Functional evidence for the involvement of microtubules and dynein motor complexes in TRIM5α-mediated restriction of retroviruses

Running title: Role of the cytoskeleton in TRIM5α function.

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Abstract

The tripartite motif (TRIM) family of proteins includes the TRIM5α antiretroviral restriction factor. TRIM5α from many Old World and some New World monkeys can restrict the human immunodeficiency virus type 1 (HIV-1), while human TRIM5α restricts N-tropic Murine Leukemia Virus (N-MLV). TRIM5α forms highly dynamic cytoplasmic bodies (CBs) that associate with and translocate on microtubules. However, the functional involvement of microtubules or other cytoskeleton-associated factors in the viral restriction process had not been shown. Here, we demonstrate the dependency of TRIM5α-mediated restriction on microtubule-mediated transport. Pharmacological disruption of the microtubule network using nocodazole or disabling it using taxol decreased restriction of N-MLV and HIV-1 by human or simian alleles of TRIM5α, respectively. In addition, pharmacological inhibition of dynein motor complexes using erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and siRNA-mediated depletion of the dynein heavy chain (DHC) similarly decreased TRIM5α-mediated restriction. The loss in restriction resulting from either the disassembly of microtubules or the disruption of dynein motor activity was seen for both endogenous and over-expressed TRIM5α and was not due to differences in protein stability or cell viability. Both nocodazole treatment and DHC depletion interfered with the dynamics of TRIM5α CBs, increasing their size and altering their intracellular localization. In addition, nocodazole, taxol and DHC depletion were all found to increase the stability of HIV-1 cores in infected cells, providing an alternative explanation for the decreased restriction. In conclusion,
association with microtubules and the translocation activity of dynein motor complexes
are required to achieve efficient restriction by TRIM5α.

Importance:
The primate innate cellular defenses against infection by retroviruses include a protein
named TRIM5α, belonging to the family of restriction factors. TRIM5α is present in the
cytoplasm where it can intercept incoming retroviruses shortly after their entry. How
TRIM5α manages to be present at the appropriate subcytoplasmic location to interact
with its target is unknown. We hypothesized that TRIM5α, either as a soluble protein or a
high-molecular-weight complex (the cytoplasmic body) is transported within the
cytoplasm by a molecular motor called the dynein complex, itself known to interact with
and move along microtubules. Our results show that destructuring microtubules or
crippling their function decreased the capacity of human or simian TRIM5α to restrict
their retroviral targets. Inhibiting dynein motor activity, or reducing the expression of a
key component of this complex, similarly affected TRIM5α-mediated restriction. Thus,
we have identified specific cytoskeleton structures involved in innate antiretroviral
defenses.
Introduction

Members of the tripartite motif (TRIM) family of proteins have been described to exhibit antiviral properties (26, 35, 77, 79). The best-known member is TRIM5α, first characterized as a factor from Rhesus macaque (rhTRIM5α) that potently inhibits human immunodeficiency virus 1 (HIV-1) (72). Other TRIM5α orthologs from some New World and Old World monkeys also provide protection against HIV-1 infection (25, 37, 70). Human TRIM5α (huTRIM5α), while not having the ability to restrict HIV-1, protects against N-tropic murine leukemia virus (N-MLV) and equine infectious anaemia virus (EIAV) (25, 37, 57, 60, 81). Expression of TRIM5α is induced by type 1 interferons, supporting their role as innate immunity effectors (3, 15). In addition, TRIM5α acts as an innate sensor of retroviral infections, triggering an antiviral signalling pathway that can lead to interferon production (58, 75).

Retroviral restriction is initiated by specific recognition of the N-terminal domain of incoming retroviral capsid (CA) proteins by the B30.2/PRYSPRY domain of TRIM5α (66, 73). TRIM5α binds to intact CA cores rather than monomeric CA proteins (23) and as a result of this interaction, replication is impaired by several effector mechanisms [reviewed in: (42, 51, 76)]. So far two major TRIM5α-mediated blocks have been described. The first one is accelerated disassembly of the retroviral CA core accompanied by a decrease in amounts of reverse transcription products (8, 56, 61, 73). As a consequence of the disassembly induced by TRIM5α, core components such as the viral RNA and integrase are solubilized or degraded (38). This restriction effector mechanism also involves the degradation of TRIM5α by the proteasome in presence of

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the restricted virus (62). Accordingly, treatment with proteasome inhibitors restores seemingly normal disassembly of the viral core and rescues the production of viral cDNA (2, 19, 38). However, proteasome inhibition does not fully rescue infectivity in restrictive conditions, pointing to the existence of a second, proteasome-independent restriction mechanism. The precise mechanism of this second block is still unclear but access of the viral DNA to the nucleus is inhibited (2, 80). This could be related to the “sequestration” of incoming retroviruses in CBs formed by TRIM5α proteins (14, 17).

TRIM5α contains a coiled-coil domain responsible for protein dimerization and a B-box domain important for the higher order organized states that probably promote the formation of CBs (36, 40). CBs were described as dynamic structures constantly associating and disassociating with each other, exchanging TRIM5α proteins with a pool of proteins diffused in the cytoplasm (13), and their size depends on the level of TRIM5α expression (55). The role of CBs in retroviral restriction is still unclear and some reports refute their relevance in this process (55, 69). Indeed, no CBs have been detected at endogenous TRIM5α expression levels, and it is possible that some observed CBs are artifacts stemming from protein over-expression (69). On the other hand, TRIM5α CBs were found to co-localize with ubiquitin (14), proteasomal subunits (17, 44) and p62/Sequestosome-1 (52). p62 is an important adaptor protein with a role in cell signalling and protein degradation [reviewed in (46, 68)]. Additionally, TRIM5α proteins form bodies that enclose incoming restriction-sensitive viruses and closely resemble pre-existing CBs (14). Collectively, these observations suggest that TRIM5α CBs are relevant to restriction mechanisms.
The microtubule network, a component of the cellular cytoskeleton, is made of highly dynamic filaments built of tubulin $\alpha/\beta$ heterodimers and plays multiple roles in the cell, including intra-cellular transport, organelle positioning and cell division (18). Microtubules provide platforms for molecular motors, which enable active transport through the dense cytoplasm of the cell. The dynein motor complex [reviewed in (29)] is a microtubule-associated molecular motor that transports various cellular cargos towards the microtubule-organizing center (MTOC) at the minus-end of microtubules. The MTOC is found in the vicinity of the nucleus except during cell division. Several viruses, including HIV-1, were described to recruit dynein motor complexes for their transport during the early stages of their replication [reviewed in (21, 31, 47)]. TRIM5$\alpha$ CBs also associate with microtubules and their movements along these filaments have been observed (13). However, a functional role for this association has not been demonstrated, nor have the molecular motors responsible for TRIM5$\alpha$ movement been identified. Here we asked whether the integrity of microtubules was functionally important for restriction to occur. In addition, we investigated the role of the dynein motor in this process. Using pharmacological and genetic approaches coupled with imaging analyses, we provide evidence that both microtubules and dynein motor activity are important for the restriction process mediated by TRIM5$\alpha$. 
Materials and Methods

Cells, pharmaceuticals and antibodies. Human embryonic kidney 293T cells, human epithelial carcinoma HeLa cells, human U373-derived MAGI cells, Rhesus macaque kidney FRhK-4 cells and feline renal CRFK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C, 5% CO₂. All cell culture reagents were from HyClone (Thermo Scientific, Logan, UT). HeLa cells stably expressing FLAG-tagged TRIM5 proteins were generated by retroviral transduction as described previously (8, 59). Nocodazole, paclitaxel (taxol), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), phenazine methosulfate (PMS), heparin sodium salt and cycloheximide were provided by Sigma (St Louis, MI). Anti-DHC rabbit polyclonal antibodies were from Santa Cruz (Dallas, TX). The HRP-conjugated mouse anti-actin antibody was from Sigma. Capsid (CA, p24) was detected using a mouse monoclonal antibody (clone 183) from the AIDS Research and Reference Reagent Program. The FLAG epitope was detected using the M2 mouse monoclonal antibody (Sigma) or the M2 rabbit polyclonal antibody from Cell Signaling (Danvers, MA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies used as secondary antibodies in western blots were all from Santa Cruz.

Plasmid DNAs and retroviral vectors production. The plasmid encoding a GFP-tagged version of α-tubulin (63) was a gift from Ali Saib. To produce viral vectors,
10-cm culture dishes of sub-confluent HEK293T cells were co-transfected using polyethylenimine (25,000 kDa, Polyscience) with the appropriate plasmids as follows. For wild-type (WT) or the G89V CA mutant of HIV-1CMV-GFP, we used 10 µg of pTRIPCMAV-GFP, 10 µg of WT or G89V pΔR8.9 and 5 µg of pMD-G; for N-MLVGFp and B-MLVGFp, we used 10 µg of pCNCG, 5 µg of pMD-G and 10 µg of pCIG3N (N-MLVGFp) or pCIG3B (B-MLVGFp); for HIV-1NL4-3-GFP, we used 10 µg of pNL-GFP and 5 µg of pMD-G; for SIVmac-GFP, 10 µg of pSIVmac-GFP and 5 µg of pMD-G (4-8, 50, 59, 67, 85). pNL4.3IRES-GFP (32) encodes a version of the HIV-1 NL4-3 strain (1) expressing GFP in addition to the viral proteins, and was a gift from David N. Levy. Production of the corresponding virus (HIV-1NL4-3-IRES-GFP) was done by transfecting 10 µg of the plasmid in sub-confluent HEK293T cells in a 75-cm flask. Media were changed 16 h post transfection and virus-containing supernatants were collected after an additional 32 h of culture. All viral stocks were clarified by centrifugation for 5 min at 400 rcf. All viral vectors were titrated in permissive CRFK cell using GFP expression as a marker of successful transduction. In some experiments, reverse transcriptase activity was measured on virus stocks using the EnzCheck kit (Molecular Probes) according to the manufacturer’s instructions.

Viral challenges, pharmacological treatments and RNA interference. Cells were seeded in 24-well plates at 1x10^5 cells/well (HeLa, CRFK, MAGI) or 5x10^4 cells/well (FRhK-4), and were challenged the next day with various retroviral vectors (HIV-1CMV-GFP, HIV-1G89V, HIV-1NL4-3-GFP, SIVmac-GFP, N-MLVGFp or B-MLVGFp) or a replication-competent retrovirus (HIV-1NL4-3-IRES-GFP). When applicable, cells were pre-
treated for 15 min with nocodazole, taxol or EHNA, and infections were then performed in presence of these drugs. Media were changed after 16 h, and 48 h post-infection cells were trypsinized and fixed in 2 % formaldehyde (Fisher Scientific) in phosphate buffer saline (PBS). The % of GFP-positive cells were then determined by analyzing 1x10^4 to 3x10^4 cells on a FC500 MPL cytometer (Beckman Coulter) using the CXP software. For the siRNA treatments, 2x10^5 cells were plated in each 3.5-cm of a 6-well plate and transfected with 40 nM of siRNA using DharmaFECT 1 (Dharmacon). The siRNA against heavy chain of dynein (DHC) was previously described (39) and targets the following sequence: 5’GATCAAACATGACGGAATT. The control siRNA (purchased from Dharmacon) was designed to target a luciferase sequence (5’CGTACGCGGAATACTTCGATT) absent in the human genome. 48 h post transfection, cells were seeded in 24-well plates and were challenged the next day with retroviral vectors as described above.

Stability assay. 1x10^6 HeLa cells stably expressing FLAG-rhTRIM5α were seeded in 6-well plates one day prior to the experiment. For the siRNA treatments, cells were transfected 48 h before seeding, as described above. Cells were pre-treated for 1 h with 100 µg/ml of cycloheximide, then treated with the indicated drugs without removing cycloheximide and harvested at the indicated time points. Drug concentrations were as follows: nocodazole, 0.1 µM; taxol, 0.1 µM, EHNA, 600 µM. Cells were lysed in cold stability buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP-40) supplemented with Complete protease inhibitor cocktail (Roche, Bale, Switzerland) and processed for
western blotting. rhTRIM5α was detected using the anti-FLAG rabbit polyclonal antibody.

Viability assay. 1x10^4 HeLa or 5x10^3 FRhK-4 cells were seeded per well in 96-well plates. The next day, cells were washed with PBS and wells were replenished with 100 µl of DMEM without phenol red supplemented with serum and serial dilutions of the tested drugs. After 16 h of incubation, 100 µl of the PBS solution containing 50 µg of XTT and 6 µg of PMS were added. Cells were incubated 2- 4 hours at 37°C, then the absorbance was read at 490 nm on a Synergy HT (BioTek) plate reader and corrected for background value determined on blank samples.

Fate-of-capsid assay. To analyze post-entry capsid disassembly, a protocol adapted from Stremlau et al (57) was used as described earlier (37). Briefly, 3x10^6 HeLa cells seeded in 10-cm dishes were infected with HIV-1CMV-GFP at a multiplicity of infection (MOI) of ~2 as calculated on the permissive control cells. 2 h later, virus-containing supernatants were removed and cells were rinsed once in PBS followed by a gentle trypsinization treatment (1:1 trypsin:PBS mixture for 10 sec at room temperature). Fresh medium containing the appropriate drugs was then added, and cells were incubated at 37°C, 5% CO2 for an additional 4 hours. Cells were then trypsinized and resuspended in ice-cold lysis buffer (100 µM Tris-HCl pH 8.0, 0.4 mM KCl, 2 µM EDTA, Roche’s Complete protease inhibitor) and disrupted with a dounce homogenizer. Whole cell Lysate (WCL) samples were collected at this point. In order to remove cell debris and nuclei, lysates were centrifuged for 5 min at 1,000 rcf, 4°C, and then layered on top of a
50% sucrose cushion prepared in STE buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Particulate viral cores were sedimented by ultracentrifugation in a Sorval WX Ultra 100 ultracentrifuge at 175,000 x g for 2 h at 4°C. Pellets were resuspended in denaturing gel loading buffer and processed for CA western blotting together with whole cell lysates. Post-centrifugation supernatants were collected from the fraction above the sucrose cushion, excluding the sucrose:supernatant interface.

**Immunofluorescence (IF) microscopy.** For the analysis of the localization patterns of LAMP-1 and microtubules, 2x10⁵ cells (HeLa) or 1x10⁵ cells (FRhK-4) were seeded on glass coverslips placed in 3.5-cm wells. For siRNA treatments, cells were transfected 48 h prior to seeding as described above. For the cells expressing GFP-tubulin, 2 µg of the plasmid were transfected per well 24 h prior to seeding. The day after seeding, cell were fixed (siRNA treatment) or incubated for 2 h with drugs (nocodazole, taxol, EHNA) and then fixed. Fixation was done for 10 min in pre-warmed 4% formaldehyde-DMEM at 37°C, then cells were washed once in PBS, blocked in 1X blocking solution (Roche) and stained or not for LAMP-1 for 1 h at room temperature. Primary rabbit LAMP-1 antibodies were described previously (39) and diluted 1:1000. LAMP-1 was revealed using the AlexaFluor594-conjugated donkey anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Cell nuclei were stained with DAPI (Invitrogen), then washed with PBS and mounted on glass slides using ImmunoMount (Thermo-Fisher Scientific). Microscopy was performed using a microscope (DM16000B; Leica) equipped with a spinning disk confocal head (WaveFX; Quorum Technologies, Guelph, Ontario), a 63X (1.4 numerical aperture oil immersion) plan apochromat.
objective lens, and an EM charge-coupled device camera (ImageEM; Hamamatsu Photonics, Boston, MA). The Volocity Imaging software (v4.3.2; PerkinElmer, Waltham, MA) was used as acquisition software. Image analyses were performed using the Imaris software v7.4 (Bitplane Inc., South Windsor, CT). We estimated the effect of EHNA treatment or DHC depletion by counting the % of cells (n=100-300 in two experiments) exhibiting predominant LAMP juxtanuclear staining versus cells exhibiting mostly peripheral staining, as described earlier (39).

For the analysis of TRIM5α CBs and of microtubules, HeLa cells in 3.5-cm wells were transfected or not with siRNAs directed against DHC or Luciferase as described above. The next day, cells were additionally transfected or not with 2 μg of pGFP-tubulin per well, using polyethyleneimine. The next day, 2x10^5 of these cells were seeded on glass coverslips placed in 3.5-cm wells. 24 h later, the cells were treated or not with nocodazole for 4 h and then fixed and processed for IF staining. Fixation was done for 10 min in 4 % formaldehyde-DMEM in 37°C, followed by three washes with ice-cold PBS. Cells were then permeabilized by treatment with 0.1 % Triton X-100, 0.1 mM sodium citrate for 1-2 min on ice. Cells were then washed again three times with PBS and treated with 10 % normal goat serum (Sigma) containing 0.3 M glycine (Sigma) for 30 min at RT. This was followed by a 4-hours incubation with a murine antibody against the FLAG epitope diluted 1:400 in PBS containing 10% normal goat serum. Cells were washed five times and fluorescently stained with the Alexa594-conjugated goat anti-mouse antibody (Molecular Probes) at a 1:200 dilution. Cells were washed five times in PBS before mounting in Vectashield (Vector Laboratories, Burlington, Ontario). Hoechst33342 (0.8 μg/ml; Molecular Probes) was added along with the penultimate PBS.
wash to reveal DNA. Z-stacks were acquired on the AxioObserver Microscope (Zeiss, Toronto, Ontario) equipped with the Apotome module and median Z-stacks were retained for analysis. For the analysis of TRIM5α CB sizes, FLAG foci in a given cell were manually outlined in the AxioVision software for calculation of the surface. A minimum of 100 and up to 214 CBs from 10 randomly chosen cells were included in the analysis. For the analysis of TRIM5α CB localization, the cell’s edge was outlined and for each FLAG foci in a given cell, we measured the closest distance to the nuclear membrane and the closest distance to the plasma membrane, using Axiovision. A minimum of 85 and up to 440 CBs from a minimum of 5 randomly chosen cells were included in the analysis. To avoid human bias, CBs size and localization analyses were performed blindly by students not otherwise involved in this project, using image files that had coded names. For the analysis of TRIM5α-microtubules co-localization, images were acquired using the Apotome in Raw Data mode to allow for subsequent deconvolution, which was done using the AxioVision software (Carl-Zeiss).
Results

Nocodazole and taxol treatments rescue infectivity of retroviruses restricted by endogenous TRIM5α. In order to determine whether the microtubule network has a functional role in retroviral restrictions mediated by TRIM5α, we used nocodazole and taxol (paclitaxel), pharmacological agents that prevent the polymerization of microtubules (43) or block their dynamics by preventing disassembly (34), respectively. First we tested the effect of nocodazole and taxol on restriction of HIV-1 or N-MLV at multiple drug concentrations (Fig. 1). In this set of experiments, we infected human HeLa cells with N-MLV<sub>GFP</sub>, a huTRIM5α-sensitive N-tropic MLV vector expressing GFP and, as a control, its restriction-insensitive B-tropic counterpart (B-MLV). Similarly, we infected Rhesus macaque FRhK-4 cells with a rhTRIM5α-sensitive HIV-1 vector expressing GFP or, as a control, with SIV<sub>mac239-GFP</sub>, a rhTRIM5α-insensitive simian immunodeficiency virus (SIV) strain mac239-based vector also expressing GFP. These infections were done using virus doses that had been previously determined to result in 0.1% to 1% infected (GFP-positive) cells in the absence of drug. In these experimental conditions, we found that both nocodazole and taxol increased GFP transduction by the restricted virus while having little effect on infection by the unrestricted control virus (Fig. 1). Specifically, nocodazole treatment of cells increased N-MLV infection of HeLa cells by up to 45.2-fold, and was active at a large range of concentrations peaking at about 0.1 µM (Fig. 1A). Nocodazole also increased the permissiveness of FRhK-4 cells to infection by HIV-1<sub>NL43-GFP</sub> by up to 8.5-fold (Fig. 1B). In these cells, nocodazole was active at higher concentrations (> 0.5 µM). Nocodazole slightly increased infection by the
unrestricted retroviral vector (B-MLV<sub>GFP</sub> or SIV<sub>mac-GFP</sub>) in both cell lines, but the magnitude of this effect reached ≤ 4-fold and ≤ 2-fold in HeLa and FRhK-4 cells, respectively (Fig. 1). This subtle enhancement in infection might be the result of cell cycle arrest mediated by nocodazole (24). Similar to nocodazole, taxol increased N-MLV infection of HeLa cells by up to 33.1-fold (Fig. 1A). This enhancement was seen for all drug concentrations tested in HeLa cells, peaking at ~0.1 µM. Taxol increased HIV-1 infection of FRhK-4 cells by up to 7.9-fold (Fig. 1B), and similar to nocodazole, higher concentrations of the drug (≥ 1µM) needed to be used in this cell line in order to observe an effect on restriction. Taxol slightly increased the infection of FRhK-4 cells by SIV<sub>mac-GFP</sub> (Fig. 1B) but, conversely, decreased B-MLV infectivity in HeLa cells at concentrations > 6 µM (Fig. 1A).

Partial inhibition of TRIM5α-mediated restriction by disruption of the microtubule network. The experiments shown in Fig. 1 indicated that disruption of microtubules by nocodazole or taxol treatments could increase infection by TRIM5α-sensitive viruses but not TRIM5α-insensitive ones. However, these results did not allow us to quantify the extent of inhibition conferred by nocodazole or taxol treatments, relative to the magnitude of restriction itself. They also did not allow us to analyze whether the effect seen was dependent on the MOI used. Therefore, we performed virus dose-dependent experiments using fixed drug concentrations. We used nocodazole and taxol concentrations that corresponded to their peak of anti-restriction activity as determined in Fig. 1. When the virus amounts used were normalized according to infectivity in non-restrictive CRFK cells (CRFK Infectious Units [IU]), we found N-
MLV\textsubscript{GFP} to be to ~500-times less infectious than B-MLV\textsubscript{GFP} in HeLa cells (Fig. 2A), reflecting the expected level of N-MLV restriction by endogenous huTRIM5\textalpha{} in these cells (37). Permissiveness to the huTRIM5\textalpha{}-insensitive B-MLV vector was not significantly affected by either nocodazole or taxol treatments, regardless of the virus amounts used (Fig. 2A). In contrast, N-MLV infectivity was increased by up to 65-fold following nocodazole treatment and 71-fold following taxol treatment (Fig. 2A). In other words, nocodazole and taxol reduced restriction of N-MLV to only about 20-fold in HeLa cells. In these cells, the effects of nocodazole and taxol on N-MLV were the greatest when a relatively small amount of virus was used (5 CRFK IU), while there was only a ~7-fold increase in N-MLV infection at MOIs 10-times higher (Fig. 2A). These observations probably reflect the fact that saturation of endogenous huTRIM5\textalpha{} by large amounts of N-MLV capsids partly suppresses restriction in the absence of pharmacological treatment.

As expected, HIV-1\textsubscript{NL4-3-GFP} was strongly restricted (~1000-fold) relative to SIV\textsubscript{mac-GFP} in macaque FRhK-4 cells, when the two viruses were normalized according to their infectious titers in CRFK cells (Fig. 2B) (5, 9). Nocodazole and taxol had no effect on infection by SIV\textsubscript{mac-GFP}, regardless of the amount of virus used (Fig. 2B). In contrast, both nocodazole and taxol increased infection by HIV-1\textsubscript{NL4-3-GFP} in these cells by 16- to 17-fold at low MOIs. When the virus dose used was > 100 CRFK IU, the enhancing effect of nocodazole and taxol was smaller, which again was probably due to saturation of TRIM5\textalpha{} by incoming capsids. The cyclophilin A (CypA) binding loop of CA is a major determinant of HIV-1 sensitivity to restriction by TRIM5\textalpha{} (41), and the CA-G89V mutant, which abrogates CypA binding (83), is known to be less susceptible to restriction.
Thus, we hypothesized that nocodazole and taxol treatments would have a smaller enhancing effect on CA-G89V HIV-1 compared to its WT counterpart. Indeed, we found that in subsaturating conditions (<200 CRFK IU), nocodazole and taxol increased WT HIV-1CMV-GFP by ~10-fold (5.6- to 16-fold) and ~7.5-fold (4.7- to 10.5-fold), respectively (Fig. 2C). In contrast, nocodazole and taxol increased infectivity of the mutant virus by only ~3.7-fold (2.3- to 5.0-fold) and ~3.4-fold (2.0- to 5.7-fold), respectively (Fig. 2C).

In order to verify that nocodazole and taxol had the expected effect on the microtubule network in the cell lines used, we transfected a construct expressing a GFP-\(\alpha\)-tubulin fusion in HeLa and FRhK-4 cells and then treated the cells with the same concentrations of nocodazole and taxol as used in Fig. 2A-C. The cells were then processed for IF analysis (Fig. 2D). Nocodazole prevents polymerization of microtubules (43) and consequently, microtubules appeared shortened and/or disassociated; most of the signal was diffuse and distributed throughout the cytoplasm (Fig. 2D). Taxol, on the other hand, binds to microtubule polymers to prevent their disassembly (34), resulting in the formation of abnormal microtubules bundles (30), a phenotype that was observed in both HeLa and FRhK-4 cell lines (Fig. 2D). Collectively, the data in Figures 1 and 2 show that disrupting the dynamics (either assembly or disassembly) of microtubules results in a decrease of restriction by endogenous TRIM5\(\alpha\) without inhibiting it completely. Interestingly, when drug concentrations were optimized, we observed that nocodazole and taxol had very similar effects on N-MLV or HIV-1 (Fig. 2A-C), suggesting that disruption of the microtubule network inhibited TRIM5\(\alpha\) regardless of the drug’s mechanism of action.
Pharmacological inhibition of dynein function rescues the infectivity of TRIM5α-restricted retroviruses. TRIM5α CBs are associated with microtubules and their movements within the cytoplasm seem to be at least partly dependent upon them (13). However, the molecular motors driving TRIM5α CBs movements along microtubules are not known. We hypothesized that dynein played a role in TRIM5α localization and contributed to its antiretroviral activity. We first infected HeLa cells with N- or B-MLV<sub>GFP</sub> viral vectors in the presence of increasing concentrations of EHNA (Fig. 3A), a drug that inhibits the ATPase activity associated with the heavy chain of axonemal and cytoplasmic dyneins (54, 84). In the presence of EHNA, N-MLV<sub>GFP</sub> infectivity increased by up to 22-fold compared to the vehicle control, while the EHNA treatment caused a drug concentration-dependent decrease in permissiveness to B-MLV (Fig. 3A). In macaque FRhK-4 cells, EHNA increased permissiveness to HIV-1<sub>NL43-GFP</sub> by up to 18.5-fold in a drug concentration-dependent fashion (Fig. 3B). By contrast, EHNA slightly decreased (less than 2-fold) infection by the restriction-insensitive SIV<sub>mac-GFP</sub>. We then performed the reverse experiments, infecting the cells at a fixed EHNA concentration but using multiple MOIs. Viruses were equalized as before, based on their titers in the non-restrictive CRFK cells. In HeLa cells, treatment with 600 µM of EHNA had no effect on B-MLV<sub>GFP</sub> infectivity, but it increased permissiveness to N-MLV<sub>GFP</sub> infection by up to 22-fold, depending on the MOI (Fig. 3C). In FRhK-4 cells (Fig. 3D), EHNA (1.2 mM) enhanced HIV-1<sub>NL43-GFP</sub> infection at subsaturating MOIs (≤100 CRFK IUs) by 7.5-fold on average (3.2- to 13.5-fold). In contrast, EHNA caused a reduction in SIV<sub>mac-GFP</sub> infectivity to ~0.4-fold the untreated control (Fig. 3D). Thus, the magnitude of
HIV-1 enhancement by EHNA in FRhK-4 cells is probably underestimated in this experiment due to the negative effect of the drug on infectivity, as seen with SIV_{mac-GFP}.

In order to verify that the EHNA treatments indeed affected dynein function, we analyzed the subcellular distribution of LAMP-1, a marker for late endosomes (22). Impairment of dynein function causes a shift of late endosomes towards the cell periphery, as previously described by us (39) and others (12). Cells were stained for LAMP-1 following treatment or not with 600 µM (HeLa) or 1.2 mM (FRhK-4) of EHNA. We then counted the number of cells exhibiting a juxtanuclear localization of LAMP-1 versus the ones with peripheral localization (Fig. 3E). In the DMSO-treated control cells, the localization of LAMP-1 was predominantly juxtanuclear. Specifically, 92 % ±2.1 (standard deviation) HeLa cells and 87 % ±1.5 FRhK-4 cells had juxtanuclear LAMP-1. As exemplified in Fig. 3E, EHNA treatment caused a significant decrease in juxtanuclear LAMP-1, to 11 % ±4.6 in HeLa cells and 8.6 % ±1.7 in FRhK-4 cells. In conclusion, EHNA can rescue both N-MLV and HIV-1 from restriction by different orthologs of endogenously expressed TRIM5α, but does not totally block restriction.

**Depletion of dynein heavy chain counteracts TRIM5α-mediated retroviral restriction.** In order to directly test the hypothesis that dynein function is important for TRIM5α-mediated restriction, we depleted DHC by transfection of a siRNA, as described previously (39). Knocking down DHC is known to disrupt all dynein-mediated transport activities, and it can also affect the assembly of microtubules by inhibiting the anterograde transport of microtubule complexes (28). HeLa cells were transfected with a siRNA targeting DHC or with an irrelevant siRNA targeting luciferase (Luc). Knockdown of DHC was efficient (Fig. 4A), resulting in an 85.4% (±6.7%) reduction in...
protein levels as estimated by western blotting in 4 independent experiments. In addition, DHC depletion caused a redistribution of LAMP-1 from juxtanuclear to peripheral (Fig. 4B), similar to what we had previously published (39). Specifically, 86.6% of HeLa cells transfected with the control siRNA had juxtanuclear staining, compared with 35.5% for the cells transfected with the siRNA targeting DHC. As shown Fig. 4C, DHC knockdown caused a significant increase in HeLa permissiveness to N-MLV<sub>GFP</sub> infection at all MOIs examined (4.6-fold on average; range, 4.0- to 6.0-fold), while having no effect on infection by B-MLV<sub>GFP</sub>. Thus, dynein motor complexes are involved in the restriction of N-MLV by endogenous huTRIM5α. Since dynein’s transport function is dependent on microtubules, we predicted that combining pharmacological disruption of microtubules and DHC depletion would have non-additive effects. Therefore, we analyzed the permissiveness to infection by N-MLV<sub>GFP</sub> and B-MLV<sub>GFP</sub> of cells depleted or not for DHC, in the presence of nocodazole or taxol. As shown in Fig. 4D, nocodazole alone increased N-MLV infection by an average of 23-fold (range, 11- to 34-fold) while dual treatment with nocodazole and DHC siRNAs resulted in a slightly smaller increase in N-MLV<sub>GFP</sub> infectivity (13-fold on average; range, 8- to 19-fold). Treatment with taxol resulted in an average increase of 16.7-fold (range: 13- to 24-fold) in permissiveness to N-MLV<sub>GFP</sub>, and the enhancement effect was only slightly bigger (23-fold; range, 18- to 28-fold) when DHC depletion was combined with taxol treatment (Fig. 4E). Altogether, the results in Fig. 4D and 4E show that disruption of the microtubule network and DHC depletion had non-additive effects on the restriction of N-MLV by endogenous huTRIM5α. None of the treatments or combinations of treatments had a significant effect on the infectivity of the non-restricted B-MLV<sub>GFP</sub> control (Fig. 4C-E).
Inhibition of HIV-1 restriction by over-expressed rhTRIM5α in human cells

is counteracted by depletion of dynein heavy chain or microtubules disruption. The experiments shown in Fig. 1-4 indicate that restriction of HIV-1 or N-MLV by endogenous huTRIM5α or rhTRIM5α cells is partly suppressed by DHC knockdown or by pharmacological disruption of microtubules using nocodazole or taxol. We therefore decided to determine whether these interventions would also inhibit HIV-1 restriction in cells in which exogenous FLAG-tagged rhTRIM5α was over-expressed. rhTRIM5α was expressed in HeLa cells through retroviral transfer, and non-transduced cells were eliminated by puromycin treatment. We infected the transduced cells with the restriction-sensitive HIV-1 vectors HIV-1CMV-GFP and HIV-1NL43-GFP as well as the rhTRIM5α-insensitive SIVmac-GFP (Fig. 5A). The two vectors used differ in that HIV-1CMV-GFP does not carry the viral products Vpr, Nef, Vif and Vpu, and no viral proteins are expressed following integration (49). In contrast, HIV-1NL43-GFP encodes all HIV-1 proteins with the exception of Env and Nef, and viral proteins are expressed in infected cells (27).

Restriction was observed for both viruses in HeLa-rhTRIM5α cells, compared with SIVmac-GFP and after normalization of viral stocks according to their titers on CRFK cells (Fig. 5A). Specifically, HIV-1CMV-GFP and HIV-1NL43-GFP were restricted ~40-fold and between ~48- and 78-fold, respectively. Nocodazole and taxol increased permissiveness to HIV-1CMV-GFP by 10.1-fold and 9.8-fold, on average, and they increased permissiveness to HIV-1NL43-GFP by an average of 17.8-fold and 15.6-fold, respectively (Fig. 5A). None of the drug treatments had a significant effect on infection by SIVmac-GFP, although taxol seemed to increase HIV-1 infectivity at relatively low MOIs and decrease
it at relatively high MOIs (Fig. 5A). Next, we used FLAG-rhTRIM5α transduced HeLa
cells transfected with the control (luciferase-targeting) siRNA or with the siRNA
targeting DHC (Fig. 5B). In this experiment, restriction of HIV-1NL43-GFP by rhTRIM5α
was particularly high (>1,000-fold). We found that cells depleted of DHC were more
permissive to infection by HIV-1NL43-GFP than cells transfected with the control siRNA
(an 11.1-fold increase on average). In contrast, DHC knockdown slightly decreased
infection by the control SIVmac-GFP vector (Fig. 5B).

In the next set of experiments (Fig. 5C-F), we compared the effect of
nocodazole, taxol and DHC depletion on the permissiveness to HIV-1 of cells transduced
with FLAG-rhTRIM5α or with a non-restrictive control (huTRIM5α). The expression
levels of huTRIM5α and rhTRIM5α were found to be comparable (Fig. 5F). Cells were
infected at an MOI leading to approximately 1% infected cells in the absence of
treatment, as in Fig. 1. Addition of nocodazole increased permissiveness of HeLa-
rhTRIM5α to HIV-1CMV-GFP by 5.3-fold ±0.21 (Fig. 5C), while it had a much smaller
effect on HeLa transduced with the “empty” vector (1.5-fold ±0.04) or HeLa transduced
with huTRIM5α (1.46-fold ±0.10). Likewise, treating the cells with taxol increased
permissiveness to HIV-1CMV-GFP by 3.9-fold ±0.33 (Fig. 5D), while it slightly decreased
infection of HeLa-vector and HeLa-huTRIM5α cells (0.67-fold ±0.13 and 0.63-fold
±0.12, respectively). Depleting DHC similarly increased permissiveness to HIV-1CMV-GFP
in cells expressing rhTRIM5α (4.2-fold ±0.38) while having no effect in cells expressing
the human ortholog (Fig. 5E). In conclusion, microtubule disruption with nocodazole and
taxol treatment and DHC depletion specifically inhibited the restriction of HIV-1 by
exogenously expressed rhTRIM5α in human cells.
Nocodazole and taxol inhibit TRIM5α-mediated restriction of an HIV-1 vector bearing autologous envelope proteins. The experiments shown in Fig. 1-5 were all performed using VSV G-pseudotyped vectors. TRIM5α-mediated restriction is not known to be affected by the mode of virus entry, nonetheless we decided to verify that nocodazole and taxol would inhibit the TRIM5α-mediated restriction of a virus bearing its autologous envelope. For that, we used a fully infectious HIV-1 clone (HIV-1NL43-IRES-GFP) encoding GFP in addition to all the other viral proteins. Human MAGI cells (78), which are U373 (glioblastoma) cells stably expressing the HIV-1 receptors and thus are permissive for HIV-1 infection, were retrovirally transduced to express huTRIM5α or rhTRIM5α. rhTRIM5α was expressed at higher levels as compared to huTRIM5α (Fig. 6B), which may reflect intrinsic differences in expression levels of the two proteins, since similar observations were made in other cellular contexts (8, 59). HIV-1 was restricted ~41-fold in the cells transduced with rhTRIM5α compared to control (“empty” vector-transduced) cells, while as expected, it was not restricted in cells transduced with huTRIM5α (not shown). Cells transduced with either of the two TRIM5α orthologs or transduced with the empty vector were then infected with HIV-1NL43-IRES-GFP using amounts of the virus leading to approximately 0.1% infected cells and in the presence or absence of drug treatment. As shown Fig. 6A, nocodazole and taxol increased the capacity of HIV-1 to infect cells transduced with rhTRIM5α by 6.1-fold ±1.7 and 12.1-fold ±4.5, respectively. The drugs had no significant effect on HIV-1 infectivity in the non-restrictive control cells. Thus, integrity of the microtubule network is important for efficient restriction by TRIM5α regardless of the mechanism of virus entry.
Loss of restriction is not caused by decreased TRIM5α stability nor by decreased cellular viability. Next, we investigated a possible impact of the treatments on the turnover/stability of TRIM5α. HeLa cells stably expressing FLAG-rhTRIM5α were either subjected to treatment with vehicle, nocodazole, taxol or EHNA, or transfected with DHC or control siRNAs, using the same conditions as before. De novo mRNA translation was inhibited using cycloheximide, and we monitored the decrease in protein levels for TRIM5α and for actin as a control (Fig. 7A-B). We found that the turnover of TRIM5α was not affected by the various treatments used. EHNA slightly decreased TRIM5α levels at a single time-point (1 h of cycloheximide treatment) while DHC depletion also slightly decreased TRIM5α levels at 3 and 4.5 h of cycloheximide treatment (Fig. 7A). However, the results derived from several experiments revealed that these effects were not statistically significant (Fig. 7B).

Nocodazole, taxol and EHNA disrupt essential cellular functions and it was thus important to insure that the loss of restriction observed in our experimental conditions was not an artifact caused by gross cytotoxic effects. In all our infectivity assays, drugs are added for only 16 h and then removed, since only the first hours of infection are relevant to mechanisms of TRIM5α-mediated restriction. Thus, we examined cellular viability following 16-hour treatments of HeLa and FRhK-4 cells with increasing concentrations of nocodazole, taxol and EHNA (Fig. 7C). Viability was monitored using the XTT assay, a colorimetric method to measure cellular metabolic activity, specifically the activity of dehydrogenase enzymes (65). Both nocodazole and taxol caused a progressive but relatively modest loss in viability, obviously reflecting a concentration-
dependent inhibition of cell division (33, 71). At the relatively low concentrations used for infectivity assays, nocodazole caused a ~19% decrease in the viability of HeLa cells and a ~32% decrease in the viability of FRhK-4 cells (Fig. 7C). Similarly, taxol caused a ~23% decrease in the viability of HeLa cells and a ~20% decrease in the viability of FRhK-4 cells at the concentrations used in our restriction assays (Fig. 7C). In both cases, the concentrations used were well below the threshold at which the decrease in viability becomes sharper, probably reflecting the occurrence of cytotoxic mechanisms in addition to the inhibition of cell division. Based on our XTT data, we would estimate these cytopathic effects to take place at above ~30 µM for nocodazole, ~20 µM for taxol. We obtained a different pattern for EHNA: in both HeLa and FRhK-4 cells, viability decreased slowly to reach 85-90% of the control levels at 1 mM of the drug, but the drop in viability was much sharper at higher concentrations, and viability was fully lost at ~5 mM (Fig. 7C). At the concentrations used in our infectivity assays (600 µM in HeLa cells, 1.2 mM in FRhK-4 cells), EHNA caused a ~12% decrease in viability in HeLa cells, 8% in FRhK-4 cells (Fig. 7C). Thus, even though these concentrations are close to the concentrations at which viability starts declining sharply, cellular viability is still at the level of untreated cells, and it is unlikely that the effects of EHNA on restriction result from its cytotoxicity.

DHC depletion, nocodazole treatment and taxol treatment stabilize post-entry HIV-1 cores. A characteristic effect of TRIM5α-mediated post-entry retroviral restriction is the increase in the CA core disassembly of restriction-sensitive retroviruses, i.e. a decrease in core stability. We used the well-established fate-of-capsid assay (8, 53, 56, 57).
TRIM5α-mediated core disassembly. HeLa cells expressing rhTRIM5α or mock-transduced with the empty vector were exposed to HIV-1NL4-3-GFP in the presence or the absence of nocodazole, taxol or DHC siRNA. The efficiency of DHC knockdown was the same as in Fig. 4A (not shown). Cells were lysed and core-associated (pelletable) CA was isolated by ultracentrifugation through a sucrose cushion. The relative amounts of core-associated, soluble (post-centrifugation supernatant-associated) and total (whole cell lysate, WCL) CAp24 were assessed by densitometry of western blots from three independent experiments (Fig. 8). The amounts of pelletable CA recovered were estimated relative to total CA or relative to soluble CA. As a control, cells were infected with a vector devoid of envelope proteins and thus incompetent for entry. Both virus preparations were normalized using a reverse transcription assay to insure equal virus input. In the absence of the VSV G envelope, no or little CA signal was detectable in the infected cells, confirming that the CA detected was not associated with unfused viral particles (Fig. 8A-C). As expected and in the absence of treatment, the relative amounts of pelletable CA cores were reduced in cells expressing rhTRIM5α, compared to the control cells transduced with the empty vector (Fig. 8A-C). Specifically, the pellet/WCL CA ratio was reduced by 85.9% (±9.0), 70.4% (±14.0) and 75.7% (±23.0) in the experiments shown Fig. 8A, 8B and 8C, respectively. Similarly, the pellet/supernatant CA ratio was reduced by 85.0% (±12.7), 72.2% (±11.5) and 88.4% (±9.1) in cells expressing rhTRIM5α (Fig. 8A-C, right panels).

We observed that all the treatments used had a stabilizing effect on post-entry CA cores, both in permissive (control) cells and in restrictive (TRIM5α-expressing) cells.
However, the increase in stability mediated by DHC depletion and taxol treatment was greater in rhTRIM5α-expressing cells than in control cells, consistent with the ability of these treatments to rescue infection. Specifically, DHC depletion increased the pellet/WCL CA ratio by 1.28-fold in control cells while this increase was of 3.10-fold in rhTRIM5α-expressing cells (Fig. 8A). Similarly, DHC depletion increased the pellet/supernatant CA ratio by 2.11-fold in control cells and by 5.78-fold in rhTRIM5α-expressing cells (Fig. 8A). Taxol treatment increased the pellet/WCL CA ratio by 1.61-fold in control cells while the corresponding increase in rhTRIM5α-expressing cells was 2.52-fold. Similarly, taxol increased the pellet/supernatant CA ratio by 1.46-fold in control cells and 3.87-fold in rhTRIM5α-expressing cells (Fig. 8B). We obtained different effects for nocodazole, since treatment with this drug sharply increased CA core stability both in permissive and restrictive conditions (Fig. 8C). Specifically, nocodazole increased the pellet/WCL CA ratio by 3.94-fold in control cells and by 2.83-fold in rhTRIM5α-expressing cells. Similarly, the pellet/supernatant CA ratio was increased by 3.77-fold in control cells and by about the same amount (4.05-fold) in rhTRIM5α-expressing cells treated with this drug (Fig. 8C). Therefore, disrupting the microtubule network or the dynein heavy chain motor has a stabilizing effect on post-entry HIV-1 cores, which may decrease their susceptibility to TRIM5α-mediated restriction. In addition, the magnitude of this stabilizing effect was greater in cells expressing rhTRIM5α under treatment by taxol or DHC siRNAs, which is consistent with these interventions specifically increasing HIV-1 infectivity in restrictive conditions.

Nocodazole treatment increased post-entry HIV-1 cores stability equally in permissive and restrictive conditions, but its effects in permissive conditions was also much stronger.
Effect of nocodazole treatment and DHC depletion on the size and localization of TRIM5 CBs. The capacity of nocodazole treatment and DHC depletion to reduce TRIM5α-mediated restriction could stem from effects on the dynamics of TRIM5α, which would affect the size and/or localization of CBs. HeLa cells stably expressing FLAG-tagged rhTRIM5α were treated with nocodazole or depleted for DHC and then stained for FLAG (Fig. 9A, B). The settings were adjusted to reveal CBs but not the diffuse FLAG signal. The size (area) of CBs (Fig. 9C) and their relative distances to the nucleus and to the “edge-of-cell” (Fig. 9D) were calculated as detailed in “Materials and Methods”. Nocodazole treatment and DHC knockdown increased the average size of rhTRIM5α CBs by 33.9±7.8 % and 52.6±8.9 %, respectively (Fig. 9C). In addition, rhTRIM5α CBs were found to be 31.5±3.7 % closer to the nucleus following nocodazole treatment (Fig. 9D). In contrast, DHC depletion caused a significant rhTRIM5α CBs localization shift (14.2±2.8 %) towards the plasma membrane (Fig. 9D). The results presented in Fig. 9 show that the microtubule network is important for the dynamics of TRIM5α CBs. Depletion of DHC and disruption of microtubules can have distinct effects on TRIM5α CBs, but the increased peripheral localization of CBs seen upon DHC depletion suggests that dynein motor complexes are responsible for their retrograde transport. To test more directly whether dynein is involved in the association of TRIM5α CBs with microtubules, we co-transfected HeLa cells stably expressing FLAG-rhTRIM5α with siRNAs targeting either DHC or an irrelevant control and with a plasmid.
expressing GFP-α-tubulin, then stained for FLAG (Fig. 9E-F). Images were acquired with the Apotome system and deconvolved to facilitate analysis, and co-localization was quantified from multiple randomly chosen cells. The depletion of DHC resulted in a modest (23 % ±0.3) yet statistically significant decrease in the co-localization of rhTRIM5α CBs and microtubules (Fig. 9G). Altogether, these results suggest that dynein motor complexes are involved in the association of TRIM5α CBs with microtubules and in the retrograde transport of either TRIM5α CBs.
Discussion

In this work, we examined the importance of microtubules and of the microtubule-associated dynein motor complexes in the restriction of HIV-1 and N-MLV by TRIM5\(\alpha\). Pharmacological and siRNA-mediated depletion studies clearly show that the capacity of TRIM5\(\alpha\) to efficiently restrict incoming retroviruses is dependent on intact microtubules and a functioning dynein motor complex (Fig. 1-6). This was true regardless of (i) the retrovirus and TRIM5\(\alpha\) ortholog studied, (ii) whether TRIM5\(\alpha\) was expressed at endogenous levels or over-expressed through stable transduction, and (iii) the mechanism of virus entry. Nocodazole and taxol, two drugs that disrupt the structure of the microtubule network through different mechanisms, had very similar, sometimes identical effects on retroviral restriction levels (Fig. 2, 4). DHC depletion generally had a smaller effect on restriction than treatment with nocodazole or taxol (Fig. 4), which may be due to the observed incomplete knockdown (Fig. 4A). However, DHC depletion did not synergistically inhibit TRIM5\(\alpha\) in the presence of either nocodazole (Fig. 4D) or taxol (Fig. 4E), implying that these treatments all affected the same pathway. Finally, the observed impact of microtubules or dynein disruption on virus restriction was not caused by gross differences in expression levels or TRIM5\(\alpha\) stability (Fig. 7A-B) and was not a putative artifact stemming from cytotoxicity (Fig. 7C).

Destabilization of the viral CA core upon interaction with TRIM5\(\alpha\) is a hallmark of restriction and occurs in the hours following virus entry. We observed that DHC depletion, nocodazole treatment and taxol treatment all increased the relative amounts of pelletable CA, both in permissive and restrictive conditions. This indicates that these
treatments increase the stability of post-entry HIV-1 CA cores. To our knowledge, this
effect of interventions altering the cytoskeleton on HIV-1 disassembly had never been
investigated before. Although the mechanism behind HIV-1 post-entry CA core
stabilization is unclear, it could contribute to making HIV-1 partly resistant to TRIM5α,
by counteracting the destabilizing effect of TRIM5α (10). The increase in the relative
amounts of core-associated CA induced by DHC depletion or taxol treatment was greater
in cells expressing rhTRIM5α compared to control cells (Fig. 8A, 8B). This is consistent
with these treatments specifically increasing HIV-1 infectivity in restrictive conditions.
The results with nocodazole were more ambiguous, as this drug strongly increased the
relative levels of core-associated CA (a 3- to 4-fold increase) both in permissive and
restrictive conditions (Fig. 8C). This apparent lack of specificity of nocodazole in this
assay might be due to compounding effects of the drug. For instance, nocodazole could
increase the apparent stability in permissive conditions through an additional mechanism
that does not take place in restrictive conditions, hence masking the restriction-specific
effect that is seen with DHC siRNAs or taxol. Consistent with this, nocodazole increased
the relative amounts of core-associated CA in permissive cells about twice as much as
DHC depletion or taxol treatment did.
Nocodazole treatment increased the size of TRIM5α CBs and caused them to be
positioned closer to the nucleus (Fig. 9), which is consistent with previous observations
by Diaz-Griffero et al. (20). These data suggest that nocodazole interferes with the
dynamics of TRIM5α CBs, perhaps by preventing the exchange of TRIM5α proteins
between diffuse cytoplasmic population and CBs-associated population (13). DHC
depletion increased the average size of rhTRIM5α CBs (Fig. 9C), but also caused CBs to
accumulate towards the cell periphery (Fig. 9D). In addition, there was a modest but
significant decrease in the association between microtubules and rhTRIM5α (Fig. 9G).
Altogether, these results suggest that the dynein motor translocates rhTRIM5α CBs on
microtubules and is at least partly responsible for their retrograde transport. When DHC
is depleted, rhTRIM5α CBs may shift towards the periphery of cells due to interactions
with other molecular motors, such as the reverse polarity motor kinesin, which performs
anterograde transport and can transport the same cargos as dynein does (48).
Alternatively, DHC depletion could enhance the association of rhTRIM5α with late
endosomes, as was observed previously for components of the HIV-1 ribonucleoprotein
such as Gag and the genomic RNA (39). Nocodazole treatment and DHC depletion
showed distinct phenotypes with regard to TRIM5α-mediated CA core disassembly (Fig.
8) and TRIM5α CBs localization (Fig. 9D), yet their effects on restriction are clearly not
additive (Fig. 4). Thus, it is likely that these phenotypic differences simply reflect
different mechanisms of action by which the two treatments inhibit a single pathway.
Perhaps surprisingly, the various interventions used in this study generally had
little effect on the transduction of HIV-1, SIVmac and MLV vectors in permissive
conditions. Nocodazole was originally reported to modestly inhibit the early stages of
HIV-1 infectivity in MAGI cells (11). However, other studies demonstrated a lack of
HIV-1 sensitivity to this drug in human embryo kidney 293T cells (16) and in CEM T
cells (82). At first glance, such a lack of inhibition by nocodazole contradicts a role for
microtubules in HIV-1 post-entry intracellular transport (45). However, the existence of a
population of nocodazole-resistant microtubules, which are less dynamic, has been
described (64), raising the possibility that HIV-1 uses these specific microtubules for its
transport. Likewise, depleting DHC had a small effect on the infection of human permissive HeLa cells by an SIVmac vector (Fig. 5B) and no effect was seen on infection of HeLa cells by B-MLV (Fig. 4C) or HIV-1 (Fig. 5E) vectors. To the best of our knowledge, the only published evidence of a functional role for dynein motor complexes in HIV-1 post-entry transport consisted in the injection of an antibody interfering with dynein motor function, leading to a ~50% decrease in HIV-1 movements toward the nucleus (45); however, this observation was not supported by infectivity data. Clearly, the precise mechanism of retroviral capsids transport toward the nucleus is yet to be determined (47). Regardless, our results show that disruption of the microtubule network and DHC depletion affect TRIM5α-mediated restriction without strongly impairing infectivity in permissive cells, firmly establishing specificity. The effects seen on restriction probably stem from effects on TRIM5α localization (Fig. 9), on CA stability (Fig. 8), or a combination of both.

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References


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**Figure 1:** Drug concentration-dependent enhancement of permissiveness to infection by TRIM5α-restricted retroviral vectors in human and simian cells. HeLa cells (A) and FRhK-4 cells (B) were infected with single doses of the indicated viral vectors in the absence or presence of increasing amounts of nocodazole or taxol for 16 h. The amounts of viruses used were adjusted to obtain in the vicinity of 0.1 to 1 % of infected cells in the absence of the drug and yielded 0.09% (N-MLV\textsubscript{GFP}), 0.38% (B-MLV\textsubscript{GFP}), 0.85% (HIV-1\textsubscript{NL4-3-GFP}) and 1.23% (SIV\textsubscript{mac-GFP}) infected cells. The % of infected (GFP-positive) cells were determined by flow cytometry 2 d post infection and results are presented as -fold changes in infectivity relative to the relevant untreated controls.

**Figure 2:** Pharmacological disruption of microtubules decreases endogenous TRIM5α-mediated retroviral restriction. (A-C) Effect of nocodazole (noc) and taxol (txl) on restriction. Human HeLa cells (A) or macaque FRhK-4 cells (B, C) were infected with multiples doses of N-MLV\textsubscript{GFP}, B-MLV\textsubscript{GFP}, HIV-1\textsubscript{NL4-3-GFP} or SIV\textsubscript{mac-GFP} as indicated. In (C), FRhK-4 cells were infected with WT HIV-1\textsubscript{CMV-GFP} or with the CA-G89V mutant of this vector. Infections were performed for 16 h and in the absence of drug or in the presence of either nocodazole or taxol. Nocodazole was used at 0.1 µM in HeLa cells and 6 µM in FRhK-4 cells, and taxol was used at 0.1 µM in HeLa cells and 2 µM at FRhK-4 cells. The x-axis in each graph represents the amounts of virus used expressed in infectious units (IU) based on infectious titers calculated for each virus in permissive feline CRFK cells. Infected (GFP-expressing) cells were detected by flow cytometry 2 d
post-infection. (D) IF microscopy analysis of microtubules in treated cells. HeLa and FRhK-4 cells were transfected with GFP-α-tubulin, and 2 d later subjected to two-hour drug treatments using the same concentrations as above, and then fixed. GFP fluorescence was observed by IF microscopy, along with DNA which was stained using DAPI (blue staining). The bar on the images represents 10 µm. A representative image from each condition is presented.

**Figure 3: Pharmacological disruption of dynein motor function decreases endogenous TRIM5α-mediated retroviral restriction.** (A, B) Dose-dependent effect of EHNA on restriction. Human HeLa cells (A) and macaque FRhK-4 cells (B) were infected for 16 h with a single dose of the indicated viruses in the presence of increasing concentrations of EHNA. The amounts of viruses used were the same as in Fig. 1. Infected (GFP-expressing) cells were detected by flow cytometry 2 d post infection. (C, D) Virus dose-dependent effect of EHNA on restriction. Human HeLa cells (C) and macaque FRhK-4 cells (D) were infected for 16 h with multiples doses of the indicated viruses in the presence or absence of EHNA at 600 µM (HeLa) or 1.2 mM (FRhK-4). The x-axis represents the amounts of virus used expressed in infectious units (IU) based on infectious titers calculated for each virus in permissive feline CRFK cells. Infected (GFP-expressing) cells were detected by flow cytometry 2 d later. (E) IF microscopy analysis of LAMP-1 distribution. HeLa and FRhK-4 cells were treated for 2 h with EHNA or left untreated, then fixed and stained for the lysosomal marker LAMP-1 (red) and DNA (blue). Cell edges are outlined and examples of localization shift caused by impaired
Figure 4: DHC depletion decreases TRIM5α-mediated retroviral restriction and has no effect on cells treated with nocodazole or taxol. (A) Western blot analysis of DHC expression in HeLa cells 48 h after transfection of the indicated siRNAs. Actin was analyzed as a loading control. (B) IF microscopy analysis of LAMP-1. LAMP-1 was stained 72 h post siRNAs transfection (red) and DNA was stained using DAPI (blue). Cell edges are outlined and a LAMP1 distribution shift caused by impaired dynein function is indicated by a white arrow. A representative image is shown for each condition. The bar on the image panels represents 10 μm. (C) Effect of DHC knockdown on restriction. Human HeLa cells were transfected with siRNAs against dynein heavy chain (siDHC), or against luciferase (siLuc) as a control. 72 h later, cells were infected for 16 h with multiple doses of N- or B-MLV GFP. Infected cells were detected by flow cytometry 2 d after infection. The x-axis shows the amounts of virus used expressed in CRFK infectious units. (D, E) Effect of combining siRNA transfections with 0.25 μM of nocodazole (D) or 0.1 μM of taxol (E). Infections were performed and analyzed as in panel C, and panels C-E all share the same legend.

Figure 5: Inhibition of HIV-1 restriction by rhTRIM5α exogenously expressed in HeLa cells. (A) Effect of nocodazole (noc) and taxol (txl) on the restriction of HIV-1 vectors at multiple virus doses. HeLa cells stably expressing FLAG-tagged rhTRIM5α (rhT5α) were infected for 16 h with multiple amounts of the indicated vectors in the
presence or absence of 0.1 µM of nocodazole or taxol. Virus doses were normalized according to titers in CRFK cells. % of infected cells were analyzed 2 d post infection.

(B) Effect of DHC depletion on the restriction of HIV-1NL43-GFP. HeLa cells stably expressing rhTRIM5α (rhT5α) were transfected with siRNAs targeting DHC (siDHC) or, as a control, luciferase (siLuc). The efficiency of DHC knockdown was similar to that in Fig. 4A (not shown). Cells were then infected for 16 h with HIV-1NL43-GFP or SIVmac-GFP, and the % of infected cells were determined 2 d later. (C, D) Effect of nocodazole or taxol treatment on the infectivity of HIV-1CMV-GFP in cells expressing HIV-1 restrictive or non-restrictive TRIM5α alleles. HeLa cells transduced as indicated with FLAG-tagged huTRIM5α (huT5α), rhTRIM5α (rhT5α) or with the empty vector were treated or not with 0.1 µM of nocodazole (C) or taxol (D). Cells were simultaneously infected in triplicates with single doses of HIV-1CMV-GFP adjusted to yield ~1-10% of infected cells in the absence of the drug. Infectivities obtained in the absence of drugs were as follows: for (C), HeLa [vector], 1.23 ± 0.09 %; HeLa [huT5α], 2.14 ± 0.81 %; HeLa [rhT5α], 0.82 ± 0.06 %; for (D), HeLa [vector], 6.84 ± 0.14 %; HeLa [huT5α], 6.58 ± 0.27 %; HeLa [rhT5α], 2.79 ± 0.19 %. Infected cells were detected by flow cytometry 2 d post infection and results are presented as -fold changes relative to the relevant untreated controls (relative change in infectivity). (E) Effect of DHC depletion on restriction. HeLa cells stably expressing huTRIM5α or rhTRIM5α were transfected with siRNAs against DHC (siDHC) or against luciferase (siLuc) as a control and infected 72 h later in triplicates with single doses of the viral vector HIV-1CMV-GFP for 16 h. Virus doses were adjusted to obtain ~1% of infected cells for the siLuc control and yielded 1.61% ± 0.29 infected HeLa [huT5α] cells and 0.30 ± 0.08 % infected HeLa [rhT5α] cells. Infected cells were
detected by flow cytometry 2 d post infection and results are presented as -fold changes relative to the relevant untreated controls (relative change in infectivity). *** indicates $P < 0.0001$ in a Student’s t-test. (F) Western blotting analysis of cells stably transduced with FLAG-tagged huTRIM5α and rhTRIM5α, with actin as a loading control.

Figure 6: Nocodazole and taxol inhibit TRIM5α-mediated restriction of non-pseudotyped HIV-1. (A) MAGI cells transduced with FLAG-tagged huTRIM5α (huT5α), rhTRIM5α (rhT5α) or with the empty vector were treated or not with 0.25 µM nocodazole (noc) or 0.1 µM taxol (txl). As a control, cells were also infected in presence of the fusion inhibitor heparin (20 µg/ml). Cells were infected in triplicates with single doses of HIV-1NL43-IRES-GFP. The amounts of virus used were adjusted to obtain ~0.1% of infected cells in the absence of the drug and yielded 0.22% ± 0.025 infected MAGI [vector] cells, 0.16% ± 0.03 infected MAGI [huT5α] cells and 0.043% ± 0.006 infected MAGI [rhT5α] cells. Supernatants were replaced with fresh medium containing heparin 16 h post infection in order to prevent re-infections. Infected (GFP-positive) cells were detected by flow cytometry 2 d post infection and results are presented as -fold changes in the % of infected cells relative to the relevant untreated controls (relative change in infectivity). * and ** indicate $P = 0.0134$ and $P = 0.0068$ in a Student’s t-test, respectively. (B) Western blot analysis of FLAG-huTRIM5α and FLAG-rhTRIM5α expression in stably transduced MAGI cells. Actin was analyzed as a loading control.

Figure 7: Effect of treatments on TRIM5α stability and cell viability. (A) Effect on rhTRIM5α protein levels. HeLa cells stably expressing FLAG-tagged rhTRIM5α were
treated with nocodazole (0.1 µM), taxol (0.1 µM), EHNA (600 µM) and concomitantly treated with cycloheximide. Alternatively, cells were transfected with luciferase- or DHC-targeting siRNAs like before and treated with cycloheximide 72 h later. The efficiency of DHC knockdown was similar to that in Fig. 4A (not shown). Protein lysates were prepared at the indicated time points following the beginning of drug treatments. Lysates were subjected to western blotting to detect FLAG-rhTRIM5α and actin. One representative experiment out of 3 independent experiments is shown. (B) Analysis of FLAG-rhTRIM5α protein turnover. FLAG-rhTRIM5α bands detected by western blotting were quantified by densitometry and normalized to actin levels and then to the value obtained at the ‘0’ time point; ctl, control; noc, nocodazole; txl, taxol. Average data from 3 independent experiments with standard deviations are shown. The P-values indicated on the graphs were calculated using a Student’s t-test for the three conditions at which an effect could be observed: EHNA treatment (1 h) and DHC siRNA transfection (3 h and 4.5 h). (C) Cell viability assay. HeLa and FRhK-4 cells were treated with multiple concentrations of the indicated drugs for 16 h and cell viability was then determined using the XTT assay and normalized using the value obtained in absence of drug as a reference. Vertical lines indicate the drug concentrations used in this study, for each combination of drug and cell line.

Figure 8: DHC depletion, taxol treatment and nocodazole treatment increase the stability of HIV-1 cores in permissive or restrictive conditions. (A) HeLa cells stably expressing rhTRIM5α (rhT5α) or transduced with the empty vector were transfected with the indicated siRNAs and 72 h later infected with HIV-1NL43-GFP or an entry-incompetent
version of this vector lacking the VSV G envelope protein. Supernatants were replaced by fresh medium 2 h post infection and cells were lysed after an additional 4 h of incubation. Pre-cleared lysates were layered on sucrose cushions and particulate viral CA cores were pelleted by ultracentrifugation. Proteins in post-centrifugation pellets (PEL), supernatants (SUP) and whole cell lysates (WCL) were analyzed by western blotting to detect CA (p24). Bands corresponding to p24 were quantified by densitometry and plotted as pellet/WCL and pellet/supernatant ratios, relative to the non-restrictive untreated control. Average data from 3 independent experiments with standard deviations are shown. -fold changes and P-values (calculated using a Student’s t-test) are shown for specific pairs of data. (B) Same as (A), but cells were treated or not with 0.1 µM taxol (txl) during the 2 h of infection and during the 4 h of incubation. (C) Same as (B), but cells were treated or not with 0.1 µM nocodazole (noc).

Figure 9: Influence of DHC depletion and nocodazole treatment on the localization and dynamics of rhTRIM5α cytoplasmic bodies (CBs). (A, B) IF microscopy. HeLa cells transduced with FLAG-tagged rhT5α and control cells transduced with the empty vector were (A) seeded on glass coverslips and 24 h later treated or not with 2 µM of nocodazole for 6 h, or (B) transfected with 40 nM of siRNAs targeting DHC or Luc and 48 h later seeded on glass coverslips and incubated for an additional 24 h. Cells were fixed and immunostained for FLAG (red). DNA was stained with Hoechst33342 (blue). Representative images are shown. (C) Sizes of CBs. All CBs were outlined in cells from a minimum of 5 randomly chosen fields and their area was calculated using an image analysis software (AxioVision). Red bars show the standard error of the mean (SEM).
*** indicates $P \leq 0.0001$ in a Student’s t-test analysis. (D) The relative localization of all TRIM5α CBs from a minimum of 5 randomly chosen cells was calculated using the formula $x/(x+y)$, where $x$ is the shortest distance to the nucleus and $y$ is the shortest distance to the cell’s edge. SEM are shown as red bars, and *** indicates $P \leq 0.0001$. (E-G) Co-localization of rhT5α CBs and microtubules. (E) HeLa cells transduced with FLAG-tagged rhTRIM5α were transfected with siRNAs targeting Luc or DHC and 24 h later cells were additionally transfected with a plasmid expressing GFP-α-tubulin. The next day, cells were seeded on glass coverslips and grown for 24 h. Cells were fixed and immunostained for FLAG (red) and DNA (blue). (F) Same as (E), except that cells were transduced with the empty vector and were transfected with the luciferase-targeting siRNA. Representative images are shown. Boxes in the top right corner show enlarged regions outlined on the images, and white arrows show examples of GFP-tubulin/FLAG-TRIM5α co-localizations. (G) Events of co-localization were quantified as a % of total CBs in 10 randomly chosen fields. Bars represent the mean values from analyzed cells with SEM (** indicates $P < 0.005$).
**Figure A:**

Bar graph showing the relative change in infectivity of HIV-1NL43-IRES-GFP in control, noc, txl, and heparin conditions. The bars represent the infectivity levels with error bars indicating standard deviation. The treatments include MAGI [vector], MAGI [huT5α], and MAGI [rhT5α].

**Figure B:**

Western blot images showing protein expression levels of vector huT5α and rhT5α. The blot images are labeled with α-FLAG and α-actin.