Poster Session Number: P-F1:
Abstract: 1360

Mechanisms by which Negative Elongation Factor (NELF) Establishes and Maintains HIV Latency.

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ABSTRACT

Background: Understanding events which contribute to HIV latency are critical for improving current treatments and potentially eradicating HIV infection. Events that contribute to HIV transcriptional latency include repressive chromatin structure, transcriptional interference, the inability of Tat to recruit P-TEFb and poor processivity of RNA polymerase II (RNAP II). Previously, we have established an important role for promoter proximal pausing in regulating HIV transcription. In this study we investigate whether RNAP II pausing is operative in the context of different cell models of latency, including transcriptional interference. Our data support that RNAP II pausing via NELF recruits co-repressor complexes and transcription factors which influence T cell commitment to repress HIV transcription.

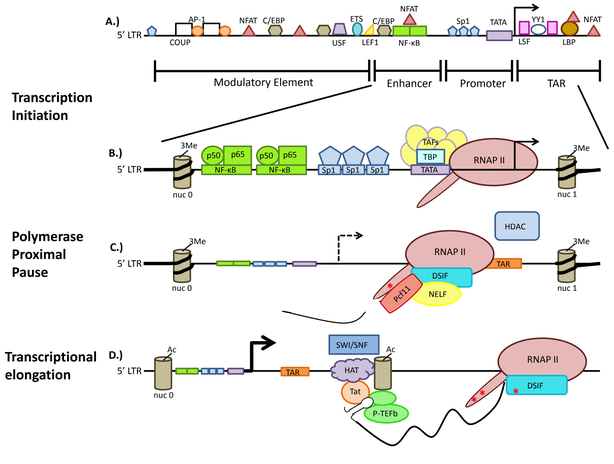
Methodology: We have identified novel NELF interacting factors via Mass Spec analysis and have used immunoprecipitation and ChIP analysis to verify physical and functional interactions. We utilized multiple infected cell models including HIV infected Jurkat T cells, the latently infected ACH2 cell line, latently infected transcriptional interference cell lines and primary CD4+ T cells to further investigate NELF's involvement in HIV transcriptional latency.

Results: We have identified novel NELF interacting partners NcoR1-GPS2-HDAC3, a known co-repressor complex, and Bcl11b, a T cell commitment factor. HDAC3, GPS2 and Bcl11b bind latent HIV LTR. When the latent HIV LTRs are induced with PMA binding of these factors to the LTR is diminished. In addition, we have investigated NELF's involvement in regulating HIV transcription in cell lines where the latent HIV LTR is integrated into a transcriptionally active gene, resulting in transcriptional interference of the provirus. Treatment of these cell lines with a HDAC inhibitor such as TSA did not induce HIV transcription, however, when we knock down NELF in this model HIV transcription is induced. Furthermore, overcoming HIV proviral transcriptional interference correlates with diminished NELF binding to the HIV LTR.

Conclusions: Our data indicate that NELF is coordinating multiple mechanisms responsible for transcriptional silencing of the HIV LTR. Key factors involved in NELF induced repression of the provirus are NcoR1-GPS2-HDAC3 and Bcl11b. NELF coordinates chromatin organization through the NcoR1-GPS2-HDAC3 complex and the recruitment of Bcl11b. These data support that RNAP II pausing is a major checkpoint in the establishment of HIV latency and a potential target by which to purge latent HIV reservoirs.

BACKGROUND

Regulation of HIV Transcription Initiation, Polymerase Proximal Pausing, Chromatin Regulation and Elongation.

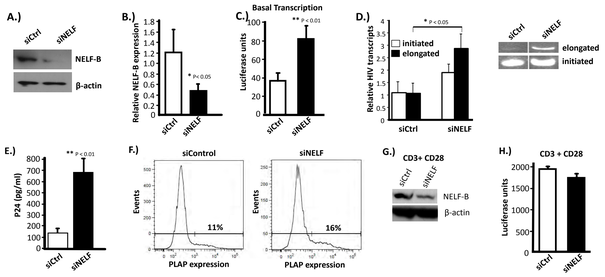


Transcription Schematic: Regulation of HIV transcription initiation and elongation. **A.)** HIV LTR organization. This only represents a small subset of cis-elements and transcription factors, which bind these sites. **B.)** Cellular transcription factors are recruited to LTR elements and initiation complex forms at the transcriptional start site. Nucleosomes are posttranslationally modified favoring a condensed chromatin structure that impedes RNAP II transcriptional elongation. **C.)** RNAP II processes a short distance downstream from the transcriptional start site when DSIF and NELF induce a pause in transcription. Pcf11 reinforces this block in elongation by prematurely terminating the transcription of the short nascent RNA product. HDAC recruitment to the paused complex reinforces a transcriptionally repressed chromatin state. The red asterisk depicts phosphorylation of RNAP II CTD at serine 5 position. **D.)** RNAP II elongation complex is released from the transcriptional pause by the recruitment of P-TEFb, which mediates hyperphosphorylation of the CTD at serine 2 position and phosphorylation of DSIF, which induces NELF disassociation from the complex (red asterisks indicate key phosphorylation events). The recruitment of chromatin remodeling machinery such as HATs and PBAF SWI/SNF facilitates acetylation of nucleosomes, which displaces the blocking nucleosome and supports transcription elongation.

RESULTS

Figure 1

Nelf Limits HIV Transcription and Replication in Primary CD4+ T Cells



This data has been published in J. Biol. Chem. 2013 Sep 6;288(36):25995-6003. doi: 10.1074/jbc.M113.496489

Figure 1: Human primary CD4+T cells infected with HIV-LUC were transfected with siControl (siCtrl) or siNELF-B. **A.)** NELF depletion was verified at 48 hpk by immunoblot. **B.)** q-PCR for NELF-B mRNA transcripts. **C.)** 48hpk luciferase activity was measured to monitor HIV transcription. **D.)** mRNA was isolated from HIV-Luc infected cells and used to measure initiated (+1 to +40) and elongated transcripts (+5396 to +5555), initiated and elongated data were normalized and set equal to 1 in siCtrl treatment, siNELF data reflects increased transcription over normalized siCtrl levels. **E.)**Cell culture supernatants from CD4+ T cells were used to measure virus particle release via ELISA 48 hpk. **F.)** CD4+ T cells were infected with HIV-PLAP with VSV-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 MFI value for siControl treatment is 6624, for siNelf treatment it is 7174. **G.)** 48hpk HIV-LUC infected CD4+ T cells were activated with anti-CD3 and anti-CD28 antibodies for 4 h, 12-16 hrs post stimulation whole cell lysates were immunobloted 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.

Figure 2

HIV Integration into Host Genes

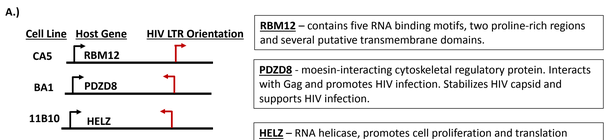


Figure 2: A.) Schematic of HIV provirus integration sites relative to neighboring cellular promoter for CA5, 11B10 and BA1 latently infected Jurkat cells. **B.) – D.)** Cell lines were treated with DMSO vehicle control or PMA (10ng/ml) + PHA (2ug/ml) for 24 hours, mRNA samples were collected and analyzed for gene expression of specific host gene and elongated HIV. **E.)** BA1 cell line was left untreated or treated with PMA (10ng/ml) + PHA (2ug/ml) for 8 hours, cells were then subject to formaldehyde crosslinking and sonication. Sheered chromatin was immunoprecipitated with a non-specific antibody (Flag) or specific antibodies for RNAP II and NelfD. ChIP samples were analyzed via qPCR with HIV specific transcriptional start site primers.

Figure 3

Nelf Limits HIV Transcription in Cells that Exhibit Transcriptional Interference

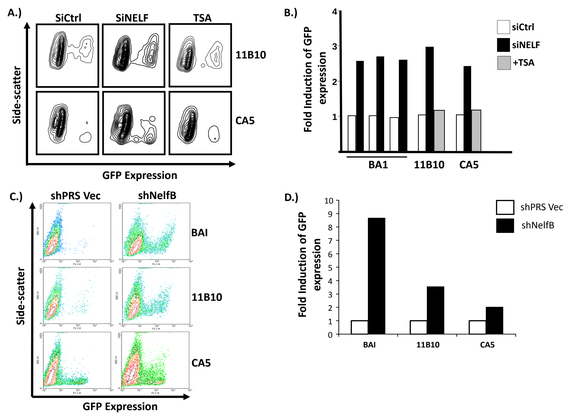
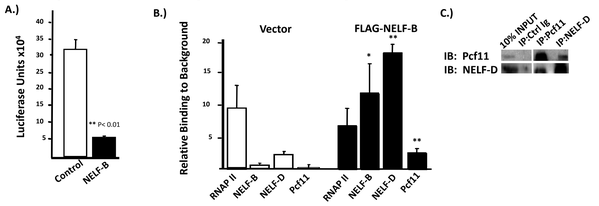


Figure 3: Cell lines were transfected with siControl or siNelf-B siRNA or treated with TSA for 72 hours. **A.)** GFP expression was measured by flow cytometry. **B.)** The fold induction of GFP expression over the siControl sample is graphed for multiple treatments of each cell line. Cell lines were transduced with shPRS Vector or shNelf-B specific Lentivirus, 72 hours post transduction **C.)** GFP expression was measured by flow cytometry. **D.)** The fold induction of GFP expression over the shPRS Vector sample is graphed for the representative experiment.

Figure 4

Overexpression of Nelf Represses HIV Transcription and Recruits Pcf11 to the HIV LTR.

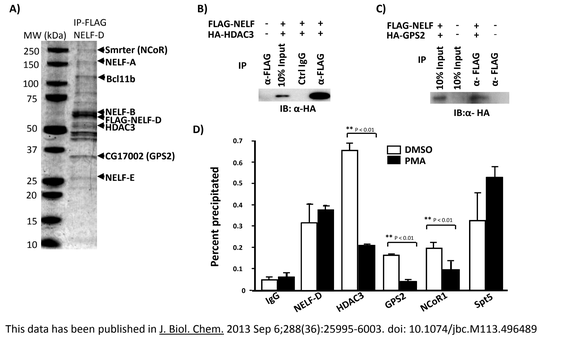


This data has been published in J. Biol. Chem. 2013 Sep 6;288(36):25995-6003. doi: 10.1074/jbc.M113.496489

Figure 4: HEK293T cells were transfected with 5ug HIV-Luc and pcDNA3 vector control or pcDNA3-FLAG-NELF-B. **A)** Luciferase assays were performed 48 h post-transfection to measure HIV transcription. **B)** 48 h post transfection, ChIPs were performed using FLAG, NELF-D, RNAP II and Pcf11 antibodies as indicated and primers that spanned -45-+72 of the HIV LTR. **C)** Jurkat T cells were lysed and precleared lysates were used for immunoprecipitation using a non-specific 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 IB was run on a single gel and processed as a single image. Lanes were rearranged for presentation purposes but were not individually modified.

Figure 5

Nelf Functionally Interacts with the NCoR1-GPS2-HDAC3 complex which Binds the Proviral LTR and Limits HIV Transcription



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Figure 5: A.) Nuclear extracts were prepared from FLAG-NELF-D transgenic *Drosophila* embryos and the epitope tag was used to immunoprecipitate 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 parenthesis. **B.)** & **C.)** HEK293T cells were transfected with indicated vectors or pcDNA3 control vector. Whole cell extracts were used for immunoprecipitation using a non-specific antibody and anti-FLAG antibody, or FLAG resin which pulls down NELF. Immunoprecipitates were immunoblotted with anti-HA antibody, which detects HA-HDAC3 and HA-GPS2. Data represent three or more independent experiments. **D.)** ChIP using chromatin prepared from untreated or PMA treated ACH-2 cells. Antibodies are indicated below the abscissa. Data are from a single experiment performed in triplicate and error bars represent the standard error between these data points. These data are representative of at least three independent ChIP experiments. * P < 0.05, ** P < 0.01.

Figure 6

Bcl11b Represses HIV Transcription

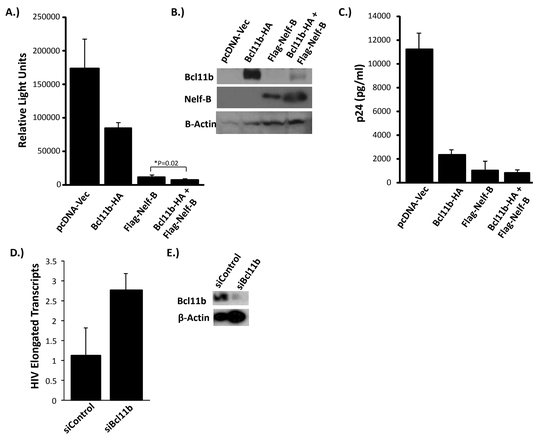


Figure 6: HEK293T cells were transfected with 2ug of pNL43-Luc DNA along with 2ug of pcDNA3-Vector or Bcl11b-HA or Flag-Nelf-B or both Bcl11b-HA + Flag-Nelf-B. At 48 hours post transfection **A.)** cells were lysed to measure HIV transcription via Luciferase expression. **B.)** Whole cell lysates were used to measure protein expression. **C.)** Viral supernatants were used to measure cell free p24 protein via ELISA. The latent cell line model Ach2 was transfected with siControl or siBcl11b specific siRNA, at 24 hours post transfection **D.)** mRNA was isolated and used to measure expression of HIV elongated transcripts. **E.)** Whole cell lysates were used to verify protein knockdown of Bcl11b.

Figure 7

Bcl11b Represses HIV Transcription in the CA5 Cell Line Model of Transcriptional Interference

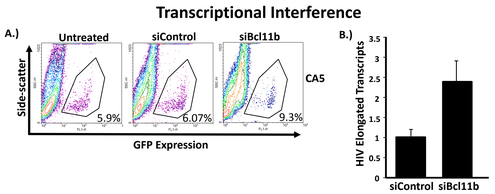


Figure 7: The CA5 cell line was transfected with siControl or siBcl11b specific siRNA. At 24 hours post transfection **A.)** GFP expression of HIV was measured by flow cytometry. **B.)** mRNA was isolated to measure elongated HIV transcripts.

SUMMARY

- Proximal pausing of RNAP II on the HIV-LTR is a key checkpoint controlling HIV transcription within multiple cell models of HIV latency which include CD4+ primary T cells, cell line models of transcriptional interference, and T cell latent HIV line Ach2.
- Releasing the paused polymerase by limiting Nelf results in the rebound of HIV transcription in cell line models of transcriptional interference irrespective of the control and activity level of the neighboring gene promoter.
- We have identified a functional interaction with Nelf and NCoR1-GPS2-HDAC3 corepressor which binds the transcriptionally silent HIV-LTR and are released upon reactivation of transcription.
- We have identified a novel Bcl11b interaction with the paused Nelf-RNA polymerase complex and have found Bcl11b to be a repressive factor in HIV transcription.

ACKNOWLEDGEMENTS

We would like to thank Dr. Olaf Kutsch (University of Alabama at Birmingham) for generously supplying the CA5, 11B10 and BA1 cell lines along with his insightful comments and critical data analysis. We would like to thank our long-term collaborator Dr. David Gilmour (Penn State) for his expertize and continual support. We would like to thank Dr. Chanhyo Lee for his experimental and intellectual efforts on the project. Flow cytometry was performed with assistance of the Boston University Medical Campus Flow Cytomety Core Facility. Mass Spec was performed at the Boston University Medical Campus Proteomics Core under the direction of Dr. Martin Steffen. This work was supported by NIH grants AI062467 and AI077463