Viral and Host Determinants of Primate Lentivirus Restriction by Old World Primate TRIM5alpha Proteins

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Viral and Host Determinants of Primate Lentivirus Restriction by Old World Primate TRIM5alpha Proteins

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A dissertation presented
by
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to
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Viral and Host Determinants of Primate Lentivirus Restriction by Old World Primate TRIM5alpha Proteins

Abstract

The host restriction factor TRIM5α mediates a post-entry, pre-integration block to retroviral infection that depends upon recognition of the viral capsid by the TRIM5α PRYSPRY domain. The two predominant alleles of rhesus macaque TRIM5α (rhTRIM5α^{Q} and rhTRIM5α^{TFP}) restrict HIV-1, but cannot restrict the macaque-adapted virus SIVmac239. To investigate how TRIM5α recognizes retroviral capsids, we exploited the differential sensitivities of these two viruses to identify gain-of-sensitivity mutations in SIVmac239, and we solved the structure of the SIVmac239 capsid N-terminal domain. When mapped onto this structure, single amino acid substitutions affecting both alleles were in the β-hairpin. In contrast, mutations specifically affecting rhTRIM5α^{TFP} surround a highly conserved patch of amino acids that is unique to capsids of primate lentiviruses. This “patch” sits at the junction between the binding sites of multiple cellular cofactors (cyclophilin A, Nup-358 cyclophilin A-like domain, Nup-153 and

CPSF6). Differential restriction of these alleles is due to a Q/TFP polymorphism in the first variable loop (V1) within the PRYSPRY domain. Q reflects the ancestral state (present in the last common ancestor of Old World primates) and has remained unmodified in all but one lineage of African monkeys, the Cercopithecinae. While Q-alleles can be found among some Cercopithecinae primates, in others Q has been replaced by a G or overwritten by a two amino acid insertion (giving rise to TFP in macaques). In one lineage, the Q to G substitution was later followed by an adjacent 20 amino acid duplication. We found that these modifications in TRIM5α specifically impart the ability to restrict Cercopithecinae SIVs without altering β-hairpin recognition. At least twice Cercopithecinae TRIM5αs independently evolved to target the same conserved patch of amino acids in capsid. Based on these findings, we propose that the β-hairpin is a retrovirus associated molecular pattern widely exploited by TRIM5α proteins, while recognition of the cofactor binding region was driven by the emergence of the ancestors of modern Cercopithecinae SIVs. Distribution on the Cercopithecinae phylogenetic tree indicates that selection for these changes in TRIM5α V1 began 11-16 million years ago, suggesting that primate lentiviruses are at least as ancient.

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Chapter 1: Introduction

Plastic Proteins and Monkey Blocks: How Lentiviruses Evolved to Replicate in the Presence of Primate Restriction Factors

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1. A. RETROVIRUSES AND PRIMATE LENTIVIRUSES

The Retroviridae

Primate lentiviruses and retroviruses. The *Retroviridae* can be subdivided into two subfamilies: the *Spumaretrovirinae* and the *Orthoretrovirinae*. The *Spumaretrovirinae* are comprised of a single extant lineage, the spumaviruses (foamy viruses). In contrast, the *Orthoretrovirinae* include six extant lineages: alpha-, beta-, gamma-, delta-, epsilon- and lenti–retroviruses [1,2]. Primate lentiviruses include the human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2 respectively) and the simian immunodeficiency viruses (SIVs). Primate lentiviruses are a member of the *Lentivirus* genus of the *Retroviridae* family of viruses [1,2]. Primate lentiviruses are a monophyletic lineage within the *Lentivirus* genus. All extant retroviruses express the same three proteins, the group specific antigen (Gag) polyprotein, the polymerase (Pol) and an envelope protein (Env) (Table 1-1). Among viruses of the *Orthoretrovirinae* Gag is a polyprotein that is cleaved to create the structural proteins of the virion, including the matrix, capsid and nucleocapsid proteins [2]. Among The Pol gene encodes for the viral enzymes whose activity are defining features of the *Retroviridae*, reverse transcriptase and integrase [1,2]. These proteins convert the RNA genome into DNA and

facilitate its integration into the target cell's DNA. The Env protein is the viral glycloprotein that facilities entry into the target cell [2].

Table 1-1. Primate lentiviruses have an expanded repertoire of accessory genes.
Abbreviations. Viruses: Murine leukemia virus (MLV), human T cell lymphotropic virus type 1 (HTLV-1), maedia-visna virus (MVV) human immunodeficiency virus type-1 (HIV-1), simian immunodeficiency virus of African green monkeys (SIVagm), simian immunodeficiency virus of rhesus macaques (SIVmac).

Virus	Type	Genus	Structural Genes	Regulatory Genes	Accessory Genes	Counteracts
MLV	Simple	*Gammaretrovirus*	*gag*		*glyco-gag (?)*	
		(rodent)	*pol*			
			env			
HTLV-1	Complex	*Deltaretrovirus*	*gag*	*tax*	*hbx*	
		(primate)	*pol*	*rex*	*orfi-p12*	
			env		*orfii-p13*	
MVV	Complex	*Lentivirus*	*gag*	*tat*	*vif*	*APOBEC3*
		(ovine)	*pol*	*rev*		
			env			
HIV-1	Complex	*Lentivirus*	*gag*	*tat*	*vif*	*APOBEC3*
		(primate)	*pol*	*rev*	*vpr*	
			env		*vpu*	*BST2*
					nef	
SIVagm	Complex	*Lentivirus*	*gag*	*tat*	*vif*	*APOBEC3*
		(primate)	*pol*	*rev*	*vpr*	*SAMHD1*
			env		*nef*	*BST2*
SIVmac	Complex	*Lentivirus*	*gag*	*tat*	*vif*	*APOBEC3*
		(primate)	*pol*	*rev*	*vpr*	
			env		*vpx*	*SAMHD1*
					nef	*BST2*

Retrovirus replication

Replication cycle. Retroviral infection begins when the viral glycoprotein engages its receptor on the target cell. Primate lentiviruses require the sequential binding of a primary receptor, CD4, followed by binding of the co-receptor, such as the chemokine receptor CCR5 [3-10]. Upon receptor (and co-receptor in this case) engagement, a series of conformational changes in the viral glycoprotein facilitates the fusion of the viral envelope with the target cell plasma membrane. This results in the deposition of the viral capsid, which contains the viral genomes, reverse transcriptase and integrase, into the cytoplasm (Figure 1-1A).

Upon deposition of the capsid into the cytoplasm of the target cell, the reverse transcriptase begins to convert the RNA genome into a double stranded DNA genome. Reverse transcription is thought to occur within the viral capsid [11-13]. At the completion of reverse transcription a complex consisting of the newly synthesized viral DNA genome, integrase and other viral components must access the cellular genomic DNA. Upon accessing the target cell's nuclear DNA, integrase facilitates the integration of the viral DNA genome [2] (Figure 1-1 A). Unlike may other retroviruses that require the breakdown of the nuclear membrane during cell division to access the nuclear DNA, lentiviruses can access the DNA of non-dividing cells via the nuclear pore [2,14,15]. It should be noted that both the alpharetrovirus Rous Sarcoma Virus

(RSV) and the betaretrovirus mouse mammary tumor virus (MMTV) have been reported to infect non-dividing cells to differing efficiencies [16,17].

For lentiviruses, the provirus (integrated viral genome) encodes transcriptional activators. These facilitate the production of spliced and unspliced messenger RNAs [2]. For primate lentiviruses the production of viral RNA species is enhanced by the trans-activator of transcription (Tat) protein. Nuclear export of unspliced or minimally spliced RNA transcripts is enhanced by the regulator of expression of virion (Rev) protein [2,18]. Some other retroviruses encode analogs of these proteins in their 3' half to perform similar functions (e.g Tax and Rex in the human T-lymphotropic virus, HTLV-1). For primate lentiviruses unspliced RNA transcripts serve as viral genomes as well as mRNAs for the production of Gag and Gag-Pol. Subgenomic (Spliced) viral RNAs act as messenger RNAs encoding for the production of all other viral proteins aside from Gag and Gag-Pol [2,18]. Viral proteins and genomes co-assemble usually at the plasma membrane [19-22]. Finally, virions bud from the cell and go on to infect other cells (Figure 1-1A).

Figure 1-1. Primate lentivirus replication, host restriction factors and viral countermeasures. (A) A diagram of the lentiviral replication cycle. Steps in the replication cycle are numbered as follows: 1. The virion binds CD4 on the cell surface via its envelope protein. 2. Co-receptor binding following CD4 engagement by the viral envelope protein. 3. Fusion of the viral membrane with the cellular membrane. 4. Deposition of the viral capsid into the cytoplasm. 5. Reverse transcription. 6. Nuclear import. 7. Integration. 8. Transcription of vrial RNAs (genomes and mRNA), nuclear export, and translation of viral proteins. 9. Virion assembly. 10. Virion budding. 11. Virion release and additional rounds of infection. Green lines indicate host genomic DNA and red lines denote viral DNA/RNA (B) Host restriction factors antagonize lentivirus replication. Trim5α blocks the completion of reverse transcription by destabilizing the viral capsid. SAMHD1 prevents the completion of reverse transcription by starving the reverse transcription complex of dNTPs. BST2 acts a molecular tether that prevents viral release and traps virions on the cell surface. APOBEC3G acts in the next infected cell where it induces hypermutation of the viral genome during reverse transcription. (C) Lentiviruses have evolved viral countermeasures to antagonize restriction factors. Viral proteins are indicated in red. Primate lentivirus Vpx and Vpr proteins have evolved to degrade SAMHD1 to increase available dNTP pools. Viral Vpu/Nef/Env have independently evolved to remove BST2 from sites of budding through a combination of degradation, sequestration and mislocalization. The Vif protein degrades APOBEC3 proteins in the cytoplasm to prevent their incorporation into virions. Notably, there are no reported examples of a virally encoded antagonist of Trim5α. To replicate in Trim5α expressing cells viruses must adapt their capsid proteins to avoid recognition.

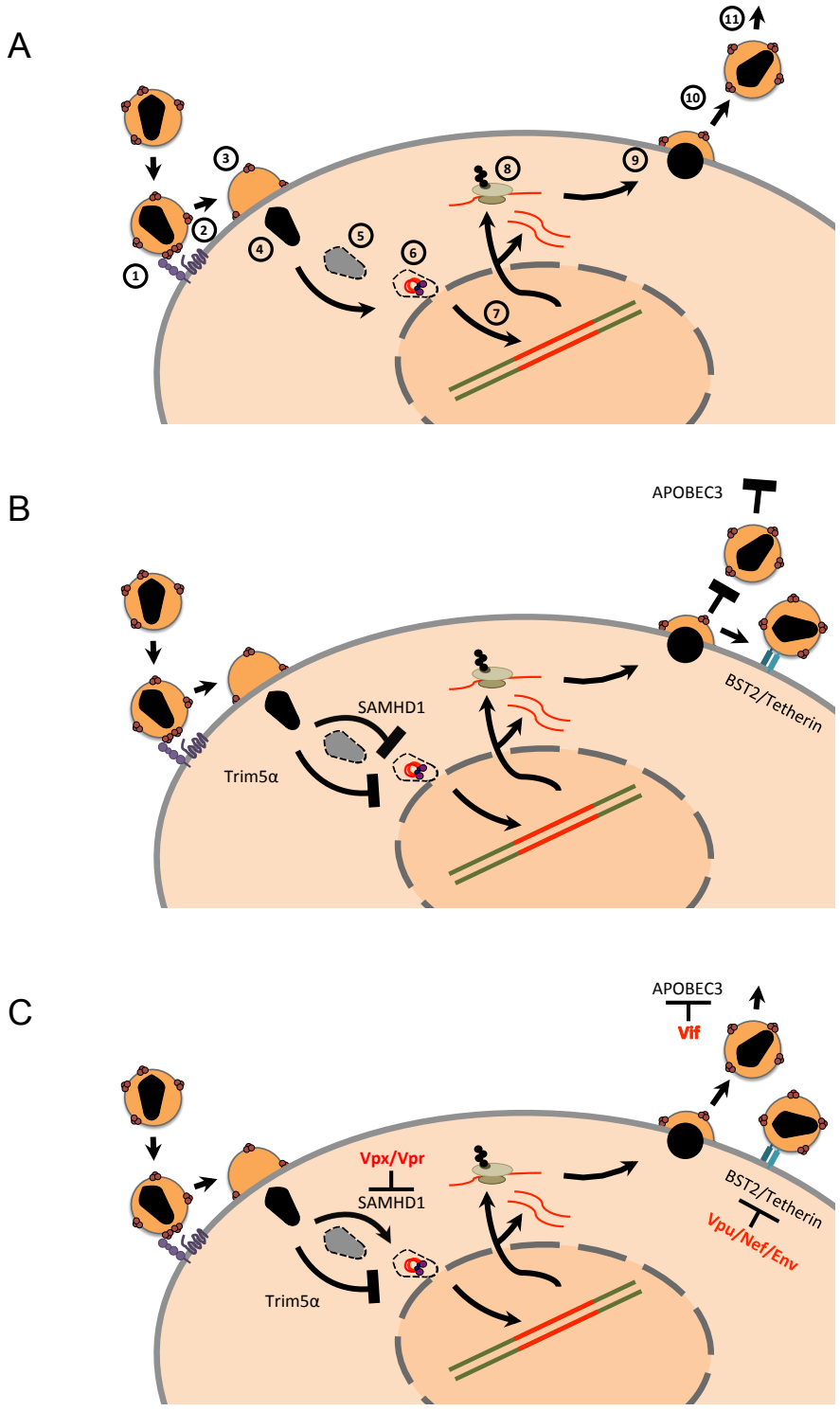

Figure 1-1. Primate lentivirus replication, host restriction factors and viral countermeasures: continued

Lentivirus accessory genes. Primate lentiviruses have had considerable evolutionary success, as evidenced by the fact that over 40 Old World primate species harbor at least one unique primate lentivirus [23-28]. The viral phylogeny is incongruent with that of the host phylogeny [23-25,27-29]. This indicates that primate lentiviruses have a longstanding evolutionary history of cross-species transmission. Replication in their native host species or a naïve species occurs despite the presence of rapidly evolving host restriction factors. This suggests that primate lentiviruses have evolved the required genetic plasticity to overcome restriction factors.

In addition to the core set of genes, *gag*, *pol* and *env* (Table 1-1), lentiviruses encode a variable number of proteins that serve a variety of modulatory functions. Null mutations in these accessory genes often result in attenuation of replication *in vivo*, even when there is little or no effect on virus replication in cell culture [30]. The accessory genes are clustered in the 3' half of the viral genome along with *env*, and separate from *gag*, *pro* and *pol* (Figure 1-2). It is tempting to speculate that physical separation of genes encoding conserved functions (structural proteins and enzymes) from variable functions (accessory proteins and surface glycoproteins) allows greater adaptive flexibility.

The accessory proteins are the most divergent lentivirus proteins (only the Env protein displays similar levels of diversity) (Figure 1-2). The complement of accessory genes is not

identical for all primate lentiviruses – for example, several do not have a *vpx* gene (e.g., HIV-1, SIVcpz) and many do not have *vpu* (e.g., SIVsmm, SIVmac) (Figure 1-2). If the comparison is expanded to include the "non-primate" lentiviruses (e.g., lentiviruses found in cats, cattle, horses and small ruminants), a distinct set of accessory genes is found, with only *vif* being common to both primate and non-primate lentiviruses (Table 1-1). Thus, lentivirus accessory genes vary in primary sequence and overall composition. The requirement for vif is not absolute among extant lentiviruses, it is notably absent from the equine infectious anemia virus (EIAV). Such evolutionary plasticity is consistent with the notion that accessory genes help determine species-tropism and may play a role in cross-species transmission and emergence of lentiviruses.

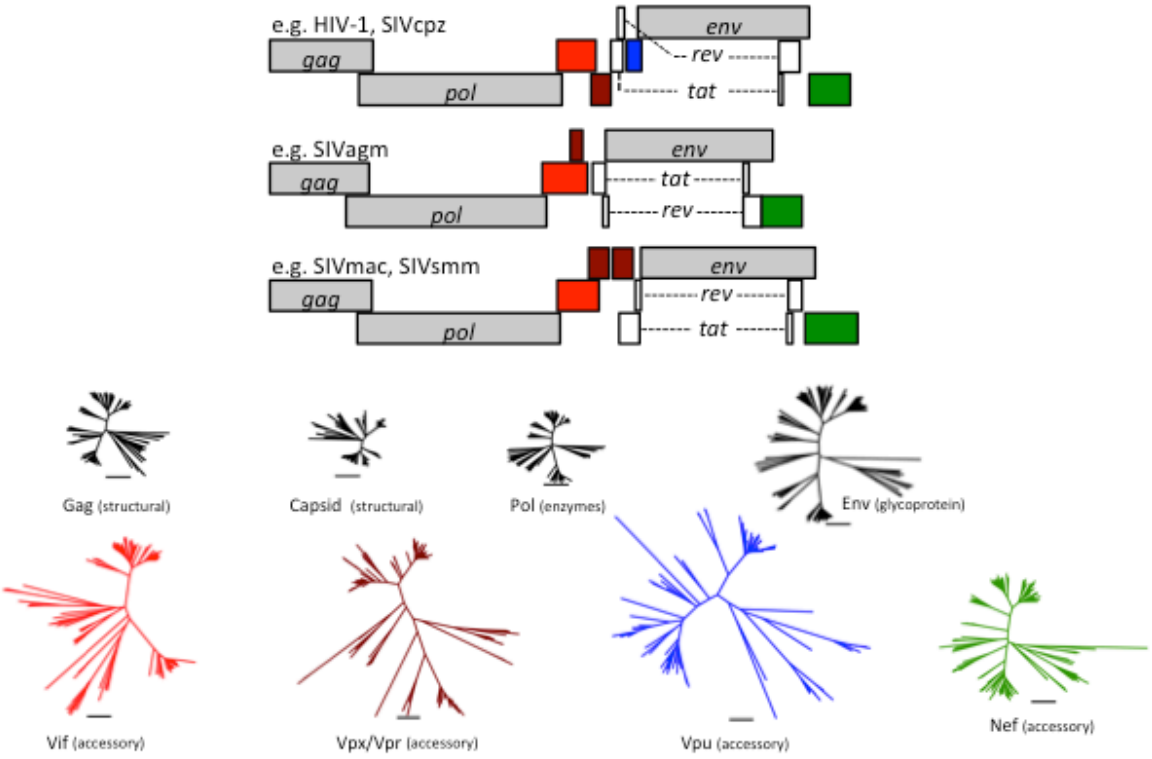

Figure 1-2. Accessory proteins are the most diverse of the primate lentivirus proteins. (A) Genomes of primate lentiviruses. Schematic representations of the three major types of genome organization found among primate lentiviruses. Genes encoding structural proteins (*gag*, *pol*, and *env*) are shown in gray. The regulatory genes, *tat* and *rev*, are in white. Accessory genes are color-coded to match the phylogenetic trees in the lower panel. (B) A comparison of genetic diversity among primate lentivirus proteins. Note that the accessory proteins are much more diverse than the structural proteins. Neighbor-Joining trees were generated using sequence alignments of primate lentiviruses available from Los Alamos National Labs (http://www.hiv.lanl.gov/). The Vpx and Vpr proteins are paralogs that arose by duplication during evolution of the primate lentiviruses and were therefore combined into a single tree. Scale bars below each gene indicate 20% diversity doi:10.1371/journal.ppat.1004017.g001

1. B. THE CROSS-SPECIES TRANSMISSION OF PRIMATE LENTIVIRUSES

Primates and the cross-species transmission of primate lentiviruses

Primates and primate lentiviruses. Within the past century humans have been infected with SIVs from wild chimpanzees, gorillas and sooty mangabeys on no less than 13 occasions [28]. Two independent cross-species transmissions of SIVs from captive macaques have also occurred as a result of laboratory accidents [31,32]. Of the 15 known SIV to human cross-species transmission events that have occurred in the past 100 years, only one has led to a global HIV-1 pandemic (HIV-1 Group M), while others have led to limited regional epidemics and in some cases have never spread beyond a single infected individual [25,28]. The factors that influence whether a virus infects a single individual or spreads to 60 million individuals have yet to be fully determined. However, through the study of primates and their lentiviruses a number of viral and host genetic determinants that influence this process have been identified.

Primates and the primate lentiviruses have proven to be a powerful system to study the process of cross-species transmission, adaption to the novel host and the dissemination of a virus throughout a population. To date, evidence of current SIV infection has been found in at least 45 primate species [25,27,28]. In nature, these viruses have only been isolated from

African primates. A majority of these reported SIVs have at least one reported full-length genome sequence. From these data it is clear that there are at least 40-50 genetically distinct primate lentiviruses circulating among African primates. SIVs are named for the species from which they are isolated, often by denoting the virus as "SIV", followed by three letters that are related to the species common name or genus and species. For example, an SIV isolated from a rhesus macaques is called SIVmac, while an SIV isolated from a pig-tailed macaque, *macaca nemestrina,* is SIVmne [33]. There is considerable genetic diversity among the primate lentiviruses or even within a single species' HIV/SIV. To put this diversity in context, there is less antigenic diversity in the glycoproteins of all known hepatitis C (HCV) genotypes (1-7) than what is found within the *env* gene of the pandemic strain of HIV-1, group M (Figure 1-3).

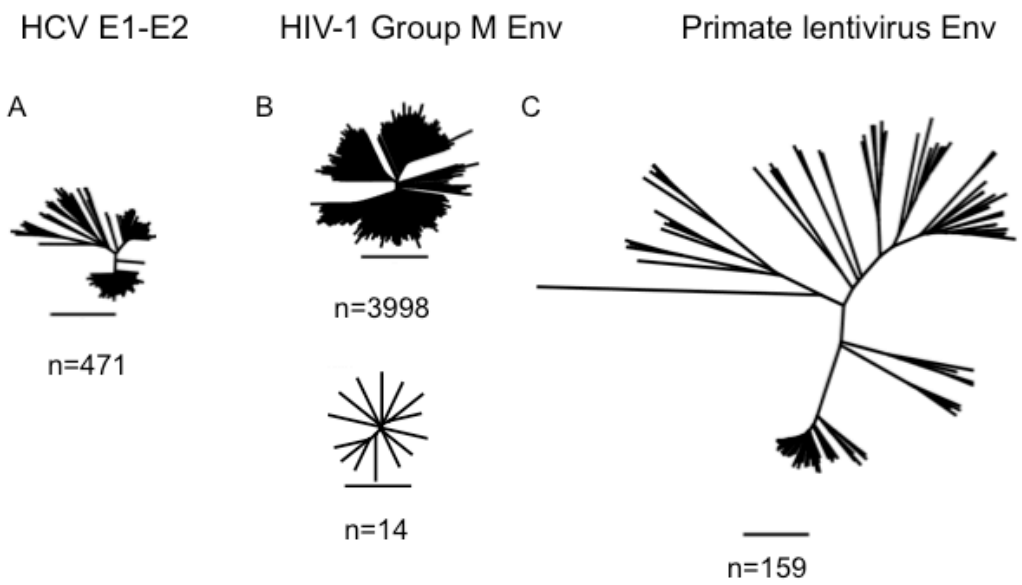

Figure 1-3. Primate lentiviruses are genetically diverse. A comparison of the genetic diversity among the glycoproteins of viruses known to establish chronic infection in their hosts: Hepatitis C, HIV-1 and primate lentiviruses. (A) Diversity of all Hepatitis C groups (groups 1-7) envelope proteins E1 and E2. The number of sequences included is 471 (n=471). (B) Envelope (GP160) diversity of HIV-1 group M. Top. Diversity of the envelope within the global HIV-1 pandemic. This diversity exceeds that off all Hepatitis C groups. The number of sequences used to generate the tree is 3998 (n=3998). Bottom. The diversity of the 14 (n=14) group M HIV-1 isolates that are included in C. The diversity of these 14 isolates is less than all Hepatitis C groups together. (C) Envelope diversity across primate lentiviruses. The diversity among primate lentivirus envelopes exceeds the diversity found in A and B. Scale bars indicate 20% diversity. Neighbor-Joining trees were generated using the curated protein sequence alignments of primate lentiviruses and Hepatitis C available from Los Alamos National Labs (http://www.hiv.lanl.gov/) and (http://www.hcv.lanl.gov).

An evolutionary history of cross-species transmission. The taxonomic order Primates includes prosimians, monkeys and apes/humans (Figure 1-4). Primate species have a well-defined phylogeny spanning at least 80 million years of evolution [34,35] and reviewed in [36]. Excluding humans (which originated in Africa), extant primate species naturally inhabit three continents, South America, Asia and Africa (including Madagascar), while evidence of extinct primates are found in Europe and North America [37]. Prosimians are only found in Asia and Africa [37]. Prosimians occupy two branches of the primate phylogeny that are basal to the monkeys and the apes/humans. New World monkeys are found in South America while Old World monkeys are found in Africa and Asia [37]. The separation of New World and Old World monkeys occurred approximately 40-45 million years ago [35]. The natural habitats of extant apes include Africa and Asia [37]. Apes/humans and Old World Monkeys last shared a common ancestor approximately 25-35 million years ago [34,35] and reviewed in [36]. The term Old World Monkey excludes apes/humans, while the term Old World primates includes apes/humans and monkeys. Due to their relation to humans and their medical importance, a number of primate genomes have been reported. For many more primate species there is a wealth of reported sequences for chromosomal regions or for targeted genes. Importantly, samples and cell lines remain accessible for targeted gene sequencing. Together, the well

established phylogeny and the wealth of sequence information have been indispensable for studying the evolution of genes that have the capacity to block primate lentivirus infection.

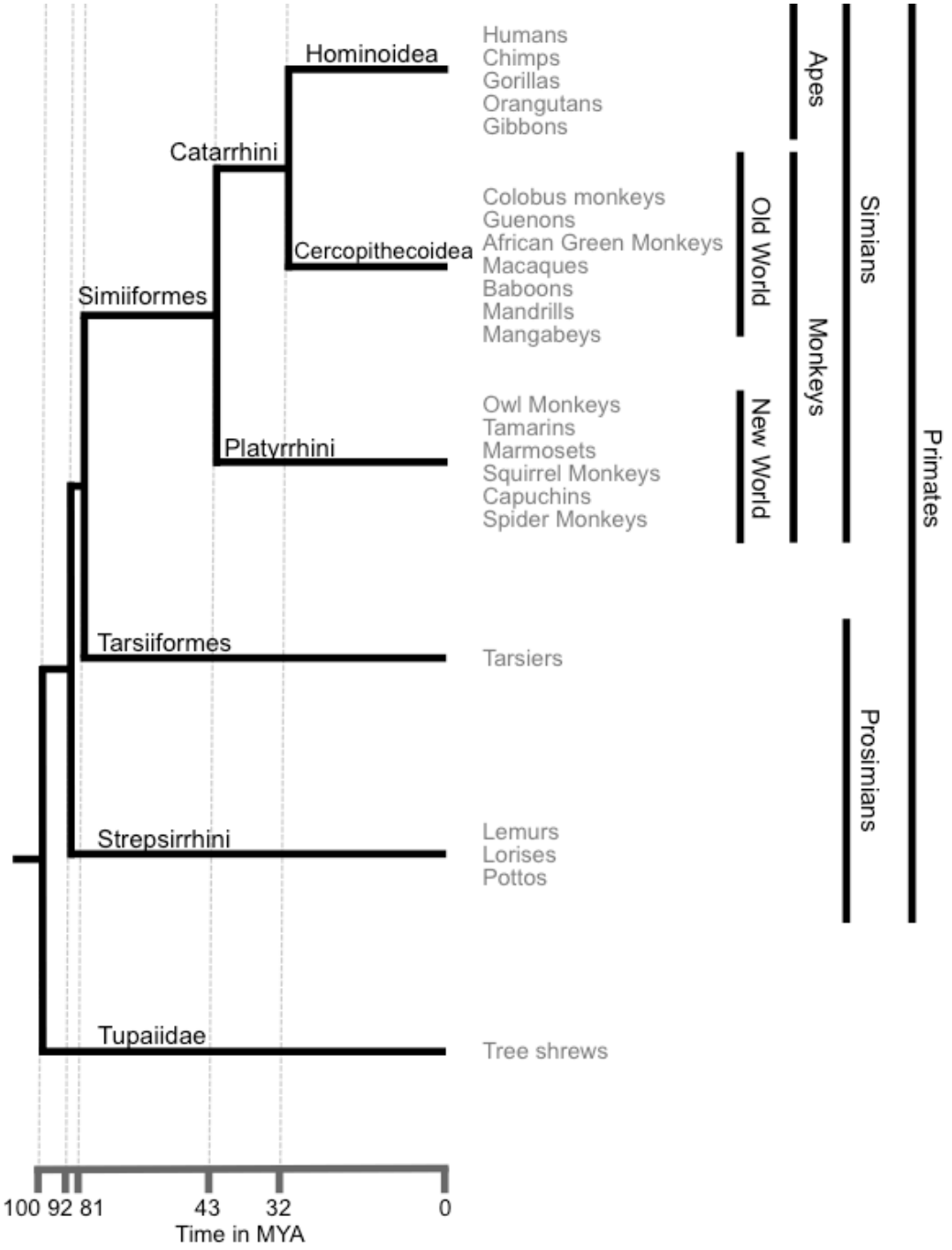

Figure 1-4. Primate phylogeny. A phylogenetic tree illustrating primate phylogeny and classification. Common names of species occupying each branch are provided. Estimated dates of divergence are indicated with the time scale bar. Dates of divergence are based on [35], phylogenetic tree adapted from [34].

Our collective knowledge of African primates and their primate lentiviruses has allowed for the construction of robust phylogenies. From these phylogenies it is apparent that the host (primate) phylogeny does not match that of the viral phylogeny [26,27,29] (Figure 1-5). This observation suggests that rather than diverging with their hosts, primate lentiviruses have a long evolutionary history of cross-species transmission. Each incongruence represents a separate viral cross-species transmission event between primates occupying different branches within the host or viral phylogeny. It is therefore likely that primate lentiviruses are particularly well suited to cross between species and rapidly adapt to a new host. It also implies that the cross-species transmission events resulting in HIV infection in humans is the continuation of an ancient pattern.

Counting the total number of cross-species transmission events based on the observed discordance between the host and virus phylogenies grossly underestimates the actual number of successful cross-species transmission events. From phylogenetic trees, it is impossible to determine whether related viruses infecting closely related species are the result of a cross-species transmission or co-divergence. For example, humans and chimpanzees are closely related species and they harbor closely related primate lentiviruses. Based on the comparison of the viral and host phylogenies it is unclear whether HIV-1 in humans is the result of co-divergence or cross-species transmission. It is only from other observations that we know that

SIVcpz was transmitted to humans at some point in the early twentieth century, rather than co-diverging from a last common ancestor 5-8 million years ago [28,38-40]. Similar conclusions have been reached for the 4 distinct SIVagm viruses which endemically infect the 4 subspecies of African green monkey [41]. Due to heavy surveillance we know that within the past century humans have been the recipients of at least 15 cross-species transmission events [26-28,31]. Though limited, these observations imply that cross-species transmission almost certainly occurs more often than the comparative virus-host phylogeny suggests.

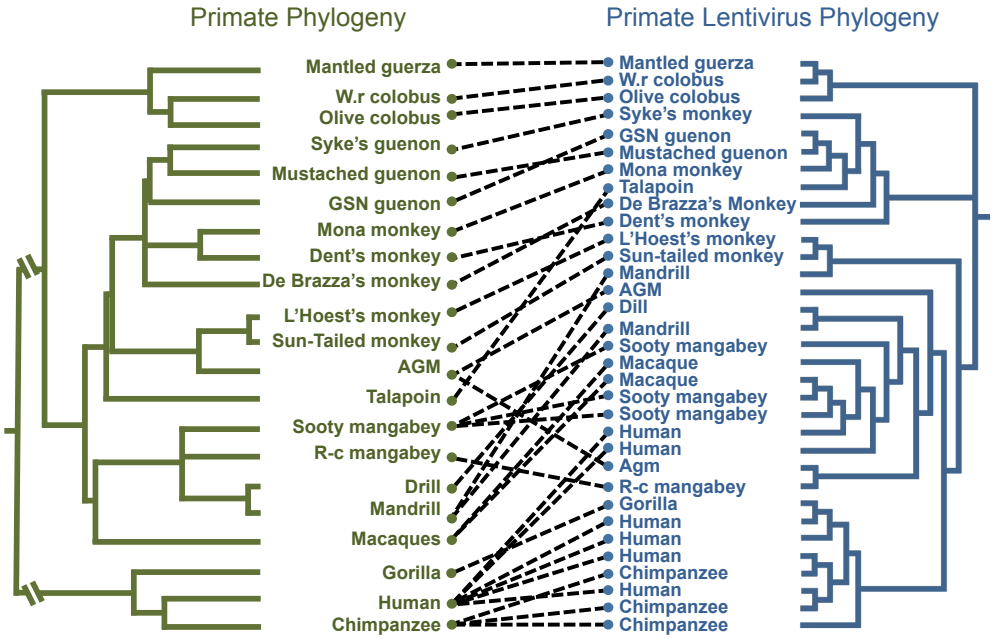

Figure 1-5. A history of cross-species transmission. The primate phylogeny (left) is incongruent with that of their SIVs (right). Lines connect species and their SIVs. The following abbreviations were used: R-c mangabey for red-capped mangabey, AGM for African green monkey, W.r colobus for Western red colobus, GSN guenon for Greater spot-nosed guenon. The primate phylogenetic tree is adapted from [34]. The SIV Neighbor-Joining trees was generated using the curated primate lentiviruses Pol alignment available from Los Alamos National Labs (http://www.hiv.lanl.gov/).

Primate lentiviruses are likely ancient. While the current distribution and genetic diversity found among primate lentiviruses suggests that they are ancient, no direct evidence supports their presence before the last Ice Age, 10,000 years ago [42,43]. Rising sea levels at the end of the last Ice Age isolated multiple SIVs and their host species on Bioko Island 10,000 years ago. Phylogenetically, these SIVs are interleaved with the primate lentiviruses isolated on the African mainland [42]. This observation suggests that SIVs were already widespread and genetically diverse at this time. Despite a lack of direct evidence, a growing number of observations support the notion that the relationship between African primates and primate lentiviruses is far more ancient. However, these age estimates can vary wildly depending on the method used to date their existence. Molecular clock estimates based on viral sequences almost unanimously conclude that SIVs are between tens of thousands to hundreds of thousands of years old [41-43]. In contrast, through phylogenetic analysis of novel host gene variants with the capacity to antagonize lentivirus replication in Old World monkeys the emergence of primate lentiviruses has been dated to 5-10 million years ago [44-51]. These observations fit with the observations made from the detection of ancient endogenous lentiviral sequences in the genomes of European brown rabbits, Malagasey lemurs and weasels, which demonstrates that lentiviruses existed and formed independent lineages at least 8-12 million years ago [52-55].

Primates and their lentiviruses offer a unique temporal view into to the process of cross-species transmission, adaptation, and dissemination of a virus throughout a population. The known histories of primate lentiviruses exceeds at least 5 orders of magnitude (1-10,000 years). The presence of primate lentiviruses on Bioko island suggests that some primate species like Drils, Guenons, and Colobus monkeys have harbored their SIVs for at least 10,000 years [42]. SIVcpz in chimpanzees arose through the cross-species transmission and recombination of two viruses related to those found in modern day guenons and mangabeys [29]. Molecular clock estimates date the emergence of SIVcpz to 300-1000 years ago [41]. The transmission of SIVcpz from chimpanzees to humans, which resulted in the emergence of epidemic HIV-1, occurred approximately 100 years ago [39,40]. The accidental infection of captive rhesus macaques with SIVsmm from sooty mangabeys resulted in SIVmac [56,57]. This virus freely circulated for 15-20 years before it was eradicated from United States primate colonies. This pathogenic SIVmac and the SIVmac239 molecular clone represent approximately 15-20 years of adaptation [23,56-58]. Experimental cross-species transmissions in laboratories involving sooty mangabeys, macaques, African green monkeys and their SIVs illustrate how viruses adapt to a new species on yearly or sub-yearly timescales [59-64].

Time and pathogenesis. There is a correlation between the amount of time a species has harbored a particular SIV and the clinical outcome of SIV infection. Specifically, despite high

viral loads, long-standing hosts tend to have apathogenic/asymptomatic SIV infections [24,25,28]. In captivity, SIV infection of African green monkeys with SIVagm or sooty mangabeys with SIVsmm is often accompanied by little to no pathogenesis [25]. This is in contrast to host species that have recently acquired a particular SIV. These species typically progress to an immunosuppressed sate [24,25]. Chimpanzees are believed to have acquired SIVcpz within the past 1000 years [41]. In nature, chimpanzees infected with SIVcpz have significantly shorter lifespans and lowered reproductive success than uninfected wild chimpanzees [65,66]. Humans acquired the pandemic lineage of HIV-1 group M approximately 100 years ago and usually progress to AIDS in the absence of medical intervention [26,28,38-40,43,67]. Infection of rhesus macaques with SIVmac results in simian AIDS, a condition similar in its symptomology to human AIDS [58,68]. While few in number, these examples suggest that successful cross-species transmission of primate lentiviruses can be coupled with pathogenic potential.

1. C. GENETIC BARRIERS TO THE CROSS-SPECIES TRANSMISSION OF PRIMATE LENTIVIRUSES

Adaptive immunity and passive genetic barriers

The evolution of barriers to cross-species transmission. Considering that primate lentiviruses have an evolutionary history that is laden with cross-species transmission events, together with the observation that successful cross-species transmission events can result in pathogenic infection, it is therefore very likely that the dynamic movement of primate lentiviruses between African primate hosts has driven the evolution of genes with anti-lentiviral activity. Accordingly, primate lentiviruses must have adapted (and must continue to adapt) to overcome barriers to replication in both their natural and acquired hosts following cross-species transmission events.

Adaptive immunity. The genes responsible for antigen recognition for the adaptive immune system have rapidly evolved [69]. Diversity within loci such as the major histocompatibility complex (MHC) locus has been maintained through balancing selection [69-75]. The MHC is responsible for the presentation of peptides from within the cell to the adaptive immune system. This includes peptides from pathogens that replicate intracellularly. Currently, it is impossible to determine whether lentivirus infection has contributed to the observed accelerated rates of evolution at this locus in Old World primates. Regardless, whether incidental or due directly to recurrent selection by primate lentiviruses, the adaptive immune system constitutes a significant barrier to the successful cross-species transmission of primate lentiviruses. Control of viral replication by cytotoxic CD8+ T-cells is observed in African green

monkeys that are experimentally infected with SIVmac [63]. Cytotoxic CD8+ T-cells have been implicated as factors in controlling the replication of modified HIV-1 viruses in pig-tailed macaques [76,77], as well as chimeric SIV-HIV (SHIV) viruses in various macaque species [78-83].

MHC influences the outcome of primate lentivirus infection when viruses are transmitted between closely related species or even between members of the same species. Rhesus macaques and crab-eating macaques shared a last common ancestor approximately 1 million years ago [34-36,84,85]. Experimental infection of crab-eating macaques with the rhesus macaque virus SIVmac239 can result in lower viral loads and slower disease progression than rhesus macaques inoculated with the same virus [86]. Differences between the MHC repertoire of rhesus and crab-eating macaques can lead to increased cytotoxic CD8+ cell responses and lowered viral loads [86-88]. Even within species with recently acquired primate lentiviruses the chance that a virus adapted to all possible MHC haplotypes is low due to balancing selection of diversity within the MHC locus. Indeed, both in humans and rhesus macaques the presence of specific HLA types is strongly associated with lower viral loads or even elite control, in addition to heterozygous advantage [87,89-91].

Passive barriers to viral entry. Primate lentiviruses utilize CD4 as their primary receptor [3,4,92]. Among primates, in particular Old World primates, the CD4 gene has evolved

under positive selection [93,94]. Based on the co-crystal structure between the HIV-1 glycoprotein soluble subunit GP120 and CD4, positively selected sites map to the D1 domain. The D1 domain makes extensive contacts with the CD4 binding pocket of the HIV-1 GP120 [95]. Furthermore, the specific amino acids substitutions that have been selected for alter the electrostatic surface of the GP120 binding interface in a primate lineage specific manner [94]. On this interface at least one site of difference between humans and macaques carries a functional consequence. The difference between the human N39 or macaque I39 largely accounts for the observed 20-50 fold reduction in the infectivity of primary transmitted founder HIV-1 viruses on macaque CD4 expressing cells [96]. Notably, HIV-1 envelopes have adapted *in vivo* to efficiency utilize the macaque receptor [96].

Upon CD4 engagement primate lentiviruses require a co-receptor to license the envelope for fusion [97]. While different proteins can serve as a co-receptor, the co-receptor is almost always a chemokine receptor. Among primate lentiviruses, including HIV-1, there is a very strong bias for the usage of CCR5 [98]. In at least three independent cases during Old World primate evolution, deletions in CCR5 have evolved that greatly reduce or entirely block cell surface expression. Both sooty and red-capped mangabey species share a 24 base pair in-frame deletion (CCR5Δ24) that prevents surface expression [99,100]. An additional variant with a novel 2 amino acid deletion is also found in sooty mangabeys [101]. This deletion reduces cell

surface expression. Similarly, approximately 10% of Caucasian humans have a CCR5 allele that is defective for cell surface expression due to a frame shift mutation that results in a 32 amino acid truncation [102,103]. Both sooty and red-capped mangabeys are believed to have harbored their respective SIVs long enough to have coevolved to a state of apathogenesis. It is also likely that in nature they are exposed to other SIVs. Therefore, there exists the likelihood that these adaptations could have been selected by primate lentiviruses. For humans the identity of the agent that selected for the 32 amino acid deletion remains controversial [104,105]. Nonetheless, the presence of this polymorphism constitutes a significant barrier to HIV-1 infection and has been exploited to cure HIV-1 infection [102,103,106-108].

For each of these examples, the reduction or lack of CCR5 expression has selected for viruses that utilize alternative co-receptors. Among red-capped mangabeys, the CCR5Δ24 polymorphism exists at a very high frequency. As a result, the entire SIVrcm lineage has evolved to use another chemokine receptor (CCR2b) as a primary co-receptor [99]. In sooty mangabeys the presence of two independent CCR5 alleles with deletions that limit surface expression has selected for viruses that have gained the ability to use a number of alternative co-receptors. These alternative co-receptors are either chemokine receptors or orphan G protein-coupled receptors [98,101]. In humans, the significant reduction of CCR5 expressing

cells during the late stages of AIDS progression is correlated with the emergence of viruses utilizing the chemokine receptor CXCR4 [109-113].

While the barriers to successful cross-species transmission imposed by the above examples are mechanistically different, they share a common quality. The evolution of these genes (MHC, CD4, CCR5) is subject to evolutionary constraints far beyond their interactions with primate lentiviruses. Evolutionary pressures on the MHC are exerted by a multitude of pathogens. While there can be enrichment for specific MHC alleles, positive selection and balancing selection of MHC alleles have clearly selected for diversity within the MHC locus [69,70,74,114]. Furthermore, since the MHC presents self-peptides in addition to pathogen peptides there is likely selection against promiscuous peptide presentation which can result in autoimmune disease or hypersensitivity reactions [115-117]. Interestingly, the MHC variants most associated with control of HIV-1 infection are also associated with autoimmunity [118]. While no apparent phenotype is associated with the loss of cell surface expression of CCR5, its presence across mammalian species argues that CCR5 must carry some selective advantage. Perhaps the clearest example of evolutionary constraints comes from CD4. The primary purpose of CD4 is to interact with MHC class II on antigen presenting cells. This interface is the same one exploited by primate lentivirus envelope proteins [95,114,119]. Therefore, despite selective pressures from primate lentiviruses there is likely a greater selective pressure to

maintain the interaction with MHC class II [93,94]. Together, these observations predict that the most potent blocks to cross-species transmission events would be mediated by proteins whose evolution is not constrained by other cellular/organismal functions.

Intrinsic immunity and restriction factors

Intrinsic immunity. Intrinsic immunity is distinct from adaptive and innate immunity. In the context of this thesis, the name is used to encompass a number of complex phenotypes, all of which describe an observed cellular resistance to the replication of specific viruses. It does not rely on pre-exposure to viruses or specific classes of specialized effector cells and is thus distinct from adaptive immunity. Intrinsic immunity differs from innate immunity in that it does not describe a general anti-viral state but rather a specific cellular resistance to specific viruses. In addition, the mediators of intrinsic immunity actively antagonize viral replication while the innate immune system signals to stimulate the production of antiviral factors. This property, together with the observation that the mediators of intrinsic immunity are dominant acting, separates intrinsic immunity from virus-cell incompatibles or other passive blocks to viral infection. The mediators of intrinsic immunity are often referred to as host restriction factors.

Restriction factors. Restriction factors are cellular factors that block virus infection, either by direct interaction with viral factors or by rendering the cellular environment incompatible with viral replication. Well-characterized primate lentivirus restriction factors include SAMHD1 [120,121], BST-2/tetherin [122,123], APOBEC3G [124] and TRIM5α [125] (Figure 1-1B). In general, viruses evolve resistance to the restriction factors of their natural hosts, but may still be sensitive to homologs of the same restriction factors from other organisms. Thus, restriction factors are potentially major determinants of virus host-range in nature. Much of the evidence favoring this hypothesis comes from comparative studies of the primate lentiviruses.

The term “restriction factor” is not intended to reflect any additional relationships such as genetic relatedness or mechanism of action and in fact the mechanisms by which each restriction factor blocks viral replication is unique. These proteins are also genetically unrelated. The identified host restriction factors for primate lentiviruses have come from different gene families, for example the cytidine-deaminase family [124,126,127], tripartite motif family [128,129], or sterile alpha motif and HD-phosphohydrolase families of proteins [120,121,130].

While there is no formal definition for what constitutes a host restriction factor, generally well-studied restriction factors share many common attributes. In the context of primate lentivirus infection these factors generally: are dominant acting, actively antagonize viral

processes (or proteins), have primarily evolved to meditate antiviral activity (thus, restriction factors most likely evolved without the evolutionary constraints of performing other cellular functions), and evolved under positive selection (or sites therein). Finally, it is usually found that viruses have evolved resistance to their host's restriction factors or that other related viruses evolve resistance to a host's restriction factors following a cross-species transmission event.

SAMHD1: The myeloid block to reverse transcription

SAMHD1. Despite the expression of the receptors that HIV-1 does not efficiently infect resting CD4 T-cells or myeloid cells. In contrast, HIV-2 and some SIVs achieve significantly higher titers on these cells [131-137]. HIV-1 does not encode a Vpx protein, however the addition of Vpx-containing virus like particles to HIV-1 infections (or HIV-1 derived gene therapy vectors) can dramatically boost HIV-1 titers [138]. SAMHD1 was immunoprecipitated from human myeloid cells using the Vpx proteins of HIV-2/SIVs [120,121]. SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase [120,121,139,140].Its enzymatic activity depletes dNTP pools within terminally differentiated target cells, such as macrophages, thereby starving the reverse transcription complex of dNTPs [139,141] (Figure 1-1B). Supplementation of SAMHD1 expressing cells with exogenous dNTPs can partially restore HIV-1 titers [141-143].

Vpx, and some SIV Vpr proteins, have anti-SAMHD1 activity [120,121,144,145]. Both Vpx and Vpr are incorporated into virions during assembly and are delivered to the target cell during entry. These virion-delivered Vpx/Vpr proteins engage cellular ubiquitin ligase machinery, specifically the CUL4 complex, to degrade SAMHD1 in the target cell [120,121] (Figure 1-1C). Presumably, this tips the balance of dNTPs in the target cell such that the concentration is above the Km of the reverse transcriptase [141]. A few amino acid positions of SAMHD1 have been shown to have evolved under positive selection [144,145]. These sites occur at the N and C termini of the protein. This finding fits with the observation that different SIV Vpx/Vpr proteins engage different regions of SAMHD1 [144-146].

Curiously, HIV-1 (and some other primate lentiviruses) does not encode Vpx or its functional equivalent [120,121,144-146]. Wild type HIV-1 is poorly infectious in cells that naturally express active SAMHD1 proteins [120,121,139-142,145,147-153]. Therefore, it does not overtly appear that HIV-1 has a mechanism to antagonize SAMHD1 restriction. In contrast, antagonism of SAMHD1 is a conserved property among primate lentiviruses that have a Vpx protein and among those with related Vpr proteins [144-146]. This argues that antagonism of SAMHD1 is biologically relevant, however its relevance *in vivo* is yet to be defined. Interestingly, SIVmac239 viruses modified to lack Vpx, Vpr or Vpx-Vpr, are pathogenic in rhesus macaques [154]. It should be noted that in nature SIVs with anti-SAMHD1 activity are found in hosts in

which the virus has coevolved to a state of apathogenesis [24,25,144-146]. Correspondingly, these animals do not have the same patterns of chronic immune activation observed in humans infected with HIV-1 or macaque species infected with SIVmac [25]. Therefore, SAMHD1 antagonism may be more important in situations where CD4+ T-cells are resting and there are less activated CD4+ monocytes to infect.

BST2: The block to virion release

BST2/tetherin. Production of HIV-1 molecular clones lacking a functional Vpu protein is blocked at a late stage of the budding process in some human cells [155-158]. Thus, under specific conditions Vpu is required for efficient virion release. Cells that are not permissive for efficient virion release have been shown to accumulate fully matured virions at the plasma membrane and in clathrin coated pits [155,156]. The factor inhibiting release was shown to be dominant acting as demonstrated by heterokaryons between permissive and non-permissive cells [159]. Virions can be released from the cells following protease treatment, indicating that the restricting factor was a protein and was present on the cell surface [158]. Finally, it was shown that treatment with interferon could render once permissive cells non-permissive [160]. The above observations led to the identification of BST2 (tetherin/CD317/HM1.24) as the

mediator of these phenotypes [122,123]. BST2 has been shown to act as a molecular “tether” that traps fully budded virions on the surfaces of infected cells [122,123] (Figure 1-1B). The accessory gene Vpu efficiently removes BST2 from the plasma membrane, trapping it in intracellular compartments while also targeting it for degradation [161,162] (Figure 1-1C).

Most primate lentiviruses do not encode a Vpu gene. This includes HIV-2, which like HIV-1 which infects humans. However, nearly all primate species encode functional BST2 genes [122,123,163-166]. The Vpu independent mechanisms employed by primate lentiviruses to overcome the BST2 mediated block showcases the inherent plasticity of primate lentiviral proteins. Most other primate lentiviruses, including SIVmac, use Nef to overcome the BST2 mediated block [163-165] (Figure 1-1C). Nef interacts with the cytoplasmic tail of BST2 to trap it in a perinuclear compartment. The amino acids in the cytoplasmic tail of BST2 that make up the Nef binding site were lost during *Homo* evolution [163,164,167]. This renders human BST2 resistant to Nef antagonism.

The HIV-1 virus is a recombinant between two other SIVs. The parental viruses of HIV/SIVcpz/SIVgor are most closely related to SIVs found in modern day red-capped mangabeys (SIVrcm) and those found in modern day greater-spot-nosed monkeys/mustached guenons (SIVmus/SIVgsn) [29]. The Vpu gene of HIV/SIVcpz/SIVgor is derived from the SIVmus-SIVgsn lineage while the HIV/SIVcpz/SIVgor Nef is related to those found in modern

day SIVrcm isolates. In the SIVmus/SIVgsn lineage Vpu antagonizes mustached and greater spot-nosed guenon BST2s [165]. In the SIVrcm lineage Nef antagonizes red-capped mangabey BST2. The recombination event that generated the HIV/SIVcpz/SIVgor lineage probably occurred in an ape, most likely a chimpanzee [29]. Species-specific differences in the transmembrane domain of chimpanzee BST2 prevented the Vpu gene of the recombinant virus from counteracting ape BST2s. Instead, Nef evolved to acquire the ability to antagonize chimpanzee BST2 [165]. When this virus was transmitted to humans lacking the Nef binding motif, the recombinant virus re-adapted to utilize Vpu as a BST2 antagonist [165,168]. Interestingly, among the 4 HIV-1 groups (M, N, O, P) there is a correlation between the ability to antagonize BST2 and the success of the virus in humans [165,168,169].

HIV-2 is the result of the cross-species transmission of SIVs from sooty mangabeys to humans. The HIV-2 Nef protein is also unable to counteract human BST2. Instead, a successful lineage of HIV-2 has acquired the ability to antagonize human BST2 mediated restriction via its Env protein [170] (Figure 1-1C). The interaction causes the entrapment of human BST2 in a perinuclear compartment similar to what is observed with Nef proteins [163,170]. SIVmac clones lacking a *nef* gene have been used as a live-attenuated vaccine model in macaques (SIVmac239ΔNef) [171]. At a low frequency these viruses can re-acquire virulence in adult macaques [172-175]. One viral lineage that re-acquired virulence adapted to overcome the

rhesus macaque BST2 mediated block in the absence of Nef. In a case of convergent evolution, this virus also gained the ability to antagonize rhesus macaque BST2 via changes in its Env protein [174,176].

From these *in vivo* examples it appears that there is a significant selective pressure to overcome the BST2 mediated block. This may be related to the direct inhibition of viral release from infected cells. However, animals vaccinated with SIVmac239ΔNef also have higher titers of antibodies that mediate antibody-dependent cell-mediated cytotoxicity, possibly due to the increased accumulation of virions on the cell surface [177]. In parallel, *in vitro*, HIV-1 viruses expressing mutant Vpu proteins that are incapable of antagonizing human BST2 have increased sensitivity to antibody-dependent cell-mediated cytotoxicity [178].

APOBEC3 Family: A block to infection upon a second round of replication

APOBEC3G. Almost all lentiviruses have a *vif* gene. Vif is an acronym for viral infectivity factor, which came from the observation that viruses lacking Vif fail to establish spreading replication in human peripheral blood mononuclear cells (PBMC) and some human T-cell lines [179-183]. Perplexingly, the virions produced from these cells were apparently indistinguishable from wild type viruses but yielded dramatically lower titers. Heterokaryon fusions between

permissive and non-permissive cells confirmed the presence of a dominant acting, producer cell mediated, block to infection [181,183]. Using the differential permissibility between two related cell lines, the RNA transcript encoding APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like G) was identified as the coding RNA responsible for the observed block to infection [124].

In the absence of Vif, APOBEC3G has been shown to incorporate into assembling virions in the producer cell [124]. Reverse transcription in the cytoplasm of the next target cell produces a minus-strand DNA intermediate, which is attacked by APOBEC3G (Figure 1-1B). Deamination of cytosines to uracils in the viral minus-strand DNA produces C-to-U mutations, resulting in the accumulation of G-to-A substitutions in the coding strand, often referred to as hypermutation [127,184-186]. Additional deamination independent mechanisms have also been observed [187]. Other APOBEC3 family members have been shown to have anti-lentiviral activity, in particular APOBEC3D, APOBEC3H and APOBEC3F ([188,189] and reviewed in [190-192]). The presence of Vif prevents the incorporation of APOBEC3 into virions [124] (Figure 1-1C). This is accomplished via the depletion of APOBEC3 family members from the producer cell by ubiquitin mediated degradation, mediated by a Vif complex that includes CBFβ and CUL5 in complex with RBX2, ELOB and ELOC and the target APOBEC3 [193-196].

APOBEC3G was the first primate lentivirus host restriction factor to be identified. The observations made from the characterization of the Vif-APOBEC3G interaction have helped define primate lentivirus restriction factors. Importantly, it was demonstrated that APOBEC3G was a dominant acting factor that inhibited lentiviral infection [124,181,183]. Viruses, including HIV-1, have evolved to overcome their host's APOBEC3G. The interaction between the Vif proteins and primate lentiviruses was shown to be species specific [197-200]. For example, HIV-1 Vif is incapable of degrading rhesus macaque or African green monkey APOBEC3G [198,201]. This phenotype is due to the accelerated rate of evolution of APOBEC3G, which has been shown to have evolved under strong positive selection [202,203]. Indeed at least some of the positively selected sites and insertions that have been selected for cluster to a single common Vif-APOBEC3G interaction face [44,199,200,204-206]

TRIM5α: A capsid-dependent post entry block

TRIM5α. TRIM5α is a broadly restrictive retrovirus-specific restriction factor. Both the nature of the interaction between retroviruses and TRIM5α and how retroviruses and TRIM5α have co-evolved are fundamentally different than the three other restriction factors discussed above. TRIM5α specifically targets the retroviral capsid shortly after it is deposited into the cell

following membrane fusion [125,201,207]. TRIM5α binding usually results in the premature uncoating of the viral capsid and degradation of its contents [12,208,209]. How TRIM5α recognizes divergent retroviruses is unknown, but likely involves multimerization on the surface of incoming capsids through a series of high-avidity/low-affinity interactions [208,210-213]. Unlike the other restriction factors, TRIM5α has not been shown to directly restrict any non-retrovirus viruses. Thus, it appears to target a highly conserved pattern that is unique to retroviruses. Among the Retroviridae, including the complex and accessory gene rich primate lentiviruses, no viral antagonists of TRIM5α have been reported. Instead, resistance (or sensitivity) to TRIM5α is determined directly by variation in the viral capsid.

Viral strategies for evading restriction

Common mechanisms to evade restriction factors. The Vif and Vpx/Vpr proteins use similar mechanisms to overcome restriction by APOBEC3 and SAMHD1, respectively. In both cases, the viral protein couples its target to ubiquitin ligase complexes, resulting in proteasome-mediated degradation of the restriction factor [192,214]. Vpu also engages cellular ubiquitin ligase complexes, which may contribute, in part, to removal or sequestration of BST-2/tetherin from the cell surface [122,123,161,162]. Vpr is a paralog of Vpx, and like Vpx, it interacts with

the cellular ubiquitin ligase machinery [120,121,215]. The cellular target of HIV-1 Vpr remains to be discovered, although in some SIV lineages Vpr has anti-SAMHD1 activity [145].

Similar to some of the activities of Vpu, primate lentivirus Nef/Env proteins modulate cell-surface expression of many cellular proteins, and for some SIV strains, Nef is the primary antagonist of BST-2 [163,164]. Interestingly, when SIVsmm jumped to humans and became HIV-2, the Nef protein could not interact with human BST-2 [28,163,164]. Consequently, the Env protein of HIV-2 evolved the capacity to counteract human BST-2 [170]. Similarly, when a SIVmac lacking a *nef* gene was passaged in macaques, BST2 imposed selection pressures that selected for changes in Env that impart anti-BST2 activity [174,176]. These envelopes induce the mislocalization and entrapment of BST2 in a perinuclear compartment [176].

Restriction factors target general viral signatures. SAMHD1, BST-2 and APOBEC3G are evolutionarily and mechanistically distinct from one another, but share a common feature – the means by which they inhibit viruses are relatively non-specific. For example, SAMHD1 inhibits viral replication indirectly by limiting the availability of precursors of DNA synthesis [120,121,139,141,214], potentially affecting any virus for which DNA synthesis is essential [147]. Likewise, BST-2 can, in theory, “capture” any membrane-enveloped structure (e.g., a virion) as it buds from the cell surface [192,214]. APOBEC3G acts on single-stranded DNA produced during reverse-transcription, but there is no evidence that the enzyme has a selective

preference for viral DNA over other single-stranded DNAs. In other words, none of these factors targets a specific viral protein and consequently, viral resistance does not result from escape mutations in a binding site or epitope. Instead, all three factors are targeted by viral accessory proteins, and interactions with these viral antagonists are primary determinants of viral sensitivity to restriction.

1. D. TRIM5α.

Identification of TRIM5

The monkey block. Experiments to develop a non-human primate model for HIV-1 infection began to define a post entry block to lentivirus infection that would later be attributed to TRIM5α. In contrast to chimpanzees, experimental cross-species transmissions of HIV-1 to captive monkeys demonstrated that they were resistant to HIV-1 infection [216-219]. Cultured monkey peripheral blood monocytes and monkey cell lines demonstrated that the observed resistance to HIV-1 was an intrinsic cellular property. The isolation of an SIV from captive macaques in the United States (SIVmac) demonstrated that monkeys were not generally

resistant to lentiviruses [58]. Together, HIV-1, SIVmac and primate cells (including those of humans) were used to characterize the lentivirus susceptibility factor (LV1).

Before the gene responsible for the LV1 block was identified, the viral and cellular determinants were established. Viruses expressing heterologous envelopes demonstrated that the block was independent of receptor usage or entry pathway [201]. Consistent with this observation, the Gag protein was shown to govern sensitivity to the block [201]. The virally encoded determinant was serially mapped from Gag, to capsid-p2 and finally to the first 204 amino acids of the capsid protein [201,220,221]. Additional evidence for a post entry block came from the study of reverse transcription intermediates. These indicated that in restrictive cells the accumulation of late reverse transcription products was inhibited [207,222,223]. All other downstream events in the viral replication cycle were also blocked. These observations suggested the block occurred in the cytoplasm of the target cell. Heterokaryons generated from non-permissive and permissive cells indicated that the block was dominant and not due to species dependent host factor incompatibilities [223-225]. Further support for an active dominant block came from the observation that infectivity of a restricted virus could at least in part be rescued using high-titered viruses or by saturating target cells with other restricted viruses [223,224,226].

The differential infectivity of HIV-1 and SIVmac on primate cells was used to identify TRIM5 as the cellular mediator of the LV1 block [49,125]. The restriction factor TRIM5α was discovered by screening human cells transduced with a rhesus macaque cDNA library for cells that resisted HIV-1 replication [125]. A similar pattern of restriction was observed in cells derived from owl monkeys, a New World primate species. Most other New World monkey species are permissive for HIV-1 infection but not for SIVmac239 [227-229]. A unique property of the block to infection in owl monkey cells led to the independent identification of TRIM5. The addition of cyclosporine, an inhibitor of cyclophilin A, could render owl monkey cells permissive to HIV-1 infection [230]. Cyclophilin A is an HIV-1 cofactor that binds to the HIV-1 capsid [231-233]. Furthermore, mutations in capsid that disrupt the interaction of HIV-1 with cyclophilin A also rescued HIV-1 infectivity in owl monkey cells [230]. These properties were used to identify a unique mRNA transcript in owl monkey cells that encoded a TRIM5α-cyclophilin A fusion protein. This fusion protein arose from a retrotransposition event that inserted a cyclophilin A mRNA into the owl monkey TRIM5α locus [49].

TRIM5 is a member of the tripartite motif containing super family of genes. The tripartite motif is composed of an invariant sequence of domains, a RING, followed by at least one B-Box and then a coiled coil domain [128,129] (Figure 1-6). In contrast to the predictable pattern of domains within the tripartite motif, TRIM proteins encode a wide variety of C-terminal domains.

The C-terminal domains are believed to give the TRIM proteins their substrate binding specificities. The alpha isoform of TRIM5, like a subset of TRIM proteins, encodes for a C-terminal PRYSPRY/B30.2 domain [128] (Figure 1-6). This arrangement is shared among at least 14 known human TRIM proteins. TRIM5s modified to lack a C-terminal PRYSPRY or the delta isoform lack the capacity to restrict viruses [125].

While initially discovered by its anti-lentiviral activity, TRIM5α is broadly active against many different retroviruses. Primate TRIM5αs have been shown to restrict retroviruses from at least four of the seven retroviral genera [125,229,234-236]. Remarkably, there are multiple examples of a single TRIM5α ortholog with the ability to restrict viruses from at least three retroviral genera [229,236,237]. These viruses may have last shared a common ancestor in excess of a hundred million years ago [238]. This observation supports the notion that TRIM5α must recognize a broadly conserved pattern common to retroviruses. Curiously, primate TRIM5αs often fail to restrict all viruses within a single retrovirus genus or may differentially restrict closely related virus [60,61,125,234,235,239,240]. Retroviral restriction by TRIM5α is not a property specific to primates. Endogenous expression of TRIM5α in many mammalian species has been demonstrated to have anti-retroviral activity [227,241,242].

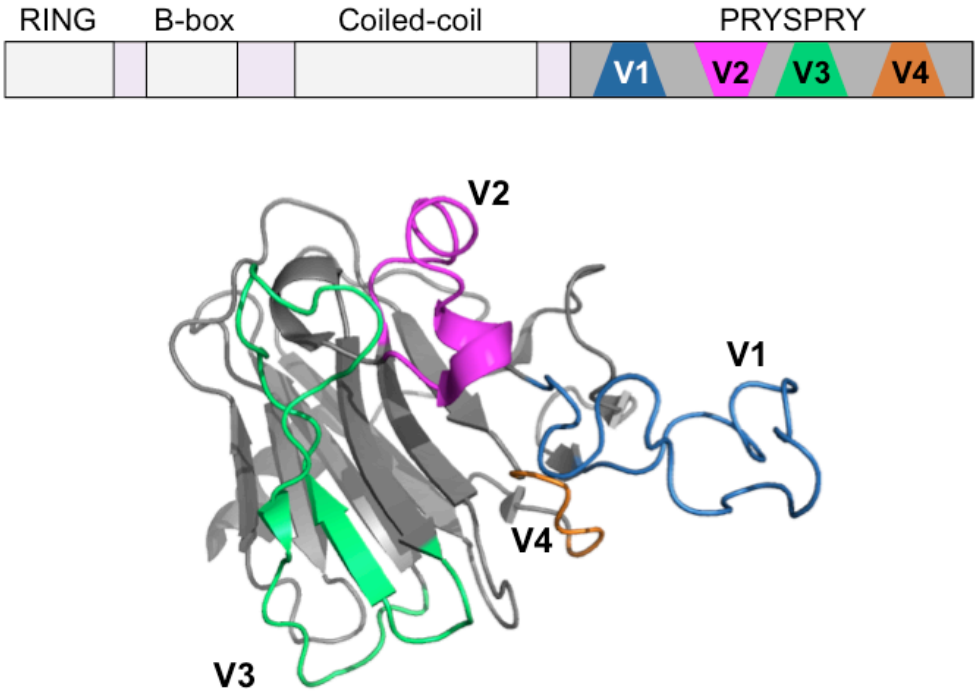

Figure 1-6 Trim5α and the PRYSPRY domain. Top. A schematic representation of Trim5α. The variable loops within the PRYSPRY (V1-4) are highlighted for emphasis. Bottom. Structure of the rhesus macaque PRYSPRY domain (PDB:2LM3). Variable loops are labeled and colored according to the top schematic. Structural images created in PyMol

Evolution of the TRIM5α PRYSPRY domain

The variable loops have evolved to target retroviral capsids. Comparisons between the amino acid sequences of TRIM5α PRYSPRY domains have identified a pattern of alternating conserved and variable regions. Among TRIM5α PRYSPRY domains there are four stretches of amino acids that differ in their length and amino acid composition. For this reason, these variable stretches are referred to as variable loops and are designated V1 to V4 [243,244]. Regions of conservation flank these variable stretches. This pattern is also observed among other closely related TRIM proteins. For example, TRIM5 is found on human chromosome 11 in a cluster of highly related but functionally distinct TRIMS: TRIM22, TRIM6 and TRIM34. These genes are thought to have arisen via duplication of an ancestral gene. Among these four TRIMs, the constant regions are nearly identical while the variable regions differ in both length and sequence. It is therefore likely that different evolutionary constraints act upon the variable and conserved regions. Both TRIM5α and TRIM22 have reported antiviral activities and both have been shown to have evolved under strong positive selection [243,245]. All of the positively selected sites within the PRYSPRY domain can be mapped to the variable regions.

Reported structures of PRYSPRY domains put these observations in context. Representative structures of this domain, including that of rhesus macaque TRIM5α, indicate that they share a conserved architecture [211,246-250]. The conserved regions of the PRYSPRY domain map to a structurally conserved β-barrel core. The variable regions map to a series of loops that extend off of the β-barrel core. The structure of TRIM21 bound to its natural ligand, IgG-Fc, serves as a conceptual framework for how the PRYSPRY domain of TRIM5α may engage retroviral capsids. In this structure the variable loops are the critical mediators of substrate binding [248].

The evolution of the TRIM5α PRYSPRY domain across primates is consistent with the notion that TRIM5α has evolved to restrict retroviruses. Retroviruses are a diverse viral family, and as evidenced by HIV-1, they have the capacity to rapidly evolve. Fittingly, the variable loops within the PRYSPRY domain of TRIM5α have been shown to have rapidly evolved under positive selection [243]. Few amino acid differences are present within the conserved β-barrel core, while the sequences of the variable loops differ in both length and amino acid composition [243,244,251]. While the variable loops harbor the majority of positively selected sites, it should be noted that insertions and deletions are typically excluded from standard measures of positive selection. However, the maintenance and further evolution of insertions in TRIM5α indicate that they have been selected for, likely by retroviruses. Based on the primate phylogeny it is likely

that the diversity seen among primate TRIM5α orthologs is the result of different selective events. These selective events were probably the result of different retroviruses.

Multiple lines of evidence have converged to implicate the involvement of the TRIM5α variable loops in capsid targeting. Molecular approaches have involved mutagenesis or the production of chimeric TRIM5α proteins by swapping select regions or entire variable loops between TRIM5αs [211,243,252-267]. All of these approaches have demonstrated that modulation of the variable loop sequences can dramatically impact restriction. For example, a single amino acid substitution in human TRIM5α can confer the ability to restrict SIVmac239, a virus that is normally resistant to human TRIM5α [252,268]. Similarly, TRIM5αs with chimeric variable loops (exchanged between two TRIM5α) have been shown to restrict viruses in a pattern more similar to the loop donor than the parental loop recipient [243,252-254,257,266]. Scanning mutagenesis has identified patches of amino acids within the variable loops that are required for capsid binding and restriction [259,265]. Biochemically, NMR titration experiments with a soluble rhesus TRIM5α PRYSPRY domain and HIV-1 capsids have found chemical perturbations in V1 and V2 consistent with conformational changes induced by capsid binding [211].

Capsid encoded determinants of restriction

Difficulties in studying the TRIM5α-capsid interaction. While the TRIM5α variable loops are clearly involved in capsid recognition, it is unclear how they recognize retroviral capsids. Efforts to elucidate the interaction between capsid(s) and the PRYSPRY domain have been impeded by the very nature of the interaction. For example, TRIM5α destabilizes capsid complexes [208,213,269-271], making the pre-restriction complex difficult to isolate. The interaction between TRIM5α and retroviral capsids is thought to be made up of a network of high-avidity low-affinity interactions [210,211,246,271]. The binding site of the PRYSPRY domain may include multiple capsid monomers spanning multiple hexamers/pentamers [208,210,246,269,272]. Retroviral capsids have variable morphology and composition and the binding of TRIM5α is likely templated by the capsid lattice [20,210,273,274]. Finally, there is considerable genetic diversity found among the TRIM5αs and retroviral capsids used to study the interaction. For example, it is currently unclear how results obtained using human TRIM5α and the gammaretrovirus murine leukemia virus relate to data obtained with rhesus TRIM5α and the lentivirus HIV-1.

The interaction between TRIM5α and retroviral capsids is thought to be a high-avidity low-affinity interaction. Sedimentation assays demonstrated that that TRIM5α does not co-sediment with monomeric capsid (or does so very weakly) but does co-sediment with virions [208,213]. This argues that the interaction may span multiple capsid monomers in the context of

the higher ordered capsid lattice. TRIM5α has also been reported to exist in dimeric and other higher order states [210,235,255,267,269,271,275-281] .Mutations that disrupt the dimerization or oligomerization of TRIM5α have established a correlation between the ability to appropriately multimerize and the ability to restrict retroviruses [271,277-279]. Two observations make the most compelling argument for the high avidity-low affinity interaction model. First, *in vitro* TRIM5 (modified to be soluble) has been demonstrated to have an inherent property to self assemble into a hexameric array [210]. The presence of a planar hexameric capsid array can serve as a guide for TRIM5α lattice formation [210]. Second, NMR titration experiments involving soluble rhesus PRYSPRY domains and HIV-1 capsid N-terminal domains have measured a weak dissociation constant (in excess of 400 μM) [211,212].

Capsid features that modulate restriction. The interaction between TRIM5α and capsid has been largely defined through mutagenesis. Mutations in capsid have been shown to modulate TRIM5α sensitivity [61,234,235,240,256,258,282-299]. While these efforts to map the interaction between capsid and TRIM5α have used different viruses and different TRIM5αs, some general trends are apparent. A majority of the mutations that modulate TRIM5α sensitivity can be mapped to the N-terminal domain of capsid for both primate lentiviruses (HIV-1/HIV-2/SIVmac/SIVsmm/SIVagm) and gammaretroviruses (N-MLV/B-MLV/MoMLV). Specifically, sites that modulate TRIM5α sensitivity are enriched on surfaces that would make

up the exterior of the capsid. On the surface of a single capsid monomer, the sites that modulate TRIM5α sensitivity can be over 25 Å apart, yet have the same restricted phenotype. These observations imply that for some viruses there may be multiple determinants of restriction spread over the capsid surface. Recent NMR studies have confirmed that the PRYSPRY domain of rhesus TRIM5α can weakly interact with the N-terminal domain of the HIV-1 capsid [211]. In these NMR experiments the sites in the capsid most perturbed by the presence of TRIM5α were on the capsid surface [212].

TRIM5CypA. The naturally occurring TRIM5α-cyclophilin A gene fusion demonstrates that TRIM5α can act as a surface recognition molecule. The productive interaction of cyclophilin A with a loop on the lentivirus capsid surface has been exploited in the form of TRIM5-Cyclophilin A fusion proteins multiple times over the course of primate evolution [45-50,231-233,300]. Owl monkeys in the New World and macaques in the Old World express TRIM5Cyp proteins [45-50]. Evidence exists that suggests two additional TRIM5-cyclophilin A gene fusions may have existed millions of years ago, but have been lost in modern day primates [45]. All four of these fusion proteins are the result of independent retrotransposition events that have inserted cyclophilin A mRNAs into the TRIM5 locus. In the modern day TRIM5Cyp fusion proteins, splicing replaces the C-terminal PRYSPRY domain with a C-terminal cyclophilin A domain, effectively exchanging one viral recognition domain for another [46-51,251]

The probable targets of TRIM5α. Taken together, the fact that TRIM5Cyp can restrict viruses solely through capsid surface interactions, the prevalence and density of mutations modulating TRIM5α sensitivity on the capsid surface, and recent findings using NMR, it is likely that TRIM5α recognizes a unique retrovirus specific pattern on the capsid surface (reviewed above). These studies support a series of major conclusions. First,it appears to generally take less mutagenic steps to confer the restricted phenotype upon a resistant virus than it does to confer the resistant phenotype upon a restricted (a virus can be made sensitive by a single amino acid substitution, but there are fewer examples of a single amino acid substitution rendering a virus resistant). For many naturally restricted viruses, multiple, possibly independent, determinants of restriction are spread across the capsid surface. If the surface of the capsid N-terminal domain is a major determinant of restriction, then the pattern created by the higher ordered arrangement of capsid surface features must in some way explain the discrepancy between the observed breadth of restriction and the specificity of restriction. Put simply, the capsid must be both a rapidly evolving and conserved target.

1. E. CAPSID AS A RETROVIRAL TARGET FOR TRIM5α

The unique interaction between TRIM5α and capsid

The capsid is a retrovirus specific target. It can be argued that the viral signatures that are targeted by the aforementioned restriction factors can be divided into two categories; those that target general viral processes and those that directly target a retroviral-specific signature. The restriction factors SAMHD1, APOBEC3-family members, and BST2 exert their restrictive potential by acting on general processes. SAMHD1 prevents DNA synthesis by lowering cellular dNTP pools, APOBEC3 proteins target single stranded DNA, and BST2 tethers enveloped viruses [122-124,127]. Due to the generality of the viral signatures they target, these proteins have also been shown to act directly upon representative viruses outside of the Retroviridae. SAMHD1 has been shown to restrict DNA viruses like poxviruses and herpesviruses in myeloid cells [147,301]. While the *in vivo* consequences of APOBEC3 family members against non-retroviruses has yet to be realized, they have been shown to have activity against a variety of DNA viruses including human papillomaviruses [302,303], hepadnaviruses (HBV) [304], herpesviruses [305] and parvoviruses [306,307] under some conditions. BST2 has been shown to have a broad range of restriction for enveloped viruses, and the broadest range of restriction for any of the discussed factors. Viruses restricted by BST2 include RNA viruses such as filoviruses [308,309], New and Old World arena viruses [310,311], and DNA viruses such as herpesviruses [312,313],

Among the discussed lentiviral restriction factors, TRIM5α is unique in that it directly targets a pattern created by the higher-ordered arrangement, or lattice, of capsid monomers within the core. Therefore, the pattern created by the retroviral capsid lattice must have at least four important properties. First, the pattern created by the higher ordered capsid structure must be unlike any other higher ordered protein arrangement naturally found in cells. Second, the pattern created by the capsid lattice must reflect a general pattern that is unique to the *Retroviridae*. This is evidenced by the ability of TRIM5αs to restrict viruses from multiple retroviral genera while simultaneously lacking direct restriction activity against any other known viral family. Third, for a factor like TRIM5α to have evolved, its target must be stable through deep evolutionary timescales. Finally, for a restriction factor like TRIM5α to have been continuously selected for and upon, escape from restriction must involve multiple simultaneous mutations or escape mutations must be accompanied with a high fitness cost. Fittingly, its target, the retroviral capsid, fulfills all of these criteria.

The involvement of capsid at most steps of retroviral infection

Assembly. The structural components of retroviral virions are encoded by the *gag* gene. While all retroviruses encode for a Gag protein, the Gag proteins of the *orthoretrovirinae* are cleaved into three major proteins. These proteins form three distinct layers within the virion. The

matrix protein, which is myristoylated, forms a structured, yet incomplete lattice, along the inner leaflet of the inner viral membrane [20-22,273,314]. The capsid protein condenses to form a higher-ordered protein shell around the inner components of the virion. The inner components include two viral genomes (normally identical or near-identical to each other) , which are coated in the final major Gag derived protein, the nucleocapsid protein. Also within the higher-ordered capsid shell are the reverse transcriptase and integrase enzymes. While the composition of cleavage products varies between retroviral genera the order and timing of each cleavage event are critical for the production of infectious virions [20,22,273].

Estimates for the number of gag proteins within the virion range from 2500-5000 [315-318]. The number of capsid monomers within the virion is identical due to the one to one ratio of capsid proteins to gag proteins. Of these 2500-5000 capsid monomers within the virion, atomic resolution models of the HIV-1 capsid core suggest that it is comprised of approximately 1,056-1,356 capsid monomers [319,320]. These models predict there to be 166-216 capsid hexamers and exactly 12 pentamers within the capsid. The 12 pentamers create the necessary curvature to close the otherwise hexameric lattice [274]. The asymmetric distribution of these 12 pentamers give lentiviral capsid cores their distinctive morphology that can be described as a fullerene cone [274]. Multiple functional surfaces of the capsid protein contribute to a carefully orchestrated series of cleavage events and conformational changes that liberate capsid proteins

from their gag precursor and facilitate the condensation into a fully assembled fullerene cone [19,20,273,274,320,321].

Reverse transcription. Following fusion, the core is deposited into the cytoplasm of the target of the cell [97]. The diffusion of dNTPs through the core is thought to trigger reverse transcription [13]. Reverse transcription is believed to occur to completion, or near completion within the capsid shell [11]. This likely serves two purposes. The first is that the capsid core traps the two viral genomes, reverse transcription intermediates, reverse transcriptase and integrase within a protein shell. This keeps their local concentration high and prevents their diffusion and the dissociation of the reverse transcription complex into the cytoplasm. Evidence from TRIM5α restriction demonstrates that the capsid becomes permeabilized resulting in the release of the capsid core's internal components [11,12,208,322]. This is correlated with the block to late reverse transcription products. Second, the capsid core prevents reverse transcription intermediates from being sensed by pattern recognition receptors of the innate immune system [323]. Importantly, the capsid is not an inert protein shell; rather it is a mediator of critical interactions with cellular cofactors.

The capsid co-factor cyclophilin A. Primate lentivirus capsids have been shown to efficiently bind to a number of cellular cyclophilins [231-233,291,324,325]. Cyclophilins make up a superfamily of proteins that are present among all known cellular organisms [326].

Functionally, they catalyze trans to cis isomerization of prolines relative to the peptide backbone (peptidyl-prolyl isomerase activity), thus facilitating protein folding [326]. Of the reported cyclophilin-capsid interaction partners two have been shown to positively influence primate lentivirus infection in human cells [231,233,327]. The first is cyclophilin A (PPIA) which was identified as the first capsid interacting protein [231] (the second is a cyclophilin A-like domain in Nup358, discussed below). It has been shown to bind an elongated loop between α-helix 4 and α-helix 5 that extends off of the capsid protein of lentiviral capsid proteins [232,233,300]. Due to its interaction with cyclophilin A, this loop is colloquially referred to as the cyclophilin A binding loop [300]. This loop is structurally unique to the capsid retroviruses of lentivirus genus [300,324,328-333]. Fittingly, the capsid-cyclophilin A interaction is also unique to this genus. Importantly among most lentiviruses, including endogenous lentiviruses dated to be at least 5-8 million years old, this loop has a proline rich sequence capable of binding cyclophilin A [232,300,324,327,333,334]. The high affinity interaction between capsid and cyclophilin A leads to efficient incorporation into virions in the producer cell [232,233]. While reports exist for the requirement for cyclophilin A in both the producer cell and the target cell, it appears that the stronger phenotype occurs in the target cell [335-346]. In human cells the presence of cyclophilin A in the target cell is generally associated with an increase of infectivity for HIV-1. One explanation for this interaction may be that it helps to shield it from human TRIM5α

[292,339,347-350]. However, the requirement for cyclophilin A to enhance the infectivity of HIV-1 is not absolute among human cell lines [282,287,335,339,340,344,349,351-357]. The pattern of cell lines requiring cyclophilin A at least in part matches cell lines which express cytosolic DNA innate sensors. Therefore the phenotype may at least in part be explained by the observation that cyclophilin A binding helps to shield the reverse transcription complex from innate sensors [323].

Capsid facilitates nuclear import. Lentiviruses can efficiently infect non-dividing cells [2,14,15,18]. While numerous viral proteins have been implicated in this phenotype, the capsid protein has been shown to be the major determinant [358]. Some details of the mechanisms underlying this phenotype have come from a number of RNA interference screens [359-362]. Collectively, these screens have implicated the requirement of a number of nuclear pore proteins, karyopherins, and karyopherin cargos for the efficient infection of cells by HIV-1.

The coordinated interactions of host proteins with lentiviral capsids shuttle primate lentivirus capsids through a preferred nuclear import pathway. This pathway not only results in efficient trafficking through the nuclear pore, but also influences the selection of integration sites. Sampling of thousands of independent integration sites has shown that HIV-1 (and presumably primate lentiviruses) are biased towards transcriptionally active regions of DNA and transcriptionally active gene clusters [204]. Perturbation of the preferred nuclear import pathway

either through depletion of the above cellular factors or through capsid mutations that disrupt the interaction with the above factors can bias HIV-1 integration target site selection away from its wild type pattern [204,327,363,364]. The transcriptional environment into which a lentiviral provirus inserts can influence viral gene expression [204]. Fittingly, deviations from the preferred pattern of integration are correlated with reduced replication [327,365].

Nup358/RANBP2. Two independent RNA interference screens implicated the involvement of nuclear pore protein Nup-358/RANBP2 in efficient HIV-1 infection [359,360]. This protein has a cyclopilin A-like domain that extends from the nuclear pore into the cytoplasm [327]. The HIV-1 capsid protein, and the capsid proteins of other lentiviruses can bind to this cyclophilin A-like domain via the 4-5 loop (cyclophilin A binding) [327]. The capsid-Nup-358 interaction has been implicated in the efficient nuclear trafficking of the pre-integration complex as well as the appropriate integration target site biases that exist for the lentivirus genus of the *Orthoretrovirinae* [327]. The relevance of the interaction between lentivirus capsids and the cyclopilin A-like domain of Nup358 has recently been called into question [366]. While the cyclopilin A-like domain of Nup358 may not be strictly required for nuclear import, it does indeed interact with the 4-5 loop of the HIV-1 capsid and the capsids of other (but not all) lentiviruses [327,366]. It is currently unclear which (if any) additional specific contacts between Nup358 and capsids contribute to lentivirus infection.

CPSF6. Cleavage and polyadenylation specific factor 6 (CPSF6), is a proven capsid interacting protein [367]. The interaction of capsid with CPSF6 was fortuitously identified in a cDNA screen to identify restriction factors. A prematurely truncated and mis-spliced CPSF6 mRNA gave rise to a cDNA that potently restricted HIV-1 [367]. This cDNA encoded an mRNA that was lacking an arginine-serine rich domain that is required for nuclear localization [368]. Cytosolic localization is associated with primate lentivirus restriction [367,369,370]. CPSF6 directly binds lentiviral capsids. This interaction is mediated by a putative capsid interacting domain. This domain contains a capsid binding peptide that is required for efficient HIV-1 nuclear import [363,370]. The structure of the CPSF6 capsid binding peptide in complex with the HIV-1 capsid N-terminal domain has been determined and shown to bind in a groove along one side of the monomeric capsid protein [363]. Both the structure and amino acid composition of this pocket are highly conserved across primate lentiviruses [363].

Nup-153. Two of the four RNA interference screens have implicated the involvement of Nup-153 as a mediator of HIV-1 infectivity [359,360]. The requirement for Nup-153 has been confirmed for HIV-1 and extended to include additional primate lentiviruses [371]. Specific capsid mutations in addition to chimeric HIV/MLV viruses established that the capsid protein governs sensitivity to Nup-153 depletion [371]. The FG repeats at the C-terminus of Nup-153 have been demonstrated to directly bind to lentivirus capsids. This interaction has been mapped

to the same binding pocket within the N-terminal domain of capsid that binds the CPSF6 capsid binding peptide [372].

Host factor shield. While interactions with the above factors shuttle the preintegration complex through the nuclear pore, some of these co-factor interactions also shield it from innate sensors [323]. The interactions with cyclophilin A (and possibly the cyclophilin A-like domain of Nup358) have been shown to reduce the detection of reverse transcription intermediates in the cytoplasm [323]. The interaction of human CPSF6 with HIV-1 can pause reverse transcription [323]. This is thought to delay reverse transcription until is within proximity of the nuclear pore, reducing the potential exposure of the reverse transcription intermediates from innate sensors [323]. These coordinated interactions with cellular co-factors prevent innate signaling and the establishment of an antiviral state by the production of interferon stimulated gene products (including restriction factors like BST2 [122,123]). Interferon signaling also establishes a local anti-viral environment. Since primate lentiviruses more efficiently spread through cell-to-cell transmission, interferon signaling in one cell may render adjacent cells more resistant to infection.

The retroviral capsid is a conserved target

Evolutionary constraints. As highlighted by the above examples, the capsid protein of primate lentiviruses either actively participates or at minimum influences almost every step of the replication cycle. As reviewed above multiple functional surfaces on the capsid protein are dynamically required to orchestrate a number of conformational rearrangements and inter- and intra- molecular interactions not limited to its role in Gag-dimerization, maturation, regulated uncoating, and its interactions with various cellular cofactors that ultimately lead to proper nuclear import and integration target site selection. Likely due to its relatively small size of approximately 230 amino acids, these functional surfaces are often used for multiple purposes. For example, the 4-5 loop productively interacts with cycolyphin A [231,232,300,327] and the cyclophilin A-like domain of Nup-358. At least for the assembly of the betaretrovirus Mason-Pfizer Monkey Virus, the capsid surfaces also mediate critical contacts prior to the condensation of the capsid [321]. These interactions are also believed to exist for other retroviruses including the primate lentiviruses [321]. The same binding groove mediates interactions with CPSF6 and Nup-153 [363,372]. The first three alpha helices in the capsid C-terminal domain participate in contacts required for Gag-Gag dimerization prior to assembly. In the context of the hexamer/pentamer they participate in intra-hexamer contacts that effectively cradle the N-terminal domain of an adjacent capsid monomer and between capsid hexamers/pentamers they are the major mediators of the inter-hexamer contacts that provide both the stability and

flexibility needed to form the core [19,20,22,319,320,373,374]. Therefore, the capsid would be predicted to have a multitude of evolutionary constraints.

Structural conservation. The amino acid sequences of the capsid proteins of orthoretroviruses bear little sequence identity. The notable exception to this observation is an approximately 22 amino acid stretch within the C-terminal domain that participates in Gag-Gag and multiple capsid-capsid interactions. The high degree of conservation between retroviruses and some retrotransposons has led to this stretch of amino acids being termed “the major homology region” or MHR. Despite the clear lack of sequence identity, the wealth of reported orthoretroviruses capsid structures demonstrate that the overall capsid structure is highly conserved [300,328,329,331,338,375]. To put this observation in context, conservative estimates postulate that the last common ancestor of all extant members of the *Orthoretrovirinae* existed between 70-100 million years ago. Despite the fact that among the orthoretroviruses the amino acid sequence of the N-terminal domain is far more variable than that of the C-terminal domain, reported structures of viruses from 5 of the 6 *Orthoretrovirinae* genera illustrates that the N-terminal domains share a common fold [300,328,329,331,338,375]. This conservation makes the structures of these capsids superimposable (Figure 1-7B-D). In particular, the presence of a β-hairpin at the extreme N-terminus and a core composed of 5 α-helices are invariant features of *Orthoretrovirinae* capsids (Figure 1-7B). Hexameric capsid

structures have been reported for both B and N tropic MLV in addition to HIV-1. Together they demonstrate that the higher ordered capsid species are also highly conserved. [319,330,373,375] (Figure 1-8). Perhaps this conservation is fitting considering the difference between the fullerene cone of lentiviruses and the sphere of gammaretroviruses is the distribution of pentamers in the capsid [20,274,330].

Figure 1-7. Structural conservation across Orthoretrovirinae capsid N-terminal domains. (A) The HIV-1 capsid N-terminal domain (PDB: 1GWP). Notable features are labeled: The β-hairpin (BHP), α-helices 1-7 (αH), the 4-5 loop (4-5L) and 6-7 loop (6-7L). A structurally conserved core of 5 α-helices is shown with a black line. (B) Conserved features of Orthoretrovirinae capsid N-terminal domains. Structures of divergent Orthoretrovirinae capsids aligned to HIV-1 (red). The variable surface features have been removed to display the conserved 5 α-helix core and β-hairpin (boxed). (C) Structural heterogeneity is segregated from structural conservation. Structures of divergent Orthoretrovirinae capsids aligned to HIV-1. Variable surface structures are boxed. (D) Structures of Orthoretrovirinae capsids aligned to HIV-1 (red) and separated by retrovirus genus. (PDB: Lenti-HIV-1-1GWP, Lenti-HIV-2-2WLV, Lenti-PSIV-2XGU gamma-N-MLV-1U7K, gamma-B-MLV-3BP9, alpha-RSV-1D1D, delta-1QRJ, beta-M-PMV-2KGF beta-JSRV-2V4X). Images created in PyMol.

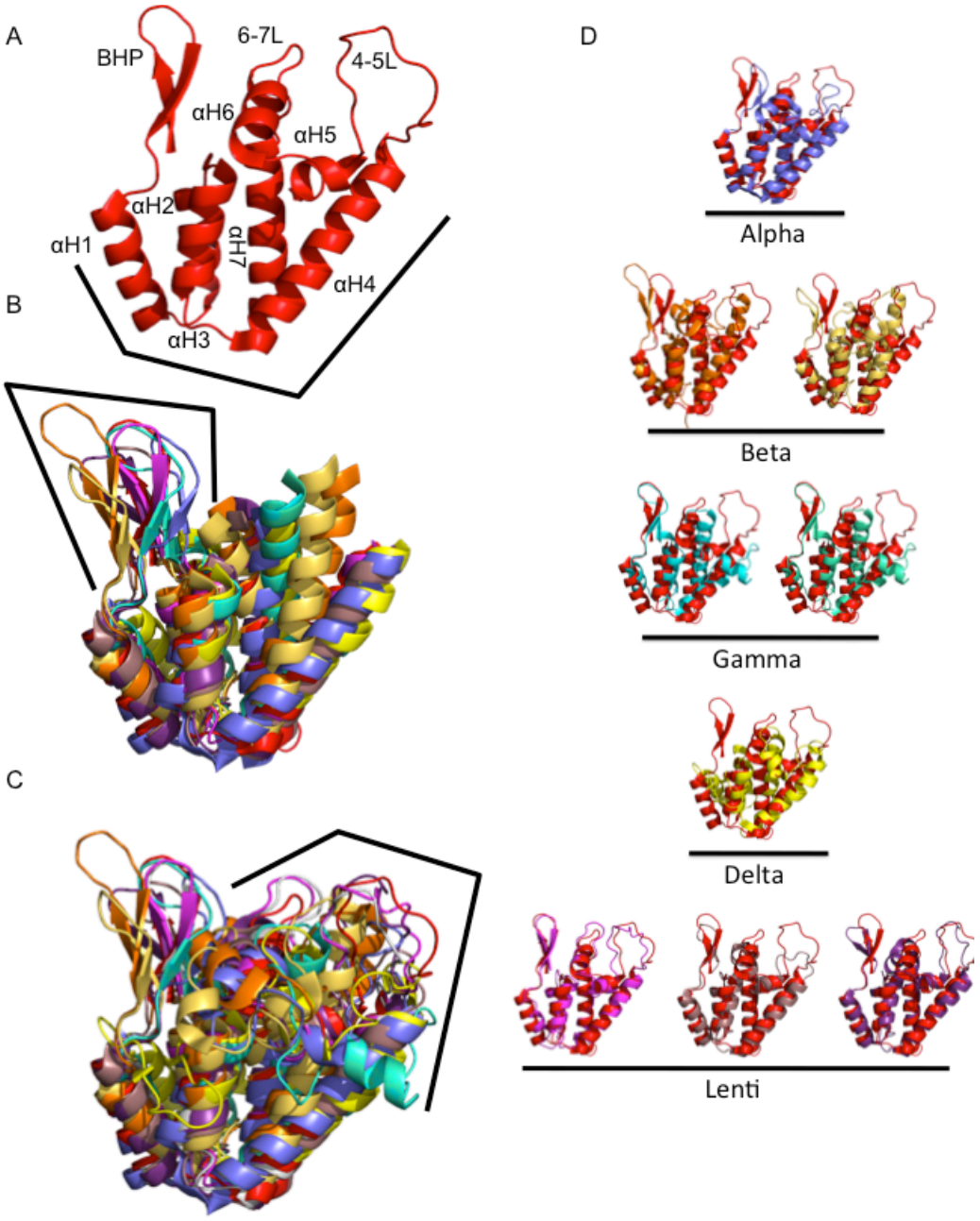

Figure 1-7. Structural conservation across Orthoretrovirinae capsid N-terminal domains. Continued.

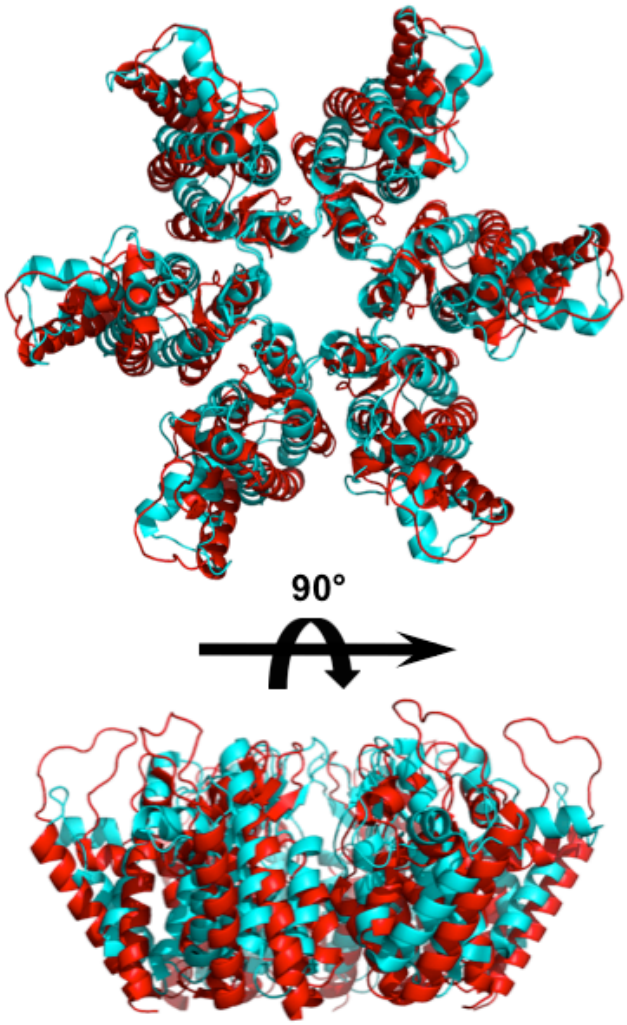

Figure 1-8. Conservation of higher ordered capsid structures. Hexameric capsid structures from the lentivirus HIV-1 and gammaretrovirus N-tropic MLV are superimposable. Top. A top view of the superimposed structures. Bottom. A side view of the superimposed structures. PDB: 1U7K and 3GV2. Red: HIV. Cyan: MLV. Images created in PyMol

The sites of major structural deviation found among the capsids of orthoretroviruses are on the capsid surface. Variation among structural features is found on the opposite side of the capsid surface from the highly conserved β-hairpin [300,328,329,331,338,375]. (Figure 1-7B-C). Therefore the capsid surface has segregated regions of structural conservation and structural heterogeneity. While variable between *Orthoretrovirinae* genera, these regions tend to be structurally conserved within any single retrovirus genus. Reported *Orthoretrovirinae* capsid structures differ in the presence of one or two surface α-helices. These surface α-helices also differ in their orientation relative to the conserved 5-α-helix core. Variation is found in the length of the linker between the final surface helix and the last α-helix of the N-terminal domain (between helix 6 and 7 of primate lentivirus capsids). Finally, there is considerable heterogeneity in the length and the conformation of an extended loop bridging α-helix 4 and the first surface α-helix. This corresponds to the 4-5 loop for primate lentiviruses [300,328,329,331,338,375].

These variances in structure are not without biological consequence. The corresponding variable structures of lentiviruses are the same structures that facilitate the infection of non-dividing cells (nuclear import), a phenotype that is a defining property of lentiviruses. Specifically, the elongated 4-5 loop which extends off of the capsid surface into the cytoplasm binds cellular cyclophilin A as well as the cyclophilin A-like domain of the nuclear pore protein

Nup-358 [232,300,327]. Similarly, the primate lentivirus surface α-helices 5 and 6 create a binding pocket and coordinate waters that facilitate the binding of the capsid binding loop of CPSF6 [363,370]. Importantly, the orientation and sequence of the HIV-1 α-helices 5 and 6 are predicted to be critical for this interaction. This same binding pocket also binds the FG-repeats of the nuclear pore protein Nup-153 [372]. Considering that the lentivirus-specific structural features are involved in efficient nuclear import it is possible that the corresponding structural features of other retrovirus capsids mediate contacts with cellular cofactors. Perhaps the structural similarities between the HIV-1 and Rous sarcoma virus capsid N-terminal domains account for the observation that Rous sarcoma virus can weakly infect non-dividing cells [16]. Interestingly, the betaretrovirus MMTV has recently been reported to infect non-dividing cells [17]. The mechanism by which MMTV infects non-dividing cells appears to be different from the canonical HIV-1 nuclear import pathway [17].

Fragility of capsid. As outlined above, the capsid participates in a number of dynamic interactions that are required for optimal viral infectivity. Selection to maintain these interactions would therefore impart a great number of evolutionary constraints upon the capsid protein. Indeed, the capsid protein is the most conserved Gag protein within any one *Orthoretrovirinae* genus. The capsid of the gammaretrovirus Moloney murine leukemia virus (MoMLV) has been shown to be the least tolerant gag protein to insertional mutations [376]. Further characterization

of insertion and point mutants within the MoMLV capsid demonstrated that most mutations interfere with assembly [376,377]. Similarly, in the context of mutation, the HIV-1 capsid has been reported to be extremely fragile. Specifically, the HIV-1 capsid is the least tolerant viral protein ever analyzed by random single amino acid mutagenesis [378]. An overwhelming majority of these mutations were shown to disrupt one or more steps in the assembly and condensation of fullerene cone shaped core. These results are largely consistent with earlier observations made from alanine-scanning mutagenesis for the HIV-1 capsid protein [379].

1. F. CONCLUDING REMARKS

Summary and findings

Summary. TRIM5α has evolved to restrict retroviruses through its interaction with the viral capsid. The qualities of orthoretrovirus capsids match the qualities that one would predict to be necessary to drive the evolution of an antiretroviral factor such as TRIM5α. Orthoretrovirus capsids are an evolutionarily stable target that are generally intolerant to random mutations. The evolution of *Orthoretrovirinae* capsids is constrained by the capsid protein's direct involvement or influence upon most steps of viral replication cycle. Conservation is seen at both the

structural level across the *Orthoretrovirinae* and the sequence level within any one *Orthoretrovirinae* genus. In the absence of obvious sequences common amongst TRIM5α resistant or TRIM5α restricted viruses from across the *Orthoretrovirinae*, conserved structures may account for the observed breadth of restriction. In parallel, sequence conservation among *Orthoretrovirinae* genera, or subgroups within, such as primate lentiviruses, may account for the specificity of restriction or resistance to TRIM5α orthologs observed for some viruses. Finally, based on the relationship between TRIM5α and retroviruses, it is likely that retrovirus infection has driven the evolution of TRIM5α while the presence of TRIM5α has likely driven the evolution of retroviral capsids.

Findings. Similar to a great body of previous work, I set out to define a binding site for rhesus TRIM5α on the HIV-1 capsid protein. As an ultimate test of our understanding of this interaction I sought to engineer a rhesus TRIM5α-resistant HIV-1 capsid. Unlike previous approaches, I took a gain-of-sensitivity approach rather than assaying for a gain of resistance. Specifically, I generated a series of mutations in the rhesus macaque adapted SIVmac239 virus. These mutant SIVmac239 viruses were modified to express chimeric capsids proteins in which whole regions or single amino acids were replaced with the corresponding sequences of the HIV-1 molecular clone, HIV-1nl4.3. This allowed us to separate multiple determinants of restriction across three major capsid surface features (β-hairpin, 4-5 loop and α-helix-6). This

led to the generation of the first reported HIV-1 capsid resistant to rhesus macaque TRIM5α. Furthermore, we identified two targets for rhesus TRIM5α: one in the structurally conserved β-hairpin and another in a patch of amino acids on the capsid surface that are both highly conserved in sequence and structurally unique to lentiviruses [380]. Conservation of this site may be due to its position at the junction of the Cyclophilin A/Nup-358 cyclophilin A-like domainand the CPSF6/Nup-153 binding sites [300,327,363,372].

Rhesus macaques have two TRIM5α variants, one reflective of an ancestral and ancient state, while the other was more recently acquired during macaque evolution [251]. We observed that only the more recently evolved variant gained the ability to restrict viruses with mutations in lentivirus specific features (conserved surface patch). However, both alleles were capable of restricting viruses with mutations in the highly conserved β-hairpin. This led us to propose an evolutionary model in which all Old World monkey TRIM5αs have an intrinsic ability to target the pan-*Orthoretrovirinae* feature, the β-hairpin. We proposed that this accounts for the observed breadth of TRIM5α restriction that can encompass multiple retroviral genera. Furthermore, we suggest that this association allows other regions of TRIM5α to evolve virus-specific contacts, such as the primate lentivirus specific conserved surface patch.

We and others noted that the site that functionally differentiates the two rhesus macaque alleles has been heavily modified across members of a single clade of African primates, the

Cercopithecinae [61,208,228,243,251,257]. To test our proposed evolutionary model and to understand the viral factors driving the evolution of primate TRIM5α we studied this rapidly evolving site within TRIM5α. We reconstructed an ancestral TRIM5α as it may have existed 11-16 million years ago, before the selective events took place. Into this isogenic and ancient backbone we introduced these evolutionarily acquired changes. These modifications led to the specific gain of ability to restrict Cercopithecinae SIVs. The restriction of non-SIV viruses was largely unaffected. Our observations imply that specific Old World monkey TRIM5αs evolved to restrict viruses from a specific primate lentivirus lineage. Using the known primate phylogeny we have concluded that selection by a specific lineage of primate lentiviruses occurred 11-16 million years ago. Thus, we speculate that Cercopithecine SIVs and primate lentiviruses are at least as ancient.

Chapter 2

Gain-of-Sensitivity Mutations in a TRIM5-Resistant Primary Isolate of Pathogenic SIV Identify Two Independent Conserved Determinants of TRIM5α Specificity

Gain-of-Sensitivity Mutations in a TRIM5-Resistant Primary Isolate of Pathogenic SIV Identify Two Independent Conserved Determinants of TRIM5α Specificity

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Contributions: Welkin E. Johnson and I both wrote the manuscript. I performed most of the experiments except: Andrea Kirmaier assisted with infections and FACS harvests, Crystallography was preformed by Aaron G. Schmidt. FACS was run by the staff of the BC and NEPRC flow cytometry cores. Figures 2-8,2-9 and table 2-2 were made by Aaron G. Schmidt. I want to thank the beamline staff at APS 24-ID-E and Uhnsoo Cho for help with data collection. Ruchi M. Newman made the TRIM5α expressing cell lines and contributed to Figure 2-1. Alison L. Wyand assisted in cloning chimeric viruses.

2. A. ABSTRACT

Retroviral capsid recognition by TRIM5 blocks productive infection. Rhesus macaques harbor three functionally distinct *TRIM5* alleles: *TRIM5*α^{Q}, *TRIM5*α^{TFP} and *TRIM5*^{CypA}. Despite the high degree of amino acid identity between *TRIM5*α^{Q} and *TRIM5*α^{TFP} alleles, the Q/TFP polymorphism results in the differential restriction of some primate lentiviruses, suggesting these alleles differ in how they engage these capsids. Simian immunodeficiency virus of rhesus macaques (SIVmac) evolved to resist all three alleles. Thus, SIVmac provides a unique opportunity to study a virus in the context of the TRIM5 repertoire that drove its evolution in vivo. We exploited the evolved rhesus TRIM5α resistance of this capsid to identify gain-of-sensitivity mutations that distinguish targets between the *TRIM5*α^{Q} and *TRIM5*α^{TFP} alleles. While both alleles recognize the capsid surface, *TRIM5*α^{Q} and *TRIM5*α^{TFP} alleles differed in their ability to restrict a panel of capsid chimeras and single amino acid substitutions. When mapped onto the structure of the SIVmac239 capsid N-terminal domain, single amino acid substitutions affecting both alleles mapped to the *β*-hairpin. Given that none of the substitutions affected TRIM5α^{Q} alone, and the fact that the *β*-hairpin is conserved among retroviral capsids, we propose that the *β*-hairpin is a molecular pattern widely exploited by TRIM5α proteins. Mutations specifically affecting rhesus TRIM5α^{TFP} (without affecting *TRIM5*α^{Q}) surround a site of conservation unique to primate lentiviruses, overlapping the CPSF6 binding site. We believe targeting this site is an

evolutionary innovation driven specifically by the emergence of primate lentiviruses in Africa during the last 12 million years. This modularity in targeting may be a general feature of TRIM5 evolution, permitting different regions of the PRYSPRY domain to evolve independent interactions with capsid

2. B. INTRODUCTION

The anti-retroviral activity of TRIM5α was discovered in a screen to identify rhesus macaque cDNAs conferring resistance to HIV-1 replication [125]. Antiretroviral activity has since been demonstrated for a large number of primate TRIM5 orthologs, including prosimians, as well as homologs from cow and rabbit [241,242,264,381]. While no single ortholog of TRIM5 universally restricts all retroviruses, the collective breadth of restriction, coupled with the observation that some orthologs can restrict viruses from two or more genera, suggests that TRIM5 recognizes a conserved, pathogen-associated molecular pattern common to members of the *Retroviridae* [229,236,381].

TRIM5α is composed of four domains: the RING, the B-Box and the Coiled-coil domains, which make up the tripartite RBCC of TRIM proteins, and a C-terminal PRYSPRY domain [128,129]. The PRYSPRY domain is thought to recognize the viral capsid [125,208,213]. In the case of lentiviruses, the cone-shaped capsid is composed of 12 pentamers and approximately

200 hexamers, each in turn comprised of identical copies of monomeric capsid (CA) protein [274,319]. An HIV-1 CA monomer has two α-helical domains connected by a flexible linker [382]. The N-terminal domain makes up the outer surface of the capsid and mediates interactions with cellular cofactors [231,232,327,363,367,371,383].

Comparisons between reported CA structures from viruses representing five *Orthoretrovirinae* genera show that the overall architecture of the N-terminal domain is conserved, despite little conservation of protein sequence. All reported retroviral N-terminal domain structures contain a conserved five α-helix core, from which a conserved surface feature, the β-hairpin, protrudes into the cytoplasm. Structural variation can be found among additional features on the CA surface. These differences include the presence and arrangement of 1-2 additional α-helices and/or the presence of an extended loop connecting helices 4 and 5 (4-5 loop) [328-331,338,384].

Reports suggest that multiple sites within retroviral CAs modulate TRIM5α sensitivity [61,234,235,240,256,258,282-299]. The majority of these sites map to the N-terminal domain and are enriched within the CA surface features. Perplexingly, engineered CA mutations, naturally occurring variants, and escape mutations can have similar phenotypes even when separated by distances in excess of 25 Å. Understanding how these sites relate to one another

is critically important for defining how TRIM5α recognizes retroviral capsids, and how viruses evolve to evade TRIM5α restriction.

We previously reported that the *TRIM5* locus of rhesus macaques (*Macaca mulatta*) is highly polymorphic, and that the different allelic lineages of rhesus *TRIM5* (*rhTRIM5*) have been maintained by long-term balancing selection [46,251]. Based on functional assays and gene association studies, rhTRIM5 alleles can be grouped into 3 classes, rhTRIM5α^{TFP}, rhTRIM5α^{Q} and rhTRIM5^{CypA} [47,48,50,51,61,257,385]. When tested against a panel of primate lentiviruses, the 3 alleles give differing patterns of restriction [61,251,257] – an indication that rhTRIM5 has at least 3 distinct (or incompletely overlapping) targets on the lentiviral CA protein.

SIVmac emerged in captive macaque colonies in the 1970s, most likely the result of an unintentional interspecies transmission of SIV from sooty mangabeys (SIVsm) [23,58,386,387]. We previously reported that SIVsm isolates are resistant to rhTRIM5α^{Q}, but sensitive to rhTRIM5α^{TFP} and rhTRIM5^{CypA} alleles [61]. Because rhTRIM5α^{TFP}, rhTRIM5α^{Q} and rhTRIM5^{CypA} likely have deferring targets within CA and because all are present at moderate-to-high frequency, emergence of SIVmac in rhesus macaque colonies required adaptations permitting simultaneous resistance to all three. Thus, comparisons between SIVmac and other restricted isolates provide a unique opportunity to understand the basis of recognition by TRIM5α proteins

and to identify specific features of CA that determine sensitivity and resistance to rhTRIM5α-mediated restriction.

The structural basis for CA recognition by rhTRIM5^{Cyp} is clear: the cyclophilin A domain (CypA) specifically binds the 4-5 loop [300,333]. In contrast, rhTRIM5α^{TFP} and rhTRIM5α^{Q} interact with capsids via a C-terminal PRYSPRY domain, but the basis for capsid recognition by TRIM5 PRYSPRY domains remains poorly understood. There are several factors that complicate studies of the interaction. For example, TRIM5α destabilizes capsid complexes [208,213,269-271], the nature of the interaction is believed to be high avidity and low affinity [210,211,246,271], the interaction site may extend beyond a single CA monomer or hexamer [208,210,246,269,272], retroviral capsids and presumably the TRIM5α lattice surrounding them have variable morphology and composition [20,210], and there is considerable diversity among TRIM5α orthologs and retroviral CA sequences.

To investigate how TRIM5α recognizes retroviral CAs, we combined genetic, phylogenic and structural investigations with an alternative mutational strategy to separate and map the determinants for the differential restriction of HIV-1 and SIVmac by rhTRIM5α alleles. The resolution of our mapping, together with the structural determination of the SIVmac239 CA N-terminal domain and consideration of primate lentivirus diversity, allowed us to identify two conserved CA surface elements that appear to be targets of rhTRIM5α recognition. The first, the

β-hairpin, is a structural feature that is present in all reported retroviral CA structures. Mutations in the β-hairpin affected targeting by both rhTRIM5α^{Q} and rhTRIM5α^{TFP} alleles. The second element, a patch of highly conserved amino acids among primate lentivirus CAs, may be a unique target of the more recently evolved rhTRIM5α^{TFP} allele. Strikingly, this patch is a surface-exposed extension of the recently identified CPSF6 binding site [363]. Therefore, similar to the exploitation of the interaction between cyclophilin A and Nup358 by TRIM5^{CypA}, it appears that rhTRIM5α^{TFP} has evolved to target the binding site of a required cellular cofactor. Taken together, the observations made from investigating the differential breadth and specificities of rhTRIM5α alleles have revealed a complex evolutionary relationship between retroviruses and TRIM5α orthologs.

2. C. RESULTS

Differential restriction by the rhesus TRIM5α^{Q} and TRIM5α^{TFP} alleles.

Differential restriction by rhTRIM5α^{Q} and rhTRIM5α^{TFP} has been mapped to a length polymorphism in the PRYSPRY domain (TFP339-341Q) [257]. Despite the fact that the protein sequences are >98% identical, the rhTRIM5α^{Q} and rhTRIM5α^{TFP} alleles yield different patterns of restriction when tested in parallel against divergent retroviruses [61,251,257,385]. We tested both alleles against multiple primate lentiviruses and found that even among these related viral

strains, the rhTRIM5α^{Q} and rhTRIM5α^{TFP} alleles give different patterns of restriction (Figure 2-1). Specifically, rhTRIM5α^{Q} restricted a human viral isolate, HIV-1nl4.3, but failed to restrict any of the lentiviruses isolated from Cercopithecine primates (SIVmac239 from rhesus macaques, SIVsmE041 and SIVsmE543-3 from sooty mangabeys, and SIVagmTAN-1 from African green monkeys) or HIV-2ROD (which originated by cross-species transmission of SIVsm [26]). In contrast, rhTRIM5α^{TFP} restricted HIV-1nl4.3, SIVsmE041, SIVsmE543-3, SIVagmTAN-1 and to a lesser extent, HIV-2ROD. Only the rhesus macaque isolate, SIVmac239, was resistant to both alleles. Thus, while both alleles are functional, the differing patterns of restriction are consistent with the hypothesis that rhTRIM5α^{Q} and rhTRIM5α^{TFP} proteins differ in the way they recognize primate lentivirus CAs.

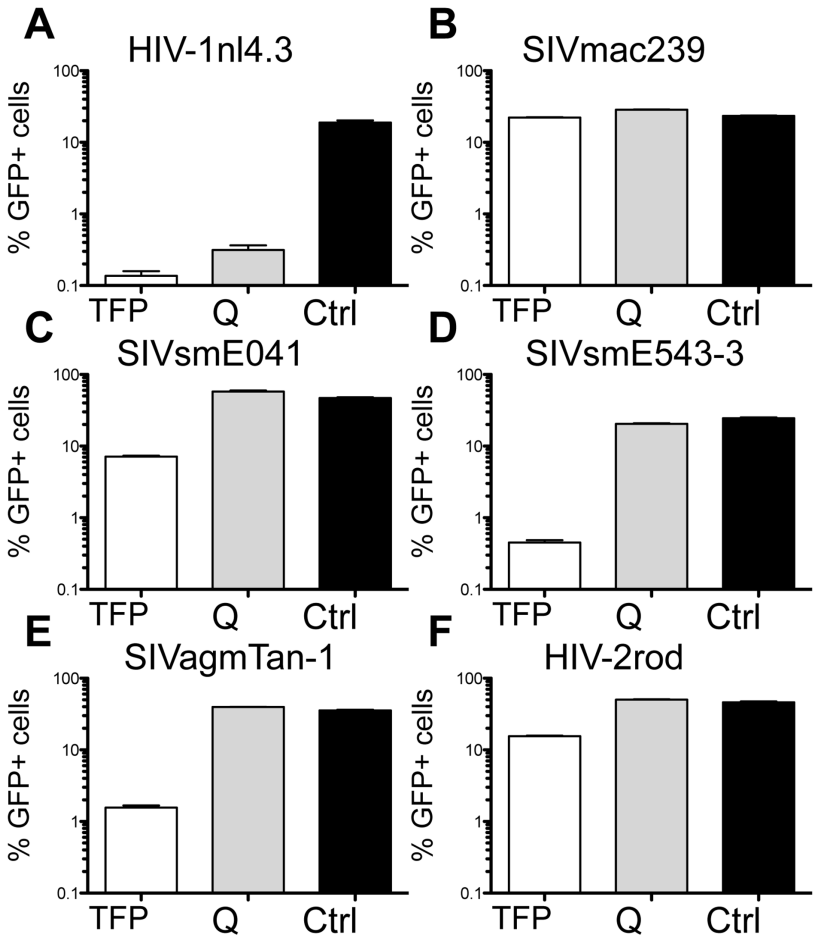


Figure 2-1. Differential restriction of primate lentiviruses by rhesus TRIM5α^{TFP} and TRIM5α^{Q} alleles. GFP reporter viruses were used to infect CRFK cells expressing the rhesus TRIM5α^{TFP} allele mamu3 (TFP) and the rhesus TRIM5α^{Q} allele mamu4 (Q). Infectivity on empty vector control cells is shown (Ctrl). (A) HIV-1nl4.3. (B) SIVmac239. (C) SIVsmE041. (D) SIVsmE543-3. (E) SIVagmTan-1. (F) HIV-2Rod. Infections were done in triplicate. Error bars indicate SEM. These results are representative of at least 3 independent experiments.

Individual surface elements of capsid determine restriction by TRIM5α.

HIV-1 and SIVmac239 had opposite restriction profiles when tested for restriction on rhTRIM5α expressing cells. HIV-1nl4.3 was restricted by both rhTRIM5α^{TFP} and rhTRIM5α^{Q} alleles, whereas SIVmac239 was resistant to both alleles. At least three lines of evidence support the existence of multiple sites of rhTRIM5α recognition within the HIV-1 CA. First, HIV-1 is restricted by both rhTRIM5α^{TFP} and rhTRIM5α^{Q} alleles while other tested primate lentiviruses are resistant to the rhTRIM5α^{Q} allele. Second, attempts to evolve an HIV-1 with resistance to rhTRIM5α have not yielded fully resistant viruses [294], while other viruses have successfully evolved resistance to rhTRIM5α-mediated restriction with genuine escape mutations both *in vitro* and *in vivo* [61,240]. Third, mutagenesis approaches in which elements of the SIVmac239 CA were inserted into the HIV-1 CA resulted in rhTRIM5α restricted viruses [282,283,289,290]. With 79 amino acid differences between the two viruses (Figure 2-2A), we hypothesized that isolating each determinant would allow us to resolve the specific amino acids involved in rhTRIM5α recognition at each target site. We therefore chose to take an alternative approach, based on identifying gain of sensitivity mutations of the inherently rhTRIM5α-resistant SIVmac239 CA. We inserted individual features of the HIV-1nl4.3 CA into the SIVmac239 CA and measured the impact on restriction.

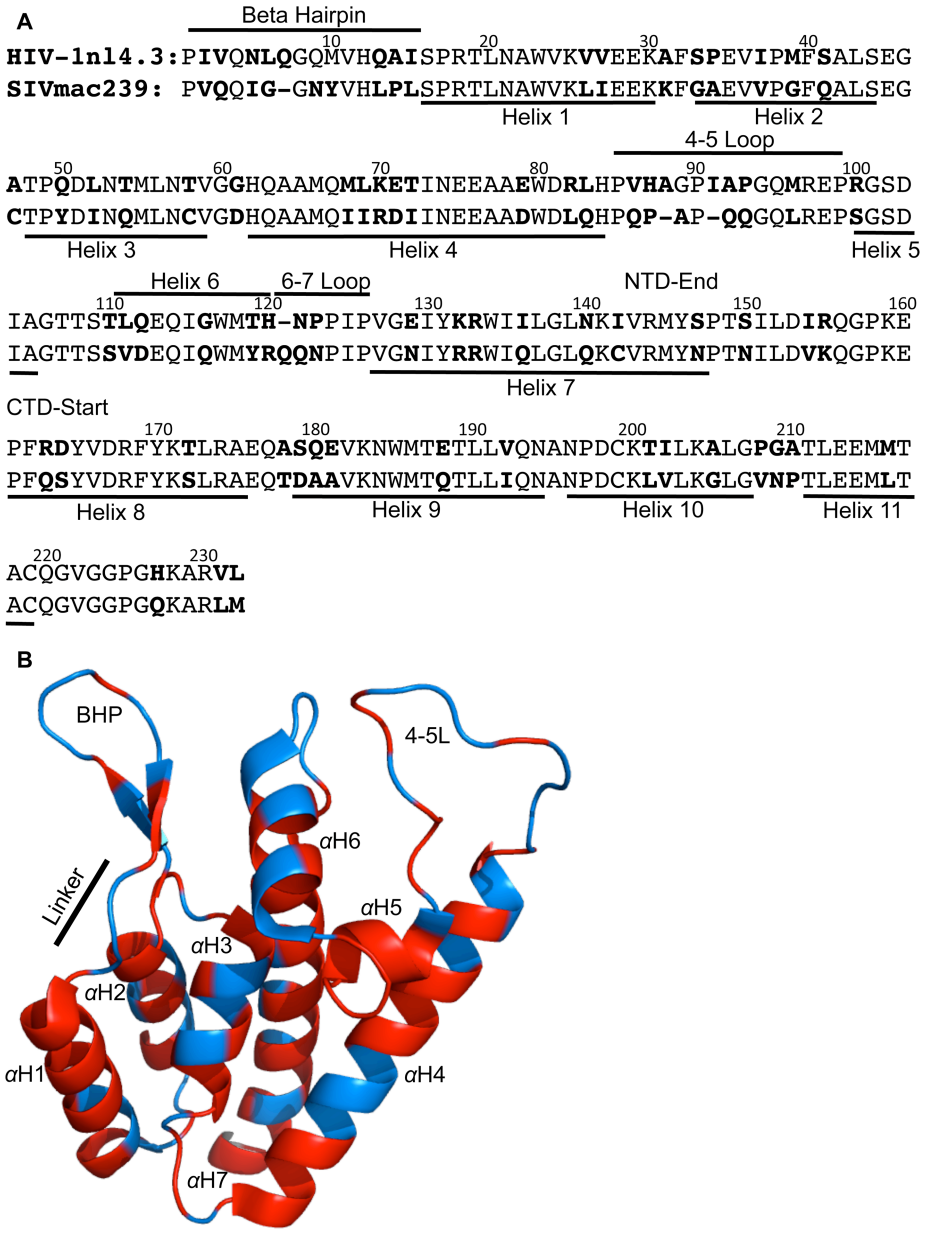


Figure 2-2. Distribution of amino acid differences between HIV-1nl4.3 and SIVmac239. (A) Sequence alignment of HIV-1nl4.3 and SIVmac239 CAs. Surface features are indicated on the top, internal α-helices on the bottom. Amino acid differences between the two viruses are in bold type. (B) Structure of the HIV-1nl4.3 N-terminal domain (PDB: 3GV2). The β-hairpin (BHP), the linker connecting the β-hairpin to helix 1 (linker), helix 6 (h6) and 4-5 loop (4-5L) are indicated. Additional α-helices are numbered αH1-αH7. Residues that are identical in HIV-1nl4.3 and SIvamc239 are in red, residues that differ are in blue.

The ability of TRIM5α orthologs to restrict highly divergent retroviruses with little to no sequence identity suggests TRIM5α may target conserved, structural elements of CA. All reported retroviral N-terminal domain structures have a conserved five α-helix core. To determine whether differences within the five α-helix core impact rhTRIM5α recognition, we generated SIV-HIV$_{interior}$, by replacing most of the five α-helix core of SIVmac239 with that of HIV-1nl4.3. This virus retained the SIVmac239 residues at the first and last amino acid of each α-helix (Figure 2-3). We then tested this virus for restriction by all verified rhTRIM5α^{TFP} (n=3) and rhTRIM5α^{Q} (n=2) alleles. This mutant was 2.3-fold more sensitive to rhTRIM5α^{TFP} than the SIVmac239 parent (Figures 2-4A-C and Figure 2-5). This differed markedly from SIV-HIV$_{surface}$, in which three surface elements, the β-hairpin, 4-5 loop and helix 6, were derived from HIV-1nl4.3. This virus was restricted by all rhTRIM5α alleles tested, at levels similar to HIV-1nl4.3 (Figures 2-4A-D).

Because SIV-HIV$_{surface}$ was phenotypically similar to HIV-1nl4.3, we asked whether a reciprocal chimera was sufficient to render HIV-1nl4.3 restriction resistant. Therefore, we replaced the HIV-1nl4.3 CA surface features with the three SIVmac239 surface features (the β-hairpin, 4-5 loop and helix 6) to create HIV-SIV$_{surface}$ (Figure 2-3). This HIV-1 variant differed from HIV-1nl4.3 by 28 amino acids and was highly resistant to restriction by rhTRIM5α^{TFP} and rhTRIM5α^{Q} alleles (Figure 2-4E). Within the linker that connects the β-hairpin to helix 1,

HIV-1nl4.3 and SIVmac239 differ at three positions (amino acids 13-15) (Figure 2-2 and Figure 2-3). Using a second HIV-1-SIV chimera, HIV-$SIV_{surface25}$, we determined that these three differences do not influence restriction (Figure 2-4F). To our knowledge, HIV-$SIV_{surface}$ and HIV-$SIV_{surface25}$ represent the first description of an HIV-1 strain resistant to all allelic classes of rhTRIM5. Titration of these viruses and abrogation assays confirm that resistance was not due to saturation of rhTRIM5α in the target cell lines (Figures 2-5 and 2-6).

Figure 2-3. Amino acid alignment of chimeric viruses.

Amino acid sequences of chimeric viruses used in this manuscript aligned to SIVmac239. Black lettering indicates unique SIVmac239 amino acids. Red lettering indicates unique HIV-1nl4.3 amino acids. Gray dots indicate conserved positions between SIVmac239 and HIV-1nl4.3. Hyphens were inserted to preserve the alignment in cases of insertions/deletions. Numbered rows correspond to the following viruses: 1. SIV-$HIV_{Interior}$ 2. SIV-$HIV_{surface}$ 3. SIV-HIV_{bhp} 4. SIV-$HIV_{bhpQ7\Delta,}$ 5. SIV-$HIV_{4\text{-}5L}$ 6. SIV-HIV_{h6} 7. HIV-$SIV_{surface}$ 8. HIV-$SIV_{surface25}$ 9. SIV_{V2I}10. SIV_{Q3V} 11. SIV_{I5N} 12. SIV_{G6L} 13. $SIV_{\Delta 7Q}$ 14. SIV_{N9Q} 15. SIV_{Y10M} 16. SIV_{Q86V} 17. SIV_{P87H} 18. $SIV_{\Delta 88A}$ 19. SIV_{A89G} 20. $SIV_{\Delta 91I}$ 21. SIV_{Q92A} 22. SIV_{Q93P} 23. SIV_{L96M} 24. SIV_{S100R} 25. SIV_{S110T} 26. SIV_{V111L} 27. SIV_{D112Q} 28. SIV_{Q116G} 29. SIV_{Y119T} 30. SIV_{R120H} 31. SIV_{R120N} 32. $SIV_{Q121\Delta}$ 33. SIV_{Q122N} 34. SIV_{N123P}

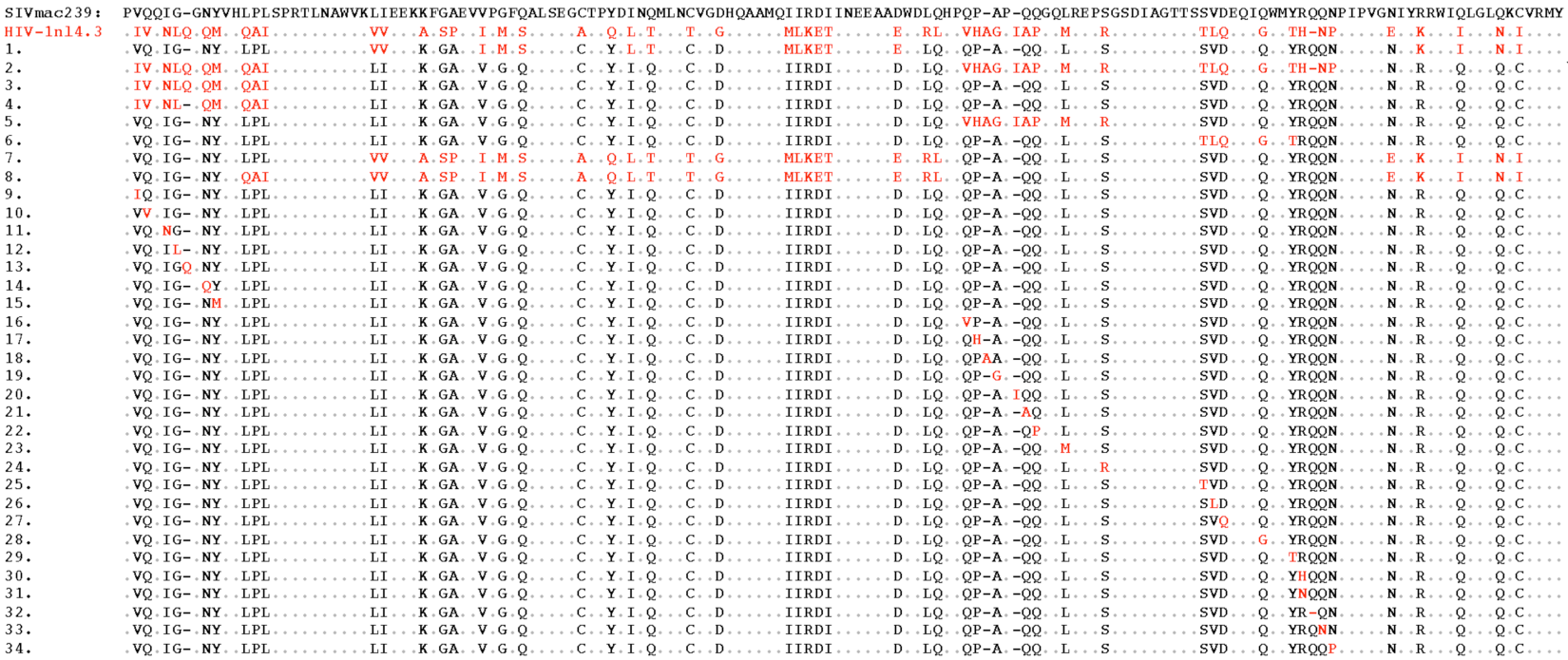


Figure 2-3. Amino acid alignment of chimeric viruses: continued.

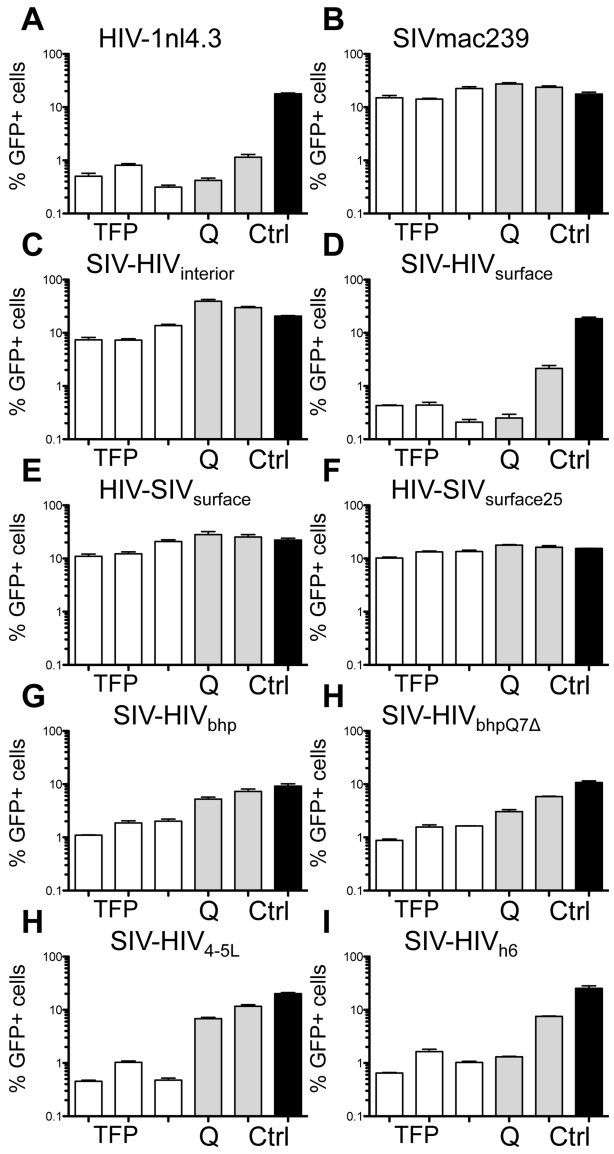


Figure 2-4. Rhesus TRIM5αs recognize the capsid surface.

The indicated GFP reporter viruses were used to infect CRFK cells expressing rhesus TRIM5α^{TFP} alleles mamu1, mamu2 and mamu3 (TFP) and the TRIM5α^{Q} alleles mamu4 and mamu5 (Q). Infectivity on empty vector control cells is shown (Ctrl). (A) HIV-1nl4.3. (B) SIVmac239. (C) SIV-HIV$_{interior}$. (D) SIV-HIV$_{surface}$. (E) HIV-SIV$_{surface}$. (F) HIV-SIV$_{surface25}$. (G) SIV-HIV$_{bhp}$. (H) SIV-HIV$_{bhpQ\Delta7}$.(I) SIV-HIV$_{4-5L}$. (J) SIV-HIV$_{h6}$. Infections were done in triplicate. Error bars indicate SEM. These results are representative of at least 3 independent experiments.

Virus	Capsid Production ug/ml	Volume Used (ul)	ng capsid used	%GFP+ Cells Control Cells	%GFP+ Cells mamu1 cells	%GFP+Cells mamu4 cells
SIVmac239	2.06	2.0	4.1	20.73	18.34	30.24
HIV-1nl4.3	2.81	15.8	44.5	17.83	0.50	0.42
SIV-HIVinterior	0.31	185.0	27.8	20.63	7.41	39.3
SIV-HIVsurface	0.15	92.0	28.5	18.5	0.43	0.25
SIV-HIVbhp	0.37	450	166.5	9.20	1.1	5.20
SIV-HIVbhpQ7Δ	0.09	30.0	2.7	10.74	0.88	3.05
SIV-HIV4-5L	2.88	1.8	5.1	20.0	0.45	6.81
SIV-HIVh6	0.92	36.5	33.6	25.37	0.65	1.31
HIV-SIVsurface	1.20	450.0	535.5	22.17	11.02	28.17
HIV-SIVsurface25	0.96	223.0	211.8	15.45	10.19	17.85
SIV-V2I	1.10	3.5	3.85	19.71	13.93	27.63
SIV-Q3V	1.01	228	250.8	16.23	3.74	6.09
SIV-I5N	1.39	12.5	17.4	18.42	22.17	29.88
SIV-G6L	0.80	25	20	15.86	2.33	5.16
SIV-Δ7Q	0.54	7.8	4.5	21.02	2.43	31.56
SIV-N9Q	1.38	2.3	3.2	17.62	9.25	26.69
SIV-Y10M	2.10	2.2	4.6	21.78	5.40	32.37
SIV-Q86V	1.90	2.7	5.1	23.91	6.80	35.53
SIV-P87H	1.60	4.4	7.1	21.16	3.21	31.49
SIV-Δ88A	1.55	12.5	19.4	20.99	16.42	31.86
SIV-A89G	2.15	5.2	11.2	16.74	3.56	25.36
SIV-Δ91I	2.01	3.3	6.6	22.67	4.03	33.50
SIV-Q92A	3.48	1.9	6.6	18.69	18.23	28.62
SIV-Q93P	5.46	3.3	18.0	20.38	7.54	32.63
SIV-L96M	3.58	3.1	11.2	25.34	3.18	36.27
SIV-S100R	1.93	3.2	6.1	17.84	1.53	28.62
SIV-S110T	1.94	2.2	4.3	16.90	8.97	23.71
SIV-V111L	2.11	1.9	4.1	14.27	2.50	12.27
SIV-D112Q	1.16	4.1	4.8	23.49	3.02	25.42
SIV-Q116G	2.69	1.7	4.6	19.11	6.11	27.88
SIV-Y119T	0.89	45.0	40	20.26	24.02	31.29
SIV-R120H	0.45	ND	ND	ND	ND	ND
SIV-R120N	0.51	300.0	150	31.46	38.54	42.54
SIV-Q121Δ	1.22	25.0	30.3	23.34	10.88	35.30
SIV-Q122N	3.20	2.0	6.4	19.83	23.56	28.96
SIV-N123P	0.21	ND	ND	ND	ND	ND

Figure 2-5. Characterization of viruses. The infectivity and capsid antigen content of viruses presented in this manuscript are provided. Capsid antigen concentration was determined by p24 and p27 antigen capture ELISA (Advanced Bioscience Laboratories, Rockville MD.). All viruses in which the C-terminal domain was derived from HIV-1 were used with p24 antigen capture kit, while all viruses in which the C-terminal domain was derived from SIVmac239 were tested using a p27 antigen capture kit.

Figure 2-6. Surface feature chimeras do not abrogate TRIM5α activity. Two independent saturation controls were done to insure that attenuated viruses did not abrogate TRIM5α activity. (A) Titration curves on CRFK-Neo control cells (Black lines I-IV) and mamu1 (rhTRIM5α^{TFP}) expression cells (red lines I-IV) were carried out. Data points are the average of 3 infections. Error bars indicate the S.E.M. 50,000 cells were seeded in a 24 well plate in 0.5 ml of media. Infections were carried out in 0.2 ml media and harvested for FACS 40 hours post infection. (I) SIVmac239. (II) HIV-1nl4.3. (III) HIV-$SIV_{surface}$. (IV) HIV-$SIV_{surface25}$. Notably there is little or no deviation between the apparent infectivities of SIVmac239, HIV-$SIV_{surface}$ and HIV-$SIV_{surface25}$ on control cells and on mamu1 expressing cells at every concentration of virus tested. There is a very large difference between the apparent infectivity of HIV-1nl4.3 on control cells and mamu1 (rhTRIM5α^{TFP}) cells. (V) Graphs I-IV graphed together. Importantly, despite the attenuation of HIV-$SIV_{surface}$ and HIV-$SIV_{surface25}$ their curves fall inside the saturating curve for HIV-1nl4.3 on mamu1 cells. (B) Two color abrogation assays were conducted under identical conditions to those in Table 2-1 and Figures 2-1 and 2-4. Cells were harvested at 30 hours post infection. Identical amounts of HIV-1, SIVmac239-S100R, HIV-$SIV_{surface}$ and HIV-$SIV_{surface25}$ to those used in Figure 2-4 and Table 2-1 were used. Additionally, the same concentration (ng of capsid) as the most attenuated mutant, HIV-$SIV_{surface}$, was used for the two rhTRIM5α restricted viruses HIV-1nl4.3 and SIVmac239 S100R. Cells were co-infected with a fixed concentration of a HIV-1 CFP reporter virus. Values for GFP and CFP positive cells are separated into two columns (“GFP” and “CFP”) for ease of viewing, but the values are from the same co-infection. Under all conditions an enhancement of infectivity for the CFP reporter virus on restrictive cells mamu1 and mamu4 (rhTRIM5α^{TFP} and rhTRIM5α^{Q}) was not observed. Therefore, despite high concentrations of virus, our experimental conditions did not saturate TRIM5α. Bar graphs represent the average of 3 independent infections. Error bars indicate the S.E.M

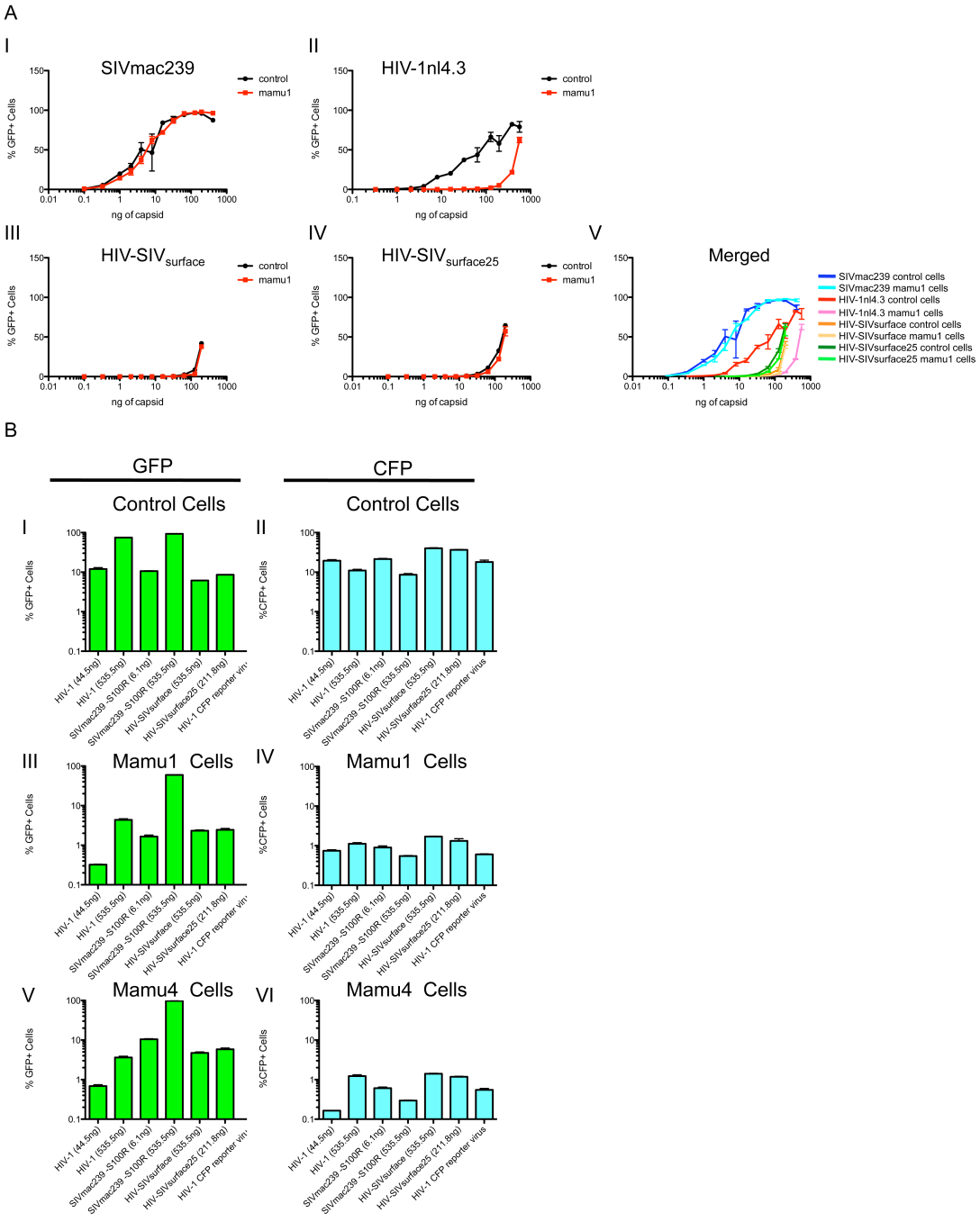


Figure 2-6. Surface feature chimeras do not abrogate TRIM5α activity: continued

To examine the individual contributions of each of the three surface features to restriction, we produced a series of SIVmac239 CAs each grafted with a single HIV-1nl4.3 surface feature. To take into account the fact that the β-hairpin is one amino acid shorter in SIVmac239, we generated two SIV variants: SIV-HIV$_{bhp}$, with a full length HIV-1nl4.3 β-hairpin, and SIV-HIV$_{bhpQ7\Delta}$, with a single amino acid deletion in the HIV-1nl4.3 β-hairpin. We also generated SIVmac239 variants with the HIV-1nl4.3 4-5 loop or helix 6 (SIV-HIV$_{4\text{-}5L}$ and SIV-HIV$_{h6}$, respectively). Rhesus TRIM5α^{TFP} alleles restricted all four of these viruses (SIV-HIV$_{bhp}$, SIV-HIV$_{bhpQ7\Delta}$, SIV-HIV$_{4\text{-}5L}$, and SIV-HIV$_{h6}$). With the exception of SIV-HIV$_{h6}$, the chimeras had little effect on restriction by rhTRIM5α^{Q} (Figure 2-4G-J). Together, these mutants suggest that the HIV-1 restriction-sensitive and SIVmac239 restriction-resistant phenotypes involve contributions from all three capsid surface features.

Capsid mutagenesis reveals differences in restriction by TRIM5α^{TFP} and TRIM5α^{Q}.

Based on results obtained from the HIV-SIV$_{surface25}$ chimera, we generated a series of SIVmac239 CA mutations in which the amino acid at each of the 25 positions of interest was substituted with the amino acid found at the homologous position in HIV-1nl4.3 (Figures 2-2A, 2-4F, 2-3, 2-5 and Table 2-1). The length of the SIVmac239 and the HIV-1nl4.3 CAs sequences are different. Relative to the other there are insertions and deletions that are distributed across

the capsid surface features. Therefore all mutant viruses are named according to the sequence alignment in Figure 2-2A. Table 2-1 details the relationship between the mutant SIVmac239 virus numbering and the numbering for the HIV-1nl4.3 and SIVmac239 CAs. Two of the 25 mutations in the SIVmac239 CA, R117H and N123P, resulted in loss of infectivity. Although a His is found at position 117 in HIV-1nl4.3, an Asp is more common among HIV-1 isolates. We found that an SIVmac239 in which R117 was substituted with Asp instead of His retained infectivity (Figure 2-5).

The 24 infectious SIVmac239 variants with single amino acid substitutions in CA were tested for sensitivity to restriction by rhTRIM5α^{TFP} and rhTRIM5α^{Q}. Restriction was quantified by determining the level of infectivity relative to SIVmac239 (Table 2-1). Only two mutant viruses single amino acid substitutions (SIVmac239$_{Q3V}$ and SIVmac239$_{G6L}$), both in the β-hairpin, resulted in gain-of-sensitivity to both rhTRIM5α^{TFP} and rhTRIM5α^{Q}. There were 12 additional mutations that caused gain-of-sensitivity to rhTRIM5α^{TFP}, but not to rhTRIM5α^{Q}. These mutations were spread among all three CA surface features. Together these results indicate that the targets of the two alleles partially overlap, and that the overlap involves elements within the β-hairpin. The observation that a large number of residues outside of the β-hairpin exclusively affect rhTRIM5α^{TFP} without altering rhTRIM5α^{Q} sensitivity raises the possibility that rhTRIM5α^{TFP} either has a larger footprint on the CA surface than rhTRIM5α^{Q}, or that it has the

capacity to target more than one determinant in CA. Most notably, there were no mutations that affected only the rhTRIM5α^{Q} allele (that is, none of the mutations tested caused gain-of-sensitivity to rhTRIM5α^{Q} but not to rhTRIM5α^{TFP}). This trend was mirrored among the 14 other viruses tested, including both naturally occurring viruses and chimeric viruses generated for this study (Figures 2-1 and 2-4).

Table 2-1. Single amino acid mutants reveal differences in restriction by TRIM5^{TFP} and TRIM5^{Q}. The amino acid numbering of mutant viruses corresponds to the alignment in Figure 2-2A. Numbering of mutated residues corresponding to the SIVmac239 capsid (Accession number M33262) and HIV-1nl4.3 (Accession number M19921.2) are also provided. All values are shown as fold-restriction relative to parental SIVmac239. The values are the result of 3 independent experiments, each done in triplicate. The error represents the standard deviation between these 9 infections. N.D - mutant was not infectious and was not analyzed.

Mutant virus	SIVmac239 Residue	HIV-1nl4.3 Residue	TFP	Q
V2I	V2	I2	1.21 ±0.08	1.02 ±0.14
Q3V	Q3	3V	5.58 ± 0.92	5.42 ±0.05
I5N	I5	N5	0.70 ±0.05	0.86 ±0.08
G6L	G6	L6	8.37 ±1.46	6.59 ±2.28
Δ7Q	Δ	Q7	7.73 ±1.35	0.96 ±0.15
N9Q	N8	Q9	1.82 ±0.21	1.09 ±0.20
Y10M	Y9	M10	3.41 ±0.07	0.94 ±0.11
Q86V	Q85	V86	3.14 ±0.31	0.99 ±0.05
P87H	P86	H87	5.73 ±1.06	0.95 ±0.17
Δ88A	Δ	A88	1.12 ±0.10	0.95 ±0.04
A89G	A87	G89	4.08 ±0.50	0.94 ±0.14
Δ91I	Δ	I91	4.53 ±0.56	0.91 ±0.22
Q92A	Q89	A92	0.95 ±0.20	1.00 ±0.21
Q93P	Q90	P93	2.53 ±0.28	1.01 ±0.24
L96M	L93	M96	7.12 ±0.58	1.03 ±0.08
S100R	S97	R100	10.80 ±2.46	0.95 ±0.20
S110T	S107	T110	1.6 ±0.23	1.01 ±0.18
V111L	V108	L111	6.01 ±0.09	2.11 ±0.37
D112Q	D109	Q112	7.15 ±0.76	1.39 ±0.25
Q116G	Q113	G116	2.83 ±0.16	1.05 ±0.15
Y119T	Y116	T119	0.73 ±0.03	0.94 ±0.07
R120H	R117	H120	N.D.	N.D.
R120N	R117	H120	0.71 ±0.08	1.04 ±0.14
Q121Δ	Q118	Δ	1.79 ±0.13	0.91 ± 0.13
Q122N	Q119	N121	0.75 ±0.07	1.01 ±0.09
N123P	N120	P122	N.D.	N.D.

Structure of the SIVmac239 CA N-terminal domain.

To provide a relevant structural context for evaluating the mutagenesis results, we determined the structure of the SIVmac239 CA N-terminal domain (Figures 2-7A, 2-8, 2-9 and Table 2-2). The SIVmac239 CA N-terminal domain was very similar to reported structures of HIV-1 (PDB: 2X2D) (RMSD at Cα positions: 2.29Å) and HIV-2 (PDB: 2WLV) (RMSD at Cα positions: 1.42Å) (calculations used SuperPose [388]) . In particular, the five α-helices of the SIVmac239 N-terminal domain core did not deviate from those of HIV-1 or HIV-2, consistent with the observation that the SIV-$HIV_{interior}$ chimera remained largely resistant to restriction (Figure 2-4C).

Table 2-2. Crystallography refinement statistics.

Table generated by Aaron G. Schmidt

	SIVmac239 CA NTD
Data Collection	*APS ID-24-E*
Resolution, Å	*50-2.9 (2.95-2.90)*
Wavelength (Å)	*0.9792*
Space Group	*P212121*
Unit cell dimensions (a, b, c), Å	*24.61, 56.66, 102.57*
Unit cell angles (α, β, γ) °	*90,90,90*
mean I/σ	*17.17 (3.0)*
Rsym	*0.063 (0.365)*
Completeness, %	*99.46 (95.10)*
Number of reflections	*15,453*
Redundancy	*4.4*
Refinement	
Resolution, Å	*2.9*
Number of reflections:	
Working	*3,345*
Free	*152*
Rwork, %	*25.4*
Rfree, %	*29.2*
Ramachandran plot,	
% favored	*89.1 (123/138)*
% disallowed	*0.72 (1/138)*
Rmsd bond lengths, Å	*0.0111*
Rmsd bond angles, °	*1.37*
Average B-factor	*109.3*

Note: values in parentheses denotes highest resolution shell

Figure 2-7. Structure of the SIVmac239 capsid N-terminal domain:
(A) Structure of the SIVmac239 CA N-terminal domain at 2.9 Å resolution. There was no clear density for Pro88, and thus, it was omitted from the structure. A dashed line is used to indicate its place. (B) Comparison of the SIVmac239 β-hairpin and 4-5 loop to all other wild type HIV-1 and HIV-2 X-ray structures deposited in the PDB. HIV-1 structures are colored dark gray, except PDB: 2X2D, which is colored red (and used in all subsequent comparisons). HIV-2 structures are colored light gray, and the SIVmac239 N-terminal domain is colored blue. (C and D) Locations of amino acids mutations associated with rhesus TRIM5α^{Q} (C) and rhesus TRIM5α^{TFP} (D) restriction from Table 2-1. Blue spheres indicate amino acid differences that do not impact TRIM5α restriction. Orange spheres show the location of mutations associated with 2.5-5 fold gains in sensitivity to rhesus TRIM5α relative to SIVmac239. Red spheres indicate positions associated with >5 fold gains in sensitivity to rhesus TRIM5α. Images created in PyMol.

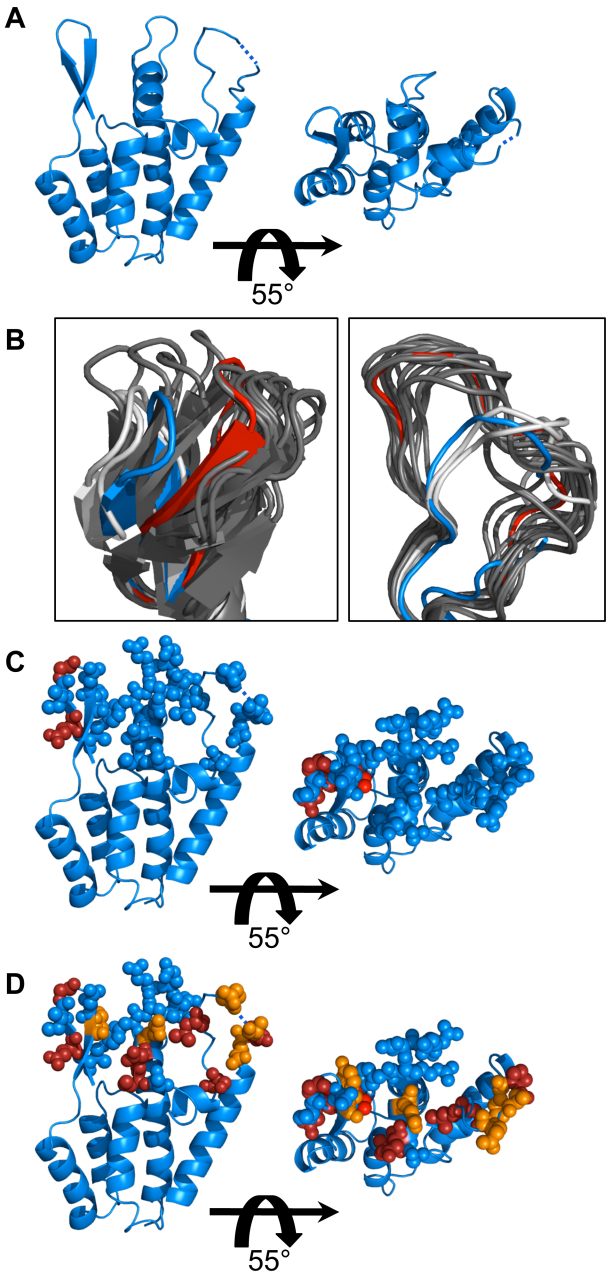


Figure 2-7. Structure of the SIVmac239 Capsid N-terminal domain: continued.

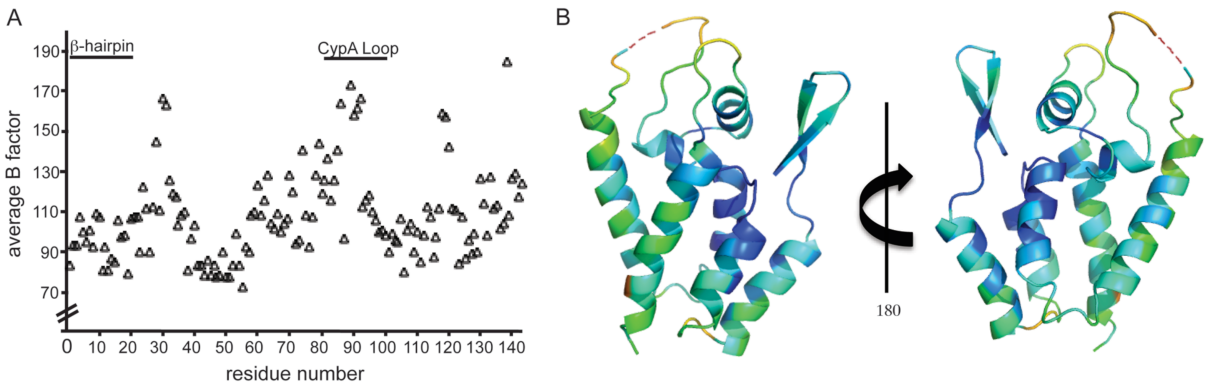


Figure 2-8. B-factor analysis of SIVmac239 structure. Average B-factor plot of each residue included in the final model. The *β*-hairpin and 4-5 loop are delineated as reference points. (B) Visual "heat-map" of average B-factors. Dark blue's indicate lowest B-factors dark red indicates the highest B-factors. Residue 88 was removed from the structure due to lack of clear density and is indicated by the dashed red line. Images created in PyMol. Figure and legend generated by Aaron G. Schmidt

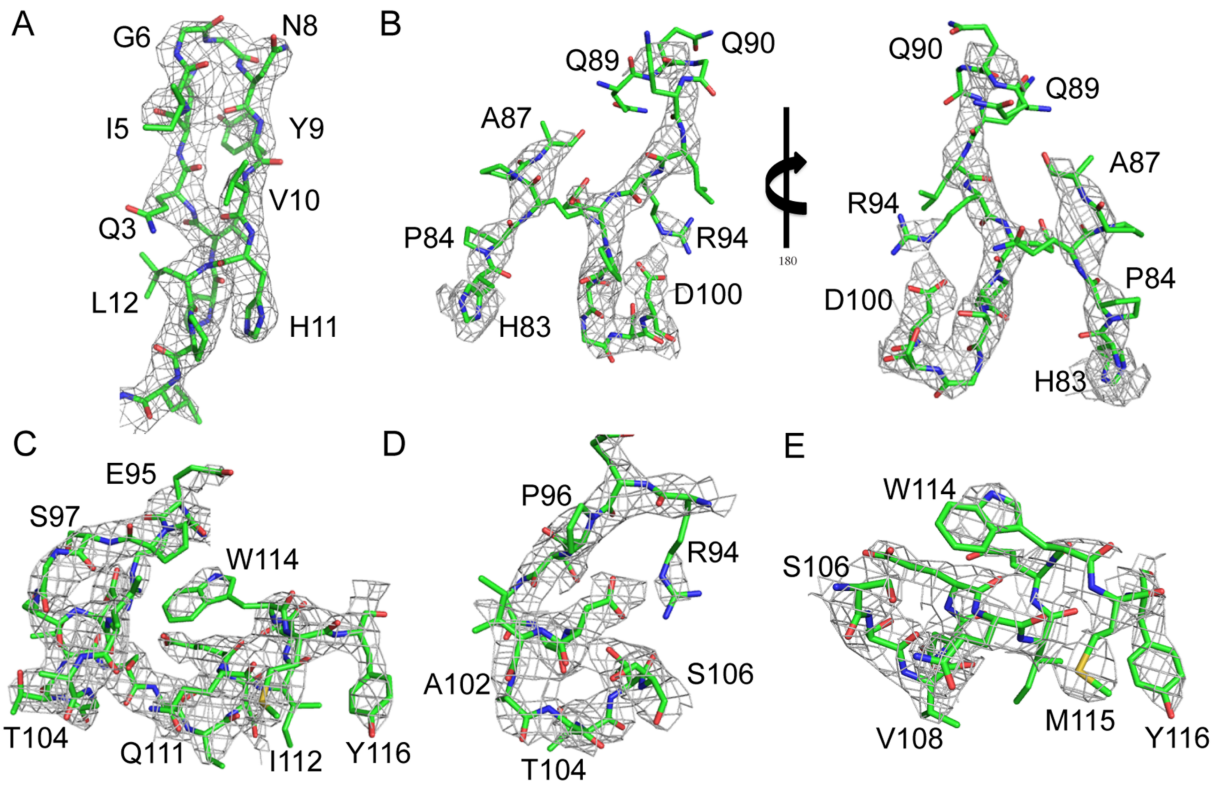


Figure 2-9. Electron density maps of key regions in the SIVmac239 structure. (A) the *β*-hairpin, residues 1–14. (B) the 4-5 loop, residues 83–100—residue 88 has been removed from the structure as there was no clear electron density (C) the "conserved patch" residues 95–116 (D) isolated residues 94–106 and (E) 106–116. All images are 2Fo-Fc maps and are contoured at 1.5**σ** throughout for consistency. Structure factors and the final model have been deposited in the Protein Data Bank accession 4HTW. All images created in PyMol Figure and legend generated by Aaron G. Schmidt.

Since the amino acids governing rhTRIM5α restriction mapped to the CA surface, we were particularly interested in structural differences between SIVmac239 and HIV-1 in the β-hairpin, 4-5 loop and helix 6. We compared the SIVmac239 CA N-terminal domain structure to all of the previously reported wild type HIV-1 and HIV-2 CA N-terminal domain structures in which the surface features were properly folded (Figure 2-7B and Figure 2-9). This dataset includes structures of CA monomers, CA monomers from cyclophilin A bound HIV-1 CAs, HIV-1 hexamers and HIV-1 pentamers. From this analysis, we found a clear distinction between the HIV-1 structures and those of the more closely related HIV-2 and SIVmac239. Specifically, the 4-5 loops and β-hairpins formed two clusters; one composed of HIV-1 structures, and the other composed of SIVmac239 and HIV-2 structures. Measurements between the HIV-1 Cα of Gly94 or Gln95 and the corresponding Gly91 and Gln92 of SIVmac239 indicate that these two groups are separated by 3.3-11 Å in the structural alignment. Similarly, measurements between the Cα of HIV-1 Gly8 and the homologous SIVmac239/HIV-2 Gly7 show the two groups are separated by 4-8.5 Å in the structural alignment (Figure 2-7B). These CA structural differences may help to explain the observed changes in restriction between the reciprocal SIV-$HIV_{surface}$ and HIV-$SIV_{surface}$ chimeras (Figures 2-4D and 2-4E)

To determine the spatial arrangement of the single amino acid substitutions associated with rhTRIM5α restriction, we mapped the restriction data for rhTRIM5α^{Q} and rhTRIM5α^{TFP} onto

the structure of the SIVmac239 N-terminal domain (Figures 2-7C and 2-7D respectively) as well as the structure of the HIV-1 CA hexamer (Figure 2-10). The two individual point mutations associated with rhTRIM5α^{Q} restriction were confined to the β-hairpin and were within 10 Å of each other. This differed from rhTRIM5α^{TFP}, which in addition to being affected by the same two sites in the β-hairpin, also recognized amino acid substitutions outside the β-hairpin, spanning approximately 30 Å of the CA surface.

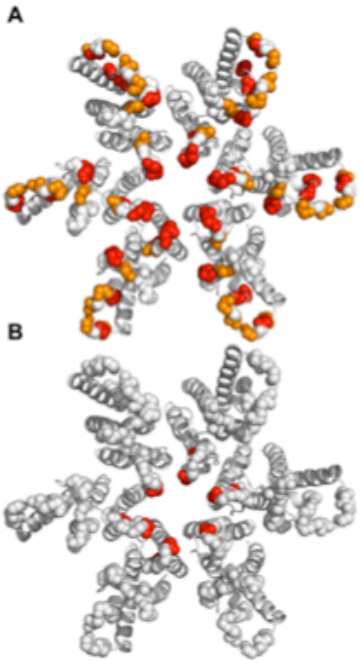


Figure 2-10. Mutations modulating TRIM5α sensitivity mapped to the HIV-1 hexamer. Mutations from Table 2-1 mapped to the HIV-1 hexamer structure 3GV2. Restriction data for mutant viruses tested against the rhesus TRIM5α^{TFP} allele mamu1 (A) and the rhesus TRIM5α^{Q} allele mamu4 (B). Positions that were mutated on the capsid surface and were <2.5 fold more sensitive to TRIM5α restriction than SIVmac239 are shown in gray spheres Orange spheres show the location of mutations associated with 2.5-5 fold gains in sensitivity to rhesus TRIM5α. Red spheres indicate positions associated with >5 fold gains in sensitivity to rhesus TRIM5α. Images created in PyMol.

Residues influencing rhesus TRIM5α^{TFP} sensitivity surround a conserved capsid patch.

In contrast to rhTRIM5α^{Q}, we found that rhTRIM5α^{TFP} restricts at least three phylogenetically distinct primate lentiviruses: HIV-1, SIVagmTan, and SIVsm (Figure 2-1). While single amino acid substitutions affecting rhTRIM5α^{Q} were confined to the β-hairpin, substitutions that increased sensitivity to rhTRIM5α^{TFP} were spread across the N-terminal domain surface (Figures 2-7C and 2-7D). Based on these two observations, we hypothesized that rhTRIM5α^{TFP} may have evolved to target a conserved element(s) unique to the primate lentivirus CA N-terminal domain. To identify uncharacterized sites of primate lentivirus conservation, we generated an alignment of CA N-terminal domains using one representative virus from eleven different primate lentivirus lineages (Figure 2-11). We then scored the number of unique amino acids found at each position, and mapped the results onto the SIVmac239 structure (Figure 2-12A).

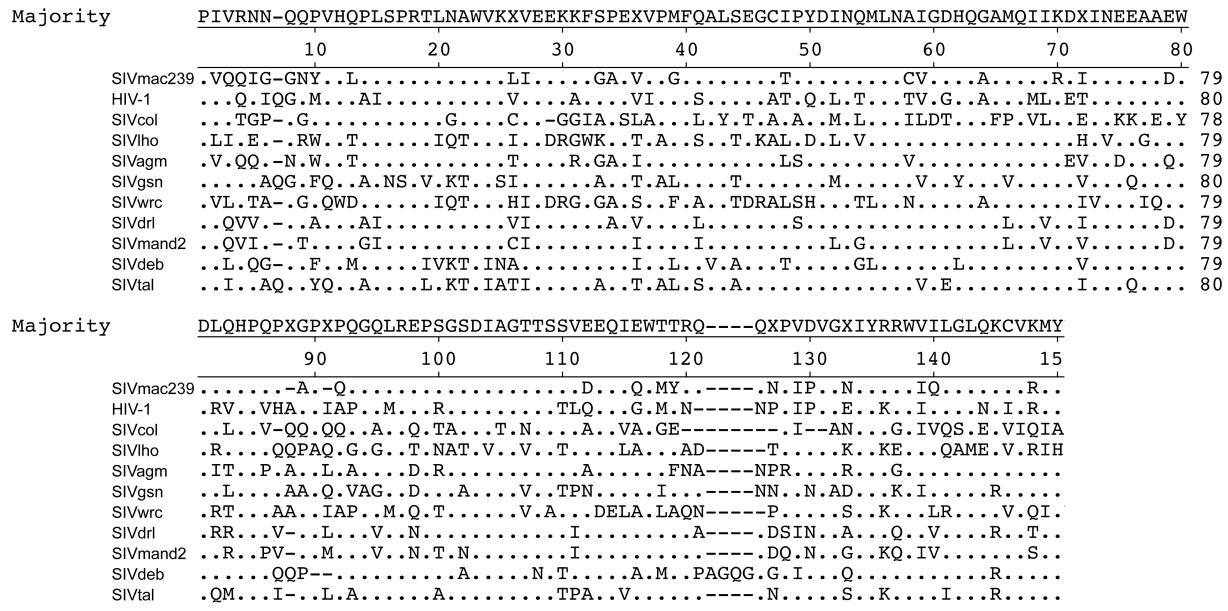
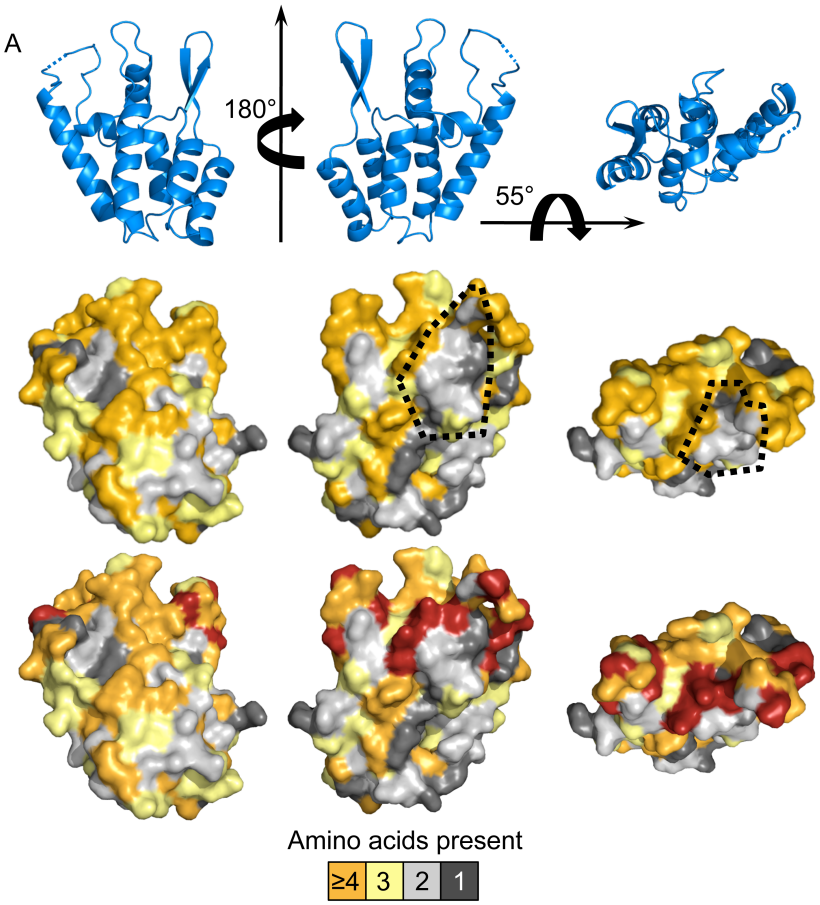


Figure 2-11. Amino acid alignment of divergent primate lentiviruses.

Primate lentiviruses from eleven different lineages are aligned corresponding to the published alignment found in the Los Alamos Sequence database. The majority sequence is depicted at top. An “X” indicates that there is no amino acid majority at that position. Accession numbers: SIVmac239-M33262, HIV-1-K03455, SIVcol-AF301156, SIVlho-AF075269, SIVagm-U58991, SIVgsn-AF468658, SIVwrc-AM745105, SIVdrl-AY159321, SIVmand2-AY159322, SIVdeb-AY523865, SIVtal-AM182197

Figure 2-12. Mutations modulating rhesus TRIM5α^{TFP} restriction ring a conserved surface patch: (A) Top row: Orientations of the SIVmac239 capsid used for Figure 2-12A. Middle row: Surface representation of the SIVmac239 capsid N-terminal domain colored to reflect amino acid conservation across divergent primate lentiviruses. The number of unique amino acids found at each position in an amino acid alignment of eleven divergent primate lentiviruses (Figure 2-11) was scored and colored according to the legend: Orange ≥4 unique amino acids at the specified position, yellow 3 unique amino acids at the specified position, light gray 2 unique residues at the specified position and dark gray 1 amino acid (100% conservation) at the specified position. The location of the conserved surface patch is indicated by dashed lines. Bottom panel: Locations of mutations that are associated with a >2.5 fold gain in sensitivity to rhTRIM5α^{TFP} are shown in dark red. (B) Atomic view of the conserved surface patch. For reference the SIVmac239 and HIV-1 (2X2D) ribbon diagrams are shown in light blue and pink, respectively. The amino acids that make up the conserved surface patch are shown in sticks that are colored according to the capsid ribbon diagram, SIVmac239 in light blue and HIV-1 in light red. Variable positions shown to modulate rhesus TRIM5α^{TFP} sensitivity are colored in dark blue (SIVmac239) and dark red sticks (HIV-1) for emphasis. Images created in PyMol.



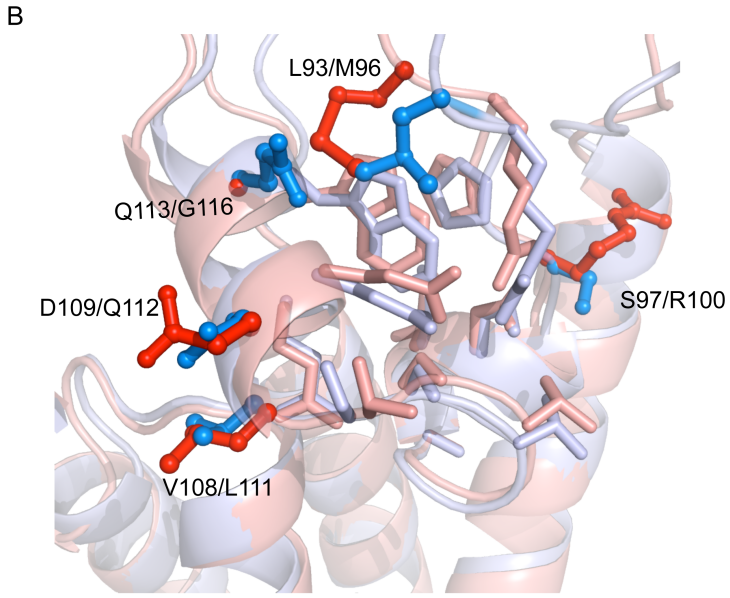


Figure 2-12. Mutations modulating rhesus TRIM5α^{TFP} restriction ring a conserved surface patch: continued.

Despite significant sequence diversity among primate lentiviruses, we found a cluster of conserved residues on the CA surface. This site overlapped with the structurally conserved C-terminus of the 4-5 loop, and helices 5 and 6. In SIVmac239, this patch is composed of residues Lue93, Arg94, Pro96, Gly98, Asp100, Ile101, Ala102, Gly103, Thr105, Ser106, Ser107, Glu110, Gln112 and Trp114 (Figures 2-12, 2-8, 2-9, and 2-11). This patch of conservation extends into a larger site of conservation formed by α-helices 3, 4 and 5. This site of conservation has recently been identified as the binding site for nuclear import factor CPSF6 [363]. Mutation SIVmac239 viruses that were restricted rhTRIM5α^{TFP} greater than 2.5-fold include SIVmac239$_{S100R}$, SIVmac239$_{V111L}$, SIVmac239$_{D112Q}$ and SIVmac239$_{Q116G}$, which positionally ring the boundaries of this patch, and SIVmac239$_{Q86V}$, SIVmac239$_{P87H}$, SIVmac239$_{A89G}$, SIVmac239$_{\Delta 91I}$, SIVmac239$_{Q93P}$ and SIVmac239$_{L96M}$ which correspond to amino acids in the 4-5 loop just above the patch (Table 2-1, Figure 2-12 and Figure 2-9). In the immediate vicinity of the surface exposed conserved patch there were three observed trends for amino acid substitutions that influenced rhTRIM5α^{TFP} restriction: 1) mutations in the variable regions of the 4-5 loop, 2) amino acid differences at the periphery of the surface patch, and 3) amino acid differences extending into the surface patch.

There were five amino acid substitutions within the highly variable regions of the 4-5 loop that had an impact on rhTRIM5α^{TFP} restriction. The SIVmac239 4-5 loop, like that of HIV-2, is

positioned further over the conserved surface patch than that of most HIV-1 loops. (Figures 2-7B and 2-12B). It has been documented that amino acid substitutions can alter the conformation or the dynamics of the 4-5 loop [334,389]. It is therefore possible that the mutations in SIVmac239$_{Q86V}$, SIVmac239$_{P87H}$, SIVmac239$_{A89G}$, SIVmac239$_{,91I}$ and SIVmac239$_{Q93P}$ may alter the conformation or dynamics of the 4-5 loop in such a way as to enhance rhTRIM5α recognition of the conserved surface patch.

Structurally, the surface patch was conserved across SIVmac239, HIV-1 and HIV-2. The C-terminus or the 4-5 loop, helix 5 and helix 6 were in very close agreement with the structures of HIV-1 and HIV-2, indicative of strong selection to preserve the overall architecture and amino acid composition of this site. Rather than changes to the structure or sequence of the patch, a majority of substitutions that altered rhTRIM5α^{TFP} sensitivity were found at its periphery. For example, we found that altering Ser97 in SIVmac239 to the corresponding HIV-1 Arg had the largest effect of any single substitution tested. An Arg at this position is found in an overwhelming majority of reported SIVsm sequences, and importantly, the Arg to Ser mutation was found to be a critical adaptive change acquired by SIVsm to evade rhTRIM5α^{TFP}-mediated restriction *in vivo* [61]. In HIV-1 and HIV-2 an Arg at this position contributes to a hydrogen bond bridging the base of the 4-5 loop. In SIVmac239 the corresponding Ser97 does not participate in a similar contact, but rather, it appears to engage in additional contacts within helix 5 which are

not observed in HIV-1 or HIV-2. SIVmac239 Asp109 and HIV-1 Gln112 are oriented similarly, however the presence of an acidic group would alter the chemical environment at the periphery of the patch (Figure 2-12B). There was no obvious difference to explain why the V111L mutant in helix-6 was six-fold more sensitive to restriction than parental SIVmac239. Perhaps slight differences between the side-chains of these residues can impact rhTRIM5α^{TFP} restriction.

Two substitutions that were associated with increased rhTRIM5α^{TFP} sensitivity extend into the conserved surface patch itself. We found that substituting the Leu at position 93 (which sits over the surface patch) for the less-bulky Met residue resulted in a 7-fold gain in sensitivity to rhTRIM5α^{TFP} (Figure 2-12B and Table 2-1). Notably, Leu93/Met96 cover Trp114 and Arg94, both of which are absolutely conserved among primate lentiviruses. Finally, SIVmac239 residue Gln113 reaches deeper into the patch than the corresponding Gly116 in HIV-1nl4.3 (Figure 2-12B).

Together, mutagenesis and structural data suggests that rhTRIM5α^{TFP} targets a surface-exposed patch of CA that is conserved in both structure and sequence across primate lentiviruses. Furthermore, differences between SIVmac239 and HIV-1 at the periphery of this patch account for their differential sensitivity to rhTRIM5α^{TFP}. At the same time, TRIM5α^{TFP} and TRIM5α^{Q} are both affected by changes in the β-hairpin, suggesting that restriction by both alleles involves recognition of this conserved feature of retroviral CAs

Evolution of TRIM5α^{TFP}.

To reconstruct the evolutionary origins of the Q/TFP polymorphism, we analyzed multiple primate TRIM5α sequences. We found that Gln341 in rhTRIM5α is present at the homologous location in TRIM5α of hominoids (*Homo sapiens* and *Pan troglodytes*), colobines (*C. guereza* and *P. nemaeus*) and macaques (*M. mulatta* and *M. fasicularis*) (Figure 2-13). In contrast, the insertion is found only in Papionins, including sooty mangabeys (*Cercocebus atys*), baboons (*P. anubis*), geladas (*T. gelada*), mandrills (*M. sphinx*), Barbary macaques (*M. sylvanus*), rhesus macaques (*M. mulatta*) and crab-eating macaques (*M. fasicularis*). Therefore, the insertion most likely originated in a common ancestor of the Papionini. Strikingly, a 60-nucleotide insertion/duplication at an identical position is found in TRIM5 of Cercopithecini (*E. patas* and other *Cercopithecus* species.). We therefore cannot rule out an earlier origin of the insertion in a common ancestor of the Cercopithecini and Papionini. Together, these observations give a range of insertion times between 9.8 and 11.6 million years ago (Figure 2-13) [84]. Thus, Gln341 is the ancestral state at this position, and TFP is the evolutionarily derived state – consistent with our hypothesis that rhTRIM5α^{TFP} alleles may be the result of selection to recognize the CA of primate lentiviruses.

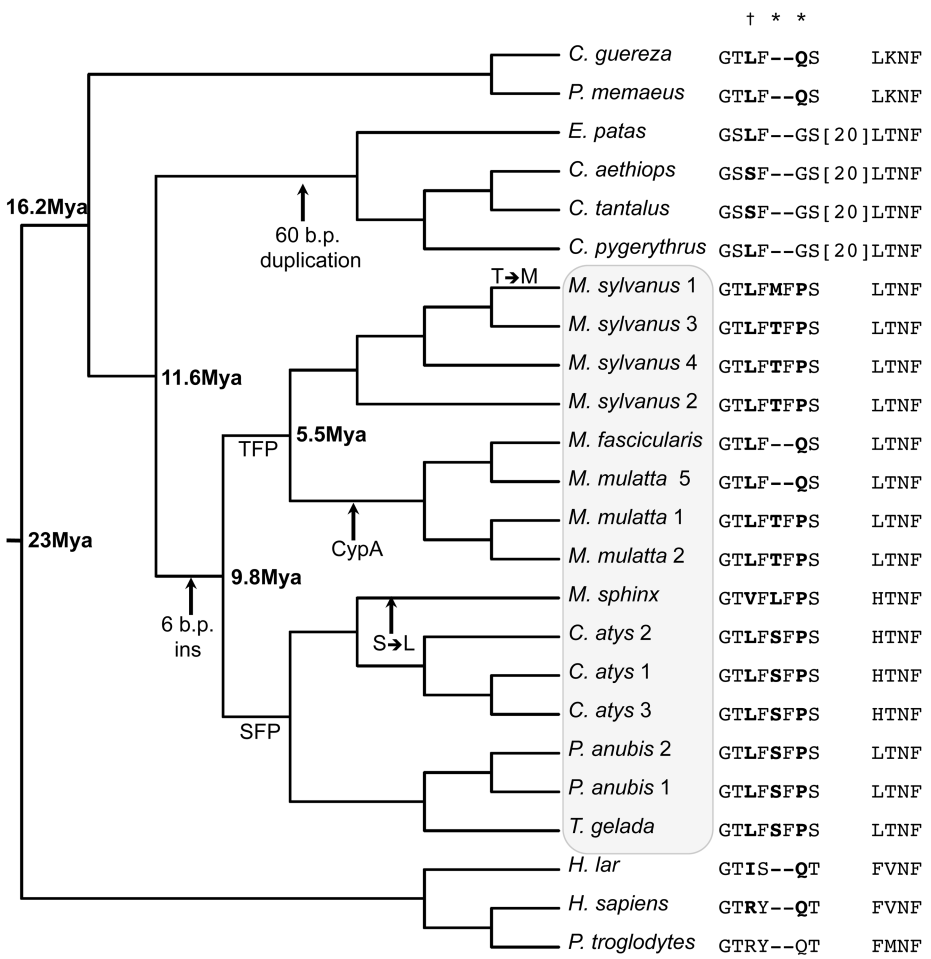


Figure 2-13. Evolutionary origins of the TRIM5α^{TFP} allele. A Cladogram depicting the evolutionary relationships among TRIM5 coding sequences from 16 extant primate species. Major divergence times are in bold, approximate dates of events discussed in the text are indicated with arrows. For each species/allele, the amino acid sequence corresponding to residues 335-346 (relative to rhesus TRIM5) is shown; species names followed by numbers indicate multiple alleles. Residues with dN/dS >1 and a high posterior probability of positive selection are indicated by † (posterior probability >99%) or * (posterior probability >95%).

We also noted considerable variation in the first codon of the inserted element itself, finding (in addition to TFP) orthologs encoding SFP, MFP and LFP among extant species (Figure 2-13). To ask whether this variation is consistent with continued positive selection since the time of insertion, we calculated dN/dS for each codon in the PRYSPRY domain using an alignment representing sixteen species of old world primate, including 4 species for which multiple haplotypes are available (*M. mulatta, M. sylvanus, P. anubis* and *C. atys*). We identified five codons in the PRYSPRY (332, 334, 337, 339 and 341) with high posterior probabilities of positive selection, including two in the 6 b.p. insertion itself (339 and 341), a pattern consistent with sequences evolving under continuous or repeated cycles of positive selection.

2. D. DISCUSSION

Rhesus macaques have three functionally distinct TRIM5 alleles, rhTRIM5α^{TFP}, rhTRIM5α^{Q}, and rhTRIM5^{CypA} [46-48,50,51,251]. Of these, the structural basis for recognition of CA by rhTRIM5^{Cyp} is best understood, and is attributed to interactions between the CypA domain and the 4-5 loop [300,333]. In contrast, CA recognition by C-terminal PRYSPRY domains, such as those found in rhTRIM5α^{TFP} and rhTRIM5α^{Q}, is not well understood. Using genetic, mutagenic, and structural approaches we found evidence that restriction by rhTRIM5α

proteins involves at least two structurally conserved elements of the primate lentivirus CA N-terminal domain.

There are four possible phenotypes for viruses that encounter rhTRIM5α^{TFP} and rhTRIM5α^{Q} alleles: resistance to both, sensitivity to both, and sensitivity to one or the other but not both. We observed only three of the four possibilities: resistance to both (SIVmac239), sensitivity to both (HIV-1nl4.3), and sensitivity to rhTRIM5α^{TFP} but resistance to rhTRIM5α^{Q} (SIVagmTAN, SIVsmE04, SIVsmE543 and HIV-2Rod) (Figure 2-1). We did not observe the converse, resistance to rhTRIM5α^{TFP} combined with sensitivity to rhTRIM5α^{Q}. Moreover, none of the 34 chimeric viruses assayed displayed a rhTRIM5$\alpha^{TFP\text{-}res}$/rhTRIM5$\alpha^{Q\text{-}sens}$ phenotype, and there are no reports of other retroviruses displaying a rhTRIM5$\alpha^{TFP\text{-}res}$/rhTRIM5$\alpha^{Q\text{-}sens}$ phenotype. In fact, the only mutations in SIVmac239 that resulted in sensitivity to rhTRIM5α^{Q} also resulted in sensitivity to rhTRIM5α^{TFP} (Figures 2-4, 2-5 and Table 2-1).

The substitutions that increased sensitivity to both alleles map to the β-hairpin of CA. Structurally, the β-hairpin is the most conserved retroviral surface feature and is present in structures from five different genera [328-331,338,384]. Thus, it appears that the β-hairpin is a retrovirus-associated molecular pattern by which TRIM5α evolved to “recognize” retroviruses. In support of these hypotheses, we note that experimental evolution of a rhTRIM5α^{TFP}-resistant N-MLV in cell-culture selected for a single change in the β-hairpin of the MLV capsid [240].

When we superimposed the MLV and lentiviral CA structures, the identified resistance mutation in MLV overlaps with Y9, a residue we identified in the SIVmac239 β-hairpin that modulates recognition by rhTRIM5α^{TFP} (Figure 2-14).

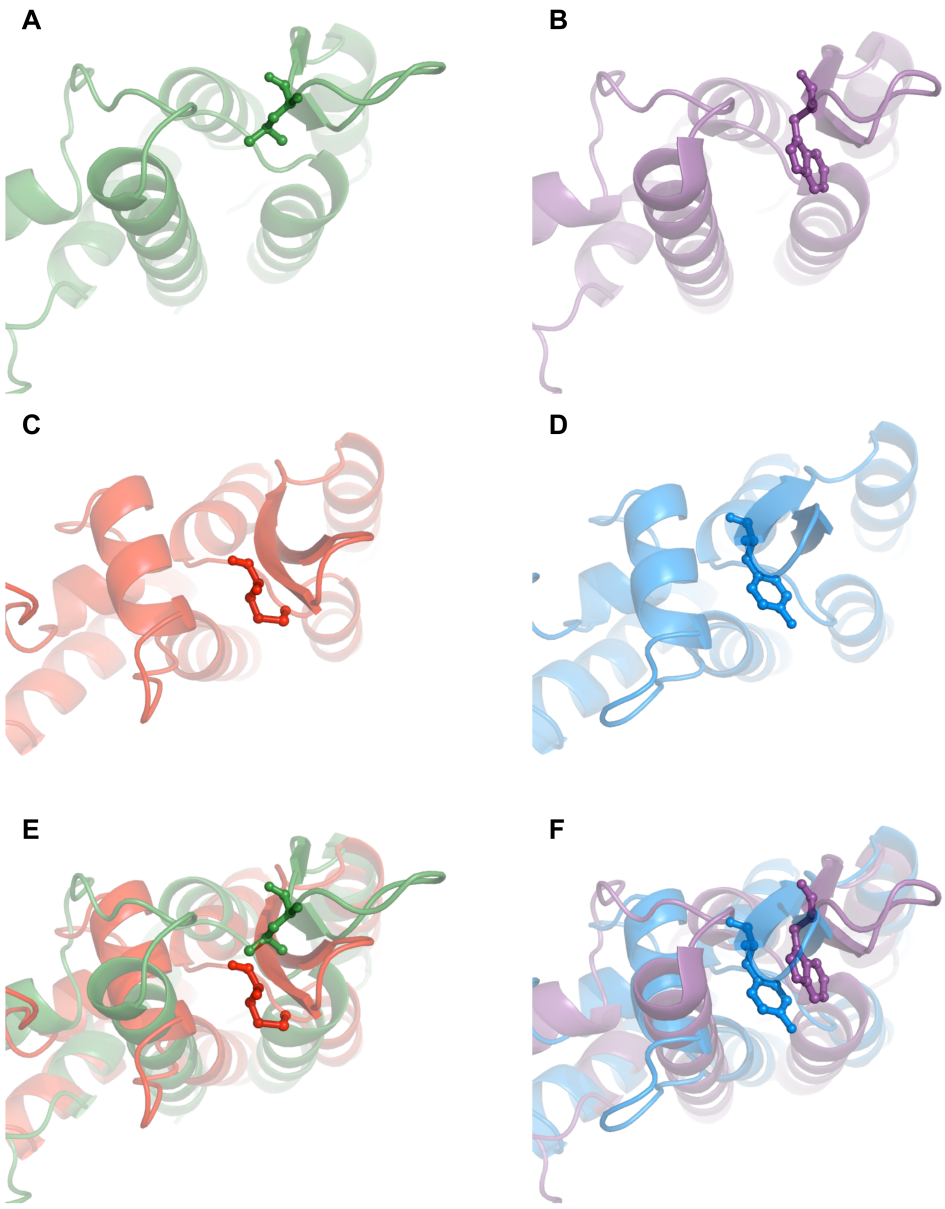


Figure 2-14. Structural comparison between SIVmac239 and MLVs with differential restriction by rhesus TRIM5α. (A) β-hairpin of N-Tropic MLV (PDB: 1U7K) with residue L10 shown in sticks and spheres (B) β-hairpin of the N-MLV L10W mutant (PDB:2Y4Z) that is rhesus TRIM5α^{TFP} resistant, 10W shown in sticks and spheres. (C) β-hairpin of HIV-1 (PDB:2X2D) with M10 shown in sticks and spheres. (D) SIVmac239 β-hairpin Y9 shown in sticks and spheres. (E) Structural alignment of rhesus TRIM5α sensitive N-MLV with HIV-1. (F) Structural alignment of the rhesus TRIM5α resistant N-MLV L10W with SIVmac239. Images created in PyMol.

In addition to substitutions in the β-hairpin that increased sensitivity to both rhTRIM5α^{Q} and rhTRIM5α^{TFP}, there were twelve additional mutations specifically associated with rhTRIM5α^{TFP} restriction (Table 2-1). We interpret this to mean that the rhTRIM5α^{TFP} allele has retained the CA-recognition capacity of rhTRIM5α^{Q}, but has evolved to interact with an additional target or targets in the lentiviral CA. These mutations map to surface features that distinguish primate lentivirus CAs from other retroviral CAs. Specifically, these substitutions ring a spatially clustered group of amino acids that are conserved across primate lentiviruses, altering this site at its periphery.

Interestingly, these mutations also overlap the binding sites of lentivirus-specific cellular cofactors, including CypA, NUP358 and CPSF6; notably, when these factors are fused to a TRIM5 RBCC, the resulting fusion proteins function as restriction factors [300,333,363,370,390]. Primate lentiviruses have extended 4-5 loops that productively interact with at least two cellular cyclophilins, CypA and the CypA-like domain of a nuclear import factor, NUP358 [232,327]. In nature, these interactions have been independently exploited at least four times during primate evolution in the form of TRIM5-CypA fusion proteins, two of which have been maintained in modern day lineages of owl monkeys and macaques [45-51,391]. SIVmac239 residue Ala86 corresponds to Gly89 in the HIV-1 CypA binding motif, while SIVmac239 Gln88 and Gln89 are previously identified sites of an adaptive change permitting SIVmac to resist rhTRIM5^{CypA}

restriction [61,232]. We demonstrate that both of these sites influence rhTRIM5α^{TFP} restriction (Table 2-1). Resistance mutations to both rhTRIM5^{CypA} and rhTRIM5α^{TFP} may explain why SIVmac239 does not utilize Nup358, which is required by other primate lentivirusess for efficient nuclear import and optimal target site integration [327].

The conserved surface patch is an extension of the CPSF6 binding site, which is conserved among primate lentiviruses [363]. Our data suggest that this site is targeted by the rhTRIM5α^{TFP} PRYSPRY domain (Figure 2-12). We therefore propose that the targeting of this site is analogous to exploitation of the CypA binding site in the 4-5loop by rhTRIM5^{CypA}, since rhTRIM5α^{TFP} also exploits a critical, conserved CA interface that is necessary for its interaction with a host co-factor that facilitates lentiviral replication.

Recent structural determination of the rhesusTRIM5α PRYSPRY domain shows the four discrete variable regions are arranged on the surface of a β-sandwich core [211,246]. Ohkura *et al*. reported that the variable regions may make independent contributions to CA recognition [266]. Thus, differences in targeting by the rhTRIM5α^{Q} and rhTRIM5α^{TFP} proteins may reflect contributions from different regions of the PRYSPRY domain. For example, the TFP insertion in variable region 1 (V1) may directly confer specificity for the conserved face of lentiviral CAs, whereas the interactions of both rhTRIM5α^{TFP} and rhTRIM5α^{Q} with the β-hairpin may involve contributions from one or more of the other variable loops.

The original insertion in V1 that gave rise to rhTRIM5^{TFP} in modern macaques arose after the *Cercopithecinae-Colobinae* split, but prior to divergence of the *Macaca* and *Papio* lineages, providing an estimate for the time of insertion between 9.8 to 11.6 million years ago [84]. In contrast, the TRIM5^{CypA} allele has only been found in Asian macaques, but not in Barbary macaques or any other old world primates [46-48,50,51,392], and may therefore have arisen less than 5-6 million years ago, after the lineage leading to Asian macaques (*Macaca sp.*) diverged from the African lineages [84]. These dates, and the observation that rhTRIM5α^{TFP} and rhTRIM5^{CypA} target lentiviral-specific features of CA, constitute indirect but compelling evidence that viruses related to modern primate lentiviruses were infecting ancestral primates as far back as 12 million years ago, driving selection of TRIM5 variants with enhanced capacity to restrict lentiviral replication. Recently, similar conclusions were independently obtained from a study of APOBEC3G variation in Old World monkeys [44]. Endogenous lentiviral sequences found in the genomes of European brown rabbits [52], Malagasey lemurs [53] and weasels [54,55] support the conclusion that lentiviruses were extant at this time, and structural studies indicate that the CA proteins of at least two of these (RELIK and pSIVgml) were very similar to modern lentiviruses [324].

The natural history of African primate lentiviruses, and the species that harbor them, suggests lentiviruses were a driving force for the selection and maintenance of TFP-like

TRIM5α alleles during the last 12 million years. Based on these observations, we propose an evolutionary model in which different regions of the PRYSPRY can evolve independently to recognize different features of retroviral CAs (Figure 2-15). β-hairpin recognition was conserved between the ancestral TRIM5α^{Q} allele and the evolutionary derived rhTRIM5α^{TFP} allele. Therefore, it is likely that the region encompassing the Q/TFP polymorphism in variable loop 1 (V1) does not contribute to β-hairpin recognition. Instead, this region may be free to make additional contacts with the CA. Due to its dynamic and unstructured nature, V1 may readily tolerate mutations and insertions (such as the 6-nucleotide insertion) affording the molecule enhanced evolutionary plasticity [211,246]. The SIV-HIV$_{h6}$ mutant was restricted by rhTRIM5α^{Q}, implying that the rhTRIM5α^{Q} PRYSPRY could recognize one edge of the conserved surface patch (Figure 2-15A). The modern day presence of TRIM5α orthologs with the 6-nucleotide insertion indicate that the insertion event conferred a selective advantage (likely against primate lentiviruses). The simplest explanation is that the insertion allowed V1 to make additional contacts or possibly even extend beyond helix 6 and further into the conserved surface patch. We have shown that the first and last positions of the rhesus TFP polymorphism have been under positive selection, indicative of continued refinement of its ability to recognize the conserved surface patch over evolutionary time.

This model is likely a snapshot of a larger evolutionary scenario in which an ancestral PRYSPRY domain may first have acquired the ability to recognize a highly conserved retroviral CA element (such as the β-hairpin). On top of this intrinsic recognition ability, modularity of TRIM5α proteins allowed them to explore additional targets on the CA surface in response to pressures from specific viruses or viral families, perhaps by taking advantage of inherent plasticity within the variable loops (Figure 2-15B). Such a process, played out over the course of tens of millions of years of evolution, could help to explain both the collective breadth and species-specificity of modern primate TRIM5α proteins.

Figure 2-15. A Proposed model for the evolution of novel TRIM5α variants. The rhTRIM5α^{Q} alleles and the rhTRIM5α^{TFP} alleles share the ability to recognize lentiviral β-hairpins. The rhTRIM5α^{TFP} alleles evolved to recognize the conserved surface patch. We believe this observation underscores an inherent uncoupling between capsid recognition modules within the PRYSPRY domain. The β-hairpin is a conserved feature found in all reported retroviral capsid structures, and therefore a convenient target for host proteins that evolve to recognize a broad range of retroviruses. We believe β-hairpin targeting is a conserved feature of TRIM5α proteins and allows for the evolution of specificity of capsid recognition. (A) Evolution of conserved surface patch recognition. The rhTRIM5α^{Q} allele is capable of strongly recognizing the β-hairpin (dark red) and able to engage in a weaker contact (pink) with helix 6, at one edge of the conserved surface patch. Recognition of these two features is conserved between rhTRIM5α^{Q} and rhTRIM5α^{TFP} alleles and therefore unaffected by the Q/TFP polymorphism. The region of the PRYSPRY that encodes for the intrinsic β-hairpin recognition module is colored light blue and the module that can adapt to specific viruses is colored dark blue. We propose that the polymorphic region of variable loop 1 (V1) is uncoupled from intrinsic β-hairpin recognition (by of another region within the PRYSPRY) allowing it to tolerate mutations such as the 6 nucleotide insertion. In this model, the rhTRIM5α^{TFP} allele engages in similar contacts as the rhTRIM5α^{Q} allele, but has gained the ability to target the conserved surface patch (dark red). (B) The ability to recognize a conserved retroviral element, even if it allows very weak associations retroviral capsids, can allow for the selection of additional capsid binding modules within the PRYSPRY domain allowing it to adapt to specific retroviral pressures. Hypothetical adaptation to different retroviral targets are depicted as differently colored PRYSPRY domains. Together this process could lead to the breadth and specificity observed among TRIM5α orthologs. For simplicity this model is depicted with one PRYSPRY domain recognizing one capsid monomer, although the stoichiometry or orientation of TRIM5α binding is not know at this time.

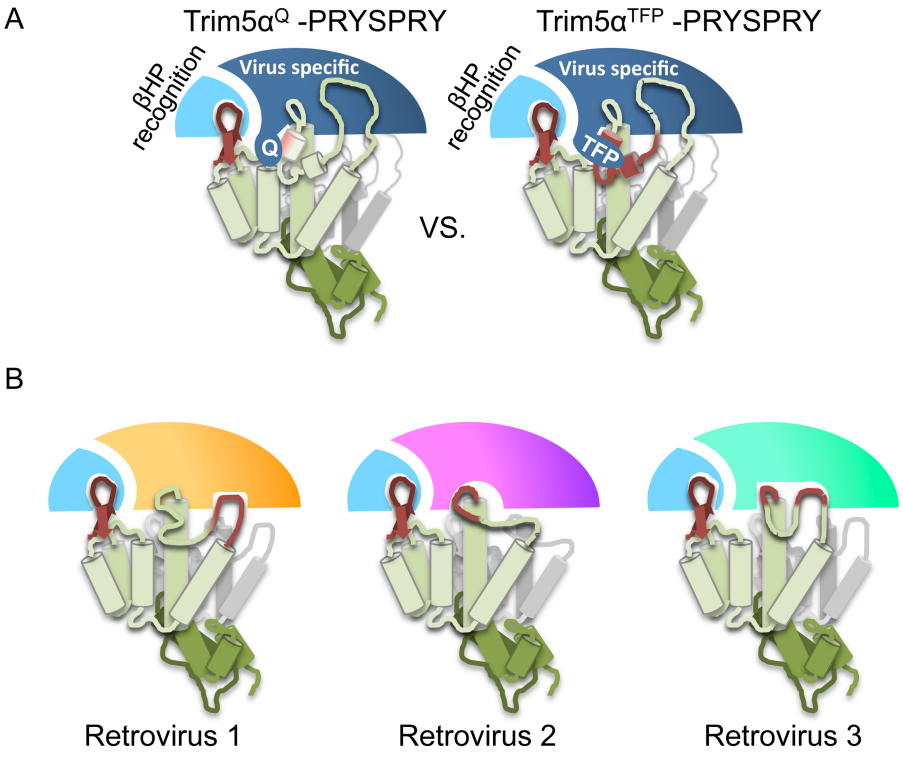


Figure 2-15. A Proposed model for the evolution of novel TRIM5α variants. Continued.

2. E. METHODS

Cell Lines

Crandell-Rees Feline Kidney (CRFK) cells and Human Embryonic Kidney 293T/17 (HEK293T/17) cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/10% FBS. CRFK cell lines stably expressing N-terminally HA-tagged TRIM5 orthologs were previously described [61]. Stable cell lines were maintained in DMEM/10% FBS supplemented with 0.5 mg/ml G418. All cultured cells were maintained at 37°C with 5% CO_2.

Virus Production

All single-cycle viruses were produced in HEK293T/17 cells by cotransfection of the appropriate viral plasmid and pVSV-G (Clontech Laboratories, Mountain View, CA), using the GenJet transfection system (SignaGen; Ijamsville, MD). Culture supernatants containing the single-cycle, GFP/EGFP expressing, VSV-G-pseudotyped virions were titered on untransfected CRFK cells; supernatant volumes resulting in approximately 25% GFP/ EGFP+ CRFK cells were used for infectivity assays on the cell lines expressing the indicated TRIM5α. Information regarding viral infectivity appears in Figure 2-5.

The CFP expressing HIV-1 lentiviral vector was made from 293T transfection of a 3:2:1 plasmid ratio of pNL-ECFP/CMV-WPREDU3 [229], pCD/NL-BH*DDD [393] and pVSV-G (Clontech Laboratories, Mountain View, CA) (pNL-ECFP/CMV-WPREDU3 and pCD/NL-BH*DDD were kindly provided by Dr. Jakob Reiser, Louisiana State University Health Sciences Center).

Infectivity Assays

Stably expressing TRIM5 CRFK cells were seeded at a concentration of $5x10^4$ cells per well in 12-well-plates and infected with the appropriate amount of VSV-G pseudotyped, single-cycle, GFP/EGFP expressing viruses. All infections were done in triplicate. After 2 days, expression of GFP/EGFP was analyzed by fluorescence-activated cell sorting (FACS) performed on a FACSCaliburTM flow cytometer (BD, Franklin Lakes, NJ), and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Viral titers were determined using the appropriate p24 (HIV-1) or p27 (SIVmac) antigen capture kit from Advanced Bioscience Labs (Rockville, MD). Information regarding viral titers appears in Figure 2-5.

Protein Expression and Purification

A codon optimized N-terminal fragment of the SIVmac239 capsid corresponding to residues 1-144 was synthesized with a C-terminal factor Xa cleavage site and 6x-His Tag by GENEART

(Regensburg, Germany). Using engineered *XbaI* and *XhoI* sites the N-terminal fragment was cloned into pET303 (Invitrogen) and expressed from BL21(DE3) *E. coli* cells. The SIVmac239 capsid was purified by Ni-NTA agarose (Qiagen) followed by gel filtration chromatography on a Superdex 200 column (GE Healthcare). The C-terminal 6x-His tag was removed by treatment with factor Xa (New England Biolabs), re-purified by orthogonal Ni-NTA agarose chromatography and gel filtration chromatography.

Crystallization

Purified SIVmac239 capsid protein was crystallized by the hanging drop method over a reservoir solution containing 10 %(w/v) PEG 2000 MME, 10 mM nickel chloride and 100 mM Tris, pH 8.5 at 24°C. Crystals were harvested from 0.2µl drops and cryoprotected by addition of 10-15% PEG 400 or glycerol to the reservoir solution, then flash cooled in liquid nitrogen. Protein concentration ranged from 10-15 mg/ml.

Structure Determination and Refinement

We recorded diffraction data at beamline 24-ID-E at the Advanced Photon Source. Data sets from individual crystals were processed with HKL2000 [394]. Molecular replacement (MR) was carried out with PHASER [395] using the HIV-2 capsid as an initial search model. One molecule

of SIVmac239 completes the asymmetric unit. Refinement was carried out using PHENIX [396,397] and all model modifications were done in COOT [398]. Initial rigid body refinement followed by simulated annealing and positional refinement was done. The 4-5 loop (residues 83-97) was initially removed from the model and rebuilt into modest density. There was no clear density for residue proline 88 and it was omitted from the structure. The model was further refined by additional cycles of positional and B-factor refinement, followed by TLS. The quality of the data was assessed using MolProbity [399]. Data collection and refinement statistics can be found in Table 2-2. Coordinates and diffraction data have been submitted to the PDB, accession number: PDB:4HTW.

Sequence Analysis

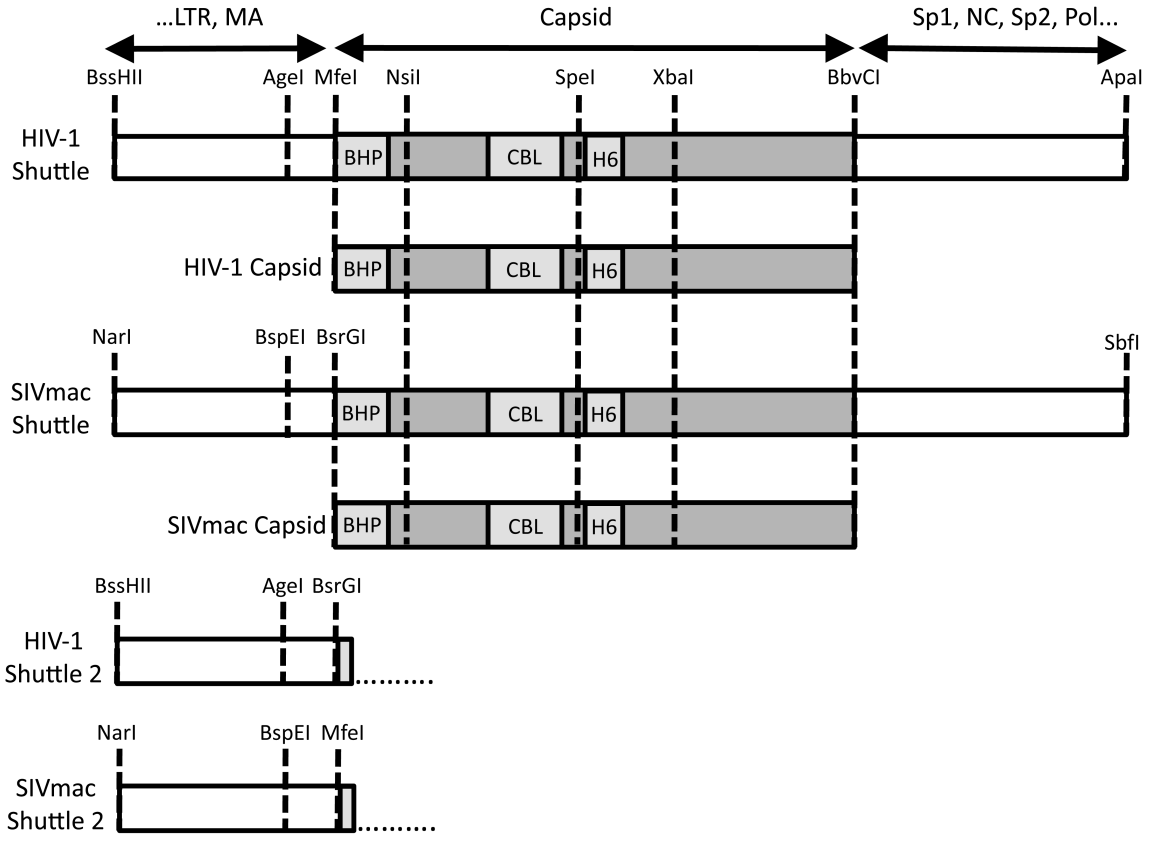
TRIM5α sequences were identified by BLAST search of the non-redundant nucleotide database, aligned in Geneious Pro v.5.5.4 using the Translation Align option. The alignment was adjusted manually, converted back to nucleotide and the best-fit tree identified with MrBayes. dN/dS analysis was performed with CODEML in v4.4 of PAML [400].

Plasmids and Mutagenesis

The SIVmac239-based retroviral vector pV1EGFP (gift from Hung Fan, University of California, Irvine, CA) was previously modified to contain a functional gag-pol ORF [61]. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-deltaE-EGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano [401]. All single cycle chimeric viruses are in either the pV1EGFP-SIV or HIV-1nl4.3 pNL4-3-deltaE-EGFP background as indicated. To facilitate the rapid production of chimeric viruses, a capsid and gag shuttle vector system was engineered through DNA synthesis by GENEART (Regensburg, Germany). Silent nucleotide changes within the capsid allowed for chimerization between capsids from either virus (Figure 2-16). All chimeric capsids with the exception of single amino acid point mutants were produced through gene synthesis by GENEART (Regensburg, Germany) and were then cloned into the proper viruses using our shuttle vector system. Single amino acid substitutions on the SIVmac239 surface were made using site directed mutagenesis. The S100R mutant was described in a previous publication [61].

A CFP expressing HIV-1 derived lentiviral vector was created for abrogation assays. A CFP gene was introduced into using AgeI an XhoI sites into pNL-EGFP/CMV-WPREDU3 [229], a vector based on pNL-EGFP/CMV (which features the WPRE element for increased mRNA stability and a deleted U3 region for added safety).

Figure 2-16. Schematic of synthesized genes and cloning strategy used to generate chimeric viruses: All constructs were synthesized by GENEART (Regensburg, Germany). Numbering corresponds to the standard HXB2 and SIVmac239 numbering, respectively. For efficient exchange of capsids between viruses and chimerization within capsids silent nucleotide changes were made in both viruses creating identical restriction sites. Naturally occurring restriction sites at the ends of the shuttle vector are used for insertion into the proper parental virus. Amino acid differences at the N-terminus of the CA protein did not allow us to use a single common enzyme for this site. Instead SIVmac239 constructs use a BsrGI site while HIV-1nl4.3 constructs use an MfeI site. Two additional shuttle vectors were made to accommodate either N-terminus in both SIVmac239 and HIV-1nl4.3 backbones.



HIV-1 Modifications

Site	Occurrence	WT Sequence	Modified Sequence	HXB2 Numbering
BssHII	Natural	GCGCGC	-	711-716
AgeI	Added	ACAGGA	ACCGGT	997-1002
MfeI	Added	CTATAG	CAATTG	1187-1192
NsiI	Natural	ATGCAT	-	1247-1252
SpeI	Natural	ACTAGT	-	1507-1512
XbaI	Added	TCTGGA	TCTAGA	1635-1640
BbvCI	Added	GCTGAAG	GCTGAGG	1879-1885
MfeI	Removed	CAATTG	CAACTG	1968-1973
ApaI	Natural	GGGCCC	-	2006-2011

SIVmac239 Modifications

Site	Occurrence	WT Sequence	Modified Sequence	SIVmac239 Numbering
NarI	Natural	GGCGCC	-	823-828
BspEI	Added	AGCGGC	TCCGGA	1437-1442
BsrGI	Added	AGTACA	TGTACA	1460-1465
NsiI	Added	ATGCCT	ATGCAT	1516-1521
SpeI	Natural	ACTAGT	-	1770-1775
BsrGI	Removed	TGTACA	TGTATA	1801-1806
XbaI	Natural	TCTAGA	-	1901-1906
BbvCI	Added	GCAGAAG	GCTGAGG	2145-2151
SbfI	Natural	CCTGCAGG	-	3140-3147

Figure 2-16. Schematic of synthesized genes and cloning strategy used to generate chimeric viruses. Continued.

Chapter 3

Evolution of Lentivirus-Specificity in Old World Monkey TRIM5αs Establishes 11-16 Million Years of Continuity Between Ancient and Modern SIVs

Evolution of Lentivirus-Specificity in Old World Monkey TRIM5αs Establishes 11-16 Million Years of Continuity Between Ancient and Modern SIVs

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Manuscript in preparation.

Contributions: Welkin E. Johnson and I both wrote the manuscript. I performed most of the experiments except: Andrea Kirmaier assisted with infections and FACS harvests. Patrick Autissier performed all of the flow cytometry.

3. A. ABSTRACT

TRIM5α is a host encoded protein that has evolved to block retroviral infection. TRIM5α inhibits replication at the reverse transcription step by directly binding to retroviral capsids and inducing their premature uncoating. Retroviral recognition by TRIM5α is mediated by the C-terminal PRYSPRY domain. Within this domain, a series of four variable loops are highly enriched for positively selected sites and polymorphisms, including single nucleotide and length variations. We observed a unique pattern of evolution in the first variable loop (V1) of Cercopithecinae primate TRIM5αs. At a specific position within V1, a single Gln has been maintained over 30+ million years of Old World primate evolution. In contrast, among the Cercopithecinae, this Gln has been replaced by a substitution and modified by two independent insertions. Phylogenetic analysis suggests modifications to the ancestral Gln began 11-16 million years ago in two different Cercopithecinae lineages. These modifications were then followed by further bursts of evolutionary refinement. To determine the selective forces that drove these adaptive changes we reconstructed an ancestral Cercopithecinae TRIM5α as it may have existed prior to the replacement of Gln. Into this Trim5α we introduced the acquired Cercopithecinae V1 modifications. We then screened this panel of ancient and modern day V1-matched Cercopithecinae Trim5αs against a diverse panel of extant retroviruses. Strikingly, the evolutionarily acquired Cercopithecinae modifications specifically conferred the ability to restrict

present day Cercopithecinae SIVs but did not affect the restriction of other retroviruses. Further characterization of Cercopithecinae TRIM5αs suggests that targeting a single site in capsid that sits at the junction of four positively acting lentivirus-specific cellular cofactor binding sites was twice acquired in two independent Cercopithecinae lineages. Taken together, our findings suggest these evolutionary innovations reflect the emergence of SIVs among Cercopithecinae primates 11-16 million years ago. For the first time our findings establish a connection between ancient and modern SIVs among primates. The recurrent selection we observed suggests that like modern SIVs, these ancient SIVs repeatedly crossed between species and caused disease. Therefore, the events leading to HIV infection in humans are likely the continuation of an 11-16 million year old pattern of transmission within and between host lineages.

3. B. INTRODUCTION

Within the past century, simian immunodeficiency virus (SIV) from chimpanzees have been transmitted to humans on at least 3 occasions, one of which resulted in the global HIV-1 epidemic [26,28,29,43]. Multiple independent cross-species transmission events involving SIVs from sooty mangabeys resulted in HIV-2 in humans and SIVmac in captive macaque colonies [23-26,28]. These recent examples of cross-species transmission, adaptation and the dissemination of a virus throughout a population are a continuation of an ancient pattern. Over 40 primate lentiviruses (HIVs and SIVs) have been identified, all from African primates [23-25,27]. Like HIV and SIVmac, these viruses also share a history of cross-species transmissions, evidenced by the fact that the primate lentivirus phylogeny is incongruent with that of their hosts [23,26,27,29,38,56,57].

While the clinical outcome of primate lentivirus infection varies, there is a general trend in which pathogenic infections are associated with a recent acquisition of a primate lentivirus. For example, higher mortality rates are observed among humans infected with HIV-1 or HIV-2, chimpanzees infected with SIVcpz, rhesus macaque species infected with SIVmac and pig-tailed macaques infected with SIVagm [23-25,38,58,64,66]. In contrast, primates believed to be long-standing hosts of a particular SIV typically do not present any overt clinical symptoms of infection [24,25,27]. The evolutionary timescales for the process of coevolution between primate

lentiviruses and hosts are largely unknown. While endogenous lentiviruses in rabbit, ferret and lemur genomes establish that lentiviruses existed 5-12 million years ago, it is not clear when the primate lentiviruses first appeared as a distinct lineage. The molucular dating of selective events in antiretroviral genes may provide compelling, yet indirect evidence of the existence of ancient lentiviruses [44].

TRIM5α is a host restriction factor that can suppress the cross-species transmission of primate lentiviruses [61,77]. It mediates a potent post-entry pre-integration block to retroviral infection that is dependent upon recognition of the viral capsid [125]. TRIM proteins are named for their shared tripartite domain structure composed of a RING, B-box and coiled-coil domains [128,129]. The antiviral α isoform of TRIM5 encodes for a C-terminal PRYSPRY/B30.2 domain that acts as a viral recognition domain [125,208]. Among primates, the TRIM5α PRYSPRY domain has evolved under strong positive selection [243,251,380]. A majority of these positively selected sites reside within the PRYSPRY's four variable loops (V1 to V4) [211,243,380]. These variable loops are thought to directly mediate contacts with retroviral capsids (CA).

The majority of positively selected sites in Old World primate TRIM5αs map to the first variable loop (V1) [211,243,380]. While the sequence of V1 varies between Old World primates, length variations are only found among Cercopithecinae primates [243,244,251]. Importantly, insertions/deletions (indels) are excluded from standard measures of positive selection.

However, the maintenance of inserted amino acids in multiple Cercopithecinae primate species implies that they may have conferred selective advantages. Among Cercopithecinae primates, two independent insertions (a 20 amino acid and a 2 amino acid insertion) have occurred at or adjacent to TRIM5α position 339 in V1. Among all non-Cercopithecinae Old World primates, the homologous position 339 invariably encodes a Q. We refer to this site and adjacent positions as the V1-patch. Recently, we proposed that the 2 amino acid insertion as it is found in rhesus macaque TRIM5α (which replaced Q with TFP at positions 339-341) evolved to target a highly conserved cluster of amino acids in lentivirus capsids [380]. We therefore hypothesized that variation at position 339 among Cercopithecinae primates may be the result of selective pressure exerted by ancient primate lentiviruses. Establishing a correlation between selective events in the evolution of V1 in Cercopithecinae TRIM5α and specificity for modern SIVs of Cercopithecinae primates would provide evidence that a common ancestor of SIVs existed 11-16 million years ago.

Using phylogenetic techniques we reconstructed the evolutionary history of the TRIM5α V1 patch. We established that a Q (henceforth “V1-Q”) corresponding to position 339 in Cercopithecinae TRIM5αs represents the state that was present in the ancestor of all Old World primates 30+ million years ago [34,35]. TRIM5α V1-Q has remained unmodified in all but one African primate lineage, the Cercopithecinae, which includes: guenons, African green monkeys,

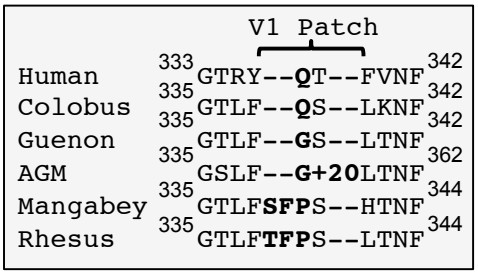
baboons, mangabeys and macaques. While TRIM5α alleles with V1-Q have been preserved among Cercopithecinae primates, Q has also been replaced by a Q to G substitution (V1-G) and overwritten by a two amino acid insertion (V1+2aa). Moreover, sometime subsequent to the Q to G substitution, a 20 amino acid insertion occurred adjacent to the G (V1-G+20). We determined that selection against V1-Q began 11 and 16 million years ago in two different Cercopithecinae lineages [34,35]. Using a reconstructed ancestral TRIM5α, predicted to have existed prior to the evolution of the V1-patch modifications, we demonstrate that these modifications were sufficient to specifically impart the ability to restrict extant Cercopithecinae SIVs. We interpret our findings to mean that ancient Cercopithecinae SIVs were present in Cercopithecinae primates at least 11-16 million years ago. Importantly, this is the first report linking ancient and modern SIV lineages. We observed recurrent selection in multiple independent Cercopithecinae lineages. This implies that like modern primate lentiviruses, these ancient Cercopithecinae SIVs were crossing between species and causing disease [34,35]. Furthermore, TRIM5αs from two independent lineages of modern day Cercopithecinae primates appear to have converged upon targeting a lentivirus specific site that sits at the junction of four cellular cofactor binding sites [232,300,327,363,372]. Thus, it appears that Cercopithecinae primate TRIM5αs have independently evolved to recognize a highly conserved capsid interface [363,380].

3. C. RESULTS

Dating selective events in the Cercopithecinae TRIM5α variable loop 1 patch

Among reported Old World primate TRIM5α sequences, the length of variable loop 1 (V1) is constant in all but one primate lineage, the Cercopithecinae. Among some Cercopithecinae primate TRIM5αs at least two independent insertion events appear to have occurred at or adjacent to TRIM5α position 339. Due to the length polymorphisms in this region we will refer to this site, centered on Cercopithecinae TRIM5α postion 339, as the V1-patch (Figure 3-1). To reconstruct the evolutionary events that have led to the diversification of the Cercopithecinae V1-patch we generated a sequence alignment and a corresponding phylogenetic tree of Old World primates for which TRIM5α sequences have been reported (Figure 3-1). To build a more robust phylogeny we additionally sequenced previously unreported TRIM5αs from individuals representing five additional Cercopithecinae species. These included four guenon species, *Cercopithecus wolfi* (Wolf's Guenon n=3), *Cercopithecus cephus* (mustached guenon n=1), *Cercopithecus ascanius* (Schmidt's guenon n=2), *Cercopithecus neglectus* (De Brazza's monkey n=1), and a mangabey *Cercocebus torquatus* (Red-Capped Mangabe*y* n=1). The V1-patch features of 28 Old World primate species are indicated on the phylogenetic tree in Figure 3-1.

A



B

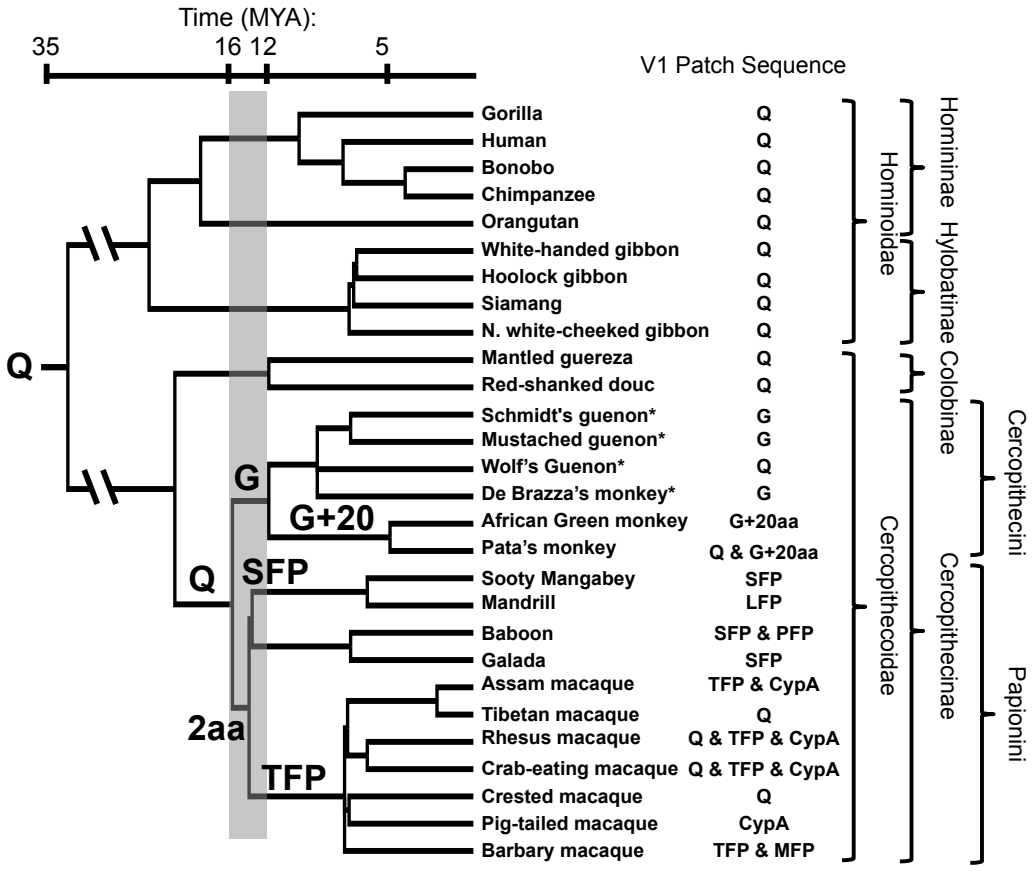


Figure 3-1. Phylogeny and TRIM5α V1 patch sequences of Old World primates. (A) Sequence alignment of select OWM Trim5alpha variable loop 1 sequences. The rapidly evolving V1 patch is indicated and novel sequences are bolded. (B) Phylogeny of select Old World primates and key evolutionary events in Trim5α variable loop one are indicated on the tree. Common species names are indicated at the branch tips. An "*" indicates full length Trim5α sequences reported in this manuscript. Relevant sequences in variable loop one are indicated. The super families, families and tribes of these primate species are indicated.

From the phylogeny and TRIM5α sequence information we were able to infer the series of events that led to the present day V1 patch diversity. Examples of TRIM5αs with a Q (V1-Q) in the V1-patch corresponding position 339 in Cercopithecinae TRIM5αs are present in every Old World primate lineage (Figure 3-1). Therefore, we infer that V1-Q is the ancestral residue at this position, and reflects the sequence that was present in the last common ancestor of all Old World primates. While V1-Q has been replaced in some Cercopithecinae TRIM5αs, V1-Q is still found in TRIM5αs from multiple Cercopithicini and Papionini species. We conclude that V1-Q represents the most likely ancestral state of TRIM5α position 339 in all extant Cercopithecinae species. V1-Q, together with other V1-patch variants, has been preserved through long term balancing selection within the Cercopithecinae. Thus, V1-Q has remained unmodified for over 30+ million years in every major Old World primate lineage except the Cercopithecinae, where it co-exists with other variants such as V1-G, V1-G+20aa, and V1-Q+2aa [34,35].

In contrast to the highly conserved V1-Q found in all Old World primate lineages, diversity found within the V1-patch is a unique feature of Cercopithecinae TRIM5αs (Figure 3-1). The Cercopithecinae include two tribes, the Cercopithicini and Papionini. Cercopithicini V1-patch variants all share a common V1-Q to G substitution (V1-G), while Papionini V1-patch variants all share a two amino acid insertion (V1-Q+2AA). No evidence of either feature exists outside of the tribe in which it is found (Figure 3-1). We hypothesize that these present day

variants within the V1-patch are due to the continued selection upon two tribe specific V1 modifications.

Two major V1-G orthologs are present among Cercopithicini TRIM5αs. The first is the unmodified V1-G that is found in some guenon species. The second is a modified form of V1-G that is found in African green monkeys and some Pata's monkeys, in which there has been a duplication of sequence resulting in the insertion of 20 additional amino acids (V1-G+20aa) (Figure 3-1). Inter- and Intra- species differences in these 20 residues indicate that the inserted sequences have continued to evolve after the duplication event. The presence of G at the homologous position in the TRIM5α of guenons, African green monkeys and some Pata's monkeys allowed us to date both evolutionary events. We infer that V1-G was present between 8 and 16 million years ago, while the insertion event leading to G+20 likely occurred between 5 and 14 million years ago (Figure 3-1).

In the Papionini, there are examples of TRIM5α V-Q+2aa orthologs in every genus. This indicates that the insertion was at least present in the last common ancestor of all extant Papionini species 8-15 million years ago (Figure 3-1). Following the insertional event, this modified patch continued to evolve, resulting in the V1-SFP/TFP/PFP/LFP/MFP derivatives currently found in nature. Rapid selection has obscured the sequence of the initial insertion and some of its potential evolutionary intermediates. One possible evolutionary pathway is illustrated

in figure 3-2. In short, the insertion arose from a duplication of adjacent sequence resulting in V1-QFQ, which became PFP followed by SFP. TRIM5α-V1-TFP could have arisen from either PFP or SFP. Together, the V1-SFP in baboons and mangabeys and V1-TFP in numerous macaque species are the only V1-2aa derivatives found in multiple species. Thus, V1-SFP and V1-TFP are the most commonly found V1+2aa patch features found in nature (Figure 3-1).

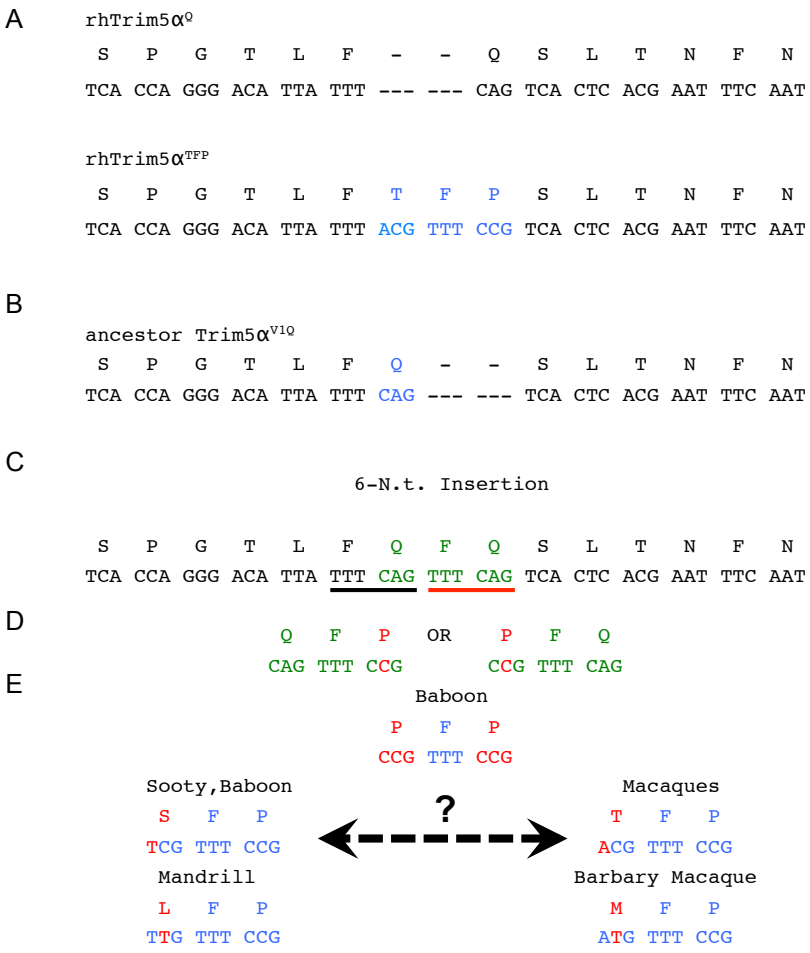


Figure 3-2. Proposed order of evolutionary steps for the evolution of the two amino acid insertion. A Q at position 339 was overwritten by a two amino acid insertion in an ancestor common to all extant Papionini species. (A) The sequences of rhesus Trim5α V1-Q (top) and rhesus TRIM5α V1-TFP are provided for reference. (B) The sequence of the predicted ancestral TRIM5α V1-Q. (C) We propose that the initial insertion event was due to the duplication of two codons corresponding to TRIM5α positions 338 and 339 (FQ) (underlined in black). Residues 339-341 would then encode for QFQ (underlined in red). (D) Single nucleotide substitutions in codons 339 and 341 alter the sequence of 339-341 to encode for QFP or PFQ. (E) A second single amino acid substitution results in PFP. This variant has been found in baboon TRIM5α sequences. (F) Single amino acid substitutions in codon 339 result in SFP, TFP, MFP, LFP. All of these sequences are found among the Papionini primates. It is unclear whether TFP arose from PFP or SFP. Black lettering corresponds to unmodified sequences that are present in modern day Cercopithecinae primates. Red lettering indicates nucleotide substitutions that alter the above sequence. Blue lettering indicates that the corresponding sequences are found in nature. Green lettering indicates hypothetical intermediates.

When TRIM5α protein sequences are compared among Old World primates, diversity within the V1 patch is unusual. However the timing and phylogenetic relationship between V1 patch modifications are quite remarkable. In contrast to V1-Q which has remained constant for 30+ million years, the V1 patch was twice modified in two independent Cercopithecinae lineages in the space of 1.1-4 million years [34,35]. These V1 modifications then continued to evolve. These evolutionary signatures may signify the emergence of a significant source of selective pressure that was acting upon multiple independent primate lineages.

The Cercopithecinae variable loop 1 patch specifically affects the restriction of modern day Cercopithecinae SIVs

We predict that the last common ancestor of all Cercopithecinae TRIM5αs had a Q at the position corresponding to 339. Using phylogenetic methods we reconstructed the sequence of this ancestral TRIM5α. A number of substitution models and methods for generating phylogenetic trees were used. All predictions of the last common node to all Cercopithecinae TRIM5αs agreed on a single sequence within the PRYSPRY domain. In the rest of the protein, only the residue at position 69 (in the linker between the ring and b-box domains) was ambiguous. This position was predicted to be either an R or a Q; an R is found among the

modern hominoidea, colobinae and the Cercopithecinae species *Macaca sylvanus* (Barbary macaque), while all other Cercopithecinae encode for a Q at this position.

Next, we synthesized a cDNA encoding the predicted ancestral TRIM5α of Cercopithecinae monkeys (ancTRIM5α^{V1Q}) and tested its ability to restrict a diverse panel of retroviruses (Figure 3-3). We also modified ancTRIM5α^{V1Q} to compare the impact of each Cercopithecinae V1 modification on restriction in an isogenic context (i.e., in the absence of other evolutionary modifications). Derivatives of ancTRIM5α^{V1Q} included the Cercopithicini V1 modifications, V1-G and V1-G+20, which we refer to as ancTRIM5α^{QV1G} and ancTRIM5α$^{QV1G+20}$. The 20 inserted amino acids exactly recreate the original duplication (such that the duplicated sequences are still identical) and do not include the additional diversification seen in modern day TRIM5α orthologs. We also made ancTRIM5α^{V1Q} derivatives with additional V1 patch modifications seen/predicted to have been in Papionini species, including QFQ, PFP, SFP and TFP (ancTRIM5α^{QV1QFQ}, ancTRIM5α^{QV1PFP}, ancTRIM5α^{QV1SFP} and ancTRIM5α^{QV1TFP}). All of these HA-tagged TRIM5αs were used to generate stably expressing CRFK cell lines.

Cell lines that stably express modern day HA-tagged Cercopithecinae TRIM5αs were also generated. These included TRIM5αs with the V1-G modification from Mustached guenons (mus), De Brazza's monkeys (deb) and two alleles from Schmidt's guenon (Sch1 and Sch2). TRIM5α V1-G+20s were cloned from vero (AgmV) and COS-1 cells (AgmC). TRIM5αs with the

V1-SFP modification came from sooty and red-capped mangabeys (ceat-1 and Rcm respectively). Rhesus TRIM5α (mamu1) was the modern day V1-TFP derivative. Cercopithecinae TRIM5α orthologs with an unmodified V1-Q were included from rhesus macaque (mamu5) and Wolf's guenon (wlf) TRIM5αs. Human TRIM5α (V1-Q) was included as a non-Cercopithecinae out-group.

A diverse panel of retroviruses was assembled to assay the restrictive properties of these 19 TRIM5α proteins. The viruses tested included: the avian alpharetrovirus Rous sarcoma virus (RSV); two murine gammaretroviruses, the N-tropic and B-tropic strains of murine leukemia virus (MLV), the Cercopithecinae betaretrovirus Mason-Pfizer monkey virus (MPMV), the non-primate lentiviruses feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV), and two human lentiviruses, human immunodeficiency virus -1, strain nl4.3 (HIV-1nl4.3) and human immunodeficiency virus -2, strain Rod (HIV-2rod). Simian immunodeficiency viruses (SIVs) isolated from Cercopithecinae primates were also included. Infectious SIVs were produced from either authentic molecular clones or modified molecular clones engineered to express the capsids of other Cercopithecinae SIVs. These included the Cercopithicini SIVs from mustached guenons (SIVmus) and African green monkeys (SIVagmTan-1, SIVagmVer, and SIVagmGrv) and Papionini SIVs included the macaque-passaged sooty mangabey SIV

(SIVsmE543), the rhesus macaque SIV (SIVmac239), the stump tailed macaque SIV (SIVstm) and an SIV from a red-capped mangabey (SIVrcm).

The 19 TRIM5α expressing cell lines were assayed for the ability to restrict each of the above 16 viruses. All TRIM5αs were functional, and each TRIM5α-expressing cell line was able to restrict viruses from multiple retroviral genera (Figure 3-3). With the exception of RSV, every virus was restricted by at least one of the TRIM5α proteins tested. Except for the SIVs, the retroviruses were either almost always resistant to a majority of the 19 TRIM5αs, or almost always sensitive to a majority of the 19 TRIM5αs (Figure 3-3). For example, N-MLV was sensitive to all 19 TRIM5α proteins, while RSV was resistant to all 19. Including the ancTRIM5α^{V1Q} and its derivatives, the Cercopithecinae TRIM5αs shared a capacity to restrict N-MLV, FIV and EIAV. With the exception of wlfTRIM5α^{Q}, these TRIM5αs also all restricted HIV-1 (Figure 3-3). Therefore, the capacity to restrict these viruses is an ancient and conserved property of all Cercopithecinae TRIM5αs. Importantly, these results demonstrate that restriction of these retroviruses are independent of the sequences found in the V1 patch.

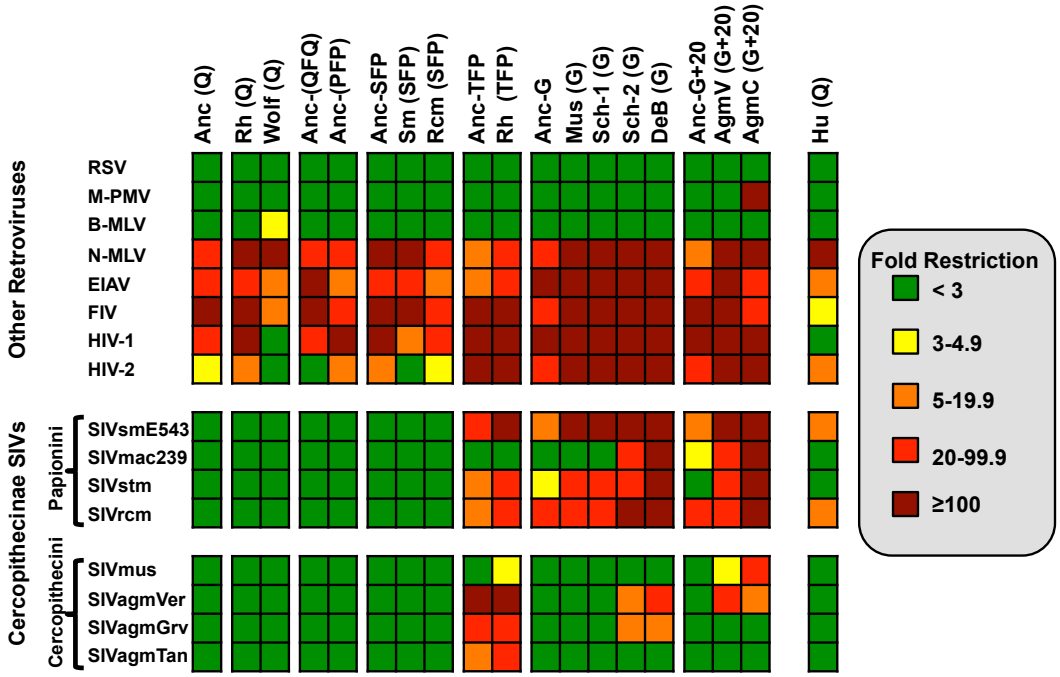


Figure 3-3. Patterns of restriction by ancient and modern Trim5αs. The restrictive potential of ancestral, modified ancestral, and modern Trim5αs was measured against a panel of non-lentiviruses and lentiviruses. A Heat map showing the level of restriction of the indicated virus with the corresponding Trim5α variant. Each data point is the average of at least three independent experiments.

The 8 Cercopithecinae SIVs were variably restricted by the 19 TRIM5αs (Figure 3-3). In contrast to the 8 non-SIV retroviruses tested, there was a strong correlation between the restriction of Cercopithecinae SIVs and the sequence of the V1 patch. The sufficiency of the V1 patch to confer the ability to restrict SIVs was demonstrated by the gain of SIV restriction by the modified ancestral TRIM5αs (ancTRIM5α^{QV1TFP}, ancTRIM5α^{QV1G} and ancTRIM5α$^{QV1G+20}$) over the non-restrictive, unmodified ancestral TRIM5α (ancTRIM5α^{V1Q}). The patterns of resistant or restricted viruses for the ancestral TRIM5αs resembled those of the present day TRIM5αs with matched V1 patch sequences (Figure 3-3). For example, both ancient and modern Cercopithecinae TRIM5αs with either V1-Q or V1-SFP lacked the ability to restrict Cercopithecinae SIVs. In contrast, both ancTRIM5α^{QV1TFP} and rhTRIM5α^{TFP} were broadly restrictive against SIVs and had nearly identical patterns of restriction. Similarly, ancTRIM5α^{QV1G} gained the ability to restrict a subset of SIVs matching those of musTRIM5α^{G} and sch1TRIM5^{G}. Finally, ancTRIM5α$^{QV1G+20}$ restricted a subset of SIVs that were also restricted by agmCTRIM5α^{G+20} and agmVTRIM5α^{G+20} (Figure 3-3). Specificity was further demonstrated by three observations: first, ancestral TRIM5αs never restricted viruses that their matched, modern day TRIM5α was unable to restrict; second, not every V1 modification led to SIV restriction; third, restriction was not observed when an SIV was tested on the TRIM5α from its natural host (regardless of V1 modificaiton) (Figure 3-3).

Convergent evolution in Papionini and Cercopithecini TRIM5αs capsid recognition

A majority of the tested TRIM5αs restricted HIV-1nl4.3 but not SIVmac239. We previously generated a series of viruses with chimeric SIVmac239-HIV-1nl4.3 CAs to map the CA encoded determinants of restriction by rhTRIM5α^{Q} and rhTRIM5α^{TFP} [380]. We used this pre-existing library of CA mutants to map the determinants of restriction for 12 representative TRIM5αs. These included 9 TRIM5αs that restricted HIV-1nl4.3 but not SIVmac239 (ancTRIM5α^{V1Q}, ancTRIM5α^{QV1SFP}, ancTRIM5α^{QV1TFP} ancTRIM5α^{QV1G} smTRIM5α^{SFP}, rhTRIM5α^{Q}, rhTRIM5α^{TFP}, musTRIM5α^{G} and sch1TRIM5α^{G}). We also tested ancTRIM5$\alpha^{QV1G+20}$, which restricted SIVmac239 5 fold, because it was the only TRIM5α with the V1-G+20 feature that did not restrict both viruses in excess of 100-fold. We also included wlfTRIM5α^{Q}, which did not restrict either virus, as a control for non-specific restriction, and huTRIM5α^{Q} as a non-Cercopithecinae TRIM5α for comparisson.

We previously demonstrated that CA surface elements (β-hairpin, 4-5 loop, helix 6 and 6-7 loop) largely governed restriction, and that the resistant or restricted phenotype could be transferred between viruses by the exchange of these features [380]. To determine whether recognition of the CA surface was an ancient and conserved property of Cercopithecinae TRIM5αs, we assayed the 12 TRIM5αs for their ability to restrict HIV-1nl4.3, SIVmac239, SIVmac239 with the HIV-1nl4.3 CA surface (SIV-HIV$_{surface}$) and HIV-1nl4.3 with the SIVmac239

CA surface (HIV-$SIV_{surface25}$). For all TRIM5αs, SIV-$HIV_{surface}$ was as sensitive to restriction as HIV-1nl4.3. Conversely, HIV-$SIV_{surface25}$ resisted restriction, similar to SIVmac239. These results demonstrate that CA surface recognition is conserved between ancient and modern primate TRIM5αs (Figure 3-4).

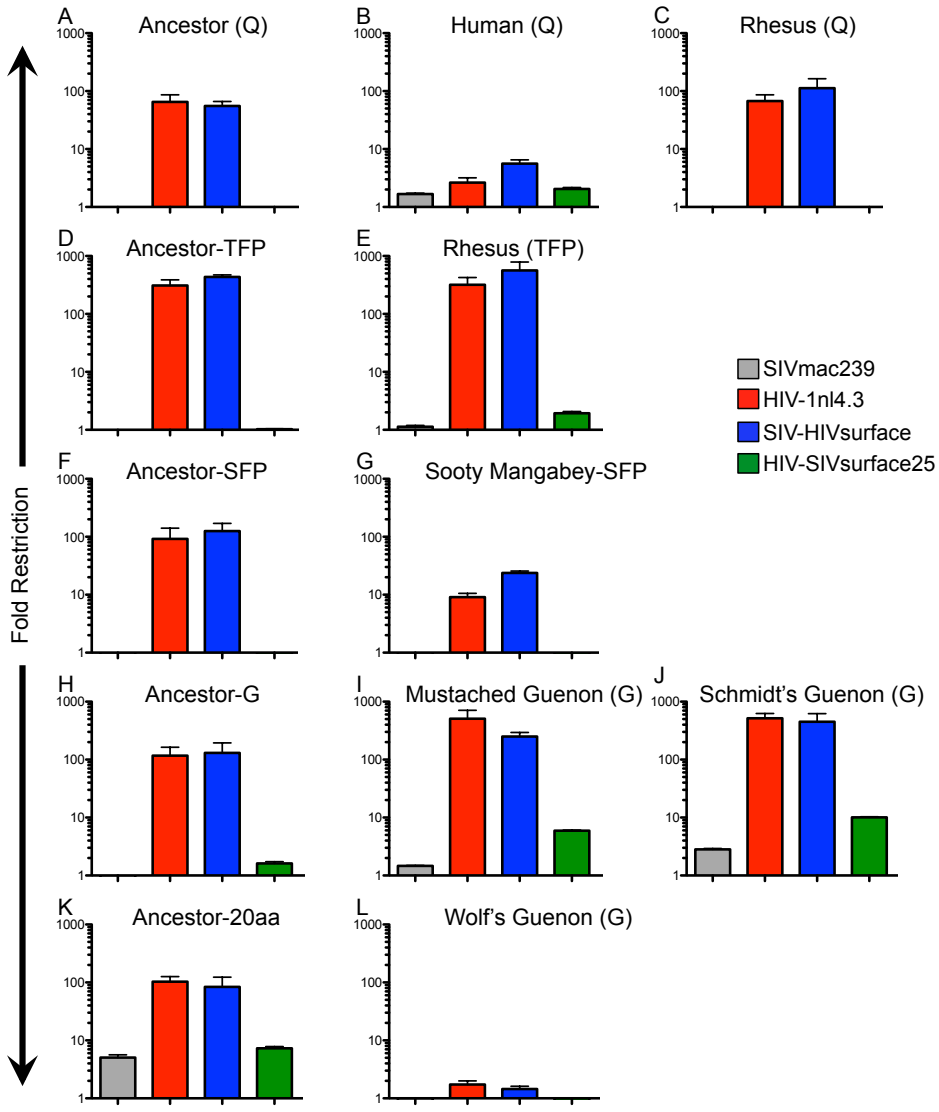


Figure 3-4. Capsid surface features are the major determinant of Old World monkey TRIM5α restriction. A subset of Trim5αs that differentially restrict HIV-1nl4.3 and SIVmac239 were identified. We tested these Trim5αs with chimeric viruses to determine whether the major determinant of this phenotype was the surface of the capsid protein. Human and Wolf's guenon Trim5αs which did not restrict either virus were also included. Cell lines were infected with wild type SIVmac239, HIV-1nl4.3, SIV with the HIV-1 surface (SIV-HIVsurface), HIV with the SIV surface (HIV-SIVsurface25), or with an interior chimera in which the internal alpha-helices of SIVmac239 were replaced with those of HIV-1 (SIV- HIVinterior). These viruses were previously described [380]. Fold restriction was graphed for each virus.

There are 25 amino acid differences between HIV-1nl4.3 and HIV-$SIV_{surface25}$. To map the specific sites that modulate restriction we tested a series of 25 SIVmac239 variants in which the amino acid at each of the 25 positions was substituted with the amino acid found at the corresponding position in HIV-1nl4.3 (Table 3-1) [380]. Of these viruses, 23 were infectious and were assayed for sensitivity to restriction by the 12 TRIM5αs (Figure 3-5). Nine of the 12 TRIM5αs tested restricted the two viruses with mutations in the β-hairpin ($SIVmac239_{Q3V}$ and $SIVmac239_{G6L}$) greater than 5 fold (Figure 3-5). A 10th, schTRIM5α^{G} only restricted the $SIVmac239_{G6L}$ mutant virus greater than 5 fold. Restriction of these viruses is unlikely to be artifactual, as they were not restricted by wlfTRIM5α^{Q}. These results support our published prediction that β-hairpin recognition is an ancient and conserved property of Old World primate TRIM5αs [380].

In contrast to the observations in Figures 3-3 and 3-4, we noticed that the ancestral TRIM5αs generally restricted fewer mutant viruses than the modern day TRIM5αs with the same V1 feature (Figure 3-5). This may reflect the stringency of assaying for the impact of a single amino acid substitution in an otherwise resistant CA. It is possible that restriction of SIVs or the surface swapped viruses is due to multiple determinants on the CA surface. Interestingly a virus with a mutation in helix 6 $SIVmac239_{V111L}$, was variably restricted by the ancestral TRIM5αs. While ancTRIM5α^{Q} and ancTRIM5α^{QV1G} restricted this virus, ancestral TRIM5αs with

V1 loop length alterations (V1+2aa and V1 G+20) lost the ability to restrict this virus (Figure 3-5). This may indicate that upon CA binding, V1 is positioned in the vicinity of helix-6.

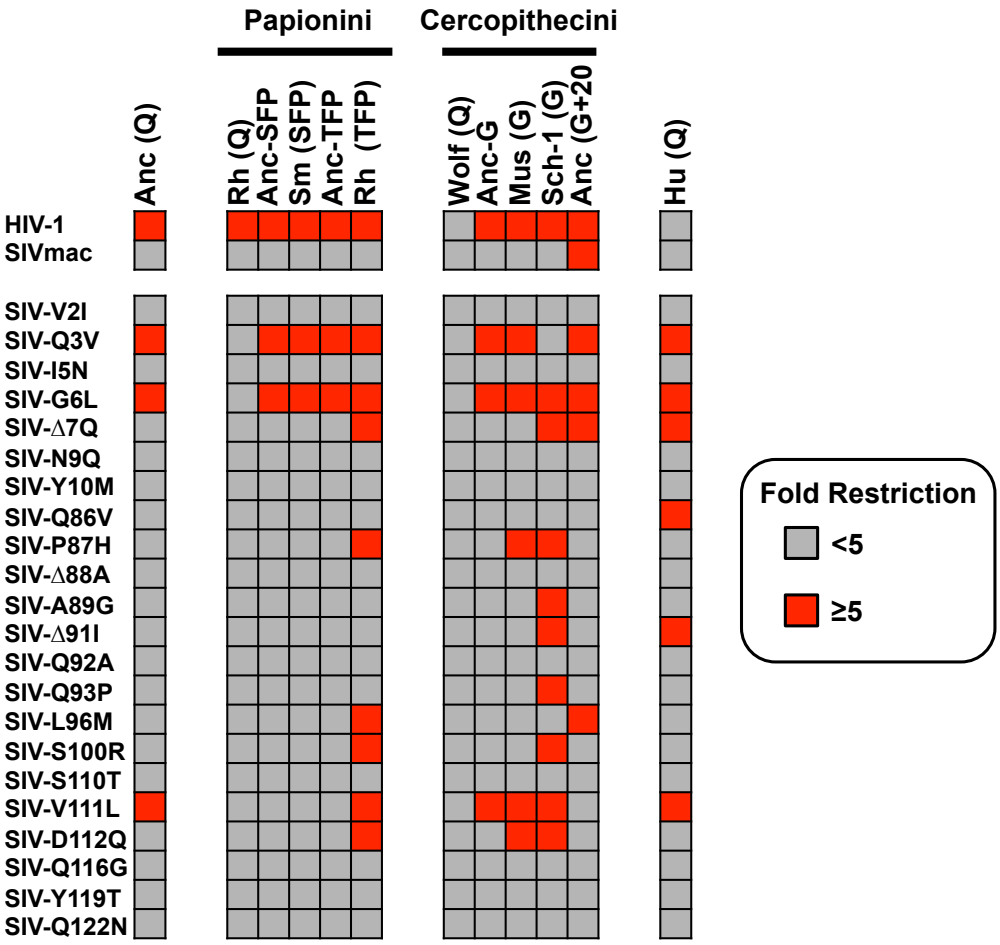


Figure 3-5. Capsid targeting of select Old World monkey TRIM5αs. The Trim5αs were screened against a panel of SIVmac239 viruses with single amino acid substitutions on the capsid surface (Table 3-1). These Trim5αs are separated by tribe. Viruses restricted greater than 5 fold by the indicated Trim5αs are colored red. Gray squares indicate less than 5 fold restriction. Trim5α-Ancestor-20aa restricts SIVmac239 approximately 5-7 fold, for this TRIM5α only, red boxes correspond to modified viruses that were restricted greater than 18 fold and gray squares indicate less than 18 fold restriction.

Table 3-1. Relationship between mutant virus numbering SIVmac239 and HIV-1nl4.3. The amino acid numbering of mutant viruses corresponds to the alignment in Figure 2-2A. Numbering of mutated residues corresponding to the SIVmac239 capsid (Accession number M33262) and HIV-1nl4.3 (Accession number M19921.2) are also provided.

Mutant virus	SIVmac239 Residue	HIV-1nl4.3 Residue
V2I	V2	I2
Q3V	Q3	3V
I5N	I5	N5
G6L	G6	L6
Δ7Q	Δ	Q7
N9Q	N8	Q9
Y10M	Y9	M10
Q86V	Q85	V86
P87H	P86	H87
Δ88A	Δ	A88
A89G	A87	G89
Δ91I	Δ	I91
Q92A	Q89	A92
Q93P	Q90	P93
L96M	L93	M96
S100R	S97	R100
S110T	S107	T110
V111L	V108	L111
D112Q	D109	Q112
Q116G	Q113	G116
Y119T	Y116	T119
R120H	R117	H120
R120N	R117	H120
Q121Δ	Q118	Δ
Q122N	Q119	N121
N123P	N120	P122

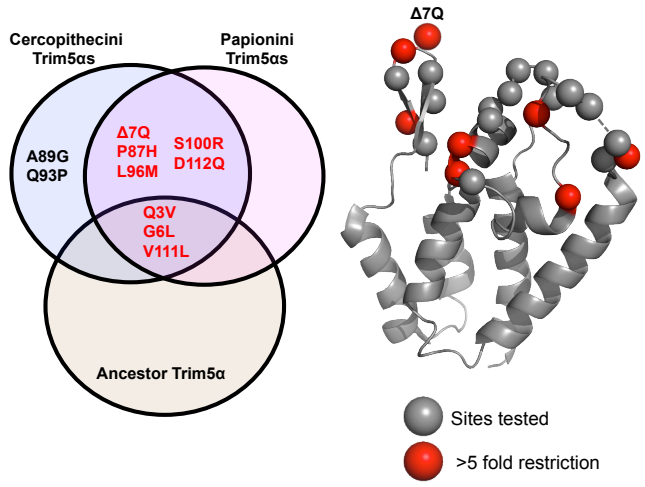
The modern TRIM5αs that restricted SIVmac239 point mutant viruses in addition to SIVmac239$_{Q3V}$ and SIVmac239$_{G6L}$ were the same TRIM5αs that restricted Cercopithecinae SIVs (Figures 3-3 and 3-5). While each TRIM5α restricted a unique subset of SIVmac239 point mutant viruses, it appears that Papionini (rhTRIM5α^{TFP}) and Cercopithecini TRIM5αs (musTRIM5α^{G} and sch2TRIM5α^{G}) have independently evolved to target similar or overlapping CA features. All three restricted SIVmac239$_{\Delta 7Q}$, a virus with an insertion in the β-hairpin (Figure 3-5 and 3-6A). The three TRIM5αs also shared the capacity to restrict SIVmac239$_{P87H}$ and SIVmac239$_{D112Q}$. In addition, rhTRIM5α^{TFP} and sch2TRIM5α^{G} also restricted SIVmac239$_{S100R}$, a key site of escape mutations from rhTRIM5α^{TFP} restriction *in vivo* [61]. Of note, ancTRIM5α$^{QV1G+20}$ also gained the ability to restrict SIVmac239$_{\Delta 7Q}$ and SIVmac239$_{L96M}$ (Figure 3-5 and 3-6A).

Positionally the mutations in viruses SIVmac239$_{P87H}$, SIVmac239$_{L96M}$, SIVmac239$_{S100R}$, and SIVmac239$_{D112Q}$ map to the periphery of a site that is highly conserved across primate lentiviruses [380]. The conservation of this site may be related to its position at the junction between two critical CA interfaces that mediate interactions with cellular cofactors that specifically interact with lentiviruses. These cofactors facilitate efficient nuclear import and shield the reverse transcription intermediates from innate sensors (Figure 3-6B) [231-

233,300,323,327,363,372]. Taken together, the correlation between restriction of SIVs and the spatial arrangement of sites that modulate restriction suggests that these TRIM5αs have evolved to target viruses similar to present day Cercopithecinae SIVs. Furthermore, it appears that both Papionini and Cercopithecini TRIM5αs have independently evolved to exploit the same conserved site that is both structurally and functionally unique to lentiviral capsids.

Figure 3-6. Convergent evolution in capsid targeting among Old World monkey TRIM5αs. Both Cercopithecini and Papionini TRIM5αs have independently gained the ability to restrict viruses with mutations that sit at the junction of two sites that mediate interactions with cellular cofactors. (A) Left a Venn diagram showing the overlap between mutations resulting in restriction for the ancestral Trim5α-V1Q and Cercopithecini and Papionini Trim5αs from figure 3-5. Mutations resulting in restriction by at least two groups of Trim5αs are colored red. Right. Amino acid substitutions colored red mapped to the SIVmac239 capsid N-terminal domain structure (PDB: 4HTW). (B) Relationship between mutations modulating Trim5α sensitivity and cellular cofactor binding sites mapped to the HIV-1 hexamer (3GV2 and 2X2D). Notably, the ability to recognize mutations at SIVmac239 amino acid positions P86, L93, S97, D109 were all independently gained in Cercopithecini and Papionini Trim5α evolution. These sites, in addition to V111L, sit at the junction of the CPSF6/Nup-153 and Nup-358 CypA like domain/CypA binding sites. Amino acids involved in these interactions are colored according to the key. Structural Images created in PyMol.

A



B

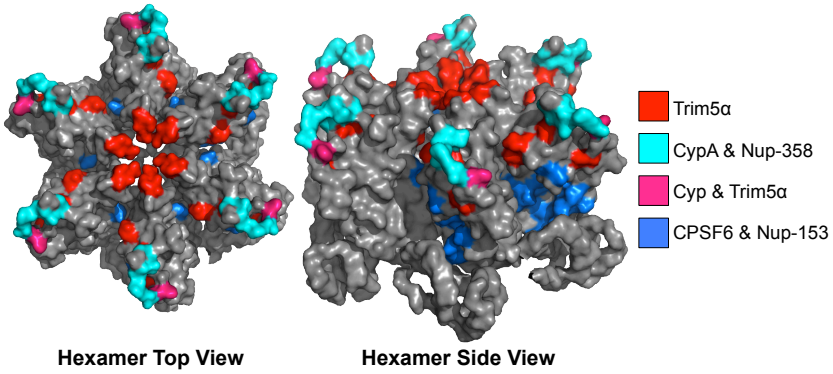


Figure 3-6. Convergent evolution in capsid targeting among Old World monkey TRIM5αs. Continued.

3. D. DISCUSSION

The *TRIM5* gene of Old World primates has evolved under strong positive selection [243]. Among Old World primates, V1 in the PRYSPRY is the most rapidly evolving feature [243,380]. This loop not only contains a prominent cluster of positively selected sites, but Cercopithecinae primates also harbor lineage specific length variations [243,244]. Analytically, these variances in length are typically excluded from standard measures of positive selection. However, the maintenance and the continued evolution of these length variants across multiple Cercopithecinae linages argues that these modifications were strongly and repeatedly selected for, by retroviruses.

We focused on a unique site of recurrent evolution among Cercopithecinae TRIM5αs that we refer to as the V1 patch. While this position was included in larger previously described V1 patches, we specifically focused on modifications that altered the sequence of Cercopithecinae TRIM5α position 339 or immediately adjacent residues [243,254,265]. Based on our present understanding of primate phylogeny and TRIM5α sequence information, we conservatively estimate that this patch began to diversify approximately 11-16 million years ago [34,35]. The diversification of this site stands in stark contrast to the Hominoidea and Colobinae TRIM5αs which have remained invariant for 30+ million years [34,35]. In contrast, this patch appears to have been twice modified in two independent Cercopithecinae lineages within a

narrow 1.1-4 million year window. Both of these tribe specific modifications have continued to evolve.

We tested the restrictive properties of a predicted ancestral TRIM5α that represents the sequence of TRIM5α as it existed just prior to the diversification of the V1 patch. This TRIM5α, which we called ancTRIM5α^{Q}, was functional against a diverse panel of retroviruses, but lacked the ability to restrict Cercopithecinae SIVs (SIVmac239, SIVsmmE543, SIVagmTan-1, SIVagmGrv, SIVagmVer, SIVmus, SIVstm, SIVrcm). We introduced the Cercopithecinae V1 patch derivatives into this TRIM5α and observed a specific gain in the ability to restrict these same Cercopithecinae viruses. Importantly, these gains matched those of present day TRIM5αs with the same V1 sequence (ex. ancTRIM5α^{TFP} and rhTRIM5α^{TFP} had very similar patterns of restriction) (Figure 3-3). For both ancient and modern TRIM5αs there was no correlation between the sequence of the V1 patch and the restriction of retroviruses other than the SIVs. In contrast, V1 patch sequences were strongly correlated with the ability to restrict specific Cercopithecinae SIVs. We did not observe a gain of restriction among an even more diverse panel of 8 non-Cercopithecinae SIV retroviruses.

Among the 12 TRIM5αs tested, almost all were capable of restricting two mutant SIVmac239 viruses (Q3V and G6L) with substitutions in the β-hairpin (Figure 3-5 and 3-6A). This supports our previously proposed model in which the β-hairpin, a highly conserved

structure in retroviral capsids, is part of a retrovirus-specific pattern widely exploited by TRIM5α proteins [380]. With the exception of SIVmac239$_{V111L}$, SIVmac239 viruses with mutations outside of the β-hairpin were only restricted by those TRIM5αs that also restricted Cercopithecinae SIVs (Figures 3-3 and 3-5). Our data using mutant viruses suggests that both Papionini and Cercopithecini TRIM5αs have independently evolved to target similar regions of the primate lentivirus capsids (Figure 3-6A). When these positions are mapped to the structure of the HIV-1 hexamer, they occupy a single face of the capsid protein (Figure 3-6B). Substitutions affecting restriction are largely absent on the opposite CA face (Figure 3-6B). The positioning of these sensitizing mutations in capsid is significant. Most of these sites are found at the junction of two cofactor binding sites that are important for optimal lentivirus replication – the binding sites for CypA/Nup358 CypA-like domain and CPSF6/Nup-153 [300,327,363,372]. The broader implication of these observations is that primate TRIM5αs may have widely evolved to specifically exploit a conserved site that governs lentivirus specific interactions with cellular cofactors. These findings suggests that unlike at least one element of TRIM5α which has evolved to generally recognize retroviruses through the β-hairpin, the V1 loop evolved to make very specific viral interactions. Together, these observations support our previously proposed model for the evolution of novel TRIM5α variants among Old World primates [380].

Our observations strongly suggest that the rapid evolution of the TRIM5α V1 patch was driven by ancient retroviruses whose CAs share a common set of attributes with present day Cercopithecinae SIVs, distinct from any of the other retroviruses tested. The most parsimonious explanation for our observations is that these evolutionary innovations were selected for by ancient Cercopithecinae SIVs. Thus, the earliest selective events may signify the emergence of SIVs among the Cercopithecinae. This conclusion, for the first time, establishes continuity between ancient and modern SIV infection among Cercopithecinae primates. Temporally, we can date waves of selection in multiple independent Cercopithecinae lineages to similar periods of time. From these observations it is easy to envision an evolutionary scenario where ancient SIVs were dynamically transmitted between species resulting in pathogenic infections. This would mean that the repeated patterns of cross-species transmission, viral adaptation, and viral dissemination observed in nature today are the continuation of an ancient 11-16 million year old pattern. Furthermore, it implies that the propensity to cross between species may be an inherent property of primate lentiviruses.

The patterns of pathogenesis associated with Cercopithecinae SIVs support our hypothesis that Cercopithecinae SIVs drove the evolution of the V1 patch. The only documented pathogenic SIV infections have arisen from the successful cross-species transmission of Cercopithecinae SIVs [24-29,58,59]. This includes the HIV-1/SIVcpz/SIVgor lineage which is a

recombinant between two Cercopithecinae SIVs [29], and the SIVmac viruses which came from sooty mangabeys. Thus it is conceivable that the cross-species transmission of ancient SIVs carried the same pathogenic potential. In contrast, many modern Circopithecinae species that are predicted to have harbored specific SIVs for a sufficient period of time to have coevolved to a state of apparent apathogeneses (e.g. sooty mangabeys and African green monkeys) are also found in the Cercopithecinae [24,25].

Currently, no direct evidence linking ancient SIVs to present day SIVs exists. However, our predictions fit within the greater context of both known and predicted (pre)history of the lentiviridae. Direct molecular evidence from three endogenized lentiviruses indicates that at least three extant lentiviral lineages existed on multiple continents 5-15 million years ago [52-55]. Distinct Cercopithecinae SIV lineages are believed to have existed prior to the isolation of Bioko Island from the African continent 10,000 years ago [42]. Indirect evidence of ancient lentiviral infection in Old World primates has largely come from the study of patterns of selection in genes with known anti-lentiviral activity. While these studies have found compelling evidence to support the existence of ancient lentiviruses, they cannot unambiguously determine if primate lentiviruses were the selective force. Binding to Cyclophilin A is a ubiquitous property of lentiviral capsids [291,324,402]. The pro-viral interaction between CA and Cyclophilins may have been exploited by an ancient TRIM5-CypA fusion protein dated to approximately 30 million years ago

[45]. Similarly, studies of APOBEC3G have identified selective events dated to 5-12 million years ago that disrupt the interaction with SIV Vifs [44]. However, as with cyclophilin A, other lentiviruses encode Vif proteins that may exploit and therefore select upon a common or overlapping interface. Importantly, restriction by TRIM5α is the result of a specific and productive interaction between TRIM5α and CA. Our conclusions are based upon assaying for this specific interaction, which was only observed with Cercopithecinae SIVs, giving us higher confidence that these evolutionary derived changes were specifically selected for by ancestors of these viruses.

Finally, we acknowledge that it has become very common in the field of indirect paleoviriolgy to infer ancient molecular interactions based solely on modern data; the risk is that alternative hypotheses including convergent or parallel evolution involving unidentified or extinct viruses cannot be ruled out. However, in our study we consistently observed a strong correlation between the independently derived V1 patch changes and the restriction of Cercopithecinae SIVs, and at the same time, we observed a clear lack of correlation among a diverse panel of other retroviruses, including other primate lentiviruses from apes. Our conclusions are also based upon assaying for the outcome of a very specific interaction between CAs and TRIM5αs. Taken together our findings strongly support the conclusion that ancient Cercopithecinae SIVs started driving the evolution of TRIM5α V1 approximately 11-16 million years ago, and that the

extant versions of V1 found in nature today are the direct result of continuous adaptive refinement [34,35].

3. E. METHODS

Cell lines

TRIM5α variants were isolated from: African green monkey kidney cell lines COS-1 and Vero, grown in DMEM/10% FBS.Skin fibroblast cell lines derived from primate species were obtained through Coriell Cell Repositories (Camden, NJ) and cultured according to specification. *Cercocebus torquatus* (Red-Capped Mangabey, PR00485), *Cercopithecus cephus* (mustached guenon, PR00531), *Cercopithecus ascanius* (Black-cheeked white-nosed monkey PR00566 and PR00634), *Cercopithecus neglectus (*De Brazza's monkey, PR01144), *Cercopithecus wolfi* (Wolf's guenon PR00486, PR00530 and PR01241). Crandell-Rees feline kidney (CRFK) cells and human embryonic kidney 293T/17 (HEK293T/17) cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/10% FBS. CRFK cell lines stably expressing N-terminally HA-tagged TRIM5 orthologs were maintained in DMEM/10% FBS supplemented with 5 µg/ml Puromycin.

RNA Isolation

RNA was extracted using Trizol regaent (Ambion/life technologies). cDNA was prepared using a Transcriptor First Strand cDNA Synthesis Kit (Roche) using an anchored-oligo($dT)_{18}$ primer. TRIM5α cDNAs were amplified and N-terminally HA-tagged using TRIM5-F-GCGGAATTCGCCACCATGTACCCATACGACGTCCCAGACTACGCTGGCGGCGCTTCTGGAATCCTGCTTAATGTAAAG AND TRIM5-R-ACCATCGATGGCTCAAGAGCTTGGTGAGCACAGAGTC primers. PCR amplicons were directly coloned into pLPXC (Clonetech) using EcoRI and ClaI sites.

Plasmids

TRIM5αs were cloned into pLPXC using EcoRI and ClaI sites. Retroviral GFP reporter viruses were produced from the following plasmids. HIV-1 was produced from the following reagent that was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-deltaE-EGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano [401]. The pV1EGFP derivatives encoding the 5' region of SIVmac239, SIVsmE543, SIVstm/37.16 were previously described [403].N-tropic or B-Tropic MLVs from either pCIGN or pCIG-B and pLXIN-EGFP (gifts of Jonathan Stoye, Medical Research Council, London, U.K.). Rous sarcoma virus (RSV) [404] (Addgene plasmid 13878, courtesy of Constance Cepko, Harvard Medical School, MA) Equine infectious anemia virus (EIAV) pEV53D and pEIAV-SIN6.1 CGFPW (Addgene plasmids 44168 and 44171 courtesy of John Olsen, University of North Carolina) [405,406].

Feline immunodeficiency virus (FIV) pFP93 and pGINSIN (gifts from Eric Poeschla, Mayo Clinic) [407,408]. The first 205 amino acids of the pNL43DeltaE-GFP Capsid were replaced with the following SIV capsids: SIVrcm (AF349680), SIVagmVerv (L40990), SIVagmGrv (M66437) and SIVmus-1 (AY340700). These capsids were synthesized as Strings by GeneArt/Life Technologies and cloned into pNL43DenvGFP using a previously described shuttle vector [380] HIV-1nl4.3-SIVmac239 chimeric viruses were previously described [380].

Virus production

All single-cycle viruses were produced in HEK293T/17 cells by cotransfection of the appropriate viral plasmid(s) and pVSV-G (Clontech Laboratories, Mountain View, CA), using GenJet (SignaGen; Ijamsville, MD). Viral supernatants were tittered on CRFK cells; supernatant volumes resulting in approximately 25% GFP/EGFP+ CRFK cells were used for infectivity assays on the cell lines expressing the indicated TRIM5α.

Infectivity assays

Stably expressing TRIM5 CRFK cells were seeded at a concentration of 5×10^4 cells per well in 24-well-plates and infected with the appropriate amount of VSV-G pseudotyped, single-cycle, GFP/EGFP expressing viruses. After 2 days, expression of GFP/EGFP was analyzed by fluorescence-activated cell sorting (FACS) performed on a FACSCaliburTM flow cytometer (BD,

Franklin Lakes, NJ), and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Phylogenetic Reconstruction

A comprehensive nucleotide alignment for 78 full-length Old World Primate TRIM5αs was generated. A maximum likelihood phylogenetic tree was generated using the HKY85 substation model [409] and 1,000 bootstrap replications. This tree was used as the input for ancestral node reconstruction via FASTML server (http://fastml.tau.ac.il/) [410-412]. The node corresponding to the split between Papionini and the Cercopithecini TRIM5αs was used as the ancestral TRIM5α. Node predictions were done in the absence of indel reconstruction. A predicted Q was placed position 339.

Chapter 4: General Discussion

4. A. LENTIVIRUSES ARE ANCIENT

Evidence of ancient primate lentiviruses. In addition to primates, extant lentiviruses exist in feline, equine, bovine, ovine and caprine species. Each of these groups harbor phylogenetically distinct lentivirus lineages, with the exception of ovine and caprine lentiviruses which form a single lentivirus lineage. Endogenous lentivirus sequences have been found in the genomes of lagomorphs, mustelids and Malagasy lemurs [52-55]. These sequences indicate that distinct lentivirus lineages have existed for at least 8-12 million years. Among all known lentiviruses, both extant and endogenous, the endogenous lemur virus PSIV is most closely related to modern primate lentiviruses [53,324]. Based on the lemur phylogeny and molecular clock estimates, this virus is predicted to have colonized lemur genomes 5-8 million years ago. It should be noted that this estimate is for the time of endogenization and does not date the emergence or extinction of this viral lineage.

The isolation of Madagascar from Africa occurred approximately 130 million years ago, while Prosimians likely colonized Madagascar approximately 70 million years ago [413]. It is unclear whether PSIV was present prior to the primate colonization of Madagascar, carried with the first primates to Madagascar, or introduced to the Malagasy ecosystem at a more recent date [53]. In the phylogeny of lentiviruses, PSIV is basal to extant primate lentiviruses. While the genomes of theses proviruses are degraded, this virus appears to lack many of the

characteristic accessory genes of present day primate lentiviruses. Structurally, the capsid N-terminal domain is similar to other capsids from lentiviruses [324]. While in some respects it is more similar to HIV-1 and HIV-2 than other lentivirus capsids, it also lacks some features commonly found in present day primate lentiviruses such as an elongated 4-5 loop [324]. The existence of PSIV in some lemur genomes establishes that viruses related to extant primate lentiviruses existed 5-8 million years ago it is unclear whether PSIV represents their predecessors or whether it represents a separate evolutionary branch.

A pattern of recurrent positive selection in the APOBEC3G gene of Cercopithecidae primates (Old World monkeys but not apes) is consistent with the presence of an antagonizing Vif protein [44]. These positively selected sites are found on a common Vif interaction face of APOBEC3G [44,197,199,200,205,206]. Using the primate phylogeny some selective events have been dated to 5-12 million years ago, which is consistent with the ages of known endogenous lentiviruses [44,52-55]. While it is likely that a Vif protein selected for these adaptations, it is unclear whether the Vif protein came from viruses related to modern day primate lentiviruses. A Vif gene is present among all extant lentivirus lineages with the exception of equine lentiviruses (EIAV) [1,2,18]. While divergent, these Vifs are thought to have shared a common ancestor and therefore may exploit similar or overlapping interfaces on APOBEC3G.

For example, selection by a Vif protein found in felines may alter the binding surface used by primate lentivirus Vif proteins.

To date, no direct evidence exists linking ancient primate lentiviruses to the extant primate lentiviruses found in Africa today. Our results reveal a strong correlation between specific, evolutionarily derived changes in the TRIM5-V1 loop of Cercopithecinae monkeys that occurred between 11-16 million years ago and the restriction of modern SIVs from Cercopithecinae hosts. Importantly, these changes did not correlate with restriction of retroviruses representing any other genus, or non-Cercopithecinae lentiviruses. Rather than assaying for the failure of two proteins to interact (like Vif-APOBEC3) we observed specific gains in ability to restrict a single group of viruses, the Cercopithecinae SIVs. The most parsimonious explanation for these findings is that ancient Cercopithecinae SIVs were present in Cercopithecinae primate species at least 11-16 million years ago. Therefore, primates and primate lentiviruses may have been coevolving for at least this long. For selection to have occurred, ancient SIV infection must have been pathogenic. Together with the observation that selection occurred in two independent Cercopithecinae lineages, the Papionini and the Cercopithecini, our findings imply that like modern day SIVs, these ancient SIVs spread through cross-species transmission.

Implications of the ancient origin of primate lentiviruses. Our observations provide evidence supporting the notion that SIVs have infected Cercopithecinae primates for the past 11-16 million years. However this is a single SIV lineage among a single group of primates. At least three major primate lentivirus lineages exist: SIVs similar to SIVsm/SIVrcm, SIVs similar to SIVgsn/SIVmus/SIVmon and SIVs similar to SIVscol/SIVwrc/SIVolc [27]. Numerous recombinant lineages including that of SIVcpz/SIVgor/HIV-1 are also present [27,29]. It is therefore very likely that the last common ancestor of all primate lentiviruses is older than the single SIV lineage that we predict was present among Cercopithecinae SIVs 11-16 million years ago.

We observed a pattern of strong and recurrent selection in a single gene, TRIM5α, in response to Cercopithecinae SIVs. If the relationship between primates and their SIVs is ancient then it is likely that other genes have also evolved to antagonize primate lentivirus infection. These genes may include other restriction factors, innate sensors or maybe even genes of the adaptive immune system. Unique Old World primate gene variants that have arisen in the past 11-16 million years should be considered in the context of ancient primate lentivirus infection. It is also accepted that primates which apathogenically harbor SIVs must have coevolved with a particular primate lentivirus for “a long period of time” [23-27]. Our findings suggest that these

timescales may span many millions of years. The genetic determinants of this apparent commensal relationship are not understood, but are of great medical interest.

If primate lentiviruses are indeed at least 11-16 million years old, then the *Lentivirus* genus of retroviruses must be far more ancient. At these time scales molecular clock estimates break down and cannot provide accurate divergence dates [414]. Without evidence of endogenous lentiviruses we can only speculate about the true age of the *Lentiviruses*. It appears that lentiviruses are endogenized at lower frequencies than viruses from some other retrovirus genera. Nonetheless, considering the genetic diversity both at the sequence level as well as in the genome structure of known lentiviruses it is clear that this retroviral genus must be significantly older than the Cercopithecinae SIVs.

4. B. A WORKING MODEL FOR TRIM5α BINDING

TRIM5α recognizes the capsid surface. In Chapter 2 we proposed an evolutionary model to describe how novel TRIM5α variants, like rhesus TRIM5α^{TFP}, could have evolved [380]. We believe that the breadth of TRIM5α restriction may be related to detection of the β-hairpin. This structural feature is conserved across the capsids of the *Orthoretrovirinae* [328-331,338,384]. We hypothesized that contacts with this conserved structural feature could allow other regions within the PRYSPRY domain to evolve additional contacts with the capsid protein.

These contacts could be specific for certain viruses or groups of viruses. Together, this modular recognition could account for the observed breadth and specificity of TRIM5α mediated restriction. In Chapter 3 we tested this model by mapping the determinants of restriction for other Old World monkey TRIM5αs. These findings were consistent with our proposed evolutionary model [380]. Evidence from a diverse panel of 11 Old World Primate TRIM5αs, suggests that the capsid surface is the major determinant of resistance or sensitivity. Of these 11 different TRIM5αs, 9 restricted two SIVmac239 viruses with single amino acid substitutions in the β-hairpin (Q3V and G6L), and a 10th restricted one of these viruses (G6L). Mutations outside the β-hairpin that resulted in restriction encircle a highly conserved patch of amino acids that sits at the junction of two cellular co-factor binding sites, CPSF6/Nup-153 and cyclophilin A/Nup-358 cyclophilin A-like domain [300,327,363,372]. Targeting of this site independently evolved in two primate lineages [47-51,61,257,385]. Importantly, both natural and synthetic TRIM5-fusion proteins that exploit either cofactor binding site are broadly restrictive against lentiviruses but not other retroviruses [47,48,50,51,61,257,327,370,372,385].

Natural exploitation of cofactor binding sites. Both the structural basis and evolutionary forces that have selected for TRIM5Cyp proteins are clear. Structurally, the cyclophilin A domain binds the proline-rich 4-5 loop of lentivirus capsids [232,300,333,334,363]. Evolutionarily, the interaction of lentiviruses and cyclophilin A is an ancient and conserved

property [260,291,324]. Two endogenous lentiviruses thought to have existed in an extant form at least 5-12 million years ago also engage cyclophilin A and can be restricted by TRIM5Cyp fusion proteins [52,53,324]. For modern lentiviruses the interaction with cyclophilin A in the cytoplasm as well as the cyclophilin A-like domain of Nup-358 are required for optimal infectivity and viral spread in tissue culture [232,233,323,327]. To date, no other *Orthoretrovirinae* capsid outside of the *Lentiviruses* has been shown to efficiently interact with cyclophilin A. Thus, the fusion of TRIM5 to a CypA domain leads to the generation of a broadly restrictive anti-lentivral factor.

Intelligent design of synthetic TRIM5s. Similarly, synthetic TRIM5α- fusions of lentivirus specific capsid interacting proteins can also generate broadly restrictive anti-lentiviral proteins. Like naturally occurring TRIM5Cyp proteins, these synthetic restriction factors owe their pan-lentivirus breadth of recognition to the conservation of capsid co-factor binding sites. Fusion of cellular cyclophilin A (PPIA) or the CypA-like domain of Nup-358 toTRIM5 creates artificial TRIM5CypA fusion proteins that bind to lentivirus capsids in a similar manner as naturally occurring TRIM5Cyps [327,333,334,390,415,416]. The capsid binding domain of CPSF6 has also been fused to the tripartite motif of TRIM5 resulting in a broadly restrictive anti-lentivirus factor [370]. Presumably, at least part of the interaction involves the binding of the capsid binding peptide to a highly conserved pocket on the side of the capsid protein [363]. This

pocket is an extension of the conserved surface patch we identified in Chapter 2. In a structure of the capsid protein in complex with the CPSF6 capsid binding peptide, the peptide adopts a conformation strikingly similar to a small molecule inhibitor, PF-3450074, that modulates capsid stability and virion infectivity [363,417-419]. PF-3450074 was used as the final proof that the FG-repeats of Nup-153 bind the same binding pocket. Fittingly, the fusion of the FG-repeats of Nup-153 to the tripartite motif of TRIM5α also yielded a broadly restrictive anti-lentiviral factor [372].

In regard to their interactions with lentivirus capsids, natural and synthetic TRIM5 fusion proteins share a number of commonalities. First, restriction, albeit broad, is limited to lentiviruses. Second, they all exploit highly conserved features of the lentivirus capsid protein. These sites are conserved because they mediate multiple interactions with cellular co-factors. Furthermore, mutations in both the cyclophilin A binding loop as well as the CPSF6/Nup-153 capsid binding loop also impact virion assembly, nuclear import, and integration site biases [327,364,371,372,378,379]. Third, these interactions are fairly well characterized because they involve the interaction of a single capsid monomer and a single capsid interaction domain. Fourth, single amino acid substitutions are largely sufficient to disrupt restriction, without entirely ablating virus infectivity in tissue culture [49,327,363,367,372]. Similarly, SIVmac evolved resistance to rhesus TRIM5Cyp through alterations in the 4-5 loop [61]. This virus has become

cyclophilin A and Nup-358 independent while maintaining pathogenicity [60,232,233,327,333,403].

A model for TRIM5α recognition. Aspects of the TRIM5α-capsid interaction are both similar to and distinct from the above factors. For example, like the above TRIM5-fusion proteins, the breadth of restriction by any TRIM5α ortholog is broad. However, unlike the above factors, this breadth encompasses retroviruses from multiple retroviral genera. For both, breadth of restriction is a product of a highly conserved target. While the TRIM5-fusion proteins bind a single capsid monomer, it is hypothesized that the binding site of TRIM5α includes binding sites on multiple capsid monomers in the context of the capsid lattice [208,210,213,420]. Our results in Chapter 2 along with the observations of many other groups support the notion that TRIM5αs recognize multiple features of the capsid protein (reviewed above). This observation is mirrored by the fact that single amino acid substitutions rarely render restricted capsids resistant to TRIM5α mediated restriction. This is in contrast to the TRIM5-fusion proteins that recognize a single target on capsid and whose recognition can be disrupted by singe amino acid substitutions. Our observations together with a great body of work including how these factors bind has led us to propose how TRIM5α recognizes retroviral capsids.

We believe that the Old World monkey TRIM5α PRYSPRY domain makes at least one contact with the β-hairpin. The β-hairpin is a conserved structural feature that is present in

viruses from at least 5 *Orthoretrovirinae* genera [328-331,338,384]. Thus, recognition of the β-hairpin could account for the observed breadth of TRIM5α mediated-restriction that can span multiple retroviral genera. In the structure between TRIM21 and the IgG-Fc, the variable loops corresponding to TRIM5α V2 and V3 do make contacts with β-sheets and a small loop with some β-sheet characteristics [248].

In the context of the hexamer the β-hairpins of the monomers form a ring around the 6-fold axis of symmetry. Due to their proximity to one another we cannot distinguish whether capsid recognition is dependent upon one or more β-hairpins in a single hexametric unit. The distance between β-hairpins of two adjacent capsid hexamers is too great for a single PRYSPRY domain to engage both simultaneously; the maximum length of the PRYSPRY domain is approximately 45 Å while the distance between β-hairpins in adjacent hexamers is approximately 90 Å. However, TRIM5α binds to capsids as a dimer and this could allow for the PRYSPRY domain of each monomer to engage β-hairpins on adjacent hexamers [210,421].

This model is supported by two studies in which N-MLV evolved resistance to rhesus and human TRIM5α [239,240]. In general, it appears that less acquired mutations were required to resist rhesus TRIM5α (which may have evolved to target primate lentiviruses) than human TRIM5α. However, in both experiments mutations in both the β-hairpin as well as the corresponding regions of N tropic MLV to the conserved surface patch were selected for during

the escape from human/rhesus TRIM5α restriction [239,240]. Interestingly, while similar escape mutations were selected for in the β-hairpin, escape mutations were varied in the homologus structures to the lentivirus conserved surface patch. We interpret this overlap to mean that the β-hairpin is indeed the source of the observed breadth of restriction. Furthermore, this model of binding supports the notion that specific capsid-TRIM5α contacts can evolve in addition to the β-hairpin.

While the β-hairpin accounts for the observed breadth of restriction, it cannot account for the specificity of restriction. Specificity of the TRIM5α-capsid interaction is demonstrated in two ways, both suggesting that TRIM5αs target lineage specific capsid features. First, TRIM5αs often fail to restrict closely related viruses such as N-tropic and B-tropic MLVs. Second, some TRIM5αs have the ability to broadly restrict a number of viruses within a single retroviral genus. This is seen for rhesus TRIM5α^{TFP}, which in Chapters 2 and 3 restricted every primate lentivirus except the macaque adapted SIVmac239. Our observations from Chapter 2 allowed us to conclude that rhesus TRIM5α^{TFP} evolved to target a patch of amino acids on the capsid surface that are highly conserved in both composition and structure across primate lentiviruses. In Chapter 3 we found that both Papionini and Cercopithecini TRIM5αs have independently evolved to target this exact site.

Our results with Cercopithecinae TRIM5α proteins are consistent with the hypothesis that the PRYSPRY domains of TRIM5α recognize the capsid surface. We propose that capsid recognition involves both specific contacts with the capsid β-hairpin and with the conserved surface patch that sits at the junction of two cofactor binding sites. When sites modulating TRIM5α sensitivity are mapped onto an HIV 1 capsid hexamer they form a pinwheel pattern that radiates from a central ring formed by the β-hairpins (Figure 4-1A). Cofactor binding sites closely match this pattern (Figure 4-1A). Importantly, the pinwheel analogy is used to emphasize that the arrangement of both sites modulating interactions with cellular cofactors and those that modulate sensitivity to TRIM5α radiate from a central β-hairpin ring with the same handedness. When this pattern is placed in the context of known structures (capsid lattice, PRYSPRY domains, the B-box and coiled-coil) a potential model for TRIM5α becomes apparent.

Structures of the TRIM5α B-box and coiled-coil form an antiparallel dimer [421,422]. This observation is supported by electron microscopy studies [210,422]. When two rhesus TRIM5α PRYSPRY domain structures are docked into the structure of the B-box and coiled-coil (through a shared helix) the two PRYSPRY domains are found within close proximity [422], and the two PRYSPRY domains are antiparallel relative to each other. In this model, the distance between PRYSPRY domains is too small to bridge β-hairpins of adjacent hexamers. However, if the B-

box + coiled-coil structure is placed across the 2-fold axis of symmetry of a single capsid hexamer, the PRYSPRY domains overlap with the β-hairpins of adjacent capsid monomers (Figure 4-1B). In this model, the antiparallel arrangement of the PRYSPRY domains matches the handedness of the sites that modulate TRIM5α sensitivity (Figure 4-1B). Based on the relative sizes of the primary components, a PRYSPRY domain can sit centered on the β-hairpin of one capsid monomer and the first variable loop (V1) can fill the volume of the gap between that monomer an adjacent capsid monomer (Figure 4-1B). This arrangement places V1 near the conserved surface patch that sits at the junction between the cellular cofactor binding sites of the second capsid monomer. Due to the handedness of the mutations that modulate TRIM5α sensitivity, the antiparallel arrangement of the PRYSPRY domains allows the second PRYSPRY domain to make the same contacts on the opposite side of the capsid hexamer.

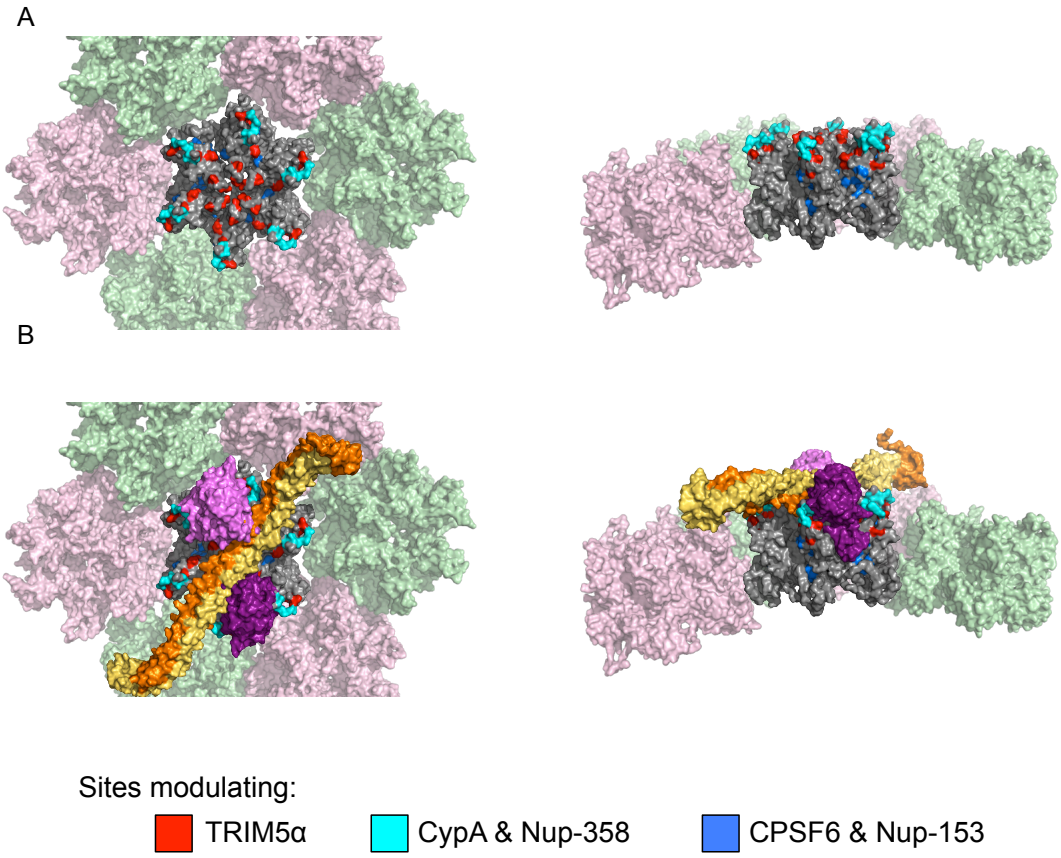
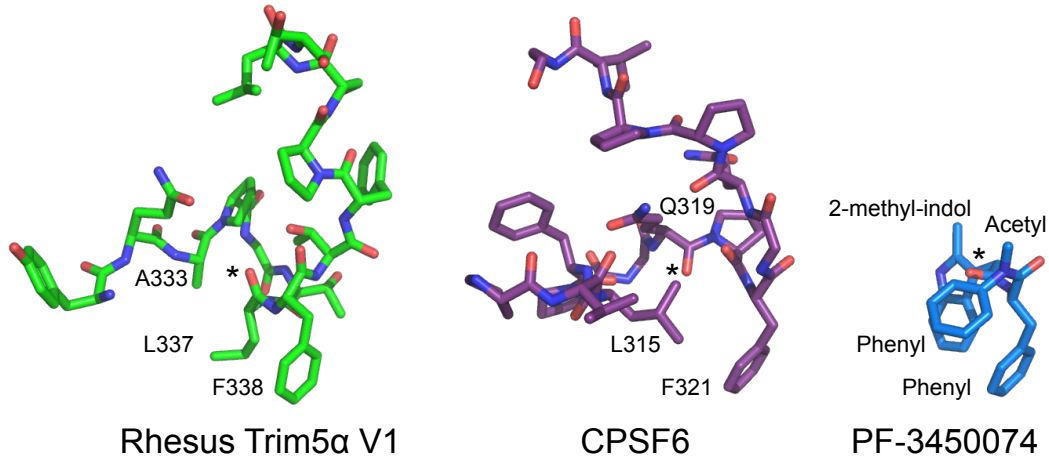


Figure 4-1. Model for TRIM5α Binding. A. A single capsid hexamer (gray)in the context of a capsid lattice (pink and green). Top (left) and side (right) views are shown. In the side view two capsid hexamers have been removed to view the gray hexamer. Sites that modulate interactions with capsid binding proteins are colored according to the key below. B. A model TRIM5α Binding based on structures of the B-Box and coiled-coil dimer (gold and orange) and two PRYSPRY domains dark (dark purple and magenta). PDB 3GV2, 2X2D, 3J3Y, 4TN3, 2LM3. Images generated in PyMOL.

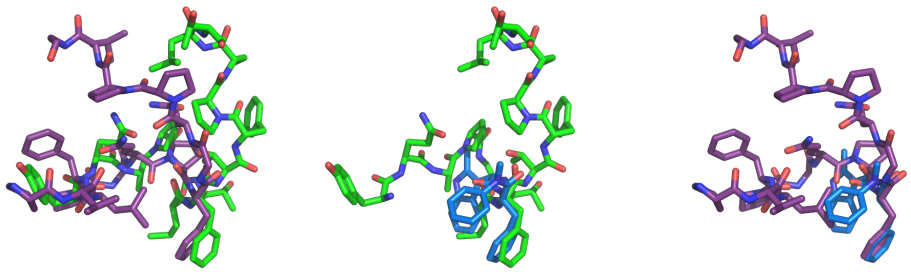
The conserved surface patch sits at the junction between the cyclophilin A/Nup358 cyclophilin A-like domain and the CPSF6/Nup-153 binding sites [300,327,333,334,363,372]. Selective pressure to preserve interactions with these cofactors likely explains the conservation of this region. Considering that mutations outside of the cyclophilin A binding loop modulate TRIM5α sensitivity we do not believe that the PRYSPRY domain binds to capsid by directly engaging the 4-5 loop in a manner reminiscent of cyclophilin A. However we have noted similarities between the V1 loop of Cercopithecinae TRIM5αs and factors that bind to the CPSF6/Nup-153 binding site. Convergent structures among the interaction partners of this binding pocket have been previously observed [363]. In complex with capsid, the capsid binding peptide of CPSF6 adopts a conformation very similar that of the small molecule inhibitor PF-3450074 [363,417]. A phenylalanine at position 321 in CPSF6 makes extensive contacts with the hydrophobic interior of the CPSF6 binding pocket (Figure 4-2A). Similarly a phenyl group of PF-3450074 occupies the same position when in bound to the HIV-1 capsid. In the capsid-bound CPSF6 peptide the side chain for L315 makes very similar hydrophobic contacts within HIV-1 α-helix 4 as a second phenyl group of PF-3450074. The acetyl group of PF-3450074 mimics the peptide oxygen of CPSF6-Q319. In both instances these oxygens form a hydrogen bond with HIV-1 capsid residue K70.

The V1 loop of rhesus macaque TRIM5α is predicted to be highly dynamic and the reported structure was solved by NMR in the absence of capsid [211]. Even in the absence of its capsid ligand this loop adopts a conformation similar to that of the CPSF6 capsid binding peptide and PF-3450074 [211,363,417] (Figure 4-2A). The structures of all three capsid interacting factors can be aligned (Figure 4-2B). In this proposed model of rhesus TRIM5α binding F338 can be aligned to CPSF6 F321 and the phenyl group of PF-3450074. Notably, this phenylalanine is absolutely conserved across Old World monkey TRIM5αs. The R-groups of rhesus TRIM5α L337 and the methyl group of A333 form a hydrophobic surface similar to that of the 2-methyl-indol group of PF-3450074. The peptide oxygen of L337 is also positioned similarly to the acetyl group of PF-3450074 and the peptide oxygen of CPSF6 Q319 (Figure 4-2B). Among Old World monkey TRIM5αs L337 is also highly conserved. Therefore, it is possible that upon capsid binding V1 can adopt a conformation that can mimic these capsid binding factors.

A



B



C

```
                      * **     *****
Human Nup-153 SSAFQFGSSTTNFNFTNN
Rhesus Q      GTL..---.L....YCTG
Ancestor Q    GTL..---.L....YCTG
Guenon G      GTL.G---.L....YCTG
Rhesus TFP    GTL.T.-P.L....YCTG
Agm G+20      G.S.G---.L....YCTG
Human Q       GTRY.---TFV...YCTG
```

Figure 4-2. Similarities between capsid interaction proteins and rhesus TRIM5α. (A) Structural similarities between variable loop one (V1) of rhesus TRIM5α, the CPSF6 capsid binding peptide and PF-3450074 (PDB: 2LM3, 4B4N, 2XDE). Key amino acids and functional groups are labeled. An "*" denotes an oxygen that is predicted to make contacts with the HIV-1 capsid. (B) A pairwise structural alignment of the structures in A. Left. Rhesus TRIM5α V1 and the CPSF6 capsid binding peptide. Middle. Rhesus TRIM5α V1 and PF-3450074. Right. The CPSF6 capsid binding peptide and PF-3450074. (C) Sequence similarity between rhesus TRIM5α V1 and residues 1423-1440 Nup-153. Multiple primate TRIM5α V1 sequences aligned to Nup-153. Asterisks indicate sites of identity or similarity, "." indicate identity and "-" denote gaps. Structural Images created in PyMol.

The FG-repeats of Nup-153 are also predicted to bind to this same site [372]. Its interaction can be outcompeted by PF-3450074 *in vitro*. We have also noticed that the amino acid sequence of rhesus TRIM5α V1 and other old world monkey TRIM5αs are similar to a stretch of amino acids in Nup-153. Rhesus TRIM5α residues 335-351 can be aligned to Nup-153 residues 1423-1440 (Figure 4-2C). These residues are found in the segment of Nup-153 that appears to mediate its interaction with HIV-1 [372]. Interestingly, the stretch of amino acids in TRIM5α that resembles Nup-153 has been duplicated in the G+20 insertion found in African green monkeys and Pata's monkeys [243,244].

Our assignment of V1 to this cofactor binding site is highly speculative. However, the commonalities between V1 and known interactors of this binding pocket are striking. These observations fit with our mutagenic data from Chapters 2 and 3. From our observations, V1 is clearly involved in making primate lentivirus specific capsid contacts. This proposed model of binding matches observations from NMR titration experiments between the N-terminal domain of the HIV-1 capsid and the rhesus PRYSPRY domain [211,212]. The addition of capsid causes chemical perturbations in amino acids in V1 [211]. Conversely, the addition of the rhesus PRYSPRY domain to HIV-1 capsid N-terminal domains causes chemical shifts on the capsid surface around the conserved patch [212]. Perhaps a structure of V1 in complex with capsid will one day support our speculations.

4. C. OTHER CAPSID TARGETING RESTRICTION FACTORS

FV1 and MX2. The exact qualities of capsid that permitted the evolution of a factor such as TRIM5α have also facilitated the evolution of other capsid targeting anti-retroviral factors. Many of the evolutionary constraints acting upon primate lentiviruses are also likely present for capsids of other members of the orthoretroviridae. Despite the absence of well characterized capsid binding cellular cofactors, the dynamic exchange of inter- and intra- capsid interactions during assembly surely imparts a number of constraints. The sum of these constraints has resulted in a viral capsid protein that has been largely intolerant of mutation [273,376-379]. While the capsid sequences of members of each *Orthoretrovirinae* genus are divergent, their capsids are structurally conserved [324,328-331,338]. This conservation extends to the hexamers that make up all but 12 of the higher ordered building blocks of capsid [330,373,375]. A growing body of evidence, including the results presented here, supports the notion that the pattern created by the higher ordered arrangement of the capsid lattice is a highly conserved and uniquely retroviral pattern. The stability of this structure over deep evolutionary time has been exploited by other anti-retroviral factors.

The cross-strain transmission of the gammaretroviruses (Friend murine leukemia virus) between laboratory strains of mice identified a number of restriction factors, before the discovery of the first primate lentivirus, HIV-1 [423-429]. One particular block, FV1 (named for

its restriction of Friend murine leukemia virus) [428-431], acts in a way reminiscent of TRIM5α. A more accurate statement is that TRIM5α acts in a manner reminiscent of FV1. It should be noted that the FV1 block was identified and characterized before the presence of TRIM5α was fully appreciated. The characterization of the FV block laid much of the conceptual framework that would later be applied to define the LV1 block and identification of TRIM5α.

Through genetics it was determined that FV1 was a heritable, dominant acting, intrinsic property of cells from resistant mice [428-431]. The block was shown to be saturable and dependent on the sequence of the viral capsid [432-437]. Importantly, the block was dependent upon the higher ordered arrangement of capsid in the context of the capsid core [438,439]. FV1 acts at a post-entry and pre-integration step that is distinct from that of TRIM5α [226,440-442]. The concept that different inbred mouse strains harbored distinct alleles with unique anti-gammaretrovirus specificities preceded many of the properties later discovered for TRIM5 [428,429,443]. Interestingly, FV1 is not a TRIM protein but rather a coopted remnant of an endogenous retrovirus *gag* gene [444].

Despite the fact that FV1 bears no genetic similarity to TRIM5, both appear to engage capsids in a similar manner. Mutations in MLVs affecting both TRIM5 and FV1 sensitivity map to the capsid surface, which is nicely illustrated by Ohkura and collogues in 2011 [239,240]. While the dimensions are different from TRIM5α, FV1 forms a regular lattice on MLV capsid tubes

[319,445]. Furthermore, both TRIM5α and FV1 form dimers that are largely alpha helical and antiparallel [421,422]. While FV1 was once thought to only act on gammaretroviruses, it has recently been demonstrated that some variants can restrict supmaretroviruses and lentiviruses [446]. Thus, like TRIM5α, FV1 orthologs have the capacity to broadly restrict retroviruses.

In addition to FV1, the recently characterized anti-lentiviral protein MX2 is believed to target lentiviral capsids [447-450]. Mutations throughout the capsid protein can modulate sensitivity to MX2 [447-450]. Notably some mutations are in positions that may not be accessible for a factor as large as MX2 to bind in the context of the fullerene cone of TRIM5α [320,449,451]. MX2 is also predicted to adopt a long alpha helical structure, perhaps similar to FV1 and TRIM5α [421,422,448,451]. While immunoprecipitation experiments hint that monomeric capsid protein can interact with exogenously expressed MX2 [448], it will be interesting to see how MX2 mediated restriction compares to FV1 and TRIM5α.

Thus it appears that the evolutionary stability of the capsid structure has been widely exploited by antiretroviral factors. As evidenced by the overwhelming number of endogenized retroviruses in the genomes of eukaryotes and the number of characterized extant retroviruses, it is clear that the *Retroviridae* has been a successful viral genus [452-454]. The evolutionary history of the *Retroviridae* and eukaryotes may extend in excess of 400 million years [238].

Considering the evolutionary stability of *orthoretrovirinae* capsids, it would not be surprising to this author if additional capsid dependent blocks to retrovirus infection are identified.

4.D. TRIM5α: THE BIG PICTURE

Integrated immunity. It is important to note that host restriction factors are not insular acting proteins as they were discussed above. Rather, they contribute to a multilayered organismal defense system. At least two restriction factors, TRIM5α and BST2, have the capacity to signal through the same cascades as innate sensors [272,455,456]. This is likely due to the specificity of the processes they target: retroviral capsids and viral budding. Signaling through these pathways not only upregulates the expression of other restriction factors and other antiviral factors in the infected cell but also in adjacent cells due to autocrine and paracrine signaling of the interferon pathway.

TRIM5α and BST2 may also modulate the adaptive immune response. Aside from the requirement of certain cytokines for the maturation of the immune response, these factors may influence the T-cell and B-cell responses. BST2 entrapment of virions on the cell surface can theoretically expose viral antigens to B-cells [122,123]. In macaques vaccinated with SIVmac239ΔNef, which is unable to counteract rhesus BST2, there are higher titers of antibodies that mediate antibody-dependent cell-mediated cytotoxicity [177]. The higher

antibody-dependent cell-mediated cytotoxicity titers correlate with protection from SIV infection *in vivo*. Similarly, modified HIV-1 viruses lacking the ability to antagonize BST2 have increased levels of antibody-dependent cell-mediated cytotoxicity *in vitro* [178].

We and others have demonstrated that single amino acid substitutions in an otherwise TRIM5α resistant capsid are sufficient to lead to viral restriction (reviewed above). Therefore, a TRIM5α that does not restrict a virus may still place a "cap" upon capsid diversity *in vivo*. In instances where regions with the potential to be recognized by TRIM5α overlap with cytolytic T lymphocyte epitopes, escape mutations may be limited or come at the cost of sensitizing a resistant capsid to TRIM5α mediated restriction. Known cytotoxic T lymphocyte epitopes overlap the conserved capsid surface patch and β-hairpin in both humans and rhesus macaques [457-467]. Indeed, some escape mutations in cytotoxic T lymphocyte epitopes lead to increased human TRIM5α sensitivity [468-470]. Natural killer cells can select for viral peptides that bind to inhibitory KIRs when presented on MHC to prevent natural killer cell mediated killing. Specific KIRs in humans can select for changes in the β-hairpin [471]. Understanding the impact of a TRIM5α mediated "cap" on capsid diversity may be critical for the development of vaccines against primate lentiviruses.

Human TRIM5α. At times it is tempting to place our observations in the context of humans and human viruses, however *homo sapiens* are indeed primates whose evolutionary

history is hardly unique among the other approximately 500 primate species [37]. At times human TRIM5α is discussed as being inferior to some primate TRIM5αs primarily because it cannot restrict HIV-1 and a handful of common SIV molecular clones. Human TRIM5α has clearly evolved with purpose [243]. Thus, while HIV-1 has evaded human TRIM5α mediated restriction we will never know of the pandemics it has prevented. Human TRIM5α is capable of restricting isolates of HIV-2 [288,348,472-475]. Specific mutations in the HIV-2 capsid are associated with lower viral loads i*n vivo* [288]. These viruses are more sensitive to human TRIM5α than capsids associated with high viral loads i*n vivo* [288]. Furthermore, while human TRIM5α does not restrict most HIV-1 isolates, its presence likely places a "cap" on viral diversity. While HIV-1 is usually weakly restricted by human TRIM5α in overexpression experiments our observations as well as others support the notion that very few amino acid substitutions would be required for HIV-1 to become restricted. Thus, human TRIM5α may play an important role *in vivo*.

Human TRIM5α, like that of non-Cercopithecinae TRIM5αs, has V1-Q in the rapidly evolving patch of amino acids (Chapter 3). However, in contrast to other TRIM5αs with this feature, human TRIM5α has the capacity to restrict Cercopithecinae SIVs. Human TRIM5α restricted SIVs from both sooty and red-capped mangabeys. Thus, human TRIM5α is more restrictive than TRIM5αs from wolf's guenon, sooty mangabey, red-capped mangabey, rhesus

TRIM5α^{Q} (Chapter 3). Importantly, the activity of human TRIM5α cannot be discounted when studying human retrovirus infection.

Heterozygous advantage. Along similar lines we cannot impose observations made from studying a single primate species upon all primates. In contrast to many primate species there appears to be less allelic diversity among human restriction factor genes. In contrast to humans, during Cercopithecinae primate evolution multiple TRIM5α variants were maintained through long term balancing selection (Chapter 3 and [251]). Within a single species, rhesus macaques, there are three TRIM5 allelic classes that differ in their capsid binding specificities [46,47,51,61,251,257,380]. These allelic classes can be further subdivided into a number of distinct alleles [251]. A moderate to high frequency of distinct TRIM5α alleles are also found in other macaque species, sooty mangabeys, and likely Schmidt's guenons (Chapter 3 and [46-48,51,243,251,257,476-479]). It is important to note that large genotyping surveys for other primate species have not been conducted. However, from these observations there appears to be recurrent selection to maintain multiple TRIM5α alleles. Our findings in Chapters 2 and 3 indicate that rhesus macaque and Schmidt's guenon TRIM5αs restrict different subsets of viruses or mutants thereof. Similar findings have been observed for sooty mangabey TRIM5α alleles [251].

The high frequency of TRIM5α alleles in captive rhesus macaques influenced the emergence of SIVmac [60,403]. This virus has clearly made multiple adaptations to evade at least two rhesus TRIM5 variants. This may have come at the expense of interacting with the Nup-358 cyclophilin A-like domain [61,327]. TRIM5α genotype influences viral loads of animals inoculated with SIVsmm viruses [60,61,385]. Heterozygous advantage for TRIM5 has been demonstrated *in vivo* [61]. The strongest reduction of viral loads in SIVsmm infected rhesus macaques is found in animals with two different restrictive alleles, rhTRIM5α^{TFP} an rhTRIM5^{CypA} [60,61,385]. Furthermore, experimental infections recreating the initial SIVsmm-rhesus macaque transmission that gave rise to SIVmac indicate that some animals with restrictive alleles can suppress viral loads below the limit of detection [61,480]. These observations together with the selection to maintain multiple TRIM5α alleles suggests allelism among restriction factors may influence cross-species transmission events or the emergence of new viruses in nature. Thus, as we embark upon conservation efforts for endangered primate species, efforts to persevere allelic diversity of restriction factors should be considered.

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