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Negative Elongation Factor (NELF) Coordinates RNA Polymerase II Pausing, Premature Termination, and Chromatin Remodeling to Regulate HIV Transcription*

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Background: Multiple mechanisms contribute to HIV latency, including NELF-mediated RNA polymerase II (RNAP II) pausing.
Results: Paused RNAP II recruits a transcription termination factor and a transcriptional corepressor complex to the HIV promoter.
Conclusion: Paused RNAP II couples premature transcription termination and chromatin remodeling to maintain HIV latency.
Significance: Paused RNAP II may be targeted to purge latent HIV infection.

A barrier to eradicating HIV infection is targeting and eliminating latently infected cells. Events that contribute to HIV transcriptional latency include repressive chromatin structure, transcriptional interference, the inability of Tat to recruit positive transcription factor b, and poor processivity of RNA polymerase II (RNAP II). In this study, we investigated mechanisms by which negative elongation factor (NELF) establishes and maintains HIV latency. Negative elongation factor (NELF) induces RNAP II promoter proximal pausing and limits provirus expression in HIV-infected primary CD4$^+$ T cells. Decreasing NELF expression overcomes RNAP II pausing to enhance HIV transcription elongation in infected primary T cells, demonstrating the importance of pausing in repressing HIV transcription. We also show that RNAP II pausing is coupled to premature transcription termination and chromatin remodeling. NELF interacts with Pcf11, a transcription termination factor, and diminishing Pcf11 in primary CD4$^+$ T cells induces HIV transcription elongation. In addition, we identify NCoR1-GPS2-HDAC3 as a NELF-interacting corepressor complex that is associated with repressed HIV long terminal repeats. We propose a model in which NELF recruits Pcf11 and NCoR1-GPS2-HDAC3 to paused RNAP II, reinforcing repression of HIV transcription and establishing a critical checkpoint for HIV transcription and latency.

The success of highly active antiretroviral therapy has shifted the focus of HIV drug discovery from treatment to eradication of infection. Long-lived latently HIV-infected cells, which lead to the rebound of virus replication following interruption of highly active antiretroviral therapy, present a major barrier to eliminating HIV infection. These latent reservoirs, which include quiescent memory T cells and tissue-resident macrophages (1–3), represent a subset of cells with decreased or inactive proviral transcription. Studies with chronically and acutely infected cells show that mutations in Tat, sites of provirus integration, absence of cellular transcription factors, and miRNA machinery contribute to post-integration latency (3–5). Whether there are common regulatory events that control HIV expression in the context of different latently infected cell populations needs to be determined if strategies to target and mobilize latent provirus are to be devised.

The upstream LTR of the HIV provirus controls transcription by functioning as an enhancer and promoter, recruiting host transcription factors necessary to initiate transcription (6, 7) and coactivators, such as histone acetyltransferases and Swi/Snf complexes that regulate the chromatin structure of integrated provirus (5, 8). However, recruitment of these factors to the HIV LTR is not sufficient for efficient transcription because provirus transcription is also controlled at the level of transcriptional elongation. HIV encodes a transcriptional activator, Tat, that enhances processive transcription by associating with transactivation response element (TAR), a RNA stem loop structure within the 5′ nascent transcript, and recruiting positive transcription factor b (P-TEFb)[4] to the RNAP II elongation complex (9, 10). P-TEFb, which is composed of CycT1 and Cdk9, modifies RNAP II activity by hyperphosphorylating the carboxy-terminal domain of RNAP II. In the absence of Tat,

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[4] The abbreviations used are: P-TEFb, positive transcription factor b; RNAP II, RNA polymerase II; DSIF, DRB sensitivity-inducing factor; NELF, negative elongation factor; PLAP, placental alkaline phosphatase; LUC, luciferase; HDAC, histone deacetylase; Pcf11, Pre-mRNA-cleavage complex II factor; NCoR1, nuclear corepressor; Gps2, G protein pathway suppressor 2; HDAC3, histone deacetylase 3.

HIV transcription elongation is inefficient, and short transcripts accumulate (9, 10). These short transcripts and the identification of a site in this region where purified RNAP II pauses elongation indicate that transcription of the integrated provirus is repressed by proximal RNAP II pausing and premature termination (11, 12). The promoter-proximal pause is executed by the negative elongation factors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) (13–15), whereas pre-mRNA-cleavage complex II factor (Pcf11) plays a critical role in premature termination (16, 17). NELF and Pcf11 have been shown to limit HIV transcription in cell line models of latency (17, 18). An additional checkpoint for HIV transcription is at the level of chromatin. Repression of HIV transcription is associated with a positioned nucleosome at the transcription start site, and induction of HIV transcription correlates with histone modifications and displacement of this nucleosome (5, 8, 19). Whether RNAP II processivity is coupled to chromatin organization has not been investigated.

We demonstrate that NELF limits HIV transcription in HIV-infected primary $CD4^+$ T cells and that NELF physically and functionally interacts with Pcf11 and the nuclear corepressor (NCoR1)-G protein pathway suppressor 2 (Gps2)-histone deacetylase 3 (HDAC3) repressor complex, thus coupling the processes of RNAP II pausing, premature termination, and chromatin modification to repress HIV transcription.

EXPERIMENTAL PROCEDURES

Cells—Jurkat E6.1 T cells (ATCC), ACH-2 Cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health), and primary human cells were grown in RPMI 1640 medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.2 M L-glutamine. HEK293T cells (ATCC) were cultured in complete DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin. Peripheral blood mononuclear cells were isolated from deidentified blood purchased from NY Biologicals by Ficoll/histopaque gradient (Sigma-Aldrich) and $CD4^+$ T cells were positively selected using a Dynal isolation kit (Invitrogen, catalog no. 113.21D).

For T cell activation, 1×10^6 cells were cultured with 0.1 μg/ml mouse anti-human CD3 antibody (BD Biosciences, catalog no. 555336) and 1.0 μg/ml mouse anti-human CD28 antibody (BD Biosciences, catalog no. 555725) for 30 min and cross-linked with 5 μg/ml goat anti-mouse IgG antibody (Sigma, catalog no. M 4280).

Transfections, Retrovirus Packaging, and Infections—For packaging HIV, 5×10^5 HEK293T cells were transfected using calcium phosphate with 15 μg of pNL4–3-Luc(+) Env(–) Nef(–) (HIV-LUC) or pHXBnPLAP-IRES-N+ (HIV-PLAP) (AIDS Research and Reference Reagent Program, Ref. 20), 3 μg of RSV-Rev, and 3 μg of vesicular stomatitis virus G, as described previously (21). Calcium phosphate transfection was also used for overexpressing proteins in HEK293T cells. HIV-LUC lacks envelope and supports a single round of infection (22). HIV-LUC transfection efficiency was assessed by luciferase activity (luciferase kit, Promega, Madison, WI) and p24 ELISA. HIV-PLAP is a replication-competent virus, and infectious titers were monitored by p24 or flow cytometry measuring placental alkaline phosphatase (PLAP) surface expression with an anti-PLAP antibody (Sigma).

2×10^7 Jurkat cells were infected by culturing with 10 ml of supernatants containing HIV-LUC for 12–16 h. Cells were allowed to recover for 12 h before transfection of siRNA. Prior to infection, CD4+ T cells were activated with phorbol 12-myristate 13-acetate and phytohemagglutinin, rested for 12 h, and spinoculated with 10 ml HIV-LUC supernatant plus 1 μg/ml polybrene for 2 h at 1200 rpm ($290 \times g$). Cells were washed in media and cultured in 5% FCS RPMI.

SMARTpools (Dharmacon) of at least four siRNAs for each specific target were transfected into cells 24 h post-infection. Cells were washed with serum-free RPMI, 20 mM HEPES, resuspended in 600 μl of HEPES RPMI plus 5 μl of 100 μM siRNA, and electroporated using a T820 square pulse electroporation system (BTX, San Diego, CA) at 1 pulse for 20 msec, 300 V in a 4-mm cuvette. To measure HIV release from infected cells, supernatants were collected at the indicated times, diluted with PBS, and p24 ELISA was performed using the PerkinElmer Life Sciences ELISA kit.

pcDNA3-FLAG-NELF-B (23) was provided by Dr. Rong Li (University of Texas Health Science Center), pCIN4-FLAG-HDAC3 (24) was provided by Dr. Robert Roeder (Rockefeller University), and pcDNA-HA-Gps2 (25) was provided by Dr. Valentina Perissi (Boston University School of Medicine). HDAC3 was subcloned into the BamHI-XbaI sites of pcDNA3 using primers that introduced the restriction sites and then HA-tagged. The primers used were as follows: 5′-CGGGATCCATGGCCAAGACCGTGGCCTATTTC-3′ (forward) and 5′-GCTCTAGATTAAGCGTAATCTGGAACATCGTATGGGTAAATCTCCACATCGCTTTCCTTG-3′ (reverse).

Quantitative Real-time PCR—RNA was prepared by resuspending cells in TRIzol, and cDNA was generated using reverse transcriptase and random primers (Invitrogen). 1–2 ng cDNA was used in quantitative real-time PCR reactions using SYBR Green reagent (Qiagen). Initiated transcripts (+1 to +40) were amplified using 5′-AGAGCTCCCAGGCTCA-3′ and 5′-GGGTCTCTCTGGTTAGA-3′. Elongated transcripts (+5396 to +5531) were amplified using 5′-GACTAGAGCCCTGGAAGCA-3′ and 5′-GCTTCTTCCTGCCATAGGAG-3′. β-actin mRNA was amplified using a Quantitect primer assay (Qiagen). PCR was carried out for 50 cycles, and the relative expression was calculated using the ΔΔCt method (26), normalizing specific amplification of the transcripts of interest to the β-actin control amplification for each specific sample. The product detected in the siControl was a calibrator, and the transcript levels in samples were calculated as fold changes in comparison to siControl.

Immunoprecipitation and Immunoblots—Whole cell extracts were prepared by resuspending cells in lysis buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1.0 mM EDTA (pH 8.0), 2.0 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X 100, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture III (Calbiochem)). Samples were spun for 10 min at 4 °C at 13,000 rpm and precleared with protein A/G beads (Santa Cruz Biotechnoology,

catalog no. sc-2003) for 30 min at 4 °C, followed by centrifugation. Supernatants were incubated with anti-NELF-D (Proteintech), anti-Pcf11 (17), anti-FLAG, anti-HA, or rabbit IgG-coated protein A/G beads for 2 h at 4 °C. The beads were collected, washed three times with lysis buffer, suspended in SDS-PAGE loading buffer, and heated for 5 min at 100 °C before resolving on 8% SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) by electroblotting. Membranes were blocked with 5% nonfat milk and incubated with the indicated antibodies to detect proteins.

Chromatin Immunoprecipitations—ChIP assay has been described in previous publications (17, 18). Briefly, cells were cross-linked using 11% formaldehyde solution (prepared from 37% formaldehyde and 10% methanol) in 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-HCl (pH 8) to the final concentration of 1%. The reaction was quenched with a final concentration of 240 mM glycine. Cells were washed, resuspended in sonication buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 0.5 mM PMSF, 1%SDS) and sonicated on ice for 30 cycles of 10 s on and 30 s off. Chromatin was diluted in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 0.5 mM PMSF, 0.1% SDS, and 1.1% Triton X-100 and incubated with 1 μg of the indicated antibodies for 16 h at 4 °C. Protein A/G beads were added for 2 h, followed by two washes each with low-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl), high-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 0.1), and 500 mM NaCl) and LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, and 10 mM Tris-HCl, 1 mM EDTA) (27). Complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$, reverse-cross-linked at 65 °C for 4 h, and treated with proteinase K for 1 h at 45 °C. DNA was extracted using phenol-chloroform and ethanol-precipitated. Real-time PCR analysis using SYBR Green reagents used the primers 5′-GAGCCCTCAGATCCTGGATA-3′ and 5′-AGGCTTAAGCAGTGGGTTCC-3′ to amplify −45 to +72 bp of HIV-LTR.

Mass Spectrometry—Nuclear extracts were prepared from transgenic *Drosophila* embryos that expressed FLAG-tagged NELF-D, and the epitope tag was used to immunoprecipitate complexes. Proteins were identified as reported previously (28). Briefly, proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining. All visible bands were excised and subjected to in-gel tryptic digestion (29). Tryptic peptides were extracted from the gel, resuspended in 0.5% acetic acid, and separated using reverse phase liquid chromatography. Mass spectra were recorded by a ThermoFinnigan LTQ ProteomeX ion trap mass spectrometer and analyzed using SEQUEST using standard thresholds, and each spectrum considered a match was inspected visually.

RESULTS

NELF Limits HIV Transcription in Primary T Cells—Our previous studies demonstrating that NELF limits HIV transcription utilized latently HIV-infected premonocytic U1 cells, which carry two copies of provirus that harbor Tat mutations (18). It is possible that Tat mutations contribute to the lack of RNAP II processivity observed in U1 cells (30). We wanted to determine whether RNAP II pausing had a role in limiting HIV transcription in primary $CD4^+$ T cells. To disrupt RNAP II pausing, siRNA was used to deplete NELF in infected primary T cells. $CD4^+$ T cells from peripheral blood of healthy donors were infected with NL4-3-luciferase (HIV-LUC) to generate an unbiased heterogeneous pool of HIV-infected primary T cells. Infected cells were transfected with siControl RNA or siRNA specific for NELF-B, which disrupts the NELF complex (31–33). Knockdowns were confirmed by immunoblot analyses and RT-PCR (Figs. 1, *A* and *B*). Forty-eight hours post-knockdown, luciferase assays were performed to measure HIV transcription. Even though these cells represented an unselected population that should include cells with a range of provirus transcription and few latently infected cells, diminishing NELF increased HIV transcription by more than 2-fold (Fig. 1*C*). Furthermore, depletion of NELF increased provirus transcriptional elongation, as determined by measuring the levels of initiated transcripts (+1 to +40) and elongated transcripts (+5396 to +5531) (Fig. 1*D*). The levels of initiated transcript were comparable in siControl and siNELF-treated cells, indicating that RNAP II was present at the transcriptional start site, whereas more elongated transcripts were seen in siNELF treated cells, consistent with RNAP II pausing limiting HIV transcription in primary T cells. These changes in provirus transcription corresponded to approximately a 7-fold increase in HIV release, as measured by p24 in the supernatant (Fig. 1*E*). To gain insights into how silencing NELF induces HIV transcription in the cell population, we infected $CD4^+$ T cells with a HIV-PLAP reporter virus that expresses PLAP on the surface of HIV-positive cells (20) and then transfected these infected cells with siControl or siNELF. PLAP was assessed by flow cytometry. A modest 45% increase in HIV-expressing cells was observed (Fig. 1*F*), suggesting that the induction of transcription in part reflected the activation of infected cells not previously expressing HIV. Activating infected cells with anti-CD3 plus anti-CD28 antibodies, which did not rescue NELF expression in siRNA-treated $CD4^+$ T cells (Fig. 1*G*), enhanced HIV transcription, monitored by luciferase (Fig. 1*H*), regardless of whether cells were treated with siControl or siNELF-B. These data indicate that RNAP II pausing is a critical checkpoint for basal HIV transcription but is bypassed when conditions favor HIV transcription elongation. Therefore, NELF-mediated RNAP II pausing limits provirus transcription in primary $CD4^+$ T cells.

RNAP II Pausing Is Coupled with Premature Termination in Limiting HIV Transcription—We showed previously that both NELF and Pcf11 limited HIV transcription in U1 cells (17, 18). We were interested in exploring whether NELF and Pcf11 act independently or cooperatively to regulate HIV transcription in primary cells. We utilized siRNAs to diminish both Pcf11 and NELF in primary $CD4^+$ T cells. RT-PCR and immunoblot analyses indicated that expression of Pcf11 and NELF were consistently decreased by 40–60% (Figs. 2, *A–C*). Attempts to increase the efficiency of these knockdowns promoted cell death, suggesting that these are essential factors. Measuring initiated and elongated HIV transcripts from $CD4^+$ T cells infected with HIV-LUC showed that depletion of Pcf11, or both NELF and Pcf11, increased processive transcription compared with siControl-treated cells (Fig. 2*D*). Moreover, depleting

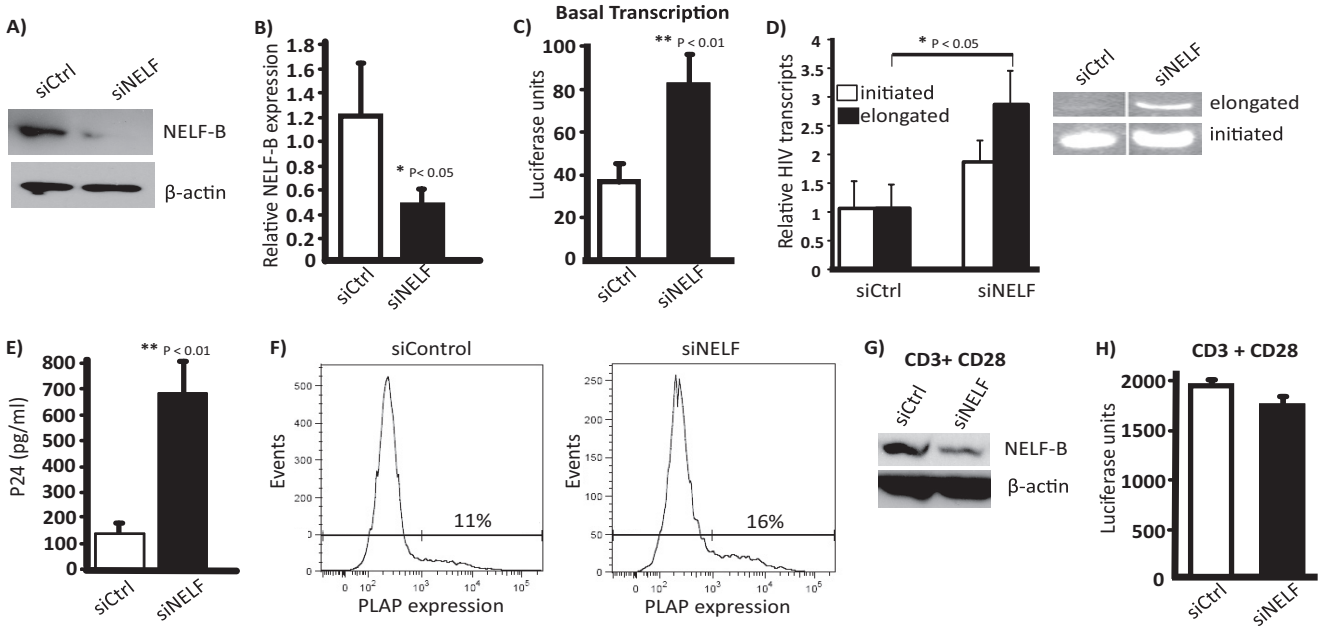

FIGURE 1. **NELF limits HIV transcription and replication in primary CD4$^+$ T cells.** Human primary CD4$^+$ T cells infected with HIV-LUC were transfected with siControl (*siCtrl*) or siNELF-B. NELF depletion was determined at 48 h post-knockdown by immunoblot analysis using NELF-B antibodies (*A*) and quantitative real-time PCR for NELF-B mRNA transcripts (*B*). *C*, 48 h post-knockdown, luciferase activity was measured to monitor HIV transcription. *D*, RNA was isolated from HIV-LUC-infected cells and reverse-transcribed, and initiated transcripts (+1 to +40) and elongated transcripts (+5396 to +5555) were detected by quantitative real-time PCR. The *right panel* shows ethidium bromide-stained PCR products from a single infection. Presented data were run on the same gel and processed as a single image. Lanes were rearranged for presentation purposes but were not individually modified. The *left panel* summarizes data from three individual infections. The initiated and elongated PCR products from siNELF-treated primary T cells were normalized to siControl products that were set equal to 1. *E*, p24 ELISA of cell culture supernatants from CD4$^+$ T cells measuring the release of virus particles 48 h post-knockdown. *F*, CD4$^+$ T cells were infected with HIV-PLAP pseudotyped with vesicular stomatitis virus G. 48 h post-infection, the cells were transfected with siControl or siNELF-B. 48 h post-transfection, cells were stained with anti-PLAP, and FACS was used to assess the HIV-infected cell population. The mean fluorescence intensity for siControl and siNELF were 6624 and 7174, respectively. *G*, 48 h post-knockdown, HIV-LUC-infected CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 antibodies for 4 h. 12–16 h post-stimulation, whole cell lysates were immunoblotted to detect NELF-B protein levels. *H*, luciferase activity was measured to monitor HIV transcription in siCtrl or siNELF-treated cells following CD3 + CD28 activation. Data are mean ± S.D. and representative of experiments using T cells isolated from three or more individual donors.

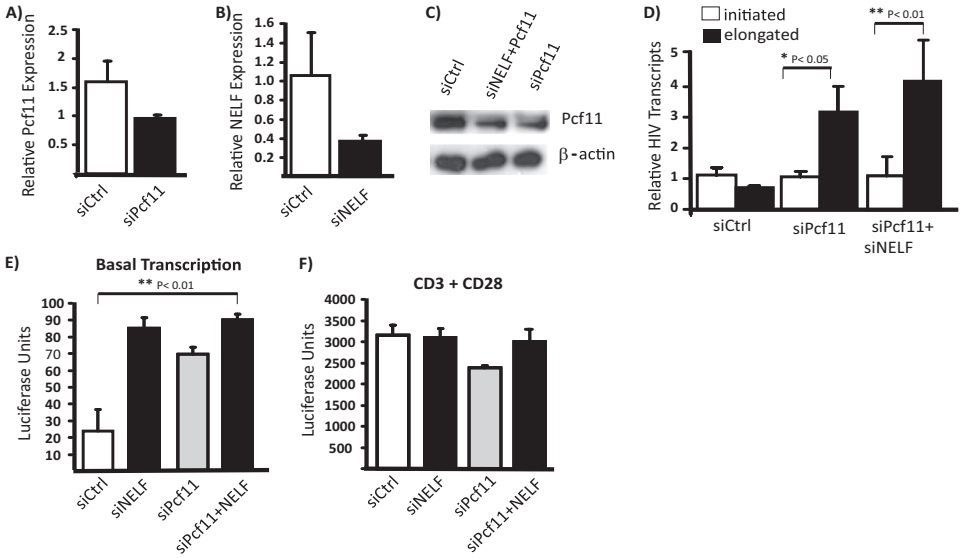

FIGURE 2. **NELF and Pcf11 repress HIV transcription elongation in T cells.** Primary CD4$^+$ T cells infected with HIV-LUC for 24 h were treated with siCtrl, siNELF-B, or siPcf11 for 48 h. *A* and *B*, quantitative real-time PCR analysis of Pcf11 and NELF mRNA following siRNA transfections. *C*, immunoblot analysis of cells treated with siNELF and siPcf11 and probed with an anti-Pcf11 antibody. *D*, cDNA was prepared 48 h post-knockdown, and initiated and elongated transcripts were determined using quantitative real-time PCR. *E*, luciferase activity of HIV-LUC-infected primary T cells transfected with siControl, siNELF-B, and/or siPcf11 was measured 48 h post-knockdown. *F*, infected CD4$^+$ T cells treated with siRNAs were activated with anti-CD3 and anti-CD28 antibodies for 4 h, and luciferase activity was measured 12 h after stimulation. These data are from at least three independent infections and knockdowns performed in triplicate. Primary cells were obtained from at least three different donors.

NELF alone, Pcf11 alone, or both resulted in comparable increases in HIV expression, as measured by luciferase activity (Fig. 2*E*). These results demonstrate roles for NELF and Pcf11 in limiting basal HIV transcription in primary T cells. Because depleting both NELF and Pcf11 did not further enhance HIV transcription, these factors appear to act in the same biochem-

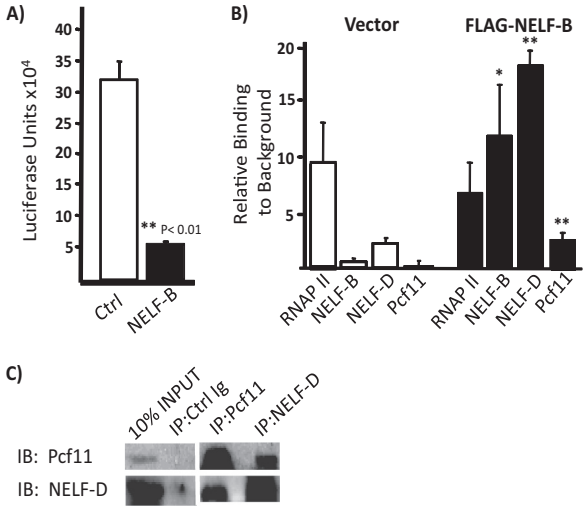

FIGURE 3. **NELF and Pcf11 physically interact.** *A*, HEK293T cells were transfected with 5 μg of HIV-LUC and pcDNA3 vector control or pcDNA3-FLAG-NELF-B. *A*, luciferase assays were performed 48 h post-transfection to measure HIV transcription. These data are from triplicate transfections and are representative of three independent experiments. *B*, 48 h post-transfection, ChIPs were performed using FLAG, NELF-D, RNAP II, and Pcf11 antibodies, as indicated, and primers that spanned −45 to +72 of the HIV LTR were used for real-time PCR to detect factor association with the HIV LTR. These data represent triplicate ChIPs and are representative two experiments. *C*, Jurkat T cells were lysed, and precleared lysates were used for immunoprecipitation using a nonspecific antibody (*Control Ig*), anti-Pcf11, or anti-NELF-D antibodies. Immunoprecipitated extracts and 10% input controls were immunoblotted (*IB*) with Pcf11 and NELF D antibodies. Each immunoblot analysis was run on a single gel and processed as a single image. Lanes were rearranged for presentation purposes but were not individually modified. These data are representative of three coimmunoprecipitations (*IP*).

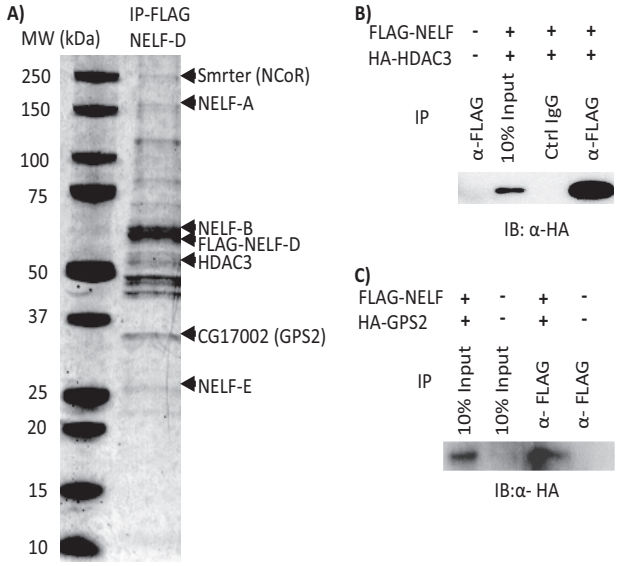

FIGURE 4. **Identification and function of the NELF-NCoR1-Gps2-HDAC3 complex.** *A*, nuclear extracts were prepared from FLAG-NELF-D transgenic *Drosophila* embryos, and the epitope tag was used to immunoprecipitate (*IP*) NELF complexes. Proteins were resolved by SDS-PAGE on 4–20% gels (Invitrogen) and visualized by Coomassie Blue staining. Bands were excised and digested with trypsin, and proteins were identified by mass spectrometry. Bands identified are indicated by *arrowheads* with human orthologs in *parentheses*. *B* and *C*, HEK293T cells were transfected with the indicated vectors or pcDNA3 control vector. Whole cell extracts were used for immunoprecipitation using a nonspecific antibody and anti-FLAG antibody or FLAG resin that pulls down NELF. Immunoprecipitates were immunoblotted (*IB*) with anti-HA antibody that detects HA-HDAC3 and HA-GPS2. Data represent three or more independent experiments.

ical pathway. Activating NELF- and/or Pcf11-deficient cells through CD3 plus CD28 led to an increase in HIV transcription that was comparable with siControl-treated cells, suggesting that both these proteins function to regulate basal proviral transcription and that their repressive activities are overcome by T cell activation (Fig. 2*F*). To explore NELF-Pcf11 functional interactions, we transiently expressed NELF-B in HEK293T cells. NELF-B was sufficient to inhibit HIV transcription (Fig. 3*A*) and facilitate the recruitment of other NELF factors as well as Pcf11 to the HIV LTR without a concomitant increase in RNAP II (Fig. 3*B*). These data suggest that NELF and Pcf11 repress HIV transcription by interacting with each other. To examine whether NELF and Pcf11 physically interact in the context of a T cell, Jurkat T cells were lysed, and Pcf11 and associated proteins were immunoprecipitated with a Pcf11-specific antibody. As shown in Fig. 3*C*, NELF-D coimmunoprecipitated with Pcf11. This interaction was validated by immunoprecipitating NELF-D to pull down Pcf11. Collectively, these data suggest that NELF recruits Pcf11 to the paused RNAP II to prematurely terminate transcription, thus reinforcing repression of HIV transcription.

NELF Interacts with the NCoR1-Gps2-HDAC3 Complex—The ability of NELF to interact with Pcf11 raises the possibility that NELF may recruit additional transcriptional repressors to the HIV LTR. Mass spectrometric analysis was used to identify potential factors that interact with NELF and contribute to HIV transcriptional repression. We took advantage of previously described transgenic *Drosophila* lines that expressed FLAG-tagged NELF subunits (34), assuming that key proteins that regulate RNAP II processivity are functionally and structurally conserved in flies and humans. Nuclear extracts from *Drosophila* embryos were immunoprecipitated using the epitope tag to enrich for NELF complexes (Fig. 4*A*). The immunoprecipitations from the different transgenic *Drosophila* lines yielded similar protein, as assessed by SDS-PAGE electrophoresis and Coomassie Blue staining (34). Furthermore, NELF subunits were efficiently coimmunoprecipitated with the FLAG antibody. For example, as shown in Fig. 4*A*, NELF-A, NELF-B, and NELF-E were all immunoprecipitated by FLAG-NELF-D, verifying that subunits known to be associated with the NELF complex were pulled down. Because the FLAG-NELF-D immunoprecipitations provided consistent protein yields and pulled down the other NELF subunits in proper stoichiometry, we used these extracts for the mass spectroscopy analysis. We were particularly interested in potential corepressors that interact with NELF and contribute to the maintenance of a repressed HIV transcriptional state. Potential transcriptional repressors that were identified included Smrter, CG17002, and HDAC3. The respective human orthologs of these proteins, NCoR1, GPS2, and HDAC3 have been demonstrated to form a corepressor complex (24). NCoR1 mediates transcriptional repression by nuclear receptors in part by recruiting and activating HDAC3, whereas GPS2 not only activates HDAC3 but inhibits Ras/MAPK signaling, potentially bridging chromatin changes with signal transduction (24). Furthermore, HDAC3 has been implicated in establishing and maintaining HIV latency (35, 36). Therefore, we investigated the physical and functional

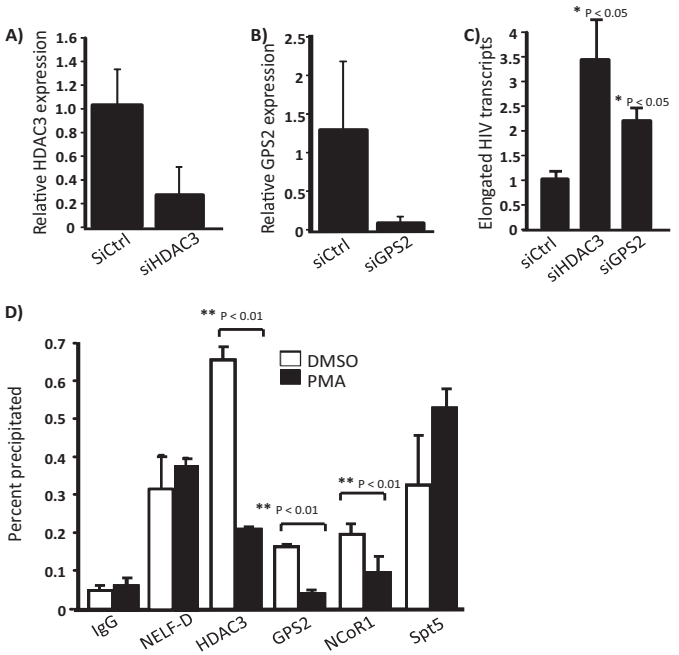

FIGURE 5. **NCoR1-Gps2-HDAC3 binds the proviral LTR and limits HIV transcription.** *A* and *B*, ACH-2 cells were transfected with siHDAC3 or siGPS-2, and mRNA transcripts of each molecule were measured 48 h post-transfection. *C*, HIV transcription was monitored 48 h post-transfection by quantitative real-time PCR for elongated HIV transcripts. Experiments were performed in duplicate, and data represent three independent knockdowns. *Error bars* are S.D. between duplicate data points. *, $p < 0.05$ as compared with the siControl transcripts. *D*, ChIP using chromatin prepared from untreated or phorbol 12-myristate 13-acetate-treated ACH-2 cells. Antibodies are indicated below the abscissa. Data are from a single experiment performed in triplicate, and *error bars* represent S.E. between these data points. These data are representative of at least three independent ChIP experiments. *DMSO*, dimethyl sulfoxide; *PMA*, phorbol 12-myristate 13-acetate.

interactions between this complex and NELF in human cells. Coimmunoprecipitation experiments in transfected HEK293T cells confirmed that NELF physically interacts with HDAC3 and GPS2 (Fig. 4, *B* and *C*). However, we were unable to demonstrate physical interactions between NELF and NCoR1 (data not shown). It should also be noted that Pcf11 was not detected by mass spectroscopy analysis, whereas NELF-D and NELF-E both pulled down Pcf11 from *Drosophila* extracts, reinforcing that NELF complexes with Pcf11 (data not shown).

Previous studies have shown HIV transcriptional repression to be regulated by proximal paused polymerase and chromatin reorganization in the ACH-2 T cell line (18, 37), a chronically infected cell line that can be induced to express HIV provirus. To investigate the role of the NCoR1-GPS2-HDAC3 complex in limiting HIV transcription, we used RNAi to diminish the expression of either HDAC3 or GPS2 in ACH2 cells. Depleting HDAC3 or GPS2 in ACH2 cells (Fig. 5, *A* and *B*), enhanced HIV transcription 2- to 4-fold in the absence of T cell activation, as measured by elongated HIV transcripts (Fig. 5*C*), supporting the conclusion that these factors are repressive to HIV proviral transcription. To determine whether NELF and NCoR1-GPS2-HDAC3 were associated with the repressed provirus LTR, chromatin was prepared from ACH-2 cells, and ChIPs were performed with antibodies against NELF-D, NCoR1, GPS2, and HDAC3. Fig. 5*D* shows that these factors occupied the 5′ HIV LTR. The observation that NCoR1 and HDAC3 bind repressed provirus LTRs is consistent with previous reports (35, 36, 38). Furthermore, activation of these cells with phorbol esters that induce HIV transcription diminished binding of NCoR1-GPS2-HDAC3 at the LTR (Fig. 5*D*). In contrast, the levels of NELF, which has been shown to be bound to transcriptionally active promoters (32, 39), and Spt5, which functions as a positive regulator (40), were not significantly changed by phorbol 12-myristate 13-acetate treatment. Taken together, these data suggest that NCoR1-Gps2-HDAC3 complex contributes to the repression of HIV transcription and, through interaction with NELF, couples RNAP II processivity with chromatin-mediated repression.

DISCUSSION

We show that NELF and Pcf11 interact to repress HIV transcription in $CD4^+$ T cells by regulating promoter proximal pausing and premature termination. Depleting NELF or Pcf11 in primary T cells increases HIV transcription, consistent with previous reports using cell lines (14, 17, 18), indicating that RNAP II and premature transcription termination have a general role in limiting HIV transcription. In addition, we suggest that NELF interacts with the NCoR1-Gps2-HDAC3 complex, providing a mechanism that couples promoter-proximal pausing, premature termination, and chromatin organization.

These data validate a critical role for NELF in limiting HIV transcription and suggest that it is required for the maintenance of HIV latency. Diminishing NELF in a heterogeneous population of infected primary cells, which included latently infected cells, enhanced HIV transcription. NELF directly regulates RNAP II processivity by interacting with a RNAP II-DSIF complex (34). The association of NELF and DSIF limits RNAP II processivity, which is overcome by P-TEFb-mediated phosphorylation of RNAP II, NELF, and DSIF (41, 42). Even though promoter-proximal pausing is an important determinant of HIV transcription, NELF and DSIF do not disengage paused RNAP II. The association of RNAP II with DNA is a stable interaction and requires active termination of transcription and eviction of RNAP II. Pcf11, which was originally identified as a protein complex involved in 3′ end processing of mRNA and transcription termination of protein-encoding genes (43–46), has been shown to be associated with promoter regions of several genes, including the HIV LTR (17, 18, 47, 48). Importantly, Pcf11 dissociates transcriptionally engaged RNAP II from DNA (16, 49). Our data suggest that Pcf11 targets paused RNAP II for termination by directly interacting with NELF. Coupling pausing and premature termination would favor a model in which NELF and Pcf11 act in the same biochemical pathway or belong to a multisubunit complex. This is consistent with our findings that NELF and Pcf11 coimmunoprecipitate and that depleting both NELF and Pcf11 does not further enhance HIV transcription elongation over depleting either protein alone. NELF-Pcf11 interactions could be further stabilized by physical interactions with the RNAP II carboxy-terminal domain and the nascent RNA.

Repression of HIV transcription has been associated with a nucleosome positioned at the transcription start site, and induction of HIV transcription correlates with histone modifications and displacement of this positioned nucleosome (5, 8,

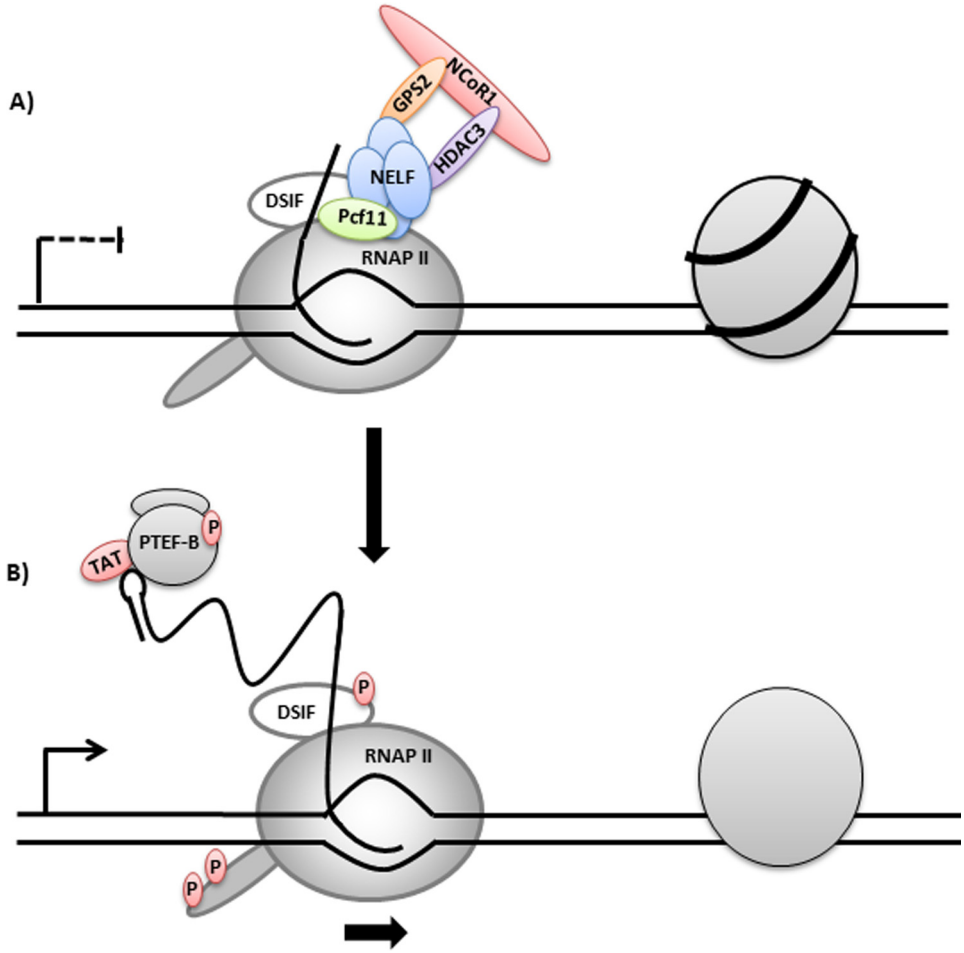

FIGURE 6. **Model highlighting how NELF and RNAP II pausing coordinates repression of HIV transcription.** See "Discussion" for details.

19). HIV transcription is activated by agents that inhibit histone deacetylases (HDAC), suggesting a critical role for chromatin in the repression of HIV transcription and latency (19, 50, 51). There have been several reports and clinical trials evaluating HDAC inhibitors as a means to purge the latent reservoir (52–57). HDACs are in part recruited to the HIV LTR through their interaction with transcription factors, including p50-p50 NF-κB homodimers, CBF, Sp1, and Myc (58–61). Our data suggest that pausing of RNAP II also facilitates the recruitment of corepressors that include HDAC. The coordinate regulation of RNAP II pausing and chromatin was first suggested when it was observed that diminishing NELF expression enhanced H3 and H4 acetylation and increased the restriction enzyme accessibility of the region protected by a positioned nucleosome (18). We show that NELF physically and functionally interacts with the corepressor complex NCoR1-GPS2-HDAC3. That this complex is relevant for repression of HIV transcription is suggested by binding of these factors at the HIV proviral LTR and the induction of HIV transcription when HDAC3 or GPS2 are diminished by siRNAs. This complex was originally identified as a transcriptional corepressor responsible for unliganded nuclear receptor transrepression (24). In addition, studies have shown that inhibition of HIV expression by nuclear receptors correlates with NCoR binding the LTR (38) and that HDAC3 is critical for repressing HIV transcription (35, 36). NCoR1 enhances HDAC3 activity, whereas GPS2 has been reported to inhibit Ras/MAPK signaling (24), which activates HIV transcription (62, 63). Therefore, recruitment of this complex to the HIV LTR would repress HIV transcription by altering chromatin as well as compromising signals necessary for efficient transcription. Additional corepressor complexes, such as Sin3A or co-repressor element-1 silencing transcription facto (CoREST), may recruit other HDACs to the HIV LTR (64, 65). It is interesting to note that several viral factors have been documented to interact with NCoR1-GPS2-HDAC3, including HTLV-1 Tax, bovine papillomavirus E2, and murine γ herpesvirus gene 50 (66–70). In the context of HIV, Vif has been shown by mass spectroscopy to interact with this complex (66). It is tempting to speculate that Vif may regulate transcriptional repression, possibly through targeted degradation of NCoR1-GPS2-HDAC3, to facilitate efficient HIV transcription, although the functional significance of these interactions and how it impacts virus replication, has yet to be determined.

We propose a model in which negative elongation factors are operative in a common pathway that limits HIV transcription and governs latency in infected primary $CD4^+$ T cells (Fig. 6*A*). NELF represses HIV transcription by at least two mechanisms: recruitment of Pcf11 and recruitment of the NCoR1-GPS-2-HDAC3 repressor complex. We propose that NELF allows for the coupling of these two mechanisms to facilitate strong

repression of HIV transcription, although additional experiments are required to determine whether this is a tripartite complex associated with the latent LTR or two independent mechanisms of repression. T cell activation induces signals that override NELF/Pcf11- and NELF/NCoR1-GPS2-HDAC3-mediated inhibition and, ultimately, enhances Tat-mediated recruitment of P-TEFb to the promoter, alleviating RNAP II pausing by phosphorylation of the RNAP II carboxy-terminal domain, NELF, and DSIF (Fig. 6*B*). This potential coupling of premature termination, promoter-proximal pausing, and post-translational modifications of the nucleosome has more general implications for the control of transcriptional elongation and provides a means to reinforce repression but allow for rapid induction of transcription. The HIV LTR offers a powerful tool to fully characterize the biochemical mechanisms operative in RNAP II pausing and how RNAP II initiation and chromatin intersect to regulate transcription processivity. More importantly, understanding the interplay between RNAP II pausing, premature termination, and chromatin organization may lead to new strategies to mobilize HIV from cellular reservoirs harboring latent HIV.

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