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SPECIES SPECIFIC DIFFERENCES IN TRIM5α FUNCTION AND GENOMIC COMPOSITION

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By

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M.S., Tulane University, 2002

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ABSTRACT

SPECIES SPECIFIC DIFFERENCES IN TRIM5α FUNCTION AND GENOMIC COMPOSITION

WILLIAM E. DIEHL

It has recently become apparent that the alpha splice variant of tripartite motif-containing protein 5 (TRIM5α) can impose a major barrier to cross-species retroviral infection. This antiviral activity is most prominently exerted shortly after viral entry into a target cell and results in an abortive infection prior to viral genome integration into the host chromatin. Work from other groups has shown that TRIM5α is capable of restricting infection by a variety of Gammaretroviruses, Lentiviruses, and Spumaviruses. In this dissertation molecular virological approaches were used to demonstrate that a prototype Betaretrovirus, Mason-Pfizer Monkey Virus (M-PMV), encounters a virus specific post-entry block to replication in cells from three New World monkey species. In two cases TRIM5α was identified as being responsible for this block, thus expanding known TRIM5α antiviral activity to Betaretroviruses.

In primate genomes, TRIM5 is found in conjunction with the related genes TRIM6, TRIM22 and TRIM34. Similar to TRIM5, TRIM22 has been shown to possess antiviral activity and both genes have been identified as having faced strong selective pressures during primate evolution. In contrast, the neighboring genes have been under purifying selection. Using genomics approaches it was discovered that elevated rates of transposable element fixation has occurred within the introns of TRIM5 and TRIM22, including the fixation of multiple endogenous retroviral (ERV) long terminal repeat (LTR) elements in the first intron of these genes. Using molecular biological techniques it was further shown that differential fixation of one such LTR element has resulted in a species-specific regulation of TRIM22 in response to p53 activation. As TRIM5 and TRIM22 are found adjacent each other in the genome and are situated such that they share a 5 kilobase (kb) promoter region, it is likely that the various LTRs fixed in these genes similarly modulate gene expression. These results are suggestive of a scenario where primate genomes have co-opted inserted endogenous retroviral elements as a mechanism for rapid alteration of gene expression, presumably to match the tissue tropism of a pathogenic virus challenge.

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INTRODUCTION

Retroviridae

The *retroviridae* are a family of viruses that infect vertebrates, mainly birds and mammals. Viral particles are generally between 80 and 120 nanometers in diameter and are surrounded by a membrane that contains a viral envelope (Env) protein, which mediates entry into a target cell. Retroviral virions contain two copies of the viral single-stranded positive sense ribonucleic acid (RNA) genome. The principal characteristics shared by members of this family of viruses are the ability to generate a deoxyribonucleic acid (DNA) copy of the viral genome from the encapsidated viral RNA, and to integrate this viral genomic DNA into the cellular chromosomal DNA. Retroviral genomic DNA is called the provirus. The generation of DNA from genomic RNA and subsequent integration are both performed by products of the viral polymerase (*pol*) gene; reverse transcriptase (RT) and integrase (IN), respectively [239]. Retroviral group-specific antigen (gag) genes give rise to at least three proteins that encircle viral genomic RNA, giving structure to the virus. A feature distinguishing members of the *retroviridae* family from other enveloped viruses is that viral particles undergo a process of maturation following release from the cell, whereby viral precursor proteins, including Gag, are cleaved by a viral protease (Pro). Gag, Pro, Pol, and Env proteins are produced by all retroviruses, and viruses that only produce this group of viral proteins are called 'simple retroviruses'. In addition to producing these required proteins, retroviruses can encode other 'accessory proteins' that assist in viral replication or help in evasion of the immune response. Viruses containing accessory genes are called 'complex retroviruses'.

Retroviruses can be divided into seven distinct genera, based on the nucleotide sequence similarity of their reverse transcriptase genes: Alpharetroviruses, Betaretroviruses, Gammaretroviruses, Deltaretroviruses, Epsilonretroviruses, Lentiviruses, and Spumaviruses. Evolutionary relationships between these groups are shown in Figure 1. Alpharetroviruses are a group of infectious retroviruses that infect birds and include members having both simple and

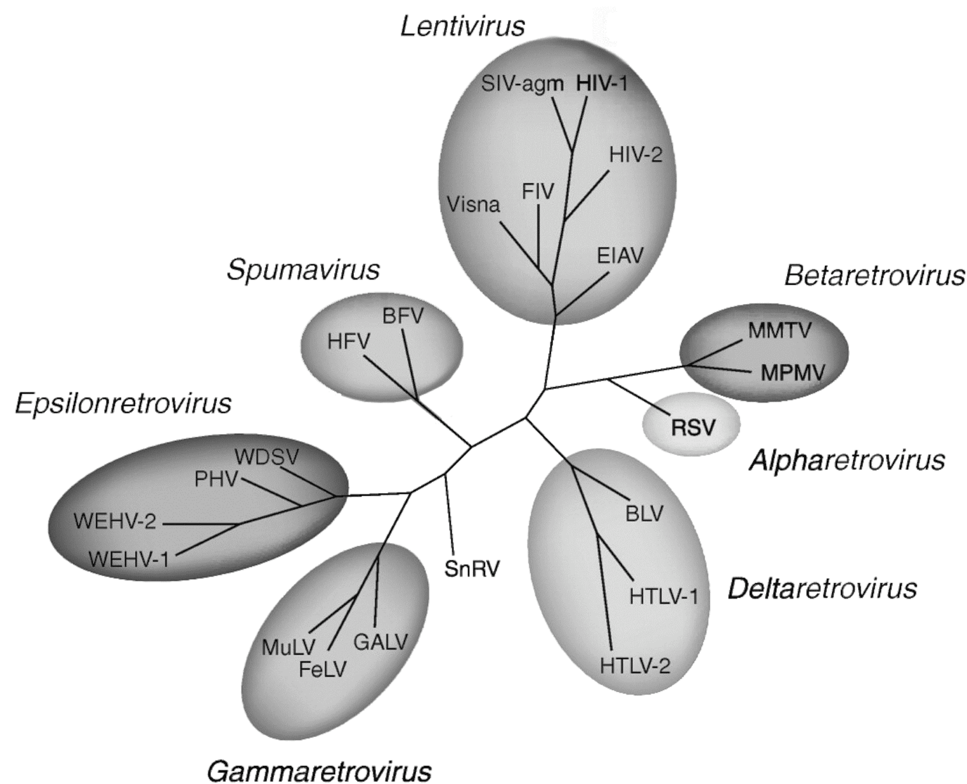

Figure 1. *Retroviral phylogeny.* Phylogenetic analysis of the amino acid residues contained within domains 1–4 and part of domain 5 of the reverse transcriptase. Depicted is an unrooted neighbor-joining phylogenetic tree, constructed using the Phylogeny Inference Package (PHYLIP) package (Felsenstein J. "PHYLIP Version 3.57c.", University of Washington, Seattle 206, USA, 1995). BFV: Bovine foamy virus; BLV: Bovine leukemia virus; EIAV: Equine infectious anemia virus; FeLV: Feline leukemia virus; FIV: Feline immunodeficiency virus; GALV: Gibbon ape leukemia virus; HFV: Human foamy virus; HTLV: Human T-lymphotropic virus; MMTV: Mouse mammary tumor virus; MPMV: Mason-Pfizer monkey virus; MuLV: Murine leukemia virus; PHV: Perch hyperplasia virus; RSV: Rous sarcoma virus; SFV: Simian foamy virus; SIV: Simian immunodeficiency virus; SnRV: Snakehead retrovirus; SRV: Simian retrovirus; STLV: Simian T-lymphotropic virus; WDSV: Walleye dermal sarcoma virus; WEHV: Walleye epidermal hyperplasia virus.

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complex genomes. A prototypic virus of this genus is the Avian Leukosis Virus (ALV), a simple retrovirus that infects chickens and can cause lymphoblatsic and erythroblastic tumors as well as osteopetrosis. Betaretroviruses are a group of both simple and complex retroviruses that infect divergent mammalian species including mice, sheep, and primates. Mason-Pfizer Monkey Virus (M-PMV), a simple retrovirus that is the causative agent of simian acquired immunodeficiency-like syndrome in juvenile macaques is a prototypic member of this genus. Gammaretroviruses are

a group of generally simple retroviruses from divergent vertebrate species including birds, reptiles, mice, cats, and primates. The prototypic member of this genus is Murine Leukemia Virus (MLV), a simple retrovirus that can cause leukemias and lymphomas in mice. Deltaretroviruses are a group of complex retroviruses responsible for B- and T-cell lymphomas and leukemias in primates and cows. The prototypical member of this genus is the Human T-lymphotropic Virus 1 (HTLV-1), which can cause myopathies in addition to T-cell lymphomas and leukemias in humans. Epsilonretroviruses are a genus comprised of complex retroviruses that infect and cause tumor growth in fish. The prototypic member is Walleye Dermal Sarcoma Virus (WDSV). Lentiviruses are a diverse group of complex retroviruses that cause immunodeficiencies, anemia, and neuropathies in primates, cats, cows, horses, and sheep. The prototypic member of this genus is Human Immunodeficiency Virus 1 (HIV-1), the causative agent of Acquired Immunodeficiency Syndrome (AIDS). Lastly, Spumaviruses are a group of complex retroviruses that infect multiple mammalian species including cats, cows, and several primate species. These retroviruses cause abnormal vacuolar cytopathology in tissue culture, but none have been shown to lead to overt disease in their native hosts.

Conserved Retroviral Genetics

Retroviruses of different genera have differently organized genomic structures, yet all encode a set of four essential gene products: Gag, Pro, Pol, and Env. These proteins perform the necessary structural, enzymatic, and membrane fusion functions essential for viral replication. The other feature common to all retroviruses is the presence in the proviral DNA of identical long terminal repeat (LTR) sequences flanking the coding region of the viral genome. These are formed from the unique 3’ (U3), repeat (R), and unique 5’ (U5) regions at the ends of the viral RNA (see below) during reverse transcription. The genome and RNA structures as well as coding regions for M-PMV are shown in Figure 2.

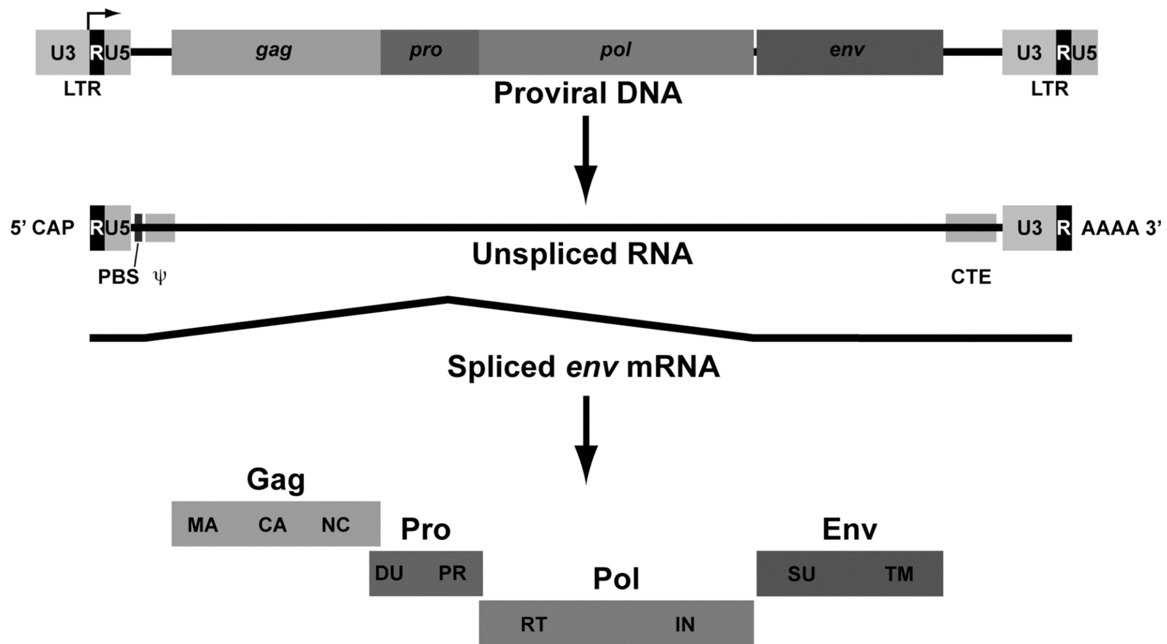

Figure 2. *Schematic representation of the Mason-Pfizer Monkey Virus (M-PMV) genomic RNA and coding structure.* Top: Proviral M-PMV genomic DNA. Middle: M-PMV RNA transcripts including the unspliced genomic/gag-pro-pol mRNA and the spliced env mRNA. Bottom: Proteins products of M-PMV with the depiction of their reading frame indicated by vertical offset. Gag: group-specific antigen; Pro: protease; Pol: polymerase; Env: envelope; MA: matrix; CA: capsid; NC: nucleocapsid; DU: dUTPase; PR: protease; RT: reverse transcriptase; IN: integrase; SU: surface subunit of Env; TM: transmembrane subunit of Env; LTR: long terminal repeat; U5; unique 5' sequence; R: repeat sequence; U3: unique 3' sequence; PBS: primer binding site; ψ: encapsidation signal; CTE: constitutive transport element.

LTR

The length of viral LTR sequences varies greatly between viruses, yet all provide transcriptional regulation, polyadenylation signals, and interacting sequences for the viral integrase. Each LTR can be broken into three segments based on their presence in the viral RNA. The U5 region is present only in the 5' end of the viral RNA, the R region is present in both the 5' and 3' ends of the viral RNA, while the U3 region is only present in the 3' end of the viral RNA (Fig. 2). As might be expected based on their locations in the RNA state, transcription initiates at the junction of U3 and R, while polyadenylation occurs at the junction of R and U5. The U3 region contains the majority of the transcriptional regulatory sequences and is where the

complement of host, and sometimes viral, transcription factors assemble. The factors controlling viral transcription differ for each retrovirus, and often between isolates of the same virus. Due to alterations in transcription factor binding, small sequence differences in the viral LTR between isolates can lead to dramatic alterations in viral pathogenicity.

gag

The *gag* gene encodes a polyprotein that forms the structural foundation of retroviral virions. While infectious viral particles require the other genes to be present, in the absence of any other viral proteins Gag molecules can catalyze the assembly of enveloped particles lacking both viral RNA and Env, called virus-like particles (VLPs). In most retroviruses, Gag molecules are produced from an unspliced RNA transcript and once produced, Gag molecules are trafficked to sites of viral assembly and homomultimerize to form thick-walled spherical protein shells of the immature virion. During assembly, viral genomic RNA is specifically packaged into the Gag shells. The specificity of viral RNA packaging being controlled by interactions between the Gag proteins and specific sequences in the viral RNA, called the encapsidation signal sequence or ψ. Following assembly, the virus particle buds through the plasma membrane where it acquires an envelope and associated Env proteins. Upon release, the viral protease becomes activated and cleaves the immature viral proteins, a process termed maturation. During this, the Gag polyprotein is cleaved into the major structural proteins of the infectious virion: matrix (MA), capsid (CA), and nucleocapsid (NC). A schematic representation of a mature retroviral virion is shown in Figure 3. As seen in this schematic, MA forms a spheroid outer-most shell of the virion located immediately underneath the lipid bylayer. CA forms an inner proteins shell, called the viral core. NC tightly associates with the viral RNA, but viral NC/RNA complexes do not form distinct macromolecular structures visible by electron microscopy. In Figure 3, the viral CA core is shown in a stylized pseudo-icosahedral form. In actuality, retroviral CA proteins assemble into a diverse array of distinct higher-order structures including centrally located round or partially angular cores (MLV and ALV), bar shaped cores (M-PMV), and conical cores (HIV-1) [589].

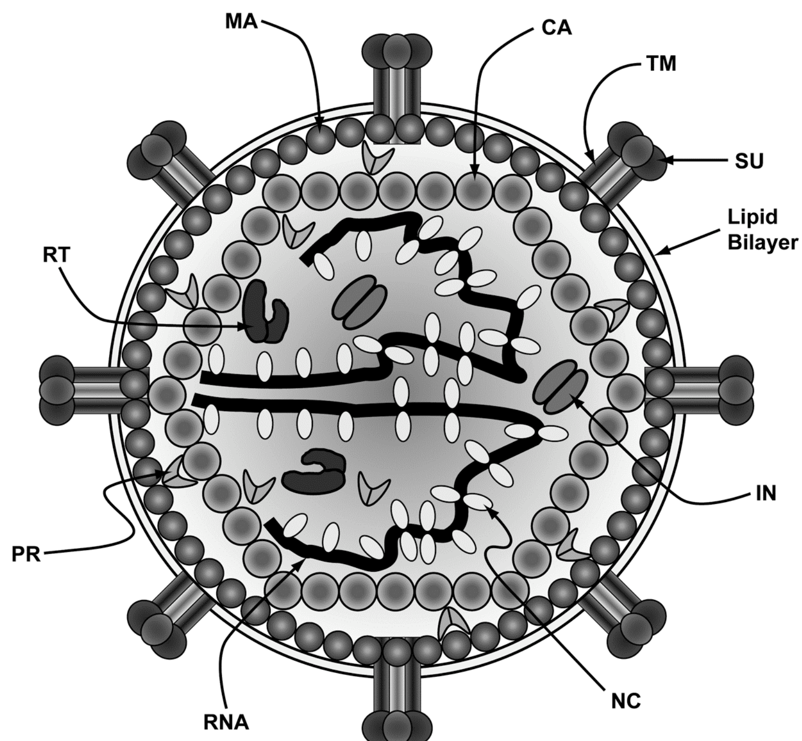

Figure 3. *Schematic of a mature particle from a hypothetical retrovirus.* MA, matrix; CA, capsid; NC, nucleocapsid; RT, reverse transcriptase; IN, integrase; PR, protease; SU, surface sub-unit; TM, transmembrane sub-unit.

Remarkably, unusual CA structures such as multiple CA cores within a single virion and multilayered cores have also been observed [35, 68, 69, 81]. Although, such peculiar CA structures may be artifacts of viral protein over-expression during viral production.

pro

In all retroviral genomes, the gene found adjacent to *gag* is the viral protease, *pro*, which is usually produced as a fusion protein in conjunction with Gag. While the location of this gene is conserved throughout the *retroviridae*, individual genera utilize different strategies for the production of Pro. Alpharetroviruses encode *pro* in-frame with *gag* such that Pro is produced with every Gag molecule produced. Betaretroviruses, such as M-PMV, and Deltaretroviruses encode *pro* in the -1 reading frame in relation to *gag* (Fig. 2) and production of Gag-Pro is achieved by -1 frame-shifting of the translating ribosome. Furthermore, in Betaretroviruses, *pro* is encoded separately from *pol*, with *pol* being in the -2 reading frame as compared to *gag*. This organization results in approximately 10-15% Gag-Pro and 1-2% Gag-Pro-Pol being produced as

compared to Gag [248]. Gammaretroviruses and Epsilonretroviruses encode *pro* (and *pol*) in frame with *gag* and utilize translational read-through of the *gag* stop codon to produce the Pro and Pol. Lentiviruses utilize a similar -1 frame-shifting mechanism as Betaretroviruses. However, instead of coding Pro and Pol separately as Betaretroviruses do, Lentiviruses encode Pro and Pol together in the -1 reading frame compared to *gag*. In Spumaviruses pro and pol are found in the +1 reading frame compared to gag. As opposed to other retroviruses, that produce Pro and Pol as a fusion protein with Gag, members of the Spumavirus genus produce a Pro-Pol protein independent of Gag from a spliced messenger RNA (mRNA). Irrespective of how a virus produces Pro, it is packaged into the assembling virus and upon viral release its activity is induced leading to cleavage of itself and other viral proteins during viral maturation. Loss of function of this protein results in accumulation of extracellular immature virions that are non-infectious.

<u>*pol*</u>

The *pol* gene of all retroviruses encodes at least three proteins, reverse transcriptase (RT), Ribonuclease H (RNase H), and integrase (IN). These proteins provide the enzymatic activities responsible for generation of viral genomic DNA as well as the subsequent integration of this DNA into the host chromosome. RT is responsible for generation of the viral genomic DNA using the packaged viral RNA as a starting template. RNase H catalyzes the degradation of the viral genomic RNA in concert with synthesis of the genomic DNA. IN catalyzes the double stranded cleavage of host chromosomal DNA and ligation of the viral genomic DNA into the subsequent DNA break. The series of events leading to the generation of proviral DNA from viral genomic RNA will be discussed in more detail below. As suggested above, all retroviruses encode the *pol* gene separately from *gag*, with most being translated via either ribosomal slippage or ribosomal readthrough of a stop codon. This coding strategy results in a relatively low abundance of Pol in the form of Gag-Pro-Pol molecules that are incorporated into virions via Gag-Gag interactions between Gag-only molecules and Gag-Pro-Pol molecules. The exception to

the Gag-Pro-Pol strategy of Pol production is found in Spumaviruses, members of which utilize RNA splicing to generate Pol and rely upon interactions between Gag and the Pro-Pol molecules [308] as well as interactions between Pol and viral RNA [222] for efficient incorporation into assembling virions.

env

Retroviral *env* genes encode type I transmembrane proteins responsible for receptor-mediated fusion of the viral and cellular membranes. All known retroviruses produce Env from a spliced mRNA (Fig. 2). Translation of these proteins occurs on the endoplasmic reticulum (ER) generating a relatively long N-terminal domain inside the ER lumen, a single transmembrane region, and, for most retroviruses, a relatively short cytoplasmic C-terminal domain. After translation, Env proteins are trafficked through the ER and Golgi apparatus where they are glycosylated and cleaved into two subunits, the surface subunit (SU) and transmembrane subunit (TM), which are typically non-covalently bound. This cleavage process activates the fusion potential of the protein. Following cleavage activation, Env proteins are specifically incorporated into budding virions. Many retroviruses, such as M-PMV, have sequences in the cytoplasmic tail that inhibit the receptor binding and membrane fusion potential of their Env proteins. In viruses that have such a cytoplasmic tail, the inhibitory sequences are cleaved off during viral maturation by the viral protease, resulting in the activation of their full fusion potential [70, 459].

Retroviral Accessory Genes

In addition to the genes common to all retroviruses discussed above, many retroviruses encode genes that play crucial roles during replication of that virus or assist in the evasion of the host's immune system.

sag

Mouse mammary tumor virus (MMTV), a Betaretrovirus, encodes a well-characterized accessory gene in the U3 region of the 3' LTR known as the superantigen (*sag*) [292]. MMTV is transferred to susceptible offspring via maternal milk and following transfer the virus is able to infect B-cells

in the gut-associated lymphoid tissue (GALT) [273]. In the GALT, Sag is presented in conjunction with the major histocompatibility complex (MHC) class II protein and together they stimulate T-cells to proliferate and release cytokines. The production of which generates an expanded pool of target lymphoid cells capable of trafficking to the mammary tissue and seeding it with virus [150, 180, 181, 224, 225, 273]. Demonstrating the importance of this accessory gene, mice infected with viruses lacking Sag are unable to transmit MMTV to their offspring [616].

nef

Lentiviruses encode an accessory protein originally referred to as the "negative factor" (Nef) that in fact provides several important functions, the loss of which results in reduced viral pathogenicity [121, 136, 233]. One function Nef serves is to down regulate the cell surface expression of several crucial immunomodulatory proteins including CD4 [170], CD8 [539], MHC type I [500] and type II [487], and the co-stimulatory molecule CD28 [549]. HIV-1 Nef has also been shown to alter cellular signal transduction pathways including the activation of p21 activated kinase 2 (PAK-2). PAK-2 activation can lead to activation of an infected T-cell [456] and modulation of the CD40L pathway [550]. These changes result in the induction of chemokines and other factors that can recruit resting T-cells and prime them for HIV infection [488]. Nef has also been shown to get packaged into HIV virions and enhance viral infectivity, likely by inducing the reorganization of the cortical actin network in target cells [84, 103].

vpr and *vpx*

HIV-1 encodes the viral protein R (*vpr*) accessory gene, which has been shown to possess a variety of viral and cellular functions. HIV-1 and other Lentiviruses have the ability to infect non-dividing cells and Vpr has been shown to facilitate this by assisting in nuclear import of the pre-integration complex (PIC) following reverse transcription [113, 223]. In addition to this, Vpr may indirectly regulate viral transcription. Vpr has been shown to induce G_2 arrest in cells by induction of double stranded breaks in cellular DNA [512] or non-genotoxic activation of the ataxia telangiectasia and Rad3-related protein (ATR), a sensor of replication stress [472].

Transcription from the HIV-1 LTR is upregulated in cells arrested at the G_2/M checkpoint and this can result in enhanced viral particle production [178, 237, 658]. Furthermore, Vpr is a potent inducer of cell death by apoptosis, which is likely the result of prolonged G2/M arrest [10, 250, 644]. It is possible that Vpr could induce apoptosis in a more direct manner as it has been demonstrated that Vpr can induce permeabilization in purified mitochondria, a key step in apoptosis [249, 584].

An ancestral duplication event has resulted in human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus from sooty mangabeys (SIVsm) encoding two *vpr* paralogs, *vpr* and viral protein x (*vpx*) [506, 569]. Together, these two genes have been shown to play largely similar roles in the viral lifecycle of SIVsm as *vpr* alone does in the HIV-1 viral lifecycle [155, 161, 188, 200, 435, 449, 504, 532, 643].

vpu

Viral protein unique (Vpu) is an accessory protein encoded only by HIV-1 and some lineages of SIV [Reviewed in 350]. This protein has been shown to exist as homo-oligomeric type 1 membrane protein. The Vpu from HIV-1 has been shown to possess two main functions: controlling the trafficking and proteasomal degradation of CD4 from the ER [48, 358], and enhancing the release of virus from the cell surface by inhibiting the retention function of the cellular factor bone marrow stromal antigen 2 (Bst2), also known as Tetherin [285, 410, 540, 556]. Vpu mediates the down regulation of CD4 expression by directly interacting with CD4 molecules in the ER and targeting them for ubiquitin-mediated proteasomal degradation [48, 494]. CD4 is targeted for destruction via direct interactions between a specific recognition motif in Vpu and β-transducin repeat containing protein (β-TRCP), a component of an E3 ubiquitin ligase complex [60, 358]. Vpu exerts its anti-tetherin activity by blocking this protein from becoming incorporated into the viral membrane during budding. This function will be discussed in greater detail below. Vpu can also form ion channels in membranes [496] and this function

may enhance the effectiveness of viral release [143, 495]. However, the significance of ion channel formation on HIV-1 pathogenesis or lifecycle is not fully understood.

rex and *rev*

Often divergent viruses encode unique accessory proteins that serve similar roles in the viral lifecycle. The accessory genes 'regulator of viral expression' (*rev*) and 'regulator of expression from x' (*rex*), from Lentiviruses and Deltaretroviruses respectively, use identical strategies to perform similar RNA export functions. All retroviruses need to export unspliced viral mRNA so that it can be packaged as genomic RNA into assembling virions. Simple retroviruses, such as M-PMV, only produce unspliced or singly spliced *env* mRNAs (Fig. 2) and these retroviruses typically utilize RNA-based mechanisms to exploit cellular export mechanisms to get the unspliced viral RNA into the cytoplasm [66]. Complex retroviruses often produce a variety of different singly and multiply spliced mRNAs. Instead of utilizing RNA sequences for export of unspliced RNA, such viruses often encode accessory proteins to serve this function. In Lentiviruses and Deltaretroviruses, *rev* and *rex* encode viral proteins that interact with cis-acting sequences in the viral RNA and mediate the export of unspliced and singly spliced viral RNA from the nucleus [146, 228, 244, 298, 347, 520, 562].

tat, *bel1* (also known as *taf* or *tas*), *tax*

Different retroviruses can also encode unique accessory genes that serve analogous functions, but utilize different mechanisms in doing so. A good example of this is the transcriptional activation function performed by the 'trans-activator of transcription' (Tat), Bel1 (also refered to as Taf or Tas), and 'trans-activator from x' (Tax) proteins from Lentiviruses, Spumaviruses, and Deltaretroviruses, respectively. Tat stimulates lentiviral transcription through its ability to bind the secondary and tertiary RNA structures of the trans-activation response (TAR) element. These structures are present in short initiated, but paused, RNA transcripts. Upon binding this element, Tat recruits positive acting factors that act to modify inhibitory molecules as

well as RNA Polymerase II. Combined, modification of these proteins results in the relief of the block to transcriptional elongation [467].

The Bel1 accessory protein in Spumaviruses is a DNA binding protein that binds to the viral promoters and, in conjunction with cellular cofactors, stimulates transcription from the viral promoters [22, 220]. In contrast to Tat or Bel1, Tax is not a binding protein for either DNA or RNA, it enhances viral transcription by recruiting and modulating the activity of the cellular transcription factors that control expression from the viral promoters (both LTR and internal) [274].

vif, bet, dUTPase

In at least one instance, retroviruses have acquired multiple mechanisms in order to achieve a universal objective: protecting against the presence of uracil in their DNA genomes. DNA acquires uracil by two distinct mechanisms misincorporating dUTP instead of dTTP during DNA synthesis or deamination of cytosines. The presence of dUTP instead of dTTP, regardless of mechanism leading to its presence, is an undesirable situation for the virus. However, only the presence of dUTP resulting from cytosine deamination results in viral mutagenesis. Retroviral reverse transcriptases, like most other DNA polymerases, are unable to discriminate dUTP and dTTP. This leads to to incorporation of dUTP during DNA synthesis and results in A–U mismatched pairs. This type of mismatch is recognized by cellular DNA repair enzymes during DNA synthesis and the DNA strand is repaired to the proper A–T pairing. Thus, this type of mismatch is not in and of itself mutagenic. However, presence of A–U mismatches can enhance genomic instability [138, 163] and alter gene expression by preventing binding of transcription factors to their target sites [152, 583].

Most actively dividing cells have relatively low cellular concentrations of dUTP [568]. However, some cell types targeted by retroviruses have elevated levels of dUTP, this includes quiescent T-cells and primary macrophages [15, 118, 235]. It appears that retroviruses have acquired the deoxyuridine triphosphatase (dUTPase) gene on three independent occasions, in the

ERV-L lineage of endogenous retroviruses [34, 115], in non-primate Lentiviruses [139, 372, 376], and in Betaretroviruses [39]. In each of these instances dUTPase is expressed as a fusion protein with Gag and is thus packaged into virions where it is proximally located to RT and the viral RNA. The lentiviral dUTPase has been studied in some detail and it has been found that dUTPase is not required for viral replication in actively dividing cells, but is necessary for infectivity in non-dividing natural target cells such as macrophages [299, 535, 559, 574, 593]. HIV-1 infects non-dividing cells, but does not encode a viral dUTPase. Instead, HIV-1 has evolved a mechanism for packaging a cellular uracil-DNA glycosylase (UNG2) potentially through interactions with viral IN [454, 605]. The presence of this packaged UNG2 is required for HIV-1 infectivity in non-dividing cells [453].

In addition to the mechanisms described above, HIV-1 and other primate Lentiviruses have devised a separate strategy for preventing uracil accumulation during reverse transcription, which occurs as a result of cytosine deamination. Mammalian hosts of these viruses express specific cellular apolipoprotein B mRNA editing enzyme (APOBEC) proteins that get incorporated into retroviral virions [507, 593, 604] and cause cytosine deamination of DNA reverse transcriptase products [212, 307, 352, 652]. Cytosine deamination of reverse transcription products can result in decreased infectivity as a result of lethal and sub-lethal mutagenesis [212, 307, 352, 652]. Additionally, it appears that cellular factors recognize deaminated RNA resulting in active degradation by an unknown mechanism [352, 359]. HIV-1 viral infectivity factor (Vif) has been shown to protect from the effects of APOBEC mediated inhibition of viral replication by directly inhibiting its deaminase activity [492], excluding APOBEC from being packaged into virions [359], and depleting APOBEC from infected cells either through proteasomal degradation of APOBEC proteins [100, 183, 379] or inhibition of APOBEC translation [272, 382, 538]. Similarly to Vif, the Bet accessory protein of feline foamy virus (FFV) has been shown to protect FFV from APOBEC's antiviral activity, likely by excluding APOBEC from virions [337].

Retroviral Lifecycle

The lifecycle of retroviruses is a wholly unique and elegantly complex process. An overview of the steps in the retroviral lifecycle is depicted in Figure 4 and proceeds as follows:

i. Binding of the viral Env to the cellular receptor and subsequent fusion of the viral and cellular membranes
ii. Internalization, transport, and uncoating of the viral core
iii. Reverse transcription of the single stranded viral RNA genome into double stranded DNA
iv. Access of the viral genomic DNA to the nucleus/host chromatin
v. Integration of the viral genome into the host chromosomal DNA
vi. Transcription of the viral genome
vii. Splicing and nuclear export of viral RNA
viii. Translation and post-translational processing of viral genes
ix. Assembly of progeny and viral RNA packaging into virions
x. Budding and release of virions
xi. Virion maturation through proteolytic processing of viral proteins

Entry

The retroviral lifecycle begins when the SU domain of a mature extracellular retroviral particle binds to its cognate receptor, displayed on the surface of a cell. Retroviruses of different lineages utilize distinct cellular receptors. For instance, the cellular receptor for M-PMV and other simian retroviruses (SRV) in the Betaretrovirus genus has been determined to be the RD114 receptor (RDR) a neutral amino acid transporter with multiple membrane spanning domains [457, 522]. HIV-1, however, utilizes CD4 as its primary receptor and either CXCR4 or CCR5 as a co-receptor [137, 147, 171, 516]. Upon binding of the SU domain to its receptor, the TM domain

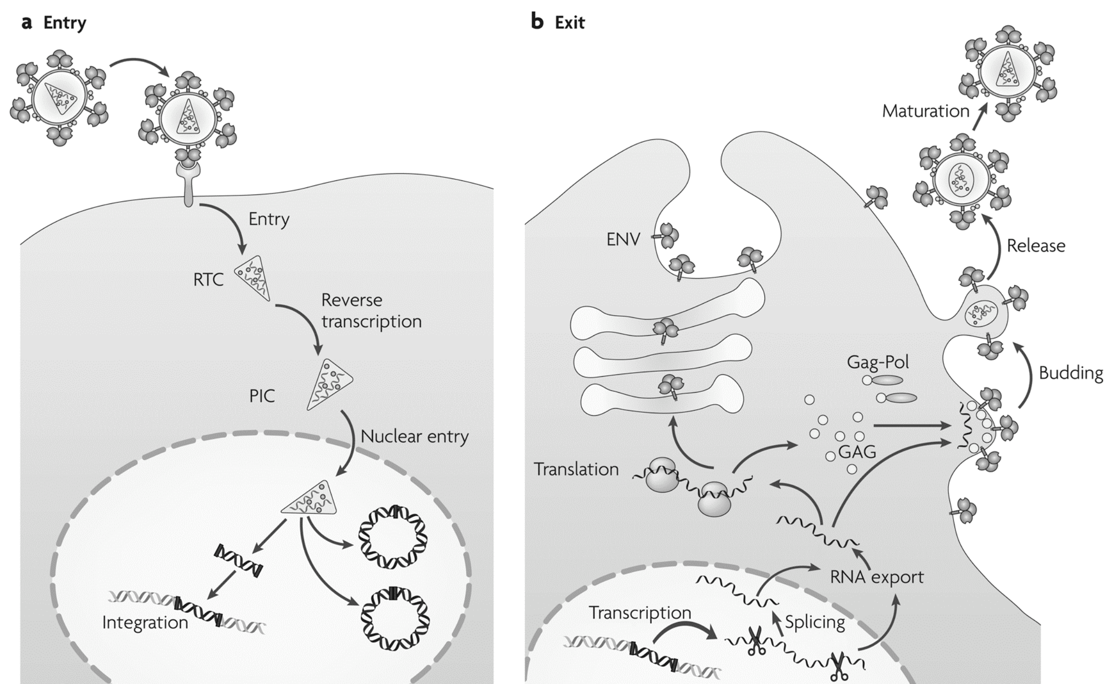

Figure 4. *Lifecycle of a typical retrovirus.* Panel A shows the processes from the point of entry through integration. Panel B depicts processes from transcription to viral release and maturation. RTC, reverse transcriptase complex; PIC, pre-integration complex; Gag: group-specific antigen; Gag-Pol: Gag-polymerase poly-protein; Env: envelope.

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undergoes a series of conformational changes that ultimately results in fusion of the viral membrane with the cellular membrane. For most retroviruses, these steps have been shown to be independent of a change in pH and thus fusion events are likely to occur at the cell surface [362, 373, 536]. Membrane fusion results in the release of the viral RNA-containing CA core into the cytoplasm of the cell.

Uncoating

The sequence of events that occur following viral entry into the cell are among the poorest understood of any during the viral lifecycle and much of this knowledge is derived from studies on HIV-1 or MLV. The lack of understanding of post-entry events is generally due to the

fact that abortive infection during post-entry steps is the norm. Particles destined for integration are thus a comparatively rare product and difficult to specifically assay.

It is known that for most retroviruses, reverse transcription does not begin until after entry and proceeds concomitantly with uncoating and trafficking. This reaction takes place in a large, 100nm wide and 400 to 700nm long, macromolecular complex called the reverse transcription complex (RTC) that includes NC, RT, and viral RNA, at the very minimum [62]. Viral RTCs also likely contain CA, IN, and Nef, Vif, and Vpr, in the case of HIV-1. CA mutations that affect the stability of the viral cores result in infections that abort at various times during reverse transcription [158]. IN has been implicated in diverse functions during this stage of the viral lifecycle indicative of inclusion in the RTC, including uncoating [306, 368, 402], reverse transcription [129, 141, 341, 368, 573, 619], and nuclear import of viral genomic DNA [165, 243, 573]. Nef deleted virions have been found to abort infection prior to completion of reverse transcription [3, 102, 499] and that inclusion of Nef increases infectivity several-fold [103, 388, 531]. Similarly, Vif appears to protect from the cytosine deaminase activity of APOBEC during reverse transcription [272, 507, 508]. Finally, Vpr has been shown to assist in nuclear import of HIV-1 following reverse transcription [113, 223]. Owing to the fact that this complex is many times larger than the CA cores found in a virion and the activity of RNase H would prevent significant accumulation of nucleic acid content, it is highly likely that many host cell proteins are included in this complex. Indeed many cellular proteins have either been identified as associating with RTC complexes of HIV-1 or have been found to be important in the reverse transcription process [65, 205, 290, 313, 314, 657].

In order for efficient viral infectivity, the RTC needs to properly traffic from the cytoplasmic membrane to the nucleus [73, 119]. The cortical actin network must be traversed by an incoming virus and thus likely represents a significant barrier to successful infection. Accordingly, it has been shown that disruption of this network is an important step for a virus to achieve [84, 637]. Additional indirect evidence of the importance of traversing the cortical actin

network comes from experiments pseudotyping HIV-1 with the vesicular stomatitis virus glycoprotein (VSV-G) instead of the native Env. The VSV-G protein mediates entry into the cell in a pH-dependent manner from endosomes, and thus bypasses the cortical actin network. Pseudotyping virions with the VSV-G envelope overcomes early blocks to infection seen in viruses either containing specific Gag mutations or lacking Nef [2, 72, 93, 342]. At points beyond the cortical actin network, experiments using fluorescent microscopy have shown that the trafficking of the RTC is dependent upon both actin and microtubules [16, 374] and that the RTC can be attached to microtubules via stalk-like projections [374]. During the concurrent processes of traversing the cytoplasm and undergoing reverse transcription, the RTC undergoes a poorly understood process of 'uncoating' where the CA cores dissociate from the protein–nucleic acid complexes. As alluded to above, the initial steps of infection have been found to be a rather inefficient process, with only a fraction of viral particles completing reverse transcription [245, 558, 599].

Reverse transcription

The process of creating a final double stranded viral genomic DNA copy from the starting viral RNA is a complex process requiring multiple distinctly ordered steps. This process results in the duplication of the U3 and U5 regions of the viral LTR. The most important stages during the reverse transcription process are depicted in Figure 5. Reverse transcription is initiated from the free 3'-OH of a transfer RNA (tRNA) primer. This tRNA binds to a primer-binding site (PBS) immediately adjacent to the 5' LTR. From this point, reverse transcription proceeds through the R region in the 5' end of the viral RNA, creating a hybrid RNA-DNA molecule known as the minus-strand strong-stop DNA. In concert with the synthesis of DNA, the activity of the RNase H domain of RT cleaves the template RNA into short oligos.

For reverse transcription to proceed, the strong-stop DNA must anneal to the 3' end of the viral RNA, through the complimentarity of the R region of the LTRs. The strong-stop DNA

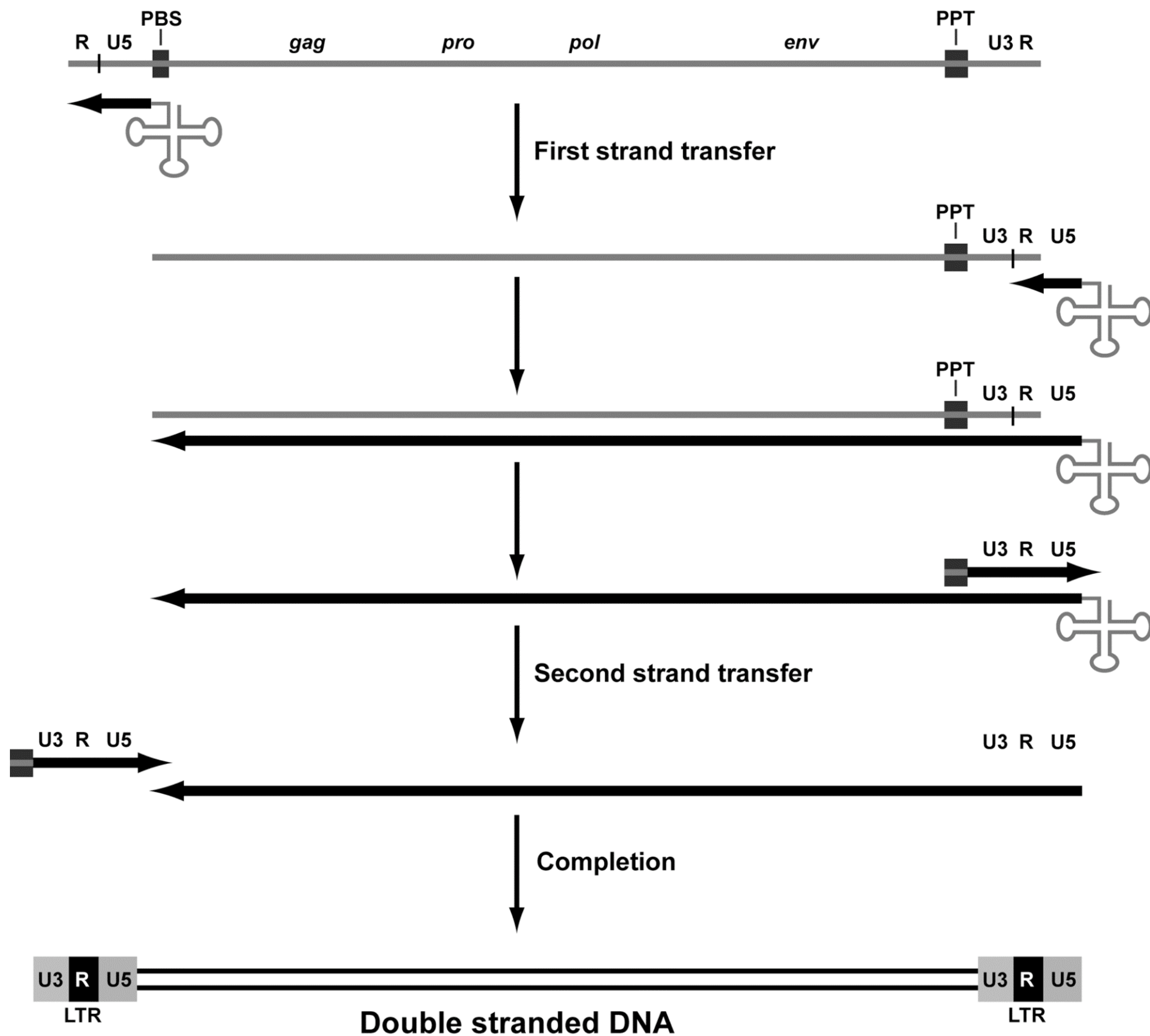

Figure 5. *Major steps during reverse transcription.* Gray lines represent RNA, Black lines represent DNA. LTR: long terminal repeat; U5; unique 5' sequence; R: repeat sequence; U3: unique 3' sequence; PBS: primer binding site; PPT: polypurine tract; Gag: group-specific antigen; Pro: protease; Pol: polymerase; Env: envelope.

acts as a primer for the initiation of reverse transcription of the long minus-strand DNA, which terminates at the 3' end of the PBS. The primer for synthesis of the positive strand DNA is produced as a result of inhibition of the RNase H activity of the RT. The polypurine tract (PPT) is resistant to the RNase activity, resulting in a short RNA oligo remaining bound to the long minus-strand DNA. The free 3'-OH of the PPT RNA serves as an initiation point for positive strand synthesis, which proceeds into the tRNA primer. The short positive strand then transfers to the 5'

end of the genome through the binding of a DNA extension, derived from the tRNA primer and complimentary to the PBS sequence in the minus-strand. Positive strand synthesis is then able to proceed to completion followed by completion of the minus-strand DNA, including removal of the PPT RNA [174].

Nuclear Import

Before integration can take place, it is absolutely necessary for the viral DNA containing PIC to gain access to the cellular DNA. However, the process by which this occurs is poorly understood. Simple retroviruses likely lack a mechanism to directly gain access to the nucleus. These viruses appear to have strict mitotic requirements in order for integration to occur [318, 385, 469, 581]. In scenarios where simple retroviruses enter into a non-dividing cell, creation of linear double-stranded DNA occurs, but this accumulates in the cytoplasm. The RT products remain in the cells and infection can be rescued if the cells undergo mitosis prior to their destruction. However, for the simple retroviruses where this has been assessed, viral DNA has a relatively short half-life in the cytoplasm [12, 385].

In contrast to the simple retroviruses, it has been shown that Lentiviruses and Spumaviruses are capable of infecting non-dividing cells [75, 317, 477, 601]. As the nuclear envelope of cells only breaks down during mitosis, infection of non-dividing cells necessitates an active mechanism for crossing the nuclear membrane. It was originally believed that the ability to cross the nuclear membrane was an additional feature of Lentiviruses and that these viruses retained the ancestral ability to integrate during mitosis [626]. However, several studies have shown that HIV-1 requires that the PIC passes through the nuclear envelope for integration in both dividing and non-dividing cells [245, 275, 468].

Multiple viral proteins and nucleic acid structures have been implicated in the nuclear importation of HIV-1 PICs. The first identified factor was the viral MA protein, which contains two nuclear localization signals (NLS) [74, 134, 203, 401, 591]. This protein is largely thought to dissociate from the CA cores upon entry, but a fraction is found associated with the viral DNA in

PIC preparations [76, 387]. A satisfying mechanism for MA-mediated nuclear import has yet to be elucidated and it has been shown that viruses containing mutations in all sequences implicated in nuclear import are still capable of successful replication, although the efficiency was much reduced [159, 458]. Thus, it is likely that MA is one of multiple factors required for efficient nuclear import, but is a non-essential component of this process. Vpr has also been implicated in nuclear importation of HIV-1 PICs. Several reports have shown that Vpr enhances HIV-1 replication, most significantly in macrophages, and that this enhancement is likely due to the enhancement of nuclear import of the PIC [113, 223, 416]. In contrast to MA, a clear mechanism has been proposed for this activity as it has been demonstrated that Vpr can interact with importin α [420, 450, 588]. However, similar to MA, Vpr is not an absolute requirement for nuclear import as viruses lacking Vpr are competent for replication in macrophages, albeit at a reduced rate [223]. HIV-1 IN has also been shown to contain at least one NLS [61, 573] and has been implicated in facilitation of nuclear import [165]. Multiple mechanisms may be responsible for this activity, as IN has been shown to interact with importin α1 [18, 61, 165], importin α3 [13], importin 7 [14], as well as TNPO3/transportin-SR2 [104]. HIV-1 CA may also play a role in nuclear importation [296, 310, 625], and mutations in HIV-1 CA result in altered utilization of import pathways [310]. The role of CA in nuclear import is however difficult to tease out as such studies are likely obfuscated by perturbations in the reverse transcription and/or uncoating steps of infection.

Finally, the central DNA flap of HIV-1 has been shown to facilitate nuclear import [17]. This structure is formed as a result of the unique reverse transcription strategy of HIV-1, and related Lentiviruses, which use a second PPT located in the central portion of the viral genome. The central flap is proposed to facilitate uncoating of the PIC at the nuclear pore during the importation process [649]. Additional studies are clearly needed in order to better elucidate the contributions each of these factors have on nuclear importation.

Integration

The viral IN is responsible for catalyzing the process of inserting the viral genome into the cellular genome [437, 455, 501]. Retroviral integration results in a consistent product with the process always yielding an intact retroviral genome containing intact LTRs. The only loss of information, compared to the unintegrated viral DNA, is a removal of several (usually two) nucleotides at the very termini of the viral LTRs. In the host genome, there is a duplication of several nucleotides, which flank the retroviral genome post-integration. This precision is provided by an ability of IN to bind specifically to short imperfect inverted repeat sequences, termed attachment (*att*) sites, near the termini of the viral LTRs [79, 108, 111, 586]. These sites are largely conserved throughout retroviridae and many transposable elements, although small changes in nucleotide sequence can be tolerated [112]. Much of what we know about the biochemical process of integration comes from studies performed *in vitro* [71, 162]. Such studies have demonstrated that integration proceeds in a two-step process. First, nucleotides (usually two) are trimmed from the 3' ends of the blunt-ended double-stranded DNA reverse transcription product, resulting in 5' extensions at both termini of the viral DNA. The second step is a transfer of the viral DNA into the cellular genomic DNA. To do this, the freshly processed 3' hydroxyl groups are directed by IN to undergo a nucleophilic attack on the phosphodiester bonds of the cellular DNA. This results in ligation of the 3' ends of the viral DNA to the cellular DNA [162]. IN does not, however, join the 5' terminal extensions of the viral genome to the cellular DNA. IN activity results in a gap of a small number of nucleotides following these 5' termini and host DNA repair enzymes likely catalyze the repair of this gap.

Many studies have been performed characterizing the integration preferences for individual retroviruses. Human and primate Lentiviruses have a distinct preference for integration in actively transcribed genes [63, 106, 107, 117, 315, 316, 335, 391, 489, 595, 618]. An examination of a reconstituted human endogenous retrovirus K (HERV-K), a gammaretrovirus-like ERV, found a distinct preference for the integration in transcriptional units and gene rich

regions as well as in or near actively transcribed genes and their promoters [64]. MLV tends to integrate near the transcriptional start site of genes and near CpG islands, but not within long interspersed repeat (LINE) elements [4, 316, 391, 595, 618]. A study of foamy virus integration revealed a similar pattern to that of MLV, with a weak preference for integration near CpG islands and against integration within LINE elements. However, this virus also showed a preference against integration within genes and a distinct preference toward integration near previously existing integration sites [570]. ALV and HTLV integrate in a nearly random fashion, with only weak preferences for integration within genes [26, 123, 391, 404, 570]. The integration pattern of MMTV was observed to be even more random than that of ALV and HTLV, but with no detectable preference for integration within genes [145].

In the case of HIV-1, IN has been shown to associate with lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) and this interaction enhances the activity of IN, *in vitro* [80, 97-99, 211, 219, 336, 344, 377, 436, 575, 639]. In cells lacking LEDGF/p75 the integration target site preference of HIV-1 changes to a pattern more similar to that of MLV. In this instance, HIV-1 integration tends to occur around the transcriptional start sites of genes [106, 363, 513]. Thus, it is likely that the target site specificity observed for other retroviruses is a result of interactions between IN and unknown cellular proteins.

Transcription and RNA export

Upon integration into the host chromatin, the late stages of the viral lifecycle begin where retroviruses largely rely on normal cellular machinery in performing the steps required for production of progeny viruses. Production of viral RNA is the first step in the late stages of the viral lifecycle. As discussed above, transcription initiates at the U3/R junction of the 5' viral LTR and proceeds to the R/U5 junction of the 3' LTR. For most retroviruses, initiation of the RNA transcript is the major step in RNA production. The exception to this rule comes from Lentiviruses, which display a two-step transcriptional strategy with a near absolute requirement for the viral Tat protein in order for transcriptional elongation. As with most viral promoters, in

most circumstances retroviral LTR promoter activity is exceptionally robust. However, as retroviruses rely upon host cell factors for controlling transcription from their LTRs, their promoter activity can be greatly influenced by the cell type and physiological state of the host cell as well as the location of integration [6, 144].

Like most cellular promoters, retroviral LTR promoters contain a TATA box, a CCAAT box, or both. These DNA elements contain recognition sequences for transcription factor IID (TFIID) and CCAAT enhancer binding protein (CEBP), respectively. TFIID and CEBP are integral members of the multi-protein complex required for activation of the RNA polymerase II (RNAP II) and subsequent initiation of transcription. Along with the TATA and CCAAT boxes, retroviral LTRs contain enhancer sequences; regions with multiple closely spaced, often overlapping, transcription factor recognition sequences. LTR sequence as well as expression levels and activation state of the transcription factors present in the host cell govern the occupancy of the individual TF binding sites of the viral LTR. Distinct patterns of TF binding are observed in cells of different type or physiological state, and the binding of multiple transcription factors often does not yield an additive effect on transcription. Rather, a more complex interplay between factors is typically the result [174, 185, 189, 400, 463, 528].

The primary transcript produced from viral LTR transcription is utilized as both the viral genomic RNA as well as the source of information for production of the viral structural and catalytic proteins. Eukaryotic cells have redundant mechanisms in place for the specific export of spliced mRNA from the nucleus, while retaining unspliced RNA inside the nucleus [21, 58, 91, 157, 164, 206, 230, 426]. Yet, the primary transcript of all retroviruses contains at least one splice donor and one splice acceptor site [206]. Retroviruses have, therefore, developed strategies to circumvent the nuclear retention of unspliced genomic RNA. Lentiviruses accomplished this by encoding an accessory protein, Rev, with the primary function of facilitating the export of unspliced viral RNA [146]. Rev binds to a secondary/tertiary RNA structure in the viral RNA, the Rev-responsive element (RRE) [355, 646, 647]. It has been shown that this association actively

inhibits splicing of the RNA [91, 207, 284]. Rev contains a canonical nuclear export signal (NES) that provides the ability to shuttle in and out of the nucleus using the chromosome region maintenance 1 (CRM1) – Exportin1 pathway. Through its interaction with viral RNA, Rev exploits the CRM1/Exportin pathway for the export of unspliced viral RNA [151, 156, 281, 332, 349]. Unlike HIV-1, M-PMV does not encode an accessory protein to enhance the export of its unspliced viral RNA. Instead, M-PMV utilizes a short cis-acting RNA sequence, called the constitutive transport element (CTE), in bypassing the splicing requirement [66]. The M-PMV CTE provides this activity by mediating direct interactions with the Tip-associated protein (Tap; also known as nuclear RNA export factor 1 [NXF1]). Tap-mediated export of RNA is believed to be the main cellular export pathway for spliced mRNAs, and the interaction between the M-PMV CTE and Tap allows for the utilization of this pathway by the unspliced M-PMV RNA [194]. Similar RNA-based elements are presumed to exist in other simple retroviruses [282, 424, 571]. However, the binding factors and pathways utilized by these retroviruses have yet to be described.

Assembly and Release

Following export of the viral RNA from the nucleus, translation of the viral Gag, Gag-Pro-Pol, and Env, as well as any accessory genes takes place. As described above, retroviruses utilize divergent mechanisms for the production of the Gag-Pro, Gag-Pro-Pol or Gag-Pol precursor proteins. Once produced, Gag-containing proteins are able to auto-assemble into viral particles and Gag alone is sufficient for assembly of these VLPs. Viral assembly occurs at distinct locations within the host cell, the site of which can vary between different retroviruses. Most retroviruses, including MLV and HIV, undergo C-type assembly with formation of retroviral particles occurring at the cytoplasmic membrane. These viruses traffic Gag monomers or Gag oligomers to the plasma membrane where a region in the N-terminus (MA domain) of the Gag precursor protein is able to interact with the cellular membrane [260]. This domain can be composed of hydrophobic or basic residues, and may include a myristic acid modification of the

N-terminus [174, 226]. It has been demonstrated that the N-terminal domain of MA interacts with a phosphoinositide, $PI(4,5)P_2$, which is preferentially found in the plasma membrane as compared to other cellular membranes [105, 428, 475]. It appears the membrane interaction of these Gag molecules nucleates Gag-Gag assembly [259], following which assembly proceeds via intersubunit interactions [45, 369]. At a point after the initiation of the particle assembly, viral RNA associates with the growing virion through specific interactions between Gag and the ψ sequence in the viral RNA [262]. Some retroviruses, typically of the Betaretrovirus and Spumavirus genera, assemble viral particles inside of the cell and these intact, but non-enveloped, particles are trafficked to the plasma membrane for envelopment and release [465]. This assembly process is referred to as either B- or D-type assembly and M-PMV is the protypic virus using this mechanism of assembly. As with the C-type retroviruses, viruses that utilize B- or D-type assembly mechanisms RNA packaging occurs during Gag multimerization and is reliant upon interactions between the ψ sequence and Gag. Also similar to C-type viruses, interactions between the plasma membrane and the N-terminal domain of Gag, including a myristic acid moiety, are responsible for the envelopment of viral particles [533, 534]. Following completion of assembly and envelopment, the viral particle is pinched off from the cellular membrane and released into the extracellular space.

After release, the viral protease becomes activated and catalyzes viral maturation. During and after assembly, the Gag molecules form a hexameric lattice. It has recently been demonstrated that the spacer peptides between CA and NC (SP1) provide the crucial interactions forming the hexameric CA structures of immature HIV-1 virions. In the immature form, the C-terminal domains (CTD) of adjacent CA hexamers governs spacing and structure of the lattice. During maturation, the SP1 sequences are removed following protease cleavage of Gag. This results in the reorganization of the hexameric lattice, with newly formed contacts between CTDs forming the basis of CA hexamer interactions and contacts between the N-terminal domains

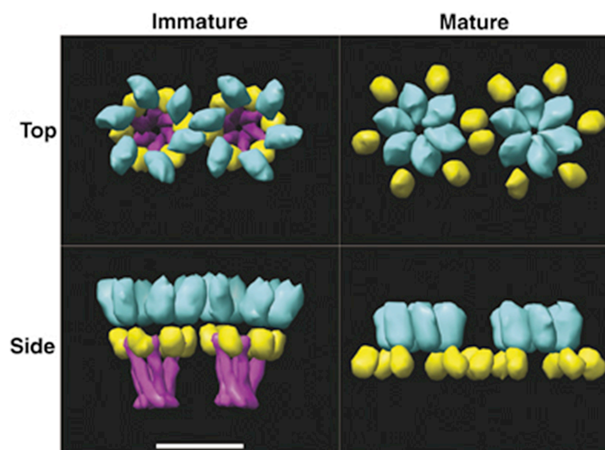

Figure 6. *Model for the hexameric structure of HIV-1 capsid domains in the immature and mature lattices.* Top and side views (top and bottom, respectively) of low-pass filtered atomic models of the immature (left) and mature (right) lattices (CA NTD in cyan, CA CTD in yellow, and SP1 in magenta). In the immature lattice, hexamers are held together from below by the SP1 bundle and spaced by CA CTD interactions. The CA NTD is not essential for assembly of the immature lattice. Maturation removes SP1 and causes formation of the CA N-terminal β-hairpin, which induces hexameric ring formation in that domain. Thus in the mature lattice, hexamers are held together instead from above, by CA NTD, but are again spaced by CA CTD interactions. Scale bar 8 nm.

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(NTDs) of CA responsible for forming the lattice structure [615]. A model of the CA interactions in the immature and mature particles proposed by E.R. Wright et al. is shown in Figure 6.

Restriction Factors and Evasion Mechanisms

In mammals, retroviral infection is a nearly universal phenomenon, with infection resulting in the permanent inclusion of the provirus in the genome of the host cell, be it either somatic or germ line. Infection of somatic cells typically results in the life-long infection of a host and often an accompanying viral induced pathology, while infection of the germ line can result in vertical transmission of the retrovirus to the offspring of a host. Due the negative outcomes associated with retroviral infection, these viruses can impose significant selective evolutionary pressures on affected host species.

The extended time of infection, failure of viral clearance, and vertical transmission of these viruses illustrate the general inability of both the innate and adaptive immune system in countering retroviral infection. As a means of resisting infection by retroviruses, mammalian hosts have evolved a series of germ-line encoded dominant proteins whose expression inhibits retroviral replication at specific stages of the retroviral lifecycle. Such proteins are called restriction factors and this general mechanism for resisting viral infection has been referred to as intrinsic immunity [47]. Restriction factors can be conserved across extended evolutionary lineages or unique to specific sub-populations within a species and can exert their inhibitory effects at virtually any stage of the retroviral lifecycle. Characterized restriction factors as well as dominant blocks to retroviral replication suspected to be mediated by restriction factors will be discussed below. Where known, viral evasion mechanisms employed to escape the effects of these factors will also be addressed.

Fv4

Some of the earliest studies examining genetic predisposition to retroviral infection were performed in mice using a Friend murine leukemia virus (Fr-MuLV) challenge. These studies identified a number of distinct blocks to Fr-MuLV infection [328]. Of these, restriction factors were later found to be responsible for two of blocks to Fr-MuLV infection, Friend virus susceptibility 4 (Fv4) and Friend virus susceptibility 1 (Fv1). Characterization of Fv4 revealed that this factor was able to block replication *in vitro* and *in vivo* [264, 423, 547], suggesting either the presence of an inhibitory factor or the lack of a required factor in the resistant mice. Later, endogenous expression of a MLV Env was correlated to susceptibility and a defective proviral genome was cloned from the resistant mice. This genome was found to lack *gag* and much of *pol*, but an intact *env* open reading frame remained, which contained a handful of nucleotide substitutions compared to functional MLV *env* genes [241]. Expression of the Env from this clone was found to interfere with the Fr-MuLV receptor, resulting in a block to viral entry [242,

265]. Transgenic mice expressing this factor were found to be resistant to Fr-MuLV infection, demonstrating that Fv4 is sufficient in mediating the block to Fr-MuLV replication [331].

Fv1

The other restriction factor found to control susceptibility to MLV infection, Fv1 [327, 447, 448], has been the subject of intensive long-term study [reviewed in 173]. This factor was found to be present as two alleles, $Fv1^n$ and $Fv1^b$, based on their identification in NIH/swiss and Balb/c, respectively. Three variants of MLV were identified based on their resistance to the different Fv1 alleles. N-tropic MLV was identified as a variant that could replicate in mice homozygous for the $Fv1^n$ allele, but was blocked in mice with the $Fv1^b$ allele. B-tropic MLV variants were identified based on the ability to replicate in the presence of the $Fv1^b$ allele, but whose replication was blocked by the $Fv1^n$ allele. Heterozygous $Fv1^n/Fv1^b$ mice were found to be resistant to both N- and B-tropic MLV viruses. Finally, an MLV variant was identified with resistance to both the $Fv1^n$ and $Fv1^b$ alleles, known as NB-tropic MLV.

Infection of MLV variants sensitive to Fv1 restriction was found to be blocked at an early stage of the viral lifecycle in resistant cells, regardless of the Fv1 allele expressed. Infection of sensitive variants proceeds through reverse transcription, generating full-length viral genomic DNA. However, DNA products indicative of nuclear import, either integrated proviral DNA or circular viral DNA, were not found in restrictive cells circular DNA [Reviewed in 173, and 175].

Genetic studies mapped the determinant of restriction to the CA protein of MLV [124], specifically to amino acid residue 110 [293]. Further, it was demonstrated that the restrictive phenotype could be overcome by pretreatment with excessive levels of a similarly sensitive virus. However, only CA in the context of infecting virions was found capable of abrogating the block imposed by Fv1. Expression of either the Gag precursor or CA molecules of sensitive viruses in the target cells did not relieve the block [28, 135]. This is suggestive of a conformational dependent presentation of the Fv1 CA determinant. Upon cloning, it was found that Fv1 encodes a Gag-like molecule with similarity to the HERV-L and MuERV-L family of endogenous

retroviruses [34, 43]. A detailed mechanism of how this restriction factor exerts its influence is currently unknown. However, it is likely that Fv1 restriction follows a similar mechanism as TRIM5α when the proteasomal pathway is blocked (discussed below).

TRIM5

A block to MLV infection similar to that observed for Fv1 was also recognized to exist in human cells. In these, the infection of N-tropic MLV was restricted, while both B- and NB-tropic MLV were found to be insensitive to this block. This restriction observed for MLV in human cells was given the name Ref1, for restriction factor 1. Identical to Fv1, the viral determinant for Ref1 was mapped to amino acid 110 of the CA protein [567]. However, the block to N-MLV in these cells was observed to occur at an earlier post-entry stage than Fv1. Negative-strand strong-stop DNA products could be detected shortly after infection, but little accumulation of full-length RT products was found to occur [42, 567]. A similar post-entry block was identified for different lentiviruses in primate cells of non-native species. HIV-1 infection was found to be inhibited in cells from Old World monkeys and owl monkeys, but not other New World monkey species [41, 116, 231, 398, 511]. In contrast, macaque SIV (SIVmac) was found to be blocked in New World monkey cells, but not in cells from Old world monkeys [41, 116]. As with the Ref1 block to MLV, it was found that the blocks to lentiviral infection resulted in abortive infection characterized by little accumulation of full-length DNA [231, 232, 511]. This block to lentiviral infection in simian cells came to be known as Lentivirus susceptibility factor 1 (Lv1). The viral determinants of this restriction were localized to the CA protein, although unlike MLV multiple amino acid residues were found to modulate the sensitivity of HIV-1 to Lv1 restriction [116, 216, 433].

The gene responsible for the Lv1 block to HIV-1 in Old World primates was identified as TRIM5α [541], a member of the tripartite motif (TRIM) family of genes. The unifying feature of this family of genes is the presence of three protein domains in tandem: an N-terminal really interesting new gene (RING) domain, followed by at least one B-box domain, and a coiled-coil

domain [464]. TRIM5α was quickly thereafter determined to also be responsible for the Ref1 restriction of MLV in human cells [217, 278, 444, 633]. A novel fusion protein between TRIM5 and cyclophilin A (CypA), TRIM-Cyp, was identified as being responsible for the block to HIV-1 in owl monkey cells [418, 484]. This protein fuses the tripartite motif portion of TRIM5 to the known HIV-1 CA binding protein CypA. Consistent with previous studies of Lv1 and Ref1 restriction, the early studies of TRIM5-mediated viral restriction identified CA as the viral target for TRIM5 [484, 541]. Biochemical studies of this interaction suggest that functional interaction between TRIM5α and restriction sensitive virus results in the acceleration of uncoating and/or destruction of CA protein. [92, 542]. Endogenously, TRIM5α may utilize a proteasome-based mechanism for achieving viral restriction [11]. However, inhibition of proteasomal activity does not releave the block to viral infection. Instead, the timing of viral inhibition changes to a point following reverse trancrition but prior to nucear import [617]. Together these findings suggest that strong interaction between TRIM5α and sensitive viral CA cores alone leads to a in the loss of viral infectivity, and proteasomal activity accelerates this process. A more detailed discussion of TRIM5α, its antiviral protential, and evolutionary history can be found below.

Macrophage late post-entry restriction factor

It has been recognized for some time that Vpx is required for the efficient infection of myeloid-derived cells (macrophages, monocytes, and monocyte derived dendritic cells) by HIV-2 and related strains of SIV [172, 187, 188, 277, 346, 354, 481, 577, 613]. Recently, the block to lentiviral infection in these cells was found to occur at a very early post-entry stage, displaying reduced production of negative-sense strong-stop DNA products in the absence of Vpx [37, 161, 188, 276, 532]. The lentiviral restriction in these cells was determined to be a dominant phenotype, as heterokaryons of macrophages and permissive COS cells maintained the restrictive phenotype [504].

Vpx from HIV-2 and SIV have been shown to interact with the Cullin4 (Cul4)/DNA

damage binding protein 1 (DDB1) ubiquitin ligase complex via direct interactions with DDB1 and Cul4 associated factor 1 (DCAF1) [37, 236, 504, 532]. Several findings demonstrate the functional importance of this interaction with the Cul4/DDB1 ubiquitin ligase complex. First, either mutation of the DCAF1 interacting region of Vpx or siRNA-mediated knockdown of DCAF1 or DDB1 results in a restrictive, Vpx null-like, phenotype [37, 504, 532]. In contrast, treatment with the proteasome inhibitor MG132 largely relieves the restrictive phenotype for SIVmac lacking Vpx [188]. While the factor responsible for the myeloid block to lentiviral infection has yet to be identified, the mechanism for overcoming this factor appears to involve ubiquitin-mediated proteasomal degradation of the restriction factor using the Cul4/DDB1 complex.

APOBEC

As noted above, mammalian cells encode a family of proteins that function to deaminate cytosine residues. In humans, this family includes activation-induced cytosine deaminase (AID), APOBEC1, APOBEC2, APOBEC4 and a series of APOBEC3 (A3) genes. The APOBEC3 family of genes is located on chromosome 22 and includes APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G, APOBEC3H [252]. As described above, members of the APOBEC family of proteins possess antiviral activities, with APOBEC3 family members exhibiting the most potent anti-retroviral activity. In searching for the factor responsible for the block infection of to *vif* null HIV-1 virus, APOBEC3G that was identified [507]. This continues to be the best characterized APOBEC3 family member although other APOBEC3 proteins are known to block retroviral infection. In spite of the efforts to characterize APOBEC3-mediated retroviral restriction, there remains much debate about the specific mechanisms governing its antiviral properties.

As noted, while much of the research to date has focused on APOBEC3G, several members of the APOBEC3 family have been shown to possess antiviral activity. APOBEC3B, APOBEC3DE and APOBEC3F are the family members that most consistently display this anti-

retroviral activity. The presence of antiretroviral activity in APOBEC3B, APOBEC3DE, APOBEC3F, and APOBEC3G proteins corresponds to the presence of two catalytic domains in these proteins. The N-terminal catalytic domain is typically catalytically inactive in the antiviral APOBEC3 genes. The exception being APOBEC3B, where both domains have been found to have catalytic activity [Reviewed in 179]. APOBEC3G has an innate binding affinity for nucleic acids, both RNA and DNA, and it has been shown that APOBEC3G will bind single-stranded and double-stranded RNA, single-stranded and double-stranded DNA, as well as RNA/DNA hybrid. It has been shown that the affinity of APOBEC3G for binding to RNA is mediated by the inactive N-terminal catalytic domain, while the active catalytic domain preferentially binds DNA [56, 202, 247, 258, 407, 412, 430].

In contrast to its broad binding affinity, APOBEC3G only demonstrates catalytic activity on single-stranded DNA [247, 252, 280, 295, 546, 596, 641], with preferential deamination of cytosine residues present in C-C dinucleotides being observed for this protein [30, 212, 307, 352, 546, 641, 652]. However, the specificity of APOBEC3G for deaminating C-C dinucleotides deaminase is markedly lower than the exquisite specificity exhibited by APOBEC1 [451, 555]. APOBEC3G deamination activity has been shown to result in a gradient of mutations, with the highest density of deamination found in the 5' end of the genome and the lowest density observed in the 3' end. Furthermore, hotspots of deamination activity are observed in the HIV genome at three locations: in the region corresponding to the negative-strand strong-stop DNA (between the R region of the LTR and the PBS), and in the two PPT regions [545, 620, 641]. These phenotypes correspond to the length of time that different regions of the viral genome remain as single-stranded DNA [545, 641].

Viral death by mutagenesis is a rather conceptually straight-forward result of APOBEC activity. However, viral attenuation by hyper-mutagenesis is unlikely to be solely responsible for the results observed in many studies that were conducted using indicator cell lines based on Tat transactivation of a viral LTR controlling expression an indicator protein. In such systems, it

would not be anticipated that viral integration and expression of *tat* would be significantly inhibited, especially not in as dramatic of a fashion as what is observed. Consistent with the reduction in infectivity, several studies have found that the presence of Vif affects production of full-length reverse transcripts [182, 405, 527, 592]. One explanation for the finding that the loss of Vif results in reduced full-length RT products has been that cellular UNG2, specifically packaged into virions, mediates the degradation of deaminated reverse transcriptase products [95, 356, 493, 605, 628]. However, some reports have questioned the requirement for UNG2 in the process of degradation of deaminated RT products [266, 303]. The different findings of these studies may stem from the reliance on APOBEC over-expression systems. It has been reported that exaggerated amounts of APOBEC3G are packaged into virions produced under such conditions and that this may affect downstream restrictive phenotypes [179]. In spite of the uncertainty regarding mechanism of action, APOBEC3 proteins appear to inhibit viral replication in two distinct ways: crippling the virus as a result of the induced mutations and additionally causing the specific degradation of the deaminated viral reverse transcription products.

Another area of debate regarding the mechanism of action of APOBEC-mediated viral inhibition is the requirement of a functional catalytic domain for the reduction in infectivity. Several reports, each employing over-expression systems, have found that mechanisms exist for APOBEC3G inhibition of infectivity independent of catalytic activity [50, 234, 412]. However, each of these reposts proposed a unique mechanism for such an effect. Additionally, other studies have questioned these findings as a potential artifact of super-physiological expression of APOBEC3G [reviewed in 179, 234, 393]. One such study found that with APOBEC3G expression at or near physiological levels, the catalytic activity of APOBEC is required for viral inactivation [393]. Additional research should help illuminate the mechanism of this important aspect of APOBEC-mediated viral inhibition.

Regardless of the ultimate mechanism of action, APOBEC proteins must be packaged into assembling virions in order to exert their antiviral affect. It has been estimated that, in the

absence of Vif, fewer than 10 APOBEC3G molecules are packaged into HIV-1 virions produced in peripheral blood mononuclear cells (PBMCs) and that this is sufficient to render the viral particles non-infectious [622]. In addition to HIV, other retroviruses have been found to incorporate APOBEC3 proteins, which results in reduced viral infectivity. Viruses found to be sensitive to APOBEC-mediated restriction include SIV [55, 120, 352, 359, 490, 640], MLV [51, 120, 212, 288, 352], EIAV [352], M-PMV [130], xenotropic murine leukemia virue-like virus (XMRV) [537], and foamy viruses [122]. These studies found that, in general, viruses were able to evade packaging of APOBEC3 proteins from their original host species, whereas viruses were unable to prevent packaging of APOBEC3 proteins from non-native species. It has been demonstrated that evasion of APOBEC3 by MLV and M-PMV occurs via specific exclusion of cognate APOBEC proteins from their virions [130, 131]. In the case of MLV this escape function appears to be dependent upon the glycosylated form of Gag [289]. It is presumed that other simple retroviruses may escape APOBEC-mediated restriction in a similar manner. APOBEC3 proteins are able to incorporate into completely unrelated viruses, indicating a conserved mechanism for packaging. However, the mechanism determining specific packaging of APOBEC3G is not fully understood. APOBEC3G has been shown to interact with both RNA [247, 252, 279, 280, 560, 596, 597, 641] and the NC domain of the Gag precursor protein [7, 77, 90, 279, 352, 359, 485, 648]. Additionally, the RNA binding but catalytically inactive N-terminal catalytic domain appears to be important for packaging into virions [204, 240, 247, 407, 430]. Taken together, it appears likely that a complex of APOBEC3G, RNA and NC is packaged into virions [247]. However, the nature of the RNA requirement is under debate with evidence available for the requirement of genomic viral RNA [279, 280, 548] and other evidence implicating a need for packaging of the cellular 7SL RNA [596, 597], which is normally a component of the signal recognition particle but has been shown to be specifically packaged into virions [280, 427, 596]. Additional studies should lead to a clearer understanding of how APOBEC3G is incorporated into virions.

Lentiviruses encode the Vif protein whose primary function appears to be inhibiting APOBEC-mediated viral inhibition. The reduction in infectivity associated with loss of this function lead to the eventual discovery of APOBEC3G. Similar to the species-specific exclusion of APOBEC3 observed for simple retroviruses, the Vif proteins from different Lentiviruses inhibit APOBEC3G in a species-specific manner [359, 517, 518]. Amino acid 128 of APOBEC3G has been shown to modulate this interaction [55, 353, 490, 623]. It is not entirely clear which regions of Vif interact with APOBEC3G as mutational studies have implicated much of the N-terminal half of the 192 amino acid protein in modulating this interaction [360, 381, 474, 491, 603]. In addition to the direct interaction requirements contained in the N-terminal portion of the protein, Vif oligomerization is likely also required for interactions with APOBEC [386, 629]. Binding of Vif has been shown to result in the ubiquitin-mediated proteasomal degradation of APOBEC proteins [114, 270, 271, 325, 360, 379, 508, 538, 604, 642] by recruiting a Cullin 5 E3 ubiquitin ligase complex that polyubiquitinates the protein [114, 287, 360, 379, 380, 508, 538, 642]. Additionally, there appears to be degradation-independent mechanisms for the exclusion of APOBEC3G from virions as evidenced by the finding that a degradation resistant form of APOBEC3G is also excluded from virions by Vif [429]. However, the specific mechanism for this ubiquitin-independent activity is as yet unknown.

p21

Few cells expressing HIV-1 receptor and co-receptor have been found to be resistant to HIV infection, one of which is CD34+ hematopoietic stem cells (HSCs) [509, 590, 600]. These cells express low levels of CD4 receptor and it was shown that this resulted in a primary block to infection that could be largely overcome by pseudotyping the HIV-1 virions with the VSV-G glycoprotein [509]. However, later experimentation with VSV-G pseudotyped HIV vectors demonstrated that siRNA knockdown of a cyclin-dependent kinase (CDK) inhibitor, p21 (also known as Cip1 and Waf1), resulted in a significant enhancement of viral transduction [653]. This was expanded upon to show that the p21-mediated block increased circular viral DNA in the

nucleus and resulted in fewer integrants [654], suggesting successful nuclear import but a failure of integration. The expression of other known mediators of intracellular viral restriction, such as TRIM5, were shown to be unaffected by p21 knockdown, suggesting that they are not involved in the restrictive phenotype. While it was shown that this effect was specific to HIV-1 infectivity, the effect of p21 was, however, not shown to be as a result of a direct interaction between this protein and HIV-1 PICs. One of the normal physiological roles of p21 is to regulate the cell cycle progression in certain cell lineages, including HSCs [96, 621]. HIV-1 and other Lentiviruses have been shown to more efficiently infect cycling cells than non-dividing [645]. While Zhang et al. demonstrate that division in p21 knockdown cells does not occur until at least 42 hours after siRNA transfection, it is possible that the enhanced viral infectivity results from the role p21 plays in controlling cell cycling [654]. An indirect mechanism of action is supported by recent findings in macrophage cells. It was demonstrated that p21 was involved in inhibiting lentiviral infection of macrophages following cellular activation by ligation of activating immunoglobulin G constant fragment receptors (FcγR). In this system, it was observed that p21 affects HIV-1 as well as SIVmac and HIV-2 infection, and that this is not due to a direct interaction with viral PICs [38]. Taken together, it is clear that p21 modulates cellular susceptibility to lentiviral infection. However, it is possible that this p21-mediated inhibition of infection is the result of pleiotropic cellular effects and not specific viral restriction, a possibility previously speculated upon [46].

PBS-mediated transcriptional repression

Some years ago it was observed that, in spite of normal integration of proviral DNA into the cellular DNA, embryonic carcinoma cells were unable to support replication of MLV and that this was the result of a lack of viral transcription in the embryonic carcinoma cells [554]. However, if embryonic carcinoma cells were differentiated from their pluripotent state, the transcriptional block for newly integrated MLV proviruses was lost [554]. A subsequent study demonstrated that MLV proviruses remained transcriptionally inactive following differentiation if

they had integrated when cells were in their pluripotent form, and that it took additional treatment with the DNA demethylating agent 5'azacytadine to restore transcription [421]. The differing phenotypes between these two sets of experiments suggests a two step process of initial recognition and inhibition by a factor or factors present in pluripotent cells followed by DNA methylation dependent long-term inactivation of the viral promoter [421].

Subsequent studies in the pluripotent embryonic carcinoma cells found a multifaceted block to MLV transcription, with a combination of reduced binding of transcription factors to the viral LTR enhancer elements [229, 334, 339, 528, 572] and trans-acting negative factors [5, 25, 148, 154, 184, 339, 572] resulting in the final potency of the block. Specific viral recognition was found to be mediated through interactions with the PBS of the proviral DNA [25, 148, 338, 339, 446]. The initial findings were extended to show inhibition of MLV transcription in embryonic stem cells [191] and hematopoietic cells [201]. Additionally, it was observed that alternate viral PBS sequences could be specifically recognized, including the proline tRNA utilizing primer-binding site (PBS^{Pro}) [394, 627].

Recently, TRIM28 (also known as Kap-1) was discovered to be a required factor in the transcriptional repression in embryonic cells of retroviruses utilizing PBS^{Pro}, $PBS^{Lys1,2}$, and PBS^{Phe} [473, 610, 611]. However, this protein has no known DNA binding activity. Rather, studies have found that TRIM28 interacts with DNA bound Krüppel associated box (KRAB) zinc finger DNA-binding proteins (KRAB-ZFP) [160, 609]. Transcriptional repression appears to be mediated through the further recruitment of effector molecules of transcriptional silencing [304, 497, 498, reviewed in 579, 608]. In the case of MLV, the KRAB-ZFP protein, ZFP809, binds to the PBS^{Pro} and leads to the recruitment of TRIM28 [609]. This then leads to a further recruitment of heterochromatin-associated protein (HP1) which potentiates long-term silencing of MLV transcription via formation of heterochromatin [608]. Taken together, these results indicate the following general mechanism of PBS-mediated transcriptional inhibition: a KRAB-ZFP acts as a sequence-specific binding protein recognizing a single class of viral PBS, the KRAB-ZPF/DNA

complex is then recognized and bound by TRIM28, which then recruits effector molecules for short-term transcriptional repression and long-term silencing by formation of heterochromatin.

ZAP

In a complimentary DNA (cDNA) library screen for factors that inhibit MLV infection of typically permissive cells the zinc-finger antiviral protein (ZAP) was identified as a factor responsible for a decrease in viral RNA production [169]. ZAP is a CCCH-type zinc finger protein shown to bind to AU-rich elements in the 3' untranslated region (UTR) of some mRNAs, causing these RNAs to be targeted for specific degradation [85, 86, 300, 301]. In cells expressing ZAP, the amount of MLV RNA present inside the nucleus was found to be similar to that of the parental (permissive) cells, whereas in the same ZAP-expressing cells viral RNA was found to be greatly diminished in the cytoplasm [169]. ZAP was shown to interact, via its CCCH zinc finger, with the 3'LTR of MLV, in spite of the apparent lack of ZAP-interacting AU-rich elements in the MLV LTR [196]. ZAP has been shown interact with members of the exosome [197], a conserved ribonuclease complex found in both the nucleus and cytoplasm of eukaryotic cells [8, 142, 390]. Knockdown of these ZAP interacting proteins using siRNA was found to resulted in an increase in MLV RNA in the cytoplasm, suggesting that exosomes are involved in the degradation of cytoplasmic MLV RNA [197]. In addition to this antiretroviral activity, ZAP has can inhibit replication of other RNA viruses. It has been shown that ZAP blocks replication by members of the negative stranded *filoviridae* (Ebola virus) and positive stranded *alphaviridae* (Sindbis virus) by specifically binding to, and causing the degradation of, viral RNA [44, 196, 397, 656]. These findings demonstrate a clear role for ZAP-mediated specific degradation of viral RNA prior to nuclear export and suggest that ZAP may represent a universal obstacle for viral replication.

Tetherin

Tetherin (Bst-2) was discovered as a result of studies examining the replication defects of Vpu-null HIV-1 viruses. Initial reports described a replication defect to Vpu null HIV-1 in HeLa cells that occurred at the stage of viral release. This was characterized by fully assembled, and

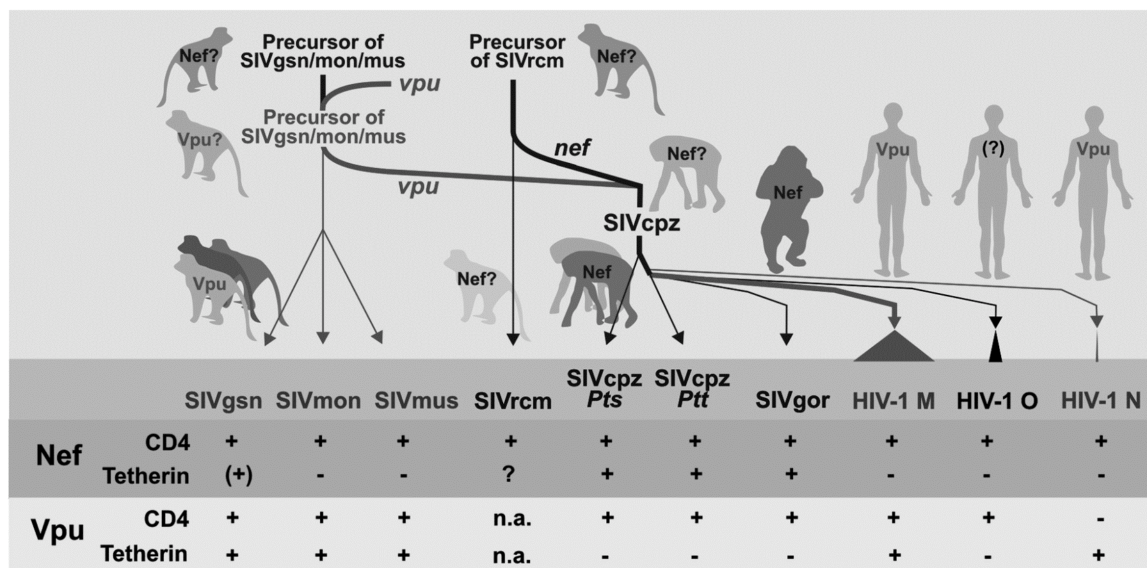
typically matured, virus being attached to the surface of the cell. However, this phenotype was not found to be universal, as HIV-1 released normally from COS cells in the absence of Vpu [186, 285, 540, 556]. When heterkaryon fusions were made between HeLa and COS cells it was found that the block to viral release was caused by a dominant factor in the HeLa cells [582]. Later it was found that the block to viral release could be induced in normally permissive cells through treatment with type I interferons (IFN) [409]. The virus attached to the cell surface was found to be fully infectious upon detchment, either by physical shearing or subtilisin treatment [268, 408].

Tetherin was identified, independently by two labs, as being the factor responsible for mediating the block to release in Vpu deficient HIV-1 [410, 580] and later it was demonstrated that this protein forms a physical connection between the viral and cellular membrane [153, 208, 441]. Tetherin is a particularly unusual type II transmembrane protein containing a short cytoplasmic N-terminal domain, followed by a transmembrane domain, and a coiled-coil extracellular domain, which is joined to a C-terminal glycosylphosphatidylinositol (GPI) anchor [297]. This structure creates a protein with two membrane-associated domains where the majority of the protein resides in the extracellular space. This is a seemingly perfectly design for connecting two membrane bound objects together, extracellularly. In order to confirm that it is the unusual protein architecture, and not the primary sequence, that is responsible for the antiviral activity, a Tetherin-like molecule was artificially constructed by replacing the protein domains with like domains from unrelated proteins. It was found that such a synthetic molecule possessed antiviral function, demonstrating the importance of the protein structure of Tetherin for its antiviral function [441]. Following its discovery, it was rapidly realized that Tetherin was able to inhibit the release of other viruses. Indeed it has been demonstrated that Tetherin inhibits viral release of all genera of retroviruses (including endogenous retroviruses) as well as members from other families of enveloped viruses such as *herpesviridae*, *arenaviridae*, and *filoviridae* [19, 186, 193, 261, 268, 357, 370, 410, 478].

As might be expected based on the protein structure of Tetherin and the events leading up to its discovery, Vpu was found to directly interact with Tetherin [133, 246]. This interaction was found to involve the transmembrane domains of both proteins [198, 329, 378, 438, 471, 561, 585]. It was shown that Vpu was unable to interact with non-hominid Tetherin and a single amino acid substitution in the transmembrane domain was found to disrupt this interaction [198, 329, 378, 431].

The mechanism of anti-Tetherin activity is still an area of intense study and little consensus. Initially it was believed that Vpu mediates its anti-Tetherin activity similarly to its down-regulation of CD4 expression, namely by recruiting β-TRCP and targeting it for ubiquitin-mediated proteasomal degradation. However, in a series of studies examining this possibility none showed a total reversal of Vpu's anti-Tetherin activity when β-TRCP-mediated proteasomal degradation was blocked [132, 176, 198, 246, 351, 392]. More recent studies have indicated that Vpu redirects Tetherin trafficking in the cytoplasm away from the cell surface and into a perinuclear organelle. This relocalization does not appear to affect Tetherin steady-state protein levels, turnover rate, or internalization from the cell surface [177, 218, 630]. Thus, it appears likely that interaction between Vpu and Tetherin may result in a two-pronged interference mechanism; redistribution of the cellular Tetherin away from sites of viral assembly or release followed by the subsequent targeted degradation of Tetherin.

As described above, Tetherin has a broad ability to impair viral release, yet not all Lentiviruses encode Vpu. Studies in HIV-2 uncovered alternative mechanisms for Tetherin antagonism, in this case mediated by the viral Env [1, 59, 199, 305]. The specificity determinant of this interaction was found to localize in the extracellular region of the TM subunit of Env [305]. Following interaction between HIV-2 Env and Tetherin, a similar outcome to Vpu-Tetherin interaction is observed. This is characterized by an accumulation of Tetherin in the trans-golgi network (a peri-nuclear location). This redistribution was found to be dependent upon the well-characterized adaptor protein 2 (AP-2) binding motif in the cytoplasminc tail of HIV-2



		SIVgsn	SIVmon	SIVmus	SIVrcm	SIVcpz *Pts*	SIVcpz *Ptt*	SIVgor	HIV-1 M	HIV-1 O	HIV-1 N
Nef	CD4	+	+	+	+	+	+	+	+	+	+
	Tetherin	(+)	-	-	?	+	+	+	-	-	-
Vpu	CD4	+	+	+	n.a.	+	+	+	+	+	-
	Tetherin	+	+	+	n.a.	-	-	-	+	-	+

Figure 7. *Schematic representation of the proposed evolution and transfer of vpu and nef during zoonotic transfer of Lentiviruses in Old World primates.* Top: Arrows indicate zoonotic transfer of lentiviral strains or accessory genes during lentiviral evolution. Bottom: Autologous CD4 and Tetherin regulatory functions of Nef and Vpu in these species. Reprinted from Cell Host & Microbe, Vol 6(5), D. Sauter et al., Tetherin-Driven Adaptation of Vpu and Nef Function and the Evolution of Pandemic and Nonpandemic HIV-1 Strains, Pages 409-421, 2009, with permission from Elsevier.

Env [305]. Additional work involving tantalus monkey SIV (SIVtan) has shown that the Env from this virus functions in a similar manner to that of HIV-2. Similar to HIV-2, the TM domain of the SIVtan Env was found to interact with the ectodomain of Tetherin, resulting in the redistribution of Tetherin to the endosome [199].

Finally, in a diverse group of SIV viruses Nef has been shown to mediate Tetherin antagonism [256, 481, 651]. A recent elegant study demonstrated that this anti-Tetherin activity of Nef is present in some lentiviral strains containing Vpu, including chimpanzee SIV (SIVcpz) (Fig. 7). This data supports the conclusion that the Tetherin antagonism functions of Nef are ancestral to that of Vpu and that the anti-Tetherin activity of HIV-1 Vpu evolved in humans, perhaps allowing for broad dissemination of such Tetherin-escape viruses in the human population. Finally, the Tetherin antagonism activity of Vpu appears to have evolved

independently in certain strains of SIV, such that the anti-Tetherin functions of Vpu and Nef appears to be mutually exclusive functions of these proteins [481].

TRIM5α

Structure/Function

The human genome encodes approximately 70 unique members of the TRIM family of genes. Members of this family of genes are defined by the presence of three sequential protein domains: a RING finger domain at the N-terminus followed by either a B-Box2 domain or tandem B-Box1/B-Box2 domains followed by a coiled-coil domain. The C-terminal portion of these proteins can consist of one or more of a select group of domains. The majority of the TRIM family members in humans contain either a PRY or a PRY-SPRY (B30.2) domain [480]. TRIM5α is the longest protein isoform expressed from the TRIM5 gene and consists of a RING finger, a B-Box2, a coiled-coil, and a B30.2 domain (Fig. 8). TRIM5α mRNA is generated from eight exons with exons 2-4 encoding the tripartite motif portion of the protein and exons 7 and 8 encoding the B30.2 domain. Of note, in the genomic configuration the exons encoding the tripartite motif are separated from those encoding the B30.2 domain by an unusually long intron over 10,000 nucleotides in size. TRIM5α exists in cells as homo-multimers that can organize into higher order multimers [217, 254, 302, 389, 411, 418, 444, 484, 633]. In cells, TRIM5α localizes to the cytoplasm and forms distinct puncta known as cytoplasmic bodies in over-expression studies [83, 523]. The cytoplasmic bodies are highly dynamic structures with TRIM5α molecules regularly shuttling between the free cytoplasmic pool of TRIM5α protein and these structures [83]. However, these bodies are not required for viral restriction [523].

The RING and B-Box2 domains each coordinate two zinc ions, and this association aids in proper protein folding as well as function [57, 227, 365, 366]. The N-terminal RING finger domain of several TRIM proteins have been shown to possess ubiquitin ligase activity, with proteins containing this domain shown to catalyze the transfer of ubiquitin to other proteins as

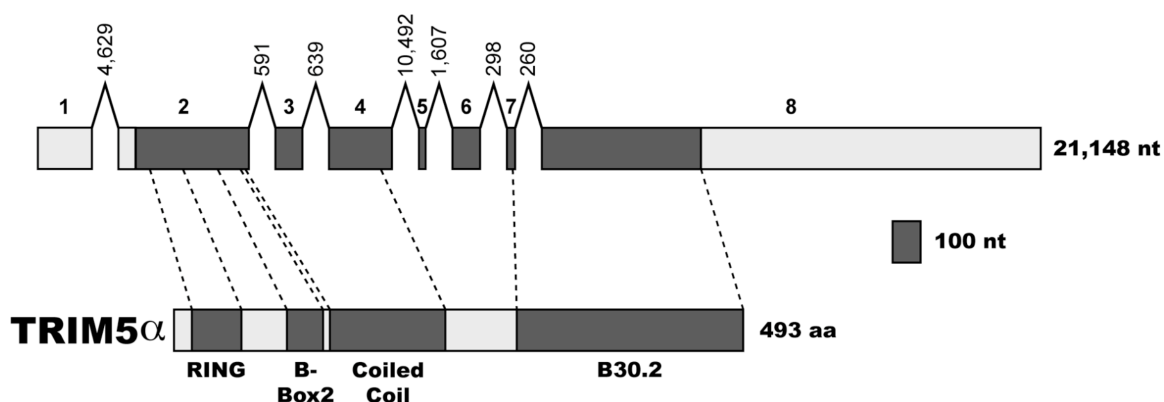


Figure 8. *Representation of the TRIM5 coding structure and protein domain architecture.* Top: Genomic and mRNA features of TRIM5 including the proportional representation of exonic structure and lengths of the introns. Light boxes represent untranslated regions and dark boxes represent coding regions. Bottom: TRIM5α - the longest of four reported protein isoforms produced from the TRIM5 gene. It is composed of a RING finger, B-Box2, and coiled-coil domains common to all tripartite motif (TRIM) genes followed by a B30.2 domain at the C-terminus. Other TRIM5 isoforms are spliced such that they lack the B30.2 domain.

well as themselves [Reviewed in 383]. TRIM5α has been demonstrated to have a short intracellular half-life due to poly-ubiqutination followed by ubiquitin-mediated proteasomal degradation [126, 624]. Mutational studies of TRIM5α have demonstrated that deletion of the RING domain or point mutants abolishing ubiquitin ligase activity have several effects on TRIM5 biology. First, the ubiquitination status of TRIM5 becomes altered such that poly-ubiquitination of TRIM5 is absent while mono- and di-ubiqutinated TRIM5 remains [126]. Second, the intracellular stability of TRIM5α increases in the absence of a functional RING domain [126, 541]. Together, these findings point to auto-ubiquitination functions of the RING finger domain of TRIM5. Finally, the TRIM5 proteins lacking a functional RING domain are still capable of viral restriction functions, albeit with a reduced efficiency.

B-Box domains have been shown to mediate protein-protein interactions [286, 322, 552] and have been implicated in regulation of protein localization [566]. In spite of this, the biology of these domains remains poorly understood. In TRIM5α, deletion of the B-Box2 domain or mutation of crucial residues result in reduced binding to viral CA cores and the loss of antiviral

activity [253, 321, 440, 541]. The observed decrease in magnitude of antiviral activity of these B-Box2 mutants often far exceeds the change in CA binding [125, 322]. Changes in this domain have also been shown to affect the higher-order multimerization of TRIM5α, which correlates with a reduction in CA binding activity. This is especially pronounced where weak interactions exist in the native protein [321, 322].

The coiled-coil domain consists of multiple α-helical domains that can interact with other coiled-coil domains to form multimeric complexes. Preferentially, but not exclusively, these complexes form homo-multimers [254, 383, 389, 419, 464]. Mutational studies have shown that the coiled-coil domain is absolutely essential for retroviral restriction [254, 440]. Additionally, TRIM5α proteins that contain heterologous multimerization domains instead of the coiled-coil domain are unable to exert antiviral activity [254, 321, 389]. Evolutionary studies [413] and recent biological evidence suggests that this domain influences the specificity of the antiviral activity of the protein [348].

The antiviral specificity determinants of TRIM5α are contained within the B30.2 domain. The importance of this domain in determining retroviral interactions was recognized in early studies, both as a result of targeted domain swapping experiments [541] as well as the observation that in owl monkey TRIM5 a retrotransposition event has resulted in the natural replacement of the B30.2 domain with Cyclophilin A (CypA), a known CA binding protein [418, 484]. There is now abundant genetic, biochemical, and structural evidence for the direct interaction between the B30.2 domain of TRIM5α and incoming viral CA, where the CA binding activity is functionally replaced by the CypA domain in TRIM-Cyp proteins [53, 251, 254, 323, 425, 432, 442, 443, 483, 484, 502, 542]. In the fully folded protein, the B30.2 domain has been shown to fold into a structure with two β-sheets lying on top of each another. The central portion of this structure displays high hydrophobicity, with the amino acids involved in these hydrophobic interactions being well conserved both across species and between B30.2 domain-containing proteins [195,

251, 367, 425, 483, 524, 614]. Extending from this hydrophobic core are loops of variable length and composition [251, 524, 614, 631]. The specific features of these loops have been shown to modulate interactions with virus such that restriction can be modulated by a single amino acid substitution [323, 403, 425, 483, 524]. However, TRIM5α proteins with wildly different loop composition can restrict the same virus [Reviewed in 29, 415]. Together, the evidence suggests that these loops form the bulk of the CA binding interface.

Specificity of TRIM5-virus interactions

Prior to the identification of TRIM5α, the antiviral activity of the factor responsible for the Lv1 block was known to act in a species and virus specific manner [116, 216, 231, 232, 433, 511]. Following the discovery of TRIM5α, multiple groups worked toward characterizing this virus-host interaction [217, 278, 413, 425, 440, 444, 483, 523, 541, 633]. Early studies focused on the ability of various primate TRIM5α proteins to block infection by a variety of Lentiviruses as well as MLV. These viruses were likely chosen as the result of the prior characterization of Lv1 and Ref1 inhibition of these viruses. These studies showed that a primate TRIM5α protein could be identified with specific antiviral activity against each virus tested (Table 1). However, viruses were found to vary greatly in their sensitivities to the tested TRIM5α molecules and conversely TRIM5α proteins were found to vary in their ability to protect against the challenge viruses. For example, HIV-1 was found to be sensitive to the actions of TRIM5α from species outside of the hominoid lineage and MLV was found to be sensitive to most TRIM5α molecules tested. Whereas, the different strains of SIV tested were largely resistant to hominid and Old World monkey TRIM5α activity, but susceptible to New World monkey TRIM5α activity. On the opposite side of the equation, of the TRIM5α molecules tested, the hominoid TRIM5α molecules demonstrated the least breadth of action against the tested viruses, while the TRIM5α from African green monkeys (AGM) and rhesus macaques displayed the greatest breadth. Finally, it is

	Species	HIV-1	HIV-2	SIVmac	SIVagm	EIAV	N-MLV
Hominoid	Human	+	-	+/-	-	++	++
	Chimpanzee	+		-	-		++
	Gorilla	++		++			++
	Orangutan	+		-	-		++
Old World monkey	Rhesus macaque	++	+	+	++	++	++
	Pigtail macaque	++		-			++
	Cynomologous monkey	++	++	-			
	African green monkey (AGM)	++	++	++	-	++	++
	AGM tantalus	++		++	-		++
	AGM pygmaeus	++		+	+		++
	Sooty mangabey	++		-			++
New World monkey	Spider monkey	++		++	++		++
	Red-chested tamarin	+		+			+
	Cotton top tamarin	++		++			++
	Emperor tamarin	++		++			++
	Squirrel monkey	-		++	+		-

Table 1. *Viral restriction by primate TRIM5α proteins.* ++: robust restriction; +: weak restriction; -: no restriction; +/-: weak restriction in some reports, no restriction in other reports. Information presented is compiled from Newman, R.M. and Johnson, W.E, 2007 [415] and Bauman, J.G., 2006 [29].

important to note that the viruses tested that were of primate origin were found to be specifically resistant to the antiviral activity of the TRIM5α protein from their natural host species.

Work that preceded the discovery of TRIM5 [116, 216, 433] as well as subsequent characterization of the TRIM5α-virus interaction [502, 510, 526, 541, 578, 636] convincingly demonstrated that CA is the viral target of TRIM5α. However, this poses a dilemma in explaining the restrictive phenotypes observed for TRIM5α. Namely, how does one TRIM5α interact with the CA molecules from multiple retroviruses that have low amino acid conservation? In an attempt to explain this question, Mische et al proposed a model where TRIM5α recognizes a

complex epitope present in the tertiary/quaternary structure on the CA core [389]. The CA cores from multiple retroviruses, including HIV-1, MLV, and ALV, have been shown to assemble into hexameric lattice quaternary structures (Figs. 6 and 9) [87, 166, 319, 396]. Thus, retroviruses likely present similar quaternary surface structures for recognition by TRIM5α. Lending support to this model is the fact that the majority of the CA residues shown to modulate TRIM5α susceptibility are exterior surface exposed [432]. The implication of such a model is that the incoming viral capsid structure can be considered a pathogen-associated molecular pattern (PAMP), with TRIM5α being a type of pattern recognition receptor (PRR). Furthermore, as all retroviral CA cores are comprised of similar hexameric CA lattices, TRIM5α likely has the capability of interacting with a broad array of retroviruses.

Initial studies only examined members of the Lentivirus and Gammaretrovirus families of *retroviridae* for sensitivity to TRIM5. The work presented in Chapter 1 of this dissertation was conducted to address the question of whether other families of retrovirus were similarly sensitive to TRIM5 restriction. In this study, we tested Betaretrovirus sensitivity to primate TRIM5α proteins using M-PMV as a prototypic Betaretrovirus.

<u>TRIM5 Genomic Structure</u>

The TRIM5 gene is located in the midst of an olfactory receptor gene dense region of the genome on chromosome 11 in humans. In the human genome, TRIM5 is found adjacent to three related TRIM genes; TRIM6, TRIM22, and TRIM34. Based on phylogenetic analyses [480], TRIM6 appears to be the ancestral gene with the rest of the members of this TRIM gene cluster arising as a result of sequential gene duplication events. In primates, these genes are arranged such that TRIM6, TRIM34 and TRIM22 are in the same orientation. TRIM5 is found between TRIM34 and TRIM22, but in the opposite orientation and situated such that TRIM5 and TRIM22 share an approximately 5 kb promoter region (Fig. 10). The genetic architecture described above

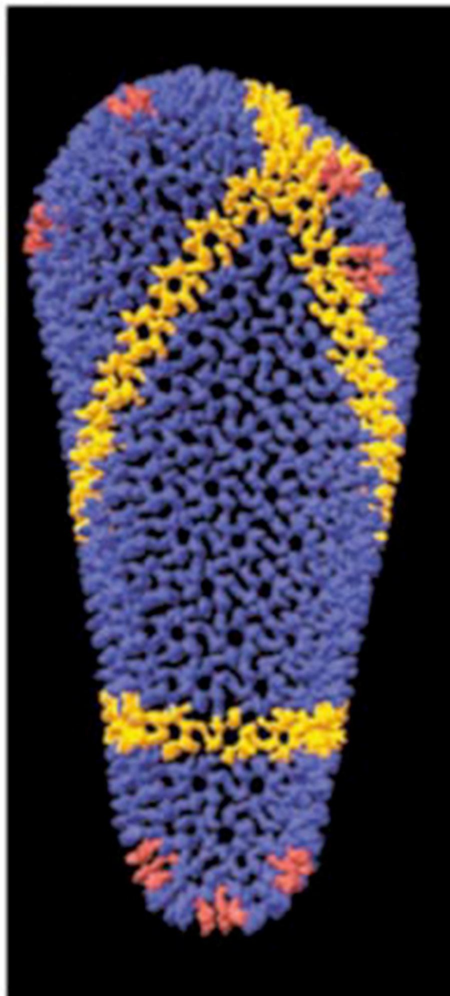


Figure 9. *Stereo model of a mature HIV-1 capsid core.* Pentameric defects are shown in red, and a contiguous line of CA hexamers is highlighted in gold to illustrate how the spiral gradually changes diameter and pitch, and eventually reverses helical hand. Figure created by docking molecular models into an idealized fullerene cone of 1,572 CA subunits[6], and calculating a mask at 8 Å around the Cα atoms.

Adapted from [319] with permission from Macmillan Publishers Ltd: Nature. Su Li et al.; Image reconstructions of helical assemblies of the HIV-1 CA protein; Nature **407**, 409-413 (September 2000), copyright 2000.

and shown in Figure 10 is unique to primates. Most other mammals have TRIM5 oriented in the same direction as the other TRIM family members of this locus [Reviewed in 257].

For any animal, the selective pressure of obtaining food is exceptionally high, which has resulted in the genomic olfactory receptor gene content being highly divergent between species, highly polymorphic within populations, and the olfactory receptor regions of the genome being highly plastic [Reviewed in 213, and 417]. Perhaps as a result of the plasticity of the surrounding olfactory receptor gene rich genomic region, the TRIM6/34/5/22 locus is also highly variable in mammals. Multiple TRIM5 orthologs are found in cows, mice, and rats, but these species lack a TRIM22 ortholog [482, 553]. In contrast, cats and dogs genomes contain both TRIM5 and TRIM22 orthologs, but in both of these species it has been reported that the TRIM5 gene does not

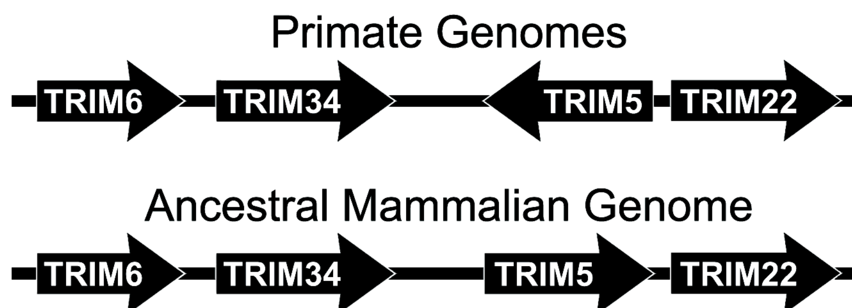


Figure 10. *Composition and structure of the of TRIM5 genomic locus.* Top: Graphical representation of the location and orientation of TRIM5, TRIM6, TRIM22 and TRIM34 genomic locus in primates. Bottom: Representation of the hypothetical structure of the ancestral mammalian TRIM5 genomic locus. In both cases, olfactory receptors are found adjacent to TRIM6 and TRIM22 (not shown).

produce a TRIM5α protein [375, 482]. Furthermore, no TRIM5 ortholog was found in the opossum genome [482], so it is likely that TRIM5 is specific to eutherian mammals and that this gene and its genomic locus have undergone significant diversification since its creation.

<u>TRIM5 Evolution</u>

TRIM5 has undergone an amazingly complex evolutionary history. The first evidence of this was the discovery of the TRIM-Cyp gene in owl monkeys. This gene arose as the result of the LINE-mediated retrotransposition of a cyclophilin A mRNA transcript between the seventh and eighth exons of TRIM5α [418, 484]. The resulting chimeric gene subsequently became fixed in all owl monkey species tested to date [466]. This process of generating new exons by utilization of heterologous genetic material contained within the genome is known as 'exon shuffling' [Reviewed in 340]. Illustrating the rarity of this process, at the time of its discovery, owl monkey TRIM-Cyp was the only case of exon shuffling to have been observed in vertebrates. However, since that time other instances of exon shuffling have been described, with one such instance found to have taken place in the TRIM5 gene of Old World primates. In this instance, a CypA mRNA was retrotransposed downstream of TRIM5 in the macaque lineage. The CypA retrotransposition is accompanied by a mutation in the splice acceptor site preceding exon 7 of

what would be TRIM5α, resulting in the expression of a similar TRIM-Cyp as is found in owl monkeys [67, 324, 414, 587, 607].

Further evidence of the history of strong evolutionary pressures in this gene are evidenced by the exaggerated rates of nonsynonymous changes compared to synonymous changes (dN/dS) in the coding region of TRIM5 [483, 524]. This is generally indicative of evolutionary pressures selecting for altered protein sequences, and is referred to as positive selection. In TRIM5, the nonsynonymous changes are concentrated in the eighth exon, and more specifically in three variable regions of this exon. These variable regions encode the variable loops of the B30.2 domain that form the TRIM5-virus interface, which have been shown to modulate viral restriction patterns [403, 425, 483, 524]. Recently, it was reported that similar selective evolution has also taken place in the virus interface of the macaque TRIM-Cyp [452].

In addition to TRIM5, elevated levels of nonsynonymous change are also found in TRIM22 [482], a gene found to encode a protein possessing antiviral activities [27, 140, 167, 563]. As with TRIM5, the nonsynonymous changes predominate in the variable regions of the B30.2 domain. An interesting interplay between TRIM5 and TRIM22 was observed [482]; primate lineages with elevated TRIM5 dN/dS rates were found to have low dN/dS rates in TRIM22, and the opposite is true – elevated TRIM22 dN/dS rates correlated to low TRIM5 dN/dS rates. In contrast to TRIM5 and TRIM22, the other genes in the TRIM5 genomic locus, TRIM6 and TRIM34, have low dN/dS ratios [320, 482], indicating a pressure to maintain a protein sequence over time, referred to as negative selection.

Relatively short in-frame insertions and deletions of coding sequence have accompanied the elevated rates of nonsynonymous substitutions in the B30.2 domain of TRIM5. In the African green monkey lineage of Old World primates, a 20 amino acid expansion is found in the v1 region of the B30.2 domain, while in the spider monkey lineage of New World primates a 55 amino acid expansion is found in the v3 region of the B30.2 domain [483, 524]. The expansion in African green monkeys has been shown to confer the ability to restrict SIVmac [403].

As a final example of the strong selective forces having been placed on TRIM5, it was found that TRIM5 has been under balancing selection during primate evolution [82, 413]. This is the process of maintaining multiple alleles of a gene in the population of a given species. In the best characterized instance of this phenomenon, it was found that multiple high-frequency TRIM5 alleles exist in both rhesus macaques and sooty mangabeys [413]. In rhesus macaques, these alleles can be separated into three functional alleles, based on their ability to inhibit the replication of HIV-1, HIV-2, EIAV, and FIV [606]. Furthermore, functional differences have been observed between these alleles in how well they are able to inhibit SIVmac replication and pathogenesis *in vivo* [283, 330, 470]. The other identified case of balancing selection in TRIM5 appears in human and great ape populations where several nucleotide polymorphisms in the first intron are present at high frequencies of the population [82]. These polymorphisms fall in an endogenous retroviral LTR element and are hypothesized to influence transcriptional regulation. In both identified instances of balancing selection, a subset of the identified sites were also found to be polymorphic in related species, indicating that the polymorphism has been under balancing selection since before the two species diverged. It is estimated that divergence of humans from chimpanzees as well as rhesus macaques from sooty mangabeys occurred between 6 and 10 million years ago [82, 413], demonstrating the longevity of such selective forces.

Transcriptional regulation

Control of transcriptional regulation of TRIM5, and the other TRIM genes of the TRIM5 locus, has not been the subject of intensive investigation. Initially, human TRIM5 expression was described as being “ubiquitous” [464]. This initial assessment was found to be misleading, as TRIM5 (and TRIM22) were shown to be differentially expressed in human tissues [482]. Furthermore, it was discovered that TRIM5 is upregulated in response to IFN treatment and that this responsiveness is controlled by an interferon sensitive responsive element (ISRE) in the TRIM5 core promoter [20]. All four TRIM genes in the TRIM5 locus were subsequently demonstrated to be IFN regulated, although only TRIM5 is predicted to have an ISRE in its core

promoter [88, 89]. Recently, the first exon of TRIM22 has been shown to contain an ISRE that governs IFN responsiveness of this gene [168]. Finally, it is known that TRIM22 is upregulated in response to p53 activation and that the p53 binding site responsible for this regulation is located in the first intron [269, 422, 598].

Three lines of evidence suggest that the mRNA expression levels of the antiviral TRIM genes may impact antiviral activity. First, it has been reported that the TRIM5 mRNA levels in human PBMCs correlate with the risk of becoming infected with HIV-1 [503] and increased TRIM22 expression levels have been correlated with decreased HIV-1 viral load and higher CD4 T-cell counts [519]. Second, TRIM5α expression is upregulated in vitro following IFN treatment and this results in an enhancement of TRIM5α antiviral activity [89]. Finally, overexpression of TRIM34 can result in a block to SIVmac infection, while endogenously expressed TRIM34 does not affect SIVmac infectivity [320, 650].

The experiments presented in chapter 2 were conducted in order to gain a better insight into the transcriptional regulation of the antiviral TRIM genes. As primate TRIM5 and TRIM22 genes share a short, largely conserved, core promoter region attention was focused on the non-coding regions of these genes.

IDENTIFICATION OF POST-ENTRY RESTRICTIONS TO MASON-PFIZER MONKEY VIRUS INFECTION IN NEW WORLD MONKEY CELLS

by

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ABSTRACT

TRIM5α has been shown to be a major post-entry determinant of host range for Gammaretroviruses and Lentiviruses, and more recently Spumaviruses. However, the restrictive potential of TRIM5α against other retroviruses has been largely unexplored. We sought to determine whether or not Mason-Pfizer Monkey Virus (M-PMV), a prototype Betaretrovirus isolated from rhesus macaques, was sensitive to restriction by TRIM5α. Cell lines from both Old World and New World primate species were screened for their susceptibility to infection by vesicular stomatitis virus G protein (VSV-G) pseudotyped M-PMV. All of the cell lines tested that were established from Old World primates were found to be susceptible to M-PMV infection. However, fibroblasts established from three New World monkey species specifically resisted infection by this virus. Exogenously expressing TRIM5α from either tamarin or squirrel monkeys in permissive cell lines resulted in a block to M-PMV infection. Restriction in the resistant cell line of spider monkey origin was determined to occur at a post-entry stage. However, spider monkey TRIM5α expression in permissive cells failed to restrict M-PMV infection and interference with endogenous TRIM5α in the spider monkey fibroblasts failed to relieve the block to infectivity. Our results demonstrate that TRIM5α specificity extends to Betaretroviruses, and suggest that New World monkeys have evolved additional mechanisms to restrict the infection of at least one primate Betaretrovirus.

INTRODUCTION

Infectious Betaretroviruses have been isolated from a number of diverse mammalian species, including mice [52, 384], cats [371], sheep [505], and a number of primate species from both the Old World [36, 101, 564] and New World [110, 221, 557, 565, 612]. The prototypical members of this retroviral genera are mouse mammary tumor virus (MMTV) and Mason-Pfizer Monkey Virus (M-PMV). Both of these viruses have been shown to cause disease in their natural

hosts; MMTV was identified based on its ability to cause mammary carcinoma in mice [52], and M-PMV has been shown to cause an AIDS-like disease in juvenile rhesus macaques [364].

In addition to these infectious Betaretroviruses, a large number of Betaretrovirus-like (type II) endogenous retroviruses have been detected in the genomes of mammals [530]. In fact, the only retroviral sequences that appear to have been added to the human genome since speciation are members of the human mouse mammary tumor virus-like 2 (HML-2) group of the Betaretrovirus-like human endogenous retrovirus K (HERV-K) elements. Some of these HML-2 integrants are polymorphic amongst human populations and, based upon homology of the long terminal repeat (LTR) sequences, several proviruses appear to have been added to the genome less than one million years ago [24, 33, 238, 576]. In contrast to this strong evidence for recently active betaretroviral infection in humans, there is no clear evidence that retroviruses of other genera have been introduced to the human genome since speciation. Among hominids, this bias towards recent addition of betaretroviral-like sequences is unique to humans, as the genomes of both chimpanzees and gorillas contain more than 100 copies of the Gammaretrovirus-like PtERV1, which is completely absent from the human genome [255, 638]. These retroviral relics are of importance because they are clear evidence of previous retroviral infections, and as such likely represent ancestral selective pressures.

TRIM5α has clearly been shown to be a major factor in determining the host range of both Gammaretroviruses and Lentiviruses in primates [217, 278, 444, 525, 633], and more recently this finding has been extended to Spumaviruses [632]. TRIM5α has been proposed to interact with capsid (CA) determinants of sensitive viruses and this interaction results in an abortive infection prior to nuclear entry [502, 541]. Recognition of TRIM5α-sensitive viral cores occurs soon after entry into the cell [116], and restriction results in production of normal to reduced levels of early reverse transcriptase products, but greatly reduced levels of full-length DNA [116, 541]. The actual mechanism of TRIM5α-mediated restriction and the requirements

for cellular cofactors remains incompletely understood. However, restriction appears to cause premature dissociation of viral cores [542], and may involve the degradative functions of the proteasome [11].

Studies have shown that TRIM5α has been under strong selective pressure over much of the course of primate evolution [483, 543], and that the TRIM5 locus is highly divergent [482]. All of these data suggest TRIM5α has evolved as an anti-retroviral agent and that cycles of retroviral challenge and subsequent selection for protective TRIM5α alleles has shaped primate evolution [483, 524]. An example of this type of selection, in response to a gammaretroviral pathogen, may have resulted in the susceptibility of humans to HIV infection [267]. This highlights the importance of studying TRIM5α and other restriction factors in general.

One of the aspects of TRIM5α biology that has yet to be fully explored is the breadth of activity of this protein. In the only study, to date, to test a Betaretrovirus for sensitivity to TRIM5α-mediated restriction, Lee and Bieniasz found that a reconstituted a HERV-K(HML-2) was not restricted by the tested TRIM5α proteins [311]. In the current study, we used M-PMV to more fully explore the possibility that TRIM5α can mediate post-entry blocks to betaretroviral infections. Cells isolated from 11 different species including human, Old World and New World monkeys, and prosimians were screened for infectivity using M-PMV engineered to be capable of a single round of infection pseudotyped with the VSV-G protein. We found that cells from three New World monkey species (squirrel, tamarin, and spider monkeys) showed a greater than 20-fold reduction in M-PMV infectivity, and time course experiments showed the block to be prior to completion of reverse transcription. When exogenously expressed in permissive cells, TRIM5α from squirrel and tamarin monkeys were found to restrict M-PMV infection. In squirrel and tamarin monkey fibroblasts small interfering RNA (siRNA) knockdown of TRIM5α and expression of dominant-negative TRIM5 both relieved the block to M-PMV infection. Conversely, expression of spider monkey TRIM5α in permissive cells failed to block M-PMV

infection and neither siRNA knockdown nor dominant-negative antagonism of TRIM5α in spider monkey fibroblasts relieved the barrier to M-PMV infectivity. These results demonstrate that New World monkey TRIM5α proteins have the capability of inhibiting betaretroviral infection and that these primates likely have multiple mechanisms for restricting betaretroviral infection.

MATERIALS AND METHODS

Cell culture

Information regarding cells used in this study is shown in Table 1. Penicillin (10U/ml) and streptomycin (10μg/ml) were added to all cultures. HeLa cells expressing the TRIM5α protein from human, rhesus macaque, African green monkey, squirrel monkey, tamarin monkey, and spider monkey [525] (kindly provided by Dr. Byeongwoon Song, Emory University) were cultured in the presence of 1μg/ml of puromycin. All TRIM5α-expressing Crandell feline kidney (CRFK) cells were maintained under the selection of 5μg/ml of puromycin.

Table 1. *Cells lines used in this study.*

Name	Species	Common name	Origin	Source	Culture Conditions
CRFK	*Felis catus*	Feline	Renal Fibroblast	ATCC	DMEM, 10% FBS
HOS	*Homo sapiens*	Human	Osteosarcoma	ATCC	DMEM, 10% FBS
HeLa	*Homo sapiens*	Human	Cervical Carcinoma	ATCC	DMEM, 10% FBS
293T	*Homo sapiens*	Human	Fetal Kidney	ATCC	DMEM, 10% FBS
CV-1	*Chlorocebus aethiops*	African Green Monkey	Kidney	ATCC	DMEM, 10% FBS
Cos-1	*Chlorocebus aethiops*	African Green Monkey	Kidney	ATCC	DMEM, 10% FBS
DBS-FRhL-2	*Macaca mulatta*	Rhesus	Foreskin Fibroblast	ATCC	α-MEM, 10% FBS
PR00559	*Papio hamadryas*	Guinea Baboon	Skin Fibroblast	Coriell	α-MEM, 15% FBS
AG07100	*Lemur catta*	Ring-tailed Lemur	Skin Fibroblast	Coriell	α-MEM, 10% FBS
AG06115	*Callicebus moloch*	Dusky Titi Monkey	Skin Fibroblast	Coriell	α-MEM, 10% FBS
AG05311	*Saimiri sciureus*	Common Squirrel Monkey	Skin Fibroblast	Coriell	α-MEM, 15% FBS
AG05308	*Saguinus labiatus*	Red-chested Mustached Tamarin	Skin Fibroblast	Coriell	α-MEM/DMEM (1:1), 20% FBS
AG05356	*Lagothrix lagotricha*	Common Woolly Monkey	Skin Fibroblast	Coriell	α-MEM, 10% FBS
AG05352	*Ateles geoffroyi*	Black-handed Spider Monkey	Skin Fibroblast	Coriell	α-MEM, 10% FBS

Viral production

Viral stocks were generated by calcium phosphate-mediated transfection of 293T cells [361, 460]. Briefly, $1x10^7$ 293T cells were plated in 150-mm^2 plates and transfected in the presence of 25mM chloroquine the following day. For the production of VSV-G pseudotyped green fluorescent protein (GFP)-expressing M-PMV virus, 21μg pSARM-EGFP [413, 533], an M-PMV proviral vector with the viral *env* gene replaced by EGFP, and 2μg pLP/VSV-G (Invitrogen) were transfected per 150-mm^2 plate. For the production of VSV-G pseudotyped Moloney Murine Leukemia Virus (MoMLV), 21μg pLXSG (kindly provided by Dr. Paul Lewis, Oregon Health Sciences University), 14μg pCS2+-mGP [625] Moloney *gag*/*pol* expression vector, and 7μg pLP/VSV-G were transfected per 150-mm^2 plate. For the production of VSV-G pseudotyped GFP-expressing HIV-1, 21μg pNL-EGFP/CMV-WPREΔU3, a vector based on pNL-EGFP/CMV [462] (which features the woodchuck post-transcriptional regulatory element (WPRE) for increased mRNA stability and a deleted U3 region for added safety), 14μg pCD/NL-BH*ΔΔΔ [655], and 7μg pLTR-G [461] were transfected per 150-mm^2 plate (all vectors kindly provided by Dr. Jakob Reiser, Louisiana State University Health Sciences Center). For the production of VSV-G pseudotyped GFP-expressing SIVmac239, 21μg pSIV/GFPΔenv [116] (kindly provided by Dr. Paul Bieniasz, Aaron Diamond AIDS Research Center) and 7μg pLP/VSV-G were transfected per 150-mm^2 plate.

In all cases, the medium was changed 16 hours post-transfection, and virus-containing medium was collected after an additional 48 hours. This virus-containing medium was spun at 500*g* for 5 minutes, passed through a 0.45-μm pore size filter, and stored at –80°C. During the course of our experiments it was observed that titers of M-PMV decreased around 200-fold after being frozen and thawed. Similar to previous results with other viruses [594], we found that addition of 10% dimethyl sulfoxide (DMSO) to the virus-containing medium prior to freezing prevented this loss of viral titer (data not shown).

Viral infectivity assays and transductions

Cells were seeded the day before infection at $5x10^4$ cells per well of a 6-well plate except in the case of 293T and CV-1, for which $1x10^5$ cells were plated. The following day, cells were exposed to 500μl total volume of virus-containing growth medium supplemented with 8μg/ml polybrene for a total of 16 hours at 37°C. Following this incubation period, the medium was replaced with fresh growth medium. For analysis by flow cytometry, 60-72 hours after exposure to virus, cells were trypsinized, spun at 500*g* for 5 minutes, and resuspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} supplemented with 2% FBS. Flow cytometry was conducted using the FACSCalibur system (BD Biosciences); at least 50,000 gated events were collected for each sample. Data analysis was conducted using the FlowJo software, Macintosh version 8.1.1 (Tree Star, Inc.). All viral stocks were titrated on the highly permissive CRFK cells using this method. Based on the CRFK infectivity functional multiplicities of infection (MOI) were determined.

TRIM5α cloning and expression

Total RNA was isolated from AG05311 (squirrel monkey), AG05352 (spider monkey), and AG05356 (woolly monkey) cells using TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was generated using anchored-oligo$(dT)_{18}$ primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche). Squirrel monkey TRIM5α was amplified using 'Squirrel F' (5'-GAGCAGGAATTCGCCACCATGTACCCATACGACGTCCCAGACTACGCTTCCAGAATCCTGGGGAGTATAAAGG-3') and 'Squirrel R' (5'-GAGCAGATCGATGGCTCAAGACCTTGGTGAGCACAGAGTCATGG-3'), spider monkey TRIM5α was amplified using 'Spider F' (5'-GAGCAGGAATTCGCCACCATGTACCCATACGACGTCCCAGACTACGCTTCCGAAATCCTGTTGAATATAAAGGAG-3') and 'Spider/Woolly R' (5'-

GAGCAGATCGATGGCTCAAGAGCTTGGTGAGCACAGAGTCATGG-3'), woolly monkey TRIM5α was amplified using ‘Woolly F’ (5'-GAGCAGGAATTCGCCACCATGTACCCATACGACGTCCCAGACTACGCTTCCGAAATCCTGGTGAATATAAAGGA-3') and ‘Spider/Woolly R’. All forward primers were designed to include a Kozak consensus sequence [294] and a sequence corresponding to the hemagglutanin (HA) tag [149]. Polymerase chain reaction (PCR) was conducted using pfu polymerase (Stratagene), and the resulting product was digested with EcoRI and ClaI and cloned into the MLV-based expression vector pLPCX (Clontech) cut with EcoRI and ClaI. Clones were sequenced; the TRIM5α sequences correspond to the accession numbers AY843517 (squirrel monkey), AY843516 (spider monkey), and AY843520 (woolly monkey) previously reported for these species. VSV-G pseudotyped TRIM5α-expressing MoMLV stocks were prepared in the same manner as GFP-expressing MoMLV virus described above, replacing pLXSG with a pLPCX-TRIM5α vector.

The generation of CRFK cells expressing the TRIM5α gene from human, rhesus, sooty mangabey, baboon, titi, and tamarin has been previously described [267, 333, 483]. To generate CRFK cells expressing woolly monkey, spider monkey, or squirrel monkey TRIM5α, CRFK cells were transduced with TRIM5α-expressing MLV in 6-well plates as described above. Two days after transduction, these cells were split and placed under selection with 5μg/ml of puromycin. Two weeks later, the puromycin-resistant cells were plated at low density (~200 cells/plate) in 150-mm^2 plates. Clones were picked from well-isolated colonies and expanded. TRIM5α expression was verified by Western blot analysis using monoclonal antibodies against the HA epitope (Covance) and β-actin (Sigma-Aldrich). Of the clones that express TRIM5α, two were tested for antiviral activity. In all cases similar results were obtained from both clones (data not shown).

Vesicular stomatitis virus infections

A viral stock of VSVΔG*-G [551], a vesicular stomatitis virus (VSV) derivative capable of a single-round infection that contains GFP in place of the G protein, was kindly provided by Dr. John Altman (Emory University). Cells were seeded the day before infection at $5x10^5$ cells per well of a 6-well plate and 16 hours later were exposed to a total volume of 500μl of virus-containing DMEM, supplemented with 8μg/ml polybrene and 2% FBS, for a total of 2 hours at 37°C. Following this incubation period the medium was replaced with fresh growth medium. Four hours later cells were trypsinized, fixed with PBS containing 4% paraformaldehyde, washed 3x with PBS supplemented with 2% FBS, and analyzed by flow cytometry.

Quantitative real-time PCR analysis of reverse transcription products

TaqMan primer/probe sets were developed to selectively amplify regions in either the strong-stop DNA RT product or the *gag* portion of the full-length RT product. The primers 'ssDNA F' (5'-CCACCATTAAATGAGACTTGATCAG-3'), 'ssDNA R' (5'-GGAGGGAGTGGGAATTGAAG-3'), and 'ssDNA probe' (5'-ACACTGTCTTGTCTCCATTTCTTGTGTCTCTTG-3') were used to quantify the strong-stop DNA product. The primers 'gag F' (5'-GCTTGGAAGATGAGGCAGCGAAAT-3'), 'gag R' (5'-ATTACAGTGGGTGCGGAAGGAGTA-3'), and 'gag probe' (5'-TAATCCCGATTGGCCTCCCTTCCTAA-3') were used to quantify full-length RT products. The strong-stop DNA probe was tagged at the 5' end with 6-Carboxyfluorescein (6-FAM), and the *gag* probe was tagged at the 5' end with hexachloro-6-carboxyfluorescein (HEX); both probes contained a 3' Iowa Black FQ non-fluorescent quencher (Integrated DNA Technologies).

One day prior to infection, $3x10^5$ cells were seeded in 60-mm^2 plates. VSV-G pseudotyped M-PMV-EGFP was DNase treated for 1 hour at 37°C with 1U TURBO DNase (Ambion) per 10μl virus. Cells were incubated in the presence of an approximate MOI of 0.35 of DNase-treated virus at 4°C for 2 hours in a total volume of 500μl CO_2-independent medium

(Gibco) supplemented with 2% FBS and 8μg/ml polybrene. Following this incubation, virus-containing medium was replaced with culture medium, and cells were placed at 37°C. At varying times thereafter, cells were harvested; total DNA was isolated using the Qiagen DNeasy kit. Real-time PCR reactions contained 1x FastStart TaqMan Probe Master Mix (Roche) with 400nM ROX passive reference dye, 250ng DNA, 250nM probe, and either 60nM *gag*-specific primers or 80nM strong-stop DNA specific primers. A standard curve was generated by two-fold serial dilutions of pSARM-EGFP. PCR reactions were performed in 384-well plates using the 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following reaction conditions: 5 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 59°C, and 30 seconds at 72°C. DNA harvested from cells incubated with virus lacking glycoprotein was used as a control for these experiments. In this control, quantitative real-time PCR (qPCR) analysis of this DNA detected approximately 1000 copies of strong-stop DNA at 0 hours and less than 200 copies at later time points, while less than 30 copies of gag DNA were detected at all times analyzed (data not shown).

<u>Generation of TRIM5 dominant-negative expressing cells</u>

Dual-expressing lentiviral vectors, based on the pNL-EGFP/CMV-WPREΔU3 vector, were generated in order to express dominant-negative forms of New World monkey TRIM5 proteins, which lack the B30.2 domain. These vectors were designed with the cytomegalovirus (CMV) promoter driving expression of a transcript containing the dominant-negative TRIM5, an internal ribosomal entry site (IRES), and yellow fluorescent protein (YFP). The encephalomyocarditis virus (EMCV) IRES was amplified from pIRESneo2 (Clontech) using the primers 'IRES F1' (5'-CTGCAGGATCCTCGCGATCGATGTCGACCGCCCCCCCCCCCCTAACGTTACTGGCCGAAGCC-3') and 'IRES R' (5'-CTTGCTCACCATGGTGGCGGCGCGCCTATTATCATCGTGTTTTTCAAAGGAAAACCAC

GTCCCCGT-3'). YFP was amplified from pEYFP-Golgi (Clontech) using the primers 'eYFP F' (5'-GATGATAATAGGCGCGCCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCAC-3') and 'eYFP R' (5'-ACCTCACTCGAGCGGCCGCTTACTTGTACAGCTCGTCCATGCCGAGAGTGAT-3'). A second round of PCR was performed utilizing the IRES and YFP PCR products as templates using the primers 'IRES F2' (5'-ACCTCAGCTAGCGCTACCGGTGAATTCCCGGGCCTGCAGGATCCTCGCGATCGATGTCGACCGCCC-3') and 'eYFP R'. The vector pNL-CXIYWΔ was generated by digesting the 1382 nucleotide IRES-YFP PCR fragment with NheI and XhoI and cloning it into pNL-EGFP/CMV-WPREΔU3 that was also digested with NheI and XhoI.

To generate the spider monkey TRIM5 dominant-negative vector, a TRIM5 fragment was digested from the pLPCX-based TRIM5α-expression vectors using EcoRI and EcoRV. To generate the dominant-negative tamarin monkey TRIM5 vector, the tamarin TRIM5α gene was amplified from AG05308 cellular RNA using the primers 'Squirrel F' and 'Squirrel R' as described above, followed by digestion with EcoRI and EcoRV. The EcoRI-EcoRV TRIM5 fragments were cloned into pNL-CXIYWΔ cut with BamHI that was filled in with Klenow, and EcoRI. This cloning strategy results in a truncated TRIM5 with an in-frame stop codon after amino acid 323. Clones were verified by sequencing.

VSV-G pseudotyped virus was generated from these vectors as described for GFP-expressing HIV. The resulting viruses were concentrated by ultracentrifugation for 2.5 hours at 100,000 *g* and used to stably transduce CRFK, TRIM5-expressing CRFK, or fibroblast cells. Western blotting was used to detect expression of HA-tagged TRIM5 dominant-negative protein, β-actin, as well as YFP (using rabbit polyclonal anti-GFP (Clontech)) in total protein extracts from transduced cells. YFP was found to be efficiently expressed via the IRES in CRFK cells, but was poorly expressed in the New World monkey fibroblasts.

TRIM5 dominant-negative expressing cells were infected with GFP-expressing challenge viruses as described previously. Analysis of GFP and YFP expression was performed using an LSRII flow cytometer (BD Biosciences) with the following filter configuration: 505LP – 510/21 for GFP detection and 525LP – 560/40 for YFP detection. This configuration allows for discrimination between YFP and GFP, in spite of their large spectral overlap. For each sample, at least 100,000 gated events were collected.

RESULTS

Cell lines from New World monkeys resist infection by M-PMV

In this study we investigated whether species-specific post-entry barriers to M-PMV infection exist by using a VSV-G pseudotyped, GFP-expressing M-PMV (M-PMV-EGFP) to screen a variety of cell lines for infectivity. M-PMV-EGFP expresses GFP instead of the viral envelope glycoprotein [413, 533]. When complemented *in trans* by an appropriate viral envelope glycoprotein the resulting virus is capable of entering a target cell, integrating into the chromosome, and expressing viral proteins, but infectious virus is not produced (data not shown). The use of GFP as a reporter gene, in conjunction with detection using flow cytometry, allows for the discrimination between establishment of infection, as determined by percent GFP-positive cells, and levels of viral gene expression, as determined by the intensity of GFP fluorescence. Viral stocks were pseudotyped with VSV-G in order to identify post-entry restrictions to M-PMV since it has been shown that VSV-G is able to mediate infection in a broad range of species and tissues [40, 78, 210, 332].

This VSV-G pseudotyped M-PMV-EGFP was used to infect cells isolated from humans, four Old World monkey, five New World monkey, and one prosimian species (Table 1 and Figure 1A). The cells were incubated overnight with an approximate MOI of 0.35; this MOI was used in order to minimize multiple integrations and remain within the linear range of flow cytometric analysis. All of the cell lines tested displayed mean fluorescent intensity (MFI) values

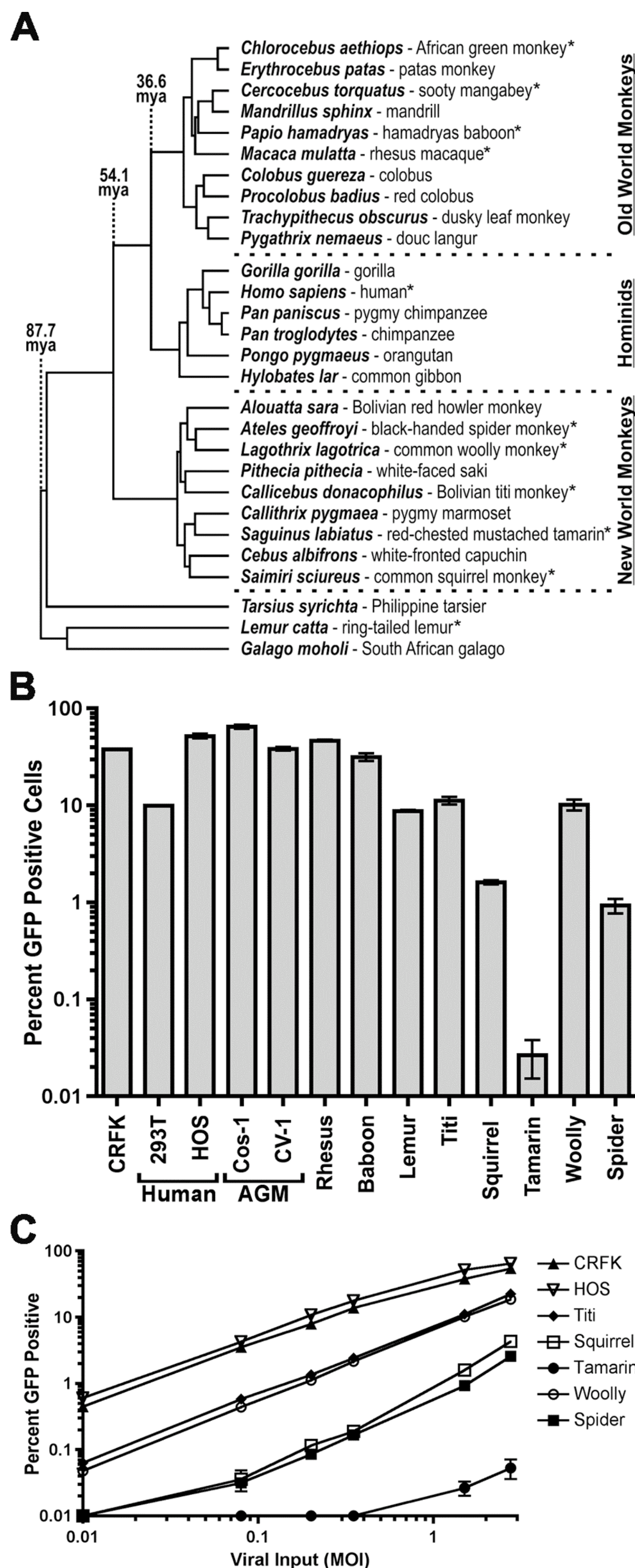

Figure 1. *M-PMV infectivity is reduced in New World monkey cell lines.*
(A) Phylogenetic tree showing the relationship of primate species, adapted from Bininda-Emonds et al. 2007 [49]. * indicates that cells from this species were examined in this study. Mya; millions of years ago.
(B) Results of transductions using VSV-G pseudotyped M-PMV-EGFP. Cells from the indicated species were incubated with approximately 0.35 MOI of virus overnight and analyzed for GFP expression by flow cytometry 48 hours later.
(C) Cells from the indicated species were incubated with increasing amounts of VSV-G pseudotyped M-PMV overnight and analyzed for GFP expression by flow cytometry 48 hours later.

between 190 (293T cells) and 725 (squirrel monkey fibroblasts) in GFP-positive cells, indicating comparable levels of viral gene expression in all cells once the virus entered and integrated.

However, the cells varied greatly in the efficiency of infection. The cell lines that were screened were classified into four categories of sensitivity (Figure 1B): those that were as permissive as CRFK, those that were 3- to 5-fold less permissive than CRFK, those that were 25- to 50-fold less permissive than CRFK, and those that were over 1000-fold less permissive than CRFK. All cells from Old World monkeys and one of the human cells lines (HOS) were readily infected with M-PMV. The other human cell line (293T) along with fibroblasts isolated from lemur, titi, and woolly monkeys displayed a slight (3- to 5-fold) decrease in M-PMV susceptibility. In contrast, fibroblasts derived from squirrel and spider monkeys displayed a marked decrease (25- to 50-fold) in M-PMV sensitivity. Finally, fibroblasts from tamarin monkeys were found to be highly resistant to M-PMV infection with greater than 1000-fold reduction in GFP-positive cells.

We also assessed M-PMV infectivity over a broad range of viral inputs in CRFK, HOS, and fibroblasts derived from New World monkeys (Figure 1C). Cells were incubated overnight with between 0.01 and 2.75 MOI of VSV-G pseudotyped M-PMV-EGFP, and infection was quantified by flow cytometry 48 hours later. The 3- to 5-fold M-PMV infectivity defect observed in titi and woolly fibroblasts and the stronger (25- to 50-fold) block in squirrel and spider monkey cells were observed at every viral input tested. In cells of the last two species, significant M-PMV infection was only observed at the highest virus inputs. In contrast, fibroblasts from tamarin monkeys exhibited infection levels near background (0.01% GFP positive cells) at all viral inputs tested. These results show that cells of New World monkey origin block M-PMV infection to varying degrees.

The block to M-PMV in squirrel, tamarin, and spider monkey fibroblast cells is virus specific

To determine the extent to which the VSV-G protein mediates entry into cells of different origins, the same panel of fibroblasts was infected with GFP-expressing VSV. Approximately 1 MOI of this virus, which is only capable of a single round of infection, was used to infect these cells (Figure 2A). Infection of CRFK and HOS cells resulted in approximately 62% of cells being GFP positive. However, infections of the primate fibroblast lines resulted in 20% to 27% GFP-

positive cells. Therefore, VSV appears to exhibit a 2.5- to 3-fold reduction in infectivity in these New World monkey fibroblasts. Similar results were observed when infections were carried out at an MOI of 0.1 (data not shown). Furthermore, all of the New World monkey fibroblast cells, with the exception of tamarin fibroblasts, produced as much GFP (as measured by MFI) as CRFK or HOS cells. GFP-positive tamarin fibroblasts were found to be 5- to 10-fold less bright than GFP-positive CRFK cells (data not shown), but this expression defect was distinct from establishment of infection. Thus, while there appears to be a small defect in VSV entry in the New World monkey fibroblasts, and for tamarin fibroblasts a second level of VSV restriction at the stage of viral gene expression, these defects do not account for the 25- to 1000-fold defect in M-PMV infectivity observed in squirrel, tamarin, and spider monkey cells.

Refractivity of M-PMV in the resistant New World monkey cells could also reflect a non-specific insensitivity to retrovirus infection. To control for this, MoMLV infectivity in these cells was therefore assessed by infecting cells with approximately 0.35 MOI of VSV-G pseudotyped GFP-expressing MoMLV (Figure 2B). All of cell types tested show a reduced susceptibility (3- to 11-fold) when compared to the very permissive CRFK. However, the pattern of reduced susceptibility was different from that seen in M-PMV infections.

In order to discern virus-specific differences, a ratio of M-PMV to MoMLV infectivity (designated $IR_{(M\text{-}PMV/MLV)}$) was determined for each of these cell lines (Figure 2C). An $IR_{(M\text{-}PMV/MLV)}$ value less than 1 indicates an M-PMV specific block in the cells, an $IR_{(M\text{-}PMV/MLV)}$ value above 1 suggests a MoMLV specific block, and $IR_{(M\text{-}PMV/MLV)}$ values near 1 indicate no specific block could be determined. Squirrel, tamarin, and spider monkey fibroblasts were found to have IR values of 0.5, 0.007, and 0.2, respectively; all of these values were determined to be significantly lower than 1 ($p < 0.05$) using Tukey's test. This data demonstrates that squirrel,

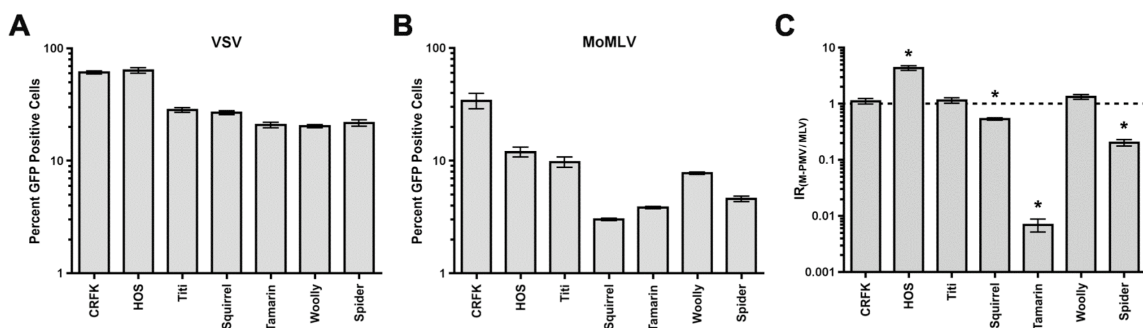

Figure 2. *Observed infectivity differences are specific to M-PMV.*
(A) Results of infections using VSVΔG*-G, a vesicular stomatitis virus derivative capable of a single-round infection that contains GFP in place of the G protein. Cells were incubated with approximately 1 MOI of virus for 2 hours and analyzed for GFP expression 5 hours later. (B) Results of transductions using VSV-G pseudotyped, GFP-expressing murine leukemia virus. Cells were incubated overnight in the presence of approximately 0.35 MOI of virus and analyzed for GFP expression 48 hours later. (C) The average infectivity ratio of M-PMV and MLV ($IR_{M\text{-}PMV/MLV}$) in selected cell lines. * denotes cell lines that have IR values that differ, in a statistically significant manner, from 1. Data shown are the means +/- SEM of representative experiments, each with n = 3.

tamarin, and spider monkey fibroblasts are not generically resistant to retroviral infection, but specifically resist M-PMV infection.

New World monkey fibroblasts display a post-entry block to M-PMV infection

To determine where the block to M-PMV infection occurred in the three non-permissive cell lines, qPCR was used to detect initial products of reverse transcription (minus-strand strong-stop DNA) as well as those representing complete transcription of the genome (*gag*) at varying times after M-PMV infection in CRFK cells, squirrel, tamarin, and spider monkey fibroblasts. Infections were synchronized by incubating fibroblasts or CRFK cells with approximately 0.35 MOI of DNase-treated VSV-G pseudotyped M-PMV-EGFP at 4°C, which allows the virus to adsorb to the cell surface but not enter or start reverse transcription. After adsorption, the medium was replaced, and the cells were incubated at 37°C for varying times. Total DNA was then harvested and strong-stop DNA or *gag* reverse transcription products were quantified. The results of strong-stop DNA product quantification are shown in Figure 3A. Readily quantifiable amounts of strong-stop DNA products were detected in squirrel, tamarin, and spider monkey fibroblast

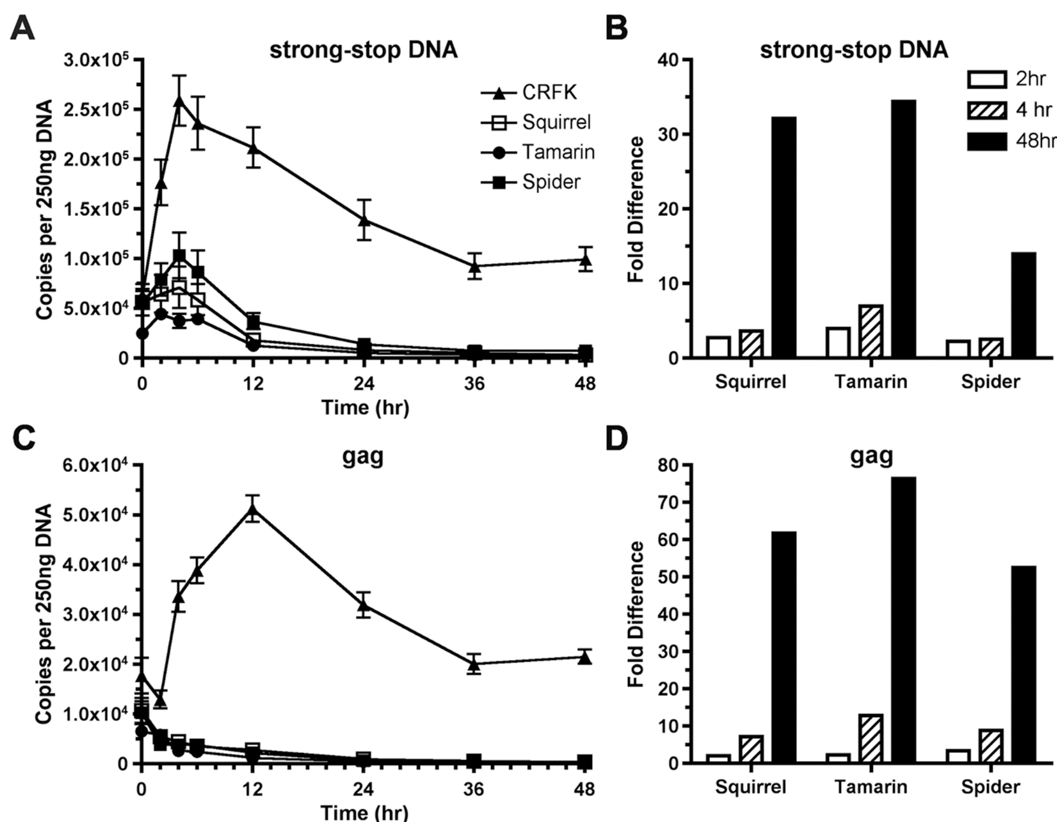

Figure 3. *Quantitative PCR analysis of reverse transcriptase products in primate fibroblast cells.*
$3x10^5$ cells were incubated with approximately 0.35 MOI of DNase-treated VSV-G pseudotyped M-PMV–EGFP virus for 2 hours at 4°C, after which the cells were returned to 37°C and total DNA was isolated at the indicated times. (A) Early reverse transcriptase products (strong-stop DNA) were detected in CRFK cells (filled triangles), spider monkey fibroblasts (filled squares), squirrel monkey fibroblasts (open squares), or tamarin monkey fibroblasts (filled circles) using quantitative real-time PCR. (B) Fold change in quantified strong-stop DNA products compared to CRFK cells at 2 hours (white), 4 hours (cross-hatched), and 48 hours (black) after start of infection. (C) Late reverse transcriptase products (*gag*) were detected in CRFK cells (filled triangles), spider monkey fibroblasts (filled squares), squirrel monkey fibroblasts (open squares), or tamarin monkey fibroblasts (filled circles) using quantitative real-time PCR. (D) Fold change in quantified *gag* products compared to CRFK cells at 2 hours (white), 4 hours (cross-hatched), and 48 hours (black) after start of infection. Data are the means +/- SEM from duplicate experiments; total n = 12.

cells early (2–6 hours) after infection. The amounts of strong-stop DNA detected in these fibroblast cells are however significantly reduced compared to that detected in CRFK cells. This decrease in detected strong-stop DNA is seen at all times examined, but the difference between the levels of strong-stop DNA quantified in CRFK cells compared to what is detected in the fibroblast lines increases over the time period examined. Figure 3B shows a 2.5- to 7-fold

reduction in strong-stop DNA levels in fibroblast lines at early times post-infection, and a much greater (13- to 35-fold) reduction in strong-stop DNA levels 48 hours after infection.

The levels of full-length reverse transcriptase products were also quantified, and the results are shown in Figure 3C. Reduced levels of late reverse transcriptase products were observed at all time points in the three New World monkey fibroblast cell lines compared to that of CRFK cells. At 2 hours post-infection there is a 2.1- to 3.4-fold reduction (Figure 3D); this difference increases to between a 7- and 13-fold reduction by 4 hours, and further to a 50- to 75-fold reduction by 48 hours post-infection. Combined, these qPCR data argue for two separate blocks to VSV-G pseudotyped M-PMV infection in these cells: a minor defect to viral entry attributable to VSV-G, and a post-entry block that manifests itself several hours after the initiation of infection. This post-entry block occurs after the initiation of reverse transcription but before reverse transcription can be completed, resulting in production of early reverse transcriptase products, but much reduced levels of late reverse transcriptase products. The timing and phenotype of this post-entry restriction to M-PMV infection of squirrel, tamarin, and spider monkey fibroblasts is reminiscent of the TRIM5α-mediated restriction of HIV-1 [511, 541].

TRIM5α from squirrel and tamarin monkeys, but not spider monkeys, restricts M-PMV infection

Due to the TRIM5α-like characteristics of the post-entry restriction to M-PMV infection in squirrel, tamarin, and spider monkey fibroblast cells, we explored the possibility that TRIM5α from these species accounts for the restriction to M-PMV infection. CRFK cells stably expressing human, rhesus macaque, sooty mangabey, baboon, titi monkey, or tamarin monkey TRIM5α from a retroviral vector have been previously described [267, 333, 483]. Additional CRFK clones stably expressing squirrel, woolly, or spider monkey TRIM5α from a pLPCX retroviral vector were generated for this study. Comparable levels of TRIM5α were expressed in these cells (Figure 4A and data not shown). It should be noted that the mass differences in TRIM5α detected

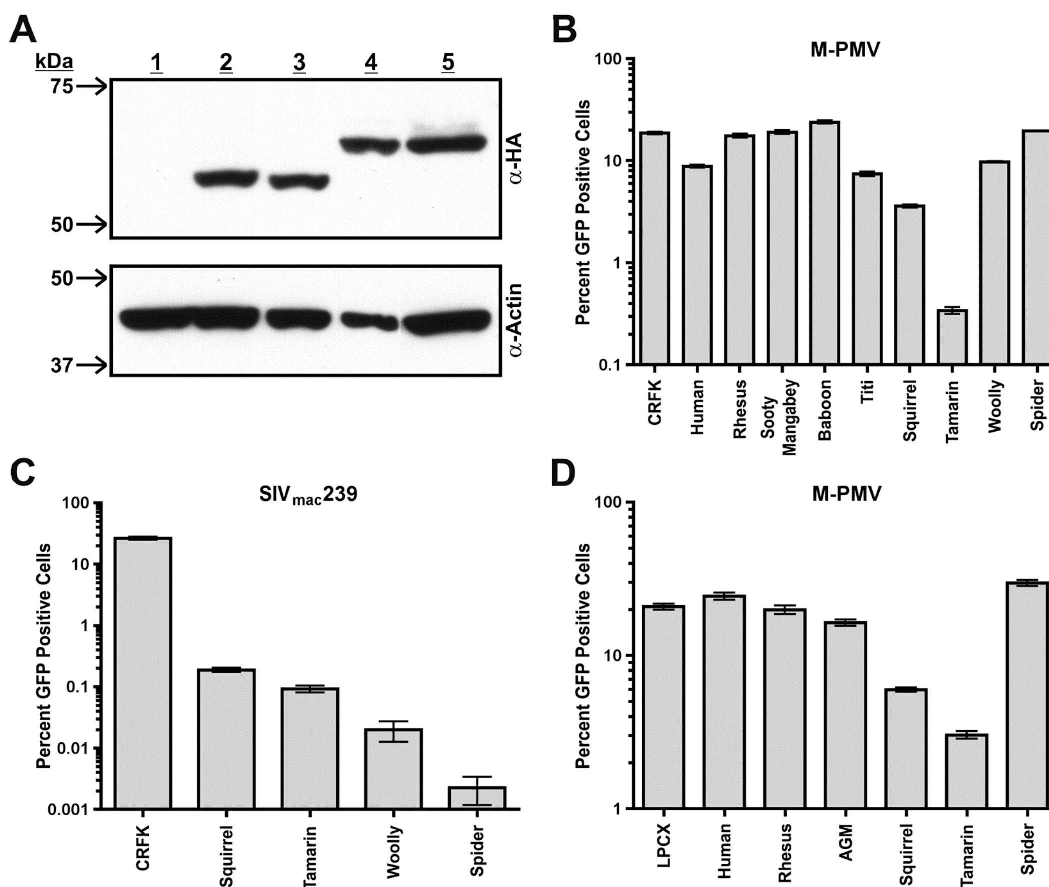

Figure 4. *Exogenous expression of tamarin and squirrel monkey TRIM5α inhibit M-PMV infection.*
(A) 30μg of total protein from CRFK cells (lane 1), or CRFK cells stably expressing HA-tagged TRIM5α from tamarin monkey (lane 2), squirrel monkey (lane 3), woolly monkey (lane 4), or spider monkey (lane 5) were subject to Western blot analysis for either the HA epitope (upper panel) or β-actin (lower panel). (B) CRFK cells expressing TRIM5α from the indicated species were incubated with approximately 0.25 MOI of VSV-G pseudotyped M-PMV-EGFP overnight and analyzed for GFP expression by flow cytometry 48 hours later. (C) CRFK cells expressing TRIM5α from the indicated species were exposed to approximately 0.35 MOI of VSV-G pseudotyped SIV/GFPΔenv overnight and analyzed for GFP expression 48 hours later. (D) HeLa cells stably transduced with TRIM5α from the indicated species or an empty vector control (LPCX) were exposed to approximately 0.25 MOI of VSV-G pseudotyped M-PMV-EGFP overnight and analyzed for GFP expression 48 hours later. Data show the means +/- SEM of representative experiments, each with n = 3.

in these blots is consistent with sequence data, in that the TRIM5α genes from woolly and spider monkeys have a tandem triplication in the v3 region of the B30.2 domain compared to the TRIM5α genes from squirrel and tamarin monkeys [483, 524].

The TRIM5α-expressing CRFK cells were incubated with VSV-G pseudotyped

M-PMV-EGFP overnight and analyzed for GFP expression 48 hours later. The results of a representative infection are shown in Figure 4B. Similar levels of GFP-positive cells were observed in CRFK cells expressing human, titi, and woolly monkey TRIM5α, consistent with the results from infection of the fibroblast lines. In contrast, cells expressing squirrel monkey TRIM5α showed a 5-fold reduction and tamarin monkey TRIM5α-expressing cells showed a 50-fold reduction in M-PMV infectivity. On the other hand, expression of spider monkey TRIM5α in CRFK cells did not inhibit M-PMV infection (this result was consistent in two independently isolated cell clones, data not shown). This data implicates TRIM5α as the factor responsible for the post-entry restriction to M-PMV in squirrel and tamarin monkey fibroblast cell lines. Based on the potency of the TRIM5α-mediated restriction of M-PMV in CRFK cells, and the qPCR data in New World monkey fibroblasts, the combination of a weak VSV-G mediated entry defect and a much stronger TRIM5α-mediated post-entry restriction can account for the bulk of the infectivity decrease observed in tamarin fibroblasts. Similarly, the 25-fold reduction in M-PMV infectivity in squirrel monkey fibroblast cells is the result of a weak defect in VSV-G mediated entry combined with a slightly more potent TRIM5α-mediated post-entry restriction.

In order to confirm the functionality of the spider monkey TRIM5α protein, CRFK cells expressing the different New World monkey TRIM5α proteins were challenged with 0.35 MOI of SIVmac239-based GFP-expressing virus [116]. SIV is known to be sensitive to restriction by the TRIM5α protein from many New World monkey species, including squirrel, tamarin, and spider monkeys [425, 523, 525, 636]. Figure 4C shows that CRFK cells expressing TRIM5α from these New World monkey species exhibit at least a 150-fold reduction in the infectivity of SIVmac239 when compared to the parental CRFK cells, confirming the functionality of the TRIM5α protein expressed in each of these cell lines.

The specificity of these TRIM5α proteins was further examined in challenges with VSV-G pseudotyped, GFP-expressing HIV-1 and MoMLV; a summary of these results is shown

in Table 2. MoMLV is not blocked by any of the New World TRIM5α proteins tested, whereas HIV-1 is restricted by the TRIM5α proteins from woolly and spider monkeys but appears insensitive to the squirrel or tamarin monkey TRIM5α proteins. The block to M-PMV infection in spider monkey fibroblasts was the only instance where expression of TRIM5α in CRFK cells did not reproduce a block to infectivity.

In order to confirm the weak restriction of M-PMV by squirrel monkey TRIM5α and rule out the possibility that spider monkey TRIM5α was inactive against M-PMV because of the cellular context in which it was expressed, HeLa cells expressing the same primate TRIM5α proteins (kindly provided by Dr. Byeongwoon Song, Emory University) were tested for susceptibility to M-PMV infection (Figure 4D). Expression of squirrel monkey TRIM5α in HeLa cells resulted in a 3.5-fold reduction in M-PMV infectivity, expression of tamarin monkey TRIM5α resulted in a 7-fold reduction in M-PMV infection, and no reduction in M-PMV infectivity was seen in cells expressing the spider monkey TRIM5α (Figure 4D). These results confirm the observations from TRIM5α-expressing CRFK cells, although the potency of restriction is reduced. This may result from the formation of TRIM5α heterotrimers in these cells, which also endogenously express the human TRIM5α protein. Thus it appears that expression of the TRIM5α protein from squirrel or tamarin monkeys is capable of blocking M-PMV infection, whereas a novel mechanism appears to be responsible for the restriction of M-PMV infection in spider monkey cells.

TRIM5α restricts M-PMV during early post-entry stages

In order to verify that the phenotype of the restriction observed in TRIM5α-expressing CRFK cells was similar to that seen in the fibroblast cells, qPCR was used to detect strong-stop DNA and *gag* reverse transcript products. In CRFK cells and squirrel monkey TRIM5α-

Table 2. *Correlation of viral infectivity in New World monkey fibroblasts and CRFK cells expressing TRIM5α from these New World monkeys.*

Species	Cell Type	M-PMV	HIV	SIV	MLV
Human	HOS	-[a]	-	-	-
	CRFK-hu	-	ND	ND	ND
Titi	Fibroblast	-	-	-	-
	CRFK-Titi	-	ND	ND	ND
Squirrel	Fibroblast	++	+	++++	+
	CRFK-SqM	+	-	+++	-
Tamarin	Fibroblast	+++	-	++++	+
	CRFK-Tam	++	-	+++	-
Woolly	Fibroblast	-	++	++++	+
	CRFK-WM	-	+	++++	-
Spider	Fibroblast	++	+++	++++	+
	CRFK-SpM	-	++	++++	-

[a] - indicates less than a 5 fold reduction in infectivity when compared to CRFK infectivity. + indicates between 5-fold and 20-fold reduction in infectivity. ++ indicates between 20- and 100-fold reduction in infectivity. +++ indicates between 100- and 1000-fold reduction in infectivity. ++++ indicates greater than 1000-fold reduction in infectivity. ND; not determined.

expressing CRFK cells equivalent levels of early reverse transcriptase products are produced, but these products are mostly lost over the first 24 hours in squirrel monkey TRIM5α-expressing cells (Figure 5A). By 48 hours post-infection there is 2.5-fold less strong-stop DNA in these cells compared to CRFK cells. In tamarin TRIM5α-expressing cells there was already a 3- to 4-fold reduction in the amount of strong-stop DNA products between 2 and 6 hours post-infection compared to CRFK cells, indicating that the tamarin TRIM5α blocks M-PMV at a slightly earlier stage in these cells. The difference in infectivity further increased to a 14-fold by 48 hours after infection.

In contrast, the levels of *gag* products are reduced 3-fold at 4 hours post-infection in cells expressing either squirrel or tamarin monkey TRIM5α proteins (Figure 5B). In tamarin monkey TRIM5α-expressing cells, the difference in *gag* products increases to 35-fold at 48 hours post-

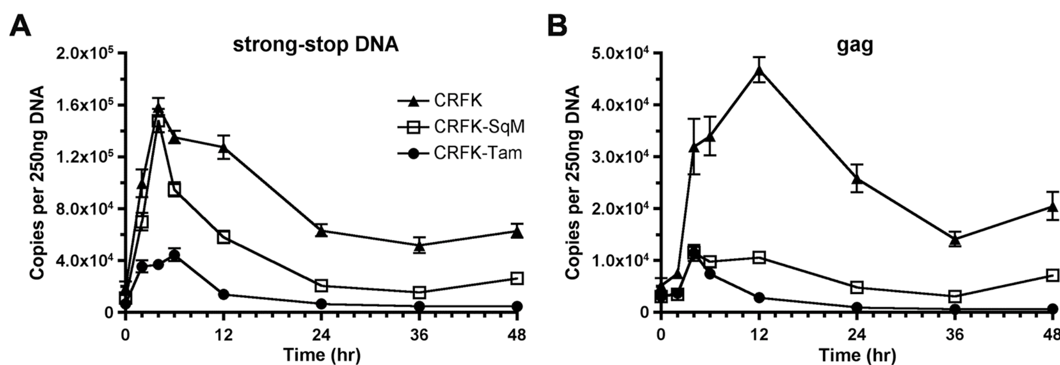

Figure 5. *Quantitative PCR analysis of reverse transcriptase products in TRIM5α expressing CRFK cells.*
CRFK (filled triangles) or CRFK cells expressing squirrel monkey TRIM5α (open squares), or tamarin monkey TRIM5α (filled circles) were incubated with approximately 0.35 MOI of DNase-treated VSV-G pseudotyped M-PMV-EGFP virus for 2 hours at 4°C, after which the cells were returned to 37°C. Total DNA was isolated at the indicated times and used to detect early (strong-stop DNA) and late (*gag*) reverse transcriptase products. Data are the means +/- SEM of a representative experiment with n=6.

infection, whereas the difference in *gag* products only slightly increases over this time in squirrel monkey TRIM5α-expressing cells. These qPCR results are strikingly similar to those obtained in squirrel and tamarin monkey fibroblasts, in that late reverse transcription is affected more than early.

Inhibition of TRIM5α in Spider monkey cells does not relieve the observed restriction of M-PMV infection

The data presented above point to a post-entry block to M-PMV infection in spider monkey fibroblasts that occurs at a similar point in the viral lifecycle as TRIM5α-mediated restriction. The inability of the TRIM5α from these cells to block M-PMV in permissive cells suggests that either a species-specific cofactor is required for TRIM5α activity or that a novel restriction factor is functioning in spider monkey fibroblasts. In order to differentiate between these possibilities, we have investigated the effect of inhibiting TRIM5α activity in these cells.

Initial experiments employing siRNAs targeting spider or tamarin monkey TRIM5α expression, although capable of modestly increasing (17.5-fold and 7.5-fold respectively)

susceptibility to infection by highly sensitive SIV, showed no effect on M-PMV infection of spider monkey cells and only increased M-PMV infection 1.5-fold in tamarin monkey cells (data not shown). We therefore utilized expression of a dominant-negative TRIM5 to more effectively inhibit the function of this protein.

i) *Truncated forms of New World monkey TRIM5 can counteract the antiviral activity of TRIM5α exogenously expressed in CRFK cells.*

Bicistronic lentiviral vectors were constructed that express YFP and truncated forms of the spider and tamarin monkey TRIM5 proteins, which lack the B30.2/SPRY domain. Similar truncated proteins have been shown to act as dominant-negative inhibitors of TRIM5α-mediated anti-HIV activity in cells from several different species of Old World monkeys [343].

To verify that the lentiviral vectors produced both the truncated forms of TRIM5 and YFP, CRFK cells were transduced with empty (NL-CXIYWΔ) vector or vectors expressing truncated forms of spider or tamarin TRIM5. Western blot analysis of total protein extracts taken from these cells (Figure 6A) show an HA-tagged protein of a size consistent with that predicted for the truncated forms of TRIM5 (~39 kDa). In addition, transduced cells produced detectable levels of YFP, although more YFP is produced from the empty vector than those expressing the mutant versions of TRIM5. Similar results were observed with flow cytometric detection of YFP, where empty vector transduced cells were 5- to 10-fold brighter than TRIM5-expressing vector transduced cells. We believe this difference in expression is due to more efficient IRES-independent initiation of YFP translation in the empty vector.

To evaluate the dominant-negative potential of these truncated forms of TRIM5, CRFK cells stably expressing spider or tamarin monkey TRIM5α (CRFK-SpM and CRFK-Tam respectively) were further transduced with either empty vector or vectors expressing a truncated form of the cognate TRIM5. These cells were then tested for their sensitivity to infection by VSV-G pseudotyped SIV, which is sensitive to TRIM5α from both of these species. Infectivity

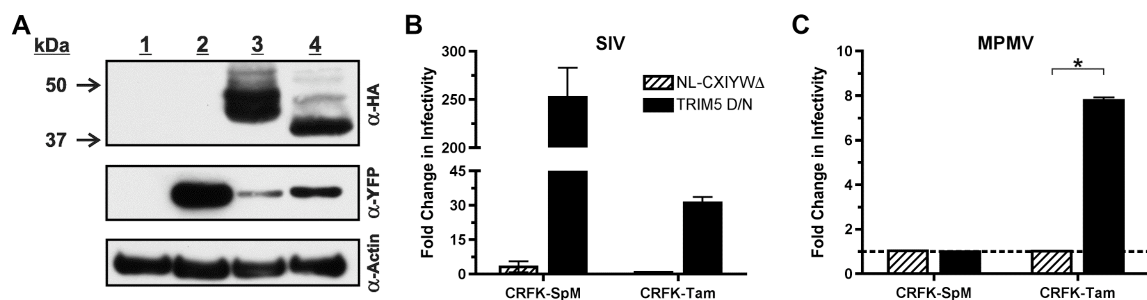

Figure 6. *Truncated forms of TRIM5 have dominant-negative activity in TRIM5α expressing CRFK cells.*
(A) Total protein from mock transduced CRFK cells (lane 1), or CRFK cells transduced with NL-CXIYWΔ (lane 2), HA-tagged dominant-negative TRIM5α from spider monkey (lane 3), or tamarin monkey (lane 4) was subjected to Western blot analysis for the HA epitope (upper panel), YFP using a polyclonal anti-GFP antibody (middle panel), or β-actin (lower panel). (B and C) CRFK cells stably expressing spider or tamarin monkey TRIM5α were transduced with either the empty (NL-CXIYWΔ) vector or a lentiviral vector expressing a truncated form of the cognate TRIM5. (B) The transduced cells were exposed to approximately 0.35 MOI of VSV-G pseudotyped SIV/GFPΔenv overnight and analyzed for YFP and GFP expression 60 hours later. Fold difference of SIV infectivity (GFP positive) in TRIM5 dominant-negative transduced (YFP positive) cells compared to infectivity in untransduced (YFP negative) cells. (C) Cells were exposed to approximately 0.5 MOI of VSV-G pseudotyped M-PMV overnight and analyzed for GFP expression 60 hours later. Fold change in M-PMV infectivity (GFP positive) in TRIM5 dominant-negative transduced (YFP positive) cells compared to infectivity in untransduced (YFP negative) cells. All data represents the mean +/- SEM with an n = 3. * denotes a *p* value calculated to be less than 0.05 using Tukey's test. D/N; dominant-negative.

was assessed using a two-color flow cytometric assay employed previously by Bock et al. [54, 633]. In this assay, the percentage of TRIM5 dominant-negative/YFP positive cells infected with the GFP-expressing challenge virus was quantified and compared to the percentage of untransduced/YFP negative cells infected with the GFP-expressing challenge virus. As seen in Figure 6B, transduction of CRFK-SpM cells with the vector expressing a truncated form of spider monkey TRIM5 resulted in a 250-fold increase in SIV infection and CRFK-Tam cells expressing the truncated form of tamarin TRIM5 were 30-fold more sensitive to SIV infection, compared to untransduced cells. No difference was observed in SIV sensitivity when cells were transduced with the empty vector. These results clearly demonstrate that expression of the truncated forms of New World monkey TRIM5 can interfere with TRIM5α function.

The cells were also probed for their sensitivity to VSV-G pseudotyped M-PMV infection. As shown in Figure 6C, expression of the dominant-negative form of tamarin monkey TRIM5 resulted in a 7.8-fold increase in M-PMV infectivity, to a level similar to that observed in parental CRFK cells. Expression of the dominant-negative spider monkey TRIM5 did not affect M-PMV infectivity in CRFK-SpM cells, which are as permissive as parental CRFK to M-PMV infection. This is consistent with additional control experiments, where expression of truncated TRIM5 proteins in CRFK cells lacking exogenous TRIM5α expression had no effect on the infectivity of any virus tested (data not shown). Together this data demonstrates that the TRIM5 dominant-negative proteins function specifically to inhibit TRIM5α activity.

ii) *Expression of dominant-negative TRIM5 in spider monkey fibroblasts does not increase M-PMV infectivity in these cells*

Spider and tamarin monkey fibroblast cells were transduced with the lentiviral vectors described above in order to determine whether expression of the dominant-negative proteins could overcome the block to M-PMV infection. Total protein extracts isolated from these cells were subjected to Western blot analysis (Figure 7A), which showed that the truncated form of TRIM5 was expressed in both fibroblast lines. While transduction with the empty vector resulted in detectable levels of YFP expression, it was undetectable in cells transduced with the TRIM5 dominant-negative expressing vectors. Thus, it appears that in these New World monkey cells the EMCV IRES utilized to produce YFP failed to efficiently promote internal ribosomal entry. Due to this, YFP was quantified but not used as a marker of lentiviral transduction in all further experiments with these cells.

Transduced fibroblasts were first challenged with the potently restricted SIV in order to examine TRIM5 dominant-negative efficacy in these cells. As seen in Figure 7B, expression of dominant-negative TRIM5 proteins in spider or tamarin monkey cells results in much greater sensitivity to SIV infection. A 500-fold increase in SIV infectivity was seen when dominant-negative spider monkey TRIM5 was expressed in spider monkey fibroblasts and a 90-fold

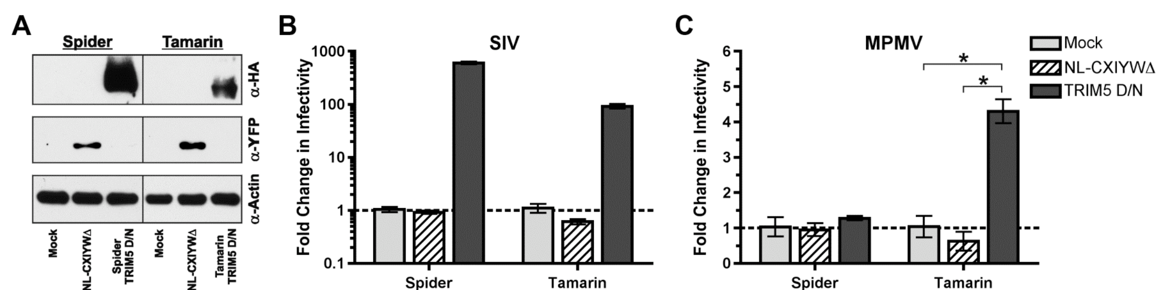

Figure 7. *Expression of dominant-negative spider monkey TRIM5 does not enhance M-PMV infectivity in spider monkey fibroblasts.*
Spider and tamarin monkey fibroblasts were each transduced with TRIM5 dominant-negative expressing lentiviral vectors, empty (NL-CXIYWΔ) vector, or mock transduced. (A) Total protein from these cells was subjected to Western blot analysis for the HA epitope (upper panel), YFP (middle panel), or β-actin (lower panel). (B) These cells were then exposed to approximately 1 MOI of VSV-G pseudotyped SIV/GFPΔenv overnight and GFP expression was analyzed 60 hours later. Fold change in SIV infectivity was calculated using average infectivity in mock transduced cells as the control. (C) These cells were exposed to approximately 1 MOI of VSV-G pseudotyped M-PMV-EGFP overnight and GFP expression was analyzed 60 hours later. Fold change in M-PMV infectivity was calculated using average infectivity in mock transduced cells as the control. All data represents the mean +/- SEM with an n = 3. * denotes a *p* value calculated to be less than 0.05 using Tukey's test. D/N; dominant-negative.

increase was noted when tamarin monkey fibroblasts were transduced with dominant-negative tamarin monkey TRIM5. In contrast, no increase in SIV sensitivity was seen when these cells were transduced with the empty vector. Thus, while these are heterogeneous populations of transduced and untransduced cells it is clear that expression of dominant-negative forms of the cognate TRIM5 results in strong suppression of TRIM5α antiviral activity.

The lentiviral transduced cells were further assessed for their susceptibility to M-PMV infection. When challenged with VSV-G pseudotyped M-PMV, expression of dominant-negative tamarin TRIM5 in tamarin monkey fibroblasts resulted in a 4.2-fold increase in M-PMV infectivity (Figure 7C), which was calculated to be statistically significant ($p < 0.05$) using Tukey's test. This confirms that TRIM5α is involved in the post-entry block to M-PMV in tamarin monkey fibroblasts. However, it should be noted that this represents a rather modest increase in infectivity considering the greater than 90-fold increase observed with SIV. Thus it is likely that additional determinants of restriction exist in these cells.

In contrast to the results in tamarin fibroblasts, there was no significant difference in M-PMV infectivity in spider monkey fibroblasts transduced with the TRIM5 dominant-negative expression vector or the empty vector (Figure 7C), even though SIV infection was enhanced 500-fold in the presence of the dominant-negative TRIM5 protein. Thus the results suggest that TRIM5 is not involved in the post-entry restriction observed in spider monkey cells.

DISCUSSION

Previous reports have shown that TRIM5α proteins from various species can restrict infection by members of the Gammaretrovirus, Lentivirus and Spumavirus genera of retroviruses [217, 278, 444, 525, 541, 632, 633]. Lee and Bieniasz have investigated TRIM5α-mediated restriction of the human endogenous Betaretrovirus HERV-K(HML-2) [24, 33, 238, 576], but found that this virus was insensitive to human TRIM5α activity [311]. Here, we demonstrate that the TRIM5α proteins from squirrel and tamarin monkeys are able to inhibit infection of M-PMV, a prototypical Betaretrovirus. In CRFK cells exogenously expressing the TRIM5α protein encoded by the tamarin monkey we observed a high level of restriction (>50-fold inhibition) and the block was demonstrated to be at an early point in the infection cycle, prior to completion of reverse transcription. This is analogous to the results reported for TRIM5α inhibition of primate Lentiviruses in Old World monkey cells [116, 541]. Moreover, inhibition of TRIM5α function in tamarin monkey fibroblast cells significantly enhanced M-PMV infectivity, confirming that endogenous TRIM5α plays a role in restricting primate Betaretrovirus infection in these cells. Thus, our data provide additional evidence that TRIM5α has evolved as an anti-retroviral protein [267, 482, 483, 525] with the potential to block infection by a broad range of retroviruses [415].

In order to exert its anti-viral activity, TRIM5α is hypothesized to target the CA core of an incoming retrovirus since substitutions in this Gag domain of HIV by that of SIV can modulate susceptibility to TRIM5α [217, 433] and even single amino acid changes can perturb this interaction [432]. Similarly, substitution of one or more amino acids in the SPRY domain of

TRIM5α can alter the viral specificity [543, 634]. The CA proteins from SIVmac239 and M-PMV share less than 20% identity and yet infection by both viruses is restricted by the TRIM5α proteins from both squirrel and tamarin monkeys. Given the exquisite specificity of this interaction, it is remarkable that a single TRIM5α protein can restrict multiple retroviruses of different genera with such highly divergent CA proteins (Table 2 and [415]). Moreover, the ability to recognize diverse CA molecules lends credence to the hypothesis that TRIM5α trimers interact with complex topological structures of three-fold pseudosymmetry present on the surface of the retroviral CA cores [389]. Under such a model, similar topological features may be presented on the surface of CA cores irrespective of the primary sequence differences of the CA proteins themselves.

Three retroviruses have been isolated from New World monkey species: an endogenous Betaretrovirus was found in squirrel monkeys [110, 221], an infectious Gammaretrovirus was isolated from woolly monkeys [557, 612], and an endogenous Gammaretrovirus was discovered in owl monkeys [564]. The squirrel monkey retrovirus (SMRV) is believed to be a recent addition to the squirrel monkey genome based on the fact that it is activated following treatment with the DNA damaging agent 5-iododeoxyuridine [221] and viral RNA does not significantly hybridize to DNA isolated from the closely related capuchins (Figure 1A and [110]). The demonstration that TRIM5α proteins of squirrel and tamarin monkeys are able to specifically block M-PMV infection may reflect prior selective pressure imposed by the relatively recent exposure of these species to members of the Betaretrovirus genus. Interestingly, Lee and Bieniasz found that HERV-K(HML-2) showed a greater than 10-fold reduction in infectivity in squirrel monkey cells compared to CRFK cells [311], raising the possibility that squirrel monkey TRIM5α is also able to restrict HERV-K(HML-2) infection.

The inability of TRIM5α from titi, woolly, and spider monkeys to block M-PMV infection may reflect an evolutionary history shaped by gammaretroviral infection, evidenced by the isolation of

at least one infectious Gammaretrovirus from woolly monkeys [612]. Based on the fixation of a peculiar retrotransposition of cyclophilin A into the owl monkey TRIM5 locus [418, 484], which results in the production of a TRIM5-cyclophilin A fusion protein (TRIM-Cyp), it is clear there have been virus-specific events in New World monkeys that have shaped the evolution of their extant viral resistance genes. This is an unexpected evolutionary adaptation since cyclophilin A has only been shown to bind to lentiviral capsids, and yet to date no members of the lentiviral genus have been isolated from any species of New World monkeys. In this case it is possible that the endogenous retrovirus isolated from owl monkeys may have played a role in selecting for this TRIM-Cyp, although it is equally likely that this virus exploited an inability to be recognized by the owl monkey TRIM-Cyp in order to establish itself in the owl monkey genome. However, given the general dearth of information regarding retroviral infection in New World primates and the possibility that the virus responsible for exerting selective pressure was unable to establish germline transmission, correlating a TRIM5α activity in these species with specific retroviruses remains a difficult endeavor.

In addition to the TRIM5α-associated restriction of M-PMV infection seen in squirrel and tamarin monkeys, we show that there is an M-PMV-specific post-entry block to infection in spider monkey fibroblasts. This block could not be relieved by expression of a dominant-negative TRIM5 protein, even though expression of the same protein enhanced susceptibility to SIV infection in these cells more than 500-fold. Based on qPCR data, this block appears to be at a similar stage of infection to that observed in squirrel and tamarin monkey fibroblast cells. However, because the block in spider monkey fibroblasts could not be ascribed to TRIM5α we believe that these cells express a novel restriction factor capable of resisting M-PMV infection. Further, it is possible that a similar factor may contribute to the high level of resistance observed in tamarin monkey fibroblasts, since expression of dominant-negative TRIM5 in these cells incompletely reversed the block to M-PMV infection. This TRIM5α-independent restriction of

M-PMV therefore represents an opportunity to identify and more fully understand additional host-virus interactions during the early stages of retrovirus infection.

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ALTERED TRANSCRIPTIONAL REGULATION OF MEMBERS OF THE *TRIM5*, *TRIM22*, *TRIM34*, *TRIM6* LOCUS AS A RESULT OF DIFFERENTIAL FIXATION OF ENDOGENOUS RETROVIRAL ELEMENTS IN THE NON-CODING REGIONS OF THESE GENES

by

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ABSTRACT

At least two genes in the *TRIM6*/*TRIM34*/*TRIM5*/*TRIM22* locus, *TRIM5* and *TRIM22*, are interferon inducible and have demonstrable antiviral activity. There is compelling evidence that TRIM5 and TRIM22 have evolved under recurrent episodes of positive selection over the course of the last 35 million years of primate evolution. The coding region of TRIM5, which can inhibit the replication of a diverse range of retroviruses, has undergone unusually high rates of nonsynonymous nucleotide replacements, in-frame insertions and deletions, and other more exotic indicators of strong positive selective pressure.

To date, evolutionary studies of the genes in the *TRIM6*/*TRIM34*/*TRIM5*/*TRIM22* locus have largely focused on the coding sequence of these genes. In order to assess the evolutionary contributions that changes in the non-coding regions of these genes, we analyzed the transcribed regions, including introns, of *TRIM5*, *TRIM22*, *TRIM6* and *TRIM34* genes from three divergent primate lineages (human, rhesus macaque, and squirrel monkey). We found that the antiviral *TRIM5* locus displays the highest interspecies variability, much of which is due to the differential insertion and deletion of transposable elements. In particular, fixation of long terminal repeats (LTRs) from multiple, ancient, endogenous retroviruses was found to be concentrated in the first introns of *TRIM5* and *TRIM22*. Using quantitative RT-PCR and chromatin immunoprecipitation, we demonstrate that differential fixation of one such endogenous retroviral LTR results in species-specific differential transcriptional regulation of the gene.

Thus, in addition to the well-established effect amino acid changes have on antiviral activity, our results raise the possibility that retroelement-mediated, species-specific differences in transcriptional regulation may represent another mechanism for cross-species differences in *in vivo* antiviral potency of these genes.

AUTHOR SUMMARY

Numerous studies have demonstrated that TRIM5 and TRIM22 are intracellular inhibitors of viral replication, meaning these genes must be expressed in cells targeted for viral

replication. Viruses shown to be susceptible to the antiviral effects of these genes infect very different target cells, yet both genes have been shown to have distinct tissue expression patterns. Here, we examined the genomic sequences of these genes from representative primate species for evidence of changes that might lead to altered transcription of the genes in these species. We discovered that multiple endogenous retroviral elements containing transcriptional regulatory sequences have become fixed in the non-coding regions of *TRIM5* and *TRIM22* during primate evolution, and that presence of these elements can alter TRIM gene transcription. These findings suggest that *TRIM5* and *TRIM22* have co-opted endogenous retroviral elements to rapidly adapt their expression to new viral pathogens.

INTRODUCTION

Greater than 70 tripartite motif-containing (TRIM) genes are encoded in the human genome, each characterized by the ordered presence of an N-terminal RING domain, followed by one or two B-box domains. One or more of a handful of domains can form the C-termini of these genes, with SPRY or PRY-SPRY (B30.2) domains present in the majority of human TRIM genes [480]. Several TRIM genes have been shown to possess anti-retroviral activity, including the B30.2 domain containing TRIM5 and TRIM22 [473, 541, 563].

The initial report demonstrating *TRIM5* antiviral activity identified the alpha splice variant of rhesus macaque *TRIM5* as responsible for a well-characterized post-entry block to HIV-1 infection in macaque cells [541]. In the time following that report, multiple laboratories have characterized the interactions between TRIM5α and retroviruses. These efforts have revealed antiretroviral activities for TRIM5α of many primate species, including members of the hominoid, cercopithecidae (Old World monkey), and platyrrhini (New World monkey) families [128, 215, 278, 291, 476, 483, 525, 543, 632, 633, 636]. This antiviral activity appears to be a general feature of this gene as orthologs from cow and rabbits display a similar ability to block retroviral infection [486, 514, 635].

A further feature of the virus-TRIM5α interaction that these studies have uncovered is that the TRIM5α protein from each species displays a very specific pattern of antiviral activity [reviewed in 415]. The specificity of productive interaction appears to be determined, on the TRIM5α side, largely by the composition of the B30.2 domain. While the viral target of TRIM5α appears to be a conformation-dependent epitope presented on viral capsid (CA) cores, which are only displayed following viral maturation [127, 253, 254, 403, 432, 440, 442, 443, 484, 502, 542, 543, 634]. As might be expected for a gene in direct competition with pathogenic invaders, TRIM5α has undergone high levels of positive selection with much of this pressure localized to several variable patches in the B30.2 domain [483, 524]. Furthermore, cDNA retrotransposition has resulted in replacement of the B30.2 domain of TRIM5α with the known CA interacting protein, cyclophilin A. This has occurred twice during primate evolution, once in owl monkeys and separately in certain macaque lineages [67, 324, 414, 418, 484, 587, 607].

TRIM5 is found in a cluster of four closely related TRIM genes, including *TRIM6*, *TRIM22* and *TRIM34*, in an olfactory receptor-rich region on chromosome 11 of the human genome (Figure 1). Phylogenetic analyses of the TRIM family suggest that the *TRIM6/34/5/22* cluster evolved by gene duplication from a common ancestral gene [480]. Among these, *TRIM22* has been reported to encode antiviral activity against HIV-1, although the two reports differ on the mechanism by which this is achieved [27, 563]. It has also been reported that TRIM22 expression can block hepatitis B virus and encephalomyocarditis virus replication [140, 167]. Consistent with the antiviral activity observed for this protein, *TRIM22* has been under strong positive selection throughout primate evolution [482]. The same study also suggests that evolutionary constraints have been placed on this locus whereby at any given time only one of these two antiviral genes (*TRIM5* or *TRIM22*) has been under positive selection. In contrast, during this time the neighboring genes, *TRIM6* and *TRIM34*, have been under consistent purifying selection.

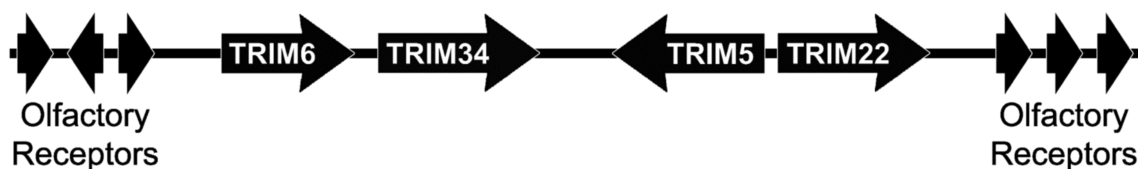

Figure 1. *Primate TRIM6/34/5/22 gene locus.*
Graphical depiction of the *TRIM6*, *TRIM34*, *TRIM5*, and *TRIM22* genomic locus of primates in the context of neighboring olfactory receptor genes.

A large proportion of the human genome is comprised of repetitive elements. Retrotransposons account for approximately 43% of the entire human genome, in the form of long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and endogenous retroviral (ERV) elements, while DNA transposons account for approximately another 2% of the genome. Many of these elements have been found to possess the potential to alter transcriptional regulation. The LINE family of autonomous retrotransposons have been found to affect transcriptional regulation by modulating normal splicing [31, 32], altering polyadenylation [439], interfering with transcriptional elongation [209], and providing novel promoter activity for neighboring genes [529]. Members of the SINE family of non-autonomous retrotranspons have been shown to affect polyadenylation [94, 309, 399], alternate splicing patterns [399], and translation efficiency [reviewed in 214]. ERV sequences in the genome are primarily represented by their long terminal repeats (LTRs) [312], which when active provided promoter and other transcriptional regulatory activity for the viruses. As such, examples have been described of ERV LTRs providing primary or alternate promoters, enhancer elements [479, 598], protein-binding sites, and polyadenylation signals [reviewed in 326].

TRIM5 and *TRIM22* produce intracellular proteins and exert their antiviral activity within the cell. However, these genes lie adjacently and in opposing orientations in the genomes of primates such that their core promoters reside in a shared ~5-kb intergenic region, which is largely conserved between humans and rhesus macaques (Fig 1 and Diehl W.E., et al. unpublished data). Thus, we analyzed the entire transcribed sequence of *TRIM5* and *TRIM22*, including intronic sequences, in

search of differences with the potential for governing differential transcriptional regulation the transcription of these genes. For comparison, similar analyses were performed on the related, but conserved, genes *TRIM6* and *TRIM34* that lie adjacent to *TRIM5* and *TRIM22*. It was observed that *TRIM5* and to a lesser extent *TRIM22* displayed an elevated rate of intronic diversity when compared to *TRIM34* or *TRIM6*. Part of his diversity arose from multiple ERV elements differentially fixed in the first introns of *TRIM5* and *TRIM22*. One of the ERV elements identified in TRIM22, LTR10D, has previously been shown to provide p53 responsive transcription in humans [422, 598]. As this element is differentially fixed in the three primate species studied, it represented a tractable model for examining of the ability of the ERV elements to modulate transcriptional regulation. Using quantitative RT-PCR on RNA from PBMCs, it was observed that TRIM22 was upregulated in response to p53 stimulation only in Old World primates, which contain the LTR10D. Based on this, we speculate that this and the multitude of other ERV sequences may provide as yet uncharacterized transcriptional regulatory properties.

METHODS

Gene identification

Genes of the TRIM5/6/22/34 cluster from humans and rhesus macaques were retrieved from the respective genomic sequence databases at the National Center for Biotechnology Information (NCBI). Genome assemblies used for these analyses are as follows: *Homo sapiens*, NCBI Build 36.3 (March 2009); *Macaca mulatta*, NCBI Build 1.1 (February 2006).

TRIM genes from squirrel monkey were identified using NCBI's Basic Local Alignment Search Tool (BLAST) [9], using the human TRIM genes as "bait" and searching the *Saimiri boliviensis boliviensis* Trace Archives. This BLAST function allows searching of sequencing information compiled as part of whole genome sequencing efforts, and other large-scale sequencing projects, as the data is being compiled but prior to the data being released in a fully annotated form. This search yielded sequencing data from BAC clone AC192681 that was

created, sequenced, and assembled as part of the NIH Intramural Sequencing Center's (www.nisc.nih.gov) Comparative Vertebrate Sequencing Initiative.

Sequence Analysis

Transcribed sequences in the TRIM5, TRIM6, TRIM22, TRIM34 cluster corresponding to the human alpha splice variant of TRIM5, isoform 2 of TRIM6, isoform 4 of TRIM34, and the sole annotated TRIM22 transcript were compiled for further examination. These correspond to Genbank mRNA accession numbers NM_033034.1, NM_058166.3, NM_021616.4, and NM_006074.3, respectively. With the exception of TRIM6, these sequences represent the longest identified transcripts. Multiple sequence alignments of these sequences were generated by hand alignment in MacClade 4.06. Repetitive elements were identified for each sequence using RepeatMasker [521] open-source version 3.2.6. RepeatMasker options used were 'default' speed/sensitivity and 'other mammal' as DNA source, except in the case of human sequences, where 'human' was selected as the DNA source.

Calculating Genetic Diversity

For pairwise comparisons, the multiple sequence alignments for each TRIM gene were broken up into pairwise alignments, and extraneous gaps were removed using the Gapstreeze program (www.hiv.lanl.gov/content/sequence/GAPSTREEZE/gap.html). Unsequenced regions in the source sequence were dealt with by assuming that the missing region exactly matched that of the second species in the comparison, this being the most conservative assumption.

In order to assess the amount and type of genetic diversity observed in the various TRIM genes, we utilized the following calculations. The percent nucleotide identity was calculated using the following formula:

$$\left(\frac{2 \ * \ \# \text{ of conserved nucleotides}}{\text{total } \# \text{ of nucleotides } [\text{taxa } 1 \ + \ \text{taxa } 2]}\right) * 100$$

The nucleotide substitution rate was calculated using the following formula:

$$\frac{\left(\frac{\#\ \text{of nucleotide substitutions (Ts + Tv) [Taxa1 + Taxa2]}}{\#\ \text{of shared nucleotides (not in indels)}}\right) * 100}{\text{millions of years since last common ancestor}}$$

with 'percentage of sequence per million years' being the unit of measurement. The rate of indel change was calculated using the following formula:

$$\frac{\left(\frac{\#\ \text{of nucleotides in indels}}{\text{total}\ \#\ \text{of nucleotides [taxa 1 + taxa 2]}}\right) * 100}{\text{millions of years since last common ancestor}}$$

where 'percentage of sequence per million years' is the unit of measurement. The rate of transposable element fixation was calculated by dividing the number of unique transposable elements in a pairwise comparison by the time since last common ancestor. In all cases, the dates used for estimation of last common ancestor are the revised dates published in Bininda-Emonds et al. 2007 [49]. Statistical significance was assessed using the Friedman test, a one-way nonparametric repeated measures analysis of variance (ANOVA) followed by a Dunn's post-test to compare all genes against one another.

<u>Human and Non-human Primate Samples</u>

Human blood samples were obtained from volunteers enrolled in the Emory University Institutional Review Board approved "Emory Vaccine Center's Healthy Adults Study" (Protocol #555-2000). Volunteers in this study were healthy adults, aged from 23 to 62, who had signed a written informed consent form.

Non-human primate blood samples were obtained in accordance with NIH guidelines from animals housed at the Yerkes National Primate Research Center. Blood was isolated as part of an Emory University Institutional Animal Care and Use Committee approved protocol. All animals included in this study were unrelated adult males with no obvious chronic illnesses. All of the rhesus macaques included in the study were of Indian origin and SIV negative.

Isolation and Culture of Blood Cells

Blood samples were collected in sodium heparin Vacutainer tubes (BD Biosciences; San Jose, CA). PBMCs were isolated following centrifugation of whole blood over a Ficoll cushion. For all experiments, freshly isolated PBMCs were plated at approximately $2x10^6$ cells per well in 6-well plates and cultured for 3 days in RPMI medium containing 15% fetal bovine serum, 10U/ml penicillin, 10μg/ml streptomycin, and 3μg/ml phytohemagglutinin (PHA). Following this stimulation, induction of p53 was accomplished by addition of either 375nM 5-fluorouracil or 0.6μg/ml doxorubicin to the culture medium. Culture of PBMCs in the presence of 0.1% DMSO served as a control since DMSO was used as the solvent for both 5-fluorouracil and doxorubicin.

Transcriptional Profiling

At various times following induction of p53, cells were harvested and RNA was isolated using TRIzol (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. A one-step, SYBR green-based quantitative RT-PCR was then performed on 50ng of input RNA. Primer sets targeting TRIM22, TRIM5, murine double minute 2 (MDM2), and β-actin were designed and validated to be gene specific, while also targeting regions conserved between all primate species included in this study. The sequences of the primers are as follows: 'TRIM22 qPCR F' (5'-ACTGTCTCAGGAACACCAAGGTCA-3'), 'TRIM22 qPCR R' (5'-CCAGGTTATCCAGCACATTCACCTCA-3'), 'TRIM5 qPCR F' (5'-TGAGGCAGAAGCAGCAGG-3'), 'TRIM5 qPCR R' (5'-AGTCCAGGATGTCTCTCAGTTGC-3'), 'rhTRIM5 qPCR R' (5'-AGTCCAGGATCTCTCTCAGTTGC-3'), 'MDM2 qPCR F' (5'-TGAACGACAAAGAAAACGCCA-3'), 'MDM2 qPCR R' (5'-CCTGATCCAACCAATCACCTG-3'), 'β-actin qPCR F' (5'-TCGACAACGGCTCCG-3'), 'β-actin qPCR R' (5'-TTCTGACCCATGCCCA-3'). All qPCR reactions were performed with 125nM forward primer and 250nM reverse primer concentrations with the following conditions:

55°C for 10 minutes (RT step) and 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, gene-specific annealing temperature for 30 seconds, 72°C for 30 seconds followed by melting curve analysis. The annealing temperatures were as follows: 64°C for TRIM22, 56.5°C for TRIM5, 61°C for MDM2, 57°C for β-actin. Relative fold change in gene transcription was calculated via the ΔΔC(t) method using DMSO treatment values as the baseline.

Genotyping

Human and rhesus macaque subjects had their p53 coding sequence and TRIM22 LTR10D genotyped. The full p53 coding sequence was PCR amplified from unstimulated PBMC RNA following cDNA generation using an oligo d(T) primer. PCR amplification of p53 was performed using the following primers at 200nM concentrations: 'p53 F' (5'-GGCTGGGAGCGTGCTTTC-3') and 'p53 R' (5'-CACAACAAAACACCAGTGCAGGC-3'). Reactions were carried out with the high-fidelity Phusion enzyme (New England Biolabs, Ipswich, MA) with the following thermocycler conditions: 95°C for 2 minutes followed by 35 cycles of 95°C for 15 sec, 62.5°C for 15 seconds, and 72°C for 1 minute 30 seconds and a final 7-minute extension at 72°C. Direct sequencing of this p53 PCR product was performed using the original PCR primers as well as the following primers: 'p53 480-500 seq F' (5'-CCTCAACAAGATGTTTTGCCA-3'), 'p53 576-598 seq R' (5'-TGTGCTGTGACTGCTTGTAGATG-3'), 'p53 1021-1039 seq F' (5'-AACAACACCAGCTCCTCTC-3'), and 'p53 1081-1099 seq R' (5'-CACGCCCACGGATCTGAAG-3').

For the genotyping of the intronic LTR10D, unstimulated human and rhesus PCMCs were used as a source of genomic DNA, which was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. This was used as source material for PCR amplification of the LTR element using 'TRIM22 LTR10D F' (5'-GACCATTCATTTCTTCAATCTAGGTAC-3') and the previously described 'TRIM22 ChIP R'

primer. PCR was performed with the Phusion enzyme with the following thermocycler conditions: 95°C for 2 minutes followed by 35 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C for 1 minute 30 seconds and a final 7-minute extension at 72°C. Direct sequencing of the PCR product was performed using the PCR primers as well as 'TRIM22 ChIP F'.

Rhesus macaques also had their TRIM5 coding sequences genotyped using previously described methods [414, 607].

Sequence Information

Sequences corresponding to the different rhesus macaque LTR10D alleles reported here have been deposited to GenBank with the following (temporary) accession numbers: allele 1, HM104186; allele 2, HM104187; allele 3, HM104188; allele 4, HM104189.

Human and rhesus macaque p53 nucleotide sequences were *in silico* translated and hand aligned. Amino acid sequences generated in this study correspond to the previously described human p53 72P and 72R alleles and rhesus macaque sequence (GenBank accession numbers NP_000537, AAD28628, and NP_001040616, respectively). While only one predicted amino acid sequence was observed, three rhesus macaque p53 alleles were identified. The allele 1 sequence corresponds to that of the rhesus genome sequence (GenBank accession #NM_001047151), and the other two alleles only differ by synonymous polymorphisms. The sequences corresponding to alleles 2 and 3 have been deposited to GenBank with the accession numbers HM104190 and HM104191, respectively.

Assessing p53 binding to chromatin

A total of 1.2×10^7 PBMCs were treated with 375nM 5-fluorouracil or 0.1% DMSO for 9 hours. Following this, protein and chromatin were cross-linked by treatment with 0.5% formaldehyde for 10 minutes at 37°C. Following cell lysis, the chromatin was sheared to fragments with average sizes between 250 and 500 nucleotides by sonication. Lysates were subjected to three 10-second pulses with a power setting of 30% using a 550 Sonic Dismembrator

(Fisher Scientific, Pittsburgh, PA). Chromatin immunoprecipitation (ChIP) was performed using a commercial ChIP kit (Millipore, Billerica, MA) according to the manufacturer's recommendations. Chromatin specifically bound to p53 or Histone H3 was enriched using either a monoclonal anti-p53 antibody, clone DO-1 (Active Motif, Carlsbad, CA), or a rabbit purified polyclonal anti-Histone H3 Immunoglobulin G (IgG) (Millipore, Billerica, MA) according to manufacturers' suggestions. Efficiency of chromatin capture was assessed by qPCR via the SYBR green method using the following primers: 'TRIM22 ChIP F' (5'-CAAAATAAGGAAAGGAATGTGAGTTGGTAC-3') and 'TRIM22 ChIP R' (5'-ATCAAAATGACAGAATAGGAATGTGGG-3'). All reactions were performed using 10% of the immunoprecipitated DNA with 100nM primer concentrations and the following thermocycler conditions: 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, followed by melting curve analysis. Relative binding was calculated via the ΔΔC(t) method using the following formula: $(2^{(\text{p53}\Delta C(t) - \text{Histone H3}\Delta C(t))}) * 100$.

RESULTS

TRIM5 displays an increased genetic diversity compared to related *TRIM* genes, resulting from sequence insertions or deletions in introns

TRIM5, and to a lesser extent *TRIM22*, encode proteins with antiviral activities [27, 128, 140, 167, 215, 278, 291, 476, 483, 525, 543, 563, 632, 633, 636]. The coding sequences of both genes also display the hallmarks of recurrent episodes of positive selection, such as elevated rates of fixation of nonsynonymous nucleotide substitutions [482, 483, 524]. Given the unusual evolutionary history of *TRIM5* and *TRIM22*, we sought to extend these analyses to the non-coding regions of the genes. The findings for these two genes were compared to that for the analogous regions of the related *TRIM6* and *TRIM34* loci, which have been under purifying selection during primate evolution. In order to do this, orthologous primate *TRIM5*, *TRIM6*, *TRIM22*, and *TRIM34* genomic sequences were retrieved from the human and rhesus macaque

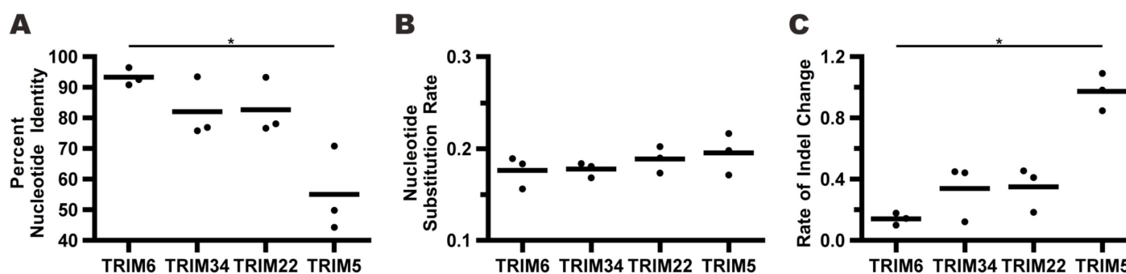

Figure 2. *TRIM genes are variably conserved as a result of differential rates of indel turnover.*
Transcribed genomic sequences of the *TRIM5*, *TRIM6*, *TRIM22*, and *TRIM34* genes were hand aligned. Using the formulas presented in the Methods section, the nucleotide alignments were used to calculate the following statistics for each pair of nucleotide sequences: the percent nucleotide identity (A), the nucleotide substitution rate (B), and the rate of indel change (C). The rate of change depicted in panels B and C is calculated as percent per million years. Black circles indicate separate pairwise sequence comparisons, and the bars represent mean values. Statistical significance was calculated using the Friedman test, followed by Dunn's post-test to compare all genes against one another. A *p* value of less than 0.05 is denoted by *.

genome sequencing databases as described in the Methods section. In order to identify squirrel monkey *TRIM* gene sequences, the longest transcribed regions of human *TRIM5*, *TRIM6*, and *TRIM22* were used as bait in BLAST searches of the *Saimiri sciureus sciureus* trace archive database. For *TRIM34*, the same *Saimiri* database was queried using the *TRIM34* "middle transcript", as annotated in the NCBI human genome build 36.3, as bait.

Following identification and trimming, a multiple sequence alignment was generated for the transcribed genomic region of each *TRIM* gene, including all introns (Supplemental files 1-4). These alignments were then used to calculate the percent nucleotide identity between orthologous sequences of each *TRIM* gene. As can be seen in Figure 2A, *TRIM5* is clearly less conserved than the other *TRIM* genes in this analysis, with on average only 55% nucleotide identity between species. In contrast, on average 93% of the nucleotides in *TRIM6* and approximately 82% of the nucleotides in both *TRIM34* and *TRIM22* are conserved across species. As might be expected due to relative size differences and limited evolutionary constraints, most of the variability observed in all of these genes lies within introns (data not shown). To determine the major source of increased genomic variability in *TRIM5*, we separated the observed variability into differences

resulting from nucleotide substitutions and those derived as a result of insertions or deletions (indels). We found that over the course of primate evolution, all four *TRIM* genomic regions have undergone similar rates of divergence due to nucleotide substitutions (Fig. 2B). In contrast, indel accumulation accounts for the majority of the interspecies divergence between these sequences (Fig. 2C).

<u>Elevated rate of transposable element fixation in *TRIM5*</u>

The elevated rate of indel accumulation in the most genetically diverse genes suggested an involvement of transposable elements. To test this possibility, transposable elements present in each sequence were identified using RepeatMasker [521] and quantified (Fig. 3). This analysis revealed similar numbers of transposable elements present in the *TRIM5* and *TRIM22* genes studied, and that both of these genes harbor more transposable elements than either *TRIM6* or *TRIM34* (Fig. 3A). The differences seen in the number of transposable elements found in these genes could represent fixation events that occurred either prior to or following divergence of these species. In order to assess this, pairwise comparison of genomic sequences was performed to identify lineage-specific transposable elements. The number of unique transposable elements identified in the pairwise comparisons of each gene are shown in Figure 3B. This analysis revealed that the number of lineage-specific transposable elements largely corresponds to the relative conservation of the coding sequence, such that increasing numbers of transposable elements were found proceeding from the *TRIM* gene with the most conserved coding sequence (*TRIM6*) to that with the most divergent coding sequence (*TRIM5*) [320, 482]. Similar to the absolute number of transposable elements, on average *TRIM5* contains the greatest number of lineage-specific transposable elements. *TRIM22* was not observed to have a significantly elevated number of lineage-specific transposable elements when compared to *TRIM34* or *TRIM6*. To correct for the fact that the three species under study diverged from one another at different times, the number of unique transposable elements in a pairwise comparison was divided by the number of years (in millions) since the last common ancestor. This provides an estimate of the rate of

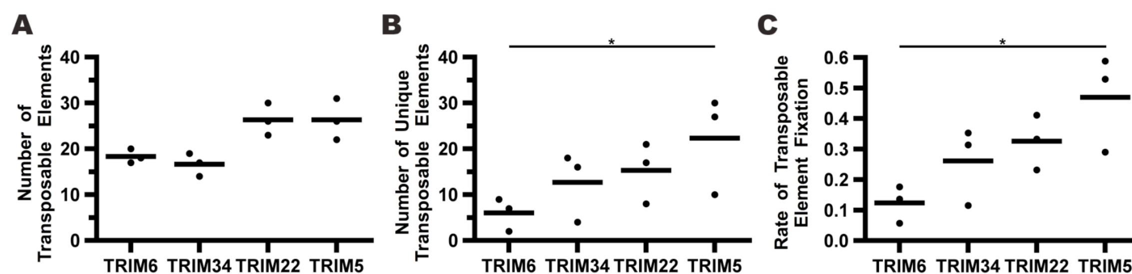

Figure 3. *TRIM5 and TRIM22 contain more transposable elements than TRIM6 or TRIM34.* The absolute number of transposable elements present in the *TRIM* genes was counted for each species, and the results are depicted in panel A. Black dots represent the number of transposable elements found in a given primate species. The quantitation shown in (A) was performed without regard for identity or conservation of the elements present; therefore, the number of novel transposable elements was considered. In panel B, the number of unique transposable elements present in pairwise sequence comparisons is shown for each *TRIM* gene. In order to correct for the time of divergence of these species, the rate of transposable element fixation was calculated by dividing the number of unique transposable elements in each pairwise comparison by the number of years (in millions) since the last common ancestor [49]. In panels B and C, black dots indicate one pairwise comparison number of elements in one comparison, and in all panels the black bar represents the mean value. Statistical significance was calculated using the Friedman test, followed by Dunn's post-test to compare all genes against one another. A *p* value of less than 0.05 is denoted by *.

transposable element fixation in each of these genes. The results (Fig 3C) largely mirror the uncompensated quantification of lineage-specific elements. Thus, while *TRIM5* and *TRIM22* harbor similar absolute numbers of transposable elements in the species studied (Fig. 3A), *TRIM22* appears to have maintained a greater percentage of transposable elements fixed prior to divergence of the catarrhini and platyrrhini lineages. Conversely, a higher percentage of the transposable elements harbored within *TRIM5* were found to be unique to a given species.

Lineage-specific fixation and loss of LTRs in *TRIM5* and *TRIM22*

The transposable elements identified by RepeatMasker were then mapped back onto the multiple sequence alignments. The position and orientation of these transposable elements in relation to the exon/intron structure of the four *TRIM* genes are depicted in Figure 4. This analysis makes starkly clear the difference in genetic variability between these genes and highlights the source of this variability.

Figure 4

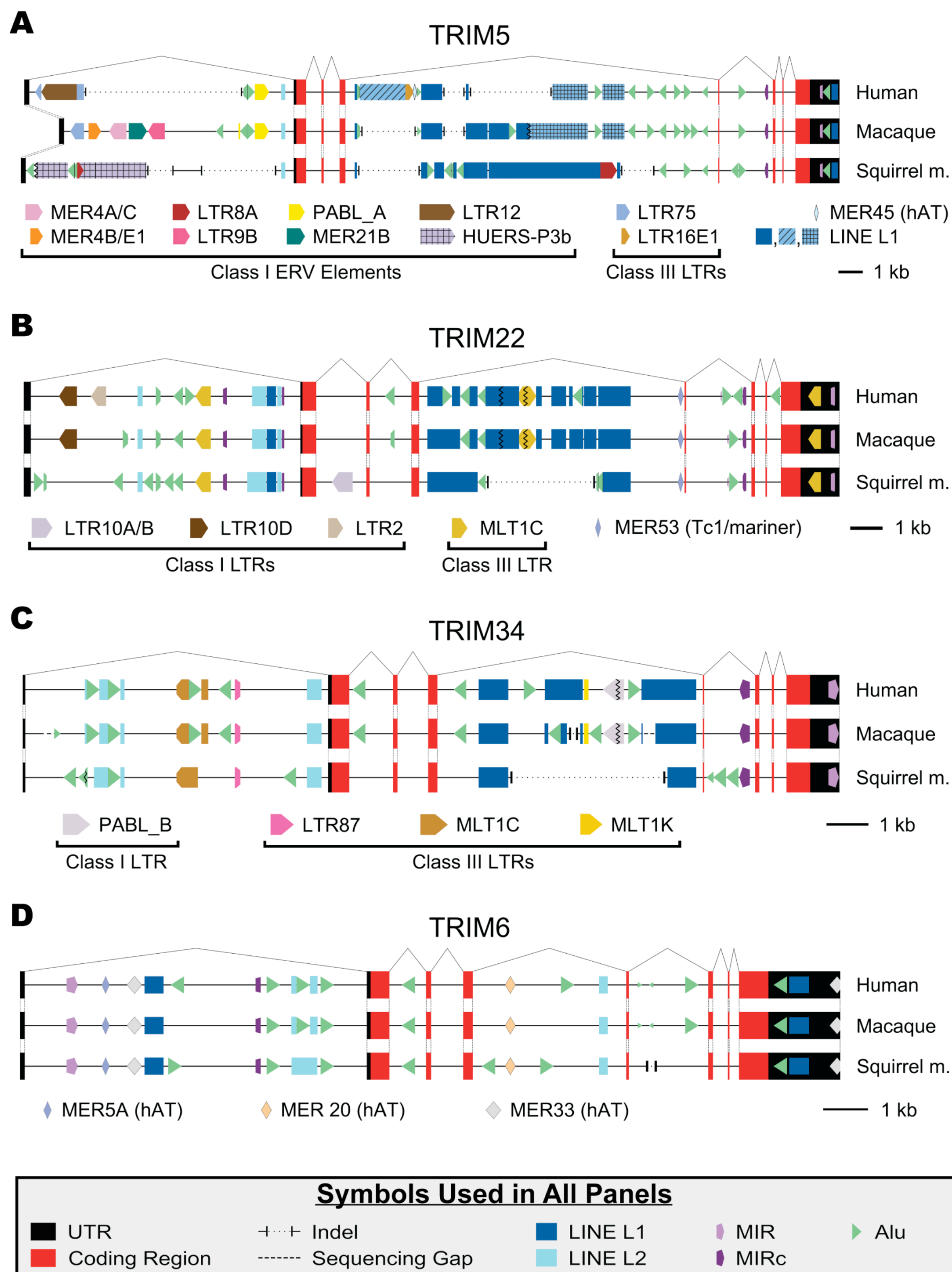

Figure 4. (Previous page) *Graphical depiction of the genomic structure and location of transposable elements in the analyzed TRIM genes.*
RepeatMasker was used to identify repetitive elements present in the genomic *TRIM* gene sequences, and these elements were mapped onto the multiple sequence alignments. Graphical representations of the various transposable elements found in *TRIM5* (A), *TRIM22* (B), *TRIM34* (C), or *TRIM6* (D) are shown in relation to the exon/intron structure, with the appropriate scale shown in each panel. In all panels, the topmost structure represents the exon/intron structure of the gene, and all subsequent structures superimpose the unique transposable elements or deletions specific to the indicated primate species. Symbols representing transposable elements only found in a given gene are presented immediately below that panel, while symbols common to all genes analyzed are shown at the bottom of the figure.

Transposable element composition

We found that the non-coding regions of the genes in the *TRIM6/34/5/22* cluster harbor representatives of each major class of transposable element, including LINEs, SINEs, and ERV retrotransposable elements as well as DNA transposons. Our analysis identified numerous LINE L1-derived sequences in *TRIM5*, *TRIM22* and *TRIM34*, largely localized to the fourth intron of these genes. Each of these LINE L1 sequences exists as 5' truncation fragments, generated as a result of premature termination during reverse transcription. LINE L1 elements appear in the fourth intron of *TRIM34*, prior to the gene duplication events that gave rise to *TRIM5* and *TRIM22*. Furthermore, *TRIM5* and *TRIM22* show an increase in the LINE L1 content of the fourth intron, with this increase proportional to the amount of selective pressure placed upon these genes [320, 482]. The expanded LINE L1 content in these antiviral genes is somewhat surprising as LINE L1 elements have been shown to have a negative effect on gene transcription levels [209, 439], and their presence inside transcribed genes is generally selected against [345].

Strikingly, the four *TRIM* genes display marked differences in the number of ERV LTR elements. We found that the number and diversity of the LTR content of each gene is proportional to the reported strength of the positive selective pressures placed on the gene over primate evolution, as assesses by the non-synonymous-to-synonymous (dN/dS) rate ratio calculated for the coding region of each gene [320, 482]. Thus, LTRs were identified found in

any of the orthologous *TRIM6* sequences, four LTR elements were found in *TRIM34*, and six LTR elements are present in *TRIM22*. Finally, orthologous *TRIM5* sequences were found to contain ten sequences of ERV origin, including nine solitary LTRs and the internal sequence from one non-autonomous ERV that contains a central LTR element in addition to the terminal LTRs (Fig. 4A-D). The LTR elements in *TRIM34* are largely conserved across the primate species included in this study, with the only difference in content resulting from a large deletion event that occurred in the fourth intron (Fig. 4C). In *TRIM22*, three MLT1C, class III LTR elements are shared across species, although a deletion in the fourth intron of squirrel monkeys removes one of the elements. Additionally, one LTR element (LTR10D) is shared by Old World primates, while humans and squirrel monkeys each encode one additional unique element (Fig. 4B). The various *TRIM5* genes examined here contain a total of ten LTR elements. Of these, only two (LTR75 and PABL_A) are found in more than one species, and neither element is strictly conserved (Fig. 4A). With the exception of the HUERS-P3b, all of these elements represent solitary LTR elements formed from homologous recombination-mediated removal of the internal retroelement sequence. HUERS-P3b elements are non-traditional, replication-defective endogenous retroviral-like element comprised of various fragments from replication-competent ERVs and containing a central LTR8A element. The HUERS-P3b sequence present in squirrel monkey *TRIM5* is in the negative orientation with respect to the *TRIM5* gene and is highly divergent relative to the described consensus. This element lacks a 5' LTR, has a central deletion that removes most of the LTR8A element, and is missing ~2.5 kb of the 3' terminus. Because LTRs play a transcriptional regulatory role in the retroviral life cycle, it is possible that each LTR element retains promoter or enhancer functions that could modulate the expression of the associated gene.

Genetic diversity

This analysis serves to highlight the extraordinary evolution of complexity in the *TRIM* genes. The genetic diversity, largely represented by the numbers of lineage specific transposable

elements, observed in the entire genomic sequence correlates with the presence and strength of diversifying pressure deduced from the coding region of these genes [320, 482]. Previous work has described the strong positive selective pressures placed upon the *TRIM5* coding region; from the current genomic analysis it is also clear that much of the sequence making up the first and fourth introns of this gene has been replaced over the course of primate evolution. In the first intron, only two regions of clear homology between all three primate species exist: a ~185-nucleotide region immediately following the first exon and ~730 nucleotides immediately adjacent to the second exon that includes sequence of LINE L2 origin. The fourth intron of *TRIM5* has undergone extensive gain and loss of LINE L1 content during primate evolution, with only a small region approximately 1 kb in size conserved between humans and squirrel monkeys.

In stark contrast to the extreme diversity in *TRIM5* is the high degree of conservation observed in the *TRIM6* genomic sequences, where only eight Alu elements and one relatively small deletion have arisen in the different primate lineages. This conservation of genomic sequence mirrors the previously described conserved nature of the coding sequence for this gene [320, 482]. *TRIM22* and *TRIM34* have undergone intermediate levels of intronic genetic turnover. In each case a considerable portion of this turnover can be attributed to a large deletion event occurring in the fourth intron of the gene. These deletion events result in the loss of LINE L1 content, and it is possible that these deletion events became fixed in New World monkeys because the loss of this LINE L1 content led to an increase in transcript levels. A feature distinguishing *TRIM22* from *TRIM34* is the presence of lineage-specific LTR element insertions into the first and second intron of the former, while the LTR elements present in the latter became fixed prior to Old and New World primate divergence.

Differential transcriptional regulation of *TRIM22* mediated by an LTR10D insertion

Transcriptional regulation of the *TRIM* genes involved in this study has not been the subject of detailed investigation. Of these genes, the regulation of *TRIM22* transcription has been the best characterized; we have therefore focused functional studies on this gene. It has been

reported that expression of human *TRIM22* is upregulated upon p53 induction, that a p53-binding site is located in the first intron of this gene, and that mutations abolishing p53 binding to this site result in the loss of p53 responsiveness [422]. Following this, the p53-binding site was found to be located in an LTR10D element, and that a network of p53-regulated genes exists as the result of fixation events involving either the LTR10 or MER61 families of ERV elements [598]. While these elements can be found in both Old and New World primate genomes, an LTR10D is only present in TRIM22 of Old World primate lineages (Fig. 4B). Based on the previous reports and our results demonstrating the presence of this element in Old World primates and absence of this element in New World primates, we hypothesized that the TRIM22 from Old World primates would respond to p53 induction by upregulating transcriptional levels, while TRIM22 in New World monkeys would be refractory to p53 stimulation. In order to test this hypothesis, TRIM22 expression changes were assessed following induction of DNA damage in peripheral blood mononuclear cells (PBMC) from healthy humans, uninfected rhesus macaques, and uninfected squirrel monkeys.

Following isolation from whole blood, PBMCs from humans and non-human primates were PHA stimulated for 72 hours and then treated for 24 hours with DNA-damaging agents 5-fluorouracil or doxorubicin, to induce p53 activation, or with vehicle (DMSO) as a control. RNA was harvested from these cells, and a quantitative one-step RT-PCR designed to function equivalently in all three of these species was used to detect changes in TRIM22 mRNA expression (Fig. 5A). In accordance with previous work [269, 422, 598], TRIM22 expression levels in human PBMCs displayed a significant increase in expression of either 2.4- or 4.09-fold following DNA damage induced by 5-fluorouracil and doxorubicin, respectively. Similar results were observed in rhesus macaque PBMCs, where TRIM22 expression was found to significantly increase 1.36- and 2.64-fold, respectively. No significant change in TRIM22 RNA levels was observed in squirrel monkey PBMCs, although treatment with 5-fluorouracil tended to reduce TRIM22 expression. Thus, the general pattern of TRIM22 expression level changes following

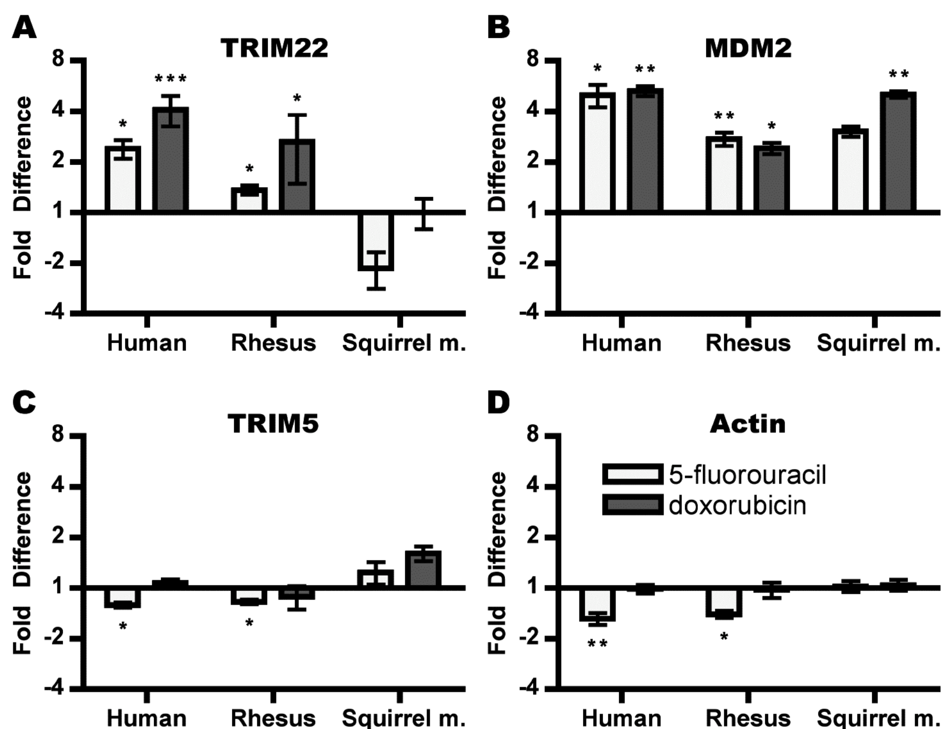

Figure 5. *Transcription of TRIM22 from different primate species is differentially regulated following p53 induction.*
Following a 3-day stimulation with PHA, PBMCs from humans, rhesus macaques, or squirrel monkeys were treated with DMSO, 5-fluorouracil, or doxorubicin for 24 hours. Total RNA was harvested and levels of *TRIM22* (A), *MDM2* (B), *TRIM5* (C), and *β-actin* (D) mRNA were assessed using SYBR green-based qPCR with 50ng per reaction input RNA. Shown are the fold changes measured following drug treatment compared to DMSO controls, as calculated using the ΔΔC(t) method. Error bars represent ± SEM with *n* = 7 human subjects, *n* = 6 rhesus subjects, and *n* = 4 squirrel monkeys subjects.

p53 induction was consistent with the presence or absence of the p53-binding site containing LTR10D element in a given species.

In order to verify that the changes to TRIM22 expression were specific for this gene, RNA levels were measured for the control genes MDM2, TRIM5, and β-actin. MDM2 is a well-studied inhibitor of p53 activity whose expression is directly controlled by p53 activation [23, 263, 445], while transcription of TRIM5 and β-actin have been shown to be largely unaffected by DNA damage [269]. Thus, MDM2 expression serves as a positive control for p53 induction, while TRIM5 and β-actin serve as indicators of global transcriptional changes. When MDM2 expression levels were assessed, it was observed that this gene was upregulated in the PBMCs of

all three species (Fig. 5B), demonstrating that the differential transcriptional regulation seen for TRIM22 was not due to a failure in inducing p53 using the DNA-damaging agents. In contrast, TRIM5 and β-actin expression levels remained largely unchanged in PBMCs following DNA damage (Fig. 5C-D). These results clearly demonstrate that TRIM22 is specifically upregulated in human and rhesus PBMCs following p53 induction, and that the p53-binding site in the first intron does not mediate transcriptional regulation of neighboring genes.

Unexpectedly, the increase in rhesus macaque *TRIM22* transcription was not as robust as that observed in human PBMCs. The reduced upregulation of *TRIM22* coincides with reduced *MDM2* upregulation in rhesus macaques compared to humans. In order to elucidate the potential role of unrecognized genetic variability on this observation, both the *p53* cDNA and the intronic LTR10D were sequenced from all human and macaque subjects (Table 1). This revealed that the human study participants had identical LTR10D sequences, while *p53* alleles encoding variants that differ at amino acid 72 were identified in these individuals. The 72P and 72R allelic variants of human p53 (Fig. S1) have been shown to function differently in a variety of assays [reviewed in 192, 602], including differences in the ability to upregulate transcription of a group of known p53-responsive genes [544]. In this study, however, the *p53* genotype of the participants did not appear to correspond to any variation observed (data not shown), although the number of individuals in each group prevented rigorous statistical analysis. Previous sequencing results have shown that the p53 amino acid sequence of rhesus macaques differs by 17 or 18 amino acid residues from that of humans (Fig. S1). In addition to this, three *p53* genotypes were observed among macaques involved in this study, all of which encode the same amino acid sequences. The macaque LTR10D sequence differs from the human element at 36 nucleotides, including two within the p53-binding domain (Fig. S1). These two nucleotide differences both represent G-to-A transitions, which have arisen in ape species. The G-to-A change at position 379 is present in both chimpanzee and human sequences, while the change at position 387 is human specific (data not shown). On top of these differences, four genotypes were found to exist in the rhesus macaque

Table 1. *Summary of demographic and genetic information for human and non-human subjects.*

Species	ID	Sex	Age	Ancestry	*p53* genotype	LTR10D genotype	*TRIM5* genotype
Human	210	M	61	Europe	72P / 72P[a]	WT	Not Tested
	335	F	27	Africa	72P / 72P	WT	Not Tested
	10	M	52	Africa	72P / 72R	WT	Not Tested
	206	M	30	Europe	72P / 72R	WT	Not Tested
	318	M	23	Europe	72P / 72R	WT	Not Tested
	243	M	42	Africa	72R / 72R	WT	Not Tested
	253	M	49	Europe	72R / 72R	WT	Not Tested
Rhesus	RHg7	M	10	Indian	1 / 3[b]	1 / 2[c]	TFP / ΔΔQ[d]
	RMj2	M	20	Indian	1 / 2	1 / 1	TFP / TFP
	RSt4	M	15	Indian	1 / 2	1 / 4	TFP / CypA
	RNq3	M	17	Indian	1 / 2	1 / 1	TFP / TFP
	RQn5	M	13	Indian	1 / 1	1 / 3	TFP / CypA
	RVe7	M	10	Indian	1 / 3	2 / 3	ΔΔQ / CypA
Squirrel monkey	2878	M	11	*S. sciureus*	Not Tested	N/A[e]	Not Tested
	2903	M	11	*S. sciureus*	Not Tested	N/A	Not Tested
	3075	M	10	*S. sciureus*	Not Tested	N/A	Not Tested
	3102	M	10	*S. sciureus*	Not Tested	N/A	Not Tested

[a] 72P and 72R refer to the amino acid residue predicted based on nucleotide sequencing to be found at position 72 of the α isoform of p53.
[b] Allele 1 is represented by the rhesus macaque genome sequencing. Alleles 2 (GenBank HM104190) and 3 (HM104191) are characterized by the presence of synonymous mutations in the coding sequence. Thus, all alleles encode identical primary amino acid sequences.
[c] LTR10D alleles are shown in Supplemental Table 1.

LTR10D region, with at least two nucleotide substitutions distinguishing one allele from another (Fig. S2 and Table S1). None of the polymorphic sites were located in the p53-binding site, and allelic variation in the LTR10D did not appear to be responsible for the difference in *TRIM22* upregulation between rhesus macaques and humans.

As this difference in magnitude of *TRIM22* upregulation was not specific to a particular *p53* or LTR10D genotype, we tested the ability of p53 to bind to the *TRIM22* intronic LTR10D in human and rhesus PBMCs. A p53-specific chromatin immunoprecipitation was performed on DNA from human or rhesus PBMCs stimulated for 9 hours with either DMSO or 5-fluorouracil (Fig. 6). In human PBMCs, treatment with 5-fluorouracil resulted in approximately a 10-fold

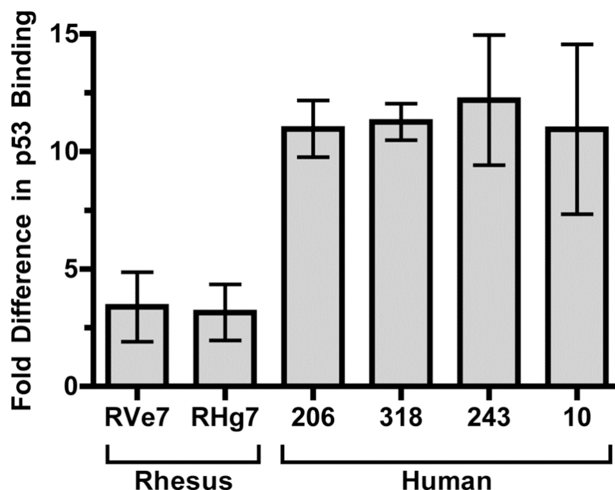

Figure 6. *Reduced binding of p53 to the LTR10D element within the first intron of rhesus macaque TRIM22.*
Following a 3-day stimulation with PHA, PBMCs from humans or rhesus macaques were treated with DMSO or 5-fluorouracil for 9 hours. Chromatin immunoprecipitation was performed on fixed, sheared DNA from these cells using an anti-p53 monoclonal antibody and a purified anti-Histone H3 antisera. The relative amount of *TRIM22* intronic LTR10D capture was assessed using 10% of the captured DNA by SYBR green-based qPCR via the ΔΔC(t) method and the following formula: $(2^{(\text{anti-p53}\Delta C(t)) - (\text{anti-histone H3 } \Delta C(t))}) * 100$. Bars represent the fold difference of intronic LTR10D bound by p53 following 5-fluorouracil treatment compared to DMSO treatment. Error bars represent ± SEM of *n* = 3 replicates from a representative experiment.

increase in p53 binding to the LTR10D element. However, a similar treatment in rhesus PBMCs resulted in only a 3-fold increase in p53 binding. These results emulate the difference in *TRIM22* upregulation shown in Figure 5A, leading us to believe that it is a combination of differences in primary amino acid sequence in p53 in addition to the two nucleotide differences in the p53-binding site of the LTR10D that are responsible for the difference in *TRIM22* upregulation between human and rhesus.

DISCUSSION

Lineage-specific gain and loss of ERV LTR elements in TRIM5 and TRIM22

Pathogenic invaders can impose major selective pressures on genes involved in the antimicrobial response. *TRIM5* and the genomic locus in which it resides stand as examples of the results of such selective pressures. Evidence of this is embodied by: the variation in copy number

of *TRIM5*, *TRIM22*, and *TRIM34* between species [483, 514, 553], elevated rates of change in the coding sequence of *TRIM5* and *TRIM22* [482, 483, 524], maintenance of balanced polymorphisms as well as cross-species sharing of polymorphisms in *TRIM5* [82, 413], and convergent evolution of exon replacement via cDNA retrotransposition [67, 324, 414, 418, 484, 587, 607]. These genetic signatures bear witness to the strength of the pressures placed upon *TRIM5* and the surrounding genomic locus during mammalian evolution.

In the *TRIM* genes examined, it was observed that the amount of nucleotide divergence observed in the intronic sequences generally corresponds to the dN/dS rate ratio previously reported for these genes [320, 482]. *TRIM6*, the most ancestral *TRIM* gene in this locus and the gene with the lowest dN/dS rate ratio, was observed to have on average 93% nucleotide identity between humans, macaques, and squirrel monkeys. *TRIM5* genomic sequences had dramatically lower conservation, with only 55% nucleotide identity between the species included in this study, and this gene has been shown to have the highest dN/dS rate ratio. This high level of diversity correlated with elevated rates of transposable element turnover, much of which was due to alterations in either ERV LTR content in the first intron or LINE L1 content in the fourth intron. In between these extremes, *TRIM22* and *TRIM34* were both found to have around 82% nucleotide identity in these species. Both of these genes feature a large deletion removing much of the LINE L1 content in the fourth intron. However, of these genes only *TRIM22* contains lineage-specific LTR insertions in the first intron.

It may be argued that positive selection on the coding regions of *TRIM5* and *TRIM22* has indirectly lead to the differences in fixation of transposable elements observed. At least in regards to the LTR elements differentially fixed in the first intron of these genes several lines of evidence argue against this. First, the entireties of *TRIM5* and *TRIM22* have not under positive selection. Rather, it is the B30.2 domain that has been under strong selection, while the tripartite motif portion undergone more of a neutral selective pressure[483, 524]. The LTR elements in the first intron are spatially separated from the regions facing the strongest selective pressures generally

by over 10 kb. Additionally, the LTR elements are buffered from the selective pressure on the B30.2 domain by the exons encoding the tripartite motif domains, which have been under neutral selective pressure during primate evolution. Finally, *TRIM6* and *TRIM34* possess similar gene structures, but have not undergone the same turnover of transposable elements, arguing for specificity of this phenotype for the antiviral *TRIM* genes. Thus, we interpret the differential fixation of LTR elements in the first intron of *TRIM5* and *TRIM22*, to represent cellular exaptation of the transcriptional regulatory potential of these elements in response to evolutionary pressures on the transcriptional regulation of these genes.

LTR-mediated alteration of transcriptional regulation

The genes of the *TRIM6/34/5/22* locus lie adjacent to one another on primate chromosomes in the following order: *TRIM6*, *TRIM34*, *TRIM5*, *TRIM22*. Three of these genes, *TRIM6*, *TRIM34*, and *TRIM22*, are oriented such that transcription proceeds in the same direction, while transcription of *TRIM5* occurs in the opposite direction. As a result, in primates *TRIM5* and *TRIM22* are situated in such a manner that only ~5 kb separates the transcriptional start sites of these genes. While extensive promoter mapping studies for these genes has not been conducted, this ~5 kb region must contain the core promoters of both genes. Both of these antiviral genes function intracellularly and over the course of primate evolution likely faced multiple rounds of viral challenge from viruses with divergent tissue tropisms. *TRIM5* and *TRIM22* therefore likely faced strong evolutionary pressures to match cellular gene expression with the tissue tropism of a target virus. The relatively short intergenic region providing core promoter function for both of these genes has likely limited the evolutionary flexibility surrounding the core promoter. The fixation of LTR elements proximally to the core promoter in *TRIM5* and *TRIM22* is suggestive of adaptive evolution in response to a transcriptional regulatory pressure imposed upon these genes. A recent report has demonstrated such an adaptation for the antiviral gene *APOBEC3* in specific strains of mice. In these strains, a xenotropic murine leukemia virus insertion into the second intron has resulted in a greater than 4-fold increase in expression in the spleens of mice, and in

conjunction with other changes, this has resulted in an enhanced antiviral phenotype in these mice [479]. Additionally, a recent report has demonstrated that LTR elements in the first intron of *TRIM5* contain polymorphisms under trans-species balancing selection in humans and chimpanzees [82]. These findings strongly argue for an evolutionary benefit of maintaining such polymorphisms. Although it was not tested, these polymorphisms are presumed to act at the level of transcriptional regulation.

We focused on *TRIM22* in order to explore the potential transcriptional regulatory influence of differentially fixed ERV LTRs on the antiviral *TRIM* genes. *TRIM22* provided a particularly tractable model as previous studies have demonstrated that a p53-binding site found in the LTR10D element in the first intron is necessary and sufficient for providing p53 responsiveness to this gene in human cells [269, 422, 598]. Because this element is present in Old World primates but absent from New World primates, we induced p53 in PBMCs from humans, rhesus macaques, and squirrel monkeys and directly tested its role in regulating *TRIM22*. These experiments showed that *TRIM22* transcription is regulated by p53 in Old World primates, while New World primates lacking this element were insensitive to p53 activation. Thus, it does not appear that New World monkeys have acquired p53 responsiveness for *TRIM22* via some other mechanism. In our studies, it was also found that *TRIM5* from all species tested were p53 non-responsive. While the *TRIM5* genes from each primate species contain a unique complement of multiple different ERV insertions in the first intron, all lack an LTR10D element. Together, these findings clearly demonstrate a lineage-specific, differential regulation of transcription resulting from fixation of an ERV LTR element fixed within an antiviral *TRIM* gene. By extension, all of the LTR elements fixed in *TRIM5* and *TRIM22* have the potential to alter transcriptional regulation of these genes in response to specific stimuli, such as p53 stimulation demonstrated here, or in a tissue- or lineage-specific manner as demonstrated for murine *APOBEC3* [479]. The lineage-specific involvement of intronic LTRs in transcriptional regulation represents a

previously unrecognized resultant characteristic of the evolutionary pressures placed on these *TRIM* genes.

Evidence of post-fixation adaptive changes to the intronic LTRs of TRIM5 and TRIM22

An apparent example of continuing selective pressures being placed upon intronic LTR elements has recently been reported for LTR12D and PABL_A elements present in the first intron of hominoid *TRIM5* genes. These elements were shown to harbor polymorphisms shared between humans and chimpanzees, demonstrating millions of years of balancing evolution involving these sites [82]. While our results show a clear difference in p53 regulatory potential for *TRIM22* of Old or New World primate origin, a difference in magnitude of p53-mediated induction of *TRIM22* was also observed between Old World primates: Human *TRIM22* was found to be more robustly upregulated in response to p53 activation via DNA damage compared to what was observed in macaques. This observation correlated with a greater increase in p53 binding to the intronic LTR10D element upon p53 activation. The differences in p53 binding and transcriptional activity may be result of a combination of differences in the primary amino acid sequence of p53 as well as two nucleotide differences in the p53-binding region of the LTR10D element. The two nucleotide substitutions in the p53-binding region were sequentially fixed in the human-chimpanzee lineage. While it is unknown to what extent these substitutions have on the differences observed in p53 binding, this could represent a selection for increased p53 responsiveness of *TRIM22* in humans. Regardless, these observations are suggestive of continuing evolution in the p53-LTR10D interaction in Old World primates that has occurred after fixation of this LTR. However, at this time we are unable to rule out the possibility that the difference in magnitude of *TRIM22* upregulation following p53 activation observed between humans and rhesus macaques results from a generally reduced level of p53-mediated transcriptional activation specific to rhesus macaques.

Potential complexity of LTR-mediated transcriptional regulation in TRIM5 and TRIM22

An increase in mRNA levels following type I interferon (IFN) treatment has been demonstrated for all four *TRIM* genes included in this study [20, 88, 89]. An interferon-sensitive responsive element (ISRE) has been identified in the core promoter region of *TRIM5*, and signal transducer and activator of transcription 1 (STAT1) has been shown to bind this element following type I IFN treatment [20]. A similar binding site has also been characterized in the first exon of human *TRIM22*, immediately adjacent to the core promoter [168]. Yet, neither *TRIM6* nor *TRIM34* are predicted to have such an element in their core promoter, and their expression is still regulated by IFN treatment [88]. Another study identified the relative expression patterns of *TRIM5* and *TRIM22* in human tissues [482]. Apart from these findings, transcriptional control of the *TRIM* genes involved this study is poorly understood. The genomic sequence of *TRIM5* contains six LTR elements differentially present in humans versus macaque. None of the LTRs present in Old World primate *TRIM5* sequences are also present in squirrel monkey, which possesses its own compliment of ERV-derived sequences. In comparison to *TRIM5*, *TRIM22* represents a relatively less-complex target of study, since there are only four LTR elements differentially present in Old and New World primates. Yet, each of these LTRs represents a probable transcriptional regulatory unit with multiple transcription factor-binding sites. Predicted STAT- and interferon regulatory factor (IRF)-binding sites are common among the LTR elements present in *TRIM22* and *TRIM5*. In addition to the sites in the promoter region, LTR-based binding sites may play a secondary role in regulation of transcription of these genes following IFN treatment. Predicting such complex regulatory interactions based solely on sequence gazing is fraught with hazards as transcription factor-binding sites may or may not be conserved between species and may differ functionally based on the context that the surrounding nucleotide sequence provides or based on the cell type in question. Such a situation of multiple lineage-specific ERV insertions independently evolving following fixation has the potential for the generation of extremely complex interactions governing expression of these genes.

A potential example of such complexity comes from *in silico* predictive analysis of transcription factor-binding sites in the conserved MLT1C element from the first intron of *TRIM22* (data not shown). Such analysis identifies a near-consensus STAT-binding site in this LTR with 100% conservation between humans, macaques, and squirrel monkeys. In contrast to this conservation, the same LTR contains multiple probable transcription factor-binding sites poorly conserved between species. For instance, the MLT1C of humans and macaques contains a near-consensus binding site for the myelocytomatosis viral oncogene homolog (Myc) – Myc-associated factor X (Max) heterodimer. This Myc-Max binding site overlaps with a perfect consensus androgen receptor-binding site. In squirrel monkey MLT1C, nucleotide substitutions have resulted in a Myc-Max-binding site that is a poor match to consensus and a loss of the putative androgen receptor-binding site. This loss of an androgen receptor-binding site is accompanied by the gain of a putative estrogen receptor-binding site at the same location. While none of these putative binding sites have been shown to be functional in any context and such predictive exercises have many caveats and limitations, such analyses can serve as informative starting points for further analysis.

It has been reported that an increase in *TRIM5* transcription correlates with a decrease in HIV-1 infection risk [503]. In *in vitro* experiments, induction of *TRIM5* expression using IFN-β results in an enhanced antiviral phenotype [89]. Additionally, overexpression of *TRIM34* can result in restriction of SIV_{Mac} infection, while endogenous expression of *TRIM34* does not appear to mediate a similar block [320, 650]. These findings suggest a critical role for transcription levels of these antiviral genes in protecting against viral pathogens. Our findings of differential transcription regulated in part by LTR elements in conjunction with the discovery of shared polymorphisms in two LTR elements strongly argue for a transcriptional regulatory role for LTR elements present in *TRIM5* and *TRIM22*. We speculate that this may have arisen as a result of the strong selective pressures placed on these genes by ancient waves of viral challenge and limited evolutionary flexibility that the shared ~5-kb promoter region has afforded these genes. The

regulatory control exerted by intronic LTR elements present in *TRIM5* and *TRIM22* provide the opportunity for a complex interaction between the core promoter and the various LTR elements present in a specific gene. A better understanding of the transcriptional control of these genes will assist in understanding how *TRIM5* and TRIM22 exert their antiviral effects *in vivo*.

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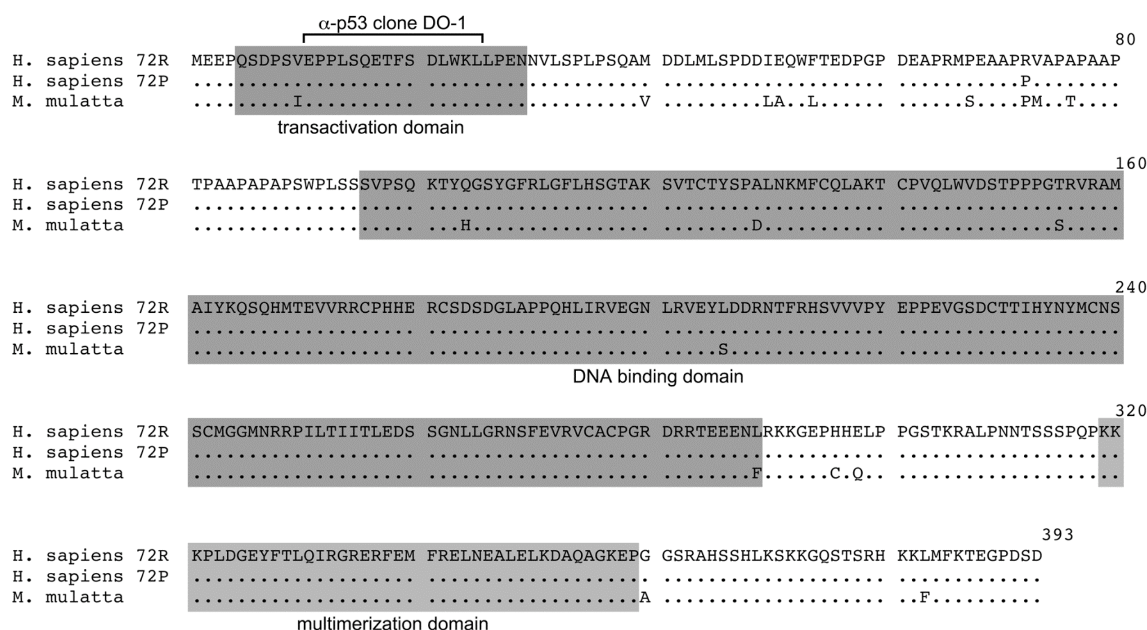

Figure S1. *Amino acid alignment of human and rhesus macaque p53.*
Amino acid residues corresponding to the transactivation domain, DNA-binding domain, and multimerization domain of p53 are highlighted in separated gray boxes. The bracketed region indicates the epitope that monoclonal anti-p53 antibody DO-1 recognizes.

Table S2. *Nucleotide differences seen in the LTR10D elements within rhesus macaque*

Genotype	nt –6[a]	nt 9	nt 173	nt 287	nt 297	nt 429	nt 479
Allele #1	C	A	C	G	T	C	G
Allele #2	C	G	C	T	C	C	G
Allele #3	T	A	C	G	T	T	G
Allele #4	C	A	T	G	T	C	T

[a] Nucleotide positions correspond to those shown in Figure 3.

```
                         -7    -1|1                                                                       73
H. sapiens               CCTTCCTTGTTGGAAATAAA GCTCAGAATCTTAAGGGAGA CGAGCACTCAAACAAAGGAT TTCTCAGCAAAGCAATTTTA
M. mulatta Allele #1     T................... .................... T................... ....................
M. mulatta Allele #2     T..............G.... .................... T................... ....................
M. mulatta Allele #3     TT.................. .................... T................... ....................
M. mulatta Allele #4     T................... .................... T................... ....................
S. sciureus              .....T.------------- -------------------- -------------------- --------------------

                                                                                                         153
H. sapiens               TTTCTGTGCAGAGGGGTGCT TCTTCTTTGCCAGTCGCCAT GAGAGCACACTAGAACAAAG GAGGAAGAAAGTTTTTATTC
M. mulatta Allele #1     C.....C.T...C....... .......G.......A.... .................... ..........T.........
M. mulatta Allele #2     C.....C.T...C....... .......G.......A.... .................... ..........T.........
M. mulatta Allele #3     C.....C.T...C....... .......G.......A.... .................... ..........T.........
M. mulatta Allele #4     C.....C.T...C....... .......G.......A.... .................... ..........T.........
S. sciureus              -------------------- -------------------- -------------------- --------------------

                                                                                                         233
H. sapiens               CTGACGCAAATCCTACCCCT TTGCCCTTTCCCCATTGGCT GGAGTCGGCCACACAATCTA AACTAGACTCAGTTGGCTAA
M. mulatta Allele #1     ....T.......T.G..... G..T.A.............. ......A............. ........C...........
M. mulatta Allele #2     ....T.......T.G..... G..T.A.............. ......A............. ........C...........
M. mulatta Allele #3     ....T.......T.G..... G..T.A.............. ......A............. ........C...........
M. mulatta Allele #4     ....T.......T.G...T. G..T.A.............. ......A............. ........C...........
S. sciureus              -------------------- -------------------- -------------------- --------------------

                                                                                                         313
H. sapiens               ACATTTTAACTTTCTTTAGA TAAGGTGGGCACGTGAGGGA GAGAGGGGAGATGGGGAAGG GGTGTGTGTGATGAGCTAGA
M. mulatta Allele #1     .................... ....A..A............ ...T................ ....C..C............
M. mulatta Allele #2     .................... ....A..A............ ...T........T....... ..C.C..C............
M. mulatta Allele #3     .................... ....A..A............ ...T................ ....C..C............
M. mulatta Allele #4     .................... ....A..A............ ...T................ ....C..C............
S. sciureus              -------------------- -------------------- -------------------- --------------------

                                                                                                         393
H. sapiens               TAACTAATCTTCTTCCAAAA TAAGGAAAGGAATGTGAGTT GGTACTGATAACGCCTGGTA CTGTGACATGTCTAGGCATG
M. mulatta Allele #1     G...........---..... .................... .................C.. .....G.......G......
M. mulatta Allele #2     G...........---..... .................... .................C.. .....G.......G......
M. mulatta Allele #3     G...........---..... .................... .................C.. .....G.......G......
M. mulatta Allele #4     G...........---..... .................... .................C.. .....G.......G......
S. sciureus              -------------------- -------------------- -------------------- --------------------
                                                                                           p53 binding region

                                                                                                         473
H. sapiens               TAGCAAAAGCAGAAAGGAAA GAGAAGAGAAGAAAAGTGAA GTGGGG------AGGGG-CA CTGTGAATTAAAGAATAAAG
M. mulatta Allele #1     .................... ..A............C.G.. A.....GGCGGG.....G.. ....................
M. mulatta Allele #2     .................... ..A............C.G.. A.....GGCGGG.....G.. ....................
M. mulatta Allele #3     .................... ..A............T.G.. A.....GGCGGG.....G.. ....................
M. mulatta Allele #4     .................... ..A............C.G.. A.....GGCGGG.....G.. ...................T
S. sciureus              -------------------- -------------------- -------------------- --------------------

                                                               516|+1    +7
H. sapiens               GATTGATCAGGCTATTTGAA GAGAAACCTTATCATACCCC ACATTCCTAT
M. mulatta Allele #1     .................... ................T... ..........
M. mulatta Allele #2     .................... ................T... ..........
M. mulatta Allele #3     .................... ................T... ..........
M. mulatta Allele #4     .................... ................T... ..........
S. sciureus              -------------------- -------------------- --------C.
```

Figure S3. *Nucleic acid alignment of the LTR10D element found within the first intron of Old World primate TRIM22.*

Nucleotide sequences comprising and flanking the Old World-specific LTR10D element were trimmed from the greater *TRIM22* nucleotide alignment. The sequence corresponding to the LTR10D element is numbered from 1 to 516; sequences upstream and downstream are numbered in reference to this element using – and + symbols, respectively. The red highlighted nucleotides correspond to the target site duplication that occurred during ERV integration. The nucleotides highlighted in green correspond to the p53-binding region. Nucleotides highlighted in blue correspond to the nucleotides found to be polymorphic in rhesus macaques.

DISCUSSION

Retroviruses are known to infect a broad range of vertebrate species, from fish to humans. In spite of eliciting both cellular and humoral immune responses against them, retroviral infections persist for the lifetime of their host. This longevity is almost certainly aided by (if it is not a result of) their integration into the host's cellular DNA. In their natural host species, retroviral infections are typically characterized by prolonged latency periods. This is often followed by the development of chronic pathologies, which include induction of tumor formation, immunodeficiency, neuropathy, anemia, arthritis, osteopetrosis, and chronic wasting syndrome. However, there is not typically an increase in the transmission of virus during the pathogenic phase of disesase [109].

Such mildly pathogenic symptoms are characteristic of an extended period of co-evolution between virus and host that has resulted the selection in both the host and virus for an evolutionarily "tolerable" interaction. On the viral side, this means that virus induced pathology should not be so severe as to kill one host prior to transmission to a new host. For viruses with low transmission frequencies, such as retroviruses [109, 190], it would therefore be advantageous to establish a chronic infection with minimal pathogenicity, a scenario which would allow for enough time to ensure that transmission occurred. On the host side, if the immune system were incapable of viral clearance, there would be selection against infections with significant pathologies that would significantly limit the reproductive fitness of infected individuals. Evolutionarily speaking, selective forces in the host largely cease to exist after reproductive age thus late-stage pathologies resulting from retroviral infections would impose minimal selective forces. As alluded to above, most retroviral infections follow the following course of infection in their natural host; minimal or no pathology during reproductive age followed by some disease state late in life. The exception to this rule are foamy viruses where infection rates can approach 100% of breeding age individuals [395], but no major pathologies have been found to develop as

a result of infection. We currently have not identified a natural host that displayed severe illness and rapid death as the typical outcome of a retroviral infection.

The clinical course of retroviral infection in non-native hosts follows a distinctly different progression. Typically, initial infection of a non-native species results in poor viral replication and perhaps even viral clearance. That there is a high genetic barrier to cross in order to establish infection in heterologous species is evidenced by the fact that the only other species that HIV-1 is known to replicate well in is chimpanzees [reviewed in 406]. Once established (ie following adaptation to growth in the new species), the lack of prior co-evolution tends to result in more severe viral pathogenesis. The differing courses of infection between native and non-native retroviral host species is typified by the difference in outcomes following infection of sooty mangabeys or macaques with sooty mangabey-derived SIV. Under such conditions, sooty mangabeys are resistant to developing clinical symptoms whereas rhesus macaques typically progress relatively quickly to disease, in spite of similar viral load levels in both species [515].

One barrier to HIV-1 and SIV infection was identified by attempts to infect cells from non-host species in vitro [231, 232, 511], and was typified by a block to infection prior to completion of reverse transcription [116, 216, 433]. The factor responsible for this block to infection was later identified as TRIM5α [541]. Shortly after this identification, it was realized that TRIM5α was also responsible for the resistance of human cells to N-tropic MLV infection [217, 278, 444, 633]. As opposed to other factors known at that time to restrict retroviral infection (eg. Fv1 and Fv4), TRIM5α activity appeared to possess antiviral activity against spectrum of diverse retroviruses. At the outset of the research presented in Chapter 1, it had been demonstrated that TRIM5α variants possessed antiviral activity against the Gammaretrovirus MLV as well as many species of Lentivirus, including HIV-1, SIVs of multiple origins, EIAV, and FIV. The observed restrictive pattern was dependent upon both species-specific determinants in the TRIM5α as well as determinants in the challenge virus [Reviewed in 29, 415]. From these

initial studies it appeared that retroviral Gag molecules had evolved to escape from the block imposed by TRIM5α in their native hosts, but remained susceptible to attack by heterologous TRIM5α proteins. From this it was proposed that TRIM5α was a general anti-retroviral factor that interacted with complex tertiary/quaternary structure determinants presented on the surface of CA cores upon retroviral entry into a target cell [389].

As retroviral CA core structure is largely conserved between retroviral species, and families, we hypothesized that TRIM5α from a subset of non-native species should possess antiviral activities against the Betaretrovirus Mason-Pfizer monkey virus (M-PMV). To test this hypothesis, we constructed a replication defective indicator virus that expresses GFP in place of the viral Env. VSV-G pseudotyped indicator virus was then used to infect a panel of fibroblast cells from primate species of New World, Old World, and Hominid lineage. It was observed that the cells from three New World primate species specifically inhibit M-PMV infection. These M-PMV resistant species are tamarin monkey, squirrel monkey, and spider monkey. Time course experiments demonstrated that this block to infection occurred early post-entry; a stage similar to previous reports describing TRIM5α activity [232, 541]. Exogenous expression of TRIM5α from these species as well as inhibition of endogenous TRIM5α activity demonstrated that TRIM5α was responsible for the post-entry block to M-PMV infection in tamarin monkey and squirrel monkey cells. Thus, the data presented in this dissertation support the hypothesis that TRIM5α is a novel type of pathogen recognition receptor (PRR) responsible for detecting the presence of retroviral capsid (CA) cores shortly after their appearance in uninfected cells. In the time since embarking on the studies presented in Chapter 1, two reports have demonstrated TRIM5α-mediated restriction of foamy virus infection [434, 632]. This is further evidence of TRIM5α functioning as a PRR with broad anti-retroviral activities. In fact, to date the only mammalian (TRIM5 appears to have arisen in eutherian mammals) retrovirus genera with no reported

sensitivity to TRIM5α activity is the Deltaretroviruses, and this likely reflects a lack of studies looking for any such activities.

While it was found that spider monkey cells imposed a block to the infection of M-PMV at the early post-entry stage of infection, it was determined that TRIM5α was not the factor responsible for this restriction. Thus, an as yet unknown mechanism accounts for the block to infectivity in spider monkey cells. It is likely that a specific factor accounts for this anti-M-PMV activity in spider monkey cells, but further studies will be required in order to address this possibility. Identification of the factor responsible for the M-PMV block, be it a inhibitory factor or the lack of a positive-acting factor, would help to shed additional light on the events in the early stages of retroviral infection of cells.

As a cytoplasmic retroviral-specific PRR possessing activity against a diverse collection of retroviruses, TRIM5α comes in direct contact with viral invaders, and has faced multiple episodes of positive selective during primate evolution [483, 524]. These episodes have alternated with episodes of positive selection on the antiviral gene TRIM22 [482], which lies in close proximity to TRIM5 and thus would be closely linked evolutionarily. Being intracellular proteins, both TRIM5 and TRIM22 exert their antiviral effects in the context of an infected cell. In the case of TRIM5α, expression levels have been correlated with likelihood of retroviral infection [503]. As both of these genes display a broad spectrum of antiviral activity, one would expect that either these genes are expressed ubiquitously or that there has been a series of selective events molding promoter activity to match gene expression with the target cell type of the virus du jour. Initial experiments suggested that these genes were expressed ubiquitously [464]. However, subsequent experiments demonstrated both tissue-restricted expression of TRIM5 and TRIM22 [482] as well as specific regulation in response to IFN (TRIM5 and TRIM22) and p53 (TRIM22) stimulation [20, 88, 89, 269, 422, 598]. Thus, we hypothesized that expression of these two genes is likely

spatially and temporally regulated, and that this regulation has evolved in response to successive waves of viral challenge.

TRIM5 and TRIM22 are situated in opposite orientations on the chromosome with only ~5 kb separating the two, such that they share a short intergenic region containing the core promoters of both genes. Upon examination, this promoter-containing region was found to largely be conserved between the primate species studied (data not shown). This suggests that changes outside of the core promoter region likely account for species-specific transcriptional regulation differences, if any exist. In order to identify such potential transcriptional regulatory sequences, the entire transcribed regions of primate TRIM5 and TRIM22 were examined, both for sequence divergence as well as differences in transposable element content. The transcribed regions of TRIM6 and TRIM34 were also examined in such fashion. These genes serve as ideal controls, as they are of similar size, have similar exon/intron structure, and are located in the same genomic lcus, but in contrast to TRIM5 and TRIM22 they have been under purifying selection during primate evolution. This analysis is, to my knowledge, the first of its kind. Here we performed multiple species comparisons of genomic sequences with the primary intent of comparing the non-coding regions and transposable element content. Others have performed multiple species comparisons of genomic DNA, but RepeatMasker was used to eliminate the complexity resulting from the presence of repetitive elements. Furthermore, typical studies of repetitive elements focus attention on one species, or compare two species. The study presented in Chapter 2 shows a three species subset of a 10 species genomic comparison that we performed. Additional novel evolutionary insights were gleaned from the larger study (manuscript in preparation).

The study presented in Chapter 2 revealed that the transcribed region of TRIM5 is very poorly conserved, with nucleotide similarity lower than 50% in comparisons between Old World and New World primates. The vast majority of this sequence divergence was observed to result from insertions and deletions in the first and fourth introns of the gene. These represent the region adjoining the promoter as well as the large intron separating the tripartite motif-encoding exons

from the virus interfacing B30.2 domain. TRIM22 and TRIM34 were observed to have similar levels of nucleotide divergence. These genes had lower divergence rates than TRIM5. However, similar to observations in TRIM5, the observed sequence divergence was largely the result of insertions and deletions in the first and fourth introns. In contrast to the other genes examined, the entire TRIM6 transcribed region was found to be well conserved between the species included in this study. Of great interest, lineage-specific endogenous retroviral LTR sequences were identified exclusively in TRIM5 and TRIM22, with the majority of these found in the first introns of these genes. Primate TRIM5s were found to contain eight unique LTR elements in their first intron, with none of these LTR elements being evolutionarily conserved from hominoids to New World primates. TRIM22 was found to have two differentially fixed LTR elements in the first intron. One of these is specific to Old World primates and the other specific only to hominoids. As LTRs serve as the viral promoters, we theorized that these ERV LTR elements play a transcriptional regulatory role for TRIM5 and TRIM22.

As a proof of concept for this hypothesis, we explored the transcriptional regulatory potential of a LTR10D element, which is present in the first intron Old World primate TRIM22 genes but is absent from New World primate TRIM22. This element had previously been shown to contain a p53-binding site that provides p53 responsiveness to the human TRIM22 gene. We demonstrated that this LTR10D element provides for lineage specific TRIM22 responsiveness to p53 activation. From this observation, we extrapolate that most or all of the LTR elements fixed in the first intron of these genes have the potential to play a role in the transcriptional regulation of the gene in which they are present. However, the number of LTR elements to be tested alone and in combination poses a daunting task in unraveling the function that each of these elements play in the regulation of gene expression. Based on the elevated sequence divergence and multiple LTR elements immediately adjacent the core promoters of TRIM5 and TRIM22, we believe that these genes are likely to be differentially expressed between humans and the non-human primate models of disease. For that reason it is important that we gain a better

understanding of the transcriptional regulatory network responsible for controlling expression of these antiviral genes and the role that lineage specific LTR elements play in this process. Future experiments should be conducted to further this aim.

From the work presented here and conducted by others, TRIM5α appears to be a PRR specific for retroviral CA cores, whose evolutionary history has been shaped by the requirement to match specificity and expression profile to match successive waves of viral challenge. Furthermore, TRIM5α is one of a growing number of germ-line encoded host factors that impose intrinsic blocks to retroviral infection and that act at the level of an individual cell, largely independent of the greater immune system. Together, these factors serve as innate barriers to retroviral infection that result in a low frequency of cross-species transmission of these viruses and a typically poor replicative potential of newly introduced retroviruses in new host species. However, retroviruses are highly plastic organisms that can evolve away from such pressures. After viral adaptation to a new host, the prevalence of allelic variants of the intrinsic immunity factors likely serve a role in limiting viral pathogenesis and evolution on a population level. Thus these factors potentially serve to hem in the emergent virus until an evolutionarily stable virus-host interaction evolves.

LIST OF ABBREVIATIONS

ψ	Encapsidation signal sequence
ΔΔC(t)	Difference in the number of PCR cycles to cross the threshold
β-TRCP	β-transducin repeat containing protein
293T	Human fetal kidney cells expressing the SV40 T-antigen
6-FAM	6-Carboxyfluorescein
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
ALV	Avian Leukosis Virus
ANOVA	Analysis of variance
AP-2	Adaptor protein 2
APOBEC	Apolipoprotein B mRNA editing enzyme
ATR	Ataxia telangiectasia and Rad3-related protein
att	Attachment sites
BFV	Bovine foamy virus
BLAST	Basic local alignment search tool
Bst2	Bone marrow stromal antigen 2 protein
CA	Capsid domain or capsid protein
cDNA	Complimentary DNA
CEBP	CCAAT enhancer binding protein
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CRFK	Crandell feline kidney cells
CRM1	Chromosome region maintenance 1
CTD	C-terminal domain of CA

CTE	Constitutive transport element
Cul4	Cullin 4 protein
CypA	Cyclophilin A
D/N	Dominant negative
DCAF1	DNA damage binding protein 1 and Cullin4 associated factor 1 protein
DDB1	DNA damage binding protein 1
DMSO	Dimethyl sufloxide
dN/dS	Rate of nonsynonymous to synonymous nucleotide change
DNA	Deoxyribonucleic acid
dUTPase	Deoxyuridine triphosphatase
ECMV	encephalomyocarditis virus
EGFP	Enhanced green fluorescent protein
EIAV	Equine infectious anemia virus
env	Envelope gene
Env	Envelope glycoprotein
ER	Endoplasmic reticulum
FcγR	Immunoglobulin G constant fragment receptors
FeLV	Feline leukemia virus
FFV	Feline foamy virus
FIV	Feline immunodeficiency virus
Fr-MuLV	Friend murine leukemia virus
FV-1	Friend virus susceptibility factor 1
FV-4	Friend virus susceptibility factor 4
gag	Group specific antigen gene
Gag	Group specific antigen protein domain
GALT	Gut-associated lymphoid tissue

GALV	Gibbon ape leukemia virus
GFP	Green fluorescent protein
HA	Hemagglutanin protein
hAT	hobo/Ac/Tam3 DNA transposon superfamily
HERV-K	Human endogenous retrovirus K
HERV-L	Human endogenous retrovirus L
HEX	hexachloro-6-carboxyfluorescein
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HML-2	Human mouse mammary tumor virus-like 2
HOS	Human osteosarcoma cells
HP1	Heterochromatin-associated protein 1
HSC	Hematopoietic stem cell
HTLV	Human T-lymphotropic virus
IFN	Type I interferon
IgG	Immunoglobulin G
IN	Integrase domain or integrase protein
Indel	Insertion or deletion of DNA sequence
IR	Infectivity ratio
$IR_{(M-PMV/MLV)}$	Infectivity ratio of M-PMV to MLV
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor protein
ISRE	Interferon sensitive response element
Kap-1	KRAB-ZFP-associated protein 1

kb	kilobase; 1000 nucleotides
kDa	Kilo-Dalton
LEDGF/p75	Lens epithelium-derived growth factor/transcriptional co-activator p75
LINE	Long interspersed nuclear element
LTR	Long terminal repeat
Lv1	Lentiviral susceptibility factor 1
Lv2	Lentiviral susceptibility factor 2
MA	Matrix domain or matrix protein
Max	Myc-associated factor X protein
MDM2	Murine double minute 2 protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIR	Mammalian interspersed repeat
MLV	Murine Leukemia Virus
MMTV	Mouse Mammary Tumor Virus
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
M-PMV	Mason-Pfizer Monkey Virus
M-PMV-EGFP	Green fluorescent protein expressing Mason-Pfizer monkey virus
mRNA	Messenger RNA
MuERV-L	Murine endogenous retrovirus L
MuLV	Murine leukemia virus
Myc	Myelocytomatosis viral oncogene homolog protein
NC	Nucleocapsid domain or nucleocapsid protein
NCBI	National Center for Biotechnology Information
nef	Negative factor gene

Nef	Negative factor protein
NES	Nuclear export signal
NLS	Nuclear localization signal
nt	Nucleotide
NTD	N-terminal domain of CA
NXF1	Nuclear RNA export factor 1; Tap
ORF	Open reading frame
PAK-2	p21 activated kinase 2 protein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Primer binding site
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PHV	Perch hyperplasia virus
PIC	Preintegration complex
pol	Polymerase gene
Pol	Polymerase protein domain
PPT	Polypurine tract
PR	Protease protein
pro	Protease gene
Pro	Protease protein domain
PRR	Pattern recognition receptor
PtERV1	Pan troglodytes endogenous retrovirus 1
qPCR	Quantitative real-time PCR
R	Repeat region of retroviral LTR
RDR	RD114 receptor

Ref1	Restriction factor 1
rev	Regulator of viral expression gene
Rev	Regulator of viral expression protein
rex	Regulator of expression from x gene
Rex	Regulator of expression from x protein
RING	Really interesting new gene protein domain
RNA	Ribonucleic acid
RNAP II	RNA polymerase II protein
RNase H	Ribonuclease H protein
RRE	Rev-responsive element
RSV	Rous sarcoma virus
RT	Reverse transcriptase domain or reverse transcriptase protein
RTC	Reverse transcriptase complex
sag	Superantigen gene of mouse mammary tumor virus
Sag	Superantigen protein of mouse mammary tumor virus
SEM	Standard error of the mean
SFV	Simian foamy virus
SINE	Short interspersed repeat
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SIVcpz	Chimpanzee simian immunodeficiency virus
SIVmac	Macaque simian immunodeficiency virus
SIVmac239	Macaque simian immunodeficiency virus molecular clone 239
SIVsm	Sooty mangabey simian immunodeficiency virus
SIVtan	Tantalus monkey simian immunodeficiency virus
SMRV	Squirrel monkey retrovirus

SnRV	Snakehead retrovirus
SP1	Spacer region 1; the peptides between HIV-1 MA and CA
SRV	Simian retrovirus
STAT1	Signal transducer and activation of transcription 1 protein
STLV	Simian T-lymphotropic virus
SU	Surface subunit of retroviral envelope protein
SV40	Simian virus 40
Tap	Tip-associated protein; NXF1
tat	Trans-activator of transcription gene
Tat	Trans-activator of transcription protein
tax	Trans-activator from x gene
Tax	Trans-activator from x protein
TF	Transcription factor
TFIIB	Transcription factor IIB protein
TM	Transmembrane subunit of retroviral envelope protein
TRIM	Tripartite motif-containing gene
TRIM-Cyp	TRIM5 cyclophilin A fusion protein
tRNA	Transfer RNA
U3	Unique 3’ region of retroviral LTR
U5	Unique 5’ region of retroviral LTR
UNG2	Uracil-DNA glycosylase 2 protein
UTR	Untranslated region
vif	Viral infectivity factor gene
Vif	Viral infectivity factor protein
VLP	Virus-like particle
vpr	Viral protein R gene

Vpr	Viral protein R protein
vpu	Viral protein unique gene
Vpu	Viral protein unique protein
vpx	Viral protein X gene
Vpx	Viral protein X protein
VSV	Vesicular stomatitus virus
VSV-G	Vesicular stomatitis virus glycoprotein
WDSV	Walleye Dermal Sarcoma Virus
WPRE	Woodchuck post-transcriptional regulatory element
XMRV	Xenotropic murine leukemia virus-like virus
YFP	Yellow fluorescent protein
ZAP	Zinc-finger antiviral protein

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