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TRIM5α-Mediated Ubiquitin Chain Conjugation Is Required for Inhibition of HIV-1 Reverse Transcription and Capsid Destabilization

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ABSTRACT

Rhesus macaque TRIM5α (rhTRIM5α) is a retroviral restriction factor that inhibits HIV-1 infection. Previous studies have revealed that TRIM5α restriction occurs via a two-step process. The first step is restriction factor binding, which is sufficient to inhibit infection. The second step, which is sensitive to proteasome inhibition, prevents the accumulation of reverse transcription products in the target cell. However, because of the pleotropic effects of proteasome inhibitors, the molecular mechanisms underlying the individual steps in the restriction process have remained poorly understood. In this study, we have fused the small catalytic domain of herpes simplex virus UL36 deubiquitinase (DUb) to the N-terminal RING domain of rhTRIM5α, which results in a ubiquitination-resistant protein. Cell lines stably expressing this fusion protein inhibited HIV-1 infection to the same degree as a control fusion to a catalytically inactive DUb. However, reverse transcription products were substantially increased in the DUb-TRIM5α fusion relative to the catalytically inactive control or the wild-type (WT) TRIM5α. Similarly, expression of DUb-rhTRIM5α resulted in the accumulation of viral cores in target cells following infection, while the catalytically inactive control and WT rhTRIM5α induced the abortive disassembly of viral cores, indicating a role for ubiquitin conjugation in rhTRIM5α-mediated destabilization of HIV-1 cores. Finally, DUb-rhTRIM5α failed to activate NF-κB signaling pathways compared to controls, demonstrating that this ubiquitination-dependent activity is separable from the ability to restrict retroviral infection.

IMPORTANCE

These studies provide direct evidence that ubiquitin conjugation to rhTRIM5α-containing complexes is required for the second step of HIV-1 restriction. They also provide a novel tool by which the biological activities of TRIM family proteins might be dissected to better understand their function and underlying mechanisms of action.

TRIM5α is a retroviral restriction factor that mediates a postentry block to infection (1, 2). The best-studied example of this restriction is the ability of the TRIM5α protein from rhesus macaques (rhTRIM5α) to potently inhibit HIV-1 infection (1, 2). As a member of the TRIM family of proteins, TRIM5α possesses the canonical RING, B-Box, and coiled-coil (CC) domains that comprise the tripartite motif (TRIM) that define this family of proteins (3). The N-terminal RING domain acts as an E3 ubiquitin ligase (4–6), and together with the B-Box domain, also functions to mediate the self-association of TRIM5α dimers (7–9). The CC domain, in cooperation with the Linker2 (L2) region, mediates the dimerization of TRIM5α monomers and the formation of higher-order assemblies (10–14). TRIM5α also possesses a C-terminal SPRY domain which is known to recognize determinants in the assembled viral core to mediate restriction (15–17). Following core binding, TRIM5α induces the abortive disassembly of the viral core (18, 19). The mechanism by which core disruption occurs is not precisely clear, although numerous studies have suggested that this process is a two-step mechanism, the second of which requires proteasome activity (20–23). Specifically, the proteasome inhibitor MG132 does not relieve the TRIM5α-mediated block to viral infection, but it does relieve the TRIM5α-mediated inhibition of reverse transcription (20, 23). Proteasome inhibitors additionally prevent disruption of the viral core, as measured in biochemical assays (22), and result in stabilization of viral particles associated with TRIM5α in cells (21). While these studies clearly suggest a role for a ubiquitin/proteasome-dependent step in the restriction process, the widespread pleotropic effects of pharmacological proteasome inhibition have prevented clear elucidation of the role of ubiquitin/proteasome in the restriction process. The effect of MG132 treatment has been recapitulated in certain RING domain mutants which still restrict infection but fail to inhibit reverse transcription or mediate core disassembly (24). However, as the RING domain also participates in TRIM5α self-association (8, 9), separating the contributions of the E3 ubiquitin ligase activity and self-associative properties of the RING domain to individual TRIM5α biological activities has been difficult.

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To overcome these limitations and to specifically assess the role of ubiquitin in TRIM5α-mediated retroviral restriction, we fused the small catalytic domain of the herpes simplex virus UL36 deubiquitinating enzyme (DUb) (25) to rhTRIM5α. DUbs have been effective tools to assess the role of ubiquitination in both cellular systems (25) and viral systems (26). Fusing UL36 DUb to rhTRIM5α generated a ubiquitination-resistant protein that allowed us to examine the role of TRIM5α ubiquitination in retroviral restriction and protein localization.

MATERIALS AND METHODS

DUb-rhTRIM5α fusion expression constructs. To engineer UL36 (DUb)-rhTRIM5α fusion genes, rhTRIM5α was amplified and fused to the 3′ end and in frame with a short linker (link) and UL36 (active or inactive form) sequences using overlapping PCR and previously published constructs as a DNA template (26). UL36-link-rhTRIM5α fusion sequences were subsequently amplified and inserted into a 3×Flag plasmid between the EcoRI and KpnI sites, which placed a 3× Flag tag at the C termini of the proteins. These constructs were used to test the expression and stability of the fusion proteins in 293T, HeLa, or TZM-bL cells. Next, rhTRIM5α, DUb-link-rhTRIM5α (active), or DUb*-link-rhTRIM5α (inactive form) were reamplified, and a flag tag sequence was inserted in frame with the 3′ end of the genes to clone in the pLVX plasmid into the BamHI site.

Cell lines, tissue culture, transfection, and selection. TZM-bL, HeLa, and 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (FBS), 1,000 U/ml penicillin, 1,000 U/ml streptomycin, and 10 μg/ml ciprofloxacin hydrochloride. Cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol.

Generation of stable cell lines. Lentivirus for transduction was produced by transfection of 293T cells with either 1 μg pLVX-FLAG-rhTRIM5α, 1 μg pLVX-FLAG-rhTRIM5α-DUb, or 1 μg pLVX-FLAG-rhTRIM5α-DUb* along with 1 μg ΔNRF (packaging plasmid lacking Nef, Vpr, Vif) or p8.9 packaging construct, and 1 μg vesicular stomatitis virus glycoprotein (VSV-g) by using polyethylenimine (PEI) (molecular weight, 25,000; Polysciences) in a 60-mm dish. Viruses were harvested 48 h after transfection, filtered through a 0.45-μm filter (Millipore), and used to transduce HeLa cells. Cells were spinoculated at 13°C for 2 h at 1,200 × *g*. Twenty-four hours after infection, vector was removed and replaced with fresh DMEM. Forty-eight hours after transduction, cells were selected in DMEM containing 5 μg/ml puromycin (Sigma-Aldrich).

Ubiquitination assays. Cells or established cell lines expressing rhTRIM5α or DUb fusion proteins were grown as described above and transfected with an equal amount of hemagglutinin-tagged ubiquitin (HA-Ub)-expressing plasmid DNA as previously described (26). Cells were harvested 48 h posttransfection, washed twice with cold phosphate-buffered saline (PBS), lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with 0.5% NP-40 and IGEPAL on ice for 30 min, and then spun down to pellet cell debris. Clear lysates obtained were used in immunoprecipitation (IP) assays using anti-HA or anti-FLAG antibody-conjugated beads (Sigma) to selectively concentrate HA-Ub modified ubiquitinated forms of rhTRIM5α or DUb fusion counterparts to determine patterns of rhTRIM5α ubiquitination in cells. Input samples and IP eluates were analyzed by odium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-Flag and/or anti-HA antibodies (Sigma). Proteins were visualized by chemiluminescence using ECL Prime (GE) on Biomax film (Kodak).

Western blotting. Whole-cell lysates were prepared by lysing cells with 0.5% NP-40 lysis buffer (1× PBS [HyClone] containing protease inhibitor cocktail [Roche]) for 10 min on ice. Following the incubation on ice for 30 min, 2× SDS sample buffer was added to the lysed cells, which were incubated at 100°C for 5 min. Samples were loaded onto a 10% or 12% polyacrylamide gel for SDS-PAGE. After separation, the proteins were transferred to nitrocellulose membranes (Bio-Rad) and were detected by incubation with monoclonal anti-FLAG M2-peroxidase (horseradish peroxidase [HRP]) clone M2 (Sigma-Aldrich) and anti-β-tubulin (University of Iowa Hybridoma Bank). Antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) or ECL Prime from GE. Chemiluminescence was detected using the Bio-Rad Chemidoc system or Biomax film from Kodak.

Infectivity assay. HeLa cell lines stably expressing each construct as well as control were seeded on a 24-well plate at 50% confluence. Previously made R7ΔEnvGFP virus was serially diluted into each well. To synchronize infection, cells were spinoculated at 13°C for 2 h at 1,200 × *g*. Twenty-four hours after the cells were infected, virus was removed and replaced with fresh DMEM. Virus infectivity was determined by measuring the percentage of cells expressing green fluorescent protein (GFP) at 48 to 72 h postinfection using a FACSCanto II flow cytometer (BD Biosciences).

Immunofluorescence. HeLa cells stably expressing the indicated rhTRIM5α fusions were allowed to adhere to fibronectin-treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (Sigma), pH 6.8. Primary antibody used for staining was monoclonal M2 mouse anti-FLAG (Sigma-Aldrich) and used at a dilution of 1:1,000. Various images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; Photometrics), using a 1.4-numerical-aperture 100× objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision). Images were analyzed using the Imaris software package (Bitplane), and the data were graphed in Prism (Graphpad Software Inc.) for statistical analysis.

Fate of capsid assay. The fate of capsid (FOC) assay was performed as previously described (18, 27). Cell lines were infected for 8 h, detached with pronase for 5 min on ice, and washed three times with ice-cold PBS. Cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl [pH 8], 10 mM KCl, 1 mM EDTA) and incubated for 15 min on ice. Cells were lysed in a 7.0-ml Dounce homogenizer with pestle B. Cellular debris was cleared by centrifugation for 3 min at 3,000 rpm. The cleared lysate was layered onto a 50% sucrose (wt/vol) cushion in 1× PBS and centrifuged at 100,000 × *g* for 2 h at 4°C in a Beckman SW41 rotor. Input, soluble, and pellet fractions were analyzed by Western blotting using anti-HIV-1 p24 antibodies.

Real-time PCR. Cells were seeded at 60% confluence and infected with R7ΔEnvGFP virus at a dilution factor of 1:2. To synchronize infection, cells were spinoculated at 13°C for 2 h at 1,200 × *g* as previously described. Ten hours after spinoculation, cells were harvested and spun down at 18°C for 5 min at 125 × *g*. Genomic DNA was extracted from cells by following the DNeasy blood and tissue kit protocol (Qiagen). The concentration of genomic DNA was determined using a NanoDrop 1000 instrument (Thermo Scientific), and genomic DNA was digested with DpnI (New England BioLabs). Reverse transcription was measured using primers specific for GFP and β-actin.

Dual-luciferase assay. 293T cells were plated in triplicate in a 96-well assay plate and transfected with 70 ng of either pLVX, rhTRIM5α, DUb, or DUb* constructs along with either 20 ng of NF-κB-firefly luciferase or AP1-firefly luciferase, 10 ng of pRL (RL stands for *Renilla* luciferase) (Promega), 1.25 μl PEI, and 10.275 μl oxidized DMEM (Cellgro). The medium was changed 24 h posttransfection, and cells were lysed 48 h posttransfection with 5× passive lysis buffer (Promega). The Dual-Luciferase reporter assay system (Promega) was used to measure control reporter (*Renilla* luciferase) and reporter (NF-κB-luciferase) activity. Relative light units for each sample were measured using the Synergy HT multimode plate reader (BioTek Instruments Corp., Winooski, VT, USA), and the ratio of NF-κB-firefly luciferase to *Renilla* luciferase or AP1-firefly luciferase to *Renilla* luciferase was determined. Luciferase substrates (100 μl/well) were added to cells using an automatic injector system. Independent experiments were compared by calculating the signaling fold change from the values for empty vector controls.

RESULTS

UL36 (DUb) fusion prevents rhTRIM5α ubiquitination in cells. To understand how ubiquitination of TRIM5α drives its biological activities, we generated rhTRIM5α fusion proteins, which fused the small catalytic domain of the herpes simplex virus UL36 deubiquitinating enzyme (DUb) (25) to the N-terminal RING domain of rhTRIM5α (Fig. 1A). For a control, we also fused a catalytically inactive form of UL36 (DUb*) to the N-terminal RING domain (Fig. 1A). To allow detection and ensure the integrity of the fusion protein, a FLAG epitope was fused to the C termini of these proteins. DUb-dependent deubiquitination of rhTRIM5α was examined directly by assessing the ability of the DUb domain to inhibit rhTRIM5α ubiquitination in TZM-bl cells. TZM-bl cells were transfected with either the FLAG-tagged versions of wild-type (WT) rhTRIM5α or the corresponding DUb and DUb* fusion constructs, and rhTRIM5α protein was immunoprecipitated. Cotransfection of hemagglutinin-tagged ubiquitin (HA-Ub) led to the appearance of ubiquitin laddering in the WT rhTRIM5α, as previously reported (6) (Fig. 1B). A similar laddering pattern was not observed for the DUb-rhTRIM5α construct, while this pattern was readily detected in the DUb*-rhTRIM5α construct (Fig. 1B). The immunoprecipitated material was additionally blotted for HA-tagged ubiquitin to confirm that this laddering pattern was indeed due to ubiquitination of WT rhTRIM5α. As expected, the WT and DUb* rhTRIM5α constructs corresponded to ubiquitin blotting patterns consistent with the modification of the parent protein, while no ubiquitin was detected in immunoprecipitations from cells expressing DUb-rhTRIM5α (Fig. 1B). A similar pattern was observed in HeLa cells stably expressing these same constructs following stable transduction with a lentiviral vector and transfection with an HA-Ub-expressing construct (Fig. 1C). In these cells, DUb-rhTRIM5α expression was slightly higher than the expression observed in the catalytically inactive DUb*rhTRIM5α cell line (Fig. 1C). WT rhTRIM5α exhibited a specific laddering pattern typical for proteins ubiquitinated at multiple sites in cells (Fig. 1C, top panel), with two reproducibly prominent bands suggesting preferential ubiquitin conjugation sites in rhTRIM5α. A strong ubiquitination signal accumulated with the DUb*-rhTRIM5α protein, in stark contrast to the active DUb-rhTRIM5α (Fig. 1C, lanes 1 to 3), a result consistent with the generation of ubiquitination-resistant rhTRIM5α in cells. In line with this result, conjugation of the first ubiquitin molecule caused the Ub-DUb*-rhTRIM5α protein to migrate to a slightly higher position than its unmodified counterpart DUb*-rhTRIM5α (labeled with an asterisk) in the input fraction (Fig. 1D, compare lanes 2 and 5). Conversely, and despite expressing at a higher level (Fig. 1D, lane 1), DUb-rhTRIM5α protein displayed only residual ubiquitination (lane 4), further confirming the efficient removal of ubiquitin following DUb fusion to rhTRIM5α.

rhTRIM5α ubiquitination facilitates cytoplasmic body formation. To understand the contribution of ubiquitination to the known biological activities of TRIM5α, we generated HeLa cell lines stably expressing the WT, DUb- and DUb*-rhTRIM5α fusions shown in Fig. 1. As observed in Fig. 1C, all three proteins were expressed at similar levels in HeLa cells (Fig. 2A), with the DUb-rhTRIM5α expressed at slightly higher levels than the corresponding DUb*-rhTRIM5α inactive form. We next used antibodies to the C-terminal FLAG epitope tag to determine how modulating ubiquitination affects rhTRIM5α localization. WT rhTRIM5α exhibited a primarily diffuse localization with some cytoplasmic body accumulation in the cytoplasm (Fig. 2B). Notably, localization of the DUb-rhTRIM5α protein was even more pronounced diffuse than that of the WT protein (Fig. 2C). In contrast, the DUb*-rhTRIM5α construct readily formed cytoplasmic bodies (Fig. 2D), despite being expressed at a lower level than the DUb counterpart in these cells (Fig. 1C). In previous studies, we have observed that increased expression levels correlated with increased cytoplasmic body formation (28). These results suggest that inhibition of rhTRIM5α ubiquitination influences its ability to form cytoplasmic bodies.

rhTRIM5α ubiquitination is required for rhTRIM5α inhibition of HIV-1 reverse transcription. Previous studies have found that proteasome inhibitors or depletion of specific E2 enzymes relieves the rhTRIM5α-mediated block to HIV-1 reverse transcription but does not relieve the block to infection (20, 23, 29). To determine whether targeted inhibition of rhTRIM5α ubiquitination with DUb proteins similarly leads to an increase in reverse transcription, we infected cells stably expressing DUb-rhTRIM5α and DUb*-rhTRIM5α. Expression of either construct potently inhibited HIV-1 infection, relative to untransduced HeLa cells (Fig. 3A). The cell line expressing DUb-rhTRIM5α exhibited slightly more inhibition of HIV-1 infection than the cell line expressing DUb*-rhTRIM5α, consistent with its slightly higher expression in stable cell lines (Fig. 1C). We also observed that appending either the DUb or DUb* protein to the N terminus slightly reduced the potency of restriction, compared to WT rhTRIM5α lacking an N-terminal modification. However, when reverse transcription (RT) was measured in these cells, DUb-rhTRIM5α exhibited substantially more RT products than the DUb*-rhTRIM5α control (Fig. 3B), despite allowing slightly less infection than the DUb*-rhTRIM5α-expressing cells. These data demonstrate that rhTRIM5α ubiquitination is not necessary for inhibition of HIV-1 infection. However, preventing rhTRIM5α ubiquitination disrupts its ability to inhibit the accumulation of reverse transcription products, consistent with another recent report (29).

Inhibition of rhTRIM5α ubiquitination prevents rhTRIM5α-mediated destabilization of HIV-1 cores. Given the ability of DUb-rhTRIM5α to inhibit HIV-1 infection, while still allowing significant levels of HIV-1 reverse transcription, we utilized immunofluorescence microscopy to examine the localization of HIV-1 cores in cells expressing DUb-rhTRIM5α, as well as the other constructs in the study. Cells were spinoculated with VSV-g-pseudotyped HIV-1. Following spinoculation, viral supernatants were replaced with fresh medium, and the infection was allowed to proceed for 4 h, at which time the cells were fixed and the localization of HIV-1 cores and rhTRIM5α was assessed using antibody to HIV-1 CA and FLAG, respectively. In untransduced HeLa cells, viral cores were readily observed in these cells 4 h after infection (Fig. 4A). In contrast, a smaller number of cores were visible in cells expressing WT rhTRIM5α, and this result was also observed in cells expressing the control DUb*-rhTRIM5α (Fig. 4A), consistent with the ability of rhTRIM5α to induce the abortive disassembly of the HIV-1 core (18). In contrast, cells expressing DUb-rhTRIM5α contained a substantial amount of HIV-1 cores (Fig. 4A). Algorithm-assisted image analysis was then used to quantify the number of viral cores per cell in each cell line 6 h after infection. This analysis confirmed that cells expressing DUb-

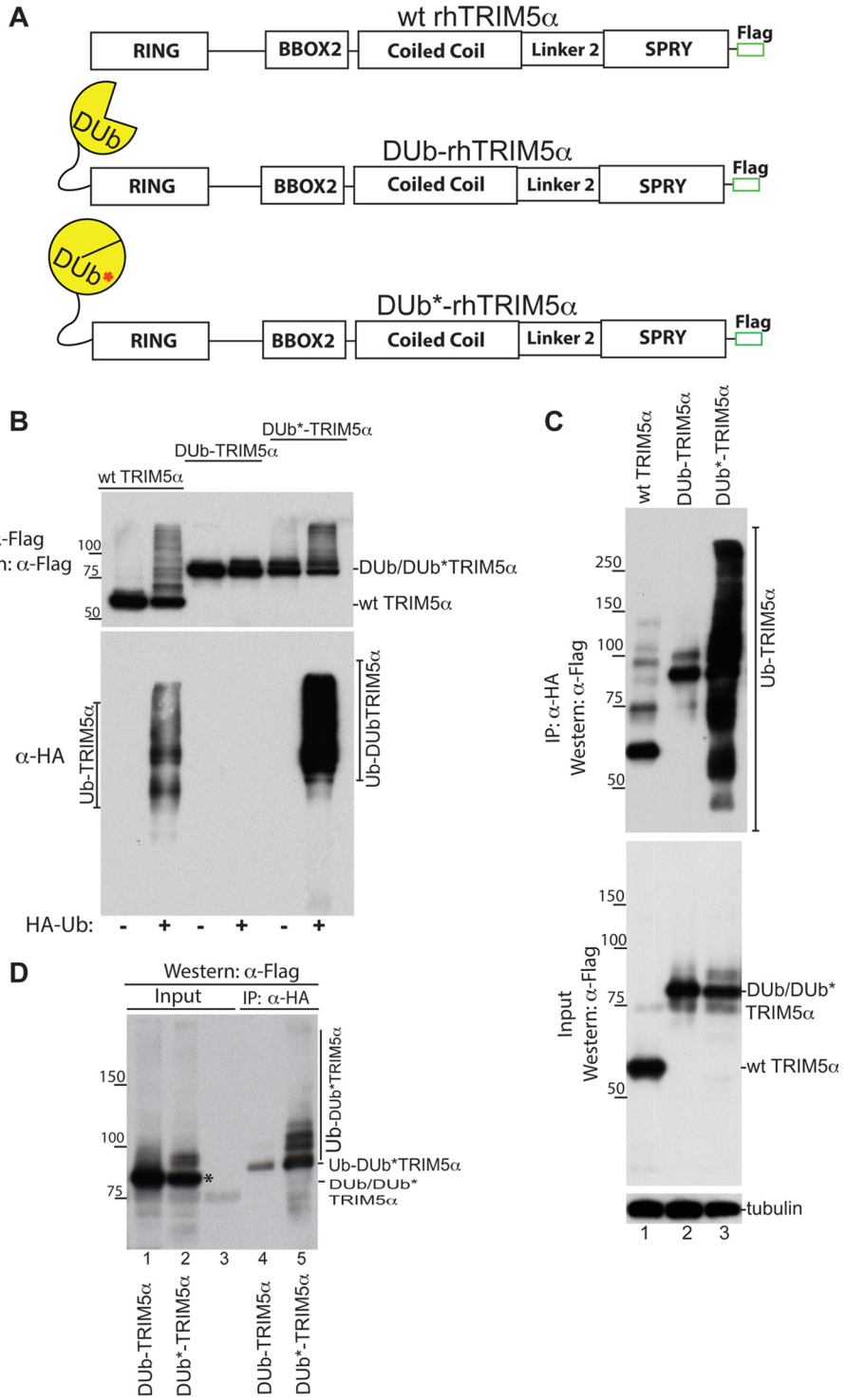

FIG 1 DUb-mediated inhibition of rhTRIM5α ubiquitination. (A) Schematic of the constructs used in this study. Enzymatically active DUb, and control, inactive DUb* domains, were added to the N-terminal RING domain of rhTRIM5α. Flag tags were appended to the C-terminal ends of the fusion proteins. (B) TZM-bl cells were transiently transfected with the indicated construct, with (+) or without (−) HA-Ub. Forty-eight hours posttransfection, rhTRIM5α was immunoprecipitated using anti-Flag antibody-coated beads and examined by SDS-PAGE (12%) and then by Western blotting using the indicated antibodies (α-Flag, anti-Flag antibody). (C) Lentiviral vectors expressing the constructs in panel A were used to generate stable HeLa cell lines expressing these proteins. Cells were transfected with HA-Ub, and rhTRIM5α proteins were immunoprecipitated using anti-HA antibody-coated beads and examined by Western blotting using the antibodies shown. (D) Experiments similar to those shown in panel C were conducted, and samples were analyzed on the same gel by SDS-PAGE (10%) and Western blotting using the indicated antibodies. The position of the unmodified DUb*-rhTRIM5α is marked with an asterisk (lane 2). The positions of the Ub-DUb*-rhTRIM5α and the higher-molecular-weight Ub-modified proteins (lane 5) are indicated, the control sample is shown in lane 3, while the DUb-rhTRIM5α samples are in lanes 1 and 4. The positions of molecular mass markers (in kilodaltons) are shown to the left of the gel. All results are representative of three or more independent experiments.

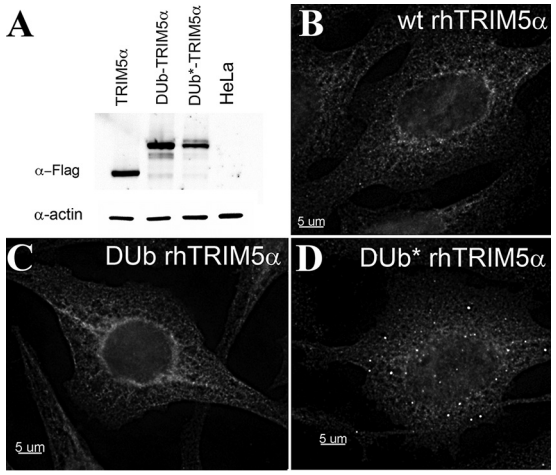

FIG 2 Ubiquitination enhances rhTRIM5α accumulation in cytoplasmic bodies. (A) Western blot of HeLa cells stably transduced with the indicated lentiviral vector. (B to D) HeLa cells stably expressing wild-type (wt) rhTRIM5α (B), DUb-rhTRIM5α (C), or DUb*-rhTRIM5α (D) were fixed, and rhTRIM5α localization was assessed using mouse anti-Flag antibody (α-Flag) and fluorescent secondary antibody against mouse IgG. Images were acquired on a wide-field fluorescence microscope, and resulting Z-stack images were deconvolved using SoftWoRx software (Applied Precision). Results are representative of three or more independent experiments.

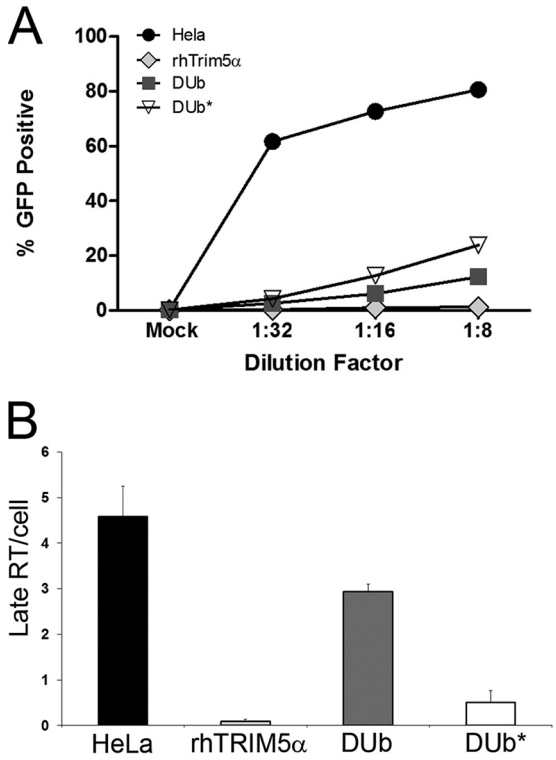

FIG 3 Ubiquitination-independent and -dependent functions of TRIM5α during HIV-1 restriction. (A) Control HeLa cells or HeLa cells stably expressing the indicated construct were infected with increasing amounts of HIV-1 GFP pseudotyped with the VSV-g envelope. GFP expression was measured 48 h postinfection by flow cytometry. (B) In parallel, the same cells were infected with HIV-1 GFP pseudotyped with VSV-g at the 1:2 dilution shown in panel A. Eighteen hours after infection, cellular DNA was harvested, and reverse transcription was assessed with quantitative PCR, as previously described (20). Error bars represent the standard errors of the means (SEM). Results are representative of three or more independent experiments.

rhTRIM5α contained significantly more HIV-1 cores than either the WT rhTRIM5α or DUb*-rhTRIM5α construct (Fig. 4B). These results indicate that inhibiting substrate ubiquitination by TRIM5α prevents HIV-1 core destabilization.

We also utilized the fate of capsid (FOC) assay to measure the amount of intact viral cores in these cells following infection. This uncoating assay relies on the centrifugation of lysates from infected cells through a sucrose cushion. As intact, assembled forms of CA, but not monomeric CA, are pelleted through this sucrose cushion, the amount of CA present in the pellet after infection allows the relative stability of viral cores between samples to be compared. As was observed when capsids were quantified by imaging, the DUb-rhTRIM5α fusion failed to induce the loss of pelletable capsid from these cells, while a pronounced loss of pelletable CA was observed in cells expressing the DUb*-rhTRIM5α or WT rhTRIM5α (Fig. 4C and D).

Inhibition of rhTRIM5α ubiquitination stabilizes the association of rhTRIM5α and HIV-1 CA in the target cell cytoplasm. To examine the fate of viral cores in cells expressing DUb-rhTRIM5α, we examined the colocalization between DUb-rhTRIM5α and CA following infection. First, we observed that HIV-1 infection induced the redistribution of DUb-rhTRIM5α from a predominately diffuse localization to a more punctate localization 4 to 6 h following infection (Fig. 5). In these cells, these individual puncta frequently, although not exclusively, colocalized with punctate viral CA staining in these cells (Fig. 5). Notably, this colocalization between DUb-rhTRIM5α and HIV-1 CA occurred in the context of small, individual puncta. This is in noticeable contrast to the association of rhTRIM5α and HIV-1 CA observed following proteasome inhibition. MG132 treatment induces the formation of larger cytoplasmic bodies than is observed in the absence of treatment, and numerous individual viral particles often accumulate within these larger accumulations of rhTRIM5α (21, 23). Rather, the association between DUb-rtTRIM5α resembled the colocalization observed between rhTRIM5α and HIV-1 CA in the absence of drug, although whereas the latter leads to the rapid loss of detectable CA (21), the association of DUb-rhTRIM5α and HIV-1 CA was more stable, such that numerous examples of colocalization were readily observed in these cells (Fig. 5). This is consistent with the observation that DUb-rhTRIM5α can inhibit HIV-1 infection (Fig. 3) but lacks the ability to induce destabilization of the viral core (Fig. 4).

Inhibition of rhTRIM5α ubiquitination reduces rhTRIM5α-mediated activation of NF-κB. Previous studies have demonstrated that TRIM5, and other TRIM family proteins, can activate signaling pathways, including NF-κB, that alter gene transcription in target cells (4, 5, 30, 31). Activation of these pathways is linked to the E3 ligase activity of TRIM5α and involves the generation of K63-linked polyubiquitin chains (4). We therefore asked whether inhibition of rhTRIM5α ubiquitination inhibits NF-κB activation using an NF-κB luciferase reporter, as performed by others (5, 30). Relative to the WT control, activation of NF-κB was reduced to background levels in DUb-rhTRIM5α-expressing cells (Fig. 6). In contrast, expression of the DUb*-rhTRIM5 construct induced NF-κB activation (Fig. 6).

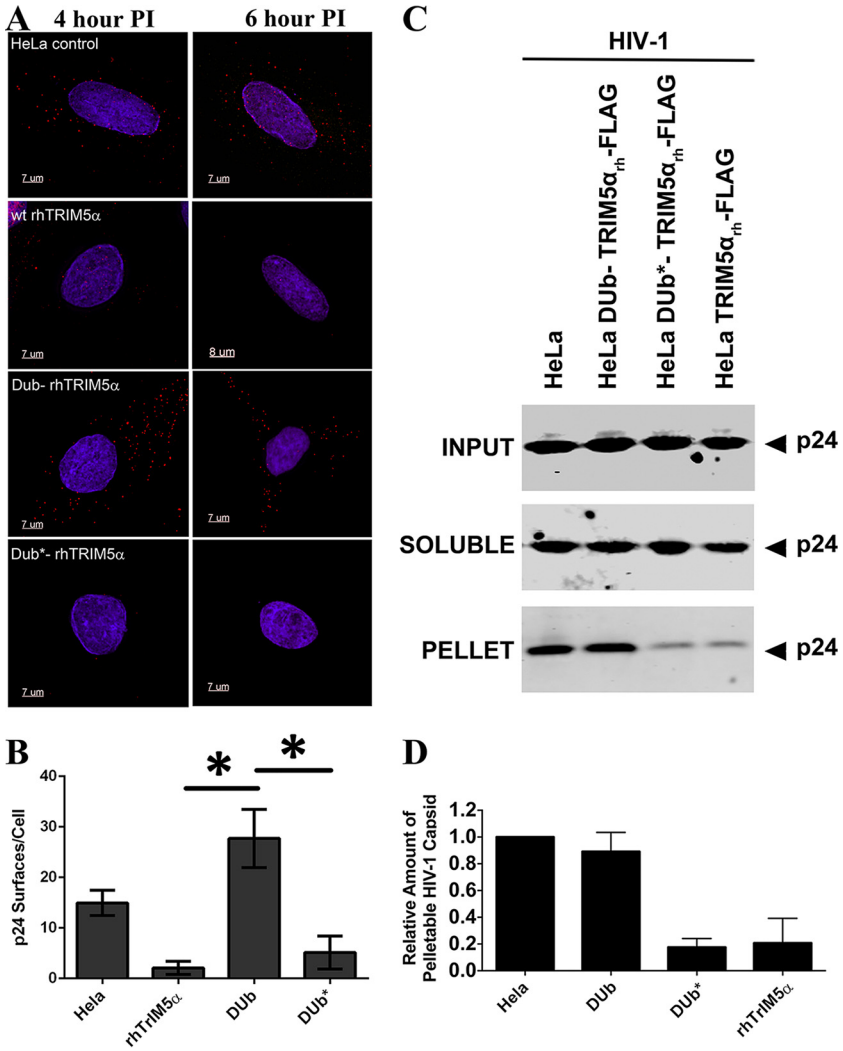

FIG 4 Destabilization of the HIV-1 core by rhTRIM5α is ubiquitination dependent. (A) HeLa cell lines stably expressing the indicated construct were spinoculated with VSV-g-pseudotyped HIV-1. Cells were incubated for an additional 4 h or 6 h, fixed, and immunostained with antibodies specific for HIV-1 CA (red). 4′,6′-Diamidino-2-phenylindole (DAPI) stain is shown in blue. PI, postinfection. (B) Magnification of the inset identified in panel A. Arrows are provided to illustrate demonstrative colocalizations between CA and DUb-rhTRIM5α. (B) The number of CA puncta 6 h after infection was calculated using Imaris software from Bitplane. The number of CA puncta observed in 10 or more Z-stack images was calculated. Error bars represent SEM. Results are representative of three or more experiments. Values that are statistically significantly different ($P < 0.001$) are indicated by a bar and asterisk. (C) HeLa cells expressing the indicated construct were infected with VSV-g-pseudotyped HIV-1 for 8 h. The cells were then lysed, and the extracts were subjected to centrifugation to determine the amount of pelletable, assembled capsid present in each cell line. A representative experiment is shown. (D) The graph represents the relative amount of pelletable capsid present in each cell line, compared to control HeLa cells, as measured by densitometry, in three experiments. Error bars represent standard deviations.

DISCUSSION

rhTRIM5α is a ubiquitin ligase, with a well-established role in restricting early events of HIV-1 infection (1). In this study, we utilized a viral deubiquitinating enzyme to generate a ubiquitination-resistant form of rhTRIM5α in order to identify ubiquitin-dependent and ubiquitin-independent activities of rhTRIM5α. Examination of DUb-rhTRIM5α fusion allowed these activities to be separated without perturbation of the cell's natural ubiquitinating enzymes or ubiquitin pool and without introducing mutations into the WT rhTRIM5α protein. More specifically, we observe that polyubiquitinated DUb-rhTRIM5α is not generated, even upon overexpressing HA-Ub in cells (Fig. 1). In contrast, strong polyubiquitination of WT rhTRIM5α and the catalytically inactive DUb-rhTRIM5α control construct was consistently detected (Fig. 1B, C, and D). Additionally, we observed more pronounced polyubiquitination of DUb*-rhTRIM5α than was observed for WT TRIM5α in stable cell lines (Fig. 1C). These ubiquitination patterns correlated notably with the localization of these constructs in stable cell lines, as fusion of rhTRIM5α to the catalytically active DUb eliminated the localization to cytoplasmic bodies in the absence of virus, while fusion to the catalytically inactive DUb* domain generated a protein with more pronounced cytoplasmic body localization than was observed for WT protein (Fig. 2A and B). This suggests that cytoplasmic body formation by rhTRIM5α, in the absence of restriction-sensitive virus, is dependent on ubiquitination. However, we did observe that the

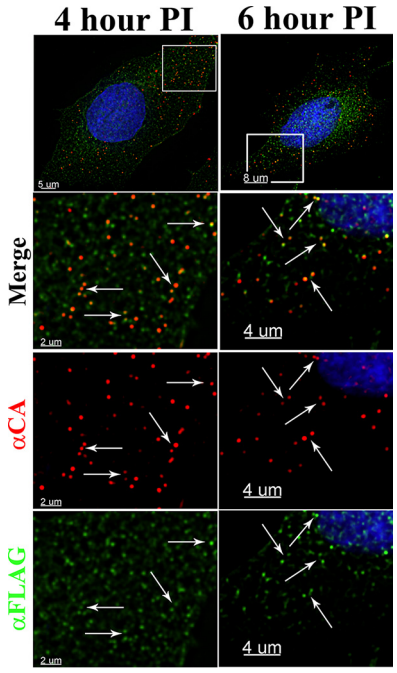

FIG 5 Colocalization of DUb-rhTRIM5α and HIV-1 CA following infection. HeLa cells stably expressing DUb-rhTRIM5α were spinoculated with VSV-g-pseudotyped HIV-1 and fixed 4 or 6 h following infection. Cells were then stained with antibodies against HIV-1 CA (red) and the FLAG epitope located on the DUb-rhTRIM5α construct (green). The cell nucleus was visualized with DAPI stain (blue). The white boxes indicated in the top panels are enlarged in the bottom three panels. Arrows indicate areas of obvious colocalization.

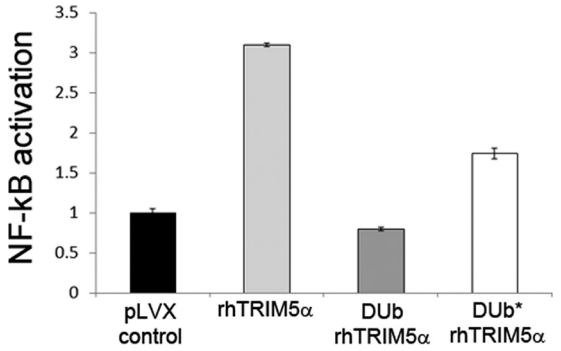

FIG 6 DUb-mediated inhibition of rhTRIM5α ubiquitination inhibits activation of NF-κB 293T cells were transfected with equal amounts of the indicated construct, a plasmid constitutively expressing renilla luciferase and a construct expressing firefly luciferase under the control of the NF-κB. Firefly luciferase expression, normalized to renilla luciferase values, is shown. Error bars represent the SEM. Results are representative of three or more independent experiments.

addition of restriction-sensitive HIV-1 induced the relocalization of DUb-rhTRIM5 to cytoplasmic assemblies which colocalized with viral cores (Fig. 4), consistent with the previous observation that restriction-sensitive cores can nucleate the *de novo* formation of cytoplasmic bodies (32) and the formation of TRIM5α assemblies on assembled CA lattices in *in vitro* systems lacking ubiquitin (33). Taken together, these results demonstrate that cytoplasmic body formation can occur via two independent pathways, and the DUb-rhTRIM5α fusion provides a tool to measure the formation of cytoplasmic bodies induced by SPRY/CA interactions, as spontaneous, ubiquitination-dependent cytoplasmic bodies were not observed in these cells. As numerous TRIM family proteins possess SPRY domains which mediate protein-protein interactions critical for protein function, this approach may have similar value in the study of other TRIM family proteins.

This study also demonstrated the role of polyubiquitination in the ability to restrict retroviral infection and specifically demonstrated that preventing TRIM5α-mediated polyubiquitination of target proteins, presumably TRIM5α itself, is required for TRIM5α-mediated inhibition of reverse transcription (Fig. 3B), but not its ability to inhibit retroviral infection (Fig. 3A). Previous studies have observed that proteasome inhibition, or specific RING domain mutations, also relieve the TRIM5α-mediated block to reverse transcription without affecting its ability to restrict infection (20, 23, 24). However, interpretation of these observations is somewhat obscure, as MG132 grossly disrupts ubiquitin homeostasis in cells, and RING domain mutations might also affect RING domain dimerization, which is important for TRIM5α self-association, as well as its enzymatic activity (8, 9). The attachment of DUb domains to the N-terminal RING domain in this study allowed specific and selective examination of the role that TRIM5α RING domain-mediated ubiquitination plays in the restriction process and clearly demonstrated that TRIM5α polyubiquitination is not required for retroviral restriction but is required for the inhibition of reverse transcription (Fig. 3). Additionally, we also observed that inhibition of TRIM5α ubiquitination prevented the activation of NF-κB signaling pathways, demonstrating that the ability to restrict retroviral infection is separable from the ability to activate signaling pathways, consistent with a recent study (29).

The finding that TRIM5α ubiquitination is required for inhibition of reverse transcription is consistent with another recent study which observed that depletion of the Ube2W E2 enzyme prevents rhTRIM5α-mediated inhibition of viral reverse transcription but not infection (29). As TRIM5α is the only known target of TRIM5α ubiquitination, it seems likely that autoubiquitination is mechanistically responsible for the inhibition of reverse transcription. However, it remains possible that other, currently cryptic cellular or viral targets of TRIM5α ubiquitination are required for the inhibition of reverse transcription. Assuming that TRIM5α autoubiquitination represents the mechanistically relevant target of TRIM5α ubiquitination activity, the simplest way to explain this would be that TRIM5α ubiquitination drives the degradation of the resulting TRIM5α assembly and the bound HIV-1 core. Both proteasomal and autophagic degradative pathways have been suggested to play a role in TRIM5α retroviral restriction (20, 23, 34). In this regard, it is notable that biochemical examination of TRIM5α autoubiquitination patterns reveal K63-linked polyubiquitin chains, rather than K48-linked chains typically associated with proteasomal degradation (29). Our observation that inhibition of TRIM5α-mediated ubiquitination prevented the ability of TRIM5α to activate NF-κB signaling pathways is consistent with the generation of K63-linked ubiquitin (35, 36), as well as other studies suggesting that TRIM5α signaling is dependent on the formation of K63-linked polyubiquitin (4, 5, 30, 31). However, a recent examination of another TRIM family protein, TRIM21, observed that proteasome recruitment to TRIM21 complexes was dependent on the formation of K63-linked ubiquitin (37), suggesting that K48-linked polyubquitination is not neces-

sarily required for proteasomal engagement of TRIM family proteins.

We have also utilized DUb-tethered TRIM5α constructs to demonstrate that TRIM5α ubiquitination is required for the ability of TRIM5α to destabilize the viral core (Fig. 4 and 5), as previously shown by the rhTRIM5α RING domain mutant Y63E (24). In fact, we observe that ubiquitination-resistant TRIM5 induced an apparent stabilization of cytoplasmic cores, compared to unrestricted control cells, ultimately resembling the core stabilization induced by the MXB restriction factor (38). These data, taken together with the inability of DUb-rhTRIM5α to inhibit HIV reverse transcription, are consistent with a two-step model of TRIM5α restriction, as rhTRIM5α ubiquitination is required for viral core destabilization and inhibition of reverse transcription, but not inhibition of infection.

Finally, the methodology used here to generate a TRIM protein resistant to polyubiquitination will likely provide an exceptional tool to examine the ubiquitin-dependent and -independent activities of other TRIM family proteins, all of which possess an N-terminal RING domain (3) which mediate diverse cellular activities, including activation of cellular pathways relevant to innate immune signaling and the development of certain forms of cancer (4, 5, 30, 31, 39).

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