TRIM34 and TRIM5a co-operatively restrict primate lentiviruses

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Abstract

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Human immunodeficiency virus (HIV) and other lentiviruses adapt to new hosts by evolving to evade host-specific innate immune proteins that differ in sequence and often viral recognition between host species. Understanding how these host antiviral proteins, called restriction factors, constrain lentivirus replication and transmission is key to understanding the emergence of pandemic viruses like HIV-1. One such restriction factor is TRIM5α, which blocks replication by multimerizing onto the HIV core, inducing aberrant capsid uncoating. TRIM5α-mediated restriction requires multimerization of TRIM5α monomers. Human TRIM34, a paralogue of the well-characterized lentiviral restriction factor TRIM5α, was previously identified by our lab via CRISPR-Cas9 screening as a restriction factor of certain HIV and SIV capsids. Notably, TRIM34-mediated restriction requires TRIM5α. Thus, we propose that TRIM34 requires multimerization with TRIM5α to restrict lentiviral capsids.

Here, we show that diverse primate TRIM34 orthologues from non-human primates can restrict a range of Simian Immunodeficiency Virus (SIV) capsids including $SIV_{AGM\text{-}SAB}$, $SIV_{AGM\text{-}TAN}$ and SIV_{MAC} capsids, which infect sabaeus monkeys, tantalus monkeys, and rhesus macaques, respectively. All primate TRIM34 orthologues tested, regardless of species of origin, were able to restrict this same subset of viral capsids. We demonstrate that TRIM5α is necessary, but not sufficient, for TRIM34-mediated restriction of these capsids, and that human TRIM5α functionally interacts with TRIM34 from different species. Finally, we find that both the TRIM5α SPRY domain, in particular the v1 loop, and the TRIM34 SPRY domain are essential for TRIM34-mediated restriction.

These data suggest that TRIM34 is a conserved primate lentiviral restriction factor and that TRIM34 and TRIM5α interact with each other and capsids. This supports a model in which TRIM34 is a broadly conserved primate lentiviral restriction factor that acts in tandem with TRIM5α, such that together, these proteins can restrict capsids that neither can restrict alone. The goals of this work are to define the range of primate lentiviruses against which TRIM34 is active, identify determinants of antiviral specificity for TRIM34-mediated restriction, to identify domains of TRIM34 and TRIM5α that are required for lentiviral restriction. Ultimately, these studies can help lead to a better understanding of TRIM34's role in host-pathogen evolutionary history.

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CHAPTER 1. INTRODUCTION

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1.1 HIV origins

Human Immunodeficiency Virus 1 (HIV-1) is a pandemic virus that is thought to have arisen about a century ago (1, 2). However, it did not spread globally until the 1970s, when it emerged as a global pandemic (3). As of 2021, the most recent year for which data are available, about 84.2 million people have acquired HIV and about 40.1 million people have died from HIV (4). Likewise, as of 2021, there are about 38.4 million people living with HIV, with about 1.5 million new acquisitions per year (4). Although there now exist safe, effective prevention and treatment protocols for HIV, about 650,000 people die from HIV-related causes each year (4). Currently, there is no vaccine nor cure for HIV. Research into the basic biology of HIV is thus essential to develop vaccines, treatments, and cures.

HIV-1 arose from cross-species transmission events of a lentivirus that infects chimpanzees, called Simian Immunodeficiency Virus of chimpanzees, or SIV_{CPZ}, into humans (**Figure 1.1**) (3, 5). At least 2 discrete transmission events occurred from chimpanzees to humans, resulting in HIV-1 groups M and N, with M becoming the major clade in the pandemic spread of HIV-1 (6, 7). HIV-1 groups O and P are relatively rare and are thought to have arisen in humans via transmission from gorillas which had themselves previously been infected by chimpanzees (6, 8). Many non-human primate species in Africa are host to endemic SIVs (9), and SIV_{CPZ}, arose in chimpanzees as a recombinant virus of SIV_{RCM} (originating from red-capped mangabeys) and SIV_{GSN}/SIV_{MUS}/SIV_{MON}, (originating from the group of monkeys known as guenons, including the greater spot-nosed monkey, the mustached monkey, and the mona monkey) (10). Human Immunodeficiency Virus type 2 (HIV-2), another lentivirus that infects humans, has independent origins from SIV_{SMM} (originating from sooty mangabey monkeys), which is also the progenitor of SIV_{MAC} (SIV from rhesus macaques) (3). The unique evolutionary

histories of these viruses are reflected in changes across the viral genomes that arose due to divergent challenges across different host species.

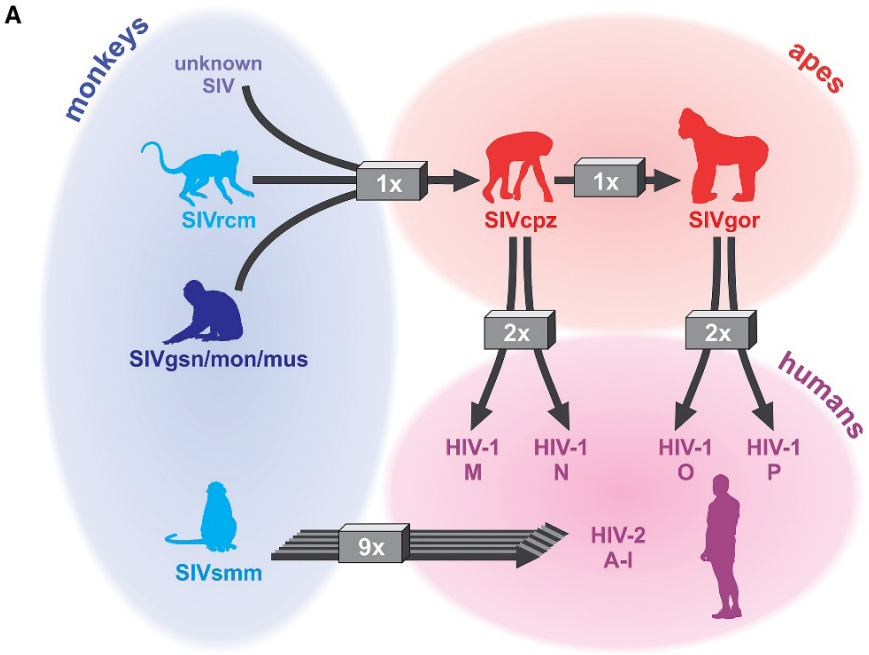

Figure 1.1. *Cross-species transmission of primate lentiviruses.* ***Figure 1.1*** *was adapted from* (5) *with permission, Cell Press license number 5583270677997.*

1.2 HIV replication

HIV belongs to the family *Retroviridae*, which is characterized by the presence of the enzyme reverse transcriptase (RT) that enables the production of DNA from viral RNA (vRNA) (11). More specifically, HIV is a member of the *Lentivirus* genus, which is characterized by a set of accessory genes not found in other retroviruses as well as the ability to infect both non-dividing and actively dividing cells, whereas other retroviruses can only infect actively dividing cells (11). The viral life cycle is reviewed extensively by Engleman and Cherepanov (12), but it will be discussed briefly herein (**Figure 1.2**) (13). HIV envelope (env) attaches to the host cell via its receptor, CD4 (14, 15), and one of two co-receptors, CCR5 (16–20) or CXCR4 (21). Different SIVs can exploit other coreceptors including, but not limited to, GPR1 (22), GPR15 (22), CCR2b (23), STRL33 (24), and CXCR6 (25) for entry—in addition to or in place of CCR5

and CXCR4. After binding, the viral membrane fuses with the host cell membrane to allow entry of the viral core, which is composed of the vRNA contained with capsid (CA), matrix (MA), and nucleocapsid (NC) proteins, into the cytoplasm. After the initiation of reverse transcription in the cytoplasm, the viral core is imported into the host nucleus via the nuclear pore complex, reverse transcription is completed in the nucleus, and uncoating occurs near the site of integration (26–28). Viral DNA is then integrated into the host cell chromosome. The process of generating a new virion begins when host transcriptional machinery transcribes vRNA from the integrated provirus. These RNA products are exported from the nucleus and translated by host translational machinery. Viral proteins and genetic material are then packaged and bud from the host cell, with the nascent viral membrane being derived from the host plasma membrane. After egress from the host cell, maturation occurs in the nascent virion, resulting in a fully-mature and infectious virus particle.

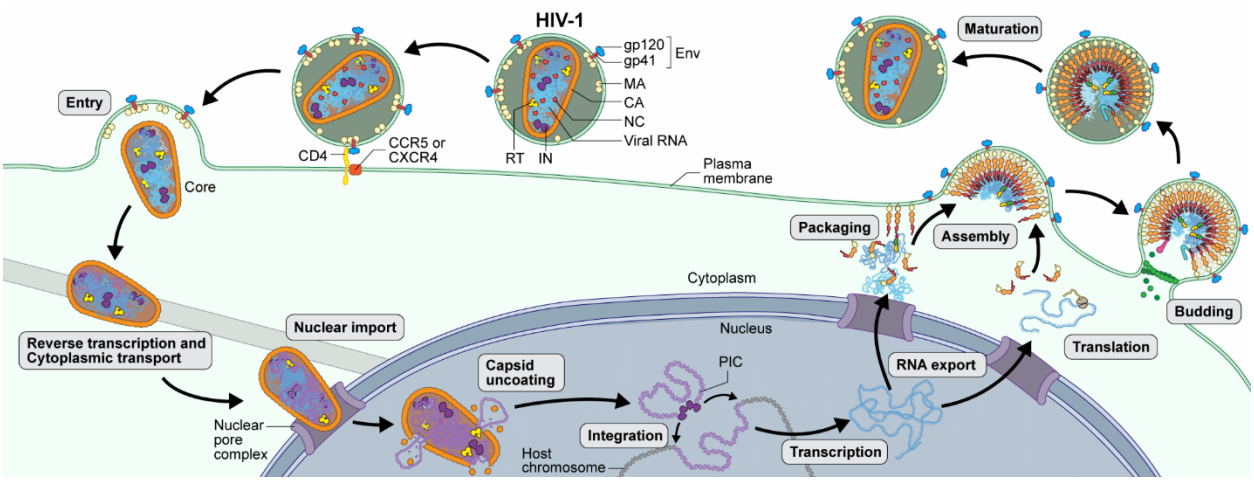

Figure 1.2*. HIV life cycle.* ***Figure 1.2*** *was reproduced from* (13) *(Janet Iwasa) with permission, Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License.*

Like other viruses with small genomes, HIV usurps the function of host proteins for its replication. Viral interactions with host proteins can have a wide range of effects on the viral life cycle. Across many stages of the viral life cycle, HIV has evolved to utilize or exploit host proteins to its own benefit (29). HIV binds and enters the cell via interactions of env with the

host receptor CD4 (14, 15) and co-receptors CCR5 (16–20) or CXCR4 (21). The viral core is than trafficked through the cell cytoplasm via microtubule networks (30). Nuclear import is mediated by the interaction of viral capsid with NUP358 and NUP153 in the nuclear pore complex (31). HIV requires host LEDGF (Lens Epithelium-Derived Growth Factor), a chromatin-binding protein, to interact with integrase to target and tether the integration complex to the host chromatin (32). Transcription initiation can occur when the viral accessory protein, Tat (33), interacts with the host proteins Cyclin T1 and Cdk9, which comprise the P-TEFb complex (34). This complex comes into physical proximity to the HIV promoter via binding the RNA TAR hairpin loop structure (34). Cdk9 then phosphorylates host RNA polymerase II allowing it to elongate productively (34). Viral mRNA is transported out the nucleus via interactions of the accessory protein Rev with the host nuclear export protein Crm1 (35). Translation is then completed by host translational machinery. Finally, viral budding and escape from the producer cell is mediated by the host ESCRT (Endosomal Complex Required for Transport) pathway (36).

1.3 The role of the capsid protein and host proteins that bind capsid

The capsid protein of HIV serves functions both early in the viral lifecycle in targeting the viral core to the nucleus, as well as structural component of the virion. The capsid of HIV is defined by its multimeric lattice structure (**Figure 1.3**) (37, 38). Capsid is encoded in the viral *gag* gene, which is translated as a polyprotein and then cleaved into individual units including the capsid protein. Each viral core is composed of about 1500 capsid monomers, which multimerize into approximately 250 hexamers and exactly 12 pentamers (37, 39). These hexamers and pentamers form the viral core surrounding the viral nucleic acids. Both proper maintenance of capsid integrity and the occurrence of proper uncoating spatially and temporally are important in a number of steps in the viral life cycle including reverse transcription and nuclear import (37). Indeed, over- or under-stabilization of capsid can have severely detrimental

effects on viral replication (40, 41). For example, Forshey *et al*. identified a number of point mutations resulting in an unstable capsid phenotype and impaired infectivity, including P38A, L136D, R143A, K170A, K203A, Q219A, and Q63A/Q67A, among others (40). Although these mutants do appear to assemble successfully into cores, some form aberrant core morphologies, whilst all are recovered at a lower rate than wild-type cores, presumably due to altered stability (40). Although relatively fewer hyperstable capsid mutants have been identified, one well-characterized hyperstable capsid mutant is E45A, which has been found to have impaired nuclear entry and decreased infectivity (40, 42). Likewise, the hyperstable A204C mutant has been shown to have reduced infectivity (43). Notably, mutants that resulted in cores recovered at a similar rate to wild type (Q4A, G116A, T119A, N139A, and P207A) were not impaired in infectivity, supporting a link between proper stabilization and infectivity (40).

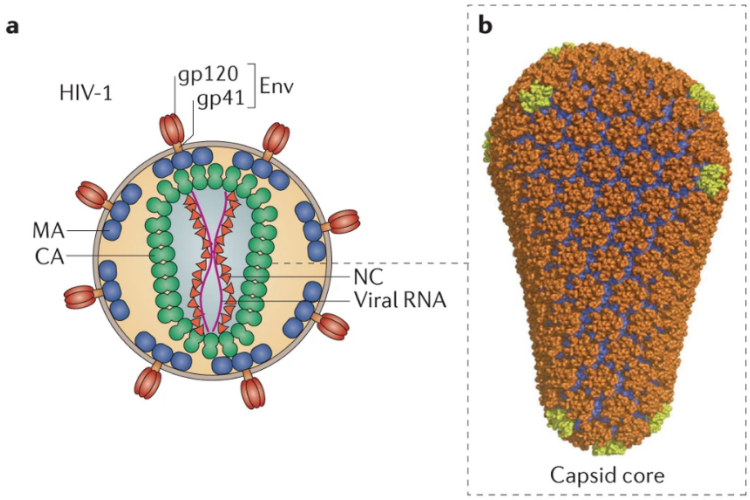

***Figure 1.3**. HIV capsid structure. **Figure 1.3** was adapted from* (38) *with permission, Springer Nature license number 5583240320980.*

Compared to other viral proteins such as *env*, which is known for its mutational resilience and flexibility (44, 45), capsid is highly genetically fragile (46, 47). Because of its abundance, importance to the viral life cycle, and relative fragility, capsid is a promising target for pharmaceutical interventions. A number of small molecule inhibitors have been identified via

compound screening (48, 49), among the first of which was a compound called PF-3450074 (PF74) (50). PF74 inhibits infectivity in a concentration-dependent fashion. At low concentrations, it competes with capsid for binding to host factors CPSF6 and NUP153, impairing nuclear entry (51–54). At high concentrations, it hyperstabilizes capsids, such that they exhibit properties similar to those of the capsid mutant E45A (50, 52, 54–58). More specifically, at high concentrations, this stabilization of the capsid lattice allows reverse transcription to occur but interferes with the uncoating process (58). More recently, newer capsid-targeting compounds such as GS-CA1 (59) and GS-6207 (60), an analogue of GS-CA1 trade named Lencapavir, have been shown to be potent antivirals. These compounds may act by inhibiting the development of mature capsid, interfering with capsid assembly, or perturbing capsid stability (48, 49). Lencapavir has recently been shown to reduce viral load rapidly and sustainably in human trials (61, 62), and has now been approved for use as a semi-annually administered therapy, raising hopes for a new class of long-acting HIV drugs (63).

Viral capsid interacts with a number of host proteins, some of which are beneficial to the virus life cycle and some of which are deleterious. Host factors that bind HIV-1 capsid can have both positive effects and negative effects on the viral lifecycle. One such capsid-binding host factor that has a positive effect on HIV replication is Cleavage and Polyadenylation Specific Factor 6 (CPSF6). The normal cellular function of CPSF6 is to recognize and cleave 3' poly(A) mRNA tails (64–66). In the context of HIV biology, CPSF6 interacts with capsid, which is important for successful viral replication as uncoating, reverse transcription, and nuclear import depend on each other in a delicate spatial and chronological balance, and premature or tardy uncoating activities can derail the viral life cycle (27, 28, 31, 67, 68). Specifically, CPSF6 interacts with capsid at the inter-molecular NTD-CTD interface (55) and can modulate nuclear import (69, 70). More specifically, CPSF6 has been shown to help target the HIV pre-integration complex to transcriptionally active chromatin for integration (71–73). This is supported by data

showing that loss of interaction with CPSF6 results in cores becoming stuck near the periphery of the nucleus. Thus, one model for how CPSF6 facilitates infection is that CPSF6 engages HIV capsid, assists it through the nuclear pore, and steers it away from heterochromatin and towards euchromatic regions in the heart of the nucleus.

CypA is a host protein with a number of putative functions in the lentiviral life cycle. CypA can be bound and incorporated into the virion of certain lentiviruses via an approximately 8 amino acid-long binding site in capsid (74–76). Not all lentiviruses can bind CypA: for example, the HIV-1/SIV_{CPZ} lineage does bind, while the HIV-2/SIV_{SMM}/SIV_{MAC} lineage does not (75, 76). It has been known for over a quarter century that CypA binding was essential to the viral life cycle in CypA-binding viruses, since treatment with cyclosporin A (CsA) results in the blockage of reverse transcription, and knockout of CypA in Jurkat cells dramatically decreases susceptibility to HIV-1 (76, 77). It has also been proposed that CPSF6 can be assisted by cyclophilin A (CypA), which helps ensure that CPSF6 binds viral cores at the correct time in the viral life cycle ensuring proper delivery of the core into the nucleus (78).

1.4 Positive selection and restriction factors

For cross-species transmission of viruses into a new host species, a primary element of host defense is the innate immune system, which includes restriction factors such as TRIM5α (Tripartite Motif 5, alpha isoform). Restriction factors are germline-encoded proteins that can act at a variety of stages in the viral life cycle and on a number of different viral components to inhibit viral entry, replication, or egress. Restriction factors such as TRIM5α counteract viral infection, and pathogens must overcome their host's immune systems in order to successfully reproduce. Hosts and pathogens exist in perpetual evolutionary conflict, each evolving to overcome the other's strategies. Frequently, rapid evolution can be observed at sites of direct host-pathogen interaction (79, 80). This is known as positive selection, which is observed when the rate of nonsynonymous mutations exceeds the rate of synonymous mutations.

Because of this evolutionary pattern, different host species have restriction factors that are orthologous but differ genetically in ways that affect their antiviral potential. Thus, restriction factors can pose an important block to cross-species transmission events. Cross-species transmission events are significant because they can result in pathogens spreading in new, susceptible populations; for example, the transmission of SIV_{CPZ} from chimpanzees into humans resulted in the HIV pandemic.

For example, TRIM5α is a well-characterized host protein that binds to multimerized retrovirus capsids and affects virus replication in a negative, rather than a positive way (**Figure 1.4**) (37). TRIM5α functions by multimerizing into higher-order structures on the viral capsid, resulting in aberrant uncoating and inhibition of the viral life cycle (37). TRIM5α's viral specificity is determined by both the host and the viral capsid in question (81–86). TRIM5α, and TRIM proteins more generally, comprise a common set of N-terminal domains (RING, B-box, and coiled-coil) and at least one variable C-terminal domain (in the case of TRIM5α, a B30.2/SPRY domain) (37). It is this SPRY domain that is the major determinant of capsid specificity for TRIM5α (81, 82, 84). Furthermore, there are a number of residues in capsid that have been found to alter TRIM5α susceptibility, including (but not limited to) P38A (87), G94D (88), and P90A (89, 90).

TRIM5α from humans is a poor restrictor of HIV-1, whereas macaque TRIM5α is a potent restrictor of HIV-1 (81, 83). In fact, a point mutation to convert a single residue of human TRIM5α to the corresponding rhesus macaque TRIM5α residue can greatly augment the restrictive potential of human TRIM5α (81, 91). This point mutation, R332P (the human arginine converted to the macaque proline at position 332), resides within the v1 (variable) loop of the TRIM5α SPRY domain which has evolved under positive selection (92). Indeed, the v1 loop in the TRIM5α SPRY domain is a major determinant of viral specificity for TRIM5α (92). In sum, interfaces of host-pathogen conflict, such as the TRIM5α v1 loop, evolve at a rate faster than

would be predicted by chance, in order to effectively suppress pathogens that are constantly evolving to escape recognition and inhibition. This type of species-dependent, highly-evolved antiviral specificity supports the idea that restriction factors can act as an important barrier to cross-species transmission events.

In addition to direct viral inhibition, TRIM5α has also been shown to possess an innate immune sensing and signaling function in the presence of viral infection. After capsid recognition by the SPRY domain, the TRIM5α RING domain can act as an E3 ubiquitin ligase which generates K63-linked ubiquitin chains (93, 94). The location of these ubiquitin chains is disputed: they may be attached to the N terminus of the TRIM5α RING domain itself (94), or they may be unassociated (93). It is also possible that both cases can occur. Regardless of anchoring status, the formation of these ubiquitin chains drives downstream events resulting in NFKb induction and subsequent immune signaling events (93–96). TRIM5α from human and a variety of nonhuman primate species has also been shown to activate AP-1 in the absence of viral infection (96). In contrast, the other, related TRIM proteins in the *TRIM5* locus do not share this effect: TRIM6 and TRIM22 do not induce no AP-1 activity, and TRIM34, the most closely-related paralogue, has minimal activity (96).

TRIM5 has also been shown to have activity in other orders of mammals. For example, rodents possess an expanded locus of TRIM5-related genes containing duplications of *TRIM12* and *TRIM30*, the rodent paralogues of *TRIM5* (97, 98). Interestingly, murine *TRIM12* orthologues were found to induce AP-1, whereas *TRIM30* orthologues did not induce AP-1 (96). Rodent *TRIM12/TRIM30* proteins have not been found to have activity against retroviruses (96, 98). However, only relatively few retroviruses have been tested, so it is possible that the rodent *TRIM12/TRIM30* locus may have evolved and expanded under pressure from other known retroviruses or from ancient, unknown retroviruses.

It was recently shown that binding of the host factor CypA actually protects capsid from inhibition mediated by the restriction factor TRIM5α (89, 90, 99, 100). Although on its own CypA has a positive effect on HIV infection, surprisingly, it also exists as a fusion protein with the restriction factor TRIM5α in some nonhuman primate species (101). In certain New World Monkey species and in the macaque lineage, CypA has been inserted at the C terminal end of TRIM5, replacing the SPRY domain (**Figure 1.4**) (37, 101–105). Like free CypA, TRIM5-CypA can bind lentiviral capsids via the CypA binding motif (37, 106). Unlike free CypA, TRIM5-CypA acts as a restriction factor rather than a dependency factor (107). Therefore, it appears that CypA's capsid-binding ability can replace the capsid-binding interface of the SPRY domains, while N-terminal domains remain intact and maintain their multimerization function (108, 109). This trait was first observed because Owl Monkey cells possessed an unexplained block to HIV-1 (105, 110). That this TRIM5-CypA fusion has arisen independently numerous times across vertebrate evolution—in New World Monkeys (105), the Asian macaque lineage (102, 104, 111–113), ray-finned fishes (114), shrews (115), and rodents (97)—perhaps suggests the repeated intrusion of viruses throughout evolutionary history and selection for this antiviral construct. Consistent CypA binding as a feature of lentiviruses across many species over millions of years may also have contributed to the selective benefit of TRIM-Cyp fusions in host genomes.

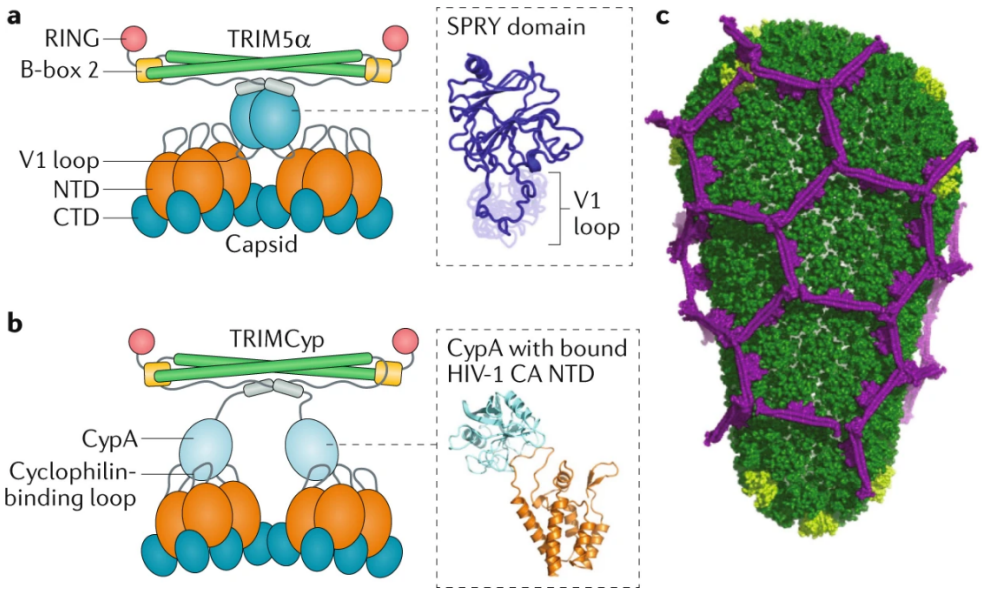

Figure 1.4*. TRIM5α structure and function.* ***Figure 1.4*** *was reproduced from* (37) *with permission, Springer Nature license number 5583240836476.*

1.5 Other Capsid-dependent restriction events, *Fv1* and *Lv1*

The first block to retroviral infection was identified in the late 1960s: *Fv1*, which inhibited Friend Murine Leukemia Virus (MLV) (116). This block was initially observed in different inbred mouse lines that displayed differential susceptibility to Friend MLV: NIH Swiss mice were permissive to viruses termed N-tropic MLV (N-MLV) and inhibitive to others termed B-tropic MLV (B-MLV), whereas BALB/c mice were permissive to B-MLV and inhibitive to N-MLV (116). The corresponding alleles were termed $Fv1^n$ and $Fv1^b$. In the mid-1990s, the responsible gene was located via positional cloning (117, 118). Based on sequence homology to the human endogenous retrovirus HERV-L, it appeared that Fv1 arose from an integration of the *gag* gene of an ancient rodent endogenous retrovirus (117). The *Fv1* inhibition was found to occur after reverse transcription and before integration (119). The block occurs via direct interaction with the viral capsid, suggesting interference with capsid integrity and uncoating (120–122). Furthermore, differential capsid susceptibility to *Fv1* between N- and B-MLV was eventually attributed to a single amino acid change at position 110 (123).

In the 1990s and 2000s, similarities were noted between the *Fv1* block to restriction and blocks observed against HIV-1 and MLV in a number of primate cell lines (110, 124–126). Specifically, it was known that some human cell lines could restrict N-MLV but not B-MLV (127). Furthermore, HIV-1 was known to be restricted in Old World Monkeys by an element that acted on capsid (125, 128, 129). However, it did notably differ from *Fv1* in that the block occurred before reverse transcription (124, 130, 131). A human allele of the murine *Fv1* could not be found, so the block was called *Ref1* (Restriction factor 1) (127). Likewise, it was thought that a similar factor existed in African green monkey cells, which could block N-MLV, HIV-1, certain SIVs, and equine infectious anemia virus (EIAV) (132). This block was termed *Lv1* (a block to Lentiviral replication) and later was recognized to be a species-specific variant of *Ref1* (86). *TRIM5* (described above) was eventually identified as the responsible gene for both of these restriction phenotypes by expression of a rhesus macaque cDNA library in human cells and a subsequent screen for clones that were resistant to HIV-1 infection (83). Notably, although the *Fv1* gene is not a *TRIM* and lives in an entirely different locus than does *TRIM5* (118), the *Lv1*/*Ref1*/TRIM5α block shares a number of similarities to *Fv1*. These include: viral sensitivity determined by the identity of position 110 in MLV (133); restriction that was saturable (134), a history of positive selection (135); and despite no sequence homology, a similar domain architecture featuring a N-terminal coiled-coil motif involved in multimerization and a C-terminal domain involved in capsid recognition and binding (136, 137).

1.6 Other Capsid-dependent restrictions, TRIM34

In primates, *TRIM5* lives in a gene locus with 3 paralogous *TRIM* family members: *TRIM34*, *TRIM6*, and *TRIM22*. *TRIM34* is the most-closely related *TRIM* gene to *TRIM5* in humans (138). TRIM34 was first identified as Ring Finger 21 (RNF21) or Interferon-Responsive Finger Protein 1 (IFP1) using degenerate primers to identify novel RING domains (139). The authors noted that it was broadly expressed across many cell types and was upregulated in the

presence of interferon-alpha. TRIM34 has been relatively poorly studied, primarily in the context of its relationship to TRIM5α, but was known not to have activity against HIV-1 (108). Recently, however, Ohainle *et al*. (99) identified TRIM34 as a restriction factor of the HIV-1 capsid mutant, N74D, via CRISPR screening (140). At the time, HIV-1 N74D was known to be more sensitive to interferon-alpha-mediated blocks relative to WT HIV-1, suggesting the presence of one or more unknown restriction factors, although it is unknown by what mechanism the N74D mutation renders this capsid susceptible (141). Ohainle *et al*. found that TRIM34 was an interferon-independent restriction factor of HIV-1 N74D, SIV_{MAC}, and $SIV_{AGM\text{-}TAN}$ (originating from tantalus monkeys) (99). The block occurred prior to reverse transcription and was dependent on TRIM5α. The topic of TRIM34-mediated restriction will be discussed subsequently in greater depth in this thesis.

1.7 Other Capsid-dependent restrictions, MxB

In addition to TRIM family capsid-binding restriction factors, there exist other host proteins that can inhibit HIV-1 and other lentiviruses via interacting with capsid. One such protein is MxB (Myxovirus Resistance B, also referred to as Mx2), which belongs to the Mx protein family. *Mx* genes are conserved across vertebrates and have expanded via ancient gene duplication and conversion events (142). Mx proteins comprise a dynamin-like large GTPase domain attached to a helical stalk by a hinge-like bundle-signaling element (143). Humans possess two Mx proteins with known antiviral function (144). Human Mx proteins can restrict a broad range of viruses, including but not limited to influenza A virus (145), Thogota virus (146), vesicular stomatitis virus (147), human parainfluenza virus (148), herpesviruses (149, 150), and hepatitis B virus (151). Human MxB is an interferon-stimulated protein that can inhibit HIV-1 infection by interfering with nuclear import (152–154). Mx proteins form dimers and higher order oligomers (143, 155), and dimerization is required for MxB antiviral activity against HIV-1 (156, 157). As with TRIM5α, restriction of HIV-1 by MxB is dependent on capsid identity,

and point mutations in capsid can radically alter susceptibility (152–154, 158, 159). Furthermore, MxB potency also seems to depend on which nuclear pore proteins are utilized for nuclear entry (160). A recent pre-print has suggested that MxB may actually act as a decoy molecule, luring HIV away from true nuclear pores and thus impeding nuclear entry (161). Finally, CypA may also be required for MxB activity, since disruption of CypA binding also resulted in abrogation of MxB activity (153, 154).

1.8 Unknown restrictions that target capsid remain to be identified

While TRIM5α was identified as the cellular component responsible for the *Lv1*/*Ref1* restriction event through cDNA screening, there exist other known blocks to lentiviral infection that depend on capsid that remain poorly understood and for which the genes responsible are not completely identified. There are at least three other blocks to lentiviral replication that have been characterized but for which a responsible host protein or proteins have not been fully or definitively identified, called *Lv2*, *Lv3*, and *Lv4*.

1.8.1 Lv2

In 2001, McKnight *et al*. (162) identified that a certain HIV-2 isolate, prCBL-23 (later termed MCR, Molecular Clone Restricted), could replicate in peripheral blood mononuclear cells (PBMCs), immortalized T cells, and certain immortalized epithelial cell lines, but was blocked in primary macrophages, immortalized fibroblasts, and other immortalized epithelial cell lines (162). This was in contrast to another HIV-2 isolate, CBL-23 (later termed MCN, Molecular Clone Nonrestricted), which could replicate well in all the cell lines tested.

Subsequently, several correlates to restriction for MCR and MCN were identified. First, by generating chimeric viruses, it was established that both gag and env were involved in the restricted phenotype (163). While MCN env pseudotyping could rescue MCR cores from restriction, MCR pseudotyping only resulted in a partial block of MCN cores (163). By sequence comparison, a single amino acid in MCR capsid, I207V (gag numbering; also enumerated as

I73V using CA numbering), was responsible for restriction (163). Despite the relatively minor change from an isoleucine to a valine, this residue seemed to be the critical determinant of susceptibility.

This restriction was proposed to be distinct from *Lv1* (which at the time was known to be similar to *Ref1* but had yet not been proven to be one in the same) because *Lv1* could not be overcome by VSV-G pseudotyping while this restriction could be (162, 163). Furthermore, N-MLV was not able to saturate this new block, whereas *Lv1* was known to be saturable by pre-treatment with N-MLV (132). Finally, the I73V/I207V capsid mutant, which was not susceptible to *Lv2*, was susceptible to TRIM5α (164). The block, now characterized but with an unknown effector, was thus termed *Lv2* following the convention of *Lv1* (163).

In support of the hypothesis that endosomal entry was required for *Lv2*, it was found that endosomal trafficking, but not acidification, were implicated in restriction (165). VSV-G pseudotyping was shown to rescue numerous other restricted HIV-1 and HIV-2 strains in addition to MCR (163, 165). Furthermore, treatment with DOTAP, a cationic liposomal transfection reagent, could also overcome the block (162). VSV-G pseudotyped viruses bypass receptor-mediated fusion and instead enter via the endocytotic pathway, while DOTAP facilitates transport of absorption of liposomal complex and subsequent escape of DNA from endosomal membranes into the cytosol. Thus, it was proposed that the block was entry-dependent and related to post-fusion trafficking events. This model was supported by later by work showing that disrupting actin enhanced MCR infectivity (166).

As with *Lv1*, there was interest in identifying the gene responsible for *Lv2*. In 2014, Marno *et al.* (167) used an siRNA screen to implicate Regulation of Nuclear Pre-MRNA Domain 2 (RPRD2), which they refer to as RNA-associate Early-stage Anti-viral Factor (REAF). The authors found that REAF knockdown rescued MCR, HIV-1, and certain susceptible SIVs from restriction by *Lv2*. They also confirmed that VSV-G pseudotyping could rescue HIV-1 from

restriction by overexpressed REAF. Somewhat surprisingly, they proposed that REAF interacts with viral nucleic acids. In a subsequent work (168), the authors identified several single amino acid mutations in capsid that are critical for REAF-mediated inhibition of HIV-1: P38A, N74D, G89V, and G94D in capsid: these residues were even stronger determinants of restriction than I73V/I207V was for HIV-2. Residue 74 had been previously implicated in CPSF6 binding (169) and more recently in TRIM34 restriction (99). G89V and G94D are located in the CypA binding site (170, 171). Notably, however, I73V/I207V did not turn out to be an important determinant of REAF susceptibility for HIV-1 (168). This could suggest that the primary determinants of susceptibility to REAF differ between HIV-1 and HIV-2 due to other, contextual, differences in capsid or other viral proteins. Alternatively, this could indicate that REAF is only one component of Lv2 and that susceptibility depends on more than one host factor.

The most recent work on REAF restriction by Gibbons *et al*. (172) suggests that HIV-1 can use *vpr* to overcome REAF-mediated restriction. This raises the question of whether HIV-2 can also use *vpr* to antagonize REAF. Furthermore, the authors argue that REAF is not interferon stimulated and not under positive selection. Since positive selection is typical of host proteins that interact directly with pathogens, the lack of positive selection could suggest that REAF works in tandem with another host factor that is under positive selection or that it indirectly inhibits virus via some other cellular process. One such host factor that could be involved in the *Lv2* block are the Interferon-induced Transmembrane proteins (IFITMs). IFITMs are a family of interferon-stimulated proteins that can inhibit infection by a wide range of viruses including, but not limited to, influenza viruses (173), flaviviruses (173), coronaviruses (174, 175), filoviruses (174), rhabdoviruses (176), alphaviruses (177), and retroviruses (173, 178), including HIV (179). They interfere with viral entry by altering membrane components and/or by altering vesicular trafficking (180). IFITM2 and IFITM3 in particular have been identified as blocks to HIV-1 replication (179). IFITM3 has also been shown to possess activity against HIV-2, SIV_{CPZ},

SIV_{MAC}, and SIV_{AGM} (originating from African green monkeys) (178). IFITMs act by impeding viral entry and localize to endocytotic compartments, but do not directly inhibit endocytosis (179), similar to the restriction described by *Lv2*. Furthermore, pseudotyped MLV pseudotyped with Influenza A virus, West Nile virus, or Dengue virus, became sensitive to IFITM-mediated restriction (173), and HIV-1 sensitivity can change based on X4 or R5 tropism as well as env identity (140, 179, 181), supporting a role for env as a determinant of susceptibility. This too mirrors the pattern observed for *Lv2*. One challenge to these studies is that because human cells contain at least 5 IFITMs with at least 3 possessing antiviral properties (182), they may compensate for each other and act as cofounding experimental factors.

TRIM proteins are also candidates for contribution to the *Lv2* block. TRIM11 was identified as a restriction factor of HIV-1 in a screen of several dozen TRIMs for antiviral activity (183). TRIM11 inhibits viral entry and affects microtubule trafficking, but it is independent of the lysosome and the proteasome, consistent with observations of *Lv2* (183, 184). The block occurs before reverse transcription and results in accelerated uncoating (184). As with *Lv2*, TRIM11 restricted HIV-1 N74D and G94V; conversely, G89V was insensitive to TRIM11 restriction (184). While HIV-1 N74D is susceptible to both TRIM34 and *Lv2*, we do not believe that TRIM34 is *Lv2* for the following reasons. First, VSV-G pseudotyped viruses are still susceptible to TRIM34, which stands in contrast to the observation that the *Lv2* block could be overcome by pseudotyping (99). Furthermore, whereas MCR and MCN produce the same total amount of reverse transcripts, but MCR is slower to accumulate these transcripts (165), TRIM34 acts before reverse transcription, although only a single timepoint was assayed to determine extent of RT (99). Finally, although HIV-1 N74D is susceptible to TRIM34 (140), HIV-1 G89V which is susceptible to the *Lv2* block is not susceptible to TRIM34 (108). A final possibility, which is not necessarily exclusive of other hypotheses, is that *Lv2* could involve a dependency

factor related to viral entry or trafficking. That is, HIV-2 strains sensitive to *Lv2* could require some cellular factor for its early life cycle, whereas MCN is independent of that factor.

In sum, the *Lv2* block seems to be entry-pathway dependent and is mediated by both the capsid and envelope identities. Some aspects of this block may be explained by REAF activity, but it is likely that there exist other, currently unidentified components as well.

1.8.2 Lv3

Prior to the characterization of *Lv2*, other cell-line dependent blocks had also been observed. In the mid-1990s, it was recognized that SIV was unable to infect some human CD4+ T cell lines that could be infected by HIV-1, while HIV-1 was unable to infect rhesus macaque cells and rhesus macaques (the block later discovered to be TRIM5α) (185). MAGI cells (HeLa/CD4 expressing a HIV-1-LTR-β-Gal reporter) (186) were known to be resistant to most primary HIV-1 isolates and only moderately susceptible to SIV_{AGM} and SIV_{MAC} (187), whereas CMMT/CD4 cells, a rhesus macaque cell line expressing human CD4, were sensitive to SIV_{SMM}, SIV_{MAC}, and SIV_{AGM} (188). Chackerian *et al.* (187) generated a macaque version of MAGI cells, termed sMAGI (simian MAGI) cells, by expressing the HIV-1-LTR-β-Gal reporter in the CMMT-CD4 line. This new cell line was susceptible to X4 and R5 SIVs as well as to some HIV-2, but not to HIV-1. Interestingly, some HIV-1 isolates were blocked before initiation of reverse transcription, while others were blocked after (185). Retrospectively, some of this block could be attributed to the presence of macaque TRIM5α, but not all of it (189).

These observations raised the question of what the minimal requirements for infection by HIV and SIV_{MNE} (originating from pig-tail macaques) in the MAGI and sMAGI cells were. They found that HIV-1 could enter sMAGI cells via the simian co-receptor, but could be blocked either before or after reverse transcription depending on the isolate (185). Productive infection of sMAGI cells by HIV-1 required the human co-receptor (185). Likewise, chimeric SHIV constructs containing HIV-1 *env*, *tat*, and *rev*, were able to productively infect sMAGI cells (185).

This suggested that some macaque viral proteins were essential for productive infection of macaque cells by HIV-1. In sum, either expression of a human co-receptor on the sMAGI cells or the expression of some macaque viral proteins were sufficient to mediate productive infection of sMAGI cells by HIV-1. This stands in contrast to the block mediated by *Lv2*, which is not co-receptor dependent (163). On the other hand, SIV_{MNE} could productively infect MAGI cells expressing CCR5, meaning that it did not require a macaque-specific co-receptor and that human cells could be productively infected by SIV in the absence of human viral proteins. Overall, these findings supported a model in which productive infection involved both the envelope and the core, similar to *Lv2*.

A decade later, Pineda *et al.* (189) sought to characterize the components of restriction of HIV-1 in sMAGI cells. By this time, TRIM5α was known, and the authors verified that part, but not all, of the previously observed inhibition was due to TRIM5α. The TRIM5α component could not be bypassed by endocytotic entry and was saturable (189). In contrast, the novel block was found to be entry strategy-dependent, as endocytic entry allowed escape from the novel block, which they term *Lv3* (189). Furthermore, *TRIM5* knockdown was not sufficient to rescue infection (189). This explains the prior finding that some viruses were blocked before reverse transcription, while others were blocked after reverse transcription. The *Lv3* component of this block was characterized as occurring after initiation of reverse transcription and being unsaturable, in contrast to TRIM5α which acts before reverse transcription and is saturable. While HIV-1 pseudotyped with SIV envelope is blocked by *Lv3*, SIV is very productive in sMAGI cells, implying that the identity of the viral core is also important to restriction. The remaining responsible component or components of *Lv3* remain as of yet unidentified

1.8.3 Lv4

The final capsid-dependent block to lentiviral infection to be discussed was first observed not due differences in species-specific cell line susceptibilities, but because of

different susceptibilities across different human cell types. It was known that SIV_{MAC} was not efficiently blocked by TRIM5α in immortalized adherent human cells such as HeLa (83, 126, 132), TE671 (126, 132, 190), and HOS (124) cell lines. However, SIV_{MAC} was actually less infectious than HIV-1 in certain human lymphocytic cell lines (110), suggesting a cell-type specific restriction activity. Pizzato *et al*. (191) verified that a block, termed *Lv4*, existed against SIV_{SMM}/SIV_{MAC}/HIV-2 lineage viruses in human B cells, T cells, myeloid cells, PBMCs, and dendritic cells, but was absent in epithelial cells. HIV-1 was robustly infectious in all of these cell lines. Substitution of HIV-1 capsid with SIV_{MAC} or HIV-2 capsid was sufficient to reduce infectivity, supporting the hypothesis that the factor was capsid-dependent. Like TRIM5α, but unlike *Lv2* and *Lv3*, *Lv4* was found to be fully entry pathway-independent. Unlike TRIM5α, the block to infection occurs after reverse transcription. By generating heterokaryons from HeLa and Jurkat cells, it was found that these heterokaryons behaved like T cells in that they could not be infected by SIV_{MAC}, supporting the hypothesis that there exists a dominant factor expressed in leukocytes.

One known restriction factor that shares some similarities with *Lv4* is TRIM34. TRIM34 is active against both VSV-G pseudotyped and non-pseudotyped viruses (99, 192). TRIM34 is also active against SIV_{MAC}, but not HIV-1 (99). However, the block to infection observed for TRIM34 occurs before reverse transcription, rather than after, although level of reverse transcripts was only measured at one timepoint, so possible differences in accumulation kinetics are not accounted for (99). Furthermore, although TRIM34 antiviral activity in epithelial cells has not, to our knowledge, been tested directly, TRIM34 was originally described in HeLa cells (139).

It is possible that some of the responsible proteins may have already been investigated in the literature but not connected with a characterized block to infection. Additionally, while most lentiviral restriction factors that are presently known appear to act alone, some, such as

TRIM34, require the presence of another protein for restriction to occur (99). It is possible that this kind of multi-protein cooperation could also occur in other restriction events. A final scenario is that two or more independently acting proteins could contribute to a given block.

1.9 Summary

I have described above the discovery, characterization, and identification of *Lv1*/*Ref1*/*TRIM5* and discussed 3 blocks to lentiviral infection that are at least partially capsid-targeting factors that have not been fully understood (summarized in **Table 1.1** and **Figure 1.5**). *Lv2* was first identified in the context of HIV-2 replication and appears to be both capsid- and envelope-dependent. Furthermore, entry mechanism seems to underpin *Lv2* susceptibility. REAF was identified as a putative agent of the Lv2 block, but likely does not completely fulfill the phenotype. Similar to *Lv2*, *Lv3* was identified because of differences in infection productivity between human and rhesus cells. Likewise, *Lv3* also appears to be mediated by both the identity of the capsid and the envelope. Unlike *Lv2* and *Lv3*, *Lv4* was first identified because of differences in viral susceptibility between kinds of human immortalized cell lines. Furthermore, *Lv4* depends only on capsid identity and is independent of entry pathway. Possible candidate genes for these hitherto unidentified restriction events could include *IFITM*s and other *TRIM* family members, however further investigation is needed to assess the plausibility of this claim. With the continued advances in genetic manipulation and next-generation sequencing technology, it may be possible to identify some of these unknown components by screening strategies. For example, by using libraries of guides against characterized interferon-stimulated genes or human TRIM-family genes, one could screen for factors that match these previously characterized but unidentified cellular factors. This type of approach would allow the possibility to discover currently unknown restriction factors in addition to ones that have previously been identified in the literature.

Restriction	Susceptible virus(es)	Characteristics
Fv1	MLV	• Susceptibility determined by CA position 110 • Block after RT • Saturable
Lv1/*Ref1*/TRIM5a	HIV-1	• Susceptibility depends on CA identity and host protein identity (SPRY domain) • Block before RT • Saturable
Lv2	Certain HIV-2 isolates, HIV-1	• Susceptibility determined by both gag and env • HIV-1 CA determinants: P38A, N74D, G89V, G94D • HIV-2 CA determinant: I73V • RT products accumulate more slowly
Lv3	HIV-1, some SIV	• Susceptibility determined by gag, env, and entry mechanism/co-receptor utilization • Block after RT
Lv4	SIV_{SMM}/SIV_{MAC}/HIV-2 lineage	• Susceptibility determined by CA • Occurs in leukocytes but not epithelial cells • Block after RT

Table 1.1*. Capsid-dependent restriction events of lentiviruses.*

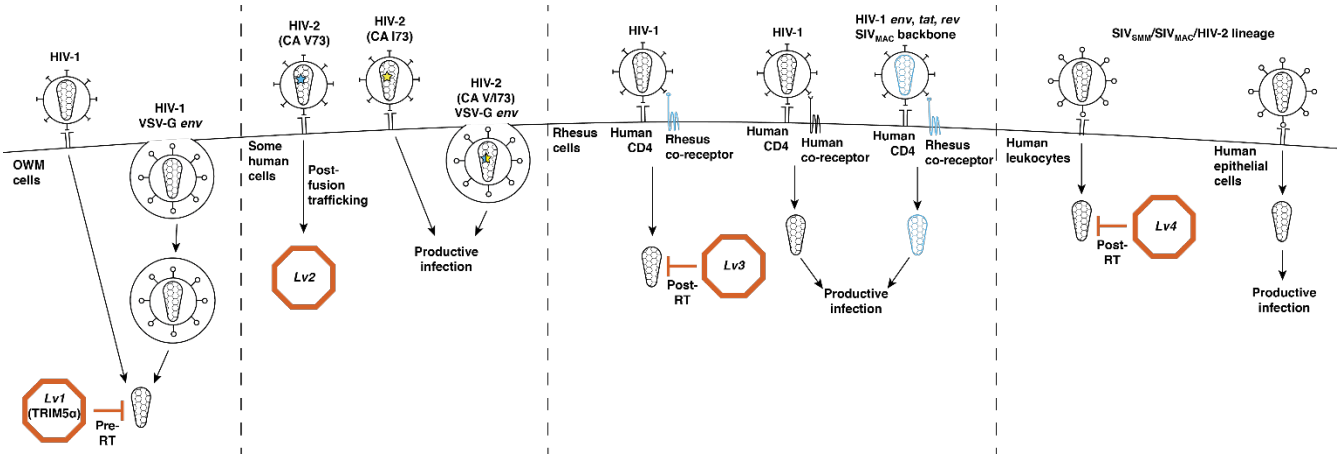

Figure 1.5*. Capsid-dependent restriction events of lentiviruses.*

In this thesis, I investigated the role of a restriction factor that targets lentiviral capsids identified by CRISPR screening, TRIM34. As with other restriction events that have been reported, TRIM34 was identified after the observation was made that a certain viral capsid mutant, HIV-1 N74D, was subject to an unknown restriction event. Since HIV-1 N74D was known to be susceptible to the unknown factor, but wild-type HIV-1 was not, it was probable that a capsid-binding factor was responsible. That capsid-binding factor was identified as TRIM34,

which turned out to depend on another capsid-binding factor, TRIM5α, for restriction activity. This thesis describes experiments aimed at understanding the function of TRIM34 and TRIM34's involvement in cross-species transmission of primate lentiviruses past and present.

CHAPTER 2. PRIMATE TRIM34 IS A BROADLY-ACTING, TRIM5-DEPENDENT LENTIVIRAL RESTRICTION FACTOR

This chapter was reproduced from the following article:

Twentyman J, Khalifeh A, Felton AL, Emerman M, and Ohainle M. Primate TRIM34 is a broadly-acting, TRIM5-dependent lentiviral restriction factor. *Retrovirology* 20, 15 (2023). doi: 10.1186/s12977-023-00629-4.

2.1 Abstract

Human immunodeficiency virus (HIV) and other lentiviruses adapt to new hosts by evolving to evade host-specific innate immune proteins that differ in sequence and often viral recognition between host species. Understanding how these host antiviral proteins, called restriction factors, constrain lentivirus replication and transmission is key to understanding the emergence of pandemic viruses like HIV-1. Human TRIM34, a paralogue of the well-characterized lentiviral restriction factor TRIM5α, was previously identified by our lab via CRISPR-Cas9 screening as a restriction factor of certain HIV and SIV capsids. Here, we show that diverse primate TRIM34 orthologues from non-human primates can restrict a range of Simian Immunodeficiency Virus (SIV) capsids including $SIV_{AGM\text{-}SAB}$, $SIV_{AGM\text{-}TAN}$ and SIVMAC capsids, which infect sabaeus monkeys, tantalus monkeys, and rhesus macaques, respectively. All primate TRIM34 orthologues tested, regardless of species of origin, were able to restrict this same subset of viral capsids. However, in all cases, this restriction also required the presence of TRIM5α. We demonstrate that TRIM5α is necessary, but not sufficient, for restriction of these capsids, and that human TRIM5α functionally interacts with TRIM34 from different species. Finally, we find that both the TRIM5α SPRY v1 loop and the TRIM34 SPRY domain are essential for TRIM34-mediated restriction. These data support a model in which TRIM34 is a broadly-conserved primate lentiviral restriction factor that acts in tandem with TRIM5α, such that together, these proteins can restrict capsids that neither can restrict alone.

2.2 Introduction

Restriction factors are a class of cell-intrinsic, germline-encoded host immune factors that can inhibit viral infection and replication. The human genome encodes for approximately 70-100 TRIM (Tripartite Motif) proteins, many of which play a role in host defense (193). The alpha isoform of TRIM5 (TRIM5a) in primates is a well-characterized example of a primate restriction factor with activity against retroviruses. The activity of TRIM5α against a given

retrovirus depends on both the species from which the TRIM5α is derived and the capsid (CA) protein of the retrovirus (83, 85, 86, 194, 195). In the context of HIV-1, TRIM5α acts directly on CA by multimerizing onto the CA lattice (194, 196). This interaction results in aberrant uncoating of CA, interrupting the viral life cycle (37, 194). TRIM5α from rhesus macaques and many other Old World monkeys has much greater antiviral activity against HIV-1 than does human TRIM5α (83, 84, 194).

TRIM proteins are composed of a common set of N-terminal domains (RING, B-box, and coiled-coil) followed by one or more variable C-terminal domains (197) . An important characteristic of TRIM5α, and TRIM proteins more generally, is their ability to oligomerize to form structures with very high binding avidity to CA (37). Homo-oligomerization of TRIM5α is essential to its ability to restrict viral CA (198–200). Furthermore, TRIM proteins have also been demonstrated to hetero-oligomerize with each other (108, 197, 201).

Previously, our lab performed a CRISPR-Cas9 screen to identify restriction factors against an HIV-1 strain with a mutation in CA, N74D, that renders it more susceptible to CA-mediated restriction factors (169). We identified TRIM34 as a restriction factor of the HIV-1 N74D capsid mutant as well as select SIV capsids (99). TRIM34 is a paralogue of TRIM5α, sharing a common domain architecture, and human TRIM34 and TRIM5α have approximately 57% amino acid identity (139). Moreover, TRIM34-mediated restriction requires TRIM5α, and TRIM34 and TRIM5α colocalize with incoming capsids (99).

While TRIM5α and TRIM34 both can interact with lentiviral CA, TRIM5α, but not TRIM34, has undergone positive selection (81, 201). A history of positive selection, in which the rate of nonsynonymous mutations exceeds the rate of synonymous mutations, is characteristic of host proteins that are in evolutionary conflict with pathogens and often occurs at sites of direct physical interface between host and pathogen (79, 80). Specifically, the v1 loop of TRIM5α's C-terminal SPRY domain has been found to have undergone rapid evolution, and this

region is responsible for viral recognition (81). Conversely, the lack of evidence for positive selection on TRIM34 suggests that TRIM34 may not contain sites of evolutionarily-important direct viral interaction.

Although currently there does not exist evidence of evolutionary conflict within TRIM34, here we show that TRIM34 antiviral activity has been broadly conserved across primate species as an antiviral gene against CA of diverse primate lentiviruses. We find that restriction by TRIM34 from primates requires TRIM5α and that the TRIM5α SPRY v1 loop is an essential mediator of restriction. These results suggest that TRIM34 relies on the capsid-binding properties of TRIM5α. However, as the TRIM34 SPRY domain is also required for restriction, our results suggest that both TRIM34 and TRIM5α contribute to capsid recognition and/or antiviral function. We propose that TRIM34, which has not undergone positive selection, is an antiviral protein that requires TRIM5α, which has undergone positive selection.

2.3 Results

2.3.1 Diverse primate TRIM34 orthologues can act as lentiviral restriction factors

Amino acid variation due to positive selection over evolutionary time in the v1 loop of TRIM5α confers different primate species with varying specificities towards retroviral capsids (81, 92). Although TRIM34 lacks evidence of positive selection found in TRIM5α, there is still diversity across different primate TRIM34 alleles. For example, relative to human TRIM34, chimpanzee, sabaeus monkey, and rhesus macaque orthologues differ by 4, 27, and 26 amino acids, respectively. While we have previously shown that human TRIM34 restricts $SIV_{AGM\text{-}TAN}$ and SIV_{MAC}, here we wished to determine whether amino acid variation in TRIM34 orthologues from divergent primates affects TRIM34's capacity to restrict different primate lentiviral capsids (99). Specifically, we tested whether a panel of four TRIM34 orthologues from diverse primate species comprising human (*Homo sapiens*), chimpanzee (*Pan troglodytes troglodytes*), sabaeus monkey (*Chlorocebus sabaeus*), and rhesus macaque (*Macaca mulatta*) orthologues would

restrict a panel of lentiviral capsids (HIV-1, SIV_{CPZ}, $SIV_{AGM\text{-}SAB}$, $SIV_{AGM\text{-}TAN}$, and SIV_{MAC}, which arise from humans; chimpanzees; two species of African green monkeys, the sabaeus monkey and the tantalus monkey; and rhesus macaques, respectively).

To test the effects of different primate TRIM34 orthologues in the absence of endogenous TRIM34, we generated a clonal *TRIM34* knockout (KO) line in the human THP-1 cell line (**Appendix A.1, Chromatogram 1**). We then introduced codon-optimized primate TRIM34 orthologues (**Appendix A.2**) using a doxycycline-inducible lentiviral vector to generate human, chimpanzee, sabaeus, and macaque TRIM34 over-expressing cell lines. These orthologues were selected because they represent a broad range of primate TRIM34 from both hominid and Old World monkeys for which we also have the corresponding lentiviral capsids. We tested an empty-vector line as a control for the absence of any TRIM34. We confirmed that expression of each of the TRIM34 orthologues is induced by doxycycline (**Figure 2.1a**), although overall steady-state expression levels varied somewhat with the macaque TRIM34 expressed at higher levels and the chimpanzee TRIM34 at lower levels.

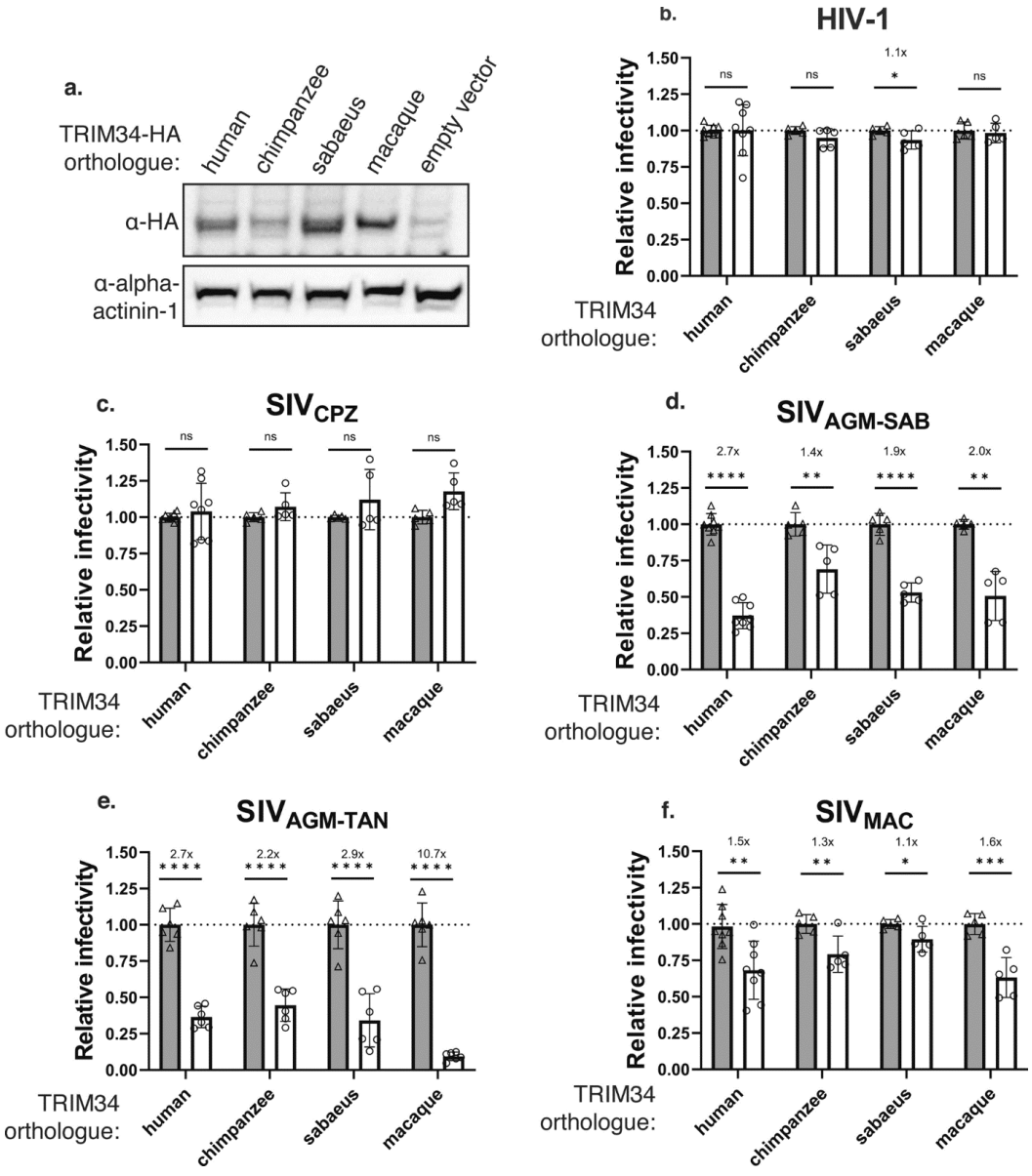

Figure 2.1. *Diverse primate TRIM34 orthologues restrict $SIV_{AGM\text{-}SAB}$, $SIV_{AGM\text{-}TAN}$, and SIV_{MAC} capsids in the presence of TRIM5α. (a) THP-1* TRIM34 *clonal KO cells were generated by electroporation of multiplexed sgRNA against TRIM34 and single cell sorting (ICE KO score = 96%,* ***Appendix A.1, Chromatogram 1****). THP-1* TRIM34 *KO cells were transduced with doxycycline-inducible HA-tagged primate TRIM34 orthologues or empty vector control. Primate TRIM34 expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. (b-f) Primate TRIM34 expression was induced in THP-1* TRIM34 *clonal KO cells. 1 day post-induction, cells were either challenged with chimeric virus particles containing HIV-1 capsids co-expressing zsGreen (b) or SIV capsids co-expressing eGFP including SIV_{CPZ} (c), $SIV_{AGM\text{-}SAB}$ (d), or SIV_{MAC} (f); or challenged with VSV-G pseudotyped $SIV_{AGM\text{-}TAN}$-luc (e). Infection was quantified 2 dpi by flow cytometry (b-d, f) or luminometry (e). Relative infectivity in induced cells (white bars, circles) is normalized to average infectivity in uninduced control cells (grey bars, triangles). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed 5-6 times across at least 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. One-sided p values were calculated by Welch's t-test. ns = nonsignificant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.* ***Figure 2.1*** *was reproduced from* (192) *with permission, Creative Commons Attribution 4.0 International License.*

We then challenged these cells to infection using a VSV-G pseudotyped lentiviral vector system, in which the CA region of a gag/pol expression construct encoded either HIV-1, SIV_{CPZ}, $SIV_{AGM\text{-}SAB}$, or SIV_{MAC} capsids, whose host species match the species of origin of the TRIM34 orthologues used (202, 203). These virus particles also encoded a fluorescent reporter. We also tested a full-length infectious molecular clone of $SIV_{AGM\text{-}TAN}$ that encodes a luciferase reporter (204). After induction with doxycycline or media-only control, infectivity was quantified 2 days post-infection (dpi) by luciferase assay ($SIV_{AGM\text{-}TAN}$) or flow cytometry (all others). Of note, we found that while none of the TRIM34 orthologues tested restricted HIV-1 or SIV_{CPZ} capsids (**Figure 2.1b-2.1c**), all of these same TRIM34 orthologues restricted $SIV_{AGM\text{-}TAN}$, $SIV_{AGM\text{-}SAB}$, and SIV_{MAC} capsids (**Figure 2.1d-2.1f**). These data suggest that, in contrast to TRIM5α, the antiviral specificity of TRIM34 does not seem to vary across TRIM34 orthologues. Rather, TRIM34 antiviral activity against the same set of lentiviral capsids is a conserved activity of all primate TRIM34 orthologues we tested. Moreover, the antiviral activity of all primate TRIM34s is specific for the same subset of lentiviral capsids. For example, both hominid lentiviruses tested were largely unaffected by all the TRIM34 orthologues: only HIV-1 was very weakly restricted by sabaeus TRIM34 (**Figure 2.1b-2.1c**). Conversely,the lentiviral capsids that were restricted by human TRIM34—$SIV_{AGM\text{-}TAN}$, $SIV_{AGM\text{-}SAB}$, and SIV_{MAC}—were also restricted by all the other primate TRIM34 orthologues tested (**Figure 2.1d-2.1f**). In sum, these data support the hypothesis that TRIM34 restriction is a conserved activity in primates with shared specificity for certain primate lentiviral capsids.

2.3.2 TRIM34 requires TRIM5α for restriction

Previously, we found that human TRIM34 requires TRIM5α to restrict HIV-1 N74D capsids (99). Given that the TRIM34 orthologues tested all show selectivity for the same subset of lentiviral capsids (**Figure 2.1**), we reasoned that TRIM5α might be responsible for the capsid specificity of TRIM34-mediated restriction. Notably, for the experiments described in **Figure 2.1**

in which all TRIM34 orthologues tested restricted the same subset of capsids, the cells used contained endogenous TRIM5α. Therefore, we next asked whether TRIM5α was more broadly required for TRIM34-mediated restriction of $SIV_{AGM\text{-}SAB}$, which was the most potently restricted capsid of the capsids tested in **Figure 2.1**. Specifically, we assayed for restriction in TRIM34-overexpressing cells in which we removed endogenous TRIM5α expression. We created pooled knockouts of *TRIM5* in the background of THP-1 *TRIM34* clonal KO cells containing doxycycline-inducible human or macaque TRIM34 (**Appendix A.1, Chromatograms 2-5**). Although we observed high KO efficiency scores for the pooled KO lines (**Figure 2.2**; legend), these pooled KO cell lines still contained some TRIM5α expression as KO is not complete at a population level. We also generated control cell lines using non-targeting control guides (NTCs); these cells still contained endogenous TRIM5α. We then infected these cells with $SIV_{AGM\text{-}SAB}$. Relative to cells missing both TRIM34 and TRIM5α (**Figure 2.2a-2.2b**; black symbols), $SIV_{AGM\text{-}SAB}$ is restricted only in the presence of both TRIM34 and TRIM5α (**Figure 2.2a-2.2b**; red symbols). Notably, this was true for both human and rhesus macaque TRIM34 (**Figure 2.2a** – human; **Figure 2.2b** – macaque). That is, human TRIM5α can fulfill TRIM34's requirement for TRIM5α, even for a TRIM34 orthologue from a different primate species. Conversely, $SIV_{AGM\text{-}SAB}$ is not restricted either by cells expressing only TRIM34 that are knocked out for TRIM5α (**Figure 2.2a-2.2b**; green symbols), nor by cells expressing only endogenous TRIM5α without induction of TRIM34 (**Figure 2a-b**; blue symbols). Although it does appear that there may be a small amount of restriction in the presence of TRIM34 only (**Figure 2.2a-2.2b**; green symbols), we attribute this to the fact that the *TRIM5* KO cells were generated as a KO pool: therefore, at a population level, some cells in this cell pool still express some endogenous TRIM5α. Overall, these data suggest that primate TRIM34 orthologues broadly require TRIM5α for restriction of lentiviral capsids.

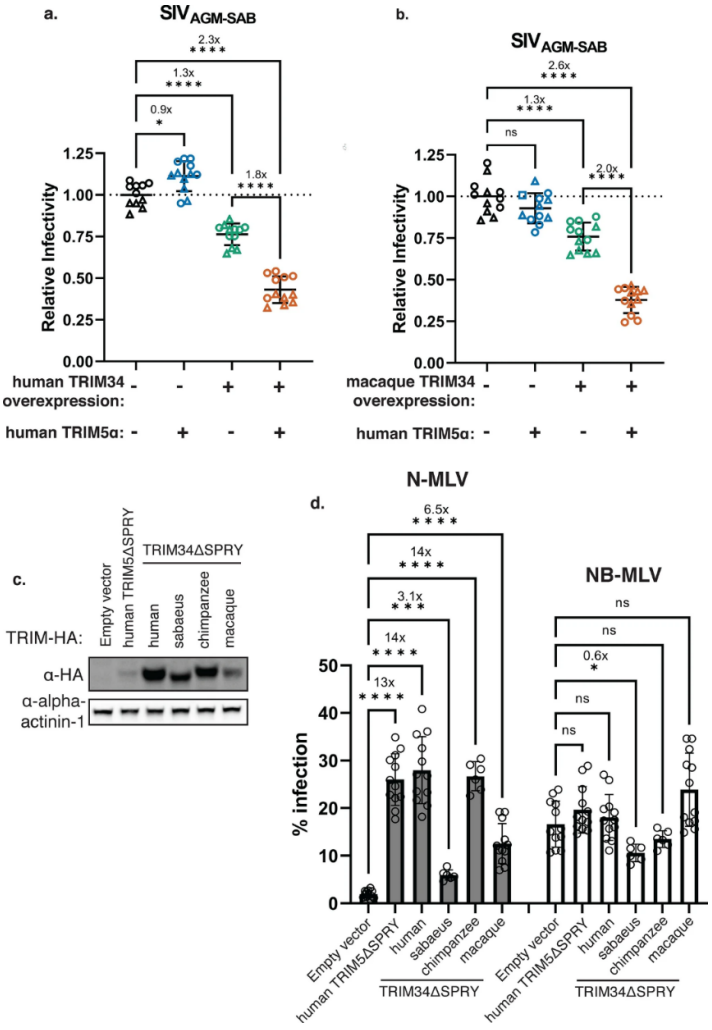

Figure 2.2*. TRIM34 requires TRIM5α to restrict SIV$_{AGM-SAB}$. (a-b) THP-1* TRIM34 *clonal KO cells containing doxycycline-inducible TRIM34 from humans (a) or rhesus macaques (b) were transduced with lentiviral vectors encoding Cas9 and 1 of 2 independent sgRNA against* TRIM5 *or a non-targeting control to generate pooled TRIM5 knockout or NTC cell lines. Pooled KO efficiency was assessed by ICE analysis (**Appendix A.1, Chromatograms 2-5**). For human TRIM34-expressing cell lines, ICE KO efficiency scores were as follows:* TRIM5 *sgRNA 1 (triangles) = 76%,* TRIM5 *sgRNA 2 (circles) = 90%. For rhesus macaque TRIM34-expressing cells, ICE KO scores were as follows:* TRIM5 *sgRNA 1 (triangles) = 83%,* TRIM5 *sgRNA 2 (circles) = 93%. Thus, there existed 4 different experimental conditions: no TRIM34 or TRIM5α expression (black symbols), only endogenous TRIM5α expression (blue symbols), only overexpressed TRIM34 (green symbols), both endogenous TRIM5α and overexpressed TRIM34 (red symbols). 1 day after doxycycline induction, cells were infected with SIV$_{AGM-SAB}$ CA particles. 2 dpi, infectivity was quantified by flow cytometry. Relative infectivity is normalized to mean infectivity in TRIM5 KO, TRIM34-uninduced cells (black symbols). Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. Fold inhibition is indicated where applicable. (c) HeLa cells were transduced to stably express primate TRIMΔSPRY orthologues or empty vector control. Expression was visualized by immunoblotting. (d) HeLa cells stably expressing primate TRIM34ΔSPRY orthologues, human TRIM5ΔSPRY, or empty vector control were infected with N-MLV (grey bars) or NB-MLV (white bars). Level of infection was quantified 2 dpi by flow cytometry. Infection of each cell line with each virus was performed a total of 6 times across at least 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. Fold rescue relative to empty vector control cells is indicated where applicable. (a-b, d) p values were calculated by Brown-Forsythe and Welch's 1-way ANOVA with Dunnett's T3 test for multiple comparisons. ns = nonsignificant, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.* ***Figure 2.2*** *was reproduced from* (192) *with permission, Creative Commons Attribution 4.0 International License.*

2.3.3 Human TRIM5α functionally interacts with TRIM34 from different primates

We next sought to assess whether TRIM34 orthologues and TRIM5α can functionally interact with each other via a TRIM5α restriction assay. Previous work has shown that dimerization and higher-order multimerization are essential for TRIM5α-mediated restriction (82, 201, 205, 206). Furthermore, TRIM5α has been shown to associate not only with itself but also with TRIM34 via two-hybrid screen and co-immunoprecipitation (108, 197, 201). To assess functional interaction of TRIM34 and TRIM5α more directly, we exploited the fact that TRIM proteins that have been deleted of their SPRY domains (TRIMΔSPRY) exert a dominant-negative effect on restriction of N-tropic MLV (N-MLV) by endogenous TRIM5α constitutively expressed in HeLa cells (108). Overexpression of human TRIM34ΔSPRY inhibits restriction of N-MLV in HeLa cells by the endogenous TRIM5α (108). This implies that TRIM34 is able to functionally interact with TRIM5α. Thus, we asked whether primate TRIM34ΔSPRY orthologues could also interact with human TRIM5α in this assay. We generated TRIMΔSPRY constructs that were truncated at the start of the SPRY domain. We overexpressed the HA-tagged human, sabaeus, chimpanzee, and macaque TRIM34ΔSPRY constructs in HeLa cells together with human TRIM5ΔSPRY as a positive control (**Figure 2.2c**). These cells were then infected with N-MLV and NB-MLV, which is not restricted by TRIM5α (127, 207). While there was no effect observed of TRIMΔSPRY constructs on NB-MLV infection (**Figure 2.2d**, white bars), we found that relative to empty vector control, overexpression of all four TRIM34ΔSPRY orthologues, as with TRIM5ΔSPRY, relieved restriction of N-MLV by human TRIM5α (**Figure 2.2d**, grey bars). These data further support our model that diverse TRIM34 orthologues and human TRIM5α can functionally interact with each other.

2.3.4 Both TRIM34 and TRIM5α SPRY domains are involved in viral restriction

Since TRIM5α is required for restriction by TRIM34 and does not seem to vary depending on which TRIM34 is used, we hypothesized that capsid recognition comes from

TRIM5α, and not from TRIM34. In the case of TRIM5α-mediated restriction, the SPRY domain, and specifically the v1 loop, determines antiviral specificity (81, 91, 92). Furthermore, altering a single amino acid in the human TRIM5α SPRY domain (arginine 332) in the v1 loop to match the corresponding macaque residue (proline) is sufficient to confer strong restriction of HIV-1 (91, 92). To ask if the TRIM5α SPRY domain is involved in TRIM34-mediated CA recognition, we used cells that were doubly knocked out for endogenous *TRIM5* and *TRIM34* (THP-1 *TRIM34 TRIM5* clonal KO cells) (**Appendix A.1, Chromatograms 6-10**). In these cells, we introduced inducible vectors expressing wild type (WT) human TRIM34 with WT human TRIM5α, TRIM5α in which the v1 loop was deleted (TRIM5α Δv1), and TRIM5α in which the arginine at position 332 was mutated to a proline (TRIM5α R332P). We expressed each TRIM individually as well as in the context of each other and confirmed expression levels by Western blot (**Figure 2.3a**). Corresponding to the results in **Figure 2.2**, we found that $SIV_{AGM\text{-}SAB}$ was restricted only in the presence of both human TRIM34 and human TRIM5α, but not when either TRIM34 or TRIM5α were expressed in isolation (**Figure 2.3b**; grey bars). Furthermore, in agreement with our previous findings (99), HIV-1 N74D, but not WT HIV-1, was restricted only in the presence of both TRIM34 and TRIM5α (**Figure 2.3c-2.3d**; grey bars).

Given that TRIM5 is required for TRIM34-mediated restriction, we next asked whether the TRIM5α v1 loop, known to be important for capsid specificity by TRIM5α, was essential for restriction by TRIM34. We found that unlike co-expression of TRIM34 with full-length TRIM5α, co-expression of TRIM34 with TRIM5α Δv1 was not sufficient for restriction of either $SIV_{AGM\text{-}SAB}$ and HIV-1 N74D (**Figure 2.33b-2.3c**; red bars). This suggests that the TRIM5α v1 loop is required for TRIM34-mediated restriction.

We then asked whether changing the identity of the TRIM5α v1 loop, but not deleting it entirely, could also alter viral specificity. To assess this question, we generated the mutant TRIM5α R332P, which converts the human residue at position 332 to the rhesus macaque

residue and has been shown to have enhanced antiviral inhibition of HIV-1 (91, 92). We co-expressed these cells with TRIM34 and infected with $SIV_{AGM\text{-}SAB}$, HIV-1 N74D, or HIV-1. In accordance with the literature, TRIM5α R332P was able to restrict HIV-1 while WT TRIM5α did not (**Figure 2.3d**; blue bars) (91, 92). Furthermore, restriction of HIV-1 by TRIM5α R332P was agnostic to the presence of TRIM34; that is, TRIM34 did not appear to augment restriction of HIV-1 by TRIM5α R332P (**Figure 2.3d**; blue bars). Similar to wild type TRIM34 and TRIM5α, TRIM34 co-expression with the mutant TRIM5α R332P allele maintained restriction of the N74D CA mutant virus (**Figure 2.3c**; blue bars). Interestingly, TRIM34 appears to be the major factor contributing to restriction of HIV-1 N74D, whereas TRIM5α R332P alone only weakly restricted HIV-1 N74D, (**Figure 2.3c-2.3d**; blue bars). This suggests that TRIM34 is critical for restriction of HIV-1 N74D, even in the presence of a TRIM5α mutant that is more potent against WT HIV-1. Finally, TRIM34 co-expressed with TRIM5α R332P restricted $SIV_{AGM\text{-}SAB}$ CA, whereas TRIM5α R332P alone did not (**Figure 2.3b**; blue bars). These findings support a role for both the TRIM5α v1 loop and for TRIM34 in viral recognition and specificity.

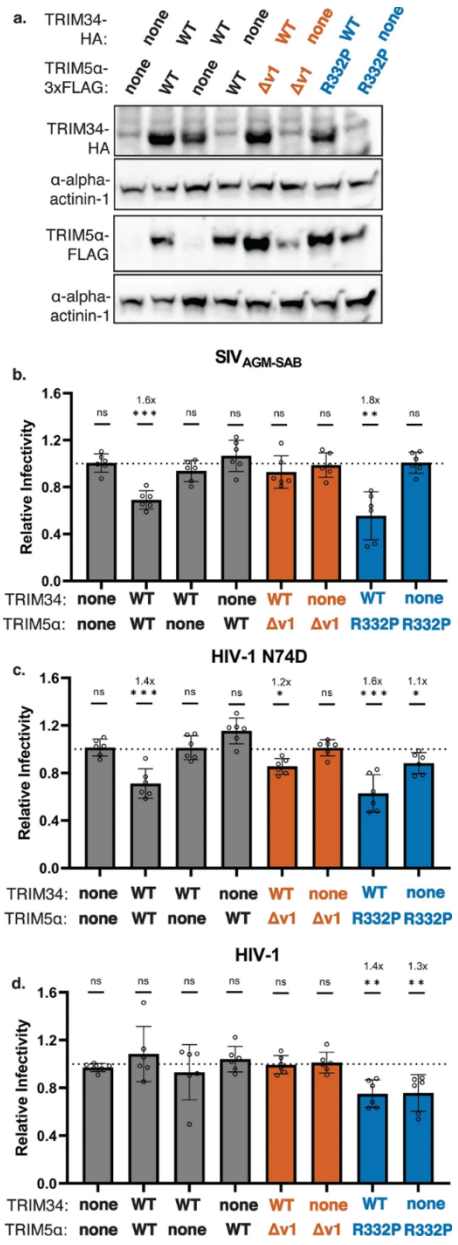

Figure 2.3*. TRIM5α v1 loop is necessary for restriction of SIV$_{AGM-SAB}$. (a) THP-1* TRIM34 *clonal KO cells were electroporated with multiplexed sgRNA against* TRIM5*. Single cell clonal lines were generated by limiting dilution to generate a THP-1* TRIM34 TRIM5 *double KO clonal cell line. KO efficiency was assessed by ICE analysis (ICE KO score = 72%,* ***Appendix A.1, Chromatograms 6-10****). Cells were doubly transduced with doxycycline-inducible, HA-tagged human TRIM34 or empty vector control in tandem with doxycycline-inducible, FLAG-tagged human TRIM5α, TRIM5Δv1, TRIM5 R332P, or empty vector control. Expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. (b-d) THP-1* TRIM34 TRIM5 *double KO clonal cells co-expressing doxycycline-inducible human TRIM34 or empty vector control and human TRIM5α (grey bars), TRIM5Δv1 (red bars), TRIM5 R332P (blue bars), or empty vector control were infected with SIV$_{AGM-SAB}$ (b), HIV-1 N74D (c), or HIV-1 (d) CA 1 day post-induction. Levels of infection were quantified 2 dpi by flow cytometry. Relative infectivity in induced cells (solid bars) is normalized to average infectivity in uninduced control cells (not shown). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. One-sided p values were calculated by Welch's t-test. ns = nonsignificant, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.* ***Figure 2.3*** *was reproduced from* (192) *with permission, Creative Commons Attribution 4.0 International License.*

2.3.5 TRIM34 SPRY domain is required for restriction

To more directly test whether the TRIM34 SPRY domain plays a role in antiviral activity and specificity, we generated a chimeric protein that encodes the TRIM34 RING, B-box, and coiled-coil domains in frame with the TRIM5 SPRY domain (TRIM34 RBCC-TRIM5 SPRY) (**Figure 2.4a**). We hypothesized that if the TRIM34 SPRY domain was required for restriction, the chimera would lose restriction relative to full-length TRIM34. We transduced this chimera into THP-1 *TRIM34* clonal KO cells and confirmed expression by Western blot (**Figure 2.4b**). Although protein expression of the chimeric construct is lower than that of WT TRIM34 (**Figure 2.4b**, compare third lane to first lane), expression is higher than that of WT TRIM5α. We assayed this construct for restriction in our THP-1 cells that lack TRIM34 expression but do contain endogenous, full-length TRIM5α. We found that in the presence of endogenous TRIM5α, the TRIM34 RBCC-TRIM5α SPRY chimera was not able to restrict $SIV_{AGM-SAB}$ CA nor $SIV_{AGM-TAN}$ (**Figure 2.4c-2.4d**). Therefore, the TRIM34 SPRY domain, in addition to the TRIM5α SPRY domain, is involved in restriction.

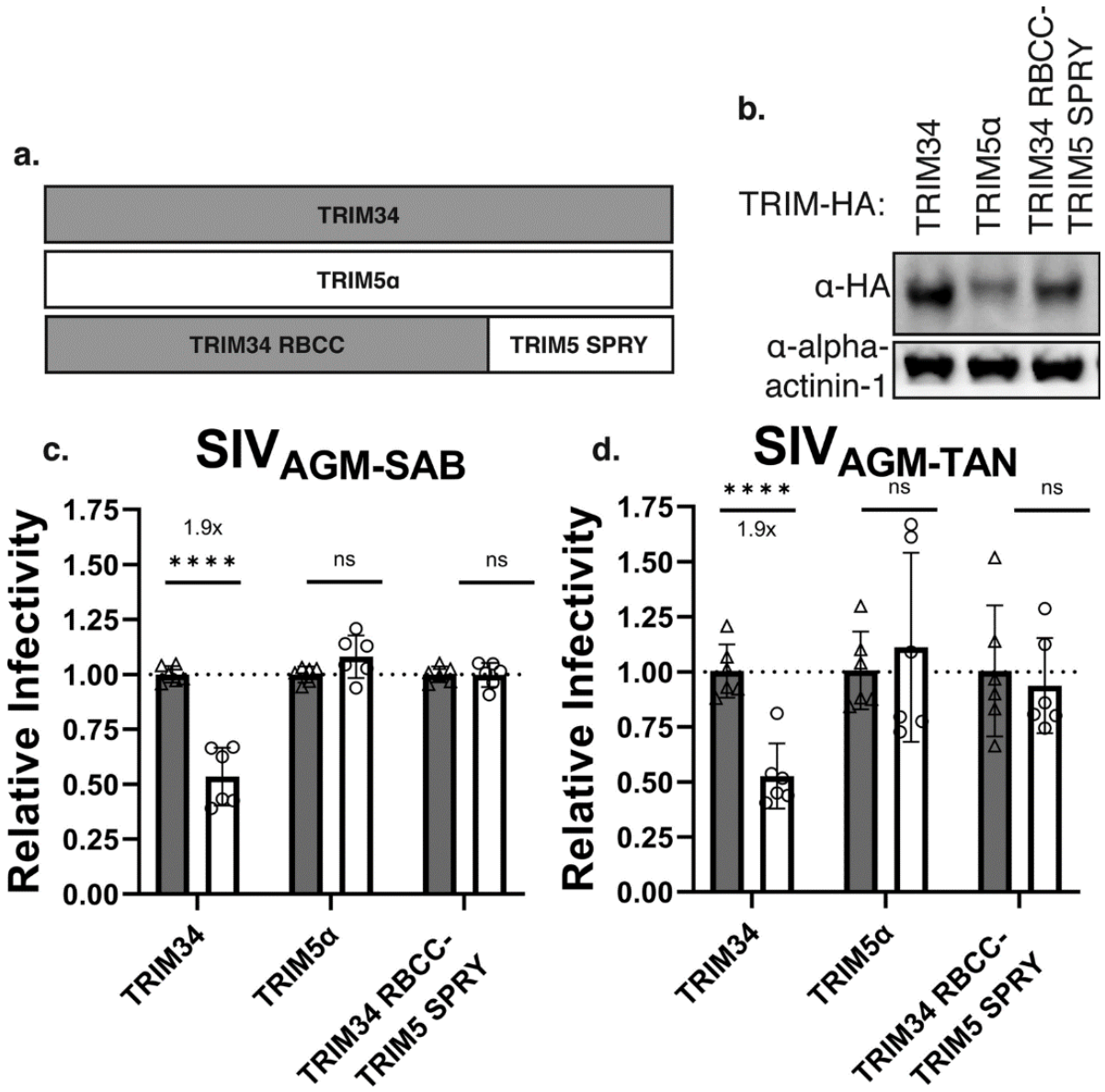

Figure 2.4. *TRIM34 SPRY domain is necessary for restriction of $SIV_{AGM-SAB}$. (a) Schematic of TRIM34 RBCC-TRIM5 SPRY chimera. (b) THP-1* TRIM34 *clonal KO cells were generated by electroporation of multiplexed sgRNA against TRIM34 and single cell sorting. Cells were transduced with doxycycline-inducible HA-tagged human TRIM34, TRIM5α, or TRIM34-TRIM5α chimeras. Expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. (c) THP-1* TRIM34 *clonal KO cells expressing doxycycline-inducible human TRIM34, TRIM5α, or TRIM34 RBCC-TRIM5 SPRY were infected with $SIV_{AGM-SAB}$ CA or $SIV_{AGM-TAN}$ particles 1 day post-induction. Level of infection was quantified 2 dpi by flow cytometry or luminometry, respectively. Relative infectivity in induced cells (white bars, circles) is normalized to average infectivity in uninduced control cells (grey bars, triangles). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. One-sided p values were calculated by Welch's t-test. ns = nonsignificant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.* ***Figure 2.4*** *was reproduced from* (192) *with permission, Creative Commons Attribution 4.0 International License.*

2.4 Discussion

Human TRIM34 was recently identified as a restriction factor of the CA mutant HIV-1 N74D and certain SIVs (99). Here we show that this antiviral phenotype is a broadly conserved function of primate *TRIM34* genes. We find that the same subset of lentiviral capsids is restricted by a diverse panel of TRIM34 orthologues, irrespective of TRIM34 species of origin. Moreover, we find that although antiviral specificity does not seem to vary with the identity of TRIM34, both the TRIM34 and TRIM5α SPRY domains are critical to restriction, suggesting that TRIM5 confers a level of specificity to the TRIM34 restriction.

Despite the lack of evidence for positive selection on TRIM34, primate TRIM34 alleles have broadly maintained the ability to restrict certain primate lentiviruses. This is surprising in light of the fact that positive selection is a marker of critical regions of direct host-viral interaction. We propose that although TRIM34 lacks this characteristic, it utilizes TRIM5α, which is a rapidly-evolving gene in primates, to restrict lentiviruses. Prior work has shown that TRIM34 and TRIM5α are capable of multimerizing *in vitro* in addition to binding capsid (108, 197, 201). In this work we demonstrate that TRIM5α can functionally interact with a number of primate TRIM34 orthologues. Indeed, we found that overexpression of TRIM34ΔSPRY was able to abrogate the restrictive capacity of TRIM5α towards N-MLV, suggesting a functional interaction between the two proteins. Human TRIM5ΔSPRY as well as human and chimpanzee TRIM34ΔSPRY had the strongest phenotype, whereas sabaeus and macaque TRIM34ΔSPRY had relatively weaker phenotypes (**Figure 2.2d**). One possible explanation for this observation is that the sabaeus and macaque constructs were expressed at relatively lower levels (**Figure 2.2c**). Another possibility is that human TRIM5α interacts most readily with itself and next most well with human TRIM34. Nonetheless, this supports a model in which TRIM5α and TRIM34 could hetero-multimerize to form a higher-order structure, similar to how TRIM5α forms homo-dimers and homo-multimers with itself mediated by the coiled-coil and B-box domains (37).

This then could enable both SPRY domains to interact with the viral capsid. Thus, we propose that despite the lack of an overt, positively-selected site of viral interface on TRIM34, TRIM34 restricts viral infection through interaction with the rapidly-evolving TRIM5 protein.

It is also possible that physical interaction between TRIM34 and TRIM5α alters the half-life of one or both proteins, affecting turnover rate or stability. Prior work has demonstrated that different TRIMs have different half-lives, and that alterations to the RING or B-box domains of TRIM proteins can affect protein turnover (201, 208, 209); thus, it is possible that interactions between different TRIMs might lead to changes in stability and restrictive capacity.

Another possibility is that TRIM34 could be filling the role of an effector molecule. Previously, the TRIM5α RING domain has been implicated as an E3 ubiquitin ligase that can both contribute to restriction directly and act as a signal transducer to initiate innate immune activation subsequent to CA sensing by the TRIM5α SPRY domain (93, 95, 96, 198, 209, 210). In the case of TRIM34, it is possible that TRIM5α is responsible for recognizing CA, while TRIM34's E3 ligase domain contributes to downstream signaling, capsid degradation, or recruitment of other molecules that aid restriction. Although Lascano *et al.* found that TRIM34 does not activate AP-1, it remains possible that it is acting by another mechanism (96). Notably, these models are not mutually exclusive and could function in tandem with TRIM34 aiding TRIM5α in more than one capacity.

Our findings suggest that the SPRY domains of both TRIM34 and TRIM5α are important for restriction. On its own, human TRIM5α is a relatively weak restriction factor. We propose that TRIM34 can assist human TRIM5α to restrict some—but not all—viral capsids. For example, TRIM5α in the presence of TRIM34 is able to restrict $SIV_{AGM\text{-}SAB}$; conversely, even in the presence of TRIM34, TRIM5α is still unable to restrict HIV-1. One possibility is that the TRIM34 SPRY domain is contributing to viral recognition. Previous work has shown that TRIM34 is able to bind HIV-1 CA-NC complexes *in vitro*, even though it does not restrict HIV-1, raising the

possibility that TRIM34 may still contribute to capsid binding (201, 211). Although the v1 loop of TRIM34 has not undergone positive selection and is only 6 amino acids long, we cannot exclude the possibility that it, or one of the other variable loops, might contribute to capsid recognition. Given the relatively high conservation of the variable loops of the primate TRIM34 alleles that we assessed (no amino acid differences in v1, v3, or v4; 1 amino acid difference in each allele in v2), this could explain the observation that all the TRIM34 alleles tested restricted the same subset of viruses. Furthermore, the observation that TRIM5α R332P—which gains restriction of HIV-1 relative to WT TRIM5α—poorly restricts HIV-1 N74D in the absence of TRIM34, supports the idea that TRIM34 could be assisting in capsid recognition beyond the capacity of TRIM5α alone. That is, TRIM34 may be contributing an additional measure of specificity for HIV-1 N74D CA beyond what is sufficient for TRIM5α R332P to restrict WT HIV-1 CA. Thus, as many TRIM5α alleles can act as potent restriction factors of a few very specific viral capsids, but a poor restriction factor of many others, we speculate that TRIM34 might act as a cofactor to enable TRIM5α to restrict viral capsids that TRIM5α is not able to restrict on its own. Of interest, we previously did not find any evidence that TRIM34 is required for TRIM5 restriction of the CypA-binding deficient P90A capsid mutants, suggesting that both TRIMs are required for restriction of only a subset of capsids (99).

It is intriguing that all the TRIM34 orthologues tested restricted the same subset of viruses: $SIV_{AGM\text{-}SAB}$, $SIV_{AGM\text{-}TAN}$, and SIV_{MAC}. Notably, only viruses originating from Old World monkeys were restricted, whereas the hominid viruses, HIV-1 and SIV_{CPZ}, were not restricted. It is possible that this is driven by the identity of TRIM5α: HIV-1 and SIV_{CPZ} are better evolved to evade restriction by human TRIM5α compared to the Old World monkey viruses (203). The three restricted viruses share only 70.6% pairwise amino acid identity across their CA regions, making it difficult to identify critical residues or motifs that determine restriction. We explored the possibility that restriction might be dependent on CypA binding, as CypA can modulate

infectivity in both restriction-dependent and restriction-independent fashions (78, 89, 90, 212–216). However, CypA binding capacity varied across the restricted viruses. For example, $SIV_{AGM\text{-}TAN}$ does bind CypA, while SIV_{MAC} and $SIV_{AGM\text{-}SAB}$ do not (75, 76, 108, 217). Furthermore, while HIV-1 is well-established to bind and incorporate CypA into virions, the CypA binding capacity of HIV-1 N74D is controversial (74, 100, 169). Thus, at minimum, restriction does not depend exclusively on CypA binding. Instead, restriction of these capsids by TRIM34 and TRIM5α may be dependent on other unknown features that distinguish these capsids. A number of blocks to HIV infection that are capsid-dependent have been characterized—for example, *Lv2*, *Lv3*, and *Lv4*—for which the responsible cellular components have either not been identified or have only been partly identified (163, 167, 168, 189, 191). It is possible that TRIM34 could contribute to one or more of these blocks.

We find that TRIM34 alleles from a broad spectrum of primates, when paired with TRIM5α, are able to restrict capsids that neither TRIM is able to restrict on its own. Despite lacking signs of positive selection characteristic of many restriction factors, TRIM34—acting in tandem with TRIM5α—can act as a barrier to cross-species transmission events. It is possible that these proteins have co-evolved such that TRIM34 can enhance or modify TRIM5α's antiviral potential. Indeed, not only do host immune proteins evolve in the context of the pathogens they counteract, but they also evolve in the context of other, complementary host proteins. Thus, our data suggest that restriction factors evolve not only in isolation in response to evolutionary pressures exerted by viral pathogens but may also co-evolve with each other resulting in more powerful antiviral activity than either could achieve on its own.

2.5 Methods

2.5.1 Cell culture

All cells were cultured at 37°C and 5% CO_2. THP-1 monocytic cells (American Type Culture Collection, Manassas, VA, #TIB-202) were cultured in Roswell Park Memorial Institute

1640 Media (RPMI 1640) (Gibco, Grand Island, NY, #118875-093) supplemented with 10% v/v fetal bovine serum (FBS) (GE Cytiva, Marlborough, MA #SH30541.03), 100 U/mL penicillin-streptomycin (Gibco #15140-122), 10mM HEPES (Gibco #15630-080), 1mM sodium pyruvate (Gibco #11360-070), 2 g/L D-Glucose (Gibco #A24940-01), and 1X GlutaMAX supplement (Gibco #35050-061). HEK 293T/17 cells (American Type Culture Collection #CRL-11268) and HeLa cells (American Type Culture Collection #CCL-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco #11965-092) supplemented with 10% fetal bovine serum, and 100 U/mL penicillin-streptomycin.

2.5.2 Cloning, plasmids, and virus production

All transfections were performed on HEK 293T/17 cells in the presence of serum-free DMEM and Trans-IT transfection reagent (Mirus Bio, Madison, WI, #MIR 2305). All transductions were performed by spinoculation at 1100 x *g* for 30 min at 30°C.

Fluorescent reporter viruses were generated by transfection of a three-plasmid system: pMD2.G (Addgene, Watertown, MA, #12259, a gift from Didier Trono) for expression of VSV-G envelope; a variable plasmid for expression of a chimeric gag/pol containing an NL4-3 backbone and HIV-1 (202), SIV_{CPZ}, $SIV_{AGM\text{-}SAB}$, or SIV_{MAC} CA (gifts from Theodora Hatziioannou) (203); and either pALPS-eGFP (Addgene #101323, a gift from Jeremy Luban) (218) or pHIV-zsGreen (Addgene #18121, a gift from Bryan Welm and Zena Werb) (219). SIV_{CPZ}, $SIV_{AGM\text{-}SAB}$, or SIV_{MAC} chimeric virus particles were made with pALPS-eGFP, and HIV-1 virus particles were made with pHIV-zsGreen. The SIV_{MAC} CA construct contained the mutation A77V. Luciferase reporter viruses were generated by transfection of a two-plasmid system: SIVagmTAN E- R- luc (204) and L-VSV-G (VSV glycoprotein expression) (220). Particles were harvested 2 days post-transfection, syringe-filtered through 0.22uM PES membranes, and frozen at -80°C. N-MLV reporter viruses were generated by transfection of a three-plasmid system: pCIG3-N for expression N-MLV gag/pol (Addgene #132941, a gift from Jeremy Luban) (89), pQCXIP-eGFP

(a gift from Jeannette Tenthorey) (92) for expression of a fluorescent reporter, and pMD2.G for expression of VSV-G envelope. NB-MLV reporter viruses were generated by transfection of a four-plasmid system: JK3 for expression of NB-MLV gag/pol (220), L-VSV-G for expression of VSV-G envelope (220), CMV-tat for transactivation (220), and pQCXIP-eGFP for expression of a fluorescent reporter.

pLentiCRISPR-v2 (Addgene #52961, a gift from Feng Zhang) constructs were generated by BsmBI (New England BioLabs, Ipswich, MA, #R0580) restriction cloning of TRIM5 guides into pLentiCRISPR-v2, a lentiviral vector encoding for Cas9 (221). pLentiCRISPR-v2 containing guides were transfected with pSPAX2 (Addgene #12260, a gift from Didier Trono) and pMD2.G to generate lentiviral particles. Particles were harvested 2 days post-transfection, syringe-filtered through 0.22 µM PES membranes, and frozen at -80°C.

HA-tagged TRIM34 and 3xFLAG-tagged TRIM5α codon-optimized expression constructs (**Appendix A.2**) were synthesized by Twist Bioscience (South San Francisco, CA). TRIM5α mutant constructs (TRIM5α v1 loop and TRIM5α R332P) were a gift from Jeannette Tenthorey (92). TRIM constructs were cloned into pLKO (a gift from Melissa Kane) (159) by SfiI (New England BioLabs #R0123S) restriction enzyme digest. pLKO-TRIM constructs were transfected along with pMD2.G and pSPAX2 to generate particles. Particles were harvested at 2 and 3 days post-transfection, cell pellets spun down at 300 x *g*, and supernatants frozen at -80°C.

HA-tagged TRIM34ΔSPRY constructs were cloned in pQCXIP (TaKaRa Bio, San Jose, CA, #631516) by SbfI (New England BioLabs #R3642S) and NotI (New England Biolabs #R3189S) restriction digest. pQCXIP-TRIM34ΔSPRY constructs were transfected along with pJK3 (MLV gag/pol), L-VSV-G (VSV-G envelope), and CMV-tat to generate particles. Particles were harvested 2 days post-transfection.

2.5.3 CRISPR Knockouts

Clonal knockout lines in THP-1 cells were generated by electroporation of multiplexed small guide RNA (sgRNA) from Gene Knockout Kit v2 (Synthego, Redwood City, CA) against *TRIM34* (guide sequences = CTTGCTTAACGTACAAG, CCACAGTCTAGACTCAA, GCAGTGACCAGCATGGG) or *TRIM5* (guide sequences = GGUAACUGAUCCGGCACACA, ACUUCUUGUGGUUUGCAGUG, CCUGGUUAAUGUAAAGGAGG). Single cell clonal lines were generated by single cell sorting (*TRIM34*) or limiting dilution (*TRIM5*). Knockout efficiency was validated by Interference of CRISPR Edits (ICE) analysis (Synthego) (222) (**Appendix A.1, Chromatograms 1, 6-10**).

Pooled knockout lines were generated by transduction of THP-1 cells with lentiviral preps containing guides delivered by pLentiCRISPR-v2. *TRIM5* guide sequences = TCACCACACGTTCCTCACAG and GTTGATCATTGTGCACGCCA. NTC guide sequences = GGGCCCGCATAGGATATCGC and TAGACAACCGCGGAGAATGC. Cells were spinoculated in the presence of 20 µg/mL DEAE-Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden, #17-0350-01) and then selected in 10 µg/mL blasticidin S HCl (Gibco #A11139-03). Knockout efficiency was validated by ICE analysis (222) (**Appendix A.1, Chromatograms 2-5**).

2.5.4 Inducible Overexpression

Doxycycline-inducible expression of TRIM34 and TRIM5α was achieved by transduction of lentiviral preps containing pLKO TRIM constructs in THP-1 cells. Cells were spinoculated in the presence of 5 µg/mL polybrene (EMD Millipore, Burlington, MA, #TR-1003-G) and then selected in 0.5 µg/mL puromycin (TRIM34) (Sigma-Aldrich, St. Louis, MO, #P8833-25MG) or 10 µg/mL blasticidin (TRIM5α). Protein expression was induced by the addition of 125 ng/mL doxycycline hyclate (Sigma-Aldrich #D9891-5G) to cells cultured in complete RPMI 1640 containing tetracycline-approved FBS (Sigma-Aldrich #F0926-50ML).

Stable expression of TRIM34ΔSPRY was achieved by transduction of lentiviral preps containing pQCXIP constructs expressing TRIMΔSPRY constructs. HeLa cells were spinoculated in the presence of 20 µg/mL DEAE-Dextran. Selection was performed in the presence of 1 µg/mL puromycin.

2.5.5 Restriction assays

1 day after induction of TRIM expression, THP-1 cells were infected with chimeric CA virus particles expressing a fluorescent reporter. Cells were spinoculated in the presence of 20 µg/mL DEAE-Dextran. 2 dpi, relative infectivity was quantified by flow cytometry using a FACSCelesta Analyzer (BD Biosciences, San Jose, CA) or Bright-Glo luciferase assay reagent (Promega, Madison, WI #E2620) using a LUMIstar Omega luminometer (BMG Labtech, Ortenberg, Germany).

2.5.6 N-MLV Restriction assay

HeLa cells that had been transduced to stably express TRIM34ΔSPRY constructs were infected with N-MLV particles expressing an eGFP reporter by spinoculation in the presence of 20 µg/mL DEAE-Dextran. Infectivity was assessed 2 dpi by flow cytometry using an LSRFortessa Cell Analyzer (BD Biosciences).

2.5.7 Western blotting

3 days after induction of TRIM expression, THP-1 cells were harvested; 1 day prior to infection, HeLa cells were harvested. Cells were washed once with PBS. Cells were then lysed on ice for 30 min in 20 mM HEPES (Fisher Scientific, Fair Lawn, NJ, #BP310-1) containing 8 M urea (Sigma-Aldrich #U-6504), 50 mM DL-Dithiothreitol (DTT) (Gold Biotechnology, St. Louis, MO, #DTT10), 0.1% w/v SDS (Fisher Scientific #BP166-500), 1.5 mM MgCl2 (Sigma-Aldrich #208337-100G), 0.5 mM CaCl2 (Sigma-Aldrich #C-3306), 50 µg/mL DNAse I (Roche Diagnostics, Mannheim, Germany, #10104159001), and 1X EDTA-free protease inhibitor cocktail (Roche Diagnostics #11836170001). 6X SDS-PAGE sample loading buffer (G

Biosciences, St. Louis, MO #785-701) containing 5% v/v 2-mercaptoethanol (Sigma-Aldrich #M3148-100ML) was added to lysates. Lysates were acid treated with 50 mM HCl (Sigma-Aldrich #320331-300ML) and then boiled at 95°C for 7 min. After cooling, lysates were neutralized with 50 mM NaOH (Sigma-Aldrich #72068-100ML). Products were resolved by SDS-PAGE on NuPage Bis-Tris 4-12% acrylamide gels (Invitrogen, Carlsbad, CA, #NP0335BOX) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, #1620115). Membranes were blocked with 2% w/v milk (Research Products International, Mt. Prospect, IL, #M17200-500.0) and 2% w/v bovine serum albumin (Sigma-Aldrich #A7906-100G) in tris-buffered saline (Fisher Scientific #BP152-5) containing 1% v/v Tween-20 (Fisher Scientific #BP152-1) (TBS-T). Primary antibodies were incubated overnight at 4°C: rabbit anti-HA high affinity at 1:500 (Roche Diagnostics #ROAHAHA), mouse anti-FLAG M2 at 1:500 (Sigma-Aldrich #F1804), and rabbit anti-alpha actinin-1 at 1:1000 (Bio-Rad Laboratories #VPA00889). Membranes were washed with TBS-T and then incubated with HRP-conjugated secondary antibodies for 1 h at RT: goat anti-rat IgG at 1:2000 (Abcam, Waltham, MA #ab97057), sheep anti-mouse IgG at 1:2000 (GE Cytiva #NA931), and donkey anti-rabbit IgG at 1:2000 (GE Cytiva #NA934V). Membranes were washed again and then incubated with SuperSignal West Pico substrate (HA and alpha actinin-1 blots) (Thermo Fisher Scientific, Waltham, MA, #34580) or Supersignal West Femto substrate (FLAG blots) (Thermo Fisher Scientific #34095) for 3 min. Membranes were imaged with a ChemiDoc MP imaging system (Bio-Rad Laboratories). Images of full membranes are available in **Appendix A.3**.

2.5.8 Statistical methods

For comparisons of 2 independent variables, one-tailed p values were computed by Welch's *t*-test. For comparisons of 3 or more independent variables, p values were computed by Brown-Forsythe and Welch's 1-way ANOVA with Dunnett's T3 test for multiple comparisons. All

calculations and data visualizations were performed using GraphPad Prism version 9.3 (GraphPad Software, San Diego, CA).

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2.7 Author contributions

Conceptualization, methodology, writing, funding acquisition: JT, ME, MO

Investigation: JT, AK, ALF

Validation, formal analysis, visualization: JT, AK

Supervision: ME, MO

CHAPTER 3. CONCLUSIONS AND FUTURE PERSPECTIVES

3.1. Summary

In this thesis, I show that a broad range of human and nonhuman primate TRIM34 orthologues can restrict certain primate lentiviruses. TRIM5α is required for this restriction. In this work I propose that together, TRIM34 and TRIM5α could act as a barrier to transmission of lentiviruses that neither could inhibit on its own. This work supports a model in which host restriction factors can evolve not only in response to pressure from pathogens but also in the context of other, complementary host immune factors. By investigating both TRIMs involved in this relationship, we can better explore how these proteins may have evolved with each other to counteract viral infection. This, in turn, can help us better understand how TRIM34 and TRIM5α might act as barriers to cross-species transmission events. Here, I present possible areas of further investigation and proposed experiments, which are summarized in **Table 3.1**.

Question	**Experimental strategy**
Is TRIM34 restriction conserved in other branches of primate evolution?	• Assess restriction in NWM and Strepsirrhini
Is TRIM34 protein level correlated to restriction potency?	• Expression level of macaque and baboon lineage TRIM34 • B-box point mutants
What determines TRIM34 specificity?	• TRIM34 v2 and v3 loop chimeras (NWM) • Chimeric TRIM5α with SPRY domain from different species
Has TRIM34 co-evolved with TRIM5α?	• Co-express TRIM34 with diverse TRIM5α, assess restriction • Co-express TRIM34 with TRIM5α chimeras or mutants that disrupt heteromultimerization, assess restriction
Can we determine a structure of TRIM34 bound to capsid (and TRIM5α)?	• CryoEM or crystallography
Does TRIM34 RING act as an E3 ubiquitin ligase, and what are its effects?	• Cell-free ubiquitination assay • Bulk RNA-sequencing
Is TRIM34 involved in signalling?	• Bulk RNA-sequencing • NFκB, AP-1 reporter assay

Table 3.1*. Future experimental strategies*

3.2. Exploring evolutionary and functional differences in other TRIM34 orthologues

I investigated a small panel of TRIM34 orthologues (human, chimpanzee, sabaeus, and macaque). These species were chosen because of the chimpanzee's evolutionary relevance to the zoonotic origins of HIV (223), the diversity of African green monkey TRIM5α alleles (81, 92), and the extensive characterization of rhesus macaque TRIM5α (37, 83, 224–226). However, there exist many other primate *TRIM34* genes whose sequences are known. Whole-genome sequences of nonhuman primate species have become drastically more available even within the last year (227), enabling more expansive exploratory research of this nature. By expanding to a more diverse set of species, TRIM34 orthologues that have different antiviral specificities or functions could be identified. Identifying differences in TRIM34 specificity or function across the primate lineage could help us better understand the role of TRIM34 in host-pathogen conflict. This could include testing TRIM34 orthologues such as: the broader *Macaca* genus (macaques) and their nearest relatives, *Papio* (baboons); New World monkeys; and the Strepsirrhini suborder (comprising lemurs and lorises). Functional testing of restriction capacity could be followed by phylogenetic analysis of restrictive and nonrestrictive TRIM34 orthologues to identify responsible genetic elements. Furthermore, expanding to more branches of the primate tree would allow us to gain a better understanding of how TRIM34 activity may have evolved across primate history.

For example, because it was observed that rhesus macaque TRIM34 (**Figure 2.1a**) has relatively elevated steady-state protein levels compared to the other orthologues tested, I could test other macaque species' TRIM34 alleles to see if they also have this phenotype. It is possible that restriction potency may correlate to amount of TRIM34 expressed. This relationship was not addressed in this work. Given that TRIM34 does not have evidence of rapid evolution at its putative capsid-binding domain that is a hallmark of other restriction factors (138), another possibility is that TRIM34-mediated restriction is modulated by protein availability

in the cytosol. To address these questions, immunoblotting could first be used to assess whether the elevated expression of rhesus macaque TRIM34 is also observed for other species of macaques of both African and Asian lineage. If all macaque orthologues demonstrate elevated TRIM34 levels, analysis could be expanded to test baboons, the next most closely related genera of Old World monkeys. Ideally, some orthologues with elevated expression and some with lower levels of expression would be identified. Phylogenetic comparisons could then be made between orthologues with lower and higher expression levels to identify responsible genetic elements. Finally, one could test whether elevated expression levels are associated with more potent restriction. Understanding whether protein levels directly correlate to strength of restriction could shed light on the mechanism by which TRIM34 restriction occurs. Currently, it is unknown what ratios of TRIM34:TRIM5α or TRIM34:capsid are required for restriction. If TRIM34-mediated restriction is dose-dependent, it could suggest a mechanism similar to that of TRIM5α, in which many individual TRIM molecules restrict by binding directly to the capsid. On the other hand, if restriction potency does not seem to depend on the amount of TRIM34 present, this could suggest that TRIM34 might be acting more as an effector molecule.

Another avenue of research would be to explore whether the TRIM34 SPRY domain contains elements that affect its ability to bind capsid. In this dissertation, it was found that both the TRIM34 and TRIM5α SPRY domains were necessary for restriction (192). Additionally, it is known that insertions and deletions in the TRIM5α v1 loop, which is an essential determinant of antiviral specificity for TRIM5α, are associated with changes in capsid binding (81, 228). TRIM34 has a smaller, more conserved v1 loop relative to TRIM5α. However, New World monkey TRIM34 orthologues possess more diversity in the v2 and v3 loops of their SPRY domains relative to Old World monkey and hominid TRIM34, and it is possible that these variable loops also contribute to capsid recognition or binding. Thus, it would be reasonable to hypothesize that these expanded regions in New World monkey TRIM34 might contribute to

restriction events not observed in the TRIM34 orthologues investigated in this work. Because New World monkeys have not recently been exposed to modern, circulating primate lentiviruses, some of their restriction factors might possess different activity relative to Old World monkeys. Chimeric proteins could then be generated to identify specific domains or regions responsible for phenotypic differences. For example, this could locate regions of TRIM34 that contribute to capsid recognition in addition to that which we propose occurs via the TRIM5α SPRY domain.

A more general strategy would be to search for antiviral activity, or the lack thereof, in primates very distantly related to hominids and Old World monkeys. Identifying antiviral activity in a more diverse set of TRIM34 orthologues would strengthen the hypothesis that TRIM34 has been broadly conserved across primate evolution as a restriction factor and that it might act as a significant barrier to cross-species transmission events. On the other hand, if it were found that TRIM34 from other primate suborders or families are not restrictive, it could suggest that TRIM34 evolved to restrict certain viral pathogens more specifically. For example, one candidate would be the Strepsirrhini, a primate suborder comprising lemurs, lorises, and bushbabies (in contrast to the Haplorrhini, which comprises monkeys and tarsiers). The Strepsirrhini have even greater diversity across species, and thus might possess TRIM34 orthologues with even more divergent function. Lemurs have adapted to highly specific niches in Madagascar over the last approximately 40-75 million years due to resource competition (229, 230). Furthermore, there exists evidence of endogenous retroviruses in several genera of lemurs (231, 232), suggesting a history of conflict with ancient retroviruses, even if there are no known modern lentiviruses that naturally infect Strepsirrhini. In light of these ancient viruses, we might expect to find signatures of host-pathogen conflict in lemurs. This possibility could be investigated first by using strategies such as positive selection analysis on *TRIM34* or *TRIM5* of lemurs. If such genes are found to have undergone rapid evolution, orthologues could be tested

for activity against extant lentiviruses. Because of lemur divergence from the Haplorrhini, we might expect to find different antiviral activity in TRIM34 originating from lemurs, and elements responsible for TRIM34 activity could be inferred based on sequence differences and then experimentally tested.

3.3. Cooperativity of other TRIM5α orthologues with TRIM34

In this work, I tested a range of TRIM34 orthologues in the presence of human TRIM5α. Next, the restrictive capacity of TRIM34 could be tested in the presence of a diverse range of TRIM5α. This could address issues such as whether the two proteins have co-evolved and which protein(s) confer antiviral specificity. Understanding which species' TRIM34 proteins are compatible with which other species' TRIM5α proteins could help elucidate how TRIM34 and TRIM5α may have co-evolved to recognize and bind capsids. While TRIM5α has undergone positive selection and shows a high degree of species specificity, TRIM34 could have evolved to be more broadly active across many primates. Together, these two proteins would allow species to inhibit a broader range of viruses without compromising TRIM5α's potent activity against specific viruses. Furthermore, TRIM34 and TRIM5α co-operation may involve the formation heteromultimers that bind and restrict capsids. This kind of co-operative heteromultimerization is a relatively unexplored area of TRIM biology, and more generally, the study of restriction factors as a whole.

Given that human TRIM5α is able to cooperate with TRIM34 from distantly related primates, it is reasonable to hypothesize that other TRIM5α might also be compatible with a range of TRIM34 orthologues. This would suggest that the two proteins evolved to cooperate with each other earlier in primate evolution. On the other hand, if TRIM34 orthologues only turn out to be compatible with more-closely related TRIM5α orthologues (for example, from the same suborder or family as the TRIM34 orthologue), this would suggest a more recent history of co-evolution. It might also be possible to make chimeras or point mutants of TRIM34 and TRIM5α

to identify specific domains or residues that are responsible for heteromultimerization. This could be assessed indirectly in a manner similar to the experiments described in **Figure 2.2.c-d**, or more directly by co-immunoprecipitation. If abrogating heteromultimerization abolishes restriction, this would strengthen the claim that the two proteins interact to restrict capsids.

It is possible that the specificity of restriction might vary in the presence of TRIM5α originating from different species. This result would suggest that TRIM5α is the primary determinant of antiviral specificity. It would be reasonable to hypothesize that this specificity might be derived from the TRIM5α SPRY domain. This could be tested by co-expressing TRIM34 with chimeras of TRIM5α in which the SPRY domain from different species' TRIM5α is switched. Further genetic comparisons of TRIM5α orthologues that permit differential restriction by TRIM34 might be able to narrow down TRIM5α-mediated specificity to smaller regions of TRIM5α (such as the v1 loop) or even individual residues. This would provide a better understanding of the mechanism by which TRIM34-mediated restriction occurs.

One possible challenge is that a number of TRIM5α orthologues have been shown to have potent activity against some primate lentiviruses (84, 203, 233). This can be controlled for by singly-expressing TRIM5α to test for restriction independent of TRIM34, but it is possible that this TRIM5α-mediated restriction might still make it difficult to detect a smaller magnitude of restriction contributed by TRIM34. One solution to this problem would be to limit the TRIM5α orthologues used to ones that demonstrate no independent restriction capacity by testing them individually, in the absence of TRIM34, first. It would be expected that some of these TRIM5α orthologues would be restrictive of some capsids, but presumably others could be identified with no measurable activity. These non-restricting TRIM5α orthologues then be tested in combination with TRIM34 to assess TRIM34 restriction.

3.4. Structure and function of TRIM34 and TRIM5α in relation to capsid

Another outstanding question regarding the relationship of TRIM34 and TRIM5α is how they physically interact with each other and with capsid. Understanding these interactions is important to understanding the function of TRIM34; that is, what the mechanism of restriction is. Studying the structural biology of TRIMs can be challenging due to issues with purifying the proteins in a soluble form; thus, using other strategies such as cell-based functional assays may be necessary. In this dissertation, I proposed that TRIM34 and TRIM5α can interact with each other as well as with capsid, however this has not been demonstrated rigorously. Other studies have shown that TRIM34 and TRIM5α can be co-immunoprecipitated with each other and with HIV-1 capsid *in vitro* (201, 205). Likewise, functional studies here and elsewhere have suggested that TRIM34 and TRIM5 can interact with each other in cellular conditions (108, 192).

Recently, a dissertation was published in which the authors attempted to characterize TRIM6 and TRIM22, which are in the same locus as TRIM34 and TRIM5α and are phylogenetically the next most closely related TRIMs in primates (234). The authors attempted crystallizing both proteins but failed due to issues with solubility and refraction. They also attempted to assess self-association, and while they demonstrated self-association for both TRIM22 and TRIM6 is mediated by their respective RING domains, the study was hampered by issues with protein stability and solubility. Expression of TRIM proteins is inherently difficult due to their tendency to form protein aggregates in the cytosol. Overall, their study demonstrates the challenges of working with these kinds of proteins.

At this time, a crystal structure or electron micrograph of TRIM34 does not exist, either free or bound to TRIM5α or to capsid. Crystal and cryo-electron microscopy structures of rhesus macaque TRIM5α bound to capsid exists; however the most complete structures were generated by making a chimera comprising a TRIM21 RING domain attached to the C terminus of TRIM5α due to issues with stability and solubility (106, 208). Additionally, there exist cryo-

electron tomography imaging of TRIM5α bound to capsid tubes (235). The macaque TRIM5α was used, rather than the human version, because of its much greater binding affinity for HIV-1 capsid. TRIM34 has been reported to associate with to HIV-1 capsid *in vitro*, even though it does not restrict (201, 211). To resolve a structure of TRIM34 bound to capsid, a high-affinity binding pair of TRIM34 and capsid would be needed. There are several strategies that could be used to identify viable candidates. One could screen many naturally occurring TRIM34 orthologues and capsids for ideal binding properties. Deep mutational scanning could be used to generate single mutants or combinatorial mutants that bind with higher affinity than their wild-type partners; although many mutants would likely have weaker affinity than the wild-type, some might be stronger. Finally, one could attempt to generate high-affinity TRIM34 chimeras or mutants as was done with TRIM5α (208). If a suitably high-affinity TRIM34 and capsid pair could be found, it would theoretically be possible to generate a crystal structure or electron micrograph of TRIM34 bound to capsid, likely also in the presence of TRIM5α. This would be a major step towards understanding if and how TRIM34 and TRIM5α heteromultimerize with each other, which is currently not well understood. It would also help address questions such as whether both TRIM34 and TRIM5α's SPRY domains directly bind capsid, and at what molar ratio TRIM34 and TRIM5α exist during restriction events.

One possible way to overcome problems with TRIM34 solubility and stability *in vitro* is the use of insect cell culture systems. These systems can be more permissive of expression of foreign DNA and can also allow generation of greater amounts of protein compared to mammalian or bacterial production systems. Nevertheless, insect cell culture systems are not a guaranteed way to generate soluble, well-behaved proteins, and other strategies may still be needed. One possibility would be to generate chimeras of TRIM34 with another TRIM that might render a protein that is easier to work with, as was done for the TRIM5-TRIM21 structure (106, 208). A possible weakness of this approach is that in my experience, chimeras of TRIM34

with other TRIMs tend not to express at all, at least in mammalian cells. During the course of this work, I attempted to generate chimeric proteins of TRIM34 and TRIM22 where each domain was individually switched with the corresponding domain of the opposite protein; none of these chimeras were able to be expressed in our cell culture system. Additionally, in **Figure 2.4**, I also attempted to generate the corresponding chimeric protein containing the TRIM5 RBCC domains and the TRIM34 SPRY domain; this chimera also could not be expressed. Thus, it may be that a combination of approaches would be needed to achieve a working system. Overall, understanding the physical structure of TRIM34, TRIM5α, and capsid in complex would allow us to pinpoint essential regions that facilitate restriction.

3.5. Potential for TRIM34 RING domain ubiquitination activity

The RING domain of TRIM5α has E3 ubiquitin ligase activity (93–95). The location of the ubiquitin chains generated is disputed—bound to the RING domain itself, free-floating, or possibly both—but it is generally understood that these chains can trigger a signaling cascade that may result in events such as proteasomal activation and upregulation of downstream effectors (93–96). The RING domains of TRIM34 and TRIM5α share 65.2% pairwise identity, and RING domains are commonly associated with ubiquitination activity. In particular, all the cysteines in the TRIM5α RING domain that are thought to be essential for E3 ligase activity are conserved in TRIM34's RING domain (236). Furthermore, TRIM22 (237),TRIM6 (238), and TRIM34 (239) have also been shown to engage in E3 ubiquitin ligase activity in the presence of other viral infections. TRIM34, in particular, was shown to induce K63-linked ubiquitination of the host protein ZBP1 during infection by influenza A (239). Thus, it is reasonable to hypothesize that the TRIM34 RING domain might also be active as an E3 ubiquitin ligase in the context of other viral infections. This is of interest because ubiquitination may have implications for protein turnover, as discussed above, as well as effects on signaling events subsequent to TRIM34-mediated restriction. It is currently unknown whether the RING domain of TRIM34 can act as an

E3 ubiquitin ligase, where possible ubiquitination might occur, and what the consequences of this activity might be. One paper has suggested that relative to TRIM5α, TRIM34 can only weakly activate AP-1 (96), but I am not aware of any other published research on the topic. This question could be further explored via techniques such as RNA sequencing (RNA-seq), which will be discussed herein.

In the course of this thesis, I attempted to assess whether human TRIM34 was competent for self-ubiquitination via immunoprecipitation; however, I was unable to achieve sufficient protein expression to ascertain this. These experiments were performed in cellular conditions using HEK 293T cells; another possibility would be to attempt to purify TRIM34 and TRIM5α and then assess ubiquitination in a cell-free system by exogenously providing an E1 activating enzyme, an E2 conjugating enzyme, and tagged ubiquitin (95, 240). This would potentially allow better control of the amount of TRIM34 and/or TRIM5α in the system. It would also confer the advantage of removing other TRIMs (and other E3 ligases) from potentially confounding the results. However, the downside to this strategy is that it can be challenging to purify recombinant TRIMs, and when TRIMs are purified they are often ill-behaved in solution. Considerations aside, defining whether TRIM34 is competent for ubiquitination would shed light on the actual mechanism of TRIM34-mediated restriction. It is possible that ubiquitination could contribute both to restriction itself and to downstream signaling events. One way to assess these possibilities, if TRIM34 were found to be able to ubiquitinate or be ubiquitinated would be to use antibodies against different ubiquitin linkages. This could help predict downstream outcomes, such as proteasomal activation, autophagy pathways, or other innate immune activity.

TRIM34 has a longer half-life than does TRIM5α, and TRIM half-life can be mediated by multiple domains, including the RING and B-box (200, 201, 208, 209). One hypothesis is that TRIM34 might act to stabilize TRIM5α in some way during restriction. I also noted that the

different TRIM34 orthologues seemed to have different expression levels, with the macaque TRIM34 generally being the most highly expressed and the chimpanzee TRIM34 generally being the least highly expressed of the orthologues we tested. It is possible that their RING domains may contribute to this difference. I attempted to test this hypothesis by generating chimeras in which the RING domains of human and macaque TRIM34 were switched. The chimeras were observed to have an intermediate expression level relative to the wild type proteins (data not shown). This suggested that it is likely that other domains, such as the B-box (200, 209) or other domains may also be involved in protein turnover. The slower turnover of TRIM34 itself could contribute to this type of activity. This could be tested by generating mutations in the TRIM34 B-box that are predicted to decrease protein half-life based on known mutations in the TRIM5α B-box that confer this phenotype (200, 209). These mutants could then be tested to see whether they are less restrictive in the presence of TRIM5α. If so, the less-restrictive TRIM34 B-box mutants could be tested for their ability to bind TRIM5α. If they cannot bind, the loss of restriction is presumably due to a total loss of interaction. If they can still bind, loss of restriction could be due to changes in turnover rate. This possibility could then be further explored by treatment with proteasomal inhibitors such as MG132. If TRIM34 turnover rate and TRIM5α binding both appear to be major factors in restriction, this could suggest that TRIM34 has a role in stabilizing TRIM5α in some way. Overall, understanding what, if any, role TRIM34 turnover rate has on TRIM5α could further elucidate the mechanism by which TRIM34 restricts capsid.

3.6. Assessing global changes in mRNA expression due to TRIM34 via RNA-seq

Ubiquitin-mediated signaling events are one among many possible outcomes of TRIM34 binding capsid. I performed bulk RNA-seq to gain a fuller understanding of the downstream effects of TRIM34. TRIM5α-mediated restriction has been shown to activate both AP-1 and NFκB pathways (93, 96) . Although TRIM34 is thought only to weakly activate AP-1, that work

was done in the absence of capsid (96). Thus, I hypothesized that in the presence of a susceptible capsid, TRIM34 could activate AP-1 and/or NFκB signalling. Additionally, if it is true that TRIM34 can either perform ubiquitination or be ubiquitinated, either by itself or by another E3 ligase, we might expect to see upregulation of other cellular processes such as autophagy or the proteasome. Finally, as I have observed that long-term ectopic expression of TRIM34 in cell culture tends to make cells nonviable, we also anticipated that we might observe some markers of cell stress or death in cells expressing TRIM34, whether or not they were infected. We will use pathway analysis to look for patterns in differential expression. RNA-seq hits can be validated by qPCR. I can then test proteins of interest *in vitro*; for example, I could generate knockouts of the genes of interest and then use a NFκB or AP-1 reporter construct to assess whether a given gene is involved in signaling events. Overall, the goal is to identify proteins or pathways that may be involved directly in TRIM34-mediated restriction or that are downstream effectors that may enhance the cellular response to viral infection. I tested THP-1 TRIM34 KO cells in which I complemented human TRIM34 or empty vector. In this background, I infected with SIV$_{SAB}$ CA or a mock treatment. 1 dpi, I lysed the cells and isolated whole-cell mRNA with an mRNeasy Plus Mini Kit (Qiagen, Germantown, MD, #74134). Columns were DNase treated (Qiagen #79254). Library preparation was performed using Takara Bio SMART-Seq v4 PLUS Kit (TaKaRa Bio #R400753) and Illumina Nextera XT Library Preparation Kit (Illumina, San Diego, CA, #FC-131-1096). Next generation sequencing was performed on a NextSeq 2000 (Illumina) on a P2 flow cell with 50 paired end reads.

Four comparisons were made: uninfected vs. infected in the presence or absence of TRIM34 and empty vector vs. TRIM34 in the presence or absence of infection. One weakness of the approach used was that the differences between the different clonal lines turned out to be greater than the differences between the experimental conditions of interest (**Appendix B.1, Figure B.1**). Thus, it was difficult to observe significant differential expression across our

experimental conditions. The only condition in which significant up- or down-regulation of genes was observed was in comparing uninfected vs. infected cells lacking TRIM34 (**Appendix B.2, Figure B.2a**). All three other conditions lacked appreciably different expression levels (**Appendix B.2, Figure B.2b-d**). This could suggest that TRIM34 is somehow suppressing signaling activity after infection with SIV$_{SAB}$ CA. However, another interpretation is that the cells were harvested too long after infection to observe differences; that is, it is possible that by 24 hours after infection, the capsid had been suppressed and thus corresponding changes in gene expression had also been muted. This scenario is supported by flow cytometry showing that the eGFP co-expressed on SIV$_{SAB}$ CA particles was decreased in infected cells containing TRIM34 compared to empty vector control. Further work could explore these possibilities by performing RNAseq again at an earlier time point in infection.

Although relatively few differentially expressed genes were identified, a few stood out in the infected cells lacking TRIM34. The genes of interest are associated with host innate immune responses to viral infection, including three interferon-induced family genes (*IFIT1*, *IFI44L*, and *IFI44*) and *TRIM22*. These hits could be validated by qPCR. If indeed these genes are upregulated only when infection occurs in the absence of TRIM34, further work could pursue the mechanism by which TRIM34 may dampen these responses. Additionally, one could investigate whether this dampening applies to other *TRIM*s or interferon-stimulated genes. Overall, these kinds of studies could provide a more complete picture of TRIM34's roles in host immunity.

3.7. Concluding remarks

Four decades after the identification of HIV as the etiologic agent of AIDS, pandemic HIV remains a public health problem. Despite major pharmacological advances in prevention and treatment, there still exists no vaccine and no cure. Thus, basic research is still essential as part of the overarching strategy to improve the repertoire of available interventions. Additionally,

as we have experienced in the past three and a half years, the threat posed by other, yet unknown zoonotic diseases is also a public health concern. As the climate changes and humans increasingly intrude into animal habitats, spillover events are likely to continue. Therefore, research that contributes to our understanding of how such diseases cross species boundaries and arise in the human population is a critical part of a holistic approach to anticipating and addressing both novel and known pathogens. The work described in this thesis attempts to probe these questions as a step towards these goals.

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APPENDIX A. SUPPLEMENTAL DATA PERTAINING TO CHAPTER 2

A.1 ICE chromatograms

Supplemental chromatograms may be downloaded from:

https://doi.org/10.6084/m9.figshare.24013050.v1

A.2 Sequences used in this study

Supplemental sequences may be downloaded from: https://static-content.springer.com/esm/art%3A10.1186%2Fs12977-023-00629-4/MediaObjects/12977_2023_629_MOESM2_ESM.txt

A.3 Full western blot membranes

Supplemental images may be downloaded from: https://static-content.springer.com/esm/art%3A10.1186%2Fs12977-023-00629-4/MediaObjects/12977_2023_629_MOESM3_ESM.pdf

APPENDIX B. SUPPLEMENTAL DATA PERTAINING TO CHAPTER 3

B.1 PCA plot

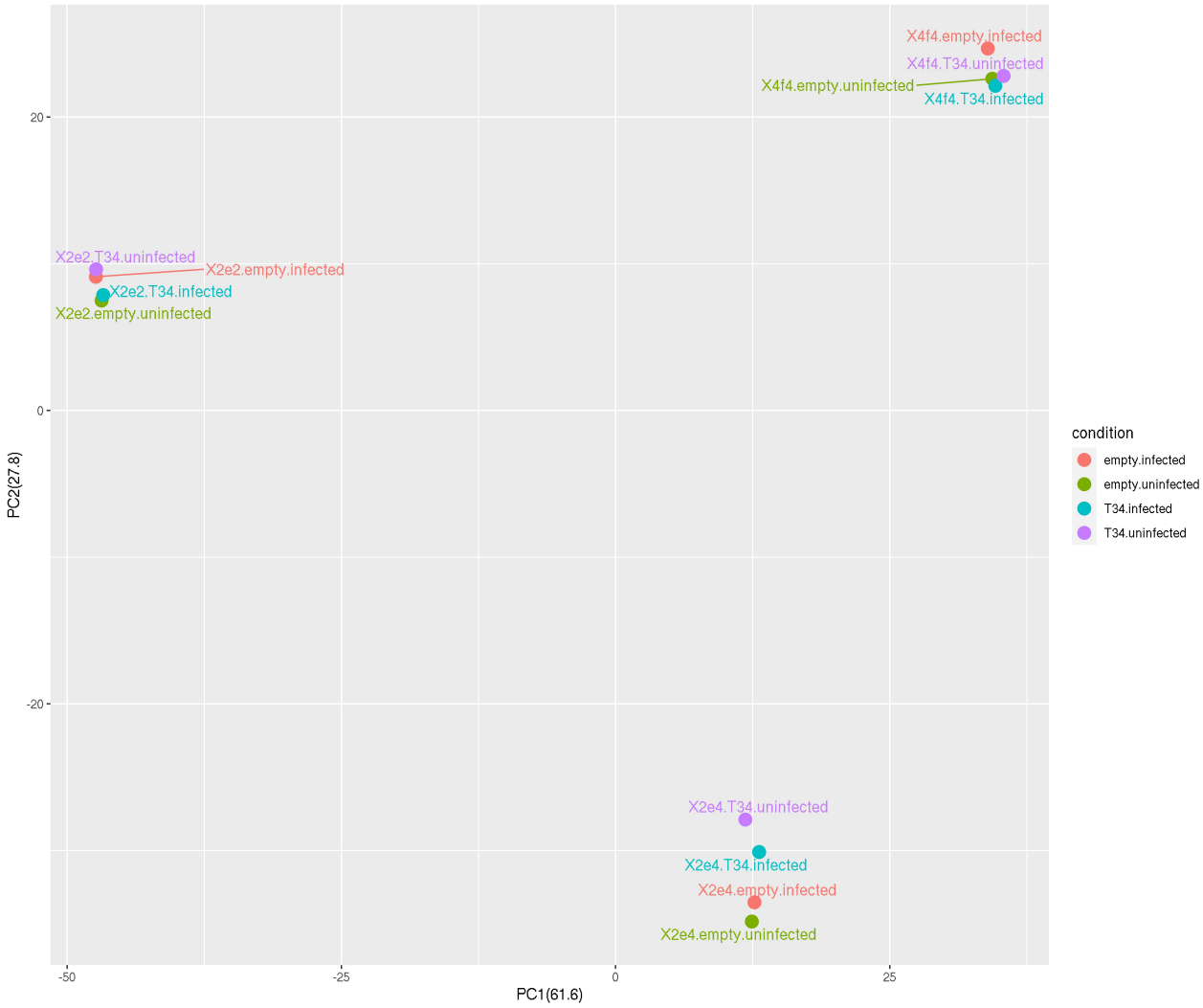

Figure B.1. *PCA plot. Each color represents a different experimental condition: no infection or infection with SIV$_{AGM\text{-}SAB}$ CA in THP-1 TRIM34 clonal KO cells that overexpress TRIM34 or contain an empty vector control. Individual clonal KO lines used are labeled 2e2, 2e4, and 4f4.*

B.2 Volcano plots

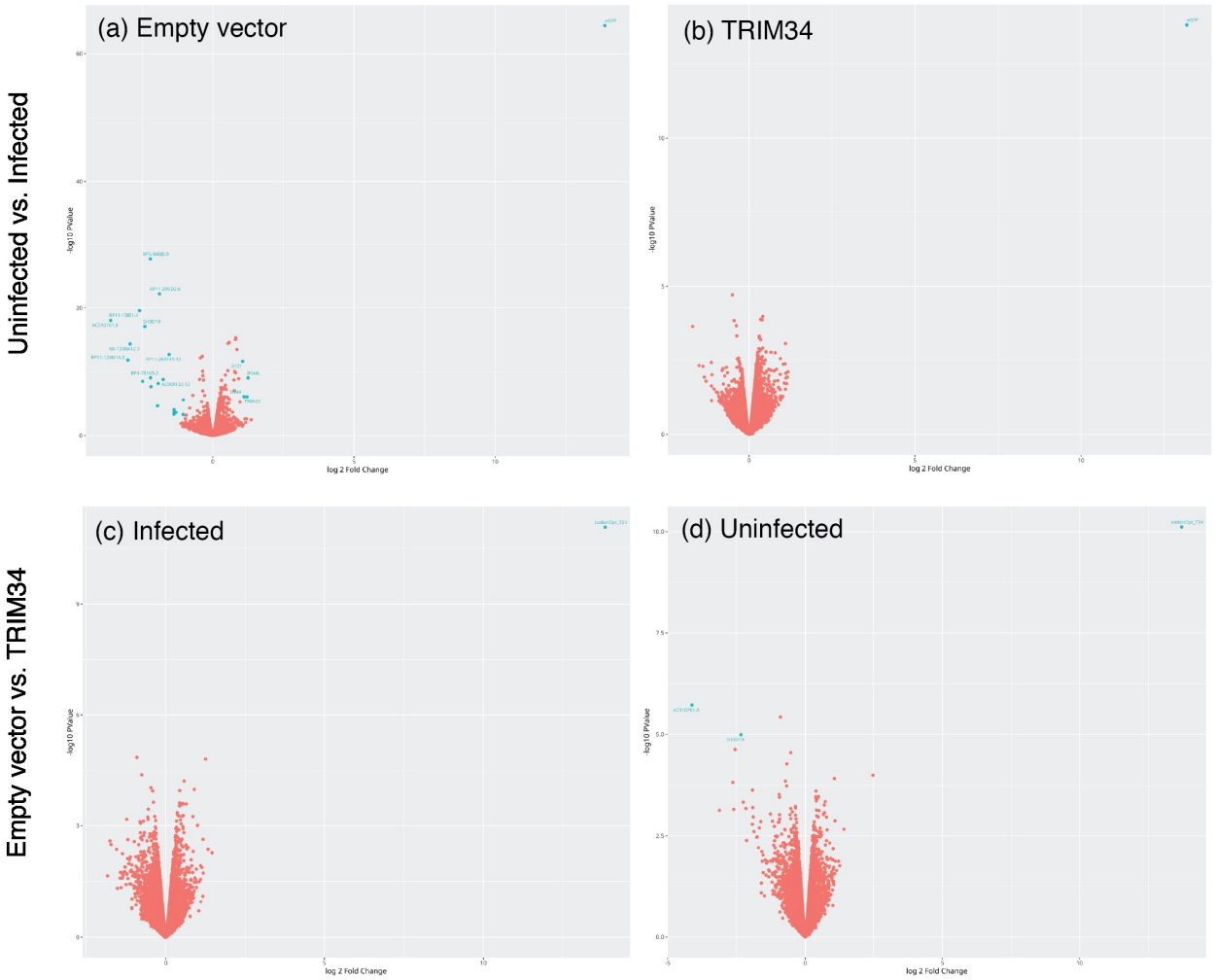

Figure B.2. *Volcano plots depicting differential gene expression. (a) and (b) represent log fold change in infected cells relative to uninfected cells; (a) represents cells containing empty vector and (b) represents cells ovexpressing human TRIM34. (c) and (d) represent log fold change in cells containing empty vector relative to those overexpressing TRIM34; (c) represents infected cells and (d) represents uninfected cells.*