The Pennsylvania State University

The Graduate School

Intercollege Graduate Degree Program in Immunology and Infectious Disease,
Huck Institute of Life Sciences

REGULATION OF HIV TRANSCRIPTION IN T CELLS

A Dissertation in

Immunology and Infectious Disease

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2010

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ABSTRACT

CD4+T cells are the primary target of HIV infection, and depletion in CD4+T cell count corresponds with disease progression leading to aquired immunodeficiency syndrome (AIDS). The current treatment for AIDS employs the administration of highly active antiretroviral therapy (HAART), which has not been successful in completely eliminating HIV infection since the virus persists in a subset of cells harboring latent provirus. A number of events contribute to HIV transcriptional latency including lack of transcription factors, repressive chromatin structure, epigenetic modifications of the DNA, the inability of Tat to recruit functional PTEFb or poor processivity of RNA polymerase II. Latent provirus can be reactivated when T cells are activated by signals downstream of the T cell receptor and the costimulatory molecule CD28. I have studied two facets of HIV transcription; how signals downstream CD28 induce HIV transcription and the role of NELF and Pcf11 in repressing HIV transcription by regulation transcription elongation.

Activation through the T cell receptor (TCR) and the costimulatory receptor CD28 supports efficient HIV transcription as well as reactivation of latent provirus. In order to characterize critical signals associated with CD28 that regulate HIV-1 transcription, I generated a library of chimeric CD28 receptors that harbored different combinations of key tyrosine (Y) residues in the cytoplasmic tail, Y173, Y188, Y191 and Y200. I found that Y191 and Y200 induce HIV-1 transcription via the activation of NF-κB and its recruitment to the HIV-long terminal repeat. Y188 modifies positive and

negative signals associated with CD28. Importantly, signaling through Y188, Y191 and Y200 is required to overcome the inhibition posed by Y173. CD28 also regulates PTEFb activity, which is necessary for HIV-1 transcription processivity, by limiting the release of PTEFb from the HEXIM1-7SK inhibitory complex in response to T cell receptor signaling. My studies reveal that CD28 regulates HIV-1 provirus transcription through a complex interplay of positive and negative signals that may be manipulated to control HIV-1 transcription and replication.

We have previously established an important role of negative elongation factor (NELF) in regulating HIV transcription in latent cell lines by inducing promoter proximal pausing. I have extended these studies by evaluating the role of NELF in infected T cell populations, including primary CD4+ T cells. Depleting NELF in HIV infected CD4+ T cells increased the release of virus. This increase in virus production corresponded to enhanced transcription elongation, emphasizing the role of NELF in maintaining a paused RNA polymerase II complex and limiting HIV transcription. Furthermore, I have shown that NELF interacts with Pcf11, a transcription termination factor. Pcf11 has been shown to cause premature termination of the paused elongation complex and I demonstrated that depletion of Pcf11 in CD4+T cells induces HIV elongation and transcription. In fact, depletion of both NELF and Pcf11 increases basal HIV transcription, suggesting that they act in concert in the same biochemical pathway. I propose that NELF recruits Pcf11 to the paused polymerase complex, coupling promoter proximal pausing with premature termination. These studies confirm that NELF mediated promoter proximal pausing and premature termination by Pcf11 are critical early check points in HIV transcription.

Overall, my studies provide new insights into the factors that regulate RNA polymerase II processivity and their overall impact on HIV transcription and latency, as well as identifying signals downstream of T cell activation which reactivate latent provirus. How these results may lead to the development of novel strategies to purge latent provirus from cellular reservoirs that harbor HIV are discussed.

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LIST OF ABBREVIATIONS

Ab……...Antibody

AICD……...Activation induced cell death

AIDS…… ……..……...Acquired Immunodeficiency Syndrome

AKT…….”Ak” transforming

AP……..…….……...Activator protein

APOBEC-3-G …Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G

ATF3……. Activating transcription factor 3

ATP……..……..……..Adenosine triphosphate

BAF……..…… Brahma associated factor

BRCA1……..…Breast cancer 1

Brd4…….…… Bromodomain-containing protein 4

BRG1…….Brahma-related gene 1

BSA……...Bovine serum albumin

CA……...Capsid

CCR5…….CC chemokine receptor 5

cAMP……..cyclic AMP

Cbl……Casitas B-lineage lymphoma

CD……..Cluster of differentiation

CD28RE……...CD28 response element

Cdc42……..Cell division cycle 42

cDNA……..Complementary DNA

C/EBP β……...CCAAT/enhancer binding protein β

CpG……Cytodine and guanine separated by a phosphate

CREB……cAMP response element-binding transcription factor

CRAC……..……...…….…..Calcium release activated calcium channel

c-Rel……...Cellular reticuloendotheliosis oncogene

CTD……...Carboxyterminal domain

CTLA-4…….……...Cytotoxic T lymphocyte associated granule serine protease 4

CTR1…….…….…… C-terminal repeat 1

CXCR4……...CXC chemokine receptor 4

DC……..Dendritic cells

DCAF1…….. Vpr binding protein

DMEM……..Dulbecco's modified Eagle's medium

DMSO……..Dimethylsulfoxide

DRB…….5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole

DSIF…….…..DRB sensitivity inducing factor

DTT……..Dithiothreitol

EDTA……..Ethylenediaminetetraacetic acid

EGTA……..Ethyleneglycolbis(2-aminoethylether)N,N,N'N'-tetraacetic acid

ELISA……Enzyme-linked immunosorbent assay

EMSA…….Electromobility shift assay

ER…….Endoplasmic reticulum

ERα…….....Estrogen receptor α

ERK…….Extracellular response kinase

ESCRT……...Endosomal sorting complex required for transport

FCS……...Fetal calf serum

FITC……Fluorescein isothiocyanate

Fyn……..Feline yes-related novel gene

GATA3……GATA transcription factor 3

GCN5……General control nonderepressible

GFP……...Green fluorescent protein

gp……..Glycoprotein

GPI……..Glycosylphosphatidylinositol

GSK……...Glycogen synthase kinase 3

Grap2…….....GRB2 related adapter protein

GTP……..Guanine triphosphate

HAART……Highly active antiretroviral therapy

HAT……...Histone acetyltransferase

Hck……. ……...…….Hemopoietic cell kinase

HDAC……Histone deacetylase

HEPES……...4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

HEXIM1…… HMBA Inducible protein 1

HIV-1…….Human Immunodeficiency Virus type 1

HIV-LTR……Human Immunodeficiency Virus-Long terminal repeat

HMBA……...…….. HexaMethylene BisAcetamide

HMG-I……High mobility group protein- I(Y)

HSF……..Heat shock factor

Hsp70……..Heat shock protein 70

ICOS……..Inducible costimulator

IL……..Interleukin

IκB……...Inhibitory κB

IKK…….. IκB kinase

IN…….Integrase

INI1……..Integrase interactor 1 protein

IRES……..Internal ribosomal entry site

ITAM……....Immunoreceptor tyrosine-based activation motif

Itk……...IL-2 inducible T cell kinase

J-Lat…….…… Jurkat- latent

JNK…….Jun N-terminal kinase

LAT……...Linker for activation of T cells

Lck……..Lymphokine-specific protein tyrosine kinase

LEDGF …… …… ……..…… lens epithelium-derived growth factor

LEF1……...Lymphoid enhancer-binding factor 1

LFA-1……Leukocyte function associated antigen 1

LPS…….….lipopolysaccharide

Luc……Luciferase

MA……..Matrix

MAPK……...Mitogen activated protein kinase

MEK1……..Mitogen activated ERK-activating kinase

MEKK……MEK kinase

MFI…….Mean fluorescent intensity

MHCI..……Major histocompatibility complex I

mRNA…….Messenger RNA

NC……Nucleocapsid

Nef…….Negative factor

NELF…….. Negative elongation factor

NFAT…….……Nuclear factor of activated T cells

NF-κB…….……...Nuclear factor κB

NP-40……....Nonidet P-40

NRE……..Negative regulatory element

Nuc-0…..……....Nucleosome 0

Nuc-1…..……Nucleosome 1

P300/CBP…….p300/CREB-binding protein

PBS……...Phosphate-buffered saline

PCAF…….p300/CREB-binding protein (CBP)-associated factor

PCR……..Polymerase chain reaction

PD-1……...Programmed cell death protein 1

PHA……...Phytohemagglutinin

PMSF……...Phenyl-methylsulfonyl fluoride

PI3K……...Phosphatidylinositol-3-kinase

PIC……....Preintegration complex

PI(4,5)P2……Phosphatidylinositol (4,5) biphosphate

PKC…….Protein kinase C

PLAP…….Placental alkaline phosphatase

PLC…….Phospholipase C

PMA……..Phorbol myristate acetate

PR…….Protease

Ptef-b…… Transcriptional elongation factor b

RNA……Ribonucleic acid

RNA Pol II…….RNA Polymerase II

RPMI…….Roswell Park Memorial Institute

RRM…….. ……..RNA recognition motif

RSV……Rous sarcoma virus

RT……Reverse transcriptase

RTC……...Reverse transcriptase complex

RT-PCR……Reverse transcriptase polymerase chain reaction

Scid-hu……Severe combined immunodeficiency-human

SDS……..Sodium dodecyl sulfate

SDS-PAGE……..Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH2……...Src-homology 2

SH3……...Src-homology 3

SKIP…… ……...splicing-associated c-Ski-interacting protein

SLP-76……SH2 domain-containing leukocyte protein

Sp1…… ……Specificity protein 1

STAT5……Signal transducer and activator of transcription

SWI/SNF……..……... SWItching/Sucrose Non Fermenting

TAR……...Transactivating region

TAT……..…… Trans-Activator of Transcription

TBP……TATA binding protein

TCR……...…….T cell receptor

TFIID (TAF1)…….....TBP associated factor

TFIIH…….Transcription Factor II H

TM……Transmembrane

TNFα……...……...Tumor necrosis factor α

TsA…….Trichostatin A

Tsg101……..Tumor Susceptibility gene 101

UTR……Untranslated region

Vif…….…… Viral infectivity factor

VLP……...Virus-like particle

Vpr…… Viral protein R

Vpu……..……..Viral protein U

VSV-G……Vesicular stomatitus virus glycoprotein

YY1……...Yin and yang 1

ZAP70……....ζ-associated protein70

ACKNOWLEDGEMENTS

I want to begin by expressing my gratitude to Andy for his guidance, mentorship and patience and most importantly for being an ideal 'guru'. He has taught me by example, and has set the bar very high! He has instilled optimism in me on several occasions, and I have learnt from is "glass is half full" ideology. My stay in the Henderson Lab was enjoyable thanks to all the cheerful and helpful Hendersonians. I would like to thank all the past members of the lab, especially Polung and Amy for helping me settle down during my initial days and providing me with the right tools to get me started. I thank my current lab members for always being supportive and patient with my eccentricities and drama. Thanks to Gill for all the scientific and non-scientific conversations that I looked forward to every day. I thank Daniele for the impromptu dinners that have uplifted my spirits on days when I most needed it and for introducing me to the American culture!

I want to acknowledge the members of my dissertation committee Dr. Avery August, Dr. David Gilmour, Dr. Pamela Hankey and Dr. Anthony Schmitt for their scientific inputs and collaborations.

This work would not have been possible without the support and blessings of my family. I am extremely thankful to my Amma and Appa for believing in me and standing by me no matter what. I would not have accomplished this if it were not for them. I also

thank my family for keeping me grounded and helping me keep things in perspective. I would especially want to thank my sisters Malathi and Vidya for their friendship and for being there for my family on occasions when I could not. I thank my nieces Jahnavi and Hamsini for being my bundles of joy and my rays of hope during the gloomy times. I also want to thank my Maa for being supportive and understanding and having faith in my decisions.

I want to thank all my friends in the US for making this my home away from home, for putting up with my antics and for being good listeners. I want to especially thank Vidya for standing by me in all my hardships and being the person that I could always count on. I thank Bhavana, Abhijit and Deepali for being my buddies from the early times and their continuous support and unparalleled friendship. I thank Manasvi for making my move to Boston easy, something that I never thought would be possible.

Last but not the least, I want to thank my husband and best friend Arnob for being patient with me, and putting up with my mood swings and idiosyncrasies. He has been with me through all the ups and downs of life and has been the rock that I could always count on. I also want to thank him for being a constant source of encouragement and my inspiration to work hard and give my very best always!

Chapter 1

Introduction

1.1 HIV/AIDS Overview

Acquired Immunodeficiency syndrome (AIDS) is a global epidemic affecting approximately 40 million people worldwide. The first documented case of AIDS was in 1981 (Stevenson, 2003). AIDS is a multifactorial systemic disease that ultimately compromises the immune system, rendering it incapable of fighting opportunistic infections and rare cancers that directly contribute to the morbidity and mortality of AIDS. AIDS is caused by the human immunodeficiency virus (HIV). The current treatment for AIDS is highly active antiretroviral therapy (HAART), a cocktail of drugs which targets and inhibits different steps of viral replication, decreasing viral load. The use of HAART has helped in treatment of AIDS, and reversal of symptoms, however, it has not been successful in curing AIDS due to the persistence of HIV in latent reservoirs. Since HAART treatment needs to be administered for the lifetime of the patient, this can lead to drug resistance and the accumulation mutations in the virus enabling viral persistence and spread (Mayers, 1997).

1.2 HIV Life cycle

HIV belongs to the lentiviral family of retroviruses. The viral particle contains two copies of single stranded RNA genome, and viral proteins, reverse transcriptase

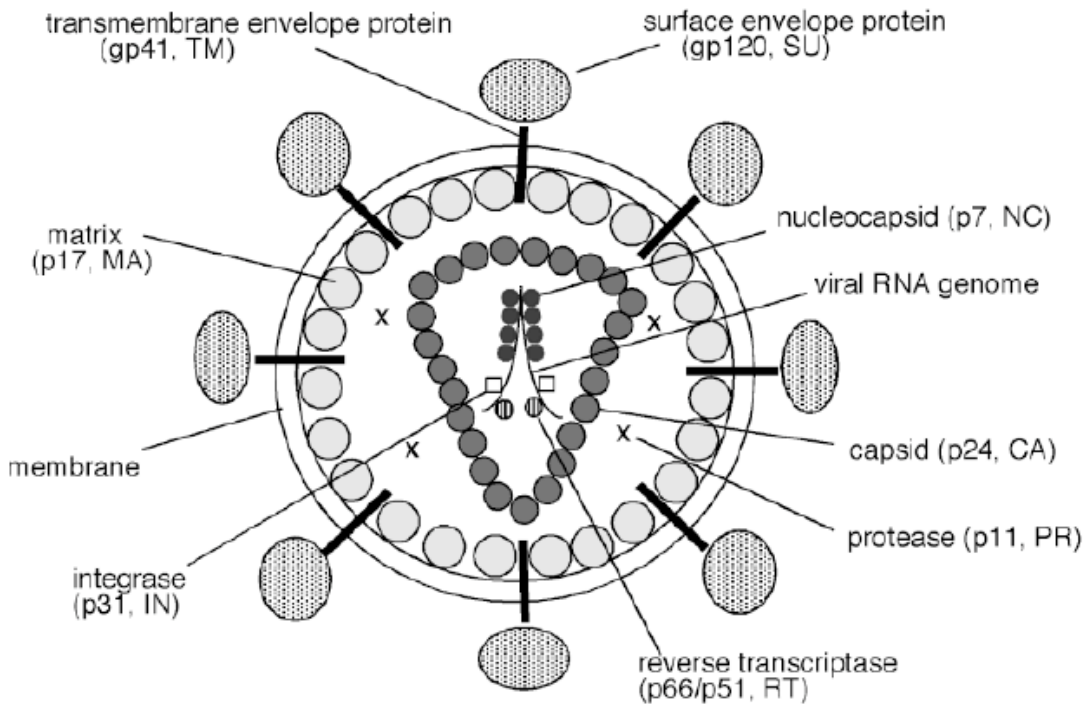


Figure 1.1: Structure of HIV **(Freed, 1998)**

(RT), integrase (IN) and protease (PR) encapsulated in the capsid. The capsid is surrounded by the viral matrix, which in turn is covered by the viral envelope (Fig 1.1).

The viral life cycle begins when the envelope binds to the cell membrane of a target cell. The envelope consists of two polypeptides, gp120 which specifically binds to CD4 on the target cell and the fusogenic peptide gp41. Upon binding CD4, gp120 undergoes a conformational change that leads to its interaction with chemokine receptors expressed on the host cell surface (Kwong et al., 1998). A number of chemokine

receptors have been identified as coreceptors for HIV in cultured cells, however, CCR5 and CXCR4 are the most widely used coreceptors by the virus (Berger et al., 1999).

Binding to the chemokine coreceptors promotes additional conformational changes exposing gp41, which mediates virus fusion and entry. Upon entry, uncoating takes place revealing the viral genome and associated viral proteins to the host cell cytoplasm. Following uncoating, reverse transcription of the ssRNA genome generates complementary DNA. Reverse transcription is mediated by the reverse transcription complex which is comprised of two strands of viral RNA, viral proteins- integrase (IN), reverse transcriptase (RT), nucleocapsid (NC), matrix (MA), viral infectivity factor (vif) and viral protein R (vpr) as well as cellular proteins including cytoplasmic histones (Karageorgos et al., 1993). The overall result of reverse transcriptase and associated factors is the generation of the complementary DNA flanked by two direct repeats or long terminal repeats (LTR) (Greene and Peterlin, 2002). The RT has a high error rate and is responsible for incorporation of mutations in the viral genome which can either cripple the virus or make the virus more potent thus altering HIV biology (Menendez-Arias, 2002). In addition, some viral genetic variants might be more resistant to antivirals than other variants, allowing them to thrive even when HAART is administered (Wainberg et al., 1993).

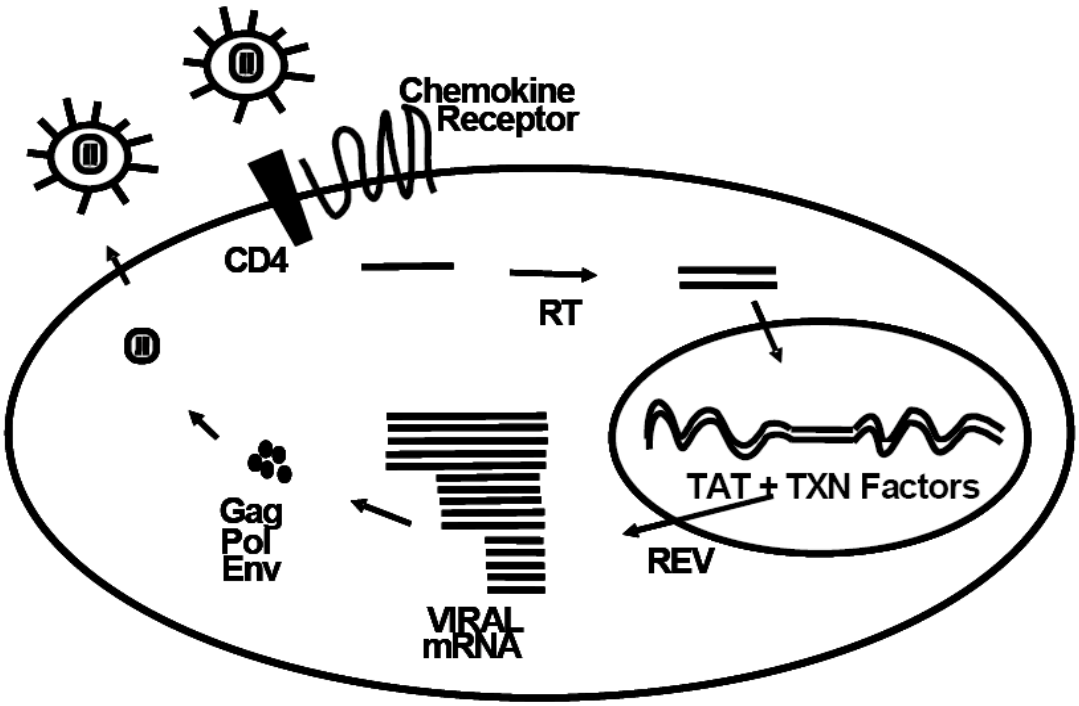


Figure 1.2: HIV life cycle

Once reverse transcription is completed, RT dissociates from the complex and the viral genome is integrated into the host genome. This is facilitated by the pre-integration complex (PIC), which contains the RT complex, minus the RT, newly synthesized cDNA and host proteins including importins, the high mobility group DNA binding proteins (HMG-1) (Miller et al., 1997), and lens epithelium-derived growth factor (LEDGF) (Llano et al., 2006). The importins and viral protein U (vpu) facilitate the nuclear import of the PIC followed by integration of the viral DNA into the host genome. Once integrated, the HIV DNA behaves like an endogenous gene, and is regulated by binding of initiation and transcription factors to the LTR which acts like a gene promoter and transcriptional regulatory element.

HIV transcription is a highly regulated process involving the viral protein trans-activator of transcription (Tat) in conjunction with several host protein complexes and transcriptional regulators (Kao et al., 1987). A number of transcription factors including nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), specificity protein 1 (Sp1), CCAAT/enhancer binding protein (C/EBP) and activating protein 1 (AP-1) bind to the LTR (Pereira et al., 2000), RNA polymerase II (RNA pol II) is recruited and transcription is initiated. Tat is an HIV encoded activator of transcription, and binds to a RNA stem loop, the Tat transactivating region (TAR), in the 5 prime region of a newly synthesized viral transcript. Tat stimulates transcription by recruiting the PTEFb complex which phosphorylates the C terminal domain (CTD) RNA pol II and facilitates processive transcription elongation (this is discussed in greater detail below).

The viral genome is approximately 9kb long and encodes nine genes (*gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*, and *tev*) that generate 19 proteins. This is achieved by use of multiple reading frames for transcription. In addition, the viral transcript is multiply-spliced and shuttled to the cytoplasm for translation and generation of viral accessory proteins nef, tat and rev. The unspliced viral transcripts are translated by the host machinery to generate the viral polypeptide. This polypeptide is cleaved to give rise to the structural proteins Gag and Env. The splicing of the viral mRNA is key in regulating the amount of spliced verses unspliced mRNA, in other words the amount of accessory proteins verses structural proteins. Splicing is controlled by the HIV protein Rev which binds to the Rev response element (RRE) in the unspliced viral transcripts and exports them to the cytoplasm for translation. Thus by regulating the location of the HIV transcript rev controls splicing. In summary, regulatory proteins tat, and rev controls the

quality and quantity of HIV transcripts produced thus being essential proteins for successful HIV replication (Greene and Peterlin, 2002).

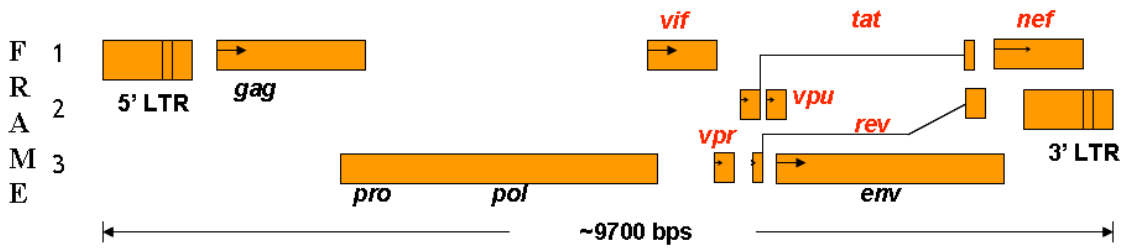


Figure 1.3: HIV genome. HIV-1 encodes for nine proteins are divided into three categories- structural proteins (gag, pol, env), regulatory proteins (tat and rev) and accessory proteins (nef, vpu, vpr and vif).

HIV assembly takes place at cholesterol rich regions in the plasma membrane where the gag-pol polyprotein, the gag precursor (Pr55Gag), env glycoprotein and the viral RNA are incorporated into a structurally defined virion (Campbell et al., 2001; Liao et al., 2001). The assembly of HIV is completed when this immature virion buds from the cell. The cellular endosomal sorting machinery, including the ESCRT-1 complex (for endosomal sorting complex required for transport) and associated proteins are recruited to budding sites via interactions with gag to facilitate HIV egress (Gomez and Hope, 2005). Post budding, the virion matures when the PR cleaves the Pr55Gag to generate four protein domains matrix (MA), capsid (CA), nucleocapsid (NC), p6 and two spacer peptides, SP1 and SP2. This cleavage event triggers the transition of an immature non-infectious particle to a mature infectious virion (Greene and Peterlin, 2002; Kinoshita et al., 1998).

1.3 Accessory proteins

The HIV accessory proteins - negative factor (nef), vif (virion infectivity factor), vpr and vpu interact with, and exploit cellular pathways to facilitate virus replication and abate host restriction (Malim and Emerman, 2008) .

Nef, is a myristoylated protein and is membrane associated. Nef binds to the cytoplasmic domain of CD4 at the plasma membrane, and targets CD4 for degradation by recruiting adaptor protein-2 (AP-2) and upregulating clathrin mediated endocytosis (Lindwasser et al., 2008). Nef also downregulates major histocompatibility complex class I (MHC I) and T cell receptor (TCR)-CD3 complex expression by altering protein trafficking (Roeth and Collins, 2006). In addition, Nef has been implicated in regulating cytoskeleton organization, chemotaxis and signal transduction (Fackler et al., 1999; Malim and Emerman, 2008; Swingler et al., 1999). Nef physically and functionally interacts with several kinases, in particular, members of the Src kinase family (Dutartre et al., 1998). This could be responsible for the multitude of nef functions; however, the significance of these interactions in regard to HIV replication and pathogenesis is still not well understood.

Vpu facilitates the proteosomal degradation of CD4 by recruiting the Cullin1-skp1 ubiquitin ligase to the cytoplasmic region of endoplasmic recticulum (ER) associated CD4 (Margottin et al., 1998; Schubert et al., 1998). Recently, Vpu has been shown to overcome an interferon-alpha induced block to virus release. Vpu enhances HIV release by targeting the intracellular restriction factor tetherin (Neil et al., 2006; Neil et al., 2007; Neil et al., 2008; Van Damme et al., 2008).

Vif also overcomes intracellular restriction factors, in this case by targeting apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC-3-G),

which limits the ability of HIV to establish infection. APOBEC is a family of editing enzymes which mutate polynucleotides by deaminating cytidine (C) to uridine (U). HIV packaged in the absence of vif, contains APOBEC-3-G and upon infection and reverse transcription, the cytosine residues are changed to uridine residues in the nascent minus strand leading to G to A hypermutation in the plus strand sequence resulting in a loss of viral genetic integrity. Vif, binds APOBEC-3-G and cullin 5-elonginB/C complex, and targets APOBEC-3-G for proteosomal degradation ensuring that the packaged virus is free of APOBEC-3-G (Malim and Emerman, 2008).

Vpr is incorporated into the virion, and has been shown to lead to cell cycle arrest at the G2 phase via its interaction with the Vpr binding protein (DCAF1): cullin family of E3 ubiqutitn ligases (DeHart et al., 2007). Vpr is also involved in the nuclear import of the PIC (Suzuki et al., 2009).

1.4 Cellular targets of HIV

HIV-1 primarily infects cells of the immune system expressing the viral receptor CD4, and coreceptors CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4). These include monocytes, macrohpages, dendritic cells (DC) and CD4+T cells which are the primary targets of HIV infection. The coreceptor used by the virus to bind gp120 determines viral tropism of the virus which could be R5 or X4 tropic. In general, macrophage infection is mediated by CCR5 whereas T cells are infected by R5 and X4 viruses.

DC and macrophages have been reported to either support HIV replication or facilitate HIV infection, whereas circulating monocytes are resistant to HIV infection.

DCs do not efficiently support HIV replication but can capture HIV and deliver it to other susceptible cells like T cells, a process known as trans-infection. Macrophages are infected by HIV but poorly express HIV leading to speculation that tissue resident macrophages are critical HIV reservoirs in various organs and tissues (Regoes and Bonhoeffer, 2005).

All subsets of CD4+T cells including naïve (CD45RA+/CD62L+) and memory (CD45RO+) CD4+T cells, are susceptible to HIV infection (Brenchley et al., 2004; Ostrowski et al., 1999; Stevenson, 2003). Infection of T cells leads to dramatic declines in T cell number and immunodeficiencies. Why HIV kills T cells is still somewhat controversial. HIV infection of T cells is cytotoxic and directly kills infected cells. Infected cells also tend to fuse or form syncytium in vitro although the physiological importance of syncytium is highly controversial (Camerini and Seed, 1990; Levy, 1993). In addition, HIV infection can indirectly target T cells to contribute to overall decline in T cell subsets. For example, HIV infection has been shown to induce apoptosis by increasing Fas ligand expression (Stevenson, 2003). Similarly, HIV has been proposed to initiate a potent lymphocyte response that may exhaust T cell populations (McCune, 2001).

1.5 T cell activation

T cell activation affects multiple steps in the HIV life cycle and is a prerequisite for productive virus replication (Jung et al., 1995; Oswald-Richter et al., 2004; Tong-Starkesen et al., 1989). Upon successful integration of virus into the host genome, T cell activation is indispensable for HIV transcription (Wong et al., 1997a). T cell activation is

necessary for naïve T cells to initiate an immune response and typically requires two signals (Ledbetter et al., 1990). The first signal emanates from the T cell receptor, which recognizes the antigenic peptide presented by MHC on antigen presenting cells (APC). This initial signal is amplified by cotimulatory signals, the best characterized being CD28 which binds its ligands CD80-CD86 (also known as B7.1 and B7.2) expressed on the APCs. In the absence of costimulation, the T cell becomes anergic by entering a state of non responsiveness (Bonnevier and Mueller, 2002; Orchansky and Teh, 1994; Powell et al., 2001). Downstream of the TCR and CD28 a multi-step signaling cascade is initiated, which eventually leads to the activation and binding of transcription factors to target cytokine genes such as interlukin-2 (IL-2), interlukin-4 (IL-4) and interferon gamma (IFN-γ).

1.5.1 TCR

The TCR is a multi-protein complex comprised of the TCR and the associated CD3 complex. TCR is composed of two separate peptide chains, T cell receptor alpha and beta (TCRα and TCRβ). This binds to the peptide bound MHC, but has a small cytoplasmic domain, incapable of intracellular signaling. Intracellular signaling in response to ligand binding is carried out by the TCR associated CD3 proteins, which contain CD3εγ and CD3εδ heterodimers and a CD3ζ homodimer, which has a total of six immunoreceptor tyrosine-based activation (ITAM) motifs. In vitro, TCR activation can be mimicked by cross-linking CD3 by using antibodies (Favero and Lafont, 1998; Qian et al., 1993).

1.5.2 CD28

The CD28 family is comprised of CD28, Inducible costimulator (ICOS), Programmed cell death protein 1 (PD-1) and Cytotoxic T lymphocyte associated granule serine protease 4 (CTLA-4) (Sharpe and Freeman, 2002). CD28 is the only costimulatory molecule which is constitutively expressed on T cells. All single mature and single positive thymocytes express CD28, whereas developing cells in the thymus do not (June et al., 1990). CD28 knock out mice and mice treated with antagonists of CD28 show a decrease in immune activation and defects in mounting immune responses to pathogens and allograft antigens (King et al., 1996; Mittrucker et al., 2001; Shahinian et al., 1993; Via et al., 1996).

CD28 is a disulphide linked homodimer of approximately 90kDa (Hara et al., 1985). It consists of an extracellular domain (134 aa), a 27 amino acid transmembrane domain and a 41 aa cytoplasmic domain. The cytoplasmic domain of CD28 is sufficient for induction of IL-2 production in the context of TCR signaling (Stein et al., 1994). There is a high degree of sequence similarity between the cytoplasmic domain of mouse verses human CD28 (Gross et al., 1990). The cytoplasmic domain consists of two proline rich regions P190YAP and P178 RRP, which can bind to SH3 domain containing proteins. It also has four tyrosine residues -Y173, Y188, Y191 and Y200 which are phosphorylated to recruit src homology 2 (SH2) containing proteins (King et al., 1997; Teng et al., 1996). The cytoplasmic domain of CD28 is highly conserved in the mouse and human underscoring that this domain is critical for normal immune function.

Figure 1.4: Cytoplasmic domain of CD28. The four tyrosine residues are in blue. The proline rich region, which binds SH3 domain containing proteins, is underlined in green.

CD28 ligands belong to the B7 family of cell surface receptors. CD80 and CD86 are expressed by professional antigen presenting cells. Both these ligands are structurally homologous and differ in their expression patterns. The expression of both these ligands is upregulated upon tissue injury, infection or the establishment of T cell-APC contact. CD80 and CD86 do seem to have partially overlapping functions, with a major difference being that CD86 has been implicated in initiating the immune response as it is expressed on resting APCs , whereas CD80 which is expressed later on activated APCs , modulates the immune response (Acuto and Michel, 2003).

One of the major functions of CD28 mediated costimulation is cell proliferation via production of IL-2 and upregulation of IL-2 receptor expression. CD28 signaling is necessary for the generation of effector CD4 T cells (T helper) and clonal expansion of the cytotoxic CD8 T lymphocytes (Alegre et al., 2001). In addition CD28 signaling increases D family of cyclin dependent kinases (cdk), and promotes the degradation of cyclin dependent kinase inhibitor (kip) (Appleman et al., 2002; Boonen et al., 1999). CD28 also upregulates the expression of an anti-apoptotic factor BCL-XL, ensuring cell survival (Boise et al., 1995).

More recently, a role for CD28 signaling in regulating the chromatin structure and epigenetic changes that contribute to cell growth and differentiation is becoming

appreciated. In naïve T cells, nuclear translocation of Brahma-related gene 1 (BRG1) associated factor (BAF), a SWI/SNF related ATP-dependent chromatin remodeling complex, ten minutes post activation has been observed (Henderson et al., 2004; Zhao et al., 1998). In addition, 60 minutes post activation chromatin remodeling at the IL-2 promoter was observed (Attema et al., 2002; Rao et al., 2003).

1.5.3 Costimulation

CD28 primarily seems to amplify the TCR signaling, however the initiation of signals via both these receptors are different. One of the major differences is the rate of interaction of the receptor with the ligand. The on-rates for TCR signaling are low for TCR-MHC interaction probably due to an "induce fit" mode of interaction, whereas CD28-B7 interactions are fast due to the "lock and key" nature of ligand binding (Acuto and Michel, 2003; van der Merwe and Davis, 2003). Also, the number of ligated CD28 receptors is larger than the number of TCR involved in the formation of an immunological synapse (IS). An IS is formed at the interface of a an interacting APC and T cell, where receptor-ligand interactions between the two cells initiates T cell signaling.

Binding of the peptide-MHC complex to TCR activates signals via ITAMs in CD3, whereas signals through CD28 are transduced through tyrosines and proline rich motifs in its cytoplasmic tail, comprised of 41 amino acids (Acuto and Michel, 2003). T cell activation by the TCR is initiated by the phosphorylation of Src kinases- Lck and Fyn. Activation of Lck via phosphorylation targets Syk family kinase ZAP-70, linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein of 76 kD, (SLP76). A multi-protein complex is formed at CD3, leading to the activation of

phospholipase C γ (PLCγ). This in turn initiates calcium signaling and Ras activation, leading to activation of low levels of AP-1 and NFAT (Acuto et al., 2003). CD28 recruits few downstream molecules including phosphoinositide-3 kinase (PI3K), IL-2 inducible T-cell kinase (ITK), Grb2 and Grb2-homologous adapter protein (Gads) to the membrane (August et al., 1994; August et al., 1997; Kim et al., 1998; King et al., 1997). PI3K is recruited only to CD28, whereas the other signaling intermediates and adapters are activated by both CD3 and CD28. CD28-mediated activation of PI3K activates protein kinase B (PKB), p38, c-Jun N-terminal kinase (JNK), serine threonine kinases, protein kinase θ (PKC θ), Cot, and guanine nucleotide exchange factor (GEF) vav (Kane et al., 2002; Lin et al., 2000; Lu et al., 1998; Michel et al., 2000). These signals act synergistically with TCR signaling to activate NFκB (Kane et al., 2002), NFAT (Diehn et al., 2002) and AP-1 transcription factors (Rincon and Flavell, 1994) in turn controlling cell proliferation, differentiation and death by regulating transcription of key genes. In HIV infected cells the transcription of the provirus is responsive to these signals.

1.6 T cell activation and HIV

Since T cell activation regulates multiple steps of the HIV life cycle, it is obvious that proteins participating in different aspects of signaling will impact virus replication. For example, the non-receptor tyrosine kinase Lck, targets gag to the plasma membrane, and inhibition of Lck promotes the accumulation of intracellular virus particles (Strasner et al., 2008). Furthermore, depletion of Itk inhibits HIV by affecting multiple stages of the viral life cycle including viral entry, which correlates with a loss in actin polarization, viral transcription and release (Readinger et al., 2008).

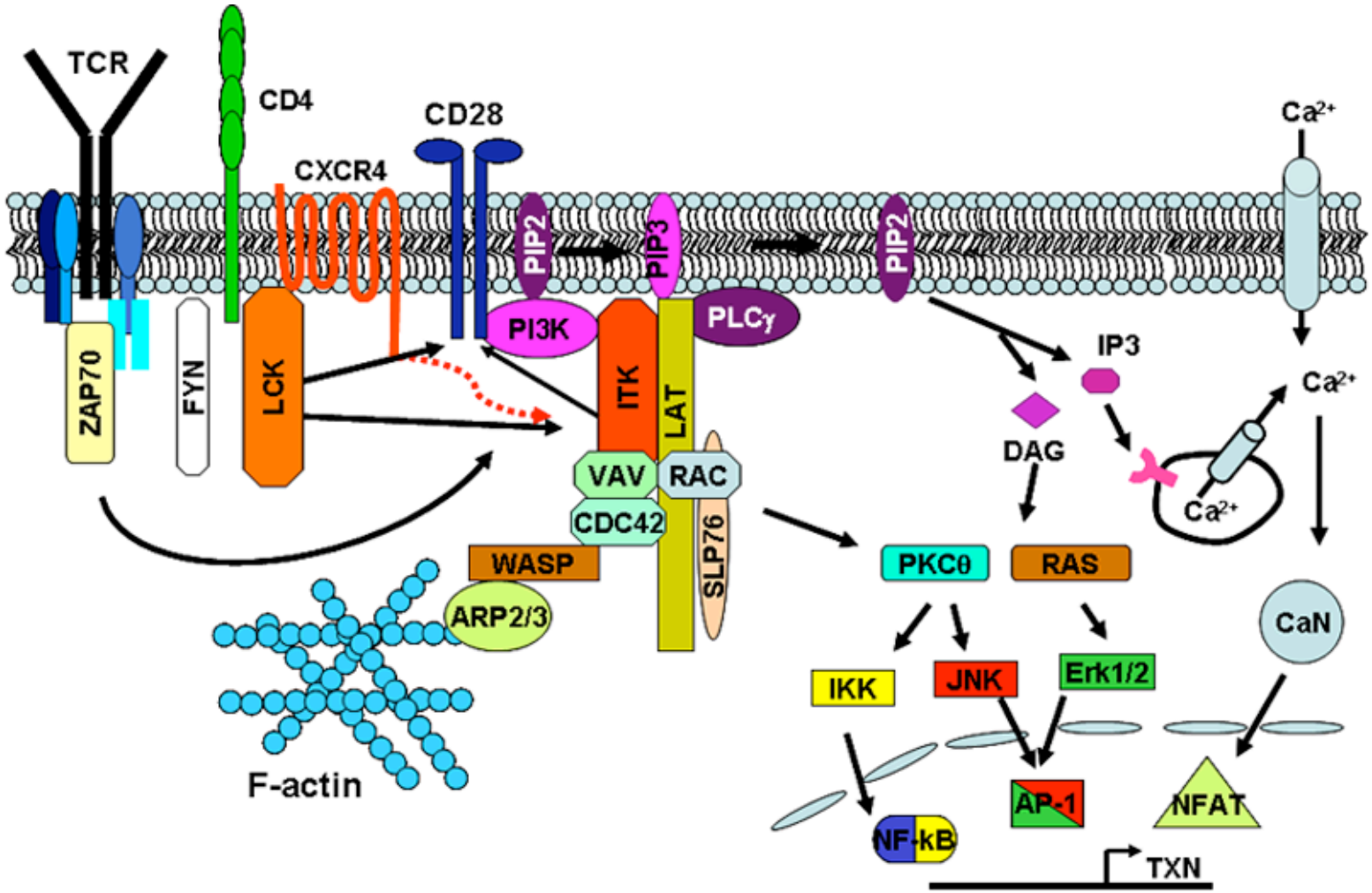


Figure 1.5: T cell activation

CD28 function is important for HIV transcription, as signaling via CD28 receptor lacking the cytoplasmic intracellular signaling domain does not lead to transcription. In fact, the four tyrosine residues in the cytoplasmic domain of CD28 are critical for mediating CD28 function. More detailed analysis of the tyrosine residues in CD28 revealed an inhibitory role for Y173 (Cook et al., 2002). Activation of PI3K disrupts Tat-PTEFb binding, thus repressing transcription. Consistent with this, inhibiting PI3K with chemical inhibitors activates HIV transcription. The loss of the Y200 receptor decreased virus transcription due to a lack of Vav and NF-κB activation (Cook et al., 2003).

It is clear that CD28 synergizes with TCR signals to induce transcription by activating distinct and overlapping pathways. TCR signaling can induce cell death in infected cells, which can be reversed by CD28 activation (Guntermann et al., 1997). Since TCR signaling leads to anergy, a state non-responsiveness, and as CD28 seems to amplify TCR signals, it is possible that suboptimal signals lead to the establishment of HIV latency (Sadegh-Nasseri et al.). Multiple signaling pathways are initiated when CD28 is activated; it is possible that incomplete signaling or lack of critical signaling intermediates would reinforce a repressed viral LTR, impeding viral transcription.

1.7 Transcriptional regulation of HIV

The activation of cellular signaling pathways results in transcription and post-translational modifications of target proteins to generate effector responses. T cell activation regulates several steps of the HIV life cycle and is indispensable for transcription.

HIV transcription is a multi-step process involving several protein complexes and regulatory pathways relying heavily on the availability of the host cellular transcriptional machinery. The virus specifically integrates into regions of the chromatin actively involved in transcription. This presents the virus better access to cellular transcriptional machinery. Transcription is initiated by the recruitment of transcription factors to the provirus LTR, including the RNA Pol II complex and chromatin remodeling factors which functionally interact to assure appropriate HIV transcription (Bieniasz et al., 1999; Coleman and Wu, 2009; Colin and Van Lint, 2009; Gaynor, 1992; Mahmoudi et al., 2006; Tong-Starkesen et al., 1989).

1.7.1 The Long Terminal Repeat

The LTR acts as the cis-transcriptional regulatory element of HIV, analogous to enhancers and promoters in a typical gene. The LTR is often divided into four functional regions; modulatory (-450 to -205), enhancer (-205 to -93), promoter (-93 to start site) and TAR (+1 to +60). Other than the TAR region which binds the HIV transactivating protein Tat, the LTR includes binding sites for a plethora of cellular transcription factors. The modulatory region has binding sites for AP-1, COUP, GATA-3, USF and NFAT (Galio et al., 1997; Lu et al., 1990; Yamamoto et al., 1991; Yang and Engel, 1993). ETS-1, LEF-1, USF-1, NF- κB, NFAT and SP-1 bind to the enhancer and promoter regions, which form the minimal promoter region required for HIV transcription (Chinnadurai, 1991; d'Adda di Fagagna et al., 1995; Du et al., 1993; Harrich et al., 1989; Holzmeister et al., 1993; Kinoshita et al., 1997; Seth et al., 1993; Sheridan et al., 1995). There are transcription factor binding sites in regions downstream of the start site (AP-1 binding

sites- +541, +572, +609; NFAT binding sites- +617), which have been shown to be important for provirus transcription by analysis of viruses containing point mutations in these sites (Van Lint et al., 1997). The availability and binding of transcription factors is also regulated in a cell or tissue specific manner. For instance, C/EBPβ regulates HIV transcription in macrophages but not in T cells (Henderson and Calame, 1997). T cell activation leads to the recruitment of transcription factors to the promoters of several genes that are transcribed upon signaling. In this context it is interesting to note that the HIV promoter is very similar to the IL-2 promoter, which suggests that the HIV promoter may mimic the IL-2 promoter to exploit T cell activation for efficient HIV transcription (Bohnlein et al., 1989; Greene et al., 1989).

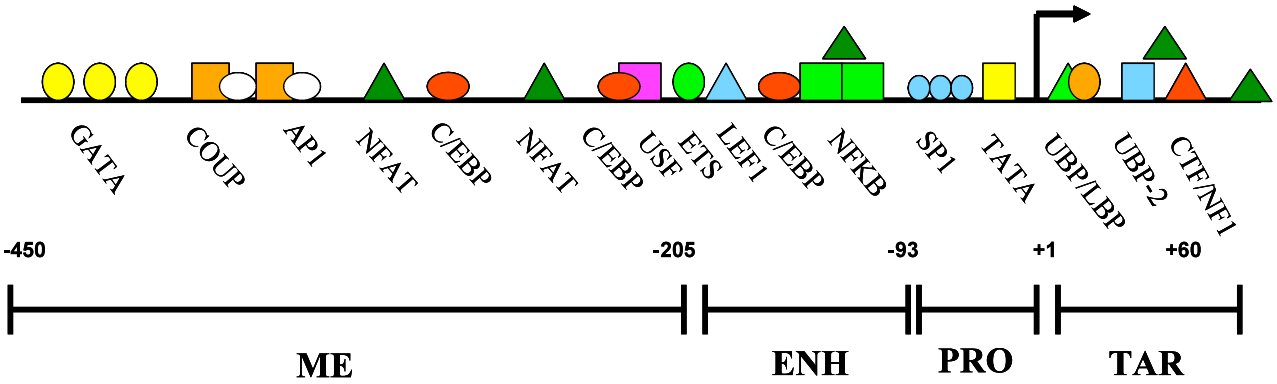


Figure 1.6: Schematic representation of the HIV LTR. The HIV LTR can be divided into four functional regions- modulatory region (-450 to -205), enhancer (-205 to -93), promoter (-93 to +1) and Transactivation response element (+1 to +60). Several transcription factors are recruited to the LTR which lead to initiation of transcription from the transcription start site at +1.

1.7.2 Recruitment of transcription factors

T cell activation is extremely important for HIV transcription and replication, primarily due to activation and recruitment of transcription factors to the proviral LTR. The LTR has multiple transcription factor binding sites as discussed in the previous

section, and some of the more critical transcription factors for HIV transcription in T cells are discussed below.

1.7.2.1 NFAT

NFAT was identified by its ability to bind to the IL-2 promoter and induce IL-2 transcription (Shaw et al., 1988). The NFAT family has five members which have a characteristic DNA binding domain, structurally similar to the rel-family of transcription factors (Northrop et al., 1994). NFAT1 and NFAT2 are primarily expressed in peripheral T cells, whereas NFAT4 is expressed in thymocytes, NFAT3 is not expressed in the immune system and NFAT5 is ubiquitously expressed. All NFAT proteins, except NFAT5 are regulated by calcium signaling (Macian, 2005). T cell signaling activates PLCγ, which hydrolyses PIP2 to produce DAG and IP3. IP3 induces the release of calcium from intracellular stores. This in turn leads to the activation of calcium release activated calcium channels (CRAC) in the plasma membrane which maintain elevated levels of calcium in the cell. Calcium binds to calmodulin, which then activates calcineurin. Calcineurin is a phosphatase which dephosphorylates NFAT. This leads to activation of NFAT and its subsequent migration into the nucleus where it binds specific sequences of DNA effecting transcription.

NFAT also interacts with other transcription factors in the nucleus, thus integrating calcium signaling with various cellular signaling pathways. During T cell activation, calcium signaling and MAPK pathways promote AP-1-NFAT-DNA complexes which induce transcription (Schneider and Rudd, 2008). It is interesting to note that AP-1 and NFAT binding sites are juxtaposed in many promoters including IL-2

and HIV (Rooney et al., 1995; Shapiro et al., 1997). Also, NFAT binds different gene promoters based on its interaction with AP-1. In fact, activation of NFAT in the absence of other signals, has been shown to lead to T cell anergy which can be overcome by Cyclosporin A, an inhibitor of NFAT (Macian et al., 2002). In a more physiologic context, as in the case of TCR signaling in the absence of costimulation, T cell anergy is due to NFAT, as activation of AP-1 requires CD28 signaling (Macian et al., 2002; Soto-Nieves et al., 2009; Wu et al., 2006).

In addition to TCR signals, CD28 can activate NFAT through PLC-γ which is induced by PI3K and TEC family kinases (Macian, 2005). By coupling different transcription factors, and different intracellular signaling pathways NFAT plays an important role in T cell development and differentiation (Hu et al., 2007; Sundrud and Rao, 2007).

NFAT has binding sites within the HIV-1 LTR, located in the modulatory region (Shaw et al., 1988), the enhancer (Kinoshita et al., 1997), and the 5' untranslated region (UTR) directly downstream of a positioned nucleosome (Van Lint et al., 1997). The NFAT sites in the modulatory region and the 5' UTR seem to have no function in HIV transcription as mutations in these sites do not alter HIV transcription in T cells transfected with LTR reporter constructs (Lu et al., 1990; Markovitz et al., 1992). The NFAT binding site in the enhancer region overlaps with the NF-κB binding sites, and is critical for HIV transcription. NFAT2 binds to the NF-κB sites in the LTR and cooperates with Tat and NF-κB to enhance HIV-1 replication (Kinoshita et al., 1997; Pessler and Cron, 2004). The synergism between NFAT and NF-κB is purely functional as there is no physical interaction between NFAT and NF-κB (Cron et al., 2000; Giffin et al., 2003).

NFAT1 also positively regulates HIV transcription (Cron et al., 2000; Robichaud et al., 2002). Furthermore, NFAT2 has been shown to overcome a block at reverse transcription, thereby creating a permissive environment for viral replication (Kinoshita et al., 1998). Treatment with Cyclosporin A, an NFAT inhibitor, limits HIV transcription, supporting an important role of NFAT in activating HIV transcription (Cron, 2001).

1.7.2.2 NF-κB

The NF-κB family consists of five proteins; RelA (p65), RelB, c-Rel, NF-κB1 (p50, precursor p105) and NF-κB (p52, precursor p100) which homo- and heterodimerise to differentially regulate genes involved in cell survival, growth and differentiation. NF-κB is particularly critical for regulating inflammatory and immune responses in B and T cells (Li and Verma, 2002). In resting cells, NF-κB is primarily retained in the cytoplasm due to its interaction with the inhibitor of κB (IκB). Disruption of this complex via phosphorylation and proteosomal degradation relieves the inhibition posed on NF-κB, allowing the dimer to translocate to the nucleus and activate transcription. This is facilitated by the inhibitor of IκB kinase (IKK) complex which contains a regulatory subunit (NEMO/IKKγ) and two catalytic subunits (IKKα and IKKβ).The IKK complex phosphorylates IκB, targeting it for proteosomal degradation.

The critical role of NF-κB in HIV transcription was established in experiments carried out to identify the factor present in activated T cells responsible for HIV transcription (Nabel and Baltimore, 1987). Activation of NF-κB has also been identified as an important mediator of costimulation via TCR and CD28 signaling (Kane et al., 2002; Verweij et al., 1991). There are two NF-κB binding sites within the HIV LTR, at -

81 to -91 and -95 to -104 relative to the transcriptional start site. In resting T cells p50 homodimers occupy the NF-κB sites, inhibiting HIV transcription by recruiting histone deacetylase 1 (HDAC)1 (Williams et al., 2006). Upon T cell activation the p50 homodimers are displaced by p65:p50 heterodimers which act as a transcriptional activator. One function of NF-κB is to recruit the histone acetyl transferase CBP/p300 to the LTR (Zhong et al., 2002) promoting the remodeling of chromatin so that the DNA is more permissive to transcription. In addition, p65 interacts with PTEFb, which enhances RNA polymerase II processivity and increases transcription elongation (Barboric et al., 2001); this will be discussed in greater detail below. NF-κB is thus a potent activator of transcription by its ability to recruit factors that alter chromatin as well as the RNA polymerase II complex (Natoli et al., 2005). More recently a histone acetyltransferase (HAT) General Control Nonderepressible (GCN5) has been shown to aid in ubiquitination and degradation of phosphorylated p65, suggesting that chromatin modifying enzymes also target and control NF-κB function (Mao et al., 2009).

1.7.2.3 AP-1

Activating protein 1 (AP-1) transcription factors are homodimers or heterodimers of Fos and Jun family proteins (Karin, 1995). TCR signaling and CD28 mediated costimulation initiates the MAPK pathway, which activates and recruits AP-1 to the nucleus (Edmead et al., 1996; Yang and Gabuzda, 1999). More specifically, costimulation activates extracellular response kinase (ERK) inducing c-fos to translocate to the nucleus and interacts with existing c-jun to form active AP-1 (Karin, 1995). In

addition, other stimuli including growth factors, cytokines and UV irradiation induce AP-1 (Angel and Karin, 1991).

In vitro gel shift experiments done in PMA induced cells demonstrated that c-Fos and Jun were bound to the AP-1 sites within the HIV LTR. Over expression of these proteins in PMA stimulated cells resulted in an increase in HIV, implicating an important role for AP-1 in regulating HIV transcription (Roebuck et al., 1996).

AP-1 also differentially associates with NFAT and NF-κB under different cellular contexts to regulate HIV transcription. T cell activation generates AP-1- NF-κB complexes that are recruited to the NF-κB binding site on the LTR resulting in a synergistic activation of HIV transcription (Yang et al., 1999).

1.7.2.4 SP1

The HIV core promoter has three tandem binding sites for specificity protein (SP) transcription factor adjacent to the TATA box(Jones et al., 1986). The SP family of transcription has 4 members, SP1-SP4, all of which have zinc finger DNA binding domains. SP1, SP3 and SP4 have an affinity for GC rich regions whereas SP2 has a higher affinity for GT rich regions (Kilareski et al., 2009). SP1 and SP4 are transcriptional activators, whereas SP3 is a repressor of transcription (Hagen et al., 1995; Hagen et al., 1994). SP1 and SP3 are ubiquitously expressed and have been implicated as being relevant for regulating HIV transcription.

SP1 has been shown to play an important role in basal and Tat mediated transcription (Harrich et al., 1989; Sune and Garcia-Blanco, 1995). Mutation of all three SP1 sites decreased HIV transcription; however, mutation of one or two SP1 binding sites

had little effect, suggesting that the three SP1 sites are redundant. SP1 has also been demonstrated to regulate NF-κB transactivation of HIV transcription (Perkins et al., 1993). In addition SP1 interacts with Cyclin T1, a subunit of PTEFb (Yedavalli et al., 2003), and TFIID stabilizing the pre-initiation complex (Gill et al., 1994). Although ubiquitously expressed, SP1 function may be regulated in the context of T cell activation (Lacroix et al., 2002). For example, post translational modification of SP1 by O-Linked *N*-acetylglucosaminylation alters its activity so that SP1 represses HIV transcription (Jochmann et al., 2009).

SP3 represses HIV transcription by competing with SP1 for binding sites on the LTR. In addition, SP3 had also been shown to repress transcription in the presence of Tat.

1.7.3 Regulation by Tat and PTEFb

Positive transcription elongation factor complex-b (PTEFb), is a complex consisting of a regulatory subunit Cyclin T1 and a catalytic subunit, the cyclin dependent kinase Cdk9 (Wei et al., 1998). Unlike many other members of the family of cyclin dependent kinases which are involved in the regulation of cell cycle, cdk9 plays a crucial role in regulating transcription of cellular genes. PTEFb phosphorylates the CTD domain of RNA Pol II, leading to activation and increased processiveness of RNA Pol II. RNA Pol II is typically phosphorylated at Ser 5 in the heptapeptide repeat of the CTD by cdk7/TFIIH. The recruitment of PTEFb to the promoter leads to phosphorylation of Ser 2 and enhanced RNA Pol II processivity (Dahmus, 1996). In addition, PTEFb phosphorylates negative factors that limit transcription elongation and are associated with RNA Pol II complex. For example, negative elongation factor complex (NELF) and DRB

sensitivity inducing factor (DSIF), both are phosphorylated by PTEFb, which leads to displacement of NELF and a switch in DSIF activity favoring efficient transcription elongation (Garriga and Grana, 2004; Peterlin and Price, 2006; Zhou and Yik, 2006). The function of PTEFb is indispensable for HIV transcription (Bieniasz et al., 1999; Mancebo et al., 1997; Zhou et al., 1998). The critical role of PTEFb in regulating HIV transcription is underscored by its interaction with tat. Tat binds to the TAR region, a viral mRNA stem loop structure formed immediately upon transcription initiation, and stimulates transcription elongation by mobilizing PTEFb to the provirus LTR (Berkhout and Jeang, 1989; Laspia et al., 1993; Zhou et al., 1998).

The activity of PTEFb is tightly regulated. About 50% of nuclear PTEFb has been shown to be sequestered in a complex with 7SK snRNP. The major components of the 7SK snRNP complex are the 7SK RNA, an abundant short RNA species, and the protein HexaMethylene BisAcetamide (HMBA) inducible protein 1 (HEXIM1). The Cyclin T1 subunit of PTEFb interacting with the 7SK snRNP is unavailable and is not recruited to the LTR therefore unable to potentiate transcription elongation (Yang et al., 2001). HEXIM1 is indispensable for the repressive function of the 7SK RNA on PTEFb (Yik et al., 2003) as HEXIM binds the 5' hairpin loop of 7SK RNA, and PTEFb binds to the 3' hairpin of 7SK RNA bound to HEXIM (Egloff et al., 2006). Thus, the inhibitory 7SK RNP complex is formed by cooperative interactions between PTEFb, HEXIM and 7SK RNA. HEXIM and 7SK RNA interact with PTEFb in a manner similar to how tat- PTEFb -TAR interact (Yik et al., 2004). In fact, Tat and HEXIM compete for binding CyclinT1 (Schulte et al., 2005) (Fig. 1.7).

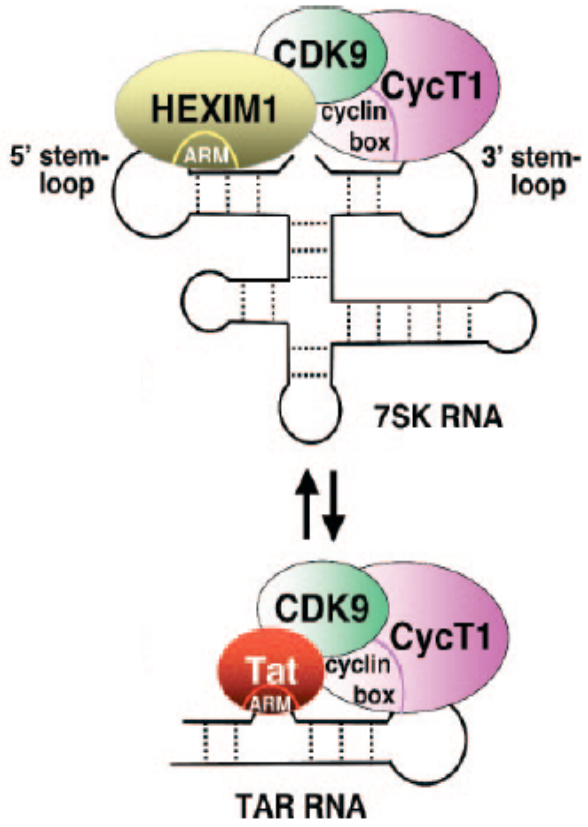


Figure 1.7: Similarity between Tat-TAR-PTEFb and HEXIM-7SK-PTEFb complexes (Zhou and Yik, 2006). PTEFb is regulated by HEXIM-7SK complex which sequesters PTEFb from sites of active transcription. The Tat-TAR-PTEFb complex is very similar to the inhibitory complex, and can compete with the 7SK RNP to bind PTEFb, resulting in HIV transcription.

The phosphorylation of Thr186 in cdk9, a conserved phosphorylation site in all the cyclin dependent kinase, leads to a conformational change, resulting in an active kinase. Cdk9 phosphorylation is essential for the formation of the PTEFb-7SK snRNP complex, which can be disrupted by phosphatase treatment of cdk9. A mutation of this threonine residue abroagates the interaction of PTEF-b with the 7SK snRNP complex. (Chen et al., 2004). This indicates that 7SK and HEXIM interact with and repress an otherwise active PTEFb, allowing for a rapid regulation of PTEFb in response to cellular signals.

PTEFb also interacts with bromodomain-containing protein 4 (Brd4), which binds acetylated histones H3 and H4 (Dey et al., 2003; Jang et al., 2005; Yang et al., 2005). The ability of PTEFb to interact with Brd4 provides a mechanism for recruiting PTEFb to sites of open chromatin structure. Thus, PTEFb exists in two different functional states or complexes in the nucleus, an active form associated with Brd4, and an inactive form associated with HEXIM and 7SK RNA. Tat may functionally replace Brd4, driving PTEFb recruitment specifically to the HIV LTR by means of its interaction with TAR (He et al., 2006).

Another factor that PTEFb interacts with is splicing-associated c-Ski-interacting protein, SKIP (NCoA-62; Drosophila Bx42; yeast Prp45p), which is required for tat mediated transactivation. SKIP interacts with Sin3A:N-CoR complexes and other co-repressors (SMRT, HDAC1/2), and plays a role in RNA splicing. SKIP associates with PTEFb in nuclear extracts and may cooperate with PTEFb in inducing transcription elongation and splicing (Bres et al., 2005; Bres et al., 2008).

In addition, PTEFb interacts with other transcription factors. Of note, in the context of HIV transcription is the interaction between NF-κB and PTEFb. NF-κB binds the HIV LTR, and its ability to interact and recruit PTEFb can explain the tat-independent transcription elongation that occurs before tat is transcribed and translated in infected cells (Barboric et al., 2001).

In summary, PTEFb seems to be in a dynamic equilibrium in the cell, and depending on the cellular needs, the signaling events in the cell, the equilibrium could be shifted to support active transcription or to arrest transcription.

1.7.4 Regulation of HIV transcription by chromatin

Transcription is governed to a large extent by the accessibility of DNA to transcription factors and the RNA Pol II machinery. DNA in the nucleus exists in a highly condensed form, by means of its association with the histone proteins. 146 bp of DNA is wound around a histone octamer containing two H2A, H2B, H3 and H4 proteins to form a nucleosome particle. Adjacent nucleosomes are connected to each other via linker DNA, a region of DNA highly accessible to other nuclear proteins, and susceptible to enzyme digestion. The nucleosomes separated by linker DNA give it a characteristic "beads on a string" appearance. During transcription, only specific regions of the chromosome, need to gain access to the transcription machinery. This is executed by multiple mechanisms which temporarily alter the nucleosome structure, by nucleosome sliding or remodeling which is brought about by ATP dependent SWI/SNF complexes. In addition, post-translational modification of histone proteins, primarily by acetylation, destabilizes higher order structures, opening up the chromatin conformation. Histones are also regulated by other post-translational modifications including phosphorylation, methylation and ubiquitination.

Recent evidence has indicated that chromatin structure of the HIV LTR is highly dynamic and provides a critical check point for HIV transcription. The HIV LTR is highly organized in regards to nucleosome positioning. Two nucleosomes, nuc-0 and nuc-1 are positioned on the HIV LTR independent of the site of integration (Sadowski and Mitchell, 2005). Nuc-0 is located -140, whereas nuc-1 is approximately +100 bp downstream of the transcription start site. The region between the two nucleosomes contains sites on the core promoter with binding sites for Sp1, NF-κB, Ets-1, USF and

TATA box binding protein (Jones and Peterlin, 1994). Nuc-1, which lies in close proximity to the transcriptional start site is remodeled upon activation of transcription, implicating an important role for nucleosome positioning in the control of HIV transcription (Van Lint et al., 1996; Verdin et al., 1993). SP1, Ets and TATA box binding protein have been shown to bind DNA irrespective of the activation status of the cell, suggesting it is not the binding of these factors, but the remodeling of the chromatin which is imperative for HIV transcription (Demarchi et al., 1993).

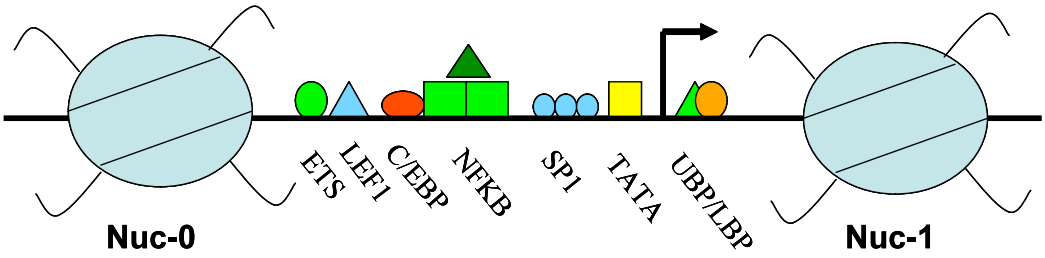


Figure 1.8: Chromatin organization of the HIV LTR. Nucleosomes associate with specific sequences of the LTR irrespective of the sites of integration. Nuc-0 is formed at -140 bp, whereas nuc-1 is assembled downstream of the transcription start site. The nucleosome free region between nuc-0 and nuc-1 contains the enhancer and promoter regions of the LTR. During transcription activation nuc-1 is rapidly remodeled which is a prerequisite for successful transcription.

HIV transcription is regulated by histone modifying enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs). The transcription factor LSF (also called UBP), binds to the HIV LTR and interacts with Yin Yang 1 (YY1) which recruits HDACs to repress transcription (Coull et al., 2000). HDACs are also recruited by the p50 homodimer of NF-κB which represses HIV transcription (Williams et al., 2006). Acetylation of histones correlates with activation of HIV transcription and virus production (Sheridan et al., 1997; Steger et al., 1998). Consistent with this model, HIV transcriptional activation is associated with the recruitment of HATs CBP, GCN5 and

p300/CREB-binding protein (CBP)-associated factor (P/CAF) to the LTR (Lusic et al., 2003; Marban et al., 2007). In addition, remodeling of suppressive chromatin structure is required for NF-κB mediated activation of HIV transcription (El Kharroubi et al., 1998). SP1 and NF-κB binding to the LTR are a prerequisite for tat-mediated induction of HIV transcription. Importantly, Tat has been shown to recruit HATs to the LTR indicating that Tat positively regulates HIV transcription by remodeling the inhibitory positioned nucleosome and enhancing RNA pol II processivity.

Histone modification and nucleosome position both contribute to chromatin environments permissive for transcription. Since these two events are not mutually exclusive, it has been difficult to define the temporal sequence of these events. Retinoic acid mediated suppression of HIV transcription in U1 cells was mapped to nucleosome-1 remodeling with not effect on histone acetylation suggesting that the remodeling of nucleosomes precedes the. acetylation of histones (Kiefer et al., 2004). In addition, the ATP dependent chromatin remodeling SWI/SNF complex is recruited to the nuc-1 via the interaction of BRG1, a part of the SWI/SNF complex with activating transcription factor 3 (ATF3), a member of the CEBP family of transcription factors. In fact, acetylation of histones at nuc-1 is important for BRG1-DNA interaction (Henderson et al., 2004). The SWI/SNF complex also synergizes with the p300 histone acetyl transferases to alter the nucleosome positioning.

T cell signals can directly influence the chromatin environment affecting the transcription of HIV and other genes. T cell costimulation via CD28 directly controls the function of p300/CBP by increasing histone H4 acetylation at the fos promoter (Nandiwada et al., 2006). In addition, the nucleosome position on the IL-2 promoter

proximal region is remodeled in response to T cell activation resulting in the transcription of IL-2 (Attema et al., 2002; Ward et al., 1998). It is likely that T cell signals will converge and regulate the chromatin organization of the HIV LTR by similar biochemical processes.

1.7.5 RNA Polymerase II Processivity and Transcription Regulation

The recruitment of transcription factors to their appropriate binding sites on the LTR leads to the assembly of the complex of proteins that initiates transcription and controls RNA elongation. First, TBP (TATA box binding subunit) of TFIID binds to the TATA box on the viral promoter and recruits TFIID to the HIV LTR. Once this step occurs, the RNA polymerase II complex and other general transcription factors, including, TFIIB, TFIIH, TFIID, TFIIE, TFIIF, are recruited to the HIV LTR to form the pre-initiation complex.

RNA polymerase II is composed of 12 subunits. The largest subunit of RNA Pol II, Rpb1, has an unusual C terminal domain (CTD) which is characterized by the presence of heptapeptide repeats (YSPTSPS). The regulation of RNA Pol II function is governed to a large extent by the phosphorylation status of the serine residues in the heptapeptide repeats. The CTD of RNA Pol II also acts as a scaffold for the assembly of other complexes involved in co-transcriptional processing (McCracken et al., 1997). The CTD gets phosphorylated by TFIIH, which includes CDK7, at ser5 of the heptapeptide repeats (Orphanides et al., 1996). TFIIH is a prerequisite for transcription and limiting TFIIH contributes to HIV latency (Kim et al., 2006). This first phosphorylation event

primes the polymerase complex to clear the promoter and enter the first phase of transcription elongation.

Upon initiation of transcription elongation, RNA polymerase II is slowed by the action of negative factors associated with the polymerase complex, NELF and DSIF. The paused RNA Pol II allows mRNA capping enzymes to be recruited to the nascent RNA via the ser 5 phosphorylated CTD, ensuring a 5' cap on the mRNA (Rodriguez et al., 2000; Schroeder et al., 2000). Serine 2 phosphorylation of the RNA Pol II CTD by PTEFb marks the progression of RNA Pol II from a "paused" state to active transcription elongation. In the absence of this second phosphorylation event, transcription elongation is aborted resulting in the production of short RNA transcripts, a hallmark of promoter proximal pausing. The presence of short transcripts is also a characteristic of asymptomatic AIDS patients as well as cells carrying latent HIV.

1.7.6 Negative elongation factors associated with RNA Polymerase II

1.7.6.1 DSIF

DSIF (DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity inducing factor) is a heterodimeric elongation factor comprised of two subunits, Spt4 and Spt5, which can repress as well as activate RNA Pol II mediated transcription (Wada et al., 1998a; Yamada et al., 2006). Transcription repression is mediated via interactions of Spt5 with RNA Pol II on several promoters (Yamaguchi et al., 1999b) including the HIV LTR. DSIF is always associated with RNA polymerase II. DSIF functions as a repressor as well as an activator with its activity being governed by PTEFb mediated phosphorylation of C-terminal repeat 1 (CTR1) of Spt5 (Ivanov et al., 2000; Ping and

Rana, 2001; Wada et al., 1998b; Yamada et al., 2006). Spt5 also plays an important role in tat mediated HIV transcription (Kim et al., 1999; Wu-Baer et al., 1998). Analysis of HIV transcription and hsp70-4 gene in zebrafish embryos have mapped the inhibitory function to the C-terminus of Spt5 (Chen et al., 2009a). Phosphorylated DSIF interacts with the Paf1 complex and Tat-SF1 to mediate efficient transcription elongation. These two proteins have been previously shown to be involved in transcription elongation. This interaction is dependent on the PTEFb mediated phosphorylation of Spt5 C terminal region (Chen et al., 2009b). Spt5 has been shown to be recruited to the HIV LTR immediately after transcription initiation, and prevents premature termination and pausing during later stages of transcription elongation by preventing polymerase pause at bent DNA and arrest sites and premature termination at terminator sequences (Bourgeois et al., 2002). In addition to regulating the activity of RNA polymerase II, DSIF also stimulates mRNA capping (Wen and Shatkin, 1999). DSIF also plays a role in transcription independent processes in cells like nucleotide excision repair and chromosome segregation (Basrai et al., 1996; Jansen et al., 2000).

1.7.6.2 NELF

NELF (negative elongation factor), is a complex of four subunits containing NELF-A, NELF-B (also known as the cofactor of BRCA1), NELF-C or NELF-D and NELF-E (Narita et al., 2003; Yamaguchi et al., 1999a). NELF alters the processivity of RNA pol II by inhibiting elongation, and by associating with the ser5 phosphorylated RNA Pol II and DSIF (Yamaguchi et al., 1999a). NELF does not bind RNA Pol II or DSIF strongly, however, it binds to the complex of DSIF and RNA Pol II (Yamaguchi et

al., 2002). This interaction is altered when PTEFb phosphorylates all the three proteins involved, dissociating NELF and resulting in productive transcription elongation of HIV (Cheng and Price, 2007; Yamaguchi et al., 1999a).

Genome wide analysis looking at the recruitment of NELF in Drosophila revealed that NELF is recruited to approximately 50% of highly expressed genes (Lee et al., 2008). Microarray analysis of Drosophila S2 cells depleted of NELF led to a down-regulation of majority of target genes, whereas only one third of the gene targets, like hsp70, were upregulated. NELF enhances transcription by maintaining an open chromatin structure around the promoter proximal region, and NELF depletion leads to a decrease in histone H3K4 trimethylation and an increase in nucleosome occupancy (Gilchrist et al., 2008).

In addition, an exon array study in T47D breast cancer cells support a role for NELF in positively regulating genes involved in cell cycle regulation. Depletion of NELF subunits downregulated genes coding for proteins involved in cell cycle progression. Reducing NELF expression also displaced RNA pol II from the promoter-proximal region of these genes, and decreased histone modifications (H3K9Ac) and (H3K36Me3) which mark transcription initiation and transcription elongation respectively, suggesting that NELF is important for transcription of these genes (Sun and Li).

A recent study in macrophages, has analyzed the role of NELF and promoter proximal pausing in transcription of immediate and late response genes in response to lipopolysaccharide (LPS) stimulation. In resting macrophages, RNA Pol II initiates transcription but pauses at promoter proximal regions at tumor necrosis factor α (TNFα) gene, until LPS activates its transcription by releasing NELF. In comparison, no NELF or

RNA polymerase II is detected near the IP-10 promoter before induction. In fact, recruitment of Pol II occurs upon LPS stimulation, which is the rate limiting step in IP-10 transcription (Adelman et al., 2009).

NELF-B binds to and represses the transcription of estrogen receptor α (ERα) via promoter proximal pausing (Aiyar et al., 2004). Analysis of transcription of jun-B, before stimulation with IL-6 revealed the presence of Pol II, NELF and DSIF at the +50 region, which when stimulated led to an accumulation of this complex at +50, but also resulted in the distribution of Pol II and DSIF in regions downstream. Depletion of NELF-E enhances the level of jun-B mRNA, under uninduced and induced conditions by overcoming the paused polymerase and by attenuating transcription after induction (Aida et al., 2006).

Similar results were obtained when the hsp70 gene Drosophila was studied. Under non-heat shock conditions, the hsp70 gene is not transcribed due to a promoter proximal pause, wherein NELF and DSIF associate with RNA Pol II at the promoter. Upon heat shock, the heat shock factor (HSF) is activated via phosphorylation and is recruited to the hsp70 promoter. This leads to recruitment of PTEFb which phosphorylates and dissociates NELF from the promoter to induce successful transcription elongation. The association of NELF-E with nascent RNA may be responsible for the pause, which is overcome when PTEFb phosphorylates NELF, DSIF and RNA Pol II (Wu et al., 2005; Wu et al., 2003).

The NELF-E subunit has the RNA recognition motif (RRM), through which it recognizes and binds to transcribing RNA. In vitro experiments showed that NELF-E could bind a fully formed TAR RNA. This interaction was lost upon phosphorylation of

NELF-E by PTEFb (Fujinaga et al., 2004). These findings imply that the promoter proximal pause occurs after the TAR element has been transcribed (Palangat et al., 1998). However, for other genes that are regulated at the level of transcription elongation Pol II proximal pausing has been mapped to +30 to +50 bp downstream of the transcription start sites (Lee et al., 2008). Tat interacts with TAR and recruits PTEFb to the LTR. However, p65, a subunit of NF-κB has also been shown to bind and recruit PTEFb to the LTR (Barboric et al., 2001).

Detailed analysis of U1 cells, a promonocytic cell line which has 2 copies of HIV with mutations in tat rendering the proviruses latent, and is a model of latent HIV infection, has revealed an important role for NELF in regulating transcription. In these cells, depletion of NELF increases HIV transcription, and virus production due to more efficient transcription elongation. Permanganate footprinting analysis of the proviral LTR promoter revealed a primary pause in transcription around the +47 site. Depletion of NELF, as well as treatment with PMA overcame this pause, leading to efficient elongation. Thus, NELF arrests transcription elongation at a site before the TAR is fully assembled. In addition, depletion of NELF increased acetylation of histone H4 and displaced nucleosome-1, suggesting that transcription elongation and chromatin remodeling are coupled events (Zhang et al., 2007a). The involvement of NELF in repressing HIV transcription is further strengthened by studies done with the receptor tyrosine kinase RON (Lee et al., 2004), which has been shown to repress HIV transcription, by decreasing the levels of NF-κB, and by increasing the recruitment of NELF to the promoter proximal region thus, maintaining a paused RNA Pol II (Klatt et al., 2008).

Overall, NELF seems to differentially regulate a subset of genes based on properties of the promoter, extracellular stimuli and intracellular signaling. In addition, NELF may interact with corepressors and coactivators to regulate the chromatin structure and nucleosome positioning. The function of NELF and its interaction with other proteins regulating the overall chromatin structure and nucleosome positioning will be important in understanding its role in HIV transcription.

1.7.7 Premature termination

Another mechanism for limiting transcription elongation is premature termination. In the case of HIV, absence of Tat leads to accumulation of short stable transcripts which represent prematurely terminated transcripts (Feinberg et al., 1991; Kessler and Mathews, 1992). Even though, NELF and DSIF cause the polymerase to pause, they cannot release the paused Pol II from the transcript. The paused polymerase complex is stable, and transcription termination is a regulated event; the RNA pol II does not just fall off the transcript. Recently, Pcf11 was identified as a factor responsible for the termination of transcripts at the Poly A tail of full length transcripts as well as transcripts engaged in a paused polymerase complex (Zhang et al., 2005; Zhang and Gilmour, 2006). A role of Pcf11 in HIV transcription premature termination in U1 cells (Verdin et al., 1993) was recently demonstrated. Depleting Pcf11 activated HIV transcription and replication. Furthermore, Pcf11 was required for DRB sensitivity of HIV transcription (Zhang et al., 2007b).

Pcf11 has been shown to be distributed throughout genes, with most Pcf11 found near the polyadenylation signal, consistent with a primary role in transcriptional

termination (Kim et al., 2004). RNA Polymerase pausing precedes transcription termination. Pcf11 interacts with the CTD of RNA Polymerase II (Sadowski et al., 2003) suggesting that Pcf11 and RNA Pol II interaction is important for Pcf11 function and transcription termination (McCracken et al., 1997). Furthermore, Pcf11 has been shown to bridge the newly synthesized RNA and the CTD of RNA Pol II (Zhang et al., 2005). It is also important to note that Pcf11 is not recruited to actively transcribing pol II (Orozco et al., 2002; Park et al., 2004). Therefore, a paused Pol II complex by recruiting Pcf11, will be cleared from the newly synthesized RNA.

We propose a model for HIV transcription in which DSIF and NELF stall the RNA polymerase II which then recruits Pcf11 to the promoter to evict the stalled pol II complexes.

1.8 HIV Latency

The treatment of HIV radically changed with the development of HAART, which is a combination of two or more drugs targeting viral proteins, primarily, protease, integrase, or RT (Colin and Van Lint, 2009). Administration of HAART to HIV infected individuals significantly decreases viral loads and increases patients CD4+ T cell count (Perelson et al., 1997). Amidst this success was the discovery of latent reservoirs in AIDS patients on HAART (Finzi et al., 1997). Within two weeks of interrupting HAART a rebound in viral loads was observed (Davey et al., 1999). It was later determined that even though HAART prevents viral replication and spread, it does not affect the viral reservoirs which can stably persist, and be reactivated upon T cell activation to produce

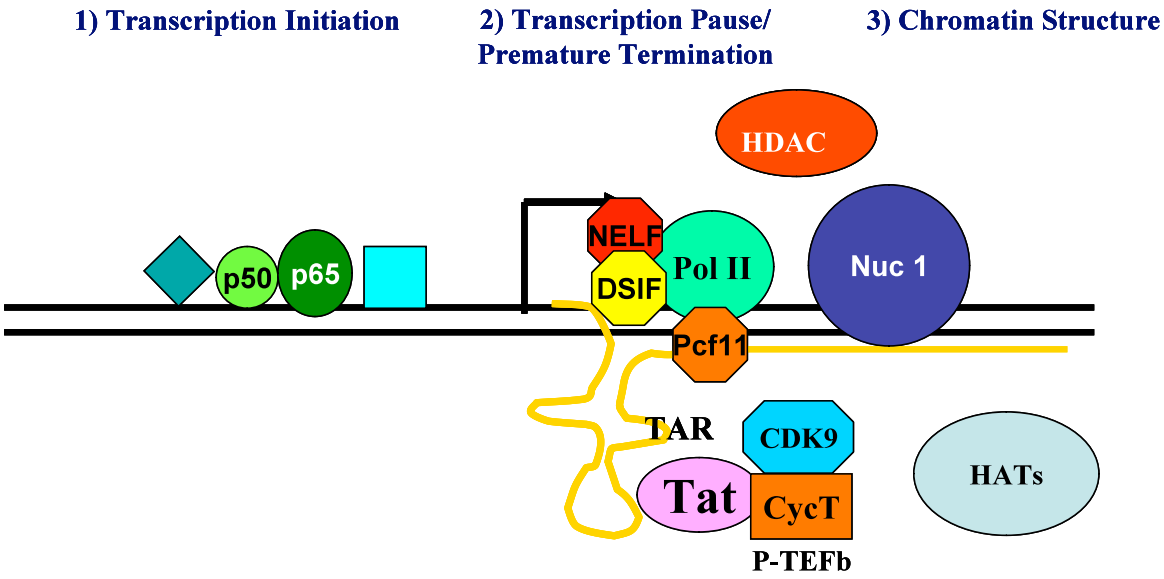


Figure 1.9: HIV transcription is regulated at multiple levels. Recruitment of transcription factors leads to initiation of HIV transcription. This results in the recruitment of RNA polymerase II whcih is regulated by the phosphorylation of it CTD. Ser 5 phosphorylation of CTD leads to promoter clearance, but is unable to support efficient transcription elongation due to its association NELF and DSIF. Recruitment of PTEFb via NF-κB, SP1 or Tat lead to serine 2 phosphorylation of RNA Pol II CTD as well as phosphorylation of NELF and DSIF. This abolishes promoter proximal pausing by NELF and premature termination by Pcf11, resulting in transcription elongation. Chromatin modifications resulting in an open chromatin structure, by post translation modification of histone tails as well as remodeling of nuc1 are essential for HIV transcription.

infectious HIV (Chun et al., 1997; Wong et al., 1997b). Latent virus was also described in AIDS patients not on any therapy (Chun et al., 1995). The cellular sources of the latent HIV are still not known but are thought to be tissue macrophages and, in particular, memory CD4 T cells. Memory T cells, which are long-lived quiescent cells, potentially persist for several years with an average half-life of approximately 40 months. Based on

this, it is estimated that an average of 60 years of HAART therapy would be required to eliminate the reservoir of latent HIV provirus (Pierson et al., 2000).

A recent study has identified two different kinds of memory CD4+T cells harboring latent HIV based on expression of CD45RA, CCR7 and CD27; Central memory (T_{CM}) and transitional memory (T_{TM}). The T_{CM} cells are maintained through T cell survival, have minimal exposure to any antigen driven immune response, and are representative of memory cells persisting in HAART treated patients. On the other hand, T_{TM} cells harbor HIV in cells expressing higher levels of IL-7, leading to homeostatic proliferation thereby ensuring their extended survival and persistence. This is representative of aviremic patients with low CD4+T cell counts. Therefore, the viral reservoirs in resting CD4 T cells are maintained by at least two mechanisms; long-term survival of infected T_{CM} cells as well as the homeostatic proliferation of infected T_{TM} cells (Chomont et al., 2009).

HIV latency can occur early in the HIV life cycle prior to integration or post-integrations once the provirus has been established into the host genome (Marcello, 2006). The pre-integration latency is primarily due to blocks in the initial steps of the viral life cycle which inhibit the integration of viral DNA into the host genome (Pierson et al., 2002a). Mechanisms that contribute to pre-integration latency include problems uncoating. For example, TRIM5α, inhibits nuclear import of the pre-integration complex. In addition, limiting amounts of dNTPs can abrogate reverse transcription resulting in latency (Gao et al., 1993). However, the clinical relevance of pre-integration latency is not clear since the persistence of unintegrated virus is not more than 4 weeks (Gillim-Ross et al., 2005; Kelly et al., 2008; Saenz et al., 2004). The rebound in virus production

observed when HAART is withdrawn from patients is due to post-integration viral latency. Since HIV is already integrated into the host genome, the virus can be activated when the cellular environment is permissive for HIV provirus transcription, as would be in the absence of HAART.

Post integration latency is maintained via multiple mechanisms. Since HIV transcription is regulated at multiple levels, inhibition of any of these events could potentially lead to latency. HIV selectively integrates into actively transcribed genes suggesting that the provirus will be located into regions of relatively open chromatin (Ciuffi and Bushman, 2006; Lewinski et al., 2006; Schroder et al., 2002). The persistence of a latent provirus when it is flanked by sites of active transcription is puzzling. This can be explained by the phenomenon of transcriptional interference, a mechanism that exists when two promoters lie in close proximity (Adhya and Gottesman, 1982; Duverger et al., 2009). It is believed that when the provirus is adjacent to an actively transcribed gene, promoter read through could occur, resulting in the displacement of transcription factors from the LTR, inhibiting formation of the pre-initiation complex (Greger et al., 1998). This read through also leads to transcription of integrated HIV, though it is eventually spliced out (Lenasi et al., 2008). Transcriptional interference can also be caused when two genes are in the opposite orientation, and the polymerase cannot efficiently transcribe (Crampton et al., 2006). In addition to the site of integration, multiple steps involved in HIV transcription have been shown to contribute to HIV latency. For instance, transcription factor recruitment and availability especially p50 homodimer via its interaction with HDACs may play a role in latency. In addition, the absence of nuc-1 remodeling and acetylation of histones might govern aspects of HIV latency (Colin and

Van Lint, 2009; Dahl et al.). Tat function is also a major determinant of latency, and mutations perturbing tat-TAR binding have led to the establishment of cell line models of latency. ACH2 cells have a point mutation C37 to T in TAR, altering the base pairing in the hairpin structure, making it unresponsive to tat (Emiliani et al., 1996). In U1 cells, latency is due to a H13L mutation in tat, which can be reversed by overexpression of recombinant tat (Emiliani et al., 1998). Analysis of HIV isolates obtained from AIDS patients have also shown accumulation in tat mutations which alter its transcriptional transactivation, indicating that tat is subject to diversification in response to immune selection. It is possible that as AIDS progresses mutations in tat are favored to establish latent reservoirs of virus (Yukl et al., 2009).

1.9 Summary and Hypothesis

CD4+T cells are the primary target of HIV infection, and a decline in these target cells is a hallmark of AIDS. The treatment of AIDS with HAART has not been completely successful due to the persistence of latent reservoirs of HIV provirus in quiescent CD4+T cells. Latency is established due to blocks in various steps of virus transcription, which can be overcome by T cell activation. The identification of mechanisms responsible for establishment of HIV provirus latency, and primary signals and pathways involved in reactivation of latent virus will be instrumental in understanding HIV biology and designing strategies to combat HIV successfully.

I propose that T cell activation initiates signals that activate as well as inhibit HIV transcription. I was interested in defining these signals to determine pathways that may be targeted to control HIV replication. I initially set out to characterize how different signals

emanating from CD28 regulate HIV transcription. Using cell lines expressing chimeric receptors where the CD28 cytoplasmic domain is fused to the CD8 extracellular domain, I studied the effect of various tyrosine residues in the cytoplasmic domain of CD28 on HIV transcription. I have showed that Y173 represses HIV transcription via the PI3K which inhibits HIV transcription by controlling the availability of PTEFb. Other tyrosine residues, Y191 and Y200 promote HIV transcription by direct activation and recruitment of NF-κB to the LTR, with a concomitant increase in the recruitment of RNA Pol II.

In addition, I examined the role of promoter proximal pausing and how it limits HIV transcription in different cell populations. My data confirmed that NELF limits HIV transcription by limiting transcription elongation by maintaining RNA Pol II in a paused complex. In addition, NELF interacts with Pcf11, a transcription termination factor, coupling promoter proximal pausing and premature termination. Depletion of NELF and Pcf11 restored transcription emphasizing the importance of these proteins in establishment of viral latency.

My results suggest a model for HIV latency where transcription is repressed due to inefficient transcription elongation. NELF establishes a primary checkpoint in viral transcription, by preventing RNA Polymerase II processivity and by reinforcing the pause by maintaining a repressive chromatin environment. In addition, NELF recruits Pcf11 which causes premature termination resulting in the release of short transcripts, a characteristic of latent cells. T cell activation leads to recruitment of transcription factors, especially NF-κB which induce HIV transcription by recruiting PTEFb to the LTR. In addition, T cell activation increases the recruitment and processivity of Polymerase II by

inhibiting PTEFb-HEXIM1 interaction, making PTEFb available for upregulating transcription.

Chapter 2

Combinatorial signals from CD28 differentially regulate HIV transcription in T cells

This chapter is modified from the following publication

Combinatorial signals from CD28 differentially regulate human immunodeficiency virus transcription in T cells. Natarajan M, August A, Henderson AJ. J Biol Chem. 2010 Jun 4:285(23):17338-47.

2.1 Introduction

One of the major blocks to eradicating human immunodeficiency virus (HIV) infections with highly active anti-retroviral therapy (HAART) has been the inability of this treatment to eliminate cellular reservoirs harboring latent provirus (Bagasra, 2006; Coiras et al., 2009; Richman et al., 2009). T cells are a major target for HIV-1 infection and T cell signal transduction has been demonstrated to impact multiple steps of HIV-1 replication including provirus transcription (Gruters et al., 1991; Readinger et al., 2008; Strasner et al., 2008; Tyagi and Karn, 2007). Characterizing T cell signaling regulatory networks that govern T cell function and HIV-1 transcription is critical for understanding the molecular mechanisms that directly contribute to the establishment, maintenance and

breaking of proviral transcription latency (Brooks et al., 2003; Williams and Greene, 2007).

HIV provirus transcription is controlled by the upstream long terminal repeat (LTR), which includes cis-elements that are recognized by cellular transcription factors, including NF-κB, AP-1 and NFAT that are induced in response to TCR/CD28 engagement (Pierson et al., 2000; Rohr et al., 2003). These transcription factors recruit coactivators including histone acetyltransferases (HATs) and the ATP-dependent chromatin remodeling Swi/Snf complexes that influence the chromatin structure of integrated provirus (Henderson et al., 2004; Lee et al., 2002; Lusic et al., 2003; Pumfery et al., 2003; Van Lint et al., 1996). Furthermore, the LTR forms an RNA stem loop structure, TAR, which the HIV transactivator, Tat, binds. Tat enhances RNA polymerase II (Pol II) processivity by recruiting P-TEFb to the HIV-LTR (Bieniasz et al., 1999; Zhang et al., 2000). The availability of P-TEFb, which is negatively regulated through association with the HEXIM1/7SK RNA particle, is also controlled by cellular signals (Barboric et al., 2007; Yik et al., 2004). Therefore, it may be possible to manipulate specific signaling cascades to control HIV transcription and improve the efficacy of current anti-viral regimens.

Efficient T cell activation requires signals from the T cell receptor (TCR) as well costimulatory molecules including CD28, which enhances TCR activation, promotes cell survival and increases cytokine production (Lenschow et al., 1996; Slavik et al., 1999; Wang and Chen, 2004; Ward, 1996). CD28 possesses no enzymatic activity and mediates signaling by recruiting other proteins to tyrosines and proline rich motifs within its cytoplasmic domain. CD28 has four signaling tyrosine residues (Y) in the cytoplasmic

tail of CD28 at position Y173, Y188, Y191 and Y200, which are required for appropriate T cell activation, induction of cytokine gene expression, cytoskeleton reorganization and immunological synapse formation (Sadra et al., 2004; Teng et al., 1996). Key signal transduction events associated with CD28 include activation of Itk, Vav and Rho/Rac GTPases, PKC theta and transcription factors such as NF-κB, AP-1 and NFAT (August and Dupont, 1994; August et al., 1994; Cook et al., 2003; Hehner et al., 2000; Nolz et al., 2007; Park et al., 2009; Readinger et al., 2008).

We have previously shown that signaling associated with CD28 positively and negatively regulates HIV-1 provirus transcription. Specifically, we demonstrated that Y200 positively regulated HIV transcription by initiating Vav-1 and NF-κB signaling, whereas, recruitment of PI3K to the Y173 residue inhibited the ability of Tat to bind P-TEFb and HIV-1 transcription (Cook et al., 2003; Cook et al., 2002). How these apparently opposing signals are coordinated to lead to induction of HIV-1 transcription, as well as the role of the other tyrosines in modulating HIV-1 transcription in response to CD28, has not been extensively investigated. Using chimeric CD28 receptors harboring mutations in different key tyrosines in the cytoplasmic domain, we show that CD28 induces HIV transcription through distinct but cooperative activities associated with the individual tyrosines.

2.2 Material and Methods

2.2.01 Cell lines and primary cell.

Jurkat E6.1 T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.2 M L-glutamine. Human embryonic kidney 293T cells were also obtained from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin. Peripheral blood mononuclear cells were isolated from whole blood by ficoll/histopaque gradient (Sigma-Aldrich) and $CD4^+$ T cells were positively selected using the Dynal isolation kit (Invitrogen; 113.21D).

2.2.2 CD8/28 chimeric receptor mutants

The 8WT, YFFF, FFYF, FFFY, YFFF, YFYY, YYFY expression vectors have been described previously (Cook et al., 2003; Cook et al., 2002; King et al., 1997; Teng et al., 1996) and these key residues are shown in figure 1. To generate receptors, FYYF and YFFY, the plasmids corresponding to pMHneo FFFY and pMHneo YFFF were digested with ApaI and HindIII (New England Biolabs). Two fragments were generated, a 400bp fragment containing CD8α and the nucleotide sequence coding for tyrosine 173 of the cytoplasmic tail of CD28, and a 6.8 kb fragment containing the rest of CD28 and the pMHNeo backbone. The 400bp fragment from pMHneo FFFY and 6.8 kb fragment from pMHneo YFFF were gel purified and ligated to generate pMHneo FYYF using T4 DNA ligase (Invitrogen). The pMHneo YFFY was generated similarly by ligating the 400bp fragment isolated from pMHneo YYYF and the 6.8 kb fragment from pMHneo FFFY vectors. Additional mutants were generated by site directed mutagenesis. Primers

were designed (Table 1) to mutate key tyrosines to phenylalanines. Primers are listed in Table 1. For PCR reactions template plasmid DNA and appropriate primers were amplified using Vent polymerase (New England Biolabs) following standard protocols. The PCR products were digested with DpnI to eliminate donor plasmid and transformed into competent *E. coli* DH5α cells. Positive clones were confirmed by sequencing.

2.2.3 Generation of CD8/CD28 cell lines

CD8/28 expression constructs were introduced in Jurkat E6.1 T cells using electroporation. 3 x 10^7 cells were washed and resuspended in 750 μL of serum free RPMI containing 20 mM HEPES. 15 μg of plasmid DNA was then added to these cells and electroporated using a T280 square electroporation system (BTX, San Diego, CA). Cells were given 1 pulse for 65 ms at 215 V in a 4-mm cuvette, and then recovered in complete RPMI. 48 h post-transfections cells were put on selection by including 1 mg/mL G418 in the growth media. After three weeks cells expressing the chimeric receptors were positively selected for CD8 using the Dynal isolation kit (Invitrogen) to generate a polyclonal pool of cells. Several independent pools for each receptor were generated to assure that there was no bias from an individual transfection and selection protocol. We also generated clonal cell lines and these behaved identically to the CD8/CD28 pooled cell lines (Cook et al., 2003; Cook et al., 2002) and data not shown).The expression of the chimeric receptors was verified by western blot (data not shown) and flow cytometry.

Table 2.1 Primers for CD28 site-directed mutagenesis

Chimeric mutant	Initian Template	Forward primer	Reverse primer
pMHneo FYFF	pMHneo FYYF	5'-GCAAGCATTACCAGCCCTTTGCCCCACC-3'	5'-GGTGGGGCAAAGGGCTGGTAATGCTTGC-3'
pMHneo FFFF	pMHneo FYFF	5'-GCAAGCATTTCCAGCCCTTTGCCCCACC-3'	5'-GGTGGGGCAAAGGGCTGGAAATGCTTGC-3'
pMHneo YFYF	pMHneo FFYF	5'-GCTCCTGCACAGTGACTACATGAACATGACTCC-3'	5'-GGAGTCATGTTCATGTAGTCACTGTGCAGGAGC-3'
pMHneo YYFF	pMHneo FYFF	5' GCTCCTGCACAGTGACTACATGAACATGACTCC-3'	5' GGAGTCATGTTCATGTAGTCACTGTGCAGGAGC-3'
pMHneo FYFY	pMHneo YYFY	5' GCACAGTGACTTCATGAACATGACTCC-3'	5' GGAGTCATGTTCATGTAGTCACTGTGC-3'
pMHneo FFYY	pMHneo YFYY	5' GCACAGTGACTTCATGAACATGACTCC-3'	5' GGAGTCATGTTCATGTAGTCACTGTGC-3'

2.2.4 Flow cytometry

For flow cytometry 2 x 10^6 cells were washed and resuspended in 100 µL of staining media (PBS containing 2% serum). Cells were incubated with 2 µL anti-CD8α-PE (BD 555635) and anti-CD28-FITC (BD 555728) for 45 min on ice. Cells were washed three times with staining media and fixed with 2% paraformaldehyde. Fluorescence was measured using Becton Dickinson FACScan at the Flow Core Facility at Boston Medical Center.

2.2.5 Generation of HIV-1 Infectious Titers and Infections

0.5 x 10^6 293T cells were plated in a 6 well plate 24 h prior to calcium phosphate transfections, which were performed using 15 µg of pNL4–3-Luc(+) Env(–) Nef(–) (Henderson et al., 1995) or pHXB-PLAP-Env Nef(+) (Chen et al., 1996) (obtained from NIH AIDS Research and Reference Reagent Program) and 3 µg of RSV-Rev, 3 µg LTR VSV-G. 293T transfection efficiency for pNL4-3-Luc was assessed by determining

luciferase activity using a Promega luciferase kit (Madison, WI), whereas p24 ELISA were performed for the pHXB-PLAP virus. Supernatants were collected and filtered through a 0.45-micron disc prior to infection. Jurkat cells were infected with this virus for 12-16 h. Cells were then recovered and cultured in complete RPMI.

2.2.6 Activation of T cells

Jurkat T cells were washed and resuspended in 5% FCS RPMI. 1 x 10^6 cells were plated in each well of a 24 well plate. Cells were either left unactivated, or activated with 0.1 μg/ml anti-human CD3 alone (BD 555336), anti-CD3 and 1.0 μg/ml anti-human CD28 (BD 555725) or 1.0 μg/ml anti-human CD8α antibodies (BD 555630) for 30 minutes. 5 μg/ml of goat anti-mouse antibody (Sigma M 4280) was added to crosslink the receptors. Following 8 hours of stimulation, Jurkat cells were harvested and luciferase activity measured. In experiments using Cyclosporin A (CsA), infected Jurkat T cells were recovered and activated in the presence of 500 ng/mL CsA or vehicle control.

2.2.7 Immunoprecipitation and Immunoblots

Jurkat T cells were serum starved for 12-16 h, activated with antibodies as described above for 5 min prior to preparing protein extracts with 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 phenylmethylsufonyl fluoride and protease inhibitor cocktail III (Calbiochem). Lysates were precleared by incubating with protein A/G beads (Santa Cruz Biotechnology, sc-2003) for 30 min at 4ºC before incubating with primary anti-Vav (Santa Cruz

Biotechnology, sc 132). Protein A/G beads were added to the antibody-lysate mix for 1 h at 4ºC, beads were washed three times with lysis buffer, and then suspended in SDS PAGE loading buffer. The samples were heated for 5 min at 100ºC before loading onto a 10% SDS PAGE gel. Proteins were transferred to a PVDF membrane (Millipore) by electroblotting. Western blot analysis was carried out using a phosphotyrosine antibody (Transduction Laboratories 610024). The blot was stripped and reprobed with a Vav antibody. CyclinT1 (Santa Cruz Biotechnology, sc 8127) and HEXIM1 (Abcam, ab28016) immunoprecipitations were also carried out with the same protocol; however, nuclear extracts (described in the next section) instead of total protein extracts were used. In the immunoprecipitation experiments done with the PI3K inhibitor, 50 μM of LY294002 (Promega) was introduced 30 minutes before activation to the culture. Western blots were quantified by densitometry. The ratio of CyclinT1 over HEXIM was calculated for all samples in figure 3A and ratio of HEXIM over CyclinT1 was calculated for figures 3B, C and D. The numbers depicted in the figures represent ratio of immunoprecipitation in each lane verses immunoprecipitation from the unactivated lane.

2.2.8 Electrophoretic Mobility Shift Assay

Jurkat cells were activated for 8 hours and nuclear extracts were isolated by resuspending 1 x 10^6 cells in low salt buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF) for 15 minutes. 0.5 % NP-40 was added to rupture the cell membranes and the nuclei were pelleted and incubated in a high salt buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1 mM DTT and 1.0 mM PMSF) to isolate the nuclear extract. Electrophoretic

mobility shift assays (EMSAs) were carried out by incubating 5 μg of protein from nuclear extracts with 4 μg of poly dIdC (Amersham Biosciences), 0.25 mM HEPES (pH 7.5), 0.6 M KCl, 9.0% glycerol, 1.0 mM EDTA, 7.5 mM dithiothreitol, 50 mM $MgCl_2$. Reaction mixtures were preincubated with 100-fold excess of specific or nonspecific competitors, or 0.5 μg of polyclonal antibodies against NF-κB subunits p50 (Santa Cruz sc-7178) and p65 (Santa Cruz sc-109). Samples were loaded onto a 6% polyacrylamide gel and electrophoresed at 120 V in 0.5x Tris borate-EDTA. Probes for EMSA were generated by annealing oligonucleotides representing the HIV-1 NF-κB sites (5'-AGCTCCTGGAAAGTCCCCAGCGGAAAGTCCCTT-3' and 5'-AGCTAAGGGACTTTCCGCTGGGGACTTTCCAGG-3'). SP1 probe was used as nonspecific competitor (sense sequence 5'- GATCATTCGATCGGGGCGGGGCGAGC-3' and antisense sequence 5'- GATCGCTCGCCCCGCCCCGATCGAAT-3'). Probes were generated by end filling with the Klenow fragment of *E. coli* polymerase in the presence of [α-P^{32}]dCTP.

2.2.9 Transfection of LTR reporter constructs

Fifteen μg of pGL2 LTR luc and pGL2-mκB-LTR luc contructs (kindly provided by Dr. Suryaram Gummuluru, Boston University (Gummuluru and Emerman, 1999)) were electroporated into 20 x 10^6 Jurkat E6.1 cells using the T280 BTX electroporator. The cells were recovered in 5% FCS RPMI for 16 h. 1 x 10^6 cells were either left untreated or activated with 0.1 μg/ml anti-human CD3 or 0.1 μg/ml anti-human CD28 and 1.0 μg/ml anti-human CD28 and luciferase assays were performed 6 h post activation as described above.

2.2.10 Chromatin Immunoprecipitation

1 x 10^8 cells were infected with pHXB-PLAP virus for 5 days. Cells were then activated with 0.1 µg/mL anti-CD3 and 1.0 µg/ml anti-CD8 or anti-CD28 antibodies for 30 min. 5.0 µg/ml of goat anti- mouse was then added and cells were activated for 6 h. Cells were cross linked using 11% formaldehyde solution (prepared from 37% formaldehyde, 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% for 10 minutes at room temperature. The reaction is quenched by adding 2 M glycine to a final concentration of 240 mM. Cells were washed with PBS and resuspended in 1 ml 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, 10 seconds on, 30 seconds off. 100µL of sonicated chromatin was diluted 10 fold with dilution buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM PMSF, 0.1% SDS) and incubated with 1 µg antibody Pol II (sc-899) p65 (sc-109) for 16 h at 4ºC. Protein A/G beads were then added for 2 h. The beads were then washed twice each with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 0.1, 500 mM NaCl) and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and TE. Complexes were eluted with 1% SDS, 0.1 M $NaHCO_3$. The crosslinks were reversed at 65ºC for 4 h, followed by addition of proteinase K for 1 hour at 45ºC. The DNA was extracted using phenol chloroform, and precipitated with ethanol. Quantitative real time PCR analysis was carried out using SYBR green reagents and the primers 5'-

TGCATCCGGAGTACTTCAAGA-3' and 5'-GAGGCTTAAGCAGTGGGTTC-3' which amplify -150-+76 of the HIV-LTR, and 5'- GACTAGAGCCCTGGAAGCA-3' and 5'- GCTTCTTCCTGCCATAGGAG-3' which amplifies +5396 to +5531 region of HIV.

2.2.11 Statistical Analysis

Statistical analysis was carried out using Student t test. A two-tailed distribution was performed on paired samples, comparing CD3 responses to CD3+CD8 responses. Values less than 0.01 were considered significant.

2.3 Results

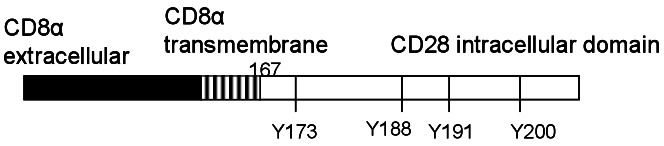
2.3.1 Specific tyrosines within the CD28 cytoplasmic tail regulate HIV-1 transcription.

To study the role of CD28 in regulating HIV transcription, we employed a strategy that was previously described (Cook et al., 2003; Cook et al., 2002; Teng et al., 1996), in which we generated CD8/28 chimeric receptors with mutations in key tyrosine residues. The chimeric receptors were designed such that the cytoplasmic domain of CD28 was fused to the transmembrane and extracellular domain of CD8α, which we refer to henceforth as CD8/CD28. This chimeric receptor forms a dimer similar to CD28 and functions identical to endogenous CD28 (Teng et al., 1996). In addition, the generation and expression of these CD8/CD28 chimeras allow their expression in cells along with

endogenous CD28, and the direct comparison of mutant and WT CD28 signals in the same cells (Cook et al., 2003; Cook et al., 2002; Teng et al., 1996). As shown in figure 1A, we changed individual or different combinations of tyrosines in the cytoplasmic tail of CD28 to phenylalanine (F). Polyclonal populations of the Jurkat E6.1 T cell line expressing these chimeric receptors were generated and stable expression of CD8/CD28, as well as endogenous CD28 receptors, was evaluated by flow cytometric analysis (Fig. 2.1). This analysis confirmed the stable expression of the chimeric receptors in Jurkat T cells, without altering the expression of endogenous CD28. The expression of chimeric receptors at the cell surface suggests that inspite of the mutations, the chimeric receptors fold properly and our this also experimental results are purely due to the function of the receptor, rather than due to the lack of a properly folded receptor.

This panel of Jurkat T cells expressing CD8/CD28 chimeras was infected with NL4-3 luciferase virus to evaluate the function of different tyrosines in HIV-1 transcription. Using the HIV-luciferase clone, which lacks envelope and supports only a single round of infection, allowed us to focus on CD28 signaling and HIV-1 transcription, rather than potential effects CD28 signaling has on virus replication and spread. Infected Jurkat T cells were stimulated through the TCR using anti-CD3 antibodies and either the chimeric CD8/CD28 receptor (using anti-CD8α antibodies) or the endogenous CD28 receptor (using anti-CD28 antibodies), which served as a control to ensure that the different T cell lines were capable of being activated. Controls included stimulating cells through the CD28, CD8/CD28 and CD3 receptors alone. Consistent with our previous studies the

Table 2.2 List of all the CD8/28 chimeric mutants used in this study



Mutant				
8 wt	Y	Y	Y	Y
AllF	F	F	F	F
Y188	F	Y	F	F
Y191	F	F	Y	F
Y200	F	F	F	Y
Y173F	F	Y	Y	Y
Y200F	Y	Y	Y	F
YYFF	Y	Y	F	F
YFYF	Y	F	Y	F
YFFY	Y	F	F	Y
FYYF	F	Y	Y	F
FYFY	F	Y	F	Y
FFYY	F	F	Y	Y
YYFY	Y	Y	F	Y
YFYY	Y	F	Y	Y
Del 167	No intracellular domain			

chimeric receptor CD8/CD28 WT, which retains all functional tyrosines, leads to transcriptional activation similar to that mediated by the endogenous CD28 receptor (Cook et al., 2003; Cook et al., 2002; Teng et al., 1996). Receptors lacking the cytoplasmic domain, del167, and the All F mutant, where all four tyrosines in the cytoplasmic tail of CD28 were mutated to phenylalanines, did not induce HIV-1 transcription confirming an indispensable role for these tyrosines in the cytoplasmic tail of CD28 (Fig. 2.2) (Cook et al., 2003; Cook et al., 2002; Teng et al., 1996).

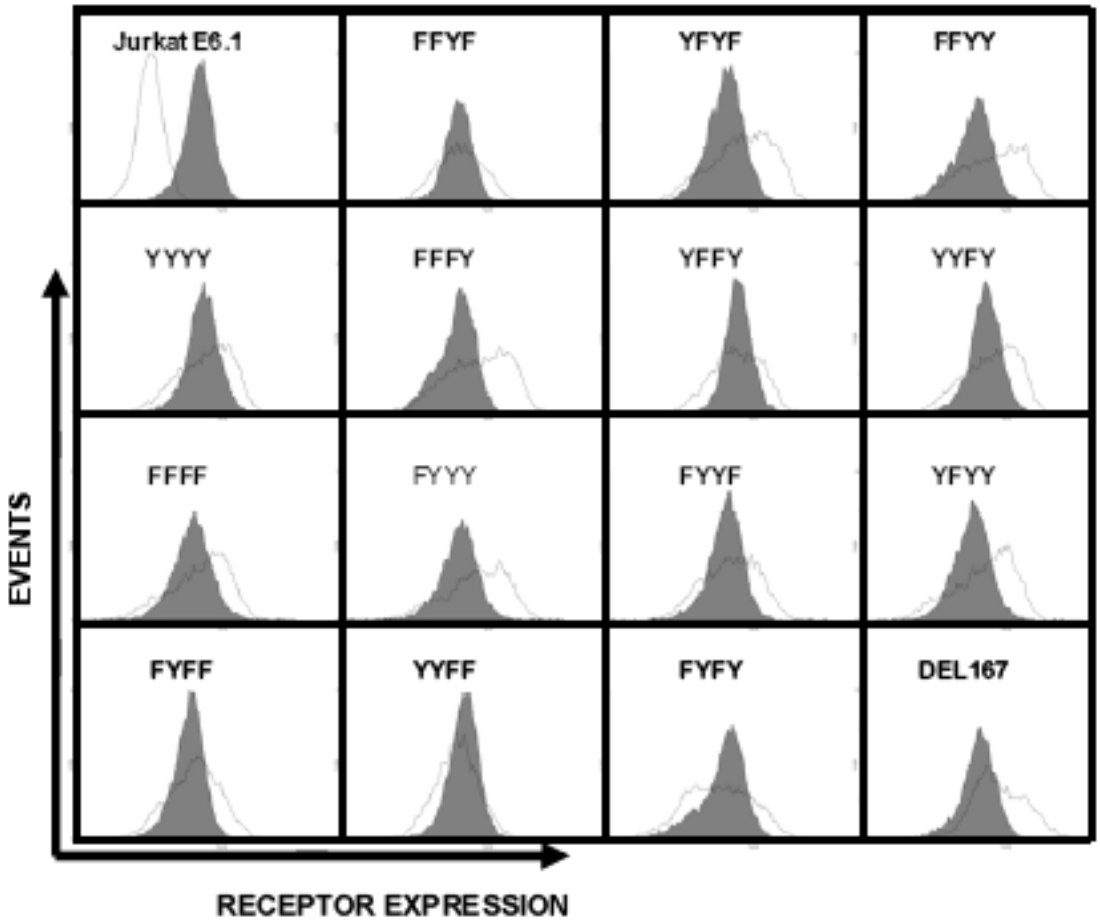


Figure 2.1: Expression of CD8/28 chimeric receptors. Expression of CD8/28 chimeric receptor (black line) and the endogenous CD28 (filled grey histogram) was determined by flow cytometry in either Jurkat T cells or Jurkat T cells stably expressing all the chimeric receptors used in this study. Cells were stained with PE-conjugated CD8α antibody, and FITC-conjugated CD28 antibody.

Previous studies have shown that the Y173 residue negatively regulates HIV-1 transcription whereas signaling through Y200 was necessary for HIV-1 transcription (Cook et al., 2003; Cook et al., 2002). We were interested in determining the functional interplay between these two apparently opposite activities mediated through Y200 and Y173, as well as the integration of signals downstream of other tyrosines within the CD28 cytoplasmic domain. We initially examined the ability of CD28 receptors with one functional tyrosine, Y173 (YFFF), Y188 (FYFF), Y191 (FFYF) or Y200 (FFFY), to

support HIV-1 transcription. As expected, the YFFF receptor was unable to activate HIV transcription, consistent with our previous report that implicated Y173 signaling as inhibitory (Cook et al., 2002). Furthermore, the FYFF receptor did not support HIV-1 transcription indicating that, signals downstream of Y188 were not sufficient to induce HIV-1 transcription. However, the FFYF and FFFY receptors activated HIV-1 transcription to levels comparable to the wild-type CD8/CD28 or the endogenous CD28 receptors, indicating that Y191 and Y200 positively regulate HIV transcription in the absence of other tyrosine residues in the signaling domain of CD28. These data suggest that Y191 and Y200 have distinct functions from Y173 and Y188. Our data also indicate that signaling through Y191 and Y200 are critical in governing the overall positive signaling associated with CD28 costimulation in HIV-1 transcription.

We were interested in understanding how the positive signals associated with Y191 and Y200 overcome the inhibition imposed by Y173 within the context of the WT receptor. To address questions regarding how these signals are integrated or functionally cooperate to control the transcriptional response of HIV provirus we examined the activity of chimeric receptors that had a functional Y173 combined with different distal tyrosines: Y173 and Y200 (YFFY), Y173 and Y188 (YYFF) and Y173 and Y191 (YFYF). Signaling through YFFY and YFYF did not support activation of HIV-1 transcription, suggesting that positive signals downstream of Y191 and Y200 were not sufficient to overcome the repressive activity associated with Y173. Surprisingly, Y188, which was not capable of inducing HIV-1 transcription when it was the only tyrosine within the CD28 cytoplasmic tail was able to partially overcome the repressive activity of Y173 resulting in 70% activity compared to the endogenous CD28 receptor (Fig. 2.2).

Therefore, none of the tyrosines individually were able to completely overcome the negative signals associated with Y173, implying that the ability of CD28 to activate HIV-1 transcription is a combinatorial event that requires cooperative activities of Y188, Y191 and Y200. These data also suggest that Y188 has a modulatory role in CD28 costimulation by interacting and modifying the activity of other tyrosines within the cytoplasmic tail including Y173.

Our previous data examining the YYYF receptor, showed that Y188 and Y191 were not able to cooperate to activate HIV-1 transcription in the presence of Y173 (Cook et al., 2002). To investigate what combination of tyrosine residues in CD28 could overcome the inhibition posed by Y173, we studied the functions of the receptors containing the combination of Y173, Y188 and Y200 (YYFY) and the combination of Y173 Y191 and Y200 (YFYY). YYFY was compromised in its ability to activate HIV-1 transcription as it led to only 55% of the endogenous CD28 response. Activation of cells via YFYY receptor did not activate HIV-1 transcription (40% of endogenous receptor activation, similar to stimulation via the TCR alone) despite Y191 and Y200 being shown to be sufficient to activate HIV-1 transcription when present by themselves in the receptor. Even though Y188 does not appear to directly activate HIV-1 transcription, this residue is indispensable for coordinating signals that induce HIV-1 transcription.

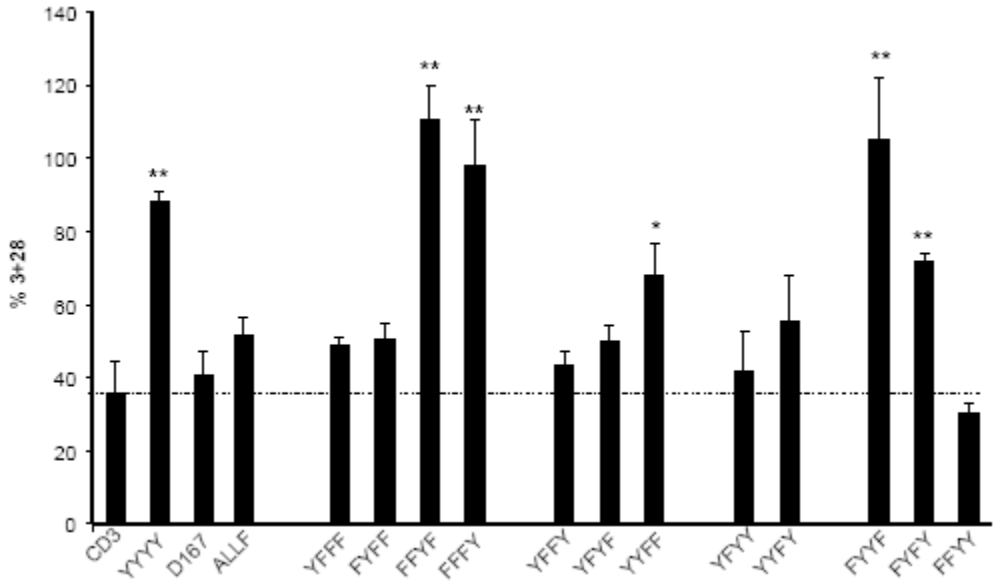


Figure 2.2: Specific tyrosine residues in the cytoplasmic domain regulate HIV-1 transcription. Jurkat cells expressing the indicated CD8/28 chimeric receptors were infected with NL4-3 luciferase virus for 16 h. Post infection cells were either left unstimulated, or stimulated with 0.1 µg/ml of anti-CD3 plus 1.0 µg/ml of anti-CD8α, or with 1.0 µg/ml of anti-CD28, followed by 5 µg/ml of secondary antibody (goat anti-mouse) to crosslink the receptors. Eight hours post-activation the cells were lysed and luciferase activity was measured. The luciferase activity is shown as a percentage of endogenous response, wherein the CD3+CD28 response of each cell line was set to a 100%. The dotted line represents the luciferase activity corresponding to activation through CD3 alone. The data shown were done in triplicates and represents one experiment out of a total of three independent infections. A two-tailed T-test was performed on paired samples comparing CD3 responses to CD3+CD8 responses. *$p<0.01$; **$p<0.005$.

Whether Y188 also modifies the activity of Y191 and Y200 was examined with chimeric receptors that had two functional tyrosines in the absence of the inhibitory signals associated with Y173: Y188 and Y191 (FYYF), Y188 and Y200 (FYFY) and Y191 and Y200 (FFYY). The FYYF receptor was able to fully activate HIV-1 transcription upon stimulation through the TCR and the CD8/CD28 receptor. FYFY was also able to activate HIV-1 transcription, however, it was reduced approximately 20% compared to the FYYF, CD8/CD28 wild-type or the endogenous receptor. Surprisingly, the FFYY receptor that includes Y191 and Y200, which alone are sufficient to induce

HIV-1 transcription, does not activate HIV-1 transcription (Fig. 2.2). This suggests that Y191 and Y200 do not cooperate to activate HIV-1 transcription. Furthermore, these data are consistent with Y188 modifying signals downstream of the other tyrosines in the intracellular domain of CD28.

2.3.2 CD28 signaling does not activate PTEFb

HIV-1 transcription is in part regulated by RNA Polymerase II (RNA Pol II) processivity (Zhang et al., 2007a; Zhou and Yik, 2006). In the absence of Tat, there is an accumulation of short initiated transcripts as a result of RNA Pol II pausing and premature termination that occurs due to the lack of P-TEFb (Biglione et al., 2007; Toohey and Jones, 1989). Tat enhances transcription processivity by binding the TAR element and recruiting P-TEFb to the HIV-LTR. P-TEFb is comprised of two subunits, CyclinT1 and Cdk9, and its availability to bind promoters is regulated by its association with the HEXIM/7SK RNA particle complex (7SK RNP). Release of P-TEFb from the repressive 7SK RNP permits P-TEFb to target and activate RNA Pol II complexes (Barboric et al., 2007; Yik et al., 2004). We examined if CD28 signaling regulates P-TEFb availability and activity and determined whether this influences HIV transcription. We assessed the ability of the different cell lines expressing chimeric receptors to modulate CyclinT1-HEXIM interactions. Activation of the wild type chimeric receptor (YYYY) as well as the endogenous CD28 receptor mediated release of P-TEFb from HEXIM (Figs. 2.3A, B).

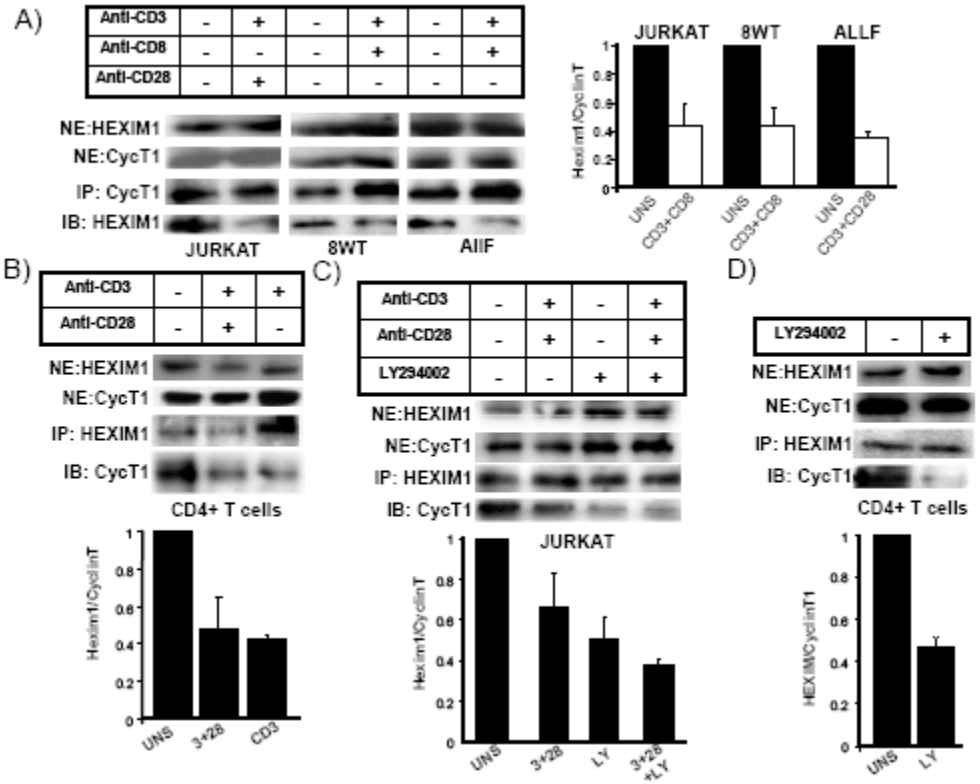


Figure 2.3: TCR signals negatively regulate CyclinT1-HEXIM1 interaction. A) Jurkat cells stably expressing the indicated CD8/28 chimeric receptors were serum starved for 12-16 h prior to activating with 0.1 µg/ml anti-CD3 and 1.0 µg/ml anti-CD8, whereas wild type Jurkat T cells were activated with 0.1 µg/ml anti-CD3 and 1.0 µg/ml of anti-CD28 for 30 min before adding 5 µg/ml of goat-anti-mouse for 4 h. Nuclear extracts were prepared and CyclinT1 immunoprecipitation was carried out. The samples were run on a SDS-PAGE gel and western blot analysis assessed the amount to CyclinT1, and an anti-HEXIM1 western blot was done to determine the amount of HEXIM1 associated with CyclinT1. Blots were quantified in the panel on the right. B) $CD4^+$ T cells isolated from human peripheral blood, and were left unstimulated or activated with 0.1 µg/ml anti-CD3, 0.1 µg/ml anti-CD3, or 1.0 µg/ml of anti-CD28 alone. Receptors were cross linked by adding 5 µg/ml of goat-anti-mouse for 4 h. Nuclear extracts were prepared and an anti-HEXIM1 immunoprecipitation was carried out. Western blot analysis determined the amount of HEXIM1 immunoprecipitated and the amount of CyclinT1 associated with HEXIM1. Blots were quantified in the lower panel. C) Nuclear extracts were prepared from Jurkat cells activated through the CD3 and CD28 receptors as described above and an anti-HEXIM1 immunoprecipitation was carried out. Western blot analysis determined the amount of HEXIM1 immobilized as well as CyclinT1 associated with HEXIM1 (quantified in the panel below). D) $CD4^+$ T cells were treated with 50 µM LY294002 for 4 hours prior to preparing nuclear extracts. HEXIM1 was immunoprecipitated and Western blot analysis determined levels of HEXIM1 and CyclinT1 (quantified in the panel below). For all panels, WCE extracts were prepared and probed with the indicated antibodies as an input control. Data shown are from a single experiment, and quantification is performed from at least two independent experiments.

Furthermore, the all F receptor and activation with anti-CD3 alone induced release of P-TEFb. These data indicate that TCR signaling is sufficient for releasing P-TEFb from the 7SK RNP. This was also observed in primary $CD4^+$ T cells isolated from peripheral blood, which showed a decrease in HEXIM1 associated CyclinT1 upon activation through the TCR (Fig. 2.3B).

PI3K is recruited to the YMNM motif in CD28, which includes Y173. We have previously shown that PI3K inhibits P-TEFb-Tat interactions and HIV-1 transcription (Cook et al., 2002). Although CD28 signaling did not influence P-TEFb release from the HEXIM-7SK RNP complex, we wanted to determine if CD28 might negatively regulate P-TEFb by promoting the formation of P-TEFb-7SK RNP. Treating Jurkat T cells with the PI3K inhibitor LY294002 decreased CyclinT1-HEXIM interactions both before and after CD3 + CD28 stimulation (Fig. 2.3C). Since Jurkat T cells have a defect in PI3K signaling due to a defect in PTEN, we also assessed the effect of inhibiting PI3K in $CD4^+$ T cells (Xu et al., 2002). Inhibition of PI3K in primary T cells decreased the association of CyclinT1 and HEXIM (Fig. 2.3D). Our data indicates that active PI3K signaling promotes interactions between CyclinT1 and HEXIM, thus sequestering P-TEFb from Tat and inhibiting HIV-1 transcription. These data suggest that CD28, through the recruitment of PI3K to Y173, limits HIV-1 transcription by inhibiting the release of P-TEFb.

2.3.3 CD28-mediated induction of HIV transcription requires NF-κB activation

To gain a better understanding of the differential role of specific tyrosines in CD28 signaling we examined if specific signaling events were associated with individual

tyrosine residues. Vav, a guanine nucleotide exchange factor (GEF) for the Rho GTPase, is known to be a target of T cell activation and has been suggested to be downstream of CD28 signaling (Marinari et al., 2002). We have shown that Vav is activated by the FFFY CD8/28 chimeric receptor and, in the absence of Y200, Vav and Rac1, as well as NF-κB, are not efficiently activated (Cook et al., 2003). These observations indicate that Vav and Rac are key regulatory events for inducing HIV-1 transcription. Jurkat cells expressing CD8/28 FYFF, FFYF and FFFY receptors were stimulated by cross-linking TCR and CD28 receptors and Vav activation and tyrosine phosphorylation was assessed by immunoblotting. Following T cell activation FFYF and FFFY, which fully support HIV transcription, induced Vav phosphorylation (Fig. 2.4A). However, FYFF, which is compromised in its ability to activate HIV transcription, also activated Vav (Fig. 2.4A), indicating that, although activation of Vav is downstream of CD28 signaling, it is not sufficient to induce HIV-1 transcription mediated by T cell activation. We also inhibited NFAT signaling with Cyclosporin A, (CsA). As expected, inhibition of NFAT led to a decrease in overall HIV-1 transcription, but CD28 signaling still enhanced HIV-1 transcription, with an approximately three fold induction in both CsA treated and untreated cells, indicating that the activation of NFAT is primarily regulated by the TCR (Fig. 4B). In addition, we examined the MAPK signaling pathways, ERK1/2, JNK and p38, and did not detect differential activation of these molecules upon costimulation via CD28 (Fig. 4C).

A consequence of T cell signaling is the activation of transcription factors, which in turn bind regulatory cis-elements within promoters and enhancers to regulate

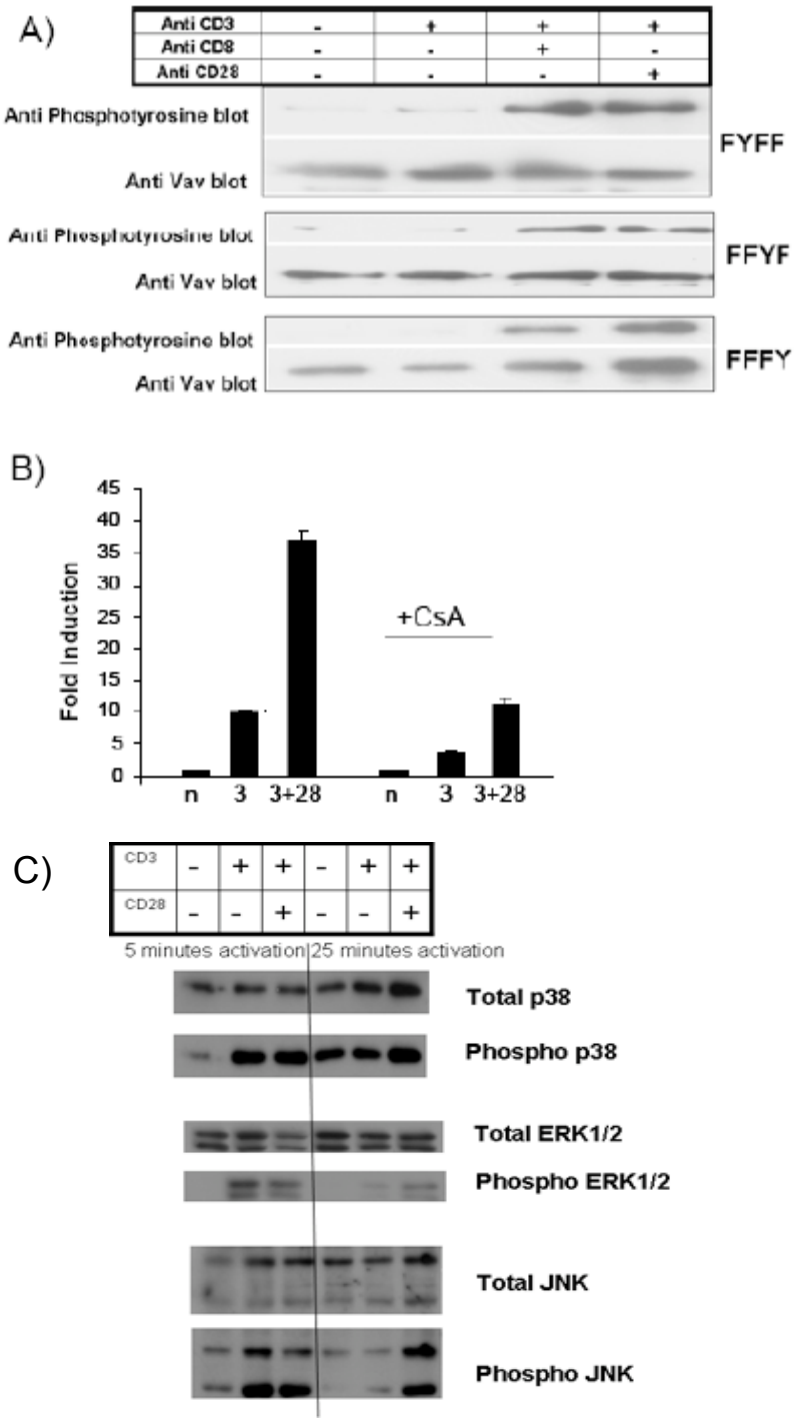


Figure 2.4: Vav activation is not necessary for induction of HIV transcription. A) Jurkat T cells stably expressing the CD8/28 chimeric receptors FYFF, FFYF and FFFY were activated as indicated with antibodies against CD3, CD28 and CD8, for 5 min after crosslinking the receptors. Vav was immunoprecipitated from cell lysates and Vav phosphorylation was determined by anti-phosphotyrosine western blot. Vav phosphorylation downstream the chimeric receptors (CD8 activation) were compared

directly to the endogenous CD28 receptor. B) Jurkat T cells were infected with NL4-3 luc virus, and activated with anti-CD3 and CD28 antibodies in the presence of Cyclosporin A. Six hours post activation luciferase assay was performed. The unstimulated response was set as 1 and fold activation has been plotted. C) Jurkat T cells were activated with CD3, and CD3+28 for 5 or 25 minutes. Whole cell lysates were prepared and analysed for the expression of phospjorylated and total p38, ERK1/2 and JNK.

transcription. The HIV LTR has binding sites for multiple transcription factors, including NF-κB, NFAT and AP-1 (Alcami et al., 1995; Cron et al., 2000; Shapiro et al., 1997). We wanted to examine if the activities of the chimeric receptors on HIV transcription reflected differential activation and recruitment of transcription factors to the HIV LTR. Since costimulation was only modestly affected by CsA and MAPK pathways were not differentially activated by the CD28 mutants, which are upstream of NFAT and AP-1, respectively, we focused on the activation of NF-κB, which has been shown to be indispensable for HIV-1 transcription (Alcami et al., 1995). Electrophoretic mobility shift assays were performed using nuclear extracts isolated from activated Jurkat T cells stably expressing CD8/28 chimeric receptor mutants to measure NF-κB binding activity following T cell activation. Receptors that had Y191 (FFYF) and Y200 (FFFY) induced NF-κB binding, whereas FYFF,YFFY, or the all F receptors were unable to activate NF-κB, correlating with the ability of the former receptors to support HIV transcription (Fig. 2.5A). The specificity of NF-κB binding was confirmed using unlabeled competitor oligonucleotides (Fig 2.5A). We also verified the identity of the NF-κB subunits by performing supershift assays, performing the gel shifts in the presence of anti-p65 and anti-p50 antibodies (Fig. 2.5B). Anti-p50 antibody generated supershifted complexes and anti-p65 disrupted binding of NF-κB consistent with the conclusion that the activated NF-κB complexes are comprised of p50 and p65 subunits. These data suggest that

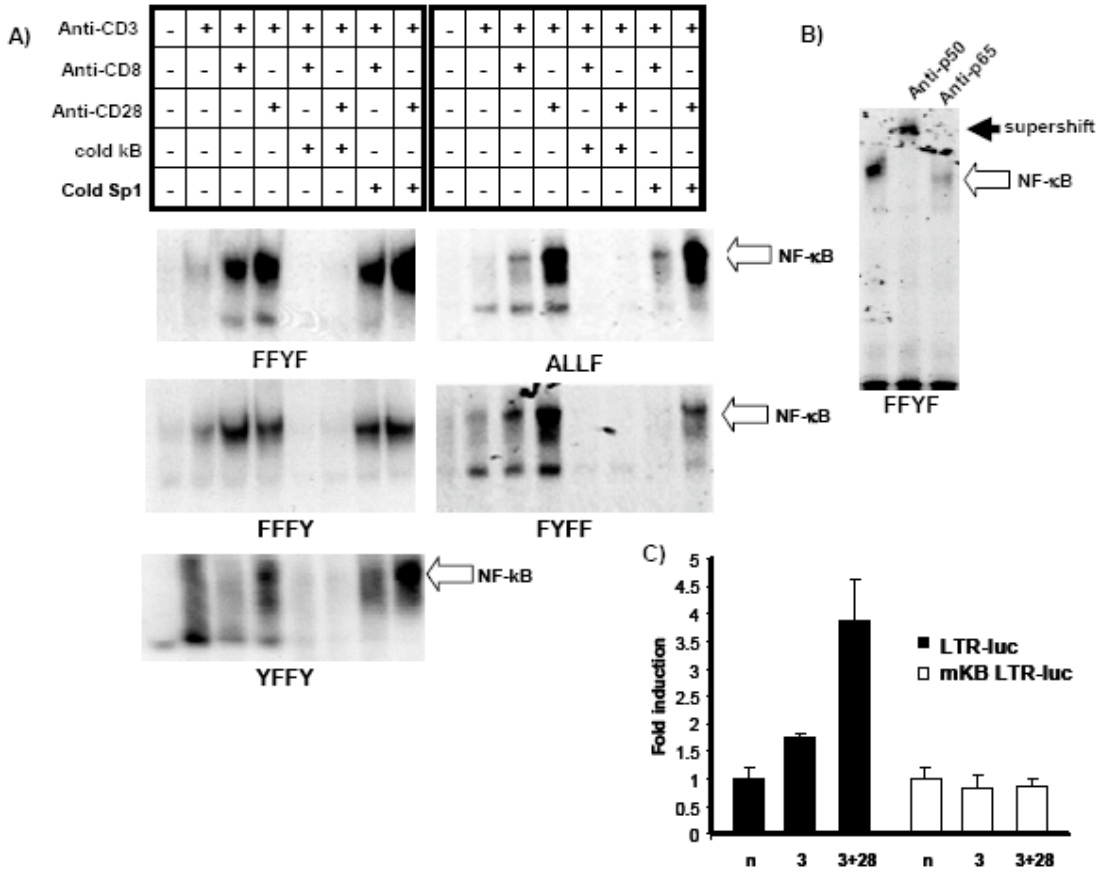


Figure 2.5: NF-κB activation is indispensable for CD28-mediated HIV-1 transcription. A) Jurkat cells stably expressing the CD8/28 chimeric receptors FYFF, FFYF, FFFY and YFFY were activated as described above. Nuclear extracts were prepared and incubated with radiolabelled NF-κB probe for 20 min, and samples were run on a 6% polyacrylamide gel. Reactions were also performed in the presence of 100 fold excess of specific competitor or non-specific competitor. B) EMSAs were performed as in (A) in the presence of anti-p50 and anti-p65 antibodies. Gel shifted complexes are denoted by arrowheads, supershifted complexes are indicated by bolded arrowheads. Data shown are from a single experiment that is representative of three independent experiments.C) Jurkat cells were electroporated with wild-type LTR-luciferase and mKB-LTR luciferase constructs. Cells were either left unstimulated, or stimulated with 0.1 µg/ml of anti-CD3, or 0.1 µg/ml of anti-CD3 and 1.0 µg/ml of anti-CD28, followed by 5 µg/ml of secondary antibody (goat anti-mouse) to crosslink the receptors. Eight hours post-activation the cells were lysed and luciferase activity was measured. The luciferase activity is shown as fold induction wherein luciferase units corresponding to the unstimulated samples were set to 1.

activation of NF-κB is critical for CD28-mediated HIV-1 transcription.

In order to verify a role of NF-κB in CD3 + CD28-mediated HIV transcription, Jurkat T cells were transiently transfected with luciferase reporters under the control of either wild-type HIV LTR or a LTR harboring mutated NF-κB (mκB) binding sites (mκBLTR). LTR-Luc was induced by approximately 4 fold when cells were activated through CD3 plus CD28 compared to cells treated with either anti-CD3 alone or were not activated (Fig 2.5C). Consistent with NF-κB being necessary for transcriptional activation, CD3 plus CD28 signaling did not induce the mκB-LTR (Fig 2.5C). These data suggests that the activation and binding of NF-κB to the HIV LTR is indispensable for HIV transcription.

To verify that CD28 signaling leads to changes in NF-κB binding at the HIV-LTR of HIV-1 infected T cells, we performed chromatin immunoprecipitation (ChIP) experiments. Jurkat T cells expressing CD28 chimeric receptors were infected with pHXB.2 and activated using anti-CD3, anti-CD8 or anti-CD28 antibodies. Chromatin was prepared and p65 binding at the HIV-LTR was measured. ChIP results indicate that recruitment of p65 to the HIV-LTR is compromised when costimulatory signals are initiated by the FYFF receptor (Fig. 2.6A), whereas, recruitment of p65 to the LTR was comparable to the endogenous CD28 receptor when cells were activated through the FFYF and FFFY chimeric receptors. We also examined the distribution of RNA Polymerase II (RNA Pol II) at the HIV-LTR, verses +5396 bp downstream of the transcriptional start site to determine RNA Pol II processivity. RNA Pol II was detected

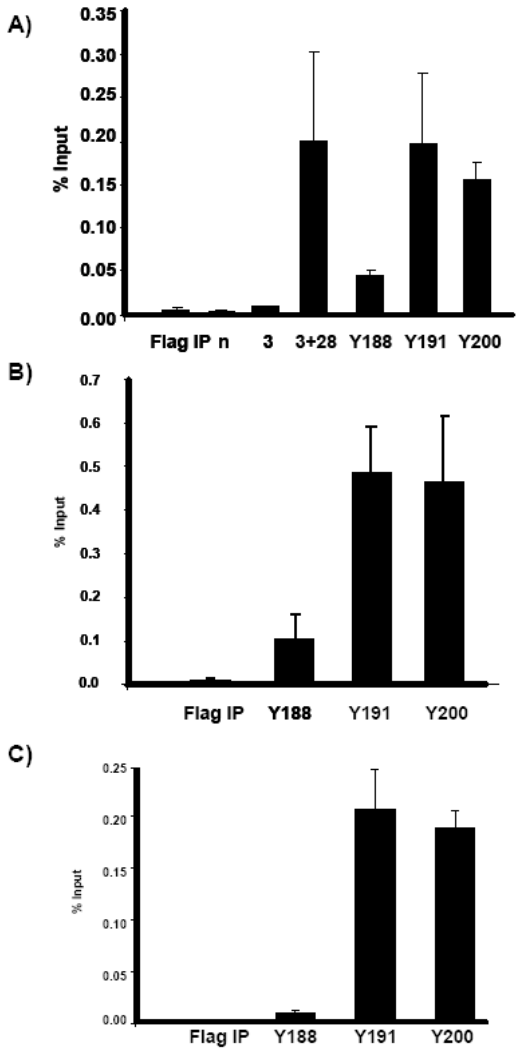


Figure 2.6: CD28 mediated signals induce recruitment of NF-κB to proviral LTR. A) Jurkat cells expressing the indicated CD8/28 chimeric receptors were infected with pHXB-PLAP virus. Infected cells were then activated for 6 h, and chromatin was isolated. Chromatin immunoprecipitations were performed using p65 antibody. FLAG antibody was used as a non specific antibody control. Real time PCR was carried out for the -150 - +76 region of the HIV LTR, and the data are plotted as a percentage of input immunoprecipitated in each reaction. B) Jurkat cells expressing the indicated CD8/28 chimeric receptors were infected with pHXB-PLAP virus. Infected cells were then activated for 6 h, and chromatin was isolated. Chromatin immunoprecipitations were performed using RNA Pol II antibody. FLAG antibody was used as a non specific antibody control. Real time PCR was carried out for -150 - +76 region of the HIV LTR, and the data are plotted as a percentage of input immunoprecipitated in each reaction..C) Jurkat cells expressing the indicated CD8/28 chimeric receptors were infected with pHXB-PLAP virus. Infected cells were then activated for 6 h, and chromatin was isolated. Chromatin immunoprecipitations were performed using RNA Pol II antibody. FLAG antibody was used as a non specific antibody control. Real time PCR was carried out for +5396 to +5531 region of the HIV, and the data are plotted as a percentage of input immunoprecipitated in each reaction

at both the LTR and downstream sequences in cells activated through the FFFY and FFYF receptors, whereas RNA Pol II was not detected at the LTR and +5396 in activated FYFF cells (2.6B & C). Overall, the levels of Pol II associated with downstream HIV sequences directly reflected levels of Pol II at the LTR indicating that Pol II recruitment and not RNA Pol II processivity are regulated by CD3 + CD28 signaling. Taken together, these data indicate that the ability of CD28 to enhance NF-κB signaling and RNA Pol II recruitment are primarily responsible for inducing HIV transcription in response to CD3+CD28 activation.

2.4 Discussion

We have studied CD28 signaling and its impact on HIV transcription, with particular focus on the interplay between signals arising downstream from the four tyrosine residues present in its cytoplasmic domain. Our study reveals a complex interplay of signals downstream of these tyrosine residues that are integrated to enhance HIV transcription.

Examining individual tyrosines we confirmed an inhibitory role for signals emanating from Y173 and positive activity for Y200 in CD28 signaling (Cook et al., 2003; Cook et al., 2002; Teng et al., 1996). In addition, Y191 is sufficient to activate CD28-dependent HIV-1 transcription similar to Y200. However, when different combinations of tyrosines were examined the complexity of CD28 signaling is revealed. Y188, which does not induce HIV-1 transcription, modulates the negative activity of Y173 as demonstrated by the ability of the YYFF mutants to induce HIV transcription. Y188 also dampens the Y200 response since FYFY does not enhance HIV-1 transcription to the same level as WT CD28, whereas Y188 does not alter Y191 signaling capability. Therefore, we

propose that by modulating positive and negative signals, Y188 may set signaling thresholds for HIV transcription. The indispensable role of Y188 for regulating HIV transcription is also supported by the observation that the YFYY receptor is unable to support HIV transcription. In addition, Y191 and Y200, which alone are sufficient for induction of HIV transcription, do not cooperate but rather neutralize each other when in receptors that contain both Y191 and Y200 emphasizing the need for all functional tyrosines in the receptor to assure proper CD28 signaling and function in HIV transcription. Overall, signals downstream to Y191 and Y200 cooperate in the presence of Y188 to overcome the inhibitory signals posed by Y173 to activate HIV-1 transcription. How Y188 is modulating CD28 signaling is not clear, however, it is not simply altering the recruitment of PI3K to CD28 since similar levels of PI3K are associated with Y173 in the context of Y188 or the F188 mutation (Fig. 2.8).

PI3K is recruited to the YMNM motif of CD28, and mutation of the Y173 to F173 abolishes the recruitment and activation of PI3K (Cai et al., 1995). Our previous studies have shown that Y173 inhibits HIV-1 transcription in a Tat dependent manner by negatively regulating the Tat-P-TEFb complex (Cook et al., 2002). Our current study indicates that PI3K diminishes the availability of P-TEFb by stabilizing interactions between CyclinT1 and HEXIM1. Therefore, PI3K mediated signals appear to favor the sequestration of P-TEFb in the 7SK RNP, decreasing the availability of P-TEFb for recruitment to the HIV-LTR. Furthermore, we observed a minimal role for CD28 in activating P-TEFb, with release of P-TEFb being primarily controlled by TCR signaling. This is consistent with previous reports indicating that Ca^{2+} signaling released P-TEFb from 7SK RNP (Chen et al., 2008). Our data seem to be in conflict with recent data from

Contreras et al, who showed that PI3K/AKT increases active P-TEFb and HIV transcription induced by HMBA treatment (Contreras et al., 2007). It is not apparent why there is a discrepancy between our data although it could reflect differences in signals induced by HMBA verses CD28, cell model systems, or temporal differences in our assays. It should be noted that a recent paper by Chen et al has also suggested a minimal role for PI3K in P-TEFb activation, which supports our findings. The use of PI3K and AKT inhibitors did not reverse the UV induced disruption of the P-TEFb-HEXIM associationin HeLa cells (Chen et al., 2008).

Activation of NF-κB is indispensable for induction of HIV transcription. Residues that lead to NF-κB signaling such as Y191 and Y200 promote NF-κB binding, as well as recruitment of NF-κB and RNA Pol II to the HIV-1 LTR, whereas, Y188 does not activate NF-κB or HIV-1 transcription. Furthermore, activation of Vav is not sufficient for activation of NF-κB, as Y188 is capable of inducing Vav phosphorylation but not HIV-1 transcription. Our results would suggest that NFAT and MAPK pathways have a minimal role in CD28-mediated HIV transcription and would implicate roles for other signaling pathways. PKC theta (Coudronniere et al., 2000; Dienz et al., 2003; Sanchez-Lockhart et al., 2008; Villalba et al., 2002), CARMA1 (Lee et al., 2005; Wang et al., 2004), and Cot kinase (Lin et al., 1999) are downstream of CD28 and potential mediators of NF-κB signaling. The role of these factors and how they are integrated by CD28 signaling requires additional investigation.

Recent studies have used similar approaches to examine the role of CD28 signaling in regulating T cell activation and, in particular induction of IL-2 transcription. In summary, these reports suggest that the Y173 residue and PI3K are dispensable for IL-2

transcription (Dodson et al., 2009; Gogishvili et al., 2008; Sadowski et al., 2008; Teng et al., 1996) whereas, Y191 and Y200 positively regulate IL-2 (Teng et al., 1996), correlating with CD28-mediated NF-κB activation. Furthermore, in general, mutations in the CD28 cytoplasmic tail that failed to activate IL-2 transcription did not support efficient HIV-1 transcription in response to CD3 + CD28 signaling. However, IL-2 and HIV-1, despite being activated by overlapping signals in response to T cell activation, do exhibit some unique responses to CD28 signaling. For example, the FYYF chimeric receptor does not robustly induce IL-2 transcription but is capable of fully activating HIV-1 transcription (data not shown). The responses of IL-2 and HIV-1 transcription to CD28 signaling may reflect differential requirements for gene activation, such as, a lower signaling threshold for induction HIV-1 transcription.

In summary, we show that CD28-mediated induction of HIV-1 transcription is the result of coordinated positive and negative signals that ultimately lead to NF-κB activation. We propose a model wherein TCR signaling positively regulates P-TEFb, whereas, CD28 signaling primarily leads to the activation of NF-κB and recruitment of RNA polymerase II. In addition, HIV-1 transcription can also be limited by CD28 signaling which promotes the sequestration of functional P-TEFb in the 7SK RNP (Fig. 7) via PI3K, suggesting a negative feedback mechanism for CD28 to control T cell activation. Our data, showing that NF-κB is limiting in HIV-1 transcription, and that it is a key target of CD3 +CD28 signaling, is consistent with several groups that have demonstrated a role for NF-κB in the reactivation of latent HIV-1 transcription in cell lines and primary cell models (Brooks et al., 2003; Burke et al., 2007; Duverger et al., 2009; Sadowski et al., 2008; Williams et al., 2007). It is important to note that a recent

report has suggested that NF-κB may have a minor role in reactivating HIV-1 expression in latently infected primary T cells that phenotypically resemble central memory cells, and that NFAT and p38 MAPK are the end-points of the CD3 + CD28 signaling cascade responsible for HIV-1 transcription (Bosque and Planelles, 2009). Whether these apparently contradictory data reflect multiple mechanisms of latency or cell specific responses to CD3 + CD28 signaling are unclear, but they do underscore the need to further characterize the biochemical events necessary for activation of HIV-1 transcription if strategies are to be developed to mobilize latent HIV-1 from latently infected T cell populations.

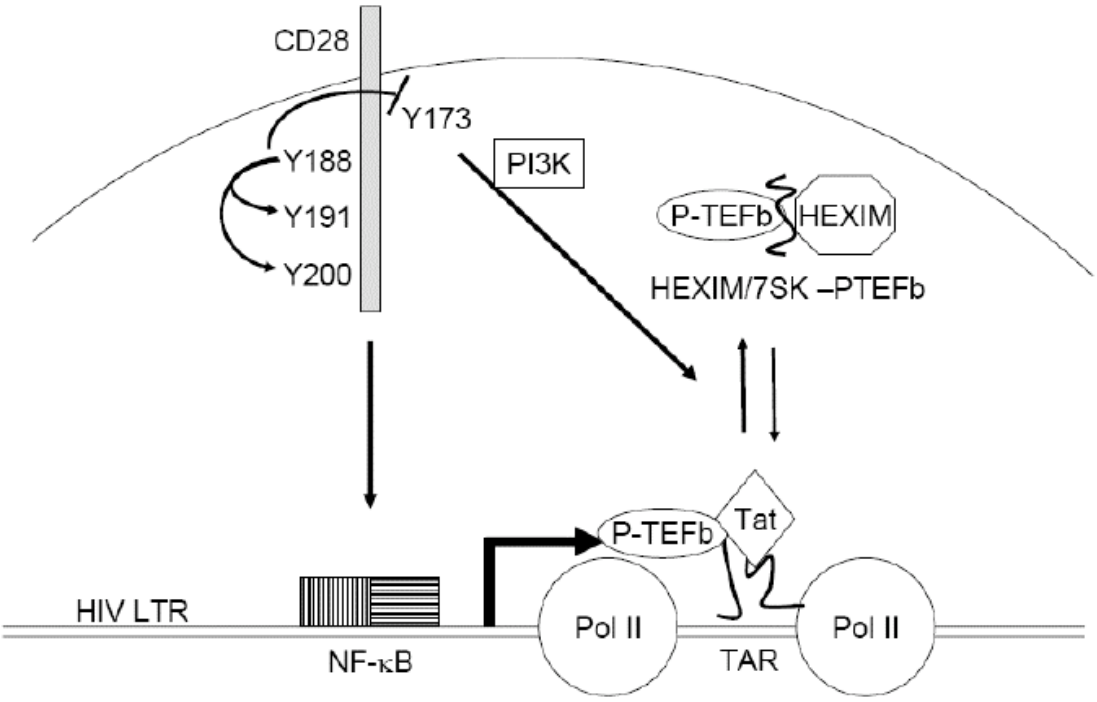


Figure 2.7: Model for CD28-mediated control of HIV-1 transcription. CD28 enhances HIV transcription by inducing NF-κB activation. Y188 modulates negative signals from Y173 and positive signals emanating from Y191 and Y200. Recruitment of PI3K to Y173 increases P-TEFb association with the HEXIM/7SK RNP to negatively regulate HIV-1 Tat-dependent transcription.

Figure 2.8: PI3K associates with CD28 containing Y173. Jurkat T cells and CD8/28 expressing stable Jurkat cells were activated with CD3+CD28 or CD3+CD8 for 5 minutes. Whole cell lysates were precleared and CD8 immunoprecipitation was performed. Samples were analysed by western blots analysis to evaluate the recruitment of p85 to CD28.

Chapter 3

Repression of HIV transcription by the Negative elongation factor complex (NELF) and the transcription termination factor Pcf11 in CD4+T cells

3.1 Introduction

The current treatment for AIDS is highly active antiretroviral therapy (HAART), which is a combination of two or more drugs inhibiting viral proteins required at different stages of the HIV life cycle, thus inhibiting virus replication. Even though HAART decreases viral load, with a concomitant increase in the number of CD4+T cells, it does not cure AIDS. This is due to the persistence of latent HIV in cellular reservoirs that re-establishes AIDS upon cessation of HAART (Colin and Van Lint, 2009; Pierson et al., 2000). HIV has been suggested to exist in quiescent cells in a non-integrated form, although this is a short lived population of cells that does not significantly contribute to long term latency (Pierson et al., 2002b; Williams and Greene, 2007; Zhou et al., 2005). In cells with established infection, a small percentage of cells either do not support or actively repress HIV transcription. Several steps that control HIV transcription including sites of integration, virus mutations, availability of transcription factors, chromatin organization and/or recruitment of negative transcription factors contribute to latency.

Importantly, a subset of these cells can be induced to express HIV, providing a source of virus upon the interruption of anti-viral treatment (Colin and Van Lint, 2009; Mok and Lever, 2007; Williams and Greene, 2007). Identifying mechanisms that actively establish latently infected cell populations will provide potential new targets to mobilize repressed provirus making HAART more effective.

RNA polymerase II promoter proximal pausing by NELF and DSIF is a major rate-limiting step in HIV transcription (Rao et al., 2006). In vitro, transcription from the HIV LTR in the absence of chromatin generated transcripts that were 500 bp from the start site, suggesting that Pol II processiveness and premature termination limit provirus transcription (Marciniak and Sharp, 1991). In vivo, HIV infected cells have been shown to possess shorter transcripts approximately 60 nucleotides long (Feinberg et al., 1991). The presence of short stable viral transcripts in HIV infected individuals further indicates that promoter proximal pausing and premature termination are major determinants of viral latency. Hence, it is of great interest to understand the underlying mechanisms governing polymerase pausing and premature termination.

The promoter proximal pause is executed by negative elongation factors, DSIF and NELF which associate with serine 5 phosphorylated RNA pol II (Cheng and Price, 2007; Fujinaga et al., 2004; Ivanov et al., 2000). Under non-permissive conditions, transcription is terminated which results in the release of short viral transcripts. The processivity of RNA Pol II is enhanced by PTEFb mediated ser2 phosphorylation of RNA Pol II as well as the the phosphorylation of negative elongation factors associated with the paused polymerase II complex (Wada et al., 1998b; Yamaguchi et al., 1999a).

Phosphorylation by PTEFb releases NELF from this complex and modifies DSIF so that it promotes transcriptional processivity (Zhou and Yik, 2006).

NELF is a complex of four subunits-NELF-A, NELF-B, NELF-C/D and NELF-E and the assembly of the entire complex is involved in NELF function (Yamaguchi et al., 2002). NELF has been shown to cause polymerase pausing, followed by premature termination and release of the paused viral transcript, how these two processes are coupled is not known. A transcription termination factor, Pcf11, has recently been shown to play a critical role in premature termination of the paused elongation complex in HIV (Zhang and Gilmour, 2006; Zhang et al., 2007b).

We have previously used a chronically infected cell line as a model for HIV latency to show that NELF and Pcf11 limit HIV transcription by regulating transcription elongation (Zhang et al., 2007a; Zhang et al., 2007b). These studies extend those observations by confirming an important role for NELF and Pcf11 in limiting HIV transcription in multiple cell lines including infected primary CD4+T cells. Depletion of NELF or Pcf11 leads to an induction of HIV provirus transcription due to productive transcription elongation and virus production. Finally, we show that NELF and Pcf11 physically interact suggesting a coupling of Pol II promoter proximal pausing and premature termination to limit HIV transcription and promote latency.

3.2 Material and methods

3.2.01 Cell lines and primary cell

Jurkat E6.1 T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 medium supplemented with 5% FCS,

100 U/ml penicillin, 100 µg/ml streptomycin and 0.2 M L-glutamine. The HIV-GFP latent cell lines BAI, CA5 and 11B10 were obtained from the lab of Dr. Olaf Kutsch at the University of Alabama at Birmingham and were grown in 10% FCS RPMI. Human embryonic kidney 293T cells were also obtained from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin. Peripheral blood mononuclear cells were isolated from whole blood by ficoll/histopaque gradient (Sigma-Aldrich) and $CD4^+$ T cells were positively selected using the Dynal isolation kit (Invitrogen; 113.21D). The latent cell lines were a generous gift from Dr. Olaf Kutsch.

3.2.2 Flow cytometry

2×10^6 cells were washed and resuspended in 200 µL of PBS and 200 µL of 4% Paraformaldehyde. The GFP fluorescence was measured using Becton Dickinson FACScan at the Flow Core Facility at Boston Medical Center.

3.2.3 Transfections in 293Ts and generation of HIV-1 Infectious Titers

0.5×10^6 293T cells were plated in a 6 well plate 24 h prior to calcium phosphate transfections, which were performed using 15 µg of pNL4–3-Luc(+) Env(–) Nef(–) (Henderson et al., 1995) (obtained from NIH AIDS Research and Reference Reagent Program) and 3 µg of RSV-Rev, 3 µg LTR VSV-G. 293T transfection efficiency for pNL4-3-Luc was assessed by determining luciferase activity using a Promega luciferase kit (Madison, WI), and p24 ELISA were performed. Supernatants were collected and filtered through a 0.45-micron disc prior to infection.

3.2.4 Infection of Jurkat T cells

Jurkat cells were infected with virus by resuspending $20X10^6$ cells in 10 mL pNL4-3 luc virus for 12-16 h. Cells were then recovered for 4-20 hours before transfection of siRNA.

3.2.5 Infection of CD4+T cells

$40 X 10^6$ CD4+T cells were activated with PMA and PHA for 2- 20 hours and recovered for 4-20 hours in media. Cells were infected by spinoculating with 10mL of pNL4-3 luc virus and 1µg/mL of polybrene for 120 minutes at 1200 rpm. Cells were washed and resuspended in 5% FCS containing RPMI and cultured for 24 hrs and then treated with siRNA.

3.2.6 Activation of T cells

24- 48 hours post knock down Jurkat and CD4+T cells were washed and resuspended in 5% FCS RPMI. $1 x 10^6$ cells were plated in each well of a 24 well plate. Cells were either left unactivated, or activated with 0.1 µg/ml anti-human CD3 (BD 555336) and 1.0 µg/ml anti-human CD28 (BD 555725) for 30 minutes. 5 µg/ml of goat anti-mouse antibody (Sigma M 4280) was added to crosslink the receptors. Following 24-48 hours of stimulation, Jurkat cells were harvested and luciferase activity measured.

3.2.7 Transfection of siRNA

24 h post infection $10- 15 X 10^6$ Jurkat and CD4+T cells were washed with serum free RPMI containing 20mM HEPES. Cells were resuspended in a volume of 600µL of

HEPES RPMI, and 5µL of 100µM si-RNA was electroporated using a T280 square electroporation system (BTX, San Diego, CA). Cells were given 1 pulse for 20 ms at 300 V in a 4-mm cuvette, and then recovered in complete RPMI. Most experiments were performed 72-96 hour post knockdown. In experiments done in resting CD4+T cells, siRNA was transfected 96 hours post infection.

3.2.8 RT PCR

Cells were pelleted and cells were resuspended in 1mL trizol for 2-5 minutes. 200 µL of chloroform was added and the samples were mixed and centrifuged at 10,000 rpm for 15 minutes at 4 ºC. The aqueous phase was transferred to another tube and RNA was precipitated by adding 500µL of isopropanol for 18h at -80ºC. Samples were spun at maximum speed for 15 minutes at 4 ºC. The samples were washed with 75% Ethanol, and the pellets were air-dried. RNA was dissolved in 30 µL of DEPC water. The RNA was quantified and cDNA was synthesized using SS RT enzyme and random primers as per manufacturer protocol. 1µL of cDNA was used in a 15µL real time PCR reaction, using Sybr green reagent. 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' and commercially available β-actin primers (biorad). The PCR was carried out for 50 cycles, and the relative amounts of transcripts were calculated using the ΔΔCt method. Relative levels of transcript for each sample were calculated by using β-actin as an internal control. Further, the amount of

product seen in the si-ctrl treated samples was used as a caliberator and the transcripts levels in samples were calculated as fold changes in comparison to si-ctrl.

3.2.9 p24 assay

Culture supernatants were collected from 293T cells 48 hours post transfection. In experiments in Jurkat T cells and CD4+T cells, supernatants were collected 72 and 96 hour post knock down, from cells that were not activated or activated for 48 hours. The supernatant was diluted with PBS, and a p24 ELISA was carried out using the Perkin Elmer ELISA kit. A standard curve was also read on each plate, in order to quantify the amount of p24 released.

3.2.10 Immunoprecipitation and Immunoblots

20 X 10^6 Jurkat T cells were lysed in 200µL of 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 phenylmethylsufonyl fluoride and protease inhibitor cocktail III (Calbiochem). The samples were spun for 10 minutes at 4ºC at 13000 rpm. The supernatants were collected and precleared by incubating with protein A/G beads (Santa Cruz Biotechnoology, sc-2003) for 30 minutes at 4ºC before incubating with primary anti-NELF D , anti-Pcf11 or Rb IgG coated Protein A/G beads for 2 hours at 4ºC, beads were washed three times with lysis buffer, and then suspended in SDS PAGE loading buffer. The samples were heated for 5 minutes at 100ºC before loading onto a 8% SDS PAGE gel. Proteins were transferred to a PVDF membrane (Millipore) by electroblotting. Western blot analysis was carried out.

3.2.11 Chromatin Immunoprecipitation

293Ts were transfected with pNL4-3 luc and pCDNA3 vector or pCDNA3-FLAG-NELFb for 48 hours. Cells were cross linked using 11% formaldehyde solution (prepared from 37% formaldehyde, 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% for 10 minutes at room temperature. The reaction is quenched by adding 2 M glycine to a final concentration of 240 mM. Cells were washed with PBS and resuspended in 1 ml sonication buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM PMSF) and sonicated on ice for 30 cycles, 10 seconds on, 30 seconds off. 100µL of sonicated chromatin was diluted 10 fold with dilution buffer and incubated with 1 µg antibody Pol II (sc-899), FLAG (sigma), NELF-D (Proteintech) for 16 hours at 4ºC. Protein A/G beads were then added for 2 h. The beads were then washed twice each with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 0.1, 500 mM NaCl) and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and TE. Complexes were eluted with 1% SDS, 0.1 M $NaHCO_3$. The complexes were reverse cross linked at 65ºC for 4 h, followed by addition of proteinase K for 1 hour at 45ºC. The DNA was extracted using phenol chloroform, and precipitated with ethanol. Quantitative real time PCR analysis was carried out using SYBR green reagents and the primers 5'-TGCATCCGGAGTACTTCAAGA-3' and 5'-GAGGCTTAAGCAGTGGGTTC-3' which amplify -150 to +76 of the HIV-LTR.

3.3 Results

3.3.1 NELF represses HIV transcription

Previous studies in U1 cells demonstrated a role for NELF in repressing HIV transcription (Zhang et al., 2007a). We were interested in determining whether NELF is sufficient for repressing HIV transcription. To examine a direct role for NELF in HIV transcription we cotransfected 293T cells with pNL4-3 luciferase, a HIV clone which lacks envelope and contains a luciferase reporter gene, with pCDNA3-FLAG NELF-B, or pCDNA3 vector control. Lysates were monitored for luciferase activity 48 h post transfection to determine HIV transcription. Overexpression of NELF significantly reduced HIV transcription by 80 % (Fig. 3.1A). These data were confirmed by measuring the amount of HIV produced by the transfected cells using a p24 ELISA (Fig. 3.1B). As expected, overexpression of NELF resulted in a 60% decrease of p24, supporting the conclusion that NELF limits HIV transcription.

Since overexpression of a single subunit of NELF led to a decrease in HIV transcription it was important to verify that inhibition of virus expression was due to a direct recruitment of the NELF complex to the HIV LTR. We performed chromatin immunoprecipitation on chromatin isolated from 293T cells cotransfected with HIV and either pCDNA3 or pCDNA3-FLAG-NELF. We used FLAG antibody for immunoprecipitating NELF-B, and evaluated the association of NELF-B with the promoter region corresponding to -45 to +72. We also performed ChIP within this same LTR region for endogenous NELF-D, another subunit of the NELF complex and RNA a

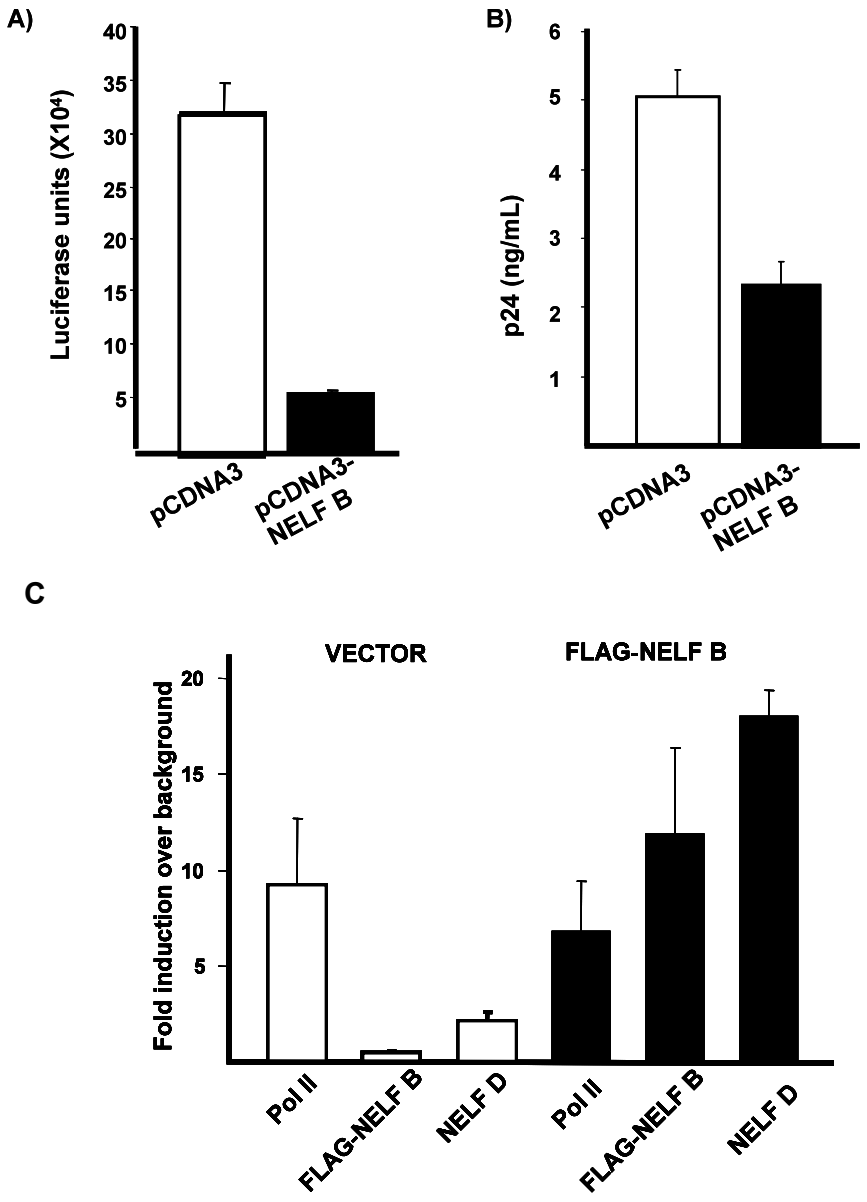


Figure 3.1: Overexpression of NELF represses HIV transcription. 293T cells were transfected with a pNL4-3 luc and pCDNA.3 vector control or pCDNA.3-FLAG NELF B. A) Luciferase assay was performed 48 hours post transfection to measure HIV transcription. B) 48 hours post transfection culture supernatants were harvested and the virus production was assessed by measuring the viral protein gag (p24) by an ELISA. C) 48 hours post transfection, Chromatin Immunoprecipitation assay was performed, using FLAG, NELF-D and RNA Polymerase antibodies to analyze their recruitment to -45 - +72 region of HIV LTR.

Polymerase II (Fig. 3.1C). In the absence of NELF, the HIV LTR associates with RNA Polymerase II, and very low levels of NELF-D. The FLAG immunoprecipitation serve as a negative control since these cells do not express FLAG-NELF B. In cells overexpressing FLAG-NELF-B, enhanced NELF-B and NELF-D binding to the HIV LTR, was observed suggesting that the repression of transcription is due to the recruitment of the NELF complex at the LTR. The association of RNA Polymerase II with the LTR did not change in response to NELF-B overexpresssion indicating that NELF does not influence the recruitment of Pol II but targets Polymerase processivity.

3.3.2 Depletion of NELF in latent cell lines increases HIV expression

Our previous studies demonstrating that NELF limits HIV transcription utilized U1 cells, which has two copies of latent provirus that harbor Tat mutations and this may contribute to the lack of Pol II processivity observed in this cell line (Zhang et al., 2007a). We were interested in determining whether NELF had a more general role in limiting HIV transcription. We employed a library of clonal Jurkat T cells generated by Kutsch et al that have been latently infected with HIV-GFP clone (Duverger et al., 2009). Previous studies by the Kutsch laboratory has shown that HIV transcription as monitored by GFP is inducible in these cells and that promoter interference is the primary mechanism responsible for repressing HIV transcription. We examined the role of NELF in regulating latency in three of these Jurkat cell lines (BAI, 11B10 and CA5). NELF-B expression was reduced using siRNA and GFP expression was monitored 72 hours post knock down by flow cytometry (Fig. 3.2). There was a small percentage of cells that stochastically express HIV, however when siNELF was introduced into these cells there

was a reproducible 2-3 fold increase in GFP positive cells. Although HDAC inhibitors, such as TSA, have been reported to induce HIV transcription, these cell lines were not

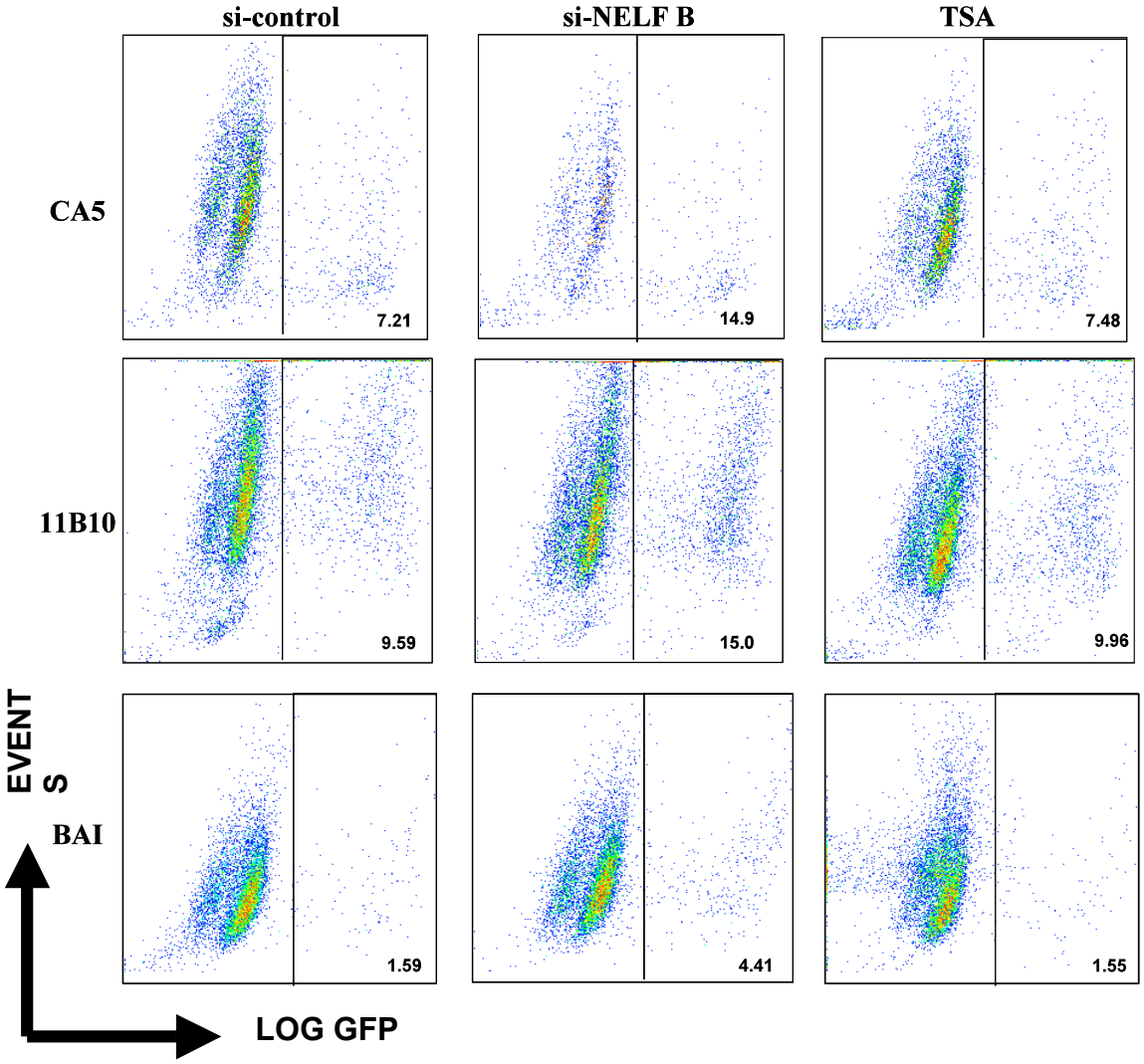


Figure 3.2: NELF limits HIV transcription in cells that exhibit transcriptional interference. CA5, 11B10 and BAI, are clones of Jurkat T cells latently infected with HIV GFP. Cells were treated with sicontrol, siNELF-B and TSA for 72 hours. GFP expression was measured by flow cytometry. The gate used to distinguish GFP+ and GFP- population is marked on each individual profile, and percentage of GFP+cells is labeled in the lower right hand corner.

responsive to TSA treatment consistent with previous reports from Kutsch et al and suggesting a limited role for chromatin in regulating latency in the context of these cell lines. More importantly, depletion of NELF reverses latency, confirming an important role for promoter proximal pausing in repression of HIV transcription even in the context of transcriptional interference.

3.3.3 Depletion of NELF in primary CD4+T cells induces HIV transcription elongation

Since depletion of NELF in different latent cell line models lead to an induction of HIV transcription we examined the role of NELF in a polyclonal population of infected T cells. Initially, we infected Jurkat T cells with VSVG- pseudotyped pNL4-3 luc virus. The pNL43-Luc virus lacks an env gene so that it only supports a single round of infection allowing us to study exclusively the effect of NELF on HIV transcription. HIV infected cells were treated with siRNA specific for NELF-B, or si-ctrl RNA. Knock downs were confirmed by western blots as well as RT-PCR (Figs. 3.3B and 3.3D). 48 hours post knock down, luciferase assays were performed to measure HIV transcription. In cells with diminished NELF, a two-fold increase in HIV transcription was observed (Fig. 3.3A). This is consistent with the role of NELF in limiting HIV transcription.

Since NELF plays an important role in promoter proximal pausing, we wanted to see if the depletion of NELF leads to an induction in HIV transcription by alleviating the paused polymerase II complex. Therefore, the levels of short initiated transcripts, (+1 to +40) which represent the prematurely terminated transcripts were compared to elongated transcripts (+5396 to +5531) (Fig. 3.3C). Using RT-PCR the amount of initiated

transcript was comparable in si-ctrl and si-NELF treated cells, however, more elongated transcripts were seen in si-NELF treated cells.

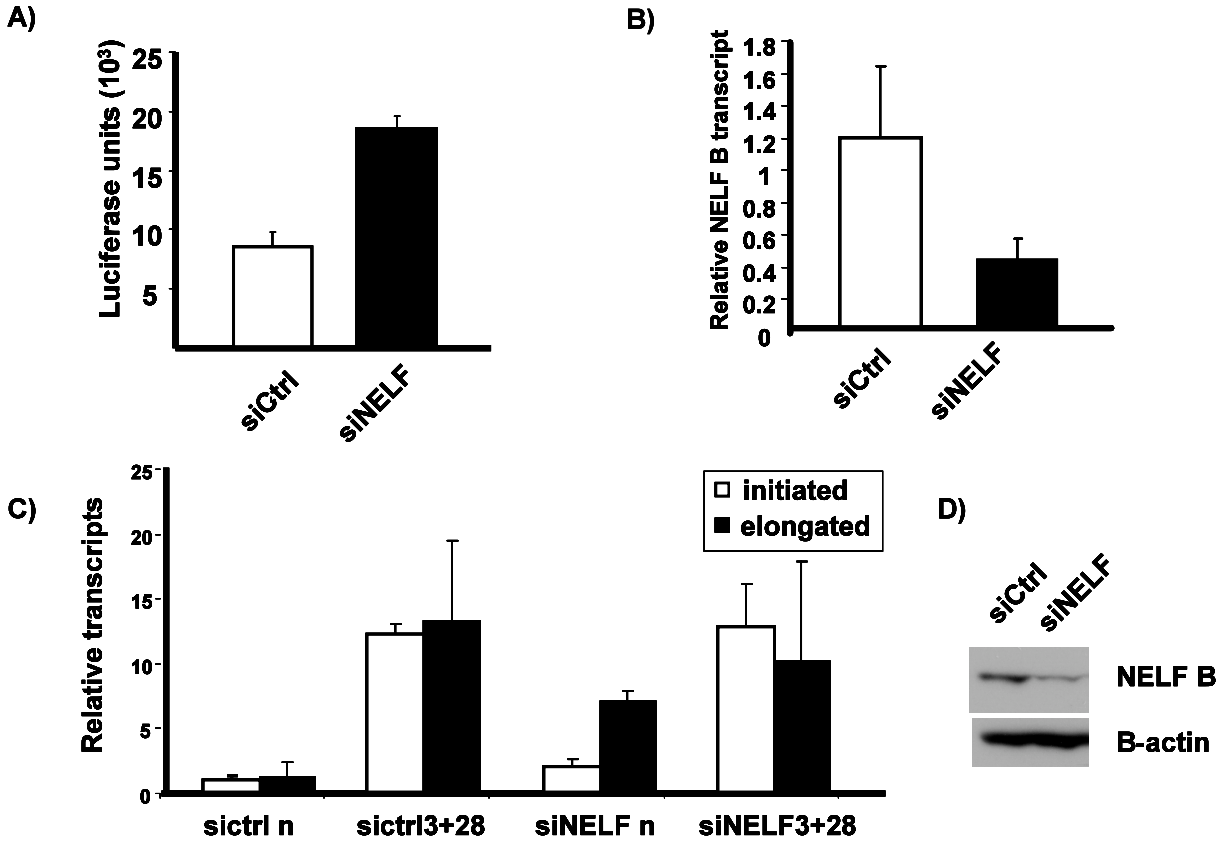


Figure 3.3: Depletion of NELF induces HIV transcription in infected Jurkat T cells. Jurkat E6.1 cells were infected with NL4-3 luc virus for 12- 16 hours. 24 hours post infection, cells were transfected with non-specific siRNA or siRNA specifically targeting NELF-B. A) 48-72 hours post knock down cells were lysed and luciferase activity was measured. **B)** NELF depletion was determined by Real time PCR. C) Cells were left unactivated or activated with 0.1 g/mL anti-CD3 and 1.0 g/mL of anti-CD28 for 6 h and cellular RNA was isolated and cDNA was prepared. The level of short initiated transcripts and elongated transcripts was determined by using primers to amplify +1 to +40, and +5396 to 5555 respectively, using real time PCR. D) Depletion of NELF was also confirmed by western blot analysis.

Subsets of these cells were activated with anti-CD3+anti-CD28 antibodies to induce T cell activation, and levels of initiated and elongated transcripts were measured. Activation of si-ctrl and si-NELF B treated cells led to comparable levels of both transcripts indicating that NELF affects HIV transcription under basal conditions, but does not have a significant impact on cells that support efficient HIV transcription (Fig. 3.3C). This is consistent with the notion that CD3+CD28 signaling functions to override inhibition by NELF.

We also verified the role of NELF in regulating HIV transcription in primary CD4+T cells. CD4+ T cells were positively selected from peripheral blood of healthy donors and infected cells with NL4-3 luciferase virus, to generate a heterogeneous pool of HIV infected primary CD4+T cells. Infected cells were then transfected with si-ctrl or si-NELF-B and luciferase assays were measured 48-72 hours post knock down. NELF si-RNA decreased not only NELF-B but also NELF-D suggesting that decreasing one subunit alters the NELF complex in general (Fig. 3.4E). A two fold increase in HIV transcription was seen in cells treated with si-NELF (Fig. 3.4A). T cell activation diminished the effect of NELF on HIV transcription, as no difference in HIV transcription was noted between si-ctrl and si-NELF treated cells (Fig. 3.4B). The unstimulated cells seem to represent a quiescent/resting population, as T cell activation significantly increased HIV transcription. Comparison of the initiated and elongated transcripts revealed an increase in transcription elongation in NELF-depleted cells that produced more elongated transcripts (Fig. 3.4C). We also quantified the effect of NELF depletion on HIV transcription by measuring the amount of p24 released from HIV

infected CD4+T cells. Consistent with the observed induction in transcription, a two-fold increase in HIV released was seen in NELF depleted cells (Fig. 3.4D).

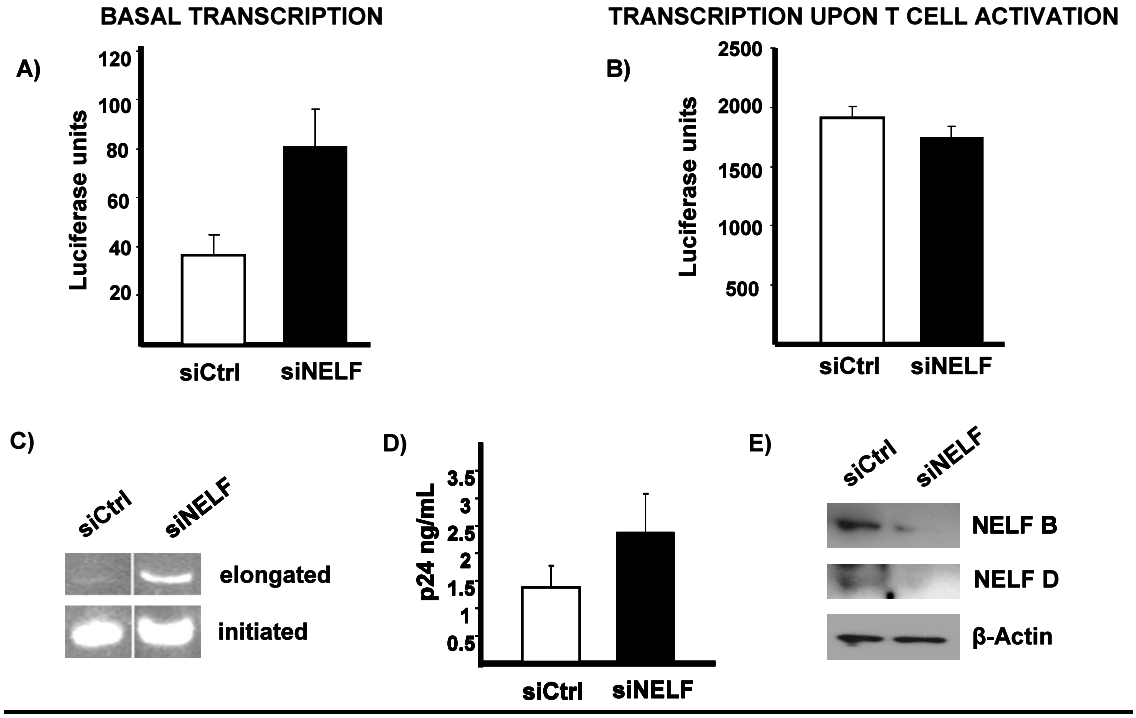


Figure 3.4: NELF represses HIV transcription and elongation in primary CD4+T cells. CD4+T cells were isolated using positive selection from PBMC's obtained from healthy donors. Cells were activated with PMA+PHA for 12-16 hours. Cells were recovered for 12 hours and then infected with NL4-3(env-) luciferase virus. 12-14 hours post infection cells were transfected with siControl and siNELF B. A) 72-96 hours post knock down luciferase activity was measured. B) 72-96 hours post knock down cells were activated with 0.1 µg/mL anti-CD3 and1.0 µg/mL anti-CD28 antibodies for 4-6 hours. Cells were lysed and luciferase activity was measured. C) Cellular RNA was also isolated and cDNA was prepared. The level of short initiated transcripts and elongated transcripts was determined by suing primers to amplify +1 to +40, and +5396 to 5555 respectively using real time PCR. The PCR products were run on a 6%PAGE gel and stained with Ethidium Bromide. D) Culture supernatants were processed for a p24 ELISA assay. E) The extent of NELF depletion was determined by western blot analysis using NELF D and NELF B antibodies.

3.3.4 NELF interacts with Pcf11

NELF and DSIF interact with RNA polymerase II to stall transcription (Zhou and Yik, 2006). However NELF and DSIF are not capable of disengaging and releasing the Pol II complex from the nascent transcript. Promoter proximal pausing leads to premature termination of transcription which recycles the transcription machinery by releasing the short transcript. Pcf11, has been shown to prematurely terminate HIV transcripts in U1 cells (Zhang et al., 2007b). We wanted to test if NELF and Pcf11 physically interact. Jurkat T cells were lysed and immunoprecipitation was carried out using Pcf11 or a non-specific antibody to isolate endogenous Pcf11 and Pcf11 interacting proteins. As shown in Fig 3.5 NELF D co-immunoprecipitates with Pcf11 (Fig. 3.5A). This interaction was validated by immunoprecipitating NELF D to pull down Pcf11 (Fig. 3.5B). These data indicate that NELF recruits Pcf11 to the paused polymerase II complex.

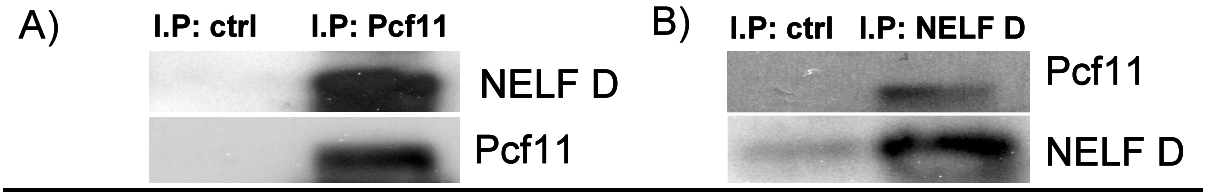


Figure 3.5: NELF and Pcf11 interact. Jurkat T cells were lysed and precleared lysates were used for immunoprecipitation using a non-specific antibody, anti-Pcf11 (left) and anti-NELF D (right) antibody. The immunoprecipitates were subject to western blot analysis using Pcf11 and NELD D antibodies.

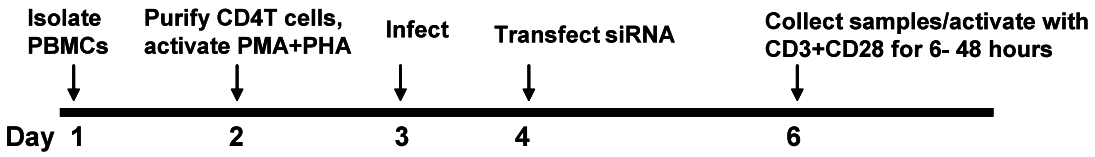
3.3.5 NELF and Pcf11 interact to inhibit HIV transcription

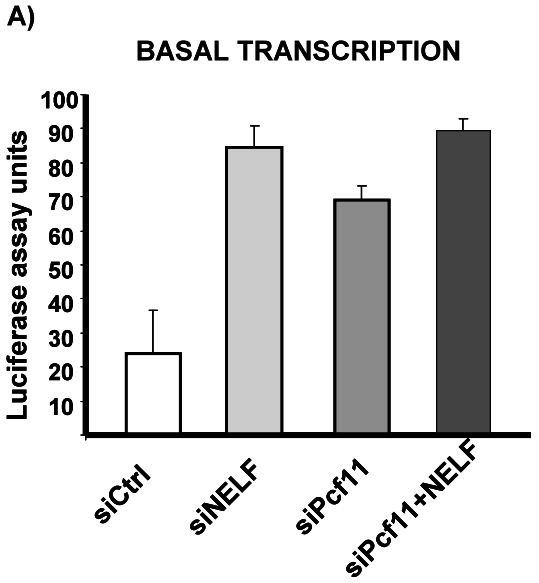
Since NELF and Pcf11 physically interact, we wanted to determine the functional consequence of this interaction. We depleted NELF, Pcf11 or both in primary CD4+T cells infected with HIV-luc. The extent of Pcf11 and NELF depletion was verified by RT- PCR and western blot analysis (Figs. 3.6 F and G). We measured luciferase activity

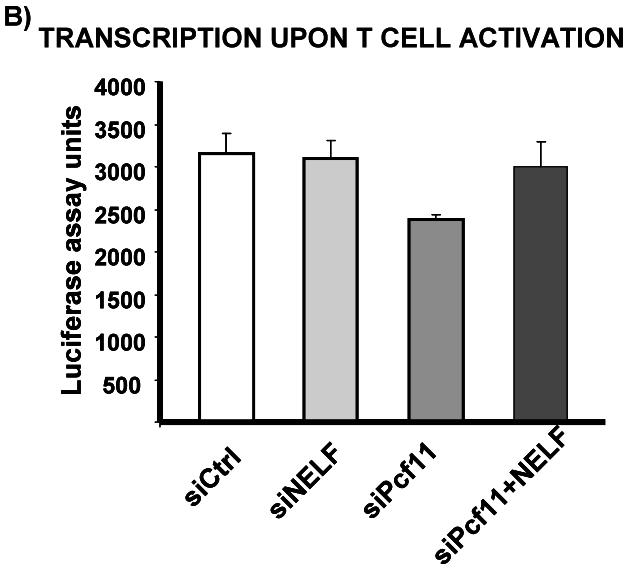
after 72 hours post knockdown. Depletion of NELF, Pcf11 or both, led to a three-fold increase in HIV transcription as compared to control cells (Fig. 3.6A). This confirms a role for NELF and Pcf11 in limiting HIV transcription in primary cells. However, since depleting both NELF and Pcf11 does not further enhance HIV transcription, these factors appear to act in the same biochemical pathway to repress transcription. We also quantified the effect of NELF and Pcf11 on HIV production by measuring the amount of p24 released. Consistent with the transcriptional response, we see a two fold increase in HIV replication when cells are depleted of NELF, Pcf11 or both (Fig. 3.6C). Activation of these cells, led to an increase in viral transcription which was comparable to si-ctrl treated cells suggesting that both these proteins function to regulate basal transcription, and their repressive activities are overcome by T cell activation (Fig. 3.6B).

To confirm a role for Pcf11 and Pcf11-NELF complex in polymerase II pausing, Jurkat T cells were infected with HIV-luc and treated with si-ctrl, si-Pcf11 or si-Pcf11 and si-NELF. RT-PCR analysis was performed to look at initiated and elongated transcripts. As in the case NELF knock down, depletion of Pcf11 or both NELF and Pcf11 led to an increase in transcription elongation as compared to si-ctrl treated cells.

We have also examined the effect of NELF and Pcf11 in resting CD4+ T cells. CD4+T cells were kept in culture for 7 days and were infected with HIV-luc. The cells were treated with si-RNA and 7 days post infection luciferase assays were performed. In these cells, depletion of NELF, Pcf11 or both led to a 3-fold increase in HIV transcription. Activation of these cells induced in HIV production that was independent of NELF and Pcf11 depletion (Fig. 3.7). This suggests that Pcf11 and NELF may contribute to the establishment or maintenance of latency.


Isolate PBMCs
Purify CD4T cells, activate PMA+PHA
Infect
Transfect siRNA
Collect samples/activate with CD3+CD28 for 6- 48 hours
Day 1
2
3
4
6


A)
BASAL TRANSCRIPTION
Luciferase assay units
100
90
80
70
60
50
40
30
20
10
siCtrl
siNELF
siPcf11
siPcf11+NELF


B)
TRANSCRIPTION UPON T CELL ACTIVATION
Luciferase assay units
4000
3500
3000
2500
2000
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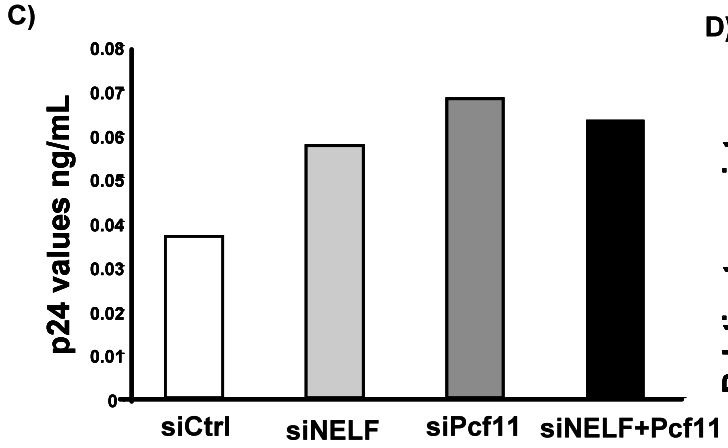
1000
500
siCtrl
siNELF
siPcf11
siPcf11+NELF


C)
p24 values ng/mL
0.08
0.07
0.06
0.05
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siCtrl
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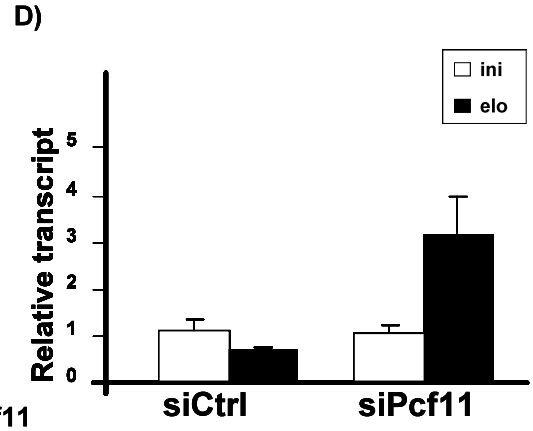
siPcf11
siNELF+Pcf11


D)
ini
elo
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4
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0
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siPcf11

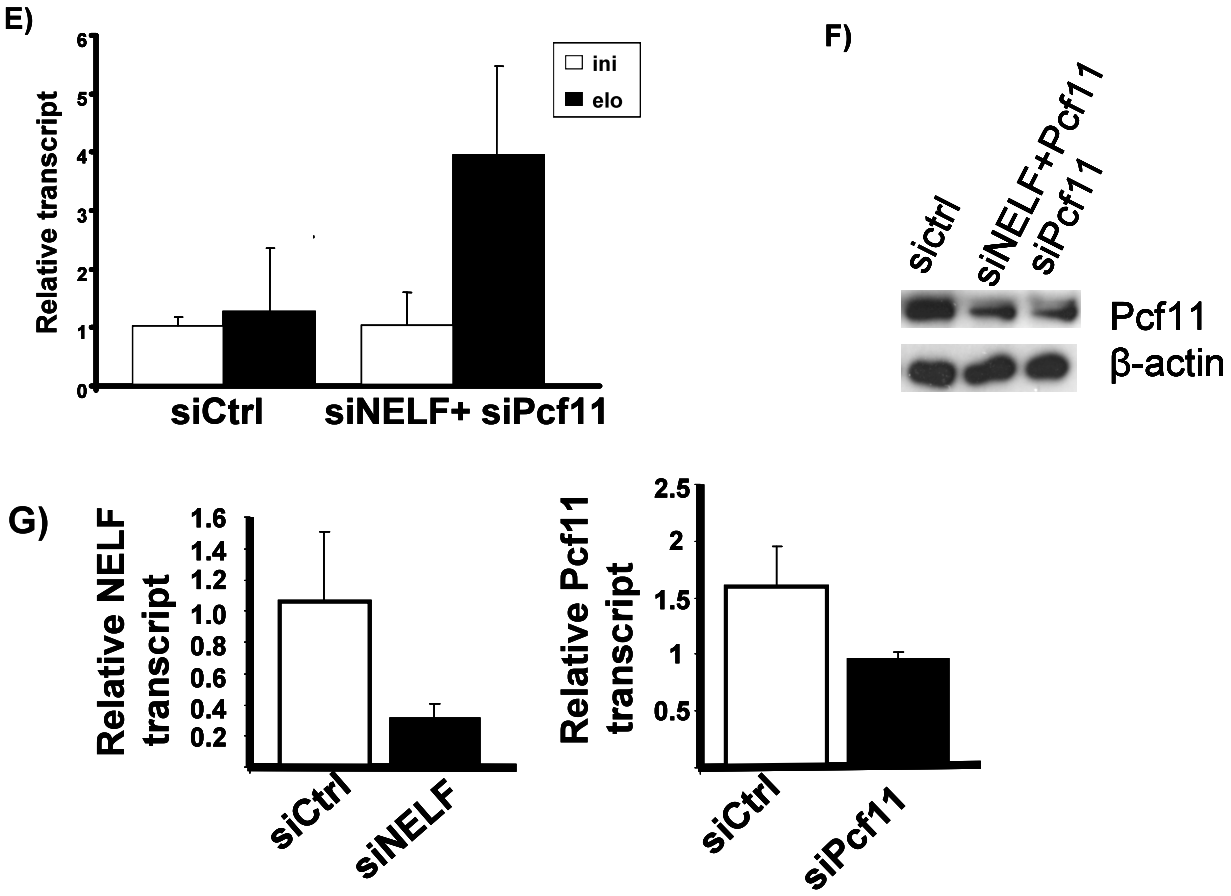


Figure 3.6: NELF and Pcf11 repress HIV transcription and elongation in primary CD4+T cells. CD4+T cells were isolated using positive selection from PBMC's obtained from healthy donors. Cells were activated with PMA+PHA for 12-16 hours. Cells were recovered for 12 hours and then infected with NL4-3(env-) luciferase virus. 12-14 hours post infection cells were transfected with siControl, siNELF, siPcf11, siNELF and Pcf11. A) 72-96 hours post knock down luciferase activity was measured. B) 72-96 hours post knock down cells were activated with 0.1 µg/mL anti-CD3 and 1.0 µg/mL anti-CD28 antibodies for 4-6 hours. Cells were lysed and luciferase activity was measured. C) 72-96 hours post knock down viral release was assessed by measuring p24 released in culture supernatants. D) Jukat T cells were infected with NL4-3 luc, and treated with siCtrl and siPc11 for 48-72 hours. Cellular RNA was also isolated and cDNA was prepared. The level of short initiated transcripts and elongated transcripts was determined by using primers to amplify +1 to +40, and +5396 to 5555 respectively using real time PCR. E) Jukat cells were infected with NL4-3 luc, and treated with sictrl and siPc11 and siNELF for 48-72 hours. Cellular RNA was also isolated and cDNA was prepared. The level of short initiated transcripts and elongated transcripts was determined by using primers to amplify +1 to +40, and +5396 to 5555 respectively using real time PCR. F) Extent of Pcf11 Knock down in CD4+T cells was estimated by western blot analysis. G) The extent of Pcf11 and NELF knock down was measured by Real time PCR analysis.

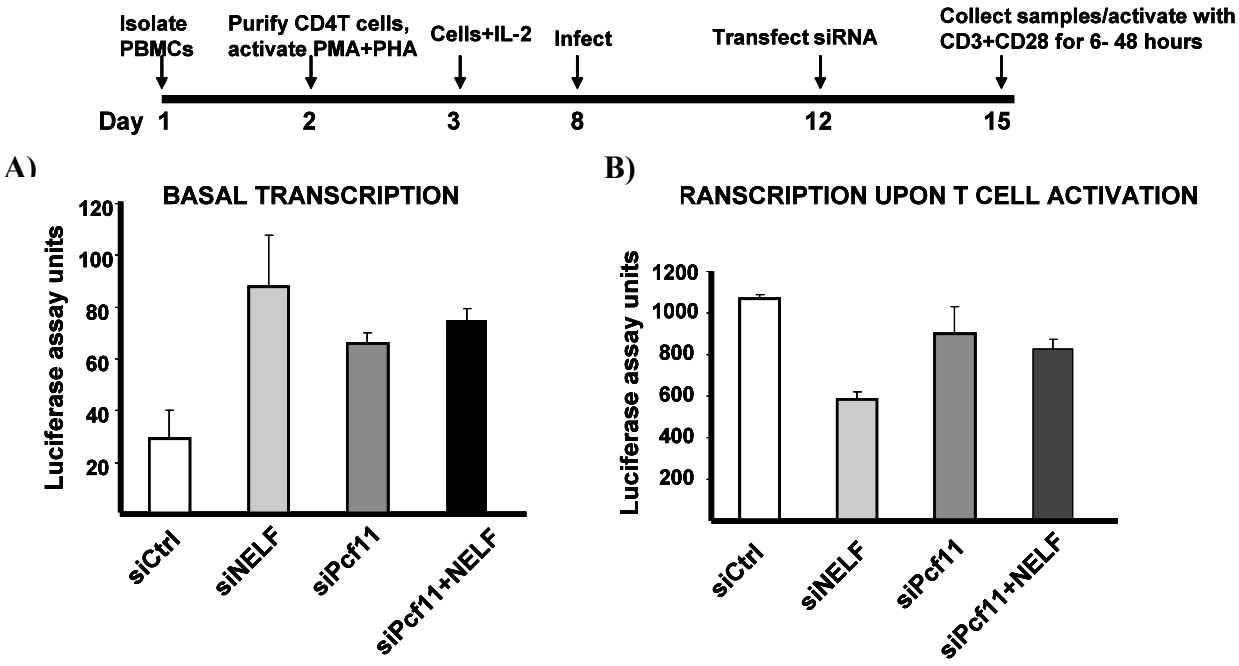


Figure 3.7: NELF and Pcf11 repress HIV transcription and elongation in resting primary CD4+T cells. CD4+T cells were isolated using positive selection from PBMC's obtained from healthy donors. Cells were activated with PMA+PHA for 12-16 hours. Cells were recovered for 12 hours and then kept in media containing IL-2 for 5 days. Cells were infected with NL4-3(env-) luciferase virus, 4 days post infection cells cells were transfected with siControl, siNELF B, siPcf11, siNELF and Pcf11. were transfected with siControl and siNELF B. A) 72-96 hours post knock down luciferase activity was measured. B) 72-96 hours post knock down cells were activated with 0.1 µg/mL anti-CD3 and1.0 µg/mL anti-CD28 antibodies for 4-6 hours. Cells were lysed and luciferase activity was measured.

3.4 Discussion

We have shown that NELF and Pcf11 repress HIV transcription in CD4+T cells by regulating promoter proximal pausing and premature termination. Depletion of NELF in Jurkat T cells, CD4+T cells and latent T cell lines results in an increase in HIV transcription. The NELF complex is recruited to the LTR where it interacts with RNA

Pol II and represses transcription by maintaining a paused RNA Pol II complex. Depletion of NELF leads to processive elongation resulting in the induction of transcription. In addition, depletion of Pcf11, a protein responsible for premature termination leads to an increase in transcription. Depletion of both NELF and Pcf11 does not enhance transcription beyond levels of transcription achieved by individual knock down of NELF and Pcf11. We also show that NELF and Pcf11 physically interact to couple promoter proximal pausing and premature termination.

Numerous proteins affect different steps involved in regulation of HIV transcription. This includes the binding of transcription factors such as NFAT, NF-κB and AP-1 to the LTR, recruitment of RNA Polymerase II, nucleosome positioning, the chromatin conformation and the viral protein tat (Lusic et al., 2003; Pumfery et al., 2003; Rohr et al., 2003; Van Lint et al., 1996). Tat, is a transcription transctivator due to its ability to recruit PTEFb to the LTR. Tat binds to the RNA stem loop structure which is assembled at +60 nt of the newly synthesized viral mRNA and recruits PTEFb which promotes processive transcription (Karn, 1999). PTEFb is also recruited to the LTR via its interactions with transcription factors NF-κB and SP1 (Barboric et al., 2001; Yedavalli et al., 2003). NELF mediated pause has been mapped to +47 nt, which is before the TAR element is formed, suggesting that promoter proximal pausing by NELF occurs prior to Tat mediated activation of HIV transcription (Zhang et al., 2007a). Thus we have established an important role for promoter proximal pausing and premature termination as critical rate limiting steps in HIV transcription independent of tat function.

NELF directly regulates polymerase processivity by interacting with ser5 phosphorylated RNA Pol II and DSIF. The association of NELF and DSIF limits the

processivity of RNA Pol II which is overcome by PTEFb mediated phosphorylation of RNA Pol II, NELF and DSIF (Yamaguchi et al., 2002; Yamaguchi et al., 1999a). In addition, NELF inhibits transcription by repressing chromatin acetylation and preventing nucleosome remodeling (Zhang et al., 2007a). It is conceivable that NELF recruits/interacts with co-repressor complexes and other chromatin modifying enzymes to maintain a non-permissive environment for transcription elongation thus reinforcing a paused elongation complex. Furthermore, NELF interacts with RNA Pol II only when RNA Pol II is in a complex with DSIF (Yamaguchi et al., 2002). Thus NELF could also be interacting with other proteins which would stabilize the NELF- Pol II association.

Even though promoter proximal pausing is an important determinant of HIV transcription, NELF and DSIF cannot disengage the paused complex. Since transcription termination is not a product of the polymerase falling of the RNA, premature termination is a checkpoint in HIV transcription. This is carried out by Pcf11, a protein that interacts with CTD of RNA Pol II, as well as nascent RNA to cause premature termination (Zhang et al., 2005; Zhang and Gilmour, 2006; Zhang et al., 2007b). Pcf11 is a transcription termination factor which was initially identified by its role in terminating transcription at the poly A tail at the end of a newly synthesized transcript (Sadowski et al., 2003; West and Proudfoot, 2008). Its role in premature termination is consistent with its role in termination of fully formed transcripts, as its interaction with RNA and RNA Pol II are involved in both cases. In the absence of Pcf11, premature termination of HIV does not occur, resulting in processive transcription elongation. This establishes premature termination as a major determinant of HIV transcription.

Depletion of both NELF and Pcf11 did not enhanced transcription, suggesting that both proteins function in the same biochemical pathway to limit HIV. Both these proteins are critical for this step, as disrupting even one component has the same effect as disrupting both components. Considering the role of Pcf11 and NELF, it suggests that promoter proximal pausing and premature termination are coupled events. In addition, the increase in HIV transcription in the presence of sufficient NELF but depleted Pcf11 suggests that Pcf11 directly regulates NELF function. This is further supported by our findings that NELF and Pcf11 co-immunoprecipitate, suggesting that both these proteins are in the same complex. We propose that Pcf11 stabilizes the NELF-Polymerase II complex. In the absence of Pcf11 this complex is perturbed resulting in the dissociation of NELF from the complex, leading to enhanced transcription.

NELF and Pcf11 depletion affect basal transcription, suggesting that these factors play an important role in maintenance of latency. Additional factors involved in transcription elongation have also been shown to be down-regulated to support a paused elongation complex under basal conditions. Low levels of PTEFb are maintained in monocytes by a specific micro-RNA (miR-198) which reduces CyclinT1. Upon activation, the microRNA is down-regulated resulting in the formation of PTEFb (Sung and Rice, 2009). In addition, activation of CD4+T cells leads to dissociation of nuclear PTEFb from the inhibitory 7SKsnRNP complex, resulting in an increase in “active” PTEFb.

It is possible that NELF and Pcf11 could act as general host factors that regulate HIV latency as NELF and Pcf11 limit transcription in different model systems of latency. The reactivation of virus from latent Jurkat T cells, and resting CD4+T cells further

strengthen a role for NELF and Pcf11 in governing HIV latency. When T cell activation signals are provided, NELF and Pcf11 do not limit HIV transcription. This suggests that T cell activation signals override the inhibition posed by these two proteins, consistent with the idea that activation of PTEFb can alleviate pausing by phosphorylation of RNA Pol II CTD, NELF and DSIF. This phosphorylation could hinder the interaction of Pcf11 with NELF leading to the disruption of the complex resulting in the recycling of the transcriptional machinery and release of the paused transcript.

We propose that promoter proximal pausing and premature termination are coupled events that serve as early checkpoints in viral transcription. We present a model where NELF interacts with RNA Pol II and DSIF on the HIV LTR in resting CD4+T cells inhibiting transcription elongation due to a promoter proximal pause. Depletion of NELF results in an increase in viral transcription due to the lack of promoter proximal pausing. NELF recruits Pcf11, which stabilizes the paused elongation complex and dismantles the paused polymerase complex by interacting with RNA pol II as well as the nascent RNA. Pcf11 releases the pause by causing premature termination that releases the initiated transcript from the elongation complex, and recycles the transcription machinery. T cell activation induces HIV transcription via Tat-mediated recruitment of PTEFb, which phosphorylates the CTD of RNA Pol II, DSIF and NELF. The promoter proximal pause is disturbed and transcription elongation proceeds resulting in HIV transcription.

The evidence for the coupling of premature termination and promoter proximal pausing has implications for transcription for a number of other cellular genes as well. In Drosophila, transcription of the hsp70 gene is caused by promoter proximal pausing

(Gilmour, 2009). In addition, Pcf11 has also been implicated in premature termination (Zhang et al., 2005; Zhang and Gilmour, 2006). We speculate that Pcf11 and NELF interaction that appear to be relevant for HIV transcription may also have more general implications for genes that can be rapidly induced.

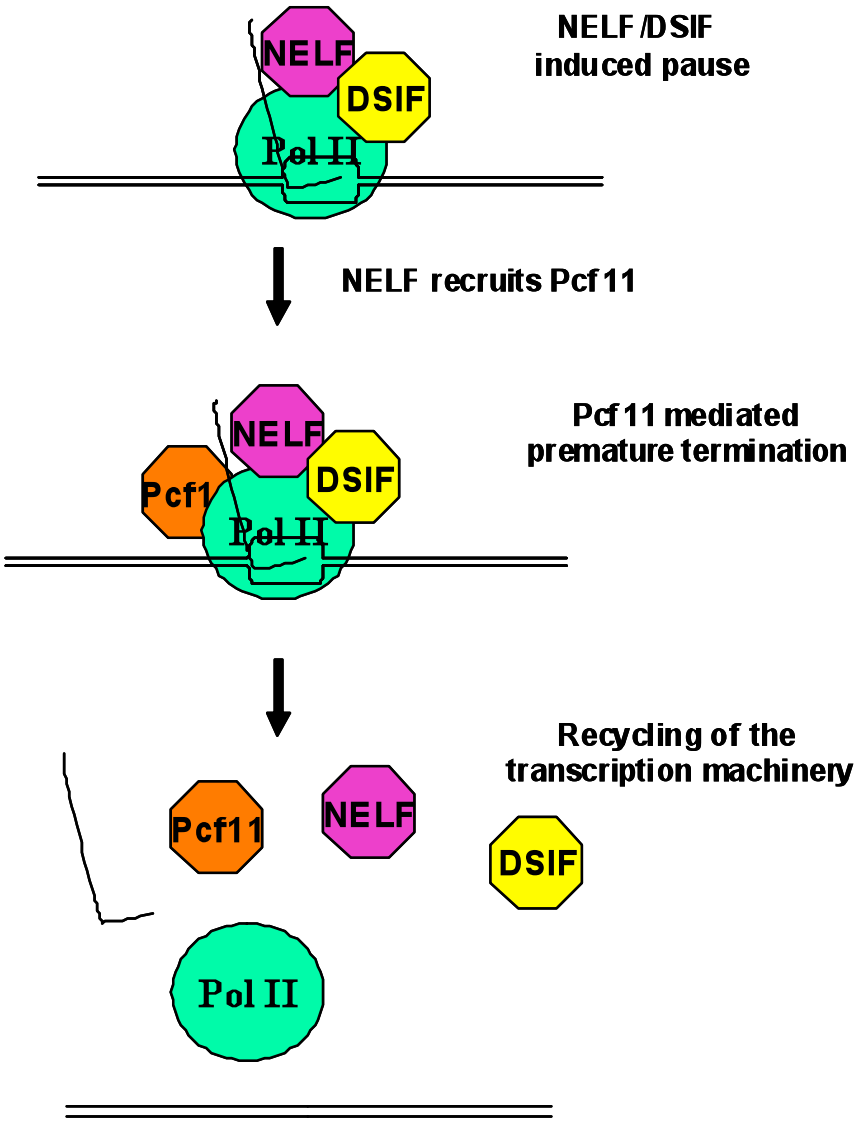


Figure 3.8: Model for NELF and Pcf11 mediated promoter proximal pausing and premature termination in regulation of HIV transcription. NELF and DSIF associate with ser2 phosphorylated RNA Pol II and inhibit transcription elongation by promoter proximal pausing. NELF recruits Pcf11 to the paused elongation complex where Pcf11 dismantles the paused complex, releasing the premature transcript and recycling the transcription machinery.

Chapter 4

Discussion

Onset of AIDS correlates with a decrease in CD4+ T cell counts, and is characterized by secondary infections which the host cannot combat due to a malfunctioning immune system (Levy, 2009). HAART has been successful in reducing virus loads, and re-establishing T cell homeostasis. However, the success of HAART has been overshadowed with the identification of HIV reservoirs which emerge from latency upon withdrawal of treatments (Bagasra, 2006). Since HAART targets viral proteins involved in viral replication, it does not affect cells carrying the latent provirus. Thus the integrated provirus can stably exist in cells, and reemerge from latency in the absence of HAART, when the cellular environment is conducive for transcription and replication (Coiras et al., 2009; Richman et al., 2009). In this study we have examined two facets of HIV transcription, the factors regulating establishment of latency, and signals involved in reactivation of virus. The results obtained provide new insights into HIV transcription as well as general gene regulatory mechanisms that exist in cells.

4.1 Role of CD28 on HIV transcription

T cell activation is a prerequisite for HIV infection and transcription. We have examined the role of the costimulatory molecule, CD28 in regulating HIV transcription.

We focused on the tyrosine residues present in the cytoplasmic domain of CD28 which are required for CD28 function. We employed CD8/28 chimeric receptors to map the function of individual or multiple tyrosine residues. Analysis of different combinations of tyrosine residues led to varying levels of HIV transcription, indicative of the complex interplay of signals originating from different tyrosine residues. In spite of the overall positive effect of CD28 on HIV transcription, CD28 has the capability to positively and negatively regulate HIV.

The tyrosine residues in the cytoplasmic domain of CD28 are indispensable, as mutation of all four tyrosine results in a receptor that does not activate HIV transcription. Loss of function of any of the tyrosine residues abrogates transcription, with the exception of Y173. Individual tyrosines, Y191 and Y200 activate HIV transcription in the absence of other signals, whereas Y188 seems to have a regulatory role. Y173 inhibits HIV transcription via the activation of PI3K, which negates positive signals from distal tyrosines Y191 and Y200. In fact, signals from the three distal tyrosines are required to overcome the inhibition posed by Y173. PI3K and its downstream signals could either directly inhibit recruitment of proteins to CD28, or could inhibit signaling pathways that originate from distal tyrosine residues.

The positive, negative and regulatory signals which originate from different tyrosine residues in the cytoplasmic domain of CD28 synergize with and amplify TCR signaling. This is achieved by the activation and recruitment of NF-κB (p50:p65) and RNA Pol II to the LTR. We know that Y191 and Y200, are both sufficient for CD28 function as both are capable of attaining these two end points. It is possible that these two tyrosines recruit and trigger different signaling proteins which may or may not initiate

similar signaling cascades to result in activation of NF-κB. Our data suggests that these two tyrosines are not redundant, as their signaling potential is different when in the context of Y188.

It will be imperative to identify proteins that are recruited to the different tyrosine residues, and then study the effect of PI3K on the recruitment of these signaling proteins. It will also be essential to determine if Y191 and Y200 activate similar signaling pathways leading to activation of NF-κB. The function of Y191 and Y200 in T cell activation could also indicate their ability to synergize in the formation of the immunological synapse (IS). PKCθ, which gets activated by TCR signaling, is recruited to the IS only upon costimulation, which eventually leads to activation of NF-κB (Huang et al., 2002). Y191 and or Y200 could be involved in recruitment of PKCθ to the IS. Alternatively, activation of MAPK pathways could be responsible for NF-κB activation.

It may be of interest to take advantage of this panel of CD8/CD28 chimeric receptors to study transcriptional regulation of other genes involved in T cell response, maturation and differentiation. In particular, analysis of cytokines IL-4 and IL-5 which are induced upon costimulation, resulting in the generation of T helper 2 (T_H2) cells, may be instrumental in understanding the role of CD28 signaling in T cell differentiation (Rulifson et al., 1997; Tao et al., 1997).

Since partial signaling from TCR leads to anergy, a state of unresponsiveness, it will be important to evaluate whether CD28 plays a quantitative or qualitative role in enhancing TCR signaling. It is very clear that CD28 amplifies the TCR signals, leading to activation of transcription factors and recruitment of Pol II. Inefficient T cell activation could be due to low signal strength during TCR signaling, which is overcome in the

presence of CD28 signals which amplify TCR signaling and activate specific pathways reaching thresholds of signals required for transcription.

The complexity underlying CD28 function, and the distinct and overlapping nature of signals arising from receptor ligation is indicative not only of its important role but also uncovers the regulatory networks which can be fine-tuned to govern the overall response to T cell activation.

T cell activation also regulates other aspects of the virus life cycle, a subset of which are mediated by non receptor tyrosine kinases like Itk and Lck. Lck promotes gag targeting to the plasma membrane, whereas Itk regulates HIV entry, transcription and egress (Readinger et al., 2008; Strasner et al., 2008). HIV also manipulates T cell signals to ensure efficient virus replication. One such mechanism is the involvement of the viral accessory protein, nef in downregulating Cbl-b (Yang and Henderson, 2005). Cbl is a E3 u biquitin ligase and targets proteins, such as Vav, Zap70 involved in T cell activation, for proteosomal degradation. Thus nef sensitizes T cells for activation, lowering the threshold required for HIV transcription. Due to these effects of nef on T cell activation the virus used in our study lacks nef. The current library of chimeric receptors can be utilized to identify additional pathways that may be targeted by nef by comparing the effect of signaling through the different receptors on nef+ and nef- virus.

4.2 T cell activation and regulation of PTEFb

In addition to activating transcription factors, T cell activation regulates RNA Polymerase II function through the control of PTEFb. PTEFb, a complex of cyclin T1 and cdk9, exists primarily in either an active or inactive state in the nucleus. PTEFb is

sequestered away from sites of transcription in a complex with the 7SK snRNP, which is comprised of HEXIM1 and 7SKsnRNA. PTEFb when in a complex with Brd4, or when in associated with tat in HIV-infected cells is actively recruited to promoters, where it phosphorylates CTD of RNA Pol II at ser 2 residues (Yang et al., 2001; Yik et al., 2003).

In this study, we show that TCR signaling as well as costimulation perturbs the interaction of PTEFb with HEXIM1, making it available for transcription elongation. How T cell activation alters PTEFb -7SK-snRNP interaction needs to be determined. Since both the components of the 7SK-snRNP are essential for its function, changes in either one would alter the 7SK-snRNP complex (Yik et al., 2003). Both HEXIM1 and 7SKRNA are abundantly expressed, hence regulation is probably mediated by post translational modifications of complex components. Post translational modifications of HEXIM1 have not been reported. However, 7SK RNA has been shown to be regulated. The stability of 7SK RNA is essential for 7SK-RNP, and is maintained by the association of 7SK RNA with La-related protein 7 (LARP7) which prevents 7SK from degradation. Depletion of LARP inhibits PTEFb-7SK-RNP association (He et al., 2008).

Additionally Cyclin T1 and Cdk9 are post-translationally regulated which influence the ability of PTEFb to interact with 7SK-snRNP. Acetylation by p300 of cyclin T1 perturbs the interaction of PTEFb with 7SK-snRNP (Cho et al., 2009; Kaehlcke et al., 2003). It is of interest that p300 has been shown to be activated by CD28 mediated costimulation, implicating this as one mechanism of regulating PTEFb upon T cell activation (Nandiwada et al., 2006). Furthermore, phosphorylation of Threonine 186 of cdk9 is essential for the interaction of PTEFb with 7SK-snRNP, and dephosphorylation of this residue results in recruitment of PTEFb to sites of transcription (Chen et al., 2004).

It is conceivable that T cell activation regulates the status of this residue altering PTEFb location. Treatment of cells with HMBA, or UV leads to calcium influx, leading to activation of calcineurin and PP1 (protein phosphatase 1) which dephosphorylate the thr186 of cdk9 resulting in the disruption of 7SK snRNP (Chen et al., 2008). It is interesting to note that calcineurin is activated upon TCR activation (Smith-Garvin et al., 2009). Thus it is conceivable that T cell signals could prime PTEFb via activation of calcineurin and PP1. We also show that PI3K represses HIV transcription by stabilizing the PTEFb- 7SK snRNP complex. It is possible that PI3K signals regulate the function of the kinase responsible for phosphorylation of thr186 of cdk9. Detailed analysis of T cell activation and PI3K pathway will help in identifying proteins responsible for phosphorylation and dephosphorylation of thr186 in cdk9.

In monocytes which do not support provirus transcription, cyclinT1 expression is inhibited by a specific microRNA, miR198 (Sung and Rice, 2009). The differentiation of monocyte to macrophages results in downregulation of miR198, increasing CyclinT1 levels which correspond to induction of virus transcription. Activation of T cells could also be regulating PTEFb function via upregulating the expression of its components. This is supported by a recent study showing that primary CD4+T memory cells have low levels of cyclinT1 expression which is upregulated upon T cell activation, suggesting that latency is maintained due to low levels of PTEFb (Tyagi et al.).

Since T cell activation, has the ability to positively and negatively regulate PTEFb, detailed analysis of T cell signaling pathways and identification of target proteins and microRNAs will expand our understanding of PTEFb regulation. In addition to a role of PTEFb in T cell activation and HIV transcription, PTEFb also plays a crucial role in

regulating apoptosis. PTEFb dysregulation has been implicated in several cancers including breast cancers (Turano et al., 2006; Wittmann et al., 2003). Identifying signals involved in PTEFb function will enable us to develop tools required to perturb these interactions for use in therapeutics in a variety of immune and cancer based diseases.

4.3 Promoter proximal pausing and premature termination in HIV transcription

The transcription of HIV provirus is regulated at multiple levels. We have focused on the role of promoter proximal pausing and premature termination in HIV transcription in CD4+ T cells. Our study is the first implicating an important role for NELF in regulating HIV transcription in CD4+ T cells. NELF associates with RNA Pol II phosphorylated on ser5 and DSIF on the LTR to prevent transcription elongation. The recruitment of PTEFb, phosphorylates NELF, DSIF and ser 2 residues in the heptapeptide repeat region of the CTD of RNA Pol II, leading to dissociation of NELF from the elongating complex resulting in processive transcription (Yamada et al., 2006; Yamaguchi et al., 2002; Yamaguchi et al., 1999a).

Depletion of NELF in CD4+ T cells led to an induction of HIV due to increased transcription elongation. Previous work on U1 cells using permanganate footprinting analysis has identified the pause site on HIV to be at +47 nt from the start site (Zhang et al., 2007a). This precedes formation of the TAR element and potentially disrupts Tat binding and recruitment of PTEFb. The notion that NELF mediated pausing is at a site before TAR is fully formed suggests that this is a primary checkpoint in early HIV transcription that precedes tat function. It will be important to verify these results by

studying the effect of NELF on HIV with deletions abrogating the formation of the TAR element.

If NELF and DSIF are inducing RNA pol II pausing, what mechanisms dismantle and recycle the transcription machinery and release the premature transcript? Based on my results, I propose that promoter proximal pausing and premature termination are coupled via the interaction of NELF and the transcription termination factor Pcf11. Depletion of either Pcf11, or Pcf11 and NELF induce HIV transcription, suggesting that both these proteins act in concert in the same pathway to limit HIV transcription.

The observation that disrupting just one protein involved in promoter proximal pausing, and premature termination leads to productive elongation suggests that these two steps are early checkpoints for HIV transcription. We propose that NELF and DSIF associate with Pol II to create the pause. Pcf11 is then recruited to the elongation complex via its interaction with NELF. Once recruited Pcf11 interacts with CTD of RNA Pol II as well as nascent RNA to release the transcript and recycle the transcription machinery. The role of Pcf11 in premature termination at promoter proximal pause site is consistent with the role of Pcf11 in transcription termination of full length transcripts, where Pcf11 is recruited to stalled Polymerase complex at the poly A tail at the 3' end of genes (Buratowski, 2005; Rosonina et al., 2006).

Alternately, it is possible that the NELF-DSIF-RNA Pol II complex is not very stable, as NELF does not interact stably with DSIF or RNA Pol II, but binds to a complex of RNA –Pol II and DSIF. Pcf11 via its interaction with NELF, CTD of RNA Pol II and RNA may stabilize the paused elongation complex, and then trigger premature termination releasing the nascent transcript, and recycling the transcriptional machinery.

This is supported by a recent study showing Pcf11 confers DRB sensitivity, to HIV transcription, suggesting an important role in regulating PTEFb. Treatment of cells with DRB represses transcription due to inhibition of PTEFb, which is lost when Pcf11 is depleted. Instead, induction of transcription, is observed when Pcf11 is depleted (Zhang et al., 2007b). Our data showing the physical interaction between Pcf11 and NELF offers an explanation for this observation. In the absence of Pcf11, the paused complex is unstable, and dissociates from the LTR, overriding the involvement of PTEFb.

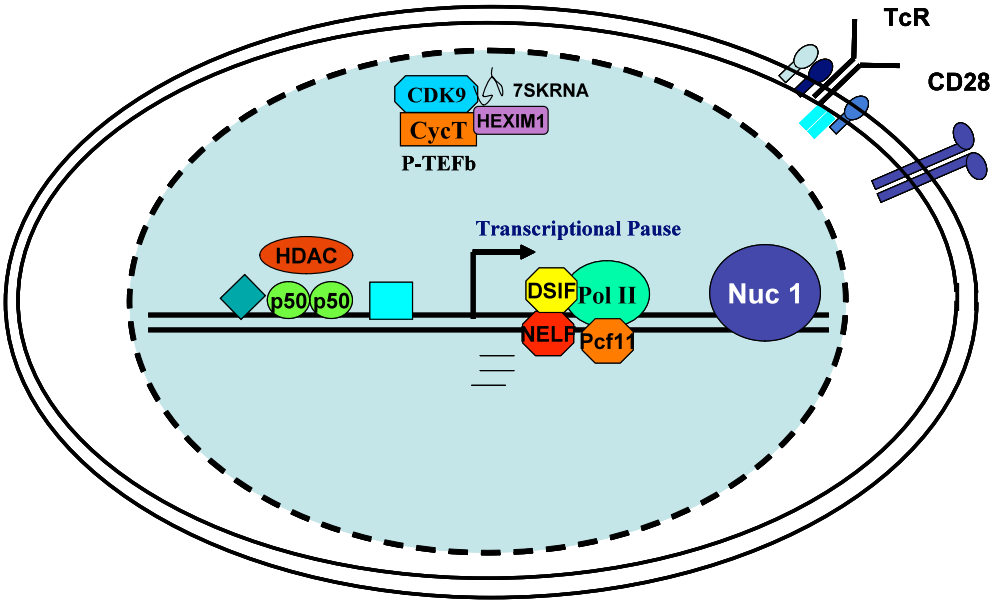
I propose that T cell activation overcomes both these events. It is conceivable, that upon T cell activation, PTEFb is recruited to the LTR via its interaction with NF-κB, Brd4 and/or other factors. PTEFb phosphorylates NELF and RNA Pol II, dissociating NELF and Pcf11 leading to transcription elongation. This is the primary checkpoint for transcription elongation in the absence of tat. Once NELF mediated promoter proximal pause is overcome, viral transcription progresses to generate early viral proteins, tat and rev. Once Tat levels reach a critical threshold, it would bind the TAR and be the primary factor recruiting PTEFb to the LTR.

The effect of NELF on Pcf11 recruitment to the LTR and vice versa needs to be examined. In vitro experiments on immobilized DNA templates, looking at elongation efficiency using purified complexes isolated from cells lacking NELF, Pcf11 or both, will reveal their contribution to transcription elongation. The biochemical basis for Pcf11 and NELF interaction need to be further explored, especially in the context of T cell signaling and in the presence of DRB. Identification of post translational modifications of NELF and Pcf11, and how these impact functional and physical interactions between these

proteins need to be explored. This will be crucial in delineating the events that lead to promoter proximal pause and premature termination.

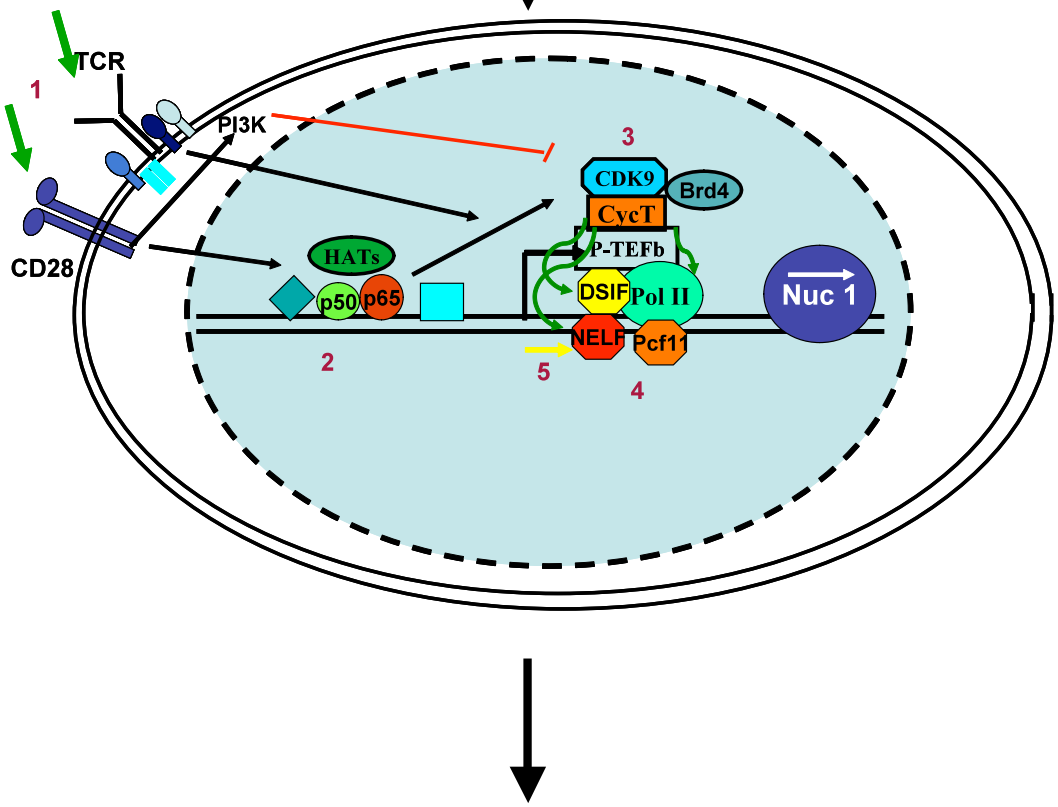
Recent studies suggest that NELF participates in differential complex formation. Genome wide analysis of NELF in Drosophila S2 cells showed recruitment of NELF to 50 % of genes (Lee et al., 2008). However, depletion of NELF led to downregulation of majority of these genes, and only few targets like hsp70 were upregulated. Exon array analysis in T47D cancer cell line revealed an important role for NELF in supporting transcription of genes involved in cell cycle (Sun and Li). This suggests that NELF can support transcription, in contrast to its established function in repressing transcription of hsp70, HIV and jun-B. It is possible that this differential function of NELF is due to its interactions with other transcriptional regulators which either positively or negatively impact transcription (like co-activators and co-repressor complexes). Pcf11, might be one of the many proteins NELF interacts with to modulate or fine tune levels of gene expression. It is conceivable, that these interactions may be regulated by extracellular stimuli, cellular signals and properties and sequence of the target gene in question. The existence of NELF in different functional complexes also indicates that NELF and its function in promoter proximal pausing is a regulated event. Identifying signals that govern NELF function and its interaction with other regulators in the context of physiological stimuli on different target genes will be important in delineating NELF function and regulation.

A)



T cell activation

B)



C)

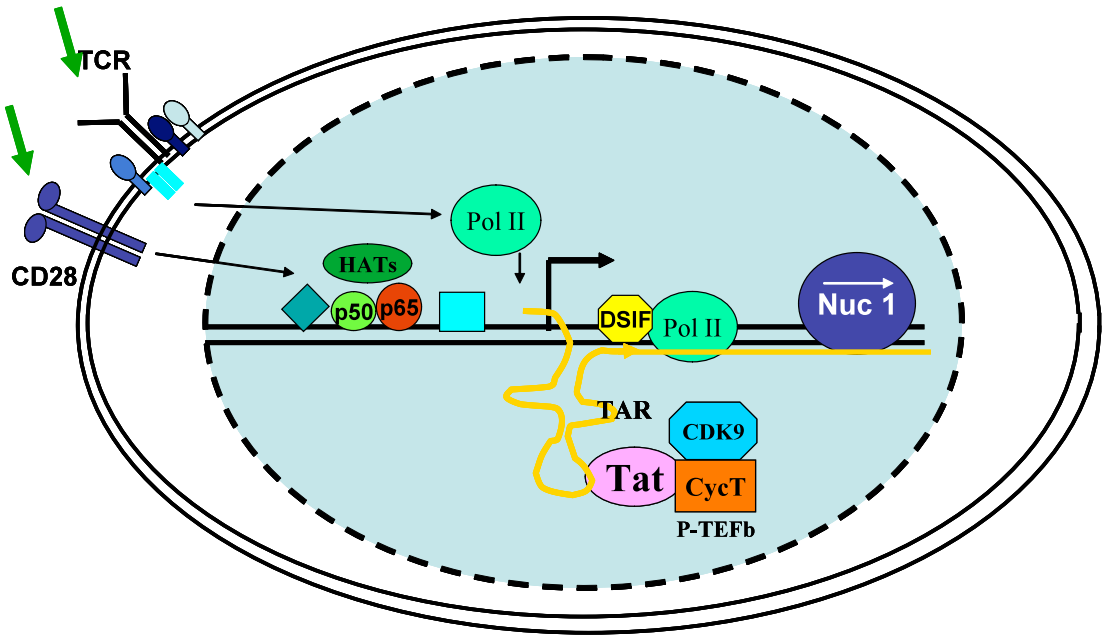


Figure 4.1: Regulation of HIV transcription in resting cells and the consequence of T cell activation. A) In infected CD4+T cells, under basal conditions HIV transcription is repressed due to absence of activated transcription factors, and the unavailability of PTEFb due to its interaction with HEXIM1 and 7SK RNA. HIV transcription is further inhibited due to the function of NELF and DSIF which interact with serine 2 phosphorylated RNA Pol II and limit transcription by facilitating promoter proximal pausing. NELF recruits Pcf11 to the elongation complex, where Pcf11 dismantles the paused polymerase complex, terminating the premature transcripts and recycling the transcriptional machinery. B) When T cells are stimulated via the TCR and costimulatory molecule CD28 (1), or other agents, T cell signaling cascades result in the activation and recruitment of transcription factors (2), especially NF-κB (p50:p65) to the LTR. T cell activation signals with the exception of PI3K (3), results in the disruption of the PTEFb –HEXIM-7SK RNA complex, enabling recruitment of PTEFb to the LTR via interactions with NF-κB. PTEFb, phosphorylates serine 5 residues in the CTD of RNA Pol II, as well as NELF and DSIF (4). This results in dissociation of NELF from the Polymerase, and allows DSIF to support transcription elongation. Thus promoter proximal pause is overcome and efficient transcription elongation proceeds resulting successful transcription (5). C) T cell activation also increases the recruitment of RNA Pol II to the promoter resulting in amplification of HIV transcription. HIV transcription results in the production of tat, which sustains HIV transcription by recruiting PTEFb to the LTR.

Promoter proximal pausing may also directly contribute to HIV latency, by repressing transcription elongation. This is supported by the presence of short stable transcripts in latently infected cells. The difference in NELF function on different genes suggests that repression of HIV transcription by promoter proximal pausing is a specific and regulated event. Identification of signals that overcome the pause may help in identifying mechanisms that can be used to target latent virus. CD4+T cells isolated from AIDS patients on HAART, should be analyzed to see if depletion of NELF and/or Pcf11 in the presence of HAART leads to a rebound of virus to evaluate the role of NELF and Pcf11 in latency. Some cells may be less permissive to HIV transcription because they have programs that favor the establishment of paused pol II. For example, we have shown that receptor tyrosine kinase RON, expressed on tissue resident macrophages, inhibits HIV transcription by promoting the assembly of NELF at the HIV LTR (Klatt et al., 2008). Therefore, macrophages that express RON may be more likely to establish latently infected reservoirs than activated T cells which favor robust transcriptional elongation.

4.4 NELF as a target for drug development

HAART does not target cells harboring repressed integrated HIV provirus. The ideal strategy to completely eliminate HIV from patients would involve purging the latent provirus to initiate virus production in otherwise latent cells in conjunction with HAART. Compounds that target transcription factors, coactivators and coreppressors are currently being tested to purge latent provirus (Coiras et al., 2009; Colin and Van Lint, 2009). Despite early in vitro data indicating clinically approved HDACi such as VPA could

mobilize latent HIV, human clinical trails have been less encouraging, suggesting that HDACi do not affect the size of the latent reservoir. This is not surprising as in our experiments with latent HIV-GFP expressing Jurkat T cells, transcription induction was not achieved when treated with TSA, another HDAC inhibitor (Archin et al., 2008; Lehrman et al., 2005; Reuse et al., 2009; Siliciano et al., 2007; Ylisastigui et al., 2004). Even though chromatin is an important factor governing virus transcription, it may not be the only determinant of latency. Multiple mechanisms may contribute to latency and chromatin may only be limiting in a small subset of latently infected cells. In these cell lines, as well as in pools of Jurkat and CD4+T cells infected with HIV, depletion of NELF was able to reactivate virus. I propose that NELF might be a candidate for drug design to purge latent virus.

Targeting NELF could be very effective in inducing latent HIV. This is because NELF, in addition to causing promoter proximal pause, also reinforces a closed chromatin environment by promoting maintenance of nucleosome position. Furthermore, NELF represses transcription by interacting with and recruiting transcriptional corepressors to the LTR (Lee et al, unpublished data).

APPENDIX

BLIMP mediated repression of HIV transcription

B-Lymphocyte maturation protein 1 (BLIMP) is a transcription repressor identified by its ability to bind to and repress Interferon β (IFN-β) (Keller and Maniatis, 1991; Turner et al., 1994). It is a transcription factor containing five DNA binding zinc finger motifs. It plays a critical role in differentiation of B cells and is important for maintenance of long lived plasma cells and antibody secretion (Lin et al., 1997). A role for BLIMP1 in T cells has recently emerged (Martins and Calame, 2008; Nutt et al., 2007). BLIMP is expressed at low levels in naïve CD4+ and CD8+T cells, however T cell activation induces BLIMP mRNA and protein levels (Calame, 2008). BLIMP regulates T cell homeostasis of peripheral T cells by attenuating T cell survival and proliferation (Cimmino et al., 2008). BLIMP regulates T cell function primarily by repressing IL-2 transcription (Gong and Malek, 2007; Martins et al., 2008). Since HIV transcription resembles IL-2 transcription, and both are responsive to T cell activation, we wanted to study the role if any of BLIMP on HIV transcription (Bohnlein et al., 1989; Greene et al., 1989).

Our studies show that BLIMP represses HIV transcription in a tat dependent manner. Overexpression of BLIMP resulted in a significant decrease in HIV transcription, and inhibited viral release completely (Fig. A.1). However, BLIMP did not effect the transcription of LTR-luc, suggesting the involvement of other factors in

BLIMP function. Interestingly, when we introduced tat, the tat mediated induction of LTR transcription was inhibited by BLIMP (Fig. A.2). To confirm the Tat dependent repression of HIV, we employed a NL4-3 luc construct lacking tat. BLIMP does not repress transcription of this clone of HIV, however when tat is expressed BLIMP mediate repression of HIV transcription is restored (Fig. A.3). We also examined the effect of BLIMP overexpression on infected Jurkat T cells, and observed a repression in HIV transcription in the presence of BLIMP only when T cells were activated (Fig. A.4). Also, limiting BLIMP in primary CD4+T cells induces HIV transcription, which is overcome when T cells are activated (Fig. A.5). The physiological relevance, and regulation of BLIMP function in HIV transcription needs to be further explored.

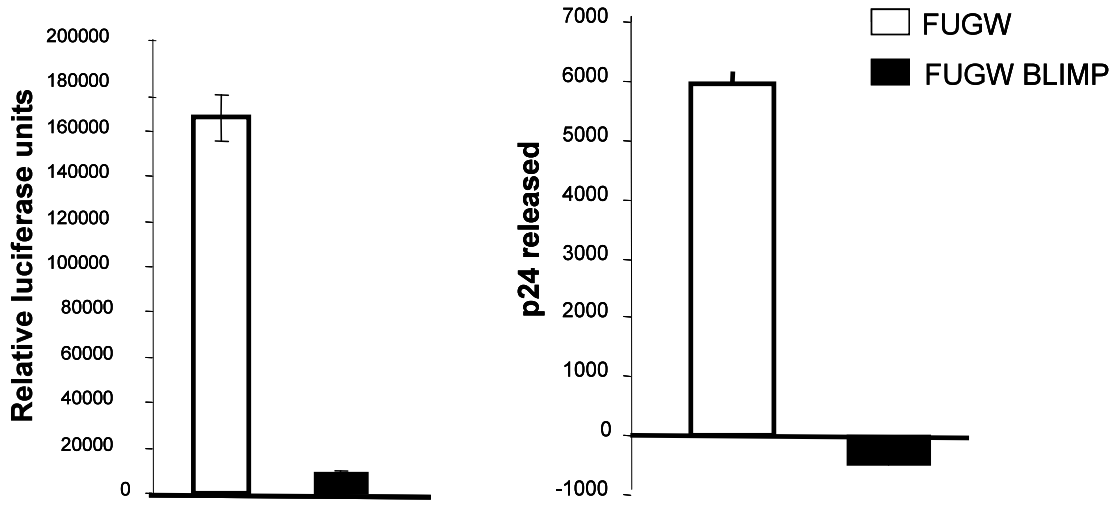


Figure A.1: Overexpression of BLIMP in 293Ts represses HIV transcription. 293T cells were transfected with a pNL4-3 luc and FUGW vector control or FUGW-BLIMP. A) Luciferase assay was performed 48 hours post transfection to measure HIV transcription. B) 48 hours post transfection culture supernatants were harvested and the virus production was assessed by measuring the viral protein gag (p24) by an ELISA.

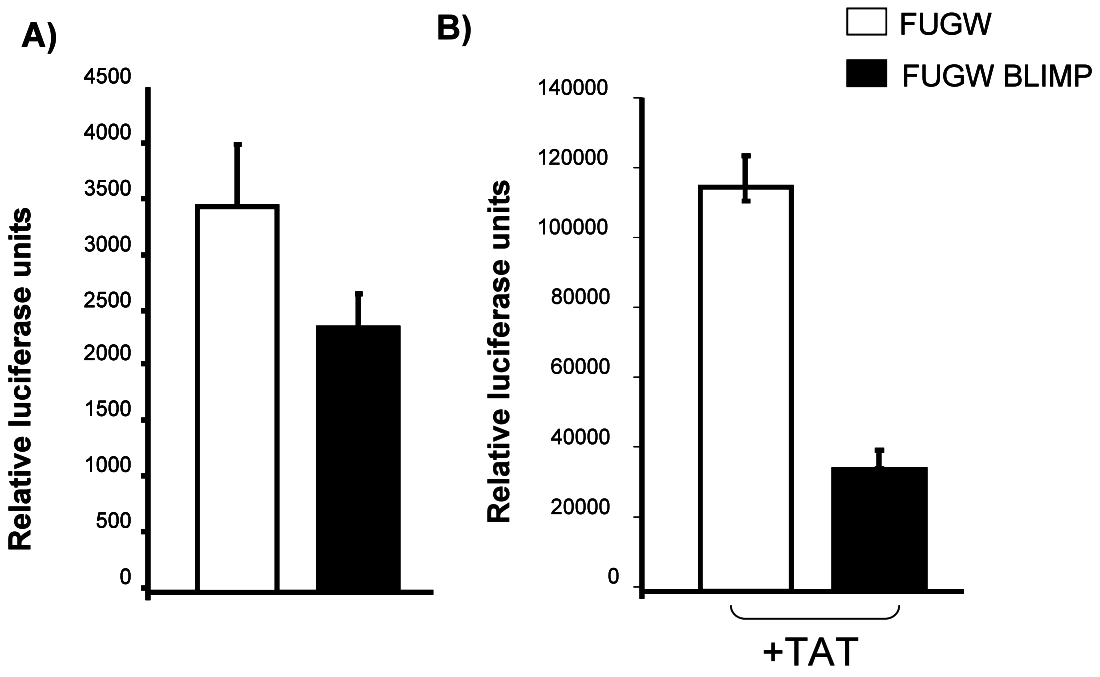


Figure A.2: BLIMP represses HIV-LTR transcription in tat dependent manner. Overexpression of BLIMP in 293Ts represses HIV transcription. 293T cells were transfected with a LTR-luc containing the TAR element and FUGW vector control or FUGW-BLIMP in the presence or absence of Tat. Luciferase assay was performed 48 hours post transfection to measure HIV transcription.

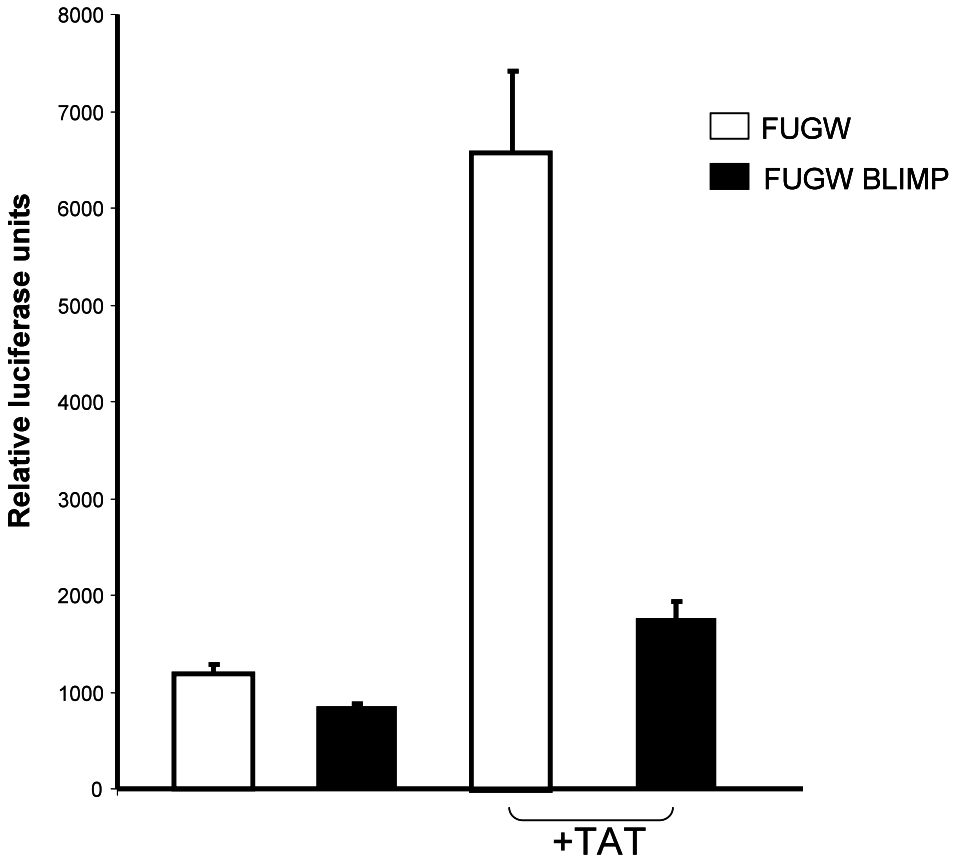


Figure A.3: BLIMP mediated repression of HIV transcription is Tat dependent. Overexpression of BLIMP in 293Ts represses HIV transcription. 293T cells were transfected with a pNL4-3 delta-tat luc and FUGW vector control or FUGW-BLIMP, with or without tat. A) Luciferase assay was performed 48 hours post transfection to measure HIV transcription. B) 48 hours post transfection culture supernatants were harvested and the virus production was assessed by measuring the viral protein gag (p24) by an ELISA.

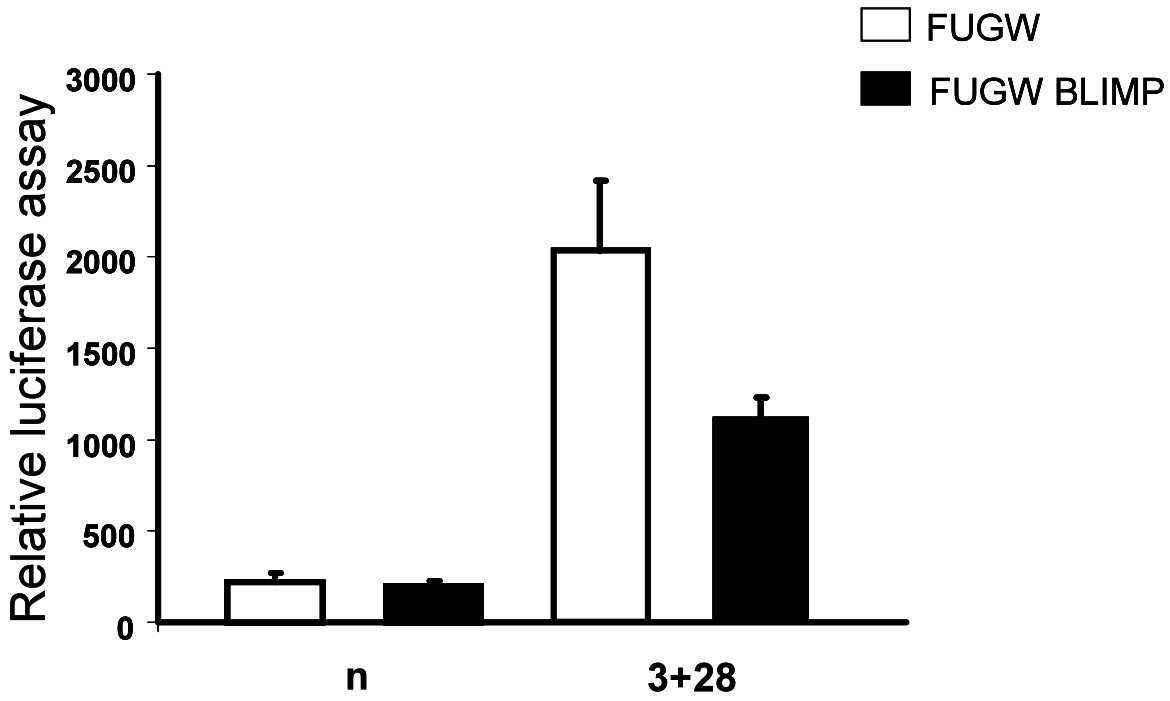


Figure A.4: Overexpression of BLIMP represses HIV transcription in activated T cells. *Jurkat T cells were infected with NL4-3 luc. 12 hours post infection, cells*

These cells were infected with either FUGW-GFP or FUGW-BLIMP-GFP recombinant viruses. Expression of GFP was measured by flow cytometry and western blot analysis (data not shown). Cells were activated with activated with 0.1 µg/mL anti-CD3 and1.0 µg/mL anti-CD28 antibodies for 4-6 hours. Cells were lysed and luciferase activity was measured**.**

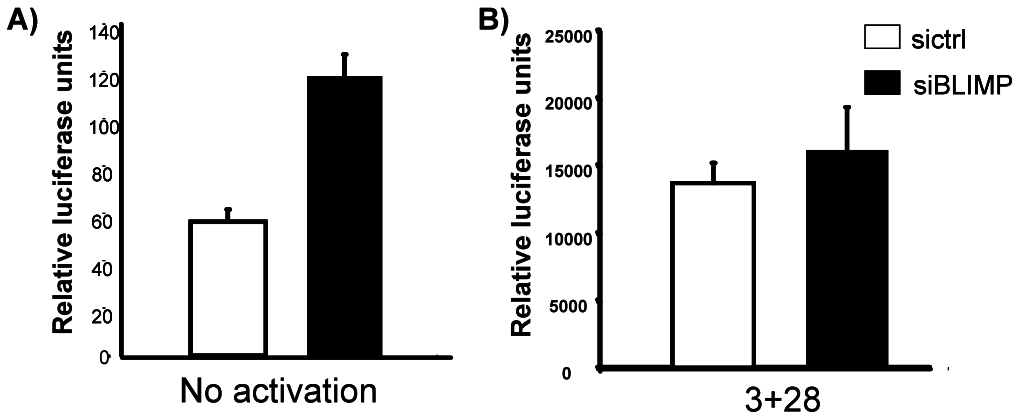


Figure A.5: BLIMP represses HIV transcription in primary CD4+T cells. CD4+T cells were isolated using positive selection from PBMC's obtained from healthy donors. Cells were activated with PMA+PHA for 12-16 hours. Cells were recovered for 12 hours and then infected with NL4-3(env-) luciferase virus. 12-14 hours post infection cells were transfected with siControl and siBLIMP. A) 72-96 hours post knock down luciferase activity was measured. B) 72-96 hours post knock down cells were activated with 0.1 µg/mL anti-CD3 and 1.0 µg/mL anti-CD28 antibodies for 4-6 hours. Cells were lysed and luciferase activity was measured.

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PUBLICATIONS

- **Repression of HIV transcription by the Negative elongation factor complex (NELF) and the terminator factor Pcf11 in CD4+T cells.** Natarajan M, Gilmour DS, Henderson AJ. *In preparation*
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