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Interferon-inducible TRIM22 contributes to maintenance of HIV-1 proviral latency in T cell lines

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ABSTRACT

The human immunodeficiency virus type-1 (HIV-1) establishes a state of latent infection in a small number of CD4$^+$ T lymphocytes that, nonetheless, represent a major obstacle to viral eradication. We here show that Tripartite Motif-containing protein 22 (TRIM22), an epigenetic inhibitor of Specificity protein 1 (Sp1)-dependent HIV-1 transcription, is a relevant factor in maintaining a state of repressed HIV-1 expression at least in CD4$^+$ T cell lines. By knocking-down (KD) TRIM22 expression, we observed an accelerated reactivation of a doxycycline (Dox)-controlled HIV-1 replication in the T lymphocytic SupT1 cell line. Furthermore, we here report for the first time that TRIM22 is a crucial factor for maintaining a state of HIV-1 quiescence in chronically infected ACH2 -T cell line while its KD potentiated HIV-1 expression in both ACH-2 and J-Lat 10.6 cell lines upon cell stimulation with either tumor necrosis factor-α (TNF-α) or histone deacetylase inhibitors (HDACi). In conclusion, TRIM22 is a novel determinant of HIV-1 latency, at least in T cell lines, thus representing a potential pharmacological target for strategies aiming at curtailing or silencing the pool of latently infected CD4$^+$ T lymphocytes constituting the HIV-1 reservoir in individuals receiving combination antiretroviral therapy.

1. Introduction

The achievement of a viral eradication ("Cure") or even a "Functional Cure" (*i.e.* silencing or decreasing the number of cells carrying replication-competent proviruses below a threshold of spreading infection after therapy interruption) for individuals infected with the human immunodeficiency virus type-1 (HIV-1) is currently prevented by the peculiarity of this virus to integrate in CD4$^+$ cells, mostly T lymphocytes, containing the best characterized viral reservoir (VR), where it can persist lifelong (Bruner et al., 2015). Although combination antiretroviral therapy (cART) suppresses virus replication very efficiently, it is otherwise ineffective in preventing the establishment of the VR even when started during primary, acute HIV-1 infection (Archin et al., 2012; Strain et al., 2005). During acute HIV-1 infection, before the onset of the adaptive immune response high levels of virus replication, measured in terms of RNA copy numbers per ml of blood (viremia), lead to a rapid loss of circulating CD4$^+$ T lymphocytes (Fauci et al., 1996). Nonetheless, a minority of infected CD4$^+$ T lymphocytes harboring integrated provirus survives both the direct or indirect viral cytopathic effect (Lassen et al., 2004). These infected cells, bearing a "resting memory" phenotype, are capable of homeostatic proliferation (Chomont et al., 2009) and thereby represent a central component of the VR (Chun et al., 1998; Sengupta and Siliciano, 2018).

At the molecular level, silencing of the HIV-1 provirus results from the combined effect of lack of positive transcription factors and dominance of negative transcription factors (Williams et al., 2006) together with epigenetic modifications. In this regard, cytoplasmic sequestration of the inducible transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Williams et al., 2007a), nuclear factor of activated T-cells (NFAT) (Kinoshita et al., 1997) and of the positive transcription elongation factor (P-TEFb) complex (Budhiraja et al., 2013; Chiang et al., 2012; Coiras et al., 2009) contributes to the

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inhibition of proviral transcription at the initiation and elongation stages, which hinders the accumulation of the viral transactivator protein Tat required for efficient HIV-1 transcription. At the epigenetic level, histone deacetylation (Mbonye and Karn, 2014; Van Lint et al., 2013) and DNA methylation of the region including the HIV-1 promoter reduce the accessibility of HIV-inductive transcription factors (Blazkova et al., 2009; Kauder et al., 2009). These latter findings, however, were not fully confirmed in resting $CD4^+$ T cells of infected individuals receiving cART, suggesting that the VR is likely maintained predominantly by mechanisms independent of methylation of the HIV-1 promoter (Blazkova et al., 2012; Ho et al., 2013; Palacios et al., 2012).

Tripartite motif-containing protein 22 (TRIM22) belongs to the family of TRIM proteins with antiviral activity against several viruses (Rajsbaum et al., 2014; Turrini et al., 2014; Vicenzi and Poli, 2018). Its E3 ubiquitin ligase activity is required for the inhibition of encephalomyocarditis virus, influenza A virus and hepatitis C virus *in vitro* (Di Pietro et al., 2013; Eldin et al., 2009; Yang et al., 2016). However, TRIM22 antiviral effects extend beyond its E3 ubiquitin ligase activity in that it restricts HIV-1 replication both at transcriptional and post-transcriptional levels, at least *in vitro* (Barr et al., 2008; Ghezzi et al., 2013; Kajaste-Rudnitski et al., 2011; Tissot and Mechti, 1995; Turrini et al., 2015). Of note is the fact that TRIM22 interference with proviral transcription occurs without interfering with either NF-κB or Tat-dependent proviral transcription (Kajaste-Rudnitski et al., 2011). In particular, we have described that, even in the absence of a direct interaction with proviral DNA, TRIM22 specifically inhibits the binding of the cellular transcription factor Specificity protein 1 (Sp1) to the HIV-1 LTR promoter region *in vitro* (Turrini et al., 2015). Furthermore, TRIM22 was shown to localize in specific nuclear structure, termed "TRIM22 nuclear bodies", also containing the HIV-1 transcriptional repressors TRIM19/Promyelocytic leukemia (PML), Cyclin T1, a crucial component of the P-TEFb complex, and Class II Transactivator (CIITA), a transcriptional repressor of HIV-1 proviral transcription (Forlani et al., 2017, 2016). Based on these observations, TRIM22 nuclear bodies have been suggested to represent an example of intracellular hub of antiviral factors, inhibiting both the basal and Tat-dependent HIV-1 transcription (Forlani and Accolla, 2017).

The potential relevance of TRIM22 in HIV-1 infection is also sustained by the observation that its expression is upregulated in peripheral blood mononuclear cells (PBMC) of individuals with both primary and chronic HIV-1 infection (Singh et al., 2011, 2014) whereas polymorphisms of its coding sequence have been associated with different outcomes of HIV-1 disease (Ghezzi et al., 2013). Furthermore, TRIM22 was found to be constitutively expressed in PBMC and cord-blood MC of HIV-infected mothers under cART (Pereira et al., 2013) as well as in macrophages isolated from Sooty Mangabeys characterized by a natural non-pathogenic simian immunodeficiency virus (SIV) infection (Mir et al., 2015). Thus, TRIM22 represents a potential candidate to significantly contribute to either the establishment or the maintenance of a state of HIV-1 latency in persistently infected $CD4^+$ T lymphocytes in individuals receiving cART.

We provide here experimental evidence supporting such an hypothesis by investigating both a Dox-dependent model of reversible proviral latency in acutely infected SupT1 cells and different T cell lines chronically infected with either a Tat/TAR proficient (J-Lat 10.6) or deficient (ACH-2) provirus following knock-down (KD) of endogenous TRIM22 gene expression.

2. Materials and methods

2.1. Cell lines and reagents

SupT1, ACH-2 and J-Lat 10.6 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with glutamine (2 mmol/liter), penicillin (100 U/ml), and streptomycin (100 μg/ml) and with 10% fetal bovine serum (FBS) from Lonza (Cologne, Germany) - complete RPMI 1640. Human Embryonic Kidney 293 T (HEK 293 T) cells and human squamous carcinoma Hep-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with glutamine (2 mmol/liter), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS (complete DMEM). Human, endotoxin-free recombinant cytokines tumor necrosis factor-α (TNF-α) was purchased from R&D Systems (Minneapolis, MN, USA); 3TC (Lamivudine), Trichostatin A (TSA), puromycin and Dox were purchased from Sigma-Aldrich (St. Louis, MO, USA) whereas panobinostat and romidepsin were purchased from Selleckchem (Munich, Germany).

2.2. Plasmids

$pLKO.1/TRIM22_{shRNA}$, $pLKO.1/random_{shRNA}$, psPax2, pMD2.G plasmids for the lentiviral vector (LV) production were previously used and described (Kajaste-Rudnitski et al., 2011). Infectious molecular clone plasmid DNA of HIV-rtTA, tetO-CMV and tetO-CMV-Sp1 were previously used and described (Das et al., 2011; Turrini et al., 2015). pcDNA-TRIM22 and pc-Luciferase (Luc) plasmids were previously used and described (Kajaste-Rudnitski et al., 2011; Turrini et al., 2015) (Marban et al., 2007).

2.3. Lentiviral vector and molecular infectious clone production

Vectors and viruses were produced by transfection of HEK 293 T cells seeded at 6×10^5 cells/ml in T75 flask by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). VSV-$pLKO.1/TRIM22_{shRNA}$ and VSV-$pLKO.1/random_{shRNA}$ vectors were produced by co-transfecting pMD2.G, psPax2, and either $pLKO.1/TRIM22_{shRNA}$ or $pLKO.1/random_{shRNA}$ at 1:3:4 ratios, respectively. Vector/virus containing supernatants were harvested 24 and 48 h post-transfection, cleared by centrifugation, filtered (0.45-μm pore size; Millex-HV polyvinylidene difluoride; Millipore, Carrigtwohill, Ireland), and stored at −80 °C. HIV-rtTA and mutant virus amount present in the stock was determined by measuring the levels of virion-associated Mg^{2+}-dependent reverse transcriptase (RT) activity present in the supernatant, as previously described (Vicenzi et al., 1994).

2.4. Generation of TRIM22-KD cells

To downregulate TRIM22 expression, SupT1, ACH-2 or J-Lat 10.6 (3×10^6 cells/condition) were transduced with VSV-$pLKO.1/TRIM22_{shRNA}$ or VSV-$pLKO.1/random_{shRNA}$ vector-containing supernatants at a 1:1 ratio by two rounds of spin-infection at 2500 rpm for 2 h at 37 °C. After the second centrifugation, transduced cells were seeded at 1×10^6 cells/ml in 6-well plates and selected by addition of puromycin (1 μg/ml) to the culture medium for 72 h. The levels of TRIM22 expression were verified by real-time PCR as described below.

2.5. Quantification of mRNAs by real-time PCR

Total RNA was extracted from SupT1, ACH-2 or J-Lat 10.6 cells by using a TRIzol Plus RNA purification kit, followed by DNase I treatment (Invitrogen). cDNA was synthesized from total RNA (1 μg) using a SuperScript first-strand synthesis system (Invitrogen) with random hexamers. PCR were performed with 50 ng of cDNA and primer pair and probes described in Table S1. To normalize the mRNA expression, either human ribosomal 18S or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA were amplified. Standard curve for TRIM22 was obtained by cloning *TRIM22* open reading frame into TOPO™-vector (Invitrogen) following the manufacturer's indication. Standard curve for 18S was purchased from Applied Biosystems. The copies of target and normalizer DNA were calculated from the relative standard curves by interpolation. All reactions were performed with an ABI 7700 Prism instrument (Applied Biosystems, Foster City, CA) using the following thermal cycling conditions: 50 °C for 2 min, 95 °C for

10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.6. Generation of latently infected SupT1 cells

72 h after puromycin selection, untransduced (UT), TRIM22-KD (KD) and control (CT) SupT1 cells were infected with the same cpm-equivalent amount of HIV-rtTA and mutant viruses by spin-infection at 2500 rpm for 2 h at 37 °C. After centrifugation, culture supernatants were replaced with Dox (1 μg/ml)-containing fresh RPMI-1640 and cells were seeded into 48-well plates for 6 days. Then, virus replication was shut-off by Dox removal with two cell washes using fresh RPMI-1640 for 30′ at 37 °C and 3TC (1 nM) addition. Twelve days after infection, cells carrying a provirus were reactivated by addition of Dox (1 μg/ml) to the culture medium in the presence of 3TC. Virus-containing supernatants were harvested every 3–4 days for 27 days and tested for RT activity as previously described. To assess potential cytotoxicity upon infection, the adenylate kinase (AK) activity was measured in the culture supernatant 3 days post-infection with the ToxiLight™ bioassay kit (Lonza) (Crouch et al., 1993).

2.7. Reactivation of latently infected ACH-2 and J-Lat 10.6 cells

Seventy-two h after puromycin selection, cells were seeded in 96-well plates at 2.5×10^5/ml, 100 μl per well. Cells were stimulated with TNF-α (1 ng/ml), TSA (1.5 μM), panobinostat (150 nM) or romidepsin (75 nM) and cultured for 72 h; panobinostat and romidepsin concentrations were selected after titration from 0.048 to 150 nM and 0.024 to 75 nM, respectively, in J-Lat 10.6 cells. Virus-containing supernatant was harvested at 24 h, 48 h and 72 h post-reactivation for RT activity assay.

2.8. FACS analysis

Flow cytometry was performed to detect either intracellular p24 Gag or green fluorescent protein (GFP) expression in ACH-2 or J-Lat 10.6 cells, respectively. Cells were collected, washed and either incubated with the anti-p24 Gag-FITC antibody (Ab, Beckman Coulter) for 15 min at room temperature (rt°) or analyzed directly. Sample data were acquired using CytoFLEX (Beckman Coulter) and analyzed with FCS express Software (De Novo Software).

2.9. Co-immunoprecipitation (Co-IP)

HEK 293 T cells were seeded at 6×10^5/ml in T75 flask. Twenty-four h later, cells were co-transfected with pcDNA-TRIM22 and Flag-HDAC-1 expressing plasmids (5 μg), either alone or in combination, and with a Luc-expressing plasmid (5 μg) as unrelated protein control. Forty-eight h after transfection, cells were lysed with Co-IP lysis buffer (50 mM Tris − HCl pH 7.4, NaCl 150 mM, EDTA 1 mM, 1% Triton x100) supplemented with complete Protease Inhibitor Cocktail (Roche) and phosphatases inhibitor PhosStop cocktail (Roche). Two mg of whole cell extract were immunoprecipitated overnight with 5 μg of polyclonal rabbit anti-TRIM22 Ab (Abnova). Immunocomplexes were then captured using Protein G Dynabeads (Invitrogen) and washed 5 times with 900 μl of Co-IP lysis buffer. Immunocomplexes were detached from Dynabeads by incubation in Laemmli buffer for 5 min at 95 °C and loaded on a SDS-PAGE for Western blot analyses as previously described (Turrini et al., 2015).

2.10. Immunofluorescence and confocal microscopy

For localization studies, Hep-2 cells cultured on glass cover slips were transfected with pcDNA-TRIM22 and Flag-HDAC-1 expressing vectors (0.2 μg each) either alone or in combination. At 24 h post-transfection, cells were washed with PBS 3 times and fixed with methanol (− 20 °C) for 4 min. Cells were washed again with PBS and incubated with a solution of 1% BSA in PBS for 1 h. Cells were then stained overnight with anti-TRIM22 polyclonal Ab (Sigma-Aldrich, HPA003575, dilution 1:169). The slides were then washed 5 times with cold PBS and incubated in the dark for 2 h, at rt° with goat anti-rabbit IgG conjugated to Alexa Fluor 546 (Thermo Scientific, diluted 1:400). After extensive washing with PBS, a fluorescein isothiocyanate (FITC)-conjugated mouse anti-Flag-M2 Ab (Sigma-Aldrich, F4049, dilution 1:200) was added for 2 h at rt°. The nuclei were stained by incubating the cells with DRAQ5 Fluorescent Probe (Thermo Scientific, diluted 1:1000), for 30 min at rt°. After washing, the slides were mounted on coverslips with the Fluor Save reagent (Calbiochem) and examined by a confocal laser scanning microscope (Leica TCS SP5; HCX PL APO objective lenses, 63X original magnification, numerical aperture of 1.25). Images were acquired and analyzed by LAS AF lite Image (Leica Microsystem) and/or Fiji (Image J) softwares.

2.11. Statistical analysis

Statistical analysis was performed using the Prism GraphPad software v. 6.0 (GraphPad Software, www.graphpad.com). Comparison between groups was performed using either one-way or two-way ANOVA; p values < 0.05 were considered significant.

3. Results

3.1. TRIM22-KD enhances Dox-dependent proviral reactivation and virus replication in SupT1 cells

We have previously characterized TRIM22 as an epigenetic repressor of HIV-1 transcription and replication in both primary PBMC (Ghezzi et al., 2013) and cell lines of both myeloid and T lymphocytic origin (Forlani et al., 2016; Kajaste-Rudnitski et al., 2011; Turrini et al., 2015). Furthermore, TRIM22 inhibited the replication of a TAR/Tat-independent, Dox-dependent virus variant (HIV-rtTA) mainly by interfering with Sp1-dependent transcription (Turrini et al., 2015). To test whether TRIM22 silencing could also influence the reactivation of latent HIV-rtTA provirus, we investigated its activity in a modified lentivirus context (Jeeninga et al., 2008). HIV-rtTA carries a mutated TAR element (bulge and loop mutations), which inactivates the Tat/TAR axis of transcriptional activation (Fig. 1A). Furthermore, the HIV-rtTA variant encodes the Dox-inducible rtTA transcriptional activator protein that was inserted at the site of the *nef* gene. Two rtTA-binding sites (tetO) were placed between the NF-κB and Sp1 binding sites in the LTR-U3 promoter region (Das et al., 2011). Therefore, the replication of rtTA is strictly controlled by the supplementation of Dox and can be switched either on or off in a reversible matter.

TRIM22 expression was firstly repressed in the SupT1 cell line by transducing a lentiviral vector expressing short-hairpin (sh) RNAs either specific for the TRIM22 mRNA or carrying a scrambled sequence as control. To select TRIM22-KD cells, transduced SupT1 cells were incubated with puromycin for 72 h (Fig. 1B). As shown in Fig. S1A, transduction with the TRIM22 shRNA vector resulted in the efficient KD of TRIM22 RNA expression. After 3 days, cells were infected with HIV-rtTA and virus replication was induced by the presence of Dox for 6 additional days when replication was halted by Dox removal and addition of the RT inhibitor lamivudine/3TC in the culture medium (Fig. 1B). Of note is the fact that the decrease of TRIM22 expression did not increase cellular toxicity (Fig. S2).

After 12 days of infection, Dox was added back to the culture medium to reactivate proviral transcription in the presence of lamivudine/3TC to prevent virus spreading (Fig. 1B) and virus replication was assessed by measuring the levels of RT activity in culture supernatants up to day 27 post-infection. A first, modest peak of virus replication was detected 6 days post-infection in TRIM22-KD, but not in control cells (Fig. 1C), consistently with the restrictive activity of TRIM22 observed in acute *in vitro* HIV-1 infection (Turrini et al., 2015). Of interest, the

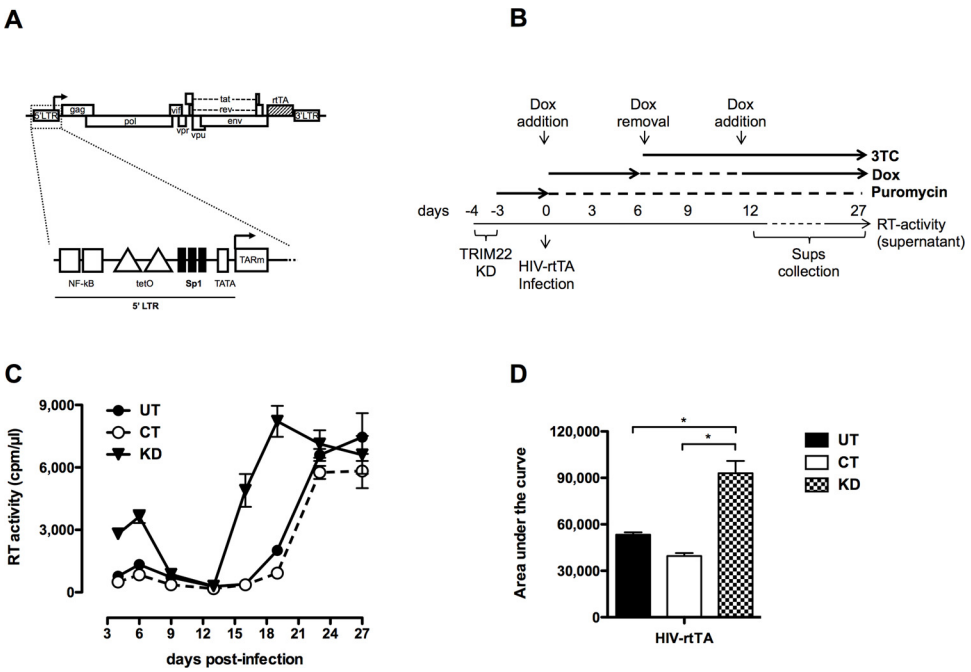

Fig. 1. Knock-down of TRIM22 enhances Dox-dependent proviral reactivation in SupT1 cells. A. Schematic structure of HIV-rtTA and the enlargement of the wild type 5′-LTR. B. Experimental protocol of HIV-rtTA latently-infected SupT1 cells. Horizontal bold arrows indicate the intervals of addition to culture media of the indicated drugs. C. Kinetics of HIV-rtTA virus replication in untransduced (UT), control-transduced (CT) and TRIM22 knock-down (KD) SupT1 cells as measured by RT activity in culture supernatants. Means ± SEM of one representative experiment out of three independent infections in triplicates are shown. D. Area under the curve of RT activity from day 12 to day 27 post-infection. Bars represent the mean ± SEM of 2 independent experiments. * indicates a p value < 0.05 as calculated by one-way ANOVA with Bonferroni post-test.

kinetics of HIV-rtTA replication observed after Dox reactivation were accelerated in TRIM22-KD cells in comparison to both UT and CT SupT1 cells (Fig. 1C). Indeed, the analysis of the area under the curve of virus replication was significantly increased in TRIM22-KD cells *vs.* the remainder conditions (Fig. 1D).

3.2. Sp1 binding sites are crucial for the reactivation of latent HIV-rtTA provirus in TRIM22-KD SupT1 cells

As TRIM22 inhibits the basal HIV-1 LTR-driven transcription by preventing the binding of the cellular transcription factor Sp1 to the viral promoter (Turrini et al., 2015), we tested whether also the reactivation of the HIV-rtTA provirus was dependent on the presence of conserved Sp1 binding sites within this region of the LTR. To this aim, we infected UT, CT and KD SupT1 cells with an HIV-rtTA variant containing a LTR with 3 tetO elements coupled to a minimal TATA-box containing cytomegalovirus promoter sequence (tetO-CMV; Fig. 2A, left panel) or with an isogenic construct in which 3 HIV-1 Sp1 binding sites had been reintroduced between the tetO and minimal promoter sequences (tetO-CMV-Sp1; Fig. 2B, left panel). Virus replication was blocked by Dox removal and addition of 3TC to the culture medium 6 days after infection, as indicated in the general experimental scheme (Fig. 1B).

Infection by tetO-CMV did not result in detectable RT levels in UT, CT or KD cells in the 6 days plus-Dox period (Fig. 2A), whereas tetO-CMV-Sp1 replication was detectable in all cells, with the highest levels of virus production obtained in TRIM22-KD cells (Fig. 2B). These results are therefore consistent with our previous observation that the presence of Sp1-binding sites in the LTR confers susceptibility to TRIM22 inhibition of HIV-1 transcription (Turrini et al., 2015). To achieve proviral HIV-1 silencing, Dox was then removed for 6 days and HIV-1 transcription and replication was reactivated by the administration of Dox at day 12 post-infection. TetO-CMV replication remained suppressed up to 16 days post-infection although low levels of RT activity were detected in TRIM22-KD cells as compared to either UT or CT cells up to 27 days post-infection (Fig. 2A). In contrast, higher RT activity levels and a significant increase of the area under the curve were observed when tetO-CMV-Sp1 was reactivated by Dox administration in TRIM22-KD cells *vs.* CT and UT controls (Fig. 2B and C, respectively).

Altogether these data support a role of TRIM22 in inhibiting the reactivation of HIV-1 expression by interfering with the binding of Sp1 to the proviral promoter.

3.3. TRIM22-KD alone induces the reactivation of proviral expression in ACH-2, but not in J-Lat 10.6 T cell lines

Next, we assessed whether TRIM22 affected HIV-1 expression in two well-established models of proviral latency in T cell lines, *i.e.* ACH-2 and J-Lat 10.6. ACH-2 was derived by cloning the cells surviving the acute $HIV_{LAI/IIIB}$ infection of the CEM-derived A3.01 cell line (Clouse et al., 1989) whereas J-Lat 10.6 was obtained from the Jurkat cell line infected with an *env*-defective HIV provirus expressing the GFP (Jordan et al., 2003). Of interest, although the provirus integrated in ACH-2 cells is characterized by a point mutation within TAR that impairs Tat responsiveness (Emiliani et al., 1996), nonetheless a robust and sustained proviral reactivation is typically induced in these cells by stimulation either with phorbol esters or with NF-κB activating cytokines, such as tumor necrosis factor-α (TNF-α) (Emiliani et al., 1996; Poli et al., 1990) or CD30 agonists (Biswas et al., 1995); more recently, proviral reactivation in ACH-2 cells was also obtained by amino acid starvation involving the inhibition of Class II HDAC-4 (Palmisano et al., 2012). Proviral reactivation in Tat/TAR proficient J-Lat 10.6 cells can also be induced by both NF-κB activating agents and by Class I HDAC inhibitors (HDACi) (Fernandez and Zeichner, 2010).

Cell lines were transduced with a lentiviral vector expressing shRNAs either specific for the TRIM22 mRNA or carrying a scrambled sequence as control. In both cell lines, TRIM22 was expressed at comparable levels in the UT and CT cells whereas TRIM22-KD significantly diminished its basal expression by *ca.* 10-fold (Fig. S1B). This effect was specific for TRIM22, as the expression of other TRIM proteins, such as TRIM19 and TRIM28, both associated with the repression of HIV-1 transcription (Lusic et al., 2013; Nishitsuji et al., 2012), and of the *TRIM5* gene adjacent to *TRIM22* in chromosome 11 (Sawyer et al., 2007) were not affected by the transduction of TRIM22 shRNA (Fig. S1C and D).

We then assessed the levels of RT activity released in culture supernatants after up to 72 h and by intracellular expression of either p24 Gag antigen in ACH-2 cells or GFP in J-Lat 10.6 cells up to 24 h (Fig. 3A). Lentiviral transduction *per se* did not affect proviral expression in ACH-2 cells, as determined by the levels of RT activity detected

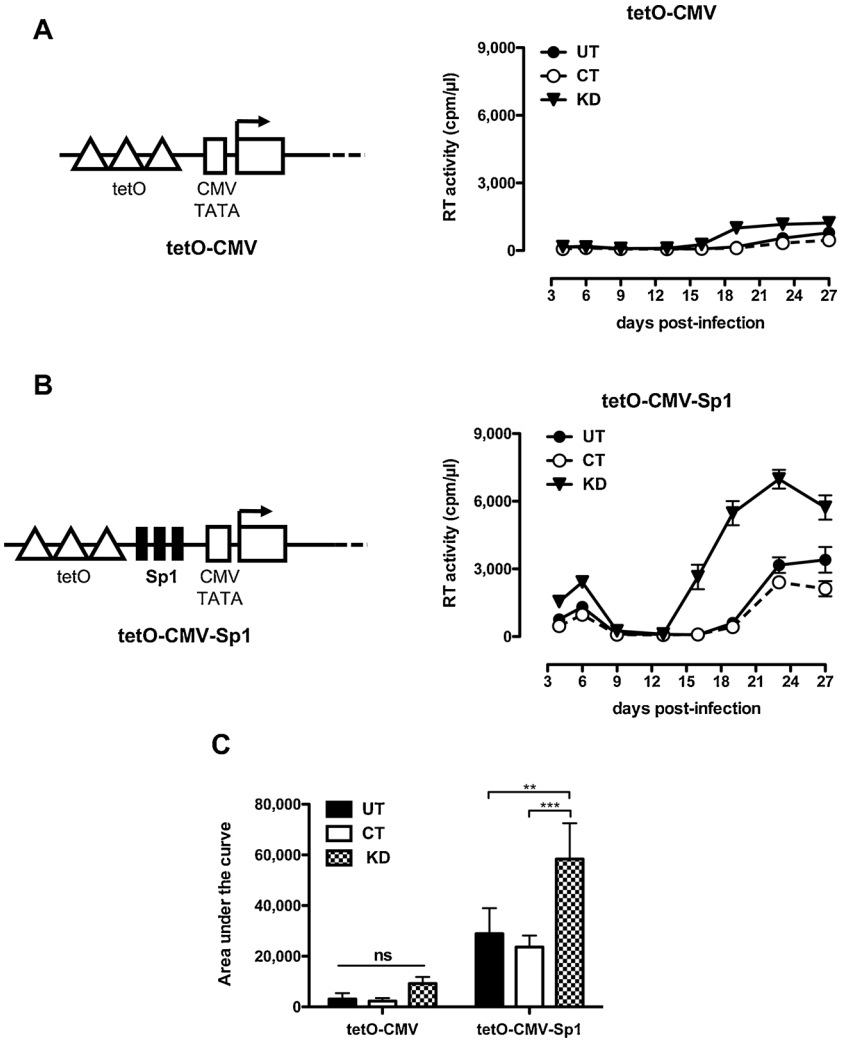

Fig. 2. TRIM22 contributes to proviral latency in SupT1 cells predominantly *via* Sp1. A. Schematic structure of tetO-CMV 5′-LTR (left panel) and kinetics of virus replication of tetO-CMV (right panel). B. Schematic structure of tetO-CMV-Sp1 5′-LTR (left panel) and kinetics of virus replication in untransduced (UT), control-transduced (CT) and TRIM22 knock-down (KD) SupT1 cells as measured by RT activity in culture supernatants. Means ± SEM of one representative experiment out of three independent infections in triplicates are shown. C. Area under the curve of RT activity from day 12 to day 27 post-infection. Bars represent the mean ± SEM of 3 independent experiments. ** indicate a p value < 0.01 and *** indicate a p value < 0.001 as calculated by two-way ANOVA with Bonferroni post-test, ns is not-significant.

in culture supernatants or by the percentage of p24 Gag$^+$ cells (Fig. 3B and C, respectively). In sharp contrast, TRIM22-KD induced a robust proviral reactivation in ACH-2 cells, resulting in significantly increased RT activity levels in culture supernatants (Fig. 3B). Furthermore, downregulation of TRIM22 resulted in *ca.* 30% p24 Gag$^+$ cells at 3 h after puromycin removal (Fig. 3C), which gradually increased up to 60% at 24 h, whereas much lower levels were detected at all time points for UT and CT cells (˜1% at 3 h and ˜18% at 24 h; Fig. 3D). In contrast to ACH-2 cells, undetectable RT activity levels were observed in J-Lat 10.6 cells, regardless of whether TRIM22 was expressed or not (Fig. 3E). Furthermore, the proportion of GFP$^+$ cells did not significantly increase in TRIM22-KD J-Lat 10.6 cells as compared to UT or CT cells both 3 h (Fig. 3F) and at later time points (Fig. 3G) after puromycin removal. As the provirus integrated in J-Lat 10.6 cells has an intact Tat/TAR axis, unlike ACH-2 cells, we also determined the effect of TRIM22-KD in the promonocytic chronically infected U1 cells in which the integrated provirus is proficient in Tat/TAR activity. As observed in J-Lat 10.6, also TRIM22-KD in U1 cells did not reactivate HIV-1 expression (data not shown).

These observations suggest that the state of proviral latency typical of both unstimulated ACH-2 and J-Lat 10.6 cell lines is maintained by at least partially distinct determinants, with TRIM22 playing a dominant role in ACH-2 cells.

3.4. TRIM22-KD potentiates TNF-α and HDACi-induced viral expression

A pivotal role in the maintenance of proviral HIV-1 latency is played by p50 homodimers that bind to NF-κB binding sites within the LTR promoter and recruit HDAC-1, ultimately repressing viral transcription (Williams et al., 2006). Activation of NF-κB heterodimers leads to squelching of p50 homodimers with the final result of triggering or potentiating proviral expression (Perkins et al., 1993; Williams et al., 2007b). Thus, we tested whether TRIM22-KD affected the latency reversal induced by TNF-α, a cytokine well-known for activating NF-κB dependent proviral expression in both ACH-2 and J-Lat 10.6 cell lines (Emiliani et al., 1996; Jordan et al., 2003; Poli et al., 1990). As expected, TNF-α stimulation reactivated HIV-1 expression in both chronically infected T cell lines that were either UT or transduced with CT vectors (Figs. 4A *vs.* 3 B for ACH-2 and Figs. 4B *vs.* 3 E for J-Lat 10.6, respectively). Of interest, a potentiation of TNF-α was observed in both ACH-2 and J-Lat 10.6 cell lines after TRIM22-KD when compared to their UT and CT counterparts (Fig. 4A and B, respectively). The observation that a synergistic proviral reactivation was observed in TRIM22-KD J-Lat 10.6 cells stimulated with TNF-α indicates that a repressive function of TRIM22 is also present in J-Lat 10.6 cells carrying a provirus characterized by a Tat/TAR proficient axis, although in a less dominant fashion than in ACH-2 cells.

We next tested whether TRIM22-KD affected the reactivation of proviruses in both ACH-2 and J-Lat 10.6 cells after stimulation with the HDAC inhibitors (HDACi) TSA, panobinostat and romidepsin, these latter two also tested in clinical trials (Rasmussen et al., 2014; Sogaard et al., 2015). All HDACi induced significant levels of HIV-1 production in TRIM22-KD ACH-2 cells at 48 and 72 h post-incubation (Fig. 4A). Concerning J-Lat 10.6 cells, TSA and romidepsin caused a modest albeit

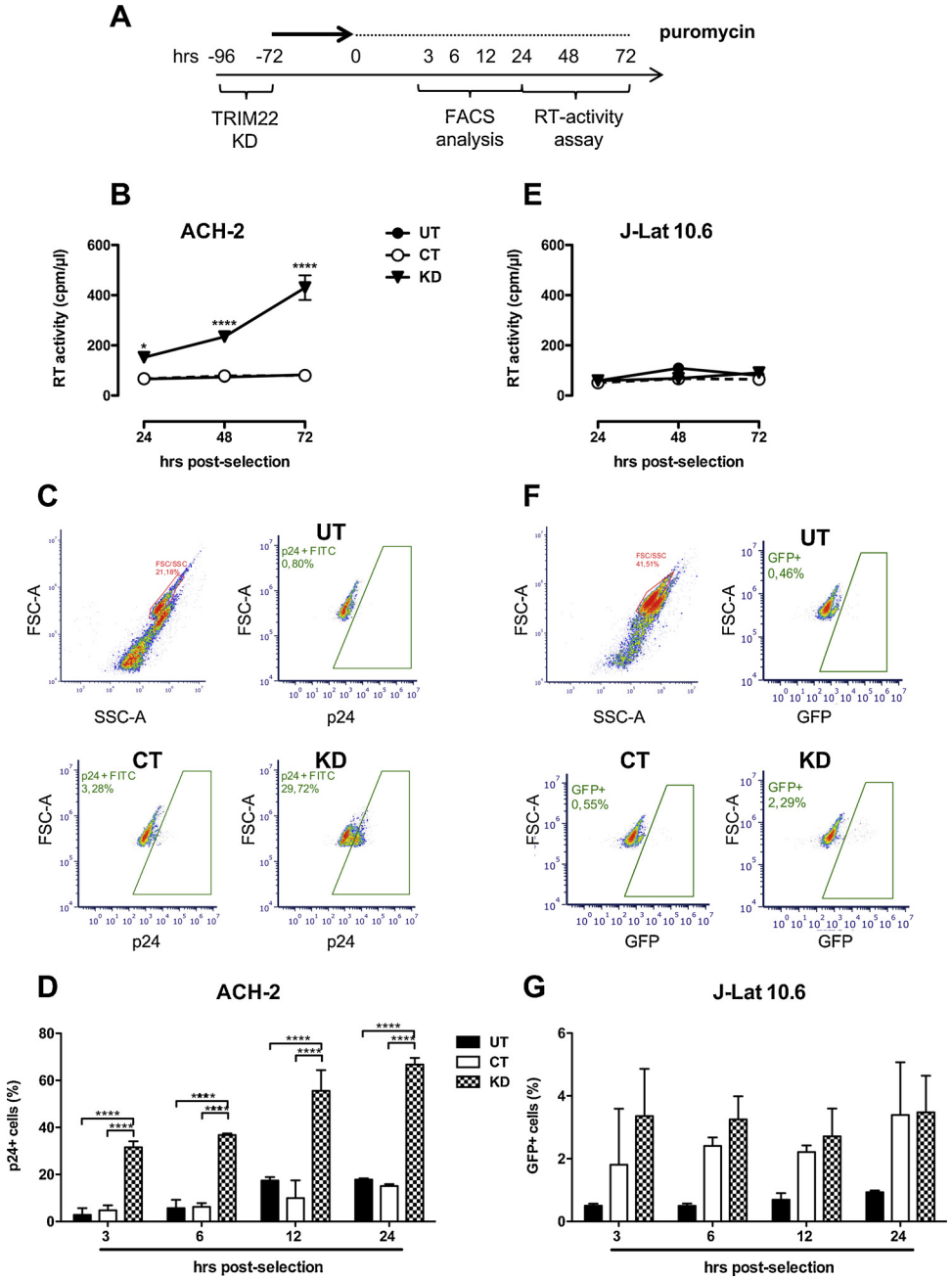

Fig. 3. TRIM22-KD reactivates proviral expression in ACH-2, but not in J-Lat 10.6 cell lines in unstimulated conditions. A. Protocol of chronically-infected ACH-2 and J-Lat 10.6 cell reactivation. Bold arrows indicate timing of puromycin treatment (72 h). Proviral reactivation was measured by intracellular p24 Gag staining or GFP expression and RT activity in culture supernatants at 24, 48, 72 h post-selection. B. Kinetics of RT activity as measured in the culture supernatant of unstimulated TRIM22-KD (KD) *vs.* untransduced (UT) or control-transduced (CT) ACH-2 cells. * indicates a p value < 0.05 and **** indicate a p value < 0.0001 as calculated by one-way ANOVA with Bonferroni post-test. C. Intracellular p24 Gag staining in ACH-2 cells 3 h after puromycin removal. D. Kinetics of intracellular p24 Gag staining in ACH-2 cells. Bars represent the mean ± SD of 3 independent experiments. E. Kinetics of RT activity measured in the culture supernatant of unstimulated J-Lat 10.6 cells. F. Green fluorescent protein (GFP) expression in J-Lat 10.6 cells 3 h after puromycin removal. G. Kinetics of GFP expression in J-Lat 10.6 cells. Bars represent the mean ± SD of 3 independent experiments.

significant increase of RT activity levels in TRIM22-KD cells *vs.* UT and CT cells whereas panobinostat had no effect (Fig. 4B).

Altogether, these results support the hypothesis that TRIM22 can cooperate with HDAC proteins in the maintenance of proviral silencing in T cell lines.

3.5. TRIM22 interacts with HDAC-1

As HDAC-1 is recruited to the HIV-1 promoter by Sp1 (Jiang et al., 2007; Marban et al., 2007; Mbonye and Karn, 2014; Van Lint et al., 2013), we next investigated whether TRIM22 could physically interact with this enzyme. To this end, HEK 293 T cells, lacking endogenous TRIM22 expression (Di Pietro et al., 2013), were transfected either with TRIM22 or Flag-tagged HDAC-1 expressing plasmids, either alone or in combination; Flag-tagged Luc plasmid was used as negative control in IP experiments. Forty-eight h post-transfection, cells were lysed and Co-IP experiments were performed in the presence of an anti-TRIM22 polyclonal Ab; both whole cell extract and IP samples were then analyzed by Western blotting. We detected similar levels of all the proteins analyzed, either when expressed alone or co-expressed (Fig. 5A, upper panel). Interestingly, HDAC-1 specifically Co-IP together with TRIM22 (Fig. 5A, Flag, lower panel, lane 6). Nonspecific bands were not detected when Flag-tagged Luc was expressed in the presence of either TRIM22 or HDAC-1 (Fig. 5A, Luc, lower panel, lanes 4 and 5, respectively).

Having shown that TRIM22 interacts with HDAC-1, we tested whether they could eventually colocalize in specific subcellular compartments. To this aim, Hep-2 cells, which do not constitutively express TRIM22 (Forlani et al., 2017), were transiently transfected with either a Flag-tagged HDAC-1 or with TRIM22 expression vectors (Fig. 5B). Their cellular distribution was assessed by both immunofluorescence and confocal microscopy. As previously shown, TRIM22 was mostly localized in specific speckled-like structures (Fig. 5B, upper panels), previously defined as “TRIM22 nuclear bodies” (Forlani et al., 2017), while

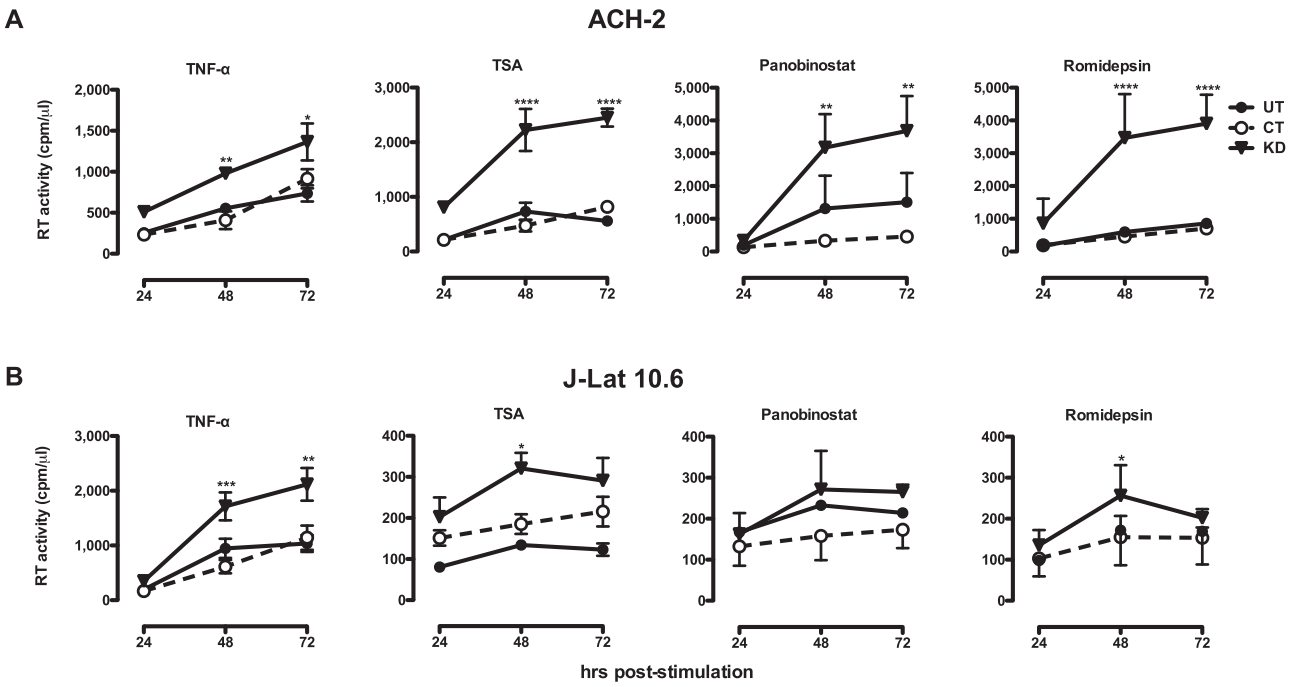

Fig. 4. TRIM22-KD potentiates virus release in both ACH-2 and J-Lat 10.6 cell lines stimulated with TNF-α and HDACi. Both ACH-2 and J-Lat 10.6 cell lines (UT, CT and KD) were selected with puromycin for 72 h; after selection, cells were stimulated with TNF-α and HDACi, *i.e.* TSA, panobinostat or romidepsin. A. Kinetics of proviral reactivation as measured by RT activity in culture supernatants of ACH-2 cells and (B) J-Lat 10.6 cells. Bars represent mean ± SEM of three independent experiments in triplicates. * indicates a p value < 0.05, ** indicate a p value < 0.01, *** indicate a p value < 0.001, **** indicate a p value < 0.0001 as determined by two-way ANOVA with Bonferroni post-test.

HDAC-1 exhibited a diffuse nuclear distribution (Fig. 5B, lower panels). Upon cell co-transfection with both plasmids, expression of HDAC-1 significantly affected TRIM22 nuclear distribution and *vice versa* (Fig. 5C). In some cells, TRIM22 staining showed the same localization pattern of HDAC-1, becoming diffuse into the nucleus (Fig. 5C, upper panels) and completely overlapping with that of HDAC-1 (Fig. 5C, upper panels, overlay). Furthermore, TRIM22 staining changed from a multi-microdot pattern to fewer and larger dots containing HDAC-1 (Fig. 5C, middle and lower panels, overlay). These results, together with the Co-IP findings, strongly suggest that TRIM22 and HDAC-1 can physically interact and associate in subcellular structures localized in the nucleus.

4. Discussion

In the present study, we have explored the potential role of TRIM22 in the control of HIV-1 proviral latency in different models of T lymphocytic cell line infection. In the SupT1 cell line, we exploited the HIV-rtTA infection system that is dependent on the supplementation of Dox to promote virus replication. By allowing the infection to undergo proviral latency upon removal of the antibiotic, viral reactivation was triggered by Dox supplementation in the presence of the RT inhibitor 3TC to prevent viral spreading. In these conditions, the shRNA-dependent downregulation of TRIM22 expression promoted an accelerated proviral reactivation, whereas tetO-CMV and tetO-CMV-Sp1 variants confirmed a central role of Sp1 for TRIM22-dependent inhibition of provirus expression. TRIM22 confirmed to be a central player in the control of proviral latency in a chronically infected cell lines carrying quasi-silent, but inducible proviruses, namely ACH-2. In particular, decreasing TRIM22 expression in ACH-2 cells was sufficient to reverse the proviral state from latent to productive, robust virus production, a phenomenon that was not observed in J-Lat 10.6 cells; nonetheless, TRIM22-KD increased TNF-α activation of the provirus in both T cell lines. A similar effect, although significantly stronger in ACH-2 than in J-Lat 10.6 cells, was observed when these cell lines were stimulated with a panel of HDACi. Overexpression experiments showed that TRIM22 interacts and colocalizes in the nucleus with HDAC-1 suggesting that both proteins could cooperate in maintaining HIV-1 expression at low levels at least in latently infected T cell lines.

We have previously reported that TRIM22 acts as a transcriptional inhibitor of both basal and stimulated HIV-1 transcription induced by phorbol ester plus ionomycin (Kajaste-Rudnitski et al., 2011). TRIM22 suppressed HIV-1 transcription by preventing the binding of the cellular transcription factor Sp1 to HIV-1 LTR, although we failed to show a direct interaction between TRIM22 and Sp1 (Turrini et al., 2015). Here we show that TRIM22 expression reduces HIV-1 replication during acute HIV-1 infection, as we have previously described (Turrini et al., 2015), but also contributes to the maintenance of a state of HIV-1 silencing (Fig. 2) in an Sp1-dependent fashion. Indeed, Sp1 has been shown to recruit both the histone acetyltransferase p300 (Billon et al., 1999), a well-known HIV-1 transactivator (Kiernan et al., 1999), but also the transcriptional repressor HDAC-1 (Jiang et al., 2007) to the HIV-1 promoter through the interaction with cellular factors such as Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) interacting protein 2 (CTIP2) or Myc proto-oncogene protein (c-Myc). HDACs are also recruited to the HIV-1 promoter by homodimers of the NF-κB subunit p50 and by the transcription factors Yin Yang 1 and C-promoter binding factor 1 (Coull et al., 2000; Jiang et al., 2007; Marban et al., 2007; Williams et al., 2006). However, as TRIM22 does not bind directly to Sp1 (although preventing its binding to the HIV-1 LTR), we propose that TRIM22 silencing might require the recruitment of a suppressive complex inclusive of HDAC-1 that impedes Sp1 binding to the HIV-1 promoter (Marban et al., 2007; Vicenzi and Poli, 2018). Indeed, we show here that TRIM22 interacts with HDAC-1 by Co-IP experiments (Fig. 5A) and it is conceivable that this interaction determines a re-colocalization of both TRIM22 and HDAC-1 at the nuclear level (Fig. 5B and C). In the absence of HDAC-1 overexpression, TRIM22 is localized in nuclear bodies that are sites of recruitment of other factors including CIITA and TRIM19/PML, both acting as suppressors of HIV-1 transcription (Forlani and Accolla, 2017; Forlani

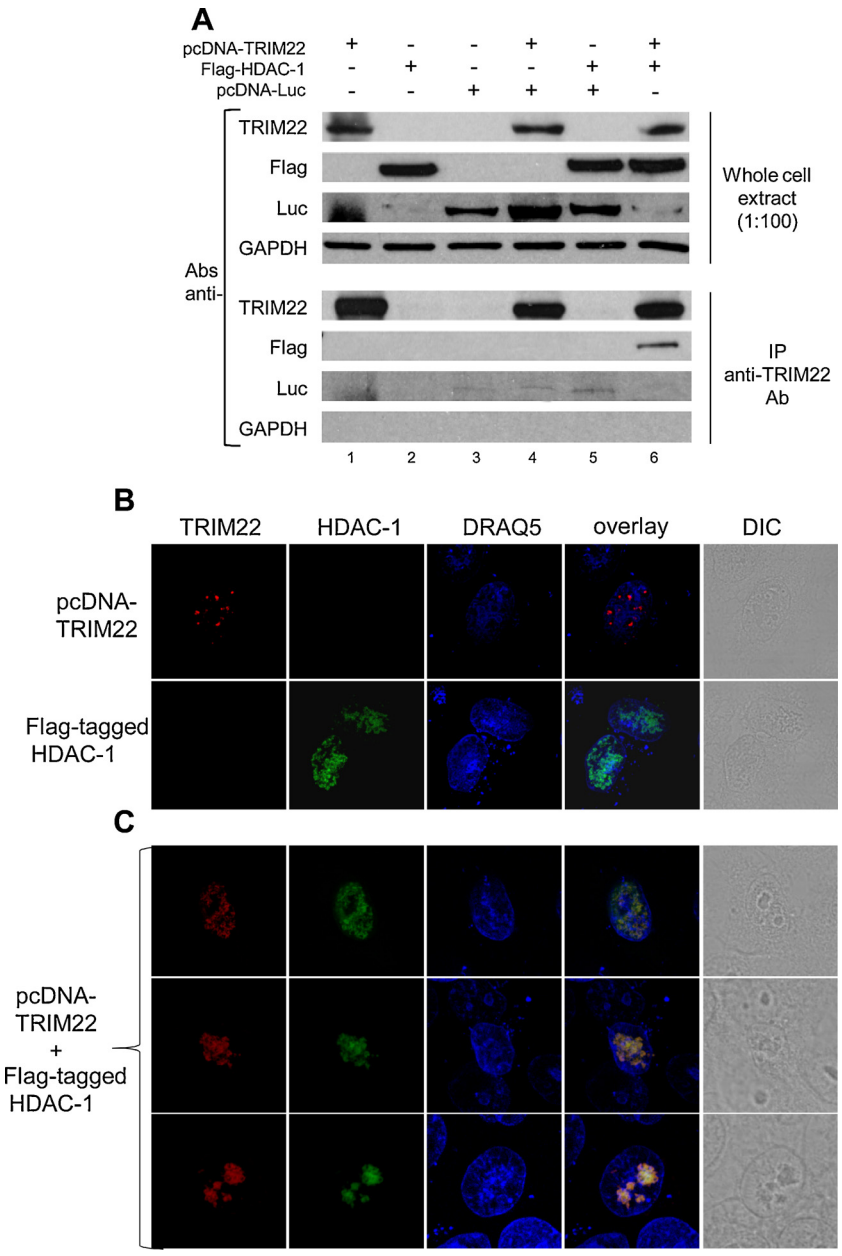

Fig. 5. TRIM22 interacts with HDAC-1. A. pcDNA-TRIM22 was co-expressed with either Flag-HDAC-1 or a control pcDNA Luc plasmid in HEK 293 T cells and Co-IP was carried out with an anti-TRIM22 antibody (Ab). Both whole cell extract (upper panels) and Co-IP products (lower panels) were analyzed by Western blotting using anti-TRIM22, anti-Flag, anti-Luciferase (Luc) and anti-GAPDH Abs. B. Hep-2 cells were transfected with either pcDNA-TRIM22 (TRIM22, red), or Flag-tagged HDAC-1 (HDAC-1, green), expressing vectors as specified on the top of the panels. Cells were fixed, stained with anti-TRIM22 rabbit polyclonal Ab followed by goat anti-rabbit AlexaFluor 546-conjugated Ab to detect TRIM22, or with fluorescein isothiocyanate-conjugated anti-Flag antibody to detect Flag-HDAC-1. Specific counterstaining of nucleus was performed by using DRAQ5 fluorescence probe (DRAQ5, blue). DIC is the differential interference contrast image. Stained cells were then analyzed by confocal microscopy, as described in section "Materials and methods". C. Hep-2 cells were co-transfected with pcDNA-TRIM22 (TRIM22, red) and Flag-tagged HDAC-1 (HDAC-1, green), expressing vectors as specified on the top of the panels. Cells were analyzed as described above. Three representative cells are shown.

et al., 2017). Furthermore, the expression of HDAC-1 changed TRIM22 localization from a multi-microdot pattern to fewer and larger dots containing HDAC-1 suggesting that TRIM22 could directly recruit HDAC-1 into nuclear bodies whereas, in the absence of TRIM22, it is present within the nucleus presumably acting throughout the chromatin. It should be underscored that HDAC-1 is also part of the catalytic core of the Mi-2 nucleosome remodeling and deacetylase (NuRD) complex that couples a chromatin remodeling ATPase and HDAC-1 and -2 deacetylase activities (Torchy et al., 2015). Therefore, TRIM22 could be also acting as stabilizer of HDAC-1 activity in the context of the NuRD complex. In this regard, TRIM22 E3 ubiquitin ligase activity (Di Pietro et al., 2013; Eldin et al., 2009) could potentially target HDAC-1 for mono-ubiquitination, although we have previously shown that this enzymatic activity is dispensable for TRIM22-dependent inhibition of HIV-1 transcription (Kajaste-Rudnitski et al., 2011).

In line with our previous observation showing that Tat transactivation is insensitive to TRIM22 inhibition (Kajaste-Rudnitski et al., 2011), the effect of TRIM22-KD on proviral reactivation was milder in a Tat/TAR responsive context like J-Lat 10.6 cells than that observed in ACH-2 cells (Fig. 3) in which viral latency and its reversal by cytokine stimulation occur independently of this axis. These findings could imply an overestimation of the role of TRIM22 in the control of HIV-1 latency and reactivation in Tat/TAR proficient proviruses. However, it should be underscored that, although as a minority of the global pool, Tat-deficient viruses exist and spread in the population (Kamori and Ueno, 2017). Tat-deficient viruses are generated as a consequence of the strong immunological pressure causing viral escape mutations in Tat (together with other viral proteins) leading to a high level of genetic variability observed within different viral clades (including B and C subtypes), as reviewed (Kamori and Ueno, 2017). Furthermore, enrichment of Tat-defective proviruses has been observed in resting $CD4^+$ T lymphocytes (Yukl et al., 2009) suggesting a potential role of these partially defective viruses in the establishment of the viral reservoir unaffected by cART. Worthy of note is also the substantial difference between ACH-2 and J-Lat cell lines as the former cells were obtained by acute infection with a fully competent $HIV\text{-}1_{LAI/IIIB}$ (Clouse et al., 1989) whereas the J-Lat cell clones were established following infection with replication-defective viruses (Jordan et al., 2001). Furthermore, J-Lat

cells are characterized by a single integration site (Symons et al., 2017) whereas ACH-2 cells have distinct integration sites (Sunshine et al., 2016) that increase in frequency along with cell proliferation (Symons et al., 2017). These results suggest that not only continuous expression of viral RNA (Butera et al., 1994), but also that low-levels of ongoing virus replication occur in ACH-2 cells. Therefore, the differences between ACH-2 and J-Lat 10.6 cells may not be confined to being Tat/TAR deficient and proficient, respectively, but they may reflect a "truly latent" (J-Lat) *vs.* "low-levels virus replication" (ACH-2) models of inducible virus expression. Thus, in the absence of proviral expression, TRIM22 deficiency might require that other agents trigger a minimal level of proviral activation to promote an increase in HIV-1 transcription, as exemplified by our results with J-Lat 10.6 cells stimulated with TNF-α or with HDACi (Fig. 4).

A potential limitation of our study is its confinement to T cell line models. However, we have previously identified two single nucleotide polymorphisms (SNPs), *i.e.* rs7935564A/G and rs1063303C/G, located within the coiled-coil domain of TRIM22 that are responsible for the loss of its transcriptional repression activity and associate with severity of HIV-1 disease (Ghezzi et al., 2013). As the coiled-coil domain of TRIM proteins is deputed to protein multimerization (Reymond et al., 2001), we can hypothesize that it may contribute to the formation of complex(es) with other transcriptional repressors. As TRIM22 expression modulates HIV-1 replication *in vivo* (Singh et al., 2011), our findings suggest that it may play a relevant role also in the control of HIV-1 latency in persistently infected $CD4^+$ T lymphocytes.

5. Conclusions

We have identified TRIM22 as a potentially novel player in maintaining a state of proviral latency, at least in different T lymphocytic cell lines following both acute and chronic infection. Thus, TRIM22 should be considered as a potential new candidate for LRA-based strategies or, *vice versa*, for strategies aiming at permanent locking of HIV-1 transcription (Darcis et al., 2017).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.05.009.

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