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VIRUS-CELL INTERACTIONS

Inhibition of a Chromatin and Transcription Modulator, SLTM, Increases HIV-1 Reactivation Identified by a CRISPR Inhibition Screen

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ABSTRACT Despite effective antiretroviral therapy, HIV-1 persistence in latent reservoirs remains a major obstacle to a cure. We postulate that HIV-1 silencing factors suppress HIV-1 reactivation and that inhibition of these factors will increase HIV-1 reactivation. To identify HIV-1 silencing factors, we conducted a genome-wide CRISPR inhibition (CRISPRi) screen using four CRISPRi-ready, HIV-1-d6-GFP-infected Jurkat T cell clones with distinct integration sites. We sorted cells with increased green fluorescent protein (GFP) expression and captured single guide RNAs (sgRNAs) via targeted deep sequencing. We identified 18 HIV-1 silencing factors that were significantly enriched in HIV-1-d6-GFP^{high} cells. Among them, SLTM (scaffold attachment factor B-like transcription modulator) is an epigenetic and transcriptional modulator having both DNA and RNA binding capacities not previously known to affect HIV-1 transcription. Knocking down SLTM by CRISPRi significantly increased HIV-1-d6-GFP expression (by 1.9- to 4.2-fold) in three HIV-1-d6-GFP-Jurkat T cell clones. Furthermore, SLTM knockdown increased the chromatin accessibility of HIV-1 and the gene in which HIV-1 is integrated but not the housekeeping gene *POLR2A*. To test whether SLTM inhibition can reactivate HIV-1 and further induce cell death of HIV-1-infected cells *ex vivo*, we established a small interfering RNA (siRNA) knockdown method that reduced SLTM expression in CD4$^+$ T cells from 10 antiretroviral therapy (ART)-treated, virally suppressed, HIV-1-infected individuals *ex vivo*. Using limiting dilution culture, we found that SLTM knockdown significantly reduced the frequency of HIV-1-infected cells harboring inducible HIV-1 by 62.2% (0.56/10^6 versus 1.48/10^6 CD4$^+$ T cells [$P = 0.029$]). Overall, our study indicates that SLTM inhibition reactivates HIV-1 *in vitro* and induces cell death of HIV-1-infected cells *ex vivo*. Our study identified SLTM as a novel therapeutic target.

IMPORTANCE HIV-1-infected cells, which can survive drug treatment and immune cell killing, prevent an HIV-1 cure. Immune recognition of infected cells requires HIV-1 protein expression; however, HIV-1 protein expression is limited in infected cells after long-term therapy. The ways in which the HIV-1 provirus is blocked from producing protein are unknown. We identified a new host protein that regulates HIV-1 gene expression. We also provided a new method of studying HIV-1–host factor interactions in cells from infected individuals. These improvements may enable future strategies to reactivate HIV-1 in infected individuals so that infected cells can be killed by immune cells, drug treatment, or the virus itself.

KEYWORDS CD4$^+$ T cells, CRISPR screen, HIV latency, HIV latent reservoir, HIV reactivation, HIV silencing factors, HUSH complex, RNA exosome, RNA processing, SLTM

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Despite effective antiretroviral therapy (ART), HIV-1 proviruses integrated into the chromatin of infected cells persist lifelong (1–3). One of the approaches for a cure is the shock-and-kill strategy (4), which aims to reactivate HIV-1 from latency, induce viral antigen expression, induce viral cytopathic effects, and expose infected cells for immune clearance. Extensive small-molecule compound library screens have identified numerous HIV-1 latency-reversing agents, some of which achieved initial success in HIV-1 reactivation *in vivo* (4–6).

HIV-1 reactivation is regulated by multiple viral and host factors at the epigenetic level, the transcriptional level, and the posttranscriptional level. Currently available latency-reversing agents disrupt epigenetic regulators through histone deacetylation inhibitors (4, 7, 8), activate transcription through noncanonical NF-κB activation (by second mitochondiral-derived activator of caspases [SMAC] mimetics) (6, 9) or protein kinase C agonists (10–13), or increase transcriptional elongation by antagonizing Brd4-mediated Tat inhibition by bromodomain and extra-terminal motif (BET) inhibitors (14, 15). Still, there is a discrepancy between HIV-1 reactivation and the elimination of HIV-1-infected cells: HIV-1 RNA expression may not result in sufficient levels of HIV-1 protein expression (16) to induce viral cytopathic effects or immune recognition. While combinations of latency-reversing agents that target different cellular pathways can synergize HIV-1 reactivation, the limited success of the shock-and-kill strategies in reducing the size of the latent reservoir suggests that additional cellular pathways that can regulate HIV-1 reactivation need to be identified and targeted for effective HIV-1 reactivation and clearance.

Epigenetic regulators such as TRIM28 (17), the human silencing hub (HUSH) complex, and SETDB1 (18–22) can silence retroviruses and retrotransposons. However, HIV-1 silencing is incompletely understood. The advancement of genome-wide gene knockdown screens has identified the impact of several cellular factors on HIV-1 reactivation (23–29). HIV-1 silencing factors suppress HIV-1 reactivation; thus, inhibiting HIV-1 silencing factors by small interfering RNA (siRNA)-mediated knockdown or CRISPR-mediated gene repression reactivates HIV-1. For example, RNA knockdown screens identified estrogen receptor 1 (ESR1) (30), TRIM28 (31), and mTOR (29) as regulators of HIV-1 latency. Among them, estrogen receptor and mTOR can be targeted by small-molecule compounds and serve as promising therapeutic targets. CRISPR screens investigating HIV-1 silencing factors, such as the H3K36me3 demethylase MINA53 (24), the zinc finger nuclease ZNF304 (28), the deubiquitinase USP14 (26), the RNA methyltransferase FTSJ3 (25), and MAT2A (27), have provided druggable targets and new mechanisms for HIV-1 latency reversal. Interestingly, the results of these siRNA or CRISPR screens do not overlap, reflecting the diverse performances of HIV-1-infected cell models (32). Ultimately, the impact of these HIV-1 silencing factors on HIV-1 reactivation needs to be tested *ex vivo*, ideally using clinical samples from ART-treated, virally suppressed, HIV-1-infected individuals, to validate whether cellular factors identified *in vitro* can reactivate HIV-1 *ex vivo*.

In ART-treated, virally suppressed, HIV-1-infected individuals, $>$90% of the HIV-1 proviruses are integrated into actively transcribed genes (33, 34). HIV-1 proviruses that are integrated into actively transcribed genes are associated with permissive chromatin, active chromatin marks, and increased responses to latency reversal (35). Unlike HIV-1 proviruses residing in repressive heterochromatin that remain silent despite maximum T cell activation, HIV-1 proviruses residing in actively transcribed genes are sources of nonsuppressible viremia or viral rebound (36). Reactivating the HIV-1 proviruses that are integrated into actively transcribed genes and inducing immune clearance of these cells may enhance HIV-1 reactivation and therefore improve the shock-and-kill strategy.

Here, we used an established, well-characterized, HIV-1-infected Jurkat cell line model with HIV-1 integrated into actively transcribed genes for a genome-wide CRISPR screen. We identified HIV-1 silencing pathways and a novel HIV-1 silencing factor, SLTM (scaffold attachment factor B [SAFB]-like transcription modulator). SLTM is an SAFB family gene that has DNA binding, RNA binding, and protein binding motifs and serves as both an epigenetic and a transcriptional regulator. While other SAFB family genes can suppress L1 retrotransposons (22), we found that SLTM has a more potent effect than other SAFB family

genes in HIV-1 suppression. We validated our results in multiple cell line clones and in $CD4^+$ T cells from ART-treated, virally suppressed, HIV-1-infected individuals. To our knowledge, this is the first time that HIV-1 silencing factors identified in genome-wide screens were validated in clinical samples by knocking down the candidate HIV-1 silencing factor directly. Our study not only provides new drug targets for HIV-1 reactivation but also bridges mechanistic studies *in vitro* to direct testing on clinical samples *ex vivo*.

RESULTS

A genome-wide CRISPR inhibition screen identified novel HIV-1 silencing factors. To identify HIV-1 silencing factors, we conducted a genome-wide CRISPR inhibition (CRISPRi) screen on four different HIV-1-d6-GFP-Jurkat T cell clones with HIV-1 integrated into actively transcribed genes, as we previously characterized (37, 38). The HIV-1-d6-GFP reporter provirus retains the full-length NL4-3 genome and preserves *cis*-acting proviral elements, such as HIV-1 long terminal repeat (LTR), trans-activation response element (TAR), Rev response element (RRE), and splice elements. In addition, this HIV-1 reporter harbors six inactivating mutations in *gag*, *vif*, *vpr*, *vpu*, *env*, and *nef* to eliminate viral cytopathic effects for long-term culture (Fig. 1A) (39). HIV-1 Env was replaced by a green fluorescence reporter (green fluorescent protein [GFP]).

The development of HIV-1 latency cell line clones, such as J-Lat (40), has provided a fundamental understanding of HIV-1 latency. The J-Lat cell line clones were particularly selected to have ~0% HIV-1 expression at baseline to allow a wide dynamic range for the measurement of HIV-1 latency reversal. The J-Lat cell line clones harbor HIV-1 proviruses integrated into either heterochromatin (such as alphoid repeats) (40) or CpG-methylated genome regions (41). While HIV-1 integration into heterochromatin may facilitate immune escape (42), in virally suppressed individuals, 90% of HIV-1 integrations are in the introns of actively transcribed genes (33, 34, 43). Furthermore, recent studies suggest that latent HIV-1 proviruses are challenging to reactivate. In contrast to true transcriptional latency, ~25% of all HIV-1 proviruses are transcriptionally active. This transcriptionally active fraction comprises the proviruses that respond to latency reversal, cause viral rebound, and expand into proliferated T cell clones (35, 44, 45). Therefore, we chose to use HIV-1 reporter cell lines having transcriptionally active HIV-1 that are integrated into introns of actively transcribed genes, as opposed to the latent J-Lat cell line clones, to examine the impact of cellular factors on HIV-1 expression.

To ensure that the CRISPRi screen identifies HIV-1 silencing factors across different HIV-1 integration sites, we used four different HIV-1-d6-GFP-Jurkat T cell clones with the HIV-1-d6-GFP reporter integrated into known integration sites (Fig. 1B). The use of four different cell line clones as biological replicates (as opposed to technical replicates of single cell line clones) enabled us to increase the generalizability of our screen. Briefly, uninfected cells were infected with HIV-1-d6-GFP pseudotyped virus (39) at a low multiplicity of infection (MOI), as we previously described (37). Three days after infection, GFP-positive cells were sorted as single cells into individual wells of 96-well plates by flow cytometry. Three weeks after sorting, cells that grew into visible clones were further propagated in individual culture flasks. HIV-1 integration sites in each HIV-1-d6-GFP-Jurkat T cell clone were examined by inverse PCR (33). We selected four cell line clones for the CRISPRi screen: 8B10 (HIV-1 integration into *VAV1* and *NFX1*), 1G2 (HIV-1 integration into *RAP1B*), 1D7 (HIV-1 integration into *SPECC1*), and 1F6 (HIV-1 integration into *PRCC*).

To create CRISPRi-ready HIV-1-infected cell line clones, we transduced the four HIV-1-d6-GFP-Jurkat T cell clones with a CRISPR-dCas9-Krab lentivirus (46). HIV-1-d6-GFP-Jurkat cell clones that were stably transduced with CRISPR-dCas9-Krab were selected by blasticidin resistance to make them CRISPRi ready. We then transduced each of the CRISPRi-ready HIV-1-d6-GFP-Jurkat T cell clones with a genome-wide single guide RNA (sgRNA) library, targeting 5 sgRNAs per gene for 18,905 genes (47). Cells transduced with sgRNA were selected by puromycin resistance. Cells were divided into two aliquots. In one aliquot, HIV-1-d6-GFP cells expressing GFP levels higher than those of nontargeting (NT) sgRNA-transduced cells were sorted by flow cytometry. The other aliquot was not sorted and served as a control. Both aliquots were genomic DNA (gDNA)

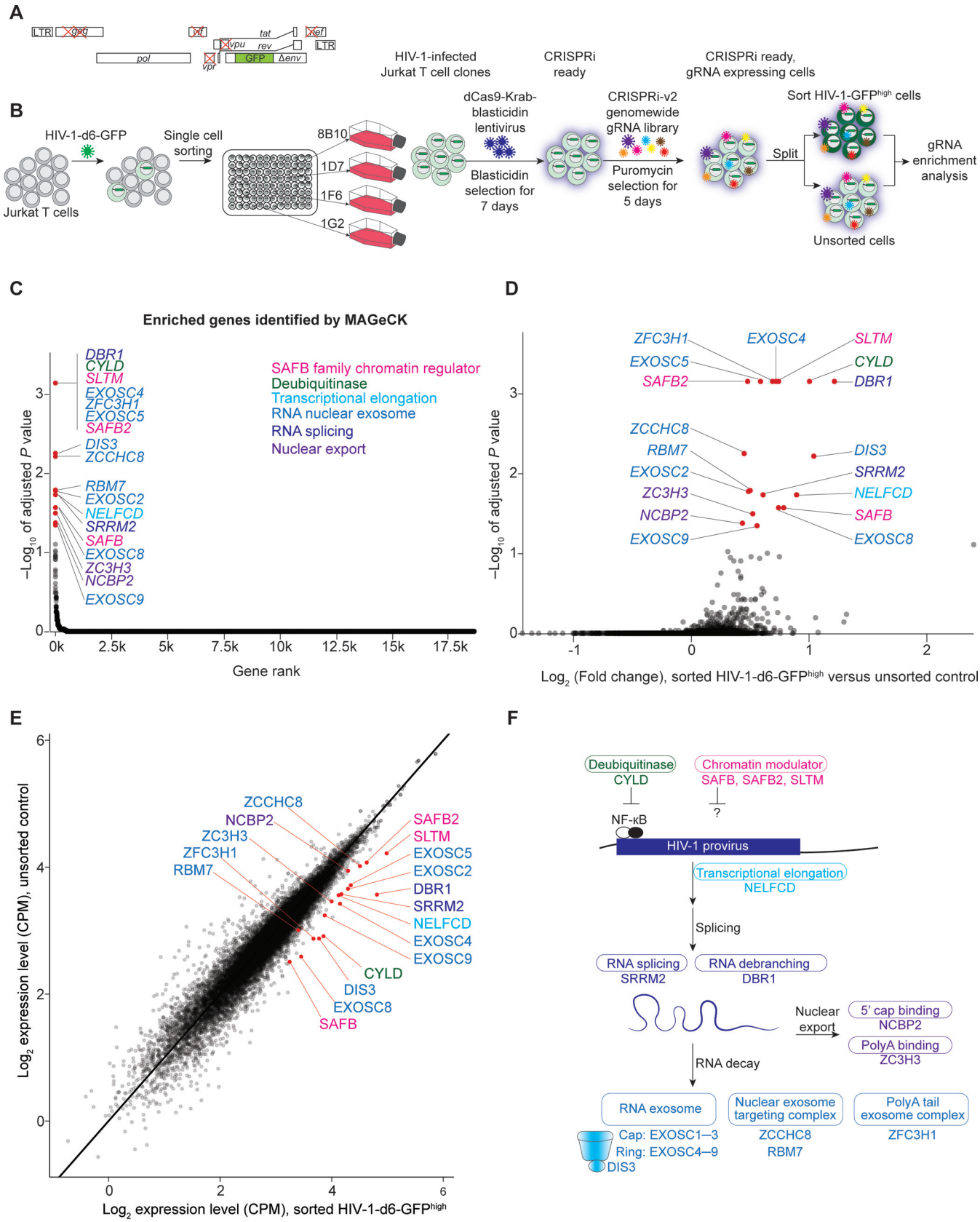

FIG 1 A genome-wide CRISPRi screen identified 18 HIV-1 silencing factors. (A) The HIV-1-d6-GFP reporter virus used in this system, with inactivating mutations in viral proteins that may cause cytopathic effects. (B) Experimental scheme of the genome-wide CRISPRi screen in four HIV-1-d6-GFP-Jurkat T

(Continued on next page)

extracted, library prepped, and sent for targeted sequencing to capture the sgRNA sequences within each population.

Using MAGeCK (48), we identified sgRNAs from 18 genes that were significantly enriched in GFP^{high} cells, indicating that knocking down the expression of these 18 genes by CRISPRi increases HIV-1-driven GFP expression (Fig. 1C to E). These 18 genes included those known to inhibit HIV-1 expression, such as the NF-κB deubiquitinase CYLD (49) (also identified in previous CRISPR screens [25, 26]) and a negative regulator of transcriptional elongation, NELFCD (50). The identification of CYLD and NELFCD indicates that our screen is capable of identifying HIV-1 silencing factors. The majority of the HIV-1 silencing factors were involved in RNA splicing (SRRM1 [51]), RNA debranching (DBR1 [52]), nuclear cap binding (by NCBP2) (53), polyadenylation (ZC3H3 [54]), and the nuclear RNA surveillance complex (such as DIS3, EXOSC proteins, ZCCHC8, RBM7, and ZFC3H1) (55) (reviewed in reference 56) (Fig. 1F). Importantly, three cellular factors were recently identified to silence retroelements in conjunction with the HUSH complex: the nuclear exosome targeting (NEXT) complex genes ZCCHC8 and RBM7 and the poly(A) tail exosome (PAXT) complex gene ZFC3H1 (57). Surprisingly, we identified all three scaffold attachment factor B (SAFB) family proteins (reviewed in reference 58), SAFB (SAFB1), SAFB2, and SLTM, in our CRISPRi screen, indicating that inhibition of these chromatin regulators increases HIV-1 expression. Of note, SAFB can bind to the heat shock protein 27 (HSP27) promoter to suppress estrogen receptor signaling or can serve as a chromatin regulator that suppresses retrotransposon (22) or HIV-1 (59) expression. The role of SLTM (SAFB-like transcription modulator) in HIV-1 infection remains unknown.

Suppression of HIV-1 silencing factors increases HIV-1 expression in three HIV-1 cell line clones. We focused on seven candidate HIV-1 silencing factors from the different pathways involved, including two from the SAFB family of chromatin regulators (SLTM and SAFB), the deubiquitinase CYLD, the transcriptional elongation inhibitor NELFCD, and the RNA processing factors DBR1, DIS3, and EXOSC4. Among them, SAFB (59), CYLD (49), and NELFCD (50, 60) are known to inhibit HIV-1 transcription and served as positive controls. An NT sgRNA served as the negative control. To validate whether knocking down HIV-1 silencing factors identified in our CRISPRi screen indeed increases HIV-1 expression, we performed validation studies in three HIV-1-d6-GFP-Jurkat T cell clones, 1D7, 1G2, and 8B10. Briefly, the CRISPRi-ready 1D7-, 1G2-, and 8B10-HIV-1-d6-GFP-Jurkat T cell clones were transduced with lentiviruses carrying one specific sgRNA and a blue fluorescent protein (BFP) reporter. We tested three sgRNAs for each of the candidate HIV-1 silencing factors, including two from the original CRISPRi screen and one additional sgRNA not included in the CRISPRi screen (see Table S1 in the supplemental material). We examined cellular viability using viability staining, sgRNA transduction using BFP reporter expression, and HIV-1-driven GFP expression using flow cytometry. We found that the downregulation of these seven candidate HIV-1 silencing factors did not significantly change cell viability (Fig. S1A to D). Knocking down these candidate HIV-1 silencing factors consistently increased HIV-1-driven GFP expression (Fig. 2A to C; Fig. S1E to H) in at least one out of the three sgRNAs tested in all three HIV-1-d6-GFP-Jurkat T cell clones, regardless of the HIV-1 integration sites in each clone (Fig. 2C to H).

SLTM suppression increases HIV-1 expression in three HIV-1 cell line clones. We next focused on the SAFB family chromatin regulator SLTM because of its DNA binding, RNA binding, and protein binding domains and due to its potential to interact with HIV-1 at both the epigenetic level (through chromatin interactions) and the transcriptional level (as a transcription regulator) (58). To examine the knockdown efficiencies of candidate cellular factors, we enriched cells that were transduced with both CRISPR-dCas9-Krab

FIG 1 Legend (Continued)
cell clones. The CRISPRi sgRNA library targets 5 sgRNAs/gene for 18,905 genes. The use of four different cell line clones provides biological replicates as opposed to technical replicates of the screen. (C) Rank of sgRNAs enriched in the GFP^{high} population in the CRISPRi screen identified by MAGeCK. CRISPRi-mediated knockdown of these genes increased HIV-1-driven GFP expression. Red dots indicate 18 sgRNAs with adjusted *P* values of <0.05. (D) Significance (adjusted *P* values) versus the level of enrichment ($\log_2$ fold change) of sgRNAs identified by MAGeCK. (E) Expression levels (normalized as counts per million) showing enrichment of sgRNA in sorted GFP^{high} cells versus the unsorted control. (F) Functional roles of the 18 HIV-1 silencing factors identified in the CRISPRi screen. Each dot indicates the aggregate score of all guides targeting that gene in the library.

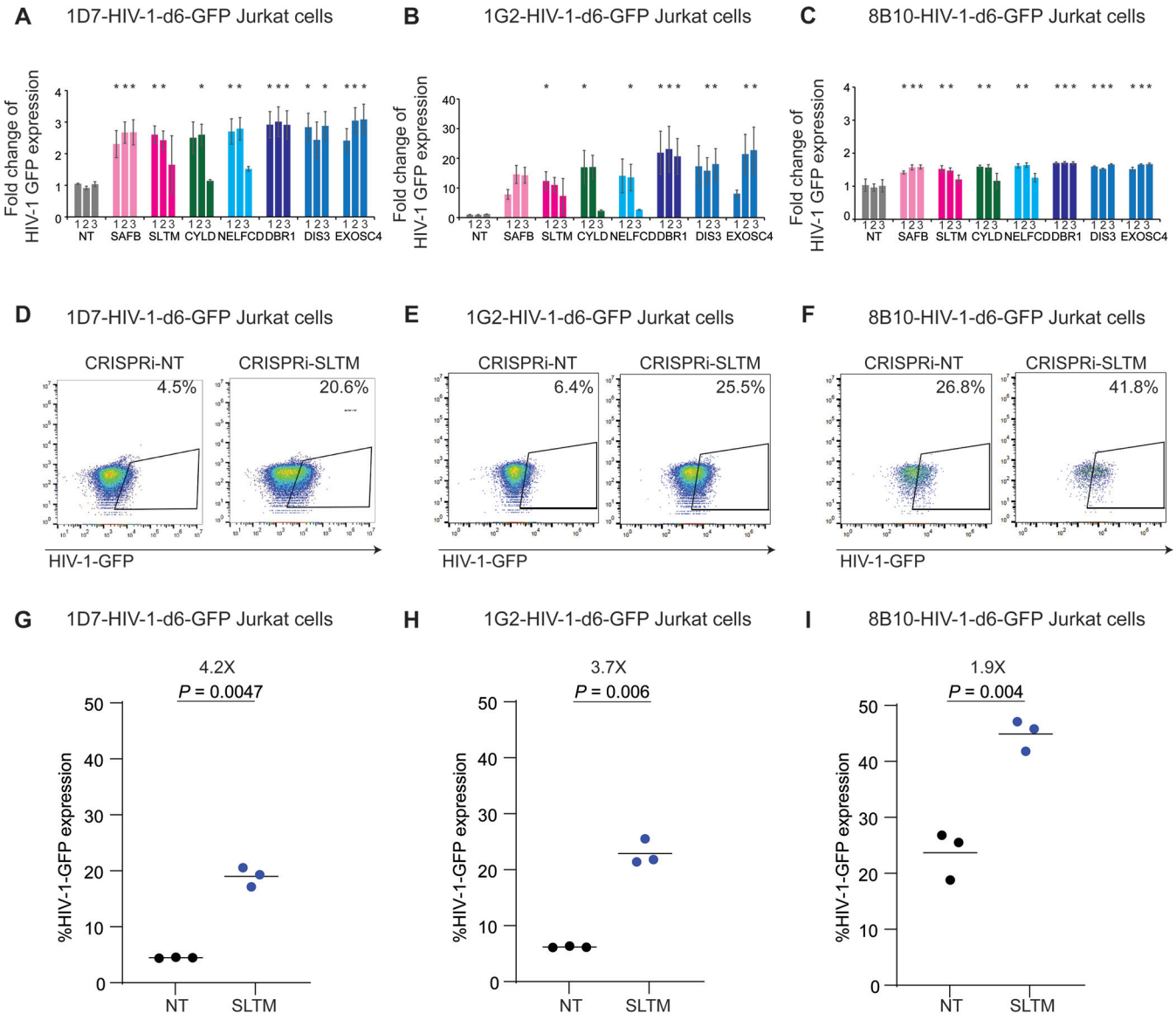

FIG 2 Knocking down HIV-1 silencing factors increases HIV-1 expression. (A to C) Fold changes in HIV-1-driven GFP expression in three different CRISPRi-ready HIV-1-d6-GFP-Jurkat T cell clones transduced with individual sgRNAs. (D to F) Representative flow cytometry plots of nontargeting (NT) and SLTM-targeting sgRNA-transduced, CRISPRi-ready, HIV-1-d6-GFP-Jurkat T cell clones showing GFP expression. (G to I) Fold increases in HIV-1-d6-GFP expression in three Jurkat T cell clones under NT and SLTM-targeting sgRNA-transduced conditions. Bar graphs show means ± standard errors of the means (SEM). *P* values were calculated by two-tailed Student's *t* test using data from two independent experiments. *, $P < 0.05$.

and sgRNA lentiviruses using blasticidin and puromycin selection. We used RNA quantitative real-time PCR (qRT-PCR) as a surrogate to measure SLTM expression, as opposed to Western blot protein expression analysis, because none of the commercially available Western blot antibodies labeled SLTM in our experiments. We found that SLTM CRISPRi knockdown led to the downregulation of SLTM RNA expression (Fig. S1I to K) and increased HIV-1-driven GFP expression (Fig. 2D to I). On average, SLTM knockdown significantly increased HIV-1-driven GFP expression by 4.2-fold (in the 1D7-HIV-1-d6-GFP-Jurkat T cell clone), 3.7-fold (in the 1G2-HIV-1-d6-GFP-Jurkat T cell clone), and 1.9-fold (in the 8B10-HIV-1-d6-GFP-Jurkat T cell clone).

SLTM suppression increases HIV-1 chromatin accessibility. To examine whether SLTM suppresses HIV-1 expression not only at the transcriptional level but also at the epigenetic level, we examined chromatin accessibility at the HIV-1 provirus and HIV-1 integration site (Fig. 3). Briefly, the 1D7-HIV-1-d6-GFP-Jurkat T cell clones were transduced with CRISPRi lentiviruses and then NT sgRNA or SLTM sgRNA and enriched by

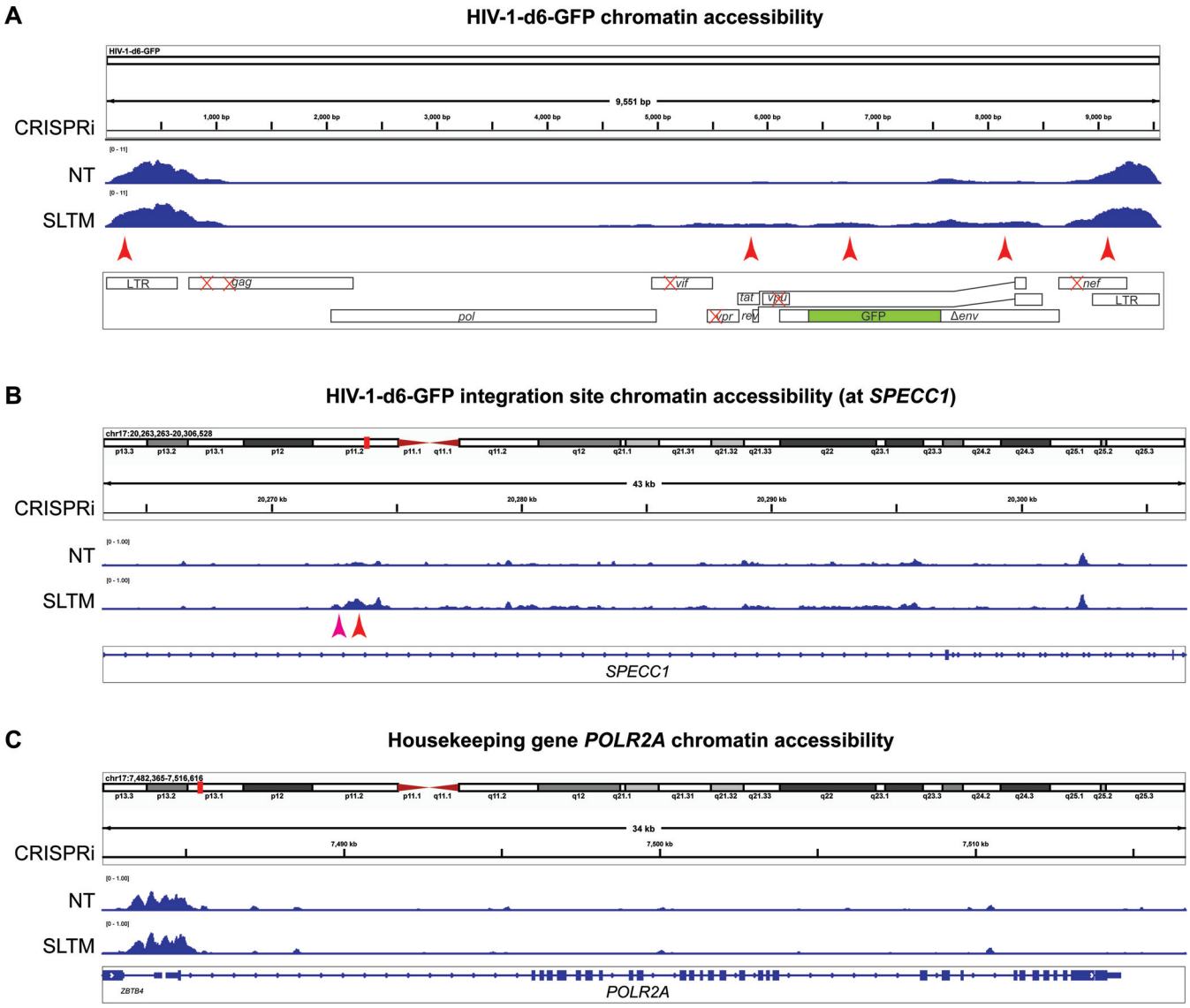

FIG 3 Knocking down SLTM increases chromatin accessibility of HIV-1 provirus. The 1D7-HIV-1-d6-GFP cell line clone was transduced with CRISPRi lentiviruses and then NT sgRNA or SLTM sgRNA and enriched by antibiotic selection. In this cell line clone, HIV-1-d6-GFP integrates into the host gene *SPECC1*. Chromatin accessibility was measured by ATAC-seq. (A) Compared with the nontargeting control, CRISPRi-mediated SLTM knockdown increases chromatin accessibility in HIV-1-d6-GFP, mainly at the 3′ genome and the U3 region of the HIV-1 LTR (red arrowheads). (B) CRISPRi-mediated SLTM knockdown increases chromatin accessibility of *SPECC1* (red arrowhead) at the HIV-1 integration site (magenta arrowhead). (C) CRISPRi-mediated SLTM knockdown does not affect chromatin accessibility in genes outside the HIV-1 integration site, such as the housekeeping gene *POLR2A*.

blasticidin and puromycin selection. In this cell line clone, HIV-1-d6-GFP integrates into the host gene *SPECC1*. Chromatin accessibility was measured by an assay for transposase-accessible chromatin via high-throughput sequencing (ATAC-seq). We found that SLTM knockdown increased HIV-1 genome accessibility at the U3 region of the HIV-1 LTR promoter and 3′ genome (Fig. 3A, red arrowheads) and the HIV-1 integration site within the host gene *SPECC1* (Fig. 3B, magenta arrowhead). The chromatin accessibility of the housekeeping gene *POLR2A* was not affected (Fig. 3C). Additionally, we observed a minimal impact on chromatin accessibility at the megabase and kilobase scales, suggesting a reasonable specificity for HIV-1 (Fig. S2). Overall, our findings indicate that SLTM knockdown not only increases HIV-1 expression at the transcriptional level but also increases HIV-1 chromatin accessibility.

SLTM knockdown reduces the frequency of HIV-1-infected cells *ex vivo*. The HIV-1-d6-GFP-Jurkat T cell clones harbor a defective HIV-1 proviral reporter, including inactivating mutations in Gag, Pol, Vif, Vpr, Vpu, Env, and Nef. Thus, HIV-1 reactivation in these

cell line clones does not induce cell death of the infected cells through viral cytopathic effects (39). In contrast, HIV-1-infected cells harboring intact and inducible HIV-1 proviruses from infected individuals may die of viral cytopathic effects upon HIV-1 reactivation due to the expression of Vif and Vpr (61, 62). We wanted to examine if HIV-1 reactivation induced by SLTM inhibition is sufficient to induce the death of HIV-1-infected cells harboring inducible HIV-1 proviruses using $CD4^+$ T cells from HIV-1-infected individuals.

Knocking down cellular factors in $CD4^+$ T cells from HIV-1-infected individuals has been extremely challenging: while CRISPR-Cas9-ribonucleoprotein-gRNA delivery into primary $CD4^+$ T cells has been previously reported, the efficiency is typically too low to reach the rare HIV-1-infected cells. CRISPR-Cas9-gRNA-lentiviral delivery, as we used in our cell line models, requires T cell activation to overcome host restriction and to achieve effective lentiviral transduction. Other siRNA-based knockdowns of cellular factors in clinical samples also require T cell activation for 3 days (31). This additional T cell activation step reactivates HIV-1 and no longer allows us to examine HIV-1 reactivation by SLTM alone. Therefore, we established an siRNA knockdown system to examine the impact of SLTM knockdown *ex vivo* without T cell activation (Fig. 4A). An NT siRNA was used as a control. We transfected $CD4^+$ T cells from 10 HIV-1-infected individuals (Table S2) with SLTM siRNA versus NT siRNA. Cells were cultured for 3 days to allow SLTM downregulation, HIV-1 reactivation, and cell death to occur. The cell culture was supplemented with enfuvirtide to block new rounds of infection. Three days after transfection, cells were plated at a limiting dilution and stimulated with phorbol myristate acetate (PMA)-ionomycin for 4 h to induce maximum HIV-1 reactivation and measure the frequency of remaining cells harboring inducible HIV-1 RNA. Of note, this method did not measure HIV-1 RNA expression levels in individual cells because there was no more than one HIV-1 RNA-positive (RNA^+) cell in $>$100,000 cells in each of the limiting dilution culture wells. Instead, similar to the measurement of the HIV-1 latent reservoir through limiting dilution culture, this method measured the frequency of HIV-1 RNA^+ cells after the duration of the culture and not the level of HIV-1 expression itself.

We next examined the efficiency of SLTM knockdown by measuring cell-associated SLTM RNA levels by qRT-PCR (Fig. 4B). We found that SLTM siRNA-transfected cells had an average of 62.0% SLTM expression compared with NT siRNA-transfected cells, indicating an SLTM knockdown of half of a log (Fig. 4C). We found no difference between the viability of cells transfected with an NT siRNA and that of cells transfected with an SLTM-targeting siRNA (Fig. 4D). Finally, we examined the frequency of HIV-1-infected cells harboring inducible HIV-1 by qRT-PCR of polyadenylated HIV-1 RNA (Fig. 4E). The frequency of cells harboring inducible HIV-1 was calculated by the infectious units per million cells (IUPM) calculator. We found that SLTM knockdown reduced the frequency of cells harboring inducible HIV-1 ($0.5577/10^6$ $CD4^+$ T cells in SLTM siRNA knockdown versus $1.4762/10^6$ $CD4^+$ T cells in NT siRNA knockdown [$P = 0.029$ by paired two-tailed Student's *t* test]). Overall, our results indicated that SLTM knockdown increased HIV-1 expression *in vitro* and reduced the frequency of cells harboring inducible HIV-1 *ex vivo*.

DISCUSSION

Using a genome-wide CRISPRi screen, we identified 18 HIV-1 silencing factors involving 4 cellular pathways that are key to HIV-1 expression, including all three members of the SAFB family of proteins (SLTM, SAFB, and SAFB2), RNA splicing (SRRM2 and DBR1), RNA nuclear export [5′ binding protein NCBP2 and poly(A) binding protein ZC3H3], and RNA decay (including 5 out of 10 RNA exosome proteins, DIS3 and EXOSC proteins 2, 4, 5, 8, and 9). We also identified cellular factors known to suppress HIV-1 expression in the screen, such as the NF-κB deubiquitinase CYLD (49) (also identified in previous CRISPR screens [25, 26]) and the transcriptional elongation inhibitor NELFCD (50), demonstrating the validity of the screen. We confirmed our results in 3 cell line clones having distinct HIV-1 integration sites, showing that SLTM increases

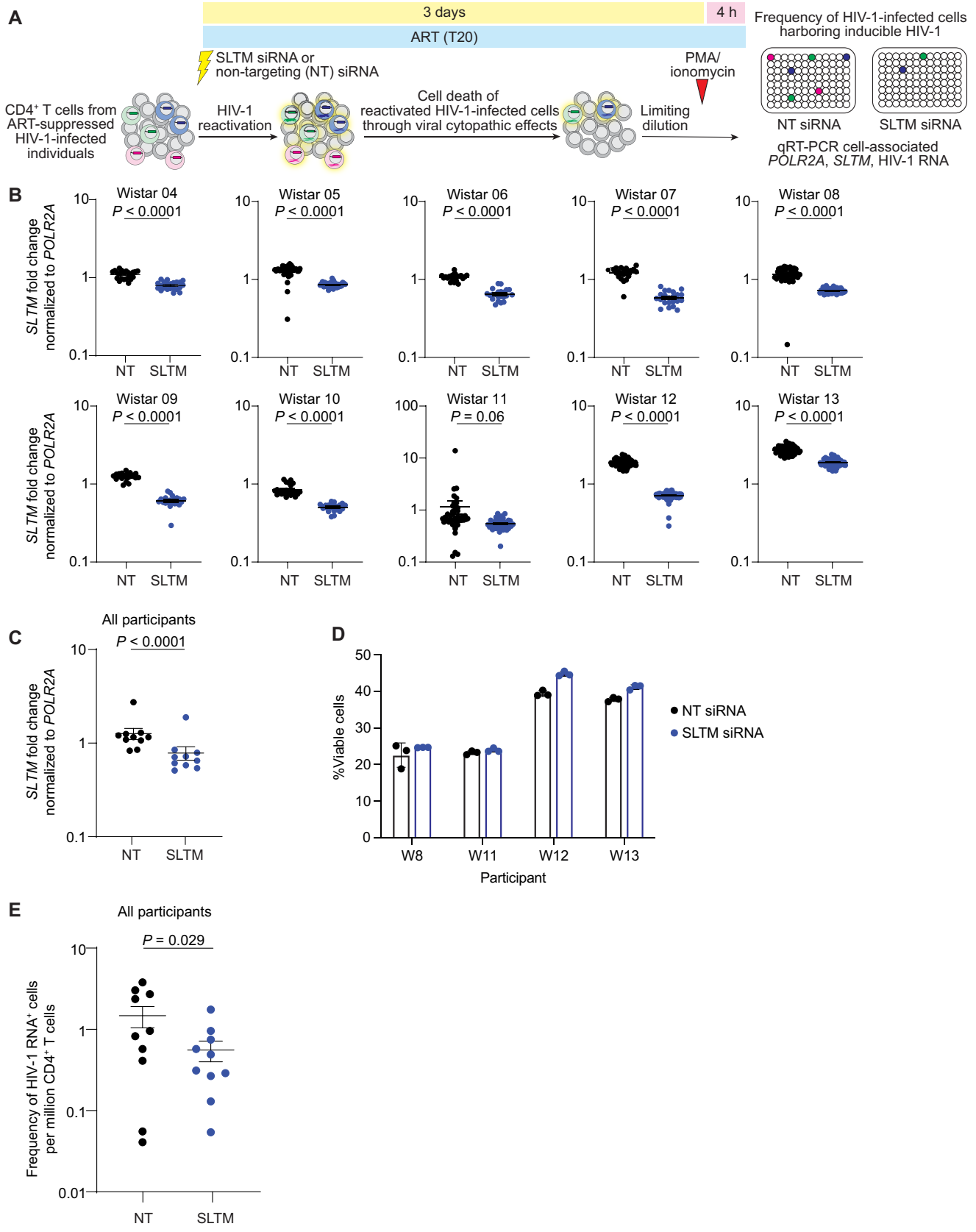

FIG 4 SLTM knockdown reduces the frequency of inducible HIV-1-infected cells *ex vivo*. (A) Experimental scheme. CD4$^+$ T cells from ART-treated, virally suppressed, HIV-1-infected individuals were transfected with NT versus SLTM siRNA. Cells were cultured for 3 days to allow SLTM knockdown, HIV-1

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HIV-1 expression without affecting housekeeping gene expression or cellular viability. Finally, we knocked down SLTM in primary CD4$^+$ T cells from ART-suppressed HIV-1-infected individuals and found that SLTM suppression reduces the frequency of HIV-1-infected cells harboring inducible HIV-1, likely through viral cytopathic effects and the death of cells having reactivated HIV-1.

We identified a chromatin and transcriptional regulator, SLTM, as a novel HIV-1 silencing factor. SLTM, SAFB, and SAFB2 are the three SAFB family proteins, which have a dual binding capacity for DNA and RNA. These proteins contain both a SAP box DNA binding motif and an RNA binding domain (RBD). SAFB family proteins bind to scaffold/matrix attachment regions of genomic DNA and regulate chromatin structural organization and gene expression regulation (63–65). SLTM, a nuclear protein, is known to dimerize with SAFB and inhibit estrogen receptor signaling (63). The overexpression of SLTM suppresses general cellular RNA synthesis and induces apoptosis (63). While SLTM shares 34% identity with SAFB, the biological impact may be distinct and has yet to be understood. For example, in a previous genome-wide CRISPR screen attempting to identify retrotransposon L1 regulators, SAFB was identified as one of the 13 L1 repressors (along with the histone methyltransferase SETDB1 and the HUSH complex proteins TASOR, MORC2, and MPP8), while SLTM was confirmed to be an L1 activator (22). Mechanisms regarding how SLTM silences HIV-1 have not yet been investigated. Given that HIV-1 silencing mechanisms are distinct from HUSH complex-mediated silencing of other retroviruses (19, 20) and that SLTM has a known role in epigenetic regulation and L1 retrovirus regulation (22), SLTM serves as a novel HIV-1 silencing factor that deserves further mechanistic investigation.

We identified RNA processing pathways, including RNA splicing (SRRM2), debranching (DBR1), nuclear export (NCBP2 and ZC3H3), RNA decay (DIS3, EXOSC2, EXOSC4, EXOSC5, EXOSC8, and EXOSC9), nuclear exosome targeting (ZCCHC8 and RBM7), and the poly(A) tail exosome complex (ZFC3H1) (reviewed in reference 56). A recent study suggests that the HUSH complex protein MPP8 interacts directly with the NEXT complex protein ZCCHC8 and indirectly with the NEXT protein RBM7 and the PAXT protein ZFC3H1 to ensure the complete silencing of retrotransposons (57). Given that our HIV-1-d6-GFP-infected Jurkat T cell models produce spliced HIV-1 RNA, unspliced HIV-1 RNA, and HIV-1-to-host chimeric RNA (37) and that knocking down these factors increases HIV-1 expression, it is likely that these RNA splicing and decay pathways serve as sensors that detect and degrade spliced, unspliced, or HIV-1-to-host chimeric RNA as innate immune defense mechanisms. Understanding whether targeting these RNA processing factors can overcome transcriptional blocks and increase HIV-1 reactivation (16) may help to more effectively target HIV-1-infected cells.

We developed an siRNA knockdown method to examine whether SLTM downregulation affects HIV-1-infected cells *ex vivo*, using CD4$^+$ T cells from ART-suppressed individuals. Previous siRNA knockdown studies (31) attempting to knock down candidate cellular factors *ex vivo* have involved phytohemagglutinin (PHA)-mediated activation of cells transfected with the target siRNA. We established a short (3-day) system that allows us to examine the impact of siRNA knockdown of candidate cellular factors on HIV-1 reactivation-induced cell death, as measured by the frequency of inducible HIV-1-infected cells by limiting dilution culture. This method will help to test the impact of other candidate cellular factors on the HIV-1 reservoir *ex vivo*.

The major limitation of this study is the lack of direct examination of physical interactions between HIV-1 silencing factors and HIV-1 genome and other cellular factors.

FIG 4 Legend (Continued)
reactivation, and viral cytopathic killing of the reactivated HIV-1-infected cells. Culture medium was supplemented with enfuvirtide to prevent new rounds of *in vitro* infection. After 3 days of culture, cells were plated at a limiting dilution (around 100,000 to 500,000 cells per well for 22 to 88 microculture wells per knockdown). Cells were treated with PMA-ionomycin to induce maximum HIV-1 latency reversal. (B and C) SLTM knockdown was measured by the level of *SLTM* RNA expression normalized to the level of *POLR2A* RNA expression. (D) Cell viability of siRNA-nucleofected (transfected by nucleofection) primary CD4$^+$ T cells as measured by a Live/Dead near-infrared kit, comparing NT and SLTM-targeting siRNAs. (E) Cell-associated HIV-1 RNA expression was measured by polyadenylated HIV-1 qRT-PCR. The frequency of inducible HIV-1-infected cells after NT versus SLTM knockdown was calculated by the IUPM calculator. *P* values were calculated by two-tailed Student's *t* test. Each dot represents an RNA measurement from individual microculture wells.

Since there are no monoclonal SLTM antibodies that can faithfully detect SLTM protein, protein-DNA, protein-RNA, or protein-protein interaction studies are challenging. The identification of the important cellular factors silencing retrotransposons at both the epigenetic level (by SAFB family proteins) and the transcription level (by the NEXT and PAXT proteins ZCCHC8, RBM7, and ZFC3H1) suggests that HIV-1 may be silenced in a way like retrotransposon silencing. More mechanistic studies are required to decipher interactions between epigenetic and transcriptional regulators of HIV-1 silencing.

Overall, our genome-wide CRISPR inhibition screen, combined with validation in HIV-1-infected T cell clones with HIV-1 in different integration sites, identified the epigenetic and transcriptional regulator SLTM as a novel HIV-1 silencing factor. Utilizing compounds that can inhibit or degrade SLTM may provide a means of increasing HIV-1 reactivation, viral protein expression, and viral cytopathic effect and enhancing immune recognition and clearance.

MATERIALS AND METHODS

Cell culture. The four HIV-1-d6-GFP-Jurkat T cell clones 8B10 (HIV-1 integration into *VAV1* and *NFX1*), 1G2 (HIV-1 integration into *RAP1B*), 1D7 (HIV-1 integration into *SPECC1*), and 1F6 (HIV-1 integration into *PRCC*) were generated as previously described (37, 38) and cultured in RPMI 1640 (catalog number 11875093; Thermo Fisher) supplemented with 10% heat-inactivated fetal bovine serum (catalog number 10082147; Thermo Fisher). Lentiviruses were generated by transfecting the Lenti-X 293T cell line (catalog number 632180; TaKaRa Bio) using Lipofectamine 2000 transfection reagent (catalog number 11668019; Thermo Fisher Scientific) and concentrated by using Lenti-X concentrator (catalog number 631231; TaKaRa Bio) or by ultracentrifugation using a 20% sucrose cushion, depending on the amount and concentration of lentiviruses needed.

Generation of CRISPRi-ready HIV-1-d6-GFP-Jurkat T cell clones. HIV-1-d6-GFP-Jurkat T cell clones were transduced with Lenti-dCas9-Krab-blast (catalog number 89567; Addgene) (46) and selected by blasticidin resistance. During blasticidin selection, 8 μg/mL of blasticidin (catalog number A1113903; Thermo Fisher Scientific) was used to supplement the culture medium for 7 days.

CRISPRi library amplification and lentiviral production. The human CRISPR inhibition pooled library hCRISPRi-v2 (catalog number 83969; Addgene) was amplified according to the recommended protocol (47). Briefly, lentiviral plasmids were electroporated into MegaX DH10B T1R electrocompetent cells (catalog number C6400-03; Thermo Fisher), expanded to a 150-mL culture, and isolated by maxiprep. The plasmid library was sequenced to ensure sufficient representation of sgRNAs in the library, with $>$70% perfectly matched guides, $<$0.5% undetected guides, and a skew ratio of $<$10 (66). Next, lentiviral particles of the library were produced by cotransfecting Lenti-X 293T cells with the hCRISPRi-v2 plasmid library, the packaging plasmid MDL g/pRRE (catalog number 12251; Addgene), an RSV-Rev plasmid (catalog number 12253; Addgene), and a vesicular stomatitis virus glycoprotein (VSV-G)-expressing envelope plasmid (catalog number 14888; Addgene) with Lipofectamine in Opti-MEM medium. The supernatant was harvested after 48 h, filtered through a 0.22-μm-pore-size filter (catalog number SCGP00525; Millipore), bottom layered with a 20% sucrose cushion, and concentrated by ultracentrifugation at 25,000 rpm at 4°C for 2 h. The lentiviral library was stored as aliquots at −80°C.

Pooled genome-wide CRISPRi screen. For each of the CRISPRi-ready HIV-1-d6-GFP-Jurkat T cell clones, 250 million cells were transduced with the hCRISPRi-v2 lentivirus library at a multiplicity of infection of 0.2. After 5 days of puromycin selection (1.5 μg/mL) (catalog number A1113803; Thermo Fisher), dead cells were removed using the EasySep dead cell removal (annexin V) kit (catalog number 17899; Stemcell). The cells were split into two aliquots. One aliquot expresses high levels of HIV-1-d6-GFP, defined as GFP expression levels higher than those of cells transduced with nontargeting sgRNA lentivirus by flow cytometry. The other unsorted aliquot of cells served as the control for comparison. For cells from the sorted HIV-1-d6-GFP^{high} cells and the unsorted control cells, the sgRNA was amplified by flanking primers and prepared for next-generation sequencing. The resulting reads were quantified against the known library guide RNA sequences, and any guides with fewer than 20 reads in at least one replicate were removed from downstream analyses. The enrichment of each sgRNA was calculated by comparing the relative abundances in the sorted and unsorted populations using CRISPRanalyzeR, version 1.50 (67), an R CRISPR analysis pipeline, and *P* values from the MAGeCK algorithm (48) are presented in all figures against DESeq2 (68)-normalized counts.

CRISPRi knockdown of individual HIV-1 silencing factors in cell line models. For candidate HIV-1 silencing factors, we used two sgRNAs per gene within the parent library and one sgRNA independent from the sgRNA library (see Table S1 in the supplemental material). Three nontargeting sgRNAs were used as controls. Individual sgRNAs were cloned into the parental plasmid pCRISPRia-v2 (catalog number 84832; Addgene) and confirmed by sequencing. Individual CRISPRi-ready HIV-1-d6-GFP-Jurkat T cell clones were transduced with lentiviruses carrying individual sgRNAs. Three days after infection, HIV-1-d6-GFP expression in cells transduced with the candidate sgRNA (as shown by positive BFP expression) was measured by flow cytometry. Cellular viability was measured by viability staining (Live/Dead fixable near-infrared dead cell stain kit, catalog number L10119; Thermo Fisher). To enrich for a pure population of CRISPRi-ready, individual-sgRNA-transduced cells, cells were cultured in the presence of blasticidin

(8 μg/mL) and puromycin (1.5 μg/mL) for 7 days. Dead cells were removed by Ficoll density gradient centrifugation (catalog number 95038-168; Cytiva).

ATAC-seq. ATAC-seq was performed using a previously described protocol (69), with modifications as described previously by Corces et al. (70). CRISPRi-ready 1D7-HIV-1-d6-GFP-Jurkat T cell clones transduced with SLTM sgRNA or nontargeting sgRNA were enriched by antibiotic selection and cultured at a low density to ensure cell viability. A total of 50,000 cells were pelleted, washed with 1$\times$ ice-cold phosphate-buffered saline (PBS), and pelleted again. Cells were lysed in a buffer containing 0.1% NP-40, 0.1% Tween 20, and 0.01% digitonin on ice for 5 min. Nuclei were washed with 1 mL of buffer, pelleted, and treated with Tn*5* transposase. Nuclei were incubated at 37°C for 30 min in an Eppendorf Thermomixer with mixing at 1,000 rpm. DNA was then isolated using a Qiagen MinElute PCR cleanup kit (catalog number 28004). The resulting DNA was amplified using NEBNext Ultra II Q5 master mix (catalog number M0544; New England BioLabs), with barcoding oligonucleotides. Libraries were sequenced to a depth of 25 million 150-bp read pairs on a NovaSeq 6000 platform. The resulting reads were trimmed to remove Nextera adapter sequences using cutadapt (v3.5) (71). Trimmed reads were mapped to hg38 using Bowtie2 (version 2.4.5) (72). Chromatin accessibility in each sample was normalized by the counts per million (CPM).

Clinical sample processing. Study participants were recruited at the Wistar Institute and processed at Yale University. Both the Yale University and Wistar Institutional Review Boards approved this study. All participants provided written consent. All HIV-1-infected individuals enrolled were on suppressive ART and maintained undetectable plasma HIV-1 RNA levels ($<$50 copies/mL) for at least 6 months prior to enrollment. Characteristics of HIV-1-infected individuals are listed in Table S2. CD4$^+$ T cells from ART-treated, virally suppressed, HIV-1-infected individuals were isolated using magnetic negative depletion (EasySep human CD4$^+$ T cell enrichment kit, catalog number 19052; Stemcell).

Measurement of RNA expression using qRT-PCR. Cell-associated HIV-1 RNA was stored in TRIzol (catalog number 15596018; Thermo Fisher) and extracted with Direct-zol-96 RNA (catalog number R2054; Zymo Research). RNA expression was measured by qRT-PCR using qScript XLT 1-step RT-qPCR ToughMix low ROX (catalog number 95134-02K; Quanta Bio) on a QuantStudio 3 real-time PCR system (Thermo Fisher) and primer and probe sets specific for polyadenylated HIV-1 RNA (73), the housekeeping gene *POLR2A* (Thermo Fisher TaqMan gene expression assays, Hs00172187_m1), and the HIV-1 silencing factor *SLTM* (Thermo Fisher TaqMan gene expression assays, Hs00932220_m1).

Measurement of the frequency of HIV-1-infected cells harboring inducible HIV-1 RNA after siRNA transfection. To knock down candidate HIV-1 silencing factors in primary CD4$^+$ T cells from ART-treated, virally suppressed, HIV-1-infected individuals, aliquots of 2 million to 5 million CD4$^+$ T cells were transfected with 1.5 μg RNA ($\sim$200 pmol) in 100 μL nucleofection reagent from the human T cell Nucleofector kit (catalog number VPA-1002; Lonza) by the Amaxa Nucleofector II (Lonza) U-014 high-viability program. Predesigned Silencer Select siRNA was obtained from Thermo Fisher (catalog numbers s36384, s36385, and s36386 for SLTM and 4390843 for nontargeting siRNA). Fresh culture medium was added 4 h after transfection. Enfuvirtide (10 μM) was used to supplement the culture medium to prevent new rounds of *in vitro* infection. Cells were cultured for 3 days to allow cellular factor knockdown, HIV-1 reactivation, and HIV-1-induced cell death to occur. By the end of the 3-day culture, cells were plated at a limiting dilution at $\sim$100,000 to 500,000 cells per well in 96-well plates, with 22 to 88 wells per participant. Next, cells were treated with PMA (50 ng/mL) and ionomycin (1 μM) for 4 h to induce maximum HIV-1 reactivation. Cell-associated RNAs in each microculture well in the 96-well plates were extracted for qRT-PCR. The frequency of HIV-1-infected cells harboring inducible HIV-1 was calculated using the IUPMStats v1.0 infection frequency calculator (74).

Statistical analysis. Two-tailed Student's *t* tests were used to compare differences between matched samples using Prism v9.3.1 (GraphPad).

Data availability. ATAC-seq reads have been deposited in the GEO under accession number GSE203235.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.3 MB.

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S.F.P., J.A.C., and Y.-C.H. designed the CRISPR screen. S.F.P. and J.A.C. performed CRISPR screen hit identification using bioinformatic methods. S.F.P. and R.N.K. performed hit validation in cell line models and clinical samples, with help from K.Y. and Y.-H.J.Y. J.A.C., A.R., and A.A.C. performed ATAC-seq. J.A.C. performed ATAC-seq analysis. L.J.M., P.T., and K.M. recruited study participants. Y.-C.H. and J.A.C. wrote the manuscript.

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