Title

MicroRNA-155 Reinforces HIV Latency*

Permalink

https://escholarship.org/uc/item/199690sw

Journal

Journal of Biological Chemistry, 290(22)

ISSN

0021-9258

Authors

Ruelas, Debbie S

Chan, Jonathan K

Oh, Eugene

et al.

Publication Date

2015-05-01

DOI

10.1074/jbc.m115.641837

Copyright Information

Peer reviewed

MicroRNA-155 Reinforces HIV Latency*□S

Debbie S. Ruelas[‡§], Jonathan K. Chan[‡], Eugene Oh[¶], Amy J. Heidersbach[§‖], Andrew M. Hebbeler[‡], Leonard Chavez[‡§], Eric Verdin[‡**‡‡], Michael Rape[¶§§], and Warner C. Greene[‡**‡‡1]

From the ‡Gladstone Institute of Virology and Immunology, San Francisco, California 94158, the §Biomedical Sciences Program and the Departments of **Medicine and ‡‡Microbiology and Immunology, University of California, San Francisco, California 94143, the ¶Department of Molecular and Cell Biology and the §§Howard Hughes Medical Institute, University of California, Berkeley, California 94720, and the ‖Gladstone Institute of Cardiovascular Disease, San Francisco, California 94158

Background: Curing HIV will require suppression of viral replication and clearance of the transcriptionally silent latent reservoir.

Results: We have discovered a novel cellular pathway involving a latency-promoting host miRNA and HIV-activating TRIM protein.

Conclusion: *miR-155* inhibits the HIV-activating effects of TRIM32 and thus may promote a return to latency in reservoir cells transiently expressing HIV.

Significance: Preventing reversion to latency could provide a novel approach for eliminating the latent reservoir.

The presence of a small number of infected but transcriptionally dormant cells currently thwarts a cure for the more than 35 million individuals infected with HIV. Reactivation of these latently infected cells may result in three fates: 1) cell death due to a viral cytopathic effect, 2) cell death due to immune clearance, or 3) a retreat into latency. Uncovering the dynamics of HIV gene expression and silencing in the latent reservoir will be crucial for developing an HIV-1 cure. Here we identify and characterize an intracellular circuit involving TRIM32, an HIV activator, and *miR-155*, a microRNA that may promote a return to latency in these transiently activated reservoir cells. Notably, we demonstrate that TRIM32, an E3 ubiquitin ligase, promotes reactivation from latency by directly modifying IκBα, leading to a novel mechanism of NF-κB induction not involving IκB kinase activation.

With the advent of combination ART,[2] hopes were high that these new and potent agents might cure HIV-infected subjects (1). However, despite consistently reducing viral loads to undetectable levels, a cure was not achieved (2) because of the persistence of latent but integrated HIV proviruses in a drug-insensitive reservoir formed at least in part by memory CD4 T cells. Considerable effort is now focused on devising ways to

attack this latent reservoir. One approach termed "shock and kill" involves the identification of a mixture of inducing agents that would be used to activate the latent virus under the cover of ART. These agents need to be strong enough to induce the virus throughout the reservoir but not so strong that a toxic cytokine storm is induced. Ideally, these agents will induce the death of the reservoir cells by a viral cytopathic effect or immune clearance.

Although the latent reservoir appears quite stable over time, it is not static. Intermittently, latently infected cells spontaneously activate and produce small amounts of virus. These viruses contribute to the low viral load observed in subjects receiving ART. Whether the transiently activated cells return to a latent state remains an intriguing but unanswered question.

The elimination of HIV latency is in its simplest terms a transcriptional problem. HIV transcription is controlled by the long terminal repeat (LTR), which contains multiple binding sites for cellular transcription factors, such as NF-κB, nuclear factor of activated T cells (NFAT), Sp1, and AP1 (3). NF-κB-inducing agents such as TNFα and prostratin are strong activators of HIV-1 transcription (4), but in keeping with the pleiotropic effects of NF-κB, these agents alter the expression of many cellular genes.

The study of HIV latency *in vivo* is complicated by the rarity of latently infected cells and a current inability to purify such cells. Many studies have relied heavily on cell-line models of chronic HIV infection. One such model is the J-Lat model of post-integration latency developed by infecting Jurkat cells using recombinant *envelope*-deleted virus containing a GFP reporter (5). Infected cells were then screened for latently infected cells that were transcriptionally inactive in the absence of cellular stimulation. Transcription of the HIV genome could be induced by a number of biological and chemical activating agents including NF-κB activators like PMA, prostratin, phytohemagglutinin (PHA), and TNFα. However, more physiological activation via the TCR complex with anti-CD3 antibodies was ineffective, likely because of down-regulation of TCR complex components. We recently developed an additional clonal

cell line, J-Lat 5A8, that retains surface CD3 expression and responds to TCR stimulation (6). Of note, when responsiveness of a variety of latency cell models including those formed in primary CD4 T cells was compared with resting CD4 T cells from aviremic patients, J-Lat 5A8 cells along with a primary CD4 T cell model developed by Dr. Sharon Lewin's laboratory displayed the strongest similarity to the reactivation profile exhibited by patient cells (7). These data also suggest that the cell signaling pathways within J-Lat 5A8 cells are similar to *ex vivo* cells from patients. In the current study, we have employed J-Lat 5A8 cells and primary CD4 T cells to study select aspects of the regulation of HIV latency.

MicroRNAs (miRNAs) are evolutionarily conserved RNAs that regulate a wide variety of biological processes including the immune response to viruses. These miRNAs correspond to 20–25-nucleotide-long non-coding RNAs that modulate gene expression through base pairing of the miRNA seed sequence to its target mRNA (usually located within the 3′-UTR). This interaction leads to either translational repression or mRNA cleavage, thereby reducing the final amount of target protein produced. Host miRNAs have inhibit HIV through cellular regulation of PCAF (8), cyclin T1 (9), and other HIV-1 factors involved in trafficking and/or importing pre-integration complexes into the nucleus (10). Cellular miRNAs also regulate HIV-1 by directly targeting the 3′-UTR of HIV-1 mRNA (11, 12). Although miRNAs clearly modulate HIV infection and replication, whether miRNAs regulate viral latency is still unclear. In this study, we identify multiple miRNAs that inhibit HIV-1 reactivation and uncover a novel miRNA-target interaction that reinforces latency in infected cells.

Tripartite motif-containing (TRIM) proteins are E3 ubiquitin ligases containing a RING finger domain, one or two B-box domains, and a coiled-coil region. TRIM32, a member of the TRIM-NHL family (named after the NCL-1, HT2A, and LIN-41 proteins), contains a C-terminal domain believed to mediate protein binding. Specifically, the NHL domain of TRIM32 binds to Ago1, which activates certain miRNAs required for neural differentiation (13). In addition, TRIM32 regulates the induction of type I IFNs and the cellular antiviral response by activating STING via Lys-63-linked ubiquitination (14). Interestingly, TRIM32 expression also activates NF-κB (15). A more recent study demonstrates that certain TRIM proteins (including TRIM32) that induce NF-κB also promote HIV-1 LTR expression (16). These studies highlight the importance of TRIM32 in NF-κB-mediated transcriptional activation of HIV-1. However, it is unknown whether TRIM32 plays a role in NF-κB signaling in a manner that antagonizes HIV latency. In this study, we explore the role of TRIM32 as an antagonist of HIV latency and counter-regulation of TRIM32 by *miR-155*.

Experimental Procedures

qRT-PCR—Total J-Lat cell RNA was purified using TRIzol reagent (Invitrogen), and the RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop). TaqMan low density array (TLDA) human miRNA assays (versions 2.1 and 3.0; Applied Biosystems) were used to calculate changes in miRNA expression between latent cells and reactivated cells collected from four independent experiments. Briefly, cDNA

was generated using the TaqMan reverse transcription kit and Megaplex primer pools A (v2.1) and B (v3.0). TLDA cards A and B were loaded with reverse transcription products and PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Fold changes in the expression of each miRNA were calculated ($\Delta\Delta Ct$ method), and differences in expression between latent and reactivated cells were analyzed using moderated t statistics. Linear contrasts were used to make all pairwise comparisons between groups. Follow-up analyses of specific miRNAs were performed using TaqMan microRNA assays. RNU6 was used as an endogenous control. TaqMan gene expression assays (Applied Biosystems) were used to quantify the expression of mRNA transcripts. The following primers and probes were used in gene expression assays: DGCR8 (Hs00256062_m1), Dicer (Hs00229023_m1), and TRIM32 (Hs00705875_s1). GAPDH or β-actin was used as an endogenous control for $\Delta\Delta Ct$ calculations.

Lentiviral Infection—Lentiviral particles were produced as described (17). For J-Lat infections, 100,000 cells were incubated with 4 μg/ml Polybrene (Sigma), RPMI, and viral suspension for ~2 h at 37 °C. After 24 h, the cells were washed and cultured in RPMI.

Lentiviral Vectors—shRNAs were cloned into the pSicoR lentiviral vector, which encodes an mCherry reporter driven by an EF-1α promoter (pSicoR-MS1). shRNAs against human DGCR8, Dicer, TRIM32, and negative control scramble were cloned into pSicoR-MS1 using the following oligonucleotide sequences: shScramble forward (TGT CAA GTC TCA CTT GCG TCT TCA AGA GAG ACG CAA GTG AGA CTT GAC TTT TTT C), shScramble reverse (TCG AGA AAA AAG TCA AGT CTC ACT TGC GTC TCT CTT GAA GAC GCA AGT GAG ACT TGA CA); shDGCR8 forward (TGA AAG AGT TTG TTA TTA ACT TCA AGA GAG TTA ATA ACA AAC TCT TTC TTT TTT C), shDGCR8 reverse (TCG AGA AAA AAG AAA GAG TTT GTT ATT AAC TCT CTT GAA GTT AAT AAC AAA CTC TTT CA); shDicer forward (TGC AGC TCT GGA TCA TAA TAT TCA AGA GAT ATT ATG ATC CAG AGC TGC TTT TTT C), shDicer reverse (TCG AGA AAA AAG CAG CTC TGG ATC ATA ATA TCT CTT GAA TAT TAT GAT CCA GAG CTG CA); and shTRIM32 forward (TGC AAA CAA ATG CTG ATA TAT TCA AGA GAT ATA TCA GCA TTT GTT TGC TTT TTT C), shTRIM32 reverse (TCG AGA AAA AAG CAA ACA AAT GCT GAT ATA TCT CTT GAA TAT ATC AGC ATT TGT TTG CA). The pCDH-TRIM32 lentiviral expression vector was constructed by performing PCR on the MGC human TRIM32 cDNA clone (accession number: BC003154, CloneID: 2906024; Thermo Scientific) using the following primer sequences: TRIM32 (EcoRI) forward (GTT TCT GAA TTC GAT GGC TGC AGC AGC AGC TTC TC), TRIM32 (BamHI) reverse (GTT TCT GGA TCC CTA TGG GGT GGA ATA TCT TCT CAG AT). PCR products were digested with EcoRI and BamHI and ligated into the pCDH-EF1α-MCS-IRES-Puro lentiviral vector.

Infection of CD4 T Cells with Dual Reporter Virus—Primary CD4 T cells and peripheral blood mononuclear cells were purified from healthy donor blood (Blood Centers of the Pacific, San Francisco, CA, and Stanford Blood Center). CD4 T cells were isolated by negative selection using the RosetteSep human CD4

T cell enrichment cocktail (StemCell Technologies). Peripheral blood mononuclear cells were purified by Histopaque-1077 density gradient. Purified CD4 T cells from peripheral blood were cultured in RPMI 1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 mg/ml). Purified CD4 T cells isolated from peripheral blood were stimulated with αCD3/αCD28 activating beads (Life Technologies) at a concentration of 1 bead/cell in the presence of 30 units/ml IL-2 (PeproTech) for 3 days. All cells were spinoculated with HIV Duo-Fluo I at a concentration of 100 ng of p24 per 1×10^6 cells for 2 h at $1,200 \times g$ at 37 °C. After spinoculation, all cells were returned to culture in the presence of 30 units/ml IL-2. Infected CD4 T cells were sorted based on GFP and mCherry fluorescence 4 days after infection using a FACSAria II (BD Biosciences), and RNA was extracted immediately using the Ambion PARIS kit (Life Technologies).

Antibodies—Protein levels were analyzed by immunoblotting using the following antibodies: DGCR8 (ab37272, Abcam), Dicer (ab13502, Abcam), TRIM32 (ab96612, Abcam), IκBα (sc-371, Santa Cruz Biotechnology), RelA (sc-109, Santa Cruz Biotechnology), β-actin (A5316, Sigma), Sp1 (sc-59, Santa Cruz Biotechnology), GAPDH (ab8245, Abcam), HSP90 (sc7947, Santa Cruz Biotechnology), and His (sc-803, Santa Cruz Biotechnology). Immunoprecipitations were carried out using protein G Dynabeads (BD Biosciences protocol) and the following antibodies: FLAG (F1804, Sigma) and HA (sc-7392, Santa Cruz Biotechnology). For ubiquitin assays, 5A8 cells were transfected, pre-treated with 1.5 μM β-lactone overnight, and lysed in NP-40 buffer supplemented with protease inhibitor and N-ethylmaleimide.

Transfections—J-Lat 5A8 cells were infected with lentivirus expressing shRNA targeting Dicer and sorted by the mCherry-positive population. These sorted shDicer-infected J-Lat cells were cultured for ∼1 week and were nucleofected (Lonza) using Solution R and the program O-028 with 2 μM miRIDIAN microRNA mimics (Dharmacon), unless otherwise noted. After 24 h, cells were stimulated with α-CD3 and α-CD28 or with TNFα overnight or for 24 h, and analyzed by flow cytometry. For immunoprecipitation experiments, 4 million 293T cells were transfected with 3.5 μg of TRIM32, IκBα, pCMV constructs, and 0.7 μg of His-ubiquitin using FuGENE HD for 24 h. For Dual-Luciferase assays, the indicated luciferase plasmid (150 ng) was transfected into HeLa cells in a 24-well plate with or without *miR-155* mimic (10 pmol) using Lipofectamine 2000 (Invitrogen). The Dual-Luciferase reporter assay system (Promega) was used to measure the levels of both firefly and *Renilla* luciferase 24 h after transfection.

Plasmid for Luciferase Assay—Luciferase reporter construct was generated using the psiCHECK2 vector (Promega). The TRIM32 3′-UTR was ligated into the XhoI/NotI sites of psiCHECK2. Sequencing was performed to confirm that construct contained the expected DNA sequence.

Plasmids in 293T Experiments—TRIM32 plasmids were a kind gift of Dr. Ki-Sun Kwon (18). IκBα plasmids were constructed as described previously (19).

In Vitro Kinase Assay—5A8 cells were unstimulated or treated (30 min) with PMA (20 nM) and ionomycin (2 μM). A total of 15 million cells were lysed in whole cell lysis buffer, and the assay was performed as described previously (4).

EMSA—Nuclear extracts were prepared (4 μg/sample) and were incubated with poly(dI-dC) and salmon sperm DNA for 15 min, followed by the addition of [γ-^{32}]ATP-labeled consensus κB or Oct-1 oligonucleotides (Promega). For cold-probe competition, 100$\times$ cold κB probes were added 10 min prior to labeled probes. Samples were separated on non-denaturing gels and analyzed by autoradiography.

Cloning, Expression, and Purification of TRIM32 and TRIM25—TRIM cDNA was cloned between the KasI and XhoI restriction sites of pPROEX-HTa. The TRIM32 I22E mutant was cloned by site-directed mutagenesis. A saturated culture of ArcticExpress (DE3) cells (Agilent) harboring pPROEX-HTa-TRIM32 was diluted to an A_{600} of ∼0.2 and grown at 30 °C for 3 h. Cells were subsequently induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (supplemented with 0.1 mM ZnSO$_4$) and grown for an additional 48 h at 12 °C. Cells were resuspended in lysis buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO$_4$, 10 mM imidazole, 10% glycerol, and 0.5 mM PMSF) and lysed by sonication. Recombinant TRIM proteins were batch-purified using nickel-nitrilotriacetic acid resin, eluted with elution buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO$_4$, 200 mM imidazole, 10% glycerol), dialyzed overnight in dialysis buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO$_4$, 10% glycerol, 1 mM DTT), and concentrated.

Cloning and in Vitro Synthesis of IκBα (NFKBIA)—IκBα cDNA was cloned between the FseI and AscI restriction sites of pCS2. 40 μl of rabbit reticulocyte lysate (Promega), 12 μl of ^{35}S-labeled amino acids (MP Biomedicals, catalogue number 0151006), and 12 μl of pCS2-IκBα (at a stock concentration of 500 ng/μl) were mixed and incubated at 30 °C for 2 h. *In vitro* transcribed/translated IκBα substrate was purified with anti-HA-agarose resin, eluted with 0.2 ml of 0.4 mg/ml 3$\times$HA peptide, and concentrated 5-fold in volume.

Ubiquitylation Assay—In a 15-μl reaction volume, 0.3 μl of 10 μM E1 (200 nM final), 0.3 μl of 235 μM UbcH5 (5 μM final), 1.5 μl of 10 mg/ml ubiquitin (1 mg/ml final), 1.5 μl of 10$\times$ ubiquitylation assay buffer (250 mM Tris 7.5, 500 mM NaCl, and 100 mM MgCl$_2$), 2.3 μl of energy mix (150 mM creatine phosphate, 20 mM ATP, 20 mM MgCl$_2$, 2 mM EGTA, pH to 7.5 with KOH), and 2 μl of ^{35}S-labeled IκBα was mixed with 1.5 μl of 20 μM TRIM25, TRIM32, or TRIM32 I22E (2 μM final) at 30 °C for 1 h. Reactions were stopped by the addition of SDS sample loading buffer, resolved on a 10% SDS-acrylamide gel, and exposed on an autoradiography screen.

Results

Knockdown of Enzymes Involved in miRNA Biogenesis Increases Reactivation of Latent HIV—Because various miRNAs block HIV replication, we hypothesized that select miRNAs promote HIV latency in infected CD4 T cells. To test this possibility, miRNA-deficient J-Lat 5A8 cells were prepared by knocking down the expression of two enzymes required for miRNA biogenesis, DGCR8 and Dicer. Knockdown was achieved by introduction of an shRNA lentiviral vector (pSicoR-MS1) containing an mCherry reporter into the J-Lat
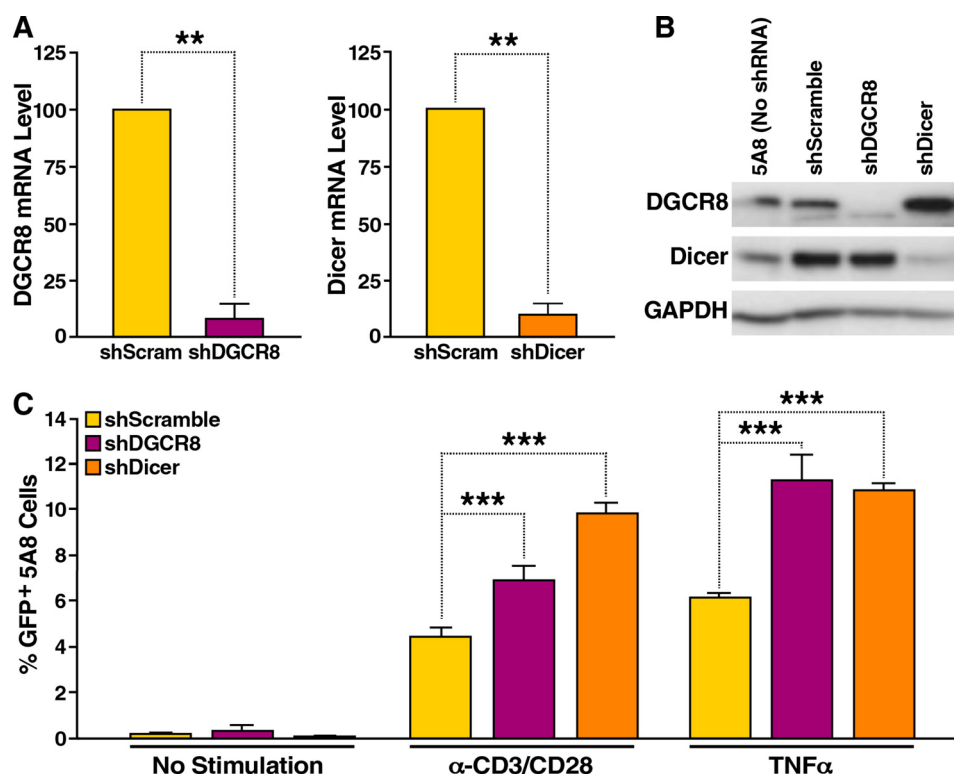
FIGURE 1. **Stable knockdown of DGCR8 and Dicer promotes HIV-1 reactivation in the J-Lat 5A8 T cell model of HIV latency.** *A*, knockdown of DGCR8 and Dicer mRNA in J-Lat 5A8 cells infected with lentiviruses expressing these specific shRNAs or a scrambled shRNA control was measured by qRT-PCR. Values are normalized to scrambled shRNA control; *error bars* represent +S.E., **, $p < 0.01$ (two-tailed *t* test), $n = 3$. *B*, knockdown as described in *A* was also measured at the protein level by immunoblotting with specific antibodies. Immunoblotting with anti-GAPDH antibodies was used to assess comparability of the different lysates. *C*, increased HIV-GFP reactivation following stable knockdown of DGCR8 or Dicer assessed by flow cytometry 24 h after stimulation. *Error bars* represent +S.E., ***, $p < 0.001$ (two-way ANOVA), $n = 3$.

5A8 cells. The mCherry-positive cells, which were successfully infected with the lentivirus, were isolated by fluorescent cell sorting followed by qRT-PCR to assess target gene expression. Knockdown of both DGCR8 and Dicer exceeded 90% in the mCherry-positive 5A8 cells as compared with levels obtained in cells receiving a negative control (5A8 shScramble) (Fig. 1*A*). Additionally, DGCR8 and Dicer levels were much lower in the knock-out cells than in cells receiving no shRNA, and further each knockdown appeared specific as expression of the counterpart microRNA biogenic enzyme was not affected, nor was the expression of GAPDH altered (Fig. 1*B*). To assess the effects of diminished miRNAs on HIV reactivation, the infected cells were cultured alone or stimulated with α-CD3/CD28 or TNFα. Both DGCR8 and Dicer knockdown cells exhibited significantly greater increases in HIV-1 reactivation with both agonists as demonstrated by increased GFP expression as compared with the shRNA scramble control knockdown (Fig. 1*C*). GFP expression in the absence of stimulation was not markedly affected by knockdown of either enzyme. These findings suggest that one or more miRNAs naturally function as inhibitors of latent HIV reactivation.

To identify the specific miRNAs that contribute to this inhibition, we used a TLDA to profile 754 unique human miRNAs (supplemental Table 1). We identified miRNAs that were differentially expressed between reactivated and latent cells (Table 1), validating top candidates in individual TaqMan assays (data not shown).

TABLE 1

TLDA identifies miRNAs involved in HIV-1 latency

J-Lat 5A8 cells were stimulated with αCD3/CD28 and sorted for either GFP-positive (reactivated) or GFP-negative cells (latent) cells. TLDA was performed, and fold changes were calculated using the ΔΔCt method. Differences in expression between latent and activated cells were analyzed using moderated *t* statistics. Samples in bold indicate the top candidates that were individually validated and carried into future experiments. RNU6 (row in italics) was used as a housekeeping control. Data represent four independent experiments.

	Average -fold difference (Log₂)	GFP − average Ct	GFP + average Ct	*p* value
hsa-miR-146a	**1.75**	**23.5**	**21.8**	**0.004**
hsa-miR-885–5p	**1.38**	**33.7**	**32.4**	**0.047**
hsa-miR-221	**1.32**	**30.9**	**29.6**	**0.000**
hsa-miR-155	**1.11**	**30.0**	**28.9**	**0.003**
hsa-miR-212	0.96	29.1	28.2	0.030
hsa-miR-150	0.87	26.1	25.3	0.026
hsa-miR-132	0.83	26.5	25.6	0.044
hsa-miR-200c	0.78	28.7	27.9	0.014
hsa-miR-149*	0.54	37.7	37.4	0.038
hsa-miR-649	0.54	40.0	39.7	0.038
hsa-let-7i*	0.53	38.8	38.5	0.037
hsa-miR-141*	0.51	39.2	38.9	0.033
hsa-miR-10b*	0.49	39.5	39.2	0.030
hsa-let-7f-1*	0.47	39.5	39.2	0.029
hsa-miR-126*	0.45	38.4	38.2	0.030
hsa-miR-571	0.44	39.4	39.2	0.032
hsa-miR-661	0.42	39.7	39.5	0.038
hsa-miR-130b*	−0.82	31.5	32.5	0.039
hsa-miR-518b	**−1.71**	**35.8**	**37.6**	**0.047**
hsa-miR-1290	**−1.99**	**30.6**	**32.8**	**0.008**
hsa-miR-505*	**−3.10**	**34.3**	**37.6**	**0.049**
hsa-miR-29b	**−4.18**	**35.3**	**39.5**	**0.007**
RNU6	*−0.09*	*15.4*	*15.4*	*0.9953*

Introducing miR-155 into Dicer-deficient Cells Decreases the Level of HIV Reactivation—To determine whether candidate miRNAs inhibited HIV-1 reactivation, each candidate miRNA
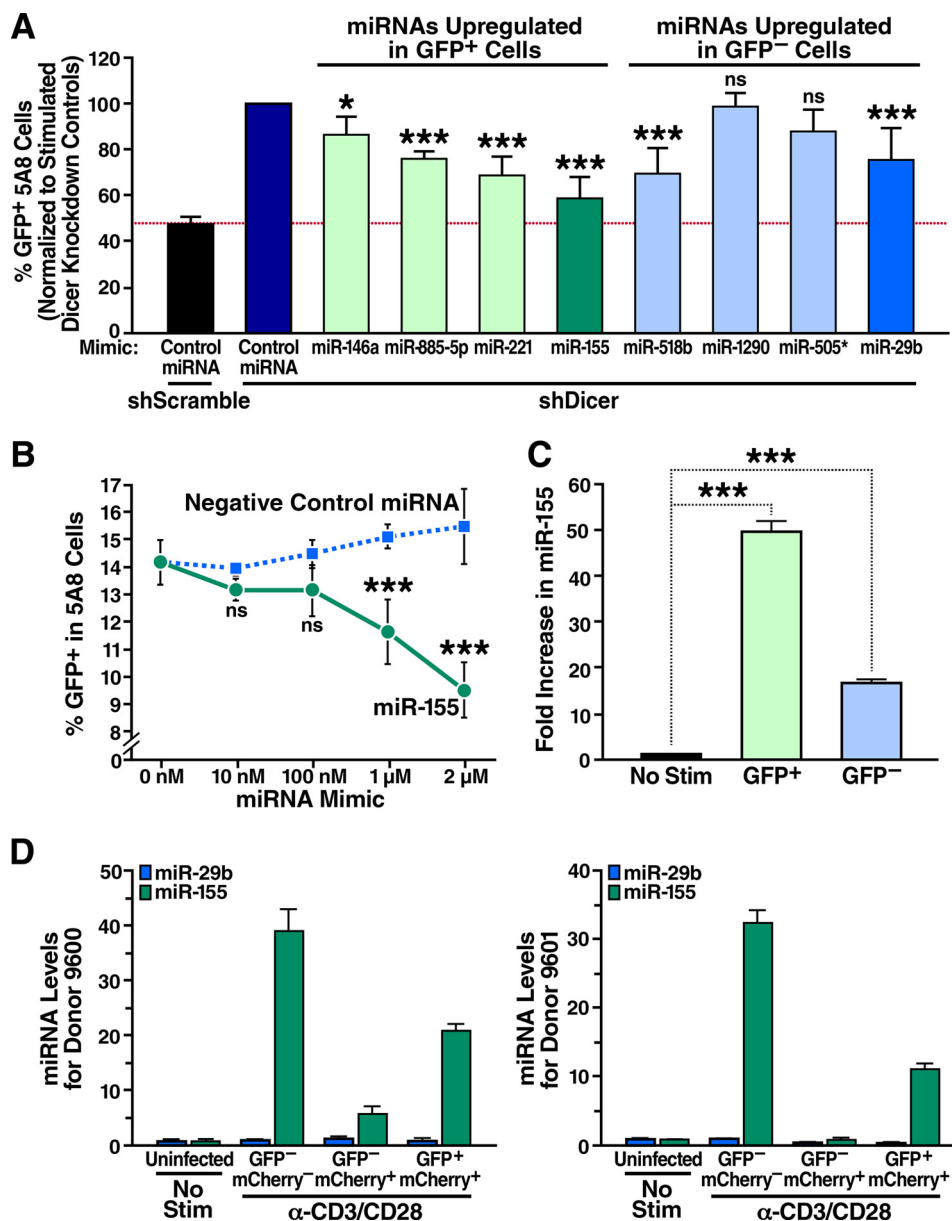
FIGURE 2. **Introduction of *miR-155* into Dicer-deficient cells reduces the level of HIV-1 reactivation from latency in J-Lat 5A8 cells.** *A*, J-Lat 5A8 shDicer cells were nucleofected with candidate miRNA mimics (or a negative control mimic) and stimulated with αCD3/CD28 24 h after nucleofection. Cells were then analyzed for GFP expression by flow cytometry ~16 h later. J-Lat 5A8 shScramble cells were nucleofected with a negative control mimic. Data shown represent the percentage of decrease in HIV-GFP reactivation as compared with introduction of a negative control mimic into the shDicer knockdown cells. *Error bars* indicate S.E., *, $p < 0.05$; ***, $p < 0.001$ (one-way ANOVA), $n = 5$–20. *ns*, not significant. Note that several of the tested microRNA mimics impaired HIV reactivation; however, *miR-155* consistently displayed the greatest effect. *B*, introduction of increasing concentrations of *miR-155* mimic decreases latent reactivation. J-Lat shDicer cells were nucleofected with either a negative control miRNA mimic or a *miR-155* mimic at various concentrations. Cells were stimulated with αCD3/CD28 antibodies 24 h after nucleofection and analyzed for GFP expression by flow cytometry ~16 h after stimulation. *Error bars* indicate S.E., ***, $p < 0.001$ (two-way ANOVA), $n = 3$. *C*, J-Lat 5A8 cells were either not stimulated (*No Stim*) or stimulated with αCD3/CD28 and sorted for GFP-positive cells at 24 h. Unstimulated and stimulated GFP-positive and GFP-negative cells were analyzed for *miR-155* levels by qRT-PCR. *Error bars* indicate S.E. (ANOVA), $n = 3$. *D*, analysis of *miR-155* and miR-29b expression in a primary CD4 T cell model of HIV latency. A dual fluorescence HIV virus was used to infect primary CD4 T cells from two donors (Donor 9600 and Donor 9601) followed by analysis of *miR-155* and miR-29b levels in unstimulated cells or αCD3/CD28 activated cells that were uninfected (mCherry-negative, GFP-negative), productively infected (GFP-positive, mCherry-positive), or latently infected (mCherry-positive, GFP-negative). miR-29b levels were low in all of the cell populations. *miR-155* was induced in activated uninfected and productively infected cells; only modest levels of *miR-155* were present in latently infected cells, and essentially no *miR-155* was detected in unstimulated cells. MicroRNAs were measured by qRT-PCR.

was reintroduced into Dicer-deficient cells. Although knocking down Dicer nearly doubled the level of reactivation in stimulated cells (Fig. 1*C*), introducing the top four candidate miRNAs from both GFP-positive and GFP-negative cells countered this effect, restoring a lower level of reactivation as compared with cells receiving a negative control miRNA mimic (Fig. 2*A*).

Introducing *miR-155* produced the greatest decline in HIV-1 reactivation (78.3% rescue) (Fig. 2*A*), occurring in a dose-related manner (Fig. 2*B*). Two of the miRNAs tested, *miR-1290* and *miR-505** (asterisk indicates passenger strand), did not significantly affect reactivation levels, indicating that this effect is specific and not associated with the introduction of every

miRNA. To further evaluate *miR-155* levels when cells were stimulated, individual TaqMan assays were performed. *miR-155* was expressed at 50-fold higher levels in GFP-positive cells (~50-fold more) as compared with unstimulated cells. *miR-155* levels were also increased in GFP-negative cells following stimulation, albeit to a lesser extent (Fig. 2*C*). Together, these findings raise the possibility that specific miRNAs abundantly expressed in reactivated cells serve to counter viral activation and perhaps promote a return to latency in cells undergoing transient virus production.

To determine the levels of *miR-155* in a model of HIV-1 latency formed with primary CD4 T cells, we employed a dual-fluorescence virus, HIV-DuoFluo, to identify latent and productively infected primary CD4 T cells occurring after HIV infection. This model has been recently described (20). In this model, HIV-DuoFluo (R7GEmC) expresses GFP under control of the HIV promoter and expresses mCherry under control of a constitutive EF1α promoter. Following integration of this virus into primary CD4 T cells, mCherry is constitutively expressed and marks all successfully infected cells. Active viral expression occurs in a subset of these cells indicated by the expression of GFP. Latently infected cells are identified by the expression of mCherry in the absence of GFP expression. CD4 T cells from two blood donors (Donor 9600 and Donor 9601) were infected with R7GEmC and sorted for uninfected cells, latently infected cells (mCherry-positive, GFP-negative), and actively infected cells (mCherry-positive, GFP-positive). Both donors exhibited high levels of *miR-155* in cells that were TCR-stimulated but uninfected. This finding is consistent with prior studies describing up-regulation of *miR-155* in activated lymphoid and myeloid cells (21, 22). Levels of *miR-155* were much higher in virus-producing cells (mCherry-positive, GFP-positive) as compared with latently infected cells (mCherry-positive, GFP-negative) (Fig. 2*D*). These findings suggest that *miR-155* may principally act within an activated cellular environment. Interestingly, *miR-29b* levels did not change in any of the tested cell populations. This microRNA has been implicated in the regulation of cyclin T1, a participant in the PTEF-b complex that functions as a key cofactor for HIV Tat (9).

MicroRNA-155 Targets TRIM32—Next, we sought to identify host genes whose action was suppressed by *miR-155* using the TargetScan prediction algorithms. These analyses identified TRIM32 as a high-value hit because it had a favorable aggregate P_{CT} and context+ score. In addition, the 3′-UTR of TRIM32 contains an 8-mer site that precisely matches the seed region (positions 1–8) of *miR-155* (Fig. 3*A*). Furthermore, TRIM32 represented an intriguing potential target because of a prior report of its direct binding to the activation domain of HIV Tat (21). To study whether *miR-155* directly binds to the 3′-UTR of TRIM32, the full 3′-UTR of TRIM32 including the predicted target site was cloned downstream of a *Renilla* luciferase reporter construct. In this assay, a decrease in luciferase activity in the presence of *miR-155* demonstrates binding of the miRNA and subsequent inhibition of luciferase expression. (Fig. 3*A*). Indeed, *miR-155* inhibited luciferase expression of the reporter containing the full 3′-UTR of TRIM32 (Fig. 3*B*). These results confirm that TRIM32 is a target of *miR-155*.

Studies were next performed to assess *miR-155* effects on endogenous TRIM32 levels in J-Lat 5A8 cells. The introduction of *miR-155* decreased levels of TRIM32 mRNA in these cells by ~70% as compared with a negative control miRNA (Fig. 3*C*). Recent studies in the Rudensky laboratory (22) have analyzed endogenous targets of *miR-155* within the entire murine transcriptome using AGO differential HITS-CLIP (dCLIP) and mRNA changes in CD4 T cells isolated from wild type or *miR-155* knock out mice. Binding maps were made accessible online for all 3′-UTRs on CLIP Base. We searched for TRIM32 in CLIP Base and found that the putative *miR-155* binding site in the TRIM32 3′-UTR is in fact bound by the *miR-155*-AGO complex in wild type CD4 T cells, but not in CD4 T cells lacking *miR-155* due to gene knock-out (Fig. 3*D*, image obtained from CLIP Base). Together, these findings provide strong evidence supporting TRIM32 as a *bona fide* cellular target of *miR-155*.

TRIM32 Regulates HIV-1 Latency—Next, we assessed the effects of shRNA knockdown of TRIM32 in latently infected J-Lat 5A8 cells. After infection with a TRIM32 shRNA, TRIM32 mRNA and protein levels were both reduced by ~40% (Fig. 4, *A* and *B*). Conversely, TRIM32 mRNA and protein levels were not reduced in cells infected with virus encoding a scrambled control shRNA (Fig. 4, *A* and *B*). In agreement with one or more miRNAs posttranscriptionally regulating TRIM32 expression, TRIM32 protein, but not mRNA, was increased when Dicer was knocked down (Fig. 4, *A* and *B*). TCR activation of J-Lat 5A8 cells with anti-CD3 and anti-CD28 antibodies decreased GFP expression when TRIM32 expression was knocked down but increased expression following Dicer knockdown (Fig. 4*C*). These findings suggest that TRIM32 promotes reactivation of latent HIV-1 and that its activity is regulated by miRNAs, notably *miR-155*.

Next, we examined the effects of lentivirus-mediated overexpression of TRIM32 (pCDH-TRIM32) in J-Lat 5A8 cells. By both qRT-PCR and immunoblotting TRIM32 levels were significantly higher in pCDH-TRIM32 cells as compared with cells infected with the empty lentiviral vector (pCDH) (Fig. 4, *D* and *E*). TRIM32 alone promoted HIV-1 reactivation in the absence of stimulation. Treatment with anti-CD3/CD28 and TNFα resulted in increased levels of latent virus reactivation in cells expressing TRIM32 (Fig. 4*F*). Together, these findings confirm TRIM32 as an HIV activator and effective host antagonist of HIV latency.

TRIM32 Promotes Reactivation of Latent HIV-1 by Stimulating NF-κB Signaling—TRIM32 has been reported to interact with the activation domain of HIV-1, HIV-2, and equine infectious anemia virus Tat proteins (21). To assess whether the activating effects of TRIM32 on latent HIV required the presence of Tat, TRIM32 was expressed in J-Lat A72 cells that lack Tat (23). TRIM32 effectively induced latent provirus expression alone or in combination with anti-CD3/CD28 antibodies or TNFα (Fig. 5*A*). These results indicate that the TRIM32 is able to activate the HIV LTR in a Tat-independent manner. Recent studies have shown that TRIM32 induces NF-κB in 293 cells (14, 16). We examined whether TRIM32 similarly activates NF-κB using Jurkat κB-dsRed cells, which contain a basal promoter and five κB enhancer elements controlling expres-
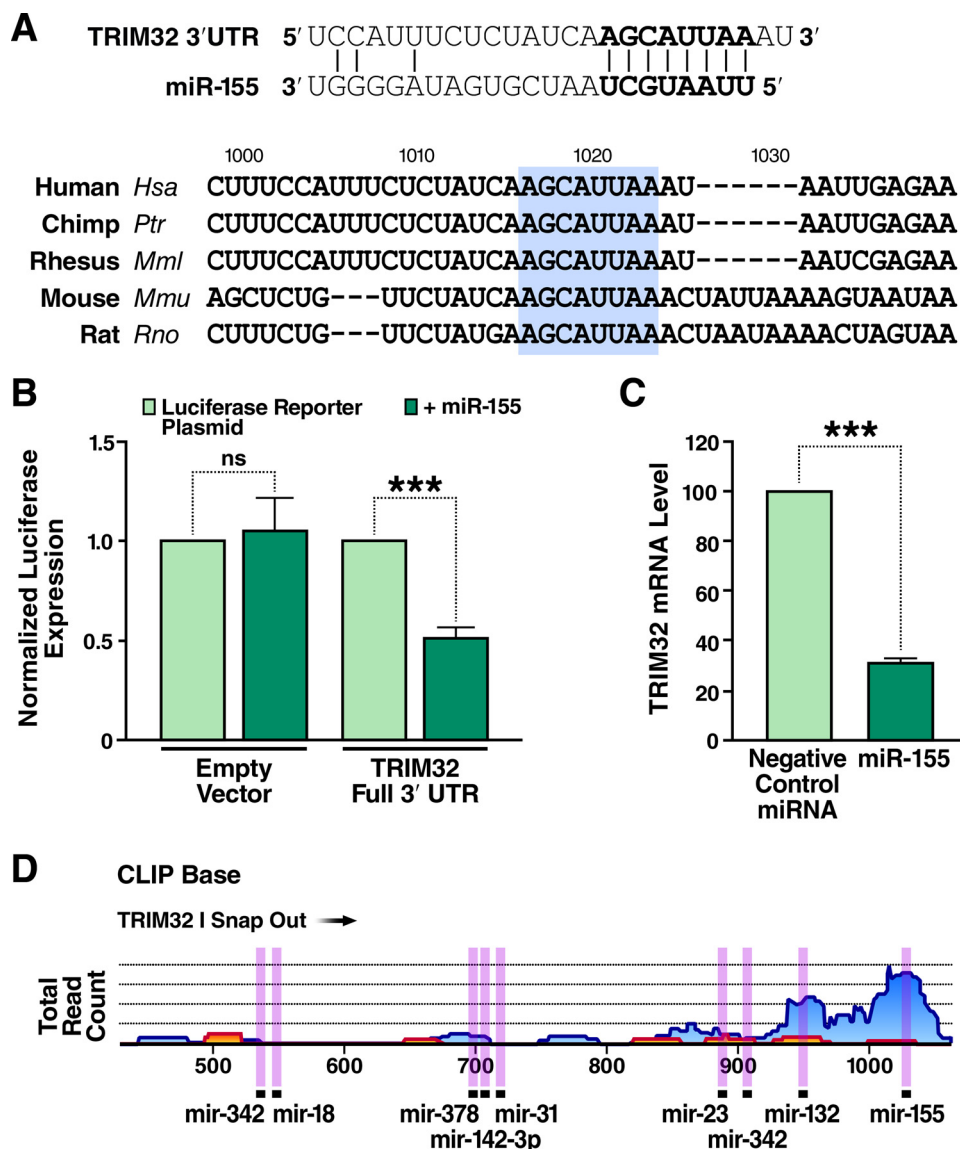
FIGURE 3. **miR-155 directly targets TRIM32.** *A*, prediction of the binding site of *miR-155*. The TRIM32 3'-UTR site is predicted to bind *miR-155* perfectly at the seed region. This region of the 3'-UTR of TRIM32 is well conserved among many mammals. *Hsa*, *Homo sapiens*; *Ptr*, *Pan troglodytes*; *Mml*, *Macaca mulatta*; *Mmu*, *Mus musculus*; *Rno*, *Rattus norvegicus*. *B*, luciferase binding assay shows that *miR-155* binds to TRIM32 3'-UTR. The full TRIM32 3'-UTR was cloned into psi-Check2, transfected into HeLa cells (with or without *miR-155*), and analyzed for levels of luciferase expression 24 h after transfection. Expression of *Renilla* luciferase was normalized to that of firefly luciferase for each sample. *Error bars* represent S.E., ***, $p < 0.001$ (one-way ANOVA), $n = 3$. *ns*, not significant. *C*, *miR-155* decreases endogenous TRIM32 levels. Negative control miRNA and *miR-155* mimics were nucleofected into 5A8 shDicer cells. Cells were collected 24 h after nucleofection, and analyzed by qRT-PCR. ***, $p < 0.001$ (two-tailed *t* test), $n = 3$. *D*, binding map demonstrates that *miR-155* binds to TRIM32 3'-UTR in wild type *miR-155* mice, but not to *miR-155* knock-out mice. The *y*-axis represents read counts, and the *x*-axis represents nucleotide position relative to the beginning of the 3'-UTR. *Blue* indicates the number of reads from miR-155 (WT) replicates, and *orange* indicates reads from miR-155-deficient (knock-out) mice. The figure was adapted from a database assembled by the Rudensky laboratory (CLIP Base).

sion of a dsRed reporter (6). When TRIM32 was expressed in these cells, the level of dsRed expression increased as compared with the empty vector control in both the absence and the presence of stimulation (Fig. 5*B*). These findings confirm the NF-κB-inducing activity of TRIM32.

Next, nuclear and cytoplasmic extracts from TRIM32-expressing cells were immunoblotted. In the absence of concomitant PMA and ionomycin stimulation, TRIM32 expression was associated with an increase in nuclear RelA expression and a decrease in cytoplasmic IκBα levels (Fig. 5*C*). Based on the pattern of nuclear Sp1 and cytoplasmic IκBα, nuclear and cytoplasmic fractions were of high quality (Fig. 5*C*). To confirm TRIM32-induced nuclear translocation of NF-κB, EMSA were

performed with nuclear extracts and ^{32}P-labeled κB and Oct1 DNA probes. Unstimulated cells expressing TRIM32 displayed increased binding activity in the form of a more slowly migrating complex (p50/RelA heterodimer) that was fully competed with unlabeled wild type κB probe but not by a mutant κB probe. (Fig. 5*D*). In contrast, Oct1 DNA binding did not change in the presence or absence of TRIM32. Together, these results indicate that TRIM32 expression induces NF-κB activation in CD4 T cells.

To further investigate the mechanism through which TRIM32 induces NF-κB, an IκB kinase (IKK) assay was performed (Fig. 5*E*). Unexpectedly, the same level of IKK activity was found in cells expressing TRIM32 *versus* the negative con-
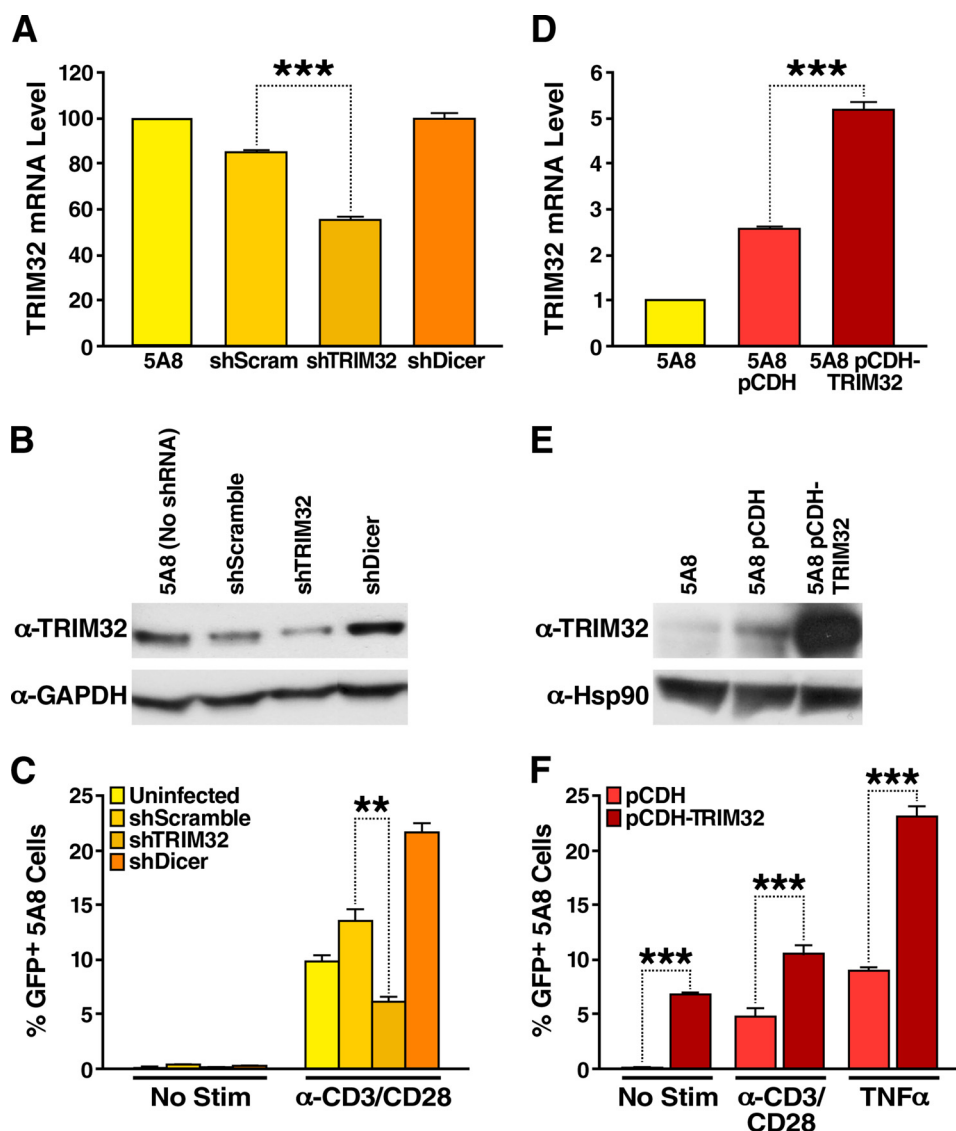
FIGURE 4. **TRIM32 regulates reactivation of latent HIV-1 in J-Lat 5A8 cells.** *A*, lentivirus expressing shRNAs targeting TRIM32, Dicer, and a control scramble shRNA were introduced into J-Lat 5A8 cells. TRIM32 levels were measured by qRT-PCR. *Error bars* indicate S.E., ***, $p < 0.001$ (one-way ANOVA), $n = 3$. *B*, knockdown of TRIM32 as described in *A* assessed at the protein level by immunoblotting with αTRIM32 antibodies. GAPHD1 was used a loading control for the various lysates. *C*, knockdown of TRIM32 reduces levels of reactivation following stimulation of J-Lat 5A8 cells. Cells were infected with lentivirus expressing the indicated shRNAs, stimulated with αCD3/CD28 for 24 h, and analyzed by flow cytometry. *Error bars* indicate S.E., **, $p < 0.01$ (one-way ANOVA), $n = 3$. *No Stim*, unstimulated cells. *D*, cells were infected with lentivirus expressing TRIM32 (pCDH-TRIM32) or empty vector (pCDH), and levels of TRIM32 were measured by qRT-PCR. *Error bars* indicate S.E., ***, $p < 0.001$ (one-way ANOVA), $n = 3$. *E*, following expression of TRIM32 protein as described in *D*, lysates were immunoblotted with αTRIM32 antibodies to assess levels of expression. Hsp90 immunoblotting was performed to control for protein loading. *F*, TRIM32 expression promotes HIV-1 reactivation alone and in combination with αCD3/CD28 or TNFα stimulation. 5A8 pCDH-TRIM32 or pCDH cells were either not stimulated or stimulated with αCD3/CD28 or TNFα as indicated. *Error bars* depict S.E., ***, $p < 0.001$ (two-way ANOVA), $n = 3$.

trol. However, brief stimulation with PMA and ionomycin increased IKK activity. Furthermore, there was no evidence of phosphorylated IκBα in the lysate of cells expressing TRIM32 (Fig. 5*E*). These findings suggest that TRIM32 acts downstream of the IKKs in the NF-κB pathway. Because our prior experiments indicated that TRIM32 expression promotes degradation of IκBα, we hypothesized that TRIM32 directly binds to and ubiquitinates IκBα. To test this possibility, TRIM32 and IκBα interaction was tested in cells expressing HA-tagged IκBα and FLAG-tagged full-length TRIM32 or FLAG-tagged mutant TRIM32 containing a deletion in the RING domain (TRIM32ΔRING), thereby removing its E3 ubiquitin ligase activity. When anti-TRIM32 antibodies were used in the

immunoprecipitations, both IκBα and the IκBα super-repressor containing S32A/S36A mutations corresponding to the key IKK phosphoacceptor sites (24) were coimmunoprecipitated. However, when anti-IκBα antibodies were used, lower amounts of the TRIM32ΔRING protein were coimmunoprecipitated as compared with wild type TRIM32 (Fig. 5*F*). The ability of TRIM32 to ubiquitinate IκBα was next tested. In the presence of the β-lactone proteasome inhibitor, full-length TRIM32 promoted ubiquitination of IκBα, whereas TRIM32-ΔRING did not. In addition, TRIM32 also promoted ubiquitination of the IκBα super-repressor, but at slightly lower levels as compared with wild type IκBα (Fig. 5*G*). To confirm these findings, we assayed the ability of recombinant TRIM32 (wild

type *versus* catalytic-dead mutant) to ubiquitinate radiolabeled IκBα *in vitro*. Wild type TRIM32 mediated ubiquitination of IκBα, whereas the I22E mutant, lacking ubiquitin transfer activity, did not (Fig. 5*H*). Furthermore, we tested the ability of another NF-κB-activating TRIM protein, TRIM25, to directly ubiquitinate IκBα. We found that recombinant TRIM25 ubiq-

uitinated IκBα *in vitro* at levels similar to that of TRIM32 (Fig. 5*I*). Together, these results suggest that both TRIM32 and TRIM25 bypass many of the usual upstream NF-κB signaling steps by directly binding to and ubiquitinating IκBα and that this mechanism may be conserved in other NF-κB-activating TRIM E3 ligases.
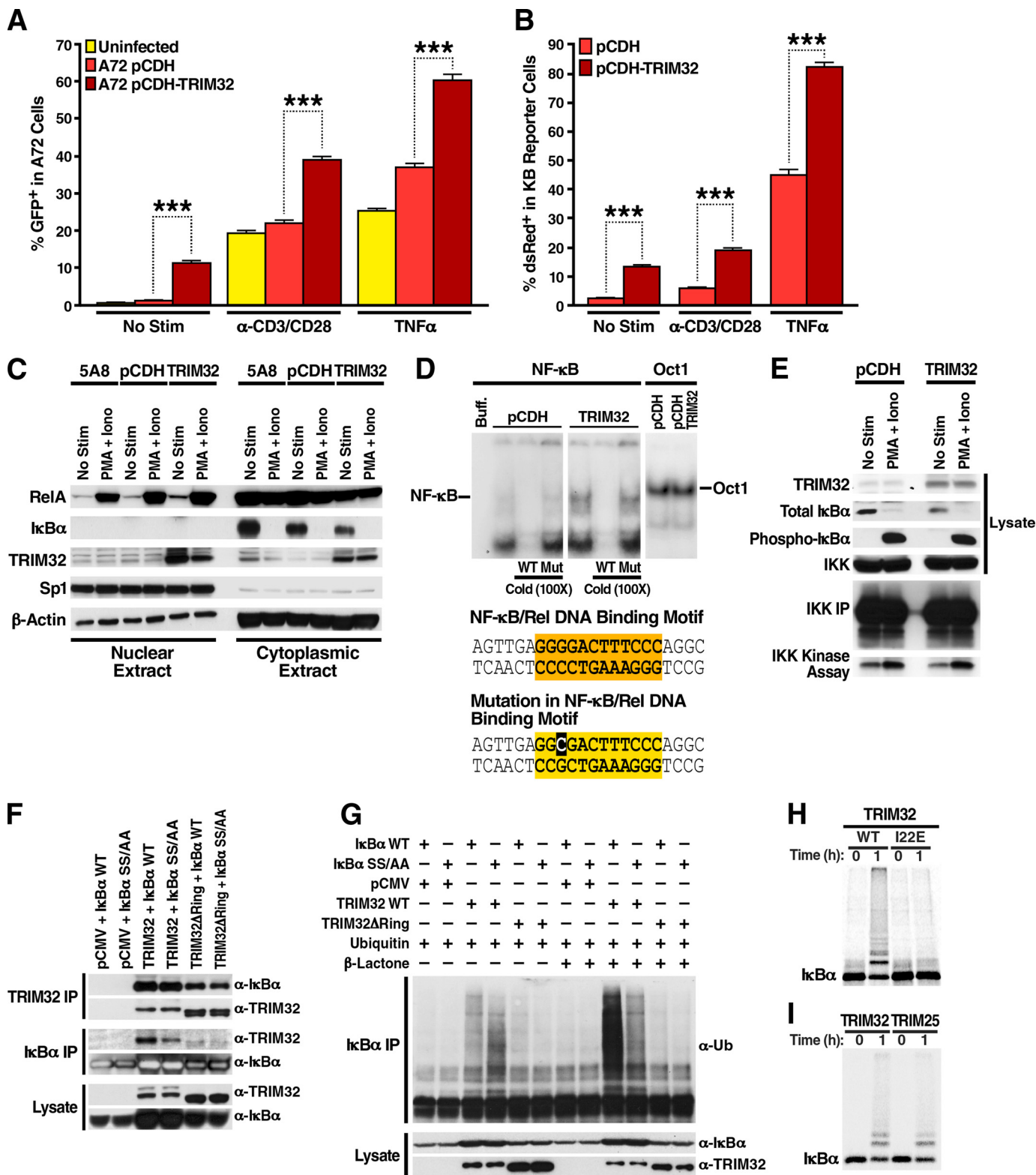
Discussion

Achieving a cure for HIV will require both the complete suppression of active viral replication *in vivo* and the clearance of the transcriptionally silent proviral reservoir. Current antiviral drugs effectively target the active virus but have no effect on the latent reservoir. One potential approach for attacking the latent reservoir is to identify combinations of agents that activate latent proviral transcription without inducing full T cell activation. These agents would promote viral replication under the cover of ART without inducing a toxic cytokine storm (25). Attacking the latent reservoir is further complicated by the fact that only a fraction of the latent proviruses appear to respond to these inducing agents (26). The variegated nature of the response suggests that repeating cycles of induction will likely be required. The half-life of productively infected cells is usually quite short, often measuring less than 24 h (27). However, recent studies suggest that induction of virus production in the latent reservoir does not result in the death of the virus-producing memory T cells (28). The production of viral proteins, however, does render this population of cells transiently visible to the immune system and thus potentially vulnerable to elimination by cytotoxic T cells or antibodies mediating antibody-dependent cellular cytotoxicity (28, 29). Latently infected cells do not appear to remain continuously latent. Rather, these cells are intermittently stimulated by cytokines or other uncharacterized signals to produce a burst of virus sometimes culminating in a visible "blip" in the viral load (30). Furthermore, low levels of virus persist in subjects on ART, below the normal detection level of standard assays (31). These viruses likely reflect intermittent production of virus by cells within the latent reservoir. However, after producing virus, these cells appear able to retreat back to a quiescent state with reestablishment of viral latency.

We have explored the potential involvement of miRNAs as key regulators of HIV latency. This notion was reinforced when knockdown of two essential enzymes required for miRNA biogenesis, DGCR8 and Dicer, was shown to lead to the activation of latent HIV proviruses. These findings are also consistent with prior studies describing an overall increase in HIV replication following knockdown of Drosha and Dicer in multiple cell lines and peripheral blood mononuclear cells (8, 12). Our findings suggest that miRNAs predominantly promote inhibition HIV-1 replication and thus function as pro-latency factors. However, we certainly do not exclude the possibility that some individual miRNAs promote viral reactivation by blocking the action of inhibitory transcription factors.

We now describe a new gene target for *miR-155* and demonstrate that this target, TRIM32, is sufficient to promote transcriptional activation of latent HIV-1. TRIM32 acts, at least in part, by a Tat-independent mechanism stimulating nuclear translocation of NF-κB. However, our studies reveal that TRIM32 activates NF-κB in a novel manner involving direct ubiquitination of IκBα. Specifically, TRIM32 induction of NF-κB proceeds independently of IKK activation within signalosomes (Fig. 6). Our studies of the potential role of microRNAs in the regulation of HIV latency have revealed an interesting network of regulation highlighting how an HIV transcriptional activator antagonizing latency through the induction of NF-κB is counter-regulated by an NF-κB-inducible microRNA that suppresses its expression.

We focused on *miR-155* in this study because, as compared with other miRNAs, it exerted the most potent blocking effects on HIV-1 reactivation in J-Lat 5A8 cells. However, our studies suggested that multiple miRNAs may play a role in blocking the reactivation of latent HIV. These other miRNAs may also be responsible for the increased levels of reactivation we demonstrate in shDGCR8 and shDicer cells following stimulation. It would be interesting to follow up on these other miRNAs as they could lead to additional miRNA host targets that regulate HIV latency. As noted previously, among all of the continuous T cell lines, J-Lat 5A8 cells most closely mirror the pattern of responsiveness found in latently infected cells from patients suppressed with ART (7). Reflecting its induction by NF-κB, *miR-155* levels are higher in activated than in resting T cells (32–35). Prior studies have implicated *miR-155* in the regulation of cellular proteins involved in trafficking and/or nuclear import of HIV pre-integration complexes including ADAM10, TNFO3, Nup153, and lens epithelium-derived growth factor (LEDGF)/p75 (10). Our findings now extend the effects of *miR-155* to a post-integration level where it may promote reestablishment of latency in reservoir cells transiently producing virus.

miR-155 is known to play a key role in the regulation of viruses other than HIV. For example, EBV strongly induces the

FIGURE 5. **Expression of TRIM32 promotes HIV-1 reactivation by inducing NF-κB activation.** *A*, J-Lat A72 cells, which lack Tat, were reactivated by pCDH-TRIM32, but not pCDH (empty vector) in the absence of other stimuli or in the presence of either αCD3/CD28 or TNFα. GFP expression was detected by flow cytometry. *Error bars* indicate S.E., ***, $p < 0.001$ (two-way ANOVA), $n = 3$. *No Stim*, unstimulated cells. *B*, TRIM32 promotes NF-κB activation in Jurkat κB-dsRed cells. Cells were infected with pCDH-TRIM32 or pCDH lentivirus and stimulated with either αCD3/CD28 or TNFα, and then analyzed by flow cytometry. *Error bars* depict S.E., ***, $p < 0.001$ (two-way ANOVA), $n = 3$. *C*, TRIM32 expression increases RelA levels in the nucleus and decreases IκBα levels in the cytoplasm. Nuclear and cytoplasmic extracts were isolated for unstimulated 5A8 cells, and 5A8 cells treated with PMA and ionomycin (*Iono*) for 30 min. *D*, TRIM32 specifically promotes the formation of nucleoprotein complexes on κB sites but not mutant (*Mut*) κB sites. 5A8 cells were infected with pCDH or pCDH-TRIM32 lentivirus, and nuclear extracts were isolated. An EMSA was performed using ^{32}P-labeled κB enhancer probes or negative control ^{32}P-labeled Oct1 probes. In addition, nuclear lysates were pre-incubated with a 100-fold excess of either unlabeled wild type or mutated κB probes. *E*, IKK activity is not increased in unstimulated TRIM32-expressing 5A8 cells as compared with pCDH controls, nor is phospho-IκBα detected. Positive controls show that IKK kinase activity is increased following 30-min treatment with PMA, and ionomycin and phospho-IκBα also increase. *F*, αFLAG immunoprecipitations (*IP*) of FLAG-tagged TRIM32 or TRIM32ΔRING and HA-tagged IκBα or IκBα super-repressor S32A/S36A (*SS/AA*) in 293T cells indicate that these proteins interact with each other. In contrast, when IκBα or its corresponding super-repressor (S32A/S36A) is first immunoprecipitated with αHA antibodies, TRIM32 is coimmunoprecipitated, but the TRIM32ΔRING binding is greatly reduced. *G*, in the presence of a proteasome inhibitor, immunoprecipitation of IκBα followed by blotting for His-tagged ubiquitin shows that wild type TRIM32 is capable of ubiquitinating IκBα (wild type and S32A/S36A) in 293T cells. The TRIM32ΔRING mutant does not produce similar ubiquitination, but this could reflect its diminished recovery in IκBα coimmunoprecipitations. *H*, IκBα is ubiquitinated by wild type TRIM32 *in vitro*, but not by an I22E mutant deficient in E2 binding. Recombinant TRIM32 (wild type or I22E) was purified and incubated with ^{35}S-labeled IκBα for 0 or 1 h. Ubiquitination reactions were visualized by autoradiography. *I*, both wild type TRIM32 and TRIM25 ubiquitinate IκBα. The experiment was performed as described for *H*.
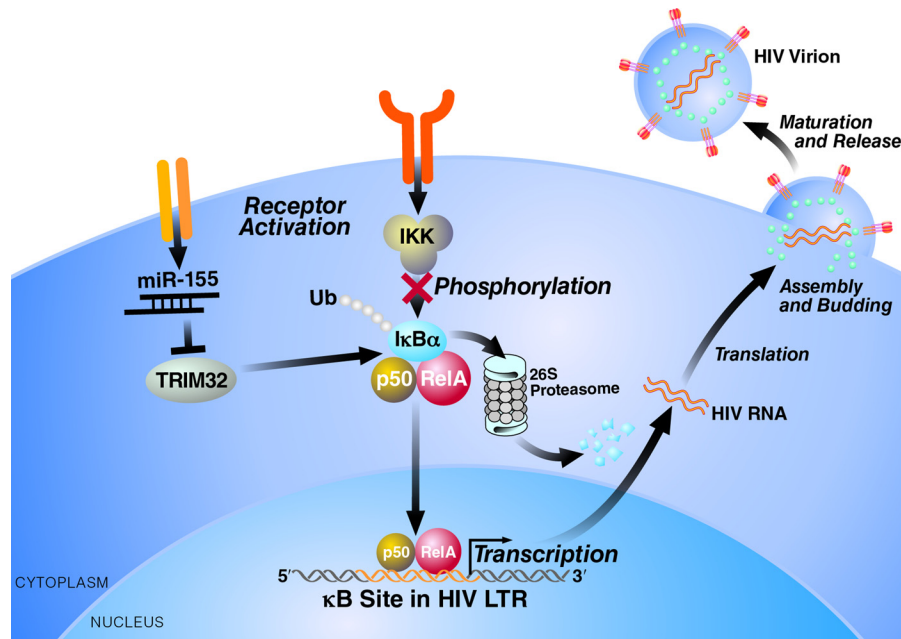
FIGURE 6. **Schematic summary.** *miR-155* directly binds to the 3′-UTR of TRIM32 and suppresses its expression. TRIM32 activates NF-κB by binding to and directly ubiquitinating IκBα in the absence of IKK activation. NF-κB p50 and RelA translocate to the nucleus and engage the duplicated κB sites in the HIV LTR, promoting HIV transcription. The ability of *miR-155* to suppress the HIV-activating effects of TRIM32 may serve to promote a retreat into latency in reservoir cells undergoing a transient cycle of virus production.

expression of *miR-155*, leading to increased growth and transformation of B cells (36, 37). In addition, Kaposi sarcoma-associated herpesvirus and Marek disease virus type 1 encode viral mimics of *miR-155*, which are thought to be involved in oncogenic transformation of infected cells (38). Kaposi sarcoma-associated herpesvirus *miR-K11* and Marek disease virus type 1 *miR-M4* both contain the full identical seed sequence of *miR-155*, and therefore likely regulate many of the same cellular targets (39, 40). Furthermore, *miR-155* regulates a variety of targets involved in mammalian immunity (41). For example, during thymic differentiation, increases in *miR-155* stimulate T regulatory cell fitness and proliferative potential by targeting the suppressor of cytokine signaling 1 (Socs1) (42). Also, *miR-155* is required for the development of inflammatory Th17 cells in experimental autoimmune encephalomyelitis (43, 44). However, the precise role of *miR-155* in immune processes is not well understood because *miR-155*, like many miRNAs, interacts with a large number targets, some of which exert opposing actions.

Although cellular miRNAs may be capable of directly targeting HIV (12), our focus was on uncovering host gene products that participate in the regulation of HIV-1 latency. In general, miRNAs are regarded as fine-tuners of gene expression (45). Having identified *miR-155* as the most HIV-repressive microRNA that was induced in our analysis, we used Target-Scan (46) for *in silico* prediction of its cellular targets. Through this process, we identified TRIM32 as a potential *miR-155* target and experimentally confirmed the ability of *miR-155* to interact with the 3′-UTR of TRIM32 to suppress its expression. Of note, the Rudensky laboratory (22) also performed experiments with 12 *miR-155* wild type mice and 12 *miR-155* knockout mice, identifying *miR-155* target genes in CD4 T cells. When we searched for TRIM32 on their CLIP database, we

found that the evolutionarily conserved TRIM32 binding sequence did bind to an AGO-*miR-155* complex *in vivo*. Taken together, these findings confirm TRIM32 as a *bona fide miR-155* host gene target.

As we predicted, when levels of TRIM32 were reduced with shRNA, lower reactivation of latent HIV was observed. Conversely, when TRIM32 was overexpressed in cells harboring latent provirus, increased viral activation was detected. Notably, TRIM32 expression induced reactivation, even in the absence of other stimuli. Interestingly, knockdown of DGCR8 and Dicer in J-Lat 5A8 cells does not result in a spontaneous increase in the level of reactivation in the absence of stimulation. Considering that the levels of both TRIM32 and miR-155 are low in unstimulated cells, we would not expect a further decrease in miR-155 levels to promote reactivation in the absence of a stimulatory signal.

These findings may be relevant in the context of aviremic patients on highly active antiretroviral therapy who commonly experience intermittent biological and statistical fluctuations in levels of viremia, termed blips (30). It is possible that the natural down-regulation of virus expression reflects changes in the levels of *miR-155* expression, which in turn could result in decreased TRIM32 expression. These events could contribute to resilencing of the virus and maintenance of the latent reservoir. Similarly, increases in *miR-155* levels during shock and kill could impair latent virus reactivation, thereby avoiding a viral cytopathic effect or allowing the cell to remain undetected by the immune system.

A prior study indicated that TRIM32 interacts with Tat but did not identify any functional consequences of this interaction. Although we certainly do not exclude a role for TRIM32-Tat interaction in HIV biology, our findings indicate that TRIM32 can function independently of Tat. Specifically, we have shown

in biochemical and functional experiments that TRIM32 expression promotes nuclear NF-κB translocation and engagement of the cognate κB enhancer sites present in the HIV-1 LTR. Consistent with our findings, a number of TRIM proteins including TRIM32 are capable of inducing the expression of NF-κB, AP-1, and activating the HIV-1 LTR in HEK293 cells (16). In that study, knockdown of TAK1 (upstream of IKK) decreased the level of NF-κB induction following stimulation with eight different TRIMs including TRIM32. Surprisingly, we observed that TRIM32 expression did not activate the IKK complex. Instead, we found that TRIM32 is able to activate NF-κB by direct ubiquitination of either wild type IκBα or IκBα super-repressor lacking IKK phospho-acceptor site promoting release and nuclear translocation of the NF-κB heterodimer. Thus, IκBα serves as a novel target of the E3 ligase TRIM32.

Supporting these findings, we have also shown that TRIM32 can directly ubiquitinate IκBα *in vitro*, whereas the introduction of a single point mutation (I22E) in TRIM32, which abrogates the activity of ubiquitin transfer without destabilizing the overall fold of the RING domain, prevents IκBα ubiquitination. Furthermore, to investigate whether this mechanism occurs in other TRIMs, we performed an *in vitro* ubiquitination assay and found that TRIM25, an NF-κB-activating E3 ligase, is also capable of directly ubiquitinating IκBα. This suggests that this mechanism may be more widespread among other TRIMs that activate NF-κB. Although over 600 human RING-based E3 ligases control many cellular processes, we have only a rudimentary understanding of their functions, substrates, and mechanisms of action (47). In the canonical NF-κB pathway, ubiquitination of IκB is normally carried out by the SCF-βTrCP E3 ligase (SKP1-CUL1-F-box ligase containing the F-box protein βTrCP) (48–50). We propose a novel form of NF-κB activation, independent of IKK activation, in which TRIM32, TRIM25, and perhaps other TRIMs directly bind to and ubiquitinate IκBα.

It is important to note that TRIM32 targets a number of substrates involved in diverse pathways (13–15, 18), and modulation of these pathways may also promote NF-κB signaling. For example, TRIM32 targets STING for Lys-63-linked ubiquitination, thereby promoting its activation and subsequent induction of NF-κB and production of IFN-β in response to Sendai virus and Herpes simplex virus 1 infection (14). This suggests that TRIM32 (via STING) plays an important role in the innate immune response against these viruses. However, in the context of HIV-1, activation of the NF-κB pathway by TRIM32 leads to the production of virus. It seems possible that the suppressive interplay of the inducible *miR-155* with the similarly inducible TRIM32 was designed as a means to control the overall stimulatory effects of TRIM32. In the case of HIV, inhibition of TRIM32 by *miR-155* may have the untoward effect of promoting a return of viral latency, thereby providing a mechanism for HIV persistence in infected reservoir cells transiently producing virus.

It is clear that a better understanding of the mechanisms that govern transcriptional activation of HIV is critical to develop new treatments that will force the reactivation of latent provirus and subsequent eradication of HIV-1 from patients. Identification of novel host regulatory pathways that govern HIV-1 transcription enhances our understanding of the molecular basis of HIV latency and may offer avenues for pharmacological intervention.

References

1. Perelson, A. S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M., and Ho, D. D. (1997) Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* **387,** 188–191
2. Chun, T. W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D., and Siliciano, R. F. (1995) *In vivo* fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat. Med.* **1,** 1284–1290
3. Colin, L., and Van Lint, C. (2009) Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. *Retrovirology* **6,** 111
4. Williams, S. A., Chen, L. F., Kwon, H., Fenard, D., Bisgrove, D., Verdin, E., and Greene, W. C. (2004) Prostratin antagonizes HIV latency by activating NF-κB. *J. Biol. Chem.* **279,** 42008–42017
5. Jordan, A., Bisgrove, D., and Verdin, E. (2003) HIV reproducibly establishes a latent infection after acute infection of T cells *in vitro*. *EMBO J.* **22,** 1868–1877
6. Chan, J. K., Bhattacharyya, D., Lassen, K. G., Ruelas, D., and Greene, W. C. (2013) Calcium/calcineurin synergizes with prostratin to promote NF-κB dependent activation of latent HIV. *PLoS One* **8,** e77749
7. Spina, C. A., Anderson, J., Archin, N. M., Bosque, A., Chan, J., Famiglietti, M., Greene, W. C., Kashuba, A., Lewin, S. R., Margolis, D. M., Mau, M., Ruelas, D., Saleh, S., Shirakawa, K., Siliciano, R. F., Singhania, A., Soto, P. C., Terry, V. H., Verdin, E., Woelk, C., Wooden, S., Xing, S., and Planelles, V. (2013) An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS Pathog.* **9,** e1003834
8. Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., Lebrigand, K., Cardinaud, B., Maurin, T., Barbry, P., Baillat, V., Reynes, J., Corbeau, P., Jeang, K. T., and Benkirane, M. (2007) Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315,** 1579–1582
9. Chiang, K., Sung, T. L., and Rice, A. P. (2012) Regulation of cyclin T1 and HIV-1 replication by microRNAs in resting CD4+ T lymphocytes. *J. Virol.* **86,** 3244–3252
10. Swaminathan, G., Rossi, F., Sierra, L. J., Gupta, A., Navas-Martín, S., and Martín-García, J. (2012) A role for microRNA-155 modulation in the anti-HIV-1 effects of Toll-like receptor 3 stimulation in macrophages. *PLoS Pathog.* **8,** e1002937
11. Huang, J., Wang, F., Argyris, E., Chen, K., Liang, Z., Tian, H., Huang, W., Squires, K., Verlinghieri, G., and Zhang, H. (2007) Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nat. Med.* **13,** 1241–1247
12. Nathans, R., Chu, C. Y., Serquina, A. K., Lu, C. C., Cao, H., and Rana, T. M. (2009) Cellular microRNA and P bodies modulate host-HIV-1 interactions. *Mol. Cell* **34,** 696–709
13. Schwamborn, J. C., Berezikov, E., and Knoblich, J. A. (2009) The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell* **136,** 913–925
14. Zhang, J., Hu, M. M., Wang, Y. Y., and Shu, H. B. (2012) TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J. Biol. Chem.* **287,** 28646–28655

15. Albor, A., El-Hizawi, S., Horn, E. J., Laederich, M., Frosk, P., Wrogemann, K., and Kulesz-Martin, M. (2006) The interaction of Piasy with Trim32, an E3-ubiquitin ligase mutated in limb-girdle muscular dystrophy type 2H, promotes Piasy degradation and regulates UVB-induced keratinocyte apoptosis through NFκB. *J. Biol. Chem.* **281,** 25850–25866

16. Uchil, P. D., Hinz, A., Siegel, S., Coenen-Stass, A., Pertel, T., Luban, J., and Mothes, W. (2013) TRIM protein-mediated regulation of inflammatory and innate immune signaling and its association with antiretroviral activity. *J. Virol.* **87,** 257–272

17. Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D. (1996) *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267

18. Ryu, Y. S., Lee, Y., Lee, K. W., Hwang, C. Y., Maeng, J. S., Kim, J. H., Seo, Y. S., You, K. H., Song, B., and Kwon, K. S. (2011) TRIM32 protein sensitizes cells to tumor necrosis factor (TNFα)-induced apoptosis via its RING domain-dependent E3 ligase activity against X-linked inhibitor of apoptosis (XIAP). *J. Biol. Chem.* **286,** 25729–25738

19. Sun, S., Elwood, J., and Greene, W. C. (1996) Both amino- and carboxyl-terminal sequences within IκBα regulate its inducible degradation. *Mol. Cell. Biol.* **16,** 1058–1065

20. Calvanese, V., Chavez, L., Laurent, T., Ding, S., and Verdin, E. (2013) Dual-color HIV reporters trace a population of latently infected cells and enable their purification. *Virology* **446,** 283–292

21. Fridell, R. A., Harding, L. S., Bogerd, H. P., and Cullen, B. R. (1995) Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins. *Virology* **209,** 347–357

22. Loeb, G. B., Khan, A. A., Canner, D., Hiatt, J. B., Shendure, J., Darnell, R. B., Leslie, C. S., and Rudensky, A. Y. (2012) Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. *Mol. Cell* **48,** 760–770

23. Jordan, A., Defechereux, P., and Verdin, E. (2001) The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *EMBO J.* **20,** 1726–1738

24. Kwon, H., Pelletier, N., DeLuca, C., Genin, P., Cisternas, S., Lin, R., Wainberg, M. A., and Hiscott, J. (1998) Inducible expression of IκBα repressor mutants interferes with NF-κB activity and HIV-1 replication in Jurkat T cells. *J. Biol. Chem.* **273,** 7431–7440

25. Archin, N. M., and Margolis, D. M. (2014) Emerging strategies to deplete the HIV reservoir. *Curr. Opin. Infect. Dis.* **27,** 29–35

26. Ho, Y. C., Shan, L., Hosmane, N. N., Wang, J., Laskey, S. B., Rosenbloom, D. I., Lai, J., Blankson, J. N., Siliciano, J. D., and Siliciano, R. F. (2013) Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* **155,** 540–551

27. Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373,** 123–126

28. Shan, L., Deng, K., Shroff, N. S., Durand, C. M., Rabi, S. A., Yang, H. C., Zhang, H., Margolick, J. B., Blankson, J. N., and Siliciano, R. F. (2012) Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity* **36,** 491–501

29. Descours, B., Avettand-Fenoel, V., Blanc, C., Samri, A., Mélard, A., Supervie, V., Theodorou, I., Carcelain, G., Rouzioux, C., Autran, B., and ALT ANRS CO15 Study Group (2012) Immune responses driven by protective human leukocyte antigen alleles from long-term nonprogressors are associated with low HIV reservoir in central memory CD4 T cells. *Clin. Infect. Dis.* **54,** 1495–1503

30. Nettles, R. E., Kieffer, T. L., Kwon, P., Monie, D., Han, Y., Parsons, T., Cofrancesco, J., Jr., Gallant, J. E., Quinn, T. C., Jackson, B., Flexner, C., Carson, K., Ray, S., Persaud, D., and Siliciano, R. F. (2005) Intermittent HIV-1 viremia (Blips) and drug resistance in patients receiving HAART. *JAMA* **293,** 817–829

31. Dornadula, G., Zhang, H., VanUitert, B., Stern, J., Livornese, L., Jr., Ingerman, M. J., Witek, J., Kedanis, R. J., Natkin, J., DeSimone, J., and Pomerantz, R. J. (1999) Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. *JAMA* **282,** 1627–1632

32. Banerjee, A., Schambach, F., DeJong, C. S., Hammond, S. M., and Reiner, S. L. (2010) Micro-RNA-155 inhibits IFN-γ signaling in CD4+ T cells. *Eur. J. Immunol.* **40,** 225–231

33. Haasch, D., Chen, Y. W., Reilly, R. M., Chiou, X. G., Koterski, S., Smith, M. L., Kroeger, P., McWeeny, K., Halbert, D. N., Mollison, K. W., Djuric, S. W., and Trevillyan, J. M. (2002) T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. *Cell. Immunol.* **217,** 78–86

34. Rodriguez, A., Vigorito, E., Clare, S., Warren, M. V., Couttet, P., Soond, D. R., van Dongen, S., Grocock, R. J., Das, P. P., Miska, E. A., Vetrie, D., Okkenhaug, K., Enright, A. J., Dougan, G., Turner, M., and Bradley, A. (2007) Requirement of *bic/microRNA-155* for normal immune function. *Science* **316,** 608–611

35. Thai, T. H., Calado, D. P., Casola, S., Ansel, K. M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J. L., Schmidt-Supprian, M., Rajewsky, N., Yancopoulos, G., Rao, A., and Rajewsky, K. (2007) Regulation of the germinal center response by microRNA-155. *Science* **316,** 604–608

36. Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M., and Lieberman, P. M. (2008) Epstein-Barr virus-induced miR-155 attenuates NF-κB signaling and stabilizes latent virus persistence. *J. Virol.* **82,** 10436–10443

37. Linnstaedt, S. D., Gottwein, E., Skalsky, R. L., Luftig, M. A., and Cullen, B. R. (2010) Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. *J. Virol.* **84,** 11670–11678

38. Gottwein, E. (2013) Roles of microRNAs in the life cycles of mammalian viruses. *Curr. Top. Microbiol. Immunol.* **371,** 201–227

39. Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W. H., Chi, J. T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B. R. (2007) A viral microRNA functions as an ortholog of cellular miR-155. *Nature* **450,** 1096–1099

40. Skalsky, R. L., Samols, M. A., Plaisance, K. B., Boss, I. W., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007) Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* **81,** 12836–12845

41. So, A. Y., Zhao, J. L., and Baltimore, D. (2013) The Yin and Yang of microRNAs: leukemia and immunity. *Immunol. Rev.* **253,** 129–145

42. Lu, L. F., Thai, T. H., Calado, D. P., Chaudhry, A., Kubo, M., Tanaka, K., Loeb, G. B., Lee, H., Yoshimura, A., Rajewsky, K., and Rudensky, A. Y. (2009) Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* **30,** 80–91

43. Murugaiyan, G., Beynon, V., Mittal, A., Joller, N., and Weiner, H. L. (2011) Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis. *J. Immunol.* **187,** 2213–2221

44. O'Connell, R. M., Kahn, D., Gibson, W. S., Round, J. L., Scholz, R. L., Chaudhuri, A. A., Kahn, M. E., Rao, D. S., and Baltimore, D. (2010) MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* **33,** 607–619

45. Bartel, D. P., and Chen, C. Z. (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* **5,** 396–400

46. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003) Prediction of mammalian microRNA targets. *Cell* **115,** 787–798

47. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78,** 399–434

48. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) Identification of the receptor component of the IκBα-ubiquitin ligase. *Nature* **396,** 590–594

49. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) The SCFβ-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination *in vitro*. *Genes Dev.* **13,** 270–283

50. Spencer, E., Jiang, J., and Chen, Z. J. (1999) Signal-induced ubiquitination of IκBα by the F-box protein Slimb/β-TrCP. *Genes Dev.* **13,** 284–294