DUB fusion proteins were differentiated into macrophages and infected with HIV-1. Colocalization between Flag and HIV-1 p24 was assessed. Representative image are shown in (A), and a magnification of a DUb-RhTRIM5α image is shown in (B)**.**

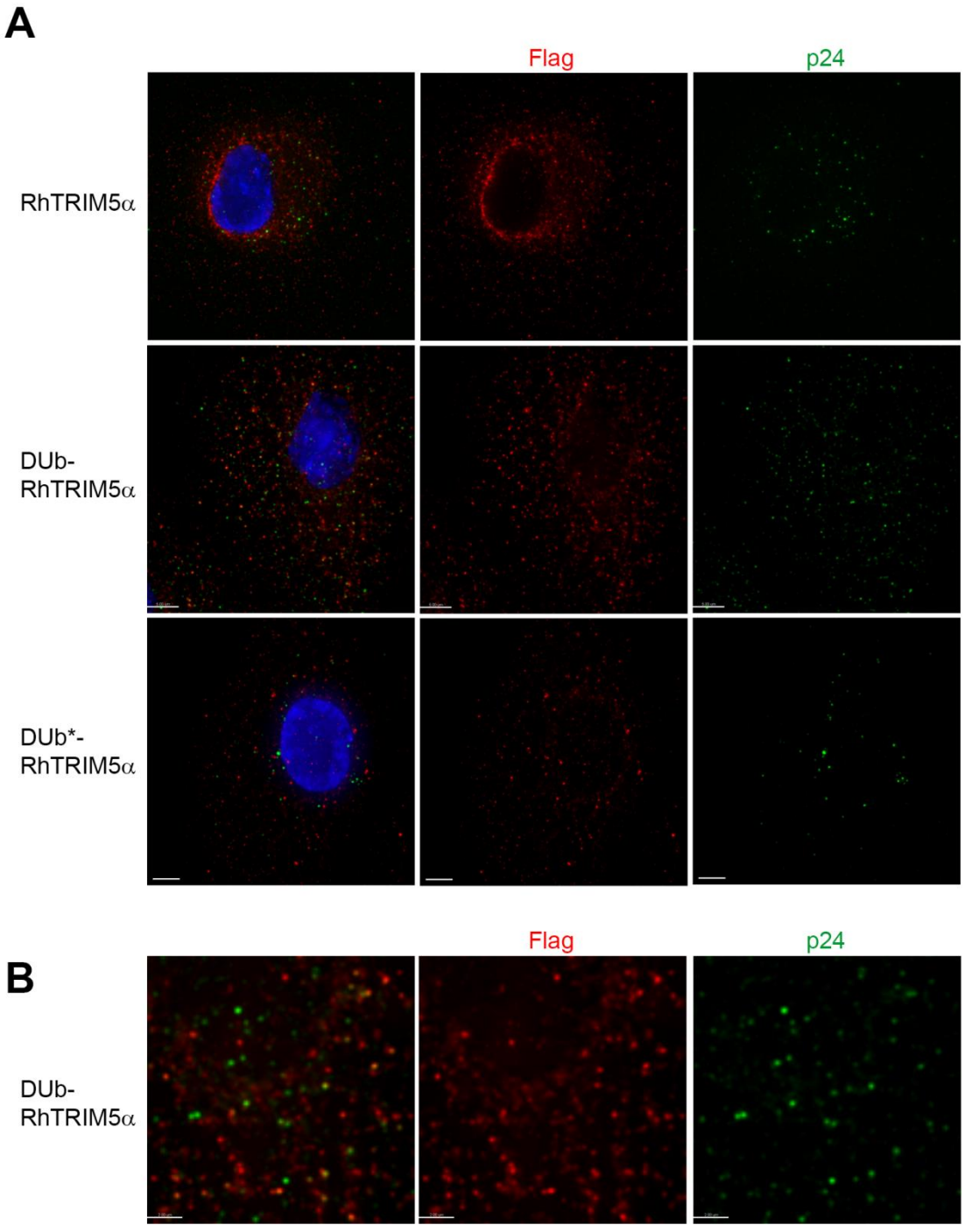


Figure 20.Co-localization of DUB-Fusion Proteins and HIV-1 p24 in A549 Cells. A549 cells expressing the indicated Flag-tagged RhTRIM5 DUB fusion proteins infected with HIV-1. Colocalization between Flag and HIV-1 p24 was assessed. Representative image are shown in (A), and a magnification of a DUb-RhTRIM5α image is shown in (B)**.**

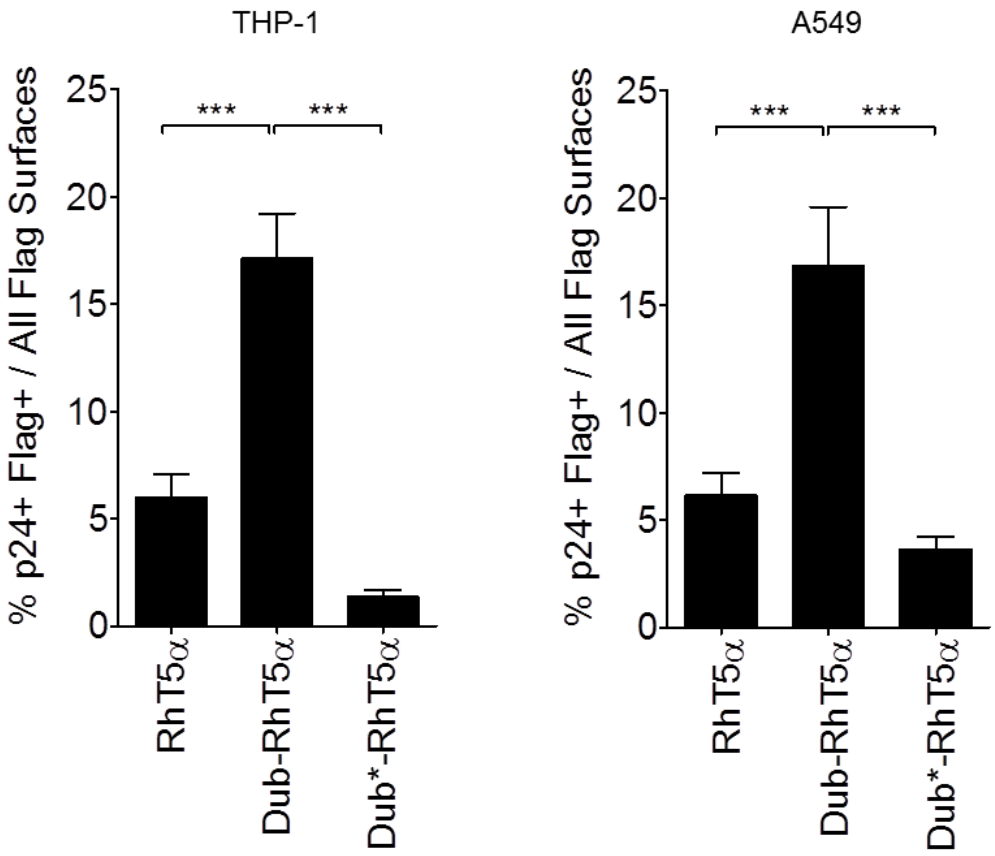


Figure 21. Inhibition of the Ubiquitin Ligase Function of TRIM5α Stabilizes the Association of RhTRIM5α with HIV-1 Viral Cores in THP-1 and A549 Cells. The degree of colocalization of between the different TRIM5α fusion proteins (Flag-tagged) and HIV-1 capsid (p24) at 6hpi in both THP-1 cells differentiated into macrophages and A549 cells was measured. Error bars represent SEM of at least 20 images taken for each cell line. Data are representative of three independent experiments. *** = $p < 0.001$ by one-way ANOVA.

Generation of Deubiquitinase-Rhesus TRIMα Fusion Proteins

To more directly identify the specific determinants of how TRIM5α recruits autophagic machinery and to determine if stabilized TRIM5α-viral core complexes are recruited to autophagosomes, we generated a panel of fusion proteins in which the catalytic domain of different deubiquitinase enzymes (DUBs), with different specificities for polyubiquitinated linkages, was fused to the N-terminal RING domain of Rhesus TRIM5α (Table 1). Our previous study utilized the HSV-1 UL36 deubiquitinating enzyme, which has been reported to cleave both K48 and K63-linked polyubiquitin chains [172, 188, 189]. The different deubiquitinase enzymes employed in the current study were chosen for their ability to cleave only a single type of ubiquitin linkage, even at high polyubiquitin concentrations *in vitro* [190]. In addition, each of these deubiquitinase-RhTRIM5α fusions was paired with a catalytically inactive deubiquitinase-RhTRIM5α fusion protein (denoted as "*") to control for the addition to the N-terminus of RhTRIM5α due to the fusion protein [171-174, 191].

Deubiquitinase Name	Lysine Linkage Specificity	Domain	Size (bp)	Mutation for Catalytic Inactivity	Reference
HSV-1 UL36 (DUb)	K48 and K63	15-260	739	-	Campbell, E.M., et al., J Virol, 2016. 90(4): p. 1849-57.
HSV-1 UL36 (DUb*)	K48 and K63	15-260	739	C40S	Stringer, D.K. and R.C. Piper, J Cell Biol, 2011. 192(2): p. 229-42.
AMSH-LP	K63	265-436	519	-	Sato, Y., et al. Nature, 2008. 455(7211): p. 358-62.
AMSH-LP*	K63	265-436	519	E292A	
OTUB1	K48	Full length	810	-	Wang, P., et al. Acta Neuropathol, 2017. 133(5): p. 731-749.
OTUB1*	K48	Full length	810	C91A	

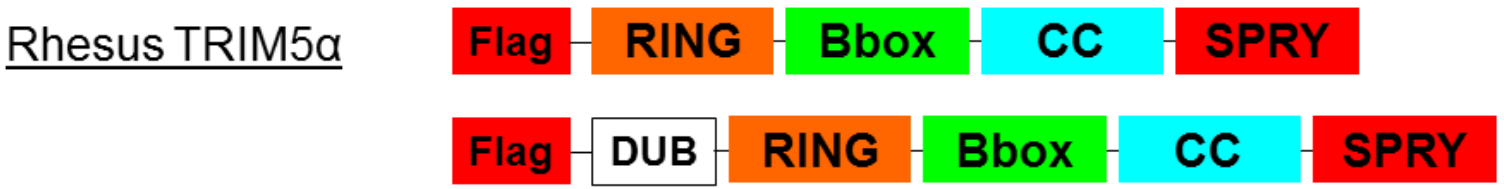
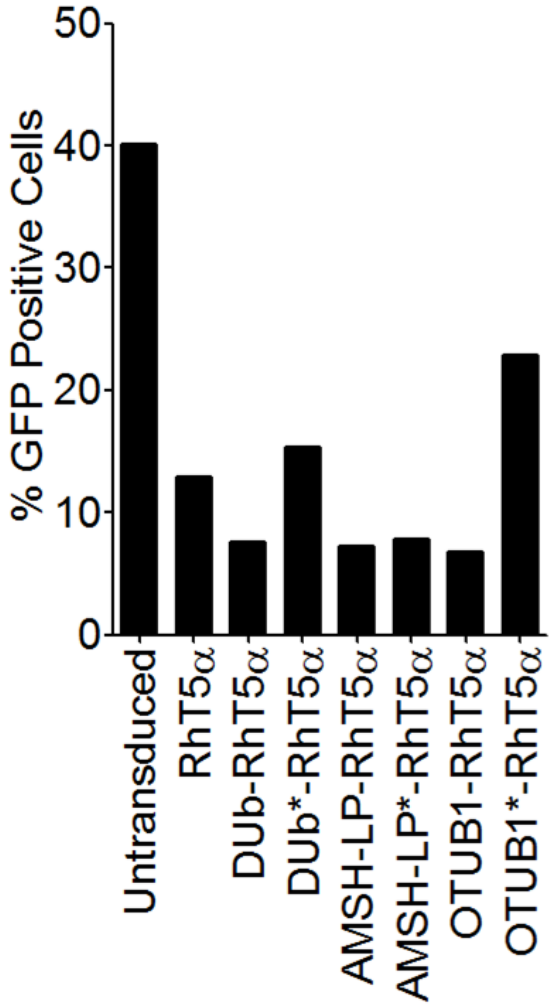


Table 1. Deubiquitinase (DUB)-RhTRIM5α Fusion Proteins Used in This Study. DUB enzymes were fused to the N-terminus of Rhesus TRIM5α, in frame between the Flag tag and the RING domain.

Deubiquitinase-RhTRIM5α Fusion Proteins Restrict HIV-1 Infection

First, these deubiquitinase-RhTRIM5α fusions were screened for their ability to restrict HIV-1 infection. A549 cells stably expressing each one of the DUB-RhTRIM5α fusion proteins were infected with an HIV-1 reporter virus, in which infected cells appear green. If ubiquitination is required for restriction, we would expect cells expressing one of active DUBs to be permissive to infection by the reporter virus. However, in all cases, both the catalytically active and inactive deubiquitinase fusion proteins retained the ability to restrict HIV-1 infection (Fig 22). It is worth noting that the catalytically inactive control for K48-specific DUB activity, OTUB1*-RhTRIM5α, was slightly more permissive to infection compared to the other cell lines. We believe this activity is due to protein folding changes that may have occurred due to the introduction of an alanine residue in the catalytic site of the DUB enzyme. Nevertheless, as all the fusion proteins were able to restrict HIV-1 infection, we concluded, from these data, that ubiquitination is not required for the restriction of infection by RhTRIM5α.



Deubiquitinase Name	Lysine Linkage Specificity
HSV-1 UL36 (DUb)	K48 and K63
HSV-1 UL36 (DUb*)	K48 and K63
AMSH-LP	K63
AMSH-LP*	K63
OTUB1	K48
OTUB1*	K48

Figure 22. Deubiquitinase-RhTRIM5α Fusion Proteins Restrict HIV-1 Infection in A549 Cells. RhTRIM5α fusion proteins were infected with a GFP reporter HIV-1 virus and the proportion of infected cells (% GFP positive) was assessed 48hpi by flow cytometry. Data are representative of two independent experiments.

K63-linked Ubiquitination Activity is Required for Restriction of Reverse Transcription by TRIM5α

During restriction, TRIM5α is known to promote the disassembly of capsids prior to the completion of reverse transcription, although this effect is abrogated in the

presence of the proteasome inhibitor MG132 [156, 157]. To determine if the restriction of reverse transcription requires ubiquitination activity, and to specifically define what type of ubiquitin linkages are critical, we infected A549 cells expressing the DUB-RhTRIM5α fusion proteins with an HIV-1 reporter virus and measured reverse transcription products (Figure 23). As expected, we observed minimal production of reverse transcription products in cells expressing RhTRIM5α, which is consistent with previous reports [85, 155]. In contrast, we observed an accumulation of reverse transcription products in cells expressing the catalytically active HSV-1 UL36 deubiquitinase fusion protein (DUb-RhTRIM5α). This enrichment is particularly evident when comparing the active DUB to its catalytically inactive control, DUb*-RhTRIM5α (Figure 23B). In this case, reverse transcription products did not accumulate, indicating that ubiquitination activity by RhTRIM5α is necessary for its restriction of reverse transcription.

Notably, the HSV-1 UL36 deubiquitinase enzyme demonstrates dual specificity to cleave both K48- and K63-linked polyubiquitin chains [172, 188, 189]. To more precisely define the ubiquitination linkages that are critical for the restriction of reverse transcription by TRIM5α, we measured reverse transcription in cells expressing K63-specific (AMSH-LP-RhTRIM5α) or K48-specific (OTUB1-RhTRIM5α) deubiquitinase fusion proteins, along with their catalytically inactive controls. Significantly, we observed an enrichment of reverse transcription products specifically in cells expressing the catalytically active K63-specific deubiquitinase, AMSH-LP-RhTRIM5α, but not in cells expressing the K48-specific DUB (Figure 23). Furthermore, this accumulation, once

again, is most apparent when comparing the active K63-specific DUB fusion to its catalytically inactive control (Figure 23B). Importantly, we observed no difference in the production of reverse transcription products in cells expressing the active or inactive K48-specific DUB fusion proteins. From these data, we conclude that RhTRIM5α requires K63-specific ubiquitination activity to restrict reverse transcription.

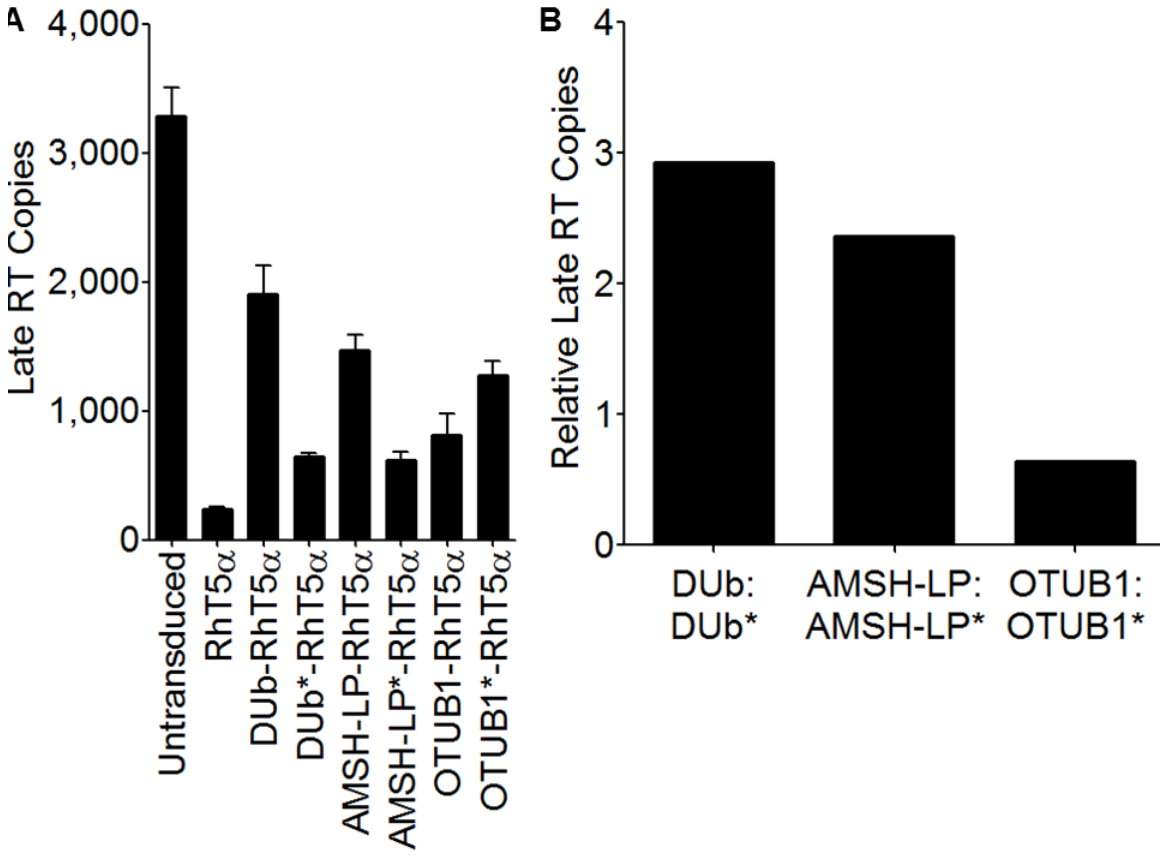


Figure 23. K63-linked Ubiquitination Activity is Required for Restriction of Reverse Transcription by TRIM5α. Deubiquitinase-RhTRIM5α fusion proteins were infected with a GFP reporter HIV-1 virus and viral reverse transcription products were measured by quantitative real time PCR. (A) Number of reverse transcription products (late RT products) accumulating in the indicated cell lines. Error bars represent the standard deviation of technical triplicates. Data are representative of at least three independent experiments. (B) Values from (A) plotted as the ratio of active DUB to its respective catalytically inactive control.

Inhibition of K63-linked Ubiquitination Produces Stable Association of RhTRIM5α with HIV-1 Cores but Impaired Association with Autophagosome Membranes

Previous reports from our lab and others determined that TRIM5α cytoplasmic bodies often colocalize with markers of autophagy, including p62/SQSTM1, LC3b, and LAMP2A [146, 164, 181]. In addition, one study mapped the interactions necessary for TRIM5α to bind to members of the mammalian ATG8 family, of which LC3b is a member, and they identified a region in the Coiled-coil domain of TRIM5α that is necessary for this interaction [146]. This was an intriguing finding, given that, as an E3 ubiquitin ligase, TRIM5α is autoubiquitinated, and the precise residues that are modified in TRIM5α have been mapped to the RING domain [103]. We previously observed that the ubiquitination activity of RhTRIM5α is required for its ability to destabilize viral cores[171], and that in the absence of ubiquitination activity, RhTRIM5α forms stable complexes with HIV-1 cores [171]. Therefore, we sought to determine if ubiquitination, and to define what type of ubiquitin linkage, is required for the stable association of TRIM5α with HIV-1 and for the recruitment of autophagic machinery to TRIM5α. To this end, we generated A549 cell lines stably expressing both YFP-tagged LC3b (YFP-LC3) and each one of the deubiquitinase-RhTRIM5α fusions. YFP-LC3 was utilized to mark autophagosome formation. LC3, a diffusely expressed cytoplasmic protein, binds to nascent autophagosome membranes, and therefore, punctate LC3 signal serves as marker of autophagosome formation. Following infection with HIV-1, we measured the degree of colocalization between the RhTRIM5α fusion proteins, HIV-1 p24 capsid protein, and/or YFP-LC3 (Figure 24-25).

Consistent with previous reports, we observed minimal stable association between RhTRIM5α and HIV-1 p24, as RhTRIM5α rapidly destabilizes viral cores (Figure 24-25). However, when K63-linked ubiquitination by RhTRIM5α is inhibited, as in the case of cells expressing DUb-RhTRIM5α and AMSH-LP-TRIM5α, we observed significant stable co-localization between TRIM5α the viral core, particularly when comparing these DUBs to their catalytically inactive controls (Figure 24-25). Importantly, we measured minimal stable co-localization between the K48-specific DUB fusion protein (OTUB1-RhTRIM5α) and HIV-1 p24, and critically there was no significant difference between the catalytically active and inactive fusions (Figure 25A). Conversely, we observed that K63-liked ubiquitination is critical for the association of RhTRIM5α with YFP-LC3, as both DUb-RhTRIM5α and AMSH-LP-TRIM5α showed minimal co-localization with LC3, in contrast to their catalytically inactive counterparts (Figure 25B). Collectively, these data indicate that the inhibition of K63-linked ubiquitination produces a stable association between RhTRIM5α and HIV-1 cores, and that K63-linked ubiquitination activity is critical for the association of TRIM5α with the autophagosome membrane marker LC3. Notably, there was minimal triple colocalization between the RhTRIM5α, LC3, and p24, suggesting that autophagic machinery is not recruited to the sites of core disassembly.

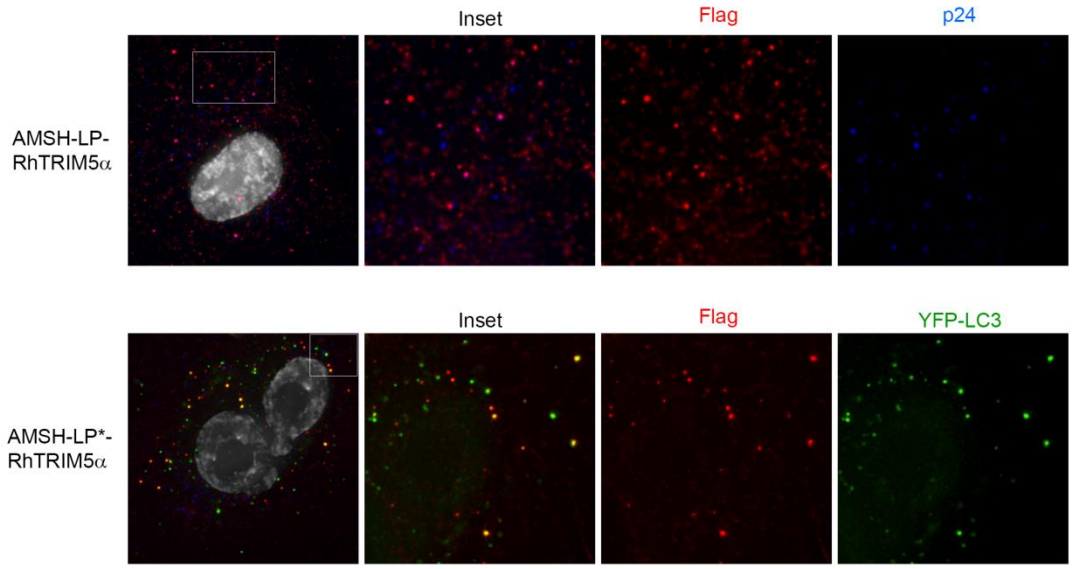


Figure 24. Colocalization of DUB-Fusion Proteins, HIV-1 p24, and/or YFP-LC3 in A549 Cells. Representative images of A549 cells expressing the indicated Flag-tagged RhTRIM5α DUB fusion proteins infected with HIV-1. Colocalization between Flag, HIV-1 p24, and/or YFP-LC3 was assessed (see Figure 24).

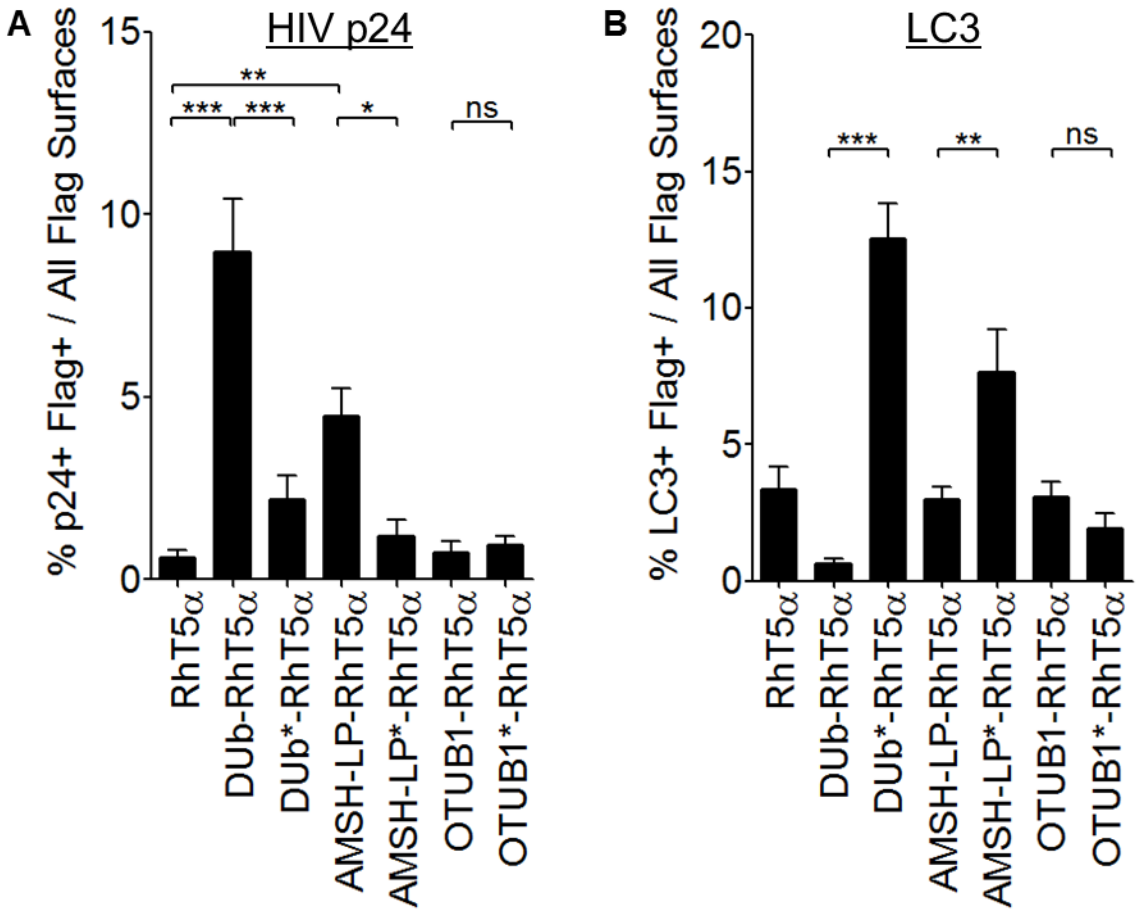


Figure 25. Inhibition of K63-linked Ubiquitination Produces Stable Association of RhTRIM5α with HIV-1 Cores but Impaired Association with Autophagosome Membranes. A549 cells expressing deubiquitinase-RhTRIM5α fusion proteins and YFP-LC3 were infected with HIV-1. The degree of colocalization of between the different TRIM5α fusion proteins (Flag-tagged) and the HIV-1 capsid (p24) (A), or YFP-LC3 (B) was measured. Error bars represent SEM of at least 20 images taken for each cell line. Data are representative of three independent experiments. *** = $p < 0.001$, ** = $p < 0.01$ by one-way ANOVA.

p62 is Critical for TRIM5α Association with Autophagosome Membranes

p62/SQSTM1 is an autophagic adaptor protein that can bind ubiquitinated substrates and direct them to autophagosomes [192]. We previously determined that p62 colocalizes with TRIM5α cytoplasmic bodies [164]. To determine if p62 is required for the association of TRIM5α with autophagosomes, we utilized CRISPR/Cas9 genome editing to target p62 or a non-targeting gRNA in A549 cells stably expressing YFP-LC3. These cells were subsequently transduced to stably express either RhTRIM5α or DUb*-RhTRIM5α, given that this was the fusion protein that most potently colocalized with YFP-LC3 (Figure 26). We quantified the association between RhTRIM5α and LC3 and observed that, in the absence of p62, there is significantly less colocalization between YFP-LC3 and RhTRIM5α, indicating that p62 is a critical mediator of the interaction between RhTRIM5α and autophagosomes (Fig 26).

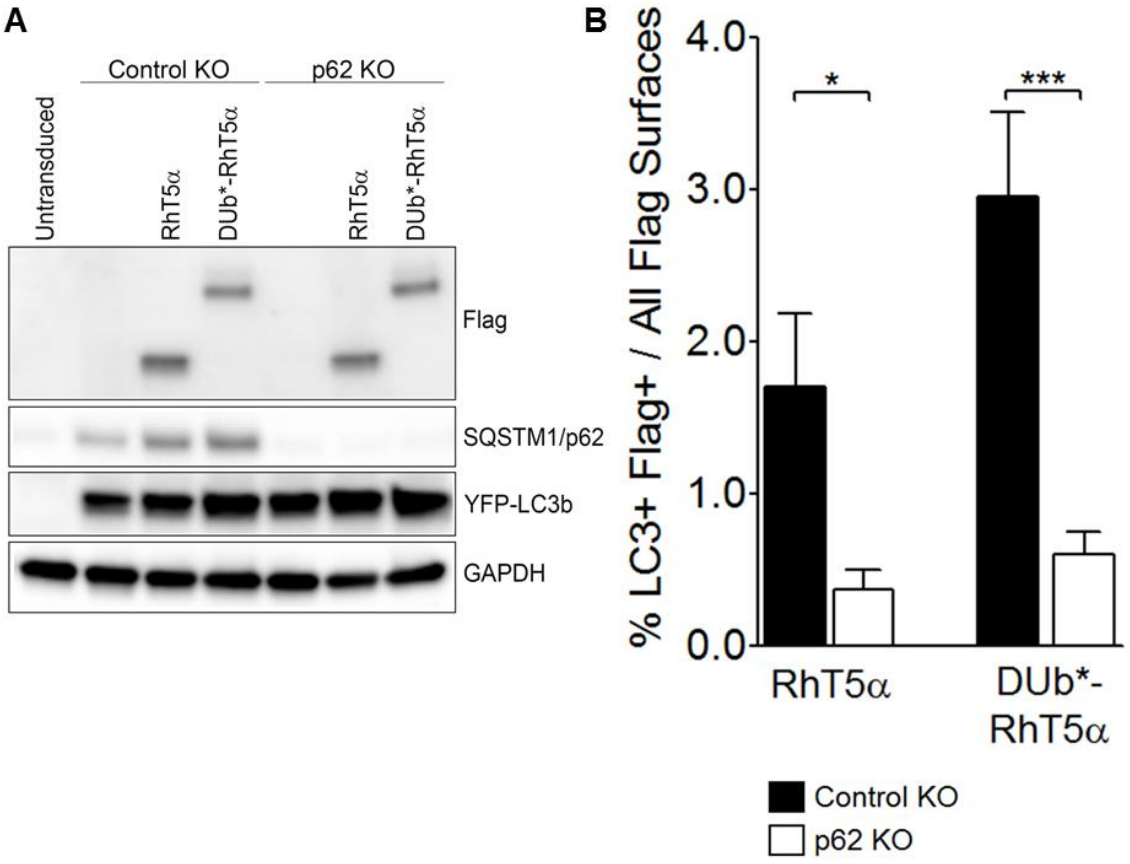


Figure 26. p62 is Critical for TRIM5α Association with Autophagosome Membranes. A549 cells were depleted of p62 via CRISPR/Cas9 genome editing. These cells were subsequently transduced to express YFP-LC3 and the indicated DUB constructs (A). (B) Following infection with an HIV-1 reporter virus, the degree of colocalization of between the different TRIM5α fusion proteins (Flag-tagged) and YFP-LC3 was measured. Error bars represent SEM of at least 20 images taken for each cell line. Data are representative of three independent experiments. *** = $p < 0.001$, * = $p < 0.05$ by Student's t-test.

CHAPTER FIVE

DISCUSSION

Summary of data

TRIM5α, as other TRIM proteins, has been shown to associate with markers of the autophagy pathway [146, 164, 181-184], and given TRIM5α's role as a retroviral restriction factor, that this association suggests a functional relationship between the autophagy pathway and TRIM5α. The goal of these studies was to examine the contributions of ubiquitination and association with autophagic effectors to TRIM5α's anti-retroviral functions.

First, we identified that the subcellular distribution of YFP-RhTRIM5α changes in the presence of BafA1 and MG132, with the accumulation of more cytoplasmic bodies of YFP-RhTRIM5α observed in the presence of autophagy inhibition compared to proteasome inhibition (Figure 13). We also determined that the YFP-RhTRIM5α cytoplasmic bodies colocalize with markers of the autophagy pathway (Figure 14). These observations provided the rationale to examine the role, if any, that autophagy plays in the restriction of retroviruses by TRIM5 proteins. For our study, we selected two important macroautophagy factors, Beclin1 and ATG5, which are critical for the nucleation and elongation of autophagosome membranes. It is worth noting that autophagy is a very complex cellular process involving many cellular proteins, and it is possible that in some cases, there may be redundant proteins that have similar effects.

Importantly, however, Beclin1 appears to be essential for macroautophagy [193] and thus was a valuable target for our assessment of autophagy in the functions of TRIM5α.

We observed that retroviral restriction was not impacted by depletion of autophagic mediators by siRNA (Figure 15). This was true in the case of endogenously expressed huTRIM5α, which still mediated potent inhibition of N-MLV infection and reverse trancription following ATG5, Beclin1 or p62 knockdown. However, these studies do not exclude the possibility that small amounts of these mediators remaining after siRNA knockdown are sufficient to preserve TRIM5α-mediated restriction. We therefore used the CRISPR-Cas9 genome editing system to deplete cells of ATG5 or Beclin1 and similarly assessed N-MLV restriction by huTRIM5α (Figure 16). Similar to our knockdown studies (Figure 15) no relief in retroviral restriction was observed (Figure 16D and F) in cells depleted of ATG5 or Beclin1. In addition, restriction of viral reverse transcription was intact following ATG5 and Beclin1 knockout (Figure 16G and H). Although we cannot discount the possibility that cells depleted of ATG5 or Beclin1 may possess alternative mechanisms of substrate degradation via autophagy, these results collectively demonstrate that restriction of retroviral infection and reverse transcription by TRIM5α is independent of ATG5 and Beclin1.

We obtained similar results when restriction of HIV-1 by RhTRIM5α (Figure 17) or TRIM-Cyp (Figure 18) was examined. Restriction of HIV-1 was not impacted by ATG5 or Beclin1 deletion (Figure 17). We did observe that ATG5 depletion caused a decrease HIV-1 infection (Figure 17B), although HIV-1 restriction remained intact in these cells (Figure 17B). These data are in apparent contrast to the findings from a

recent study, which observed that the depletion of autophagic adaptor proteins abrogated RhTRIM5α-mediated restriction of HIV-1 [146]. One noticeable difference between our studies was that the Mandell et. al. study utilized primary Rhesus fibroblasts to examine the role of autophagic adaptors in the restriction mechanism of RhTRIM5α; they observed modest restriction of HIV-1 by the primary Rhesus fibroblasts, with minimal relief of restriction observed following the depletion of rhTRIM5α by siRNA [146]. Our study dissected the role of autophagy in retroviral restriction in the context of much more potent restriction, as observed in the restriction of N-MLV by huTRIM5α and HIV-1 by RhTRIM5α or TRIM-Cyp. Thus, we suspect that the differences between our results and the Mandell et. al. study stem from the more pronounced degree of restriction observed in our studies. However, we cannot exclude the possibility that cell type or species specific differences explain the apparent discordance between our observations. Furthermore, it should be noted that the Mandell et. al. study assessed infectivity by measuring the amount of HIV-1 p24 protein in the cellular lysate of infected cells, rather than through the more conventional approach of measuring the expression of a reporter gene expressed upon viral integration. In this regard, autophagy has been implicated in regulating virus production [194], and therefore, the quantification of p24 in the cellular lysate, which could represent CA associated with the core or unincorporated CA monomers, does not directly reflect the restriction capabilities of TRIM5α and its relationship to autophagy.

Although we concluded that autophagy is not required for retroviral restriction by TRIM5α proteins, it is possible that the association of TRIM5α proteins with autophagy

markers could have some other role in TRIM5α's function. In the present study, we assessed the role of the ubiquitin ligase activity of RhTRIM5α on its restriction of HIV-1 infection, reverse transcription, stable association with viral capsids, and recruitment with the autophagosome marker LC3. We conclude that K63-linked ubiquitin ligase activity of RhTRIM5α is critical for its restriction of reverse transcription and association with LC3. In the absence of K63-linked ubiquitination, we observed a restoration of HIV-1 reverse transcription and the formation of stable complexes of RhTRIM5α and HIV-1. Finally K63-linked ubiquitination is critical for RhTRIM5α's association with LC3. The results from our study are in agreement with a recent report which identified that TRIM5α is modified by K63-linked ubiquitination *in vitro* [103]. Furthermore, the same study assessed the contribution of ubiquitination to the restriction of reverse transcription by TRIM5α and found that the restriction of reverse transcription by TRIM5α was relieved in cells expressing a K63R mutant ubiquitin, in which the formation of K63-linked polyubiquitin chains is blocked [103]. One pitfall of such an approach is that K63-linked ubiquitination is likely important for many cellular processes, and expression of this ubiquitin mutant could alter cellular pathways that could have an indirect impact on the restriction functions of TRIM5α. Our approach, in which we fused different deubiquitinases to RhTRIM5α, offered the opportunity to study ubiquitination in the context of the assembly of TRIM5α around a capsid, as this assembly has been shown to be critical to the activation of the E3 ligase function of TRIM5α [102]. Furthermore, by pairing each DUB with a catalytically inactive control protein, we were able to focus our analysis on the phenotypes associated with the enzymatic activity of

the DUB, rather than changes that could be a consequence of adding a fusion to the N-terminus of Rhesus TRIM5α. Finally, by including DUB enzymes with unique specificities for cleaving K48 or K63-linked polyubiquitin linkages, we demonstrated that the ubiquitin-dependent steps in restriction by TRIM5α specifically require its ability to generate K63-linked polyubiquitin chains. We are currently confirming the deubiquitinase activity of the each of the DUB-fusion proteins in *in vitro* studies.

Implications of the Data: TRIM5α and the Autophagy Pathway

Two recent studies have considered the importance of the autophagy pathway in facilitating core disruption by TRIM5α [146, 165]. This hypothesis is particularly attractive, given how K63-linked ubiquitin chains have been associated with the recruitment of autophagy machinery in cells [195]. In this model, TRIM5α binds to and forms an assembly around incoming viral cores, synthesizes K63-linked polyubiquitin chains [102], and recruits autophagic machinery to degrade both TRIM5α itself and its bound viral core [146, 165]. We showed that K63-linked ubiquitination is required for TRIM5α's association with autophagosome membranes (Figure 25) and its ability to restrict viral reverse transcription (Figure 23). A hypothesis one can draw from these data is that in the absence of K63-linked ubiquitination, TRIM5α is unable to recruit autophagic machinery; as result, stable complexes of TRIM5α bound to the core persist in the cytoplasm (Figure 24-25), allowing sufficient time for reverse transcription to proceed. In this case, the association with autophagosomes would be essential for the ability of TRIM5α to inhibit reverse transcription. However, we determined that the depletion of key macroautophagy factors ATG5 or Beclin1 had no impact on the ability

of TRIM5α to restrict reverse transcription [181]. These finding are in opposition to recent work highlighting the central role of specifically these two factors, along with p62, in restriction by TRIM5α [146]. While we disagree with this finding, it is possible that TRIM5α's association with the autophagy pathway may be relevant in other contexts.

Specifically, one recent study reported that HIV-1 infection is suppressed in Langerhans cells, a subset of dendritic cells found within mucosal tissues, and this restriction is mediated by TRIM5α [165]. In their model, Langerhans cells bind HIV-1 via the C-type lectin receptor Langerin, which in turn facilitates the binding of TRIM5α to the internalized virus [165]. Subsequently, TRIM5α directs the core complex to the autophagy pathway for degradation [165]. Importantly, this study found that this particular pathway of TRIM5α-mediated restriction was specific to Langerhans cells, as dendritic cells expressing a different C-type lectin receptor, DC-SIGN, were unable to direct the TRIM5α-virus complex to autophagosomes for degradation [165]. However, our studies investigating the role of autophagy in restriction determined that restriction of infection by different TRIM5 proteins occurred in the absence of ATG5 or Beclin1 (Figures 15-18) or K63-linked ubiquitination, which is critical for the association of TRIM5α with autophagosome membranes (Figure 22). This is supported by biochemical experiments which observed the spontaneous formation of TRIM5α assemblies on *in vitro* assembled capsid assemblies (Figure 8). Taken together, it is difficult to conceive how the formation of such an assembly, or the restriction to infection this assembly seems to confer, would be different in the context of Langerhans cells. However, a possible explanation for this apparent discord may be related to the ability of TRIM5α to

promote the induction of an innate signaling response upon capsid recognition [35, 102]. In this regard, it is possible that TRIM5α signaling may occur without the formation of a full, restricting TRIM5α assembly around the viral core, and it is possible that, in Langerhans cells, induction of this innate signaling response is sufficient to suppress infection and could explain the decreased infection in this cell type observed by Ribeiro and colleagues.

Nonetheless, identifying a restriction mechanism within Langerhans cells is particularly important, given their prevalence in mucosal sites, as these sites are the major route of transmission of HIV-1. It is also possible that the association of TRIM5α with autophagosomes might be more relevant for the generation of adaptive immune responses to the virus. In this case, autophagosomes containing TRIM5α-virus complexes could be fused with MHC class II containing compartments, thereby facilitating the delivery of antigens to MHC class II for presentation [196]. Therefore, association of TRIM5α with autophagosomes, in the context of infection, could be a means of bridging the innate and adaptive immune responses and should be an avenue of future investigation.

Implications of the Data: TRIM5α and the Proteasome

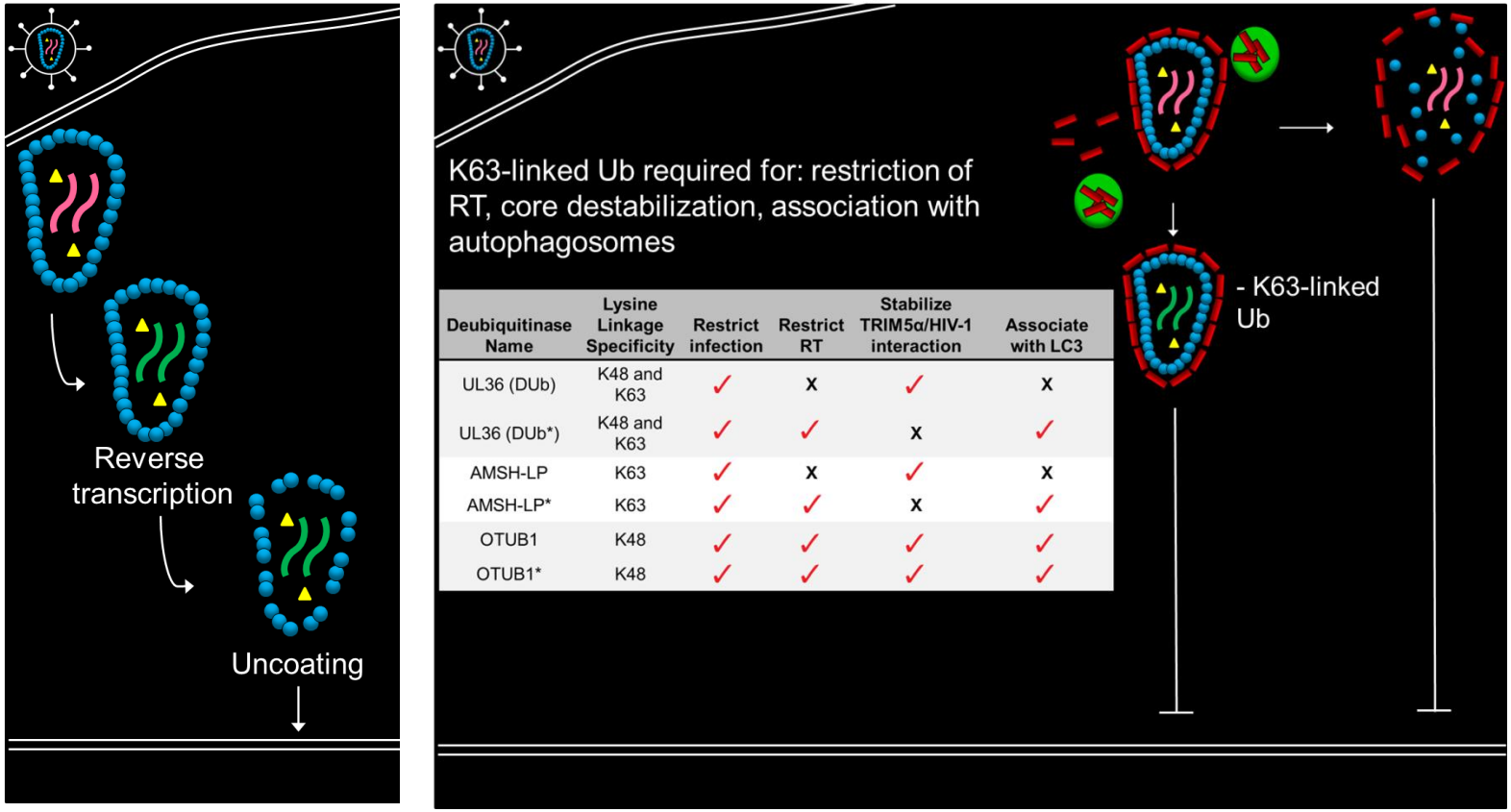
Previous reports determined that the destabilization of retroviral capsids by TRIM5 proteins is sensitive to proteasome inhibitors [156-158], and one implication of these observations is that TRIM5α utilizes proteasome machinery to destabilize retroviral capsids. However, this model raises important questions. Proteasomal degradation is generally associated with K48-linked rather than K63-linked polyubiquitin

linkages [126-128, 132-134]. It is possible that TRIM5α, which produces K63-linked polyubiquitin chains exclusively ([103] and the current study), recruits proteasomal machinery through a noncanonical mechanism. Two studies determined that while both K48- and K63-linked polyubiquitin linkages can bind to proteasomal components *in vitro* [135, 138], the processivity with which the proteasome unfolds substrates is dramatically reduced in substrates bearing K63-linked chains, compared to K48-linked chains [134]. Furthermore, individual TRIM5α proteins bind to the capsid with low affinity [114], and therefore, it is unclear how this low affinity interaction, coupled with the low processivity associated with K63-linked polyubiquitin chains, explains the efficient destabilization of the core that is observed during retroviral restriction by TRIM5α. Finally, many of the early studies investigating the role of the proteasome in restriction utilized proteasome inhibitors, such as MG132. However, several studies determined that treatment of cells with MG132 resulted in an accumulation of proteins bearing ubiquitin linkages of all types except for K63-linkages [142, 186], strongly suggesting that substrates marked with K63-linked polyubiquitin are not destined for proteasomal degradation. Collectively, these observations justify a reevaluation of the role of the proteasome in restriction by TRIM5α.

It is worth considering how our studies with the DUB-RhTRIM5α fusion proteins, which essentially recapitulated the effects of MG132 observed in earlier studies [156, 157], did so without, ostensibly, modulating the proteasome. One explanation to reconcile these two effects is that treatment with MG132 is known to deplete the cellular pool of free ubiquitin [197]. This, in turn, could potentially limit the availability of free

ubiquitin available for TRIM5α to complete its ubiquitin-dependent steps during restriction. Therefore, the effects of MG132 on restriction are likely phenotypes associated with side-effects of the drug itself rather than a disruption of a proteasome-dependent step in restriction.

The data from the current study support a model, first set forth by Barbie Ganser-Pornillos et. al., in which the formation of an assembly of TRIM5α around a retroviral core is sufficient to block infection [115]. Autophagic machinery is not required for the restriction of retroviral infection, as both ATG5 or Beclin1 knockout cells maintained potent restriction of multiple retrovirsues (Figure 15-18). Furthermore, cells expressing a RhTRIM5α fusion protein in which K63-linked ubiquitnation is inhibited maintain potent restriction of HIV-1 infection (see AMSH-LP-RhTRIM5α, Figure 22) despite minimal association with the autophagosome membrane marker LC3 (Figure 25). Importantly, core destabilization, which occurs prior to reverse transcription [155] and requires the K63-specific ubiquitin ligase activity of TRIM5α (Figure 25) does not appear to be required for the restriction of infection. This suggests that TRIM5α likely evolved multiple mechanisms of restricting retroviral infection. An early block, which requires the E3 ligase activity on the RING domain of TRIM5α, promotes the destabilization of cores prior to the completion of reverse transcription. However, infection is still blocked without the E3 ligase activity, suggesting a later block to infection. This later block is remarkably similar to the mechanism of restriction of murine Fv1, the first characterized retroviral restriction factor.



Deubiquitinase Name	Lysine Linkage Specificity	Restrict infection	Restrict RT	Stabilize TRIM5α/HIV-1 interaction	Associate with LC3
UL36 (DUb)	K48 and K63	✓	X	✓	X
UL36 (DUb*)	K48 and K63	✓	✓	X	✓
AMSH-LP	K63	✓	X	✓	X
AMSH-LP*	K63	✓	✓	X	✓
OTUB1	K48	✓	✓	✓	✓
OTUB1*	K48	✓	✓	✓	✓

Figure 27. Current Perspective of the Mechanism of Retroviral Restriction by TRIM5α. (left panel) During infection, retroviruses reverse transcribe their RNA genome (pink) into DNA (green), uncoat their capsid (blue) and traffic to the nucleus for integration. (right panel) TRIM5α [6] exists as diffuse protein and cytoplasmic bodies. K63-specific ubiquitin ligase activity of TRIM5α is critical for its association with autophagosome membrane markers and its ability to destabilize viral cores prior to the completion of reverse transcription. In the absence of K63-specific ubiquitin ligase activity, TRIM5α forms a stable association with the capsid, allowing reverse transcription to proceed, however infection is still blocked. This favors a model whereby the formation of a TRIM5α assembly around a capsid is sufficient to inhibit infection, while ubiquitin ligase activity of TRIM5α is needed to inhibit reverse transcriptio

Fv1 inhibits retroviral infection after reverse transcription but before integration, a phenotype mirrored in RhTRIM5α fusions with incompetent K63-linked ubiquitin ligase activity (AMSH-LP-RhTRIM5α, Figure 22-23). Importantly, although TRIM5α and Fv1 are very different at the primary sequence level, they each likely evolved independently in response to retroviral challenge over time [86, 198]. However, the evolution of the RING domain in TRIM5α proteins likely afforded TRIM5α the capacity to effectively and efficiently block both retroviral reverse transcription and infection.

Intriguingly, TRIM5-based capsid binding restriction factors have evolved at least twice, independently, through the retrotransposition of a Cyclophilin A pseudogene to replace the SPRY domain of TRIM5α [199-203]. While this retrotranspostion event could have occurred by chance, it is possible that the fusion of a capsid binding factor, such as cyclophilin A, to the TRIM motif conferred some selective advantage against retroviral pathogens, hence their independent evolution among different primates. In this regard, in a laboratory setting, fusion of cyclophilin A to a protein that is known to multimerize generates a capsid-binding factor that is competent to restrict HIV-1 infection [99]. If multimerization was the only requirement to generate an efficient capsid-binding restriction factor, why would TRIM-based factors, specifically, arise repeatedly? One hypothesis is that in addition to multimerization, the TRIM motif, and specifically the RING domain, confers the ability to generate an antiviral signaling response [35], and thus was selected for over time, given the evolutionary advantages associated with the expression of sensors for pathogens.Upon binding to a restriction-sensitive capsid, the ubiquitin ligase activity of TRIM5α is activated [102], and in turn

TRIM5α has been shown to generate free, unanchored K63-linked polyubiquitin chains [35]. These ubiquitin chains can subsequently bind to and promote the activation of the cytoplasmic kinase TAK1, which in turn promotes the activation and upregulation of AP-1 and NFκB-dependent genes [35]. Finally, the anti-retroviral activity of TRIM5α begins with its binding to the retroviral capsid, and because the capsid is considered "genetically fragile" and is required for many aspects of the retroviral life cycle [27, 36, 37], it is unlikely to evolve to avoid TRIM5α binding. Therefore, TRIM5α has the ability to efficiently couple its restriction function with the generation of an innate immune response, and studies to define the underlying mechanisms of restriction can help to enhance the activity of human TRIM5α against significant pathogens like HIV-1.

Future Directions

This work leaves a few important lingering questions. First, it is worth revisiting if proteasomes are indeed involved in the core destabilization function of TRIM5α. This work suggests that this destabilization occurs independently of proteasomes and supports a model whereby the destabilization of capsids may occur as a result of the dynamic movement of individual SPRY domains bound to the capsid [100]. In this model, the spring-like movement of the L2 region (Figure 7), which exists as a helix, could displace individual SPRY domains bound to the capsid and promote capsid disassembly [100]. However, within this model, it is unclear what role TRIM5α's E3 ligase activity plays, although from the current work it is clear that this ligase activity is required for capsid disassembly. It is possible that as the activation of the E3 ligase function promotes the ubiquitination of TRIM5α, this in turn imparts some

conformational change in the protein that facilitates the dynamic movement of the L2 spring, and subsequently, displacement of the SPRY domain bound to the capsid. In another model, a recent study described how a cytoplasmic polyanion, IP_6, binds to and stabilizes HIV-1 cores, in a manner that the authors suggest prevents spontaneous disassembly of the cores [204]. In this case, ubiquitination of TRIM5α bound to a capsid could promote the displacement of IP_6 and summarily, induce the disassembly of the capsid.

Furthermore, early studies describing proteasome-dependent steps in TRIM5α-mediated restriction relied on MG132 [156-158, 162]. For example one study reported that during restriction, viral core components such as the RNA genome, reverse transcriptase and integrase proteins, and the capsid itself, are likely degraded by the proteasome [158]. However, given that the same study could identify no direct ubiquitination of core components [158], and the caveats associated with the model of proteasome-dependent destabilization described above, it is unclear what role, if any, the proteasome has in TRIM5α's restriction functions.

Finally it is important to consider the implications of this work on the development of strategies to improve the activity of human TRIM5α against HIV-1. While human TRIM5α is not as potent as Rhesus TRIM5α in blocking HIV-1 infection, several lines of evidence suggest that there are opportunities to improve its functions. For example, several reports have characterized SNPs in human TRIM5α that are associated with higher viral loads and a more rapid progression towards AIDS [205-207], suggesting that wild-type human TRIM5α is exerting some protective effect that is absent in

individuals bearing these SNPs. Furthermore, a recent study described how an artificially stabilized version of human TRIM5α had the ability to restrict HIV-1 infection to a degree comparable to that performed by Rhesus TRIM5α [208]. In this vein, strategies that promote the stabilization of human TRIM5α may enhance its anti-HIV-1 activity. As a proof of this principle, it would be interesting to assess whether human TRIM5α fused to the K63-specific deubiquitinase enzyme, which we showed produces a stable complex between AMSH-LP-RhTRIM5α and HIV-1 (Figures 24-25), could impart improved restriction capabilities to human TRIM5α.

LIST OF REFERENCES

1. Riddell Jt, Amico KR, Mayer KH. HIV Preexposure Prophylaxis: A Review. *JAMA* 2018,**319**:1261-1268.

2. Bhatti AB, Usman M, Kandi V. Current Scenario of HIV/AIDS, Treatment Options, and Major Challenges with Compliance to Antiretroviral Therapy. *Cureus* 2016,**8**:e515.

3. Maartens G, Celum C, Lewin SR. HIV infection: epidemiology, pathogenesis, treatment, and prevention. *Lancet* 2014,**384**:258-271.

4. Zaidi J, Grapsa E, Tanser F, Newell ML, Barnighausen T. Dramatic increase in HIV prevalence after scale-up of antiretroviral treatment. *AIDS* 2013,**27**:2301-2305.

5. Popovic M, Sarngadharan MG, Read E, Gallo RC. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 1984,**224**:497-500.

6. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF*, et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 1984,**224**:500-503.

7. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J*, et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983,**220**:868-871.

8. Sharp PM, Hahn BH. Origins of HIV and the AIDS pandemic. *Cold Spring Harb Perspect Med* 2011,**1**:a006841.

9. Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* 2011,**25**:679-689.

10. Cohen MS, Shaw GM, McMichael AJ, Haynes BF. Acute HIV-1 Infection. *N Engl J Med* 2011,**364**:1943-1954.

11. Hladik F, McElrath MJ. Setting the stage: host invasion by HIV. *Nat Rev Immunol* 2008,**8**:447-457.

12. Ribeiro RM, Qin L, Chavez LL, Li D, Self SG, Perelson AS. Estimation of the initial viral growth rate and basic reproductive number during acute HIV-1 infection. *J Virol* 2010,**84**:6096-6102.

13. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J*, et al.* Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J Virol* 2009,**83**:3719-3733.

14. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV*, et al.* The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* 2009,**206**:1253-1272.

15. Liao HX, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD*, et al.* Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature* 2013,**496**:469-476.

16. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B*, et al.* Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006,**12**:1198-1202.

17. Cooper A, Garcia M, Petrovas C, Yamamoto T, Koup RA, Nabel GJ. HIV-1 causes CD4 cell death through DNA-dependent protein kinase during viral integration. *Nature* 2013,**498**:376-379.

18. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S*, et al.* Sex differences in the Toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. *Nat Med* 2009,**15**:955-959.

19. Wightman F, Solomon A, Khoury G, Green JA, Gray L, Gorry PR*, et al.* Both CD31(+) and CD31(-) naive CD4(+) T cells are persistent HIV type 1-infected reservoirs in individuals receiving antiretroviral therapy. *J Infect Dis* 2010,**202**:1738-1748.

20. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B*, et al.* HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat Med* 2009,**15**:893-900.

21. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T*, et al.* Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* 1999,**5**:512-517.

22. Kirchhoff F. HIV Life Cycle: Overview. In: *Encyclopedia of AIDS*. Edited by Hope TJ, Stevenson M, Richman D. New York, NY: Springer New York; 2021. pp. 1-9.

23. Koppensteiner H, Brack-Werner R, Schindler M. Macrophages and their relevance in Human Immunodeficiency Virus Type I infection. *Retrovirology* 2012,**9**:82.

24. Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, Wyatt RT. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. *Nat Rev Microbiol* 2008,**6**:143-155.

25. Doyle T, Goujon C, Malim MH. HIV-1 and interferons: who's interfering with whom? *Nat Rev Microbiol* 2015,**13**:403-413.

26. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M*, et al.* Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996,**381**:661-666.

27. Campbell EM, Hope TJ. HIV-1 capsid: the multifaceted key player in HIV-1 infection. *Nat Rev Microbiol* 2015,**13**:471-483.

28. Dharan A, Opp S, Abdel-Rahim O, Keceli SK, Imam S, Diaz-Griffero F*, et al.* Bicaudal D2 facilitates the cytoplasmic trafficking and nuclear import of HIV-1 genomes during infection. *Proc Natl Acad Sci U S A* 2017,**114**:E10707-E10716.

29. Barre-Sinoussi F, Ross AL, Delfraissy JF. Past, present and future: 30 years of HIV research. *Nat Rev Microbiol* 2013,**11**:877-883.

30. Cosnefroy O, Murray PJ, Bishop KN. HIV-1 capsid uncoating initiates after the first strand transfer of reverse transcription. *Retrovirology* 2016,**13**:58.

31. Mamede JI, Cianci GC, Anderson MR, Hope TJ. Early cytoplasmic uncoating is associated with infectivity of HIV-1. *Proc Natl Acad Sci U S A* 2017,**114**:E7169-E7178.

32. Rankovic S, Varadarajan J, Ramalho R, Aiken C, Rousso I. Reverse Transcription Mechanically Initiates HIV-1 Capsid Disassembly. *J Virol* 2017,**91**.

33. Lahaye X, Satoh T, Gentili M, Cerboni S, Conrad C, Hurbain I*, et al.* The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells. *Immunity* 2013,**39**:1132-1142.

34. Rasaiyaah J, Tan CP, Fletcher AJ, Price AJ, Blondeau C, Hilditch L*, et al.* HIV-1 evades innate immune recognition through specific cofactor recruitment. *Nature* 2013,**503**:402-405.

35. Pertel T, Hausmann S, Morger D, Zuger S, Guerra J, Lascano J*, et al.* TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 2011,**472**:361-365.

36. Forshey BM, von Schwedler U, Sundquist WI, Aiken C. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. *J Virol* 2002,**76**:5667-5677.

37. Rihn SJ, Wilson SJ, Loman NJ, Alim M, Bakker SE, Bhella D*, et al.* Extreme genetic fragility of the HIV-1 capsid. *PLoS Pathog* 2013,**9**:e1003461.

38. Peng K, Muranyi W, Glass B, Laketa V, Yant SR, Tsai L*, et al.* Quantitative microscopy of functional HIV post-entry complexes reveals association of replication with the viral capsid. *Elife* 2014,**3**:e04114.

39. Hulme AE, Kelley Z, Foley D, Hope TJ. Complementary Assays Reveal a Low Level of CA Associated with Viral Complexes in the Nuclei of HIV-1-Infected Cells. *J Virol* 2015,**89**:5350-5361.

40. Freed EO. HIV-1 assembly, release and maturation. *Nat Rev Microbiol* 2015,**13**:484-496.

41. Nielsen MH, Pedersen FS, Kjems J. Molecular strategies to inhibit HIV-1 replication. *Retrovirology* 2005,**2**:10.

42. Briggs JA, Simon MN, Gross I, Krausslich HG, Fuller SD, Vogt VM*, et al.* The stoichiometry of Gag protein in HIV-1. *Nat Struct Mol Biol* 2004,**11**:672-675.

43. Ho DD. Time to hit HIV, early and hard. *N Engl J Med* 1995,**333**:450-451.

44. Goody RS, Muller B, Restle T. Factors contributing to the inhibition of HIV reverse transcriptase by chain-terminating nucleotides in vitro and in vivo. *FEBS Lett* 1991,**291**:1-5.

45. Sluis-Cremer N, Tachedjian G. Mechanisms of inhibition of HIV replication by non-nucleoside reverse transcriptase inhibitors. *Virus Res* 2008,**134**:147-156.

46. Malim MH, Bieniasz PD. HIV Restriction Factors and Mechanisms of Evasion. *Cold Spring Harb Perspect Med* 2012,**2**:a006940.

47. Yu Q, Konig R, Pillai S, Chiles K, Kearney M, Palmer S*, et al.* Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat Struct Mol Biol* 2004,**11**:435-442.

48. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 2003,**424**:94-98.

49. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 2003,**424**:99-103.

50. Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN*, et al.* DNA deamination mediates innate immunity to retroviral infection. *Cell* 2003,**113**:803-809.

51. Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P*, et al.* Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* 2003,**302**:1056-1060.

52. Goldstone DC, Ennis-Adeniran V, Hedden JJ, Groom HC, Rice GI, Christodoulou E*, et al.* HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature* 2011,**480**:379-382.

53. Berger A, Sommer AF, Zwarg J, Hamdorf M, Welzel K, Esly N*, et al.* SAMHD1-deficient CD14+ cells from individuals with Aicardi-Goutieres syndrome are highly susceptible to HIV-1 infection. *PLoS Pathog* 2011,**7**:e1002425.

54. Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M, Srivastava S*, et al.* Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 2011,**474**:658-661.

55. Diamond TL, Roshal M, Jamburuthugoda VK, Reynolds HM, Merriam AR, Lee KY*, et al.* Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase. *J Biol Chem* 2004,**279**:51545-51553.

56. Fribourgh JL, Nguyen HC, Matreyek KA, Alvarez FJD, Summers BJ, Dewdney TG*, et al.* Structural insight into HIV-1 restriction by MxB. *Cell Host Microbe* 2014,**16**:627-638.

57. Goujon C, Moncorge O, Bauby H, Doyle T, Barclay WS, Malim MH. Transfer of the amino-terminal nuclear envelope targeting domain of human MX2 converts MX1 into an HIV-1 resistance factor. *J Virol* 2014,**88**:9017-9026.

58. Liu Z, Pan Q, Ding S, Qian J, Xu F, Zhou J*, et al.* The interferon-inducible MxB protein inhibits HIV-1 infection. *Cell Host Microbe* 2013,**14**:398-410.

59. Goujon C, Moncorge O, Bauby H, Doyle T, Ward CC, Schaller T*, et al.* Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature* 2013,**502**:559-562.

60. Jia X, Zhao Q, Xiong Y. HIV suppression by host restriction factors and viral immune evasion. *Curr Opin Struct Biol* 2015,**31**:106-114.

61. Neil SJ, Sandrin V, Sundquist WI, Bieniasz PD. An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. *Cell Host Microbe* 2007,**2**:193-203.

62. Neil SJ, Eastman SW, Jouvenet N, Bieniasz PD. HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. *PLoS Pathog* 2006,**2**:e39.

63. Sanz-Ramos M, Stoye JP. Capsid-binding retrovirus restriction factors: discovery, restriction specificity and implications for the development of novel therapeutics. *J Gen Virol* 2013,**94**:2587-2598.

64. Rowe WP, Hartley JW. Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-1 b strains of mice. *J Exp Med* 1972,**136**:1286-1301.

65. Rowe WP. Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with Fv-1 n strains of mice. *J Exp Med* 1972,**136**:1272-1285.

66. Odaka T. Inheritance of susceptibility to Friend mouse leukemia virus. V. Introduction of a gene responsible for susceptibility in the genetic complement of resistant mice. *J Virol* 1969,**3**:543-548.

67. Lilly F. Fv-2: identification and location of a second gene governing the spleen focus response to Friend leukemia virus in mice. *J Natl Cancer Inst* 1970,**45**:163-169.

68. Kozak CA, Chakraborti A. Single amino acid changes in the murine leukemia virus capsid protein gene define the target of Fv1 resistance. *Virology* 1996,**225**:300-305.

69. Hopkins N, Schindler J, Hynes R. Six-NB-tropic murine leukemia viruses derived from a B-tropic virus of BALB/c have altered p30. *J Virol* 1977,**21**:309-318.

70. Jolicoeur P, Baltimore D. Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia viruses. *Proc Natl Acad Sci U S A* 1976,**73**:2236-2240.

71. Pryciak PM, Varmus HE. Fv-1 restriction and its effects on murine leukemia virus integration in vivo and in vitro. *J Virol* 1992,**66**:5959-5966.

72. Duran-Troise G, Bassin RH, Rein A, Gerwin BI. Loss of Fv-1 restriction in Balb/3T3 cells following infection with a single N tropic murine leukemia virus particle. *Cell* 1977,**10**:479-488.

73. Pincus T, Hartley JW, Rowe WP. A major genetic locus affecting resistance to infection with murine leukemia viruses. IV. Dose-response relationships in Fv-1-sensitive and resistant cell cultures. *Virology* 1975,**65**:333-342.

74. Stoye JP, Kaushik N, Jeremiah S, Best S. Genetic map of the region surrounding the retrovirus restriction locus, Fv1, on mouse chromosome 4. *Mamm Genome* 1995,**6**:31-36.

75. Best S, Le Tissier P, Towers G, Stoye JP. Positional cloning of the mouse retrovirus restriction gene Fv1. *Nature* 1996,**382**:826-829.

76. Benit L, De Parseval N, Casella JF, Callebaut I, Cordonnier A, Heidmann T. Cloning of a new murine endogenous retrovirus, MuERV-L, with strong similarity to the human HERV-L element and with a gag coding sequence closely related to the Fv1 restriction gene. *J Virol* 1997,**71**:5652-5657.

77. Towers G, Bock M, Martin S, Takeuchi Y, Stoye JP, Danos O. A conserved mechanism of retrovirus restriction in mammals. *Proc Natl Acad Sci U S A* 2000,**97**:12295-12299.

78. Owens CM, Yang PC, Gottlinger H, Sodroski J. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol* 2003,**77**:726-731.

79. Munk C, Brandt SM, Lucero G, Landau NR. A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc Natl Acad Sci U S A* 2002,**99**:13843-13848.

80. Cowan S, Hatziioannou T, Cunningham T, Muesing MA, Gottlinger HG, Bieniasz PD. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc Natl Acad Sci U S A* 2002,**99**:11914-11919.

81. Besnier C, Takeuchi Y, Towers G. Restriction of lentivirus in monkeys. *Proc Natl Acad Sci U S A* 2002,**99**:11920-11925.

82. Hatziioannou T, Perez-Caballero D, Yang A, Cowan S, Bieniasz PD. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. *Proc Natl Acad Sci U S A* 2004,**101**:10774-10779.

83. Keckesova Z, Ylinen LM, Towers GJ. The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. *Proc Natl Acad Sci U S A* 2004,**101**:10780-10785.

84. Perron MJ, Stremlau M, Song B, Ulm W, Mulligan RC, Sodroski J. TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. *Proc Natl Acad Sci U S A* 2004,**101**:11827-11832.

85. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 2004,**427**:848-853.

86. Yap MW, Colbeck E, Ellis SA, Stoye JP. Evolution of the retroviral restriction gene Fv1: inhibition of non-MLV retroviruses. *PLoS Pathog* 2014,**10**:e1003968.

87. van Tol S, Hage A, Giraldo MI, Bharaj P, Rajsbaum R. The TRIMendous Role of TRIMs in Virus-Host Interactions. *Vaccines (Basel)* 2017,**5**.

88. Hatakeyama S. TRIM Family Proteins: Roles in Autophagy, Immunity, and Carcinogenesis. *Trends Biochem Sci* 2017,**42**:297-311.

89. Meroni G, Diez-Roux G. TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases. *Bioessays* 2005,**27**:1147-1157.

90. Esposito D, Koliopoulos MG, Rittinger K. Structural determinants of TRIM protein function. *Biochem Soc Trans* 2017,**45**:183-191.

91. Massiah MA, Simmons BN, Short KM, Cox TC. Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *J Mol Biol* 2006,**358**:532-545.

92. Ozato K, Shin DM, Chang TH, Morse HC, 3rd. TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 2008,**8**:849-860.

93. Hatziioannou T, Cowan S, Goff SP, Bieniasz PD, Towers GJ. Restriction of multiple divergent retroviruses by Lv1 and Ref1. *EMBO J* 2003,**22**:385-394.

94. Towers GJ. Control of viral infectivity by tripartite motif proteins. *Hum Gene Ther* 2005,**16**:1125-1132.

95. Nisole S, Stoye JP, Saib A. TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 2005,**3**:799-808.

96. Bishop KN, Mortuza GB, Howell S, Yap MW, Stoye JP, Taylor IA. Characterization of an amino-terminal dimerization domain from retroviral restriction factor Fv1. *J Virol* 2006,**80**:8225-8235.

97. Javanbakht H, Yuan W, Yeung DF, Song B, Diaz-Griffero F, Li Y*, et al.* Characterization of TRIM5alpha trimerization and its contribution to human immunodeficiency virus capsid binding. *Virology* 2006,**353**:234-246.

98. Perez-Caballero D, Hatziioannou T, Yang A, Cowan S, Bieniasz PD. Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *J Virol* 2005,**79**:8969-8978.

99. Yap MW, Mortuza GB, Taylor IA, Stoye JP. The design of artificial retroviral restriction factors. *Virology* 2007,**365**:302-314.

100. Lamichhane R, Mukherjee S, Smolin N, Pauszek RF, 3rd, Bradley M, Sastri J*, et al.* Dynamic conformational changes in the rhesus TRIM5alpha dimer dictate the potency of HIV-1 restriction. *Virology* 2017,**500**:161-168.

101. Maegawa H, Miyamoto T, Sakuragi J, Shioda T, Nakayama EE. Contribution of RING domain to retrovirus restriction by TRIM5alpha depends on combination of host and virus. *Virology* 2010,**399**:212-220.

102. Yudina Z, Roa A, Johnson R, Biris N, de Souza Aranha Vieira DA, Tsiperson V*, et al.* RING Dimerization Links Higher-Order Assembly of TRIM5alpha to Synthesis of K63-Linked Polyubiquitin. *Cell Rep* 2015,**12**:788-797.

103. Fletcher AJ, Christensen DE, Nelson C, Tan CP, Schaller T, Lehner PJ*, et al.* TRIM5alpha requires Ube2W to anchor Lys63-linked ubiquitin chains and restrict reverse transcription. *EMBO J* 2015,**34**:2078-2095.

104. Javanbakht H, Diaz-Griffero F, Stremlau M, Si Z, Sodroski J. The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5alpha. *J Biol Chem* 2005,**280**:26933-26940.

105. Li X, Sodroski J. The TRIM5alpha B-box 2 domain promotes cooperative binding to the retroviral capsid by mediating higher-order self-association. *J Virol* 2008,**82**:11495-11502.

106. Goldstone DC, Walker PA, Calder LJ, Coombs PJ, Kirkpatrick J, Ball NJ*, et al.* Structural studies of postentry restriction factors reveal antiparallel dimers that enable avid binding to the HIV-1 capsid lattice. *Proc Natl Acad Sci U S A* 2014,**111**:9609-9614.

107. Sastri J, Johnsen L, Smolin N, Imam S, Mukherjee S, Lukic Z*, et al.* Restriction of HIV-1 by rhesus TRIM5alpha is governed by alpha helices in the Linker2 region. *J Virol* 2014,**88**:8911-8923.

108. Langelier CR, Sandrin V, Eckert DM, Christensen DE, Chandrasekaran V, Alam SL*, et al.* Biochemical characterization of a recombinant TRIM5alpha protein that restricts human immunodeficiency virus type 1 replication. *J Virol* 2008,**82**:11682-11694.

109. Newman RM, Hall L, Connole M, Chen GL, Sato S, Yuste E*, et al.* Balancing selection and the evolution of functional polymorphism in Old World monkey TRIM5alpha. *Proc Natl Acad Sci U S A* 2006,**103**:19134-19139.

110. Ohkura S, Yap MW, Sheldon T, Stoye JP. All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *J Virol* 2006,**80**:8554-8565.

111. Rahm N, Yap M, Snoeck J, Zoete V, Munoz M, Radespiel U*, et al.* Unique spectrum of activity of prosimian TRIM5alpha against exogenous and endogenous retroviruses. *J Virol* 2011,**85**:4173-4183.

112. Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J. Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *J Virol* 2005,**79**:3930-3937.

113. Stremlau M, Perron M, Welikala S, Sodroski J. Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *J Virol* 2005,**79**:3139-3145.

114. Biris N, Yang Y, Taylor AB, Tomashevski A, Guo M, Hart PJ*, et al.* Structure of the rhesus monkey TRIM5alpha PRYSPRY domain, the HIV capsid recognition module. *Proc Natl Acad Sci U S A* 2012,**109**:13278-13283.

115. Ganser-Pornillos BK, Chandrasekaran V, Pornillos O, Sodroski JG, Sundquist WI, Yeager M. Hexagonal assembly of a restricting TRIM5alpha protein. *Proc Natl Acad Sci U S A* 2011,**108**:534-539.

116. Li YL, Chandrasekaran V, Carter SD, Woodward CL, Christensen DE, Dryden KA*, et al.* Primate TRIM5 proteins form hexagonal nets on HIV-1 capsids. *Elife* 2016,**5**.

117. Wagner JM, Roganowicz MD, Skorupka K, Alam SL, Christensen D, Doss G*, et al.* Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5alpha. *Elife* 2016,**5**.

118. Nisole S, Lynch C, Stoye JP, Yap MW. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc Natl Acad Sci U S A* 2004,**101**:13324-13328.

119. Sayah DM, Sokolskaja E, Berthoux L, Luban J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 2004,**430**:569-573.

120. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 1993,**73**:1067-1078.

121. Sawyer SL, Wu LI, Emerman M, Malik HS. Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 2005,**102**:2832-2837.

122. Song B, Gold B, O'Huigin C, Javanbakht H, Li X, Stremlau M*, et al.* The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *J Virol* 2005,**79**:6111-6121.

123. Kaiser SM, Malik HS, Emerman M. Restriction of an extinct retrovirus by the human TRIM5alpha antiviral protein. *Science* 2007,**316**:1756-1758.

124. Yap MW, Nisole S, Stoye JP. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol* 2005,**15**:73-78.

125. Diaz-Griffero F, Vandegraaff N, Li Y, McGee-Estrada K, Stremlau M, Welikala S*, et al.* Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. *Virology* 2006,**351**:404-419.

126. Yau R, Rape M. The increasing complexity of the ubiquitin code. *Nat Cell Biol* 2016,**18**:579-586.

127. Husnjak K, Dikic I. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu Rev Biochem* 2012,**81**:291-322.

128. Komander D, Rape M. The ubiquitin code. *Annu Rev Biochem* 2012,**81**:203-229.

129. Rajsbaum R, Garcia-Sastre A, Versteeg GA. TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 2014,**426**:1265-1284.

130. Ye Y, Rape M. Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* 2009,**10**:755-764.

131. Ohtake F, Saeki Y, Ishido S, Kanno J, Tanaka K. The K48-K63 Branched Ubiquitin Chain Regulates NF-kappaB Signaling. *Mol Cell* 2016,**64**:251-266.

132. Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep* 2008,**9**:536-542.

133. Ebner P, Versteeg GA, Ikeda F. Ubiquitin enzymes in the regulation of immune responses. *Crit Rev Biochem Mol Biol* 2017,**52**:425-460.

134. Reichard EL, Chirico GG, Dewey WJ, Nassif ND, Bard KE, Millas NE*, et al.* Substrate Ubiquitination Controls the Unfolding Ability of the Proteasome. *J Biol Chem* 2016,**291**:18547-18561.

135. Peth A, Uchiki T, Goldberg AL. ATP-dependent steps in the binding of ubiquitin conjugates to the 26S proteasome that commit to degradation. *Mol Cell* 2010,**40**:671-681.

136. Saeki Y, Kudo T, Sone T, Kikuchi Y, Yokosawa H, Toh-e A*, et al.* Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome. *EMBO J* 2009,**28**:359-371.

137. Park CW, Ryu KY. Cellular ubiquitin pool dynamics and homeostasis. *BMB Rep* 2014,**47**:475-482.

138. Nathan JA, Kim HT, Ting L, Gygi SP, Goldberg AL. Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *EMBO J* 2013,**32**:552-565.

139. Prakash S, Tian L, Ratliff KS, Lehotzky RE, Matouschek A. An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat Struct Mol Biol* 2004,**11**:830-837.

140. Lee C, Schwartz MP, Prakash S, Iwakura M, Matouschek A. ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol Cell* 2001,**7**:627-637.

141. Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 2009,**78**:477-513.

142. Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J*, et al.* Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009,**137**:133-145.

143. Jacobson AD, Zhang NY, Xu P, Han KJ, Noone S, Peng J*, et al.* The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome. *J Biol Chem* 2009,**284**:35485-35494.

144. Ohtake F, Tsuchiya H, Saeki Y, Tanaka K. K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains. *Proc Natl Acad Sci U S A* 2018,**115**:E1401-E1408.

145. Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. *Nat Rev Mol Cell Biol* 2008,**9**:679-690.

146. Mandell MA, Jain A, Arko-Mensah J, Chauhan S, Kimura T, Dinkins C*, et al.* TRIM proteins regulate autophagy and can target autophagic substrates by direct recognition. *Dev Cell* 2014,**30**:394-409.

147. Mizushima N. Autophagy: process and function. *Genes Dev* 2007,**21**:2861-2873.

148. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H*, et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 2007,**282**:24131-24145.

149. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A*, et al.* p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005,**171**:603-614.

150. Kimura T, Mandell M, Deretic V. Precision autophagy directed by receptor regulators - emerging examples within the TRIM family. *J Cell Sci* 2016,**129**:881-891.

151. Kimura T, Jain A, Choi SW, Mandell MA, Schroder K, Johansen T*, et al.* TRIM-mediated precision autophagy targets cytoplasmic regulators of innate immunity. *J Cell Biol* 2015,**210**:973-989.

152. Campbell EM, Perez O, Anderson JL, Hope TJ. Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha. *J Cell Biol* 2008,**180**:549-561.

153. Perez-Caballero D, Hatziioannou T, Zhang F, Cowan S, Bieniasz PD. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *J Virol* 2005,**79**:15567-15572.

154. Perron MJ, Stremlau M, Lee M, Javanbakht H, Song B, Sodroski J. The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. *J Virol* 2007,**81**:2138-2148.

155. Stremlau M, Perron M, Lee M, Li Y, Song B, Javanbakht H*, et al.* Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* 2006,**103**:5514-5519.

156. Anderson JL, Campbell EM, Wu X, Vandegraaff N, Engelman A, Hope TJ. Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *J Virol* 2006,**80**:9754-9760.

157. Wu X, Anderson JL, Campbell EM, Joseph AM, Hope TJ. Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proc Natl Acad Sci U S A* 2006,**103**:7465-7470.

158. Kutluay SB, Perez-Caballero D, Bieniasz PD. Fates of retroviral core components during unrestricted and TRIM5-restricted infection. *PLoS Pathog* 2013,**9**:e1003214.

159. Danielson CM, Cianci GC, Hope TJ. Recruitment and dynamics of proteasome association with rhTRIM5alpha cytoplasmic complexes during HIV-1 infection. *Traffic* 2012,**13**:1206-1217.

160. Lukic Z, Hausmann S, Sebastian S, Rucci J, Sastri J, Robia SL*, et al.* TRIM5alpha associates with proteasomal subunits in cells while in complex with HIV-1 virions. *Retrovirology* 2011,**8**:93.

161. Kim J, Tipper C, Sodroski J. Role of TRIM5alpha RING domain E3 ubiquitin ligase activity in capsid disassembly, reverse transcription blockade, and restriction of simian immunodeficiency virus. *J Virol* 2011,**85**:8116-8132.

162. Rold CJ, Aiken C. Proteasomal degradation of TRIM5alpha during retrovirus restriction. *PLoS Pathog* 2008,**4**:e1000074.

163. Diaz-Griffero F, Li X, Javanbakht H, Song B, Welikala S, Stremlau M*, et al.* Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology* 2006,**349**:300-315.

164. O'Connor C, Pertel T, Gray S, Robia SL, Bakowska JC, Luban J*, et al.* p62/sequestosome-1 associates with and sustains the expression of retroviral restriction factor TRIM5alpha. *J Virol* 2010,**84**:5997-6006.

165. Ribeiro CM, Sarrami-Forooshani R, Setiawan LC, Zijlstra-Willems EM, van Hamme JL, Tigchelaar W*, et al.* Receptor usage dictates HIV-1 restriction by human TRIM5alpha in dendritic cell subsets. *Nature* 2016,**540**:448-452.

166. Korolchuk VI, Menzies FM, Rubinsztein DC. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. *FEBS Lett* 2010,**584**:1393-1398.

167. Park C, Cuervo AM. Selective autophagy: talking with the UPS. *Cell Biochem Biophys* 2013,**67**:3-13.

168. Campbell EM, Dodding MP, Yap MW, Wu X, Gallois-Montbrun S, Malim MH*, et al.* TRIM5 alpha cytoplasmic bodies are highly dynamic structures. *Mol Biol Cell* 2007,**18**:2102-2111.

169. Sastri J, O'Connor C, Danielson CM, McRaven M, Perez P, Diaz-Griffero F*, et al.* Identification of residues within the L2 region of rhesus TRIM5alpha that are required for retroviral restriction and cytoplasmic body localization. *Virology* 2010,**405**:259-266.

170. Flavin WP, Bousset L, Green ZC, Chu Y, Skarpathiotis S, Chaney MJ*, et al.* Endocytic vesicle rupture is a conserved mechanism of cellular invasion by amyloid proteins. *Acta Neuropathol* 2017,**134**:629-653.

171. Campbell EM, Weingart J, Sette P, Opp S, Sastri J, O'Connor SK*, et al.* TRIM5alpha-Mediated Ubiquitin Chain Conjugation Is Required for Inhibition of HIV-1 Reverse Transcription and Capsid Destabilization. *J Virol* 2016,**90**:1849-1857.

172. Stringer DK, Piper RC. A single ubiquitin is sufficient for cargo protein entry into MVBs in the absence of ESCRT ubiquitination. *J Cell Biol* 2011,**192**:229-242.

173. Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, Ookata K*, et al.* Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* 2008,**455**:358-362.

174. Wang P, Joberty G, Buist A, Vanoosthuyse A, Stancu IC, Vasconcelos B*, et al.* Tau interactome mapping based identification of Otub1 as Tau deubiquitinase involved in accumulation of pathological Tau forms in vitro and in vivo. *Acta Neuropathol* 2017,**133**:731-749.

175. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L*, et al.* Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 1998,**72**:9873-9880.

176. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 2014,**11**:783-784.

177. Vermeire J, Naessens E, Vanderstraeten H, Landi A, Iannucci V, Van Nuffel A*, et al.* Quantification of reverse transcriptase activity by real-time PCR as a fast and accurate method for titration of HIV, lenti- and retroviral vectors. *PLoS One* 2012,**7**:e50859.

178. Campbell EM, Nunez R, Hope TJ. Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity. *J Virol* 2004,**78**:5745-5755.

179. Diaz-Griffero F, Qin XR, Hayashi F, Kigawa T, Finzi A, Sarnak Z*, et al.* A B-box 2 surface patch important for TRIM5alpha self-association, capsid binding avidity, and retrovirus restriction. *J Virol* 2009,**83**:10737-10751.

180. Wagner JM, Christensen DE, Bhattacharya A, Dawidziak DM, Roganowicz MD, Wan Y*, et al.* General Model for Retroviral Capsid Pattern Recognition by TRIM5 Proteins. *J Virol* 2018,**92**.

181. Imam S, Talley S, Nelson RS, Dharan A, O'Connor C, Hope TJ*, et al.* TRIM5alpha Degradation via Autophagy Is Not Required for Retroviral Restriction. *J Virol* 2016,**90**:3400-3410.

182. Huett A, Heath RJ, Begun J, Sassi SO, Baxt LA, Vyas JM*, et al.* The LRR and RING domain protein LRSAM1 is an E3 ligase crucial for ubiquitin-dependent autophagy of intracellular Salmonella Typhimurium. *Cell Host Microbe* 2012,**12**:778-790.

183. Zheng YT, Shahnazari S, Brech A, Lamark T, Johansen T, Brumell JH. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. *J Immunol* 2009,**183**:5909-5916.

184. Sparrer KMJ, Gableske S, Zurenski MA, Parker ZM, Full F, Baumgart GJ*, et al.* TRIM23 mediates virus-induced autophagy via activation of TBK1. *Nat Microbiol* 2017,**2**:1543-1557.

185. Bowman EJ, Siebers A, Altendorf K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci U S A* 1988,**85**:7972-7976.

186. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A*, et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 2011,**44**:325-340.

187. Yang Y, Brandariz-Nunez A, Fricke T, Ivanov DN, Sarnak Z, Diaz-Griffero F. Binding of the rhesus TRIM5alpha PRYSPRY domain to capsid is necessary but not sufficient for HIV-1 restriction. *Virology* 2014,**448**:217-228.

188. Kim ET, Oh SE, Lee YO, Gibson W, Ahn JH. Cleavage specificity of the UL48 deubiquitinating protease activity of human cytomegalovirus and the growth of an active-site mutant virus in cultured cells. *J Virol* 2009,**83**:12046-12056.

189. Wang S, Wang K, Li J, Zheng C. Herpes simplex virus 1 ubiquitin-specific protease UL36 inhibits beta interferon production by deubiquitinating TRAF3. *J Virol* 2013,**87**:11851-11860.

190. Ritorto MS, Ewan R, Perez-Oliva AB, Knebel A, Buhrlage SJ, Wightman M*, et al.* Screening of DUB activity and specificity by MALDI-TOF mass spectrometry. *Nat Commun* 2014,**5**:4763.

191. Mische CC, Javanbakht H, Song B, Diaz-Griffero F, Stremlau M, Strack B*, et al.* Retroviral restriction factor TRIM5alpha is a trimer. *J Virol* 2005,**79**:14446-14450.

192. Rogov V, Dotsch V, Johansen T, Kirkin V. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell* 2014,**53**:167-178.

193. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T*, et al.* Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* 2009,**461**:654-658.

194. Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, Dong C*, et al.* Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. *J Cell Biol* 2009,**186**:255-268.

195. Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. *Mol Cell* 2009,**34**:259-269.

196. Munz C. Antigen Processing for MHC Class II Presentation via Autophagy. *Front Immunol* 2012,**3**:9.

197. Fernandez-Garcia MD, Meertens L, Bonazzi M, Cossart P, Arenzana-Seisdedos F, Amara A. Appraising the roles of CBLL1 and the ubiquitin/proteasome system for flavivirus entry and replication. *J Virol* 2011,**85**:2980-2989.

198. Johnson WE, Sawyer SL. Molecular evolution of the antiretroviral TRIM5 gene. *Immunogenetics* 2009,**61**:163-176.

199. Liao CH, Kuang YQ, Liu HL, Zheng YT, Su B. A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. *AIDS* 2007,**21 Suppl 8**:S19-26.

200. Brennan G, Kozyrev Y, Hu SL. TRIMCyp expression in Old World primates Macaca nemestrina and Macaca fascicularis. *Proc Natl Acad Sci U S A* 2008,**105**:3569-3574.

201. Virgen CA, Kratovac Z, Bieniasz PD, Hatziioannou T. Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc Natl Acad Sci U S A* 2008,**105**:3563-3568.

202. Newman RM, Hall L, Kirmaier A, Pozzi LA, Pery E, Farzan M*, et al.* Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS Pathog* 2008,**4**:e1000003.

203. Wilson SJ, Webb BL, Ylinen LM, Verschoor E, Heeney JL, Towers GJ. Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc Natl Acad Sci U S A* 2008,**105**:3557-3562.

204. Mallery DL, Marquez CL, McEwan WA, Dickson C, Jacques DA, Anandapadamanaban M*, et al.* IP6 is an HIV pocket factor that prevents capsid collapse and promotes DNA synthesis. *Elife* 2018,**7**.

205. Nakayama EE, Nakajima T, Kaur G, Mimaya JI, Terunuma H, Mehra N*, et al.* A naturally occurring single amino acid substitution in human TRIM5alpha linker region affects its anti-HIV type 1 activity and susceptibility to HIV type 1 infection. *AIDS Res Hum Retroviruses* 2013,**29**:919-924.

206. Speelmon EC, Livingston-Rosanoff D, Li SS, Vu Q, Bui J, Geraghty DE*, et al.* Genetic association of the antiviral restriction factor TRIM5alpha with human immunodeficiency virus type 1 infection. *J Virol* 2006,**80**:2463-2471.

207. van Manen D, Rits MA, Beugeling C, van Dort K, Schuitemaker H, Kootstra NA. The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection. *PLoS Pathog* 2008,**4**:e18.

208. Richardson MW, Guo L, Xin F, Yang X, Riley JL. Stabilized human TRIM5alpha protects human T cells from HIV-1 infection. *Mol Ther* 2014,**22**:1084-1095.

VITA

Sabrina Imam was born in Queens, New York to Selina and Quazi Imam. She attended Cornell University in Ithaca, NY, where she earned a Bachelor's of Arts in Biological Sciences with a concentration in Cell and Molecular Biology. After graduation, Sabrina worked as a research technician in the lab of W. Nicholas Haining, MD, at the Dana-Farber Cancer Institute in Boston, MA, where she studied the mechanisms of dysregulation of the immune response during chronic infections. Sabrina matriculated into the Loyola University Chicago Stritch School of Medicine MD/PhD Program in 2012. After completing two years of medical school, she began her graduate education in the Department of Microbiology and Immunology under the mentorship of Edward M. Campbell, PhD.

Sabrina's dissertation work on focused on understanding the molecular determinants of TRIM5α retroviral restriction and association with autophagic effectors. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight. Upon completion of her graduate studies, Sabrina will return to medical school, where she will join the Class of 2020. After completing the MD/PhD Program, Sabrina plans to continue training to become a physician-scientist.