A functional screen identifies transcriptional networks that regulate HIV-1 and HIV-2

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The molecular networks involved in the regulation of HIV replication, transcription, and latency remain incompletely defined. To expand our understanding of these networks, we performed an unbiased high-throughput yeast one-hybrid screen, which identified 42 human transcription factors and 85 total protein–DNA interactions with HIV-1 and HIV-2 long terminal repeats. We investigated a subset of these transcription factors for transcriptional activity in cell-based models of infection. KLF2 and KLF3 repressed HIV-1 and HIV-2 transcription in CD4+ T cells, whereas PLAGL1 activated transcription of HIV-2 through direct protein–DNA interactions. Using computational modeling with interacting proteins, we leveraged the results from our screen to identify putative pathways that define intrinsic transcriptional networks. Overall, we used a high-throughput functional screen, computational modeling, and biochemical assays to identify and confirm several candidate transcription factors and biochemical processes that influence HIV-1 and HIV-2 transcription and latency.

HIV transcription | yeast one-hybrid | HIV latency

A major checkpoint for HIV replication and latency is proviral transcription. Understanding how proviral transcription is regulated is critical for developing eradication strategies. Proviral transcription is regulated by a combinatorial balance of transcription factors and coregulatory complexes coupled with the viral encoded factor Tat, which recruits the activation complex P-TEFb to the long terminal repeat (LTR) (1). The LTR includes promoter and enhancer elements with binding sites for numerous cellular transcription factors, such as NF-κB, NFAT, Sp1, and AP-1 (2, 3). HIV replicates efficiently in activated CD4+ T cells in which positive transcriptional regulators are not limiting, RNAP II is processive, and chromatin organization is favorable for transcription (4–6). If HIV-infected activated CD4+ T cells transition to a long-lived resting memory state, proviral gene expression can be repressed due to the absence of positive transcriptional regulators and the establishment of repressive chromatin modifications (7–10). These infected HIV-1+ memory cells persist, forming a latently infected reservoir that is resistant to antiretroviral therapies. A cure for HIV-1 infection will either require eliminating these latently infected cells or preventing reactivation of latent provirus, underscoring the need to fully understand events that control HIV proviral transcription.

Our current understanding of HIV transcription reflects classic molecular biology approaches used to define transcriptional elements and DNA binding proteins (5, 11–15). These approaches typically do not explore the full repertoire of transcription factors in a functional manner and rely on predicting potential DNA binding sites and/or available antibodies for chromatin immunoprecipitation (ChIP)-based screens. Furthermore, studies tend to focus on dominant actors in transcriptional regulation, robust transcriptional activators or repressors, minimizing factors that fine tune transcription or act cooperatively in larger transcriptional regulatory networks. There have been several recent examples of high-throughput screens that utilize compounds, RNA interference, single-cell RNA sequencing, and CRISPR technologies to discover pathways and factors that intersect with HIV transcription, including stress responses, estrogen receptor, the proteosome and cell metabolism (a few examples include refs. 16–23). Although these approaches have provided insights into HIV-1 replication and latency, they do not directly assess transcription factor binding and function.

To gain insights into networks and intrinsic cellular factors that control HIV transcription, we used high-throughput yeast one-hybrid assays to identify transcriptional networks that regulate HIV-1 and HIV-2. Transcription factors identified that repress HIV-1 and HIV-2 and contribute to proviral transcriptional repression in CD4+ T cells are KLF2 and KLF3, whereas a third factor, PLAGL1, preferentially activates HIV-2 transcription. Computational modeling based on documented interactions implicate upstream pathways associated with T-cell activation, proliferation, and survival. These studies expand our knowledge of the intrinsic events that differentially regulate HIV-1 and HIV-2.

Results

Yeast One-Hybrid Screen Identifies Human Transcription Factor Interactions with HIV-1 and HIV-2 LTRs. To gain insights into intrinsic transcription factor networks that mediate HIV transcription, we used an unbiased functional enhanced yeast one-hybrid (eY1H) screen, consisting of a transcription factor "prey" library and LTR "baits" (24). The transcription factor array consists of 1,086 different yeast strains expressing human transcription factors fused to the yeast Gal4 activation domain. This

Significance

Understanding mechanisms that control HIV expression will provide insight into HIV replication, latency, and pathogenesis. In particular, HIV latency, which is a major barrier to a cure, is maintained by combinatorial mechanisms that regulate transcription. A functional screen was employed to explore transcriptional networks that regulate HIV. These studies identified novel transcription factors that influence HIV transcription and provided an appreciation into intrinsic networks that influence transcriptional activation and repression of different HIV strains.

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library represents ~66% of the known repertoire of human transcription factors (24, 25). LTR bait sequences included 1 kb of sequence spanning the full proviral LTR, extending ~500 bp beyond the transcriptional start site. LTRs were cloned upstream of two reporter genes, *LacZ* and *HIS3*, and integrated into the *Saccharomyces cerevisiae* (*S. cerevisiae*) genome to generate chromatinized DNA-bait strains. Matings between prey and bait yeast strains were performed in quadruplicate, and positive hits were identified as blue X-Gal–positive colonies that grew on histidine-deficient plates. Greater than 90% of interactions detected were positive in all four colonies as previously described (24–26).

We identified binding of 42 transcription factors to three HIV-1 clade B LTRs (NL4-3, REJO, and CH058) and two HIV-2 group A LTRs (ROD9 and GH1), totaling 85 interactions (Fig. 1*A*). Transcription factors identified included several reported to influence HIV transcription, such as Sp-related factors, Ets-related factors, and interferon responsive factors (IRFs) (27–29). In addition, multiple C2H2 zinc finger–containing proteins were identified. A subset of factors, including KLF2, KLF3, KLF4, and Sp4, interacted with both HIV-1 and HIV-2 LTRs, while others interacted primarily with LTRs either from HIV-1 (GABPA, KLF15, IRF2, ZNF542, and TGIF2LX) or HIV-2 (HHEX, THRB, SOX14, ZDHHC7, HEY1, HEY2, HES5, and PLAGL1), suggesting differential binding. As a control, we screened 1,500 bp from HIV-1_{NL4-3} *gag-pol*, which did not detect any transcription factor binding, indicating the specificity of the screen for transcriptional elements.

Transcription factors identified are mostly expressed in CD4+ T cells, monocytes, macrophages, and/or dendritic cells based on expression profiles from Blueprint Epigenomics (*SI Appendix*, Table S1). We confirmed the expression of factors in uninfected CD4+ T cells by microarray analysis of unstimulated CD4+ T cells and CD3 + CD28–activated CD4+ T cells from three human donors (30). All transcription factors display some degree of differential expression in CD4+ T cells. In particular, KLF3, ZIC1, KLF2, Sp4, KLF12, and IRF2 are expressed in unstimulated CD4+ T cells relative to activated cells, while KLF4, PLAGL1, and E2F1 show increased RNA expression following CD3 + CD28 stimulation compared to unstimulated CD4+ T cells (Fig. 1*B*), suggesting that these factors are regulated by T-cell activation. Nevertheless, this does not preclude that these transcription factors have activities in unstimulated CD4+ T cells, as previously described (31).

HIV LTR Transcription Factor Networks Intersect with Key Cellular Processes. Transcription factors act downstream of cellular signaling cascades and integrate multiple biological processes, including cell division, cell stress, and DNA repair. We hypothesized that the array of transcription factors binding LTRs provide a footprint of the intrinsic cellular environment that influences HIV transcription. We performed pathway enrichment analysis for transcription factors identified to bind HIV-1 and HIV-2 LTRs by eY1H assays and combined these results with LTR interacting transcription factors reported in the literature. Given that this is a small set of genes to perform enrichment analysis, we included interactors of these transcription factors (32) that are expressed in CD4+ T cells (Blueprint Epigenome) (Fig. 2*A*). We used PANTHER (33) for pathway analysis and identified 29 enriched pathways, most of which were related to CD4+ T-cell functions, including Toll-like receptor signaling, Wnt, TGF-β, T-cell activation, and p53 (Fig. 2*B*). Pathways, such as p53, Toll-like receptor signaling, and Jak/STAT signaling, have been linked to HIV replication and persistence, providing proof-of-concept that this approach predicts events upstream of HIV transcription (34–36).

KLF2 and KLF3 Repress HIV-1 Transcription. We were interested in validating that transcription factors that were identified in the eY1H screen regulated HIV transcription. Initial experiments

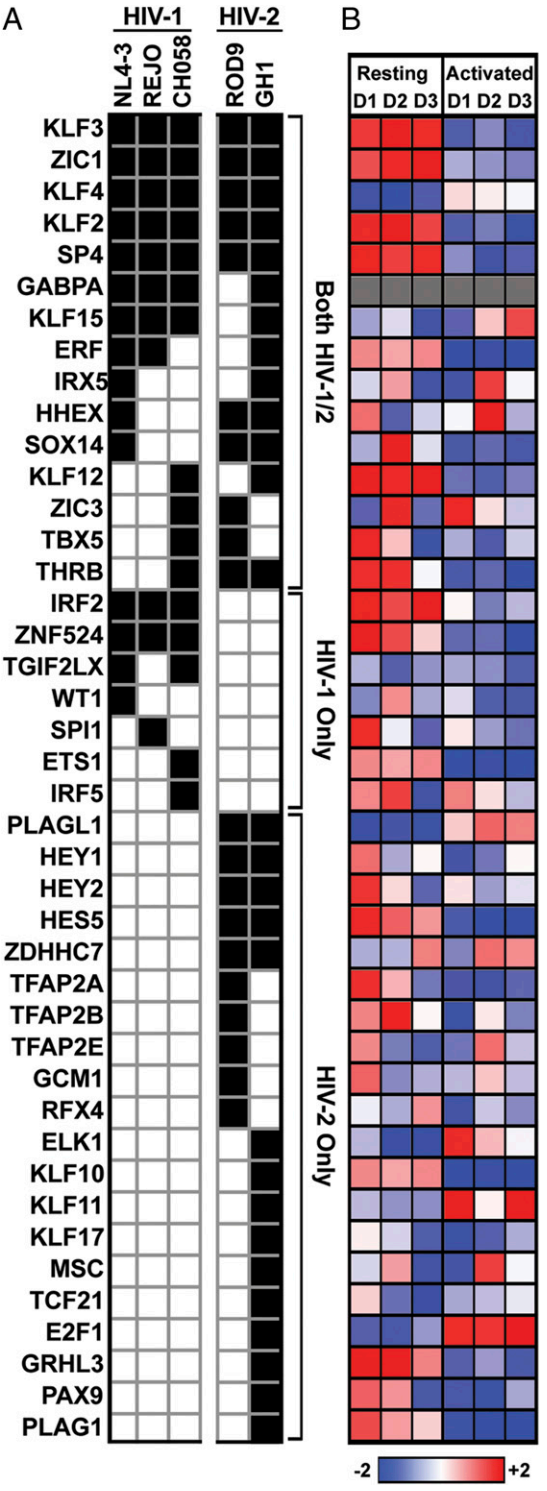

Fig. 1. Enhanced Y1H screen reveals human transcription factor interactions with HIV LTRs. (*A*) Results of eY1H assays. Each row corresponds to a protein prey, and each column corresponds to a DNA bait used in the assay. Positive interactions are represented as black squares. (*B*) Transcription factor expression in CD4+ T cells. Expression patterns generated from microarrays for CD4+ T cells from three human donors unstimulated and stimulated with anti-CD3/28–coated microbeads as described previously (30). The log2 (expression) values for each gene were *z*-score–normalized to a mean of 0 and SD of 1 within each donor to adjust for the donor effect. *z*-scores were then *z*-score–normalized across all samples in each row and trimmed to the range −2 to +2, with blue, white, and red indicating final *z*-scores of ≤−2, 0, and ≥2, respectively. Gray boxes indicate no data.

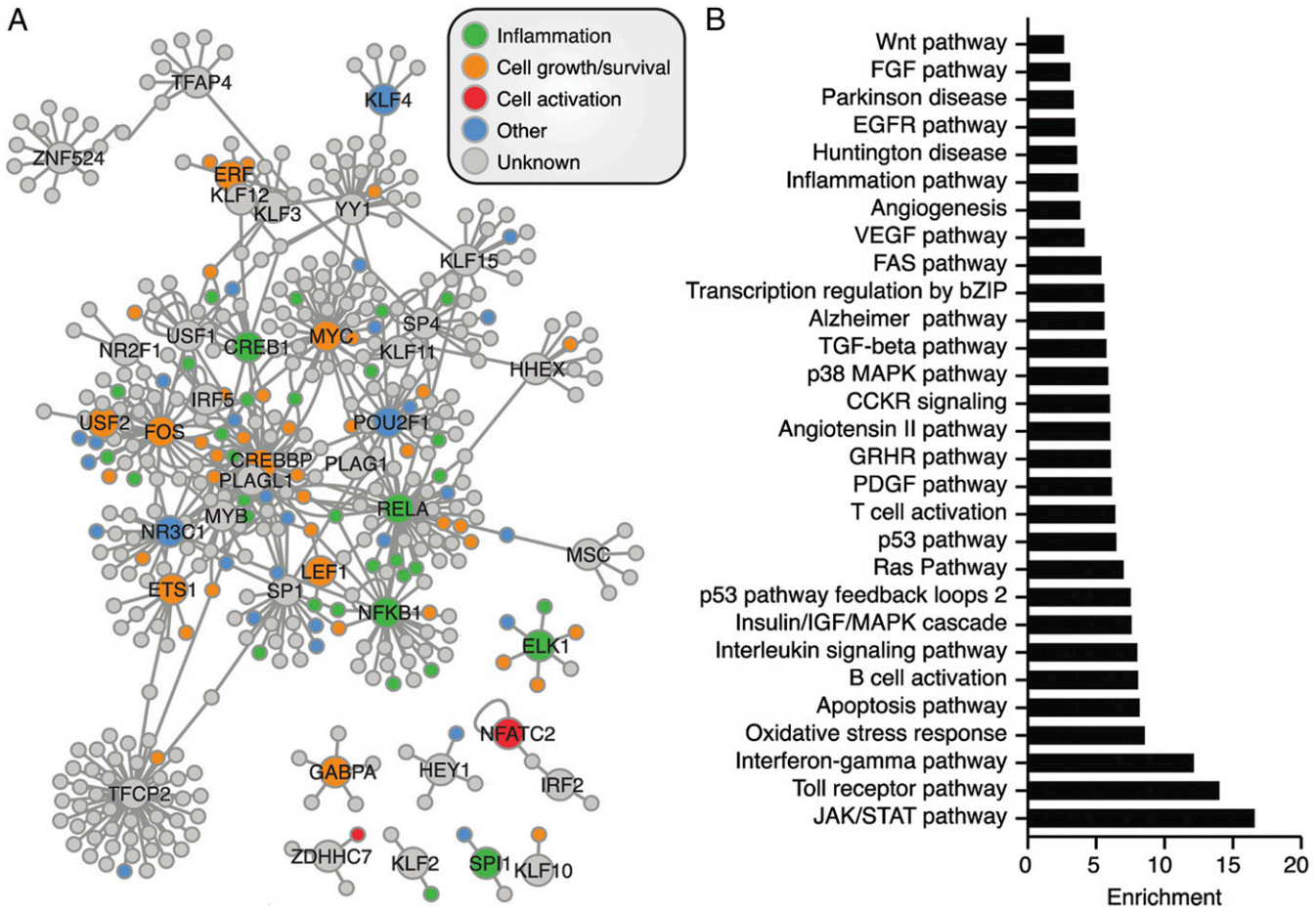

Fig. 2. Identification of transcriptional and biochemical networks. (*A*) Pathway enrichment analyses with transcription factors that interact with HIV-1 or HIV-2 LTRs by eY1H assays as well as transcription factor interactions reported in the literature. Protein–protein interactions with transcription factors reported in HuRI and the literature were also included. Transcription factors and proteins expressed in at least one CD4+ T subset with a TPM > 0 (Blueprint Epigenome) were included in the analysis. (*B*) PANTHER Pathway enrichment analysis was performed using human proteins as a background. Pathways containing at least three genes and with an FDR < 0.05 are shown.

tested a subset of factors by transient transfection with the HIV-1 and HIV-2 LTRs in human embryonic kidney 293 SV40 T-antigen (HEK293T) cells and confirmed that several of the transcription factors positively or negatively regulated LTR-mediated transcription (*SI Appendix*, Fig. S1). We focused on further characterizing KLF2 and KLF3 and their mechanisms of action in regulating HIV transcription. The rationale for focusing on these factors included the following: 1) KLF2 and KLF3 bound all five LTRs in the eY1H screen, leading us to hypothesize that they regulate HIV-1 and HIV-2 transcription; 2) KLF2 and KLF3 are Krüppel-like C2H2 zinc finger DNA-binding proteins and recognize GC-rich DNA sites which are conserved in HIV-1 and HIV-2 LTRs; and 3) KLFs have been implicated in the regulation of lymphocyte trafficking, function, differentiation, and quiescence (37). We confirmed KLF2 and KLF3 binding to HIV-1 proviral LTR following infection of unstimulated resting primary CD4+ T cells by ChIP using primers that flanked the GC-rich Sp1 binding sites within the LTR (Fig. 3 *A* and *B*). KLF2 and KLF3 LTR binding was decreased by 60 to 80% following CD3/CD28 stimulation, consistent with these factors being down-regulated in response to T-cell activation (Fig. 1*B* and *SI Appendix*, Fig. S2*A*).

KLF2 and KLF3 function as either activators or repressors of gene expression (38). Since proviruses within unstimulated CD4+ T cells are biased to be transcriptionally silent, and we observed binding of KLF2 and KLF3 in unstimulated cells, we hypothesized that these factors are transcriptional repressors. To determine the function of KLF2 and KLF3 on HIV transcription, we infected unstimulated CD4+ T cells by spinoculation as previously described (refs. 39 and 40 and *Materials and Methods*) with HIV-1_{NL4-3} and knocked-down KLF2 and KLF3 with small-interfering RNAs (siRNAs). Knockdowns of 40 to 60% of KLF2 and KLF3 were confirmed at messenger RNA (mRNA) and protein levels (Fig. 3 *C* and *D*). Approximately 5% of cells contained integrated HIV-1 provirus measured by *Alu*-PCR (*SI Appendix*, Fig. S2*B* and *Materials and Methods*). siRNA nucleofection did not affect cell viability or T-cell activation, which maintained an unstimulated CD4+ T-cell phenotype based on a lack of CD25 and CD69 expression (*SI Appendix*, Fig. S2*C*).

KLF2 or KLF3 knockdowns led to significant increases in total HIV-1 RNA expression in unstimulated resting CD4+ T cells (Fig. 3*E*). Double knockdown of KLF2 and KLF3 did not result in a significant additive induction of HIV-1 transcription, suggesting that KLF2 and KLF3 are either in the same biochemical pathway or are redundant in repressing HIV expression. We observed similar increases in HIV-2 transcription in infected unstimulated CD4+ T cells when KLF2 or KLF3 were knocked down (*SI Appendix*, Fig. S3), consistent with these factors binding and regulating HIV-2 LTRs. These data suggest that KLF2 and KLF3 directly repress HIV-1 and HIV-2 transcription.

To gain insights into whether KLF2 and KLF3 repress HIV transcription by mediating epigenetic changes on HIV LTRs,

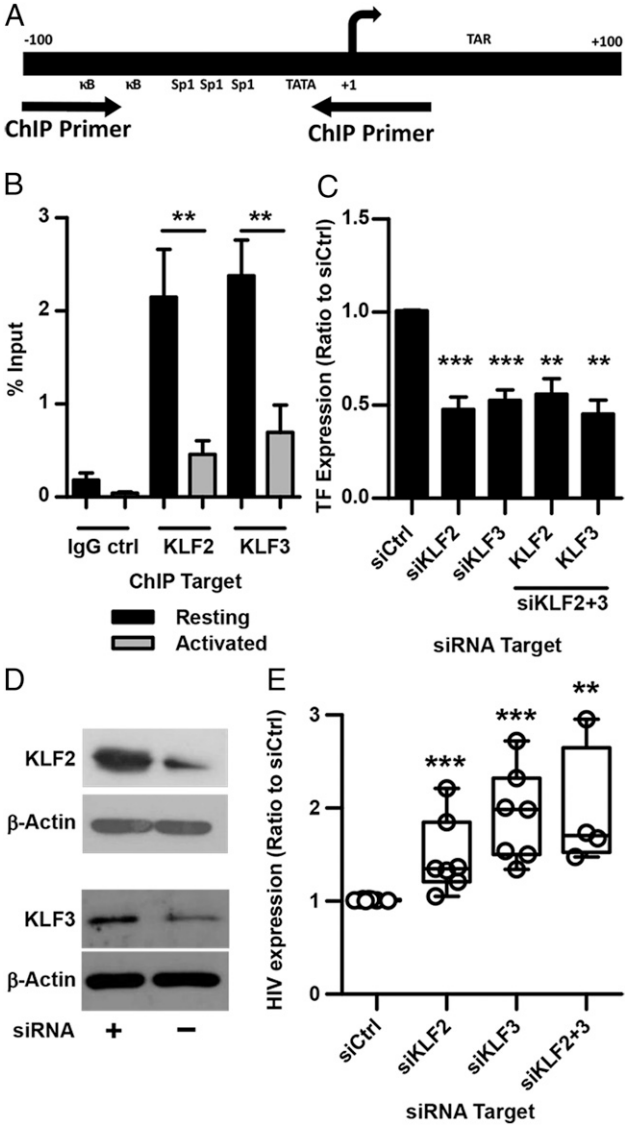

Fig. 3. KLF2 and KLF3 regulate HIV-1 transcription in CD4+ T cells. (*A*) Schematic of HIV LTR highlighting where ChIP primers bind and approximate location of previously described transcription factor binding sites for NF-κB, Sp1, and TATA as well as the transcription start site (arrow +1) and TAR element. (*B*) Unstimulated resting CD4+ T cells that were depleted of CD25+, CD69+, and HLA_DR+ cells were infected with HIV (40). After 72 h, cells were either activated with anti-CD3/CD28 (gray bars) or left unstimulated (black bars) for 24 h. ChIPs using anti-KLF2 or anti-KLF3 and mouse IgG were performed. Associated HIV-1 LTR DNA was measured by RT-qPCR and normalized against input chromatin. $n = 6$ independent ChIPs for resting cells; $n = 5$ independent ChIPs for activated cells. Data are presented as mean ± SEM. (*C*) Unstimulated resting CD4+ T cells were infected with HIV and siRNA targeting KLF2, KLF3, or nontargeting control were nucleofected into cells. Cells were harvested for HIV transcriptional analysis 48 h later. KLF2 and KLF3 mRNAs were measured by RT-qPCR in HIV-infected CD4+ T cells targeted by the indicated siRNAs. $n = 9$ independent knockdowns for siCtrl, $n = 8$ independent knockdowns for siKLF2 and siKLF3, and $n = 4$ independent knockdowns for siKLF2+siKLF3. Data are presented as mean ± SEM (Mann–Whitney *U* test). (*D*) KLF2 and KLF3 protein expression were measured by Western blot in unstimulated resting CD4+ T cells nucleofected with siKLF2 or siKLF3 ($n = 1$; representative of three experiments). β-actin was the loading control. (*E*) HIV-1 RNA expression in cells treated with control, KLF2, or KLF3 siRNAs was measured by RT-qPCR. $n = 9$ experiments for siCtrl, $n = 8$ experiments for siKLF2 and siKLF3, and $n = 4$ experiments for siKLF2 + siKLF3. Data are shown as box and whisker. Bars are the range of values, and the horizontal line is the median. Significance was determined by Mann–Whitney *U* test. $^{**}P < 0.005$, $^{***}P < 0.00005$.

these factors were overexpressed in HIV-luciferase–infected HEK293T cells. Overexpressed KLFs bound the proviral LTR and repressed luciferase expression (Fig. 4 and *SI Appendix*, Figs. S4 and S5), as was observed in primary cells. KLF2 and KLF3 mediated repression correlated with a greater than 50% decrease in histone H3 acetylation (Fig. 4*C* and *SI Appendix*, Fig. S5). Furthermore, KLF3 overexpression increased the recruitment of HDAC2 to the LTR (*SI Appendix*, Fig. S5*D*). These data suggest that KLF2 and KLF3 facilitate epigenetic changes at the HIV-1 LTR, repressing proviral transcription.

PLAGL1 Promotes HIV-2 Transcription. We identified several transcription factors that preferentially bound HIV-2 LTRs (Fig. 1*A*). PLAGL1 was an interesting candidate since it bound both HIV-2 LTRs used in this screen, is widely expressed in immune cells, and interacts with other transcriptional activators of HIV, namely, Sp1, AP-1, and PCAF/CBP/P300 (41). Furthermore, relative expression of PLAGL1 is increased upon CD3 + CD28 activation of unstimulated CD4+ T cells (Fig. 1*B* and *SI Appendix*, Fig. S2). PLAGL1, a C2H2 zinc-finger transcription factor, recognizes GC-rich DNA regions and has both transactivating and repressing activities (42–44). This factor functions in the context of cell cycle regulation and oncogenesis by regulating p21 via p53 (41, 44). PLAGL1 has not been reported to regulate HIV transcription.

Overexpressing PLAGL1 in HEK293T cells with HIV-1 and HIV-2 luciferase reporters induced expression by approximately twofold (*SI Appendix*, Fig. S4), suggesting that PLAGL1 was a transcriptional activator. PLAGL1 bound both HIV-1 and HIV-2 LTRs in infected unstimulated primary CD4+ T cells as determined by ChIP using primers that flanked the GC-rich Sp1 binding sites (Fig. 5*A*). PLAGL1 was knocked down by 50% using siRNAs in HIV-$1_{NL4\text{-}3}$– or HIV-2_{ROD9}–infected unstimulated CD4+ T cells and was confirmed at the mRNA and protein levels (Fig. 5 *B* and *D*). Despite binding the HIV-1 LTR, knocking down PLAGL1 had no effect on HIV-1 transcription, while reduction of PLAGL1 in HIV-2–infected unstimulated primary CD4+ T cells resulted in a 50 to 80% decrease in HIV-2 RNA expression in four out of five donors tested (Fig. 5*C*). Furthermore, significant reduction of HIV-2 transcription was observed in primary monocyte-derived macrophages (MDMs) derived from four additional donors in which PLAGL1 was knocked down (*SI Appendix*, Fig. S6). Taken together, this set of experiments suggest that PLAGL1 can act as an HIV-2–specific transcriptional activator.

Discussion

Our understanding of HIV-1 and HIV-2 transcription is largely based on strategies that depend on LTR transcription factor binding sites, DNA affinity capture methods, and ChIP-seq, which are not suited for capturing low-abundance or cell-specific protein–DNA interactions. Advantages of the eY1H assay include that it is independent of protein abundance, it represents 66% of the known human transcription factor repertoire, and it utilizes compound cis-elements as DNA baits. eY1H assays have been used to characterize transcription factor binding to human enhancers and promoters as well as the identification of loss and gain of protein–DNA interactions for disease-associated variants (24, 26, 45). We used this approach to characterize transcription factor binding to HIV-1 and HIV-2 LTRs to gain insights into networks that control HIV-1 and HIV-2 transcription. The eY1H screen is a discovery assay, and, although hits likely regulate HIV transcription, failure to detect transcription factor binding cannot be interpreted, as factors are not binding or are dispensable for HIV transcription. Limitations to eY1H assays include that LTR chromatization may not be recapitulated in yeast, prey transcription factors may not have proper posttranslational modifications, and the screen does not capture heteromultimeric complexes. However,

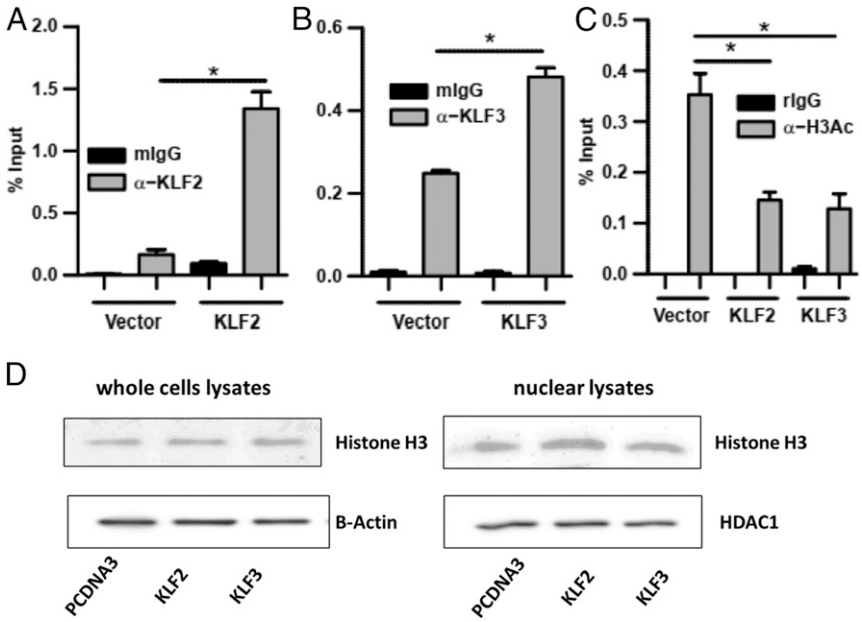

Fig. 4. KLF2 and KLF3 bind the HIV-1 LTR and correlate with H3 acetylation. HEK293T cells were infected with HIV-1 for 24 h. Expression constructs were transfected into cells and harvested 24 h later. ChIPs with antibodies specific to (*A*) KLF2, (*B*) KLF3, and (*C*) acetylated H3 and control mouse or rat IgG were performed. Associated LTR DNA was measured by RT-qPCR and normalized to input chromatin. Representative data from two experiments are shown. Data are presented as mean ± SD. $*P < 0.05$. (*D*) Western blot for H3 in cytoplasmic and nuclear lysates prepared from transfected cells. β-actin was used as loading control for cytoplasmic extracts, and HDAC1 was used as a control for nuclear extracts ($n = 1$; representative of three independent transfections).

our previous studies examining transcriptional elements by eY1H had 30 to 60% validation rates for identified factors, comparable to, or better than, other methods, including ChIP-seq and motif prediction (26, 45–47).

We assayed five LTRs and saw overlapping binding as well as unique binding of transcription factors, especially between HIV-1 and HIV-2. We propose that eY1H could be expanded to examine human isolates from a variety of clades or LTRs from different tissue sites or cell subsets to provide insights into pathogenesis and disease progression. eY1H assays allow for comparison of protein–DNA interactions in standardized conditions and have detected differences in interactions between sequences that differ in a single-nucleotide polymorphism, highlighting its sensitivity for identifying subtle differences between cis-elements (24, 45).

Transcription factors are downstream of multiple signaling cascades. We leveraged the screen results to define broader networks that influence HIV transcription by generating a CD4+ T-cell protein–protein interactome network between eY1H and literature-derived transcription factors. Factors identified by eY1H assays were associated with a cellular process critical for T-cell activation, maturation, and cell survival and directly and indirectly intersected with well-characterized biochemical processes and transcriptional regulators, including NF-κB, Sp1, and Ets factors.

A subset of transcription factors identified are preferentially expressed in unstimulated CD4+ T cells and have been described as transcriptional repressors, including KLF2 and KLF3 (48). Studies of HIV expression have focused on active transcription or induction of latent provirus, which bias the focus onto key transcriptional activators. However, it is important to consider that most tissue-specific genes in cells are not expressed and are actively repressed (49). We see modest changes in HIV transcription associated with knockdown of the repressors we have analyzed so far, but this may reflect cooperative or redundant pathways that assure gene repression. We speculate that our screen has captured repressive transcription factors that are present in resting CD4+ T cells, and that by usurping repressive transcriptional programs, HIV avoids immune recognition until CD4+ T cells are activated and provide a favorable intrinsic program for HIV transcription.

Zinc-finger proteins are highly represented in our screen with 17 of 42 transcription factors identified as zinc-finger factors and 8 of those 17 being KLF family members. The KLF family has been implicated in lymphocyte development, maturation, and exhaustion (50). Furthermore, next-generation sequencing and genome-wide association have suggested that KLF3 is a genetic locus associated with HIV-1 plasma levels (51, 52). KLF2 and KLF3 bound all HIV LTRs screened and repressed HIV transcription in unstimulated cells. KLF2 and KLF3 may be targeting Nuc-1 positioning through histone acetylation. Additionally, KLF2 prevents differentiation of activated T cells into T_{fh} cells through Blimp-1 (53), and we have shown Blimp-1 represses HIV transcription in T-cell memory subsets (54). Differential regulation of KLF2 and KLF3 in unstimulated and activated CD4+ T cells further suggests a role for these repressors in the establishment of latency in quiescent T-cell populations.

Our eY1H screen suggested that different LTRs bind unique constellations of transcription factors that regulate their proviral transcription. For example, our data suggest that PLAGL1 is a specific transcriptional activator for HIV-2; to our knowledge, there have not previously been any HIV-2–specific transcription factors described. It should be noted that although PLAGL1, which did not bind HIV-1 in the eY1H assay and selectively mediated expression of HIV-2 in primary cells, bound HIV-1 LTRs in human cells. We do not know why we observe differential transcriptional activity with HIV-1 and HIV-2 LTRs despite binding to both subtype LTRs; however, we speculate that PLAGL1 binds specifically or with higher affinity to HIV-2, whereas binding to HIV-1 LTR may be facilitated through protein–protein interactions and recruitment by neighboring transcription factors rather than directly binding the HIV-1

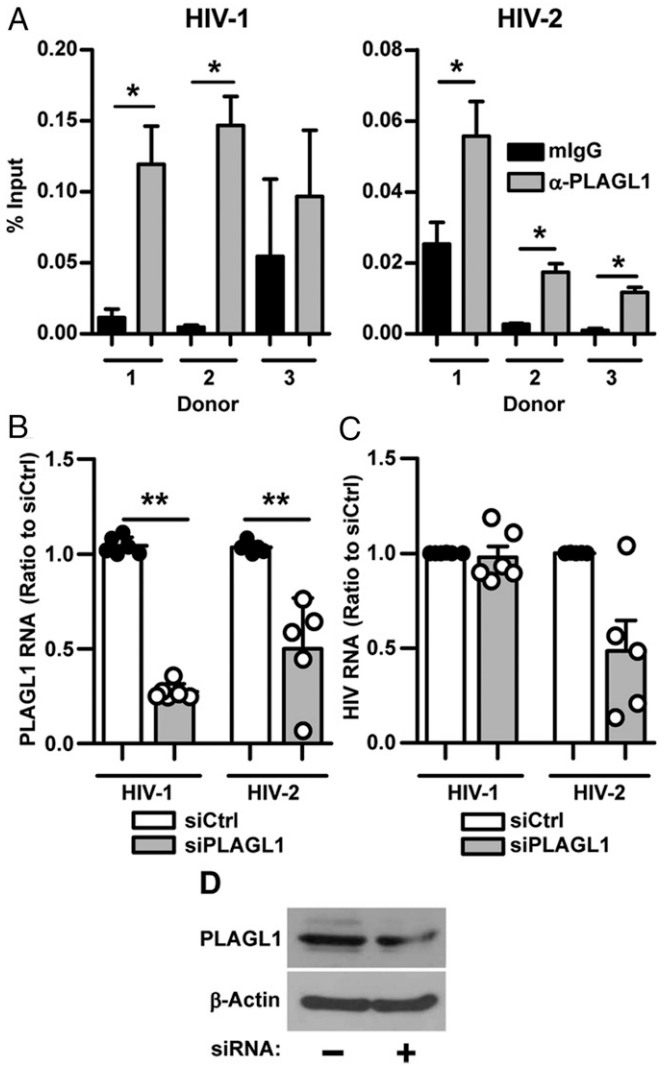

Fig. 5. PLAGL1 activates HIV-2 transcription. (*A*) Chromatin was prepared from HIV-1– and HIV-2–infected unstimulated CD4+ T cells for ChIP using mouse IgG or anti-PLAGL1. Associated LTR DNA was measured by RT-qPCR and normalized to input chromatin. Primers that flanked the GC-rich Sp1 binding sites in HIV-1 and HIV-2 were used. $n = 3$ donors are shown. Data are represented as mean ± SD. (*B*) PLAGL1 mRNA expression was measured by RT-qPCR following siRNA treatment. Data are represented as mean ± SEM. (*C*) HIV RNA expression was measured by RT-qPCR following siRNA treatment. Data are represented as mean ± SEM. (*D*) Western blot for PLAGL1 expression in unstimulated CD4+ T cells nucleofected with siPLAGL1. β-actin was used as loading control ($n = 1$; representative of three experiments). (*A*–*C*) $*P < 0.05$ and $**P < 0.005$ as determined by Mann–Whitney *U* test.

LTR. These data suggest that the HIV-2 LTR is differentially regulated compared to HIV-1, and a more detailed transcriptional analysis of mechanisms that control HIV-2 proviral expression is warranted.

In summary, we used an eY1H screen to provide an unbiased approach to understand transcriptional regulation of HIV-1 and HIV-2. Our findings describe the discovery of factors that regulate HIV-1 and HIV-2 and provide insights into intrinsic networks that influence transcriptional activation and repression of HIV proviruses.

Materials and Methods

Enhanced Yeast One-Hybrid Screen. We used eY1H assays to evaluate binding of transcription factors to HIV LTRs as described previously (24–26). The U3-R-U5 LTR bait sequences included 1 kb that spanned the full LTR extending ~500 bp beyond the transcriptional start site, were subcloned into pDONR-P4P1R and transferred to upstream of two reporter genes, *LacZ* and *HIS3*, using Gateway cloning and introduced into *HIS3* and *URA3* genomic *loci* in the *S. cerevisiae* genome (24). Transcription factor "preys" were expressed as fusion proteins with the yeast Gal4 activation domain, allowing for the detection of activators and repressors (24). A total of 1,086 yeast transcription factor prey strains were tested against LTRs in a pairwise manner as described previously (24, 25). Matings were performed in quadruplicate, and positive hits were blue X-Gal–positive colonies that grew on His-minus plates with the competitive His3p enzyme inhibitor 3-amino-1,2,4-triazole. Colonies with reporter activity above background for at least two colonies were deemed positive (24–26).

Signaling Pathways Associated with HIV Transcriptional Networks. For pathway enrichment analyses, we included the transcription factors that bound HIV-1 or HIV-2 LTRs in eY1H assays and transcription factor interactions reported in the literature. We also included protein–protein interactions with these transcription factors reported in HuRI and the literature (32). Only transcription factors and proteins expressed in at least one subset of CD4+ T cells with a TPM > 0 (Blueprint Epigenome) were included in the analysis. PANTHER Pathway enrichment analysis was performed using human proteins as a background and Fisher's exact test and false discovery rate correction for multiple hypothesis testing. Terms with at least three genes in our query set were included.

Cells. HEK293T cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 100 U/mL penicillin/streptomycin (P/S; Invitrogen), 2 mM L-glutamine (Invitrogen), 10% fetal bovine serum (FBS) (Gemini Bio-Products). Unstimulated CD4+ T cells were enriched by negative selection using the EasySep Human CD4+ T Cell Enrichment Kit (Stemcell Technologies) from peripheral blood mononuclear cells (PBMC) obtained from leukapheresis packs (New York Biologics) using Lymphoprep density gradient (Stemcell Technologies) (39, 40, 55). Unstimulated CD4+ T cells were cultured in Roswell Park Memorial Institute medium (RPMI; Invitrogen) with 100 U/mL P/S, 2 mM of L-glutamine, and 10% FBS, and their unstimulated status was confirmed by staining with CD25 and CD69 (*SI Appendix*, Fig. S2*C*). For some experiments, these cells were further enriched for unstimulated resting cells by depleting CD4+ T cells expressing CD25, CD69, and human leukocyte antigen (HLA)-DR (39, 40). Activated T cells were generated from unstimulated CD4+ T cells with Dyna anti-CD3/CD28 beads (Invitrogen) at one bead per cell for 72 h. Once the beads were removed, cells were maintained in medium supplemented with 100 U/mL IL-2 (AIDS Reagents Program) and 100 ng/mL IL-7 (Miltenyi Biotec). MDMs were differentiated from PBMCs by resuspending cells in RPMI minus serum at a density of 5×10^6 cells/mL and plated 1 mL per well in 12-well plates. Cells were incubated at 37 °C for 1 to 2 h to allow for the attachment of monocytes. Unbound cells were discarded, and attached cells were cultured in RPMI with 10% FBS, 10% human AB serum (Corning), 100 U/mL of P/S, and 2 mM L-glutamine. MDMs were differentiated for a week at 37 °C, and spent medium was removed and replaced with fresh medium every 2 to 3 d.

Transfections of HEK293T Cells. HEK293T cells were cotransfected with firefly luciferase reporters under the control of HIV-1_{NL4-3} (56) or HIV-2_{ROD9} LTRs (kindly provided by Suryaram Gummuluru, Boston University School of Medicine) and expression vectors for transcription factors using Opti-MEM (Invitrogen) and PEI Max 40,000 (Polysciences, Inc.). Transcription factor expression constructs were KLF2 (Origene, SC127849), KLF3 (Addgene, 49102), PLAGL1 (Origene, SC115928), or empty pcDNA3 vector.

Viruses and Infections. Viruses were generated by transfection of HEK293T cells with molecular clones HIV-1_{NL4-3} or HIV-2_{ROD9} (AIDS Reagents Program). For some viral stocks, an expression construct for VSV-G was cotransfected with the HIV clones. Viruses were titered in CEM-GFP cells (AIDS Reagents Program). Unstimulated CD4+ T cells purified by negative selection as described above were infected by spinoculation with an multiplicity of infection (MOI) of 0.125 (39, 40, 55). To prevent viral spread beyond the first round of infection, 1 μM protease inhibitor saquinavir (AIDS Reagents Program) was included. Infection of unstimulated cells was monitored by Alu-PCR. MDMs were infected with VSV-G pseudotyped HIV for 4 h and then washed twice with phosphate-buffered saline (PBS) + 2% FBS. Cells treated with 1 μM efavirenz (AIDS Reagents Program) served as negative controls for infection.

siRNA Knockdown of Transcription Factors. Infected unstimulated CD4+ T cells were resuspended in 100 μl 1SM buffer (57) and siRNAs targeting KLF2, KLF3, PLAGL1, or nontargeting control (Dharmacon: L-006928-00-0005, L-006987-02-0005, L-006546-00-0005, D-001810-10-05) and electroporated using the Nucleofector I (Amaxa/Lonza) program U-14. Following electroporation, cells were cultured for 24 h in RPMI, P/S, L-glutamine, and 20% FBS before adjusting the serum concentration to 10% FBS. HIV-infected macrophages were transfected with siRNA packaged in Lipofectamine 2000 (Invitrogen).

Western Blotting. For whole-cell extracts, cells were resuspended in lysis buffer (20 mM Tris HCl [pH 7.4], 1% Triton X-100 [Thermo Fisher Scientific], 10% glycerol [Thermo Fisher Scientific], 137 mM NaCl [Thermo Fisher Scientific], 2 mM ethylenediaminetetraacetic acid [EDTA; Thermo Fisher Scientific], and 25 mM β-glycerophosphate [Sigma]), protease inhibitor mixture III (MilliporeSigma), and phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific). Nuclear extracts were prepared by suspending cells in hypotonic buffer (20 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40), pelleting the nuclei by centrifugation and resuspending the pellets in extraction buffer (10 mM Tris pH 7.4, 3 mM Na3VO4, 10 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 0.5% deoxycholate, 20 mM Na4P2O7, 1 mM PMSF, and protease inhibitor mixture) and incubated for 30 min on ice. Nuclear fractions were cleared by centrifugation for 30 min at 14,000 × *g* at 4 °C, and supernatants were collected. Lysates were mixed in Laemmli's SDS-Sample Buffer (Boston BioProducts, number BP-111R) and heated at 95 °C for 5 min. Lysates were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred by electroblotting onto polyvinylidene difluoride membrane (Millipore). After blocking 1 h with 5% nonfat dry milk, blots were incubated with primary antibodies (*SI Appendix*, Table S3) or Histone H3 (Millipore Sigma product, catalog number 06-755) or HDAC 1 (mouse anti-human HDAC1, sc-81598, Santa Cruz Biotechnology) overnight at 4 °C and probed with horseradish peroxidase (HRP)-conjugated secondary antibody. Membranes were developed with the ECL Prime Western Blotting System (GE Healthcare) and visualized on X-ray film. Membranes were stripped and reprobed with anti–β-Actin (catalog number VMA00048, Bio-Rad) for loading control.

Analysis of T-Cell Viability and Phenotype by Flow Cytometry. Cells were stained with Zombie NIR (Biolegend) followed by staining for CD25 (clone 2A3, BD Biosciences), CD69 (clone FN50, Biolegend), and HLA-DR (clone L243, Biolegend). Cells were analyzed with an LSR-II SORP (BD Biosciences) and FlowJo software.

RT-qPCR. Total cellular RNA was purified by TRIzol extraction, and cDNA was generated as previously described (58). Expression of HIV was analyzed by qPCR using GoTaq PCR master mixture (Promega) and specific primers (*SI Appendix*, Table S2). qPCR was performed with a QuantStudio 3 thermocycler (Thermo Fisher Scientific). The amplification program was 15 min hot start at 94 °C, 45 cycles of 15 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and plate read. Relative levels of mRNA transcripts were calculated using the ΔΔCt method (59).

Measuring HIV DNA. Integrated HIV-1 and HIV-2 DNA within CD4+ T cells infected in vitro was measured by *Alu*-qPCR as previously described (39, 40, 58). Cells treated with 1 μM efavirenz, a reverse-transcriptase inhibitor, were included as negative controls for HIV infection and integration. Primers are listed in *SI Appendix*, Table S2. For HIV-2, serially diluted HIV-2_{ROD9} plasmid was used as a copy standard. A parallel reaction for cellular albumin was used as a control for endogenous DNA copy number (39, 40, 58).

Chromatin Immunoprecipitations. HIV-infected cells were washed with PBS and fixed with 1% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min, followed by quenching with saturating amounts of glycine (MP Biomedicals) for 5 min. Samples were washed at 4 °C with PBS to remove paraformaldehyde. Pellets were resuspended in lysis buffer (5 mM Tris HCl pH8, 90 mM KCl [Thermo Fisher Scientific], 1% Nonidet P-40 [Boston BioProducts], and 1X Halt Protease and Phosphatase Inhibitor Mixture [Thermo Fisher Scientific]), spun, and then resuspended in nuclear lysis buffer (50 mM Tris HCl pH8, 10 mM EDTA [Thermo Fisher Scientific], 0.5% SDS [EMD Millipore], 25 mM sodium butyrate [Acros Organics], and 1X Halt Protease and Phosphatase Inhibitor Mixture) and sonicated using a Bioruptor Pico (Diagenode) for 15 cycles of 1.5 min on and 30 s off for CD4+ T cells and 10 cycles of 30 s on and 30 s off for HEK293T cells. Following sonication, lysates were cleared by centrifugation and supernatants were transferred to fresh tubes. The pellet was lysed one last time with RIPA-like buffer (20 mM Tris HCl pH8, 2 mM EDTA, 0.5 mM EGTA pH8 [Thermo Fisher Scientific], 1% Triton X, 140 mM NaCl [Thermo Fisher Scientific], 0.25% sodium deoxycholate [Acros Organics], and 1X Halt Protease and Phosphatase Inhibitor Mixture) and spun, and the supernatant was combined with the rest of the sonicated chromatin. Chromatin was precleared with Protein-A sepahrose beads (Invitrogen) before immunoprecipitation. Antibodies used are in *SI Appendix*, Table S3. Antibody-chromatin complexes were bound to Protein-A sepahrose beads. The beads were pelleted and washed with low-salt buffer (20 mM Tris HCl pH8, 0.1% SDS, 1% Triton X, 2 mM EDTA, and 150 mM NaCl), high-salt buffer (20 mM Tris HCl pH8, 0.1% SDS, 1% Triton X, 2 mM EDTA, and 500 mM NaCl), lithium wash buffer (10 mM Tris HCl pH8, 10 mM, 0.25 M LiCl [Acros Organics], 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA), and Tris EDTA (TE) buffer. Chromatin was eluted in TE buffer containing 0.3% SDS and 0.47 mg/mL of proteinase K (Invitrogen) incubated overnight at 65 °C. DNA was purified using the ChIP DNA Clean & Concentrator kit (Zymo Research). DNA was analyzed by qPCR using GoTaq PCR master mixture (Promega) and primers to detect HIV DNA immunoprecipitated (*SI Appendix*, Table S2) or histone H3 modifications (54). DNA purified from the "input" sample was serially diluted to serve as a standard.

Microarray. CD4+ T-cell microarray with unstimulated and CD3 + CD28 activated CD4+ T cells was described previously (30). Microarrays and statistical support were provided by Boston University Microarray and Sequence Resource Core Facility, as previously described (30).

Statistical Analysis. All HIV in vitro experiments and technical replicates were conducted at least three times. Experiments with primary cells included cells from at least three different donors. Data are presented as mean values ± SE. *P* values were calculated based on the Mann–Whitney *U* test using GraphPad Prism software.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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