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The Role of Linker 2 (L2) Region in RhTRIM5 α Assembly and HIV-1 Restriction

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LOYOLA UNIVERSITY CHICAGO

THE ROLE OF LINKER 2 (L2) REGION IN
TRIM5 α ASSEMBLY AND HIV-1 RESTRICTION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN BIOCHEMISTRY AND
MOLECULAR BIOLOGY

BY

JAYALAXMI SASTRI

CHICAGO, IL

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To my dear parents

Education is what remains after one has forgotten
what one has learned in school.

Albert Einstein

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ABSTRACT

The cellular restriction factor TRIM5 α inhibits infection by numerous retroviruses in a species specific manner. TRIM5 α protein from rhesus macaques (rhTRIM5 α) and a related protein TRIM-Cyp from Owl monkeys restrict infection by HIV-1 while human TRIM5 α (huTRIM5 α) restricts infection by N-tropic murine leukemia virus (N-MLV) but not HIV-1. Several models have been proposed for retroviral restriction by TRIM5 proteins (TRIM5 α and TRIMCyp). These models collectively suggest that TRIM5 proteins mediate restriction by recognizing specific determinants in the viral capsid and directly binding the capsid. Following binding, the TRIM5 proteins self-associate into large assemblies around the viral capsid, which leads to either abortive disassembly of the viral capsid via a poorly understood mechanism that is sensitive to proteasome inhibitors and/or activation of innate immune signaling pathways. This study focuses on the initial step in restriction that is assembly of TRIM5 α around the HIV-1 capsid. TRIM5 α is known to form assemblies in the cytoplasm of the cell, termed as cytoplasmic bodies. We show that the ability of rhTRIM5 α to assemble into cytoplasmic bodies is required for HIV-1 restriction and the L2 region has determinants that govern rhTRIM5 α assembly. Additionally, the L2 variants that have a higher tendency to assemble into cytoplasmic bodies, exhibit increased HIV-1 restriction ability. This suggests that the tendency of rhTRIM5 α to assemble directly correlates with its HIV-1 restriction ability. We also show that the L2 region of rhTRIM5 α has a propensity to form α -helices that facilitate

rhTRIM5 α assembly, most likely, by mediating protein-protein interactions. This α -helical conformation of the L2 region seems to be stabilized in L2 variants that exhibit increased tendency to assemble but not in the variants that fail to assemble. Lastly, the L2 region forms α -helices in a concentration dependent manner and possibly acts as a molecular switch in the assembly of rhTRIM5 α .

CHAPTER I

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is a disease of the human immune system and is accompanied by a profound decrease in the number of circulating CD4+ T cells. AIDS is characterized by an increased susceptibility of patients to infection with opportunistic pathogens including bacteria, viruses, fungi and other parasites, that are otherwise evaded by the immune system. AIDS was first described in 1981 by the Centers for Disease Control (CDC), following a high incidence of unusual opportunistic infections and rare malignancies among the homosexual population. Two years later in 1983, the etiological agent responsible for AIDS was discovered, a lentivirus now known as Human Immunodeficiency Virus type I (HIV-1) [5]. Ever since the discovery of HIV-1 the reasons for its sudden emergence, spread and pathogenicity have been under investigation. In 1986, HIV-2 which is morphologically similar to HIV-1 was reported to cause AIDS in infected individuals in Western Africa. It was later found that HIV-2 was more closely related to the primate lentivirus, Simian Immunodeficiency Virus (SIV) that caused AIDS like disease in captive macaques, than to HIV-1 (Reviewed in [7]). The discovery of several similar lentiviruses in other primates and an extensive study of their phylogenetic lineage provided the first evidence that the emergence of AIDS in humans and macaques was a result of cross-species infections with lentiviruses from different

primate species (Reviewed in [7]. Eventually, it became clear that HIV-1 and HIV-2 had arisen in humans as a result of the zoonotic transfers of viruses that infect primates in Africa [8]. HIV-1 infection involves three main stages: primary infection, clinical latency and AIDS as described below and reviewed in [9].

Stages of HIV-1 Infection

Primary Infection

Primary infection with HIV-1 is associated with an acute mononucleosis-like or influenza-like clinical syndrome which appears 3-6 weeks after infection. The common symptoms include fever, swelling of the lymph nodes, throat inflammation and rash, which overlap with a number of diseases. Hence, these symptoms usually go unrecognized as the signs of HIV-1 infection. The severity and persistence of these symptoms vary. During this phase there is a burst of viral replication and increased viral load can be detected in peripheral blood and cell-free plasma about 3 weeks after infection. This is accompanied by significant decline in levels of circulating CD4+ T lymphocytes in the first 2-8 weeks following infection. These levels may recover, although not completely, as the patient enters the next stage of the disease, which is clinical latency.

Clinical Latency

The acute phase is followed by an asymptomatic phase called clinical latency. Without treatment this phase can last on an average for about 8-10 years with very few, clinical manifestations, if any, and this period varies from individual to individual. During this phase there is a steady decline in CD4+ T lymphocytes. Although this phase is known as clinical latency some amount of viral replication does occur, as virus can be detected in the peripheral blood of infected individuals. This suggests that the term “clinical latency” applies only to the lack of symptoms during this phase and does not mean viral latency.

Clinically Apparent Disease or AIDS

This phase sets in when the CD4+ T cell levels in the peripheral blood drop below 500 cells/uL followed by appearance of first symptoms. Once the levels of CD4+ T cells falls below 200 cells/uL, the patients become more and more susceptible to infections by opportunistic pathogens as well as rare malignancies. The decline in CD4+ T lymphocytes continues until virtually all cells are lost.

HIV-1 comprises of four distinct lineages namely, M, N, O and P, which are a result of independent cross-species transmission events. Group M is the pandemic form of HIV-1 and is responsible for infection of millions of people worldwide. Groups O, N and P are much less prevalent as compared to group M and are mostly restricted to

Cameroon, Gabon and neighboring countries (Reviewed in [7]). Currently, HIV-1 along with HIV-2, which is relatively less widespread, is responsible for infection of over 30 million people world-wide. According to the World Health Organization (WHO) report (<http://www.who.int/hiv/data/en/>), approximately 34 million people were living with HIV-1 infection, about 2.5 million new infections were recorded and there were about 1.7 million AIDS related deaths in 2011. In the last three decades, since its discovery, scientists have extensively studied the HIV-1 genome, the encoded proteins, their structure and function in the viral replicative cycle. These studies have provided scientists the necessary knowledge and tools to develop powerful antiviral drugs that counteract HIV-1 infection [1]. Within five years after the discovery of HIV-1 the first anti-HIV-1 drug, AZT, became available for treatment and since then several effective combinations of anti-viral drugs have been used to treat patients. The currently available highly active antiretroviral therapy (HAART) involves treatment of patients with at least three active antiretroviral medications (ARVs). Although the number of AIDS-related deaths has greatly reduced after the development of these antiretroviral drugs, HIV-1 infection still remains a worldwide pandemic. Apart from a small effect observed in one vaccination trial [10], currently there is no vaccine approved for HIV-1. These statistics of global AIDS epidemic clearly point to an urgent need for improvement in antiviral therapy as well as ready access, worldwide, to the drugs that are currently available.

HIV-1 Structure and Genomic Organization

HIV-1 is a Lentivirus that belongs to the Retroviridae family and primarily infects cells of the immune system including CD4⁺ T cells and macrophages. HIV-1 particles are roughly spherical and have a diameter of 100 to 120nm [11, 12]. They are surrounded by a lipoprotein membrane, which is derived from the host cell during budding of a progeny viral particle at the plasma membrane (Figure 1A). The viral genome is composed of two copies of positive sense single-stranded RNA (~10 kb) and encodes nine open reading frames (ORFs) (Figure 1B). Three of these ORFs encode the group specific antigen (Gag), Polymerase (Pol) and Envelope (Env) polyproteins which are eventually cleaved by the viral protease into smaller proteins (Figure 1B). The three Pol proteins, protease (PR), reverse transcriptase (RT) and integrase (IN) are enclosed in the virion and are critical for early events in infection. The two Env proteins derived from the 160kDa precursor glycoprotein, gp160, are the surface glycoprotein (SU, gp120) and the transmembrane protein (TM, gp41) which are embedded in the viral membrane (Figure 1A). These glycoproteins form heterodimers and are arranged as spikes or tripod-like structures in the viral membrane (Reviewed in [13]) (Figure 1A). Each heterodimer consists of a trimer of gp120, which forms the head, and a trimer of gp41, which forms the stem of the spike. The products of the Gag polyprotein are matrix (MA, p17), capsid (CA, p24), Nucleocapsid (NC) and p6 which make up the viral core. MA is present just below the viral membrane and forms the inner shell of the virion (Figure 1A) [11, 12].

The CA or p24 protein forms hexamers and pentamers which assemble into an asymmetrical closed shell known as the viral capsid (Figure 1A). The capsid resembles a fullerene cone [14] consisting of approximately 1500 copies of the capsid protein (reviewed in [15]). Approximately 250 hexamers and exactly 12 pentamers, seven at the broad end and five at the narrow end, need to be assembled to form a closed fullerene cone [16]. The capsid surrounds the viral ribonucleoprotein complex which comprises the viral RNA genome along with viral proteins such as NC, PR, RT, and IN [11, 12]. Additionally, viral accessory proteins such as Vif, Vpr and Nef are found in the viral particle. All the viral proteins enclosed in the viral particle are required for the early steps of viral infection. Several host proteins including actin, cyclophilin A and RNA-binding proteins have also been shown to be associated with virions and facilitate viral infection (Reviewed in [17]).

Figure 1B shows the HIV-1 genome with ORFs encoding various viral proteins. The full-length viral mRNA is translated to produce the Gag and Pol precursor proteins, which are further cleaved by the viral PR. Further splicing events give rise to several sub-genomic RNA molecules that are translated to other viral proteins [11, 12]. The HIV-1 genome also encodes regulatory and accessory proteins such as Tat, Rev, Vif, Vpr, Vpu and Nef. Tat, a transactivating protein interacts with the RNA loop structure in the 3' LTR known as Tat response element (TRE), along with other cellular proteins and induces the expression of Tat, Nef and Rev [11, 12]. Rev, another viral regulatory protein

interacts with the Rev-response element (RRE) present in the viral Env mRNA along with other cellular proteins and allows the export of unspliced viral mRNA from the nucleus to the cytoplasm [9, 11]. This allows the translation of the unspliced viral mRNA transcripts that are needed for production of progeny virions. Nef or negativity factor has a variety of potential roles including cell activation, enhanced infectivity and signal transduction [9, 11]. Other HIV-1 accessory proteins, Vif, Vpr and Vpu, are involved in viral assembly, budding and viral infectivity. The Vif protein is also involved in countering the HIV-1 inhibitory role of the cellular protein APOBEC3G, as discussed below [18, 19].

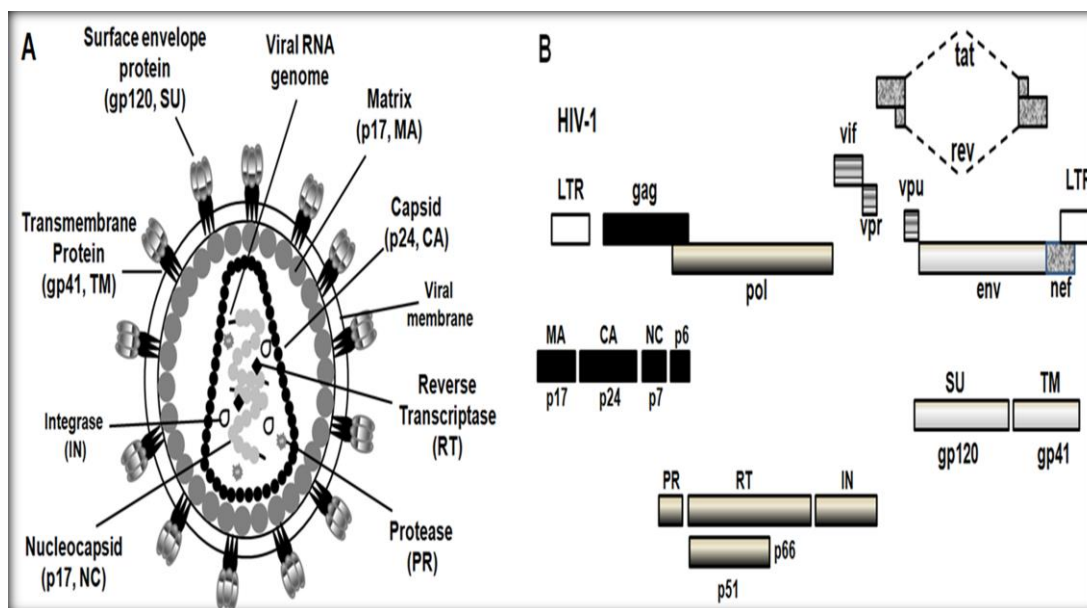


Figure 1: Representation of the HIV-1 structure and genome. A) A mature HIV-1 virion. The virions are roughly spherical and surrounded by a lipoprotein membrane. The envelope glycoproteins are embedded in the viral membrane. The viral RT, IN and PR enzymes along with the viral RNA genome are packaged in a inside a conical capsid composed of the p24 or CA proteins. The matrix forms the inner shell of the virion. B) Genomic organization of HIV-1. The RNA genome is flanked by long terminal repeats (LTRs) that mediate viral integration and regulation of the viral genome. The 3 main ORFs are the gag, pol and env that encode the polyproteins Gag, Pol and Env respectively. These polyproteins are further cleaved by the viral PR to generate structural proteins, viral enzymes and envelope proteins, respectively. The Tat, Rev and Nef mRNAs are a result of splicing of the viral transcripts and hence these genes are split in the genome. The HIV-1 genome also encodes several accessory proteins such as Vif, Vpr, Vpu and Nef.

HIV-1 Life Cycle

HIV-1 predominantly infects cells of the immune system. CD4⁺ T cells are the major targets for HIV-1 infection as they express high levels of CD4, the primary HIV-1 receptor, on their surface [20-22]. Other immune cells such as macrophages also express the CD4 receptor and co-receptors for HIV-1 and can serve as targets for HIV-1 infection (Reviewed in [23]). The HIV-1 life cycle can be divided into two phases: an early phase and a late phase (Reviewed in [24]) .

Early Phase of HIV-1 life cycle

The early phase of infection refers to steps 1 through 5 shown in Figure 2. The early phase begins when an HIV-1 particle binds a host cell following the interaction between the viral envelope glycoprotein gp120 and the cell surface CD4 receptor (Figure 2, step 1) (Reviewed in [24] and [13]). This interaction exposes a binding site for the co-receptor, typically CC-Chemokine receptor 5 (CCR5). Following binding to the co-receptor, the fusion peptide at the amino terminus of gp41 is inserted into the host cell membrane causing significant conformational changes in gp41, which ultimately result in the fusion of the viral and target cell membranes (Figure 2, step 2). The attachment and fusion steps of the HIV-1 life cycle serve as targets for a number of antiviral agents such as attachment inhibitors, CCR5 antagonists and fusion inhibitors discussed ahead. The viral core, i.e. the conical capsid containing the ribonucleoprotein (RNP) complex, is then

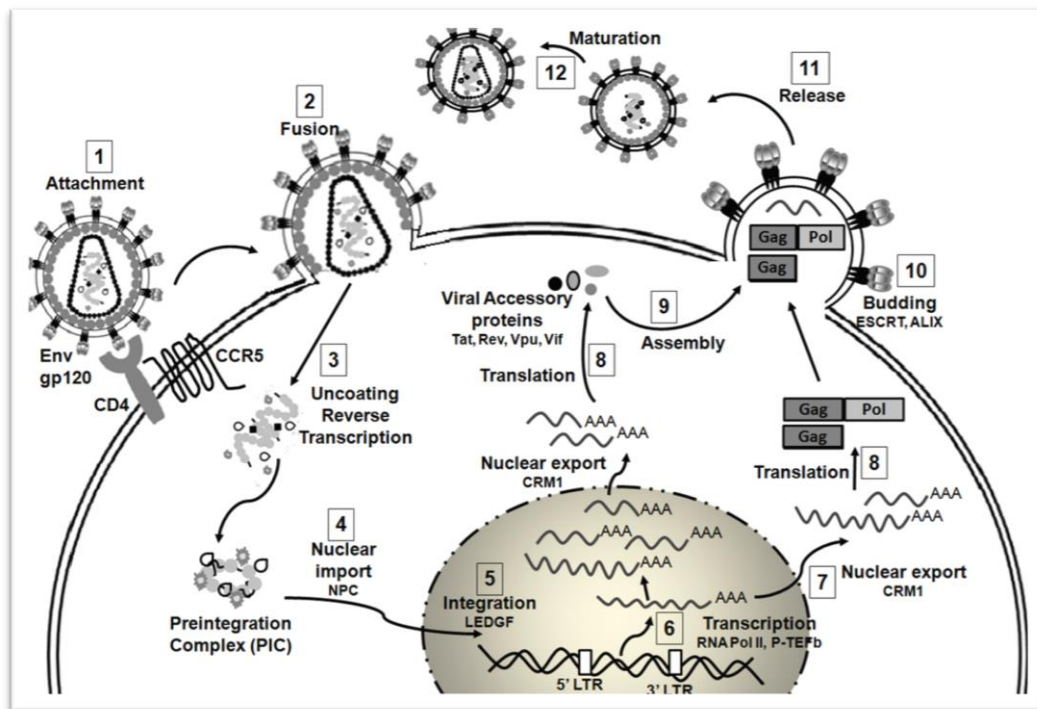


Figure 2: Schematic overview of the HIV-1 replicative cycle. The HIV-1 life cycle can be divided into two phases: early phase (steps 1-5) and late phase (steps 6-12). 1) The infection begins with the binding of the envelope glycoprotein gp120 to the cell surface CD4 receptor and the membrane spanning co-receptor (for example, CCR5). 2) This leads to fusion of the viral and cell membranes and entry of the viral core into the cytoplasm of the cell. 3) Partial uncoating of the viral capsid facilitates reverse transcription which yields the preintegration complex (PIC). 4) The PIC is actively imported into the cell nucleus with the help of nuclear import machinery of the cell. 5) The viral IN that is associated with the PIC along with the cellular protein LEDGF facilitates the integration of the viral DNA into the host cell genome, also known as the provirus. 6) Transcription of the provirus leads to production of mRNAs of various lengths, 7) the larger of which are exported from the nucleus. 8) The genome length mRNA serves as the template for translation 9) The RNA along with the viral proteins is then assembled into immature viral particles. 10) The ESCRT machinery facilitates viral budding at the plasma membrane. 11) Release of the viral particles from the host cell is accompanied or immediately followed by maturation, mediated by the viral PR, generating an infectious viral particle.

released into the cytoplasm of the host cell. The viral capsid then begins to disassemble, a process termed as uncoating, and the viral RNA genome is reverse transcribed by the viral RT to yield a double stranded DNA molecule also known as the pre-integration complex (PIC) (Figure 2, step 3). A number of viral proteins have been shown to be associated with the HIV-1 PIC including NC, MA, RT, IN and Vpr [25-28]. These studies also suggested that little or no CA was present in the HIV-1 PICs. However, it was later demonstrated that the HIV-1 CA protein is the main viral determinant required for nuclear import of PICs [29], suggesting a possible link between the processes of uncoating and nuclear import. However, the kinetics and the exact mechanism of uncoating have been unclear. A recent study by the Hope laboratory used a novel assay to determine the kinetics of uncoating and showed that the viral capsid starts uncoating within 1 hr following fusion [30]. Moreover, the process of reverse transcription appears to be tied to uncoating as indicated by the delay observed in uncoating upon treatment with the RT inhibitor nevirapine. Several cellular proteins have also been shown to interact with the PIC, which possibly facilitate proper integration of the provirus [31, 32].

The PIC is then actively translocated to the host cell nucleus through the nuclear pore complex (NPC) (Figure 2, step 4). Several cellular proteins involved in nuclear import including the nucleoporins Nup153 and Nup358/RanBP2 and the transportin TNPO3 have been shown to interact with the HIV-1 capsid and mediate HIV-1 nuclear import [33-36]. The next step is integration of the viral DNA into the host genome, which

is mediated by the viral IN (Figure 2, step 5). The cellular protein lens epithelium-derived growth factor (LEDGF) has been shown to facilitate this process [37, 38]. This step ends the early phase of the viral life cycle. Several cellular proteins such as Tripartite Motif-containing protein 5 (TRIM5) are known to inhibit this phase of the viral life cycle as discussed below.

Late phase of HIV-1 life cycle

The late phase of HIV-1 life cycle begins with transcription of the integrated viral DNA (Figure 2, step 6) (Reviewed in [13]). The viral transactivator protein Tat is required for efficient transcription of the integrated proviral DNA. The cellular positive transcription elongation factor P-TEFb and RNA Pol II are recruited by Tat to the viral trans-activation response (TAR) element to facilitate transcription elongation. Viral mRNAs are produced as a result of several alternative splicing events. The smaller mRNAs are exported out of the nucleus by CRM-1 mediated nuclear export while export of the larger unspliced mRNAs requires the viral protein Rev (Figure 2, step 7). Rev binds the Rev-response element (RRE), which lies within the env mRNA coding region, along with CRM-1. The viral mRNAs are then translated to produce viral proteins (Figure 2, step 8). These viral proteins along with the genome-length RNA assemble into progeny viral particles at the plasma membrane (Figure 2, step 9). The progeny virions are then released from the host cell by a process known as budding, processes facilitated by the cellular endosomal sorting complex required for transport (ESCRT) and ALIX

proteins (Figure 2, steps 10 and 11). Following budding from the cell membrane the virions undergo maturation mediated by the viral PR resulting in the generation of infectious viral particles (Figure 2, step 12). This process occurs concomitantly or immediately after viral release and involves PR mediated proteolysis of the Gag and Gag-Pol polyproteins to yield the MA, CA and NC, and the PR, RT and IN enzymes. Cryo-electron tomography studies have also shown that the Gag proteins undergo several structural rearrangements within immature virions during the maturation step resulting in the formation of infectious viral particles. Several steps in the HIV-1 replicative cycle can serve as targets for antiretroviral intervention as discussed below.

Potential targets for antiretroviral intervention

In the late 1980s and early 1990s, very few antiretroviral drugs existed for treatment of HIV-1. The treatment was mainly targeted towards management of AIDS-related illness and involved treatment of patients for common opportunistic infections. The first enzyme inhibitor Zidovudine or 3'-azido-3'-deoxythymidine (AZT), which targets the HIV-1 RT, was made available for use by the Food and Drug Administration (FDA) in 1987. Since then the development of inhibitors that specifically target HIV-1 enzymes such as reverse transcriptase (RT) and protease (PR), which are critical for HIV-1 replication, have revolutionized the treatment of HIV-1 infection (Reviewed in [39]). Initially, anti-HIV-1 drugs were given as monotherapy which later evolved to include a combination or cocktail of two or more antiretroviral agents (ARVs). Combination therapy greatly

enhanced the efficacy of HIV-1 treatment. The highly active antiretroviral therapy (HAART), which involves the use of a cocktail of at least three ARVs, has been instrumental in reducing the overall morbidity associated with HIV-1 infection (Reviewed in [39]). The HIV-1 life cycle provides a number of potential targets for therapeutic intervention however, only a few of them have been exploited so far. Currently, about thirty drugs that target four major steps in the HIV-1 replication cycle can be used for treatment of HIV-1 infected individuals (Reviewed in [13, 39]). These drugs can be divided into various classes based on their molecular targets and mechanism of action and are currently a part of HAART. Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) target the HIV-1 RT that is required early in the viral life cycle whereas protease inhibitors (PIs) target the HIV-1 PR that is required during late infection. Entry or fusion inhibitors block viral entry into target cells by inhibiting the viral envelope protein gp120 and the co-receptor CCR5. Some entry inhibitors target the HIV-1 transmembrane envelope protein gp41 and block the formation of the gp41 six-helix bundle, which is required for fusion of the viral and cellular membranes. The only integrase strand transfer inhibitor currently available, raltegravir, blocks the integration of viral DNA into the host cell genome by inhibiting the strand transfer activity of the HIV-1 IN. The HAART regimen typically involves the use of two NRTIs along with an NNRTI or a PI or another NRTI abacavir (Ziagen). The mechanism of action of some of these inhibitors and their respective molecular targets are discussed below (Figure 3).

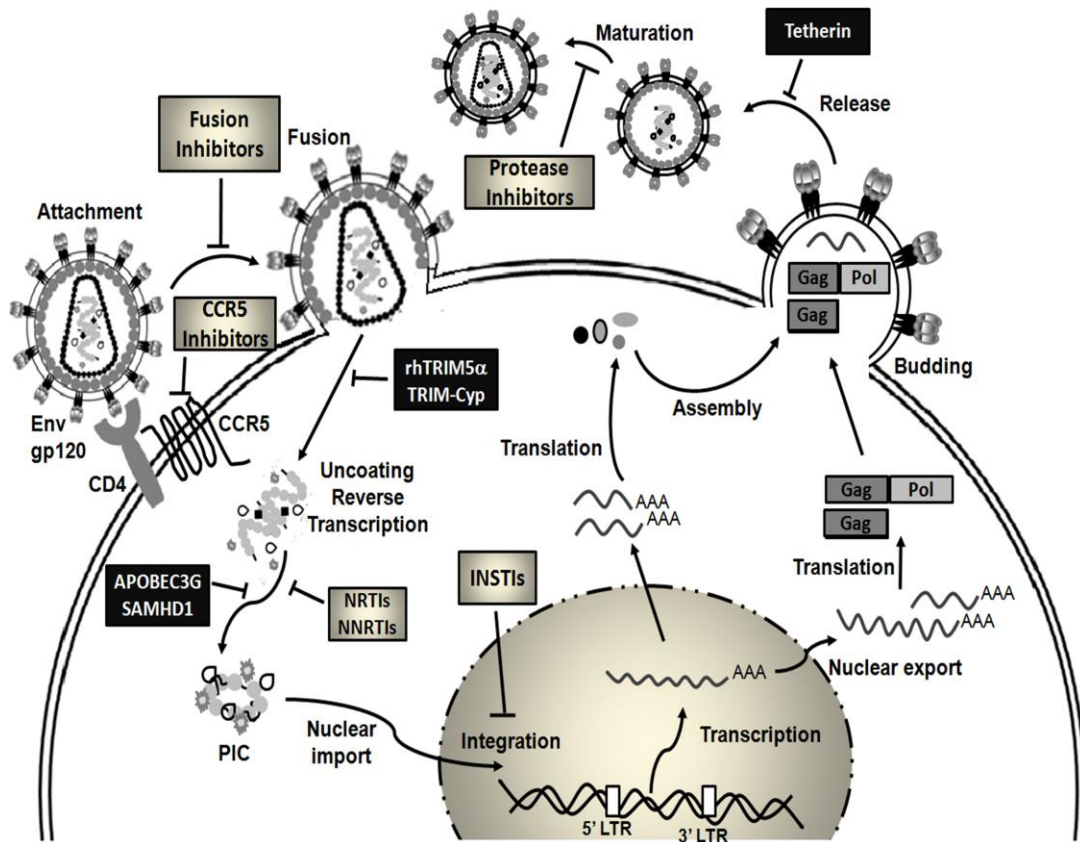


Figure 3: Potential targets for antiretroviral intervention. Several steps in the HIV-1 replicative cycle can serve as potential targets for antiviral intervention. The sites of action of clinical inhibitors are shown in grey boxes and those of host cell restriction factors are shown in black boxes. NRTIs – Nucleoside/Nucleotide reverse transcriptase inhibitors; NNRTIs – Non-nucleoside reverse transcriptase inhibitors; INSTIs – Integrase strand transfer inhibitors.

Entry and Fusion Inhibitors

Entry and fusion inhibitors prevent viral entry into target cells by inhibiting the interaction of the HIV-1 envelope glycoproteins gp120 and gp41 with the cell surface receptor CD4. High-resolution crystallography of engineered HIV-1 glycoprotein constructs has shown that the core gp120 structure is highly flexible, which allows it to undergo extreme conformational changes following CD4 binding while remaining attached to gp41. Additionally, CD4 binds gp120 in a hydrophobic cavity and development of small molecule inhibitors that mimic this interaction can have great potential in antiretroviral therapy (Reviewed in [13]). BMS-378806 is one such molecule that binds in the CD4 binding pocket of gp120 which results in a conformation change of gp120 such that it can no longer bind to the CD4 receptor. Another study used an anti-CD4 monoclonal antibody TNX-355, that inhibits the interaction of CD4 with gp120 without affecting the immunological function of CD4. Although these small molecule inhibitors have shown some clinical potential they have not been approved for use in humans (Reviewed in [39]). Wu et al. used a structure-based approach to redesign the surface of gp120 and using this construct as bait, B cells clones from AIDS patients that produced antibodies against gp120 were generated. These antibodies possess remarkably broad neutralizing activity [40, 41]. Further structural analysis revealed that these antibodies neutralize HIV-1 by engaging the CD4-binding site. Additionally, small peptides generated from the N- or C-terminal sequences of gp41 that inhibit membrane

fusion by disrupting six-helix bundle formation are being used as antiviral agents.

Fuzeon, a peptide based on the C-terminal sequence of gp41, was approved for patient use in 2003 however, the virus develops resistance to this drug relatively easily. Another set of inhibitors known as D-peptides based on the N-terminal gp41 helical structure are also act as potent inhibitors and might overcome some of the limitations of Fuzeon (Reviewed in [13]).

Reverse Transcriptase Inhibitors (RTIs)

There are two classes of RTIs namely, nucleoside/nucleotide and non-nucleoside RT inhibitors (NRTIs and NNRTIs) that inhibit DNA polymerization and are the main components of HAART (Reviewed in [13]). NRTIs lack the 3'-hydroxyl group required for the incorporation of subsequent nucleotides by the RT during reverse transcription and thus act as chain terminators by getting incorporated into the viral DNA that is being synthesized. However, the virus can easily acquire mutations that make it resistant to RTIs. For example, a single mutation in the RT of Met184 to a Val or Ile can prevent the incorporation of NRTIs such as 2',3'-dideoxy-3'-thiacytidine (3TC) in place of dNTPs. In case of AZT, the mutant RT has developed a way to excise the incorporated drug from the primer strand. NNRTIs on the other hand act as allosteric inhibitors that induce the formation of a flexible binding pocket by causing large conformational changes in the active site of RT. The displacement of the primer grip or the 3 stranded β -sheets which

consist of the catalytic triad (Asp110, Asp185 and Asp186) most likely forms the basis of NNRTI inhibition.

Integrase Strand Transfer Inhibitors (INIs)

Several small molecule inhibitors that inhibit DNA strand transfer activity of the viral IN (INSTIs) are being developed. Raltegravir (Merck) is one such clinically approved INSTI. INSTIs have a broad anti-retroviral activity (Reviewed in [13]). All INSTIs possess two moieties – 1) co-planar heteroatoms (usually three oxygen atoms) that chelate the metal ions in the active site of IN and 2) halogenated benzyl groups that interact with the penultimate viral DNA G-C base pair and the residues Pro145-Gln146 in the HIV-1 IN. This ejects the viral 3'-deoxyAdenine (which is the DNA strand transfer nucleophile) from the active site of the IN. INSTIs also compete with the target DNA for the active site of IN thus disrupting the binding of IN to the target DNA. Lentiviruses such as HIV-1 require the cellular chromatin binding protein LEDGF for integration and hence favor integration within active genes. A novel class of IN inhibitors, termed LEDGINS, are known to mimic the interaction between IN and LEDGF *in silico* and inhibit their interaction *in vitro* (Reviewed in [13]).

Protease Inhibitors (PIs)

The development of PR inhibitors greatly benefited from structure-based approaches as the structure of full-length PR had been solved long before the first PI was

developed (Reviewed in [13]). The currently used PIs bind to the active site of the HIV-1 PR and act as competitive inhibitors. These PIs were designed so that they specifically bound in the substrate binding pocket of the viral PR and contacted only those residues that are required for PR function. In such a scenario resistance mutations in the PR would be unfavorable for the virus as they would disrupt the activity of the enzyme. Some of the recently developed PR inhibitors indeed exhibit marginally improved binding profiles to drug resistant PR as compared to wild-type PR *in vitro*. Further studies are underway to increase the binding efficiency of these inhibitors to resistant PR.

HAART greatly suppresses HIV-1 replication thus reducing the HIV-1 viral load in the blood plasma below detection levels. This in turn helps replenish the levels of circulating CD4+ T-lymphocytes. When only one of the HIV-1 proteins is targeted, the virus can easily acquire mutations making it resistant to the drug. However, the use of a combination of drugs that specifically target multiple HIV-1 proteins stalls the virus from rapidly acquiring drug resistant mutations. Although HAART greatly delays the progression of AIDS and reduces the viral load in the blood stream, taking the patients off the therapy usually leads to rise in the viral load. Hence, there is a need for novel and effective antiretroviral intervention strategies.

In the past decade the focus of HIV-1 related research has shifted to understanding the role of host proteins in HIV-1 infection, both as facilitators and inhibitors. These studies involved the identification of cellular proteins that directly

interact with various components of the virus, such as cyclophilin A and LEDGF [38, 42]. Several groups have also used genome-wide RNA interference (RNAi) screens to identify cellular proteins that regulate HIV-1 infection [43-47]. A number of cellular proteins, termed “restriction factors”, that naturally possess the ability to inhibit HIV-1 infection have also been identified [18, 48-52]. The interplay of these host factors with components of HIV-1 can be exploited to develop novel treatment strategies against HIV-1.

Cellular Restriction Factors that target HIV-1

The initial evidence that host cells express inhibitors of retroviral replication originated from studies conducted in the 1960s when the Friend virus susceptibility factor 1 (Fv1) was discovered. Fv1 dictates the susceptibility of mice to two different strains of murine leukemia virus (Reviewed in [53]). Subsequently, retroviral restriction activities similar to that of Fv1 were discovered in other mammalian species, including humans, suggesting that the ability to restrict retroviruses was not unique to mice. In humans Ref1 (Restriction factor 1) was identified as the factor that inhibited infection by N-tropic murine leukemia virus (N-MLV). Using mutagenesis studies it was shown that the same viral capsid had determinants that conferred susceptibility of N-MLV to both, Fv1 and Ref1 (Reviewed in [53]). Since then several studies have identified cellular proteins that provide “intrinsic immunity” against retroviruses, including the lentivirus HIV-1, by targeting various steps in the viral life cycle. These “restriction factors” include proteins

such as APOBEC3G, SAMHD1, Tetherin and the Tripartite Motif-containing proteins 5 (TRIM5) (Reviewed in [4]). Figure 3 summarizes the steps in the HIV-1 life cycle that are targeted by these restriction factors. While some restriction factors cause hypermutation of the viral genome (APOBEC3 family proteins), others disrupt the viral capsid by directly associating with and forming large assemblies around the viral capsid soon after its entry into the cell cytoplasm, thus inhibiting subsequent steps in infection (TRIM5 α). Tetherin inhibits the release of infectious progeny virions from infected cells by physically tethering them to the plasma membrane. SAMHD1 plays an indirect role in viral inhibition by depleting intracellular pools of deoxynucleotide triphosphates thus inhibiting reverse transcription.

Identification of these restriction factors involved the use of two main experimental strategies (Reviewed in [54]). One strategy involved the use of gene arrays or cDNA subtraction, to identify genes that were exclusively expressed in cells that were resistant to viral infection but not in those that were susceptible. In this method, first the candidate genes that could be potentially involved in viral restriction are shortlisted based on specific criteria such as interferon responsiveness etc. and further validated by ectopically expressing them in susceptible cells. If a candidate gene is required for viral restriction then ectopically expressing it in susceptible cells should make them resistant to viral infection. This strategy was used in the discovery of the restriction factors APOBEC3G [18] and Tetherin [50]. The second experimental strategy, which led to the

discovery of rhesus macaque TRIM5 α [48], involved the expression of a cDNA library derived from resistant cells into susceptible cells and screening for cells that had acquired restrictive properties. The cDNA conferring viral resistance was then isolated from these cells. While on one hand the host has evolved to inhibit HIV-1 infection by expressing restriction factors, the virus has developed ways to antagonize the activity of these proteins. The steps in the HIV-1 life cycle that are targeted by these proteins and the viral proteins that antagonize the activity of these restriction factors are discussed below and shown in figure 4.

APOBEC3G

For several years it was known that HIV-1 requires its accessory protein Vif to replicate in certain human cell types (termed as non-permissive cells) but not in others (termed as permissive cells). In 2002, Sheehy et al. discovered that the non-permissive cells express a protein known as the Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G or A3G) that inhibits replication of HIV-1 and other retroviruses in these cells, but only in the absence of Vif [18]. Vif inhibits the antiviral activity of A3G by binding to it and targeting it for degradation [19]. A3G is an enzyme encoded by the APOBEC3G gene in humans and belongs to the APOBEC family of proteins. A3G gets incorporated into budding viral particles and is then transferred to the next target cell where it can exert its anti-viral effects at multiple stages of the viral life cycle (Reviewed in [54, 55]). A3G is a cytidine deaminase that converts nascent cytidines

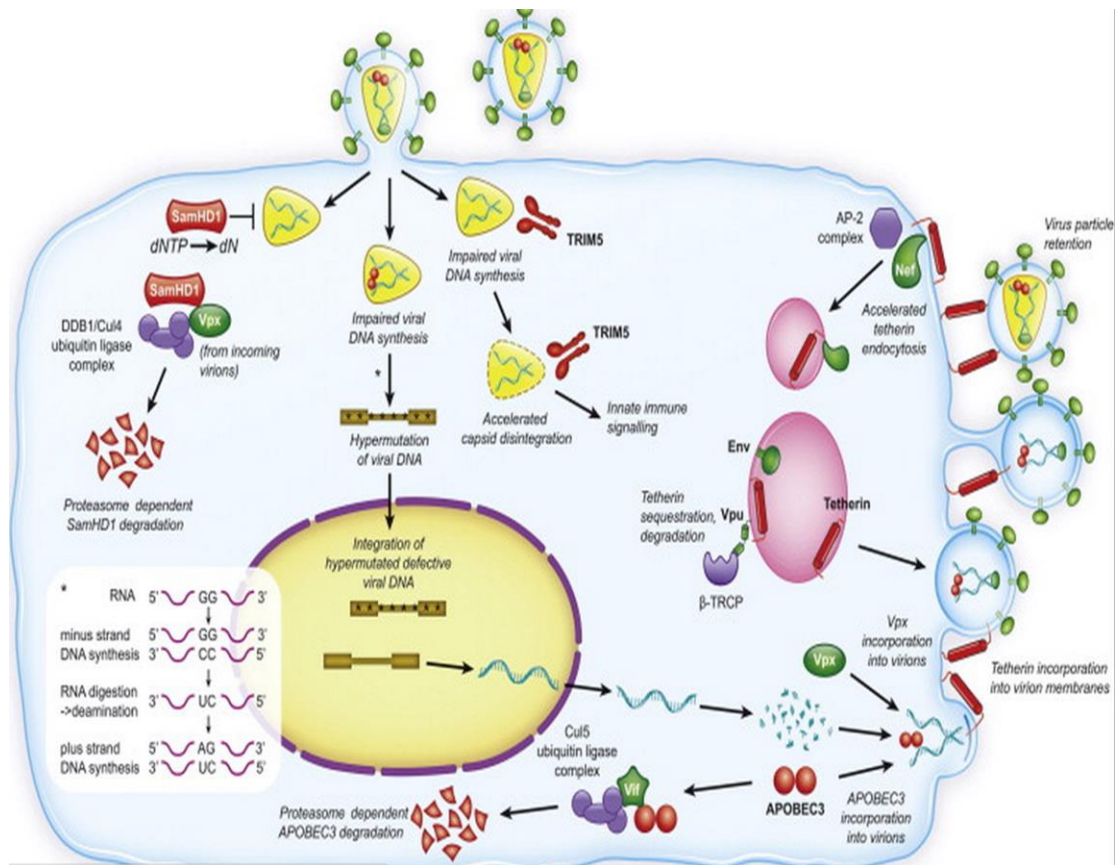


Figure 4: Overview of the cellular restriction factors that target HIV-1 and their viral antagonists. The mechanism by which the restriction factors APOBEC3G, SamHD1, Tetherin and TRIM5a target various steps in the HIV-1 life cycle is depicted. The viral accessory proteins that counteract the antiretroviral function of these restriction factors are shown. Inset – The process of APOBEC3G mediated hypermutation of the HIV-1 genome. Reprinted with permission from [4]

in the viral DNA to Uracils (dC → dU) during viral DNA minus strand synthesis [56, 57]. So when the plus strand of the viral DNA is synthesized adenosines get incorporated instead of guanines, thus resulting in G-to-A mutations in the viral DNA resulting in hypermutation of the viral genome [58-61]. A3G can also hinder the movement of RT along the RNA template during elongation by directly binding to the viral RNA in a cytidine deaminase activity-independent manner [62]. Additionally, there are also reports suggesting that A3G might interfere with integration of the viral DNA into the host genome (Reviewed in [55]).

SAMHD1

The Sterile Alpha Motif- and HD-domain containing protein 1 (SAMHD1) is a 72kDa protein that inhibits replication of HIV-1 and SIV in macrophages [51, 52]. SAMHD1 is a triphosphohydrolase that depletes the intracellular pool of nucleotides, thus inhibiting reverse transcription of the viral genome [63]. SAMHD1 consists of an N-terminal SAM domain, which typically mediates protein-protein interactions as well as RNA binding ability and a C-terminal HD domain that is conserved in metalloproteases possessing phosphohydrolytic activity (Reviewed in [4]). dGTP is known to activate the triphosphohydrolase activity of SAMHD1, which leads to the hydrolysis of deoxynucleoside triphosphates (dNTPs) to yield deoxynucleosides and inorganic triphosphate, as well as mediate SAMHD1 dimerization ([64, 65] and reviewed in [4]). While dGTP particularly acts as an activator and substrate of this triphosphohydrolase

and is also known to mediate dimerization of SAMHD1, other dNTPs are also substrates of this enzyme (Reviewed in [4]). The accessory proteins Vpx and Vpr of HIV-2 and SIV from macaques (SIVmac), and African green monkeys (SIVagm) respectively are known to counteract SAMHD1-mediated HIV-1 restriction by targeting it for proteasomal degradation [51, 66-68]. Although HIV-1 does not seem to express any protein that counteracts SAMHD1 activity, HIV-1 RT has acquired the ability to function at lower concentrations of dNTPs. It has been demonstrated that HIV-1 RTs can efficiently carry out reverse transcription in terminally differentiated macrophages (non-dividing cells) that contain much lower concentrations of dNTPs as compared to actively dividing CD4+ T cells [69]. Additionally, increasing dNTP levels in cells relieves SAMHD1-mediated restriction of RT.

Tetherin

Tetherin, also known as CD317, BST-2 or HM1.24, is a ~20kDa interferon inducible, type II transmembrane protein which is mainly localized to and shuttles between the trans-Golgi network (TGN) and the plasma membrane (Reviewed in [4]). It has a short cytoplasmic N-terminal domain and an extracellular α -helical domain that is flanked by two membrane anchors. At its C-terminus tetherin has a glycosylphosphatidylinositol (GPI) anchor. Tetherin inhibits the release of nascent but mature HIV-1 particles by trapping them at the surface of infected cells thus inhibiting further transmission of the virus [50, 70]. One study used an engineered protein

comprised of domains that resembled in structure, but had no sequence homology to the domains found in tetherin and tested its antiviral potential [71]. They found that this protein also inhibited viral release at the cell surface suggesting that tetherin does not recognize specific residues in the viral particle and mediates its activity independent of other cellular proteins. More recent studies have demonstrated that tetherin accumulates at the sites of viral budding on the cell surface and gets incorporated into the membrane of the budding viral particles [71-73]. The HIV-1 accessory protein Vpu has been shown to interact with tetherin and antagonize its antiviral activity [74-76]. Several studies have shown that Vpu inhibits tetherin by increasing its endocytosis, thus downregulating it from the cell surface and eventually leading to its lysosomal degradation [74, 77-79]. The increase in ubiquitination and recruitment of the ESCRT pathway of tetherin mediated by Vpu also seem to inhibit its antiviral activity [80, 81]. A recent study demonstrated the role of tetherin in the induction of innate immune signaling by acting as a viral sensor [82]. This study showed that following restriction of viral release, tetherin dimers cluster and recruit a signaling complex that includes TRAF6, and potentially TRAF2 and Ubc13. This results in activation of TAK1 and NF κ B, and increased expression of proinflammatory genes in the infected cells. Additionally, the restricted virions are then targeted for endosomal degradation where the viral components are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) which in turn induces IRF-mediated activation of type I IFN responses.

TRIM5 α

TRIM5 α is a ~55kDa interferon inducible protein that belongs to the Tripartite Motif (TRIM) family of proteins [83]. TRIM5 α restricts retroviruses in a post-entry and species specific manner [48] by directly binding to the viral capsid soon after it enters the cell cytoplasm. This leads to disruption of the viral capsid before reverse transcription can occur thus preventing further infection by the virus [48]. TRIM5 α is characterized by the presence of the really interesting new gene (RING) domain, B-Box2 and Coiled-coil domains, that are conserved among all TRIM family proteins, at its N-terminus [83]. Each of these domains has been shown to be required for TRIM5 α -mediated HIV-1 restriction. The RING domain is required for TRIM5 α auto-ubiquitination [84] and RING domain mutants exhibit reduced HIV-1 restriction activity [85]. The coiled-coil and B-Box2 domains mediate TRIM5 α dimerization and higher-order multimerization respectively [86-91], and are required for efficient capsid binding and retroviral restriction by TRIM5 α [85, 89, 90, 92].

At its C-terminus TRIM5 α has a B30.2/SPRY domain which directly binds the retroviral capsid. The SPRY domain has been under strong selective pressure and has regions that are highly variable among primate species [93, 94]. This domain contains the determinants that govern the recognition of a wide variety of retroviral capsids by TRIM5 α [95-100]. In Owl monkeys and certain old world monkeys the SPRY domain has been functionally replaced by a cyclophilin A (CypA) domain, as a result of two

independent retrotransposition events [49, 101, 102]. The resulting TRIM-Cyp protein also potentially restricts HIV-1 infection by directly binding to and disrupting the retroviral capsid before reverse transcription can occur [49, 92, 103-105]. Although it is known that direct interaction of the TRIM5 proteins with the retroviral capsid is required for restriction subsequent events and the mechanistic details of the restriction process are poorly understood. However, based on several biochemical and structural studies, a number of models for TRIM5-mediated retroviral restriction have been proposed. It has been demonstrated that viral restriction occurs minutes after it interacts with TRIM5 proteins [106, 107] most likely due to TRIM5-mediated disruption of the viral capsid [105, 108]. However, the TRIM-mediated block to RT, but not viral infection, can be relieved by inhibition of the proteasome using MG132 [109], suggesting the presence of a proteasome dependent step in the restriction process. Additionally, TRIM5 α has been shown to associate with components of the proteasome both in the presence and absence of restriction sensitive virus [110, 111]. However, TRIM5-mediated ubiquitination or proteasomal degradation of the viral components has not been observed; although TRIM5 α is known to undergo proteasomal degradation in the presence of restriction sensitive virus [112]. Intriguingly, Ganser-Pornillos et al. recently demonstrated that recombinant rhTRIM5 α , in which the TRIM5 α RING domain has been replaced by that of TRIM21), forms a hexameric lattice that is complementary to that formed by purified HIV-1 capsid protein [113]. The formation of TRIM5 α hexameric lattices was greatly enhanced in the presence of assembled HIV-1 capsid. B-Box2-mediated higher-order

multimerization of TRIM5 α was required for formation of these lattices [113]. This again suggests that self-association of TRIM5 α plays an important role in retroviral restriction most likely by increasing the avidity of TRIM5-capsid interaction. Moreover, a dual role for TRIM5 α as a restriction factor and an intracellular PRR has been suggested [114]. Pertel et al. showed that TRIM5 α induces the production of unanchored K63-linked polyubiquitin chains that are recognized by TAB2. This leads to TAB2 multimerization and ultimately TAK1 activation and downstream signaling pathways. Formation of these K63 ubiquitin chains was enhanced in the presence of viral capsid. This suggests that TRIM5 α -mediated HIV-1 restriction is a complex process involving multiple phases. The initial phase involves recognition and binding of the retroviral capsid, followed by disruption and/or degradation of the capsid, possibly via the proteasome. The second phase of restriction involves activation of innate immune signaling pathways ultimately resulting in an antiviral state in the cell. Thus, TRIM5 α plays a direct and indirect role in retroviral restriction. The proposed models for TRIM5-mediated retroviral restriction are discussed below.

The Tripartite Motif (TRIM) family proteins

Tripartite Motif (TRIM) proteins are found in most multi-cellular organisms [83]. Humans have over 70 known TRIMs [119-123], mice express over 60 and flies express approximately 10-20 TRIMs suggesting their widespread expression across various species [83]. TRIM family proteins are involved in a number of different cellular

processes including cell proliferation, differentiation, development, oncogenesis, apoptosis, innate immune signaling and viral restriction [120]. Most TRIM proteins act as E3 ligases for ubiquitin (Ub), small ubiquitin-like modifier (SUMO) and the Interferon-stimulated proteins of 15kDa (ISG15) and their role in the induction of innate immune signaling pathways and viral restriction is extensively being studied [120]. In their recent study, Versteeg et al. tested the ability of 75 known human TRIM proteins to induce innate immune signaling and found that almost half of these TRIM proteins were able to positively regulate signaling [117].

Domain Structure and Classification of TRIM proteins

The Tripartite Motif - Almost all TRIM proteins are defined by the presence of the Tripartite Motif also known as the RBCC motif that comprises a RING domain, one or two B-Box domains and a coiled-coil domain [83, 119, 122]. Figure 5 shows the domain organization of all TRIM proteins. The RING domain is present at the N-terminus of most TRIM proteins and has conserved cysteine and histidine residues that co-ordinate two zinc atoms [124, 125]. This domain confers E3 ubiquitin ligase and in some cases SUMO E3 ligase activity to TRIM proteins [126, 127]. The B-Box domains, that are structurally related to RING fingers [128], are also zinc binding domains and are exclusively found in TRIM proteins [120, 122]. Although the biological function of B-

Box domains is not clearly understood, these domains are known to promote protein-protein interactions thus resulting in protein multimerization, at least in some TRIMs. Moreover, B-Box domain mutations are associated with developmental abnormalities and abrogation of viral restriction by some TRIM proteins [85, 89, 90, 129]. The coiled-coil domain is predicted to be predominantly α -helical and is found in many proteins [130]. This domain mediates protein-protein interactions, mainly homo-interactions and hetero-interactions, and is required for the formation of TRIM dimers which form the building blocks of the higher-order multimers formed by most TRIM proteins [83].

C-terminal domains – TRIM proteins can be divided into eleven sub-groups based on their C-terminal domains [83, 121, 123, 131]. Since, the RBCC motif of TRIM proteins is highly conserved, the functional diversity of TRIM proteins, for most part, can be attributed to their C-terminal domains [3]. The most common C-terminal domain expressed by TRIM proteins is the PRY/SPRY domain, also known as the B30.2 domain which is involved in protein-protein interactions (Figure 5; [83, 121-123]). Other domains that can be present at the C-terminal end of TRIM proteins include the COS, fibronectin type-3 (FN3) domain, the 6-bladed β -propeller domain present in Ncl-1, HT2A, Lin-41 proteins (NHL)-repeats domain, and the plant homeodomain (PHD)-BROMO domain which is involved in chromatin binding, meprin and TRAF homology (MATH), ADP ribosylation factor-like (ARF) domain, filamin type Ig (FIL) (Figure 5;

[83, 121-123]). The C-terminal domains of TRIM proteins and their putative functions are listed below.

C-terminal domain	Function
COS	protein binding to microtubes
FN3	binding to DNA and heparin
PHD	chromatin-mediated gene regulation
Bromodomain (always located downstream of PHD in TRIM proteins)	recognition of acetylated lysine residues
PHD-Bromo	transcription repression
NHL repeats	protein-protein interactions
MATH	protein-protein interactions
ARF	intracellular trafficking

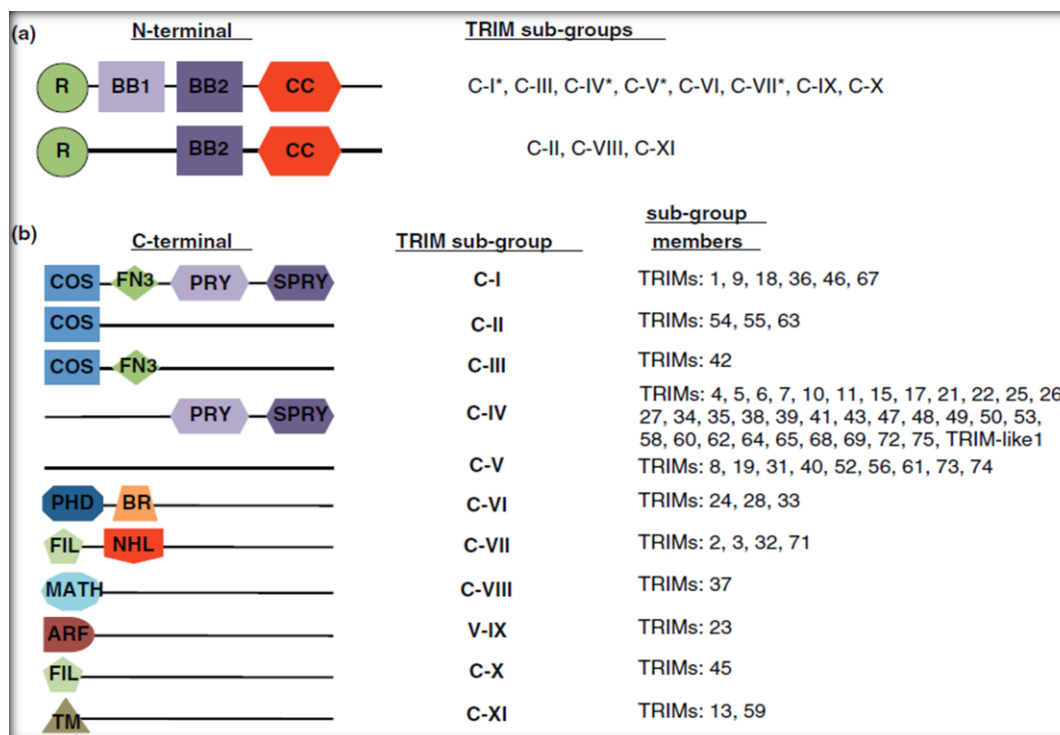


Figure 5: Schematic representation of TRIM family proteins. A) Domains present at the N-terminal region of TRIM proteins. Most TRIM proteins express the RING domain (R), B-Boxes (BB1 and BB2) and a coiled-coil domain (CC). B) C-terminal domains expressed by various TRIM proteins and the different subgroups are shown. ARF, ADP ribosylation factor-like; BR, bromodomain; COS, C-terminal subgroup one signature; FIL, filamin-type immunoglobulin; FN3, fibronectin type 3; MATH, meprin and tumor-necrosis factor receptor-associated factor homology; PHD, plant homeodomain; TM, transmembrane. Reprinted with permission from [3].

TRIM proteins as E3 ligases

Ubiquitination is a type of post-translational protein modification in which either single or multiple ubiquitin (Ub) chains are covalently attached to specific lysine (Lys)

residues of a target protein [132]. It is a multi-step process involving a Ub activating enzyme (E1), a Ub conjugating enzyme (E2) and a Ub ligase (E3) [132]. E1 activates Ub and forms an E1-Ub thioester intermediate which is then transferred to the active cysteine of E2. The ubiquitin ligase E3 then directly interacts with E2 and the target protein and facilitates the transfer of Ub to a specific Lys residue of the target protein (Reviewed in [120]). Depending on the length of the Ub chains attached, ubiquitination plays a role in a number of cellular processes. For example, mono- and di-Ubiquitination are usually associated with exocytosis and endosomal sorting whereas poly-ubiquitination usually targets proteins for proteasomal degradation (Reviewed in [120]). Similarly, the Lys of the Ub moiety used for isopeptide bond formation (such as K48, K63 and so on), also dictates the outcome of the ubiquitination process (Reviewed in [120]).

More recent studies are focused on understanding the role of TRIM proteins as E3 Ub ligases. E3 ligases can be divided into three classes based on their catalytic domains that mediate Ub transfer. These are HECT (Homologous to E6AP carboxy terminus), U-box and RING finger E3s [120]. TRIM proteins are characterized by the presence of RING finger domains at their N-terminus and act as putative E3 Ub ligases [120]. TRIM proteins mainly mediate two forms of ubiquitination reactions namely, Lys48 ubiquitination which targets proteins for proteasomal degradation, and the Lys63 ubiquitination which is involved in a variety of processes including NFkB signaling,

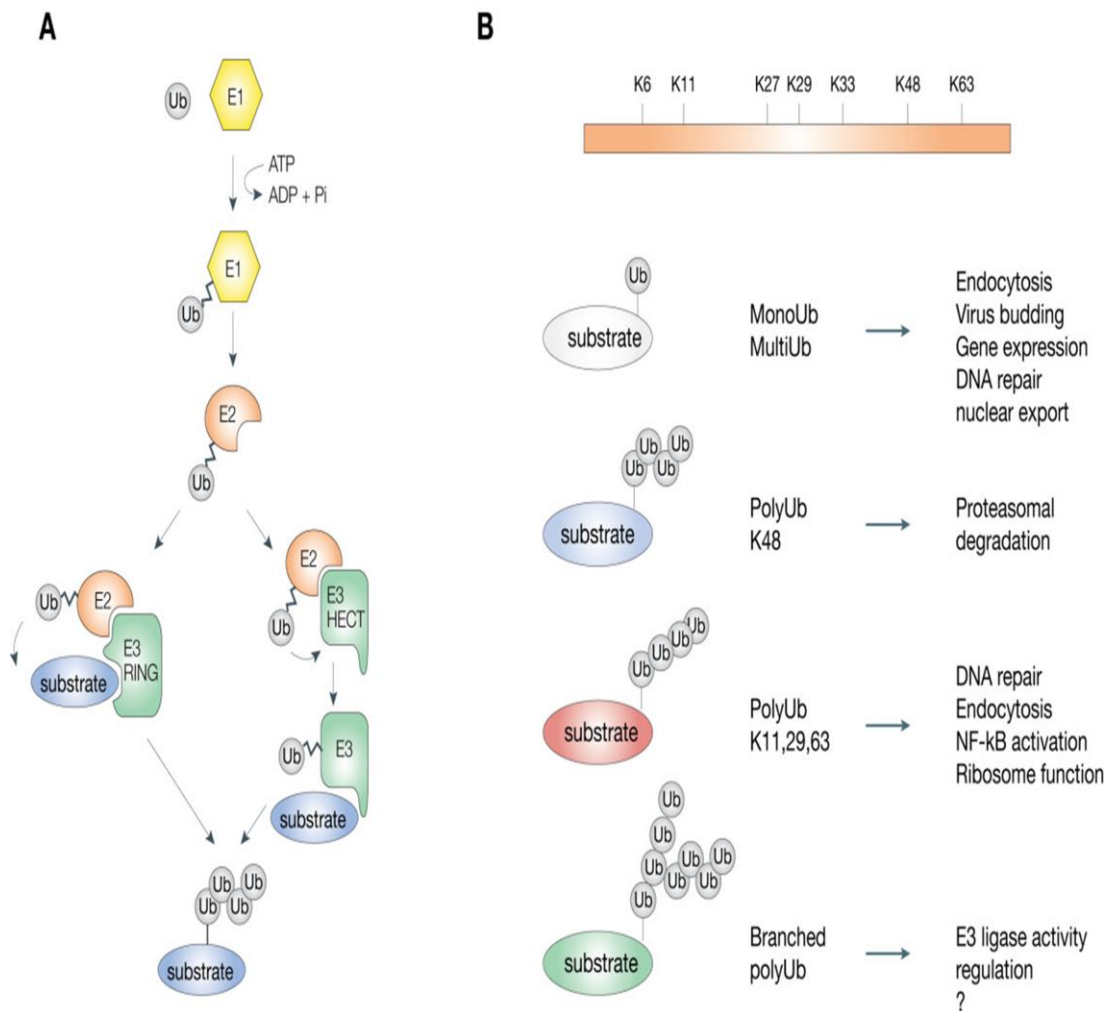


Figure 6: The ubiquitin pathway. A) Schematic representation of the ubiquitination process. The three enzyme required for substrate ubiquitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes are shown. The RING and HECT family E3 ligases are the two major classes of E3 ligases. B) Schematic representation of the different types of Ub modifications. The functional roles of these modifications are listed. The question mark indicates that the functions of branched chains are largely unknown. Reprinted with permission.

A recent screen showed that TRIM proteins interact with Ub-conjugating E2 enzymes or UBE2s and generally prefer the D and E classes of UBE2s [126]. This study also demonstrated that the specific E3 activity of these TRIM proteins depends on the specific E2 enzyme they interact with. Some TRIM proteins that have been previously shown to possess E3 ligase activity include TRIM23/ARD1, TRIM11, TRIM18/Mid1, TRIM21/Ro52, TRIM25/Efp, TRIM32 and TRIM5 α (Reviewed in [120]). This E3 ligase activity of TRIM proteins has been shown to be associated with their biological function. For example, TRIM25/Efp has been shown to regulate the protein levels of the cell cycle inhibitor, 14-3-3 σ by targeting it for proteasomal degradation [133]. Pertel et al. have that the E3 ligase activity of TRIM5 proteins plays a role in the production of unanchored K63 poly-Ub chains which further activate TGF- β -activated kinase 1 (TAK1)-mediated activation of AP-1 and NF- κ B signaling [114]. This activity of TRIM5 α was shown to be enhanced in the presence of the HIV-1 CA. In addition to acting as putative E3 Ub ligases TRIM proteins have also been implicated in other Ub-like modification processes such as SUMOylation and ISGylation. SUMOylation involves the transfer of the small ubiquitin like modifier (SUMO) and ISGylation involves the transfer of the interferon-induced 15-kDa protein (ISG15) to substrate proteins. Recently, several TRIM proteins have been shown to function as SUMO E3 ligases including TRIM1, 19, 22, 27, 32 and 36). Additionally, three SUMO interacting motifs (SIMs) were identified in TRIM5 α that are important for its antiretroviral activity [134, 135]. TRIM25, on the other hand, has

been shown to mediate ISGylation of 14-3-3 σ as well as undergo auto-ISGylation [136, 137].

Self-association of TRIM proteins and its biological significance

Most TRIM family proteins are known to self-associate and form lower and higher-order multimers that localize to different cellular compartments [83]. Most of them localize to discrete structures or assemblies in the cytoplasm or the nucleus. Cytoplasmic TRIMs usually form filaments or ribbon-like structures or in some cases localize to punctate assemblies termed as “cytoplasmic bodies” as seen in case of TRIM5 α . Nuclear TRIMs usually form punctate assemblies termed as “nuclear bodies” [83]. A classic example of TRIM that localizes to nuclear bodies is TRIM19 or PML. Some TRIM proteins that express the BROMO domain localize to specific regions of the chromatin [83]. In most cases the coiled-coil domain seems to be required for the formation of these assemblies and disruption of the coiled-coil domain often results in a diffuse localization of these proteins [83]. Coiled-coil domains are found in many cellular proteins and can be identified by the presence of the characteristic heptad repeats $(abcdefg)_n$, where typically a and d are hydrophobic amino acids and e and g are polar/charged amino acids [138]. Coiled-coil domains are predicted to form amphipathic α -helices and mediate homo-multimerization of proteins [138]. In the case of TRIM5 α and TRIM72/MG53 the coiled-coil domain is required for formation of protein dimers [71, 83, 86, 87]. The B-Box2 domain has also been implicated in mediating the self-

association of TRIM proteins and has been shown to be required for higher-order multimerization in the case of TRIM5 α [85, 89-91]. Dimerization and higher-order multimerization have been shown to be critical to the biological function of TRIM proteins. For example, in case of TRIM72/MG53, which nucleates the assembly of cell membrane repair machinery, homodimerization mediated by the leucine zipper motifs in the coiled-coil domain is essential for TRIM72-mediated cell membrane repair [139, 140]. Similarly, coiled-coil mediated dimerization and B-Box2 mediated higher-order multimerization have been shown to be required for retroviral restriction by TRIM5 α [85, 86, 89, 90, 113, 129, 141, 142]. The role of self-association in TRIM5-mediated retroviral restriction is the main focus of this dissertation and will be discussed in more detail in section 1.4.2.

Role of TRIM proteins in innate immune signaling and viral restriction

A number of TRIM proteins have been shown to possess antiviral activity [114, 129, 143-146]). In one study 36 human and 19 mouse TRIM proteins were screened for their ability to act as anti-viral factors. Of the 55 TRIMs screened, approximately 20 TRIMs inhibited various stages of the viral life cycle indicating the antiviral potential of these proteins [144]. Some TRIM proteins are capable of restricting multiple viruses for example; TRIM22 restricts HIV-1 replication by inhibiting LRT-mediated transcription, EMCV by degrading 3C protease and hepatitis B by inhibiting RNA synthesis [147, 148]. Similarly, TRIM5 α restricts a wide spectrum of retroviruses. The C-terminal SPRY

domain has determinants that govern the species specific recognition of retroviruses by TRIM5 α [94-96, 98-100, 149]. TRIM21 inhibits Adenovirus infection by acting as an intracellular IgG receptor [150, 151]. It was recently demonstrated that TRIM21 binds IgG-coated Adenoviral particles in cells and targets the virus-IgG complex for proteasomal degradation thus neutralizing the virus [151].

Additionally, the ability of TRIM proteins to initiate signaling cascades has also been implicated in viral restriction. It was recently demonstrated that about half of the 75 known TRIMs can activate innate immune responses and this ability of TRIM proteins has been linked to their antiviral potential [117, 144, 146]. For example, McEwan et al. recently showed that recognition of antibodies by TRIM21 in cells induces the formation of K63-linked polyubiquitin chains which stimulates the activation of NF- κ B, AP-1 and IRF3, 5 and 7 signaling pathways ultimately resulting in an anti-viral state in the cells [152]. This study also showed that TRIM21-mediated recognition of IgG could be stimulated by infecting the cells with both, DNA and RNA viruses as well as by intracellular bacteria suggesting that activation of innate immune signaling pathways by TRIM21 is independent of specific PAMPs. Similarly other TRIMs have also been shown to modulate intracellular signaling pathways including TLR4 [153], RIG-I [154, 155], NF- κ B [114, 156], and IRF [157-159] signaling. A recent study by the Luban laboratory provided evidence for the role of TRIM5 proteins as PRRs in the recognition of the retroviral capsid lattice [114]. This study showed that TRIM5 proteins induce

innate immune signaling by activation of NF- κ B and AP-1 signaling pathways. TRIM5 interacts with TAK-1 as well as the E2 enzyme UBC13/UEV1A and induces the production of unanchored K63 polyubiquitin chains which further activate TAK1 resulting in downstream NF- κ B and AP-1 signaling. The UBC13/UEV1A dependent E3 Ub ligase activity of TRIM5 is further enhanced upon recognition of a restriction sensitive virus.

TRIM5 proteins (TRIM5 α and TRIM-Cyp)

In 2004, using a genetic screen, Stremlau et al., identified TRIM5 α as the protein responsible for inhibiting HIV-1 infection in rhesus macaques [48]. Owl monkeys, a New World monkey species, express a restriction factor similar to TRIM5 α which also restricts HIV-1 infection [49]. Since then TRIM5 proteins (TRIM5 α and TRIM-Cyp) have been shown to restrict a variety of retroviruses in humans [97, 160], other primate species [101, 103, 160-164], cattle [165, 166], and non-primates [167, 168]. TRIM5 proteins restrict retroviruses in a species-specific manner. For example, rhesus macaque TRIM5 α (rhTRIM5 α) potently restricts infection by HIV-1 but does not restrict SIVmac [48]. Human TRIM5 α (huTRIM5 α) restricts infection by other retroviruses such as N-tropic murine leukemia virus (N-MLV) and Equine Infectious Anemia Virus (EIAV), but not HIV-1 and B-tropic murine leukemia virus (B-MLV) [160, 169-171]. The viral determinants responsible for the susceptibility of retroviruses to TRIM5-mediated restriction have been mapped to the viral capsid (CA) protein [172, 173]. In case of

TRIM5 α , the determinants in the C-terminal B30.2/SPRY domain are known to govern the ability of these proteins to specifically recognize and bind retroviral capsids [94-100, 149, 174]. TRIM5 proteins block retroviral replication soon after the viral core enters the cell cytoplasm [107], thus preventing accumulation of reverse transcripts in turn inhibited further infection [48, 108, 175, 176].

TRIM5 proteins: Domains and their Function

TRIM5 proteins contain the N-terminal RING, B-Box2 and Coiled-coil domains that are characteristic of all TRIM family proteins [83]. The RING domain has a putative E3 ubiquitin ligase activity that is required for TRIM5 α autoubiquitination [84] and HIV-1 restriction [84, 141, 142, 162, 177, 178]. Deletion of the RING domain or mutation of the two conserved Cysteine residues, that are required for zinc binding and proper folding of the RING domain, as well as mutation of residues that lie in the E2 binding interface, results in reduced auto-ubiquitination and HIV-1 restriction abilities of TRIM5 α [84, 85, 177]. The coiled-coil domain is required for dimerization [83, 86, 87, 97] and a surface-patch in the B-box2 domain mediates higher-order multimerization of TRIM5 α [89, 90]. Both dimerization and higher-order multimerization of TRIM5 α have been shown to be important for efficient capsid binding and restriction [86, 87, 89, 90, 97, 104, 141, 142, 179, 180].

The primary transcript of TRIM5 undergoes differential splicing to give rise to several isoforms of the protein. The α isoform of TRIM5, which is the largest isoform, contains a C-terminal B30.2/SPRY domain in addition to the RBCC motif [83]. The SPRY domain plays a critical role in TRIM5-mediated retroviral restriction by recognizing specific determinants in the retroviral capsid and directly binding the capsid [48]. In some cases, most notably that of Owl monkeys, the SPRY domain has been functionally replaced by the retrotransposition of Cyclophilin A (CypA) into the TRIM5 locus [49, 101, 161, 163, 181, 182]. The resulting TRIM-Cyp protein also exhibits potent antiretroviral activity against HIV-1 [49, 101].

Capsid recognition and species specific retroviral restriction

It is well established that as soon as the retroviral capsid enters the cell cytoplasm, TRIM5 α directly binds the capsid by recognizing specific capsid determinants. Several groups have demonstrated that the determinants that govern recognition of the retroviral capsid lie within the SPRY domain of TRIM5 α . This domain comprises three variable loops that have evolved to recognize specific determinants in the retroviral capsid [94, 96, 99, 100] thus resulting in the species-specific retroviral restriction. This can be best explained by the fact that while rhTRIM5 α potently restricts HIV-1, human TRIM5 α (huTRIM5 α) only weakly interferes with HIV-1 infection. Interestingly, replacing a single amino acid in the SPRY domain of huTRIM5 α confers HIV-1 restriction ability to a level that is similar to that of rhTRIM5 α (R332P) [174]. It is important to note that the

regions and amino acids that are responsible for specificity of retroviral restriction have been subjected to strong selective pressure during primate evolution [94]. In agreement of this notion, in more than one instance, the SPRY domain of TRIM5 α has been functionally replaced by the cellular cyclophilin A (Cyp A) gene in various monkey species [49, 101, 161]. Similar to the SPRY domain, CypA binds the HIV-1 capsid alone or in the context of the TRIM-Cyp protein. Although the determinants that mediate the interaction between TRIM5 proteins and the viral capsid have been identified for some time, studying this interaction using biochemical techniques has been a challenge. One reason for this is the fact that TRIM5 proteins recognize and bind the viral capsid only in the context of an intact core. Although, interaction of TRIM5 α with capsid monomers has been detected this interaction is very weak [183]. The most direct evidence for this comes from a study by the Aiken laboratory, which showed that viral cores that are unstable or not fully processed do not saturate TRIM5-mediated restriction unlike mature and hyper-stable cores. The second challenge to the study of TRIM5-virus interaction is the instability of the viral cores isolated from an active infection. However, one study demonstrated an interaction between the huTRIM5 α and detergent-stripped N-MLV virions [184]. The development of biochemical techniques allowing the assembly of purified recombinant viral cores in the recent years has provided major insight into the mechanism of TRIM5-virus interactions. Using in vitro assembled recombinant HIV-1 capsid-nucleocapsid (CA-NC) proteins or CA tubular assemblies it was shown that both rhTRIM5 α and TRIM-Cyp efficiently bind these assembled viral cores. huTRIM5 α on

the other hand only weakly binds assembled HIV capsids. Taken together, these data suggest that TRIM5 proteins recognize determinants that are exposed only in the context of an intact, mature viral core. This interaction is mediated by the C-terminal SPRY domain in case of TRIM5 α and the CypA domain in case of TRIM-Cyp. Although the C-terminal domains are absolutely required for the initial interaction with the viral capsid, self-association of TRIM5 α plays an important role in capsid binding, as described below.

Self-Association of TRIM5 α and Cytoplasmic Body formation

Protein self-association can be defined as the selective and non-covalent interaction between two or more domains of the same protein. TRIM5 α is known to self-associate to form dimers and higher-order multimers [86, 87, 141]. In cells, TRIM5 α self-association induces the formation of cytoplasmic assemblies known “cytoplasmic bodies”. TRIM5 α has been shown to exchange rapidly between cytoplasmic bodies and a diffuse pool of cytoplasmic TRIM5 α [185].

The biological significance of cytoplasmic bodies in TRIM5-mediated retroviral restriction has been controversial as two studies demonstrated that pre-existing cytoplasmic bodies are not required for HIV-1 restriction [107, 186]. One study found that treatment of cells expressing TRIM5 α with geldanamycin, a heat shock protein 90 (Hsp 90) inhibitor, prevented the formation of TRIM5 α cytoplasmic bodies [186]. This

treatment however, did not affect the ability of these cells to restrict HIV-1 infection. In the second study, a cell line stably expressing relatively low levels of TRIM-Cyp did not localize to cytoplasmic bodies but was still able to restrict HIV-1 infection [107]. Further treatment of these cells with sodium butyrate resulted in up to a 10-fold increase in TRIM-Cyp levels and in turn increased localization of TRIM-Cyp to cytoplasmic bodies. However, the localization of TRIM-Cyp to cytoplasmic bodies in these cells did not dramatically increase HIV-1 restriction. Although these studies examined cytoplasmic body formation prior to infection they did not determine if cytoplasmic bodies formed during restriction. These studies therefore demonstrated that pre-existing cytoplasmic bodies, i.e. the cytoplasmic bodies formed in the absence of virus, are not relevant for HIV-1 restriction.

Alternatively, Campbell et al. have previously shown that TRIM5 α cytoplasmic bodies are dynamic structures that turn over rapidly and traffic through the cell by travelling on the microtubule network [185]. Infection of cells stably expressing rhTRIM5 α with fluorescently labeled HIV-1 showed a dynamic interaction of the virions with TRIM5 α cytoplasmic bodies [106]. Treatment of these cells with the proteasome inhibitor MG132 prior to infection resulted in accumulation of the virions within large rhTRIM5 α cytoplasmic bodies. Live cell imaging also showed *de novo* formation of rhTRIM5 α cytoplasmic bodies around individual virions and minutes within this interaction occurred the fluorescent signal of the virus was lost [106]. These studies

suggest that TRIM5 α cytoplasmic bodies that are formed in the presence of restriction sensitive virus could be important for viral restriction. Collectively, these studies point towards a possibility that although pre-existing cytoplasmic bodies are not required for restriction, the ability of TRIM5 α to form these bodies following viral infection could be important for the restriction process by mediating interaction of TRIM5 α with the viral capsid. Self-association of TRIM5 α proteins involves multiple steps involving dimerization, higher-order multimerization and assembly and each of these steps is essential for efficient capsid binding and restriction by TRIM5 proteins [86, 87, 113, 141, 142].

Low-Order Multimerization: Dimerization - The coiled-coil domain plays a critical role in dimerization of most TRIM proteins [83] including TRIM5 α and TRIM-Cyp [87, 104]. TRIM5 α was initially reported to form trimers [87]. However, subsequent studies have demonstrated that this was an erroneous conclusion owing to an electrophoretic mobility of the glutaraldehyde crosslinked dimer that is inconsistent with its actual molecular weight. More recent studies using a recombinant TRIM5 α protein, in which the TRIM5 α RING domain has been replaced by the TRIM21 RING domain (TRIM5 α -21R), suggest that the dimer is the predominant form of the protein [141, 142]. While dimeric TRIM5 α -21R binds in vitro assembled HIV-1 capsid, the monomeric form of the protein does not [113]. Moreover, disruption of TRIM5 α dimerization by partial or

complete deletion of the coiled-coil domain results in loss of capsid binding and restriction by TRIM5 α [86].

Higher-Order Multimerization - TRIM5 protein dimers have been shown to further self-associate into higher-order multimers that can be visualized by SDS-PAGE following biochemical cross-linking of cellular proteins with reagents such as ethylene glycol-bis(succinimidyl succinate) (EGS) or glutaraldehyde [86-88]. The B-Box2 domain and the L2 region, which connects the coiled-coil and C-terminal SPRY or CypA domains, has been shown to mediate the higher-order multimerization of TRIM proteins [85, 89, 90, 129]. The B-Box2 domain of TRIM5 proteins has two conserved cysteines at positions 96 and 97 and a histidine at position 100 which are required for zinc binding and proper folding of this domain [187]. Replacement of these residues with alanine, C96A and C97A/H100A, results in abrogation of TRIM-Cyp-mediated and TRIM5 α -mediated HIV-1 restriction, respectively [85, 92]. Li et al. showed that alteration of certain residues within the B-Box2 domain reduce the HIV-1 capsid binding ability of rhTRIM5 α , thus resulting in reduced ability of the protein to restrict the virus [129]. This suggests that alteration in the B-Box2 domain possibly affect the orientation or the conformation of the SPRY domain in turn affecting capsid binding. However, mutation of the same residues within the B-Box2 domain of TRIM-Cyp had no effect on HIV-1 capsid binding or restriction by TRIM-Cyp. It is known that Cyp A has a higher affinity for the HIV-1 capsid as compared to the SPRY domain. Hence, it is possible that binding

of TRIM-Cyp to the capsid via the Cyp A domain is sufficient for restriction. However, in case of TRIM5 α since the SPRY domain has a relatively low affinity for the capsid, the B-Box2 domain is required to increase the avidity of TRIM5 α -capsid binding. In fact, Li et al. demonstrated that the B-Box2 facilitates cooperative binding to the retroviral capsid by mediating higher-order multimerization of TRIM5 α [90]. Using nuclear magnetic resonance (NMR), Diaz-Griffero et al. discovered a hydrophobic patch of residues on the surface of the B-Box2 domain which when altered results in abrogation of TRIM5-mediated HIV-1 restriction [89]. Mutation of a flanking arginine at position 121 also had a similar effect on restriction. In each case the loss of restriction ability of these mutants could be correlated to their inability to self-associate into higher order multimers as well as their reduced binding affinity for the viral capsid. A recent study aimed at determining the TRIM5 α domains that are required for self-association, showed that while the coiled-coil and B-Box2 domains are required for self-association of TRIM5 α , the RING domain possibly contributes to the efficiency of this self-association further enhancing capsid binding and restriction [91]. This study also showed that coiled-coil mediated dimerization is required for higher-order multimerization of TRIM5 α . Additionally, a region of the protein immediately downstream of the coiled-coil domain, known as Linker 2 (L2), also facilitates the efficiency of higher-order multimerization of TRIM5 α however the SPRY domain does not play a role in this process.

Assembly - While the coiled-coil and B-Box2 domains mediate low and higher-order multimerization of TRIM5 α , the formation of cytoplasmic bodies and the regions of the protein that are required for formation of these assemblies have not been determined. Furthermore, the functional significance of TRIM5 α cytoplasmic bodies is yet to be characterized. A recent study by Ganser-Pornillos et al. provides a significant insight into the mechanism of TRIM5 α -mediated HIV-1 restriction and the role for TRIM5 α assembly in the restriction process [113]. Using negative-stain electron microscopy of purified TRIM5 α -21R, a recombinant TRIM5 α protein, spontaneous assembly of this protein into two-dimensional hexagonal arrays was observed. They also showed that dimerization and higher-order multimerization of TRIM5 α -21R mediated by the coiled-coil and B-Box2 domains respectively were required for the formation of these assemblies. Formation of TRIM5 α -21R hexameric assemblies was further enhanced in the presence of assembled HIV-1 capsids, although they were not absolutely required for this process. Based on the size and dimensions of the viral capsid and TRIM5 α -21R assemblies and previous studies involving TRIM5 α self-association, they proposed a model for self-assembly of TRIM5 α around an intact viral core. This self-assembly most likely involves multiple steps including coiled-coil mediated dimerization, B-Box2 mediates higher-order self-association and finally assembly of the protein into large hexameric lattices around the viral core. This model also proposes that the B-Box2 domain mediates higher-order multimerization generates a trimeric interface of TRIM5 α dimers which facilitates the tripodial extensions required for the formation of a hexameric

lattice. Berthoux group had previously shown that both TRIM5 α and TRIM-Cyp form dimers, trimers, hexamers and more complex multimers in mammalian cells [88]. Assuming the B-Box2 domain facilitates the formation of the tripodial extensions, as the model suggests, this could explain the apparent hexamers observed by the Berthoux group [88, 113].

Proposed Models of TRIM5-mediated retroviral restriction

The regions mediating the binding and assembly of TRIM5 proteins to the retroviral capsid are well defined by several groups. Additionally, it has been demonstrated that following this interaction viral infection is inhibited before the virus can reverse transcribe its genome. However, the mechanistic details of the restriction process remain unclear. Several models have been proposed to explain TRIM5-mediated retroviral restriction as described below.

Accelerated Uncoating

Uncoating can be defined as the loss of the p24 capsid protein from the viral ribonucleoprotein complex. The accelerated uncoating model proposed by the Sodroski laboratory, suggests that TRIM5 α binds and induces accelerated uncoating of the retroviral capsid so that subsequent steps in infection are inhibited. This model is supported by data from the “fate of capsid” assay. This assay attempts to measure the total amount of intact capsids in cells expressing TRIM5 α following infection. Typically,

lysates of infected cells expressing TRIM5 α are centrifuged through a sucrose cushion and the amount of CA in the supernatant (dissociated or uncoated cores) and pellet (intact cores) fractions is determined. These studies show that TRIM5 α mediates loss of the intact pelletable capsid while the total amount of capsid in the sample remains unaffected. This suggests that rhTRIM5 α promotes the rapid and pre-mature dissociation of the viral capsid without promoting degradation of the cytosolic capsid protein. However, whether this TRIM5-mediated “accelerated uncoating” shares similarities to the natural uncoating of the viral core remains unclear. In their recent study by the Bieniasz laboratory made use of a biochemical assay to determine the fate of various components of the retroviral core when the infection was carried out in the presence and absence of TRIM5 proteins [105]. Following synchronized infection of cells VSV-g-pseudotyped retroviruses such as MLV and HIV-1, the cytosolic proteins were fractionated on linear gradients and the fates of viral core components including capsid, integrase, viral genomic RNA and RT products were monitored. They found that in the absence of TRIM5 proteins the retroviral core components formed large complexes. However, upon huTRIM5 α -mediated restriction of MLV infection the integrase and RT products could not be detected while the capsid and the viral genomic RNA were both solubilized. Similar loss of integrase and RT products as well as the viral RNA was observed upon restriction of HIV-1 by rhTRIM5 α and TRIM-Cyp. Additionally, as previously demonstrated by inhibition of the proteasome blocked these consequences of TRIM5-mediated restriction without affecting viral restriction. Thus this study shows the consequences of TRIM5-

mediated restriction on retroviral components and suggests that the proteasomes are required for disruption of the viral core by TRIM5 proteins. This data contradicts the model proposed by the Galloway laboratory suggesting that capsid degradation occurs in a proteasome independent manner but is in concordance with the Two-Step model of restriction proposed by the Hope laboratory as discussed below.

Proteasome Independent Capsid Degradation

The Galloway laboratory demonstrated that in cells expressing rhTRIM5 α there was a specific loss of the cytosolic HIV-1 capsid [188]. Separation of the cytosolic and vesicular fractions of cells expressing rhTRIM5 α after infection with HIV-1 showed that rhTRIM5 α induced the degradation of the viral capsid without affecting other components of the viral ribonucleoprotein complex. Additionally, proteasome inhibition did not affect rhTRIM5 α -mediated degradation of the capsid. Thus, this model suggests that rapid removal of the viral capsid from the viral ribonucleoprotein complex prevents subsequent steps in the infection pathway.

Two-Step Mechanism of Restriction

This model was proposed by the Hope laboratory and suggests that TRIM5-mediated retroviral restriction occurs in two distinct phases (Figure 7). The first step is sufficient to inhibit retroviral infection and involves the binding of the retroviral capsid by TRIM5 α or TRIM-Cyp via the SPRY or CypA domain respectively. The second step

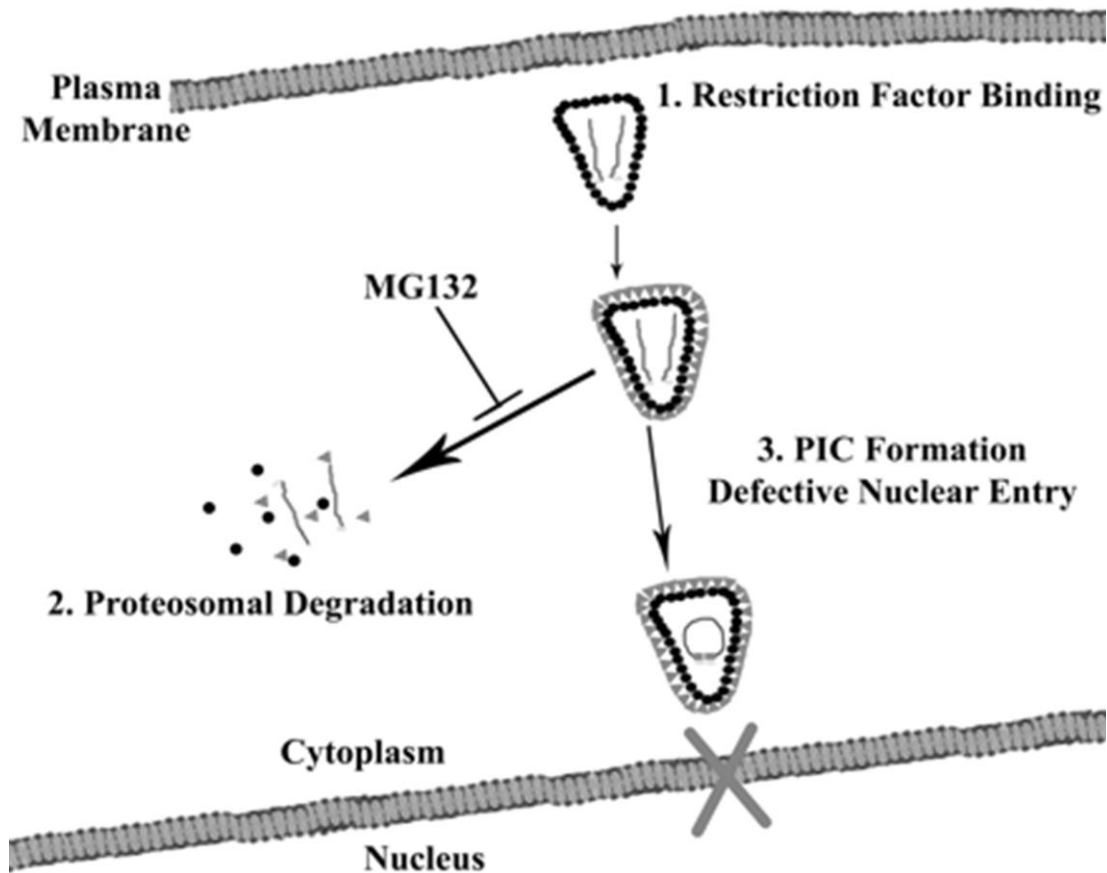


Figure 7: Two-step model of TRIM5 α -mediated HIV-1 restriction as described by [2]. 1) TRIM5 proteins recognize determinants in the HIV-1 capsid soon after its entry in the cell cytoplasm. 2) In the absence of MG132 the viral core is degraded in a proteasome dependent manner. 3) Upon inhibition of proteasomes using MG132, the core stabilizes and viral cDNA reverse transcribes. This results in the production of an integration-competent PIC intermediate whose nuclear localization remains impaired analogous to *Fv1* restriction. Reprinted with permission from [2].

involves proteasome-dependent abortive disassembly of the bound virion, which is induced by the TRIM5 proteins. The data supporting this model comes from a studies conducted by the Hope laboratory which showed that proteasome inhibition relieves TRIM5 α -mediated block to reverse transcription while not affecting the ability of the protein to restrict infection [2, 109]. In collaboration with the Engelman laboratory they also showed that inhibition of the proteasome allows RT to complete in restricted cells and these viral ribonucleoprotein complexes are capable of undergoing integration in vitro. This suggests that in vivo, the viral preintegration complexes that are produced upon proteasome inhibition, although intact, are unable to translocate to the nucleus to complete the integration step due to an interaction with TRIM5 proteins. This notion is supported by the observation that inhibition of the proteasome leads to the accumulation of fluorescently labeled HIV-1 virions within enlarged TRIM5 α cytoplasmic bodies [106]. Additionally, the Aiken laboratory has shown that addition of restriction sensitive virus to cells results in proteasome-mediated degradation of TRIM5 α [112]. Indeed, using fluorescent microscopy and biochemical assays at least two groups have observed recruitment of proteasomal subunits to TRIM5 α cytoplasmic bodies in the absence and presence of restriction sensitive virus [110, 111]. However, the exact mechanism underlying this process remains unclear.

TRIM5 α : Restriction factor and a Pathogen Recognition Receptor (PRR)

Previous studies have shown that TRIM5 α is an IFN inducible protein and can elicit an NF- κ B response [131, 189]. The Luban laboratory recently studied the role of TRIM5 α as a component of the innate immune system [114]. This study demonstrated that TRIM5 plays a multifunctional role as a capsid-specific restriction factor, as described previously, and as a pathogen recognition receptor (PRR) resulting in the activation of inflammatory genes. Based on this and previous studies the authors propose a model that suggests that TRIM5 specifically recognizes and multimerizes around the incoming retroviral core and induces the production of unanchored K63 polyubiquitin chains. These K63 polyUb chains then activate TAK1 and the transcription of inflammatory genes most likely through multimerization of the TAK1 associated Ub-binding protein TAB2 [190]. The E3 Ub ligase activity of TRIM5 is enhanced in the presence of a restriction sensitive hexameric capsid lattice. The spontaneous assembly of TRIM5 into hexagonal lattices has been shown to be enhanced in the presence of hexameric capsid lattices [113]. Moreover, the cellular factors that are required for activation of the E3 Ub ligase activity of TRIM5 and the activation of its inflammatory activity namely, UBC13/UEV1A and TAK1, also enhance TRIM5-mediated retroviral restriction. This suggests that the two functions of TRIM5 as a restriction factor and as a component of the immune system are mechanistically linked. However, the downstream substrates of TAK1 that could potentially be required for TRIM5-mediated restriction are unknown. Although these above mentioned models suggest different mechanisms of TRIM function, they are not mutually exclusive as described in figure 8.

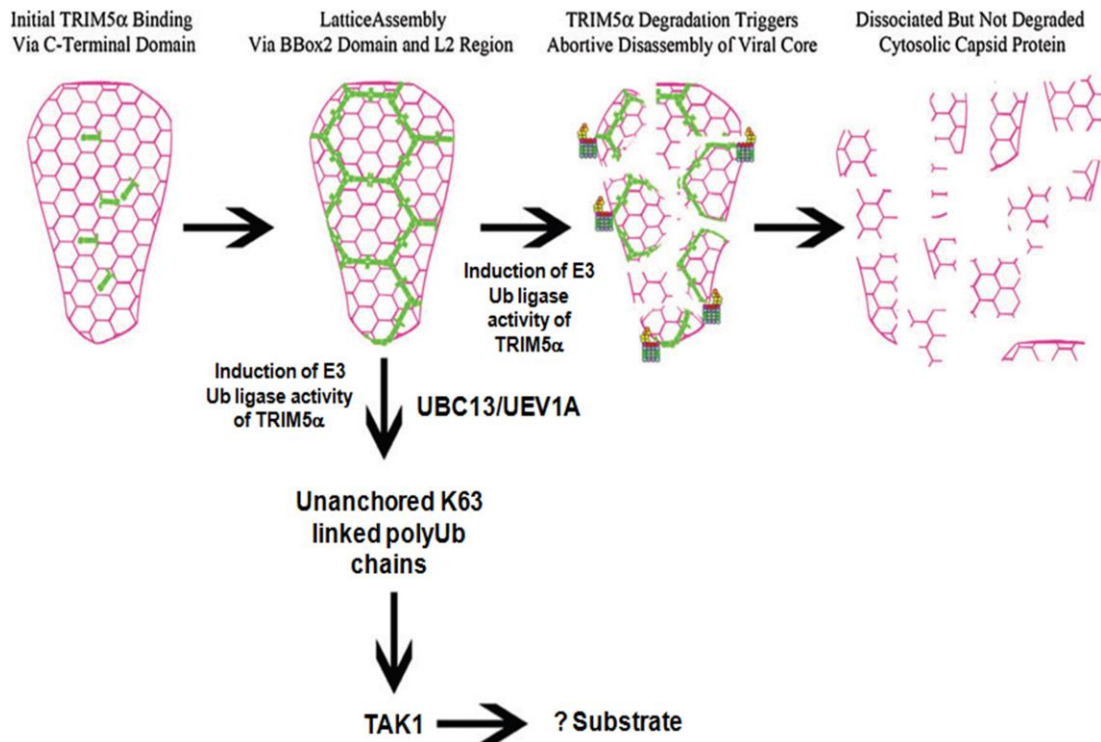


Figure 8: Current model for TRIM5 α -mediated retroviral restriction as described by Jeremy Luban [1] & Jaya Sastri and Edward M. Campbell [6]. Following entry of the retroviral core (pink) in the host cell cytoplasm TRIM5 dimers directly bind the retroviral capsid by recognizing specific determinants in the viral capsid. Following binding, TRIM5 dimers multimerize and assemble into a hexameric lattice surrounding the viral core. Given the specificity of the SPRY domain for the viral capsid proteins it is possible that multiple TRIM5 lattices assemble around a single virion. A single TRIM5 lattice (green) is shown for clarity. This in turn increases the E3 ubiquitin ligase activity of TRIM5. Depending upon its proximity to specific E2 enzymes, TRIM5 will undergo autoubiquitination and engage the proteasome and/or it will activate the TAK1 kinase and downstream signaling molecules. Proteasomal degradation of TRIM5 proteins, triggers the “abortive disassembly” of the viral core in turn blocking reverse transcription. This results in dissociation but not degradation of the capsid proteins keeping the cytosolic capsid levels unchanged. Increase in avidity of TRIM5-capsid binding, following TRIM5 assembly is critical for these processes. Reprinted with modification from [6]

CHAPTER II

MATERIALS AND METHODS

Recombinant DNA constructs

The wild-type rhTRIM5 α plasmid was a kind gift from Dr. Joseph Sodroski (Harvard school of Public Health). To generate the HA-tagged rhTRIM5 α construct and the L2 variants SmaI and EcoRI restriction sites were inserted flanking rhTRIM5 α using the primers GCCTGGCATTATGCCAG and AGCTTGCCAAACCTAC. Polymerase chain reaction (PCR) was performed and the PCR product was digested with SmaI and EcoRI and inserted into the EXN retroviral vector, also digested with SmaI and EcoRI. The EXN plasmid was generously provided by the lab of Dr. Greg Towers (Royal Free and University College, London). This EXN vector was used to derive the YXN retroviral vector, which was generated by PCR amplification of the Yellow fluorescent protein (YFP) coding region of the YFP-N1 (Clontech) plasmid, using the primers TGGATGAACTATAACAAGTGGATCCGGCCG and CGGCCGGATCCACTTGTATAGTTCATCCA. The PCR amplified YFP fragment was then digested with AgeI and BsrGI and inserted into the similarly digested EXN plasmid. To facilitate easier subsequent cloning, the BamHI site of wt rhTRIM5 α was disrupted by

SOEing PCR using the interior primers CCCCAGTATCCAAGCACTTTT and AGTGCTTGGATACTGGGGGTATGT and exterior primers GCGGCGGGATCCATGGCTTCTGGAATCCT and GGCCGGCTCGAGTCAAGAGCTTGGTGAGC. These primers introduced a silent mutation in the wt rhTRIM5 α open reading frame that eliminated the BamHI. This PCR product was then digested with BamHI and XhoI and inserted into the similarly digested YXN plasmid. Alanine mutations were introduced into wt rhTRIM5 α or rhTRIM5 α lacking a BamHI site using SOEing PCR. YFP- and HA-tagged triple alanine mutants of huTRIM5 α and Owl monkey TRIM-Cyp were cloned in a similar manner. Similarly, the CC-L2 and L2 fragments of wt rhTRIM5 α and its L2 variants were cloned into a pET-15b vector containing a 6X His-tag, generously provided by Dr. Christopher M. Wiethoff (Loyola University Chicago), using the NdeI and BamHI restriction sites so that upon translation the peptide would have an N-terminal 6X His-tag.

Cell culture, Virus/Vector production, Stable cell lines

HeLa and 293T cells were cultured in complete DMEM containing 10% fetal bovine serum, penicillin (final concentration 100 U/ml), and streptomycin (final concentration 100 μ g/ml). VSV-g pseudotyped HIV-1 reporter virus was produced by transfecting 293T cells in a 15 cm plate using Polyethylenimine (PEI) (Polysciences) transfection [191] along with 10 μ g of pVSVG and 15 μ g of the proviral construct R7 Δ Env-GFP in which the Nef gene has been replaced with GFP. Virus and vector were

harvested 48 hrs post transfection by filtering the culture media from the transfected cells through a 0.45 μm filter (Millipore). Virus infectivity was assessed by infecting equivalent numbers of cells in a 24 well plate for 14 hrs, after which virus was removed, normal medium was added and GFP expression was determined 48-72 hrs post infection using FACS Canto II flow cytometer (Becton Dickinson). Vector expressing YFP- or HA-tagged wt or L2 variant TRIM5 proteins was made in a similar way by transfecting 293T cells in a 60 mm dish using PEI along with 1 μg of the plasmid of interest, 1 μg of VSV-g and 1 μg of pCig-B. Vector was harvested 48 hrs post transfection, filtered through the 0.45 μm filter and either frozen at -80°C or used to transduce HeLa cells. To make stable cell lines HeLa cells were plated at 50% confluency and transduced with the respective vectors for 14 hrs, after which the vector was replaced with regular DMEM and 48 hrs post transduction media containing G418 drug at a concentration of 400 $\mu\text{g}/\text{mL}$ of DMEM was added to the cells. The expression of polyclonal or single colony clones were screened by immunofluorescence to ensure all cells expressed the transduced protein. Such cell lines were then analyzed by western blot analysis, and the clones expressing comparable amounts of protein were chosen for subsequent analysis.

Infectivity assay

Equivalent numbers (0.75×10^5) of HeLa cells stably expressing epitope-tagged wt or L2 variants of TRIM5 proteins plated in a 24-well plate were infected with VSV-g pseudotyped GFP reporter HIV-1 (R7 Δ EnvGFP) for 14 h, after which the virus was

removed and regular DMEM was added to the cells. Percentage of GFP positive cells was determined 48 hpi using a FACS Canto II flow cytometer (Becton Dickinson). For N- and B-MLV infections, CrFK cells were used. For TRIM-Cyp the infection was performed in the presence or absence of the drug cyclosporine A.

Immunofluorescence

HeLa cells stably expressing epitope-tagged TRIM5 proteins were plated on fibronectin-treated coverslips, allowed to adhere and fixed for 5 min. with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES, pH 6.8 [piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)] (Sigma). Cells were stained with DAPI and TexasRed Phalloidin in 1X Phosphate Buffered Saline (0.0067M PO₄; Hyclone) containing 0.1% Triton X-100 (Sigma) and 0.01% NaN₃. Cells expressing HA-tagged proteins were stained with monoclonal antibody against HA followed by staining with FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) in blocking solution containing 10% NDS, 0.01% NaN₃, 0.1% Triton X-100. Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; Photometrics), using a 1.4-numerical aperture 100× objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision).

Image Analysis

Z-stack images of each cell line were acquired using identical acquisition parameters.

The coverslips were coded such that the individual acquiring the images did not know the identity of the cell lines. Deconvolved images were analyzed for YFP-rhTRIM5 α cytoplasmic bodies and cortical actin, stained with TexasRed Phalloidin, using the Surface Finder function of the Imaris software package (Bitplane). Surfaces for cytoplasmic bodies in all samples analyzed were identified using defined fluorescence intensity and size criteria (Volume=above 0.03 or 0.066 μm^3 depending on the experiment).

45 individual images of cells expressing YFP-rhTRIM5 α (WT and L2 mutants) were obtained and deconvolved. To calculate the relative expression of YFP-labeled rhTRIM5 α proteins in each cell a 3D surface was created overlapping a single cell using the surface finder function of Imaris. The sum intensity in the FITC channel, corresponding to the total YFP fluorescence in that cell, and the surface volume, corresponding to the cell volume, were determined. The relative YFP-rhTRIM5 α protein expression in each cell was then calculated by dividing the sum intensity in the FITC channel with the total surface volume. Additionally, untransduced HeLa cells were fixed and stained in a similar manner, Z-stack images were acquired and the relative protein expression was calculated as described above and averaged (n=15). This number was then subtracted from the relative protein expression value calculated for each cell to eliminate the background fluorescence. The number of cytoplasmic bodies in each cell

was determined and normalized by the relative protein expression in that cell. The data were plotted in Prism (Graphpad Software Inc) for statistical analysis. Dunnett's Multiple Comparison test was used to determine the statistical significance of the differences between cell lines.

Western blotting

Whole cell lysates were prepared by lysing cells with NP-40 lysis buffer (100 mM Tris pH 8.0, 1% NP-40, 150 mM NaCl) containing protease inhibitor cocktail (Roche) for 15 min on ice. Coomassie Plus Bradford Assay (Thermo scientific) or Absorbance at 280nm was used to determine total protein concentration. 2× SDS sample buffer was added to the cell lysates and the samples were boiled for 5-10 min at 100 °C. Equal amount of protein was loaded onto a 10% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were separated at 200V for 30-40 min. After separation, the proteins were transferred to nitrocellulose membrane at 100V for 1 hr and detected by incubation with anti-GFP (Covance) or anti-HA (clone 3F10) conjugated to Horseradish Peroxidase (HRP) (Roche). Secondary antibodies conjugated to HRP (Thermo Scientific) were used where necessary and antibody complexes were detected using SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Scientific). For 6X His-tagged purified proteins an HRP-conjugated mouse anti-his antibody generously provided by Dr. Thomas Gallagher (Loyola University Chicago)

was used. Chemiluminescence was detected using the UVP EC3™ Imaging System (UVP LLC) or the Biorad ChemiDoc™ Imaging System.

Coomassie Staining

Protein samples from bacterial cell lysates or purified proteins were prepared by adding 2X (25mL 4X Tris. Cl/SDS, pH 6.8, 20% Glycerol, 4% SDS, 0.2% v/v β -mercaptoethanol and 0.001% w/v bromophenol blue in Milli Q) or 6X (7mL 4X Tris.Cl/SDS, pH6.8, 30% glyverol, 10% SDS, 5% β -mercaptoethanol and 0.012% bromophenol blue in Milli Q water) SDS Sample buffer and boiled for 5-10 min. at 100°C. The protein samples were separated on 10%, 13%, or 16.5% polyacrylamide gels at 100V for ~50min. The gels were then fixed in a collidal coomassie fixative (45% methanol and 1% acetic acid in Milli Q water) for at least 1 hr and stained for at least 2 hrs with Coomassie stain (170g ammonium sulfate, 1g Coomassie G250, 0.5% acetic acid and 34% methanol in 1 lt. of Milli Q water). Stained gels were washed with deionized water to remove excess stain and the gels were imaged using the Biorad ChemiDoc™ MP Imaging System.

Glutaraldehyde Crosslinking assay

Glutaraldehyde crosslinking assays were performed as previously described [85]. Briefly, cell lysates were incubated on ice for 30 min and centrifuged at 3000 rpm for 1 min to remove cell debris. The clarified lysates were divided into 20 μ L aliquots and

incubated with 0, 1, 2 and 4 mM glutaraldehyde for 5 min at room temperature.

Similarly, purified peptides were incubated with 0, 0.5, 1, 2 mM glutaraldehyde. The glutaraldehyde was saturated by adding 1 M glycine. 2× or 6X SDS sample buffer was added and the samples were boiled for 5-10 min. at 100 °C. The samples were then subjected to SDS-PAGE using 4%–15% Tris–HCl gradient gels (Ready Gels, BioRad) and subsequent Western Blot analysis or Coomassie staining.

Protein turnover assay

Cell lines stably expressing epitope-tagged wt rhTRIM5 α or the L2 variants were treated with cyclohexamide (20 μ g/mL) and cells were harvested every hour for up to 4 hrs following cyclohexamide addition. Coomassie Plus Bradford Assay (Thermo scientific) was used to determine total protein concentration. Equivalent amounts of protein from individual samples were subjected to SDS-PAGE and the YFP-TRIM5 α protein was detected by western blot.

Protein expression and Induction

BL21(DE3) cells (having the T7 promoter expression system) were transformed with pET-15b constructs containing the CC-L2 or L2 only fragments of wt rhTRIM5 α or the L2 variants, spread onto LB-Amp plates and allowed to grow overnight at 37°C. A single colony from each plate was inoculated in LB broth (1/20th volume of the larger culture) containing 100 μ g/mL Carbenicillin (Invitrogen) and the cultures were allowed to

grow overnight at 37°C. These starter cultures were transferred to 500mL LB containing 100µg/mL Carbenicillin and were grown in a 37°C shaker till the cultures reached an OD₆₀₀ of 0.6. A small aliquot was collected as the uninduced sample. 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Invitrogen) was added and the cultures were induced for an additional 3-5 hours at 37°C. Following induction a small aliquot of each culture was collected and centrifuged at 4000 rpm (centrifuge/rotor) for 20 min. Similarly, the larger induced cultures were centrifuged at 5500 rpm (centrifuge/rotor) for 20 min. The bacterial pellets were either frozen at -20°C or were used for protein purification. To test the level of protein induction and solubility of the induced proteins, the smaller aliquots of cells collected before and after induction were resuspended in 1M Tris, pH 8.0 containing the protease inhibitor cocktail (PIC) (Roche) and a small aliquot was collected as the “whole cell lysate” (wcl) fraction. Cells were then sonicated and the lysates were centrifuged at 13,000 rpm at 4°C for 30 min. Following centrifugation the supernatant (sup) and pellet fractions were separated and prepped for SDS-PAGE analysis by resuspending the pellets in 8M Urea solution and adding 2X and 6X SDS sample buffers to the pellet and sup fractions respectively. The samples were boiled at 100°C for 10 min. and analyzed by SDS-PAGE by western blot analysis and Coomassie staining as described above.

Protein Purification

Depending on the presence of the induced proteins in the sup or pellet fractions the following protocols were followed for the purification process. All steps were carried out on ice or under cold conditions.

Purification of soluble proteins

Frozen bacterial pellets were resuspended on ice in 10-12 mL of lysis buffer (50mM Na₂HPO₄, 500mM NaCl, 10mM Imidazole, 1% Triton X-100, 0.5 mg/mL Lysozyme (Invitrogen)) containing PIC. The lysates were centrifuged at 13,000 rpm at 4°C for 30 min. The sup was incubated with 600 µL of Talon metal affinity resin slurry for 1-2 hrs at 4°C with gentle mixing to allow binding of the His-tagged protein to the cobalt resin. The mixture was then passed through a 2 mL TALON disposable gravity column twice. The flow through was discarded and the column was washed with 10X volume of the resin bed volume of wash buffer (40mM Tris, 300mM NaCl, pH 8.0). The His-tagged proteins were eluted from the column using the elution buffer (50mM Na₂HPO₄, 500mM NaCl, 150mM Imidazole). Six 300 µL fractions were collected. Protein concentrations were determined by measuring Absorbance at 280nm. The purified proteins were flash frozen using dry ice or liquid nitrogen and stored at -80°C.

Purification of Insoluble proteins

Frozen bacterial pellets were resuspended on ice in 10-12 mL of lysis buffer containing PIC as mentioned above. The cells were then sonicated and the lysates were centrifuged at 13,000 rpm at 4°C for 30 min. The sup was discarded and the pellet was washed with lysis buffer containing PIC to remove cell debris and other impurities centrifuged at 13,000 rpm for 10 min at 4°C. This step was repeated 3 more times. The pellet was washed once with wash buffer 1 (50mM Na₂HPO₄, 500mM NaCl, 10mM Imidazole) containing PIC and centrifuged at 13,000 rpm for 10 min at 4°C. The pellets were then resuspended in a buffer containing 40mM Tris, 150mM NaCl, 8M Urea, pH 8.0. 500 µL of Talon metal affinity resin (Clontech) slurry was added to the resuspended pellet and the mixture was incubated at 4°C for 1-2 hrs with gentle mixing to facilitate binding of the His-tagged proteins to the cobalt resin. The mixture was passed through a 2 mL TALON disposable gravity column (Contech) twice. The flow through was discarded and the resin was washed with 10X volume of the resin bed volume of wash buffer 2 (40mM Tris, 300mM NaCl, 8M Urea, pH 8.0). The 6X His-tagged proteins were eluted from the column using the elution buffer (40mM Tris, 150mM NaCl, 8M Urea, 10mM Imidaole). Six 300 µL fractions were collected for each peptide. Protein concentrations were determined by measuring Absorbance at 280nm. The proteins were flash frozen using dry ice or liquid nitrogen and stored at -80°C.

Dialysis and Concentration of Purified Proteins

The proteins purified using the protocol for insoluble proteins, were further processed in the following manner. 2-3 protein fractions were pooled, depending on the purity of each fraction, and diluted to a concentration of 100 μ g/mL. The peptides were added to appropriate volume of chilled dialysis buffer while it is stirring to avoid protein aggregation. The diluted peptides were then injected into Slide-A-lyzer dialysis cassettes (10,000 MWCO or 3,500 MWCO) (Thermo Scientific) and dialyzed in 2 liters of dialysis buffer overnight at 4°C on a stir plate with gentle stirring. The dialyzed peptides were then concentrated using Centrifugal filter units (10,000 MWCO or 3,500 MWCO) (Millipore) following the company instructions. The concentrated peptides were flash-frozen using dry ice or liquid nitrogen and stored at -80°C or used for further analysis.

CD Spectroscopy

UV spectra (190 – 260nm) of the purified CC-L2, or synthetic L2 peptides were measured by CD spectroscopy using a JASCO J-810 Circular Dichroism Spectrometer. CD spectroscopy for the L2 peptides was performed in the presence and absence of 10% TFE. Spectra were collected in triplicate in 1mm path length cuvettes at 20°C and the resulting scans were averaged, and the buffer signal was subtracted. The specific ellipticities for each peptide spectra were calculated and computation of relative amount of helix was performed using ContinLL.

In vitro Capsid Binding Assay

293T cells were plated at 65% confluency and transfected with 3 μ g of HA-tagged wt rhTRIM5 α or the L2 variant described. 14 hours post-transfection, the cells were collected and incubated with 750 μ L of lysis buffer ((10 mM Tris-HCl pH 8.0, 10 mM KCl, 1 mM EDTA)) on ice for 15 minutes. Cells were then sonicated and the lysates were centrifuged at 14,000xg for 30 minutes. 200 μ L of the clarified lysate was then incubated with 1M NaCl and either 20mM Tris, pH 8.0 or 2.5 μ L of *in vitro* assembled HIV-1 capsid tubular assemblies (generously provided by Dr. Owen Pornillos, University of Virginia) at room temperature for 1 hour with gentle mixing every 10 minutes. A 15 μ L aliquot was collected for wcl and the remaining reaction was loaded onto a 65% sucrose cushion and centrifuged at 25,300 rpm at 4 $^{\circ}$ C for 1 hour. 50 μ L of the sample remaining on top of the cushion was collected as sup and 2X SDS sample buffer was added. The sucrose in the tube was emptied and the pellet was resuspended in 1X SDS sample buffer. The samples were analyzed by SDS-PAGE and western blotting.

CHAPTER III

HYPOTHESIS AND SPECIFIC AIMS

TRIM5 proteins (TRIM5 α and TRIM-Cyp) are known to self-associate into dimmers, higher-order multimers and form large assemblies in the cell cytoplasm, termed as “cytoplasmic bodies” [48, 185]. The functional significance of TRIM5 α cytoplasmic bodies in HIV-1 restriction has been controversial as two studies have demonstrated that preexisting TRIM5 α cytoplasmic bodies are not required for HIV-1 restriction [107, 186]. Alternatively, it has previously been observed that upon infection of cells expressing rhTRIM5 α with fluorescently labeled HIV-1, the viral particles accumulate within rhTRIM5 α cytoplasmic bodies [106]. Moreover, using live-cell imaging, the *de novo* formation of rhTRIM5 α cytoplasmic bodies around individual virions has been observed [106]. Moreover, rhTRIM5 α dimerization and higher-order multimerization, mediated by the coiled-coil [83, 87, 89, 97, 104] and B-Box2 domains [85, 89, 90, 129] respectively, are essential for viral capsid binding and restriction [89, 104, 141, 142, 179]. These studies suggest that TRIM5 α self-association facilitates binding of rhTRIM5 α to the HIV-1 capsid possibly by increasing the avidity of this interaction. However, the mechanism by which TRIM5 α self-associates into cytoplasmic bodies and their functional significance in HIV-1 restriction is poorly understood. Hence, we sought to

identify the regions of rhTRIM5 α that are required for cytoplasmic body formation. Once these regions were identified we could then mutate them and ask if they are required for HIV-1 restriction.

In order to identify the regions of rhTRIM5 α required for its assembly into cytoplasmic bodies we generated C-terminal truncation mutants of GFP-labeled rhTRIM5 α (Figure 1A). These rhTRIM5 α mutants were then transiently transfected into HeLa cells and their sub-cellular localization was observed by fluorescent microscopy. We found that the mutants lacking the residues 263-278, that lie within the L2 region of rhTRIM5 α , did not form cytoplasmic bodies and exhibited a diffuse localization in cells (Figure 1A and 1B). However, mutants that contained these residues formed punctate assemblies in the cytoplasm (Figure 1A and 1B). This suggests that the stretch of 15 residues, 263-278, that lies within the Linker 2 (L2) region has determinants that are required for rhTRIM5 α cytoplasmic body formation. Hence we hypothesized that “***The ability of rhTRIM5 α to assemble into cytoplasmic bodies is governed by determinants in the L2 region and is required for its ability to restrict HIV-1 infection***”. This dissertation focuses on understanding the mechanism underlying the assembly of TRIM5 proteins and its role in TRIM5-mediated retroviral restriction. The following specific aims were designed to test the above mentioned hypothesis.

Specific Aims 1

Determine if the Linker 2 (L2)-mediated assembly of rhTRIM5 α governs HIV-1 restriction.

Identify specific residues within the L2 region that are required for rhTRIM5 α cytoplasmic body formation and HIV-1 restriction

Determine if the tendency to assemble into cytoplasmic bodies correlates with the HIV-1 restriction ability of rhTRIM5 α

Determine if mutations in the L2 region affect the ability of rhTRIM5 α to bind assembled HIV-1 capsid

Identify the amino acid residues or secondary structural motifs of L2 that mediate rhTRIM5 α assembly around the HIV-1 core.

Identify specific residues within the L2 region that are required for assembly and HIV-1 restriction

Identify the secondary structural motifs associated with the L2 region using CD Spectroscopy

Determine if secondary structure can be induced in the L2 region in a concentration dependent manner thus acting as a molecular switch in HIV-1 restriction

Determine if the role of L2 in mediating viral restriction is conserved across species

Determine if the L2 region of HuTRIM5 α governs assembly and N-MLV restriction and identify the residues are required for these activities

Determine if the L2 region of TRIM-Cyp governs assembly and HIV-1 restriction and identify the residues that are required for these activities

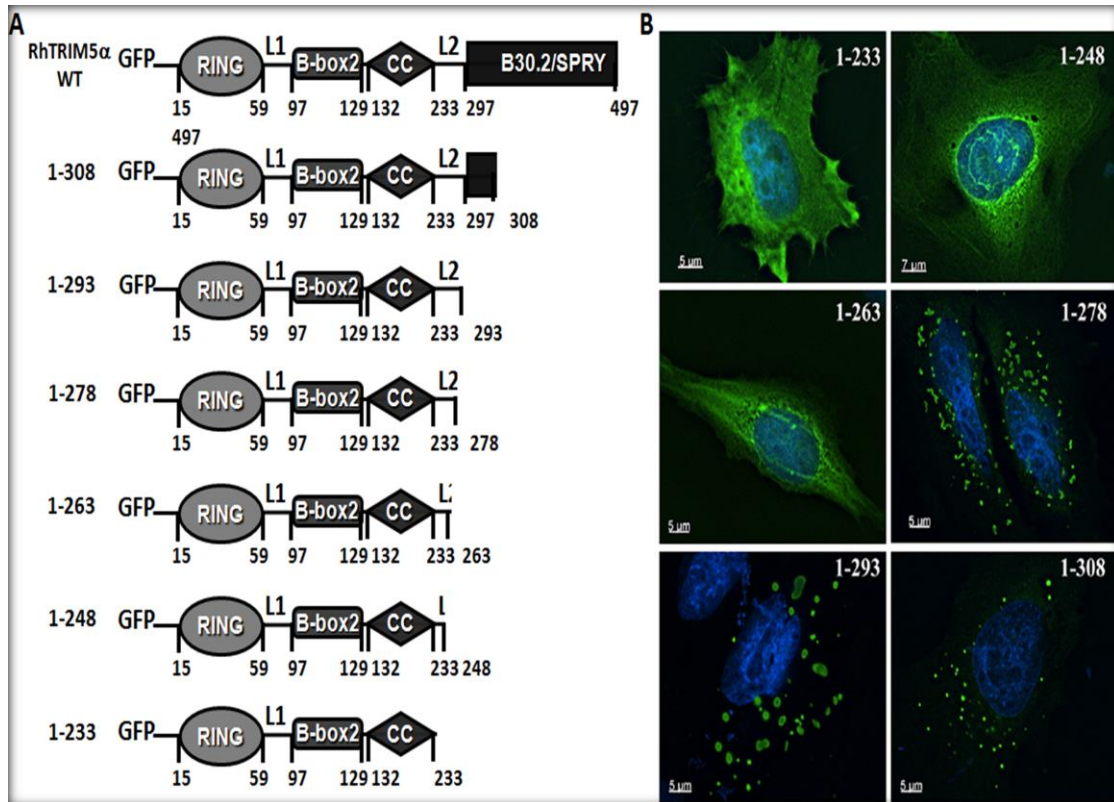


Figure 9: The L2 region has determinants that are required for rhTRIM5α cytoplasmic body formation. A) Domain structure of rhTRIM5α. Numbers outline the different domains of the protein. WT and the various C-terminal truncation mutants are shown. B) HeLa cells were transiently transfected with GFP-labeled wt rhTRIM5α or the truncation mutants. Cells were allowed to adhere to fibronectin treated coverslips, fixed and imaged using a fluorescent microscopy.

CHAPTER IV

RESULTS

The Linker 2 (L2) region has determinants that govern rhTRIM5 α assembly and HIV-1 restriction

This aim tests the hypothesis that the ability of rhTRIM5 α to form cytoplasmic bodies is required for HIV-1 restriction. Additionally, this aim focuses on identifying the residues within the L2 region that govern the ability of rhTRIM5 α to form cytoplasmic bodies.

Two discrete stretches of residues within the L2 region are required for rhTRIM5 α cytoplasmic body formation and HIV-1 restriction

Our preliminary data suggested that the 15 amino acid stretch (AA 263-278) that lies within the L2 region is required for rhTRIM5 α cytoplasmic body formation. In order to identify the specific residues that are required for rhTRIM5 α cytoplasmic body formation we performed triple alanine mutagenesis to generate L2 variants in which stretches of three residues within the stretch 263-278 were replaced with alanine (Figure 10A). We reasoned that if we could identify the L2 variants of rhTRIM5 α that failed to localize to cytoplasmic bodies, we could test their ability to restrict HIV-1 infection. The

wild type (wt) and the triple alanine L2 variants were cloned into a retroviral vector expressing a yellow fluorescent protein (YFP) tag in order to generate N-terminal YFP-fusion forms of these proteins. These proteins were then stably expressed in HeLa cells and the cell lines expressing comparable amounts of protein were chosen for subsequent experiments. We first determined the effect of these triple alanine mutations on the ability of rhTRIM5 α cytoplasmic body formation, in the context of the full-length protein. The sub-cellular localization of YFP-labeled wt rhTRIM5 α and the L2 variants was determined by fluorescent microscopy (Figure 10B). We observed that YFP-labeled wt rhTRIM5 α formed cytoplasmic bodies as previously described [185] (Figure 10B). Triple alanine mutagenesis of two stretches of residues within the L2 region, induced a diffuse localization in cells expressing these L2 mutants and had very few cytoplasmic bodies, if any (KPK266AAA (KPK) and RRV275-277AAA (RRV). Conversely, alanine mutagenesis of the residues between the two stretches above (TFH269-271AAA (TFH) and HKN271-273AAA (HKN)) generated mutants which retained the ability to form cytoplasmic bodies (Figure 10B).

We next tested the ability of these L2 mutants to restrict HIV-1 infection. Equivalent numbers of HeLa cells stably expressing YFP-labeled wt or L2 variant rhTRIM5 α proteins were infected with serial dilutions of HIV-1 expressing GFP following infection (HIV-1 GFP reporter virus) [48, 192]. The percentage of GFP

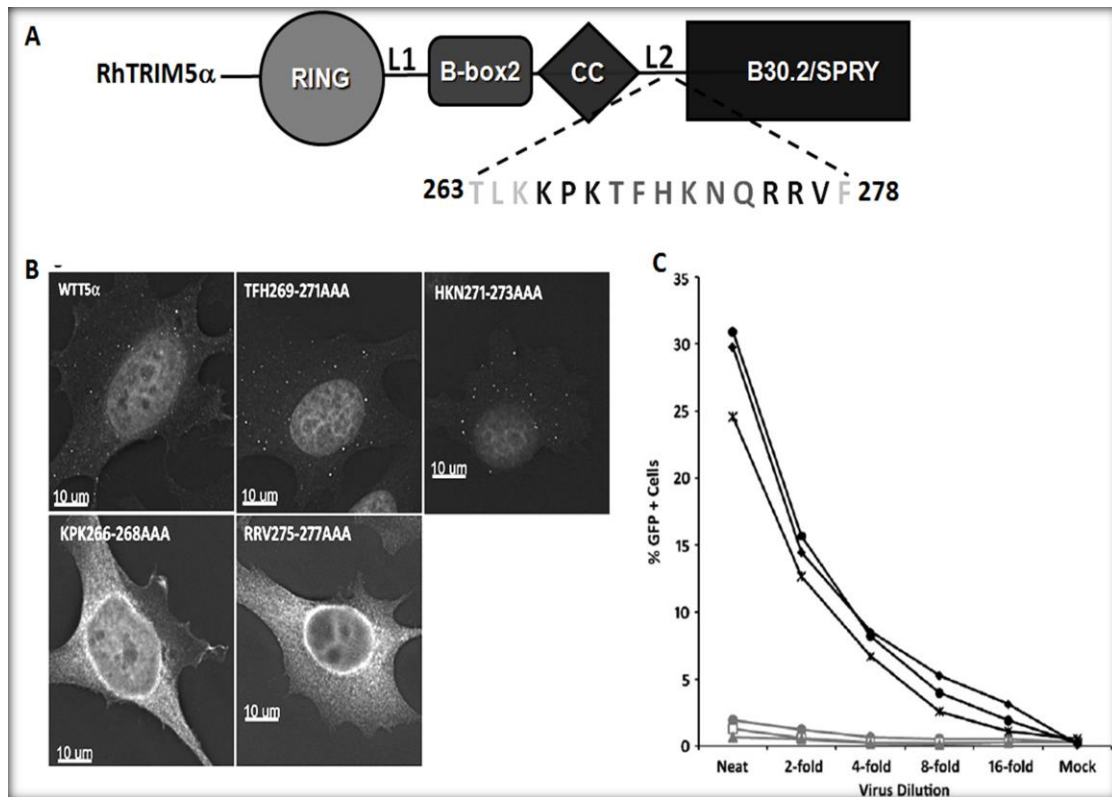


Figure 10A-C: L2-mediated assembly of rhTRIM5 α is required for HIV-1 restriction. A) Residues 263-278 within the L2 region of rhTRIM5 α are shown. Residues highlighted in black and dark grey were replaced by alanine. B) HeLa cells stably transduced with YFP-labeled wt rhTRIM5 α or the indicated L2 variants were allowed to adhere to fibronectin treated coverslips, fixed and 20 Z-stack images were acquired using a DeltaVision fluorescent microscope. Representative deconvolved images are shown. C) Equivalent numbers of the stable cells were plates in a 24-well plate and infected with serial dilutions of VSV-g pseudotyped HIV-1 expressing GFP for 14hrs. Cells were harvested 48 hpi and the percentage of GFP positive cells was determined by Flow Cytometry. Results are representative of 3 independent experiments.

positive cells was determined by FACS analysis 48 hpi. Our results show that cells expressing the KPK and RRV mutants that did not form cytoplasmic bodies, were unable to restrict HIV-1 infection and were as infected as the untransduced control HeLa cells (Figure 10C). The TFH and HKN mutants on the other hand that retained the ability to form cytoplasmic bodies, were able to restrict HIV-1 infection at least up to the levels of wt rhTRIM5 α (Figure 10C). This suggests that although preexisting cytoplasmic bodies are not required for HIV-1 restriction as previously suggested, the ability of rhTRIM5 α to assemble into cytoplasmic bodies in the presence of restriction sensitive virus is essential for its anti-retroviral activity. Additionally, two discrete stretches of residues within the L2 region are required for rhTRIM5 α cytoplasmic body formation and HIV-1 restriction. Similar results were observed with cells stable expressing HA-tagged wt and L2 variant rhTRIM5 α proteins (Figure 10D and E).

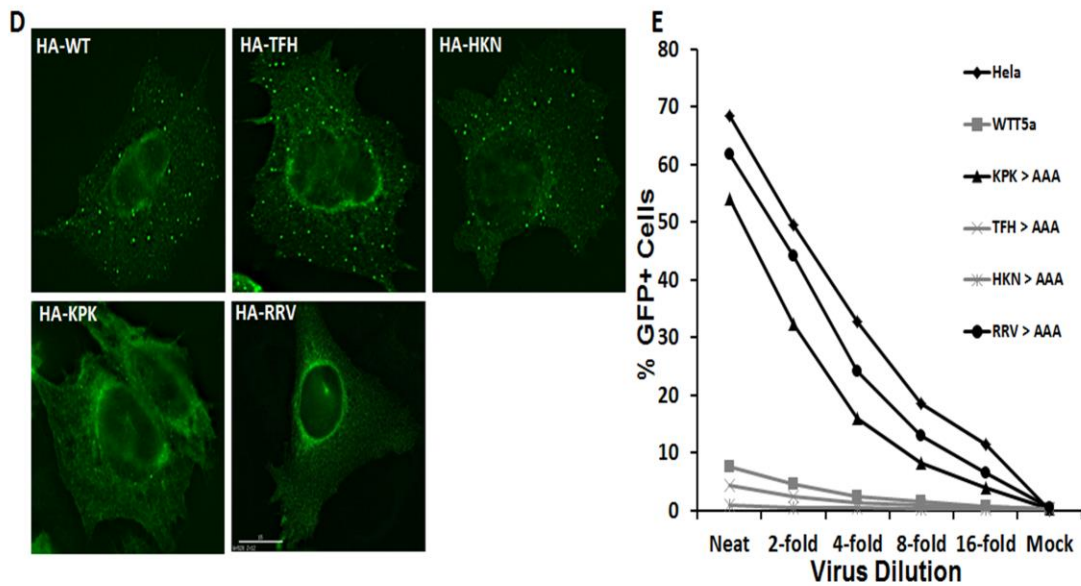


Figure 10D-E: L2-mediated assembly of rhTRIM5 α is required for HIV-1 restriction. D) HeLa cells stably transduced with HA-labeled wt rhTRIM5 α or the indicated L2 variants were allowed to adhere to fibronectin treated coverslips, fixed and 20 Z-stack images were acquired using a DeltaVision fluorescent microscope. Representative deconvolved images are shown. E) Equivalent numbers of the stable cells were plates in a 24-well plate and infected with serial dilutions of VSV-g pseudotyped HIV-1 expressing GFP for 14hrs. Cells were harvested 48 hpi and the percentage of GFP positive cells was determined by Flow Cytometry. Results are representative of 3 independent experiments.

Proteasome inhibition does not affect the ability of rhTRIM5 α L2 variants to restrict HIV-1 infection

Wu et al. demonstrated that treatment of rhTRIM5 α expressing cells with the proteasome inhibitor MG132 results in accumulation of rhTRIM5 α into cytoplasmic bodies that are larger than those observed in untreated cells [109]. This study also showed that inhibition of proteasome function using MG132 relieves rhTRIM5 α -mediated block to reverse transcription without affecting viral restriction [109]. We therefore tested the effect of MG132 treatment on the cytoplasmic body formation and HIV-1 restriction abilities of the L2 variants. HeLa cells stably expressing YFP-labeled wt and L2 variant rhTRIM5 α proteins were treated with MG132 (1 μ g/mL) for 5 hrs and their sub-cellular localization was observed by fluorescent microscopy. Cells expressing wt rhTRIM5 α upon treatment with MG132 had larger cytoplasmic bodies as compared to untreated cells (Figure 11A) as previously described [109]. Interestingly, all the L2 mutants tested, including the diffuse mutants KPK and RRV, localized to large cytoplasmic bodies following MG132 treatment (Figure 11A). Since the KPK and RRV diffuse mutants formed cytoplasmic bodies in the presence of MG132 we asked if they could restrict HIV-1 infection under these conditions. Equivalent number of cells were pre-treated with MG132 for 5 hours and then infected with serial dilutions of the HIV-1 GFP reporter virus for 5 hours in the presence of MG132. The percentage of GFP positive cells was determined by FACS analysis 48 hpi. WT rhTRIM5 α restricted HIV-1 infection both in

the presence and absence of MG132 (Figure 11B), as previously described [109]. The RRV mutant was unable to restrict viral infection upon MG132 treatment in spite of localizing to cytoplasmic bodies (Figure 11B). The KPK mutant exhibited partial restriction of the virus when treated with MG132 (Figure 11B). MG132 treatment did not affect the ability of the TFH and HKN L2 variants to restrict HIV-1 (Figure 11B). This suggests that inhibition of the proteasome does not influence the ability of the rhTRIM5 α L2 variants to restrict HIV-1 infection. The large cytoplasmic bodies observed upon MG132 treatment most likely do not participate in the restriction process. This is consistent with the hypothesis that it is not the preexisting cytoplasmic bodies but the ability of rhTRIM5 α to form cytoplasmic bodies is essential for HIV-1 restriction.

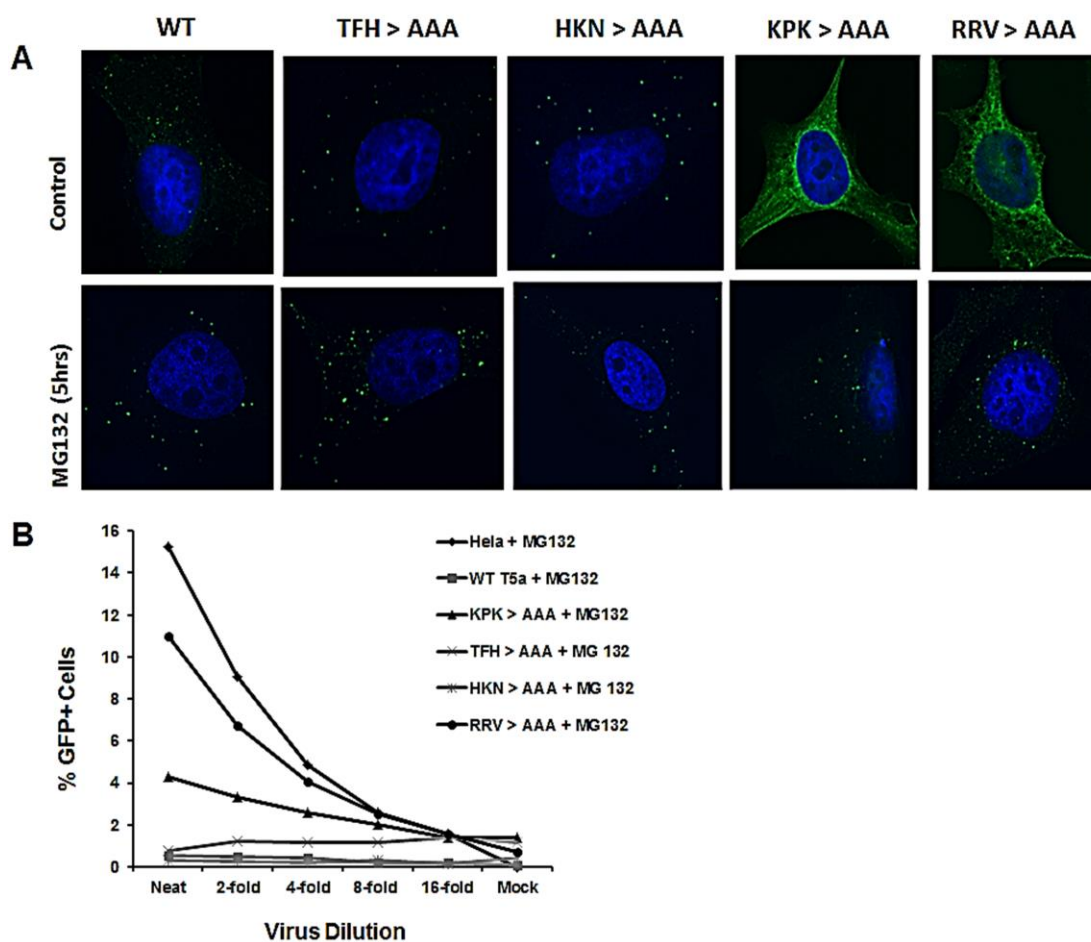


Figure 11: Proteasome inhibition does not affect the ability of rhTRIM5 α L2 variants to restrict HIV-1 infection. A) HeLa cells stably expressing YFP-labeled wt rhTRIM5 α or the L2 variants were allowed to adhere to fibronectin treated coverslips and treated with MG132 (1 μ g/mL) for 5 hrs. The cells were then fixed and imaged as described above. B) Equivalent numbers of cells were plated in a 24-well plate and pre-treated for 5 hrs with MG132 (1 μ g/mL). The cells were then infected for an additional 5 hrs in the presence of MG132 after which the virus was replaced with DMEM. 48 hpi cells were harvested and the percentage of GFP+ cells was determined by flow cytometry. Data is representative of 2 independent experiments.

L2 mutations that disrupt cytoplasmic body formation do not interfere with rhTRIM5 α dimerization and higher-order multimerization

We next assessed if the mutations that we introduced in the L2 region were grossly affecting the conformation or folding of rhTRIM5 α . Previous studies have observed lower and higher-order multimers of rhTRIM5 α on denaturing polyacrylamide gels by biochemically crosslinking cellular proteins with Ethylene glycol bis(succinimidylsuccinate) EGS or glutaraldehyde [85, 87, 141, 142]. In order to test the ability of the L2 variants to multimerize we performed biochemical crosslinking of the L2 variants with increasing concentrations of glutaraldehyde. Lysates of HeLa cells stably expressing YFP-labeled wt rhTRIM5 α or the L2 variants were treated with increasing concentrations of glutaraldehyde and the crosslinked proteins were separated on denaturing polyacrylamide gels and analyzed by western blotting. All the L2 mutants including the diffuse KPK and RRV mutants formed multimers at higher concentrations of glutaraldehyde (appear as a smear on a 4-15% gradient SDS-PAGE gel) (Figure 12). We observed a band around 150kDa (dimer) and a second, more prominent band around 250kDa (higher-order multimer) (Figure 12). This result suggests that the triple alanine mutations introduced in the L2 region do not affect the ability of rhTRIM5 α to form dimers and higher-order multimers. However, this gel does not allow for a stronger conclusion because it is known that crosslinked TRIM5 migrates with an electrophoretic mobility inconsistent with the molecular weight of the cross linked

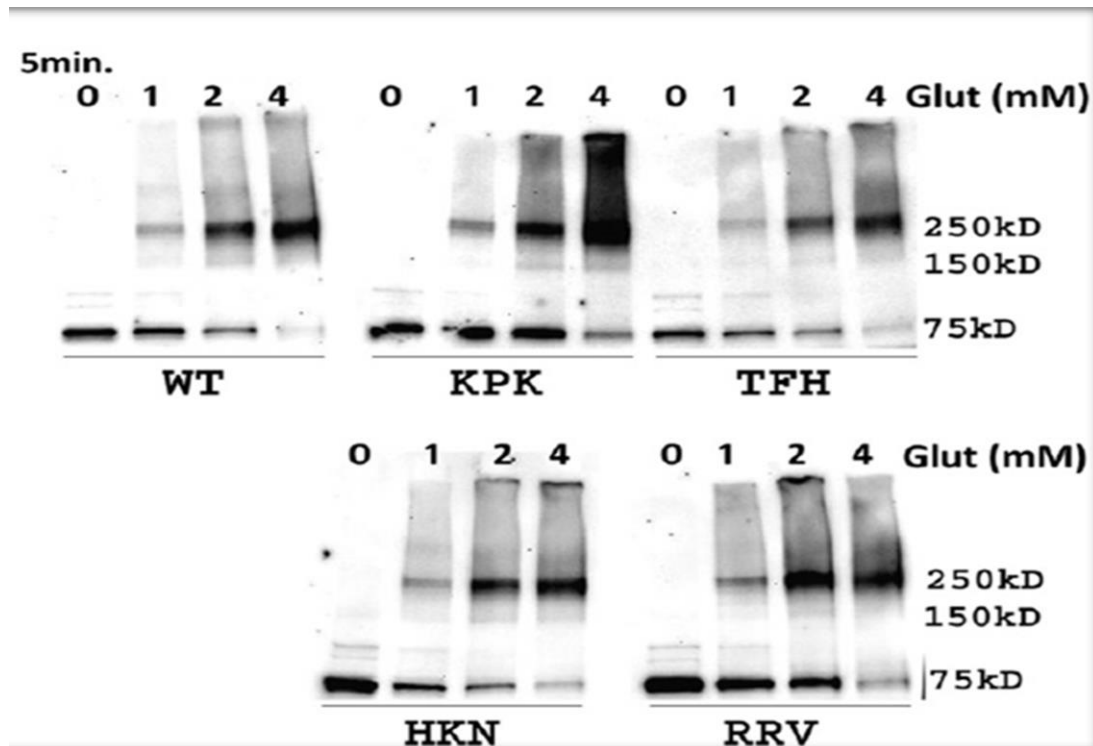


Figure 12: Mutation of residues in the L2 region does not disrupt rhTRIM5 α dimerization and higher-order multimerization. Equivalent numbers of HeLa cells stably expressing YFP-labeled wt rhTRIM5 α or the L2 variants were lysed and the lysates were centrifuged at 3000 rpm for 1 min. at 4°C. Clarified lysates were treated with 0, 1, 2 or 4 mM glutaraldehyde for 5 min. at room temperature. The glutaraldehyde was then saturated by adding 1M Glycine. The samples were then analyzed by western blotting. The sizes of the monomer (75kDa) and the different oligomeric species observed are indicated. Results are representative of 3 independent experiments.

species [86, 87]. Similar results were observed using HA-tagged wt and L2 variant rhTRIM5 α proteins by our collaborator Dr. Diaz-Griffero, suggesting that the multimeric species observed on crosslinking is not specific for one type of epitope tag.

L2 mutations that do not disrupt cytoplasmic body formation seem to enhance rhTRIM5 α assembly

In order to quantitatively compare the ability of the L2 variants to form cytoplasmic bodies to that of wt rhTRIM5 α we acquired 20 Z-stack images of each cell line using fluorescence microscopy. Cells stably expressing YFP-labeled rhTRIM5 α proteins were used for this analysis as the YFP fluorescent tag made imaging and cytoplasmic body quantification easier. We next used our image analysis software Imaris, to identify cytoplasmic bodies in each cell using fixed fluorescence and volume definitions. This allowed us to quantify the number of cytoplasmic bodies present in each cell for each cell line in an unbiased and automated manner. In agreement with the immunofluorescence data our automated image analysis showed that cells expressing the RRV mutant had no cytoplasmic bodies (Figure 13A). The KPK mutant had very few cytoplasmic bodies per cell as compared to the wt protein however; this mutant showed a statistically significant increase in body formation as compared to the RRV mutant ($p < 0.0001$). This suggests that while the KPK mutant appears to have a reduced ability to assemble into cytoplasmic bodies it retains a higher ability to form bodies than the RRV mutant. Cells expressing the TFH and HKN mutants seemed to have more cytoplasmic

bodies per cell as compared to those expressing wt rhTRIM5 α (Figure 13A). One explanation for the increase in the number of cytoplasmic bodies observed in cells expressing the TFH and HKN mutants could be that these mutants were expressed at a higher level as compared to wt rhTRIM5 α . The second possibility could be that the TFH and HKN mutants were more stable and were being turned over at a slower rate than wt rhTRIM5 α . The KPK and RRV diffuse mutants on the other hand were possibly less stable and being turned over faster than the wt protein.

Cytoplasmic body formation by the L2 mutants does not correlate with their turnover rate in cells

We first tested if the differences in the localization of the L2 mutants correlated with their relative stability in cells by analyzing the ability of these mutants to turnover in cells following cyclohexamide treatment. Cyclohexamide inhibits protein synthesis by blocking translation elongation hence, if cytoplasmic body localization of the L2 mutants correlates with their altered stability in cells, then we would expect the KPK and RRV diffuse mutants to be turned over faster than and the TFH and HKN mutants would be turned over at the same rate as wt rhTRIM5 α . However, we observed that rate of turnover of the RRV (diffuse) and TFH (forms cytoplasmic bodies) mutants was reduced as compared to wt rhTRIM5 α while that of the KPK (diffuse) and HKN (forms bodies)

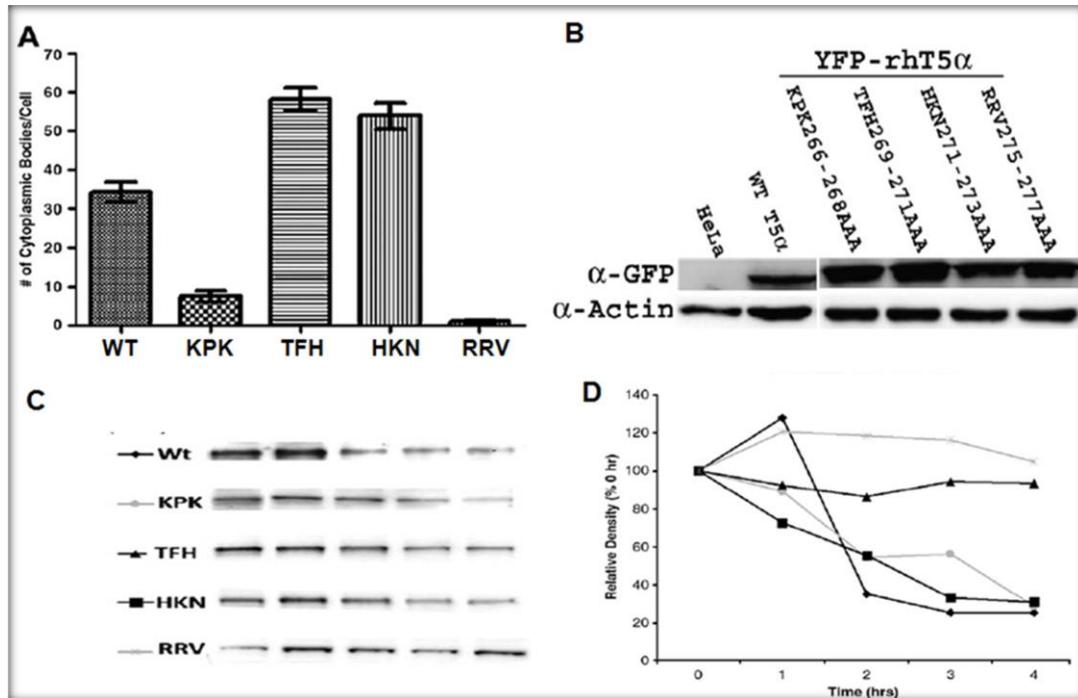


Figure 13: The increased ability of the L2 variants to assemble into cytoplasmic bodies does not correlate with their turnover. A) Quantification of the number of cytoplasmic bodies formed by wt rhTRIM5 α and the L2 variants. 20 Z-stack images of HeLa cells stably expressing YFP-labeled wt rhTRIM5 α or the L2 variants were acquired using identical parameters, deconvolved and analyzed using Imaris image analysis software. Fixed fluorescent intensity and size criteria were applied to all images to determine the total number of cytoplasmic bodies per cell. The average number of cytoplasmic bodies per cell in all 20 images is plotted for each cell line. Error bars represent the SEM. B) Representative western blot showing steady-state protein levels of the YFP-labeled wt and L2 variant TRIM5 α proteins. HeLa cells stably expressing the indicated proteins were lysed and the cell lysates were analyzed by SDS-PAGE. WT and mutant TRIM5 α proteins were detected by western blotting with a monoclonal mouse anti-GFP antibody. Actin was used as a loading control. C) HeLa cells stably expressing YFP-labeled wt and L2 variants rhTRIM5 α proteins were treated with cyclohexamide for the indicated time period after which the cells were lysed, and YFP-expression was analyzed by western blotting. Samples were normalized to include an identical amount of total protein. D) Densitometric analysis of the samples shown on the top panel. Results are representative of 3 independent experiments.

mutants was similar to wt rhTRIM5 α (Figure 13C and D). Thus, while one diffuse mutant and one mutant that formed bodies exhibited slower turnover kinetics, the other mutants seemed to turnover at the same rate as the wt protein. This indicates that the cytoplasmic body localization of the rhTRIM5 α L2 mutants does not depend on their turnover rate in cells.

The tendency of rhTRIM5 α to assemble into cytoplasmic bodies directly correlates with its HIV-1 restriction ability

We next determined the steady-state expression levels of the wt and L2 variant rhTRIM5 α proteins by western blotting and found that all the L2 variants were expressed at slightly higher levels as compared to wt rhTRIM5 α (Figure 13B). Additionally, during our image analysis we noticed heterogeneity in cytoplasmic body formation within each cell line. Typically, cells expressing more protein would have more cytoplasmic bodies as compared to those with lower protein expression. This suggested that formation of cytoplasmic bodies by rhTRIM5 α was dependent on two main factors namely, 1) the ability of the protein to form cytoplasmic bodies and 2) intracellular protein expression.

As shown in figure 10, the ability of rhTRIM5 α to form cytoplasmic bodies is governed by determinants in the L2 region (Figure 10B) and is critical for HIV-1 restriction (Figure 10C). However, the variability in expression levels observed between cells was background noise in our assay. In order to eliminate this background noise and

to confirm that the increased ability of the TFH and HKN mutants to assemble was not due to their higher expression in cells, we calculated the relative intracellular protein expression of wt and mutant rhTRIM5 α proteins in each cell. Western blot analysis measures the total protein expression of an entire population of cells. Hence, using fluorescent microscopy and our image analysis software we developed a new method to measure protein expression in each cell. Briefly, cells expressing YFP-labeled rhTRIM5 α , wt and L2 variants, were allowed to adhere to fibronectin treated coverslips, fixed and stained for cortical actin using TexasRed Phalloidin. Z-stack images were acquired for each cell line as described earlier and deconvolved images were analyzed using Imaris. We constructed 3D surfaces around individual cells by utilizing the actin staining and determined the total YFP fluorescence within each cell (Figure 14A). The total YFP fluorescence corresponds to the total YFP-rhTRIM5 α protein in that cell. We also measured the volume of that surface which corresponds to the volume of that cell. The relative intracellular protein expression was calculated by dividing the total YFP-fluorescence value by the cell volume (see Equation). The total number of cytoplasmic bodies per cell was also quantified as described in figure 13A. The total number of cytoplasmic bodies per cell was normalized to the relative protein expression for each cell line. Our results show that at similar relative expression levels the TFH and HKN mutants exhibit a significantly higher tendency to assemble into cytoplasmic bodies as compared to wt rhTRIM5 α ($p < 0.001$) (Figure 14B).

$$\text{Cytoplasmic body formation} = \frac{\text{Ability of the protein to self-associate}}{\text{Total intracellular protein concentration}}$$

$$\text{Intracellular protein concentration} = \frac{\text{Total cellular fluorescence}}{\text{Cell Volume}}$$

Since the ability of rhTRIM5 α to form cytoplasmic bodies is required for HIV-1 restriction (Figure 10C) we hypothesized that the TFH and HKN mutants, that have a higher tendency to assemble into cytoplasmic bodies, would exhibit increased HIV-1 restriction ability as compared to rhTRIM5 α . To test this hypothesis we generated seven different cell lines stably expressing either YFP-labeled wt rhTRIM5 α or the TFH or HKN L2 variants and infected them with increasing titers of the HIV-1 GFP reporter virus. An average of all seven cell lines is shown in Figure 14C. We observed that cells expressing the TFH and HKN mutants were significantly less infected as compared to those expressing wt rhTRIM5 α (Figure 14C). This suggests that the tendency of rhTRIM5 α to assemble into cytoplasmic bodies directly correlates with its ability to restrict HIV-1 infection, at least in the context of the TFH and HKN L2 variants.

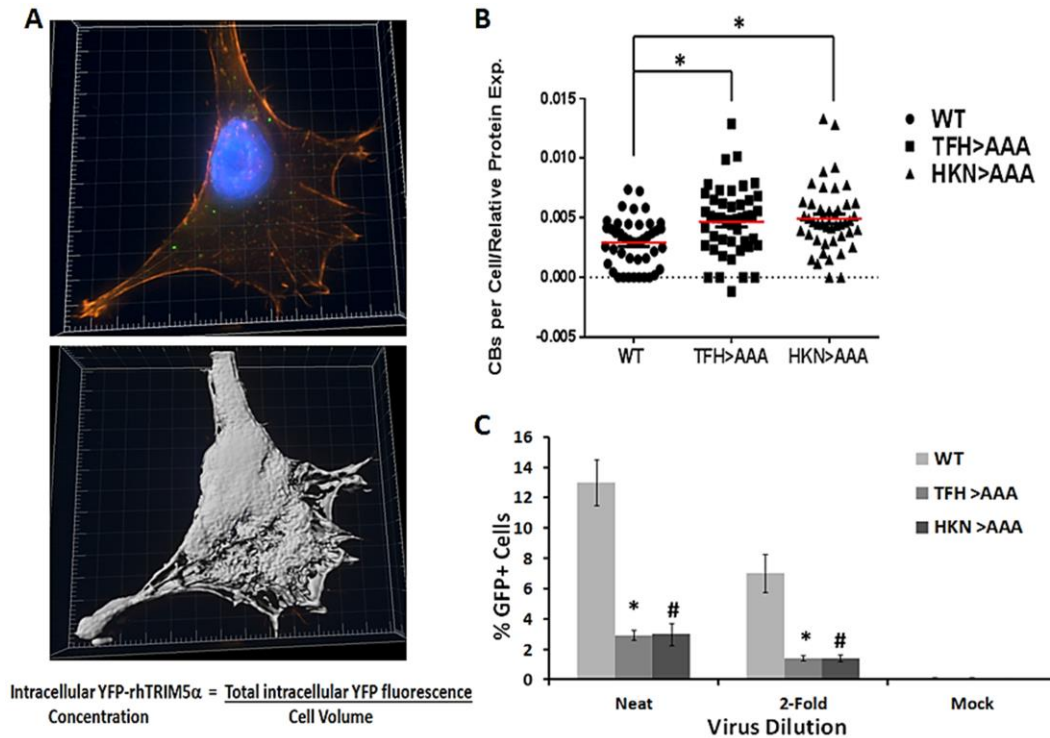


Figure 14: The tendency of rhTRIM5 α to assemble directly correlates with its ability to restrict HIV-1. A) Quantification of the number of rhTRIM5 α cytoplasmic bodies per cell normalized to its relative expression. HeLa cells stably expressing YFP-labeled rhTRIM5 α (WT, TFH or HKN) were allowed to adhere to fibronectin treated coverslips, fixed and stained for cortical actin using TexasRed Phalloidin to visualize the cell periphery. Z-stack images were acquired for each cell line (n=45/cellline) as described before and deconvolved images were analyzed using Imaris. Using fixed intensity of TRITC and size criteria a 3D surface was constructed around an individual cell (lower panel). For each surface the sum intensity in the FITC channel and volume were measured. The relative YFP-rhTRIM5 α concentration in each cell was calculated by dividing the sum YFP intensity by the volume. In the same manner untransduced HeLa cells were stained with TexasRed Phalloidin and 15 Z-stack images were acquired and analyzed. The relative YFP expression for each cell was calculated and the average was subtracted from the relative rhTRIM5 α concentration of each cell. B) The total number of cytoplasmic bodies per cell was determined as described before. The graph shows the number of cytoplasmic bodies per cell normalized by the relative protein expression for the indicated proteins. C) HeLa cells stably expressing the indicated YFP-rhTRIM5 α proteins were infected with increasing titers of the HIV-1 GFP reporter virus as described before.

Mutations in the L2 region do not affect the ability of rhTRIM5 α to bind in vitro assembled HIV-1 CA complexes.

The binding of rhTRIM5 α to HIV-1 cores has been previously demonstrated using cell lysates or purified recombinant rhTRIM5 α proteins and in vitro assembled HIV-1 CA and CA-NC complexes [15, 86, 89, 92, 193]. We used in vitro assembled tubular assemblies of purified recombinant HIV-1 capsid protein to test if the ability of rhTRIM5 α to assemble into cytoplasmic bodies influences its ability to bind the HIV-1 capsid. Stremlau et al. previously demonstrated that rhTRIM5 α -bound HIV-1 capsid can be pelleted through a 70% sucrose cushion following ultracentrifugation [108]. We used a similar assay to test if the ability of the rhTRIM5 α L2 mutants to bind in vitro assembled the HIV-1 capsid. HA-tagged wt and L2 variant rhTRIM5 α proteins incubated with the HIV-1 tubular assemblies were centrifuged through a 65% sucrose cushion. The amount of rhTRIM5 α proteins that reached the bottom of tube in the presence and absence of the HIV-1 capsid following the centrifugation was analyzed by western blotting. Our results show that HA-tagged wt rhTRIM5 α was detected in the pellet fraction collected from beneath the sucrose cushion only in the presence of HIV-1 capsid (Figure 15), as described by previous studies. The L2 mutants that retain the ability to form cytoplasmic assemblies (TFH and HKN) were also present in the pellet fraction in the presence of capsid (Figure 15). Surprisingly, the RRV mutant, that does not form assemblies was also detected in the pellet fraction (Figure 15), suggesting that mutations

in the L2 region that disrupt the ability of rhTRIM5 α to assemble into cytoplasmic bodies do not affect its ability to bind the HIV-1 capsid. This suggests that the L2 region is required but not sufficient for the formation of rhTRIM5 α assemblies around the HIV-1 core.

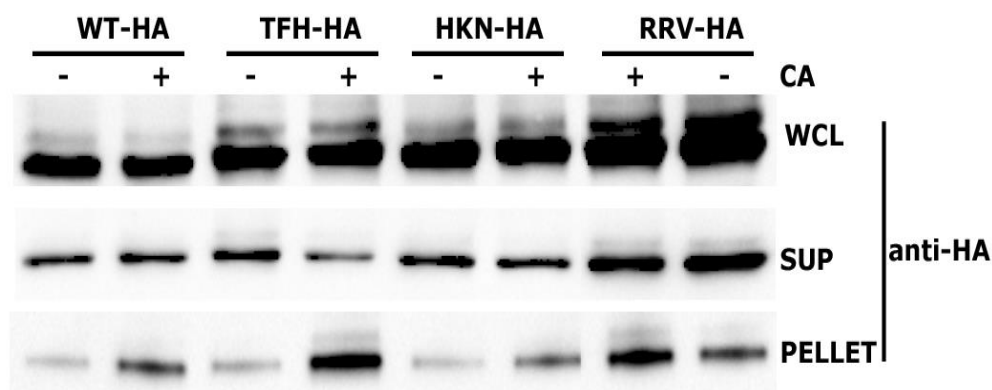


Figure 15: L2 mutations that enhance rhTRIM5 α assembly and HIV-1 restriction do not affect the ability of rhTRIM5 α to bind in vitro assembled HIV-1 capsids. In vitro TRIM5 α binding to HIV-1 CA. CA was assembled in vitro and mixed with lysates from 293T cells transfected with the relevant HA-TRIM5 α -expressing plasmid constructs. Capsids were separated from soluble proteins by ultracentrifugation through a sucrose cushion and analyzed by Western blotting using CA and HA antibodies (pellet). A fraction of the pre-centrifugation mix was analyzed by Western blot for CA content (input).

CHAPTER V

The L2 region has secondary structural motifs that mediate rhTRIM5 α assembly around the HIV-1 core

In order to further characterize the role of the L2 region in mediating rhTRIM5 α assembly and HIV-1 restriction we generated triple alanine mutants of the entire L2 region (residues 234-278) and tested the effect of these mutations on rhTRIM5 α assembly and HIV-1.

Two additional stretches of residues within the L2 region seem to facilitate rhTRIM5 α assembly and HIV-1 restriction

In addition to the KPK and RRV regions we found two other stretches of residues which when substituted with alanine (RLQ240-242AAA and LQG249-251AAA) seemed to partially disrupt the ability of rhTRIM5 α to form cytoplasmic bodies. While the RLQ and LQG L2 variants formed cytoplasmic bodies, their ability to do so was reduced as compared to wt rhTRIM5 α (Figure 16A). The other variants tested formed cytoplasmic bodies at levels similar to wt rhTRIM5 α . These cells were infected with serial dilutions of the GFP reporter virus and the percentage of GFP positive cells was determined 48 hpi as described earlier. As expected, cells expressing the RLQ and LQG L2 variants exhibited abrogated HIV-1 restriction ability as compared to wt rhTRIM5 α (Figure 16B). The L2

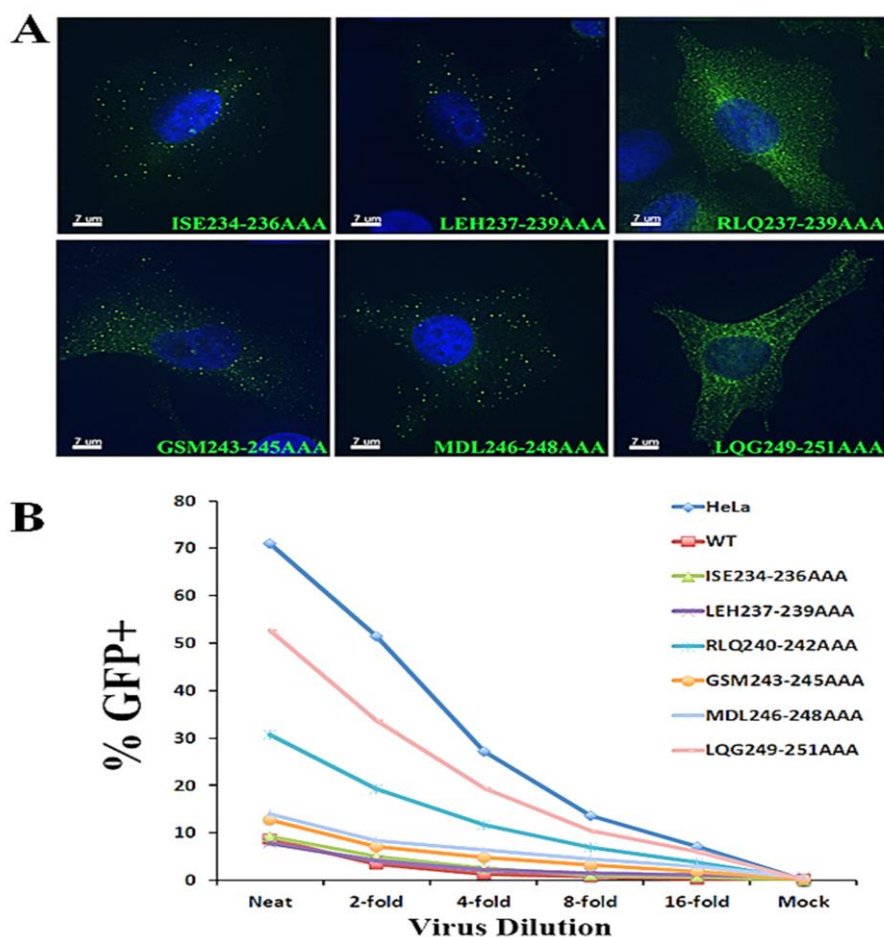


Figure 16: Identification of residues within the L2 region that partially disrupt rhTRIM5a assembly and HIV-1 restriction. A) Triple alanine mutagenesis of residues 234-251 of the L2 region. HeLa cells stably expressing YFP-labeled the indicated L2 variants were allowed to adhere to fibronectin treated coverslips, fixed and 20 Z-stack images were acquired using a DeltaVision fluorescent microscope. Representative deconvolved images are shown. B) Equivalent numbers of the stable cells were plated in a 24-well plate and infected with serial dilutions of VSV-g pseudotyped HIV-1 GFP reporter virus for 14hrs. Cells were harvested 48 hpi and the percentage of GFP positive cells was determined by Flow Cytometry. Results are representative of 3 independent experiments.

variants that retained the ability to form cytoplasmic bodies, restricted HIV-1 infection (Figure 16B).

Single residue substitutions within the L2 region do not affect the assembly or HIV-1 restriction abilities of rhTRIM5 α

In order to identify the specific residues that govern rhTRIM5 α assembly we replaced individual residues within the L2 region with alanine and determined the ability of these L2 variants to assemble into cytoplasmic bodies. HeLa cells stably expressing YFP-labeled single residue substitutions of the L2 region were generated and their sub-cellular localization was observed by fluorescence microscopy as described earlier. Surprisingly, none of the single residue substitutions tested affected the ability of rhTRIM5 α to form cytoplasmic bodies (Figure 17A). Moreover, when these cells were infected with serial dilutions of the HIV-1 GFP reporter virus, none of the mutations seemed to affect the ability of rhTRIM5 α to restrict HIV-1 infection (Figure 17B). However, as described earlier, mutation of stretches of three residues within the L2 region resulted in either a complete or partial loss of rhTRIM5 α assembly and HIV-1 restriction (RLQ, LQG, KPK and RRV variants) or in some cases enhanced these abilities (TFH and HKN variants) of the protein.

Based on these data we hypothesized that the L2 region has secondary structural motifs that are required for rhTRIM5 α assembly by mediating protein-protein interactions. Mutation of stretches of three residues either disrupts or stabilizes these motifs. This in turn results in the loss or enhancement of the tendency of rhTRIM5 α to

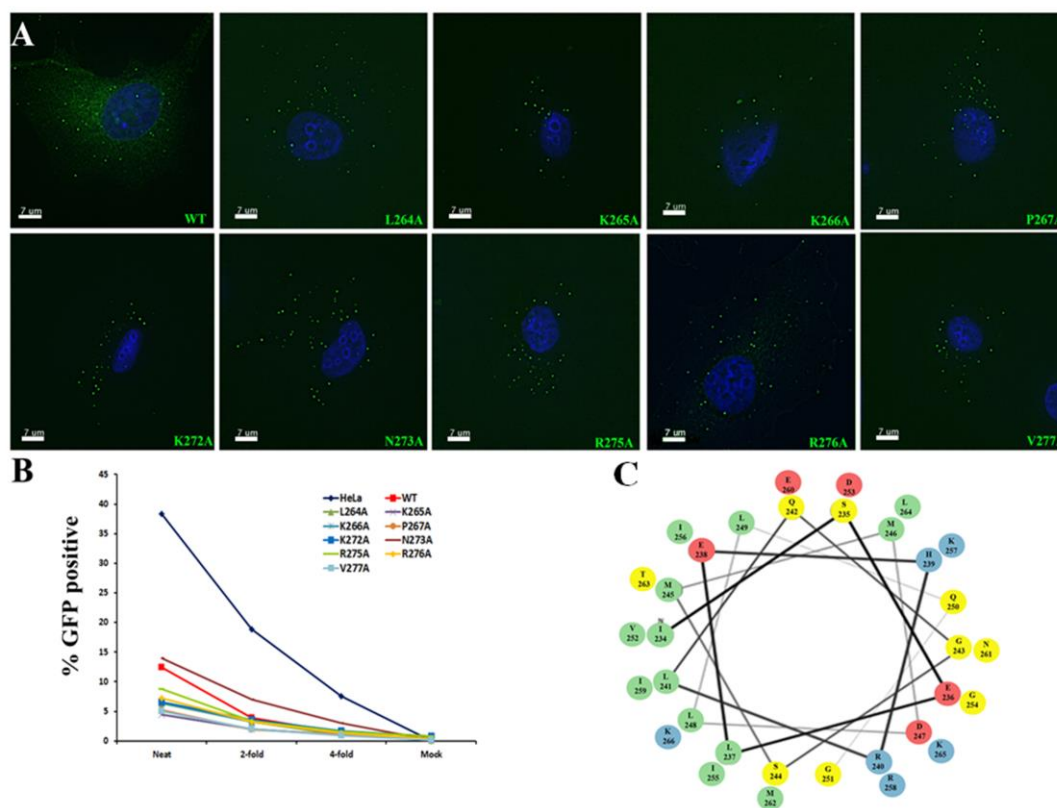


Figure 17: Substitution of individual residues within the L2 region with alanine does not disrupt rhTRIM5 α assembly or HIV-1 restriction. A) Determination of sub-cellular localization of the single alanine variants of rhTRIM5 α . HeLa cells stably expressing YFP-labeled wt or the indicated L2 variants were allowed to adhere to fibronectin treated coverslips, fixed and 20 Z-stack images were acquired using a DeltaVision fluorescent microscope. Representative deconvolved images are shown. B) Determination of the ability of single alanine L2 variants of rhTRIM5 α to restrict HIV-1 infection. Equivalent numbers of the stable cells were plated in a 24-well plate and infected with serial dilutions of VSV-g pseudotyped HIV-1 GFP reporter virus for 14hrs. Cells were harvested 48 hpi and the percentage of GFP positive cells was determined by Flow Cytometry. Results are representative of 3 independent experiments. C) Helical wheel depiction of residues 263-285 of rhTRIM5 α L2 region. Positively and negatively charged residues are shown in black and light grey respectively, nonpolar residues are shown in dark grey and polar, uncharged residues are shown in white. Data representative of 3 independent experiments.

form assemblies. Depiction of residues of the L2 region in the form of a helical wheel diagram shows two interfaces, one predominantly consisting of positively and negatively charged residues and the second consisting of uncharged polar and non-polar residues (Figure 17C). However, based on solely the primary amino acid sequence of the L2 region a secondary structure cannot be predicted for this region.

The CCL2 region of rhTRIM5 α has a predominantly α -helical conformation

We next used CD Spectroscopy to analyze the structure of the CC-L2 and L2 regions of rhTRIM5 α . We generated 6X His-tagged constructs of the CCL2 fragment (AA 134-296) of wt rhTRIM5 α or its L2 variants. These constructs were transformed into bacterial cells, the protein was induced using IPTG and individual proteins were affinity purified. We then used Circular Dichroism (CD) Spectroscopy to determine the secondary structure of these peptides. Our results show that all the CCL2 peptides, wt and the L2 variants had a predominantly α -helical conformation. Interestingly, the CCL2 peptides harboring the TFH and HKN mutations had a higher α -helical content (TFH 81%; HKN 88%) as compared to the wt peptide (74%) (Figure18). The minima at 222nm and 208nm are characteristic of proteins with α -helical conformation. On the other hand, the CCL2 peptide harboring the RRV mutation had a lower α -helical content (61%) as compared to the wt peptide (74%) (Figure18). It is interesting to note that the RRV CCL2 peptide is about 61% α -helical. Since the coiled-coil domain, which is predominantly α -helical, makes up ~63% of the entire CCL2 peptide we can assume that the coiled-coil domain alone is responsible for the α -helical conformation of the RRV CCL2 peptide.

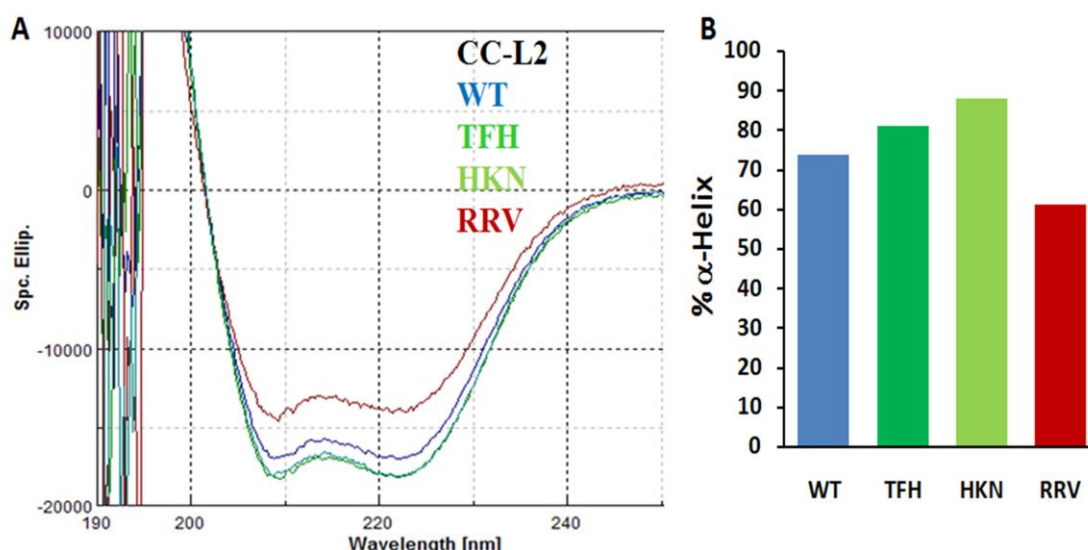


Figure 18: The CC-L2 peptide of rhTRIM5 α has an α -helical conformation. A) CD Spectroscopic analysis of purified CC-L2 peptides of rhTRIM5 α . 6X His-tagged CC-L2 peptides of the wt and L2 variant rhTRIM5 α proteins were expressed in BL21 cells and affinity purified using Talon cobalt resin. The purified peptides were analyzed by CD Spectroscopy at 20°C and the spectra were normalized by the peptide concentrations. B) The total helix content for each peptide was calculated using ContinLL. Data representative of 3 independent experiments.

The L2 region undergoes a coil-to-helix transition in the presence of 2,2,2-trifluoroethanol (TFE)

We next determined the secondary structure of the L2 region alone (AA 234-296) of rhTRIM5 α . We acquired synthetic peptides, wt and those containing the TFH, HKN and RRV mutations, from Bio-synthesis, Inc. and analyzed them by CD Spectroscopy. In

the absence of TFE each of the L2 peptides generated CD spectra that are characteristic of partially unstructured proteins (minima at 222nm and 205nm) (Figure 19A). However, in the presence of 10% TFE each of the L2 peptides generated minima at 222nm and 208nm characteristic of proteins with a predominantly α -helical conformation (Figure 19B). These results suggest that the L2 region has a propensity to form α -helices. These helices are stabilized in the presence of 10% TFE. In the context of the full-length protein, the α -helical conformation of the L2 region is most likely stabilized by the coiled-coil domain.

The L2 region forms α -helices in a concentration dependent manner

A recent study demonstrated that SP1, a small spacer present between the CA and NC in HIV-1 Gag, that is required for Gag assembly undergoes a conformation change to form an α -helix in the presence of TFE [194]. They saw a similar coil-to helix transition when high peptide concentrations were used, suggesting that SP1 assumes α -helical conformation in a concentration dependent manner thus acting as a molecular switch in Gag assembly. Since TRIM5 α forms cytoplasmic bodies in a concentration dependent manner we asked if this increased ability to assemble at higher protein concentrations was due to stabilization of α -helical conformation of the L2 region. In order to test if the L2 region formed α -helices in a concentration dependent manner, analyzed two different concentrations of the wt L2 peptide (1mg/mL and 100ug/mL) by CD spectroscopy in the presence of increasing amounts of TFE. We reasoned that if peptide concentration influenced the α -helical conformation of the L2 region then the requirement of TFE for

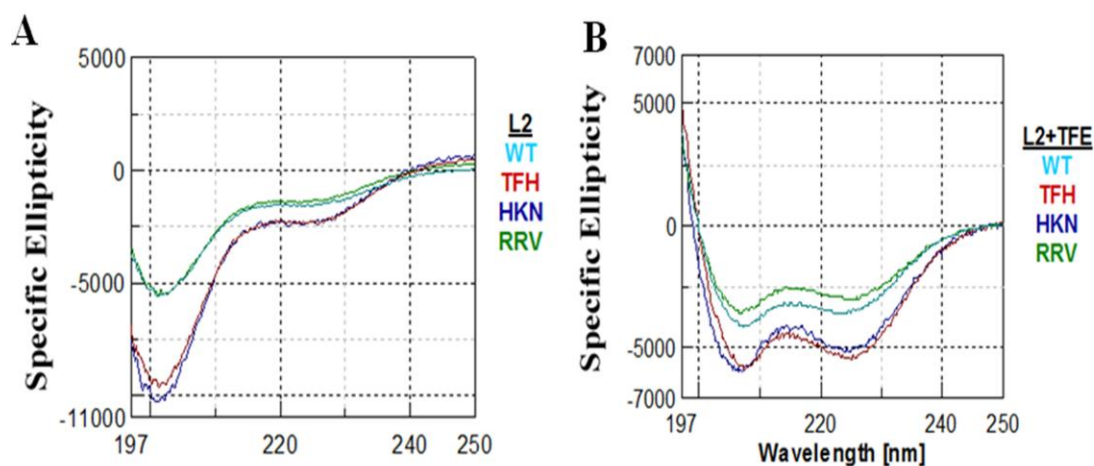


Figure 19: The L2 peptide of rhTRIM5 α undergoes a coil-to-helix transition in the presence of 10% TFE . A) CD Spectra of synthetic L2 peptides of rhTRIM5 α . The L2 peptides of wt and L2 variants of rhTRIM5 α were resuspended in appropriate buffer and analyzed by CD Spectroscopy at 20°C. The spectra were normalized by the peptide concentrations. B) 10% TFE was added to each peptide immediately before analysis. The spectra were normalized by the peptide concentrations. Data representative of 2 different experiments.

induction of α -helices would be reduced at higher concentrations of the peptide. As shown in figure 20, we determined the secondary structure of the wt L2 peptide in the presence of 0, 5, 6, 7, 8, 9, and 10% TFE. We found that in the absence of TFE the 1mg/mL peptide was about 15% α -helical. When the peptide was diluted 10-fold the α -

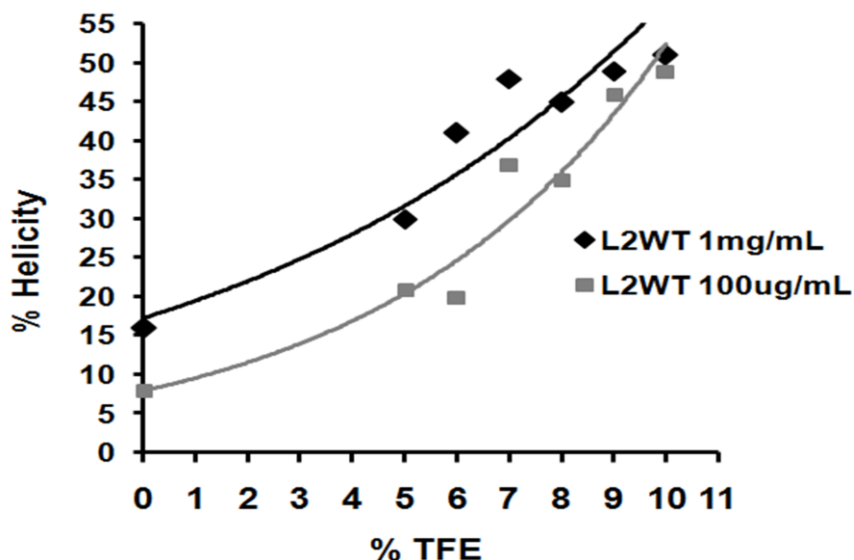


Figure 20: Concentration dependent changes in secondary structure of wt rhTRIM5 α L2 peptide. WT L2 peptide at 1mg/mL and 100ug/mL concentration were analyzed by CD Spectroscopy in the presence of the indicated amounts of TFE. CD spectra were acquired in triplicates at 20°C and normalized by peptide concentration. The total amount of helix was calculated using ContinLL. Data representative of 2 independent experiments

helicity was only ~8%. Similarly, when increasing amounts of TFE were added the 1mg/mL peptide was consistently more α -helical as compared to the 100ug/mL peptide. This suggests that peptide concentration does play a role in the induction of α -helical conformation of the L2 region. In a cell a local increase in rhTRIM5 α concentration would be observed when the protein multimerizes and assembles around the HIV-1 core.

CHAPTER VI

The role of Linker 2 region in TRIM5 α -mediated retroviral restriction is conserved across species

Other TRIM5 proteins such as huTRIM5 α and Owl monkey TRIM-Cyp are also known to localize to cytoplasmic bodies. However, the functional significance of these cytoplasmic bodies in retroviral restriction by these TRIM5 proteins is unknown. We asked if the L2 region of huTRIM5 α and TRIM-Cyp mediate retroviral restriction. An alignment of the L2 regions of huTRIM5 α and TRIM-Cyp with that of rhTRIM5 α shows that these regions share a high sequence similarity (Figure 21).

The L2 region of huTRIM5 α has determinants that are required for restriction of N-MLV infection

Human TRIM5 α potently restricts N-MLV but not B-MLV infection. To test if the L2 region is required for huTRIM5 α -mediated N-MLV restriction we generated triple alanine mutants of residues in the L2 region. We first mutated the residues corresponding to the KPK, TFH, HKN and RRV stretches in the rhTRIM5 α L2 region (Figure 22A). Crandell-Rees Feline Kidney (CrFK) cells, that lack intrinsic immunity to a number of retroviruses, were used to stably express YFP-labeled wt huTRIM5 α and the L2 variants

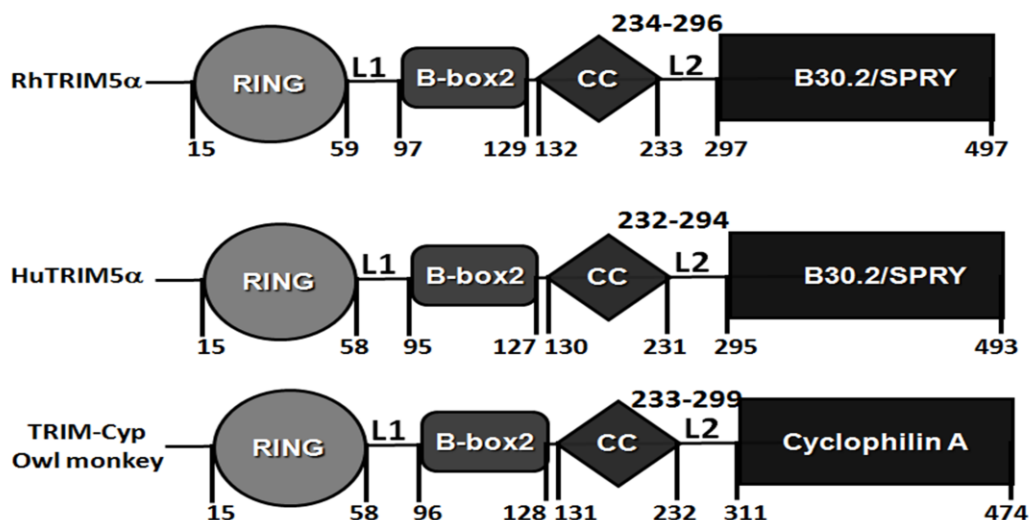


Figure 21: Representation of the domain organization of TRIM5 proteins. The N and C-terminal domains of rhesus TRIM5 α , human TRIM5 α and Owl monkey TRIM-Cyp are shown. Numbers indicate the starting and ending residues of each domain.

expressing YFP-labeled wt and mutant huTRIM5 α proteins with serial dilutions of VSV-g pseudotyped N-MLV expressing GFP upon infection (N-MLV GFP reporter virus). The cells were also infected with B-MLV GFP reporter virus as a control. Cells expressing YFP-labeled huTRIM5 α restricted N-MLV infection (Figure 22B). Cells expressing the RRV mutant on the other hand were unable to restrict N-MLV infection and level of infection in these cells was similar to that observed in case of the control CrFK cells (Figure 22B). Interestingly, this stretch of residues (RRV) is conserved between TRIM5 α proteins from rhesus macaques and humans (Figure 22A). The other L2 variants tested

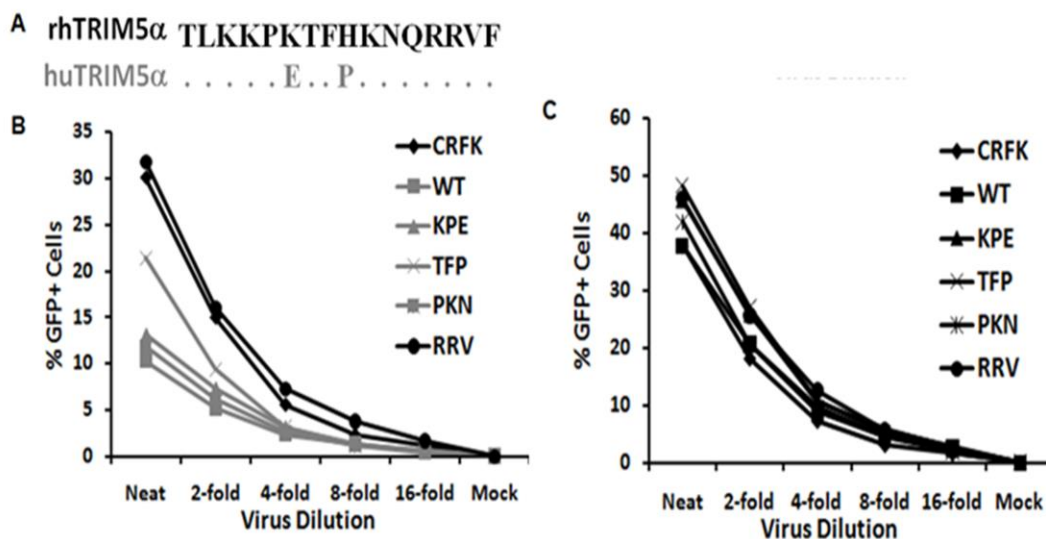


Figure 22: The role of L2 region in retroviral restriction is conserved between rhesus and huTRIM5 α proteins. A) Alignment of residues 263-278 of rhTRIM5 α and the corresponding huTRIM5 α residues is shown. Dots indicate same amino acid as in rhTRIM5 α . B and C) CrFK cells stably expressing YFP-labeled wt and L2 variants of huTRIM5 α were infected with serial dilutions of VSV-g pseudotyped (B) N-MLV and (C) B-MLV GFP reporter viruses for 14 hrs. Cells were harvested 48 hpi and percentage of GFP positive cells was determined by flow cytometry. Results are representative of 3 independent experiments.

were able to restrict N-MLV infection at levels similar to wt huTRIM5 α . Neither wt nor the L2 mutants restricted B-MLV infection (Figure 22C). Overall, these results suggest that the L2 region plays a role in huTRIM5 α -mediated retroviral restriction. The determinants that govern this ability are conserved between humans and rhesus macaques.

The L2 region is not required for TRIM-Cyp mediated HIV-1 restriction

In order to test if the L2 region of TRIM-Cyp mediates HIV-1 restriction we generated epitope-tagged triple alanine mutants of residues in the L2 region (Figure 23A) and stably expressed them in HeLa cells. The ability of these L2 variants to restrict HIV-1 infection in the presence and absence of cyclosporine A (CsA) was determined as described for TRIM5 α . CsA prevents the interaction of TRM-Cyp with the HIV-1 capsid thus relieving TRIM-Cyp mediated restriction. Equivalent number of cells expressing YFP-labeled wt TRIM-Cyp or the L2 variants was infected with serial dilutions of the VSV-g pseudotyped HIV-1 GFP reporter virus in the presence and absence of CsA. Our results show that none of L2 mutations affected the ability of TRIM-Cyp to restrict HIV-1 infection (Figure 23B), suggesting that the L2 region of TRIM-Cyp is not required for HIV-1 restriction. None of the TRIM-Cyp proteins restricted HIV-1 infection in the presence of CsA similar to the wt protein (Figure 23C). Similar observation was made by Li et al. using B-Box2 variants. They showed that the B-Box2 residues that are required for rhTRIM5 α -mediated HIV-1 restriction do not play a role in TRIM-Cyp mediated HIV-1 restriction [90]. This could be explained by the relatively higher affinity of CypA for the HIV-1 capsid as compared to the SPRY domain. Since the SPRY domain of rhTRIM5 α has a lower affinity for the HIV-1 capsid, the B-Box2 domain and L2 region

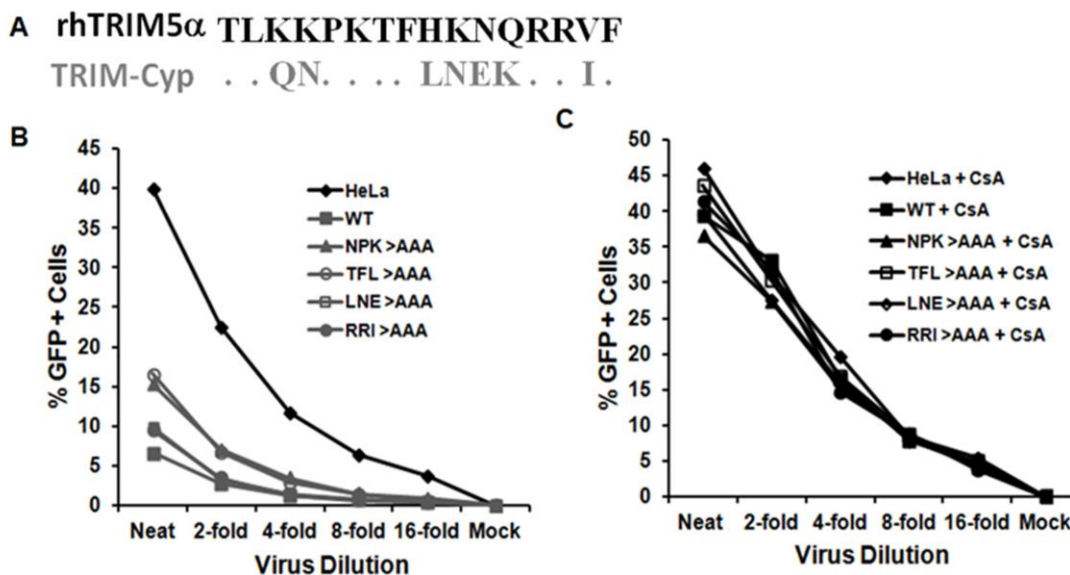


Figure 23: The L2 region does not facilitate TRIM-Cyp mediated HIV-1 restriction. A) Alignment of residues 263-278 of rhTRIM5 α and the corresponding TRIM-Cyp residues is shown. Dots indicate same amino acid as in rhTRIM5 α . B and C) HeLa cells stably expressing YFP-labeled wt and L2 variants of TRIM-Cyp were infected with serial dilutions of VSV-g pseudotyped HIV-1 GFP reporter viruses for 14 hrs. in the (B) absence or (C) presence of cyclosporin A (CsA). Cells were harvested 48 hpi and percentage of GFP positive cells was determined by flow cytometry. Results are representative of 3 independent experiments.

increase the overall avidity of the rhTRIM5 α -capsid interaction by facilitating higher-order multimerization and assembly of rhTRIM5 α respectively, around the viral core. Conversely, since the affinity of TRIM-Cyp to the HIV-1 capsid is higher the B-Box2 and L2 regions are dispensable for capsid binding.

CHAPTER VII

DISCUSSION

Summary

This study examined the role of the Linker 2 (L2) region in retroviral restriction by facilitating the assembly of TRIM5 proteins. We show that the ability of rhTRIM5 α to assemble into cytoplasmic bodies is required for HIV-1 restriction. Using triple alanine mutagenesis we have found determinants in the L2 region that govern rhTRIM5 α assembly. While some mutations disrupt rhTRIM5 α assembly into cytoplasmic bodies (KPK and RRV), others enhance cytoplasmic body formation (TFH and HKN). Moreover, the L2 variants with a higher tendency to assemble into cytoplasmic bodies, exhibit increased HIV-1 restriction ability. This suggests that the tendency of rhTRIM5 α to form assemblies correlates directly with its anti-HIV-1 activity. This study also provides evidence for the presence of secondary structural motifs in the L2 region of rhTRIM5 α . Using CD spectroscopy we show that the CC-L2 peptide of rhTRIM5 α has a predominantly α -helical conformation. Moreover, this α -helical conformation seems to be stabilized in CC-L2 peptides carrying L2 mutations that increase rhTRIM5 α assembly, but not in those carrying mutations that disrupt assembly. The L2 peptide by itself seems to be partially unstructured however it undergoes a coil-to-helix transition in the presence of TFE. This suggests that the L2 region has a propensity to form α -helices and its α -

helical conformation is stabilized in the presence of TFE. In the context of a full-length protein, the α -helical conformation of the L2 region is most likely stabilized by the coiled-coil region. Our results also indicate that the L2 region forms α -helices in a concentration dependent manner. This suggests that it is possible that the L2 region, which is capable of assuming alternative conformations, acts as a switch and undergoes a coil-to-helix transition when rhTRIM5 α multimerizes. The presence of secondary structural motifs within the L2 region has not been reported prior to this study and this finding can provide an insight into the mechanism of rhTRIM5 α assembly around the HIV-1 core, which is one of the initial steps in the HIV-1 restriction process.

The L2-mediated ability of rhTRIM5 α to assemble into cytoplasmic bodies is required for HIV-1 restriction

The role of TRIM5 cytoplasmic bodies has been controversial as two studies previously demonstrated that pre-existing TRIM5 cytoplasmic bodies are not required for HIV-1 restriction [107, 186]. These studies involved either disruption of rhTRIM5 α cytoplasmic bodies following geldanamycin treatment or increased recruitment of TRIM-Cyp to cytoplasmic bodies following treatment with sodium butyrate and testing the effect of these treatments on HIV-1 restriction. However, the formation of TRIM5 cytoplasmic bodies in the presence of restriction sensitive virus was not tested in these studies. Conversely, our triple alanine mutagenesis data shows that the L2 variants that fail to assemble into cytoplasmic bodies also fail to restrict HIV-1 infection, while those that retain the ability to form cytoplasmic body formation restrict HIV-1 at least up to WT levels (Figure 10). Moreover, Campbell et al. have previously demonstrated that

following infection, fluorescently labeled HIV-1 particles accumulate within rhTRIM5 α cytoplasmic bodies [106] and *de novo* formation of rhTRIM5 α cytoplasmic bodies around individual virions has also been observed. Based on these data we can speculate that if rhTRIM5 α cytoplasmic body formation in geladanamycin treated cells was measured following HIV-1 infection we would observe an increase in the number of cytoplasmic bodies as compared to the untreated cells. Thus, while the triple alanine mutagenesis data clearly shows a correlation between cytoplasmic body formation and HIV-1 restriction we do not believe that the pre-existing cytoplasmic bodies play a role in the restriction process. Although these studies seem controversial, collectively they suggest that although pre-existing cytoplasmic bodies are not required for HIV-1 restriction, the ability to form these assemblies is essential for restriction.

This idea is also supported by the observation that although the KPK and RRV diffuse L2 mutants form assemblies upon treatment with MG132 they fail to restrict HIV-1 infection (Figure 11). Moreover, we have generated a cell line that expresses lower levels of rhTRIM5 α and in turn has very few cytoplasmic bodies. Following infection with HIV-1 we observe an increase in the number of rhTRIM5 α cytoplasmic bodies in these cells (data not shown). Moreover, Campbell et al. have also shown that in cells rhTRIM5 α exists in two populations, 1) cytoplasmic assemblies and, 2) a fraction that is diffusely localized throughout the cell and TRIM5 α shuttles between these two populations with rapid kinetics [185]. So it is possible that rhTRIM5 α dimers recognize the incoming HIV-1 capsid cores and recruit more rhTRIM5 α dimers which undergo a

series of self-association events ultimately leading to the formation of large assemblies around the viral core.

The L2 region mediates the final step in rhTRIM5 α self-association:

Assembly

It is known that the coiled-coil and B-Box2 domains mediate TRIM5 α dimerization and higher-order multimerization [85-87, 89, 90, 141]. B-Box2 deletion mutants of TRIM5 α retain the ability to form dimers [87], suggesting that coiled-coil mediated dimerization preceded B-Box2 mediated higher-order multimerization. These TRIM5 multimers can be analyzed by biochemical crosslinking of cellular lysates with EGS or glutaraldehyde [85-87, 104]. Biochemical crosslinking of the L2 variants with increasing concentrations of glutaraldehyde showed that all the L2 variants tested could be crosslinked to dimers, irrespective of their ability to form cytoplasmic bodies (Figure 12). At higher concentrations of glutaraldehyde all the L2 variants, including the diffuse KPK and RRV variants, formed higher-order multimers. This suggests that mutation of residues in the L2 region does not disrupt the function of the coiled-coil and B-Box2 domains in mediating lower- and higher-order multimerization of TRIM5 α , respectively. These results also suggest that the L2 region facilitates the assembly of rhTRIM5 α higher-order multimers. These assemblies are manifested as cytoplasmic bodies, microscopically. Collectively, results from the crosslinking studies suggest that the self-association of rhTRIM5 α is a multi-step process. The first step is dimerization which is mediated by the coiled-coil domain. Next, the B-Box2 domain facilitates the higher-order

multimerization of rhTRIM5 α dimers and finally, the L2 region mediates the assembly of these multimers into larger cytoplasmic bodies.

In 2011, a study by Ganser-Pornillos et al. provided significant insight into the mechanism of rhTRIM5 α assembly around the HIV-1 capsid [195]. Using negative-stain electron microscopy they showed that purified, recombinant rhTRIM5 α (TRIM5 α -21R) assembles into two-dimensional hexagonal arrays. Formation of these assemblies requires protein dimerization and higher-order multimerization, and is enhanced in the presence of assembled HIV-1 capsid. This study also proposed a model for TRIM5 α assembly around the viral core by taking into account the size and dimensions of the viral and TRIM5 α assemblies. In this model the coiled-coil, B-Box2 and L2 regions are shown to facilitate TRIM5 α assembly around the viral core. The coiled-coil domain is shown to mediate formation of TRIM5 α dimers. The B-Box2 domain is shown to mediate the formation of a trimeric interface of TRIM5 α dimers thus generating tripodial protein extensions that are required for the formation of a hexameric lattice. Interestingly, in this model the L2 region has been shown to mediate protein-protein interactions between opposing TRIM5 α dimers, allowing the B-Box2 domains to align on both sides of each segment of the hexameric lattice and facilitating the formation of hexameric assemblies.

Another possibility is that the L2 region does not mediate the interaction between opposing TRIM5 α dimers but instead helps orient the B-Box2 and SPRY domains in such a manner that is most favorable for the formation of hexagonal assemblies of rhTRIM5 α . However, a better knowledge of the structural arrangement of the L2 region in the context of dimeric and multimeric TRIM5 α is critical to understand the exact

mechanism by which TRIM5 α assembles around the viral core. Nevertheless, it seems most likely that TRIM5 α binds the viral capsid as a dimer. Following this binding the L2 region undergoes a coil-helix transition which results in the orientation of the B-Box2 domains that is most favorable for the formation of assemblies of TRIM5 α . Formation of a hexagonal lattice of TRIM5 α around the viral core in this manner may exert mechanical stress on the capsid in turn resulting in abortive disassembly of the capsid.

Another possibility is that the L2 region mediates the interaction of TRIM5 α with one or more cellular proteins that facilitates the formation of TRIM5 α assemblies. Moreover, the L2 region has a number of charged residues that potentially participate in these intra- or inter-molecular interactions. Further characterization of the L2 region is required to fully understand the mechanistic basis of these interactions.

The tendency of rhTRIM5 α to assemble correlates with its ability to restrict HIV-1 infection

Comparison of the tendency of the wt, and the TFH and HKN variant rhTRIM5 α proteins to assemble, at similar protein expression levels, showed that cells expressing the TFH and HKN L2 variants had more cytoplasmic bodies as compared to those expressing wt rhTRIM5 α (Figure 13A). However, this increase was not due to their delayed turnover as compared to the wt protein (Figure 13C and D). Assuming the L2 region plays a role in protein-protein interactions, it is possible that substitution of the TFH and HKN residues with alanine results in a conformation change in the L2 region that stabilizes these interactions in turn increasing the tendency of these proteins to form assemblies. Next, since we observe a correlation between cytoplasmic body formation and HIV-1

restriction we wondered if these L2 variants, that have a higher tendency to assemble, would exhibit increased HIV-1 restriction ability. Indeed, when cells expressing these proteins were treated with increasing titers of the HIV-1 GFP reporter virus we found that cells expressing the TFH and HKN variants had significantly less infection as compared to those expressing wt rhTRIM5 α (Figure 14C). This suggests that the tendency of rhTRIM5 α to assemble into cytoplasmic bodies directly correlates with its ability to restrict HIV-1 infection. It would be interesting to test if replacing the L2 region of huTRIM5 α with that of rhTRIM5 α , particularly comprising the TFH and HKN mutations would increase its ability to restrict HIV-1 infection.

The role of L2 region in retroviral restriction is conserved between rhesus and human TRIM5 α proteins

We next tested if the L2 regions of other TRIM5 proteins, huTRIM5 α and TRIM-Cyp, are required for their antiretroviral activity. We performed triple alanine mutagenesis of the residues in the L2 regions of huTRIM5 α and TRIM-Cyp proteins corresponding to their rhTRIM5 α counterparts and tested their ability to restrict N-MLV and HIV-1 restriction, respectively. We found a stretch of three in the L2 region of huTRIM5 α that are required for N-MLV restriction, suggesting that the role of L2 region in mediating retroviral restriction is conserved across species. Interestingly, these determinants are conserved between human and rhTRIM5 α proteins. In case of TRIM-Cyp, mutation of residues in the L2 region did not abrogate HIV-1 restriction, suggesting that residues in the L2 region do not facilitate TRIM-Cyp mediated HIV-1 restriction. Similar results were obtained by the Sodroski laboratory in a study focused on

understanding the role of B-Box2 domain in TRIM5 α multimerization. They found that the B-Box2 residues that are required for HIV-1 restriction by rhTRIM5 α are not required for TRIM-Cyp mediated HIV-1 restriction [90]. This can be explained by the difference in the binding affinities of the CypA and SPRY domains for the HIV-1 capsid. CypA has a relatively high affinity for the HIV-1 capsid as compared to the SPRY domain. Hence, in case of TRIM5 α the B-Box2 domain and L2 region are required to promote cooperative binding to the HIV-1 capsid by mediating TRIM5 α self-association. In case of TRIM-Cyp, however, since the affinity of the CypA-capsid interaction is relatively high the B-Box2 domain and L2 region are dispensable for capsid binding.

The L2 region of rhTRIM5 α has secondary structural motifs that are required for assembly

In order to further characterize the role of the L2 region in rhTRIM5 α assembly and to identify specific residues that are involved in this process we performed single residue substitutions. To our surprise, none of the mutations affected rhTRIM5 α assembly or HIV-1 infection (Figure 17A and B). However, mutation of three residues resulted in either loss of or in some cases enhancement of the ability of rhTRIM5 α to assemble (Figure 10). This suggested that the L2 region possibly has secondary structural motifs that are either disrupted or stabilized upon mutation of three or more residues. To test this

hypothesis we determined the secondary structure of the CC-L2 and L2 peptides of rhTRIM5 α by CD spectroscopy. Our results show that the CC-L2 peptide of rhTRIM5 α has α -helical conformation. Importantly, the TFH and HKN CC-L2 peptides have a higher helical content and the RRV CC-L2 peptide has a lower helical content as compared to wt CC-L2 (Figure 18). This result taken together with the results from the cytoplasmic body quantification study suggest that the α -helices present in the L2 region mediate rhTRIM5 α assembly. These α -helices are possibly stabilized in the TFH and HKN mutants resulting in increased assembly. Since the CC-L2 peptide comprising the RRV mutation does form α -helices, albeit to a lesser extent (~61%) than that of wt (~74%), it is unlikely that the RRV mutation completely destabilizes the α -helix in the L2 region. It is important to note however, that the coiled-coil domain occupies about 62% of the entire CC-L2 peptide and has been predicted to be predominantly α -helical. Hence, it is reasonable to speculate that in case of RRV, the coiled-coil domain contributes almost entirely to the α -helical conformation of the CC-L2 peptide. Comparing the total helical content of the CC-only peptide to that of the RRV CC-L2 peptide at an equivalent concentration will provide further evidence for this hypothesis.

CD spectroscopic analysis of synthetic wt and mutant L2 peptides showed that the L2 region of rhTRIM5 α is partially unstructured (Figure 19A), but undergoes a coil-to-helix transition upon addition of 10% TFE (Figure 19B). This suggests that the L2 region has a propensity to form α -helices and is capable of assuming alternative conformations. We also tested if the L2 region could assume a helical conformation in a concentration dependent manner by determining the secondary structure of two different concentrations

of the wt L2 peptide in the presence of increasing amounts of TFE. This study showed that at a given amount of TFE, the peptide at a higher concentration was more helical as compared to that at a relatively lower concentration (Figure 20). This suggests that the L2 region possibly has a potential to undergo a conformation change, most likely from partially unstructured to helical, at higher peptide concentrations.

Based on these data we can propose a model for the role of L2 region in rhTRIM5 α assembly. In a TRIM5 α dimer the L2 region is most likely partially unstructured. Formation of higher-order multimers by the B-Box2 domain brings the L2 regions of individual TRIM5 α dimers in close proximity thus inducing a conformation change in the L2 region and stabilizing its α -helical conformation. The α -helices in the L2 region can then facilitate TRIM5 α assembly by mediating protein-protein interactions. The formation of these is enhanced in the presence of a restriction sensitive capsid, as clearly demonstrated by Ganser-Pornillos et al. using purified recombinant TRIM5 α protein [113]. In light of this finding we observe that the L2 region forms α -helices in a concentration dependent manner. Thus, based on this and previous studies we hypothesize that assembly of rhTRIM5 α around the HIV-1 capsid involves the following steps: 1) binding of rhTRIM5 α dimers via the SPRY domain, 2) followed by B-Box2 mediated higher-order multimerization, 3) stabilization of the α -helical conformation of the L2 region mediating protein-protein interactions between TRIM5 dimers, as suggested by Ganser-Pornillos et al. [113], and finally assembly. Another possibility is that the 2 regions mediate the interaction between three sets of dimers giving rise to three-fold symmetry, as suggested by Li et al. [91]. Alternatively, the L2 region mediates

the interaction of TRIM5 α with other cellular protein(s). However, more studies are needed to test these models to completely understand the mechanism by which the L2 region participates in protein-protein interactions and assembly of rhTRIM5 α .

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