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Virus-Specific Effects of TRIM5α_{rh} RING Domain Functions on Restriction of Retroviruses

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The tripartite motif protein TRIM5α restricts particular retrovirus infections by binding to the incoming capsid and inhibiting the early stage of virus infection. The TRIM5α RING domain exhibits E3 ubiquitin ligase activity and assists the higher-order association of TRIM5α dimers, which promotes capsid binding. We characterized a panel of RING domain mutants of the rhesus monkey TRIM5α (TRIM5α_{rh}) protein. The RING domain function that significantly contributed to retroviral restriction depended upon the restricted virus. The E3 ubiquitin ligase activity of the RING domain contributes to the potency of HIV-1 restriction. Nonetheless, TRIM5α_{rh} mutants without detectable E3 ubiquitin ligase activity still blocked reverse transcription and inhibited HIV-1 infection at a moderate level. When TRIM5α_{rh} capsid binding was weakened by substitution with a less efficient B30.2/SPRY domain, the promotion of higher-order association by the RING domain was more important to HIV-1 restriction than its E3 ubiquitin ligase activity. For the restriction of N-tropic murine leukemia virus (N-MLV) and equine infectious anemia virus (EIAV) infection, promotion of higher-order association represented the major contribution of the RING domain. Thus, both identity of the target virus and the B30.2/SPRY domain-mediated affinity for the viral capsid determine the relative contribution of the two known RING domain functions to TRIM5α restriction of retrovirus infection.

The replication of human immunodeficiency virus type 1 (HIV-1) is potently blocked in Old World monkeys at an early postentry step, prior to reverse transcription (1, 2). In 2004, TRIM5α was identified as the major host factor that mediates this block (3). Since this discovery, the TRIM5 proteins of multiple mammalian species have been characterized, and the range of restricted viruses has expanded to include a variety of retroviruses (lentiviruses, a betaretrovirus, a gammaretrovirus, and spumaviruses) (4–16). A TRIM5 variant, TRIMCyp, which arose as a result of retrotransposition events involving TRIM5 and cyclophilin A, exhibits distinct restriction activities and has been identified in New World owl monkeys and some Old World macaque species (17–22). The wide existence of restricting TRIM5 variants suggests that they are part of a novel, widespread mechanism of innate immunity.

TRIM5 proteins block viral infection in a species-specific manner. Rhesus monkey TRIM5α (TRIM5α_{rh}) potently blocks infection by HIV-1, whereas human TRIM5α (TRIM5α_{hu}) only modestly restricts HIV-1 infection but potently blocks infection of N-tropic murine leukemia virus (N-MLV) (3, 5, 8, 9, 15). The mechanism of TRIM5-mediated restriction is still not completely understood. Retroviral sensitivity is determined by viral capsid proteins; TRIM5α has been shown to bind directly to *in vitro*-assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes, which resemble authentic viral cores (23–25). Interactions between TRIM5 proteins and viral capsids promote the uncoating of sensitive viruses, as the level of particulate capsids is decreased in the cytosol of cells expressing a restricting TRIM5α protein (25, 26). Recent electron microscopic studies (27) demonstrated that purified TRIM5α proteins spontaneously form a large hexagonal lattice structure on the HIV-1 capsid, which is composed of smaller CA hexameric units. This observation has led to the hypothesis that a slight mismatch between the geometry of the TRIM5α_{rh} hexagonal lattice and that of the capsid could lead to disassembly of the capsid (27). Some investigators have reported that proteasome inhibitors relieve the TRIM5α-mediated block of viral reverse transcription but not infection, implying a two-step restriction mechanism that requires the proteasome (28, 29). Degradation of TRIM5 proteins was also observed in the presence of saturating levels of sensitive viruses, suggesting that the TRIM5α-capsid complexes are targeted for degradation; however, changes in capsid protein stability or accumulation of ubiquitinated TRIM5α proteins were not observed upon HIV-1 infection (30). Recognition of the retroviral capsid by TRIM5α has been shown to promote innate immune signaling by catalyzing the synthesis of unattached K63 ubiquitin chains (31).

TRIM5α is a member of the tripartite motif (TRIM) protein family (32). TRIM proteins all contain a RING domain, one or two B-box domains, and a coiled-coil domain. TRIM5α, in addition, contains a B30.2/SPRY domain at the C terminus (32, 33). The B30.2/SPRY domain determines viral specificity and restriction potency; sequence variations in this domain correlate with the ability of TRIM5 to recognize different viral capsids (34–40). The coiled-coil domain of TRIM5 is essential for dimerization (41). The RING and B-box 2 domains cooperatively promote the higher-order association of preformed TRIM5α dimers (42–44). Multimerization increases TRIM5α avidity for viral capsids, which po-

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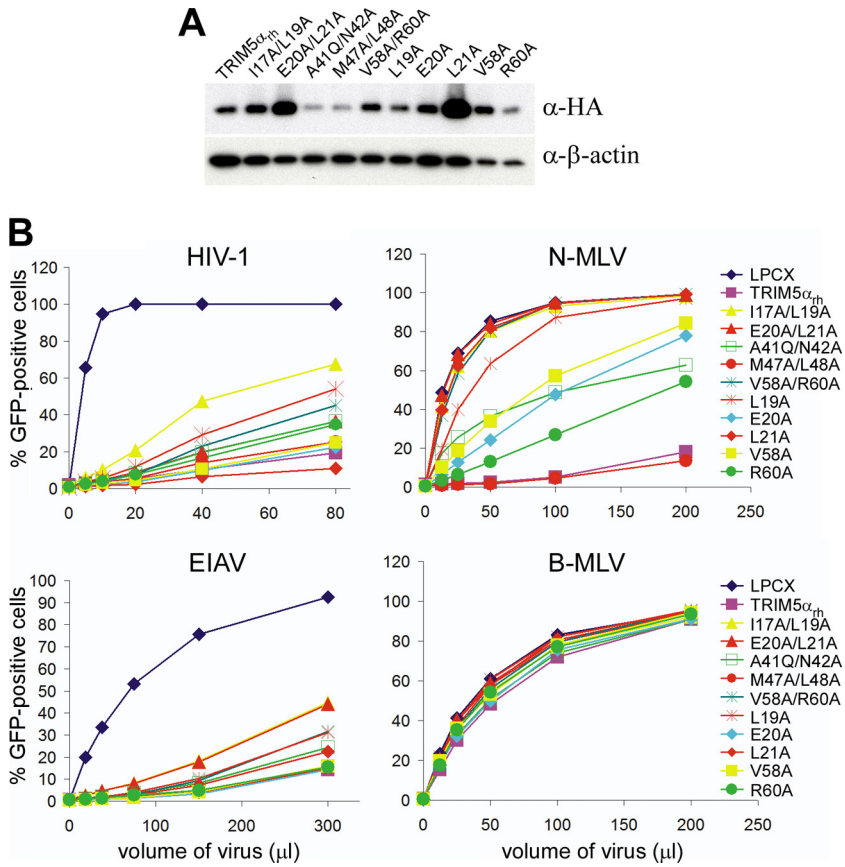

FIG 1 Expression and antiretroviral activity of TRIM5α_{rh} RING domain mutants stably expressed in Cf2Th cells. (A) Steady-state expression levels of the TRIM5α_{rh} RING domain mutants were assessed by Western blotting with an anti-HA antibody (α-HA; Roche). All of the TRIM5α_{rh} variants carry an HA tag at the C terminus. The β-actin levels in the lysates are shown as an internal control. (B) Antiviral activities of TRIM5α_{rh} RING domain mutants. Cf2Th cells expressing the different TRIM5 variants, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of recombinant HIV-1, N-MLV, EIAV, and B-MLV viruses expressing GFP. Infected GFP-positive cells were counted by FACS. The experiments were repeated four times; shown here are the results from one typical experiment.

tentiates antiviral restriction. In both *in vivo* and *in vitro* assays, the TRIM5 RING domain has been shown to have E3 ubiquitin ligase activity (23, 24, 45). The proposed involvement of the proteasome in blocking reverse transcription and the observed degradation of TRIM5 proteins upon infection of TRIM5-expressing cells with a sensitive virus both suggest that the E3 ubiquitin ligase activity of TRIM5 might play some role in restriction (28–30). However, deletion or disruption of the RING domain of TRIM5α_{rh} or TRIM5α_{hu} only partially decreased restriction of HIV-1 and N-MLV infection, respectively, arguing against a necessary role of the E3 ubiquitin ligase activity in the TRIM5α-mediated antiviral effect (35, 46, 47). More recent data suggest that the contribution of the TRIM5α RING domain to restriction depends on both the host and the viruses (48). The E3 ubiquitin ligase activity of TRIM5$_{agm}$ correlated strongly with the restriction of macaque simian immunodeficiency virus (SIV$_{mac}$) infection, blockade of reverse transcription, and premature uncoating of the SIV$_{mac}$ capsid (49). In light of these results, a reevaluation of the role of the RING domain and its associated E3 ubiquitin ligase activity in viral restriction is merited.

Although small, the TRIM5α RING domain is known to be multifunctional. Changes in this domain might simultaneously affect several properties of the protein, including the E3 ubiquitin ligase activity, higher-order self-association, protein turnover, and cellular localization (44, 46, 50). To understand the contribution of each RING-determined property to antiviral activity, and whether different viruses are affected differently by these properties, we characterized comprehensively a panel of RING domain mutants of TRIM5α_{rh}. We analyzed the correlations between these properties and the restriction of HIV-1, N-MLV, and equine infectious anemia virus (EIAV) infection. Our results indicated that the E3 ubiquitin ligase activity makes a modest contribution to the restriction of HIV-1 by TRIM5α_{rh}. However, when the TRIM5α_{rh} B30.2/SPRY domain was replaced by the TRIM5α_{hu} B30.2/SPRY domain, which binds the HIV-1 capsid less efficiently, the ability to support higher-order association represented the dominant contribution of the RING domain to HIV-1 restriction. For TRIM5α_{rh} restriction of N-MLV and EIAV, RING-mediated higher-order association contributed to restriction, whereas E3 ubiquitin ligase exerted little detectable impact on restriction. Thus, the antiviral potency of TRIM5α toward different viruses is dictated by distinct properties of the TRIM5 RING domain.

MATERIALS AND METHODS

Plasmid constructs and cell lines. All of the RING domain mutants were constructed by QuikChange mutagenesis, using as a template a pLPCX

TABLE 1 Phenotypes of TRIM5α_{rh} RING domain variants

TRIM5α_{rh} variant	Expression level[a]	E3 ubiquitin ligase activity[b]	Higher-order association[c]	HIV-1 capsid binding[d]	HIV-1 reverse transcription[e]	Infection by[f]:		
						HIV-1	N-MLV	EIAV
Wild-type	++	++++	++++	++++	+/−	+/−	+/−	+/−
I17A/L19A	+++	−	+	++	+++	++	++++	++
E20A/L21A	++++	++	+	++	++	+	++++	++
A41Q/N42A	+	++++	++++	+++	+/−	+/−	++	+/−
M47A/L48A	+	++++	++++	+++	+/−	+/−	+/−	+/−
V58A/R60A	+++	−	++	+	++	+	++++	+
L19A	++	−	+++	++++	+	++	++++	+
E20A	+++	++	++	++	+/−	+/−	+++	+/−
L21A	++++	+++	−	+	+/−	+/−	++++	+
V58A	+++	+	++	+	+/−	+/−	+++	+/−
R60A	++	−	++++	++++	+/−	+	+	+/−

[a] The expression level of each TRIM5α variant was determined as described in Materials and Methods. The level relative to that of the wild-type TRIM5α_{rh} protein is reported as follows: ++++, more than 300% of the wild-type TRIM5α_{rh} level; +++, 150 to 300%; ++, 100 to 150%; +, 40 to 55%.

[b] The ubiquitin ligase activity of each TRIM5α variant was determined as described in Materials and Methods. The E3 ubiquitin ligase activity relative to that of the wild-type TRIM5α_{rh} protein is reported as follows: ++++, 100 to 101% of the wild-type TRIM5α_{rh} E3 ligase activity; +++, 60 to 70%; ++, 40 to 50%; +, 5 to 15%; −, not detected.

[c] The higher-order association of each TRIM5α variant with the wild-type TRIM5α_{rh} protein was determined as described in Materials and Methods. The association efficiency relative to the self-association of wild-type TRIM5α_{rh} protein is reported as follows: ++++, ≥100% of the higher-order self-association of the wild-type TRIM5α_{rh} protein; +++, 80 to 95%; ++, 10 to 30%; +, 0 to 10%; −, not detected.

[d] Binding of each TRIM5α variant to HIV-1 CA-NC complexes was measured as described in Materials and Methods. The binding relative to that of the wild-type TRIM5α_{rh} protein is reported as follows: ++++, 95 to 105% of the binding observed for the wild-type TRIM5α_{rh} protein; +++, 60 to 75%; ++, 20 to 50%; +, 10 to 25%.

[e] Late HIV-1 reverse transcripts were measured as described in Materials and Methods. The reverse transcript level relative to that observed following HIV-1 infection of cells transduced with the empty LPCX vector is reported as follows: ++++, 100% of the late reverse transcript level in control pLPCX-transduced cells; +++, 70 to 80%; ++, 40 to 50%; +, 15 to 25%; +/−, 0 to 5%.

[f] The level of infection by the indicated viruses in cells expressing the TRIM5α_{rh} variants was determined as described in Materials and Methods. The level of infection, relative to the level observed in cells transduced with the empty pLPCX vector, is reported as follows: ++++, 95 to 100%; +++, 75 to 95%; ++, 55 to 75%; +, 35 to 55%; +/−, 10 to 35%.

vector encoding C-terminal hemagglutinin (HA)-tagged wild-type TRIM5α_{rh}. In some cases, the sequences encoding the B30.2/SPRY domain from TRIM5α_{hu} were substituted for the equivalent rhesus monkey TRIM5 sequence in the above vector, as previously described (51). Stable Cf2Th cell lines were established using the pLPCX vector-based retroviral gene delivery system (Clontech) and selected in 5 μg/ml puromycin, as previously described (52).

Infection of cells with viruses expressing GFP. Recombinant HIV-1, N-MLV, B-tropic MLV (B-MLV), and EIAV viruses expressing green fluorescent protein (GFP) were made as previously described (3, 9, 12). Cells were infected with various doses of GFP-expressing viruses, and the percentage of GFP-positive cells was counted 60 h after infection by fluorescence-activated cell sorting (FACS).

Assay for TRIM5α higher-order association. The association between dimers of TRIM5α_{rh} RING domain mutants and the wild-type TRIM5α_{rh} was assessed using a previously described higher-order self-association assay with some modifications (43, 44). In 6-well plates, 293T cells were transiently transfected with the empty pLPCX vector or pLPCX vectors encoding the HA-tagged TRIM5α_{rh} RING domain mutants or FLAG-tagged wild-type TRIM5α_{rh}. Cell lysates were extracted 48 h after transfection and were cleared of insoluble materials/aggregates by centrifugation. Depending on the expression level of TRIM5α proteins, some lysates were further diluted with lysates from 293T cells transfected with the empty pLPCX vector. Lysates containing HA-tagged TRIM5α_{rh} RING domain mutants and lysates containing FLAG-tagged wild-type TRIM5α_{rh} were then mixed. Samples of the mixture were taken at this point for analysis of the input TRIM5α protein level. Magnetic protein G beads (7 μl; Invitrogen), prebound with 1 μl (~5 to 6 μg) of anti-FLAG antibody (Sigma), were added to the lysate mixture and incubated on a rocker overnight at 4°C. The beads were then washed four times with 1 ml buffer (300 mM NaCl, 50 mM Tris-HCl, 1% NP-40) at 4°C for 10 min before being boiled for 5 min in 2× SDS sample buffer (125 mM Tris-HCl, 2% SDS, 16% glycerol, 3% β-mercaptoethanol, 0.01% bromophenol blue) to release the immunoprecipitated proteins. The proteins were analyzed by Western blotting, using the HA and FLAG epitopes for detection, as described previously (43, 44).

***In vitro* E3 ubiquitin ligase activity assay.** The E3 ubiquitin ligase activity of TRIM5α variants was measured using a previously described *in vitro* self-ubiquitylation assay with minor modifications (49). Cf2Th cells stably expressing the RING domain mutants were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) supplemented with 0.5 mM dithiothreitol (DTT). After preclearing by centrifugation, the cell lysates were mixed with magnetic protein G beads (7 μl; Invitrogen) prebound with 5 μg of anti-HA antibody (Sigma) and incubated overnight at 4°C on a rocker. The beads were then washed 4 times with RIPA buffer supplemented with 0.5 mM DTT and once with HEPES buffer (50 mM HEPES, 200 mM NaCl, and 0.5 mM DTT). For the self-ubiquitylation reaction, the beads were resuspended in HEPES buffer and mixed with 0.2 μM E1 enzyme, 1 μM E2 enzyme (UbcH5a), and 20 μM ubiquitin in the presence and absence of an energy-regenerating system (5 mM Mg-ATP; BostonBiochem). The reaction mixture was incubated at 37°C for 2 h. TRIM5α proteins were then detected by Western blotting with an an-

TABLE 2 *P* values for Spearman rank-order correlations between antiretroviral activities and other properties of TRIM5α_{rh} variants

TRIM5α restriction	*P* value[a]		
	HIV-1	N-MLV	EIAV
E3 ubiquitin ligase activity	**(+) 0.0191**	(+) 0.1893	(+) 0.2460
Higher-order association	(+) 0.6495	**(+) 0.0021**	**(+) 0.0165**
CA-NC binding	(−) 0.7128		
HIV-1 late RTs	**(−) 0.0097**		
Half-life	(−) 0.7932	**(−) 0.0017**	(−) 0.0553
Expression level	(−) 0.8308	**(−) 0.0084**	(−) 0.1604
Detergent insolubility	**(−) 0.0073**	(−) 0.0317	**(−) 0.0062**

[a] The *P* values from the Spearman rank-order correlation analysis on each pair of TRIM5α_{rh} properties/activities are listed. A positive sign indicates a direct relationship, and a negative sign indicates an inverse relationship. Correlations with *P* values less than 0.02 were considered significant and are highlighted in bold.

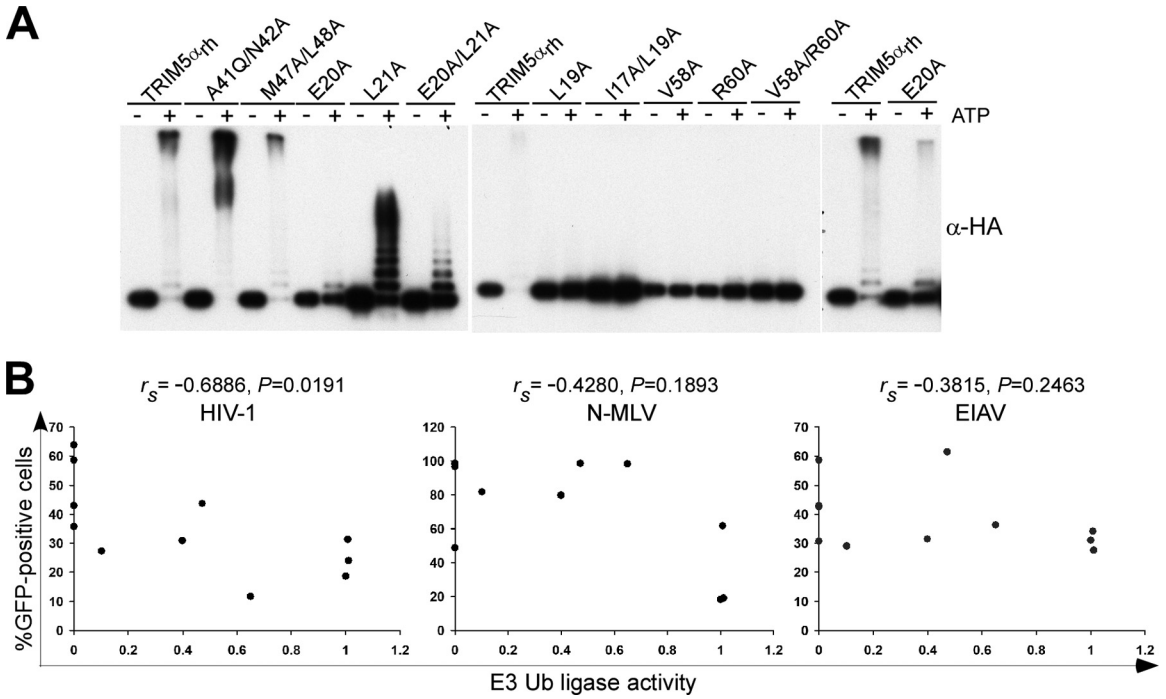

FIG 2 Relationship between E3 ubiquitin ligase activity of TRIM5α_{rh} RING domain mutants and antiretroviral activity. (A) TRIM5α_{rh} RING domain mutants immunoprecipitated from the lysates of stably expressing cell lines were used in an *in vitro* E3 ubiquitin ligase activity assay in the absence and presence of ATP. After 2 h of incubation at 37°C, the reaction mixture was examined by Western blotting with an anti-HA antibody (α-HA), which recognizes both unmodified and ubiquitylated TRIM5 proteins. The far right panel shows results of a longer exposure of the Western blot film from an independent experiment, where the ubiquitylated forms of mutant E20A are more visible. (B) Correlations between the E3 ubiquitin ligase activity of TRIM5α_{rh} RING domain variants and their restriction activities were assessed by Spearman rank-order correlation analysis. The plots show the relationship between viral infectivity in the TRIM5α_{rh}-expressing cells and the percent conversion of TRIM5α proteins to ubiquitylated forms in the *in vitro* E3 ubiquitin ligase assay. The r_s and *P* values are shown on the plots.

ti-HA antibody (Roche). For densitometric analysis, films were scanned digitally, and the relative intensities of protein bands were quantitated by using the ImageJ 1.42q software (NIH). The E3 ubiquitin ligase activity is expressed as the amount of the unmodified TRIM5α protein after the ubiquitylation reaction subtracted from the amount of input TRIM5α protein, divided by the amount of the input TRIM5α protein. For mutants that exhibited no E3 ubiquitin ligase activity, film quantitation occasionally yielded small negative values; in those cases, the E3 ubiquitin ligase activities were assigned a value of zero.

Detergent solubility assay. To assess the solubility of TRIM5α_{rh} variants in detergent buffers, 4×10^5 stable Cf2Th cells expressing TRIM5α RING domain mutants were seeded into 6-well plates. Cells were lysed the following day with 1 ml of lysis buffer (1% NP-40–phosphate-buffered saline–protease inhibitor cocktail). The detergent-soluble and -insoluble fractions represented the supernatant and the pellet obtained by centrifuging crude cell lysates at 13,200 rpm for 10 min. The pellet was dissolved in SDS sample buffer, and the amounts of proteins in both the supernatant and the pellet were examined by Western blotting with an anti-HA antibody (Roche). Detergent insolubility was defined as the ratio of insoluble protein to soluble protein, both normalized against the β-actin level in the respective fractions.

Subcellular localization using immunofluorescence confocal microscopy. The subcellular localization of TRIM5α mutants was detected with a rat anti-HA 3F10 antibody (1:100; Roche) followed by a goat anti-rat immunoglobulin G conjugated with Alexa-488 (1:200; Molecular Probes) and observed under a Zeiss LSM510 META confocal microscope, as previously described (43).

Measurement of HIV-1 late reverse transcription products. One day prior to infection, 4×10^5 stable TRIM5α-expressing Cf2Th cells were seeded into 6-well plates in triplicate. HIV-1–GFP viruses were prepared as described above and were treated with 44 U/ml of Turbo DNase I (Ambion) at 37°C for 1 h. The viruses were then added to cells at a dose that would infect 50 to 80% of cells expressing the empty pLPCX vector. Sixteen hours after infection, cells were harvested and total DNA was extracted with the DNeasy kit (Qiagen). One hundred nanograms of total DNA was used as a template for quantitative real-time PCRs under previously described conditions (53). Duplicate measurements were performed for each of the triplicate samples.

Statistics. Potential relationships between variables were examined by Spearman rank-order correlation analysis (Vassarstats.net). To account for multiple comparisons, only correlations with *P* values of <0.02 were considered significant.

RESULTS

Antiretroviral activities of TRIM5α_{rh} RING domain mutants. The solution structure of the human TRIM5α RING domain has been solved (doi:10.2210/pdb2ecv/pdb). Like other RING domains, the TRIM5α_{hu} RING coordinates two zinc ions and adopts a ββα fold (54). Based on homology with some well-characterized RING domains, several residues in the TRIM5α_{rh} RING domain (I17, L19, V58, R60, A41, and N42) were predicted to be at or near the putative E2 binding site (54). To study the contribution of these residues to the E3 ubiquitin ligase activity of TRIM5α and retrovirus restriction, we changed these residues to alanines (A) (except A41, which was changed to glutamine [Q]), either individually or in combination.

Previously, we showed that the RING domain contributes to the higher-order self-association of TRIM5α (44). A closely re-

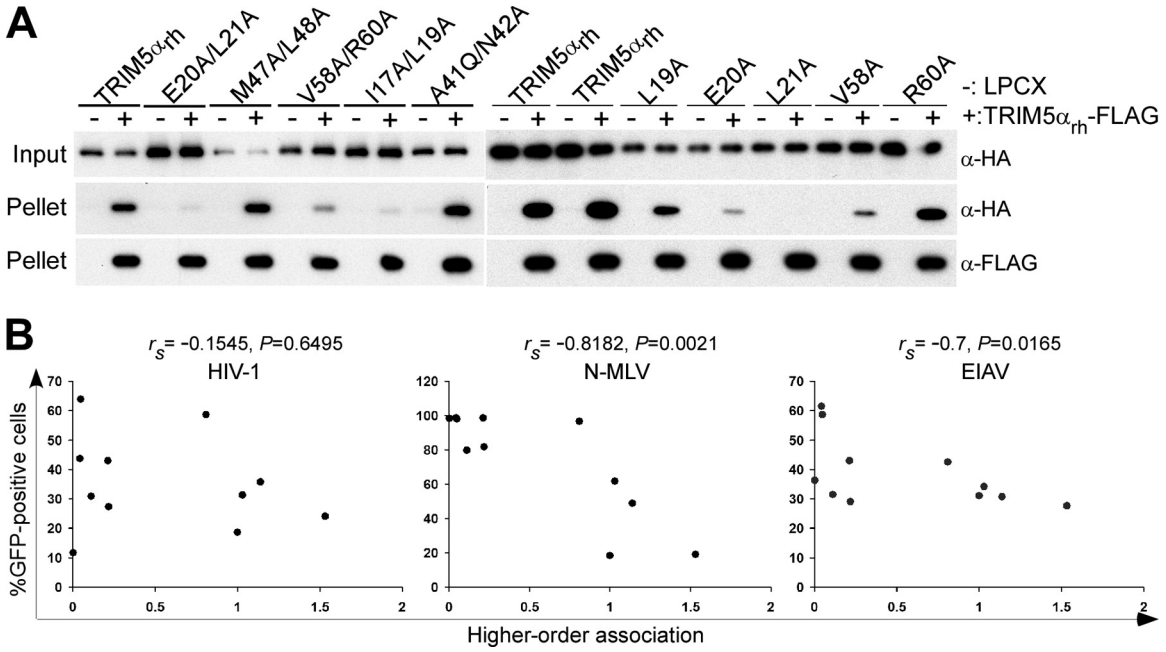

FIG 3 Relationship between higher-order association of TRIM5α$_{rh}$ RING domain variants and antiretroviral activity. (A) Coprecipitation of HA-tagged TRIM5α$_{rh}$ RING domain mutants with FLAG-tagged TRIM5α$_{rh}$ dimers. 293T cells were transiently transfected with the empty pLPCX vector or vectors expressing the indicated TRIM5α$_{rh}$ variant. The inputs of the mutants were adjusted with lysates from pLPCX-transfected 293T cells so that the concentrations of TRIM5 proteins were more comparable. Cell lysates from separate transfections were mixed as indicated and immunoprecipitated with an anti-FLAG antibody (α-FLAG). The amounts of HA- and FLAG-tagged proteins in the lysates (input) and immunoprecipitates (pellet) were analyzed by Western blotting with horseradish peroxidase-conjugated anti-HA and anti-FLAG antibodies. (B) Relationships between higher-order association of TRIM5α$_{rh}$ RING domain mutants and their restriction activities were assessed by Spearman rank-order correlation analysis. The plots show the relationship between higher-order association and the infectivity of the indicated virus in cells expressing a TRIM5α$_{rh}$ variant. Higher-order association is defined as the amount of the HA-tagged TRIM5α variant that was coprecipitated, normalized against the input of the TRIM5α variant and the amount of immunoprecipitated FLAG-tagged wild-type TRIM5α$_{rh}$ protein. The r_s and P values are shown on the plots.

lated chimeric protein, TRIM5α$_{rh}$-21R, which is a TRIM5α$_{rh}$ protein with a human TRIM21 RING domain, exhibits much less efficient higher-order self-association than wild-type TRIM5α$_{rh}$ (unpublished results). By comparing the RING domains of human TRIM21 and TRIM5α$_{rh}$, several residues (E20, L21, M47, and L48) were identified as potential contributors to higher-order self-association. These residues were also changed to alanines, either individually or in combination, to investigate the contribution of RING domain-mediated higher-order self-association to restriction.

Cf2Th cell lines stably expressing these double or single TRIM5α$_{rh}$ mutants were established, and the steady-state level of TRIM5α expression was examined (Fig. 1A). These cells were then challenged with recombinant HIV-1, N-MLV, EIAV, or B-MLV viruses expressing GFP (Fig. 1B). Some mutants, particularly E20A/L21A and L21A, were expressed at much higher levels than others, but higher expression levels did not significantly correlate with restriction potency (Tables 1 and 2). For example, the mutant M47A/L48A, although expressed at a lower level, restricted HIV-1, N-MLV, and EIAV infection as efficiently as wild-type TRIM5α$_{rh}$. Most of the RING domain mutants potently restricted HIV-1 infection, with a few mutants exhibiting very modest reductions in their anti-HIV-1 activity. All of the mutants potently restricted infection by EIAV. In contrast to the two lentiviruses, the gammaretrovirus N-MLV was able to overcome the block by many of the RING domain mutants. For example, mutant L21A blocked HIV-1 infection even more potently than wild-type TRIM5α$_{rh}$, yet exerted little effect on N-MLV infection (Fig. 1B); this observation suggests that the TRIM5α$_{rh}$ RING domain may contribute to HIV-1 and N-MLV restriction by different mechanisms. Neither wild-type TRIM5α$_{rh}$ nor any of these mutants was able to block infection by B-MLV (Fig. 1B).

E3 ubiquitin ligase activity of TRIM5α$_{rh}$ RING domain mutants. The E3 ubiquitin ligase activity of the TRIM5α$_{rh}$ RING domain mutants was examined in an *in vitro* assay, as previously described (49). The wild-type TRIM5α$_{rh}$ efficiently formed polyubiquitin ladders upon addition of ATP (Fig. 2A). Two mutants, A41Q/N42A and M47A/L48A, self-ubiquitylated as efficiently as the wild-type TRIM5α$_{rh}$ protein. All other mutants exhibited some degree of defect in their E3 ubiquitin ligase activity. The L19A, I17A/L19A, V58A, R60A, and V58/R60A mutants exhibited no detectable E3 ubiquitin ligase activity, consistent with the prediction that the E2 binding site is disrupted by these changes (54). The relationship between the E3 ubiquitin ligase activity of the TRIM5α$_{rh}$ mutants and their ability to restrict retroviral infection was investigated by a Spearman rank-order correlation analysis (Vassarstats.net). The viral infection results from four independent experiments were averaged and used in the analysis. The E3 ubiquitin ligase activity was quantified as the percentage of proteins ubiquitylated after the *in vitro* reaction. The relationships between the E3 ubiquitin ligase activity and the restriction activities are shown in Fig. 2B. A positive correlation was observed between the E3 ubiquitin ligase activity and the ability of TRIM5α$_{rh}$ to block HIV-1 infection, even though mutant

TRIM5α$_{rh}$ proteins with poor E3 ubiquitin ligase activity (e.g., V58A) still restricted HIV-1 quite efficiently. No correlation with the E3 ubiquitin ligase activity was observed for the TRIM5α$_{rh}$-mediated restriction of either N-MLV or EIAV infection (Fig. 2B).

Higher-order association of TRIM5α$_{rh}$ RING domain mutants. We previously showed that higher-order self-association of TRIM5α protein dimers promotes cooperative binding to the HIV-1 capsid (42, 43). Electron microscopy studies have demonstrated that purified TRIM5α proteins spontaneously form a hexagonal lattice structure, which is promoted by interaction with the assembled HIV-1 capsid (27). The hexagonal lattice structure likely forms as a result of TRIM5α higher-order self-association, allowing "pattern recognition" of viral capsids (27). The TRIM5α$_{rh}$ mutants dimerized efficiently, compared with the wild-type TRIM5α$_{rh}$ protein; the L19A and A41Q/N42A mutants exhibited slight decreases in dimerization, which may be related to their lower levels of expression (data not shown). As the RING domain contributes to TRIM5α higher-order self-association, we examined whether the RING domain mutants associated with wild-type TRIM5α$_{rh}$ dimers to form higher-order structures, using a previously described coimmunoprecipitation assay with differently tagged proteins (see Materials and Methods) (43, 44).

To achieve equivalent input levels of the TRIM5α$_{rh}$ variants, some of the cell lysates were diluted with the lysates from 293T cells transiently transfected with the empty pLPCX vector. Three of the mutants (M47A/L48A, A41Q/N42A, and R60A) associated with TRIM5α$_{rh}$ dimers as efficiently as the wild-type TRIM5α protein; all the other mutants demonstrated some degree of defect in higher-order association (Fig. 3A). No significant correlation was observed between higher-order association and the restriction of HIV-1 infection for this panel of mutants (Fig. 3B). In contrast, there was a very strong correlation between TRIM5α$_{rh}$ higher-order association and the restriction of N-MLV infection. A weaker correlation was also observed between higher-order association and the restriction of EIAV infection. Thus, the contribution of the TRIM5α$_{rh}$ RING domain to higher-order association is apparently more important for N-MLV and EIAV restriction than for HIV-1 restriction.

Higher-order association and capsid binding. The binding of the RING domain mutants to the HIV-1 capsid was examined by using a previously described assay in which TRIM5α cosediments with *in vitro*-assembled HIV-1 CA-NC complexes (25, 55) (Fig. 4A and B). We observed a strong correlation between capsid binding ability and higher-order association efficiency for these mutants, confirming that RING-dependent higher-order association can enhance capsid-binding avidity (Fig. 4C). For this panel of RING domain mutants, we did not observe a correlation between HIV-1 restriction and the ability of the mutant TRIM5α$_{rh}$ proteins to associate with HIV-1 CA-NC complexes (Table 2). We hypothesized that because these mutants all have a wild-type B30.2/SPRY domain from TRIM5α$_{rh}$, which efficiently binds the HIV-1 capsid, the contribution of RING-mediated higher-order self-association to HIV-1 restriction is less critical. Moreover, because the RING domain mutants defective for higher-order association tend to be expressed at higher levels in cells (Fig. 1A and Table 1), higher concentrations of these mutants may compensate for lower capsid avidity.

We asked whether the requirements of retroviral restriction for specific TRIM5α RING functions depended on capsid binding affinity, which is primarily dictated by the B30.2/SPRY domain

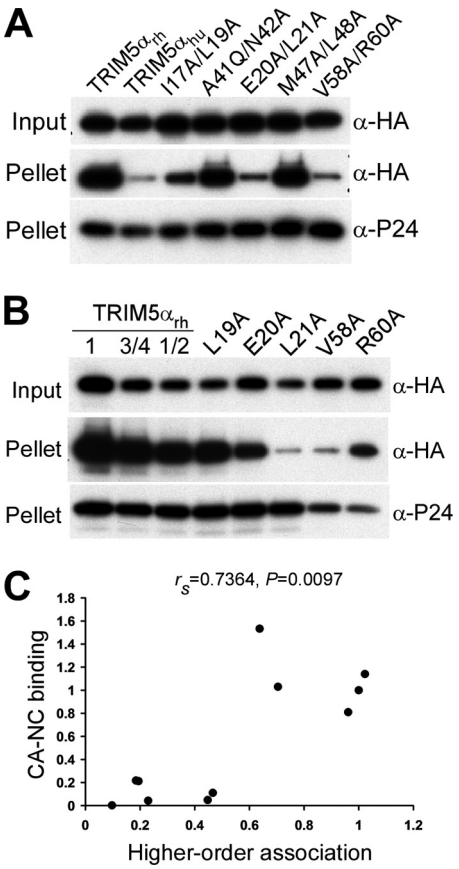

FIG 4 Binding of TRIM5α$_{rh}$ RING domain variants to HIV-1 CA-NC complexes. (A and B) Lysates from 293T cells transiently expressing the HA-tagged TRIM5α$_{rh}$ variants were incubated with assembled HIV-1 CA-NC complexes, which were pelleted through a sucrose cushion and analyzed as previously described (25). To achieve equivalent input levels of TRIM5α$_{rh}$ protein, some cell lysates were diluted with cell lysates from 293T cells transfected with the empty vector pLPCX. For comparison purposes, the cell lysates containing wild-type TRIM5α$_{rh}$ were diluted as indicated in panel B. α-HA, anti-HA antibody. (C) The relationship between higher-order association and HIV-1 CA-NC binding was assessed by Spearman rank-order correlation analysis.

(34–40). To that end, the B30.2/SPRY domain of TRIM5α$_{hu}$ was substituted for the B30.2/SPRY domain of wild-type TRIM5α$_{rh}$ and three RING domain mutants of TRIM5α$_{rh}$. TRIM5α$_{hu}$ binds the HIV-1 capsid and restricts HIV-1 infection less efficiently than TRIM5α$_{rh}$, but it restricts N-MLV infection more efficiently than TRIM5α$_{rh}$ (3, 9, 25, 55); the TRIM5α B30.2/SPRY domain determines these phenotypes (35, 36, 39, 40, 51, 55, 56). Figure 5 shows the effects of the TRIM5α variants on infection by HIV-1 and N-MLV. The TRIM5α variants were expressed at comparable levels, except for the highly expressed L21A and the L21A-huSPRY mutants (Fig. 5A); the L21A-huSPRY mutant contains the L21A change in a TRIM5α$_{rh}$ chimera with a TRIM5α$_{hu}$ B30.2/SPRY domain. As expected, the huSPRY chimeras restricted HIV-1 infection less efficiently than the TRIM5α$_{rh}$ variants. Of interest, HIV-1 inhibition by the huSPRY variants correlated with higher-order association (r_s = 0.8), and not with E3 ubiquitin ligase activity (r_s = 0.4). Thus, when the interaction of the B30.2/SPRY domain with the retroviral capsid is relatively weak, the importance of the higher-order association function of the RING domain to restriction increases.

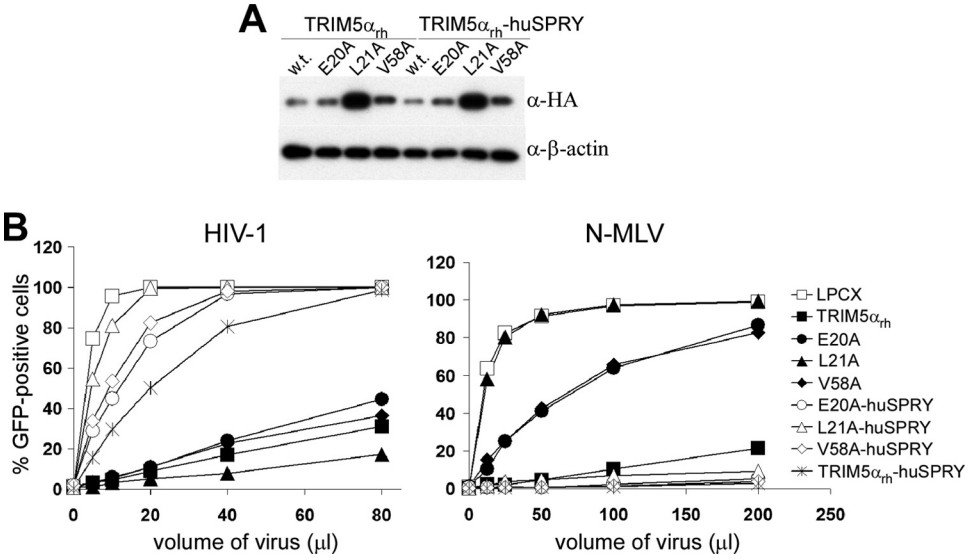

FIG 5 Antiviral activities of RING domain mutants in the context of TRIM5α$_{rh}$ and the TRIM5α$_{rh}$-huSPRY chimera. (A) The steady-state expression levels of RING domain mutants in the context of TRIM5α$_{rh}$ and the TRIM5α$_{rh}$-huSPRY chimera were assessed by Western blotting with an anti-HA antibody (α-HA; Roche). All of the TRIM5α variants carry an HA tag at the C terminus. The β-actin levels in the lysates are shown as an internal control. (B) Cf2Th cells expressing the indicated variants of TRIM5α$_{rh}$ or the TRIM5α$_{rh}$-huSPRY chimera, or control cells transduced with the empty pLPCX vector, were incubated with various amounts of recombinant HIV-1 (left panel) or N-MLV (right panel) expressing GFP. Infected GFP-positive cells were counted by FACS. The experiments were performed two times with similar results. The results of a typical experiment are shown.

The huSPRY chimeras all restricted N-MLV infection potently (Fig. 5B). In this case, the restriction potency of the chimeras correlated with higher-order association ($r_s = 1$), and not with E3 ubiquitin ligase activity ($r_s = 0.2$). Thus, promoting higher-order association appears to be the predominant contribution of the TRIM5α RING domain to N-MLV restriction, regardless of the B30.2/SPRY contribution to capsid interaction.

Formation of HIV-1 late reverse transcripts. To examine how the RING domain changes affect HIV-1 restriction by TRIM5α$_{rh}$, we studied HIV-1 late reverse transcript (RT) formation in cell lines expressing TRIM5α$_{rh}$ RING domain mutants. HIV-1 late RTs were measured 16 h after infection with HIV-1–GFP viruses at a viral dose that would infect approximately 50 to 80% of the cells transduced with the empty pLPCX vector. Most of the TRIM5α$_{rh}$ mutants potently blocked RT formation, whereas a few were less efficient in this regard (Fig. 6A). A good correlation was observed between the level of HIV-1 infection and the late RT level in cells (Fig. 6B). Of note, we did not identify any RING domain changes that uncoupled inhibition of HIV-1 reverse transcription and restriction of infection. The correlation between HIV-1 late RT formation and the E3 ubiquitin ligase activity for these TRIM5α$_{rh}$ mutants fell short of statistical significance (Fig. 6B). Indeed, despite exhibiting no detectable E3 ubiquitin ligase activity in the *in vitro* assay, some mutants (e.g., V58A, R60A) potently blocked HIV-1 late RT formation. Thus, restriction of HIV-1 infection by this panel of TRIM5α$_{rh}$ RING mutants is closely linked to disruption of reverse transcription.

The effect of protein turnover on antiviral activities and other protein properties. As changes in the RING domain have been reported to affect the turnover of TRIM5 proteins, we measured the half-lives of the RING domain mutants, as previously described (50, 52). Some changes in the RING domain significantly reduced the turnover of the TRIM5α$_{rh}$ protein; the stable, steady-state expression level of these mutants correlated with their respective half-life (Fig. 7 and Table 3). An inverse correlation was observed between the half-life of the RING mutants and higher-order association, as well as two other properties, HIV-1 CA-NC binding and N-MLV restriction, which are linked to higher-order association (Table 2 and 3).

Formation of detergent-insoluble aggregates in cells. By using a detergent solubility assay, we examined if the TRIM5α$_{rh}$ RING domain mutants could be extracted with buffer containing 1% NP-40. The wild-type TRIM5α$_{rh}$ was largely soluble in this buffer, and the majority of the protein partitioned into the supernatant (Fig. 8). Some mutants were resistant to detergent extraction, and a significant amount of the protein could be detected in the detergent-insoluble pellet fraction (Fig. 8). The tendency to form detergent-insoluble complexes was independent of the expression level or half-life, as some mutants with a high expression level (e.g., L21A) were still quite soluble (Fig. 8). Immunofluorescence staining showed that mutants with high detergent insolubility formed juxtanuclear inclusion bodies that were reminiscent of aggresomes or cellular inclusion bodies (Fig. 9) (57). The detergent insolubility index was defined as the ratio of the insoluble TRIM5α$_{rh}$ proteins in the pellet to the soluble proteins in the supernatant, both normalized to the actin level in the corresponding fractions. The detergent insolubility index inversely correlated with restriction of HIV-1 and EIAV and exhibited a trend toward an inverse correlation with N-MLV restriction (Table 2). Detergent insolubility also negatively correlated with the E3 ubiquitin ligase activities of these mutants. Detergent insolubility may reflect suboptimal folding of the RING domain, with some associated aggregation in expressing cells as well as functional impairment.

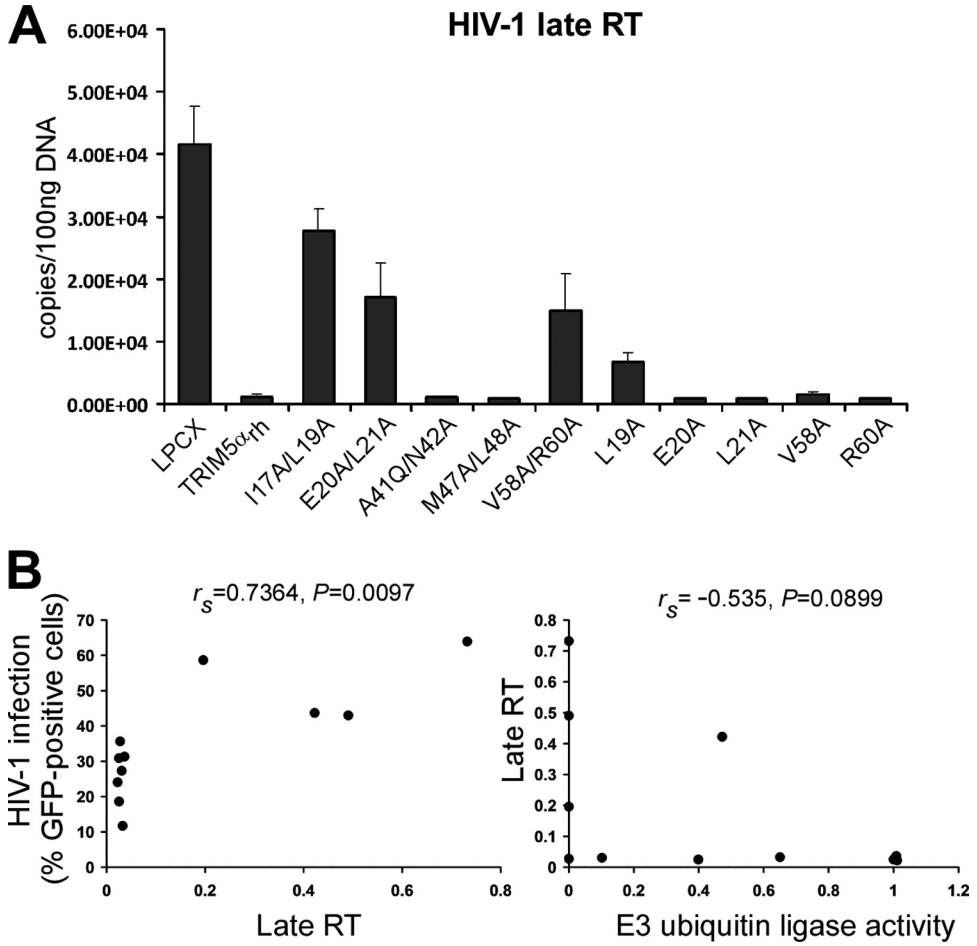

FIG 6 HIV-1 late reverse transcript formation in stable Cf2Th cell lines expressing TRIM5α_{rh} RING domain mutants. (A) Cells were infected with DNase I-treated HIV-1–GFP viruses at a dose that would infect 50 to 80% of the cell line transduced with the empty pLPCX vector. Total DNA was extracted from the cells 16 h after infection, and 100 ng DNA was used to measure the copy number of HIV-1 late reverse transcripts using quantitative real-time PCR. (B) Relationships between HIV-1 infection and late RT formation and between late RT formation and E3 ubiquitin ligase activity are plotted. The Spearman rank-order correlation coefficients and *P* values are shown on the plots.

DISCUSSION

In this study, we assayed multiple properties of a panel of TRIM5α_{rh} RING domain mutants, including two properties, E3 ubiquitin ligase activity and higher-order association, to which the TRIM5α RING domain contributes. Our results revealed the relationships among these properties and TRIM5α_{rh} restriction of infection of two lentiviruses, HIV-1 and EIAV, and a gammaretrovirus, N-MLV.

E3 ubiquitin ligase activity correlated with the potency of restriction of HIV-1 infection by TRIM5α_{rh} RING mutants, consistent with previously reported results (58). However, the E3 ubiquitin ligase function of the RING domain affected HIV-1 restriction potency over a relatively narrow range. All the mutants examined were able to restrict HIV-1 infection with at least moderate potency. Moreover, some of the E3 ubiquitin ligase-defective TRIM5α_{rh} mutants (e.g., V58A, R60A) potently blocked HIV-1 reverse transcription. Thus, RING-mediated E3 ubiquitin ligase activity, at least as measured in our *in vitro* autoubiquitylation assay, does not appear to be absolutely essential for the early blockade of HIV-1 infection. Lienlaf et al. also found that the V58A and R60A TRIM5α_{rh} mutants were defective for autoubiquitylation yet reduced HIV-1 reverse transcription by more than 10-fold (58). However, they reported only weak restriction of HIV-1 infection by these mutants, a result that is unexpected in light of the reverse transcription decrease and in contrast to the significant HIV-1 restriction activity observed in our study. Despite these differences, both studies support a role for TRIM5α_{rh} ubiquitin ligase activity in HIV-1 infection.

Some caveats apply in extrapolating our results to reach conclusions about the role of RING-mediated E3 ubiquitin ligase activity in TRIM5α_{rh} function *in vivo*. The *in vitro* autoubiquitylation assay is a sensitive and commonly used method to assess E3 ubiquitin ligase activity when the substrate is unknown (59), and the E2 enzyme used in this study (UbcH5a) was selected by screening a battery of different E2 enzymes (49). Nonetheless, given that an E3 ubiquitin ligase could potentially utilize numerous E2s *in vivo*, changes in the TRIM5α RING domain might differentially affect its interactions with a physiological E2 partner(s) (54, 60). A second caveat is that the phenotype of E3 ubiquitin ligase-defective TRIM5α mutants may depend upon the level of TRIM5α expression. Although our results are corroborated by the observation that RING-deleted TRIM5α_{rh} mutants retain some HIV-1-

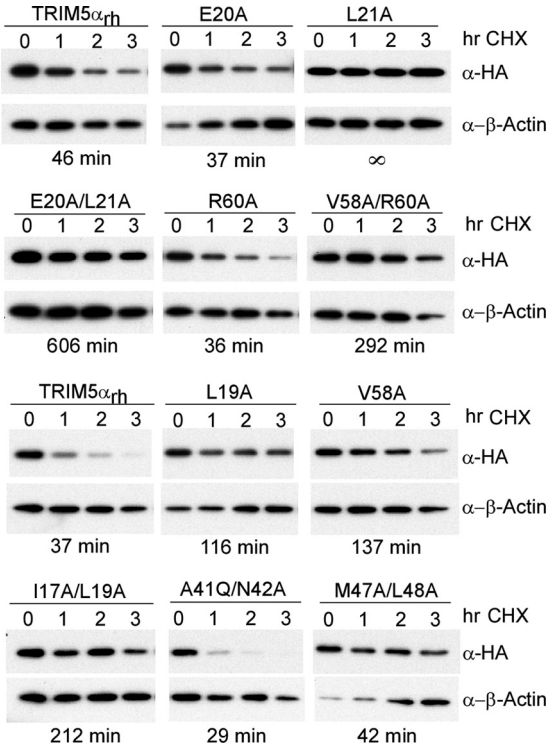

FIG 7 Turnover of TRIM5α$_{rh}$ RING domain mutants. Cf2Th cells expressing TRIM5α$_{rh}$ RING domain mutants were treated with cycloheximide at 100 μg/ml for a 3-h period. Cells were harvested and lysed at 1-h intervals. Cell lysates containing an equal amount of total protein were analyzed by Western blotting with an anti-HA (α-HA) antibody and an anti-β-actin antibody. Protein half-life was determined by plotting the log of the percent initial protein level versus time. The calculated half-life is indicated beneath the respective panel associated with each TRIM5α$_{rh}$ variant.

restricting activity (35, 46, 47), infection inhibition assays are typically performed with cells expressing higher-than-physiologic levels of mutant TRIM5α proteins. In natural target cells for HIV-1 infection, where TRIM5α expression is lower, RING-mediated E3 ubiquitin ligase activity may assume greater importance.

The precise mechanism by which E3 ubiquitin ligase activity contributes to the potency of HIV-1 restriction requires further investigation. Notably, the RING function important for HIV-1 restriction potency differed in the context of TRIM5α$_{rh}$, which binds the HIV-1 capsid tightly, and the chimeric TRIM5α$_{rh}$-huSPRY protein, in which the replacement of the TRIM5α$_{rh}$ B30.2/SPRY domain with that of human TRIM5α results in a decreased affinity for the HIV-1 capsid. In the restriction of HIV-1 infection by TRIM5α$_{rh}$-huSPRY, the contribution of the RING domain to higher-order association superseded the contribution of the RING domain to E3 ubiquitin ligase activity. Apparently, in the context of the TRIM5α$_{rh}$-huSPRY chimera, where the affinity of the B30.2/SPRY domain for the HIV-1 capsid is low, the contribution of higher-order association to capsid binding avidity becomes more important for successful HIV-1 restriction. This result is reminiscent of the effect of B30.2/SPRY-mediated capsid binding affinity on the HIV-1 restriction phenotypes resulting from changes in the TRIM5α B-box, which cooperates with the RING domain to achieve higher-order self-association (43, 51). Capsid binding affinity specified by the B30.2/SPRY domain also influenced the consequences of RING domain changes on the restriction of SIV$_{mac}$ infection by TRIM5α of African green monkeys (49).

Several of the TRIM5α$_{rh}$ RING mutants exhibited deficiencies in higher-order association, consistent with a previous study implicating the RING domain as a contributor to the higher-order association of TRIM dimers (44). Significant correlations were found between TRIM5α$_{rh}$ higher-order association and the restriction of N-MLV infection. These results clearly contrasted with those obtained for restriction of HIV-1 infection by the TRIM5$_{rh}$ RING domain variants. For example, the L21A TRIM5α$_{rh}$ mutant, which exhibited poor higher-order association, was able to restrict HIV-1 as potently as the wild-type TRIM5α$_{rh}$ protein but completely lost its ability to restrict N-MLV. Even when the N-MLV restriction potency was augmented by a B30.2/SPRY domain replacement from TRIM5α$_{hu}$, RING-determined higher-order association exhibited the strongest correlation with N-MLV restriction. The different phenotypes of the TRIM5α RING mutants with respect to HIV-1 and N-MLV restriction may reflect differences in the formation or consequences of the hexagonal TRIM5α lattice on the conical and spherical capsids, respectively, of HIV-1 and N-MLV. The relative importance of TRIM5α higher-order association and the formation of a TRIM5α lattice to N-MLV restriction are consistent with the observation that changes that allow N-MLV to escape TRIM5α$_{rh}$ restriction map over a large surface of the viral capsid (61).

Higher-order association of the TRIM5α$_{rh}$ RING domain mutants apparently exerted a larger influence than E3 ubiquitin ligase activity on the restriction of EIAV infection. Nonetheless, all of the RING domain mutants exhibited quite potent inhibition of EIAV infection. Thus, like HIV-1 restriction by TRIM5α$_{rh}$, inhibition of

TABLE 3 *P* values for Spearman rank-order correlations between TRIM5 protein properties

	P value[a]					
TRIM5α restriction	E3 ubiquitin ligase activity	Higher-order association	Detergent insolubility	CA-NC binding	HIV-1 Late-RT	Half-life
High-order association	(+) 0.4840					
Detergent insolubility	**(−) 0.0022**	(−) 0.1333				
CA-NC binding	(+) 0.9380	**(+) 0.0097**	(+) 0.8764			
Late RT	(−) 0.0899	(−) 0.1020	**(+) 0.0112**	(−) 0.2999		
Half-life	(−) 0.7056	**(−) 0.0053**	(+) 0.2131	**(−) 0.0021**	(+) 0.0706	
Expression	(−) 0.3211	**(−) 0.0002**	(+) 0.2463	**(−) 0.0073**	(+) 0.2359	**(+) 0.0165**

[a] The *P* values from the Spearman rank-order correlation analysis on each pair of TRIM5α$_{rh}$ properties/activities are listed. A positive sign indicates a direct relationship, and a negative sign indicates an inverse relationship. Correlations with *P* values less than 0.02 were considered significant and are highlighted in bold.

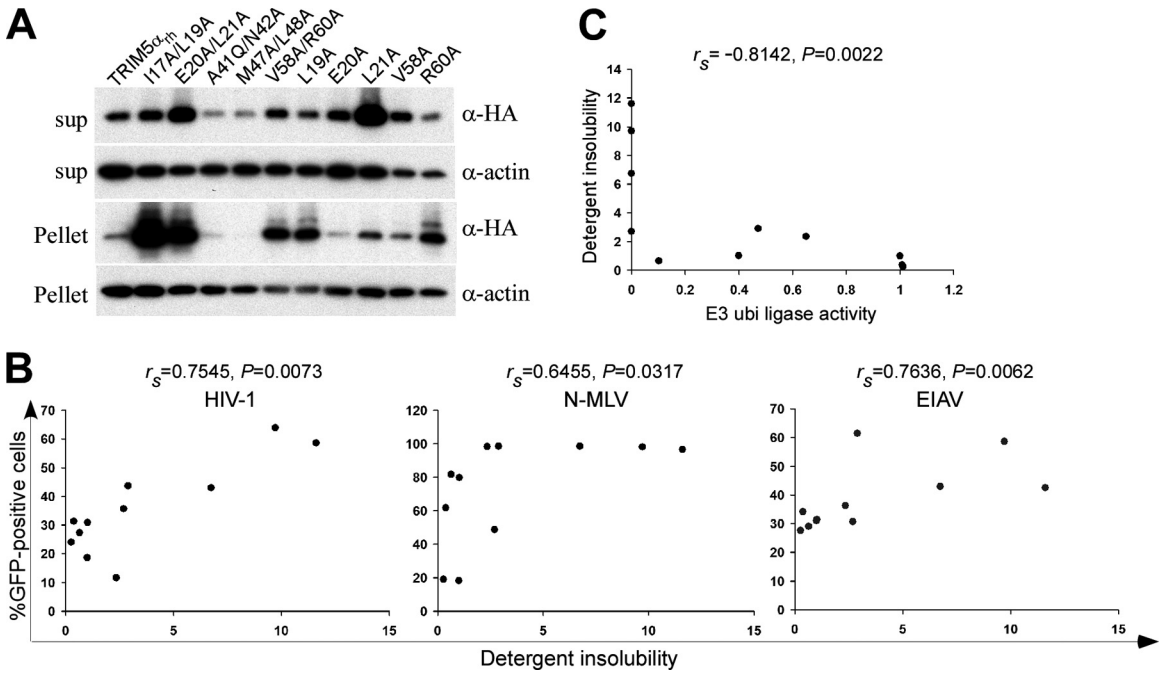

FIG 8 Detergent solubility of TRIM5α_{rh} RING domain mutants and the relationships with restriction activities and the E3 ubiquitin ligase activity. (A) Cf2Th cells stably expressing TRIM5 variants were lysed with 1% NP-40 buffer. Proteins in the supernatant (detergent-soluble fraction) and the pellet (detergent-insoluble fraction) were examined with an anti-HA (α-HA) antibody for TRIM5α_{rh} variants and an anti-β-actin antibody as a control. (B) Relationship between detergent insolubility and the restriction activities against different viruses. Detergent insolubility was defined as the ratio of insoluble proteins to soluble proteins, both normalized against the β-actin level in the respective fractions. (C) Relationship between detergent insolubility and E3 ubiquitin ligase activity.

EIAV infection by TRIM5α_{rh} in this context is not absolutely dependent on either of the RING functions measured in our assays. This is consistent with the retention of lentivirus restriction activity for TRIM5α variants completely lacking the RING domain (3, 46, 47). Perhaps, as discussed above, features of the lentivirus conical capsid diminish the requirement for RING domain function during the restriction process. The previously mentioned caveats about generalizing our results to *in vivo* lentivirus infections apply here to EIAV as well.

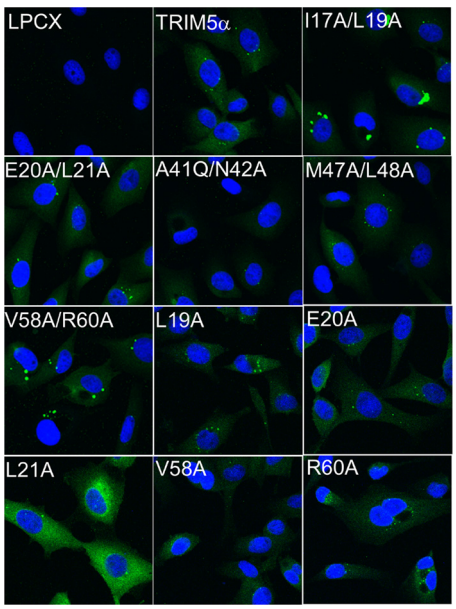

FIG 9 Subcellular localization of TRIM5α_{rh} RING domain variants. Cf2Th cells stably expressing the HA-tagged TRIM5α_{rh} variants were stained with an anti-HA antibody, followed by an anti-rat secondary antibody conjugated to Alexa-488. The TRIM5α_{rh} proteins are shown in green, and the nuclei were stained blue with 4′,6-diamidino-2-phenylindole.

Higher-order association of the TRIM5α_{rh} RING domain variants correlated significantly with TRIM5α binding to HIV-1 CA-NC complexes, as expected from the contribution of TRIM5α_{rh} higher-order structures to capsid binding (27, 43, 44). TRIM5α_{rh} mutants that associated to form higher-order structures also exhibited shorter half-lives and lower steady-state levels of expression in stably expressing cell lines. Thus, although higher-order self-association is conducive to the TRIM5α-capsid interaction, it may predispose to the formation of aggregates that need to be avoided or processed to maintain optimal cell growth (57). Neither the E3 ubiquitin ligase activity nor detergent insolubility of the TRIM5α_{rh} variants significantly correlated with the half-life or steady-state expression level of the proteins. The mechanism of TRIM5α turnover in these stably expressing cells is unclear. A slower-migrating band reminiscent of ubiquitylated TRIM5α was consistently observed in detergent-insoluble pellets (Fig. 8), even for mutants with no or very weak E3 ubiquitin ligase activity. Other E3 ubiquitin ligases in the cells likely ubiquitylate TRIM5α and could potentially play a role in its turnover.

In summary, the correlations identified in this study delineate how the identity of the targeted retrovirus influences the contributions of TRIM5α RING domain functions to antiviral restriction. Different retroviruses apparently require distinct properties of TRIM5 proteins for efficient restriction. Detailed structural information on the interactions between TRIM5α proteins and ret-

roviral capsids should shed light on how TRIM5α recognizes and disassembles the capsids of different viruses.

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REFERENCES

1. **Himathongkham S, Luciw PA.** 1996. Restriction of HIV-1 (subtype B) replication at the entry step in rhesus macaque cells. Virology **219**:485–488.
2. **Hofmann W, Schubert D, LaBonte J, Munson L, Gibson S, Scammell J, Ferrigno P, Sodroski J.** 1999. Species-specific, postentry barriers to primate immunodeficiency virus infection. J. Virol. **73**:10020–10028.
3. **Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J.** 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. Nature **427**:848–853.
4. **Fletcher AJ, Hue S, Schaller T, Pillay D, Towers GJ.** 2010. Hare TRIM5α restricts divergent retroviruses and exhibits significant sequence variation from closely related Lagomorpha TRIM5 genes. J. Virol. **84**:12463–12468.
5. **Hatziioannou T, Perez-Caballero D, Yang A, Cowan S, Bieniasz PD.** 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5α. Proc. Natl. Acad. Sci. U. S. A. **101**:10774–10779.
6. **Jauregui P, Crespo H, Glaria I, Lujan L, Contreras A, Rosati S, de Andres D, Amorena B, Towers GJ, Reina R.** 2012. Ovine TRIM5α can restrict visna/maedi virus. J. Virol. **86**:9504–9509.
7. **Johnson WE, Sawyer SL.** 2009. Molecular evolution of the antiretroviral TRIM5 gene. Immunogenetics **61**:163–176.
8. **Keckesova Z, Ylinen LM, Towers GJ.** 2004. The human and African green monkey TRIM5α genes encode Ref1 and Lv1 retroviral restriction factor activities. Proc. Natl. Acad. Sci. U. S. A. **101**:10780–10785.
9. **Perron MJ, Stremlau M, Song B, Ulm W, Mulligan RC, Sodroski J.** 2004. TRIM5α mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc. Natl. Acad. Sci. U. S. A. **101**:11827–11832.
10. **Saenz DT, Teo W, Olsen JC, Poeschla EM.** 2005. Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5α proteins. J. Virol. **79**:15175–15188.
11. **Schaller T, Hue S, Towers GJ.** 2007. An active TRIM5 protein in rabbits indicates a common antiviral ancestor for mammalian TRIM5 proteins. J. Virol. **81**:11713–11721.
12. **Si Z, Vandegraaff N, O'Huigin C, Song B, Yuan W, Xu C, Perron M, Li X, Marasco WA, Engelman A, Dean M, Sodroski J.** 2006. Evolution of a cytoplasmic tripartite motif (TRIM) protein in cows that restricts retroviral infection. Proc. Natl. Acad. Sci. U. S. A. **103**:7454–7459.
13. **Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J.** 2005. Retrovirus restriction by TRIM5α variants from Old World and New World primates. J. Virol. **79**:3930–3937.
14. **Yap MW, Lindemann D, Stanke N, Reh J, Westphal D, Hanenberg H, Ohkura S, Stoye JP.** 2008. Restriction of foamy viruses by primate Trim5α. J. Virol. **82**:5429–5439.
15. **Yap MW, Nisole S, Lynch C, Stoye JP.** 2004. Trim5α protein restricts both HIV-1 and murine leukemia virus. Proc. Natl. Acad. Sci. U. S. A. **101**:10786–10791.
16. **Ylinen LM, Keckesova Z, Webb BL, Gifford RJ, Smith TP, Towers GJ.** 2006. Isolation of an active Lv1 gene from cattle indicates that tripartite motif protein-mediated innate immunity to retroviral infection is widespread among mammals. J. Virol. **80**:7332–7338.
17. **Brennan G, Kozyrev Y, Hu SL.** 2008. $TRIM_{Cyp}$ expression in Old World primates Macaca nemestrina and Macaca fascicularis. Proc. Natl. Acad. Sci. U. S. A. **105**:3569–3574.
18. **Liao CH, Kuang YQ, Liu HL, Zheng YT, Su B.** 2007. A novel fusion gene, TRIM5-cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. AIDS **21**(Suppl 8):S19–S26.
19. **Newman RM, Hall L, Kirmaier A, Pozzi LA, Pery E, Farzan M, O'Neil SP, Johnson W.** 2008. Evolution of a TRIM5-CypA splice isoform in Old World monkeys. PLoS Pathog. **4**:e1000003. doi:10.1371/journal.ppat.1000003.
20. **Sayah DM, Sokolskaja E, Berthoux L, Luban J.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430**:569–573.
21. **Virgen CA, Kratovac Z, Bieniasz PD, Hatziioannou T.** 2008. Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. Proc. Natl. Acad. Sci. U. S. A. **105**:3563–3568.
22. **Wilson SJ, Webb BL, Ylinen LM, Verschoor E, Heeney JL, Towers GJ.** 2008. Independent evolution of an antiviral $TRIM_{Cyp}$ in rhesus macaques. Proc. Natl. Acad. Sci. U. S. A. **105**:3557–3562.
23. **Kar AK, Diaz-Griffero F, Li Y, Li X, Sodroski J.** 2008. Biochemical and biophysical characterization of a chimeric TRIM21-TRIM5α protein. J. Virol. **82**:11669–11681.
24. **Langelier CR, Sandrin V, Eckert DM, Christensen DE, Chandrasekaran V, Alam SL, Aiken C, Olsen JC, Kar AK, Sodroski JG, Sundquist WI.** 2008. Biochemical characterization of a recombinant TRIM5α protein that restricts human immunodeficiency virus type 1 replication. J. Virol. **82**:11682–11694.
25. **Stremlau M, Perron M, Lee M, Li Y, Song B, Javanbakht H, Diaz-Griffero F, Anderson DJ, Sundquist WI, Sodroski J.** 2006. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. Proc. Natl. Acad. Sci. U. S. A. **103**:5514–5519.
26. **Diaz-Griffero F, Perron M, McGee-Estrada K, Hanna R, Maillard PV, Trono D, Sodroski J.** 2008. A human TRIM5α B30.2/SPRY domain mutant gains the ability to restrict and prematurely uncoat B-tropic murine leukemia virus. Virology **378**:233–242.
27. **Ganser-Pornillos BK, Chandrasekaran V, Pornillos O, Sodroski JG, Sundquist WI, Yeager M.** 2011. Hexagonal assembly of a restricting TRIM5α protein. Proc. Natl. Acad. Sci. U. S. A. **108**:534–539.
28. **Anderson JL, Campbell EM, Wu X, Vandegraaff N, Engelman A, Hope TJ.** 2006. Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. J. Virol. **80**:9754–9760.
29. **Wu X, Anderson JL, Campbell EM, Joseph AM, Hope TJ.** 2006. Proteasome inhibitors uncouple rhesus TRIM5α restriction of HIV-1 reverse transcription and infection. Proc. Natl. Acad. Sci. U. S. A. **103**:7465–7470.
30. **Rold CJ, Aiken C.** 2008. Proteasomal degradation of TRIM5α during retrovirus restriction. PLoS Pathog. **4**:e1000074. doi:10.1371/journal.ppat.1000074.
31. **Pertel T, Hausmann S, Morger D, Zuger S, Guerra J, Lascano J, Reinhard C, Santoni FA, Uchil PD, Chatel L, Bisiaux A, Albert ML, Strambio-De-Castillia C, Mothes W, Pizzato M, Grutter MG, Luban J.** 2011. TRIM5 is an innate immune sensor for the retrovirus capsid lattice. Nature **472**:361–365.
32. **Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, Guffanti A, Minucci S, Pelicci PG, Ballabio A.** 2001. The tripartite motif family identifies cell compartments. EMBO J. **20**:2140–2151.
33. **Meroni G, Diez-Roux G.** 2005. TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases. Bioessays **27**:1147–1157.
34. **Maillard PV, Reynard S, Serhan F, Turelli P, Trono D.** 2007. Interfering residues narrow the spectrum of MLV restriction by human TRIM5α. PLoS Pathog. **3**:e200. doi:10.1371/journal.ppat.0030200.
35. **Perez-Caballero D, Hatziioannou T, Yang A, Cowan S, Bieniasz PD.** 2005. Human tripartite motif 5α domains responsible for retrovirus restriction activity and specificity. J. Virol. **79**:8969–8978.
36. **Sawyer SL, Wu LI, Emerman M, Malik HS.** 2005. Positive selection of primate TRIM5α identifies a critical species-specific retroviral restriction domain. Proc. Natl. Acad. Sci. U. S. A. **102**:2832–2837.
37. **Sebastian S, Grutter C, Strambio de Castillia C, Pertel T, Olivari S, Grutter MG, Luban J.** 2009. An invariant surface patch on the TRIM5α PRYSPRY domain is required for retroviral restriction but dispensable for capsid binding. J. Virol. **83**:3365–3373.
38. **Song B, Gold B, O'Huigin C, Javanbakht H, Li X, Stremlau M, Winkler C, Dean M, Sodroski J.** 2005. The B30.2(SPRY) domain of the retroviral restriction factor TRIM5α exhibits lineage-specific length and sequence variation in primates. J. Virol. **79**:6111–6121.
39. **Stremlau M, Perron M, Welikala S, Sodroski J.** 2005. Species-specific variation in the B30.2(SPRY) domain of TRIM5α determines the potency of human immunodeficiency virus restriction. J. Virol. **79**:3139–3145.
40. **Yap MW, Nisole S, Stoye JP.** 2005. A single amino acid change in the

SPRY domain of human Trim5α leads to HIV-1 restriction. Curr. Biol. **15**:73–78.
41. **Javanbakht H, Yuan W, Yeung DF, Song B, Diaz-Griffero F, Li Y, Li X, Stremlau M, Sodroski J.** 2006. Characterization of TRIM5α trimerization and its contribution to human immunodeficiency virus capsid binding. Virology **353**:234–246.
42. **Diaz-Griffero F, Qin XR, Hayashi F, Kigawa T, Finzi A, Sarnak Z, Lienlaf M, Yokoyama S, Sodroski J.** 2009. A B-box 2 surface patch important for TRIM5α self-association, capsid binding avidity, and retrovirus restriction. J. Virol. **83**:10737–10751.
43. **Li X, Sodroski J.** 2008. The TRIM5α B-box 2 domain promotes cooperative binding to the retroviral capsid by mediating higher-order self-association. J. Virol. **82**:11495–11502.
44. **Li X, Yeung DF, Fiegen AM, Sodroski J.** 2011. Determinants of the higher order association of the restriction factor TRIM5α and other tripartite motif (TRIM) proteins. J. Biol. Chem. **286**:27959–27970.
45. **Yamauchi K, Wada K, Tanji K, Tanaka M, Kamitani T.** 2008. Ubiquitination of E3 ubiquitin ligase TRIM5 alpha and its potential role. FEBS J. **275**:1540–1555.
46. **Javanbakht H, Diaz-Griffero F, Stremlau M, Si Z, Sodroski J.** 2005. The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5α. J. Biol. Chem. **280**:26933–26940.
47. **Yap MW, Mortuza GB, Taylor IA, Stoye JP.** 2007. The design of artificial retroviral restriction factors. Virology **365**:302–314.
48. **Maegawa H, Miyamoto T, Sakuragi J, Shioda T, Nakayama EE.** 2010. Contribution of RING domain to retrovirus restriction by TRIM5α depends on combination of host and virus. Virology **399**:212–220.
49. **Kim J, Tipper C, Sodroski J.** 2011. Role of TRIM5α RING domain E3 ubiquitin ligase activity in capsid disassembly, reverse transcription blockade, and restriction of simian immunodeficiency virus. J. Virol. **85**:8116–8132.
50. **Diaz-Griffero F, Li X, Javanbakht H, Song B, Welikala S, Stremlau M, Sodroski J.** 2006. Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. Virology **349**:300–315.
51. **Li X, Song B, Xiang SH, Sodroski J.** 2007. Functional interplay between the B-box 2 and the B30.2(SPRY) domains of TRIM5α. Virology **366**:234–244.
52. **Li X, Gold B, O'HUigin C, Diaz-Griffero F, Song B, Si Z, Li Y, Yuan W, Stremlau M, Mische C, Javanbakht H, Scally M, Winkler C, Dean M, Sodroski J.** 2007. Unique features of TRIM5α among closely related human TRIM family members. Virology **360**:419–433.
53. **Butler SL, Hansen MS, Bushman FD.** 2001. A quantitative assay for HIV DNA integration in vivo. Nat. Med. **7**:631–634.
54. **Deshaies RJ, Joazeiro CA.** 2009. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. **78**:399–434.
55. **Li Y, Li X, Stremlau M, Lee M, Sodroski J.** 2006. Removal of arginine 332 allows human TRIM5α to bind human immunodeficiency virus capsids and to restrict infection. J. Virol. **80**:6738–6744.
56. **Perron MJ, Stremlau M, Sodroski J.** 2006. Two surface-exposed elements of the B30.2/SPRY domain as potency determinants of N-tropic murine leukemia virus restriction by human TRIM5α. J. Virol. **80**:5631–5636.
57. **Kopito RR.** 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. **10**:524–530.
58. **Lienlaf M, Hayashi F, Di Nunzio F, Tochio N, Kigawa T, Yokoyama S, Diaz-Griffero F.** 2011. Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5α_{rh}: structure of the RING domain of TRIM5α. J. Virol. **85**:8725–8737.
59. **Yang Y, Lorick KL, Jensen JP, Weissman AM.** 2005. Expression and evaluation of RING finger proteins. Methods Enzymol. **398**:103–112.
60. **Wenzel DM, Stoll KE, Klevit RE.** 2011. E2s: structurally economical and functionally replete. Biochem. J. **433**:31–42.
61. **Ohkura S, Goldstone DC, Yap MW, Holden-Dye K, Taylor IA, Stoye JP.** 2011. Novel escape mutants suggest an extensive TRIM5α binding site spanning the entire outer surface of the murine leukemia virus capsid protein. PLoS Pathog. **7**:e1002011. doi:10.1371/journal.ppat.1002011.