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RESEARCH PAPER

Rab3a, a small GTP-binding protein, is required for the stabilization of the murine leukaemia virus Gag protein

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ABSTRACT

We recently identified a CD63-interacting protein to understand the role of CD63 in virion production of the human immunodeficiency virus type 1, and we have found that Rab3a forms a complex with CD63. In this study, we analysed the effect of Rab3a on virion production of the murine leukaemia virus (MLV), which is another member of the retrovirus family. We found that Rab3a silencing induced lysosomal degradation of the MLV Gag protein, and recovery of the Rab3a expression restored the level of the Gag protein through a complex formation of MLV Gag and Rab3a, indicating that Rab3a is required for MLV Gag protein expression. In contrast, CD63 silencing decreased the infectivity of released virions but had no effect on virion production, indicating that CD63 facilitates the infectivity of released MLV particles. Although Rab3a induced CD63 degradation in uninfected cells, the complex of MLV Gag and Rab3a suppressed the Rab3a-mediated CD63 degradation in MLV-infected cells. Finally, we found that the MLV Gag protein interacts with Rab3a to stabilize its own protein and CD63 that facilitates the infectivity of released MLV particles. Considering the involvement of Rab3a in lysosome trafficking to the plasma membrane, it may also induce cell surface transport of the MLV Gag protein.

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Introduction

Rab family members of small GTP-binding proteins regulate transport of intracellular vesicles [1] and are involved in the replication of the human immunodeficiency virus type 1 (HIV-1) [2]. Some of these proteins are incorporated into HIV-1 particles [3]. Rab7a [4], Rab9 [5], and Rab27a [6] are essential for HIV-1 virion production. Furthermore, Rab11-FIP1C [7] and Rab14 [8] induce the incorporation of the HIV-1 envelope glycoprotein (Env) complex into the virus particles. Rab6 is required for HIV-1 entry into host cells [9].

Recently, we demonstrated that a complex of Rab3a with CD63 is formed and induces lysosomal degradation of CD63 [10]. CD63 is efficiently incorporated into HIV-1 particles, suggesting that CD63 participates in the formation of an HIV-1 virion. Rab3a is involved in granule exocytosis in sperm [11,12] and eggs [13]. The GTP-binding form of Rab3a attaches to the vesicle membrane and promotes docking of vesicles onto the plasma membrane. Upon GTP hydrolysis, Rab3a dissociates from the vesicle membrane, thus, allowing exocytosis to proceed. Recent studies have reported that Rab3a is required for lysosome positioning and plasma membrane repair [14–16]. Although CD63 is efficiently incorporated into HIV-1 particles and binds to Rab3a, the Rab3a protein was not detected in virion-containing fractions, suggesting that only Rab3a-free CD63 is incorporated into HIV-1 particles [10]. Rab3a overexpression exerted a moderate inhibitory effect, whereas silencing its expression had no effect on HIV-1 virion production [10]. These results indicate that Rab3a has a minor role in HIV-1 virion production.

CD63 is a member of the tetraspanin family. The proteins of this family form special microdomains in the plasma membrane, which are termed as tetraspanin-enriched microdomains (TEMs) that have been reported to be involved in several biological events [17,18]. Tetraspanin proteins are known to participate in HIV-1 replication [17,18]. It has been demonstrated

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Supplemental data for this article can be accessed here.

that CD63 expressed in target cells disrupts the trafficking of CXCR4 to the plasma membrane and inhibits CXCR4-tropic HIV-1 infection [19]. CD63 reportedly diminishes the membrane fusion activity of HIV-1 envelope glycoprotein [20,21]. Incorporation of CD63 into HIV-1 particles was found to attenuate the infectivity of HIV-1 particles [22]. These reports suggest that CD63 is a negative regulator of HIV-1 infection.

However, several lines of evidence suggest that tetraspanins are essential for HIV-1 infection. CD63 silencing in target cells has been reported to inhibit HIV-1 infection [23,24]. A monoclonal antibody against CD63 attenuated CCR5-tropic HIV-1 infection in macrophages [25]. Peptides derived from the extracellular domain of tetraspanins, including CD63, inhibited HIV-1 infection [26]. Most recently, it has been reported that a CD63-high cell population displayed enrichment of HIV-1 infection using single-cell analysis, suggesting that CD63-high cells are more susceptible to HIV-1 infection [27].

Tetraspanin proteins also play an important role in virion production of HIV-1, although several contradictory reports indicate that CD63 silencing does not affect HIV-1 particle production [28,29]. HIV-1 particles are formed on the TEMs of HIV-1-producing cells [30–33]. Although CD63 is specifically localized to late endosomes/lysosomes and HIV-1 particles are formed in plasma membranes, CD63 is preferentially incorporated into HIV-1 particles [3,33–35], thus, suggesting its role in HIV-1 virion formation. Moreover, CD63 silencing decreased the level of HIV-1 Gag protein in culture supernatants, again suggesting its role in HIV-1 virion production [10,36,37]. We have previously also demonstrated that gamma–interferon–inducible lysosomal thiol reductase restricts HIV-1 virion production by digesting the disulphide bonds of CD63 and that a CD63 mutant with serine substitutions at conserved cysteine amino acid residues (CD63 TCS) also suppresses HIV-1 virion production [38]. Furthermore, knockdown of CD81, another member of the tetraspanin family, was found to significantly inhibit HIV-1 virion production [39].

Murine leukaemia virus (MLV), another member of the retrovirus family, induces leukaemia[40], immunodeficiency [41], or neurological disease [42] in susceptible mice and is, therefore, used in mouse models of human disorders. MLV is also used as a vesicle to transfer genes of interest into target cells in several biological fields [43]. Retrovirus vectors are generally constructed by the transfection of human 293 T cells. Therefore, understanding the mechanism of MLV replication in human 293 T cells would contribute towards improving MLV vectors in the efficient transduction of target cells. Although the role of Rab and tetraspanin family proteins in HIV-1 replication has been investigated vigorously, there is no clear knowledge on the proteins involved in MLV replication.

In this study, we examined the effects of Rab3a and CD63 on MLV virion production and found that Rab3a silencing significantly reduced the MLV Gag protein level and virion production, indicating that Rab3a is a critical cellular factor in MLV replication. CD63 silencing in MLV-producing cells did not affect MLV virion production but moderately decreased the viral titres, suggesting that CD63 incorporated into MLV virions enhances the infectivity of the released MLV particles. Although Rab3a induces lysosomal degradation of CD63, the complex formation of MLV Gag and Rab3a proteins disrupted CD63 degradation.

Results

Endogenous Rab3a is required for gag protein expression

To investigate whether endogenous Rab3a is involved in MLV vector production, 293 T cells were inoculated with a lentiviral vector encoding shRNA against Rab3a mRNA (shRab3a) (Supplementary Figure S1) and selected using puromycin because the lentiviral vector additionally encodes the puromycin-resistant gene. The puromycin-resistant cell pool was used in subsequent experiments. We confirmed that the endogenous Rab3a protein level was indeed reduced in the shRab3a-expressing cells (Figure 1a). The Rab3a-silenced 293 T cells were transfected with Moloney MLV (MoMLV) Gag-Pol, amphotropic Env, and LacZ-encoding MLV vector genome expression plasmids (amphotropic MLV vector construction plasmids), and culture supernatants obtained from the transfected cells were inoculated into TE671 cells to estimate transduction titres. The transduction titres were reduced to <1% by Rab3a silencing (Figure 1b). To determine whether this transduction titre reduction is induced by the reduction of the MLV Gag protein level, we performed western immunoblotting using antiMLV Gag p30 antibody. The levels of both p65 precursor (Pr65) and p30 mature proteins in the cell lysates and virion fractions were significantly decreased (Figure 1c). To determine whether Rab3a silencing also inhibits ecotropic MLV particle production, 293 T cells transduced with control or shRab3a-expressing lentivirus vector were transfected with MoMLV Gag-Pol, MoMLV Env, and LacZ-encoding MLV vector genome expression plasmids (ecotropic MLV vector construction plasmids), and culture supernatants obtained from the transfected cells

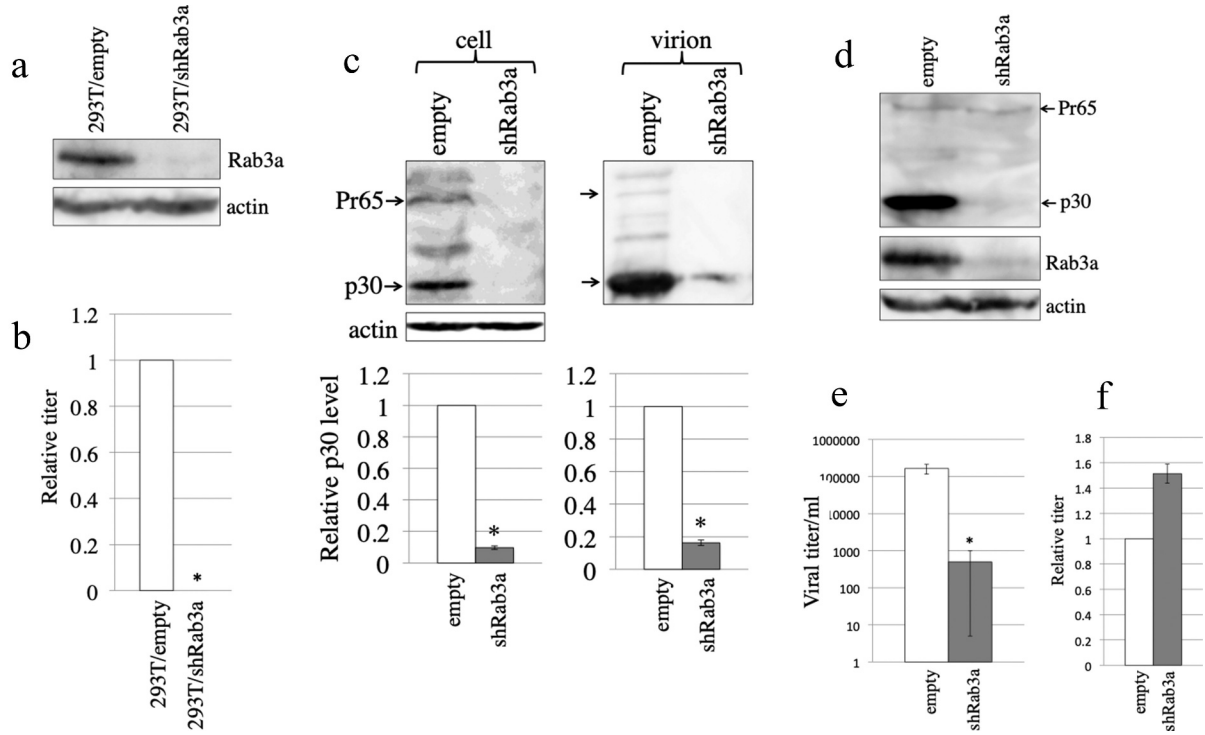

Figure 1. Rab3a silencing significantly reduces the MLV Gag protein level. (a) Endogenous Rab3a levels in the empty or shRab3a-encoding lentiviral vector-transduced 293 T cells were analysed by western blotting. (b) Rab3a-silenced cells were transfected with amphotropic MLV vector construction plasmids. Transduction titres of culture supernatants obtained from the transfected cells were measured. Transduction titres of culture supernatants obtained from the empty vector-transduced cells were always set to 1. Relative values of transduction titres in the empty vector-transduced cells ± standard deviations (SD) are indicated. This experiment was repeated three times. Asterisks indicate statistically significant differences. (c) Cell lysates and virion fractions prepared from the transfected cells were analysed by western blotting using the antiMLV Gag p30 antibody (upper panel). Levels of p30 were normalized by actin. The normalized p30 levels in the empty vector-transduced cells were always set to 1. Relative values to the normalized p30 levels in the empty vector-transduced cells ± SD are indicated (lower panel). This experiment was repeated three times. (d) Replication-competent Moloney MLV-producing TE671-mCAT1 cells were transduced with empty or shRab3a-encoding lentiviral vector. MLV Gag p30 and Rab3a levels in the transduced cells were analysed by western blotting. (e) Viral titres of the culture supernatants obtained from the transduced cells are indicated. This experiment was repeated three times. (f) Human 293 T cells transduced with empty or shRab3a-encoding lentiviral vector were inoculated with an amphotropic MLV vector. Transduction titres of empty vector-transduced cells were always set to 1. Relative values of transduction titres ± SD are indicated. This experiment was repeated three times.

were inoculated into 293 T cells expressing the mouse ecotropic MLV receptor protein (mCAT1) to estimate transduction titres. We found that Rab3a silencing also significantly attenuated the transduction titres of the ecotropic MLV vector (Supplementary Figure S2A) and Gag protein levels in the cell lysates (Supplementary Figure S2B).

When replication-competent Moloney MLV-producing TE671-mCAT1 cells were inoculated with the shRab3a-encoding lentiviral vector and selected with puromycin, the Rab3a protein level was found to decrease (Figure 1d). Rab3a silencing in Moloney MLV-producing TE671-mCAT1 cells reduced the MLV Gag p30 level in the cell lysates. The viral titres of culture supernatants were measured using the XC cell plaque assay, which showed a reduction to <0.1% due to Rab3a silencing (Figure 1e).

To analyse the impact of Rab3a expressed in target cells on sensitivity to MLV vector infection, the Rab3a-silenced 293 T cells were inoculated with the amphotropic MLV vector. Transduction titres showed no reduction due to Rab3a silencing in the target cells (figure 1f). These results indicate that endogenous Rab3a is required for Gag protein stability and, thus, MLV replication. To determine whether the importance of Rab3a for MLV replication is independent of the cell lines used, we also constructed replication-competent Moloney MLV-producing 293 T-mCAT1 cells. The levels of Rab3a in the 293 T and TE671 cells were similar (Supplementary Figure S3).

However, we could not use the cells because the growth of Moloney MLV-producing 293 T-mCAT1 cells was severely impaired.

The p30 protein levels in the virion fractions prepared from MoMLV-producing TE671 cells stably transduced with the shRab3a-encoding lentivirus vector were six-fold lower than those in fractions prepared from control Moloney MLV-producing cells (Supplementary Figure S4A). Hence, the culture supernatants from the control Moloney MLV-producing cells were diluted six-fold with fresh medium and inoculated to target cells. The viral titres of culture supernatants obtained from shRab3a-expressing cells were still <1% compared with those of the diluted culture supernatants obtained from the control Moloney MLV-producing cells (Supplementary Figure S4B). These results suggest that Rab3a is additionally required for the infectivity of released MLV particles.

To confirm the conclusion that Rab3a is important for MLV Gag protein expression, we constructed a C-terminally influenza virus haemagglutinin epitope (HA)-tagged Rab3a expression plasmid resistant to the shRab3a-mediated silencing, termed Rab3a RS-HA [10]. Compared with wild-type Rab3a, the Rab3a RS-HA expression plasmid has synonymous nucleotide substitutions but no amino acid changes in its shRab3a target sequence. Both control and shRab3a-expressing 293 T cells were transfected with the amphotropic MLV vector construction plasmids together with empty or Rab3a RS-HA expression plasmid. Consistent with the abovementioned result (Figure 1c), MLV Gag p30 levels were reduced by Rab3a silencing and recovered by the Rab3a RS-HA expression (Figure 2a). The transduction titres of culture supernatants obtained from the Rab3a-silenced cells were much lower than those of culture supernatants obtained from the control cells (Figure 2b), as mentioned earlier (Figure 1b). The transduction titres were recovered by the Rab3a RS-HA expression. Exogenous expression of Rab3a RS-HA in control 293 T cells moderately attenuated the transduction titres but did not affect the MLV Gag protein expression. These results support the conclusion that Rab3a is required for MLV Gag protein expression.

MLV gag protein is degraded in Lysosomes in the absence of Rab3a

MLV Gag protein levels were significantly reduced in the Rab3a-silenced cells. To assess whether the Gag protein is degraded in the lysosome or proteasome of Rab3a-silenced cells, we transfected the Rab3a-silenced 293 T cells with the MLV Gag-Pol expression plasmid and treated with inhibitors of lysosome (concanamycin

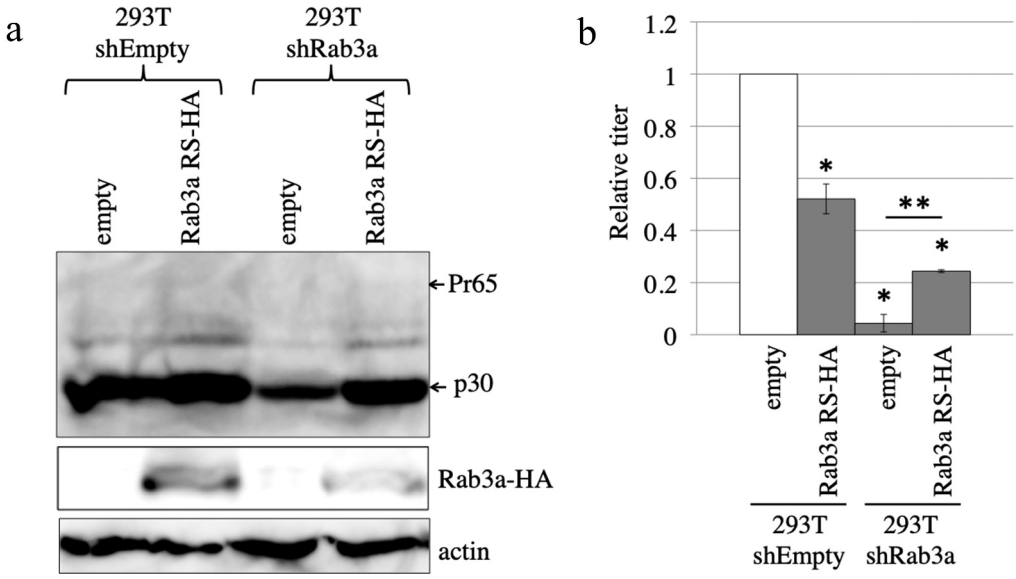

Figure 2. Rab3a is required for MLV Gag protein expression. (a) Control and Rab3a-silenced 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1 or Rab3a RS-HA expression plasmid. Cell lysates prepared from the transfected cells were analysed by western blotting using the antiMLV Gag p30, antiHA, or antiactin antibody. This experiment was repeated two times. (b) Transduction titres of the culture supernatants obtained from the transfected cells were measured. The transduction titres of the control cells transfected with pcDNA3.1 were always set to 1. Relative values to the transduction titres of the control cells transfected with pcDNA3.1 ± SD are indicated. Single asterisks indicate significant differences compared with the titres of the control cells transfected with pcDNA3.1. Double asterisks indicate significant differences compared with the titres of Rab3a-silenced cells transfected with cDNA3.1. This experiment was repeated three times.

A; CMA) or proteasome (MG-132). CMA treatment resulted in elevated MLV Gag protein levels, but this finding was not observed in the case of MG-132 treatment (Figure 3a and b). However, in control 293 T cells, MLV Gag protein levels remained unchanged by CMA or MG-132 treatment (Figure 3c and d). These results indicate that endogenous Rab3a inhibits the lysosomal degradation of the MLV Gag protein.

MLV gag forms a complex with Rab3a

The above-described results prompted us to speculate that the MLV Gag protein interacts with Rab3a. To

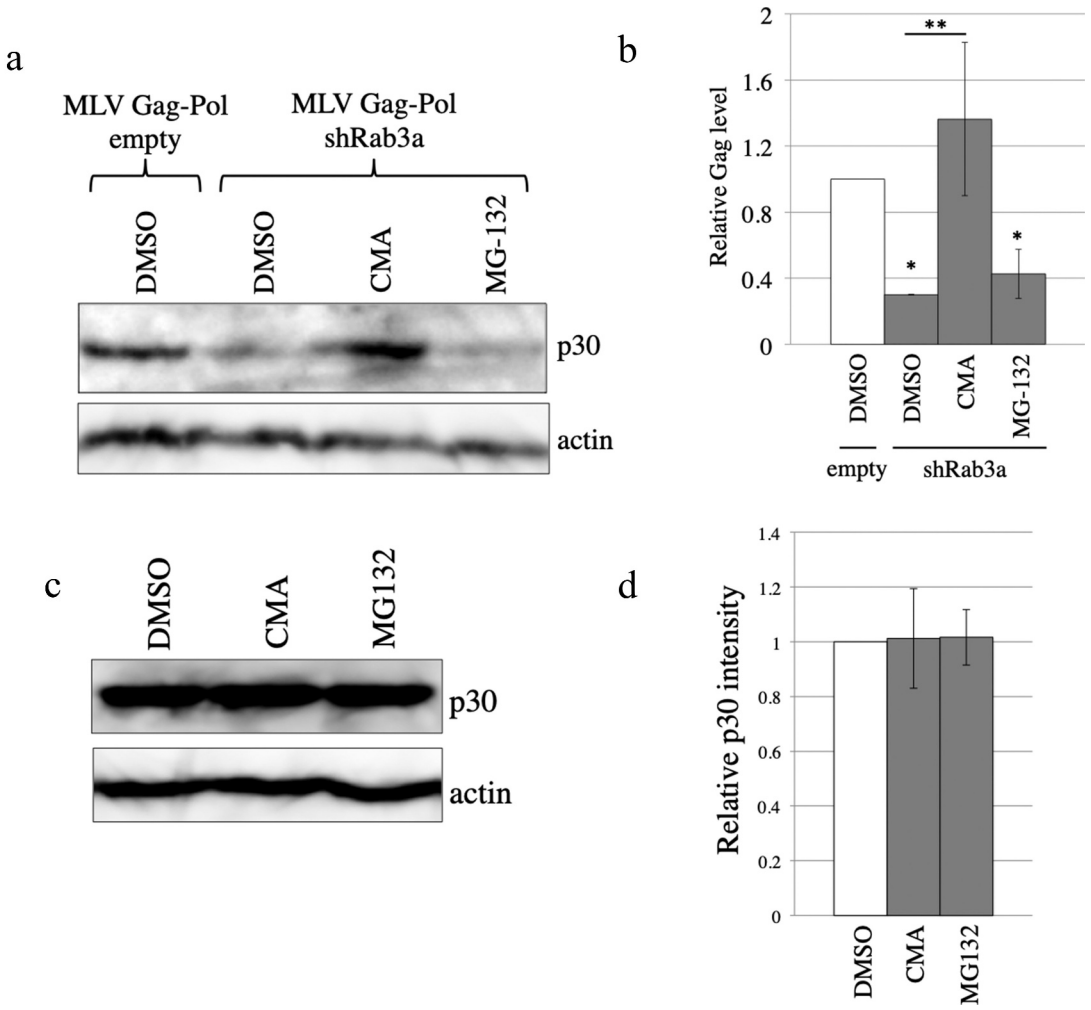

Figure 3. The MLV Gag protein is degraded in the lysosomes of Rab3a-silenced cells. (a) Control and Rab3a-silenced 293 T cells were transfected with the MLV Gag-Pol expression plasmid and treated with DMSO, concanamycin A (CMA), or MG-132. Cell lysates prepared from the treated cells were analysed by western blotting using the antiMLV Gag p30 or antiactin antibody. (b) Band intensities of MLV Gag p30 and actin proteins were measured using a densitometer, and p30 levels were normalized by actin levels. Normalized p30 levels of the control cells treated with DMSO were always set to 1. Relative values to the normalized p30 levels of the control cells treated with DMSO ± SD are indicated. Single asterisks represent significant differences compared with the p30 levels of the control cells treated with DMSO. double asterisks indicate significant differences compared with the p30 levels of the Rab3a-silenced cells treated with DMSO. This experiment was repeated three times. (c) Control 293 T cells were transfected with the MLV Gag-Pol expression plasmid and treated with DMSO, CMA, or MG-132. Cell lysates prepared from the treated cells were analysed by western blotting using the antiMLV Gag p30 or antiactin antibody. (d) Band intensities of the MLV Gag p30 and actin proteins were measured using a densitometer, and the p30 levels were normalized by actin levels. The normalized p30 levels of the control cells treated with DMSO were always set to 1. Relative values to the normalized p30 levels of the control cells treated with DMSO ± SD are indicated. This experiment was repeated three times.

evaluate our speculation, we transfected 293 T cells with the C-terminally HA-tagged Rab3a wild-type (Rab3a WT-HA) expression plasmid together with pcDNA3.1 or MLV Gag-Pol expression plasmid. The MLV Gag protein in the cell lysates prepared from transfected cells was precipitated using goat antiMLV p30 antibody, and the precipitates were analysed by western blotting using antiHA antibody. The Rab3a WT-HA protein was detected in the presence of the MLV Gag-Pol expression plasmid but not in its absence (Figure 4a). This result shows that the MLV Gag protein forms a complex with Rab3a WT-HA.

We had previously reported that Rab3a forms a complex with CD63 to induce lysosomal degradation of CD63. To determine whether the MLV Gag protein inhibits the complex formation of CD63 and Rab3a, we transfected the 293 T cells with CD63-GFP and Rab3a-HA expression plasmids together with pcDNA3.1 or the MLV Gag-Pol expression plasmid and treated the cells with CMA and MG-132 to prevent their degradation. The CD63-GFP protein in the cell lysates prepared from the transfected cells was precipitated using the antiGFP antibody, and the precipitates were analysed using the antiHA antibody. The Rab3a WT-HA protein was detected in cells transfected with pcDNA3.1 but not in the presence of MLV Gag-Pol expression plasmid (Figure 4b). This result demonstrates that the MLV Gag-Pol protein inhibits the complex formation of CD63 and Rab3a.

Rab3a protein is C-terminally geranylgeranylated, and this modification is required for its function [44]. The C-terminally HA-tagged Rab3a is not geranylgeranylated. Hence, expression plasmids of N-terminally HA-tagged Rab3a WT and RS were constructed (HA-Rab3a WT and HA-Rab3a RS). Like the C-terminally HA-tagged Rab3a RS, the N-terminally HA-tagged Rab3a RS recovered the Gag protein level (Figure 5a) and transduction titre (Figure 5b) that were decreased due to Rab3a silencing. Similarly, 293 T cells were transfected with the HA-Rab3a WT and MLV Gag-Pol expression plasmids, and the MLV Gag protein was precipitated using the MLV p30 antibody. The HA-Rab3a protein was detected in the precipitate (Figure 5c), indicating that the N-terminally HA-tagged Rab3a forms a complex with the MLV Gag protein.

Active and inactive forms of Rab3a decrease and increase MLV vector infectivity, respectively

Rab3a is a small GTP-binding protein that is involved in exocytosis. The GTP- and GDP-binding forms of Rab3a are active and inactive, respectively. Rab3a containing an asparagine substitution at its threonine

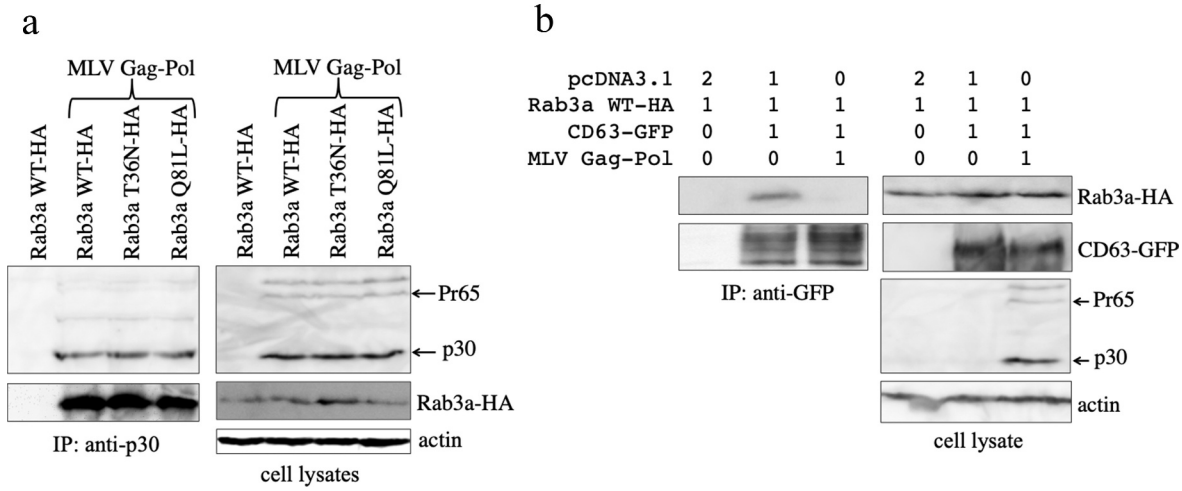

Figure 4. The MLV Gag protein inhibits the complex formation of CD63 and Rab3a. (a) Human 293 T cells were transfected with Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmid together with pcDNA3.1 or MLV Gag-Pol expression plasmid. Cell lysates prepared from the transfected cells were immunoprecipitated with the antiMLV p30 antibody. The precipitates were analysed by western blotting using the antiHA or antiMLV p30 antibody (left panels). Western blots of the cell lysates obtained using the antiMLV p30, antiHA, or antiactin antibody are also shown in the right panels. (b) Human 293 T cells were transfected with various combinations of pcDNA3.1, Rab3a WT-HA, CD63-GFP, and MLV Gag-Pol expression plasmids. Cell lysates prepared from the transfected cells were immunoprecipitated using the antiGFP antibody. The precipitates were analysed by western blotting using the antiHA or antiGFP antibody (left panels). Western blotting of cell lysates was performed using the antiHA, antiGFP, antiMLV p30, or antiactin antibody (right panels). These experiments were repeated two times.

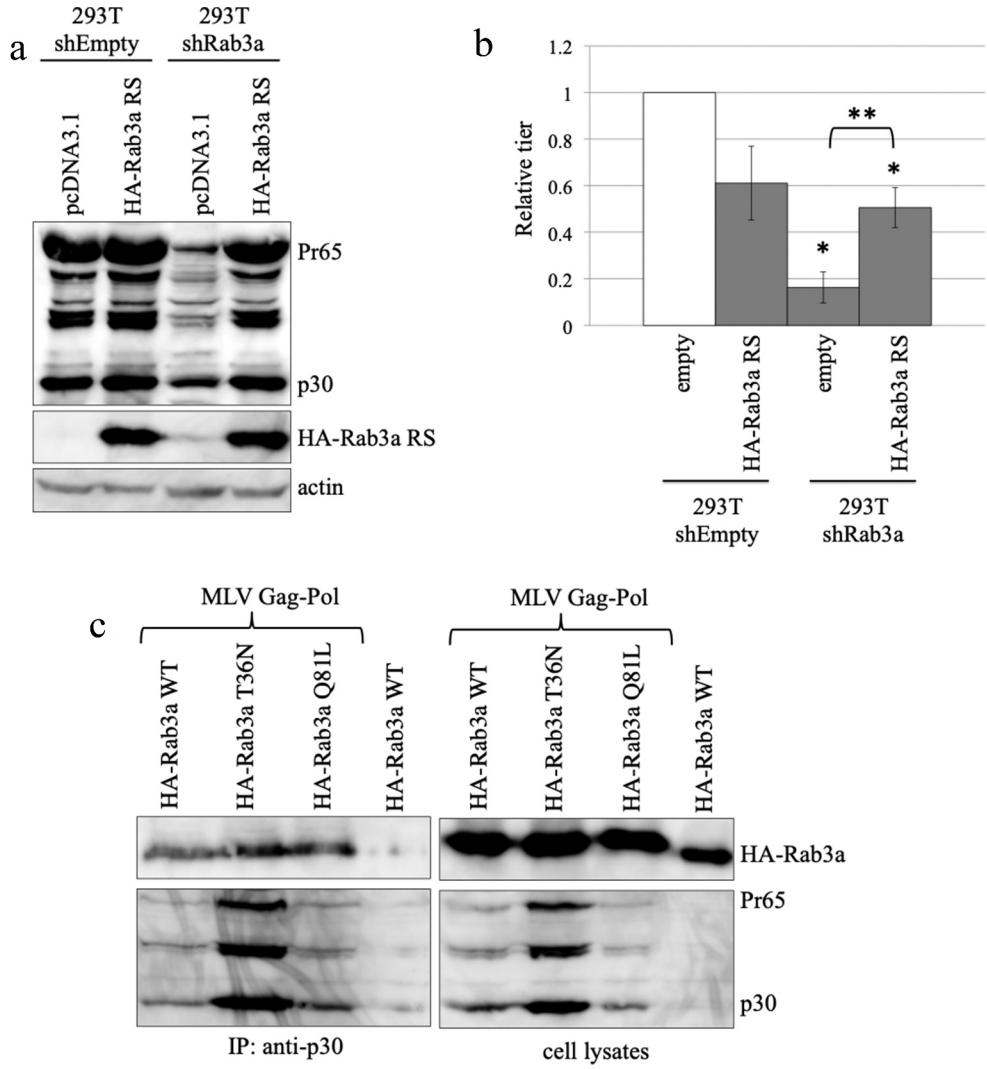

Figure 5. N-terminally HA-tagged Rab3a stabilizes and binds to the MLV Gag protein. (a) Human 293 T cells transduced with empty or shRab3a-encoding lentiviral vector were transfected with amphotropic MLV vector construction plasmids. Cell lysates prepared from the transfected cells were analysed by western blotting using antiMLV p30, antiHA, or antiactin antibody. This experiment was repeated two times. (b) Transduction titres of culture supernatants obtained from the transfected cells were measured. Transduction titres of control cells transfected with pcDNA3.1 were always set to 1. Relative values to transduction titres of control cells ± SD are indicated. This experiment was repeated three times. Single asterisks show significant differences compared with the transduction titres of the control cells. Double asterisks indicate significant differences between the two indicated groups. (c) Human 293 T cells were transfected with the indicated expression plasmids. Cell lysates prepared from the transfected cells were immunoprecipitated using the antiMLV p30 antibody. The precipitates were analysed by western blotting using the antiHA or antiMLV p30 antibody (left panels). The cell lysates were analysed by western blotting using the same antibody (right panels). This experiment was repeated two times.

amino acid residue 36 (T36N) cannot bind to GTP and, therefore, functions as a constitutively inactive mutant [45]. Another Rab3a mutant containing a leucine substitution at its glutamine amino acid residue 81 (Q81L) lacks GTPase activity and functions as a constitutively active mutant [46–49]. To evaluate the effects of these forms of Rab3a on MLV vector production, C-terminally HA-tagged T36N and Q81L Rab3a mutant expression plasmids (Rab3a T36N-HA and Rab3a Q81L-HA, respectively) were constructed [10]. The 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1, Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmid. Culture supernatants obtained from the transfected cells were inoculated into TE671 cells to estimate the transduction titres, which were found to be decreased by Rab3a WT-HA and Q81L-HA but increased by Rab3a T36N-HA (Figure 6a). However, MLV Gag protein levels in the cell lysates and virion fractions remained unchanged (Figure 6b

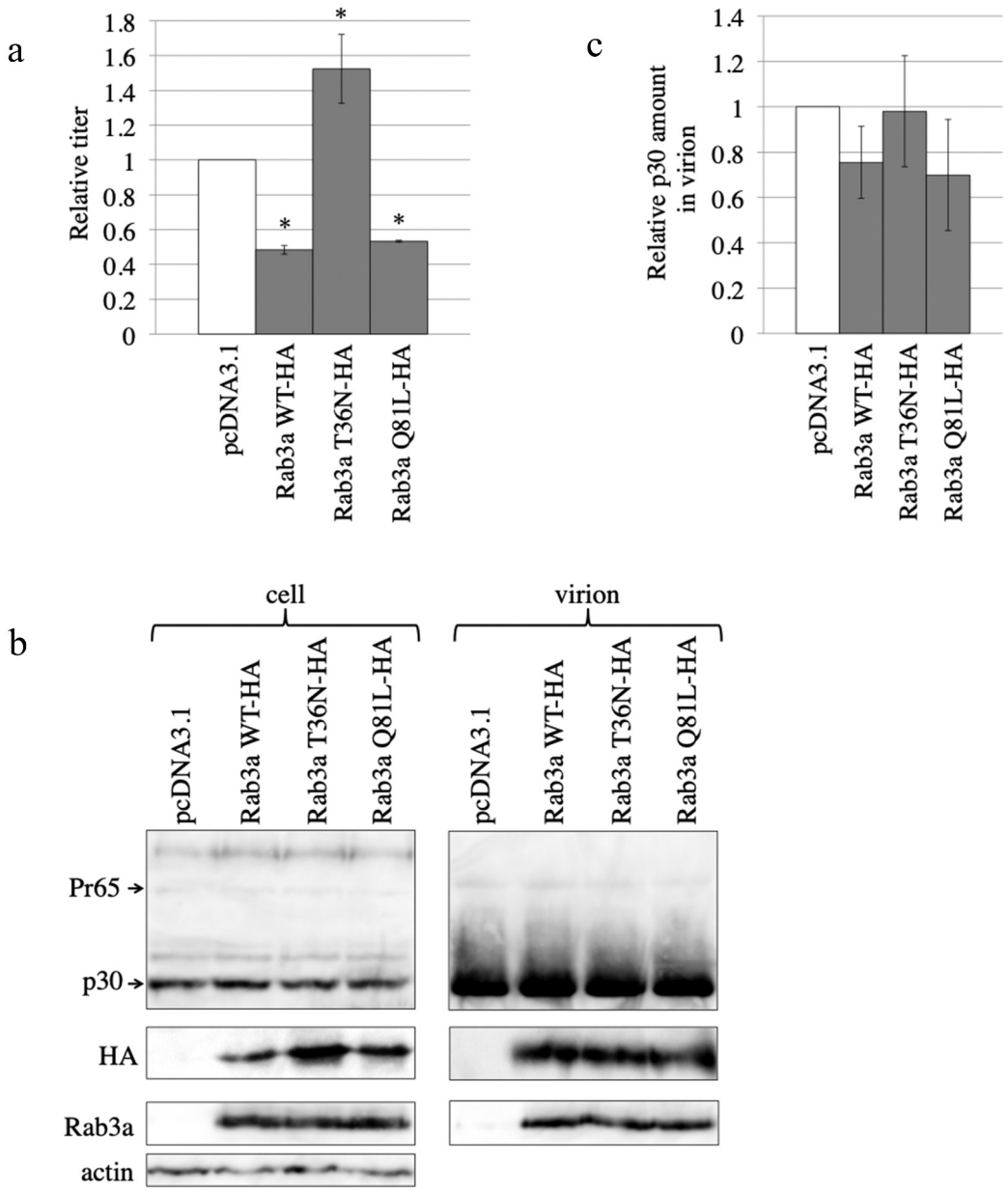

Figure 6. Active and inactive forms of C-terminally HA-tagged Rab3a decrease and increase MLV vector infectivity, respectively. (a) Human 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1, Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmids. Culture supernatants of the transfected cells were inoculated into TE671 cells, and transduction titres were measured. The transduction titres of the pcDNA3.1-transfected cells were always set to 1. Relative values to the transduction titres of the pcDNA3.1-transfected cells ± SD are indicated. This experiment was repeated three times. Asterisks indicate significant differences compared with control cell titres. (b) Cell lysates and virion pellets from the transfected cells were analysed by western immunoblotting. (c) p30 levels in virion pellets normalized by p30 levels in the cell lysates were calculated. Normalized p30 levels in the virion pellets of the pcDNA3.1-transfected cells were always set to 1, and relative values ± SD are indicated. This experiment was repeated three times.

and c). We performed direct western blotting using the antiRab3a antibody on cell lysates and virion fractions and found that the levels of endogenous Rab3a protein were much lower than those in the cells transfected with the Rab3a expression plasmid (Figure 6b). Therefore, the impact of the endogenous Rab3a protein should be much weaker than that of the overexpression of Rab3a WT-HA, T36N-HA, or Q81L-HA. These results show that the active form of Rab3a reduces the infectivity of released MLV vector

particles and, conversely, the inactive form enhances the infectivity. The C-terminally HA-tagged Rab3a WT and mutant proteins were easily detected in the virion fraction, suggesting that the Rab3a protein was incorporated into the MLV particles. This result also supports the conclusion that the Rab3a protein forms a complex with the MLV Gag protein (Figure 4a).

We also analysed the impact of N-terminally HA-tagged Rab3a T36N and Q81L mutants (HA-Rab3a T36N and Q81L). To examine the cellular localization of these mutant proteins, we transfected 293 T cells with MLV Gag-Pol expression plasmid together with HA-Rab3a WT, T36N, or Q81L expression plasmid and permeabilized them with methanol followed by treatment with the antiHA and antiMLV p30 antibodies. The MLV Gag, HA-Rab3a WT, and Q81L proteins were distributed in the cytoplasm (Figure 7a and Supplementary Figures S5 and S6). The HA-Rab3a T36N protein expression induced neurite-like extension and the protein was detected as filamentous signals colocalized with the Gag protein (Figure 7a and Supplementary Figure S7).

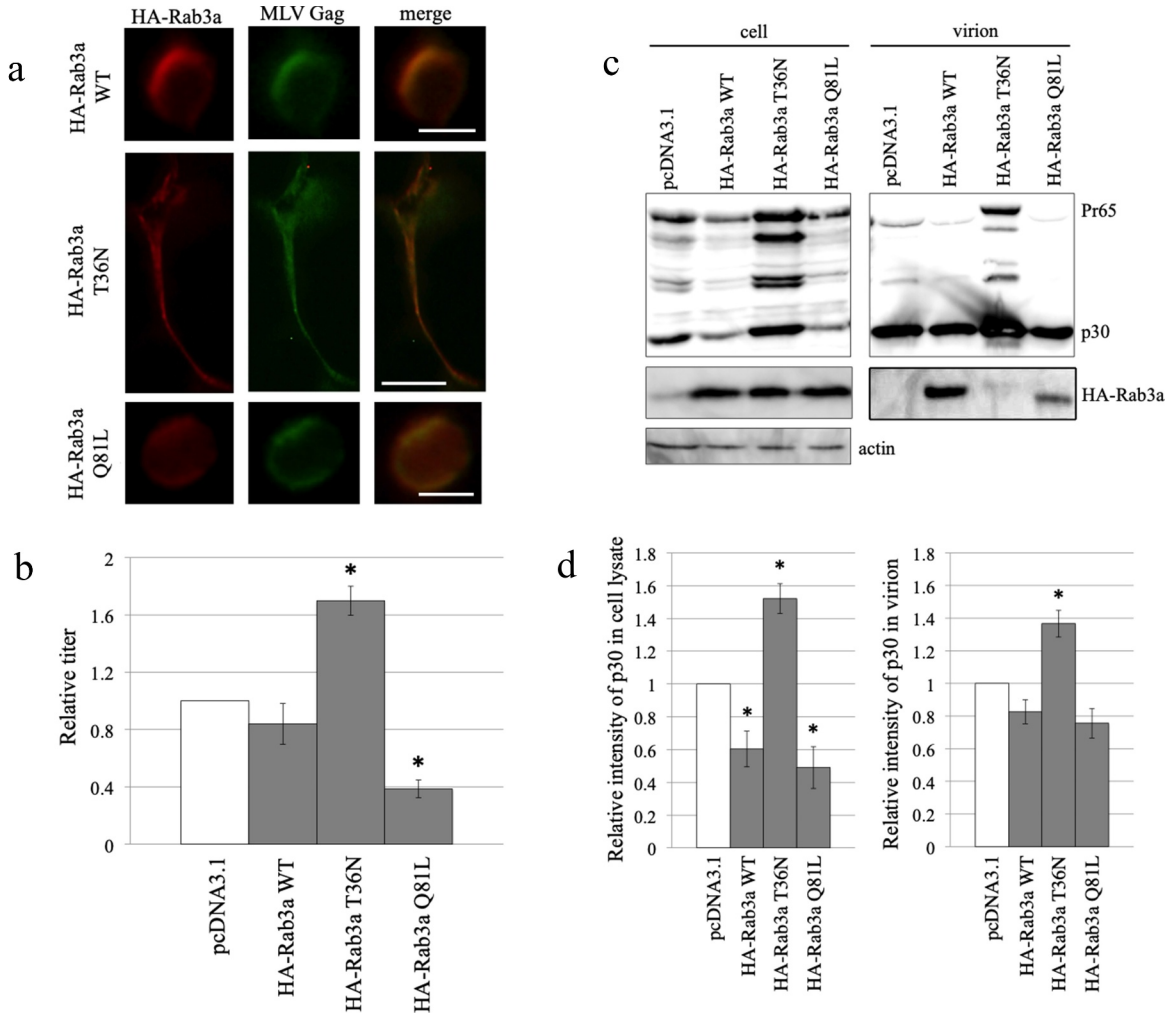

Figure 7. Active and inactive forms of N-terminally HA-tagged Rab3a decrease and increase MLV Gag protein levels, respectively. (a) Human 293 T cells were transfected with the MLV Gag-Pol expression plasmid together with HA-Rab3a WT, T36N, or Q81L expression plasmid. The transfected cells were treated with methanol and then with mouse antiHA and goat antiMLV p30 antibodies. Then, the cells were treated with Cy3-conjugated antimouse IgG (red) and FITC-conjugated antigoat antibodies (green). The treated cells were observed by confocal microscopy. (b) Human 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1, HA-Rab3a WT, T36N, or Q81L expression plasmids. Transduction titres of culture supernatants obtained from the transfected cells were measured. The transduction titres of the pcDNA3.1-transfected cells were always set to 1. relative values to the transduction titres of the pcDNA3.1-transfected cells ± SD are indicated. This experiment was repeated three times. Asterisks indicate significant differences compared with control cell titres. (c) Cell lysates prepared from the transfected cells were analysed by western blotting. (d) p30 levels normalized by the p30 levels in the cell lysates and virion fractions were calculated. Normalized p30 levels in the pcDNA3.1-transfected cells were always set to 1, and relative values ± SD are indicated. This experiment was repeated three times.

To explore the impacts of these N-terminally HA-tagged Rab3a mutants on MLV virion production, 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1, HA-Rab3a WT, T36N, or Q81L expression plasmid. The culture supernatants of transfected cells were inoculated into TE671 cells to estimate the transduction titres. Similar to the C-terminally HA-tagged Rab3a, the N-terminally HA-tagged Rab3a T36N, and Q81L mutants respectively increased and decreased the transduction titres compared with pcDNA3.1 (Figure 7b). However, HA-Rab3 WT did not reduce the transduction titres. Although C-terminally HA-tagged Rab3a proteins did not change the level of MLV Gag protein in the cell lysates and virion fractions (Figure 6b and c), N-terminally HA-tagged Rab3a Wt and Q81L reduced the Gag levels in cell lysates but not in virion fractions (Figure 7c and d). HA-Rab3a T36N increased the levels of the Gag protein in both the cell lysates and virion fractions. Interestingly, HA-Rab3a Wt and Q81 proteins, but not T36N, were detected in the virion fractions.

Mouse Rab3a forms a complex with the MLV Gag protein

The mouse is the natural host of the MLV. To examine whether mouse Rab3a is also essential for MLV Gag protein expression, a lentiviral vector encoding shRNA against Rab3a was inoculated into mouse NIH3T3 cells and selected with puromycin because the target sequence of shRNA is conserved in human and mouse (Supplementary Figure S1). No puromycin-resistant colonies were observed. Compared with human Rab3a, mouse Rab3a contains two amino acid substitutions in its C-terminal region (Supplementary Figure S1). To determine whether the mouse Rab3a protein forms a complex with the MLV Gag protein, these amino acid substitutions were introduced into the Rab3a RS-HA expression plasmid (mRab3a RS-HA). Next, 293 T cells were transfected with this mRab3a RS-HA plasmid together with pcDNA3.1 or MLV Gag-Pol expression plasmid. The MLV Gag protein was precipitated using the antiMLV p30 antibody, and the precipitates were analysed by western immunoblotting using the antiHA antibody. The mRab3a RS-HA protein was detected only in the presence of MLV Gag protein (Figure 8a), indicating that mRab3a RS-HA protein forms a complex with the MLV Gag protein.

To determine whether mRab3a RS-HA stabilizes the MLV Gag protein, the control or Rab3a-silenced 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1 or the mRab3a RS-HA expression plasmid. The culture supernatants of the transfected cells were inoculated into the 293 T cells, the transduction titres were measured, and the cell lysates prepared from the transfected cells were analysed by western immunoblotting using the antip30,

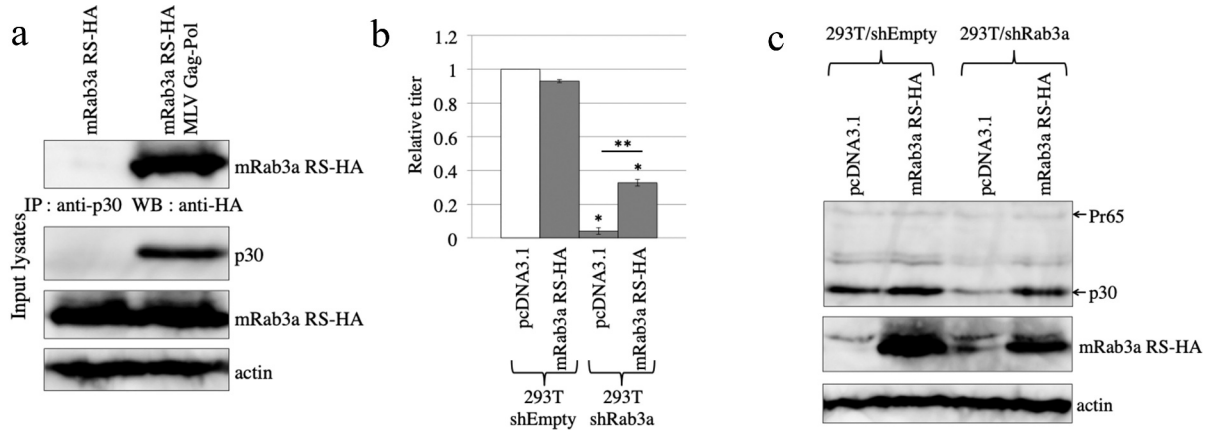

Figure 8. Mouse Rab3a stabilizes the MLV Gag protein. (a) Human 293 T cells were transfected with the mRab3a RS-HA expression plasmid together with the pcDNA3.1 or MLV Gag-Pol expression plasmid. The MLV Gag protein was precipitated by the antip30 antibody. The precipitates were analysed by western immunoblotting using the antiHA antibody (upper panel). Cell lysates prepared from the transfected cells were analysed by western blotting (lower panel). This experiment was repeated two times. (b) Control and Rab3a-silenced 293 T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1 or mRab3a RS-HA expression plasmid. Culture supernatants of the transfected cells were inoculated into 293 T cells, and transduction titres were measured. The transduction titres of the control cells were always set to 1, and relative values ± SD are indicated. Single asterisks indicate significant differences compared with control cell titres. Double asterisks represent significant differences between the two indicated groups. This experiment was repeated three times. (c) Cell lysates prepared from the transfected cells were analysed by western immunoblotting. This experiment was repeated two times.

antiHA, or antiactin antibody. We observed that mRab3a RS-HA increased the transduction titres (Figure 8b) and p30 levels (Figure 8c) in Rab3a-silenced cells, indicating that mRab3a RS-HA elevates MLV Gag protein levels and transduction titres. These findings suggest that mouse Rab3a also stabilizes the MLV Gag protein.

MLV increases CD63 Levels

To determine whether CD63 is incorporated into MLV particles, we transfected 293 T cells with a C-terminally GFP-tagged CD63 (CD63-GFP) expression plasmid together with pcDNA3.1 or amphotropic MLV vector construction plasmids. Cell lysates and virion fractions were prepared from the transfected cells. CD63-GFP was detected in the cell lysates in both the absence and presence of MLV vector construction plasmids (Figure 9a). However, in the virion fractions, CD63-GFP was detected only in the presence of MLV vector construction plasmids. This result indicates that CD63-GFP is incorporated into MLV vector particles.

We had previously reported that Rab3a decreases CD63 expression levels by inducing lysosomal

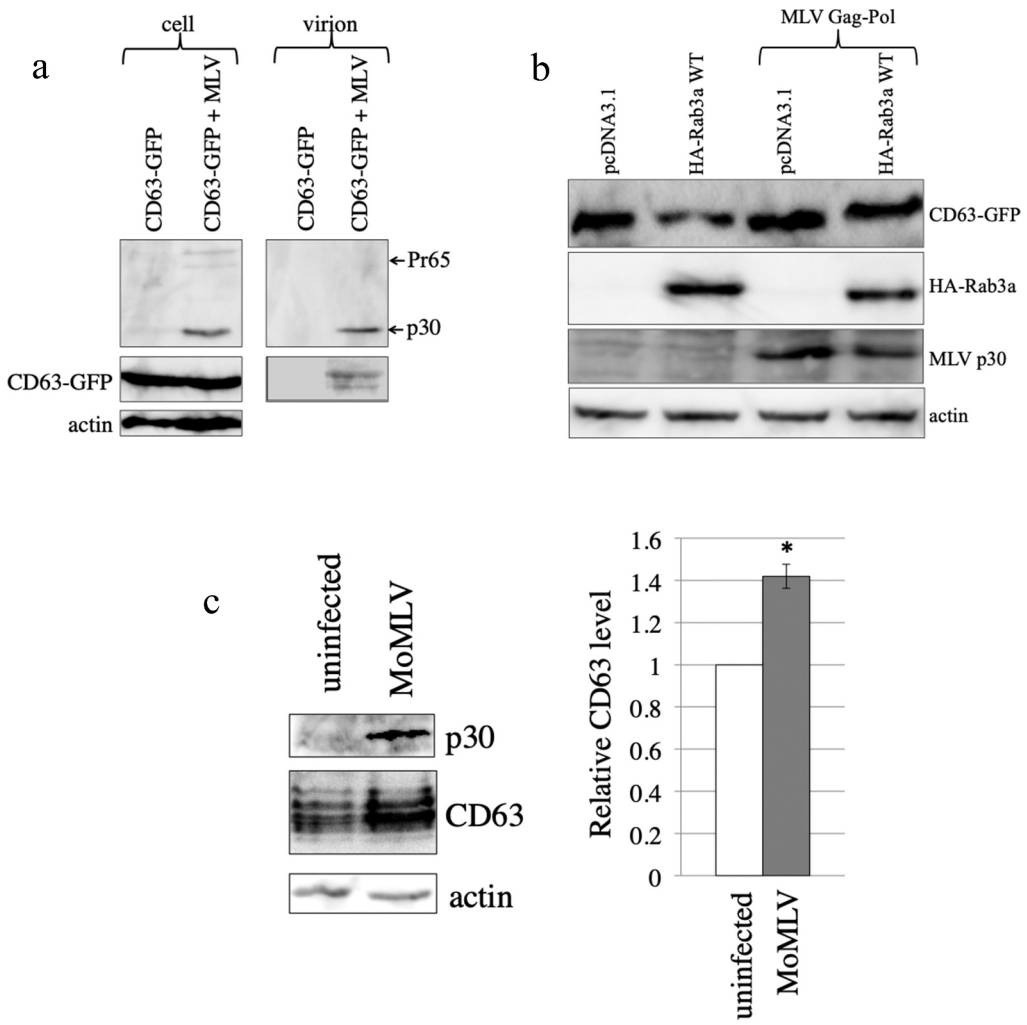

Figure 9. MLV inhibits Rab3a-mediated degradation of CD63. (a) Human 293 T cells were transfected with C-terminally GFP-tagged CD63 expression plasmid together with pcDNA3.1 or amphotropic MLV vector construction plasmids. Cell lysates and virion fractions were prepared from the transfected cells. Virion fractions were collected by centrifugation through 20% sucrose. The cell lysates and virion fractions were analysed by western blotting. This experiment was repeated two times. (b) Human 293 T cells were transfected with Rab3a WT-HA and CD63-GFP expression plasmids with or without the MLV Gag-Pol expression plasmid. Cell lysates prepared from the transfected cells were analysed by western blotting using the antiGFP, antiHA, antiMLV Gag p30, or antiactin antibody. This experiment was repeated two times. (c) Cell lysates prepared from replication-competent Moloney MLV-producing and uninfected TE671-mCAT1 cells were analysed by western blotting using the antiMLV p30, antiCD63, or antiactin antibody (left panel). CD63 levels normalized by actin levels were calculated. The normalized CD63 levels in uninfected cells were always set to 1, and relative values are indicated (right panel). Asterisks indicate significant differences. this experiment was repeated three times.

degradation of CD63 in 293 T and TE671 cells [10]. Similarly, endogenous CD63 levels were decreased in mouse NIH3T3 cells transduced with an MLV vector encoding Rab3a RS-HA (Supplementary Figure S8A). In the abovementioned experiment, it was observed that MLV Gag protein inhibits the complex formation of Rab3a with CD63. Hence, the MLV Gag protein may suppress the Rab3a-mediated degradation of CD63. To examine the effect of the MLV Gag protein on the Rab3a-mediated lysosomal degradation of CD63, we transfected 293 T cells with CD63-GFP and HA-Rab3a WT expression plasmids together with pcDNA3.1 or MLV Gag-Pol expression plasmid. Cell lysates prepared from transfected cells were analysed by western immunoblotting. Similar to the result of the previous report [10], exogenous HA-Rab3a WT expression decreased the levels of CD63-GFP in the absence of the MLV Gag-Pol expression plasmid (Figure 9b). However, in the presence of the Gag-Pol expression plasmid, the CD63-GFP level remained unchanged.

Based on the above-described result, we speculated that endogenous CD63 levels are increased in the MLV-infected cells. To evaluate this assumption, we measured endogenous CD63 levels in the control TE671-mCAT1 cells and replication-competent Moloney MLV-producing TE671-mCAT1 cells by western immunoblotting. As expected, endogenous CD63 levels in the Moloney MLV-producing cells were higher than those in the control cells (Figure 9c). Similarly, endogenous CD63 levels in the Moloney MLV-producing mouse NIH3T3 cells were higher than those in the uninfected NIH3T3 cells (Supplementary Figure S8B). Altogether, these results indicate that the complex including CD63 and Rab3a is degraded but not the Gag protein complex with Rab3a.

CD63 did not inhibit MLV Env-mediated infection

It has already been demonstrated that CD63 is incorporated into MLV particles [50]. To examine the impact of CD63 expressed in virus-producing cells on MLV Env-mediated infection, we transfected 293 T cells with amphotropic MLV vector construction plasmids together with pcDNA3.1 or CD63-GFP expression plasmid. The culture supernatants obtained from the transfected cells were inoculated into human TE671 cells to estimate the transduction titres. We detected similar transduction titres in the presence and absence of CD63-GFP (Figure 10A). We also measured MLV Gag p30 levels in cell lysates and virion fractions by western immunoblotting and found that p30 protein levels were unchanged by CD63-GFP expression (Figure 10B). Similarly, in mouse NIH3T3 cells, mouse CD63 (mCD63) expression did not affect the transduction titres and Gag protein levels (Supplementary Figures S8C and SD). These results indicate that exogenous CD63 expression does not inhibit amphotropic Env-mediated infection or MLV virion production.

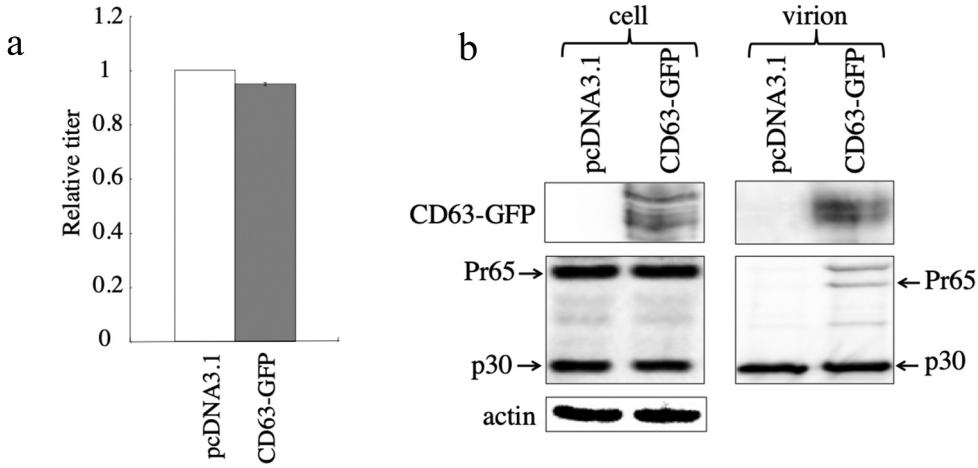

Figure 10. Exogenous CD63 expression has no effect on MLV virion production and infectivity. (a) Human 293 T cells were transfected with amphotropic MLV vector construction plasmids together with an empty or CD63-GFP expression plasmid. Transduction titres of the culture supernatants obtained from the transfected cells were measured in TE671 cells. Transduction titres of the empty plasmid-transfected cells were always set to 1. Relative values to the transduction titres of the empty plasmid-transfected cells ± SD are indicated. This experiment was repeated three times. (b) Cell lysates and virion fractions prepared from transfected cells were analysed by western blotting. This experiment was repeated two times.

CD63 is required for efficient infectivity of released MLV particles

To determine whether CD63 is required for MLV particle production, a lentiviral vector encoding an shRNA against CD63 mRNA (shCD63) was constructed in TE671 cells that endogenously expressed CD63 at a relatively higher level [10]. The control and shCD63-expressing TE671 cells were transfected with amphotropic MLV vector construction plasmids, and the culture supernatants were inoculated into 293 T cells to determine the transduction titres. We found that shCD63 decreased the transduction titres (Figure 11A). We analysed the levels of CD63, MLV Gag, and Env proteins in the cell lysates and virion fractions by western immunoblotting and found that endogenous CD63 levels were reduced in the shCD63-transduced cells (Figure 11B), confirming that CD63 was silenced. However, the levels of the MLV Gag and Env proteins in the cell lysates and virion fractions were unchanged. Similarly, in the mouse NIH3T3 cells, CD63 silencing decreased the transduction titres (Supplementary Figures S8E and SF). These results indicate that endogenous CD63 expressed in MLV vector-producing cells is required for the efficient infectivity of released MLV particles but not for MLV virion production.

In the above-described experiments, a replication-defective MLV vector was used. Hence, we next analysed the effects of endogenous CD63 on MLV virion

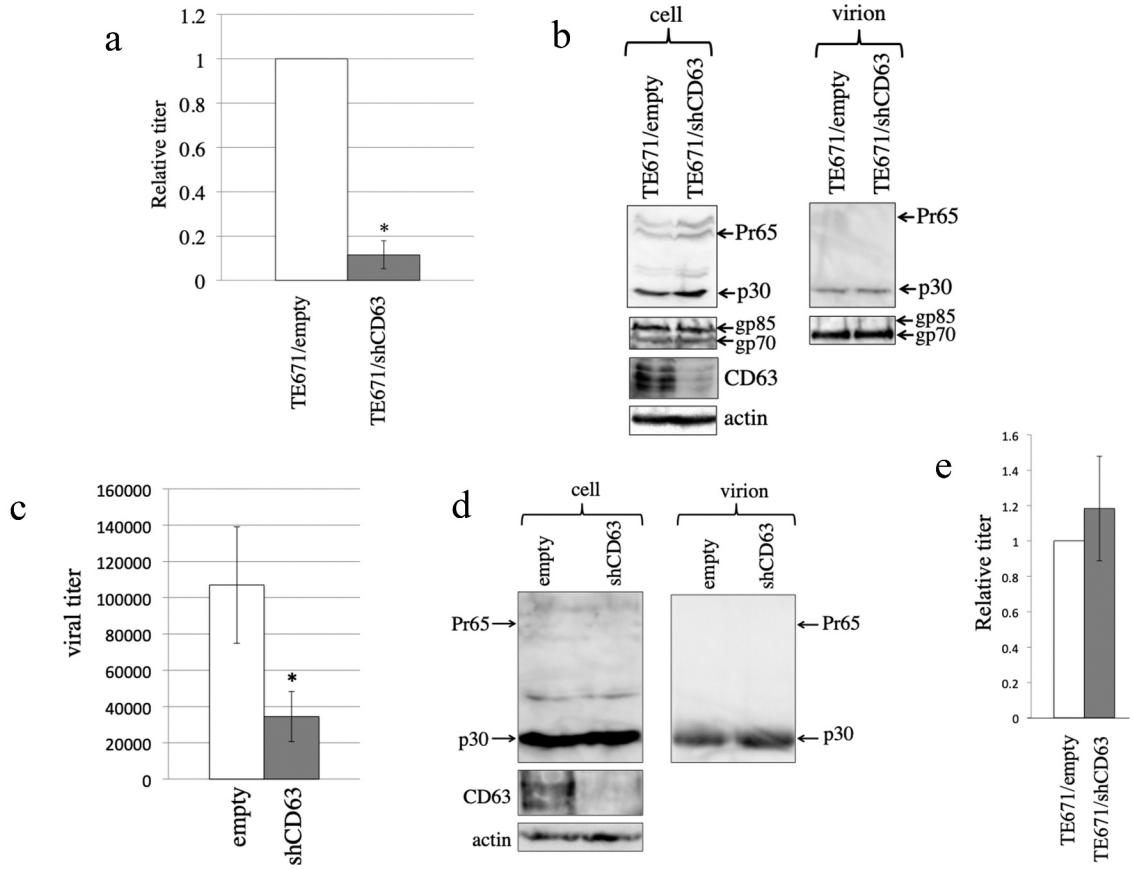

Figure 11. CD63 silencing inhibits the infectivity of released MLV particles. (a) Control and shCD63-expressing TE671 cells were transfected with ecotropic MLV vector construction plasmids. Transduction titres of the culture supernatants obtained from the transfected cells were measured. The transduction titres of the control cells were always set to 1. Relative values to control cell transduction titres ± SD are indicated. Asterisks indicate statistically significant differences. This experiment was repeated three times. (b) Cell lysates and virion fractions from the transfected cells were analysed by western blotting. This experiment was repeated two times. (c) Replication-competent Moloney MLV-producing cells were transduced with the shCD63-expressing lentiviral vector. Viral titres of culture supernatants obtained from empty or shCD63-transduced cells were measured. This experiment was repeated three times. (d) Cell lysates and virion fractions from the transduced cells were analysed by western blotting. This experiment was repeated two times. (e) Human 293 T cells transduced with empty or shCD63-encoding lentiviral vector were inoculated with an amphotropic MLV vector, and transduction titres were measured. Transduction titres of empty vector-transduced were always set to 1. Relative values to control cell transduction titres ± SD are indicated. This experiment was repeated three times.

production using replication-competent Moloney MLV. Moloney MLV-producing TE671 cells were transduced by an shCD63-encoding or control lentiviral vector and selected using puromycin. The viral titres of their culture supernatants were measured using the XC cell plaque assay [51], which revealed decreased titres due to shCD63 transduction (Figure 11C). The endogenous CD63 level was also reduced by shCD63, confirming that CD63 was silenced (Figure 11D). The levels of the Gag protein in the cell lysates and virion fractions showed no changes. To examine the effect of CD63 expressed in target cells on the sensitivity to MLV vector infection, we inoculated the CD63-silenced cells with the amphotropic MLV vector. We observed that transduction titres were not changed by CD63 silencing, which indicates that CD63 expressed in the target cells has no effect on amphotropic MLV infection (Figure 11E). These results support the conclusion that endogenous CD63 expressed in MLV-producing cells is required for the efficient infectivity of released MLV particles.

The above-described result together with the observation that CD63 is efficiently incorporated into MLV particles suggests that the MLV Gag protein is colocalized with CD63. To evaluate this hypothesis, we transfected 293 T cells with MLV Gag-Pol and C-terminally DsRed-tagged CD63 expression plasmids. The transfected cells were permeabilized with methanol and treated with goat antip30 antibody and then with FITC-conjugated antigoat IgG antibody. The MLV Gag

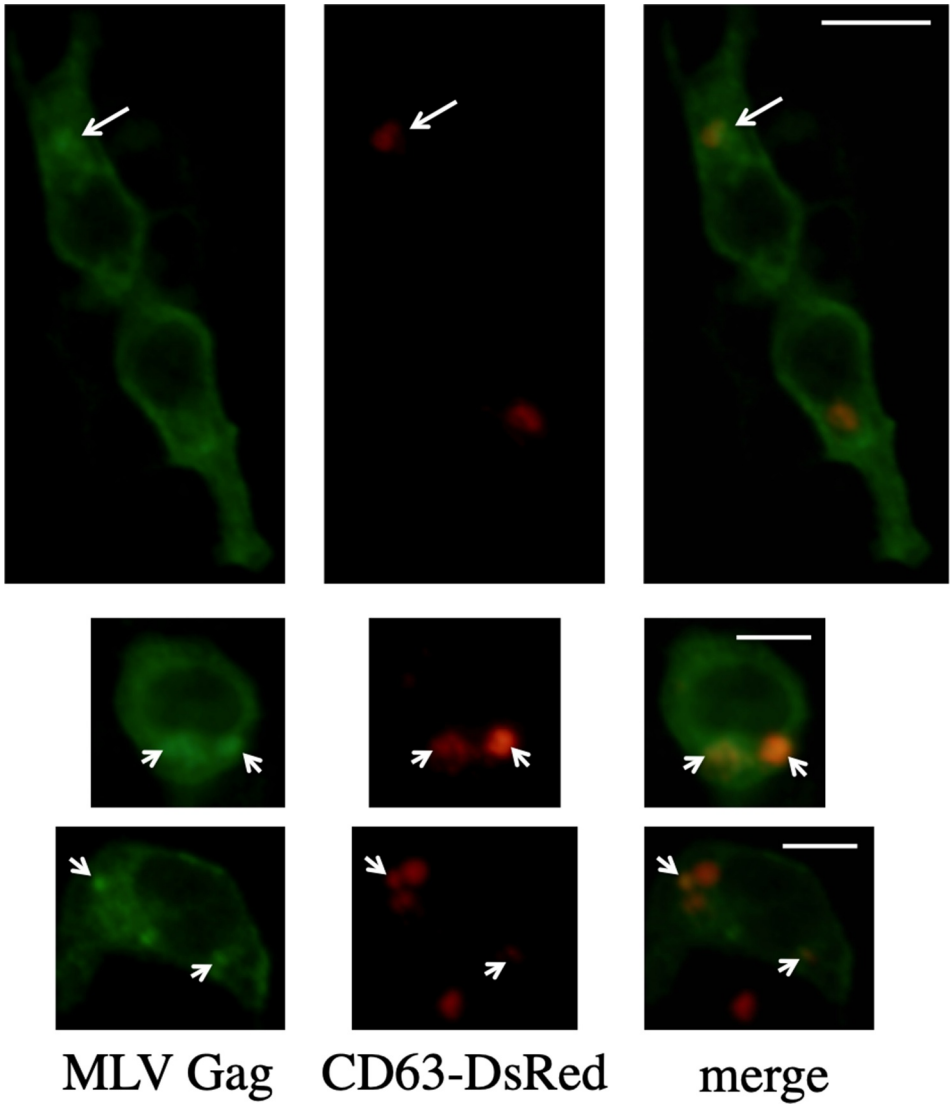

Figure 12. CD63 is colocalized with the MLV gag protein. Human 293 T cells were transfected with MLV Gag-Pol and CD63-DsRed expression plasmids and then permeabilized with methanol. The cells were treated with the goat antiMLV p30 antibody and then with the FITC-conjugated antigoat IgG antibody. The cells were observed under a confocal microscope. Arrows indicate colocalization of the MLV Gag and CD63-DsRed proteins. Scale bar, 10 μm.

protein was widely detected in the cytoplasm. Moreover, small spots with a strong green signal (Gag protein) were detected in the cells and colocalized with CD63-DsRed (Figure 12). The DsRed protein itself tends to aggregate within cells, and the detected red signals might not represent lysosomes. To explore this possibility, we transfected 293 T cells with MLV Gag-Pol and untagged DsRed expression plasmid. The DsRed protein was widely distributed in the cytoplasm and not colocalized with Gag protein spots (Supplementary Figure S9). These results indicate that the MLV Gag protein is colocalized with CD63, a late endosome/lysosome marker.

Discussion

We found that Rab3a is essential for MLV Gag protein expression and that the complex formation of Rab3a and Gag proteins inhibits the Rab3a-mediated degradation of CD63 that potentiates the infectivity of released MLV particles. The levels of the MLV Gag protein were significantly reduced in the Rab3a-silenced cells. However, treatment with a lysosome inhibitor increased the Gag protein level, indicating that Rab3a suppresses the lysosomal degradation of the Gag protein.

However, Rab3a silencing had no effect on the Gag protein level of HIV-1, another member of the retrovirus family [10]. Moreover, the HIV-1 Gag protein did not form complex with Rab3a, and the Rab3a protein was not detected in HIV-1 virion fractions. These data show that Rab3a plays a critical role in MLV replication, but not in HIV-1 replication.

CD63 silencing did not affect MLV particle production, but it attenuated the transduction titres. This result indicates that CD63 enhances the infectivity of released MLV particles. However, CD63 silencing attenuated HIV-1 virion production [10,36,37]. Although a CD63 mutant containing amino acid substitutions at conserved cysteine residues (CD63-TCS) consistently inhibited HIV-1 virion production, this effect was not observed in MLV virion production [38]. CD63 is required for the virion production of HIV-1 but not of MLV.

Rab3a is a member of the Rab3 family that includes Rab3a, Rab3b, Rab3c, and Rab3d, all of which are structurally and functionally related [52]. Therefore, it is possible that Rab3b, Rab3c, and Rab3d are also involved in MLV virion formation. We primarily used 293 T cells that were isolated from human embryo kidney. The reads per kilobase of exon per million mapped reads (RPKM) of Rab3a, Rab3b, Rab3c, and Rab3d in the kidney are 2.374, 0.338, 0, and 2.394, respectively (https://www.ncbi.nlm.nih.gov/gene/5864). Similarly, different cell types express different members of the Rab3 family. MLV may require different members of the Rab3 family according to the cell types used. Further research is needed to understand this issue.

The inactive form of Rab3a efficiently stabilizes the MLV Gag protein. HA-Rab3a T36N significantly increased the Gag protein level and transduction titre but HA-Rab3a WT and Q81L did not. However, all these Rab3a proteins formed a complex with the MLV Gag protein. HA-Rab3a T36N, but not Q81L, was localized in the cytoskeleton as already reported [53,54]. All the C-terminally tagged Rab3a proteins did not increase the level of the Gag protein. This cellular localization is essential for the efficient stabilization of the MLV Gag protein.

C-terminally HA-tagged Rab3a T36N, but not N-terminally HA-tagged protein, is incorporated into MLV particles. Because HA-Rab3a T36N is constitutively inactive and was localized in the cytoskeleton, the constitutive interaction between HA-Rab3a T36N and the cytoskeleton may inhibit its incorporation into MLV particles. However, the C-terminally HA-tagged Rab3a protein cannot bind to a lipid bilayer due to the lack of prenylation. Therefore, the protein can move freely and always interact with the Gag protein to be incorporated into MLV particles. The N-terminally HA-tagged Rab3a T36N was not incorporated into virions, but Gag protein levels were increased in virion fractions compared with those by the empty expression plasmid. Hence, in the HA-Rab3a T36N-transfected cells, the exogenous inactive Rab3a stabilizes the MLV Gag protein, and then, the Gag protein may bind to the endogenous active Rab3a protein to transfer it to the plasma membrane.

The active form of Rab3a inhibits the infectivity of released MLV particles. Exogenous expression of Rab3a WT-HA and RS-HA decreased the transduction titres but not Gag protein levels, indicating that these C-terminally HA-tagged proteins attenuate the infectivity of released MLV particles. However, HA-Rab3a WT did not affect the transduction titre and Gag level in the virion fractions. The C-terminally HA-tagged proteins, but not N-terminal HA-tagged proteins, affected the infectivity of released viral particles. Moreover, Rab3a Q81L-HA decreased the transduction titre without affecting the Gag protein level. In contrast, HA-Rab3a Q81L decreased the transduction titre and Gag protein level. These results suggest that prenylation of Rab3a is essential for the infectivity of released MLV particles but not for Gag protein stabilization.

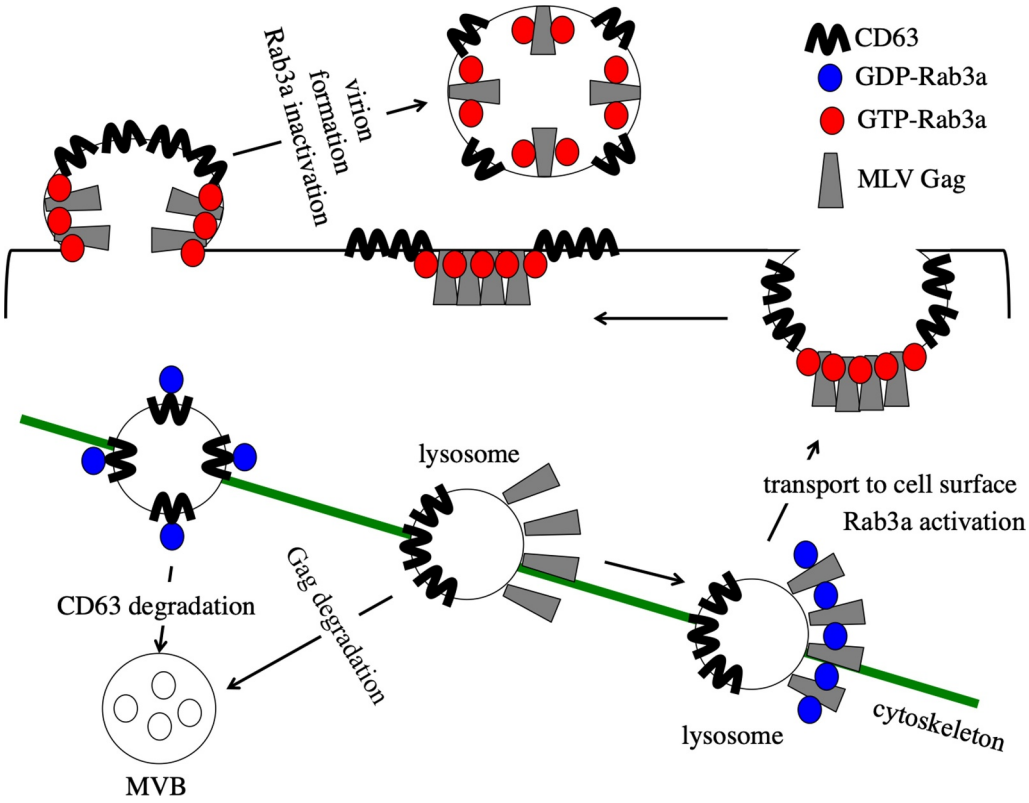

Figure 13. Speculated model of the Rab3a function in MLV gag protein transport.

CD63 promotes the infectivity of released MLV particles. Previous studies have demonstrated that HIV-1 Vpu and Nef proteins decrease the levels of tetraspanin protein [55,56]. As CD63 exerts disadvantageous functions during HIV-1 entry into host cells, HIV-1 has to downregulate CD63 expression to suppress its disadvantageous role. Conversely, CD63 expression did not inhibit the infectivity of released MLV particles. CD63 exerts advantageous functions only during MLV replication. Therefore, MLV would benefit from the upregulation of CD63 expression.

Altogether, we speculated the role of Rab3a and CD63 in MLV replication in this study (Figure 13). In uninfected cells, Rab3a forms a complex with CD63 and induces lysosomal degradation of CD63. After MLV infection, its Gag protein forms a complex with the inactive form of Rab3a to stabilize the Gag protein. The MLV Gag protein inhibits the complex formation of CD63 with Rab3a and the lysosomal degradation of CD63. The MLV Gag protein is transported to the cell surface through lysosomes by the activation of Rab3a. Through this mechanism, CD63 is simultaneously transported to the cell surface. This is followed by the formation of MLV virions in CD63-containing plasma membrane domains, and then, CD63 is incorporated into the MLV particles. The incorporated CD63 in MLV virions potentiates the infectivity of released MLV particles.

Rab3a is required for the viability of mouse NIH3T3 cells. NIH3T3 cells that stably express shRab3a were not isolated. It has been reported that Rab3a induces cell growth [57]. Therefore, Rab3a silencing might inhibit the growth of NIH3T3 cells but not that of transformed 293 T and TE671 cells.

In conclusion, Rab3a is a critical cellular factor for MLV replication. It forms a complex with the MLV Gag protein and stabilizes it. The Rab3a with the MLV Gag protein complex also inhibits the lysosomal degradation of CD63 that enhances the infectivity of released MLV particles.

Materials and methods

Cell lines

Human 293 T, human TE671, and mouse NIH3T3 cells were maintained in our laboratory since our group was started. These cells were cultured in Dulbecco's modified Eagle's medium (Wako) supplemented with 8% foetal bovine serum and 1% penicillin–streptomycin (Sigma-Aldrich) at 37°C in 5% CO_2. CD63 protein

level in TE671 cells was higher than that in 293 T cells [10]. To construct Rab3a-silenced cells, a lentiviral vector encoding shRNA against Rab3a mRNA was inoculated into 293 T or TE671 cells. The lentiviral vector additionally encodes the puromycin-resistant gene. The inoculated cells were cultured in the presence of puromycin (2 ng/ml) for 2–3 weeks, and the survived cell pool was used in this study. To construct ecotropic MLV receptor (mCAT1)-expressing TE671 cells, TE671 cells were transfected with pTargeT plasmid (Promega) encoding mCAT1 and a neomycin-resistant gene [58]. The transfected cells were selected using geneticin (400 μg/ml) (Promega), and several geneticin-resistant cell clones were isolated. To identify the cell clones that expressed mCAT1, the cell clones were inoculated with the LacZ-encoding ecotropic MLV vector [58] and the transduction titres were measured. The most susceptible cell clone (TE671-mCAT1) was used in this study. To construct NIH3T3 cells stably expressing mRab3a RS-HA (see below), NIH3T3 cells were transduced with an MLV vector encoding mRab3a RS-HA and treated with puromycin. A puromycin-resistant cell pool was used in this study.

Plasmids

A plasmid expressing C-terminally GFP-tagged CD63 was constructed in our previous study [10]. Mouse CD63 (mCD63) cDNA was isolated from mouse NIH3T3 cells by RT-PCR. The mCD63 cDNA was subcloned into the pTargeT expression plasmid (Promega) by TA ligation. Its nucleotide sequence was completely identical to the already reported sequence of mCD63. The C-terminally HA-tagged mCD63 expression plasmid was constructed as follows. The protein-coding region of mCD63 was amplified by PCR. The reverse primer contained the HA epitope sequence. The PCR product was subcloned into the pTargeT expression plasmid by TA ligation. C-terminally HA-tagged Rab3a WT, T36N, Q81L, and RS expression plasmids were constructed in another previous study [10]. N-terminally HA-tagged Rab3a expression plasmids were constructed as follows. The protein-coding region of Rab3a was amplified by PCR. The forward primer contained the HA epitope sequence. The PCR product was subcloned into the pTargeT expression plasmid. The lentiviral vector genome expression plasmid encoding shRab3a was constructed in our laboratory as follows. Two single-strand DNA fragments were synthesized. The nucleotide sequences were 5′-AAT TCG GGA CAT CAT TAA TGT CAA GTC AAG AGC CTG TAG TAA TTA CAG TTC TTT TTG C-3′ and 5′-GGC CGC AAA AAG GAC AAC ATT AAT GTC AAG CTC TTG ACC TGT TGT AAT TAC AGT TCC G-3′. The single and double underlines in these sequences indicate the restriction sites and loop sequences, respectively. The remaining regions are target sequences on Rab3a mRNA. These two single-strand DNA fragments were mixed and heated at 95° C for 2 min and then gradually cooled to 4°C for 2 h to prepare double-strand DNA. The double-strand DNA was subcloned into the pLVX lentiviral vector genome expression plasmid (TaKaRa). pLVX expresses shRNA under the control of U6 promoter. An shRNA sequence was inserted between EcoRI and NotI sites. The target sequence of shRab3a was 5′-GGACAACAUUAAUGUCAAG-3′. The original pLVX contains an unrelated sequence and expresses a short RNA. pLVX was used as the control designated as empty vector in this study. The lentiviral vector encoding shRNA against mouse CD63 was purchased from Santa Cruz Biotechnology. The target sequence has not been published. The Env expression plasmid was previously constructed in our laboratory [58]. The MLV Gag-Pol protein expression plasmid was purchased from TaKaRa and does not encode glycosylated Gag protein. The shCD63-encoding lentivirus vector was purchased from Santa Cruz Biotechnology. The target sequence has not been published.

Mutagenesis

To construct C-terminally HA-tagged mouse Rab3a (mRab3a RS-HA), PCR-mediated mutagenesis was performed on human Rab3a RS-HA. Because mouse Rab3a contains only two amino acid changes in the C-terminal region compared with that in human Rab3a, PCR was performed using human Rab3a RS-HA expression plasmid as a template. The reverse primer contained the amino acid changes. The nucleotide sequences of the forward and reverse primers were 5′-ATG GCA TCC GCC ACA GAC TCG-3′ and 5′-TCA GCA GGC GCA CTG GTG CGG TGG CGC CTG CTG GTC GGT GAG CTG GCC CTG CTT GGC-3′, respectively. The PCR product was ligated into pTargeT expression plasmid. The EcoRI-BamHI fragment that contained mRab3a RS-HA was ligated into an MLV vector genome expression plasmid (pMX-puro) [59].

Transduction

Human 293 T cells were transfected with VSV-pseudotyped HIV-1 or MLV vector construction plasmids in a 6-cm dish. The culture media of transfected cells were replaced with fresh media 24 h after the transfection. Cells were cultured for 24 h, and the culture supernatants containing HIV-1 or MLV vector particles

were collected. The culture media of target cells were completely exchanged with vector-containing supernatants and treated with appropriate antibiotics (puromycin or geneticin). When several dead cells were observed, their culture media were replaced with fresh media. When no dead cells were observed, the antibiotic-resistant cell pools were used in this study.

MLV vector

To construct an amphotropic MLV vector, 293 T cells were transfected with MLV Gag-Pol (1 μg), amphotropic Env (1 μg), and LacZ-encoding MLV vector genome (1 μg) expression plasmids using the Fugene Transfection reagent (5 μl) (Promega) in a 6-cm culture dish. The culture media were replaced with fresh media 24 h after the transfection, and the cells were cultured for an additional 24 h. The culture supernatants of transfected cells were centrifuged at 280 g for 10 min to remove cells and cell debris, and the supernatants were used as an MLV vector solution. The culture media of target TE671 cells were completely exchanged with the MLV vector solution in the presence of polybrene (4 μg/ml) in a 3-cm dish. The inoculated cells were cultured for 2 days, fixed with glutaraldehyde, and stained with X-Gal (Wako). The number of blue cells was counted in 10 randomly selected microscopic fields per 3-cm dish, and the total number of blue cells was compared. In general, 200–500 blue cells were detected (5.7×10^4 to 1.4×10^5 transduction forming unit/ml).

Replication-competent MLV

Human 293 T cells were transfected with a plasmid encoding replication-competent Moloney MLV [60]. The culture supernatant of transfected cells was inoculated into TE671-mCAT1 cells, and the inoculated cells were maintained for at least 2 weeks. The cells were transduced with lentiviral vectors. The viral titres of culture supernatants obtained from the cells were measured using the XC cell plaque assay [51].

Virion fraction

Virion fractions were collected as follows. Culture supernatants (3 ml) were centrifuged first at 280 *g* for 10 min to remove cells and cell debris and then at 1600 *g* for 4 h through 20% sucrose to remove exosomes [61]. The resulting pellets were suspended in a 30-μl RIPA buffer and used as virion fractions. The Rab3a WT-HA protein was not detected in HIV-1 virion fractions collected using this method, indicating that the virion fractions do not contain cell debris [9]. When 293 T cells were transfected with the CD63-GFP expression plasmid without the MLV expression plasmid, the CD63-GFP protein was not detected in the pellet fractions, indicating that the pellet fractions do not contain exosomes (Figure 9a).

Western immunoblotting

Cells (1×10^7) were lysed with a 100-μl RIPA buffer. Cell lysates or virion fractions (15 μl) were mixed with a 2× sample buffer (15 μl) and subjected to SDS-PAGE (Bio-Rad) together with a molecular size marker (Thermo Scientific). The proteins were transferred onto PVDF membranes (Millipore). When the membranes were treated with the mouse antiGFP (Nacalai Tesque, Inc.) (04363–66), antiHA (Covance) (16B12), antihuman CD63 (Santa Cruz Biotechnology)(sc-5275), or antiactin (Santa Cruz Biotechnology) (sc-47,778) antibody, they were also treated with the HRP-conjugated antimouse IgG antibody (Bio-Rad). These western blotting experiments were conducted on separate membranes. When the membranes were treated with the rabbit antimouse CD63 antibody (Santa Cruz Biotechnology) (sc-15,363), goat antiMLV Gag p30 antiserum (ViroMed) (81S263), or goat antiMLV SU antiserum (ViroMed)(81S262), they were also treated with HRP-conjugated protein G (Bio-Rad). The antibody-bound proteins were visualized using the ECL reagent (Bio-Rad). Protein bands were identified according to their molecular sizes (Supplementary Figure S10).

Because endogenous Rab3a protein was not detected by direct western blotting, Rab3a protein in cell lysates was concentrated by immunoprecipitation. Rabbit antiRab3a antibody (Santa Cruz Biotechnology) (sc-308) and protein G-agarose beads (Sigma-Aldrich) were added to the lysates and incubated at 4°C for 4 h. The precipitates were analysed by western immunoblotting using antiRab3a and HRP-conjugated antirabbit IgG antibodies (GeneTex). This antirabbit IgG antibody allowed the detection of only native IgG; hence, the detection of denatured IgG present in the precipitates was avoided. Images of western blotting and band intensities were obtained by FluorChem 8800 (Alpha Innotech).

Acquisition of western blot images and quantitative measurements of band intensities were performed using the FluorChemR Imaging System. Band intensities were measured before they were saturated.

Immunoprecipitation

Appropriate antibodies were added to cell lysates and incubated at 4°C for 4 h. Then, antimouse IgG

antibody- or protein G-agarose beads (Sigma-Aldrich) were added and incubated at 4°C for an additional 4 h. The beads were washed five times with a lysate buffer; then, the SDS-containing sample buffer was added. The supernatants were analysed by western blotting. HRP-conjugated secondary antibodies that do not recognize denatured IgG (GeneTex) were used in this experiment.

Confocal microscopy

The 293 T cells were transfected with the indicated expression plasmids. These transfected cells were permeabilized with methanol and treated with goat antiMLV p30 and mouse antiHA antibodies and then with FITC-conjugated antigoat IgG and Cy3-conjugated antimouse antibodies. Finally, the treated cells were observed by confocal microscopy (Olympus).

Statistical analysis

Statistical analyses were conducted in Excel. Differences between two sets of data were measured using Student's *t*-test and were considered to be significant at p values of <0.05.

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Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

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References

[1] Langemeyer L, Fröhlich F, Ungermann C. Rab GTPase function in endosome and lysosome biogenesis. Trends Cell Biol. 2018;28(11):957–970.
[2] Spearman P. Viral interactions with host cell Rab GTPases. Small GTPases. 2018;9(1–2):192–201.
[3] Chertova E, Chertov O, Coren LV, et al. Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. J Virol. 2006;80(18):9039–9052.
[4] Caillet M, Janvier K, Pelchen-Matthews A, et al. 7a is required for efficient production of infectious HIV-1. PLoS Pathog. 2011;7(11):e1002347.
[5] Murray JL, Mavrakis M, McDonald NJ, et al. Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses, and measles virus. J Virol. 2005;79(18):11742–11751.
[6] Gerber PP, Cabrini M, Jancic C, et al. Rab27a controls HIV-1 assembly by regulating plasma membrane levels of phosphatidylinositol 4,5-bisphosphate. J Cell Biol. 2015;209(3):435–452.
[7] Qi M, Chu H, Chen X, et al. A tyrosine-based motif in the HIV-1 envelope glycoprotein tail mediates cell-type- and Rab11-FIP1C-dependent incorporation into virions. Proc Natl Acad Sci USA. 2015;112(24):7575–7580.
[8] Qi M, Williams JA, Chu H, et al. Rab11-FIP1C and Rab14 direct plasma membrane sorting and particle incorporation of the HIV-1 envelope glycoprotein complex. PLoS Pathog. 2013;9(4):e1003278.
[9] Brass AL, Dykxhoorn DM, Benita Y, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science. 2008;319(5865):921–926.
[10] Kubo Y, Masumoto H, Izumida M, et al. Rab3a-bound CD63 is degraded and Rab3a-free CD63 is incorporated into HIV-1 particles. Front Microbiol. 2017;8:1653.
[11] Bustos MA, Roggero CM, De la Iglesia PX, et al. GTP-bound Rab3a exhibits consecutive positive and negative roles during human sperm dense-core granule exocytosis. J Mol Cell Biol. 2014;6(4):286–298.
[12] Quevedo MF, Lucchesi O, Bustos MA, et al. The Rab3A-22A chimera prevents sperm exocytosis by stabilizing open fusion pores. J Biol Chem. 2016;291(44):23101–23111.
[13] Bello OD, Cappa AI, de Paola M, et al. Rab3a, a possible marker of cortical granules, participates in cortical granule exocytosis in mouse eggs. Exp Cell Res. 2016;347(1):42–51.
[14] Encarnação M, Espada L, Escrevente C, et al. A Rab3a-dependent complex essential for lysosome positioning and plasma membrane repair. J Cell Biol. 2016;213(6):631–640.
[15] Raiborg C, Stenmark H. Plasma membrane repairs by small GTPase Rab3a. J Cell Biol. 2016;213(6):613–615.
[16] Vieira OV. Rab3a and Rab10 are regulators of lysosome exocytosis and plasma membrane repair. Small GTPases. 2018;9(4):349–351.
[17] Florin L, Lang T. Tetraspanin assemblies in virus infection. Front Immunol. 2018;9:1140.
[18] Suárez H, Rocha-Perugini V, Álvarez S, et al. Tetraspanins, another piece in the HIV-1 replication puzzle. Front Immunol. 2018;9:1811.
[19] Yoshida T, Kawano Y, Sato K, et al. A CD63 mutant inhibits T-cell tropic human immunodeficiency virus type 1 entry by disrupting CXCR4 trafficking to the plasma membrane. Traffic. 2008;9(4):540–558.

[20] Weng J, Krementsov DN, Khurana S, et al. Formation of syncytia is repressed by tetraspanins in human immunodeficiency virus type 1-producing cells. J Virol. 2009;83(15):7467–7474.
[21] Symeonides M, Lambelé M, Roy NH, et al. Evidence showing that tetraspanins inhibit HIV-1-induced cell-cell fusion at a post-hemifusion stage. Viruses. 2014;6(3):1078–1090.
[22] Sato K, Aoki J, Misawa N, et al. Modulation of human immunodeficiency virus type 1 infectivity through incorporation of tetraspanin proteins. J Virol. 2008;82 (2):1021–1033.
[23] Li G, Dziuba N, Friedrich B, et al. A post-entry role for CD63 in early HIV-1 replication. Virology. 2011;412 (2):315–324.
[24] Li G, Endsley MA, Somasunderam A, et al. The dual role of tetraspanin CD63 in HIV-1 replication. Virol J. 2014;11(1):23.
[25] Von Lindern JJ, Rojo D, Grovit-Ferbas K, et al. Potential role for CD63 in CCR5-mediated human immunodeficiency virus type 1 infection of macrophages. J Virol. 2003;77(6):3624–3633.
[26] Ho SH, Martin F, Higginbottom A, et al. Recombinant extracellular domains of tetraspanin proteins are potent inhibitors of the infection of macrophages by human immunodeficiency virus type 1. J Virol. 2006;80 (13):6487–6496.
[27] Rato S, Rausell A, Muñoz M, et al. Single-cell analysis identifies cellular markers of the HIV permissive cell. PLoS Pathog. 2017;13(10):e1006678.
[28] Ruiz-Mateos E, Pelchen-Matthews A, Deneka M, et al. CD63 is not required for production of infectious human immunodeficiency virus type 1 in human macrophages. J Virol. 2008;82(10):4751–4761.
[29] Krementsov DN, Weng J, Lambelé M, et al. Tetraspanins regulate cell-to-cell transmission of HIV-1. Retrovirology. 2009;6(1):64.
[30] Jolly C, Sattentau QJ. Human immunodeficiency virus type 1 assembly, budding, and cell-cell spread in T cells take place in tetraspanin-enriched plasma membrane domains. J Virol. 2007;81(15):7873–7884.
[31] Nydegger S, Khurana S, Krementsov DN, et al. Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1. J Cell Biol. 2006;173(5):795–807.
[32] Krementsov DN, Rassam P, Margeat E, et al. assembly differentially alters dynamics and partitioning of tetraspanins and raft components. Traffic. 2010;11 (11):1401–1414.
[33] Hogue IB, Grover JR, Soheilian F, et al. Gag induces the coalescence of clustered lipid rafts and tetraspanin-enriched microdomains at HIV-1 assembly sites on the plasma membrane. J Virol. 2011;85 (19):9749–9766.
[34] Meerloo T, Parmentier HK, Osterhaus AD, et al. Modulation of cell surface molecules during HIV-1 infection of H9 cells. An immunoelectron microscopic study. AIDS. 1992;6(10):1105–1116.
[35] Orentas RJ, Hildreth JE. Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. AIDS Res Hum Retroviruses. 1993;9 (11):1157–1165.
[36] Chen H, Dziuba N, Friedrich B, et al. A critical role for CD63 in HIV replication and infection of macrophages and cell lines. Virology. 2008;379(2):191–196.
[37] Fu E, Pan L, Xie Y, et al. Tetraspanin CD63 is a regulator of HIV-1 replication. Int J Clin Exp Pathol. 2015;8(2):1184–1198.
[38] Kubo Y, Izumida M, Yashima Y, et al. Gamma-interferon-inducible, lysosome/endosome-localized thiolreductase, GILT, has anti-retroviral activity and its expression is counteracted by HIV-1. Oncotarget. 2016;7(44):71255–71273.
[39] Grigorov B, Attuil-Audenis V, Perugi F, et al. A role for CD81 on the late steps of HIV-1 replication in a chronically infected T cell line. Retrovirology. 2009;6(1):28.
[40] Tsuruyama T, Hiratsuka T, Yamada N. Hotspots of MLV integration in the hematopoietic tumor genome. Oncogene. 2017;36(9):1169–1175.
[41] Kubo Y, Kakimi K, Higo K, et al. Possible origin of murine AIDS (MAIDS) virus: conversion of an endogenous retroviral p12gag sequence to a MAIDS-inducing sequence by frameshift mutations. J Virol. 1996;70(9):6405–6409.
[42] Peterson KE, Chesebro B. Influence of proinflammatory cytokines and chemokines on the neuropathogenesis of oncornavirus and immunosuppressive lentivirus infections. Curr Top Microbiol Immunol. 2006;303:67–95.
[43] Maetzig T, Galla M, Baum C, et al. Gammaretroviral vectors: biology, technology and application. Viruses. 2011;3(6):677–713.
[44] Reddy JM, Raut NGR, Seifert JL, et al. Regulation of small GTPase prenylation in the nervous system. Mol Neurobiol. 2020;57(5):2220–2231.
[45] Burstein ES, Brondyk WH, Macara IG. Amino acid residues in the Ras-like GTPase Rab3A that specify sensitivity to factors that regulate the GTP/GDT cycling of Rab3a. J Biol Chem. 1992;267 (32):22715–22718.
[46] Star EN, Newton AJ, Murthy VN. Real-time imaging of Rab3a and Rab5a reveals differential roles in presynaptic function. J Physiol. 2005;569(1):103–117.
[47] Huang CC, Yang DM, Lin CC, et al. Involvement of Rab3a in vesicle priming during exocytosis: interaction with Munc13-1 and Munc18-1. Traffic. 2011;12 (10):1356–1370.
[48] Brondyk WH, McKierman CJ, Burstein ES, et al. Mutants of Rab3A analogous to oncogenic Ras mutants. Sensitivity to Rab3A-GTPase activating protein and Rab3A-guanine nucleotide releasing factor. J Biol Chem. 1993;268:9410–9415.
[49] Ngsee JK, Fleming AM, Scheller RH. A Rab protein regulates the localization of secretory granules in AtT-20 cells. Mol Biol Cell. 1993;4(7):747–756.

[50] Segura MM, Garnier A, Di Falco MR, et al. Identification of host proteins associated with retroviral vector particles by proteomic analysis of highly purified vector preparations. J Virol. 2008;82 (3):1107–1117.
[51] Klement V, Rowe WP, Hartley JW, et al. Mixed culture cytopathogenicity: a new test for growth of murine leukemia viruses in tissue culture. Proc Natl Acad Sci USA. 1969;63(3):753–758.
[52] Schlüter OM, Khvotchev M, Jahn R, et al. Localization versus function of Rab3a proteins. Evidence for a common regulatory role in controlling fusion. J Biol Chem. 2002;277:40919–40929.
[53] Dolce LG, Ohbayashi N, Dfc DS, et al. Unveiling the interaction between the molecular motor Myosin Vc and the small GTPase Rab3A. J Proteomics. 2020;212:103549.
[54] Nishimura N, Araki K, Shinahara W, et al. Interaction of Rab3B with microtubule-binding protein Gas8 in NIH3T3 cells. Arch Biochem Biophys. 2008;474(1):136–142.
[55] Haller C, Müller B, Fritz JV, et al. Nef and Vpu are functionally redundant broad-spectrum modulators of cell surface receptors, including tetraspanins. J Virol. 2014;88(24):14241–14257.
[56] Lambelé M, Koppensteiner H, Symeonides M, et al. Vpu is the main determinant for tetraspanin down-regulation in HIV-1-infected cells. J Virol. 2015;89 (6):3247–3255.
[57] Kim JK, Lee SY, Park CW, et al. Rab3a promotes brain tumor initiation and progression. Mol Biol Rep. 2014;41(9):5903–5911.
[58] Kamiyama H, Kakoki K, Yoshii H, et al. Infection of XC cells by MLVs and Ebola virus is endosome-dependent but acidification-independent. PLoS ONE. 2011;6(10):e26180.
[59] Onishi M, Kinoshita S, Morikawa Y, et al. Applications of retrovirus-mediated expression cloning. Exp Hematol. 1996;24(2):324–329.
[60] Bacheler L, Fan H. Isolation of recombinant DNA clones carrying complete integrated proviruses of Moloney murine leukemia virus. J Virol. 1981;37 (1):181–190.
[61] Benedict CA, Tun RYM, Rubinstein DB, et al. Targeting retroviral vectors to CD34-expressing cells: binding to CD34 does not catalyze virus-cell fusion. Hum Gene Ther. 1999;10 (4):545–557.