K63-Linked Ubiquitin Is Required for Restriction of HIV-1 Reverse Transcription and Capsid Destabilization by Rhesus TRIM5 α

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ABSTRACT TRIM5 α is an antiviral restriction factor that inhibits retroviral infection in a species-specific fashion. TRIM5 α binds to and forms assemblies around the retroviral capsid. Following binding, poorly understood, ubiquitin-dependent events lead to the disassembly of the viral core, prior to the accumulation of viral reverse transcription products in the target cell. It is also known that assemblies of TRIM5 α and other TRIM family proteins can be targets of autophagic degradation. The goal of this study was to define the role of specific ubiquitin linkages in the retroviral restriction and autophagic degradation of TRIM5 α and delineate any connection between these two processes. To this end, we generated fusion proteins in which the catalytic domains of different deubiquitinase (DUB) enzymes, with different specificities for polyubiquitinated linkages, were fused to the N-terminal RING domain of Rhesus macaque TRIM5 α . We assessed the role of ubiquitination in restriction and the degree to which specific types of ubiquitination are required for the association of TRIM5 α with autophagic proteins. We determined that K63-linked ubiquitination by TRIM5 α is required to induce capsid disassembly and to inhibit reverse transcription of HIV, while the ability to inhibit HIV-1 infection was not dependent on K63-linked ubiquitination. We also observed that K63-linked ubiquitination is required for the association of TRIM5 α with autophagosomal membranes and the autophagic adapter protein p62.

IMPORTANCE Although the mechanisms by which TRIM5 α can induce the abortive disassembly of retroviral capsids have remained obscure, numerous studies have suggested a role for ubiquitination and cellular degradative pathways. These studies have typically relied on global perturbation of cellular degradative pathways. Here, through the use of linkage-specific deubiquitinating enzymes tethered to TRIM5 α , we delineate the ubiquitin linkages which drive specific steps in restriction and degradation by TRIM5 α , providing evidence for a noncanonical role for K63-linked ubiquitin in the process of retroviral restriction by TRIM5 α and potentially providing insight into the mechanism of action of other TRIM family proteins.

KEYWORDS deubiquitinating enzymes, HIV-1, retroviral restriction, TRIM5 α , ubiquitination

Host cell restriction factors are a class of proteins that inhibit viral replication by blocking specific steps of the viral life cycle. TRIM5 α is one of the best characterized antiviral restriction factors. Members of the TRIM family of proteins are defined by having a tripartite motif consisting of an N-terminal RING domain, which functions as an E3 ubiquitin ligase, one or two B-box domains, and a coiled-coil domain (1, 2).

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TRIM5 α is distinguished from other members of this family by its C-terminal PRY/SPRY domain, which allows TRIM5 proteins to directly bind to the retroviral capsid, a proteinaceous core that houses the viral genome (2, 3). Upon capsid recognition, the B-box and coiled-coil domains, which are critical for self-assembly among many TRIM family members (4–7), facilitate the assembly of TRIM5 into a multimeric lattice surrounding the viral core (8–10). Furthermore, higher-order assembly of TRIM5 also activates the E3 ubiquitin ligase function of the RING domain (11). Through the formation of this multimeric assembly, TRIM5 is able to initiate its antiviral activities, which include (i) inhibition of viral infection, (ii) inhibition of viral reverse transcription and disassembly of the capsid, and (iii) synthesis of K63-linked ubiquitin chains that activate innate signaling pathways and induce an antiviral state for the cell (3, 12–14).

Initial studies investigating the mechanism of restriction of retroviral infection by TRIM5 proteins determined that TRIM5 causes a destabilization of the retroviral capsid (3, 15), and early models invoked cellular degradative machinery, such as the proteasome or autophagy pathways, as being critical mediators of this destabilization. Several groups, including our own, have determined that there is a step in restriction that is sensitive to proteasome inhibitors; treatment of TRIM5 α -expressing cells with proteasome inhibitors prior to retroviral infection allows viral reverse transcription to proceed, though infection is still restricted, suggesting the existence of a proteasome-dependent step in the restriction process (14, 16–19). Furthermore, rhesus TRIM5 α (RhTRIM5 α), which efficiently destabilizes HIV-1 capsids under normal circumstances, forms stabilized complexes with HIV-1 virions in the presence of proteasome inhibitors (3, 17, 20). These complexes stain positively for ubiquitin (17), indicating a role for ubiquitin or ubiquitination in TRIM5 α 's antiretroviral functions.

Ubiquitin is a 76-amino-acid protein that can be covalently attached to lysine residues in target proteins, and this modification is often the signal to direct substrates to particular cellular pathways (21, 22). Ubiquitin has seven internal lysines (residues 6, 11, 27, 29, 33, 48, and 63), and these residues can be targets of ubiquitination themselves, thereby producing polyubiquitin linkages (23, 24). Polyubiquitin linkages can be anchored to a particular protein substrate, or they can be unanchored (25, 26). Importantly, different polyubiquitin linkages are associated with different cellular fates. K48- and K63-linked polyubiquitin chains are the most prevalent, representing approximately 80% of all ubiquitin linkages observed in mammalian cells (27). K48-linked polyubiquitin chains are canonically associated with directing substrates to the proteasome for degradation, while K63-linked polyubiquitin modification is associated with endosomal trafficking, intracellular signaling, and DNA repair (21–23, 28–33). As an E3 ubiquitin ligase, TRIM5 α has been shown to undergo autoubiquitination and produce anchored K63-linked ubiquitin chains *in vitro* (12). We and others have observed that TRIM5 α colocalizes with markers of the autophagy pathway (34–36), and these observations suggested a possible role for autophagy in TRIM5 α 's restriction functions. However, we previously established that restriction of retroviral infection or reverse transcription by TRIM5 proteins does not require either the ATG5 or the Beclin1 autophagy effector molecule (34). It remains unclear whether ubiquitination is required for the recruitment of the autophagic machinery to TRIM5 α assemblies and what other cellular proteins play a role in this recruitment.

Therefore, our goal was to delineate the role of ubiquitination in the antiretroviral functions of TRIM5 α and its recruitment to autophagosomes. We generated fusion proteins in which the catalytic domains of different DUB enzymes, with different specificities for polyubiquitinated linkages, were fused to the N-terminal RING domain of RhTRIM5 α (37). Using these fusion proteins as tools, we found that in the absence of K63-specific ubiquitin ligase activity, TRIM5 α forms a stable association with the capsid, allowing reverse transcription to proceed; however, infection is still blocked. These data favor a model whereby the formation of the TRIM5 α assembly around a capsid is sufficient to inhibit infection, while K63-linked ubiquitination is required for capsid disassembly and inhibition of reverse transcription. We also determined that K63-linked

ubiquitination by TRIM5 α is critical for its association with autophagosomal membranes, which also requires the autophagic adaptor p62.

RESULTS

K63- or K48-specific DUB fusions influence RhTRIM5 α polyubiquitination in cells. In seeking to define the ubiquitin-dependent steps of restriction by TRIM5 α , a recent study from our group determined that the E3 ubiquitin ligase function of TRIM5 α is required for its ability to destabilize retroviral capsids (38). TRIM5 α proteins in which the herpes simplex virus 1 (HSV-1) UL36 deubiquitinating enzyme (here referred to as UL36) was fused to the N-terminal RING domain of RhTRIM5 α (UL36-RhTRIM5 α) were able to restrict HIV-1 infection (38). However, viral cores in complex with UL36-RhTRIM5 α accumulated in the cytoplasm of infected cells, suggesting impaired destabilization of cores in the absence of competent ubiquitination (38). Importantly, cells expressing a catalytically inactive version of the DUB (denoted with an asterisk [*]), termed UL36*-RhTRIM5 α , maintained the ability to both restrict infection and destabilize viral cores (38).

To more directly identify the specific determinants of how TRIM5 α recruits autophagic machinery and to determine if stabilized TRIM5 α -viral-core complexes are recruited to autophagosomes, we generated a panel of fusion proteins in which the catalytic domains of different deubiquitinase (DUB) enzymes, with different specificities for polyubiquitinated linkages, were fused to the N-terminal RING domain of RhTRIM5 α (Fig. 1A). Our previous study utilized the HSV-1 UL36 deubiquitinating enzyme, which has been shown to cleave both K48- and K63-linked polyubiquitin chains (39–41). The different DUBs employed in the current study were chosen for their ability to cleave only a single type of ubiquitin linkage, even at high polyubiquitin concentrations (37). We therefore fused a K63-specific DUB AMSH-LP and a K48-specific DUB (OTUB1) to the N terminus of RhTRIM5 α ; the fusion proteins are denoted AMSH-LP-RhTRIM5 α and OTUB1-RhTRIM5 α , respectively (Fig. 1A). In addition, each of these DUB-RhTRIM5 α fusions was paired with a catalytically inactive deubiquitinase-RhTRIM5 α fusion protein to control for these N-terminal fusions to RhTRIM5 α .

In order to test the ability of our constructs to influence the ubiquitination of RhTRIM5 α , we transiently transfected HEK293T cells with our fusion constructs with or without hemagglutinin-tagged ubiquitin (HA-Ub) and immunoprecipitated each RhTRIM5 α with anti-FLAG antibodies as previously described (Fig. 1B and C, top left) (38). By blotting with anti-HA antibody, we were able to observe high-molecular-weight ubiquitin laddering for catalytically inactive AMSH*-LP-RhTRIM5 α , which was drastically reduced in the presence of the active AMSH fusion protein (AMSH-LP-RhTRIM5 α) (Fig. 1B, bottom). When we used K63 linkage-specific antibody for our blots, the ubiquitin ladder was not visible, which confirms that the AMSH fusion is able to remove K63-linked ubiquitin chains from RhTRIM5 α (Fig. 1B, top right). We observed a similar pattern with OTUB1-RhTRIM5 α proteins. In this case, however, the ubiquitin ladder for the active OTUB1 was modestly decreased compared to that of the catalytically inactive control. In confirmation of this result, we observed high-molecular-weight ubiquitin laddering when the catalytically inactive OTUB1*-RhTRIM5 α -immunoprecipitated product was probed with a K48 linkage-specific ubiquitin antibody, which was not present in the catalytically active counterpart (Fig. 1C, left). These results demonstrate that ubiquitination of RhTRIM5 α consists of both K63- and K48-linked ubiquitin. They also demonstrate that both AMSH-LP- and OTUB1-RhTRIM5 α fusion proteins effectively remove K63-linked and K48-linked ubiquitin, respectively, from RhTRIM5 α .

Deubiquitinase-RhTRIM5 α fusion proteins restrict HIV-1 infection. To understand the role of K63- and K48-linked ubiquitin in RhTRIM5 α -mediated restriction of HIV-1, we generated A549 and HeLa cells stably expressing these constructs. In A549 cells stably expressing RhTRIM5 α fused to three DUBs and their enzymatically inactive controls, we observed that cells expressed less of the K63-specific DUB fusions (AMSH-LP) than of fusions expressing K48-specific (OTUB1) or dually specific (UL36) DUBs (Fig. 2A), and this pattern was also observed in HeLa cells (Fig. 2B). However, this reduced

FIG 1 Deubiquitinase-RhTRIM5 α fusion proteins inhibit K63- or K48-linked polyubiquitination. (A) Schematic diagram of the RhTRIM5 α fusion proteins used in this study. The active and inactive (denoted by an asterisk [*]) DUB fusions were produced by inserting the given enzyme, in frame, between a Flag tag and the start codon for RhTRIM5 α . (B and C) HEK293T cells were transiently transfected with DUB fusion constructs with (+) or without HA-Ub (-). Cells were harvested 48 hpi and immunoprecipitated with anti-Flag antibody-conjugated beads. They were evaluated by SDS-PAGE (precast gradient gel) and Western blotting with the indicated antibodies. AMSH/AMSH*-RhTRIM5 α (B) and OTUB1/OTUB1*-RhTRIM5 α (C) ubiquitination status was evaluated with linkage-specific antibodies. The arrows mark the locations of unmodified fusion proteins. The results shown are representative of three or more independent experiments.

expression did not significantly influence the potency of restriction, as all six fusions restricted HIV-1 infection in both A549 and HeLa cells (Fig. 2C and D). In A549 cells, the catalytically inactive OTUB1*-RhTRIM5 α was slightly more permissive to HIV-1 infection, while in HeLa cells, the catalytically inactive AMSH*-LP-RhTRIM5 α construct was slightly more permissive to infection than its catalytically active counterpart.

In order to understand if the slight changes in restriction levels were indirect effects of DUB proteins altering cellular pathways, we doubly transduced HeLa cells to express DUB proteins in an inducible system (Fig. 2E) and stably express HA-tagged RhTRIM5 α without any DUB fusions. We checked the restriction by RhTRIM5 α upon induction of UL36, AMSH, and OTUB1 expression and did not observe any changes in the permissiveness to the infection (Fig. 2F).

FIG 2 Deubiquitinase-RhTRIM5 α fusion proteins restrict HIV-1 infection. (A and B) A549 (A) and HeLa (B) cells transduced to express the indicated DUB-RhTRIM5 α fusions were collected, and fusion protein expression was assessed using an anti-FLAG antibody. (C and D) Equal numbers of A549 (C) and HeLa (D) cells stably expressing the different DUB-RhTRIM5 α fusion proteins were infected with serial dilutions of HIV-1-GFP reporter virus. Infectivity was quantified by measuring the proportion of GFP-positive (GFP⁺) cells by flow cytometry at 48 hpi. (E and F) HeLa cells transduced to express inducible DUB constructs and unanchored RhTRIM5 α were incubated with/without doxycycline for 48 h to evaluate the protein expression levels by Western blotting with anti-FLAG and anti-HA antibodies (E) and to quantify infectivity (F) as described in the legend to panels C and D. Data depicted here are representative of at least three independent experiments. arb. u., arbitrary units; Untransd, untransduced.

The minor changes in the restriction levels of DUB-RhTRIM5 α fusion proteins correlate well with the expression levels. Nevertheless, as all the fusion proteins were able to restrict HIV-1 infection, we concluded that ubiquitination is not required for the restriction of infection by RhTRIM5 α .

K63-linked ubiquitination activity is required for restriction of reverse transcription by TRIM5 α . During restriction, TRIM5 α is known to promote the disassembly of capsids prior to the completion of reverse transcription, although this effect is abrogated in the presence of the proteasome inhibitor MG132 (14, 16). To determine if the restriction of reverse transcription requires ubiquitination activity and to specifically define what type of ubiquitin linkages are critical, we infected A549 and HeLa cells expressing the DUB-RhTRIM5 α fusion proteins with an HIV-1 reporter virus and mea-

FIG 3 K63-linked ubiquitination activity is required for restriction of reverse transcription by TRIM5 α . Cells stably expressing DUB-RhTRIM5 α fusion proteins were infected with a GFP-expressing HIV-1 reporter virus, and viral reverse transcription products were measured by quantitative real-time PCR. (A) Numbers of reverse transcription products (Late RT products) accumulating in the indicated A549 cell lines. Error bars represent the standard deviations of technical triplicates. (B) Late RT products were evaluated for variations in viral dilutions in HeLa cells. Error bars represent the standard deviations of technical triplicates. (C and D) Ratios of RT products of an active DUB to those of its respective catalytically inactive control for A549 (C) and HeLa (D) cells at different viral concentrations. Error bars represent the SEM of biological replicates. (E) RT products evaluated for HeLa cells transduced to express inducible DUB proteins and unfused RhTRIM5 α . Error bars represent the standard deviations of technical triplicates. (F) Ratios of late RT products of untreated to doxycycline-treated (No tx/Dox tx) cell lines. Error bars represent the SEM of biological replicates. Data are representative of at least three independent experiments. Statistical significance was determined by one-way ANOVA: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

sured the reverse transcription products (Fig. 3A and B). As expected, we observed minimal production of reverse transcription products in cells expressing RhTRIM5 α , which is consistent with previous reports (2, 3), and observed an increase in reverse transcription products in cells expressing the catalytically active HSV-1 UL36–deubiquitinase fusion protein (UL36-RhTRIM5 α) (38).

To more precisely define the ubiquitination linkages that are critical for the restriction of reverse transcription by TRIM5 α , we measured reverse transcription in cells expressing K63-specific (AMSH-LP-RhTRIM5 α) or K48-specific (OTUB1-RhTRIM5 α) deu-

biquitinase fusion proteins, along with their catalytically inactive controls. We observed an enrichment of reverse transcription products specifically in A549 cells expressing the catalytically active K63-specific deubiquitinase, AMSH-LP-RhTRIM5 α , but not in A549 cells expressing the K48-specific DUB (Fig. 3A). A similar pattern was apparent across a range of viral dilutions in HeLa cells (Fig. 3B). Furthermore, this accumulation was most apparent when comparing the ratio of the reverse transcription products in cells expressing the active DUB versus their respective catalytically inactive controls (Fig. 3C and D). In this case, we observed an enrichment of reverse transcription products in the cells expressing the dually specific or K63-specific DUB fusion proteins but not in cells expressing the K48-specific fusion proteins. This was not due to nonspecific effects of DUB expression, as we did not see any increase in viral reverse transcription in cells expressing RhTRIM5 α when individual DUB proteins were expressed as unfused proteins (Fig. 3E and F).

Inhibition of K63-linked ubiquitination produces stable association of RhTRIM5 α with HIV-1 cores but impaired association with autophagosome membranes. Previous studies have demonstrated that engagement of HIV-1 cores by RhTRIM5 α leads to the rapid loss of fluorescent signal associated with the viral core but that treatment of cells with the proteasome inhibitor MG132 or appending the UL36 deubiquitinase to RhTRIM5 α stabilizes these complexes in the cytoplasm (17, 38). We therefore sought to determine which type of ubiquitin linkage stabilized cytoplasmic RhTRIM5 α -HIV-1 complexes using A549 cells stably expressing linkage-specific DUB-RhTRIM5 α fusions. Consistent with previous reports, we observed minimal stable association between wild-type RhTRIM5 α and HIV-1 p24 (Fig. 4C), consistent with the notion that RhTRIM5 α rapidly destabilizes viral cores (17). However, when K63-linked ubiquitination by RhTRIM5 α is inhibited, as in the case of cells expressing UL36-RhTRIM5 α and AMSH-LP-TRIM5 α , we observed significant stable colocalization between TRIM5 α and the viral core, particularly when comparing the results for these DUBs and their catalytically inactive controls (Fig. 4A and C). Conversely, we measured minimal stable colocalization between the K48-specific DUB fusion protein (OTUB1-RhTRIM5 α) and HIV-1 p24, and there was no significant difference between the results for the catalytically active and inactive fusions (Fig. 4C).

We also asked if inhibiting specific ubiquitin linkages influenced the ability of RhTRIM5 α to associate with autophagic proteins, such as LC3. To this end, we generated A549 cell lines stably expressing both yellow fluorescent protein (YFP)-tagged LC3b (YFP-LC3) and each of the DUB-RhTRIM5 α fusions. LC3 localizes to autophagosome membranes, and therefore, LC3 puncta serve as markers of autophagosomes. Following infection with HIV-1, we measured the degree of colocalization between DUB-RhTRIM5 α fusions and YFP-LC3 (Fig. 4B and D).

Analysis of TRIM5 α -LC3 colocalization revealed that K63-linked ubiquitination is also critical for the association of RhTRIM5 α with YFP-LC3, as both DUB-RhTRIM5 α and AMSH-LP-TRIM5 α showed minimal colocalization with LC3, in contrast to the results for their catalytically inactive counterparts (Fig. 4B). Collectively, these data indicate that the inhibition of K63-linked ubiquitination produces a stable association between RhTRIM5 α and HIV-1 cores and that K63-linked ubiquitination activity is critical for the association of TRIM5 α with the autophagosome membrane marker LC3. Notably, there was minimal triple colocalization between RhTRIM5 α , LC3, and p24 (data not shown), suggesting that autophagic machinery is not recruited to stabilized HIV-1-RhTRIM5 α complexes when K63-linked ubiquitination is inhibited.

K63-linked ubiquitination is required for the destabilization of HIV-1 cores by TRIM5 α . Our previous study demonstrated that appending the UL36 DUB to RhTRIM5 α also prevented the abortive disassembly of the HIV-1 core, using a biochemical fate of capsid (FOC) assay (38). In this assay, viral cores are pelleted from cytoplasmic extracts via centrifugation, and the relative amounts of pelletable capsid in cells can be assessed. We therefore asked if inhibiting K63-linked ubiquitination prevented the ability of RhTRIM5 α to destabilize the HIV-1 core, leading to an increase in the corresponding amount of pelletable capsid in these cells. Consistent with our imaging-

FIG 4 Colocalization of DUB fusion proteins, HIV-1 p24, and/or YFP-LC3 in A549 cells. A549 cells expressing YFP-LC3 and the indicated Flag-tagged RhTRIM5 α -DUB fusion proteins were synchronously infected with an HIV-1 reporter virus. (B) Representative images depicting colocalization between Flag and HIV-1 p24 (A) or Flag and YFP-LC3. (C) The degree of colocalization between RhTRIM5 α (Flag) puncta and HIV-1 core (p24) was quantified for each of the DUB-RhTRIM5 α cell lines. Data are depicted as the proportion of double-positive (p24⁺ Flag⁺) puncta out of the total number of Flag puncta. Error bars represent SEM from at least 20 images taken for each cell line. Data are representative of three independent experiments. (D) The degree of colocalization between RhTRIM5 α (Flag) puncta and YFP-LC3 (p24) was quantified for each of the DUB-RhTRIM5 α cell lines. Data are depicted as the proportion of double-positive (LC3⁺ Flag⁺) puncta out of the total number of Flag puncta. Error bars represent SEM of at least 20 images taken for each cell line. Data are representative of three independent experiments. Statistical significance was determined by one-way ANOVA: ***, $P < 0.001$; **, $P < 0.01$; ns, not significant.

based assays, we observed that cells expressing AMSh-LP-RhTRIM5 α contained similar amounts of pelletable capsid as untransduced, control A549 cells (Fig. 5), while the catalytically inactive construct reduced the amount of pelletable capsid following infection, similar to the results for cells treated with 10 μ M PF74 (Fig. 5), which we have previously shown to accelerate uncoating of the HIV-1 core using this assay (42, 43). These data demonstrate that the ability of RhTRIM5 α to induce the abortive disassembly of the HIV-1 core requires K63-linked ubiquitin. Moreover, we have conducted imaging-based studies to quantify capsid proteins in cell lines expressing DUB fusion proteins. Under synchronized infection conditions, we observed increased numbers of

FIG 5 K63-linked ubiquitination is necessary for TRIM5 α -mediated disassembly of HIV-1 cores. (A and B) A549 cells expressing AMSH-LP/LP*-RhTRIM5 α were infected with VSV-G-pseudotyped HIV-1. Eight hours following infection, cells were harvested, and intact cores were isolated by ultracentrifugation through a 50% sucrose cushion. (A) Input, soluble, and pelletable fractions were assessed for p24 levels by Western blotting. Results shown are representative of three independent experiments. (B) Relative results of densitometry of Western blot bands for three biological replicates. (C) A549 cells expressing Flag-tagged RhTRIM5 α -DUB fusion proteins were synchronously infected with VSV-G-pseudotyped HIV-1, and numbers of p24 surfaces per cell were quantified for 20 images. Error bars represent SEM from three biological replicates. Statistical significance was determined by one-way ANOVA: ***, $P < 0.001$; **, $P < 0.01$; ns, not significant.

puncta for cell lines expressing UL36 (K63 and K48) and AMSH-LP-RhTRIM5 α (K63 specific) (Fig. 5C), supporting the idea of a requirement of K63-linked ubiquitination for viral core disassembly.

Ubiquitination is critical for TRIM5 α association with p62 and autophagosome membranes. We next sought to define other cellular determinants required for the recruitment of RhTRIM5 α assemblies to autophagosomes. p62/SQSTM1 (p62) is an autophagic adaptor protein that can bind ubiquitinated substrates and direct them to autophagosomes (44). We previously determined that p62 colocalizes with TRIM5 α cytoplasmic bodies (36). To determine if ubiquitination activity of TRIM5 α is required for its association with p62, we measured the degree of colocalization between the different TRIM5 α fusion proteins, specifically the dually specific and K63-specific DUB-RhTRIM5 α fusions, and p62 after infection with HIV-1. We observed that, in the absence of ubiquitination activity, there is significantly less colocalization of RhTRIM5 α proteins and p62 (Fig. 6B), indicating that the ubiquitination activity of TRIM5 α is essential for its association with autophagic adaptor proteins, such as p62. We next wanted to determine if TRIM5 α requires autophagic adaptor proteins, namely p62, for its association with the autophagic membrane marker LC3. To address this question, we utilized clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated

FIG 6 K63-linked ubiquitination is necessary for TRIM5 α association with p62 and autophagosome membranes. (A) A549 cells expressing the indicated Flag-tagged RhTRIM5 α -DUB fusion proteins were synchronously infected with an HIV-1 reporter virus. At 6 hpi, the degree of colocalization between Flag and p62 was measured. (B) Colocalization is quantified as the proportion of double-positive (p62⁺ Flag⁺) puncta out of the total number of Flag puncta. Error bars represent SEM from at least 20 images taken for each cell line. Data are representative of three independent experiments. Statistical significance was determined by one-way ANOVA: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. (C) A549 cells were depleted of p62 via CRISPR-Cas9 genome editing. These cells were subsequently transduced to express YFP-LC3 and the indicated DUB constructs. Western blot depicts the expression of Flag fusion protein (top), p62 knockout (KO) (second panel), YFP-LC3 (third panel), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control (bottom). (D) Following infection with an HIV-1 reporter virus, the degree of colocalization between the different TRIM5 α fusion proteins (Flag tagged) and YFP-LC3 was measured. Error bars represent SEM from at least 20 images taken for each cell line. Data are representative of three independent experiments. Statistical significance was determined by Student's t test: ***, $P < 0.001$; *, $P < 0.05$.

protein 9 (Cas9) genome editing to target p62 or a nontargeting guide RNA (gRNA) in A549 cells stably expressing YFP-LC3 (Fig. 6C). These cells were subsequently transduced to stably express either RhTRIM5 α or UL36*-RhTRIM5 α , given that this was the DUB fusion protein that most potently colocalized with YFP-LC3 (Fig. 4B). We quantified the association between RhTRIM5 α and LC3 and observed that, in the absence of p62, there is significantly less colocalization between YFP-LC3 and RhTRIM5 α (Fig. 6D). Taken

together, these data indicate that TRIM5 α requires ubiquitination activity for its association with the autophagic adaptor protein p62 and that, without the p62, TRIM5 α is unable to associate with the autophagosome membrane marker LC3.

DISCUSSION

In this study, we used RhTRIM5 α conjugated to deubiquitinating enzymes specific for K63- and K48-linked ubiquitin to dissect the role of these specific ubiquitin linkages in the antiretroviral effector functions of TRIM5 α and its recognition by autophagic proteins. This approach has the advantage of specifically altering the ubiquitin-dependent functions of TRIM5 α without grossly perturbing cellular degradative pathways, as occurs with pharmacological inhibitors or the expression of ubiquitin pools incapable of specific ubiquitin linkages (i.e., K48R or K63R ubiquitin).

Examination of RhTRIM5 α fused to both K63- and K48-specific DUBs, analyzed with antibodies specific for either type of ubiquitin linkage, demonstrated that both forms of ubiquitin can be conjugated to RhTRIM5 α and that DUBs specific for these linkages effectively removed these linkages from RhTRIM5 α (Fig. 1), providing evidence that both K63- and K48-linked ubiquitin can be conjugated to RhTRIM5 α . These tools provided the opportunity to interrogate the role of each type in linkage in TRIM5 α effector functions and association with autophagic pathway proteins.

Previous studies have observed that the destabilization of retroviral capsids and inhibition of HIV-1 reverse transcription by TRIM5 proteins is sensitive to proteasome inhibitors, such as MG132 (14, 16, 20), and one implication of these observations is that TRIM5 α utilizes proteasome machinery to destabilize retroviral capsids. However, proteasomal degradation is generally associated with K48-linked rather than K63-linked polyubiquitin linkages (21–23, 28–30), and our results here suggest that the same steps of restriction that are sensitive to proteasome inhibition are also sensitive to K63-specific DUB activity (AMSH-LP) (Fig. 3 and 4) but are not influenced when TRIM5 α is appended to a K48-specific DUB (OTUB1). Notably, while the manuscript was in revision, a paper by Fletcher et al. observed that TRIM5 α assembly around restriction-sensitive viral cores triggers TRIM5 α autoubiquitination with K63-linked ubiquitin, which drives the proteasomal degradation of TRIM5 α (45). Our data using DUB fusions in this study are entirely consistent with such a noncanonical function of K63-linked ubiquitin and are also consistent with our previous observation that all aspects of TRIM5 α restriction are insensitive to depletion of the key autophagic mediators Beclin1 and Atg5 (34). Such a model can also reconcile prior studies that observe that proteasomal inhibition prevents TRIM5 α -mediated core disassembly (14, 17) and the degradation of TRIM5 α observed in response to saturating levels of restriction-sensitive virus (19).

In this study, we also observe that K63-linked ubiquitin can lead to the recruitment of autophagic proteins, such as LC3 and p62 (Fig. 4 and 6). As our previous studies show that these events are separable from steps in retroviral restriction (34), as described above, it seems likely that autophagic recruitment of TRIM5 α assemblies plays a role in other aspects of TRIM5 α function. In this regard, a number of studies have demonstrated that TRIM5 α assembly can trigger innate immune activation in a manner that is also dependent on K63-linked polyubiquitin chains, which can induce the expression of genes leading to an antiviral state (13, 45, 46). We speculate that autophagic recognition of TRIM5 α assemblies ensures fine-tuning of such signaling assemblies.

As it is known that TRIM5 α is capable of autoubiquitination *in vitro* (12, 47), it seems likely that the ubiquitination patterns and dependencies we observed are due to TRIM5 α autoubiquitination, though we cannot exclude the possibility that some TRIM5 α ubiquitination occurs through another, unidentified cellular ubiquitin ligase. The idea that autoubiquitination is responsible for the effects observed is also supported by the recent study by Fletcher et al. (45).

In summary, these studies demonstrate that the ability of TRIM5 α to induce the disassembly of the HIV-1 capsid during restriction and inhibit reverse transcription is

FIG 7 Current perspective on the mechanism of retroviral restriction by TRIM5 α . (A) During infection, retroviruses reverse transcribe their RNA genome (pink) into DNA (purple), uncoat their capsid (CA) (blue), and traffic to the nucleus for integration. (B) TRIM5 α (red blocks) exists as diffuse protein and cytoplasmic bodies which colocalize with autophagosomes (green circles). Upon retroviral infection, TRIM5 α forms assemblies around the capsid, which is sufficient to inhibit infection (C). (D) K63-specific ubiquitin ligase activity of TRIM5 α is critical for its association with autophagosome membrane markers and its ability to destabilize viral cores prior to the completion of reverse transcription. In the absence of K63-specific ubiquitin ligase activity, TRIM5 α forms a stable association with the capsid, allowing reverse transcription to proceed; however, infection is still blocked. This favors a model whereby the formation of a TRIM5 α assembly around a capsid is sufficient to inhibit infection, while ubiquitin ligase activity of TRIM5 α is needed to inhibit reverse transcription. RT, reverse transcriptase.

dependent on the generation of K63-linked ubiquitin by TRIM5 α (Fig. 7). Similarly, the ability to associate with autophagosomes was dependent on both K63-linked ubiquitination and the autophagic adaptor p62, although our previous studies provide evidence that capsid disassembly and inhibition of reverse transcription are not dependent on either p62 (36) or autophagic effectors (34). This study also establishes tools that can be used to probe the role of linkage-specific ubiquitin conjugation in the cellular function of other ubiquitin ligases.

MATERIALS AND METHODS

Cells and pharmaceuticals. The HeLa, HEK293T, and A549 cell lines utilized in this study were obtained from the American Type Culture Collection. HeLa, HEK293T, and A549 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml ciprofloxacin.

Cloning and generation of stable cell lines. Generation of deubiquitinase (DUB) RhTRIM5 α fusion proteins was performed as described previously (38). Briefly, the catalytically active and inactive DUB enzymes utilized in this study were as follows: HSV-1 UL36 DUB (residues 15 to 260) (38, 40), AMSH-LP (residues 265 to 436, a kind gift from the Fukui laboratory, University of Tokyo) (48), and OTUB1 (a kind gift from the laboratory of Wade Harper [plasmid number 22551; Addgene]; mutation described in Table 1 of reference 49). Each of these DUBs was cloned, in frame, into pLVX Flag-RhTRIM5 α , in between the Flag tag and a short linker sequence before the start codon of RhTRIM5 α , using splicing by overhang extension (SOE) PCR. For inducible expression of DUB constructs, Flag-tagged UL36 (residues 15 to 260) (38, 40), AMSH-LP (residues 265 to 436), and OTUB1 were cloned, in frame, into a pLKOΔ-IP plasmid (a kind gift from the laboratory of Paul Bieniasz) (50).

To generate stable cell lines, lentivirus was prepared by transfecting equal amounts of vesicular stomatitis virus glycoprotein (VSV-G), psPAX2 (from Didier Trono, NIH AIDS Reagent Program [catalog number 11348]) (51), and the pLVX-DUB-RhTRIM5 α constructs or pLKOΔ-DUB into HEK293T cells. Viral supernatant was harvested 48 h posttransfection, filtered through 0.45- μ m filters (Millipore), and applied

to HeLa or A549 cells. Forty-eight hours after transduction, puromycin was added to the cells, and following selection, cells were collected for phenotypic analysis.

Stable expression of YFP-LC3- or HA-tagged RhTRIM5 α was achieved by cloning YFP-LC3- or HA-tagged RhTRIM5 α , as described previously (52), into retroviral vector (53) EXN, producing EXN YFP-LC3 or RhTRIM5 α . To generate stable cell lines, retrovirus was prepared by transfecting equal amounts of VSV-G, pCigB packaging plasmid, and EXN YFP-LC3 or RhTRIM5 α into HEK293T cells. Viral supernatant was harvested as described above and applied to A549 or HeLa cells. Forty-eight hours after transduction, G418 was added to the cells, and following selection, cells were collected for phenotypic analysis.

p62/SQSTM1 knockout A549 cell lines were generated using a modified version of LentiCRISPRv2 (Addgene plasmid number 52961, a gift from Feng Zhang) (54) in which the puromycin resistance cassette was replaced with a hygromycin resistance cassette, producing LentiCRISPRv2-Hygromycin. Guide RNA sequences were generated using the CRISPR design tool at <http://www.crispr.mit.edu>. The following oligonucleotides were annealed and cloned into LentiCRISPRv2-Hygromycin: 5'-CACCGTGAACACGGACACTTCGGG-3', targeting p62/SQSTM1, and 5'-CACCGGCACTACCAGAGCTAACTCA-3', targeting the control sequence. Lentivirus was prepared by transfecting equal amounts of VSV-G, psPAX2 (from Didier Trono, NIH AIDS Reagent Program [catalog number 11348]) (51, 55), and LentiCRISPRv2-Hygromycin (containing the guide RNA of interest) into HEK293T cells. Viral supernatant collection, transduction, and selection of stable cell lines was performed as described above.

Generation and quantification of reporter virus. HIV-1 reporter virus was prepared as described previously (36). Briefly, HIV-1 reporter virus was produced by polyethyleneimine (56) transfection of HEK293T cells seeded into 25-cm tissue culture plates with 10 μ g of VSV-G and 15 μ g of the proviral construct R7 Δ EnvGFP, in which the Nef gene was replaced with green fluorescent protein (GFP). Reporter virus was harvested as described above and previously (57). Titration of reporter virus was conducted using the real-time quantitative PCR (qPCR)-based, product-enhanced reverse transcription (RT) (PERT) assay with SYBR Green I (SG-PERT), as described previously (55). Briefly, lysed viral supernatant was incubated with MS2 RNA (product number 10165948001; Sigma-Aldrich), and the resulting reverse transcription products were quantified by RT-qPCR using SYBR green (catalog number 1725124; Bio-Rad).

In vitro ubiquitination assay and Western blotting. HEK293T cells were cultured as described above and were transfected with equal amounts of the pLVX-DUB or DUB*-RhTRIM5 α construct and hemagglutinin-tagged ubiquitin (HA-Ub)-expressing plasmid DNA as previously described (58). Cells were harvested 48 h posttransfection and washed twice with ice-cold phosphate-buffered saline (PBS). They were lysed in Tris-buffered saline (TBS) supplemented with 1% Triton X-100, 0.1% IGEPAL, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) on ice for 30 min and spun for 15 min at 13,000 $\times g$ for pellet cell debris. The supernatant was filtered through a 0.22- μ m filter, and clear lysates were obtained. The expression levels of fusion proteins and HA-Ub were evaluated by Western blotting. The protein contents of whole-cell lysates were quantified by using a bicinchoninic acid (BCA) protein assay kit (Thermo Fischer), and equal amounts of protein boiled with Laemmli 2 $\times$ SDS sample buffer for 5 min. They were loaded onto a precast polyacrylamide gel for SDS-PAGE (Mini-Protein TGX; Bio-Rad) and separated by electrophoresis. Proteins were transferred onto nitrocellulose membranes and detected by incubation with the following antibodies: anti-HA antibody (clone 3F10) conjugated to horseradish peroxidase (HRP) (Roche Applied Science, Indianapolis, IN, USA) and anti-Flag M2 antibody-HRP (product number A8592; Sigma). The cell lysates containing equivalent amounts of DUB fusion proteins and Ha-Ub were used for immunoprecipitations (IPs). IPs were done by using anti-Flag antibody conjugated to magnetic beads (anti-Flag M2 antibody, product number M8823; Sigma) to selectively concentrate Flag-tagged fusion proteins. The concentrated proteins were eluted with nonreducing SDS-PAGE sample buffer by boiling for 3 min. The ubiquitination status was evaluated by Western blotting with anti-HA, anti-Flag, and antiubiquitin (K48 or K63 linkage specific, catalog numbers 179434 and 140601; Abcam) antibodies.

Quantification of reverse transcription products. Viral reverse transcription products were quantified as described previously (16). Briefly, DNA was extracted from cells infected with R7 Δ EnvGFP reporter virus at 24 h postinfection (hpi) using the Qiagen DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. DNA was subsequently quantified, and equal amounts of DNA were digested with DpnI (New England Biolabs). Reverse transcription products were quantified by real-time quantitative PCR using SYBR green (catalog number 1725124; Bio-Rad) and the following primers: GFP-FWD, 5'-CAACAGCCACAACGTCTATATCAT-3'; GFP-REV, 5'-ATGTTGTGGCGGATCTGAAG-3'; GAPDH-FWD, 5'-GCACCGTCAAGGCTGAGAAC-3'; and GAPDH-REV, 5'-GCCTCTCCATGGTGGTGAA-3'.

Infection and immunofluorescence microscopy. A549 and HeLa cells were allowed to adhere to fibronectin-treated glass coverslips placed in wells of a 24-well plate. Synchronized infection was performed by spinoculation of reporter virus onto cells at 13°C for 2 h at 1,200 $\times g$, after which virus-containing medium was removed and replaced with warm medium. Coverslips containing cells were incubated at 37°C for 6 to 8 h and were subsequently fixed with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.8. Cells were permeabilized with 0.1% saponin, 10% normal donkey serum, 0.01% sodium azide in PBS. We used the following primary antibodies: rabbit anti-Flag (product number F1804; Sigma-Aldrich), mouse anti-HIV-1 p24 (catalog number sc-69728; Santa Cruz), rabbit anti-p62/SQSTM1 (catalog number 7695S; Cell Signaling), and mouse anti-p62/SQSTM1 (catalog number sc-28359; Santa Cruz) antibodies. Primary antibodies were labeled with fluorophore-conjugated donkey anti-mouse or anti-rabbit antibody (Jackson Immuno-

Research Laboratories, Inc., West Grove, PA, USA). Images were collected with a DeltaVision microscope (Applied Precision, Issaquah, WA, USA) equipped with a digital camera (CoolSNAP HQ; Photometrics, Tucson, AZ, USA), using a 1.4-numerical aperture (NA) 100 $\times$ lens objective, and were deconvolved with SoftWoRx software (Applied Precision, Issaquah, WA, USA).

Image analysis. For each cell line in each experiment, 20 to 30 z-stack images were acquired using identical acquisition parameters. Deconvolved images were analyzed using Imaris software (Bitplane). For analysis of colocalization between Flag-RhTRIM5 α or the DUB fusion proteins and markers indicated in the figures, an algorithm was designed to create a three-dimensional surface around the signal of interest (Flag), and subsequently, the maximum fluorescence intensity associated with the other channels of interest was measured. The algorithm was applied to all the images within a given experiment. Colocalization was defined as the presence of a signal intensity above the background present in the surface, and the same colocalization threshold was maintained for a given channel for all the images in a particular experiment. Both graphing and statistics calculations were performed in Prism (GraphPad Software, Inc.). As indicated on each graph shown in the figures, data are presented as the mean values and standard errors of the means (SEM), and significance was determined by Student's *t* test or analysis of variance (ANOVA) as indicated in the legends.

Fate of the capsid assay. The fate of the capsid assay was performed as previously described (3, 59, 60). Cell lines were infected for 8 h, detached with Pronase for 5 min on ice, and washed 3 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% (wt/vol) sucrose cushion in 1 $\times$ PBS and centrifuged at 100,000 $\times g$ 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.

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REFERENCES

- Ozato K, Shin DM, Chang TH, Morse HC, III. 2008. TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8:849–860. <https://doi.org/10.1038/nri2413>.
- Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. 2004. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* 427:848–853. <https://doi.org/10.1038/nature02343>.
- 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 TRIM5 α restriction factor. *Proc Natl Acad Sci U S A* 103:5514–5519. <https://doi.org/10.1073/pnas.0509996103>.
- 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 TRIM5 α self-association, capsid binding avidity, and retrovirus restriction. *J Virol* 83:10737–10751. <https://doi.org/10.1128/JVI.01307-09>.
- Javanbakht H, Yuan W, Yeung DF, Song B, Diaz-Griffero F, Li Y, Li X, Stremlau M, Sodroski J. 2006. Characterization of TRIM5 α trimerization and its contribution to human immunodeficiency virus capsid binding. *Virology* 353:234–246. <https://doi.org/10.1016/j.virol.2006.05.017>.
- 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 TRIM5 α protein that restricts human immunodeficiency virus type 1 replication. *J Virol* 82:11682–11694. <https://doi.org/10.1128/JVI.01562-08>.
- Wagner JM, Roganowicz MD, Skorupka K, Alam SL, Christensen D, Doss G, Wan Y, Frank GA, Ganser-Pornillos BK, Sundquist WI, Pornillos O. 2016. Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5 α . *Elife* 5:e16309. <https://doi.org/10.7554/eLife.16309>.
- Ganser-Pornillos BK, Chandrasekaran V, Pornillos O, Sodroski JG, Sundquist WI, Yeager M. 2011. Hexagonal assembly of a restricting TRIM5 α protein. *Proc Natl Acad Sci U S A* 108:534–539. <https://doi.org/10.1073/pnas.1013426108>.
- Li YL, Chandrasekaran V, Carter SD, Woodward CL, Christensen DE, Dryden KA, Pornillos O, Yeager M, Ganser-Pornillos BK, Jensen GJ, Sundquist WI. 2016. Primate TRIM5 proteins form hexagonal nets on HIV-1 capsids. *Elife* 5:e16269. <https://doi.org/10.7554/eLife.16269>.
- Wagner JM, Christensen DE, Bhattacharya A, Dawidziak DM, Roganowicz MD, Wan Y, Pumroy RA, Demeler B, Ivanov DN, Ganser-Pornillos BK, Sundquist WI, Pornillos O. 2018. General model for retroviral capsid pattern recognition by TRIM5 proteins. *J Virol* 92:e01563-17. <https://doi.org/10.1128/JVI.01563-17>.
- Yudina Z, Roa A, Johnson R, Biris N, de Souza Aranha Vieira DA, Tshiperson V, Reszka N, Taylor AB, Hart PJ, Demeler B, Diaz-Griffero F, Ivanov DN. 2015. RING dimerization links higher-order assembly of TRIM5 α to synthesis of K63-linked polyubiquitin. *Cell Rep* 12:788–797. <https://doi.org/10.1016/j.celrep.2015.06.072>.
- Fletcher AJ, Christensen DE, Nelson C, Tan CP, Schaller T, Lehner PJ, Sundquist WI, Towers GJ. 2015. TRIM5 α requires Ube2W to anchor Lys63-linked ubiquitin chains and restrict reverse transcription. *EMBO J* 34:2078–2095. <https://doi.org/10.15252/embj.201490361>.
- Pertel T, Hausmann S, Morger D, Züger 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, Grütter MG, Luban J. 2011. TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472:361–365. <https://doi.org/10.1038/nature09976>.
- Wu X, Anderson JL, Campbell EM, Joseph AM, Hope TJ. 2006. Proteasome inhibitors uncouple rhesus TRIM5 α restriction of HIV-1 reverse transcription and infection. *Proc Natl Acad Sci U S A* 103:7465–7470. <https://doi.org/10.1073/pnas.0510483103>.
- Yang Y, Brandariz-Nunez A, Fricke T, Ivanov DN, Sarnak Z, Diaz-Griffero F. 2014. Binding of the rhesus TRIM5 α PRYSPRY domain to capsid is necessary but not sufficient for HIV-1 restriction. *Virology* 448:217–228. <https://doi.org/10.1016/j.virol.2013.10.012>.
- 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. <https://doi.org/10.1128/JVI.01052-06>.
- Campbell EM, Perez O, Anderson JL, Hope TJ. 2008. Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5 α . *J Cell Biol* 180:549–561. <https://doi.org/10.1083/jcb.200706154>.

18. Lukic Z, Hausmann S, Sebastian S, Rucci J, Sastri J, Robia SL, Luban J, Campbell EM. 2011. TRIM5 α associates with proteasomal subunits in cells while in complex with HIV-1 virions. *Retrovirology* 8:93. <https://doi.org/10.1186/1742-4690-8-93>.
19. Rold CJ, Aiken C. 2008. Proteasomal degradation of TRIM5 α during retrovirus restriction. *PLoS Pathog* 4:e1000074. <https://doi.org/10.1371/journal.ppat.1000074>.
20. Kutluay SB, Perez-Caballero D, Bieniasz PD. 2013. Fates of retroviral core components during unrestricted and TRIM5-restricted infection. *PLoS Pathog* 9:e1003214. <https://doi.org/10.1371/journal.ppat.1003214>.
21. Yau R, Rape M. 2016. The increasing complexity of the ubiquitin code. *Nat Cell Biol* 18:579–586. <https://doi.org/10.1038/ncb3358>.
22. Husnjak K, Dikic I. 2012. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu Rev Biochem* 81:291–322. <https://doi.org/10.1146/annurev-biochem-051810-094654>.
23. Komander D, Rape M. 2012. The ubiquitin code. *Annu Rev Biochem* 81:203–229. <https://doi.org/10.1146/annurev-biochem-060310-170328>.
24. van Tol S, Hage A, Giraldo MI, Bharaj P, Rajsbaum R. 2017. The TRIMendous role of TRIMs in virus-host interactions. *Vaccines (Basel)* 5:E23. <https://doi.org/10.3390/vaccines5030023>.
25. Rajsbaum R, Garcia-Sastre A, Versteeg GA. 2014. TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426:1265–1284. <https://doi.org/10.1016/j.jmb.2013.12.005>.
26. Ye Y, Rape M. 2009. Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* 10:755–764. <https://doi.org/10.1038/nrm2780>.
27. Ohtake F, Saeki Y, Ishido S, Kanno J, Tanaka K. 2016. The K48-K63 branched ubiquitin chain regulates NF-kappaB signaling. *Mol Cell* 64:251–266. <https://doi.org/10.1016/j.molcel.2016.09.014>.
28. Ikeda F, Dikic I. 2008. Atypical ubiquitin chains: new molecular signals. "Protein Modifications: Beyond the Usual Suspects" review series. *EMBO Rep* 9:536–542. <https://doi.org/10.1038/embor.2008.93>.
29. Ebner P, Versteeg GA, Ikeda F. 2017. Ubiquitin enzymes in the regulation of immune responses. *Crit Rev Biochem Mol Biol* 52:425–460. <https://doi.org/10.1080/10409238.2017.1325829>.
30. Reichard EL, Chirico GG, Dewey WJ, Nassif ND, Bard KE, Millas NE, Kraut DA. 2016. Substrate ubiquitination controls the unfolding ability of the proteasome. *J Biol Chem* 291:18547–18561. <https://doi.org/10.1074/jbc.M116.720151>.
31. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T. 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282:24131–24145. <https://doi.org/10.1074/jbc.M702824200>.
32. Kirkin V, McEwan DG, Novak I, Dikic I. 2009. A role for ubiquitin in selective autophagy. *Mol Cell* 34:259–269. <https://doi.org/10.1016/j.molcel.2009.04.026>.
33. Lamark T, Kirkin V, Dikic I, Johansen T. 2009. NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. *Cell Cycle* 8:1986–1990. <https://doi.org/10.4161/cc.8.13.8892>.
34. Imam S, Talley S, Nelson RS, Dharan A, O'Connor C, Hope TJ, Campbell EM. 2016. TRIM5 α degradation via autophagy is not required for retroviral restriction. *J Virol* 90:3400–3410. <https://doi.org/10.1128/JVI.03033-15>.
35. Mandell MA, Jain A, Arko-Mensah J, Chauhan S, Kimura T, Dinkins C, Silvestri G, Munch J, Kirchhoff F, Simonsen A, Wei Y, Levine B, Johansen T, Deretic V. 2014. TRIM proteins regulate autophagy and can target autophagic substrates by direct recognition. *Dev Cell* 30:394–409. <https://doi.org/10.1016/j.devcel.2014.06.013>.
36. O'Connor C, Pertel T, Gray S, Robia SL, Bakowska JC, Luban J, Campbell EM. 2010. p62/sequestosome-1 associates with and sustains the expression of retroviral restriction factor TRIM5 α . *J Virol* 84:5997–6006. <https://doi.org/10.1128/JVI.02412-09>.
37. Ritorto MS, Ewan R, Perez-Oliva AB, Knebel A, Buhrlage SJ, Wightman M, Kelly SM, Wood NT, Virdee S, Gray NS, Morrice NA, Alessi DR, Trost M. 2014. Screening of DUB activity and specificity by MALDI-TOF mass spectrometry. *Nat Commun* 5:4763. <https://doi.org/10.1038/ncomms5763>.
38. Campbell EM, Weingart J, Sette P, Opp S, Sastri J, O'Connor SK, Talley S, Diaz-Griffero F, Hirsch V, Bouamr F. 2016. TRIM5 α -mediated ubiquitin chain conjugation is required for inhibition of HIV-1 reverse transcription and capsid destabilization. *J Virol* 90:1849–1857. <https://doi.org/10.1128/JVI.01948-15>.
39. Kim ET, Oh SE, Lee YO, Gibson W, Ahn JH. 2009. Cleavage specificity of the UL48 deubiquitinating protease activity of human cytomegalovirus and the growth of an active-site mutant virus in cultured cells. *J Virol* 83:12046–12056. <https://doi.org/10.1128/JVI.00411-09>.
40. Stringer DK, Piper RC. 2011. A single ubiquitin is sufficient for cargo protein entry into MVBs in the absence of ESCRT ubiquitination. *J Cell Biol* 192:229–242. <https://doi.org/10.1083/jcb.201008121>.
41. Wang S, Wang K, Li J, Zheng C. 2013. Herpes simplex virus 1 ubiquitin-specific protease UL36 inhibits beta interferon production by deubiquitinating TRAF3. *J Virol* 87:11851–11860. <https://doi.org/10.1128/JVI.01211-13>.
42. Bhattacharya A, Alam SL, Fricke T, Zadrozny K, Sedzicki J, Taylor AB, Demeler B, Pornillos O, Ganser-Pornillos BK, Diaz-Griffero F, Ivanov DN, Yeager M. 2014. Structural basis of HIV-1 capsid recognition by PF74 and CPSF6. *Proc Natl Acad Sci U S A* 111:18625–18630. <https://doi.org/10.1073/pnas.1419945112>.
43. Fricke T, Buffone C, Opp S, Valle-Casuso J, Diaz-Griffero F. 2014. B1-2 destabilizes HIV-1 cores during infection and prevents binding of CPSF6 to the HIV-1 capsid. *Retrovirology* 11:120. <https://doi.org/10.1186/s12977-014-0120-x>.
44. Rogov V, Dotsch V, Johansen T, Kirkin V. 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell* 53:167–178. <https://doi.org/10.1016/j.molcel.2013.12.014>.
45. Fletcher AJ, Vaysburd M, Maslen S, Zeng J, Skehel JM, Towers GJ, James LC. 2018. Trivalent RING assembly on retroviral capsids activates TRIM5 ubiquitination and innate immune signaling. *Cell Host Microbe* 24:761–775.e6. <https://doi.org/10.1016/j.chom.2018.10.007>.
46. Tareen SU, Emerman M. 2011. Human Trim5 α has additional activities that are uncoupled from retroviral capsid recognition. *Virology* 409:113–120. <https://doi.org/10.1016/j.virol.2010.09.018>.
47. Yamauchi K, Wada K, Tanji K, Tanaka M, Kamitani T. 2008. Ubiquitination of E3 ubiquitin ligase TRIM5 α and its potential role. *FEBS J* 275:1540–1555. <https://doi.org/10.1111/j.1742-4658.2008.06313.x>.
48. Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, Ookata K, Nureki O, Iwai K, Komada M, Fukai S. 2008. Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* 455:358–362. <https://doi.org/10.1038/nature07254>.
49. Wang P, Joberty G, Buist A, Vanoosthuysen A, Stancu IC, Vasconcelos B, Pierrot N, Faelt-Savitski M, Kienlen-Campard P, Octave JN, Bantscheff M, Drewes G, Moechars D, Dewachter I. 2017. Tau interactome mapping based identification of Otub1 as Tau deubiquitinase involved in accumulation of pathological Tau forms in vitro and in vivo. *Acta Neuropathol* 133:731–749. <https://doi.org/10.1007/s00401-016-1663-9>.
50. Kane M, Zang TM, Rihn SJ, Zhang F, Kueck T, Alim M, Schoggins J, Rice CM, Wilson SJ, Bieniasz PD. 2016. Identification of interferon-stimulated genes with antiretroviral activity. *Cell Host Microbe* 20:392–405. <https://doi.org/10.1016/j.chom.2016.08.005>.
51. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 72:9873–9880.
52. Flavin WP, Bousset L, Green ZC, Chu Y, Skarpathiotis S, Chaney MJ, Kordower JH, Melki R, Campbell EM. 2017. Endocytic vesicle rupture is a conserved mechanism of cellular invasion by amyloid proteins. *Acta Neuropathol* 134:629–653. <https://doi.org/10.1007/s00401-017-1722-x>.
53. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisiewicz J, Lori F, Flexner C, Quinn TC, Chaisson RE, Rosenberg E, Walker B, Gange S, Gallant J, Siliciano RF. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* 5:512–517. <https://doi.org/10.1038/8394>.
54. Sanjana NE, Shalem O, Zhang F. 2014. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11:783–784. <https://doi.org/10.1038/nmeth.3047>.
55. Vermeire J, Naessens E, Vanderstraeten H, Landi A, Iannucci V, Van Nuffel A, Taghon T, Pizzato M, Verhasselt B. 2012. Quantification of reverse transcriptase activity by real-time PCR as a fast and accurate method for titration of HIV, lenti- and retroviral vectors. *PLoS One* 7:e50859. <https://doi.org/10.1371/journal.pone.0050859>.
56. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381:661–666. <https://doi.org/10.1038/381661a0>.
57. Campbell EM, Nunez R, Hope TJ. 2004. Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunode-

- iciency virus type 1 infectivity. *J Virol* 78:5745–5755. <https://doi.org/10.1128/JVI.78.11.5745-5755.2004>.
58. Sette P, Nagashima K, Piper RC, Bouamr F. 2013. Ubiquitin conjugation to Gag is essential for ESCRT-mediated HIV-1 budding. *Retrovirology* 10:79. <https://doi.org/10.1186/1742-4690-10-79>.
59. Diaz-Griffero F, Vandegraaff N, Li Y, McGee-Estrada K, Stremlau M, Welikala S, Si Z, Engelman A, Sodroski J. 2006. Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. *Virology* 351:404–419. <https://doi.org/10.1016/j.virol.2006.03.023>.
60. Yang Y, Luban J, Diaz-Griffero F. 2014. The fate of HIV-1 capsid: a biochemical assay for HIV-1 uncoating. *Methods Mol Biol* 1087:29–36. https://doi.org/10.1007/978-1-62703-670-2_3.