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# Intragenic elements support the transcription of defective HIV-1 proviruses

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Dissertation

**INTRAGENIC ELEMENTS SUPPORT THE TRANSCRIPTION OF DEFECTIVE  
HIV-1 PROVIRUSES**

by

**JEFFREY KUNIHOLM**

B.S., Northeastern University, 2013

Submitted in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

2022

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*“We are trying to prove ourselves wrong as quickly as possible, because only in that way  
can we find progress.”*

*-Attributed to Richard Feynman, PhD*

*Nobel Laureate, 1965*

## **DEDICATION**

I dedicate this work to all family and friends who have supported me through my endeavors in life and research.

For my parents, Marie and James Kuniholm.

For my brother and collaborator in thought, Matthew Kuniholm.

For the love of my life, Alice.

And for my son, Barrett.

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The work presented in this dissertation would not have been possible without the support and contributions of my mentors, colleagues, friends, and family. I would like to specifically thank Elise Armstrong, MSc who generated the HIV-1 5'LTR deletion construct and contributed extensively to the conceptual formation of this project and its preparation for publishing. It was a privilege to work with Elise as she completed her master's degree and I look forward to seeing where she takes her PhD training once completed. I am thankful for the efforts of Carolyn Coote who performed CD4+ T cell isolations and infections for this work. Carolyn and Alex Olson helped establish and optimize the droplet digital PCR system for the Henderson Lab which was essential for the studies presented here. I thank Alex for his invaluable knowledge of equipment, protocols, and ordering processes for obtaining reagents. I would also like to thank Alex and Nina Lin, MD for their assistance in obtaining volunteer samples from HIV-infected individuals on antiretroviral therapy. I thank Xianbao He for his help with establishing optimized western blot protocols for the detection of HIV-1 proteins. Binita Basukala, Jonathan Kilroy, Viviana Dominguez, Andrew Ngo, and Richard Pickering, Ph.D. were thoughtful lab members and colleagues that gave useful critical feedback regarding this work. They also refeed cells or sent me data on days when I could not easily make the travels to campus from my apartment in Waltham. Kyle Pedro, Ph.D. and Luis Agosto, Ph.D. are former members of the Henderson Lab that helped guide early studies for this work and provided useful reagents and protocols. Finally, I am most thankful for the

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**INTRAGENIC ELEMENTS SUPPORT THE TRANSCRIPTION OF DEFECTIVE  
HIV-1 PROVIRUSES**

**JEFFREY KUNIHOLM**

Boston University School of Medicine, 2022

Major Professor: Andrew Henderson, Ph.D., Professor of Medicine and Microbiology

**ABSTRACT**

Human immunodeficiency virus-1 (HIV-1) establishes a persistent proviral reservoir by integrating into the genome of infected host cells. Current antiretroviral treatments (ART) do not target this persistent population of proviruses which include latently infected cells that upon treatment interruption can be reactivated to contribute to HIV-1 rebound. Deep sequencing of persistent HIV-1 proviruses has revealed that greater than 90% of integrated HIV-1 genomes are defective and unable to produce infectious virions. We hypothesized that intragenic elements in the HIV genome support transcription of aberrant HIV-1 RNAs from defective proviruses that lack long terminal repeats (LTRs). Using an intact provirus detection assay, I observed that resting CD4<sup>+</sup> T cells and monocyte-derived macrophages (MDMs) are biased towards generating defective HIV-1 proviruses. Multiplex reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) identified *env* and *nef* transcripts which lacked 5' untranslated regions (UTR) in acutely infected CD4<sup>+</sup> T cells and MDMs indicating transcripts are generated that do not utilize the promoter within the LTR. 5'UTR-deficient *env* transcripts were also identified in a cohort of people living with HIV-1 (PLWH) on ART, suggesting that these aberrant RNAs are produced *in vivo*. Using 5'

rapid amplification of cDNA ends (RACE), I mapped the start site of these transcripts within the Env gene. This region bound several cellular transcription factors and functioned as a transcriptional regulatory element that could support transcription and translation of downstream HIV-1 RNAs. Transient expression of an HIV-1 5'UTR deletion construct in HEK293T cells demonstrated that HIV-1 transcripts and proteins are still produced when the 5'UTR is absent. These studies provide mechanistic insights into how defective HIV-1 proviruses are persistently expressed to potentially drive inflammation in PLWH.

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## LIST OF ABBREVIATIONS

AIDS .....	Acquired Immunodeficiency Syndrome
APOBEC .....	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like
ARF.....	Alternative Reading Frame
ART.....	Antiretroviral Therapy
AZT.....	Azidothymidine
BU .....	Boston University
BUSM.....	Boston University School of Medicine
cART.....	Combination Antiretroviral Therapy
CFAR .....	Centers For AIDS Research
CpG.....	5'-C-phosphate-G-3'
CRISPR.....	Clustered Regularly Interspaced Short Palindromic Repeats
CRS.....	Cis-acting Repressive Sequence
CTL.....	Cytotoxic T Lymphocyte
dCa .....	Didehydro-cortistatin A
ddPCR.....	Droplet Digital PCR
DNA .....	Deoxyribonucleic Acid
ELISA .....	Enzyme Linked Immunosorbent Assay
eY1H Screen .....	Enhanced Yeast-1-Hybrid Screen
FAM.....	Fluorescein Amidites
HEX .....	Hexachloro-fluorescein
HIV-1 .....	Human Immunodeficiency Virus-1

HRP.....	Horseradish Peroxidase
IFI16.....	Interferon Gamma Inducible Protein 16
IPDA .....	Intact Provirus Detection Assay
ITP.....	Immunology Training Program
KRAB .....	Krüppel-associated Box
LRA.....	Latency Reversal Agent
LTR.....	Long Terminal Repeat
MDM.....	Monocyte-derived Macrophage
MHC-1 .....	Major Histocompatibility Complex-1
MOI.....	Multiplicity of Infection
Mx2.....	Myxovirus Resistance 2
Nuc 1 .....	Nucleosome 1
ORF.....	Open Reading Frame
PBMCs.....	Peripheral Blood Mononuclear Cells
PEI.....	Polyethylenimine
PiBS .....	Program in Biomedical Sciences
PLWH .....	People Living with HIV
PrEP .....	Pre-exposure Prophylaxis
PRR.....	Pattern Recognition Receptor
RACE.....	Rapid Amplification of cDNA Ends
RNA .....	Ribonucleic Acid
RNAPII .....	RNA Polymerase II

RRE..... Rev-responsive Element  
RT ..... Reverse Transcription  
RT-ddPCR..... Reverse Transcription Droplet Digital PCR  
SAMHD1 .sterile alpha motif domain and histidine-aspartate domain-containing protein 1  
SIV ..... Simian Immunodeficiency Virus  
STING..... Stimulator of Interferon Genes  
TAR..... Transactivation Response Element  
TLR..... Toll-like Receptor  
TRIM5 $\alpha$  ..... Tripartite Motif-containing Protein 5  
UTR..... Untranslated Region  
VSVg..... Vesicular Stomatitis Virus G Protein

## CHAPTER ONE: INTRODUCTION

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### **The Human Immunodeficiency Virus-1 Epidemic**

The impact of human immunodeficiency virus-1 (HIV-1) infection was first described in 1981 when the United States Center for Disease Control published a weekly report describing rare lung infections in five men living in Los Angeles. It would not be until 1983 that the causative agent of this acquired immunodeficiency syndrome (AIDS) would be described as a T-lymphotropic retrovirus capable of human transmission. Initial treatment for HIV-1 infection would not be approved by the Food and Drug Administration until 1987 when a nucleoside reverse transcriptase inhibitor, azidothymidine (AZT), was demonstrated to inhibit HIV-1 replication in human cells. The severe side effects of AZT and inadequate distribution of an affordable treatment option to regions with the highest prevalence of AIDS led to a significant increase in new HIV-1 infections from the 1990s to the early 2000s. During this period, roughly three million new HIV-1 infections were estimated to occur per year. At the peak of the HIV pandemic in 2005, the number of AIDS-related deaths were estimated at 1.83 million.

Since its discovery, HIV-1 is estimated to have infected 79.3 million people worldwide and is responsible for an estimated 36.3 million AIDS-related deaths

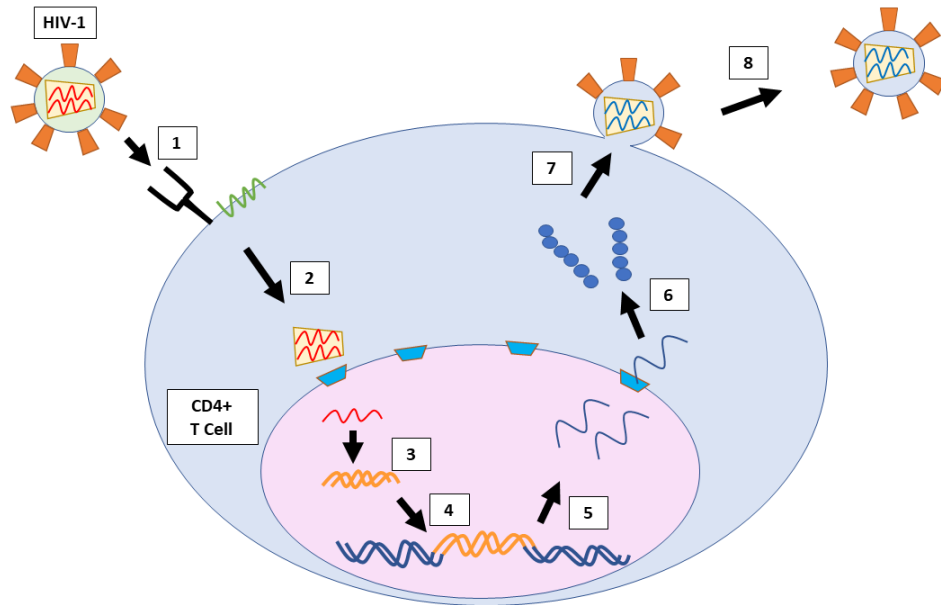
(UNAIDS 2022a). HIV/AIDS remains a leading cause of morbidity and mortality in eastern and southern Africa (UNAIDS 2022b). These regions account for more than half of all people and two thirds of all children living with HIV. Many factors contribute to the persistence of HIV-1 spread and lack of treatment in these regions. A major problem facing these African regions is a health care workforce that is inadequate in size and geographical distribution. For example, opportunities and access to antiretroviral therapies (ART) in Africa are concentrated in urban centers while most of HIV-1 cases are reported in rural areas. Reaching targeted reductions in global AIDS-related deaths and new HIV-1 infections will require a combination of increased health spending, increased testing so that people know their status, improved access to therapies, and sustained adherence to ART regimens. These improvements are focuses of the Joint United Nations Program on HIV/AIDS which has outlined a “95-95-95” goal. This goal entails enabling 95% of HIV infected individuals to know their status through testing, 95% of HIV-positive individuals to have access to antiretroviral therapies, and 95% of individuals on antiretroviral therapy to achieve viral suppression by the year 2025.

For wealthier nations, access to ART has greatly improved since the introduction of AZT, as has the options for antiretroviral drugs. The introduction of combined antiretroviral therapy (cART) entails use of pharmacological inhibitors that target the HIV-1 life cycle at multiple stages and greatly improves patient outcomes compared to monotherapy. The estimated number of new HIV-1 infections and AIDS-related deaths for Western/Central Europe and North America have experienced an 11% and 30% decrease since the year 2000, respectively. Individuals on cART benefit from longer life

expectancies than HIV-positive individuals not on therapy (The Antiretroviral Therapy Cohort Collaboration 2008). Together, these data demonstrate that an increasing number of people infected with HIV-1 are living longer in wealthier nations. Improving access to cART for Southern and Eastern African regions will increase the size of the population of people managing a chronic HIV-1 infection with cART. However, cART is not a cure and most people living with HIV will still harbor cells latently infected by HIV. The latent viral reservoir in individuals on cART remains the focus of intense study, as it enables viral rebound upon ART regimen interruption and stands in the way of a sterilizing cure for HIV.

### **HIV-1 Replication Cycle**

HIV-1 is transmitted primarily by sexual intercourse. Transmission occurs when virus in bodily fluids of an infected individual first infects immune cells of an uninfected individual at mucosal barriers (Figure 1.1). HIV-1 virions attach and fuse with host cells as a result of interactions between the viral envelope glycoprotein and the host proteins CD4 and CCR5 or CXCR4. In the mucosa, these cells include CD4<sup>+</sup> T cells, macrophages, and dendritic cells. Upon viral fusion, the contents of the HIV-1 virion are released into the cytoplasm of the host cell. This includes the viral nucleocapsid containing two copies of an RNA genome that are transported to the nucleus where nucleocapsid uncoating occurs and viral reverse transcriptase generates a DNA



**Figure 1.1: HIV-1 replication cycle.** A schematic of the HIV-1 lifecycle outlining the steps of 1. HIV-1 virion attachment 2. Uncoating of the incoming virion and shuttling of the viral capsid containing two copies of the HIV-1 genome to the nucleus. Capsid uncoating occurs in the nucleus and is coordinated with the initiation of reverse transcription. 3. Reverse transcription of HIV-1's single stranded RNA genome by viral reverse transcriptase generates a double-stranded DNA genome intermediate, referred to henceforth as a provirus. 4. Viral integrase introduces double-stranded breaks in the host cell DNA to enable the integration of HIV-1 provirus into the host genome. 5. A combination of viral and host factors coordinate the transcription of new HIV-1 RNA. 6. Viral transcripts are translated by the host cell and processed by viral protease. 7. Viral proteins are packaged into newly forming virions that bud from the cell surface. 8. New virions are released from the infected host cell.

intermediate genome. HIV-1 integrase and various host cell DNA repair factors then

work together to integrate viral DNA into the host cell DNA forming a provirus.

Integrated intact proviruses generate HIV-1 proteins and RNAs for new HIV-1 virions by

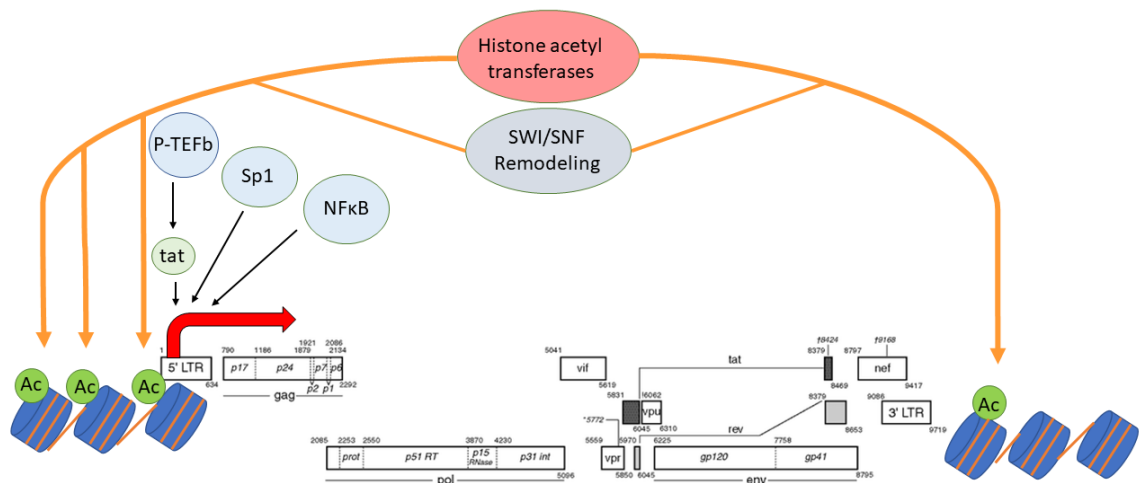
utilizing various host transcription factors and RNA polymerase II (RNAPII) to

coordinate a complex process of viral transcription. A 5' long terminal repeat (LTR) acts

as the core promoter element in the HIV-1 genome and is a site for host cell transcription

factor binding. These factors include NF $\kappa$ B and Sp1 which participate in recruiting

RNAPII to the transcriptional start site of the HIV-1 genome. Processive transcription is orchestrated in part by the viral protein Tat, which binds to the transactivating region (TAR), an RNA stem loop structure at 5' of a newly transcribed HIV-1 mRNA. Tat interacts with subunits of the SWI/SNF complex and histone acetyltransferases which are involved in nucleosome remodeling and maintaining the LTR in an open chromatin state (Figure 1.2). Accessible chromatin is necessary for the actions of HIV-1 transcriptional regulatory elements. Tat also recruits the host elongation factor P-TEFb to the 5' end of the genome to promote the processive transcription of HIV-1 provirus by enhancing RNAPII activity and releasing negative transcriptional elongation complexes. Upon completion of transcription, host translational machinery is leveraged to produce viral proteins which assemble into new virions and eventually bud from the surface of the infected host cell.

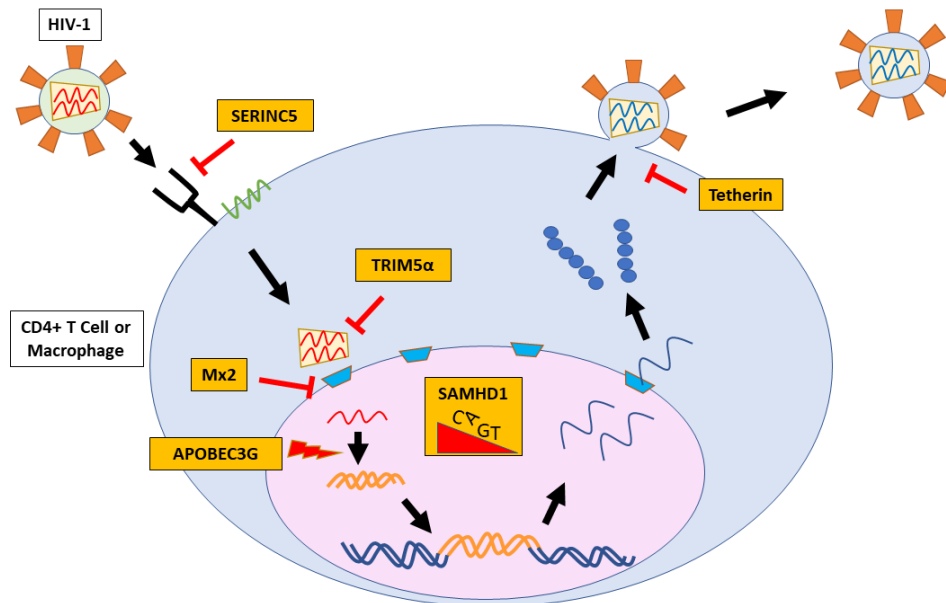


**Figure 1.2: Chromatin remodeling complexes maintain HIV-1 proviruses in a transcriptionally active state.** Histone acetyltransferases coordinate with chromatin remodeling complexes like SWI/SNF to maintain an open chromatin state at the site of HIV-1 integration and near the 5' LTR core promoter sequence. The open chromatin state in this region is necessary for access and binding by host transcriptional factors like NFκB and Sp1. These host factors are involved in the recruitment of RNA polymerase II and P-TEFb, which promotes elongation of viral transcription through interactions with RNA polymerase and release of negative transcriptional regulators.

Reverse transcription (RT) of HIV's RNA genome is a low efficiency process that introduces sequence diversity for progeny virions over the course of infection (Maldarelli et al. 2013). HIV-1 reverse transcriptase lacks proofreading ability and is error prone, introducing approximately  $1.4 \times 10^{-5}$  mutations/bp/cycle (Abram et al. 2010; Preston, Poiesz, and Loeb 1988). Furthermore, replication of the genome requires dissociation of viral reverse transcriptase and re-initiation of reverse transcription on the RNA genome template which contributes to the propensity for internal deletions, mutations, and truncated HIV-1 DNA intermediates. Recombination describes the process of template switching between the two copies of the HIV-1 RNA genome during reverse transcription and further contributes to genetic diversity and mutations (Morris et al. 1999; Song et al. 2018). The inefficiency of RT creates a mechanism to introduce genetic diversity in viral progeny and has been implicated as means for developing antiretroviral drug resistance and escape from viral immunity (Molla et al. 1996; Simon et al. 2003). However, sequence diversification comes at a cost of viral fitness and results in the generation of replication-incompetent or defective HIV-1 proviruses.

Host antiviral factors further limit viral replication at multiple stages of the viral life cycle and contribute to the generation of defective and mutated daughter HIV-1 proviruses. Innate restriction factors are active early in the process of the viral replication cycle targeting viral fusion and uncoating. SERINC5 is a host protein that is packaged into nascent virions and interferes with the envelope glycoprotein of HIV-1 virions to prevent the formation of a virus-cell fusion pore upon cellular attachment (Sood et al. 2017; Timilsina et al. 2020). Additionally, rhesus macaque tripartite motif-containing

protein 5 alpha (TRIM5 $\alpha$ ) binds to incoming viral capsid and promotes its premature disassembly preventing successful



**Figure 1.3 Viral restriction factors of the host cell reduce the efficiency of HIV-1 reverse transcription and replication.** Schematic highlighting the steps of HIV-1 replication which are influenced by innate restriction factors. SERINC5 interferes with virion fusion to the target cell. TRIM5 $\alpha$  promotes premature capsid uncoating to prevent reverse transcription and integration of the HIV-1 RNA genome. Mx2 blocks nuclear import of viral capsid. SAMHD1 keeps the concentration of free nucleotides low in quiescent cells like macrophages and resting CD4+ T cells, reducing reverse transcription efficiency. APOBEC3G introduces mutations in viral cDNA by deaminating cytidines during the reverse transcription process resulting in G to A mutations within the newly formed HIV-1 provirus. Tetherin prevents the release of virions from the infected cell surface.

reverse transcription of viral RNA genome (Black and Aiken 2010; Pertel et al. 2011).

Myxovirus resistance 2 (Mx2) is an interferon-induced restriction factor that inhibits nuclear import of the HIV-1 capsid (Kane et al. 2013). As reverse transcription proceeds, APOBEC3G, a human enzyme that carries out cytidine deamination, targets single-stranded DNA intermediates and induces guanine-to-adenine changes, effectively increasing the rate of hypermutation in proviral genomes and decreasing the efficiency of

viral replication by introducing missense mutations and premature stop codons throughout the HIV-1 genome (Mangeat et al. 2003; Harris et al. 2003; Sharma et al. 2016). Sterile Alpha Motif- and HD-domain containing protein 1 (SAMHD1), a dNTP triphosphohydrolase, reduces the concentration of intracellular dNTPs in resting CD4+ T cells and myeloid cells, thereby limiting the efficiency of reverse transcription (Goldstone et al. 2011; Baldauf et al. 2012). Tetherin is a cell surface protein that prevents viral budding from an infected cell by physically tethering virus particles to the cell surface, thus effectively lowering the amount of circulating cell-free virus particles (Perez-Caballero et al. 2009).

The activities of host restriction factors do not completely block virus replication and many of these factors are counteracted by viral proteins. For example, HIV-1 Vif binds to APOBEC3G and induces its degradation by the host cell proteasome (Marin et al. 2003). Vpx is an accessory protein expressed by HIV-2 that induces the proteasomal degradation of SAMHD1, preventing its functions as a restriction factor (Goldstone et al. 2011; Hofmann et al. 2012; Hrecka et al. 2011). The generation of defective HIV-1 proviruses occurs, in part, as a result of the competing pressures between these pro- and antiviral factors.

A combination of the inefficiency of generating the proviral genome and host restrictions facilitate the establishment of integrated defective HIV-1 proviruses which can persist in an infected cell for years depending on their transcriptional and translational competence and susceptibility to immune selection. This dissertation will focus on the viral and cellular factors that influence the intact or defective state of newly

formed HIV-1 proviruses, mechanisms of defective provirus expression, and the potential immunological consequences of this expression.

### **Latency**

A small percentage of integrated HIV-1 genomes (~2-5%) in people living with HIV (PLWH) on antiretroviral therapies are replication competent. These proviruses are harbored primarily in activated CD4 T cells and participate in the production of nascent HIV-1 virions. A minority of this integrated intact provirus population does not participate in viral replication during acute infection but, rather, enters a state of transcriptional dormancy referred to as latency (Chun et al. 1997). The factors involved in establishing latency and the frequency of cells entering a latent state during acute in vivo infection are incompletely understood and no standardized biomarkers for identifying latently infected cells have been identified. Historically, viral outgrowth assays have been used to estimate the size of the inducible intact provirus population in PLWH on cART (Finzi et al. 1997). This method involves reactivating peripheral blood mononuclear cells from infected people with broad acting latency reversing agents (LRAs) and determining the proportion of cells that produce viral p24. Though useful in measuring the degree of viral reactivation, the viral outgrowth assays do not account for proviruses that remain in a state of latency after treatment with LRAs. Comparison of various PCR-based quantifications of the provirus reservoir with viral outgrowth assays have supported that the noninduced intact provirus reservoir is significantly larger than the estimates generated using viral outgrowth assays and indicate that different depths of

latency are established given the differential susceptibility of proviruses to LRAs (Eriksson et al. 2013; Ho et al. 2013).

In vitro infections of model cell lines or primary human CD4 T cells have been used in efforts to quantify the frequency of latency establishment. These studies typically involve infecting cells with HIV, removing productively infected cells, stimulating cells with LRAs, and comparing the number of cells that reactivated virus with the total proviral reservoir which includes latently infected cells. Results from such studies suggest that approximately 1-1.5% of integrated intact proviruses remain in a state of latency (Jordan, Bisgrove, and Verdin 2003; Battivelli et al. 2018; Kwon et al. 2020). While useful in describing the relatively rare frequency of latency establishment, this estimation does not capture the complexity of infection in humans where infection dynamics vary depending on cell type, activation states, and anatomical locations.

Studies focused on factors that establish latency indicate that multiple modes of latency establishment and maintenance are active among the infected cell population. There is evidence that infection of effector CD4<sup>+</sup> T cells transitioning to a memory phenotype provides a temporal window during which provirus is reverse transcribed and integrated as a state of transcriptional quiescence is established (Dobrowolski et al. 2019; Shan et al. 2017). Alternatively, infection of memory and resting CD4 T cells can support the direct establishment of latent HIV-1 proviruses. HIV-1 preferentially integrates in transcriptionally active regions of the host genome and transcriptional interference resulting from elongating RNAPII reading through the 5' LTR of HIV-1 provirus integrated in these transcriptionally active regions has been reported as another

mechanism which contributes to latency (Schröder et al. 2002; G. P. Wang et al. 2007; Marini et al. 2015; Lenasi, Contreras, and Peterlin 2008). In chronically infected individuals on cART, provirus integration sites are found predominantly within heterochromatic regions which are less accessible to host transcriptional regulators. Increased prevalence of integration sites in these regions might reflect the sustained immune pressures against proviruses that integrate into euchromatic regions and remain transcriptionally active (Jiang et al. 2020). Thus, proviruses integrated into transcriptionally inactive regions of the genome may be “trapped” in an inactive state because of their physical location in the genome. Likewise, repressive transcriptional regulators, chromatin marks, and epigenetic silencing of latent HIV-1 proviruses correlates with a repressed transcriptional state (Battivelli et al. 2018; Matsuda et al. 2015; Tyagi, Pearson, and Karn 2010).

In general, histone methyltransferases help establish and maintain repression of the 5’LTR of HIV-1 provirus by condensing chromatin through chemical modification of histone tails, primarily through methylation of histone-associated lysine residues. This condensation precludes the recruitment and access of RNAPII and transcriptional coactivators to the viral promoter. Conversely, histone acetyltransferases target histone tails for acetylation to promote an open chromatin state and active transcription. Many chromatin remodeling factors have been implicated in the control of HIV-1 latency (Boehm et al. 2017; Imai, Togami, and Okamoto 2010; Ding et al. 2013; Friedman et al. 2011). Epigenetic control of gene expression is influenced by viral factors like Tat, which recruits histone acetyltransferases and transcriptional coactivators to the 5’LTR (Marzio

et al. 1998). Host restriction factors function to counteract epigenetic remodeling by HIV-1. For example, APOBEC3A recruits epigenetic silencing machinery to the HIV-1 5'LTR to maintain latency (Taura et al. 2019). The competing forces of viral and host epigenetic remodelers are not mutually exclusive and act in a combinatorial fashion. These mechanisms involved in maintaining and regulating HIV-1 latency influence the depth of their transcriptional repression and susceptibility to reactivation.

The mechanisms governing the reactivation of latent proviruses are incompletely understood. The initiation of cART and the maturation of the adaptive immune response drastically reduces the levels of circulating virus in infected people. Undetectable viremia in the plasma can be maintained in people living with HIV-1 for years with adherence to antiretroviral drug regimens (Rank et al. 2009; Musey et al. 1997; Jain et al. 2013). However, interruptions or cessation of cART adherence results in a rapid rebound of viremia in a matter of weeks (Davey et al. 1999; Marek Fischer et al. 2003; Fagard et al. 2003). This rebound is fueled by clonal populations of latently infected cells from diverse anatomical niches which are maintained through cellular proliferation (De Scheerder et al. 2019; Reeves et al. 2018). Differential reactivation of latent HIV-1 provirus from effector memory, transitional memory, and central memory CD4<sup>+</sup> T cell subsets has been reported and correlates with a difference in the levels of host cell factors that support viral reactivation (Pardons et al. 2019). The low inducibility of latent proviruses has been demonstrated across a variety of CD4<sup>+</sup> T cell subsets (Kwon et al. 2020). However, CD4<sup>+</sup> T cell subsets decay at differential rates, resulting in the enrichment of latent proviruses in certain cell populations over time. For example, cross sectional studies

analyzing the cell types harboring latent HIV-1 proviruses in chronically infected individuals has revealed that less differentiated memory T cell subsets like central memory cells and stem cell-like memory cells decay slower than effector T cell subsets (Jaafoura et al. 2014). Additionally, it has been shown that tissue resident CD4<sup>+</sup> T cells of lymphoid tissues are more likely to establish latent infections that are harder to reactivate upon *ex vivo* infection when compared to cells isolated from the peripheral blood (Chavez, Calvanese, and Verdin 2015). Furthermore, the degree of T cell activation and signaling strength through the T cell receptor has been shown to influence the likelihood that latent proviruses become reactivated (Gagne et al. 2019). These observations support that the likelihood of latent provirus reactivation is heterogenous among the persistent reservoir and influenced by multiple factors simultaneously.

Targeting provirus transcription has been a priority of cure research strategies since preventing the reactivation of HIV proviruses could contribute to a functional cure. For example, one strategy is to promote provirus repression without purging it from the host genome. “Block-and-lock” cure strategies have the goal of permanently preventing provirus reactivation. For example, the Tat inhibitor didehydro-Cortistatin A (dCA) prevents the epigenomic remodeling of the HIV-1 genome at the Nucleosome 1 (Nuc 1) site, a site involved in provirus transcription initiation and elongation. dCA also promotes the accumulation of repressive nucleosomes in this region. The effect of Tat inhibition on the epigenetic landscape of this region results in a deeper state of latency for integrated proviruses which are then less likely to reactivate during ART cessation or exposure to LRAs. In this dissertation I will discuss the alternative transcriptional pathways utilized

by the HIV-1 provirus which may complicate this strategy of inactivating Tat activity and 5' LTR suppression. The effect of block-and-lock approaches on defective proviruses that retain transcriptional competency has not been explored and may be an important area to study given that these defective viral genomes lack the elements targeted by conventional block-and-lock strategies to promote provirus repression.

“Shock-and-kill” cure strategies have the goal of forcing persistent proviruses out of their latent state with chemical or biological agents, thus allowing the targeting of these latently infected cells for immunological clearance (Marsden et al. 2020; Kim et al. 2022; Margolis et al. 2016). Studies have attempted to identify effective LRAs, many of which promote transcriptional activity of CD4+ T cells (Pache et al. 2015; Barton et al. 2016; Sjøgaard et al. 2015; H. Liu et al. 2021). These LRAs include histone deacetylase inhibitors which prevent transcriptional repression of HIV-1 at the epigenomic level. Another class of LRAs are Smac mimetics, which function by activating noncanonical NFκB signaling and depleting negative regulators of LTR-driven transcription to promote the expression of repressed proviruses (Pache et al. 2020, 2015; G. R. Campbell et al. 2020). Efforts to reactivate latent proviruses has revealed that only a subset of cells harboring intact provirus can be forced out of latency during treatment with strong LRAs. Direct sequencing and reconstruction of intact proviruses that fail to reactivate when stimulated with LRAs in vitro has been performed (Ho et al. 2013). Cloning of these sequences into reporter plasmids and transfection into CD4+ T cells has demonstrated that as much as 11.7% of noninduced intact proviruses isolated from peripheral blood CD4 T cells are replication competent despite their failure to reactivate when treated with

LRAs. Furthermore, the effectiveness of different LRAs varies depending on the CD4+ T cell subset that harbors intact latent provirus (Pardons et al. 2019; Grau-Expósito et al. 2019). The success of shock-and-kill cure strategies rely on pan-reactivation of latent HIV-1 proviruses and successful clearance by the immune system. This strategy does not target the large population of defective proviruses that comprise the reservoir of HIV-1 DNA detected in chronically infected individuals on cART. I will present in this dissertation, data that supports the transcriptional and translational competence of defective HIV-1 proviruses which should be considered components of the HIV-1 provirus reservoir.

Gene editing approaches have the potential to specifically target DNA sequences and introduce deletions or insertions of specific nucleotides. Clustered regularly spaced palindromic repeats (CRISPR)-cas9 systems represent one such approach that has the potential to inactivate HIV-1 proviruses and has been studied in vitro and in humanized mouse models of HIV infection (Yin et al. 2018; Ebina et al. 2013; Zhu et al. 2015). This system uses engineered guide RNA sequences to direct the cas9 nuclease to complementary target DNA where excision or editing of the DNA occurs. The design of guide RNA sequences targeting HIV-1 DNA have been demonstrated to reduce the extent of provirus integration, expression of viral genes, virus replication, and can even shrink the persistent provirus reservoir. Interference of the HIV-1 replication cycle can be achieved through targeting and inactivating different viral sequences including host transcription factor binding sites of the LTRs. This has been demonstrated using guide RNAs targeting NF $\kappa$ B binding sites of HIV-1 LTRs (Chung et al. 2020). Alternatively,

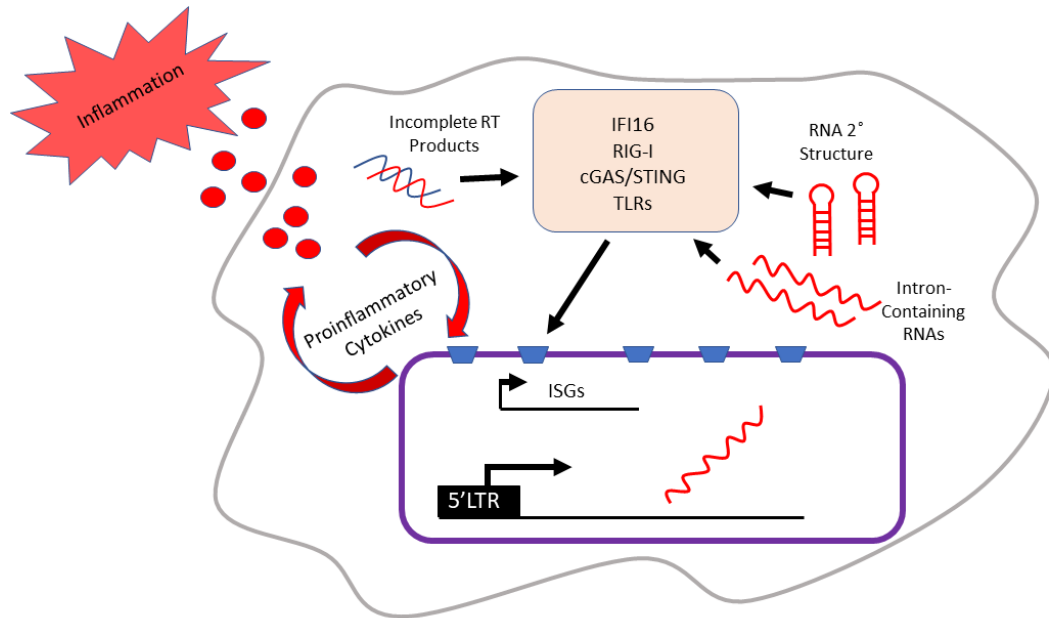
cas9 directed to viral regulatory genes like Tat and Rev has been shown to inactivate expression of provirus (Ophinni et al. 2018). Mouse models of HIV-1 infection have used cas9 targeting a region spanning the 5'LTR and gag to demonstrate that significant reduction in viral persistence and expression is possible in vivo (Dash et al. 2019; Kaminski et al. 2016). Disabled cas9 nuclease (dCas9) has also been engineered to include the repressor domain of the chromatin remodeler Kruppel-associated Box (KRAB) and demonstrated to repress HIV-1 provirus expression through targeted chromatinization (Olson et al. 2020). Thus, many approaches based on CRISPR-cas9 technology are being developed to eradicate the HIV-1 reservoir or at least prevent its expression and replication. There are important considerations for these approaches, however, and it has been demonstrated that cas9 editing of HIV provirus can promote viral escape through nonhomologous end joining repair (G. Wang et al. 2016). Furthermore, targeting LTRs of HIV can promote the formation of transcriptionally active LTR circles in infected cells (Lai et al. 2021). Importantly, viral diversity has been identified as an important consideration for the sequence-specific approach of HIV-1 genome editing (Roychoudhury et al. 2018). Again, defective HIV-1 proviruses may be missing or harboring mutated sequences targeted by engineered cas9 editing approaches. I will present in this dissertation, evidence that subsets of defective proviruses retain transcriptional and translational competence, supporting that gene editing approaches may need to be expanded to account for defective provirus species. Viral diversity is particularly important considering that viral rebound is supported by a small minority of replication-competent proviruses within the persistent provirus reservoir.

### **HIV-1 RNA and Proteins are Immunostimulatory**

HIV-1 infection is cytopathic and leads to DNA fragmentation and the downregulation of pro-survival factor bcl-2 in host cells resulting in cell death (Laurent-Crawford et al. 1991; Popovic et al. 1984). Expression of the viral proteins Vpr, Nef, and Tat promote apoptosis in infected and bystander cells (Rasola et al. 2001; Chen et al. 2002; Andersen et al. 2006). In general, most CD4+T cells infected with HIV-1 are in a resting state and nonpermissive to HIV-1 replication (Doitsh et al. 2010). Incomplete reverse transcription of the HIV-1 RNA genome results in an accumulation of intermediate viral DNA products which are recognized by intracellular pattern recognition receptors (PRRs) to promote apoptosis and pyroptosis, an inflammatory cell death pathway. The result of these cytopathic signaling pathways is a massive depletion of virus-exposed and non-productively infected CD4+ T cells, while the HIV-1 replication cycle proceeds in only a small (~5%) population of mostly activated CD4 T cells. Bystander CD4+ T cell death perpetuates the release of proinflammatory cytokines like IFN $\beta$  and IL-1 $\beta$ , contributing to systemic inflammation. Inflammatory cytokine production is also driven by antigen presenting cells that respond to infected cells and the extensive cell death that occurs during acute HIV-1 infection (Schmidt et al. 2005; Lepelley et al. 2011; Lahaye et al. 2013). Cell-cell interactions between plasmacytoid dendritic cells (pDCs) and HIV-infected CD4+ T cells results in signaling through the toll-like receptor 7 (TLR7) pathway resulting in the production of type I interferons by

pDCs. Direct infection of dendritic cells and macrophages can result in the detection of cytosolic viral DNA prior to integration through the cytoplasmic sensor cGAS and results in production of additional type I interferon. Macrophages are susceptible to HIV-1 infection and respond to HIV-1 nucleic acid species at multiple stages of viral replication.

Like resting CD4<sup>+</sup> T cells, macrophages can recognize intermediate viral DNA species in the cytoplasm using the cytoplasmic sensor interferon-inducible protein 16 (IFI16), though the extent to which these products are generated in natural infection of macrophages has not been well described (Monroe et al. 2014; Jakobsen et al. 2013). *In vitro* experiments have demonstrated that recognition of reverse transcription intermediates by IFI16 induces transcription of interferon stimulated genes through a stimulator of interferon genes (STING)-dependent pathway and regulates HIV-1 replication. Innate detection of nascent HIV-1 transcripts occurs through multiple mechanisms including RIG-I-dependent detection of secondary structured RNA and activation of MAVS-dependent signaling pathways resulting in the induction of interferon responses. HIV-infected macrophages perpetuate systemic inflammatory responses through these innate sensing pathways and have been implicated



**Figure 1.4: HIV-1 nucleic acid species are recognized by innate sensors to promote proinflammatory signaling.** Inefficient reverse transcription results in the cytoplasmic accumulation of incomplete provirus DNA which is detected by IFI16 and stimulates interferon stimulated gene expression. Newly produced HIV-1 transcripts with double stranded RNA structure or containing intronic sequences can be detected by multiple intracellular pattern recognition receptors which also contribute to proinflammatory and interferon signaling. Proinflammatory signaling by macrophages can influence the macrophage's own functional phenotype and skew them towards a proinflammatory transcriptional programming. This signaling feedback contributes to the systemic inflammation seen during HIV-1 infection.

as local harbors of HIV-1 RNA and DNA in various anatomical niches of PLWH on cART. Furthermore, HIV-1 infected macrophages are skewed away from an anti-inflammatory phenotype and towards a proinflammatory, or M1-like, phenotype, characterized by the production of proinflammatory cytokines (Porcheray et al. 2006; Cassol et al. 2009; Neff et al. 2020). Inflammatory cytokine signaling restricts HIV-1 infection and replication in macrophages but contributes to the systemic inflammation observed in infected individuals (Cassol et al. 2009; Jiménez et al. 2012; Cassetta et al. 2013). Inflammatory cytokines have been detected in the serum of people living with HIV on cART at higher levels than uninfected individuals and include  $\text{TNF}\alpha$ , IL-6, and

D-dimer (Osuji et al. 2018; Grund et al. 2016; Brites-Alves et al. 2018). Elevated levels of IL-6 and D-dimer have been correlated with increased morbidity and risk of death in PLWH on cART. Persistent viral replication and escape, the depletion of CD4+ helper T cells, and the systemic increase in proinflammatory cytokines all contribute to the functional exhaustion of cytotoxic CD8+ T lymphocytes (CTLs) over time, exacerbating immunodeficiency and increasing the chances of opportunistic infections characteristic in people with AIDS (Pantaleo et al. 1997; Appay et al. 2000; Kostense et al. 2002).

### **Dynamics of the HIV-1 Proviral Landscape**

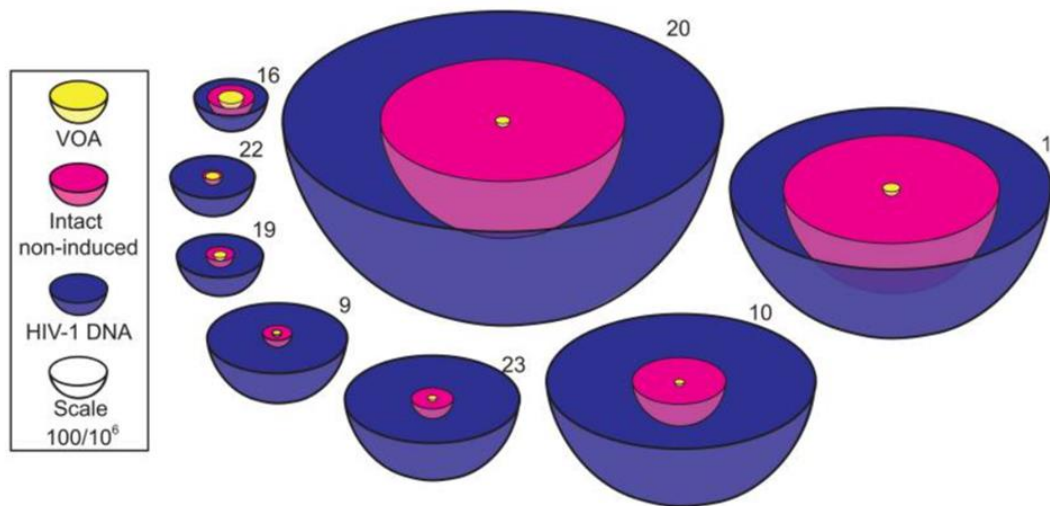
During the first weeks after exposure, dendritic cells that have captured HIV-1 virions traffic from mucosal barriers to the lymphatic tissues, sites of concentrated CD4+ T cell populations and the main site of viral infection, replication, and persistence (Masurier et al. 1998; Geijtenbeek et al. 2000; Haase 1999; Fox et al. 1991; Schacker et al. 2000). Analysis of biopsies of intestinal tissues from HIV-1 infected people and nonhuman primates infected with simian immunodeficiency virus (SIV) have revealed extensive viral replication and CD4+ T cell depletion in lymphoid tissues, particularly gut associated lymphoid tissues (GALT) (Veazey et al. 1998; Guadalupe et al. 2003; Brenchley et al. 2004). This depletion occurs during acute infection and correlates with an exponential increase of the viral load in infected individuals (Arnoczy et al. 2012; Kaufmann et al. 1998). Clinical samples from acutely infected individuals have demonstrated that the sharp increase in plasma viremia is followed by an increase in

circulating CD8<sup>+</sup> T cell numbers. Cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs) expansion initially correlates with effective targeting of infected cells and reduction of viral load. However, the evolution of the CD8<sup>+</sup> T cell response against HIV-1 occurs in parallel with the rise of viral escape mutations which subvert the adaptive immune response (Borrow et al. 1994; Mendoza et al. 2012; Appay et al. 2002; Takata et al. 2017). The persistent reactivation and adaptation of the CD8<sup>+</sup> T cell response to viral replication and evolution has been proposed to contribute to the phenotypic exhaustion described in chronically infected individuals. The sustained efficacy of cellular immunity in controlling HIV-1 infection is variable among individuals and dependent on genetic factors and the timing of cART initiation. A small group of elite controllers naturally control HIV-1 infection through enhanced viral peptide processing and presentation to cytotoxic HIV-specific CD8<sup>+</sup> T cells. However, without therapeutic intervention, most individuals will progress to end-stage disease.

cART dramatically reduces the mortality, morbidity, and risk of transmission associated with HIV-1 infection (Rank et al. 2009; Chou et al. 2019). Current cART regimens typically consist of a combination of reverse transcriptase inhibitors to prevent the early steps of HIV-1 replication (Hammer et al. 2008; Gazzard et al. 2008). Protease inhibitors are also included in many cART regimens to prevent the production of new HIV-1 virions from cells already harboring intact proviruses. Initiation of cART shortly after HIV exposure limits the size of the persistent provirus reservoir in infected individuals. Treatment also correlates with restriction of viral replication and limits the genetic diversity of the integrated provirus population to clones established early during

acute HIV-1 infection (Josefsson et al. n.d.; Siliciano et al. 2003; Frenkel et al. 2003).

Proviruses that retain transcriptional and translational activity are preferentially cleared by the adaptive immune response which shapes the proviral reservoir during sustained cART (Pollack et al. 2017). Over time, this results in the persistence of two general populations of HIV-1 provirus: 1. Intact noninduced proviruses and 2. Defective proviruses with diminished capacities for transcription and translation. Both populations are maintained by cellular proliferation which drives the maintenance and clonal expansion of persistent latent proviruses, even during cART (Reeves et al. 2018; Pinzone et al. 2019a).



**Figure 1.5: Most of the persistent HIV-1 provirus reservoir is defective or intact and difficult to reactivate.** This figure illustrates the proportional sizes provirus populations sequenced from PLWH on ART and tested for reactivation in viral outgrowth assays (VOA). 1. (yellow) Inducible intact HIV-1 proviruses that were reactivated to produce p24 by LRAs in a VOA. 2. (pink) Intact proviruses which were not induced to express viral protein after treatment with potent LRAs. 3. (blue) Total HIV-1 DNA representing both intact and defective HIV-1 proviruses. This illustration emphasizes the predominance of defective HIV-1 proviruses within the reservoir of PLWH on ART and the different depths of latency exhibited among different intact HIV-1 proviruses. Figure generated and published by Ho et al., 2013. Figure caption written by author of this dissertation.

Intact HIV-1 proviruses have been the focus of novel therapies and cure strategies because of their ability to support viral rebound, even though they only represent 2-5% of the persistent provirus population. Deep sequencing and longitudinal tracking of intact provirus sequences in CD4<sup>+</sup> T cells of PLWH before and after cART initiation suggests that most of the latent intact HIV-1 reservoir (~71% of detected sequences) is seeded by the time of ART initiation (Abrahams et al. 2019b; Brodin et al. 2016). cART enables the rebound of CD4<sup>+</sup> T cell counts as the cytopathic effects of HIV-1 replication are reduced. The differentiation of infected CD4<sup>+</sup> T cells to long-term memory cell subsets may contribute to the stability of the latent intact reservoir which is established early in infection.

Defective HIV-1 proviruses make up the largest percentage of provirus detected in peripheral blood (>90%) and harbor mutations and deletions that prevent viral replication (Ho et al. 2013; Pollack et al. 2017; Bruner et al. 2019). These include large deletions of the genome, sequence inversions, and hypermutations which can introduce premature stop codons. Limiting dilution PCR and full genome sequencing of individual proviruses has been performed on CD4<sup>+</sup> T cells from PLWH on cART to characterize the provirus reservoir in chronically infected individuals. Data generated from these studies estimate that as much as 80% of persistent HIV-1 proviruses contain large deletions (Bruner et al. 2016). An estimated 18% of proviruses from these cells contained hypermutated or inverted sequences. As with the intact proviral reservoir, these sequencing efforts have demonstrated that the defective provirus reservoir is established early during HIV-1 infection to comprise most (~93%) of the reservoir within the first

few weeks after infection (Bruner et al. 2016). Proviruses which retain their ability to transcribe HIV-1 RNAs and translate viral proteins are preferentially cleared during sustained immunological pressure. Concurrently, proviruses with minimal transcriptional or translational activity escape immune selection and clonally expand to comprise a majority of the reservoir. Over time, the persistent provirus reservoir becomes dominated by a small number of clonally expanded proviruses compared to the reservoir early after infection (Cohn et al. 2015).

Despite the benefits of cART, people managing HIV-1 infection with antiretroviral treatments still suffer from various comorbidities at higher frequencies than uninfected people. These comorbidities include an increased risk of coronary heart disease, HIV-associated neurological disorders (HAND), and other end-organ diseases (Paisible et al. 2015; Višković et al. 2018; Deeks, Tracy, and Douek 2013; Bloch et al. 2016; Simioni et al. 2010). These conditions have been associated with inflammation and an accumulation of age-related epigenetic marks in cells from the blood and brain leading to the hypothesis that HIV-1 infection promotes accelerated aging referred to as ‘inflammaging’ (Deeks, Tracy, and Douek 2013; Babu et al. 2019; Schank et al. 2021). Importantly, this phenomenon does not correlate directly with plasma viremia and is observed in people living with HIV-1 even when viremia is largely controlled by cART. cART does not prevent the transcription of HIV-1 RNA and residual transcriptional bursting, or “blips”, have been reported in people living with HIV-1 on therapy (Sörstedt et al. 2016; Crowell et al. 2020; Suzuki et al. 2021). HIV-1 DNA, RNA, and protein have been detected within immune privileged sites of PLWH on ART at concentrations higher

than peripheral blood and may represent a source of viral factors that perpetuate inflammation in tissues of infected individuals on therapy (Peluso et al. 2012; Günthard et al. 2001; Henderson et al. 2019; Wu et al. 2021). These anatomical sites include brain and lymphoid tissues which limit the access of cytotoxic lymphocytes. There are also unique challenges to delivering therapeutic agents to these sites which are protected by highly selective cell layers like the blood brain barrier (Asahchop et al. 2017). For intact proviruses, these tissue niches can act as harbors of latent HIV-1 infected cells which avoid immune clearance and enable rapid viral rebound upon ART interruption. Less is known about the prevalence of defective HIV-1 proviruses in these anatomical niches and their capacity to generate viral RNA and protein. However, given the predominance of defective HIV-1 proviruses among the CD4<sup>+</sup> T cell reservoir in the blood, it is possible that a significant proportion of cells infected with HIV-1 in local tissue niches are also defective. Thus, the mechanisms by which defective HIV-1 proviruses are expressed will be important to understand when developing new therapeutics that address the persistent inflammation observed in the growing population of people managing a chronic HIV-1 infection on cART.

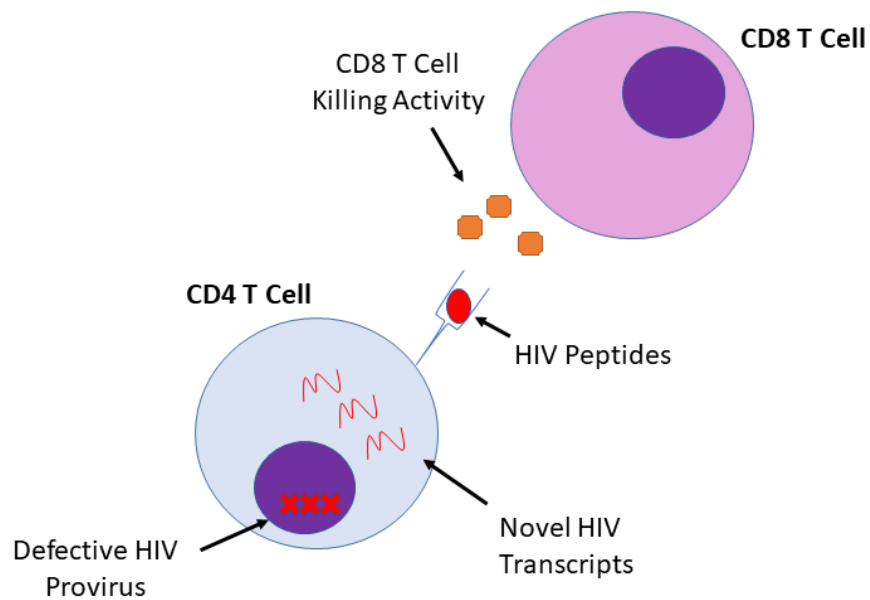
### **Expression of Defective HIV-1 Proviruses**

Despite being unable to complete replication, defective HIV-1 proviruses can overcome mutations in their genome to produce viral transcripts. Isolation of defective HIV-1 proviral clones from people living with HIV-1 on cART has allowed careful

interrogation of the transcriptional and translational capacity of defective genomes. The relevant mechanisms that allow defective proviruses to be expressed are incompletely understood and can vary depending on the particular proviral genetic defect. For instance, proviruses with mutations in their major splice donor sequence can overcome this defect by using alternative splice donor and acceptor sites (Pollack et al. 2017). Alternatively, internal sequence deletions and inversions introduced during negative strand synthesis of reverse transcription can generate novel open reading frames (ORFs) which support the generation of non-canonical HIV-1 transcripts (Imamichi et al. 2016). The processes that generate defective provirus RNAs are not well characterized. Additional non-canonical HIV-1 transcription includes antisense transcription from the 3'LTR (Mancarella et al. 2019; Vanhée-Brossollet et al. 1995; Affram et al. 2019). Whether antisense transcription is regulated by the same mechanisms as transcription from the 5' LTR is unclear. The functions of these non-canonical or cryptic viral RNAs in HIV-1 replication and pathogenesis are undefined. However, there is evidence to support that these transcripts are immunostimulatory. Unspliced, or intron-containing, HIV-1 transcripts stimulate innate immune sensing pathways and can be produced from defective HIV-1 proviruses (Imamichi et al. 2016; Akiyama et al. 2018). Specifically, such transcripts are detected by a MAVS-dependent pathway in infected macrophages upon nuclear export. HIV-1 RNA detection leads to IFN-type 1 responses and proinflammatory cytokine release by infected macrophages which can contribute to T cell dysfunction and exhaustion.

A subset of the RNAs generated by defective HIV-1 proviruses generate viral proteins (Imamichi et al. 2020; Pollack et al. 2017). Isolation and in vitro expression of

an HIV-1 provirus clone harboring a large internal deletion that removed many of HIV's accessory proteins still produced gag and nef protein (Imamichi et al. 2020). Ex vivo cloning and expression of proviruses with defects in their major splice donor sites or point mutations throughout the genome retained the ability to produce and secrete gag protein which could be detected in cell supernatants by ELISA (Pollack et al. 2017). Translation of viral proteins has the potential to drive immune dysregulation and skew the adaptive immune response in chronically infected individuals. Indeed, it has been demonstrated that these proteins are amenable to antigen processing and presentation to induce the killing activity of HIV-specific CD8<sup>+</sup> T cell clones in co-culture assays (Pollack et al. 2017). Synthesis of predicted HIV-1 peptides from the many alternative reading frames (ARFs) throughout the genome has enabled testing of their immunostimulatory capacity against HIV-specific CD8<sup>+</sup> T cells (Bansal et al. 2010a, 2015). These peptides, generated from sense and antisense ARFs, generally activate CD8<sup>+</sup> T cells from PLWH on cART and responses are greater for those with chronic infections, suggesting



**Figure 1.6 Subsets of defective HIV-1 proviruses generate novel protein-coding RNAs and HIV-1 proteins.** This schematic illustrates the evidence for defective HIV-1 provirus transcription and translation. HIV-1 proteins generated by defective HIV-1 proviruses, or using alternative reading frames, have been shown to be amenable to proteasomal processing and loading onto MHC-I molecules. Presentation of HIV-1 peptides generated from defective HIV-1 proviruses stimulate activation and killing activity of HIV-1-specific CD8<sup>+</sup> T cells.

that repeated exposure to viral protein may take place, even during sustained cART. The generation of viral transcripts and proteins from defective HIV-1 proviruses begs the question of whether these viral ligands play an immunostimulatory role in chronically infected individuals and how this may affect the host immune response.

Intragenic cis-acting elements have been described for HIV-1 and other retroviruses and represent factors that could support the expression of defective HIV-1 proviruses. The presence of intragenic transcriptional elements in the HIV-1 genome has been postulated for decades but the function and regulation of such elements have not been demonstrated (E Verdin et al. 1990; Olsen, Cochrane, and Rosen 1992; Cochrane et

al. 1991a; Lint et al. 1994). Cis-acting repressive sequences (CRS) have been described and are thought to limit HIV-1 transcription, splicing, and nuclear export (Cochrane et al. 1991a; Olsen, Cochrane, and Rosen 1992). These functions of CRS are at least partially achieved through interactions with host cellular transcription factors. Such interactions have also been described for cis-acting elements involved in regulating the alternative splicing of HIV-1 transcripts. HIV-1 envelope has been identified as a region with increased intragenic transcriptional activity and an increased prevalence of methylated CpG islands and DNase I sensitivity (Cochrane et al. 1991b; Kint et al. 2020; Kmiec et al. 2020). The transcriptional capacity of these elements in defective proviruses and their interactions with host cellular factors has not been reported. Residual transcription driven by cis-acting elements that remain active in defective HIV-1 proviruses would provide a mechanism for the generation of viral RNAs when the core promoter sequence of HIV-1 is repressed, mutated, or deleted.

### **Testing the Hypothesis that Defective HIV-1 Proviruses are Expressed using Intragenic Elements**

HIV-1 establishes a persistent infection by integrating into the host genome and HIV-1 infection can only be fully eradicated by the elimination of infected cells. Although cART restricts viral replication and disease progression, it only slowly diminishes the persistent reservoir. Understanding the mechanisms that establish and maintain persistent and latent HIV-1 infections are required for effective HIV-1 cure strategies. Persistent HIV-1 infection is also postulated to contribute to inflammation and

chronic immune activation associated with systemic diseases, even in people treated with antiretroviral drugs that have undetectable HIV-1 expression. The processes contributing to this persistent inflammation and immune dysfunction are poorly understood.

Approximately 90% of HIV-1 proviruses sequenced from peripheral blood samples from antiretroviral treated PLWH have crippling mutations including large deletions of the genome that would prevent the generation of infectious HIV-1 particles. A subset of CD4+ T cells harboring defective proviruses generate polypeptides that are presented in the context of MHC I. Cells presenting such peptides can be targeted by CD8+ cytotoxic T lymphocytes (CTLs), thereby shaping the HIV-1 provirus reservoir and potentially redirecting adaptive HIV-1 immunity. These reports indicate that subsets of defective proviruses are expressed and influence immune cell function. Importantly, what regulates the expression of defective proviruses and how they contribute to persistent HIV-associated inflammation has not been reported. Based on the persistent immune dysregulation observed in PLWH on cART and previous reports of intragenic elements that support HIV-1 transcription, I hypothesized that defective provirus transcription is regulated by intragenic transcriptional elements. Accordingly, I experimentally addressed the following hypotheses:

**Hypothesis #1: Establishment of defective HIV-1 proviruses reflects intrinsic properties of the host cell and is influenced by activation states.** I used droplet digital PCR (ddPCR) to measure the intact and defective provirus populations established in monocyte-derived macrophages (MDMs), resting CD4+ T cells, and CD4+ T cells

activated with anti-CD3/CD28 beads after HIV-1 infection. I developed a multiplex reverse transcription droplet ddPCR (RT-ddPCR) assay to simultaneously detect and quantify seven populations of HIV-1 transcripts in these infected primary cells. Multiplex RT-ddPCR allowed the detection of both canonical HIV-1 transcripts and aberrant transcripts which lacked 5' untranslated region (5' UTR) sequence.

Using these approaches, I determined that resting CD4<sup>+</sup> T cells and macrophages are biased towards generating defective proviruses. Multiplex RT-ddPCR revealed that cells harboring defective proviruses still express HIV-1 RNAs, many of which lack 5' UTR sequences. These results support that defective HIV-1 proviruses use alternative transcriptional mechanisms to produce viral transcripts in resting CD4<sup>+</sup> T cells and MDMs. I confirmed the presence of these aberrant transcripts using peripheral blood mononuclear cell (PBMC) RNA from a subset of PLWH on cART, confirming that these transcripts are produced in vivo.

**Hypothesis #2: There are intragenic elements that support the transcription of defective proviruses.** I performed 5' rapid extension of cDNA ends (5'RACE) PCR to sequence the transcriptional start sites of HIV-1 transcripts isolated from infected primary MDMs and CD4 T cells. Using a yeast-1-hybrid screen, we identified regions of the HIV-1 genome that are enriched in direct binding of human transcription factors. we used a luciferase reporter construct to measure the transcriptional activity of HIV-1 sequences throughout the genome.

5'RACE PCR mapped the transcriptional start site of aberrant HIV-1 RNAs to a ~200 base pair sequence in the envelope gene of HIV-1. This region aligned with out-of-frame start codons in the HIV-1 genome and was positioned adjacent to predicted promoter elements and TATA box sequence. This region was identified as a site enriched for binding to 14 human transcription factors in a yeast-1-hybrid screen. I cloned this HIV-1 sequence upstream of a luciferase ORF in a reporter construct and demonstrated significant transcriptional enhancement in luciferase expression over that observed with a pcDNA3.1 plasmid control upon transient transfections in HEK293T cells, supporting that intragenic sequence in Env possesses transcriptional activity independent of the 5'LTR.

**Hypothesis #3: Transcripts are generated from the intragenic promoter and translated into proteins.** To interrogate whether intragenic elements of HIV-1 retained transcriptional and translational capacity in proviruses lacking the 5'LTR, we constructed a proviral expression plasmid with deleted 5' LTR sequences. This construct only included the 3' end of the envelope gene through the 3' LTR. HEK293T cells were transfected with this deletion construct and HIV-1 transcripts were measured by multiplex RT-ddPCR. Lysates from infected cells were probed with HIV-1 antiserum to detect viral proteins.

These experiments confirmed that HIV-1 transcripts are produced using intragenic promoter elements in the HIV-1 genome when the core 5' LTR is deleted. Envelope transcripts lacking 5' LTR sequences were detected by multiplex RT-ddPCR.

Furthermore, HIV-1 proteins were detected in the lysates of cells transfected with the 5' deletion construct, supporting that a subset of the aberrant RNAs generated from defective proviruses are translated into viral proteins.

Our findings describe the cellular context in which establishment of defective HIV-1 proviruses are favored. The identification of proviral elements and transcription factors involved in the expression of aberrant HIV-1 RNAs might further our understanding of how HIV RNAs derived from defective proviruses fuel chronic immune activation and inflammation. These data can inform therapeutic strategies and treatments for PLWH on cART that suffer from chronic inflammation and its associated disorders.

## CHAPTER TWO: MATERIALS AND METHODS

Parts of this chapter were originally published in: Kuniholm J, Armstrong E, Bernabe B, Coote C, Berenson A, Patalano SD, Olson A, et al. Intragenic proviral elements support transcription of defective HIV-1 proviruses. PLOS Pathogens <https://doi.org/10.1371/journal.ppat.1009982>

### Cells

HEK293T cells (ATCC) were maintained and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 100 U/mL penicillin/streptomycin (P/S; Invitrogen), 2 mM L-glutamine (Invitrogen), and 10% fetal bovine serum (FBS; Gemini Bio-Products). CD4<sup>+</sup> T cells were enriched from peripheral blood mononuclear cells (PBMC) from leukapheresis packs (New York Biologics). Negative selection was performed using EasySep Human CD4<sup>+</sup> T Cell Enrichment Kit (Stemcell Technologies) after Lymphoprep density gradient (Stemcell Technologies) separation of PBMCs. Red blood cell lysis was performed by pelleting freshly isolated PBMCs and incubating with 155 mM NH<sub>4</sub>Cl, 12 mM NaHCO<sub>3</sub>, and 0.1 mM EDTA for 3 minutes. Resting CD4<sup>+</sup> T cells were cultured in Roswell Park Memorial Institute medium (RPMI; Invitrogen) with 100 U/mL P/S, 2 mM of L-glutamine, and 10% FBS. Flow cytometry using CD25 and CD69 markers confirmed resting CD4<sup>+</sup> T cell phenotypes (Agosto et al. 2007). CD4<sup>+</sup> T cells were activated with anti-CD3/CD28 Dynabeads (Invitrogen) at a ratio of one bead per cell for 72 h and maintained in RPMI with 100 U/mL IL-2 (AIDS Reagents Program) and 100 ng/mL IL-7 (Miltenyi Biotec). Monocyte-derived-macrophages (MDMs) were generated by positive selection of CD14<sup>+</sup> cells from PBMCs using the EasySep Human CD14 Positive Selection Kit II (STEMCELL

Technologies, Cat: 17858) and differentiating cells for at least 7 days in RPMI supplemented with 10% Human AB Serum (Millipore Sigma), 100 U/mL P/S, 2 mM of L-glutamine, and 20 ng/mL M-CSF (BioLegend, Cat: 574802) at 37°C and 5% CO<sub>2</sub>. 50% fresh media was added 2 days after plating followed by full media changes every 2–3 days to remove non-adherent cells.

### **Viruses and Infections**

Viral stocks of molecular clones HIV-1<sub>NL4-3</sub> and HIV-1<sub>NL4-3 BaL</sub> (AIDS Reagents Program) were generated by transfecting HEK293T cells using polyethylenimine (PEI) transfection reagent (Sigma, Cat: 408727). Supernatant from transfected cells was filtered at 24-hours post-transfection. Pseudotyped HIV-1<sub>VSVg-NL4-3</sub>, HEK293T cells were co-transfected with an expression vector containing VSVg and HIV-1<sub>NL4-3</sub>. HIV-1<sub>NL4-3</sub> virus stocks titers were estimated using CEM-GFP cells obtained from the AIDS Reagent Program (Gervaix et al. 1997). HIV-1<sub>NL4-3 BaL</sub> virus stocks were titrated on primary MDMs and IU/mL were estimated from IPDA results. All infections were performed using MOI < 1 (HIV-1<sub>VSVg-NL4-3</sub> MOI = 0.1, HIV-1<sub>NL4-3</sub> MOI = 0.02, HIV-1<sub>NL4-3 BaL</sub> MOI = 0.01). DNase/MgCl<sub>2</sub><sup>+</sup> was added to cells at time of infection and prior to cell lysis for DNA/RNA isolation to limit contaminating plasmid DNA. CD4<sup>+</sup> T cells were infected by spinoculation. Spinoculation involved plating 5-6 million CD4<sup>+</sup> T cells per well of a 6-well cell culture plate and adding appropriate volume of viral stock. Plates were centrifuged for 90 minutes at 1200xg to promote infection. VSVg pseudotyped virus was immediately removed after spinoculation by pelleting cells and resuspending in PBS.

Non-pseudotyped virus was allowed to incubate with cells post-spinoculation for an additional 30 minutes before pelleting and PBS wash. After PBS wash, cells were resuspended in complete media and plated in a clean 6-well plate. MDMs were infected by adding HIV-1<sub>NL4-3BaL</sub> directly to the cells at 37°C for 4 h, washed twice with PBS and maintained in fresh media. 1 µM of the non-nucleoside reverse transcriptase inhibitor Efavirenz was added prior to infection and post-infection as a negative control as indicated.

### **DNA/RNA Isolation**

DNA and RNA were isolated from cells using the AllPrep DNA/RNA Mini Kit (Qiagen, Cat: 80204). Briefly, cells were washed twice with PBS, treated with DNase for 10 min prior to lysis directly in cell culture plate (MDM) or as cell pellets (CD4<sup>+</sup> T cells, HEK293T cells) using Qiagen RLT buffer supplemented with β-Mercaptoethanol. Cell lysates were homogenized with Qias shredder columns (Qiagen, Cat: 79654) following manufacturer's protocol for AllPrep DNA/RNA Mini Kits. RNA isolation columns were treated with DNase for > 10 minutes to remove contaminating DNA.

### **Droplet digital PCR (ddPCR)**

Droplet digital PCR (ddPCR) takes advantage of microfluidics to perform thousands of separate, single-template, PCRs simultaneously. By using hydrolyzable probes flanked by primers at two relatively conserved sites of the HIV genome, PCR amplicons are standardized in size and HIV-1 genomes can be categorized as intact or

defective depending on the fluorescent signature of the droplets in which they are contained. Normalization of HIV-1 specific PCR product probe signals to a host cell gene of similar size allows estimation of the number of different provirus species among a specific number of host cells. This normalization also enables an estimation of the extent of artificial DNA shearing to be accounted for in data analysis, as all host gene signals are expected to be intact. Accordingly, I used this strategy to measure the relative quantities of intact and defective HIV-1 provirus in CD4+ T cells, macrophages, and human peripheral blood DNA samples.

#### **Intact Provirus Detection Assay (IPDA)(Bruner et al. 2019)**

Probes targeting 5' and 3' regions of the HIV provirus were designed to bind minimally variant locations as described in detailed by Bruner et al(Bruner et al. 2019). Briefly, primers and probes (see Table 2.1 and Table 2.2) to amplify signal from the psi region and RRE of the env region of the HIV-1 provirus simultaneously were used.

<b>IPDA Primers*</b>			
<b>Assay</b>	<b>Primer Name</b>	<b>Function</b>	<b>Sequence (5' to 3')</b>
Psi	Psi-Forward	Forward	CAGGACTCGGCTTGCTGAAG
	Psi-Reverse	Reverse	GCACCCATCTCTCTCCTTCTAGC
Env	Env-Forward	Forward	AGTGGTGCAGAGAGAAAAAAGAGC
	Env-Reverse	Reverse	GTCTGGCCTGTACCGTCAGC
RPP30-1	RPP30-1-F	Forward	GATTTGGACCTGCGAGC
	RPP30-1-R	Reverse	GCGGCTGTCTCCACAAG
RPP30-2	RPP30-2-F	Forward	GACACAATGTTTGGTACATGGTTAA
	RPP30-2-R	Reverse	CTTTGCTTTGTATGTTGGCAGAAA

**Table 2.1** Primer sequences used for the intact provirus detection assay (originally described by Bruner et al. (Bruner et al. 2019))

<b>IPDA Probes*</b>		
<b>Probe Name</b>	<b>Fluorophore</b>	<b>Sequence (5' to 3')</b>
Psi	FAM	TTTTGGCGTACTCACCAGT
Env	VIC	CCTTGGGTTCTTGGGA
Env Hypermutation	None	CCTTAGGTTCTTAGGAGC
RPP30-1	HEX	CTGACCTGAAGGCTCT
RPP30-2	FAM	CCATCTCACCAATCATTCTCCTTCCTTC

**Table 2.2** Probe sequences used for the intact provirus detection assay (originally described by Bruner et al. (Bruner et al. 2019))

A hydrolysable FAM probe was designed to bind Psi sequence whereas hydrolysable VIC probe was designed to bind the RRE sequence. Primers flanked probes. An unlabeled “dark probe” specific for a commonly hypermutated env sequence was included in each IPDA reaction to distinguish hypermutated provirus sequences. Proviruses containing hypermutated signals were detected as single-positive droplets containing FAM-only signal.

Droplet digital PCR reaction mixes were comprised of: 1X ddPCR Supermix (no dUTP) (Bio-Rad, Cat: 186–3024), 750 nM primers, 250 nM probes, and nuclease free

H<sub>2</sub>O to bring the final master mix volume to 16 µL per reaction. DNA was diluted to ~10–100 ng in 8 µL of nuclease free H<sub>2</sub>O per reaction. A control reaction using probes and primers for human RPP30 gene was performed to calculate shearing index and correct the estimated concentrations of intact and defective HIV-1 proviruses. Droplets were generated using Bio-Rad's QX200 Droplet Generator (Cat: 1864002).

Thermalcycling was performed on droplets containing cellular genomic DNA and ddPCR master mix using the following conditions: 95°C for 10 min enzyme activation, 94°C for 30 sec denaturation, 59°C for 1 min annealing/extension, repeat denaturation and annealing/extension 45 cycles, 98°C for 10 min to inactivate enzyme.

Droplets containing ddPCR product were assessed on the Bio-Rad QX200 Droplet Digital PCR System. Data were collected using Quantasoft Software set to copy-number variation (CNV) mode. All experiments included a no template (water only) control and/or an uninfected cell sample negative control for background probe signal. RPP30 single probe populations were used to estimate a shearing index and to correct the estimation of defective and intact proviruses. Estimations of intact and defective provirus frequencies were calculated using droplet counts of single- and double-positives determined by the Quantasoft software.

### **Multiplex Reverse Transcription Droplet Digital PCR**

For RT-ddPCR, RNA was reverse transcribed to generate cDNA with RT reaction mixtures of 14 µl of 5× SuperScript III buffer, 7 µl of 50 mM MgCl<sub>2</sub>, 3.5 µl of random hexamers (50 ng/µl), 1.35 µl of oligo dT15 (500ug/mL), 3.5 µl of 10 mM

deoxynucleoside triphosphates (dNTPs), 1.75  $\mu$ l of RNaseOUT (40 U/ $\mu$ l; Invitrogen), 2  $\mu$ l of SuperScript III RT (200 U/ $\mu$ l; Invitrogen), and nuclease-free dH<sub>2</sub>O to bring the final reaction volume to 70  $\mu$ L. RT was performed at 25°C for 10 min, 50°C for 50 min, followed by an inactivation step at 85°C for 5 min. Negative controls included duplicate reaction mixes with no RT enzyme. FAM probes were designed and directed to sites throughout expected HIV transcripts. RT-ddPCR probe design was based on those reported by Yukl et al (see Table 2.3 and Table 2.4)(Yukl et al. 2018). ddPCR probes were multiplexed as indicated to measure the frequency of probe sites simultaneously in ddPCR assays. Probe signal was analyzed using the Bio-Rad QX200 Droplet Digital PCR System set to Absolute Quantitation mode. Reaction components for single probe ddPCR mixtures and mixtures using only two differently colored probes simultaneously were comprised of: 10  $\mu$ l of ddPCR Probe Supermix (no deoxyuridine triphosphate), 900nM final concentration of forward and reverse primers, and 250nM final concentration of FAM probe.

<b>RT-ddPCR Primers</b>			
<b>Assay</b>	<b>Primer Name</b>	<b>Function</b>	<b>Sequence</b>
Long LTR	Kumar F	Forward	GCCTCAATAAAGCTTGCCTTGA
	Kumar R	Reverse	GGGCGCCACTGCTAGAGA
Nef	F8883-03	Forward	GGTGGGAGCAGTATCTCGAGA
	R9040-10	Reverse	TGTAAGTCATTGGTCTTAAAGGTACCTGAGG

**Table 2.3 Reverse transcription droplet digital PCR Primers (originally described by Yukl et al.(Yukl et al. 2018))**

<b>RT-ddPCR Probes</b>			
<b>Assay</b>	<b>Primer Name</b>	<b>Fluorophore</b>	<b>Sequence</b>
Long LTR	Kumar P	FAM	CCAGAGTCACACAACAGACGGGCAC A
Nef	P8967-50	FAM	CCAGGCACAAGCAGCATT

**Table 2.4 Reverse transcription droplet digital PCR Probes (originally described by Yukl et al. (Yukl et al. 2018))**

For multiplex RT-ddPCR reactions, an additional probe reaction was performed by adding half of the normal primer/probe concentrations (450nM primers, 125nM probes). Droplets were amplified using a 7900 Thermal Cycler (Life Technologies) with the following cycling conditions: 95C for 10 minutes, 95C for 30 seconds, 59C for 60 seconds, repeat steps 2 and 3 for a total of 45 cycles, a 98C final droplet cure step for 10 minutes, and then hold at 4C.

### **People Living with HIV-1 on Antiretroviral Therapy**

These studies utilize PBMCs from PLWH treated with ART (see Table 2.5) enrolled in a study assessing inflammation and immune dysfunction in people living with HIV and aging which was approved by Boston University IRB (IRB-33095) (Olson et al. 2021). Inclusion criteria included individuals between 18–35 and  $\geq 50$  years old who were on antiretroviral treatment for at least 6 months. Subjects with recent active infection (30 days) or on immunosuppressive therapy were excluded from enrollment. Written informed consent was obtained prior to specimen and data collection. PBMCs were isolated using Ficoll-Hypaque density gradient separation and cryopreserved at  $-150^{\circ}\text{C}$  until DNA/RNA isolation was performed.

Subject	Age	Sex	CD4 at enrollment	Nadir CD4	Years on ART
A014	66	Male	1523	508	12.4
A033	60	Female	394	394	6.4
A042	68	Female	494	619	13.2
A044	54	Female	932	192	7.2
A058	64	Male	567	567	6.7
A061	59	Female	625	543	12.5
W102	75	Male	-	182	5.1
W124	67	Male	810	284	4.4
W143	33	Male	471	270	3.2
X101	55	Female	414	259	2.7
X104	51	Male	1055	607	3.3

**Table 2.5 HIV-positive PBMC donors:** Details of participant ages, sex, CD4+ T cell counts, and years on antiretroviral therapy (Olson et al. 2021).

### HIV-1 Expression Constructs

HIV proviral fragments, designated regions 1–17 (see Table 2.6), were cloned in vector pGL4.23 (Addgene, Cat: E8411) upstream of the luciferase reporter gene. pcDNA3.1+ (ThermoFisher, Cat: V79020) was used as a negative control. The pNL4-3- $\Delta$ 5' construct was derived from pNL4-3 (GenBank: AF324493.2) using enzymatic digestion with *StuI* which cuts within the 5' cellular genomic sequence of the NL4-3 plasmid and within *env* at 6,825 bp. Following digestion, pNL4-3- $\Delta$ 5' was generated by blunt end ligation to delete the 5' end of the proviral genome. Successful deletion of 5' end was confirmed by DNA sequencing.

Sequ ence name	Regio n	Forward primer name	Forward sequence
NL43 -1	501- 1527	NL43-1- Fw	GGGACAACCTTTGTATAGAAAAGTTGACTAGGG AACCCACTGCTTAAGC

		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-1-R	GGGGACTGCTTTTTTGTACAACTTGTCTTGTTCC CTGAAGGGTACTAGTAGTCC
NL43-2	1031-2022	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-2-Fw	GGGGACAACCTTTGTATAGAAAAGTTGCAATAGC AGTCCTCTATTGTGTGCATC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-2-R	GGGGACTGCTTTTTTGTACAACTTGTCTTTTTTC CTAGGGGCCCTGC
NL43-3	1501-2537	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-3-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGGAACTA CTAGTACCCTTCAGGAACAA
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-3-R	GGGGACTGCTTTTTTGTACAACTTGTCTGCAGCC AATCTGAGTCAACAGATTTC
NL43-4	2003-3022	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-4-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGCAGGGC CCCTAGGAAAAAG
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-4-R	GGGGACTGCTTTTTTGTACAACTTGTCTCGCTGGT GATCCTTTCCATCCCT
NL43-5	2512-3549	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-5-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGAAATCT GTTGACTCAGATTGGCTGC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>

		NL43-5-R	GGGGACTGCTTTTTTGTACAAACTTGTCGCCCT GCTTCTGTATTCTGC
NL43-6	3001-4024	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-6-Fw	GGGGACAACCTTTGTATAGAAAAGTTGAGGGATG GAAAGGATCACCAGC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-6-R	GGGGACTGCTTTTTTGTACAAACTTGCTCTAAT CCCGAATCCTGCAAAGCT
NL43-7	3528-4522	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-7-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGCAGAAA TACAGAAGCAGGGGC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-7-R	GGGGACTGCTTTTTTGTACAAACTTGTCGCTGTT TCTTGCCCTGTCTCTG
NL43-8	4001-5033	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-8-Fw	GGGGACAACCTTTGTATAGAAAAGTTGAGCTTTG CAGGATTCGGGATTAGA
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-8-R	GGGGACTGCTTTTTTGTACAAACTTGTCGATGAT CTTGCTTTTCTTCTTGGCAC
NL43-9	4501-5531	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-9-Fw	GGGGACAACCTTTGTATAGAAAAGTTGCAGAGAC AGGGCAAGAAACAGC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-9-R	GGGGACTGCTTTTTTGTACAAACTTGTCGGCAA GGTGGCTTTATCTGTTTTGG

NL43-10	5007-6026	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-10-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGTGCCAA GAAGAAAAGCAAAGATCATC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-10-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTGATGA GTCTGACTGTTCTGATGAGC
NL43-11	5506-6539	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-11-Fw	GGGGACAACCTTTGTATAGAAAAGTTGCCAAAAC AGATAAAGCCACCTTTGCC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-11-R	GGGGACTGCTTTTTTTGTACAAACTTGTCCCTCAT GCATCTGTTCTACCATGTC
NL43-12	6001-7027	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-12-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGCTCATC AGAACAGTCAGACTCATCA
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-12-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTACTAC ATCTTCTTCTGCTAGACTGCC
NL43-13	6515-7525	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-13-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGACATGG TAGAACAGATGCATGAGG
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-13-R	GGGGACTGCTTTTTTTGTACAAACTTGTGTCAGGGGC ATACATTGCTTTTCTACT
NL43-14	7001-8027	<b>Forward primer name</b>	<b>Forward sequence</b>

		NL43-14-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGGCAGTC TAGCAGAAGAAGATGTAGTA
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-14-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTGCAAA TGAGTTTTCCAGAGCAACC
NL43-15	7501-8521	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-15-Fw	GGGGACAACCTTTGTATAGAAAAGTTGAGTAGGA AAAGCAATGTATGCCCT
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-15-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTGGTA GCTGAAGAGGCACAG
NL43-16	8003-9016	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-16-Fw	GGGGACAACCTTTGTATAGAAAAGTTGGGTTGCT CTGGAAACTCATTGCA
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-16-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTTAAA GGTACCTGAGGTGTGACTGG
NL43-17	8501-9709	<b>Forward primer name</b>	<b>Forward sequence</b>
		NL43-17-Fw	GGGGACAACCTTTGTATAGAAAAGTTGCTGTGCCT CTTCAGCTACCAC
		<b>Reverse primer name</b>	<b>Reverse sequence</b>
		NL43-17-R	GGGGACTGCTTTTTTTGTACAAACTTGTCTGCTAG AGATTTTCCACACTGACTAAAAGG

**Table 2.6 HIV-1 Genome Tile sequences:** Tile sequences used for luciferase expression assays and enhanced yeast-1-hybrid screen. All sequences derived from NL4-3 virus strain. Genomic coordinates are provided in addition to forward and reverse primers used to generate each tile sequence from the NL4-3 sequence template.

Transfection of HEK293T cells was performed in black-walled, clear-bottom 96-well plates. Cells were plated at a density of 20,000 cells/well 24 h prior to transfection. Cells were transfected at cell confluency of 60–70%. 300 ng of PEI and 50 ng of luciferase reporter constructs in up to 10  $\mu$ l of Opti-MEM were transfected in triplicate wells. 24 hours post transfection, growth media was removed, and cells were lysed with cell culture lysis buffer for 10 min at room temperature. Firefly D-Luciferin was added at a 1:1 volume ratio and the plate was immediately read on BioTek Synergy HT plate reader at 260 nm for 1 sec/well.

### **Enhanced yeast-1-hybrid screen**

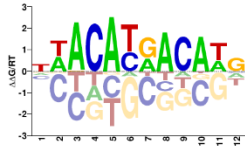

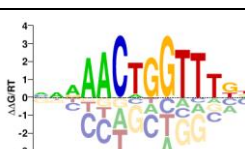

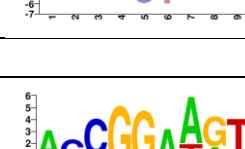
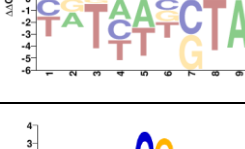
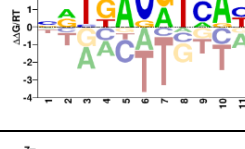
Enhanced yeast one-hybrid (eY1H) assays were performed as previously described (Santoso et al. 2020; Fuxman Bass et al. 2015; Pedro et al. 2021). Briefly, the HIV-1 NL4-3 genome was tiled into 17 overlapping fragments of 1000-1200bp in length (see Table 2.6). “Bait” HIV DNA were cloned upstream to the yeast selection marker *HIS3* or the reporter gene *LacZ*. Constructs were transformed and integrated into yeast chromatin. A library of 1,086 human “prey” transcription factors were linked with the yeast Gal4 activation domain. Binding of the transcription factors to the DNA fragment resulted in transcription of reporter genes. Positive interactions were determined as two of the four replicate colonies that survived in the absence of histidine and experienced a color change in the presence of the substrate X-Gal. More than 90% of positive interactions were identified in 4/4 colonies. Table 2.7 highlights transcription factors that bound HIV-1 tile sequences.

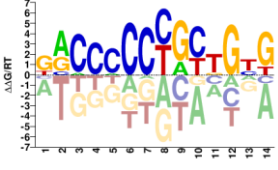
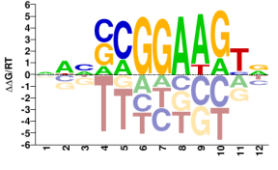
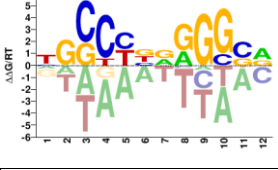
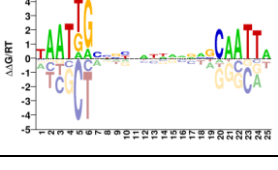
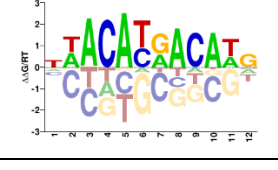
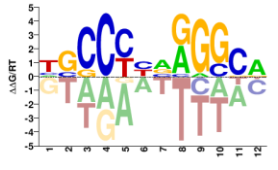
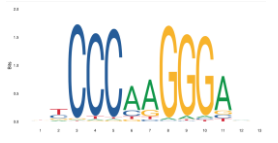
<b>Transcription Factor</b>	<b>Family</b>	<b>HIV Tile Sequence</b>
Iroquois homeobox protein 5	HD	HIV1-2, HIV1-11, HIV1-14, HIV1-15
Nuclear factor 1 A-type	MH1	HIV1-2
Iroquois homeobox protein 6	HD	HIV1-2, HIV1-11, HIV1-15
Homeobox protein Hox-C8	HD	HIV1-2
SRY-Box Transcription Factor 14	HMG	HIV1-2, HIV1-16
Nuclear factor 1 C-type	MH1	HIV1-2
PLAG1 Like Zinc Finger 1	ZF-C2H2	HIV1-2
Homeobox protein Hox-A6	HD-HOX	HIV1-2, HIV1-16
Transcription factor AP-2 beta	AP-2	HIV1-10, HIV1-14, HIV1-15
Grainyhead Like Transcription Factor 2	CP2	HIV1-10, HIV1-14, HIV1-16
Forkhead box protein H1	WH - FH	HIV1-11
Twist Family BHLH Transcription Factor 1	bHLH	HIV1-11
Class B basic helix-loop-helix protein 8	bHLH	HIV1-11
Runt-related transcription factor 1	RUNT	HIV1-11
Runt-related transcription factor 3	RUNT	HIV1-11
Zinc finger protein basonuclin-1	ZF - C2H2	HIV1-11
Zinc Finger Protein 710	ZF - C2H2	HIV1-11, HIV1-16
Musculin	bHLH	HIV1-11
Transcription factor 21	bHLH	HIV1-11
Transcription factor EC	bHLH	HIV1-11
MYC Associated Factor X	bHLH	HIV1-11
Atonal BHLH Transcription Factor 1	bHLH	HIV1-11, HIV1-14
T-Box Transcription Factor 22	T-BOX	HIV1-11
POU Class 4 Homeobox 3	HD - POU	HIV1-11
Myogenic Factor 6	bHLH	HIV1-11
Zinc Finger And BTB Domain Containing 10	ZF - C2H2	HIV1-11, HIV1-14
Glial Cells Missing Transcription Factor 1	GCM	HIV1-11
ETS2 Repressor Factor	WH - ETS	HIV1-14
CAMP Responsive Element Binding Protein 5	bZIP	HIV1-14
Zic Family Member 3	ZF - C2H2	HIV1-14
Zic Family Member 1	ZF - C2H2	HIV1-14
E74 Like ETS Transcription Factor 2	WH - ETS	HIV1-14, hiv1-16
Transcription Factor AP-2 Alpha	AP-2	HIV1-14
T Cell Leukemia Homeobox 3	HD	HIV1-14

Haematopoietically-expressed homeobox protein	HD	HIV1-14, HIV1-16
Early B-cell factor 3	IPT/TIG	HIV1-15, HIV1-16
Forkhead Box J2	WH - FH	HIV1-16
Krüppel Like Factor 4	ZF - C2H2	HIV1-16
SIX Homeobox 6	HD - SIX	HIV1-16
Spi-1 Proto-Oncogene	WH - ETS	HIV1-16
REL Proto-Oncogene, NF-KB Subunit	IPT/TIG, p53	HIV1-16
Early B-cell factor 1	IPT/TIG	HIV1-16
Nuclear Receptor Subfamily 2 Group F Member 2	ZF - NHR	HIV1-16

**Table 2.7 HIV-1 binding human transcription factors identified by yeast-1-hybrid screen:** Human transcription factors that bound HIV-1 tile sequences are listed. The transcription factor family to which each factor belongs is specified. Multiple HIV-1 tile sequences are listed for transcription factors that bound more than one tile sequence (Kuniholm et al. 2021).

Putative binding sites were predicted by FIMO software with a  $p < 0.001$  and these data are shown in Table 2.8 (C. E. Grant, Bailey, and Noble 2011).

Bait	Interacting TF	Motif Evidence Type	Motif logo	PWM Link
14	IRX5	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M03222_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M03222_2.00.txt</a>
14	TFAP2B	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02753_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02753_2.00.txt</a>
14	GRHL2	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M08129_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M08129_2.00.txt</a>
14	ATO1	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04166_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04166_2.00.txt</a>
14	ZBTB10	None		
14	ERF	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02968_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02968_2.00.txt</a>
14	CREB5	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04299_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04299_2.00.txt</a>
14	ZIC3	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02894_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02894_2.00.txt</a>

14	ZIC1	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02893_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02893_2.00.txt</a>
14	ELF2	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04722_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M04722_2.00.txt</a>
14	TFAP2A	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02762_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02762_2.00.txt</a>
14	TLX3	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M05233_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M05233_2.00.txt</a>
14	HHEX	None		
15	IRX5	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M03222_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M03222_2.00.txt</a>
15	IRX6	None		
15	TFAP2B	Direct		<a href="http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02753_2.00.txt">http://cisbp.cabr.utoronto.ca/data/2.00/DataFiles/PWMs/Files/M02753_2.00.txt</a>
15	EBF3	Direct		<a href="https://jaspar.genereg.net/matrix/MA1637.1/">https://jaspar.genereg.net/matrix/MA1637.1/</a>

**Table 2.8 Putative binding sites of transcription factors identified in the yeast-1-hybrid screen:** Binding site characteristics predicted by the FIMO software are described for HIV-1 tile sequences 14 and 15. Existing evidence for direct binding of transcription factors to a given motif is available where indicated. Motif logos describe the predominance of specific nucleotides as part of transcription factor binding motifs (C. E. Grant, Bailey, and Noble 2011).

### **5' Rapid Amplification of cDNA Ends (RACE) PCR**

5'-RACE PCR was performed on total RNA from VSVg-NL43 infected CD4+ T cells and MDMs following the manufacturer's protocol for SMARTer RACE 5'/3' Kit (Takara Bio, Cat: 634858). Random primers were annealed to template RNA using 10X Random Primer Mix, RNA (1µg), and water and incubating the mixture at 72°C for 3 min followed by cooling to 42°C for 2 min. SMARTer II A Oligonucleotide (1µL) was added to tag the 5'-end of the generated cDNA providing a universal forward primer binding site for subsequent PCRs. The RT reaction included template RNA annealed with random primers, 5X First-Strand Buffer, DTT (100 mM), dNTPs (20 mM), RNase Inhibitor (40 U/µl), Reverse Transcriptase (100U) and was performed at 42°C for 90 min and then cooled to 70°C for 10 min. This first-strand reaction product was diluted with 90 µL of Tricine-EDTA Buffer before RACE-PCR.

For 5' RACE PCR a nef specific reverse primer was designed (5'-GATGGGGTGGGAGCAGTATCTCGAGACC-3'). The RACE reaction mixture consisted of 5' RACE PCR sample: PCR-Grade H<sub>2</sub>O (15.5 µL), 2X SeqAmp Buffer (25 µL), SeqAmp DNA Polymerase (1 µL), 5' RACE-Ready cDNA (2.5 µL), 10X Universal Primer Mix (5 µL), 5' gene-specific Primer (10 µM). Negative controls included only the universal primer mix or only the gene-specific primers. 5' RACE PCR thermal cycling was: 94°C for 30 sec, 68°C for 30 sec, 72°C for 3 min, repeated for 25 cycles. 5' RACE-PCR products were separated by gel electrophoresis (1% agarose) and ~1kb PCR products were excised, pooled, and subcloned into a linearized puC19-based vector

(pRACE) using linearized pRACE vector (1  $\mu$ L), Gel-purified RACE product (7  $\mu$ L), and In-Fusion HD Master Mix (2  $\mu$ L). The ligation reaction was incubated for 15 min at 50°C before transforming Stellar Competent Cells (TakaraBio). Colonies were selected for direct bacterial colony sequencing using M13F/R primers which flanked the In-Fusion cloning site of the pRACE vector (Genewiz). Sequences generated are shown in Table 2.9.

<b>5' RACE Sequence ID</b>	<b>Starting Nucleotide Number (NL4-3, GenBank: AF3244 93.2)</b>	<b>5' RACE PCR Product HIV Sequence (5' to 3')</b>
<b>MDM-4</b>	7937	CAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAA AGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAA CTCATTTGCACCACTGCTGTGCCTTGAATGCTAGTTGGAG TAATAAATCTCTGGAACAGATTTGGAATAACATGACCTGGA TGGAGTGGGACAGAGAAATTAACAATTACACAAGCTTAAT ACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAG AATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTT GTGGAATTGGTTAACATAACAAATTGGCTGTGGTATATAA AATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGA ATAGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCA GGGATATTCACCATTATCGTTTTCAGACCCACCTCCCAATCC CGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAG GTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTGAA CGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCTGT GCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTG TAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGGA AGCCCTCAAATATTGGTGAATCTCCTACAGTATTGGAGTC AGGAACTAAAGAATAGTGCTGTAACTTGCTCAATGCCACA GCC
<b>MD</b>	7903	GCATCTGTTGCTCTCACAGTCTGGGGCATCAAAGTCTCCG GGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAA

<b>M -6</b>		CAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACCTCATTTG CACCCTGCTGTGCCTTGGAAATGCTNNTTGGAGTAATAAAT CTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTGG GACAAATAAATTAGCAATTACNCAAGCTTAATACACTCCTT AATTGAATAATCNCAAACCAGCAAGAAAAGAATGAACAA GAATTATTGGAATTATATAAATGGGCAAGTTTGTGGAATTG GTTTAACGTACCCAATTGGCTGTGGTATATAAAATTATTCA TAATGATANNANGAGGCTTGGTATGTTTTCGAATAGTTTTT GCTGTACTTTCTATAGTGAATAGAGATCTGCAGGGATATTC NCCATTATCNTTTCACACCCNCCTCCCANTCCCCAGGGGAC CCNACAGGACCGAAGGANTACAAGAANAANGTGGANAGA NAGACNNANACNGATCCNTTCNANTNNTGAACGGATCCTN NNCACTNATCTGGGACGATCTGCNNAGCCTGTGCCTCTTCA CCTACCACCGCTTGACAGACTNACTCTTNATNGTAAACNAGG ATTGTGGAACCTTCTGGNACGCNNNNNGTGNGAANCCCCCTC A
<b>M D M -8</b>	8179	GAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGT TTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATAT AAAATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAA GAATAGTTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGG CAGGGATATTCACCATTATCGTTTTAGACCCACCTCCCAAT CCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGA AGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTG AACGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCT GTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGAT TGTAACGAGGATTGTGGAACCTTCTGGGACGCAGGGGGTGG GAAGCCCTCAAATATTGGTGGAAATCTCCTACAGTATTGGAG TCAGGAACTAAAGAATAGTGCTGTAACTTGCTCAATGCCA CAGCCATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGA AGTATTACAAGCAGCTTATAGAGCTATTCGCCACATACCTA GAAGAATAAGACAGGGCTTGGAAAGGATTTTGCTATAAGA TGGGTGGCAAGTGGTCAAAAAGTAGTGTGATTGGATGGCCT GCTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAG AT
<b>M D M -9</b>	8138	ATACACTCCTTAATTGAAGAATCGCAAACCAGCAAGAAA AGAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAG TTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATA TAAAATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTA AGAATAGTTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAG GCAGGGATATTCACCATTATCGTTTTAGACCCACCTCCCAA TCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGA AGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTG AACGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCT GTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGAT

		TGTAACAAGGATTGTGGAACCTTCTGGGACGCAGGGGGTGG GAAGCCCTCAAATATTGGTGGAACTCTCCTACAGTATTGGAG TCAGGAACTAAAGAATAGTGCTGTAACTTGCTCAATGCCA CAGCCATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGA AGTATTACAAGCAGCTTATAGAGCTATTCGCCACATACCTA GAAGAATAAGACAGGGCTTGGAAGGATTTTGTATAAGA TGGGTGGCAAGTGGTCAAAAAGTAGTGTGATTGGATGGCCT GCTGTNANGGGAAAGAATGAGACGAGCTGAGCCA
<b>M D M - 11</b>	7984	ACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACCTCATT GCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAA TCTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTG GGACAGAGAAATTAACAATTACACAAGCTTAATACACTCCT TAATTGAAGAATCGCAAACCAGCAAGAAAAGAATGAACA AGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATT GGTTAACATAACAAATTGGCTGTGGTATATAAAATTATTC ATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTT TGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATT CACCATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGA CCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAG AGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCCTT AGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCA GCTACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGG ATTGTGGAACCTTCTGGGACGCAGGGGGTGGGAAGCCCTCA AATATTGGTGGAACTCTCCTACAGTATTGGAGTCAGGAACTA AAGAATAGTGCTGTAACTTGCTCAATGCCACAGCCATAGC AGTAGCTGAGGGGACAGATAGGGTTATAGAA
<b>M D M - 14</b>	8025	GCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAA TCTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTG GGACAGAGAAATTAACAATTACACAAGCTTAATACACTCCT TAATTGAAGAATCGCAAACCAGCAAGAAAAGAATGAACA AGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATT GGTTAACATAACAAATTGGCTGTGGTATATAAAATTATTC ATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTT TGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATT CACCATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGA CCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAG AGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCCTT AGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCA GCTACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGG ATTGTGGAACCTTCTGGGACGCAGGGGGTGGGAAGCCCTCA AATATTGGTGGAACTCTCCTACAGTATTGGAGTCAGGAACTA AAGAATAGTGCTGTAACTTGCTCAATGCCACAGCCATAGC AGTAGCTGAGGGGACAGATAGGGTTATAGAAAGTATTACAA

		GCAGCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAG ACAGG
<b>M D M - 15</b>	8110	CAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAA TTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGA ATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGT TTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATA ATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGC TGTACTIONTCTATAGTGAATAGAGTTAGGCAGGGATATTCAC CATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCC GACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGA GACAGAGACAGATCCATTTCGATTAGTGAACGGATCCTTAGC ACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCT ACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATT GTGGAACCTTCTGGGACGCAGGGGGTGGGAAGCCCTCAAAT ATTGGTGGAAATCTCCTACAGTATTGGAGTCAGGAACTAAAG AATAGTGCTGTAACTTGCTTAATGCCACAGCCATAGCAGT AGCTGAGGGGACAGATAGGGTTATAGAAGTATTACAAGCA GCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAGACA GGGCTTGGAAAGGATTTTGCTATAAGATGGGTGGCAAGTG GTCAAAAAGTAGTGTGATTGGATGGCCTGCTGTA
<b>M D M - 18</b>	8076	AGATTTGGAATAACATGACCTGGATGGAGTGGGACAGAGA AATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAG AATCGCAAAACCAGCAAGAAAAGAATGAACAAGAATTATT GGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAAACA TAACAAATTGGCTGTGGTATATAAAATTATTCATAATGATA GTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGTGCTGACT TTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTAT CGTTTCAGACCCACCTCCCAATCCCGAGGGGACCCGACAGG CCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGA GACAGATCCATTTCGATTAGTGAACGGATCCTTAGCACTTAT CTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACC GCTTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGGAA CTTCTGGGACGCAGGGGGTGGGAAGCCCTCAAATATTGGTG GAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTG CTGTAACTTGCTCAATGCCACAGCCATAGCAGTAGCTGAG GGGACAGATAGGGTTATAGAAGTATTACAAGCAGCTTATA GAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGCTT GGAAAGGATTTTGCTATAAGATGGGTGGCAAGTGGTCAA AAGTAGTGTGATTGGATGGCCTG
<b>M D M - 19</b>	8110	CAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAA TTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGA ATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGT TTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATA ATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGC

		TGTA CTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCAC CATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCC GACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGA GACAGAGACAGATCCATTCGATTAGTGAACGGATCCTTAGC ACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCT ACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATT GTGGAACCTTCTGGGACGCAGGGGGTGGGAAGCCCTCAAAT ATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAACTAAAG AATAGTGCTGTTAACTTGCTCAATGCCACAGCCATAGCAGT AGCTGAGGGGACAGATAGGGTTATAGAAGTATTACAAGCA GCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAGACA GGGCTTGGAAGGATTTTGCTATAAGATGGGTGGCAAGTG GTCAAAAAGTAGTGTGATTGGATGGCCTGCTGTAAGGGAA AGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAG CAGT
<b>M D M - 23</b>	7955	CTGGCTGTGGAAGATACCTAAAGGATCAACAGCTCCTGG GGATTTGGGGTTGCTCTGGAAAACCTATTTGCACCACTGCT GTGCCTTGAATGCTAGTTGGAGTAATAAATCTCTGGAACA GATTTGGAATAACATGACCTGGATGGAGTGGGACAGAGAA ATTAACAATTACACAAGCTTAATACTCCTTAATTGAAGA ATCGCAAAACCAGCAAGAAAAGAATGAACAAGAATTATTG GAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACAT AACAAATTGGCTGTGGTATATAAAATTATTCATAATGATAG TAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTT CTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCG TTTCAGACCCACCTCCCAATCCCGAGGGGACCCGACAGGCC CGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGA CAGATCCATTCGATTAGTGAACGGATCCTTAGCACTTATCT GGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGC TTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGGAAC TCTGGGACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGG AATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGC TGTTAACTTGCTCAATGCNACAGCCATAGCAGTAGCTGANG GGACANA
<b>C D4 -1</b>	8001	GGGGTTGCTCTGGAAAACCTATTTGCACCACTGCTGTGCCT TGGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTG GAATAACATGACCTGGATGGAGTGGGACAGAGAAATTAAC AATTACACAAGCTTAATACTCCTTAATTGAAGAATCGCA AAACCAGCAAGAAAAGAATGAACAAGAATTATTGGAATTA GATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAA TTGGCTGTGGTATATAAAATTATTCATAATGATAGTAGGAG GCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTTCTATAG TGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAG ACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAG

		GAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGAT CCATTTCGATTAGTGAACGGATCCTTAGCACTTATCTGGGAC GATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAG AGACTTACTCTTGATTGTAACGAGGATTGTGGAACCTTCTGG GACACAGGGGGTGGGAAGCCCTCAAATATTGGTGGAACTCT CCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTTA ACTTGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGAC AGATAGGGTTATAGAAGTATTACAAGCAGCTTATAGAGCTA TTCGCCACATACNTAG
<b>C D4 -5</b>	7789	AGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACG GTACAGGCCAGACAATTATTGTCTGATATAGTGCAGCAGCA GAACAATTTGCTGAGGGCTATTGAGGGCGCAACAGCATCTGT TGCAACTCACAGTCTGGGGCATCAAACAGCTCCAGGCAAG AATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCC TGGGGATTTGGGGTTGCTCTGGAAAACCTCATTTGCACCACT GCTGTGCCTTGGAAATGCTAGTTGGAGTAATAAATCCCTGGA ACAGATTTGGAATAACATGACCTGGATGGAGTGGGACAGA GAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGA AGAATCGCAAACCAGCAAGAAAAGAATGAACAAGAATTA TTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAA CATAACAAATTGGCTGTGGTATATAAAATTATTCATAATGA TAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGTCTGTA CTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATT ATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCCGACA GGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACA GAGACAGATCCATTCGATTAGTGAACGGATCCTTAGCACTT ATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCA CCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGG AACTTCTGGGACGCAGGGGGT
<b>C D4 -6</b>	8204	GATAAATGGGCAAGTTTGTGGAATTGGTTTAAACATAACAAA TTGGCTGTGGTATATAAAATTATTCATAATGATAGTAGGAG GCTTGGTAGGTTTAAGAATAGTTTTTGTCTGTACTTTCTATAG TGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAG ACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAG GAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGAT CCATTTCGATTAGTGAACGGATCCTTAGCACTTATCTGGGAC GATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAG AGACTTACTCTTGATTGTAACGAGGATTGTGGAACCTTCTGG GACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAACTCT CCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTTA ACTTGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGAC AGATAGGGTTATAGAAGTATTACAAGCAGCTTATAGAGCTA TTCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAG GATTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAGT

		GTGATTGGATGGCCTGCTGTAAGGGAAAGAATGAGACGAG CTGAGCCAGCAGCAGATGGGGTGGGAGCAGTATCTCGAGA CCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG AAATTGTTATCCGCTCACAATTCCANNCAACATACGAGC
<b>C D4 -7</b>	7827	CGGTACAGGCCAGACAATTATTGTCTGATATAGTGCAGCAG CAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCT GTTGCAACTCACAGTCTGGGGCATCAAACAGCTCCAGGCAA GAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCT CCTGGGGATTTGGGGTTGCTCTGGAAAACCTATTTGCACCA CTGCTGTGCCTTGAATGCTAGTTGGAGTAATAAATCTCTG GAACAGATTTGGAATAACATGACCTGGATGGAGTGGGACA GAGAAATTAACAATTACACAAGCTTAATACACTCCTTAATT GAAGAATCGCAAACCAGCAAGAAAAGAATGAACAAGAA TTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTT TAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAA TGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGT GTAATTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACC ATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCCG ACAGGCCCGAAGGAATAGAAGAAGAANNTGGAGAGAGAG ACAGAGACAGATCCATTCGATTAGTGAACGGATCCTTAGCA CTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTA CCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATTN TGGAACCTTCTGGG
<b>C D4 -9</b>	7783	GGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACG CTGACGGTACAGGCCAGACAATTATTGTCTGATATAGTGCA GCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAG CATCTGTTGCAACTCACAGTCTGGGGCATCAAACAGCTCCA GGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAA CAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACCTATTTG CACCCTGCTGTGCCTTGAATGCTAGTTGGAGTAATAAAT CTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTGG GACAGAGAAATTAACAATTACACAAGCTTAATACACTCCTT AATTGAAGAATCGCAAACCAGCAAGAAAAGAATGAACAA GAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTG GTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTC TAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTT GCTGTAATTTCTATAGTGAATAGAGTTAGGCAGGGATATTC ACCATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGAC CCGACAGGCCCGAAGGAATAGAAGAAGAANNTGGAGAGA GAGACAGAGACAGATCCATTCGATTAGTGAACGGATCCTTA GCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAG CTACCACCGCTTGAGAGA
<b>C D4</b>	7978	GGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAAC TCATTTGCACCACTGCTGTGCCTTGAATGCTAGTTGGAGT

- 10		<p>AATAAATCTCTGGAACAGATTTGGAATAACATGACCTGGAT  GGAGTGGGACAGAGAAATTAACAATTACACAAGCTTAATA  CACTCCTTAATTGAAGAATCGCAAACCAGCAAGAAAAGA  ATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTG  TGAATTGGTTAACATAACAAATTGGCTGTGGTATATAAA  ATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTAAGAA  TAGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCAG  GGATATTCACCATTATCGTTTCAGACCCACCTCCCAATCCC  GAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGG  TGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTGAAC  GGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCTGTG  CCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTGT  AACGAGGATTGTGGAACCTTCTGGGACGCAGGGGGTGGGAA  GCCCTCAAATATTGGTGGAAATCTCCTACAGTATTGGAGTCA  GGAACTAAAGAATAGTGCTGTAACTTGCTCAATGCCACAG  CCATAGCAGTAGCTGNNGGGACAGATAGGGTTATAGAAGT  ATTACAAGCAGCTTATAGAGCTNTTCGCC</p>
C D4 - 12	8087	<p>AACATGACCTGGATGGAGTGGGACAGAGAAATTAACAATT  ACACAAGCTTAATACACTCCTTAATTGAAGAATCGCAAAC  CAGCAAGAAAAGAATGAACAAGAATTATTGGAATTAGATA  AATGGGCAAGTTTGTGGAATTGGTTAACATAACAAATTGG  CTGTGGTATATAAAATTATTCATAATGATAGTAGGAGGCTT  GGTAGGTTAAGAATAGTTTTTGCTGTACTTTCTATAGTGAA  TAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAGACCC  ACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAGGAAT  AGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCAT  TCGATTAGTGAACGGATCCTTAGCACTTATCTGGGACGATC  TGC GGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGAC  TACTCTTGATTGTAACGAGGATTGTGGAACCTTCTGGGACG  CAGGGGGTGGGAAGCCCTCAAATATTGGTGGAAATCTCCTAC  AGTATTGGAGTCAGGA ACTAAAGAATAGTGCTGTAACTTG  CTCAATGCCACAGCCATAGCAGTAGCTGAGGGGACAGATA  GGGTTATAGAAGTATTACAAGCAGCTTATAGAGCTATTCGC  CACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTT  TGCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAGTGTGA  TTGGATGGCCTGCTGTNANGGNAAGAATGAGACGAGCTGA  NCCAGCA</p>
C D4 - 13	7961	<p>GTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTT  GGGGTTGCTCTGGAAA ACTCATTTGCACCACTGCTGTGCCT  TGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTG  GAATAACATGACCTGGATGGAGTGGGACAGAGAAATTAAC  GATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGCA  AAACCAGCAAGAAAAGAATGAACAAGAATTATTGGAATTA  GATAAATGGGCAAGTTTGTGGAATTGGTTAACATAACAAA</p>

		<p>TTGGCTGTGGTATATAAAAATTATTCATAATGATAGTAGGAG  GCTTGGTAGGTTTAAGAATAGTTTTTGGCTGTACTTTCTATAG  TGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAG  ACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAG  GAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGAT  CCATTTCGATTAGTGAACGGATCCTTAGCACTTATCTGGGAC  GATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAG  AGACTTACTCTTGATTGTAACGAGGATTGTGGAACCTTCTGG  GACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAAATCT  CCTACAGTATTGGAGTCNGGAACTAAAGAATAGTGCTGTTA  ACTTGCTCAATGCCACAGCCATAGCAGTAGCTGANGGGAC  AGATAGGGTTATAGAAGTATTACNAGCAGCTTATAGAGCTA  TTC</p>
<b>C D4 - 18</b>	8572	<p>ACGCAGAGGGTGGGAAGCCCTCAAATATTGGTGGAAATCTC  CTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTAA  CTTGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGACA  GATAGGGTTATAGAAGTATTACAAGCAGCTTATAGAGCTAT  TCGCCACATACCTAGAAGAATAAGACAGGGGCTTGGAAGG  ATTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAGTG  TGATTGGATGGCCTGCTGTAAGGGAAAGAATGAGACGAGC  TGAGCCAGCAGCAGATGGGGTGGGAGCAGTATCTCGAGAC  CAAGCTTGCGTAATCATGGTCATAGCTGTTTCCTGTGTGA  AATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGG  AAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGC  TAACTCACATTAATTGCGTTGCGCTCACTGCCCCGCTTTCAG  TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCA  ACGCGCGGGGAGAGGCGGTTTGCCTATTGGGCGCTCTTCCG  CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTTCGGCTGC  GGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTT  ATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGA  GCAAANNCCAGCAAAGGCCAGGAACCGTAAAAAGGCC  GCGTTGCTGGCGTTTTTCCATNGNCTCCGCCCCCTGACGA  GCATC</p>

**Table 2.9 5' RACE PCR sequences:** cDNA sequences generated from RNA isolated from HIV-1 infected MDMs and CD4+ T cells using a nef-specific reverse primer and universal 5' oligo and primer mix (see Materials and Methods).

### Transfection with HIV-1 Vectors

Transfection of HEK293T cells was performed in black-walled, clear-bottom 96-well plates. Cells were plated at a density of 20,000 cells/well 24 h prior to transfection.

Cells were transfected at cell confluency of 60–70%. 300 ng of PEI and 50 ng of

luciferase reporter constructs in up to 10  $\mu$ l of Opti-MEM were transfected in triplicate wells. 24 hours post transfection, growth media was removed, and cells were lysed with cell culture lysis buffer for 10 min at room temperature. Firefly D-Luciferin was added at a 1:1 volume ratio and the plate was immediately read on BioTek Synergy HT plate reader at 260 nm for 1 sec/well.

### **Western blot**

HEK293T cells transfected with NL-43 or pNL4-3- $\Delta$ 5' were lysed using freshly made lysis buffer (0.5% Igepal CA-630, Sigma; 50 mM Tris, pH 8.0; 150 mM NaCl; and Complete Protease Inhibitor (Millipore Sigma) in Milli-Q H<sub>2</sub>O. Lysates were mixed in Laemmli's SDS-Sample Buffer (Boston BioProducts, Cat: BP-111R) and heated at 95°C for 5 min. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred by electroblotting onto polyvinylidene difluoride membrane (Millipore). After blocking 1 h with 5% nonfat dry milk, blots were incubated with primary antibodies (Monoclonal Mouse anti- $\beta$ -Actin, BioRad, Cat: VMA00048 or Polyclonal Human Anti-HIV IgG, NIH-ARP3957, AIDS Reagent Program) overnight at 4°C and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were developed with ECL Prime Western Blotting System (GE Healthcare) and visualized by merging a calorimetric (white light) and chemiluminescent image captured using a Bio-Rad Gel Doc system.

### Statistical analysis

All *in vitro* experiments and technical replicates were conducted at least three times. Experiments with primary cells included cells from at least three different donors. Data are presented as mean values  $\pm$  standard error of the mean. *P* values were calculated based on the two tailed two sample T-test using GraphPad Prism software unless otherwise noted. \* denotes  $p < 0.05$ ; \*\* denotes  $p < 0.01$  and \*\*\* denotes  $p < 0.001$ .

## **CHAPTER 3: INTRAGENIC ELEMENTS SUPPORT THE TRANSCRIPTION OF DEFECTIVE HIV-1 PROVIRUSES**

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### **Introduction**

#### *Experimental Rationale*

HIV-1 replication in CD4+ T cells is influenced by the activation state of the host cell (Oswald-Richter et al. 2004; Pierson et al. 2002; Painter, Zaikos, and Collins 2017). Generally, activated T cells are more permissive to viral entry, completion of reverse transcription, integration of provirus, and production of nascent virions (Orendi et al. 1998; Stevenson et al. 1990; Gowda et al. 1989; Doitsh et al. 2010). Activated CD4+ T cells support HIV-1 replication with a network of host transcriptional activators and coactivators which contribute to an open chromatin organization that is permissive for transcription (Lelek et al. 2015; Alcamí et al. 1995; Kinoshita et al. 1997). Conversely, resting CD4+ T cells are comparatively nonpermissive to HIV infection and replication (Zack et al. 1990; Korin and Zack 1998; Pierson et al. 2002). This nonpermissive state results from a lack of free nucleotides and repressive host cell transcription factor activity. Together, these attributes of resting cells limit the completion of reverse transcription, integration of HIV's DNA genome intermediate, and expression of viral genes. Increased presence and activity of host restriction factors in resting CD4+ T cells

also limit the completion of the viral replication cycle (Baldauf et al. 2012; Ryoo et al. 2014; Chiu et al. 2005). Despite these blocks in replication, resting CD4<sup>+</sup> T cells can be directly infected by HIV-1. Activated CD4<sup>+</sup> T cells infected by HIV-1 during their transition to a resting or memory-like phenotype further contribute to the size of the infected resting CD4<sup>+</sup> T cell population. Extensive cell death among activated CD4<sup>+</sup> T cells results in a decrease in CD4<sup>+</sup> T cell counts during acute in vivo infection while HIV-1 infected CD4<sup>+</sup> T cells in a resting state or with memory-like phenotypes persist in the peripheral blood and tissues. I hypothesized that resting CD4<sup>+</sup> T cells would harbor more defective HIV-1 proviruses than activated cells after acute infection as a result of the quiescent host cell state and activity of restriction factors that preclude efficient replication.

Macrophages have a diverse array of immune functions including control of inflammation and antigen presentation. Macrophages are also susceptible to HIV-1 infection and studies of tissue samples from PLWH on cART and myeloid-only humanized mice have detected replication competent HIV-1 in macrophages of the brain and urethra (Honeycutt et al. 2016, 2017; Ganor et al. 2019; Koenig et al. 1986; Andrade et al. 2020). Sensing of HIV-1 entry by macrophages directly triggers a type I interferon response (Decalf et al. 2017). Nuclear export of intron-containing HIV-1 transcripts is recognized by intracellular nucleic acid sensors in macrophages and microglial cells which respond by promoting the production of proinflammatory cytokines (Akiyama et al. 2021, 2018). The host restriction factor SAMHD1, a dNTPase, is highly active in macrophages and resting CD4<sup>+</sup> T cells, restricting viral replication in these cells at the

reverse transcription step by reducing intracellular levels of dNTPs (Jáuregui and Landau 2018; Hrecka et al. 2011; Laguette et al. 2011; Lahouassa et al. 2012). Given the significance of macrophages in perpetuating proinflammatory signaling during HIV-1 infection and their central role in innate and adaptive immune responses, I decided to determine the extent of intact and defective HIV-1 provirus establishment in these cells during acute infection. I hypothesized that the relatively quiescent state of macrophages and the actions of host restriction factors make macrophages a harbor for defective HIV-1 proviruses.

The shaping of the reservoir and persistent proviral genomes has been linked to provirus expression and selection by anti-HIV immune responses. For example, it has been shown that a subset of defective viruses from individuals treated with ART can be expressed and targeted by CD8<sup>+</sup> T cells (Bruner et al. 2016; Pollack et al. 2017). However, it is not clear how these defective proviruses are transcribed, especially if a significant number of proviruses have large upstream deletions or LTR mutations. The existence of intragenic promoters has been suggested for several retroviruses (Arrigo, Yun, and Beemon 1987; M. Campbell, Eng, and Luciw 1996; Löchelt, Flügel, and Aboud 1994; Löchelt, Muranyi, and Flügel 1993). The presence of proviral cis-regulatory elements has also been proposed within the HIV-1 genome, with reports of DNase hypersensitive sites, methylated CpG islands, transcription factor DNA binding sites and modest transcriptional activation potential (Cochrane et al. 1991b; Eric Verdin 1991; E Verdin et al. 1990; Graf et al. 2000; Kint et al. 2020). In the context of intact proviruses, the 5' LTR includes the cis-regulatory elements that recruit and coordinate host

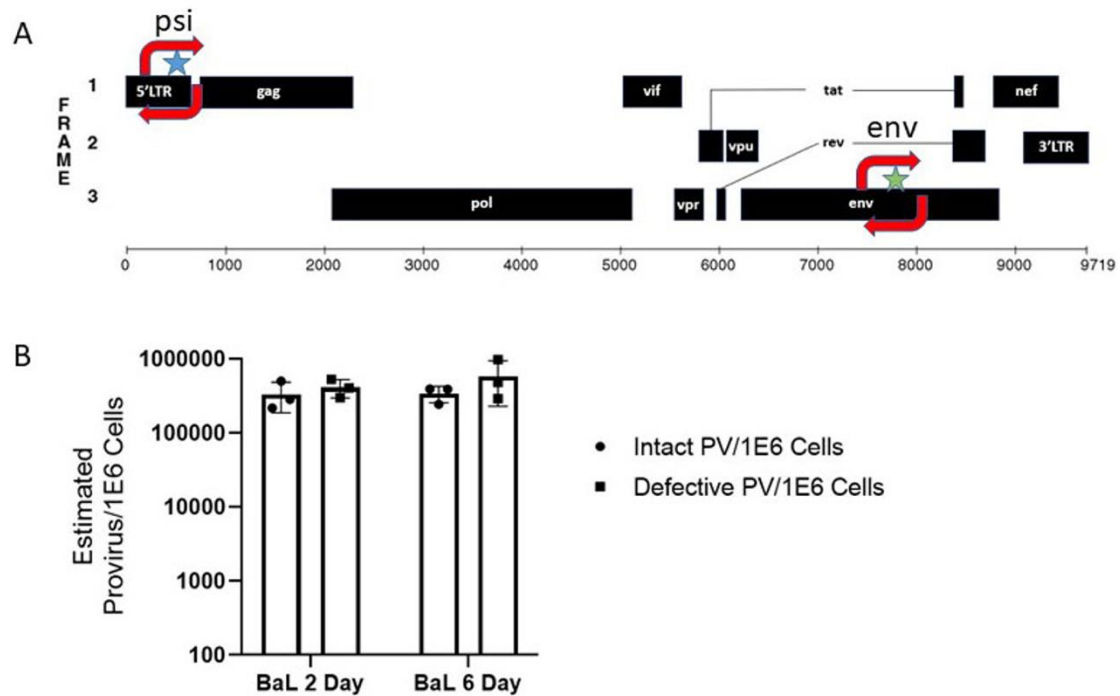
transcription factors to initiate and regulate HIV-1 transcription. The mechanisms by which HIV transcription is regulated in defective HIV proviruses that lack a functional 5' LTR remains incompletely described. We hypothesized that there are intragenic sequences that support transcription of defective HIV-1 proviruses.

## Results

### *Defective HIV proviruses are generated in both resting CD4<sup>+</sup> T cells and MDMs*

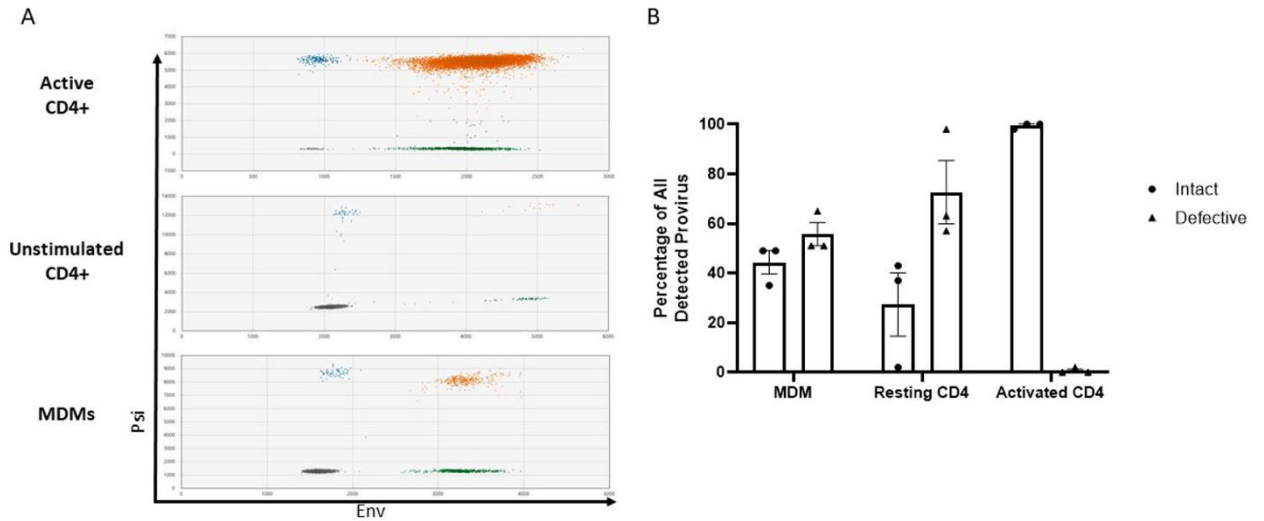
A well described intact provirus detection assay (IPDA) has been developed and described for HIV-1 by Bruner et al (Bruner et al. 2019). This assay uses probes specific for HIV-1 psi element and the rev-responsive element (RRE) of the envelope gene (see Figure 3.1A). We implemented this assay to measure HIV-1 proviruses established in acutely infected human CD4<sup>+</sup> T cells and monocyte-derived macrophages (MDMs). We hypothesized that acute HIV-1 infection of resting and quiescent cells biases infection towards defective proviruses. CD4<sup>+</sup> T cells were either unstimulated or activated with anti-CD3/anti-CD28 beads prior to infection with HIV-1<sub>VSVgNL4-3</sub>, whereas monocyte derived macrophages (MDMs) were infected with R5-tropic HIV-1<sub>NL4-3 BaL</sub>. Infected cells were cultured for at least 48-hours to allow the completion of reverse transcription and integration (see Figure 3.1B). Genomic DNA from infected cells was used as template for IPDA droplet digital PCR (ddPCR). IPDA showed that activated CD4<sup>+</sup> T cells had a higher frequency (>90%) of intact HIV provirus when compared to resting CD4<sup>+</sup> T cells or MDMs (Figure 3.2) which had on average 25% and 40% intact proviruses, respectively. Approximately 50–60% of proviruses detected in both resting CD4<sup>+</sup> T cells

and MDMs were defective with most proviruses harboring 5' mutations that prevented detection by the psi probe. The non-nucleoside reverse transcriptase inhibitor Efavirenz (EFV) was used as a negative control to assure that IPDA signals were dependent on



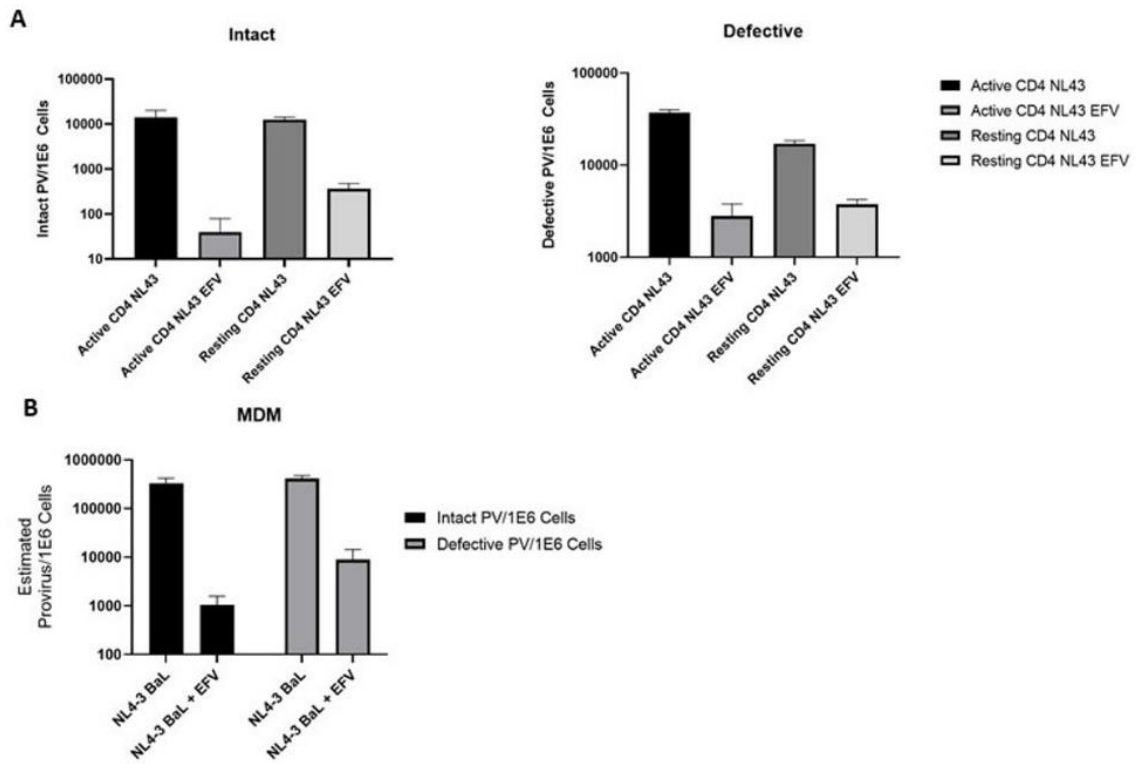
reverse transcription and were not contaminating DNA (see Figure 3.3). These results support that defective HIV proviruses are generated in both resting CD4<sup>+</sup> T cells and MDMs after an acute infection and the activation state of CD4<sup>+</sup> T cells influences efficient establishment of intact proviruses.

**Figure 3.1 IPDA and MDM infection time course:** (A) Schematic of primer (arrows) and probe (stars) binding sites used for IPDA. For details see methods (Bruner et al. 2019). (B) IPDA time course data for HIV-1 NL4-3-BaL infected MDMs. MDMs differentiated from 3 separate donors were infected as described in Methods and incubated for either 2 or 6 days before DNA isolation. IPDA was used to estimate intact and defective provirus frequencies per  $1 \times 10^6$  cells at each time point. Day 2 and Day 6 intact and defective provirus estimates were not statistically different when analyzed by Two-Sample T Test.



**Figure 3.2 Acute HIV-1 infection generates defective proviruses in MDMs and resting CD4+ T cells.**

(A) Representative raw IPDA data of (top) activated CD4+ T cells, (middle) unstimulated CD4+ T cells, and (bottom) monocyte-derived macrophages (MDMs) infected with HIV-1. Droplets are color coded based on manual gating of positive and negative probe signals (gray = empty or double mutation/deletion droplets, blue = droplets single positive for *psi*, green = single positive *env* droplets, orange = double positive *psi* and *env* intact proviral droplets). A parallel reaction to detect the host cell gene *RPP30* was used as a correction for DNA shearing. (B) Percentages of intact and defective HIV genomes detected in MDMs and CD4+ T cells following HIV-1 infection. MDMs were infected with HIV-1<sub>NL4-3-BaL</sub> for 48 hours prior to DNA isolation. Resting and anti-CD3/CD28 bead activated CD4+ T cells were infected with HIV-1<sub>NL4-3-VSVg</sub> for 72 hours prior to DNA isolation. Data represents three independent infections using cells generated from three different donors. Resting and activated CD4+ T cell sample data are participant matched.



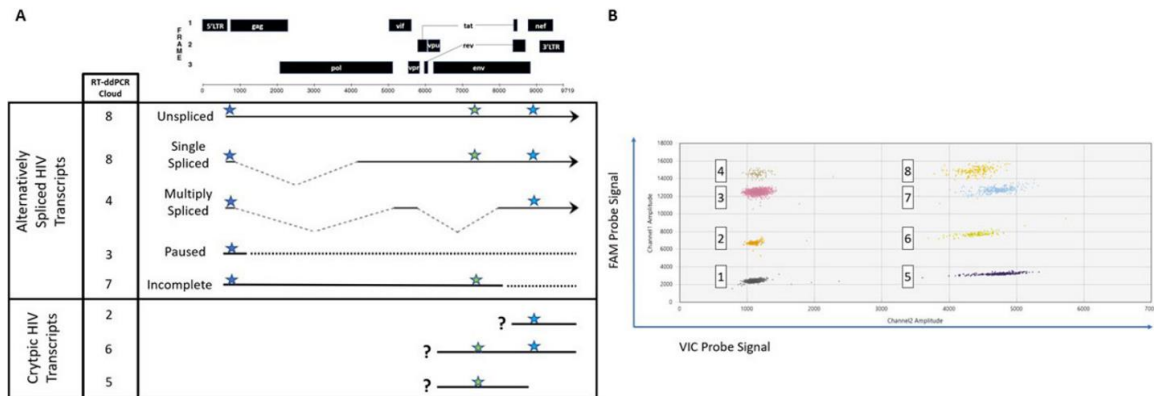
**Figure 3.3 Reverse transcriptase inhibitor Efavirenz (EFV) treatment of CD4+ T cells and MDMs prevented intact and defective HIV provirus establishment regardless of activation state.** (A) IPDA data for EFV pretreated CD4+ T cells infected with HIV-1NL4-3. For CD4+ T cells, EFV was added at a concentration of 10  $\mu$ M 30 minutes prior to spinoculation with HIV-1NL4-3. Infection was limited to a single round by addition of the viral protease inhibitor Saquinavir 30 minutes after spinoculation. Resting and activated CD4+ T cells were participant matched. Data are from 3 separate donors. (B) IPDA data for EFV pretreated MDMs infected with HIV-1NL4-3-BaL. For MDMs, EFV was added at a concentration of 10  $\mu$ M 24-hours prior to addition of HIV-1NL4-3-BaL to the cell cultures. Data are from 3 separate donors.

*Aberrant HIV transcripts are produced after acute HIV-1 infection of MDMs and CD4 T cells*

Defective HIV-1 proviruses comprise the majority of the persistent provirus in PLWH on cART. Transcription of HIV-1 RNAs from defective HIV-1 provirus clones isolated from cells of PLWH on cART has been reported by multiple groups. HIV-1 transcripts act as ligands for pattern recognition receptors (PRRs) in both CD4 T cells and MDMs. Detection by these PRRs generally initiates proinflammatory signaling pathways and promotes an antiviral type I interferon response. cART does not prevent the transcription of HIV-1 RNAs and PLWH on cART suffer from persistent systemic inflammation and a variety of inflammation-related end organ diseases. Given the observation that resting CD4 T cells and MDMs are predisposed to defective HIV-1 infection, we hypothesized that these cells produce aberrant HIV-1 transcripts which may stimulate proinflammatory signaling pathways.

Canonical HIV-1 transcription is driven by the 5' long terminal repeat (5'LTR) of HIV-1. This region, also known as the 5' untranslated region (5'UTR), is the site of host cell transcription factor binding which allows the recruitment of RNA polymerase II (Pol II). HIV-1 transcripts are subject to complex posttranscriptional splicing. Generally, multiply spliced transcripts are produced first, followed by single spliced transcripts, and then unspliced HIV-1 RNA. 5'UTR sequence harbors the transcriptional start site for traditional spliced and unspliced HIV-1 transcripts and should be detectable on all HIV-1 RNAs.

To determine the presence or absence of aberrant HIV-1 RNAs in HIV-1 infected CD4+ T cells and MDMs, I adapted a reverse transcription droplet digital PCR assay (RT-ddPCR) originally described by Yukl et al (Figure 3.4) (Yukl et al. 2018). This assay used the envelope-specific primers and probe employed for the IPDA coupled with primers and probes spanning R-U5-gag sequence (referred to in figures as “LTR”; see Figure 3.4). This 5’ probe detects HIV-1 transcripts that have successfully initiated transcription elongation of HIV-1 transcripts from the 5’ LTR. Furthermore, to capture

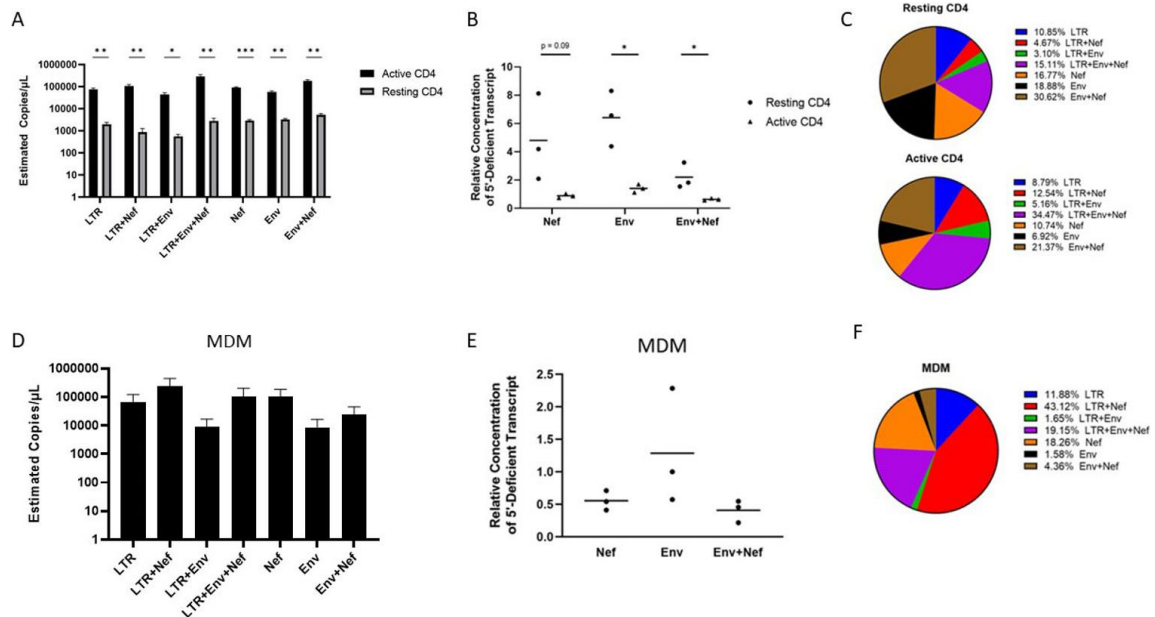


the full array of

**Figure 3.4 Multiplex RT-ddPCR probe locations and representative data.** (A) Schematic of RT-ddPCR probe binding sites aligned to the HIV genome (Yukl et al. 2018; Bruner et al. 2019). Traditional HIV transcripts are labeled with population numbers that correlate to the droplet populations shown in (B). Putative cryptic HIV transcripts lacking 5’UTR sequence are labeled as populations 2, 6, and 5. Population 1 represents empty droplets or HIV transcripts which lack all probed sequence sites. (B) Representative data acquired during multiplex RT-ddPCR. Data shown reflects the following multiplexed probe reactions: 1X concentration LTR probe reaction (y-axis, FAM), 0.5X Nef probe reaction (y-axis, FAM), and 1X Env probe reaction (x-axis, VIC). The multiplexed assay detected 8 distinct populations which were manually gated as reflected by different droplet colors and numbers above. Numbers represent droplets harboring the following distinct transcriptional species: (1) Empty droplets, (2) Nef Only Transcripts, (3) LTR Only Transcripts, (4) LTR+Nef Transcripts, (5) Env Only Transcripts, (6) Env+Nef Transcripts, (7) LTR+Env Transcripts, (8) LTR+Env+Nef Transcripts.

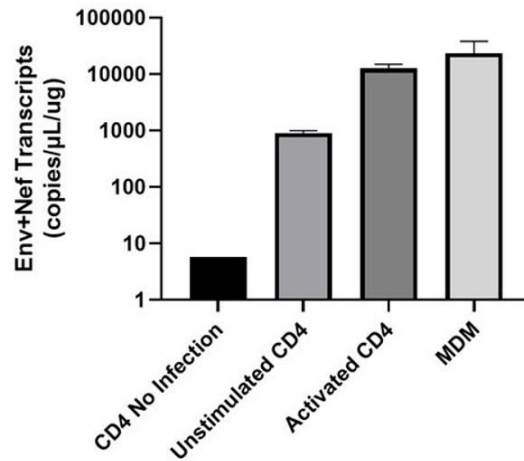
potential HIV-1 RNAs transcribed, we included nef-specific primers and probes. This probe used the same fluorophore as the LTR probe but was added at a half concentration to allow separation of signal based on amplitude (Maar D, Heredia NJ 2019). By

multiplexing the RT-ddPCR assay I was able to distinguish splice variants of canonical HIV-1 transcription initiated from the 5' LTR and HIV-1 transcripts that may not include 5'UTR sequences.



**Figure 3.5 CD4+ T cells and MDMs express aberrant HIV-1 RNAs.** (A) Multiplex RT-ddPCR was performed on RNA prepared from unstimulated and activated CD4+ T cells infected with HIV-1<sub>NL4-3</sub> by spinoculation for 90 minutes at an MOI of 0.02 and incubated for 72 hours prior to RNA isolation. HIV-1 infection was limited to a single round by addition of 10 μM saquinavir 30 minutes after spinoculation. Activated CD4+ T cells were cultured with anti-CD3/CD28 beads for 72 hours prior to spinoculation. Estimated copies/μL for transcripts were calculated with the QuantaSoft droplet reader software after manual gating of multiplex RT-ddPCR data (See Figure 3.4) and dilution factor correction. (B) Ratios of HIV 5'UTR defective transcripts relative to transcripts with intact 5'UTRs for resting and activated HIV infected CD4+ T cells. (C) Frequencies of detected HIV transcripts as a percentage of all transcripts quantified by multiplex RT-ddPCR for resting (top) and CD3/CD28 activated CD4+ T cells. (D) Multiplex RT-ddPCR performed with RNA from HIV-1<sub>NL43-BaL</sub> infected MDMs. MDMs were infected with HIV-1<sub>NL43-BaL</sub> at an MOI of 0.01 for 4 hours by adding virus directly to adherent cell cultures for 48 hours prior to RNA isolation. (E) Ratios of 5'UTR defective transcripts relative to transcripts with intact 5'UTR for HIV infected MDMs. (F) Frequencies of HIV-1 transcripts as a percentage of all transcripts quantified by multiplex RT-ddPCR for HIV infected MDMs. Data represent three independent experiments using cells from three donors. Resting and activated CD4+ T cell sample data are donor matched. Bars represent standard error of the mean. Significance values were generated using multiple unpaired Two-tailed T tests. \* denotes  $p < 0.05$ ; \*\* denotes  $p < 0.01$ , and \*\*\* denotes  $p < 0.001$ .

The multiplex RT-ddPCR identified two general sets of transcripts in CD4<sup>+</sup> T cells and MDMs; a set of RNAs that contained 5' UTR sequences and a set of RNAs that were positive for distal RNAs *env* and *nef* but lacked 5' UTRs (Figure 3.5). The relative levels of RNAs with and without 5' R-U5 sequence differed between the infected CD4<sup>+</sup> T cell populations with approximately 40% of RNAs in activated CD4<sup>+</sup> T cells lacking 5' UTRs compared to 66% of RNAs lacking 5' UTRs in resting cells (Figure 3.5A-C). These frequencies correlated with IPDA data which showed that resting CD4<sup>+</sup> T cells harbor more defective proviruses (Figure 3.2B). We also observed approximately 25% of RNAs in infected MDMs lacked 5' UTRs (Figure 3.5D-F). Using a poly d(T) primer instead of random hexamers for the RT reactions resulted in the amplification and detection of 5' UTR deficient transcripts in CD4<sup>+</sup> T cells and MDMs, indicating that a subset of these RNAs were polyadenylated (Figure 3.6). These data support that aberrant HIV transcripts are produced after acute HIV-1 infection, with their relative expression influenced by cell type and activation state.

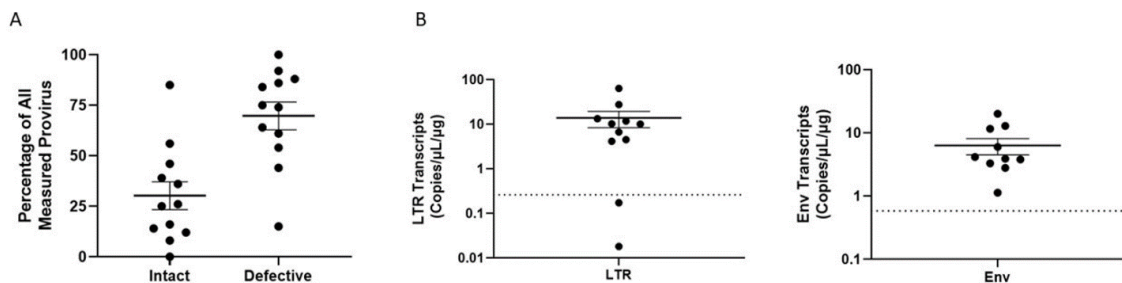


**Figure 3.6 Env+Nef HIV transcripts are polyadenylated.** RT was performed as described in Methods using a poly d(T) primer for reverse transcription of 500ng of RNA from either HIV-1<sub>NL4-3-BaL</sub> infected MDMs or HIV-1<sub>NL4-3</sub> infected CD4+ T cells which were unstimulated or activated for 72 hours using anti-CD3/CD28 beads. No Infection negative control sample is representative of N = 1. All remaining data are representative of three separate infections. CD4+ T cell data are donor matched between unstimulated and activated cells.

*Aberrant HIV-1 RNAs are detectable in a subset of chronically infected individuals on cART*

Chronically infected individuals on ART demonstrate a gradual decrease in intact HIV proviruses concurrent with selection and expansion of defective proviruses due to sustained immunological pressure and clearance (Anderson et al. 2020; Bruner et al. 2016; Pollack et al. 2017; Antar et al. 2020a). Notably, defective HIV proviruses are observed to decay at a slower rate than intact provirus clones, presumably due to their relatively low HIV-1 expression levels (Pinzone et al. 2019b; R. Liu, Catalano, and Ho 2021b; Antar et al. 2020b; MJ et al. 2020; SD et al. 2021). We posit that HIV-1 mRNA lacking 5' UTRs should be detected in people living with HIV (PLWH) on ART. Using DNA isolated from PBMCs of PLWH on ART (Table 2.5), we first confirmed the

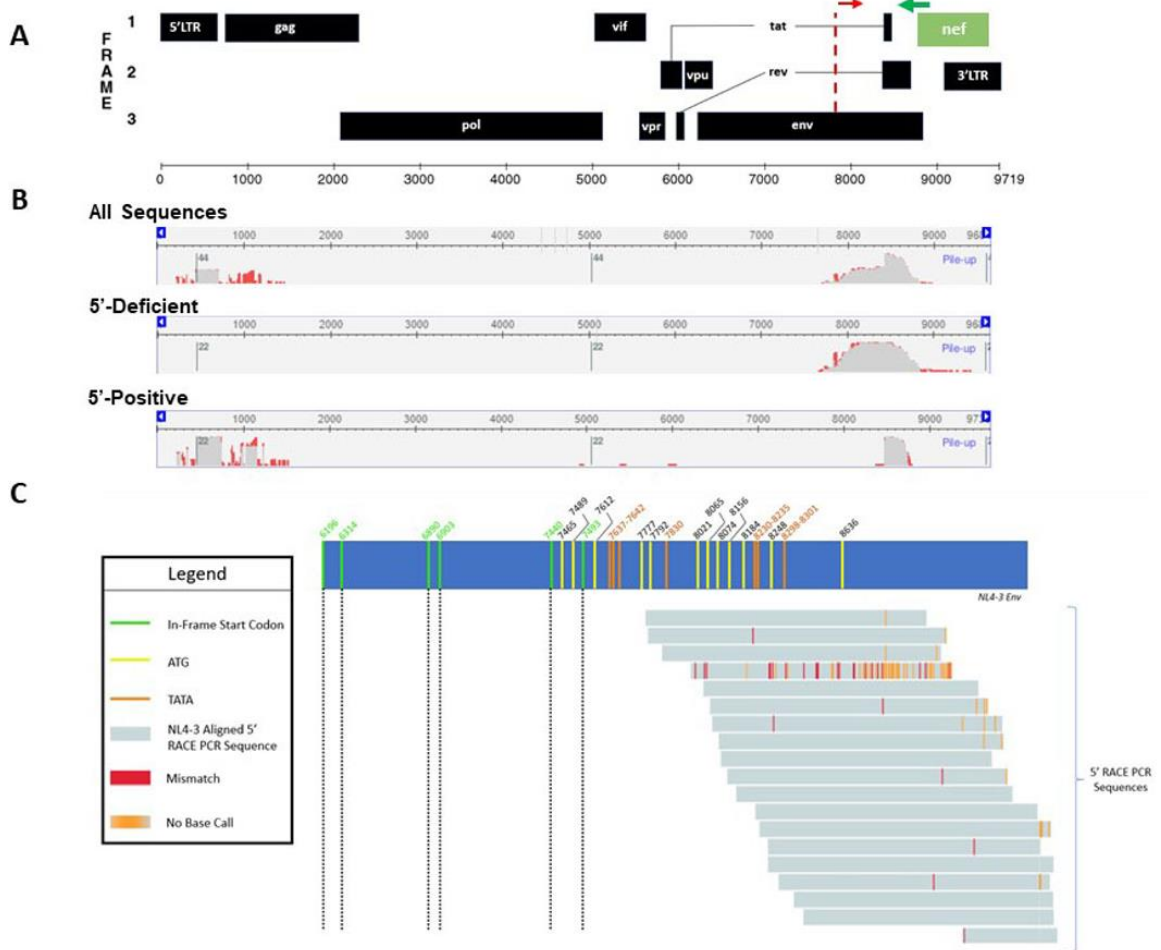
presence of HIV-1 proviruses in chronically infected individuals by IPDA with most proviruses detected in these individuals being defective (Figure 3.7A). To determine if aberrant RNAs that lack 5'-UTRs were also present in individuals chronically infected with HIV-1, multiplex RT-ddPCR was performed using RNA from PLWH donor PBMCs. Probes specific for R-U5 5' UTR sequences and *env* were used in tandem to determine the presence of one or both probe sites on HIV-1 transcripts expressed in HIV-positive donor PBMCs. Multiplex RT-ddPCR assays successfully detected HIV-1 RNAs with 5' UTR as well as *env* transcripts that lacked 5' UTRs start sites (Figure 3.7B). *Env* sequences lacking 5' UTRs were detected with signal above uninfected PBMC background controls in 10/11 RNA samples from HIV-positive PBMC donors (Figure 3.7B and Table 2.5). These data show that aberrant HIV transcripts are detected in a subset of individuals chronically infected with HIV even during ART, suggesting these RNAs are persistently expressed in PLWH.



**Figure 3.7 Detection of HIV-1 aberrant transcripts in PLWH on ART.** (A) Percentage of intact and defective HIV provirus detected in DNA samples from PBMCs of HIV-positive ART-treated individuals relative to all provirus quantified by IPDA (Olson et al. 2021). (B) Multiplex RT-ddPCR was performed to detect intact 5' UTR containing transcripts (LTR) and 5' UTR-deficient *env* transcripts (Env) using RNA samples prepared from PBMCs of HIV-positive ART-treated individuals. No RT enzyme control reactions were carried out for each assay and background signal was subtracted from experimental readings. Dots represent estimated copies/uL of single positive transcripts per μg of RNA. Dotted line represents the mean assay background signal calculated from five HIV-negative PBMC samples. Each point represents an independent participant sample. A total of 11 participant samples were assessed.

*Intragenic transcriptional elements and host cell transcription factors support expression of defective HIV-1 provirus*

To characterize transcriptional start sites of defective HIV transcripts, we performed 5' RACE PCR using RNA isolated from HIV infected activated CD4<sup>+</sup> T cells and MDMs. A universal forward primer and a gene-specific reverse primer for nef (Figure 3.8 and Methods) were used to amplify the populations of nef containing transcripts from infected cells. PCR products were separated by gel electrophoresis and ~1kb RACE PCR products were gel extracted, cloned into a pUC19-based vector, and 41 products were sequenced to map their 5' ends (Figure 3.8 and Table 2.9). MDM and CD4<sup>+</sup> T cell sequences demonstrated that 53.6% of the transcripts were splice products which initiated at the transcriptional start site within the 5' LTR sequence (Figure 3.8A-B). The remaining 46.3% of sequences initiated transcription within env, across a span of approximately 200 nucleotides located between +7760 to +8178 bp downstream of the



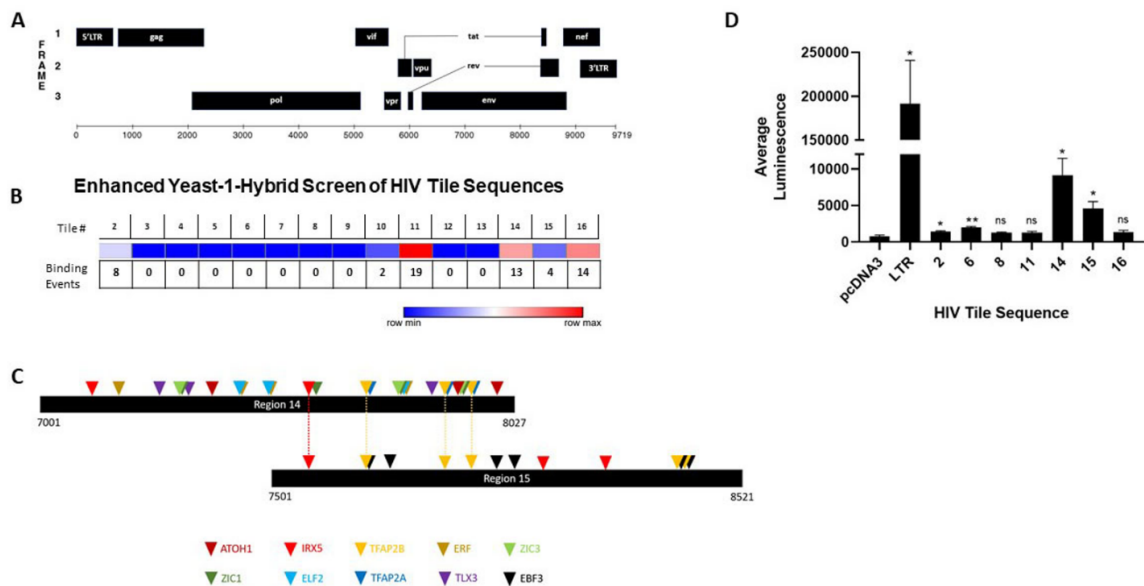
**Figure 3.8 Sequencing of 5' RACE PCR products identified HIV-1 transcripts with intragenic start sites.** (A) Schematic of HIV-1 genome with putative promoters indicated as dotted red lines as predicated by Promoter 2.0 and ElementNT V2(Knudsen 1999; Sloutskin et al. 2015). The green line represents the 5' RACE gene-specific Nef primer. (B) 5' RACE results analyzed using the NCBI Multiple Sequence Alignment Viewer. (Top) All 5' RACE sequences aligned to NL4-3 sequence (N = 41 sequences). (Middle) 5' RACE sequences which lacked 5' UTR aligned to NL4-3 sequence (N = 19). (Bottom) 5' RACE sequences which contained 5' UTR aligned to NL4-3 sequence (N = 22). (C) Schematic alignment of 5' RACE sequences lacking 5' UTR sequence (gray bars, N = 19) with the NL4-3 env gene (blue bar). Green and black dotted lines indicate in-frame start codons (numbered using GenBank: AF324493.2). Yellow lines represent out of frame start codons. Orange lines (top) represent TATA box sequences. Red lines represent sequence mismatches between 5' RACE reads and NL4-3 env. Orange lines (bottom) indicate that no nucleotide was assigned for that position during the sequencing reaction. Start codons and TATA box sequences were mapped and multiple sequence alignments were performed using Benchling Biology Software (<https://benchling.com>, 2021).

canonical 5' LTR transcriptional start site (Figure 3.8B-C and Table 2.9). These data support that transcription initiates from intragenic sites of the HIV genome.

Based on the RACE data, we used Promoter 2.0 and ElementNT V2 to predict if promoter characteristics were within the env region (Knudsen 1999; Sloutskin et al. 2015). These bioinformatic tools predicted a transcriptional start site (7800 bp, score 1.141) and TATA box motifs (7483–7488 bp and 7662–7669 bp) within the env sequence, adjacent to the start sites identified by 5' RACE (Figure 3.8 and Table 2.9). Alignment of 5' RACE PCR sequences to the NL4-3 env gene revealed a cluster of out-of-frame start codons in this region, suggesting that multiple alternative transcriptional start sites may be operative in the context of defective provirus (Figure 3.8C).

To survey direct binding of human transcription factors within the HIV proviral genome, we performed a functional enhanced yeast one-hybrid screen (eY1H) (Pedro et al. 2021; Fuxman Bass et al. 2015; Santoso et al. 2020). This method allows for high-throughput screening of host transcription factor binding events to the HIV-1 genome. In brief, HIV bait sequences were generated by tiling the HIV-1<sub>NL4-3</sub> genome into 17 overlapping fragments (Table 2.6). Fragments were cloned upstream of the two reporter genes, LacZ or HIS3. Cells were transformed with reporter constructs as well as a library representing 66% of the known human transcription factors fused to the yeast activation domain, Gal4. Transcription factor binding hits were identified as yeast colonies that grew in the absence of histidine and were blue when provided X-Gal. The eY1H screen detected 60 transcription factor-DNA interactions, representing 43 unique transcription factors which bound to six regions within the HIV-1 genome (designated 2, 10, 11, 14, 15 and 16) (Table 2.7 and Figure 3.9B). Transcription factor binding hot spots were

clustered within and near HIV env gene (Figure 3.9B-C), whereas, transcription factor binding was not detected in pol (Regions 3–9). Identified transcription factors included homeobox, basic helix-loop-helix, and zinc finger families (Table 2.7). The distribution of transcription factor binding hotspots near the HIV env gene correlated with transcriptional start sites suggested by the 5' RACE results.



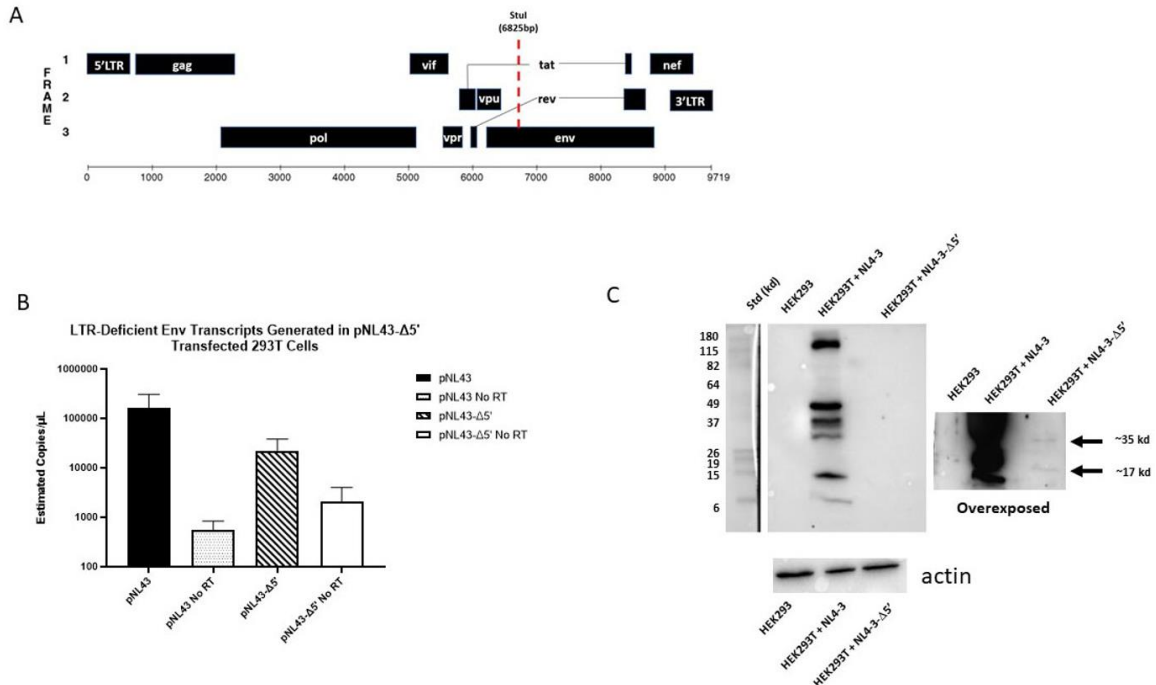
**Figure 3.9 Identification of intragenic transcriptional elements.** (A) Schematic of HIV-1 genome. (B) Heat map for transcription factor binding within the HIV-1 provirus. HIV-1 tile sequences (1–16) were used in yeast-1-hybrid screen (See Table 2.6). Dark blue represents regions lacking transcription factor binding whereas red indicates regions of transcription factor binding. Binding events represent the number of transcription factors that bound the specific HIV-1 genomic sequence. (C) Location of predicted binding sites for transcription factors identified by yeast-1 hybrid assay that bind baits 14 and 15. Motifs were identified using FIMO with a threshold set at  $p < 0.001$  ((C. E. Grant, Bailey, and Noble 2011) and Table 2.8). (D) Intragenic sequences enhance transcription. HIV-1 tile sequences were cloned upstream of a luciferase gene in pcDNA3.1 Luciferase and transfected in HEK-293T cells. Luciferase was measured and reported as fold-change in signal over cells transfected with the negative control pcDNA3.1-Luc plasmid. HIV-LTR-Luciferase was used as a positive control. These data include three independent transfections performed in triplicate. Error bars represent standard error of the mean for all values. Two-tailed unpaired T-tests were used to calculate statistical significance between raw luminescence values for HIV-1 tile sequences and negative control pcDNA3.1-Luc plasmid. \* denotes  $p < 0.05$ ; \*\* denotes  $p < 0.01$ , and \*\*\* denotes  $p < 0.001$ .

To investigate if these intragenic sequences that bound host transcription factors function as cis-regulatory elements, reporter vectors were generated with HIV-1 fragments cloned upstream of the luciferase reporter gene. Reporter plasmids were transiently transfected in HEK293T cells, and firefly luciferase was assessed 24 hours post-transfection (Figure 2.9D). Most of the intragenic sequences, including Regions 11 and 16, which bound several transcription factors in the eY1H assays, modestly influence transcription of the reporter gene. However, regions 14 and 15, which spanned sequences 7001 to 8521 bp in *env* and bound several transcription factors in the eY1H assays, exhibited 5–10 fold higher luciferase signal compared to pcDNA3 control. These sequences also spanned sites that included putative promoter elements and the RACE transcriptional start sites. The predicted transcription factor binding motifs generated using Finding Individual Motif Occurrences (FIMO) software for the sequences that span regions #14 and #15 are shown in Table 2.8 (C. E. Grant, Bailey, and Noble 2011).

*A truncated HIV-1 provirus construct expresses RNA and protein*

We generated a viral expression construct, pNL4-3Δ5' in which we deleted approximately 6,000 bp of the 5' end of the provirus, including the 5' LTR, gag and pol genes, and transfected this construct into HEK293T cells (Figure 3.10A). Transcription of *env* containing sequences was detected by RT-ddPCR a log-fold higher than the assay background (Figure 3.10B). Furthermore, 35 kd and 19 kd proteins were detected by immunoblotting when whole cell lysates from pNL4-3Δ5' transfected cells were probed with human HIV anti-serum suggesting that these RNAs are translated

(Figure 3.10C). It should be noted that immunoblots required longer exposure times to visualize the proteins expressed from pNL4-3 $\Delta$ 5', probably reflecting that these intragenic elements are much weaker than the HIV-1 5'-LTR which increased luciferase



**Figure 3.10 Truncated HIV-1 proviral construct expresses RNA and protein.** (A) Schematic of HIV-1 NL4-3 in pNL4-3 expression plasmid and location of *Stul*-restriction digest site used to generate pNL4-3 $\Delta$ 5' (bottom). (B) RT-ddPCR data from HEK-293T cells transfected with either full length pNL4-3 (black) or pNL4-3 $\Delta$ 5' (striped) for 24-hours. RNA samples were subjected to multiplex ddPCR using a probe for the 5'LTR and a probe for env and droplets were quantified as indicated above. Reaction mixtures without RT enzyme (No RT) were used as negative controls for contaminating plasmid DNA. No LTR signal was detected for HEK293T cells transfected with pNL4-3 $\Delta$ 5'. Data include 4 independent pNL4-3 transfections and three pNL4-3  $\Delta$ 5' transfections. (C) Western blots using cell lysate from HEK293T cells transfected with either full length pNL4-3 plasmid or pNL4-3 $\Delta$ 5'. Lysates were run on 12% SDS-PAGE gel, transferred to a PDVF membrane, and probed with human polyclonal anti-HIV IgG. Two exposures are shown: a short exposure demonstrating HIV-1 proteins (left) and an extended exposure which shows weaker HIV protein bands at approximately 35 kDa and 19 kDa, as indicated by the arrows (right). Non-transfected HEK293T cell lysates were also probed as a negative control. The lower panel is a separate gel from the above lysates probed with a monoclonal against  $\beta$ -actin antibody demonstrating the presence of proteins in the different lysates.

expression on the order of ~200-fold compared to pcDNA control in transfected

HEK293T cells (Figure 3.9D). Taken together, these data support that HIV-1 intragenic

sequences, including those within the env gene, act as cis-regulatory elements which support the transcription of cryptic RNAs from a subset of defective proviruses.

## Discussion

The reservoir of persistent HIV-1 proviruses is dynamic, being shaped over time by immune recognition and clearance of cells expressing HIV-1 proteins, as well as clonal expansion and homeostasis of memory T cell subsets that harbor HIV-1 proviruses (Bruner et al. 2016, 2019; Imamichi et al. 2016; Pinzone et al. 2019b; R, FR, and YC 2020). This model is supported by longitudinal tracking of the persistent HIV-1 proviruses in PLWH on ART and long-term controllers which demonstrated a relatively rapid decay of intact HIV-1 provirus compared to defective proviruses (R. Liu, Catalano, and Ho 2021a; Antar et al. 2020b; MJ et al. 2020; Jiang et al. 2020). These dynamics result in the progressive accumulation of defective HIV-1 proviral genomes which comprise the vast majority of the latent reservoir in chronically infected individuals that are undergoing treatment or are controllers of HIV infection (Pinzone et al. 2019b; R, FR, and YC 2020; Anderson et al. 2020; Z. Wang et al. 2018; R. Liu, Catalano, and Ho 2021a; Antar et al. 2020b; Jiang et al. 2020).

I hypothesized that certain cells may be predisposed to generating defective proviruses upon infection. It has been observed that quiescent and resting cell populations such as macrophages, resting CD4<sup>+</sup> cells and memory T cell subsets are difficult to infect and biased towards unproductive or latent infections (Pinzone and O'Doherty 2018; Agosto et al. 2007, 2018). I compared the fate of HIV-1 infections in macrophages,

unstimulated CD4<sup>+</sup> T cells, and CD4<sup>+</sup> T cells activated through the CD3/CD28 signaling axis. At the time of infection, macrophages and unstimulated CD4<sup>+</sup> T cells harbor more defective proviruses compared to activated CD4<sup>+</sup> T cells with up to 80% of the infected cells containing 5' deletions or mutations. These results support that upon acute infection, even in the absence of immune selection, a foundation of cells harboring defective viruses is rapidly established (Abrahams et al. 2019a; Bruner et al. 2016).

The adaptive immune system of PLWH on cART shapes the transcriptionally active persistent proviruses reservoir over time. This results in preferential clearance of persistent proviruses that express viral proteins. In agreement with this model, I confirmed that the majority of persistent proviruses detectable in the DNA of PBMC samples from PLWH on cART were defective using an IPDA. Multiplex RT-ddPCR detected HIV-1 transcripts from these same individuals which were positive for *env* sequence but lacked an associated 5'UTR sequence. This suggests that the shaping of the persistent provirus reservoir biases the reservoir towards transcriptionally silent HIV-1 proviruses but does not prevent residual expression of aberrant HIV-1 transcripts. In a model of HIV-1 RNA expression that includes 5'UTR-independent transcriptional mechanisms, aberrant HIV-1 transcripts may be spuriously expressed by the large defective HIV-1 provirus population. Expression of HIV-1 transcripts from defective HIV-1 proviruses generates questions about the host and viral factors supporting their expression, their translational competence, and possible impacts on immune function. Detection of HIV transcripts that do not contain 5'UTR sequence suggests that alternative

transcriptional mechanisms support the generation of RNA from defective HIV-1 proviruses.

Despite minimal detectable virus replication in PLWH undergoing anti-retroviral treatment, immune stimulation and inflammation persist, contributing to co-morbidities including neuroinflammation, cardiovascular disease, and signs of accelerated aging (Deeks, Tracy, and Douek 2013; Višković et al. 2018; Angel et al. 2021). Previous work has indicated that HIV-1 transcripts are produced by defective proviral clones and a subset of these transcripts can be translated into viral proteins that stimulate CD8<sup>+</sup> T cell activity (Pollack et al. 2017; Imamichi et al. 2020, 2016). Furthermore, cryptic HIV-1 peptides are produced by using alternative reading frames (ARFs) which are distributed throughout the HIV-1 genome. Previous studies have demonstrated that ARFs can be loaded into and presented by MHC-I to activate CD8<sup>+</sup> T cells, inducing cytokine release and killing activity (Champiat et al. 2012; Bansal et al. 2010b; Cardinaud et al. 2004). Taken together, these data support that defective persistent HIV proviruses influence and perpetuate immune responses against HIV. Our ability to detect HIV-1 protein from proviral sequences that lack intact 5' sequences suggest that defective viruses may provide an alternative mechanism to generate ARFs.

In addition, it has been demonstrated that partially spliced and unspliced HIV-1 RNAs are recognized by MAVS-dependent nucleic acid sensing pathways and mediate IFN-I responses (Akiyama et al. 2021; McCauley et al. 2018; Akiyama et al. 2018). The defective mRNA sequences generated from the intragenic elements that we have characterized initiate transcription within *env* and span the intron leading us to speculate

that these mRNAs could be proinflammatory. Importantly, we detect transcripts that are initiated from intragenic elements in samples from PLWH on ART. Overall, our study provides potential insight into the mechanisms and proviral elements which regulate transcription of these defective HIV-1 genomes and the potential of cryptic peptides that perpetuate HIV pathogenesis.

#### **CHAPTER 4: DISCUSSION AND FUTURE IMPLICATIONS**

Parts of this chapter were originally published in: Kuniholm J, Armstrong E, Bernabe B, Coote C, Berenson A, Patalano SD, Olson A, et al. Intragenic proviral elements support transcription of defective HIV-1 proviruses. PLOS Pathogens <https://doi.org/10.1371/journal.ppat.1009982>

Part of this writing originally submitted as part of a review article titled "Persistent HIV-1 Genomes and their Potential Impact on HIV Pathogenesis." for BMC Retrovirology, 2022.

#### **Summary of Results**

The results presented in this dissertation identify host and viral factors that support the expression of defective HIV proviruses. The first hypothesis tested whether intrinsic properties of infected cells and their associated activation state influence the proportion of intact or defective HIV-1 provirus establishment. To address this hypothesis, I compared intact and defective provirus prevalence in unstimulated resting primary human CD4<sup>+</sup> T cells to CD4<sup>+</sup> T cells activated through the CD3/CD28 signaling axis. An intact provirus detection assay (IPDA) revealed that resting CD4<sup>+</sup> T cells are biased towards establishing defective HIV-1 proviruses, whereas activated CD4<sup>+</sup> T cells are permissive to intact infection *in vitro*. I also used the IPDA to analyze the proportions

of intact and defective provirus established in human monocyte-derived macrophages (MDMs) after acute infection. Like resting CD4+ T cells, MDMs were skewed towards infection with mostly defective proviruses harboring defects in the psi packaging element at the 5' end of the genome, proximal to HIV's promoter or 5'LTR.

By modifying a previously described reverse transcription ddPCR (RT-ddPCR) protocol, I developed a multiplex assay for detecting 5' sequence-deficient HIV transcripts isolated from HIV-1 infected CD4+ T cells and MDMs. This assay identified a population of HIV-1 transcripts which expressed distal gene sequences but lacked 5' UTR sequences, suggesting that they were generated independent of an intact 5'LTR. Resting CD4+ T cells had a significantly higher proportion of these aberrant HIV-1 transcripts when compared to 5' intact transcripts supporting the conclusion that activation state alters the transcriptional mechanisms that regulate HIV-1 infected CD4+ T cells. I confirmed that the generation of these aberrant transcripts was not an artifact of acute *in vitro* infections by analyzing RNA samples from peripheral blood cells of people living with HIV on ART. I successfully detected 5'LTR-deficient *env* transcripts from these samples which were confirmed to harbor mostly defective HIV proviruses in the IPDA. These data support that aberrant HIV-1 transcripts are produced in PLWH on cART.

The detection of 5'UTR deficient HIV-1 transcripts led me to develop my second hypothesis, intragenic elements within the HIV-1 genome support transcription of defective proviruses. To determine what viral elements support transcription from defective proviruses, I mapped the transcriptional start sites of aberrant HIV-1 transcripts

detected in CD4<sup>+</sup> T cells and MDMs using 5' RACE PCR. Sequencing of 5' RACE PCR products identified a subset of aberrant HIV-1 transcripts initiated within a ~200 base pair region of the envelope gene. This region of the envelope gene contains many out-of-frame start codons, TATA-box sequence motifs, and is adjacent to a predicted promoter element. To directly interrogate the transcriptional activity of the putative transcriptional start site within the envelope gene and elsewhere in the genome, we generated a set of 17 HIV-1 tile sequences and cloned them upstream of a luciferase reporter. Transient transfection experiments with these sequences in 293T cells confirmed that the region within the envelope gene that aligned with aberrant HIV-1 transcripts mediated transcriptional activity. In collaboration with the Fuxman-Bass Lab, we performed a yeast-1-hybrid screen using HIV-1 tile sequences as bait for a library of human transcription factors conjugated to an inducible reporter system. The yeast-1-hybrid screen revealed enhanced binding of human transcription factors to the region of the envelope gene that generated aberrant HIV-1 RNAs. These data support that a combination of host factors binding an intragenic transcriptional element support the transcription of aberrant HIV-1 transcripts from proviruses with absent or defective 5'LTR sequences.

To determine if HIV-1 transcripts could be directly generated from defective proviruses lacking a 5'LTR, we generated HIV-1 expression construct that lacked 6,825 base pairs of 5' HIV-1 genome. Transient transfection of HEK293T cells and 2-color RT-ddPCR confirmed that *env* transcripts lacking 5'LTR sequence were generated in the absence of 5'LTR sequence from a putative intragenic promoter in the envelope gene.

Cell lysates from these transient transfections were analyzed by western blot using human HIV-1 antiserum confirming the translation of viral proteins. These data demonstrate that defective HIV-1 proviruses harboring large 5' deletions support the generation of HIV-1 transcripts and that are translated.

### **Defective Provirus Reservoir: Trash or Therapeutic Target?**

Development of an effective vaccine for HIV-1 will be essential for preventing the establishment of both intact and defective HIV-1 provirus populations in exposed individuals. In the absence of a vaccine, adherence to effective ART regimens limits viral pathogenesis and spread. Pre-exposure prophylactic use of cART (PrEP) has helped lower transmission rates in areas with adequate access to medical services (R. M. Grant et al. 2010; Koss et al. 2021). As access to cART improves globally, the population of individuals managing a chronic HIV-1 infection will increase, making the persistent provirus population in these individuals the final barrier to a sterilizing HIV-1 cure.

My work, and reports by others, support that the mutations and deletions observed among the large and diverse population of defective HIV-1 proviruses *in vivo* do not entirely prohibit the transcription of HIV-1 RNA or the translation of these mRNAs into proteins (Pollack et al. 2017; Imamichi et al. 2016, 2020). I have demonstrated that there are intragenic elements of the HIV-1 genome that support the transcription of HIV-1 genes independent of a functional 5'LTR promoter. This mechanism of defective provirus expression is distinct from those in previous studies which mainly identified proviruses with large internal deletions, hypermutations, or defective packaging element

sequence while maintaining a functional core promoter sequence of the 5' LTR. The results presented support that HIV-1 proviruses harboring deletions or mutations in the 5'LTR can still act as a source of viral RNAs.

The factors governing the establishment, maintenance, and reactivation of intact latent HIV-1 proviruses remain incompletely understood despite much focus on the subject. The heterogeneity of latent provirus reactivation seen after maximal *in vitro* stimulation with LRAs supports the idea that the latent reservoir is seeded at different depths (Ho et al. 2013). There is a lack of consensus regarding what factors predict whether a provirus is in a state of deep-seeded latency. This has led many groups to suggest that the dynamics of latent provirus reactivation is stochastic and influenced by a combination of many interacting factors that makes predicting the likelihood of reactivation using biomarkers difficult or impossible (Ho et al. 2013; Hill et al. 2014; Singh et al. 2010; Conway and Coombs 2011). For potential cure strategies, a stochastic model of latency reversal supports that all intact persistent proviruses must be targeted for eradication regardless of the depth of their latent state to eliminate the possibility of future viral reactivation.

Whether defective proviruses are subject to the same processes of latency establishment and reactivation as intact proviruses is unknown. In a model of stochastic defective provirus reactivation, aberrant RNAs would be expressed over time through both 5'LTR-driven transcription and, based on the data presented in this dissertation, mechanisms independent of the 5'LTR. Host transcription factor levels and activity vary among different cell types and activation states. Transcription factor dynamics influence

the establishment, maintenance, and reactivation of HIV-1 latency. Accordingly, the prevalence and activity of the human transcription factors we mapped to the transcriptionally active intragenic region of the HIV-1 Env gene may depend on cell type, activation state, and cytokine microenvironment of the infected cell. These influences may be dynamic, changing over time to influence the activity of alternative HIV-1 transcriptional mechanisms. Further study of these transcription factors in cell types that perpetuate inflammation and are associated with organ dysfunction in PLWH on cART will be useful in determining their potential roles in alternative HIV-1 transcription and immune dysfunction.

The potential for proviruses with repressed 5' LTR sequences to use alternative transcriptional mechanisms is not directly addressed in this dissertation but represents another potential source of aberrant HIV-1 transcripts. We detected viral RNAs expressed using intragenic elements of the HIV-1 genome at substantially lower concentrations than RNA expression driven by the 5'LTR. However, the defective provirus population comprises >90% of the total latent HIV-1 reservoir in PLWH on cART. Many of the aberrant HIV-1 RNAs we sequenced after mapping their transcriptional start sites by 5'RACE PCR indicated that they include intronic sequences which have been shown to act as ligands for intracellular PRRs to perpetuate proinflammatory signaling (McCauley et al. 2018; Akiyama et al. 2021, 2018). Thus, detection of aberrant HIV-1 RNAs by innate sensors could occur chronically, with subsets of the defective provirus reservoir exhibiting low HIV-1 transcriptional activity over time. Stochastic expression of HIV-1 RNAs from the mostly defective persistent HIV-1 provirus reservoir, even at a low level,

represents a potential mechanism for perpetuating proinflammatory signaling among defectively infected cells and bystander cells. Importantly, this process is unhindered by cART which does not target mechanisms of HIV-1 transcription.

### **Clinical Implications of Defective HIV-1 Provirus Expression**

Based on the data presented in this dissertation and the prevalence of defective HIV-1 proviruses in the persistent reservoir of infected individuals on therapy, I propose that residual expression of defective proviruses contributes to chronic inflammation in PLWH on ART. Short intracellular viral RNAs have been detected in CD4<sup>+</sup> T cells of PLWH on cART and correlate with CD8<sup>+</sup> T cell immune activation and exhaustion markers (Ishizaka et al. 2016). Likewise, low levels of cell-associated and cell-free viral RNA have been detected in studies of PLWH on cART for years (Zhang et al. 2008; M. Fischer et al. 2004; Dornadula et al. 1999). These observations highlight the inability of cART to prevent viral transcription and support the hypothesis that residual transcription correlates with chronic immune activation.

The ability of intact proviruses to support viral rebound from a small amount of identical provirus clones located in diverse anatomical niches indicates that transcriptionally competent HIV-1 proviruses are seeded throughout the body and persist during cART (De Scheerder et al. 2019). Immune clearance of reactivated HIV-1 proviruses limits the size of the intact provirus reservoir and is sustained during cART. As a result of this selection, defective HIV-1 proviruses expand over time to comprise the majority of the proviral landscape (Pollack et al. 2017). The data presented in this

dissertation demonstrate that defective HIV-1 proviruses are established more frequently in myeloid cell types and resting CD4<sup>+</sup> T cells than activated CD4<sup>+</sup> T cells. Microglial cells of the brain have been implicated as perpetuators of inflammation which is fueled by the recognition of HIV-1 transcripts (Ko et al. 2019; Akiyama et al. 2021; Castellano, Prevedel, and Eugenin 2017). Likewise, tissue resident macrophages have been identified as a source of HIV-1 RNA in various organs of PLWH on cART (Cribbs et al. 2015; Damouche et al. 2015; Ganor et al. 2019). The prevalence of defective proviruses among the various anatomical niches known to harbor intact HIV-1 provirus remains an important unexplored area of research. Given the predominance of defective HIV-1 proviruses in the peripheral blood of PLWH on cART, I would argue that the majority of proviruses in various anatomical niches are also likely defective. Alternative transcriptional processes, such as those described in this dissertation, make defective proviruses in these anatomical niches potential contributors to residual HIV-1 transcription and immune activation in chronically infected individuals on cART. The extent to which aberrant HIV-1 transcripts perpetuate inflammatory signaling and potentially drive the end-organ diseases reported in people managing a chronic HIV-1 infection remains unclear but may have important implications for the improvement of the health burden experienced by this population.

Cure strategies aimed at silencing the latent HIV-1 provirus reservoir through “block-and-lock” or gene editing may need to be expanded to account for the alternative transcriptional mechanisms used by defective HIV-1 proviruses. Based on the data presented here, single target inactivation of the 5’LTR will not be sufficient to completely

repress the expression of HIV-1 RNAs. While LTR-driven transcription remains the most important target for provirus repression, inclusion of alternative transcriptional start sites and regulatory sequences could improve the effectiveness of these approaches at alleviating chronic inflammatory signaling. Alternatively, for “shock-and-kill” therapies, more information is needed to determine the effects of LRAs on the defective provirus population. Immunological clearance of cells harboring intact proviruses is made possible by the recognition of viral proteins presented by the host cell. In the case of defective proviruses, reactivation could result in the generation of aberrant HIV-1 RNAs which are not competent for translation but stimulate viral RNA sensors, thereby fueling proinflammatory signaling. Alternatively, translation of aberrant transcripts may generate cryptic HIV-1 proteins and peptides that subvert the adaptive immune response by presenting proteins generated from alternative reading frames or spanning deletion junctions. Transcriptionally and translationally active defective HIV-1 proviruses complicate cure strategies aimed at repressing or eradicating the persistent HIV-1 provirus reservoir. The mechanisms described in this dissertation identify potential targets for therapeutic strategies that seek to limit the effects of alternative HIV-1 provirus expression from the mostly defective provirus population established in PLWH on cART.

## **Study Limitations and Remaining Mechanistic Questions**

The studies presented in this dissertation identify host and viral factors that support the expression of defective HIV-1 proviruses but are limited in their descriptions of individual provirus clones, transcriptional mechanisms, and use of cells from PLWH on cART. We observed a higher prevalence of defective HIV-1 provirus establishment in MDMs and resting CD4+ T cells. These cells also harbored higher proportions of 5'LTR-deficient transcripts. However, gaining insights into how defective proviruses are expressed will require methods that allow us to generate and manipulate established provirus genomes. Future studies will take advantage of engineered Jurkat and THP-1 cell lines stably expressing cas9 protein. By infecting these cells with HIV-1, transfecting with 5'LTR-targeting guide RNA sequences, and subcloning to isolate a genetically pure monoculture, the Henderson Lab will generate clonal defective HIV-1 provirus cell lines. These cell lines will allow the unambiguous characterization of HIV-1 transcripts produced from proviruses lacking a functional 5'LTR core promoter. Furthermore, a defective provirus cell line model will enable the characterization of the transcription factor complexes that enable transcription to initiate from intragenic sites of the HIV-1 genome. Co-immunoprecipitation and chromatin immunoprecipitation experiments targeting the human transcription factors that bound the transcriptionally active intragenic site of the HIV-1 genome will allow more complete characterization of the regulation of gene expression from this region.

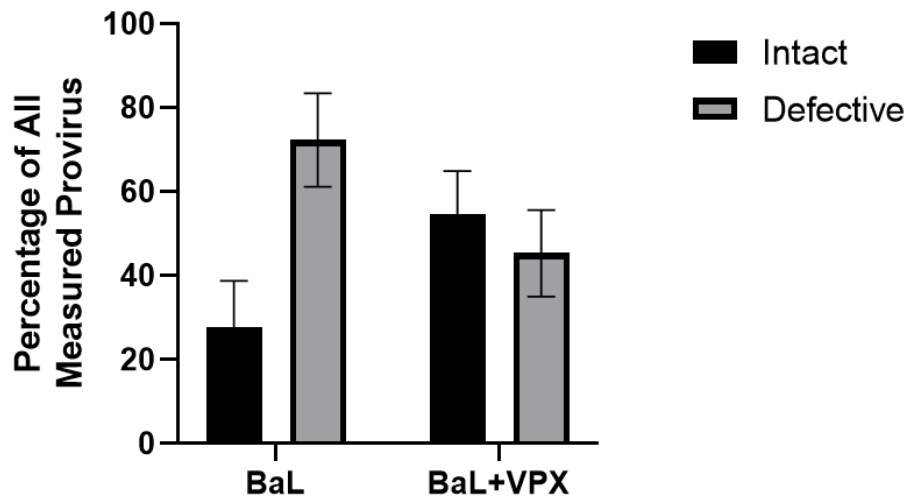
Our studies demonstrated that aberrant HIV-1 transcripts containing intronic sequence are generated by defective HIV-1 proviruses in CD4+ T cells and MDMs. Intron-containing HIV-1 transcripts have been demonstrated to perpetuate proinflammatory signaling and cytokine production in *in vitro* experiments. Demonstrating an immunostimulatory role of the aberrant transcripts identified in this dissertation are still required, and it is possible that the specific intronic sequences identified from these studies are not sufficient to initiate inflammatory signaling by intracellular PRRs. Likewise, we have demonstrated that HIV-1 human antiserum binds to proteins generated from a 5'LTR-deficient HIV-1 expression construct. However, identification of these proteins will require more high-powered techniques like mass spectrometry to confirm that they are HIV-1 proteins encoded by the aberrant HIV-1 transcripts detected in the same cells.

Most of the experiments performed in this dissertation used acutely *in vitro* infected primary cells. The landscape of defective proviruses established during the acute *in vitro* infection model will likely differ from the landscape which is established in PLWH on cART and shaped by the immune system over time. Experiments using infected cells from PLWH on cART will be most useful for determining the prevalence of different defective provirus subsets and their ability to generate aberrant viral RNAs *in vivo* where they are the most immunologically relevant. Studying a cohort of HIV-1 positive participants that report experiencing co-morbidities associated with chronic immune dysregulation and inflammation would be useful for focusing studies on proviruses and transcripts detected in individuals where these phenomena are actively

taking place. Participant-matched serum, cells, and DNA/RNA from these participants would allow correlation of different defective proviruses and aberrant transcripts with serum inflammatory cytokines, CD4<sup>+</sup>/CD8<sup>+</sup> T cell counts, and the prevalence of T cell exhaustion markers. Synthesis of the proteins encoded by the aberrant HIV-1 transcripts identified in our studies could be tested on HIV-experienced CD8<sup>+</sup> T cells from PLWH on cART to determine if these cells previously encountered these cryptic proteins *in vivo*. As an alternative approach, the Henderson Lab plans to study induced pluripotent stem cells that are differentiated into a microglia-like phenotype. Using this approach will allow characterization of the provirus and transcriptional landscape in cells most relevant to HIV-1 related comorbidities in human-derived tissues that can be difficult to sample.

## APPENDIX 1: IPDA OF HIV-1 INFECTED MACROPHAGES AFTER TREATMENT WITH VPX-CONTAINING VIRUS LIKE PARTICLES

Macrophages were treated with SIV3-derived virus like particles containing VPX 30 minutes before being infected with HIV-1NL4-3 BaL as described in Materials and Methods. DNA was collected from infected macrophage cell cultures 48-hours post-infection and used in an IPDA. Macrophages treated with VPX-containing virus-like particles harbored a higher percentage of intact HIV-1 provirus when quantified by IPDA. These data suggest that SAMHD1 activity promotes the generation of defective HIV-1 proviruses in macrophages.



**Figure A1. VPX treatment of macrophages during HIV-1 infection promotes intact provirus generation.** IPDA results for MDMs infected with either HIV-1<sub>NL4-3</sub> BaL alone (BaL) or with VPX virus-like particle pre-treatment (BaL+VPX). Provirus quantification is represented as a percentage of all proviruses detected for each sample. Data is representative of N=3 separate HIV-naïve blood donors.

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**CURRICULUM VITAE**

