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A CRISPR-screening approach to increase HIV latency reversal to improve an HIV cure strategy

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Abstract

A CRISPR-screening approach to increase HIV latency reversal to improve an HIV cure strategy

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Despite the availability of effective antivirals, HIV remains a global burden. Although virus replication can be suppressed, people living with HIV (PLWH) need to continue life-long antiviral therapy as there is currently no way to eradicate the long-lived and stable HIV reservoir that evades the immune system but retains the ability to reactivate and spread virus. Understanding mechanisms that govern HIV latency within these reservoirs, such as host and viral factors that influence transcription initiation, transcription elongation, and chromatin dynamics, has led to the discovery of a number of small molecule drugs called latency reversal agents (LRAs) capable of stimulating HIV transcription. One strategy being explored to target the HIV reservoir is called "shock-and-kill" where the silent provirus in latently infected cells is transcriptionally stimulated such that the immune system can recognize the subsequently synthesized viral proteins and clear infected cells. However, LRAs used to date in this strategy have not been potent or specific enough on their own to sufficiently activate and lead to clearing of infected cells. While pairing together LRAs that function through differing mechanisms of action have proven synergistic in their ability to activate HIV transcripts, these combinations in relevant model systems, such as *ex vivo* systems from cells from people living with HIV (PLWH), have

demonstrated there are more blocks to latency reversal present in primary cells that prevent full virus reactivation. These results highlight a need to find ways to uncover these blocks and improve reactivation.

In my thesis, I used a CRISPR-based screening approach with the LRA combination AZD5582 and I-BET151. This combination synergizes on HIV RNA synthesis by targeting the noncanonical NF-kB pathway to increase transcription initiation and targeting BET proteins to increase available cellular PTEF-b to aid in HIV specific transcription elongation in conjunction with HIV Tat. This combination was effective in in vitro models, but not in an *ex vivo* system. With this screening approach, I was able to identify a gene target, INTS12, that is a part of a larger complex called the Integrator Complex that could be targeted to improve reactivation in an *ex vivo* system. These studies provide a framework for predicting *in vivo* blocks to HIV latency reversal and strategically improving LRAs and identifying novel targets for which small molecule drugs should be developed.

Table of Contents

CHAPTER 1. INTRODUCTION 1

HIV OVERVIEW 1
FIGURE 1.1. SCHEMATIC OF THE HIV LIFECYCLE. 4
HIV LATENCY AND MAINTENANCE 4
FIGURE 1.2. OVERVIEW OF VIRAL LEVELS THROUGHOUT HIV INFECTION AND TREATMENT. 5
FIGURE 1.3. MODEL OF LATENCY ESTABLISHMENT. 6
FIGURE 1.4. LATENT RESERVOIR MAINTENANCE. 9
CONTROL OF HIV TRANSCRIPTION 10
FIGURE 1.5. SCHEMATIC OF THE HIV-1 LTR. 11
FIGURE 1.6. TRANSCRIPTIONAL CHANGES UPON LATENCY REVERSAL. 15
HIV CURE STRATEGIES 15
FIGURE 1.7: OVERVIEW OF PATHWAYS THAT LRAS TARGET. 20
DISSERTATION OVERVIEW 21

CHAPTER 2. INTEGRATOR COMPLEX SUBUNIT 12 KNOCKOUT OVERCOMES A TRANSCRIPTIONAL BLOCK TO HIV LATENCY REVERSAL 23

ABSTRACT 23
INTRODUCTION 24
RESULTS 27
LATENCY HIV-CRISPR SCREEN IDENTIFIES GENE TARGETS THAT INCREASE HIV LATENCY REVERSAL WITH AZD5582 & I-BET151 TREATMENT 27
FIGURE 1: A SCREEN TO PREDICT GENE KNOCKOUTS THAT WILL IMPROVE HIV REACTIVATION FROM THE COMBINATION OF AZD5582 & I-BET151. 28
INTEGRATOR COMPLEX SUBUNIT 12 KNOCKOUT SPECIFICALLY REACTIVATES HIV INDEPENDENTLY AND IN COMBINATION WITH AZD5582 & I-BET151 TREATMENT 30
FIGURE 2: VALIDATION OF INTS12 KNOCKOUT IN HIV LATENCY REVERSAL BOTH ON ITS OWN AND IN THE PRESENCE OF AZD5582 & I-BET151. 31
SUPPLEMENTAL FIGURE S1: ADDITIONAL VALIDATION OF INTS12 KNOCKOUT IN HIV LATENCY REVERSAL BOTH ON ITS OWN AND IN THE PRESENCE OF AZD5582 & I-BET151. 32
SUPPLEMENTAL FIGURE S2: FLOW CYTOMETRY VALIDATION OF INTS12 KNOCKOUT IN HIV LATENCY REVERSAL BOTH ON ITS OWN AND IN THE PRESENCE OF AZD5582 & I-BET151. 33
INTS12 KNOCKOUT INCREASES HIV STEADY-STATE RNA LEVELS WITH SPECIFICITY FOR THE LTR 33
FIGURE 3: INTS12KO REACTIVATES HIV ALONE AND IN COMBINATION WITH AZD5582 & I-BET151 AT THE LEVEL OF STEADY-STATE VIRAL TRANSCRIPTS. 34
FIGURE 4: SPECIFICITY OF INTS12KO FOR REACTIVATION OF HIV ON ITS OWN AND IN COMBINATION WITH AZD5582 & I-BET151. 36
CHROMATIN LOCALIZATION OF INTS12 AND RNAPII ACROSS THE INTEGRATED HIV PROVIRUS 37
FIGURE 5: INTS12 BINDS THE HIV PROMOTER AND KO WITH LRAS INCREASE OCCUPANCY OF RNAPII THROUGH THE PROVIRUS. 38
SUPPLEMENTAL FIGURE S3: INTS12 LOCALIZATION WITH A SECOND ANTIBODY. 39
INTS12 KNOCKOUT IMPROVES LATENCY REVERSAL EX VIVO IN T CELLS FROM VIRALLY SUPPRESSED PEOPLE LIVING WITH HIV (PLWH) 40

Figure 6: INTS12 knockout in an *ex vivo* primary cell system shows reactivation from knockout alone and in combination with AZD5582 & I-BET151. 42
Role of additional members of the Integrator complex in HIV latency reversal 42
Figure 7: HIV-CRISPR screen with a library that includes Integrator complex members. 43
Discussion 44
The role of Integrator complex in modulating HIV transcription 45
Ex vivo Primary Cell system 46
Synergy on multiple mechanisms 47
Methods 49

CHAPTER 3: PERSPECTIVES AND FUTURE DIRECTIONS 64

Summary 64
Figure 3.1. Model of the Integrator complex and AZD5582 & I-BET151 treatment. 66
Figure 3.2. INTS12 knockout with AZD5582 & I-BET151 treatment increases RNAPII elongation. 68
Integrator complex recruitment to the HIV LTR 70
Figure 3.3. Overview of how the Integrator complex members interact. 71
Integrator and Latency Establishment 73
CRISPR screen future directions 74
Integrator and the shock-and-kill approach as a whole 74
Bibliography 76

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Chapter 1. Introduction

There are currently about 40 million people in the world living with HIV (WHO, 2024). Untreated HIV leads to fatal opportunistic infections due to an immunocompromised state (Bekker et al., 2023) and lifelong infection that can be passed to infants during birth, expanding the burden across generations (Violari et al., 2008). Fortunately, combination antiretroviral therapy (cART) blocks the virus at various steps of the viral lifecycle (Jilek et al., 2012; Menendez-Arias & Delgado, 2022) allowing people living with HIV (PLWH) to live full lives and prevents transmission of the virus to others (Edwards et al., 2022; Palmisano & Vella, 2011). However, this treatment is not available in all parts of the world (WHO, 2024), and even in areas where it is available, people with HIV will need to adhere to treatment lifelong. Moreover, as the virus exists within a stable, latent reservoir that is largely invisible to the immune system and capable of reactivating spontaneously (Chun et al., 1999; Davey et al., 1999), HIV infection is not cured by antiretroviral therapy. Thus, the latent HIV reservoir is a major barrier that needs to be addressed to develop an HIV cure (Chun & Fauci, 1999), and consequently, HIV remains a global burden. This thesis explores improving a strategy to target this reservoir called shock-and-kill and uncovers a factor influencing HIV reactivation, the Integrator complex, that has potential to become a drug target for effecting better reservoir clearance.

HIV Overview

Human immunodeficiency virus 1 (HIV-1) is a lentivirus in the *Retroviridae* family that was discovered in 1983 when it was isolated from a person with lymphadenopathy at risk for AIDS (Barre-Sinoussi et al., 1983). Subsequently, this virus was linked to the rising acquired fatal immunodeficiency syndrome in cases around the world. HIV is transmitted by infected bodily

fluids such as blood or sexual fluids, blood transfusions, and newborns were at risk of contracting the virus during birth and breast-feeding from HIV-infected mothers (Garcia-Broncano et al., 2019; Violari et al., 2008). HIV infection progresses through an acute stage where there is massive depletion of CD4-positive cells in the gut and a dramatic rise in virus levels, followed by a lower setpoint of virus that gradually increases over many years, eventually leading to a generalized loss of immune functions and subsequently to the fatal acquired immunodeficiency syndrome (AIDS) (Mellors et al., 1996). It was through basic biology research that uncovered the steps of the HIV lifecycle which allowed for development of antivirals that now lead to viral control (although not viral elimination).

The HIV lifecycle (**Figure 1.1**) begins with HIV binding to the host cell CD4 receptor using its envelope protein gp120 on the virion surface followed by engagement of a co-receptor, CCR5 or CXCR4 (Hokello et al., 2024). Next the virus undergoes a conformational change in the envelop transmembrane protein gp41 upon binding to allow for fusion of the viral membrane with the host membrane allowing the virus to enter the cell. Through the primary receptor, CD4, HIV infects CD4+ T cells as well as macrophages, although the exact subsets of cells infected also depends on the co-receptor, CCR5 (and to the lesser extent CXCR4) (Alkhatib et al., 1996; Feng et al., 1996; Ferreira et al., 2024; Lederman et al., 2006). The process of fusion releases the viral core, containing the 2 strands of viral RNA and other viral proteins including the processed Gag proteins (MA, CA, and NC), as well as the processed Pol proteins (PR, RT, and IN) and the accessory protein Vpr into the cytoplasm (Hokello et al., 2024). Once the core is in the cytoplasm, viral RNA is converted to a double-stranded DNA copy of HIV using HIV reverse transcriptase (RT), as the viral complex of proteins and RNA is shuttled into the nucleus and the core is uncoated (Burdick et al., 2024; Hokello et al., 2024). After reverse transcription and uncoating are completed in the nucleus (Dharan et al., 2020), the viral DNA is irreversibly integrated into the host genome using the HIV integrase (IN) protein. HIV is known to preferentially integrate into sites of active transcription (Han et al., 2004) through its association

with cleavage and polyadenylation specificity factor 6 (CPSF6) that shuttles the pre-integration complex, containing viral DNA and integrase, to sites of euchromatin near the periphery of the nuclear pore (Sowd et al., 2016), and lens epithelium-derived growth factor (LEDGF/p75) directing the pre-integration complex to integrate within gene bodies at these active sites (Ciuffi et al., 2005). After viral integration, productive infections involve transcription of the viral RNA using both host machinery and the viral protein HIV Tat, which allows for translation of RNA and generation of viral proteins (Chun et al., 1995; Kao et al., 1987; Karn, 2011). HIV virions are then assembled at the cell membrane and immature virions bud from the cell. Lastly, HIV protease processes polypeptides within the virion to produce the mature HIV virion (Hokello et al., 2024).

By understanding these different steps of the lifecycle, targets for antivirals were identified that could be used to interfere with the viral lifecycle reviewed in (Sever et al., 2024). The first of these were inhibitors of reverse transcriptase itself, followed by inhibitors to the viral protease. Subsequently, other inhibitors that target key steps, in particular, inhibitors to IN. More recently, capsid binders target and prevent uncoating of the core to prevent adequate shuttling of viral DNA to the nucleus which prevent subsequent integration steps (Bester et al., 2020). While all of these antivirals work to some degree on their own, they are best taken in combination to effectively block HIV, and this treatment is called combination anti-retroviral therapy (cART) (Perelson et al., 1997). On the other hand, there are no approved drugs to target the integrated provirus itself, therefore once integration occurs there is currently no way to get the virus out of the cell (Rasmussen et al., 2022). For this reason, cART is not curative and people living with HIV will have to take antivirals for the rest of their life (Chun et al., 1999; Davey et al., 1999).

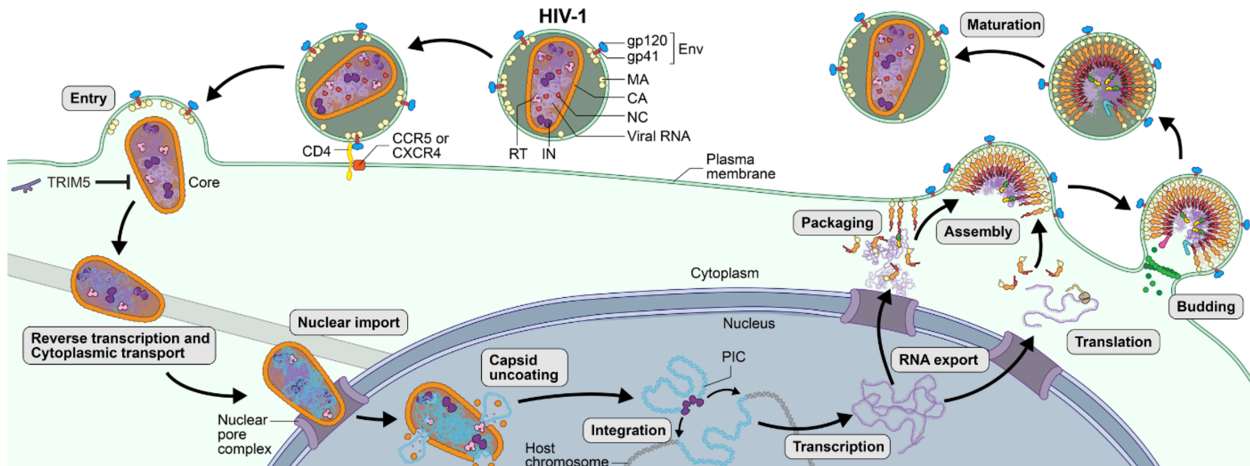


Figure 1.1. Schematic of the HIV lifecycle.
Steps of the lifecycle are shown in grey boxes. HIV enters the cell via the CD4 receptor and co-receptor where it fuses with the membrane. The viral core is trafficked to the nucleus where reverse transcription begins and after being imported into the nucleus uncoating and reverse transcription are completed. After nuclear import, the virus can integrate into the host genome. The virus then uses host machinery as well as the viral Tat protein to generate genomic and subgenomic RNAs that can be translated into viral proteins or packaged into virions. Virions assemble at the plasma membrane, bud off from the cell, and are matured by protease cleavage of the polyproteins to form infectious HIV virions. This figure is from (Iwasa, 2024), used under Creative Commons CC-BY 4.0 license.

HIV Latency and maintenance

HIV persists long-term by entering a state of latency that is undetectable to the immune system and by infecting long-lived CD4+ memory T cells that are maintained throughout a lifetime. During HIV infection, many of the cells infected actively produce HIV virions and die due to cytotoxicity of viral antigens, which explains the rapid CD4+ T cell depletion seen upon infection (Chun et al., 1999; Mbonye & Karn, 2024; Wong et al., 1997). However, a small minority of infected cells are able to enter a state of latency that is characterized by a transcriptionally silenced state (Margolis et al., 2020). Thus, after integration, viral antigens are not produced and viral DNA is not detected, allowing HIV to effectively evade the immune system (Hermankova et al., 2003). Despite this transcriptional silencing, the integrated provirus can spontaneously reactivate due to T cell activation via antigen exposure, viral or other, which can allow for HIV levels to rebound and infect new cells (Chun et al., 1999; Davey et al., 1999).

For this reason, people with HIV must remain on lifelong cART to prevent viral rebound (Figure 2).

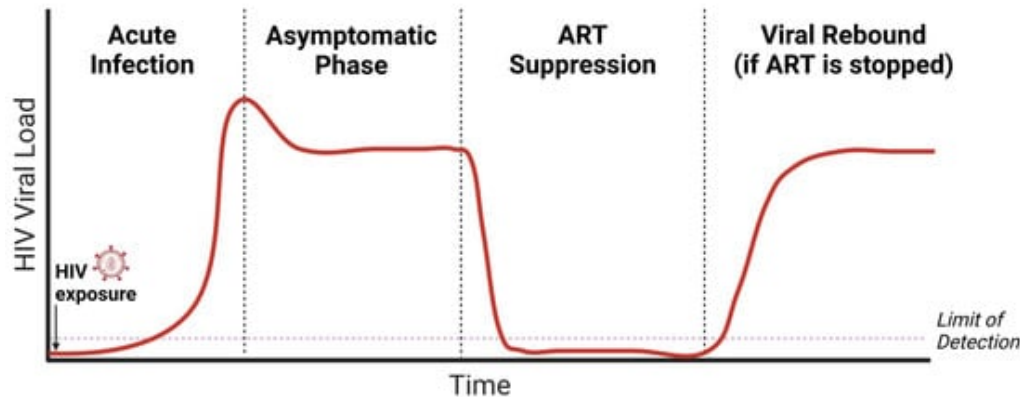


Figure 1.2. Overview of viral levels throughout HIV infection and treatment.
Acute infection is characterized by an abrupt increase in viral levels. The asymptomatic phase follows in the weeks after initial infection where infected cells producing virus are dying and depleting CD4+ T cells. This decline will continue and eventually lead to AIDS if untreated. ART suppression shows that virus levels drop dramatically and demonstrates why HIV is not transmittable in this state. However, the viral rebound phase shows what happens, in as little as 2 weeks, after stopping ART where virus levels rebound and CD4+ T cells continue their decline. Figure 1.2 was reproduced from (Chou et al., 2024) with permission, Creative Commons Attribution License 4.0 International (CC BY 4.0).

CD4+ memory T cells naturally possess a resting or quiescent state of transcription that is conducive to conferring HIV latency and these cells form the majority of the latent HIV reservoir (Chun et al., 1997; Finzi et al., 1999; Finzi et al., 1997; Josefsson et al., 2013). HIV latency is thought to be established in CD4+ T cells though basic T cell biology (Chomont et al., 2009), i.e.: the naïve CD4+ T cell is made in the thymus and can begin circulating in the body, once exposed to an antigen the CD4+ T cell becomes activated and transitions to an effector T cell that can expand to mount an immune response against the antigen, and lastly, a subset of these activated effector CD4+ T cells will enter the resting state once more and become central memory CD4+ T cells that will confer protection against the antigen upon re-exposure (reviewed in (Bekker et al., 2023). Thus, studies suggest the avenue for latency establishment involves

infection of activated CD4+ T cells and subsequent latency upon the cell transitioning to the resting state reviewed in (Sengupta & Siliciano, 2018) and shown in **Figure 1.3**. In favor of the this hypothesis, CD4+ T cells that are in the process of reverting to a resting memory state are the most permissive because in addition to higher dNTPs and CCR5 upregulation, these cells are also beginning to ramp down transcription, which can help bring the virus into latency (Shan et al., 2017). However, some studies have also suggested that the latent reservoir can be formed by infecting resting CD4+ memory T cells directly (Chavez et al., 2015).

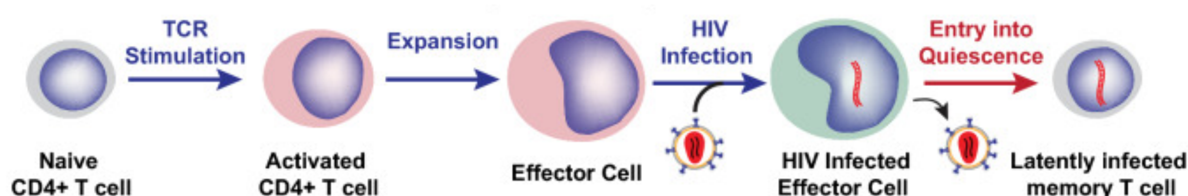


Figure 1.3. Model of latency establishment.
Schematic to describe how HIV latency is primary thought to be established. The Naïve CD4+ T cell is first activated through the T cell receptor (TCR) by an antigen. The antigen can be viral, such as human cytomegalovirus (HCMV) or HIV (Ballegaard et al., 2018), or it can be in response to other antigens, such as LPS that is a common antigen generated during HIV infection (Zevin et al., 2016). These cells will then expand and become effector cells, which are permissive to HIV infection. After infection, through natural memory T cell processes, the cell will ramp down transcription and enter a resting state, subsequently silencing HIV with it. **Figure 1.3** was modified from (Mbonye & Karn, 2024) with permission, Creative Commons Attribution License 4.0 International (CC BY 4.0).

The predominant sites of HIV infection are in lymphoid tissues (lymph nodes, GALT, and spleen) (Schacker, 2008; Tedla et al., 1999). Lymph nodes provide a unique environment due to its compartmentalization that produces different microenvironments for HIV-infected cells, where HIV-infected cells actively producing viral RNA are typically found in the B cell follicles (Sun et al., 2023). Within the lymph node, the most commonly infected cells are the T follicular helper cells (Tfh) and there is an enrichment of this cell type that is not fully understood, but it may be from heterogeneous ART penetration of the lymph node tissue potentially allowing for low an

intermittent levels of viral replication (Fletcher et al., 2014). While the CD4+ memory T cell reservoir in the peripheral blood is the most well-studied viral reservoir, HIV can also infect other CD4+ cells, such as macrophages (Banga & Perreau, 2024). Additionally, since HIV is found in a number of anatomical sites, there are also major reservoirs in the GI tract/GALT, lymph node (LN), blood, bone marrow, as well as less-studied reservoirs in the central nervous system (CNS), lung, and genitourinary systems (Banga & Perreau, 2024). Additionally, though not infected directly, follicular dendritic cells (FDCs) have been shown to contain HIV virions from binding and it is possible that these cells can retain the virus and transmit to CD4+ T cells (Busman-Sahay et al., 2021; Heesters et al., 2015).

While a majority of research on the HIV reservoir is performed with cells from the peripheral blood (Schacker, 2008; Tedla et al., 1999), it is important to note that the peripheral blood CD4+ memory T cells only accounts for <2% of the HIV in the body (Ganusov & De Boer, 2007) therefore, these other anatomical sites need to be considered when developing a cure. Fortunately, tissue lymphocytes recirculate through the peripheral blood, therefore, the peripheral blood is still a useful and relevant tool (Gowans, 1959). In addition to the interplay between various cell types within the lymph node compartments, this anatomical site is also void of cytotoxic CD8+ T cells that can detect and clear HIV-infected cells, therefore the lymph node is thought to be a sort of “sanctuary” site for the HIV reservoir and is important to consider for HIV cure strategies (Paryad-Zanjani et al., 2023; Zaman et al., 2024).

While the reservoir can exist in various cells and tissues throughout the body, when it comes to HIV cure strategies, the hypothesis is that a smaller viral reservoir will be less likely to spontaneously reactivate than a large viral reservoir. Early initiation of cART has been shown to reduce reservoir size because treatment prevents virions from seeding more cells (Buzon et al., 2014) and sequences of HIV virions at the time of cART initiation are representative of virions after long-term cART indicating successful viral suppression as there is minimal viral evolution (Abrahams et al., 2019; Margolis et al., 2020; Pankau et al., 2020; van Zyl et al., 2018). The

reservoir in the presence of cART is stable due to being maintained by memory T cell processes (Bosque et al., 2011; Chomont et al., 2009; Vandergeeten et al., 2013) reviewed in (Cohn et al., 2020)), and CD4+ memory T cells are long lived and have a half-life of 44 months (Chun et al., 1995; Churchill et al., 2016; Siliciano et al., 2003).

There are three main ways that the reservoir is thought to be maintained (**Figure 1.4**) (Cohn et al., 2020). Firstly, the integration site can influence reactivation potential. Secondly, Homeostatic proliferation, a process where cytokines stimulate the cells to divide without activating the internal provirus, can renew the infected cells in an immunologically undetectable way. Lastly, memory T cells can undergo antigenic proliferation when exposed to the antigen with which it was made and can expand cells. However, this last strategy also reactivates the internal provirus. All three of these factors may result in clonal expansion where a certain HIV clone may be overrepresented in the reservoir (Bailey et al., 2006; Finzi et al., 1999). These factors explain why there is minimal reservoir decay overtime and how HIV can persist lifelong (Crooks et al., 2015; Kandathil et al., 2016; Siliciano et al., 2003).

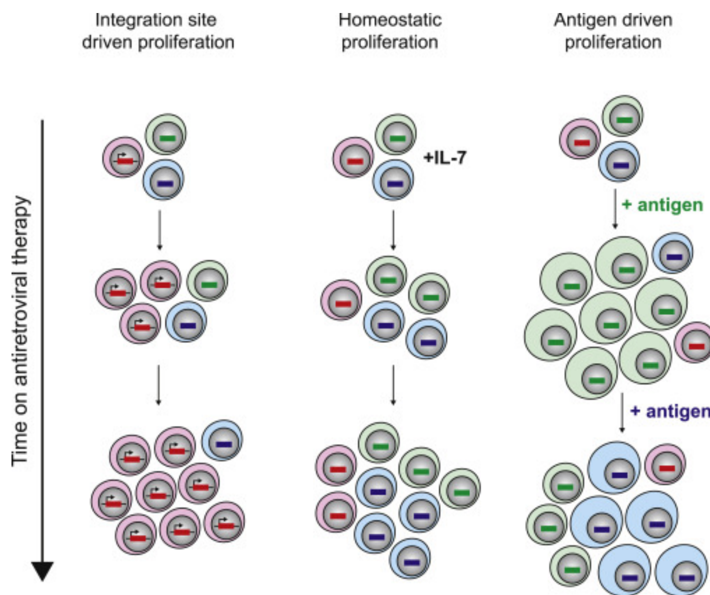


Figure 1.4. **Latent reservoir maintenance.**
During long term cART suppression viral clones have been seen to become overrepresented in the reservoir. This is thought to occur through three mechanisms: integration site dependent, homeostatic proliferation, and antigen driven proliferation. **Figure 1.4** was reproduced from (Cohn et al., 2020) with permission, Elsevier license number 5894490280033.

Because the viral reservoir is stable, it becomes imperative to develop methods to quantify the reservoir to identify how many cells need to be targeted for HIV cure efforts. To quantify the replication competent reservoir, the current gold standard is a viral outgrowth assay (VOA) where resting CD4+ T cells that contain an integrated provirus are stimulated with the mitogen phytohemagglutinin (PHA), which can maximally activate the T-cell through the T-cell receptor (Crooks et al., 2015; Eriksson et al., 2013). Upon activation, virions accumulate in the supernatant, and then are expanded through culture with CD4+ T cells from healthy donors, and then are quantified through a limiting dilution in an ELISA detecting p24gag (the capsid protein that forms the HIV core in the virion). This assay was able to quantify cells of the HIV reservoir to be $1/10^6$ of total resting CD4+ T cells (Eriksson et al., 2013). However, the total HIV reservoir has been shown to be much larger than what is induced in this assay, as when the same cells tested were further profiled using PCR looking at the sequence of HIV integrated into cells, it

was seen that there were approximately 300-fold more cells containing an integrated provirus compared to the cells that were able to reactivate (Eriksson et al., 2013). This is because most of the integrated proviruses are defective, i.e they contain either deletions (most often) or mutations in one or more of the viral genes to productively make virions (Bruner et al., 2016; Ho et al., 2013). Even if the proviruses appear intact, it's been estimated that only 10% of HIV-infected cells have intact proviruses, and of those, less than 5% are able to reactivate, pointing to differences in the chromatin landscape of proviruses or the transcriptional or metabolic state of the cell in silencing these proviruses (Ho et al., 2013; Hosmane et al., 2017). Because the reservoir is much larger than originally estimated, curing HIV becomes an even more daunting challenge. However, because only a small fraction of the reservoir can reactivate and contribute to spread and disease, targeting this fraction of the reservoir is the most important and could provide a functional cure (Sengupta & Siliciano, 2018).

Control of HIV Transcription

Since HIV latency is largely controlled at the step of transcriptional regulation, it is important to understand the factors that control viral transcription. HIV transcription initiation is principally governed by its promoter in the 5' long terminal repeat (5'-LTR) that is a binding site for cellular transcription factors (Duggan et al., 2023; Mori & Valente, 2020; Pereira et al., 2000). Several transcription factors have been shown to be necessary for HIV transcription such as nuclear factor- kappa B (NF-kB), nuclear factor of activated T-cells (NFAT), specificity protein 1 (Sp1), and the TATA-binding protein (TBP) (Li et al., 1994). Transcriptional repressors such as Yin Yang 1 (YY1) and LSF can also negatively modulate the HIV LTR (Bernhard et al., 2013; He & Margolis, 2002). Many other factors have been shown to play a role in modulating HIV transcription, some shown in **Figure 1.5** such as COUP, cMyb, AP-1, ETS-1, LEF-1, and RBF-2, and some predicted binding sites have yet to be validated (Mori & Valente, 2020).

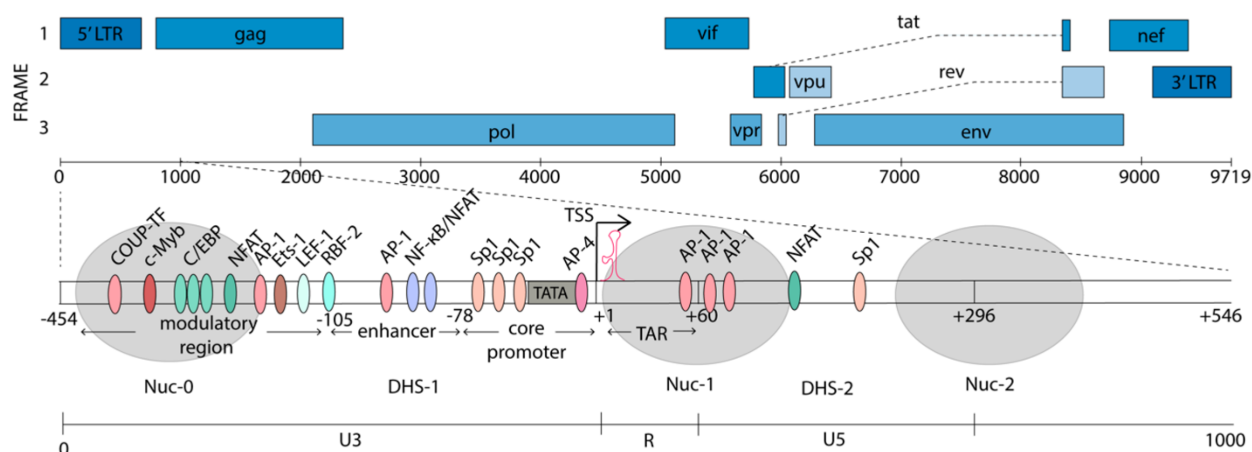


Figure 1.5. Schematic of the HIV-1 LTR.

Up top in blue is the HIV genome with all viral genes labeled and their nucleotide positioning. Below this is a zoom in of the LTR showing the transcription factor binding sites spanning three nucleosomes (Nuc-0, 1, and 2). Each transcription factor is marked by a different color, and some transcription factor sites overlap. Some regions where the LTR binds have been given names based on the observed function of the transcription factor binding sites within the region, and these names are labeled accordingly. The +1 site represents the start of HIV transcription (TSS) that is immediately preceding the sequence that generates the HIV TAR loop. On the bottom the general regions of the LTR are labeled U3, R, and U5. **Figure 1.5** was reproduced from (Mori & Valente, 2020) with permission, Creative Commons Attribution License 4.0 International (CC BY 4.0).

HIV transcription is modulated across 3 main nucleosomes, Nuc-0, Nuc-1, Nuc-2 that have been mapped onto the HIV provirus independent of integration site (Mori & Valente, 2020). Similar to host DNA, HIV DNA is wrapped around nucleosomes that are octamers of four histone pairs (H2A, H2B, H3, and H4) that have 147 bp of DNA wound around them (McGinty & Tan, 2015). The nucleosomes themselves can be regulated with remodeling factors PBAF and BAF that alter chromatin by removing Nuc-1 and making chromatin more open or maintaining Nuc-1 and making chromatin less accessible to other factors, respectively (Conrad et al., 2017; Easley et al., 2010; Mahmoudi et al., 2006; Rafati et al., 2011). Additionally, DNA sites called CpG islands that fall before and after Nuc-1 can be hypermethylated by cellular proteins DNMT1

and DNMT3a to help to maintain the Nuc-1 positioning on chromatin which helps preserve heterochromatin (Kauder et al., 2009; Nguyen et al., 2021; Verdikt et al., 2022).

In addition to chromatin remodeling and positioning, post translational modifications (PTMs) can affect HIV transcription. Each histone has a tail that can be post translationally modified and the two most well studied markers are acetylation marks deposited by histone acetyl transferases (HATs), and methylation marks that are deposited by histone methyl transferases (HMTs) (Mori & Valente, 2020). Acetylation is typically associated with active HIV transcription while methylation is typically associated with latent HIV transcription (Wang et al., 2008). One of the most well studied HATs is p300/CBP that is associated with HIV transcription activation that adds H3K27Ac to chromatin to facilitate the transition of transcription initiation to elongation (Cho et al., 2009). Chromatin marks that contribute to HIV latency are methylations such as H3K27me2/me3 and H3K9me2/3 that are deposited on chromatin by enhancer of zeste 2 (EZH2), Euchromatic histone lysine methyltransferase 2 (EHMT2) and Suppressor of Variegation 3–9 Homolog 1 (SUV39H1) (Mbonye & Karn, 2024). While acetylation and methylation are the most well-studied markers that play a role in HIV transcription and latency, other marks such as crotonylation and sumoylation have also been shown to play a role (Ait-Ammar et al., 2019; Jiang et al., 2020).

In additional to cellular transcription factors, HIV and other lentiviruses also encode a protein, Tat, that acts to facilitate efficient elongation of HIV RNA. HIV elongation without Tat has been estimated to only occur 10% of the time, whereas this number jumps to 75% with Tat present (Mori & Valente, 2020). HIV encodes a critical RNA structure called the TAR loop at the start of transcription, at the +1 site depicted in red in **Figure 1.5** and **Figure 1.6**, that can be bound by HIV Tat (Feng & Holland, 1988). Upon binding, Tat is acetylated by P300/CBP-associated factor (PCAF) that allows Tat to recruit cellular PTEF-b to the HIV 5’-LTR (Kiernan et al., 1999). PTEF-b typically binds to host Brd4 for cellular gene transcription, and HIV Tat can competitively bind PTEF-b to prioritize HIV transcription (Bisgrove et al., 2007). HIV is regulated

by pausing factors DRB-sensitivity inducing factor (DSIF) and negative elongation factor (NELF), that bind to the docked RNAPII and HIV DNA after transcription initiation, approximately 20-60 nt from the TSS (Abuhashem et al., 2022). Regulation by these factors is common to an approximated 40% of protein-coding genes (Abuhashem et al., 2022). In order for HIV to transition from the state of pausing to a state of productive elongation, the elongation factor PTEF-b needs to phosphorylate RNAP II on the C terminal domain (CTD), at Ser2, and phosphorylate pausing factors DSIF and NELF (Yamaguchi et al., 1999). Therefore, as more Tat is made, this process can occur more efficiently as a Tat feedback loop is established (Weinberger et al., 2005). Additionally, it has been shown in the absence of Tat, short abortive transcripts from HIV accumulate in the cell, which has been recently attributed to binding of the Integrator complex: a complex known to detect aberrances in the transition from transcription initiation to elongation, through ways not completely understood, and further stall transcription by antagonizing cellular PTEF-b and abort transcription through cleaving nascent HIV RNA. The Integrator complex will be discussed in more detail in Chapter 2 of this thesis.

Although it seems paradoxical that HIV integrates into actively transcribed region of the chromosome, the cells that survive to form the latent reservoir are rare (Jiang et al., 2020) and transcriptional silencing is established and maintained through multiple mechanisms (reviewed in (Mbonye & Karn, 2017)). HIV latency (**Figure 1.6**, top panel) is characterized by blocks to transcription initiation. For example, in cells that are entering a resting state, positive transcription factors NF-kB and NFAT are sequestered to the cytoplasm, which helps to promote HIV latency (Shan et al., 2017). In this state, repressive chromatin factors, BAF, EZH2, SUV39h1, 9Ga (EHMT2), YY1, and LSF prevent removal of Nuc-1 and deposit repressive methylation marks on histones or DNA to enforce a closed chromatin environment to prevent positive transcription factors from binding and to block the RNAPII from elongating (Mediouni et al., 2022). Additionally, even with these blocks in place, the pre-initiation complex may still bind the TATA box to initiate transcription. However, the paused polymerase will not transition to a

positive elongating state until PTEF-b is recruited to phosphorylate pausing factors and the polymerase, and without high levels of Tat in this phase, these promoters are largely paused and not productively elongating. Cellular PTEF-b levels are low in resting cells, which contributes to latency (Ait-Ammar et al., 2019). Furthermore, the Integrator complex can enforce pausing by terminating transcription and antagonizing PTEF-b to prevent the cell from fully transitioning into the active phases.

Changes that occur to facilitate active transcription are shown in **Figure 1.6**, middle panel. That is, upon T cells stimulation the levels of active NFAT and NF-kB increase and, active NF-kB can replace repressive NF-kB at the promoter and aid in recruitment of PTEF-b from the repressive 7SK complex, which phosphorylates negative factors and RNAPII to facilitate elongation. In this low HIV Tat stage, where some repressive factors are still present, replication is inefficient, and it is thought that HIV may rely on the host protein Brd4 to recruit cellular PTEF-b to initiate transcriptional elongation to create the initial Tat copies (Kaczmarek et al., 2013). Lastly, as shown in **Figure 1.6** bottom panel, fully active transcription of the provirus occurs when Tat levels generate a positive feedback loop that supports efficient elongation. Tat is able to competitively bind PTEF-b from host Brd4 and bring PTEF-b specifically to the HIV TAR loop for HIV transcription. PCAF activates Tat through acetylation (Kiernan et al., 1999). Additionally, repressive factors have been removed and replaced by stimulatory transcription factors p300 and PBAF.

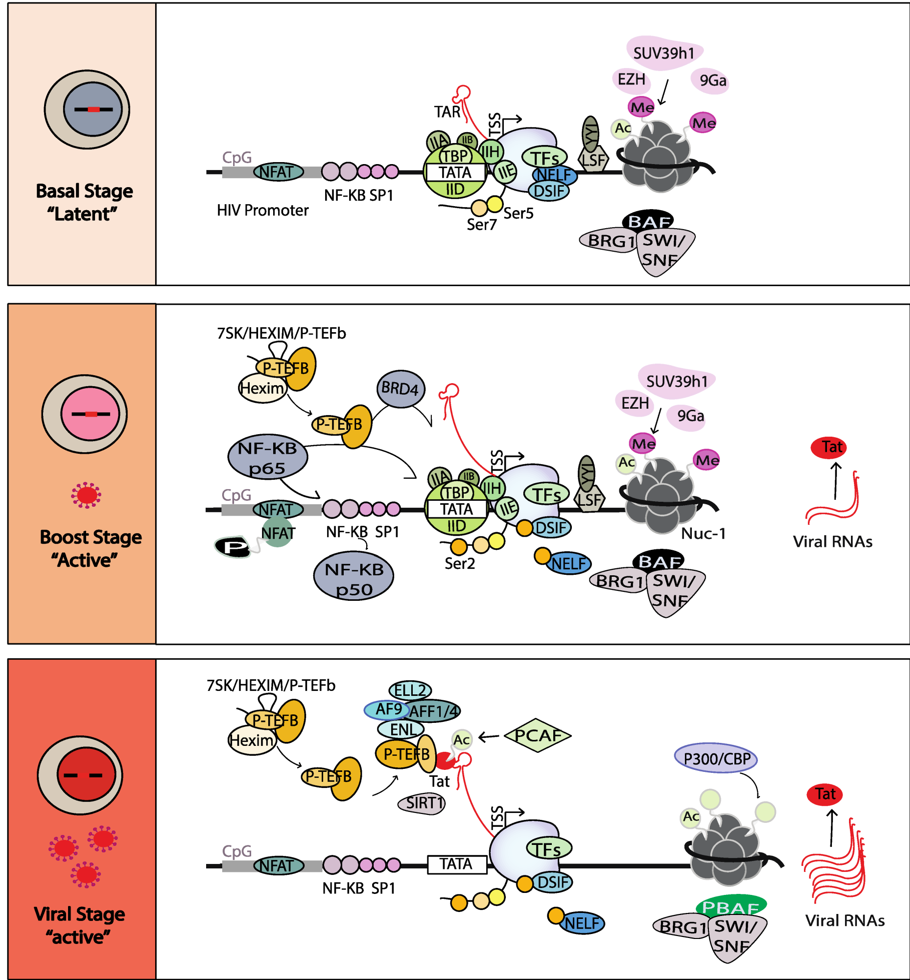


Figure 1.6. Transcriptional changes upon latency reversal.
showing how transcription state changes during latency (top panel) and reactivation (bottom two panels). Top panel depicts basal levels where repressive chromatin factors enforce latency, and positive factors are sequestered away. Here, RNAPII exists in a paused state unable to proceed to elongation and generate HIV. Middle panel shows how initiation factors can aid in simulating transcription and how the paused RNAPII proceeds to elongation in a minimal Tat environment. Bottom panel depicts the viral stage of active infection where high Tat levels lead to a positive feedback loop and stimulatory transcription factors have replaced repressive factors. This stage depicts efficient viral transcription. Figure 1.6 reproduced from (Mediouni et al., 2022) with permission, Creative Commons Attribution License 4.0 Internation (CC BY 4.0)

HIV Cure Strategies

One of the most prevalent HIV cure strategies currently being developed is called shock-and-kill (Duggan et al., 2023): a strategy where latently-infected cells are treated with small molecule drugs to stimulate virus transcription (shock), such that viral antigens are produced

that can lead to immune detection and clearing of infected cells (kill). This strategy would be done in the presence of cART, so induced virions would not productively infect and seed more cells in the viral reservoir (Mbonye & Karn, 2011). The goal behind this strategy is to reduce the size of the reservoir of intact, inducible, proviruses to reduce the probability of these proviruses becoming active if cART is discontinued. Such reduction in latent proviruses that could contribute to ongoing rounds of replication is sometimes referred to as “functional cure” (Mediouni et al., 2022). The small molecule drugs used in this strategy are called latency reversal agents (LRAs). The existing LRAs work through a variety of mechanisms and target that are informed by what is known about transcriptional control of HIV.

Latency is maintained differently depending on the model system that it is measured. Commonly used cell line models to test HIV reactivation are latently infected Jurkat T cell lines, called J-lat cells. Clones of J-lats that have the same integration site have been isolated and characterized, and it has been shown that reactivation profiles differ even amongst these clones (Spina et al., 2013). Additionally, J-lat cells reactivation profiles vary from latently infected primary cells, where J-lat cells are more readily inducible which is likely due to less effective epigenetic silencing and presence of initiation and elongation factors (Spina et al., 2013). Thus, J-lat cells can be used to preliminarily test LRAs however, further validation should be performed in primary cell systems or in vivo models. The current gold standard for testing LRAs is either in an *ex vivo* system where HIV-infected CD4 T cells are isolated from blood from virally suppressed PLWH (Falcinelli et al., 2022) which allows for measurements of virus reactivation or an *in vivo* nonhuman primate model on ART (Nixon et al., 2020) which allows for assessment of both peripheral blood and tissues and measurements of virus reactivation and the viral reservoir.

The most effective way to reactivate a latent cell to date is through TCR co-stimulation. Anti-CD3 and anti-CD28 antibodies can be used to co-stimulate the TCR directly, and this stimulation brings sequestered NFAT and NF-kB from the cytoplasm into the nucleus to initiate

transcription, it stimulates PTEF-b biogenesis that facilitates elongation, and it removes epigenetic silencing to open chromatin (Shan et al., 2017). By targeting several blocks to latency maintenance at once, this type of stimulation is potent in inducing HIV, however as a result it also nonspecifically reactivates host genes, which leads to toxicity. The TCR can also be stimulated indirectly by modulating steps downstream of the receptor for instance by toggling Protein kinase C (PKC) (**Figure 1.7C**) that can phosphorylate substrates like IkB that is essential for NF-kB pathway activation (Spina et al., 2013). Examples of an LRAs that stimulate the NF-kB pathway as PKC agonists (PKCa) are phorbol myristate acetate (PMA), Prostratin, and Ingenol (Venkatachari et al., 2015). The NF-kB pathway can also be stimulated by inhibiting PTEN and an example of an LRA that functions in this way is Disulfiram (**Figure 1.7C**) (Xing et al., 2011). Additionally, TLR agonists can also act as LRAs stimulating the NF-kB and MAPK pathway, which results in the upregulation of proinflammatory cytokines and upregulation of immune modulators (**Figure 1.7B**) however, TLRs are not highly expressed on CD4+ T cells so the effects of these drugs on CD4+ T cells is thought to be modulated through interferon and other cell types, such as dendritic cells (Duggan et al., 2023; Martinsen et al., 2020). While the potency of NF-kB-targeting LRAs are enticing for cure, many off-target effects have been documented from usage of these drugs in cancer therapeutics highlighting the need to find more specific ways to target HIV with LRAs. Even when less toxic NF-kB targeting drugs were explored, such as Disulfiram that was investigated in the context of alcoholism, despite this drug being more well-tolerated than PKCa, there was a minimal induction of HIV transcription and no effect on the reservoir size (Xing et al., 2011).

As a step towards maintaining potency but increasing specificity, another class of LRAs were investigated that also target transcription initiation, but through targeting the non-canonical NF-kB pathway (ncNF-kB) (**Figure 1.7D**). Targeting ncNF-kB affects a smaller number of genes than the canonical pathway and does so in a more gradual way therefore there are less off-target effects (Sun, 2012). LRAs that target this pathway are called SMAC mimetics because

they mimic cellular stress response proteins called SMACs, that are released from pores in the mitochondria during stress, that bind to inhibitor of apoptosis protein family members to induce apoptosis. These drugs were explored in the context of cancer therapeutics, and it was shown that by targeting IAPs in the ncNF-kB pathway with SMAC mimetics, this prevents the degradation of protein NIK that leads to the upregulation of the ncNF-kB pathway (Pache et al., 2020). Examples of this class of LRAs are AZD5582, Xevinapant, and Ciapavir (Hennessy et al., 2013; Nixon et al., 2020; Pache et al., 2020), which have been shown to increase HIV reactivation in vitro, ex vivo and in vivo systems (Duggan et al., 2023). In one promising study, AZD5582 was brought into *in vivo* NHP trials, and paired with N-803, which is an IL-15 superagonist that facilitates the recruitment of CD8 T cells and natural killer (NK) cells to lymph nodes (Dashti et al., 2023). Furthermore, Rhesus-derived antibodies that target SIV-env were added in these animals in an attempt to aid the immune-mediated clearance of infected cells. Reducing the reservoir has been a challenge in all current shock-and-kill in vivo studies, and while this approach was not able to reduce the reservoir in all tissues of the NHP, it was able to show a modest decrease in the viral reservoir in the lymph node specifically, and did not need to induce massive immune activation to do so. This study shows a proof-of-concept of the shock-and-kill approach and that we may be able to achieve clearance upon further optimization. For this reason, SMAC mimetics are one of the most promising LRAs to date.

Epigenetic marks can also be targeted to reverse latency, for instance histone deacetylases (HDACs) remove stimulatory acetyl groups and histone methyltransferases (HMTs) add repressive methyl marks and therefore, inhibiting HDAC or HMT activity has been shown to reverse latency (**Figure 1.7A**). Many HDACi are available to test due to their use in cancer therapeutics where treatment with these drugs upregulated tumor suppressor genes and thereby reduced cell proliferation (Li & Seto, 2016). HDACi were brought to HIV research once it was shown that latent cells contain high levels of HDACs and it was subsequently shown that HDACi could function as LRAs (Archin & Margolis, 2014). FDA approved HDACs are

romidepsin, panobinostat, and vorinostat (SAHA) that have been tested in clinical trials (reviewed in (Duggan et al., 2023), which showed some HIV reactivation from panobinostat and vorinostat (Kroon et al., 2020; Rasmussen et al., 2014), but variable reactivation with romidepsin (McMahon et al., 2021; Sogaard et al., 2015). No reduction to the reservoir was seen for panobinostat, and vorinostat had no impact on rebound kinetics (Kroon et al., 2020; Rasmussen et al., 2014). As for HMT inhibitors, there is one FDA-approved inhibitor called Tazemetostat that targets EZH2 in the polycomb repressive complex 2 (PRC2) (Straining & Eighmy, 2022). Clinical trials are currently underway for Tazemetostat to treat several cancer types with initial phases showing tolerable side effects (Straining & Eighmy, 2022). Other inhibitors of the PRC2 complex have also been explored for HIV reactivation from latency in cell culture models (Turner et al., 2020).

LRAs have also been made to target proteins involved in transcription elongation such as the BET proteins which read epigenetic marks on chromatin and toggle gene expression (**Figure 1.7A**, right). BRD4 is one example of a well-studied BET (bromodomain containing protein) that is targeted by pan-BETi, such as I-BET151. BRD4 is known to bind to cellular PTEF-b and bind chromatin with its bromodomains to facilitate cellular gene transcription, but the BET inhibitor binds to bromodomains preventing the recruitment of BRD4 to chromatin. It is thought that this may lead to the ability of Tat to recruit more PTEF-b for HIV-specific transcription (To et al., 2023). I-BET151 showed reactivation of HIV in vitro and in an ex vivo humanized mouse model (Boehm et al., 2013; Li et al., 2019). However, when cell types were isolated and reactivation was measured in the in vivo system, it was shown that only monocyte-derived macrophages reactivated and CD4 T cells were unaffected (Li et al., 2019). Therefore, more work needs to be done to explore the potential of this LRA in targeting the latent reservoir of CD4 T cells.

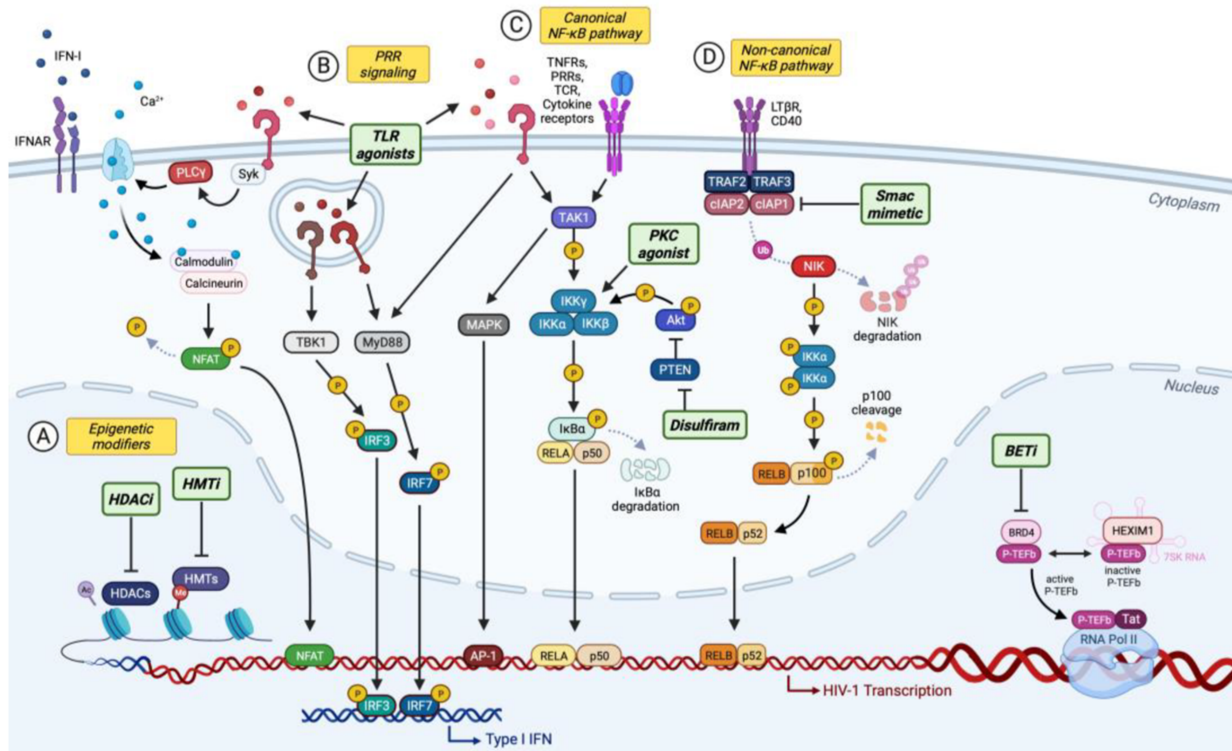


Figure 1.7: Overview of pathways that LRAs target.
A) Epigenetic modifiers, B) pattern recognition receptor (PRR), C) Canonical NF-kB pathway, D) Non-canonical NF-kB pathway**. Figure 1.7** was reproduced from (Duggan et al., 2023) with permission, Creative Commons Attribution License 4.0 International (CC BY 4.0).

Since no single LRA currently has been sufficient to potently reactivate a majority of the HIV reservoir nor has been specific enough to HIV to provide a non-toxic treatment and result in reservoir reduction, one strategy to improve both potency and specificity has been to combine LRAs with differential mechanisms of action (Duggan et al., 2023). This strategy allows for lower doses of each LRA to be used, which aids in specificity, and increases potency as the LRAs can synergize by targeting different pathways (Sengupta & Siliciano, 2018). Some known LRAs that synergize well with others are HDAC inhibitors such as SAHA with PKCa such as Prostratin (Reuse et al., 2009). Another combination being explored is SMAC mimetic, AZD5582, paired with elongation LRA, I-BET151, that is particularly appealing given the lower natural toxicity of

SMAC mimetics compared to PKCa (Falcinelli et al., 2022). This combination showed synergy in cell line models, however when it was tested in an ex vivo system with cells from PLWH, there was strong induction of transcription, leading to a buildup of gag transcripts within the cell, but lower poly adenylated and multiply spliced transcripts, which resulted in minimal detection of virus in the supernatant. These results highlighted the possibility that there were one or more additional blocks to virus reactivation to consider when using the AZD5582 and I-BET151 combination in cells from PLWH, and especially a block at the level of HIV transcription elongation. Thus, it is imperative that we develop strategies to identify targets to improve LRAs on their own and in combination so that we can identify these blocks to reactivation to improve the shock-and-kill approach in PLWH.

Current strategies towards this effort are using CRISPR-screening approaches to predict gene targets that increase HIV reactivation with or without LRAs (Dai et al., 2022; Hsieh et al., 2023; Li et al., 2020). Our lab as well as others have used screens to predict which genes to target to improve HIV latency reversal. Some genes identified have known LRAs, which helps inform which LRAs will likely synergize when paired together. Other gene targets identified are novel and predicted to improve gene reactivation either on their own or in the presence of LRAs.

Dissertation overview

HIV remains a global burden due to the inability to eliminate the latent reservoir in people living with HIV. Reservoir cells are governed by different latency maintenance pathways, which makes developing cure approaches such as shock-and-kill challenging. However, with current models in place to explore peripheral blood from PLWH and ex vivo NHP models, in addition to the LRAs that are available, and knowledge of HIV transcriptional control, we can begin to strategically improve the shock-and-kill approach. My graduate thesis focuses on improving an LRA combination, AZD5582 and I-BET151, that shows synergy by targeting

different latency maintenance pathways but fails to consistently produce virions in an *ex vivo* model of cells from PLWH. I used the CRISPR-screening approach to predict epigenetic targets that, when knocked out, will improve this LRA combination specifically with the goal of increasing potency and specificity and identifying novel gene targets for which small molecule drugs could be developed. My research found that targeting the Integrator complex could improve reactivation with AZD5582 and I-BET151 in an *ex vivo* system of PLWH. My thesis research therefore uncovers a pathway that could be targeted to improve the shock-and-kill approach to move us closer towards a functional cure where the productive reservoir is purged and people can safely be taken off cART without viral rebound and in turn, cannot spread infection to others.

Chapter 2. Integrator complex subunit 12 knockout overcomes a transcriptional block to HIV latency reversal

This manuscript is currently in press at eLife https://pubmed.ncbi.nlm.nih.gov/39257755/

Abstract

The latent HIV reservoir is a major barrier to HIV cure. Combining latency reversal agents (LRAs) with differing mechanisms of action such as AZD5582, a non-canonical NF-kB activator, and I-BET151, a bromodomain inhibitor is appealing towards inducing HIV-1 reactivation. However, even this LRA combination needs improvement as it is inefficient at activating proviruses in cells from people living with HIV (PLWH). We performed a CRISPR screen in conjunction with AZD5582 & I-BET151 and identified a member of the Integrator complex as a target to improve this LRA combination, specifically Integrator complex subunit 12 (INTS12). Integrator functions as a genome-wide attenuator of transcription that acts on elongation through its RNA cleavage and phosphatase modules. Knockout of INTS12 improved latency reactivation at the transcriptional level and is more specific to the HIV-1 provirus than AZD5582 & I-BET151 treatment alone. We found that INTS12 is present on chromatin at the promoter of HIV and therefore its effect on HIV may be direct. Additionally, we observed more RNAPII in the gene body of HIV only with the combination of INTS12 knockout with AZD5582 & I-BET151, indicating that INTS12 induces a transcriptional elongation block to viral reactivation. Moreover, knockout of INTS12 increased HIV-1 reactivation in CD4 T cells from virally suppressed PLWH *ex vivo*. We also detected viral RNA in the supernatant from CD4 T cells of all three virally suppressed PLWH tested upon INTS12 knockout suggesting that INTS12 prevents full-length HIV RNA production in primary T cells.

Introduction

Despite advancements in keeping viral loads below detectable limits, HIV-1 still exists within a latent reservoir *in vivo* and viral replication returns when anti-retroviral therapy (ART) is interrupted (Davey et al., 1999). HIV-1 predominately infects memory CD4+ T cells (reviewed in (Ait-Ammar et al., 2019; Cohn et al., 2020)) and while many acutely infected cells, particularly the more differentiated effector memory cells, die off rapidly upon infection due to cytopathic effects, some cells can revert to a resting state where the provirus becomes transcriptionally silent (reviewed in (Duggan et al., 2023; Mbonye & Karn, 2024)). These infected resting memory cells form the HIV reservoir that is largely invisible to the immune system and not sensitive to enhanced antiviral therapy (Siliciano & Siliciano, 2024). The reservoir of latently infected cells decays slowly and is maintained by both external stimuli and homeostatic proliferation even after initiation of ART (reviewed in (Cohn et al., 2020)). Thus, the major barrier to HIV cure is the existence of this ART-insensitive and long-lived latent reservoir that is largely not sensed by the immune system (Chun & Fauci, 1999), reviewed in (Duggan et al., 2023; Mbonye & Karn, 2024)).

One strategy being explored to target the latent reservoir, called “shock-and-kill”, uses small molecule compounds called latency reversal agents (LRAs) to transcriptionally activate latent cells, so that immune-mediated mechanisms can then recognize and kill infected cells (Archin et al., 2012), reviewed in (Maina et al., 2021)). This strategy would be employed in the presence of ART so that newly induced HIV virions could not infect more cells. However, inducing HIV-1 activation from latency is challenging, because the latent reservoir size varies between people and heterogenous HIV integration sites can influence reactivation of infected cells (reviewed in (Ait-Ammar et al., 2019)). Moreover, activation of latently infected resting T cells has not yet been able to induce HIV-1 replication from more than a fraction of the cells with a replication-competent provirus (Ho et al., 2013; Siliciano & Siliciano, 2024).

Transcriptional repression of HIV-1 in its latent state is multifactorial and it is known to be affected by repressive chromatin modifications and blocks to both transcription initiation and transcription elongation. Several classes of LRAs have been identified that can undo one or more blocks to latency reversal (Stoszko et al., 2019), reviewed in (Rodari et al., 2021; Singh et al., 2021)). For example, AZD5582 is an LRA that targets a transcription initiation block by activating the noncanonical NF-kB pathway (Hennessy et al., 2013; Pache et al., 2020) and has been shown to induce HIV transcription in HIV latency cell line models as well as induce viremia in animal models (Nixon et al., 2020). Another class of LRAs are BET inhibitors that undo a block to transcription elongation by targeting various bromodomain containing proteins. I-BET151 is a pan-BET inhibitor that is thought to reverse HIV latency by either blocking host Brd4 from binding to PTEF-b to facilitate PTEF-b availability for HIV Tat (Jang et al., 2005; Schroder et al., 2012; Yang et al., 2005) or more generally by increasing the amount of PTEF-b not bound to the 7SK complex (Turner, 2024). Currently, no single LRA has been potent enough to reactivate the majority of the latent proviruses nor specific enough to HIV to not cause toxicity to the host (reviewed in (Debrabander et al., 2023)). On the other hand, combinations of LRAs with different mechanisms of action have shown synergy in HIV latency reversal in different model systems including the combination of AZD5582 & I-BET151 (Dai et al., 2022; Falcinelli et al., 2022; Laird et al., 2015) reviewed in (Rodari et al., 2021).

While the combination of AZD5582 & I-BET151 is synergistic in activation of HIV in latency cell line models, and can induce robust HIV transcription initiation *ex vivo* in primary CD4+ T cells from virally suppressed PLWH, it failed to induce full-length proviral transcription in these same sample (Falcinelli et al., 2022). Thus, there exist additional blocks to HIV reactivation that even potent LRA combinations currently cannot overcome. Here we hypothesized that we could use a CRISPR screen to uncover pathways to improve LRA combinations. Previous work from our lab showed that we could use a high throughput CRISPR screening approach, called the Latency HIV-CRISPR screen, to predict gene knockouts that

improve the efficacy of a single LRA, AZD5582. Specifically, knockout of ING3, a member of the NuA4 histone acetylation complex, could improve the potency and the specificity of AZD5582, both in J-Lat model systems and in primary T cells. Thus, in this study, we used a similar strategy to identify gene knockouts to increase HIV latency reversal with the more powerful AZD5582 & I-BET151 drug combination, and uncovered Integrator complex subunit 12, INTS12, as contributing to a block in HIV reactivation, both on its own, but, especially in the presence of the AZD5582 & I-BET151 drug combination.

INTS12 is a subunit of the Integrator complex that is thought to act as a reader to link the complex to chromatin (Welsh & Gardini, 2023). The Integrator Complex functions as a genome-wide attenuator of mRNA transcription where it can antagonize transcription with both its cleavage and phosphatase modules (Hu et al., 2023), and members of Integrator have been shown to modulate transcription at the HIV LTR previously (Lykke-Andersen et al., 2021; Stadelmayer et al., 2014).

We found that knocking out INTS12 reactivates HIV on its own and improves reactivation with AZD5582 & I-BET151 treatment. RNA-seq analysis showed that INTS12 knockout (KO) more specifically reactivates HIV compared to AZD5582 & I-BET151 treatment alone and the combination of INTS12 KO with AZD5582 & I-BET151 increases the potency of reactivation. Additionally, we have evidence of an elongation block being overcome with INTS12 KO paired with AZD5582 & I-BET151 specifically, showing an increase in bound RNAPII in the gene body of HIV that is not seen with either KO or LRA treatment alone. We found that INTS12 is bound to the viral promoter and therefore may be acting directly on viral transcription. Furthermore, we used the HIV-CRISPR screen approach to probe the Integrator complex as a whole and predict Integrator members comprising the cleavage module, phosphatase module, and core to all be involved in the control of HIV-1 latency in addition to the reader, INTS12. Importantly, we see reactivation of HIV in the CD4 T cells of the majority of the virally suppressed people living with HIV (PLWH) tested *ex-vivo*, upon INTS12 KO with or without

AZD5582 & I-BET151 treatment. We also detected viral RNA in the supernatant suggestive of the presence of HIV virions released into the supernatant indicating that full length HIV transcripts are being generated.

Results

Latency HIV-CRISPR screen identifies gene targets that increase HIV latency reversal with AZD5582 & I-BET151 treatment

We used a high throughput CRISPR screen previously described by our lab (Hsieh et al., 2023) to identify gene targets that, when knocked out, would further improve latency reversal agents. The screen involves a lentiviral vector, called HIV-CRISPR, that contains two functional LTRs, a packaging signal, a guide RNA, and Cas9 to knock out a gene of interest (Figure 1A). Cells that have a latent HIV provirus are transduced with a library of HIV-CRISPR-containing guides and then, after selection for integration of the vector, the cells are treated with the combination of LRAs, AZD5582 & I-BET151 to reactivate HIV (Figure 1A). If a gene knockout improves LRA reactivation, we will detect more HIV in the supernatant compared to the DMSO control. We can determine the gene knockout responsible for HIV reactivation because the HIV-CRISPR vector will be packaged along with the reactivated provirus from the cells and become enriched in the supernatant (Figure 1A, see purple or black HIV RNA in virions). We then determine how enriched a guide is by the ratio of guides in the supernatant compared to the total guide representation in the cells and this generates a value called the MAGeCK gene score, where higher values correspond to more enrichment (i.e. HIV reactivation) (Li et al., 2014). Importantly, all guides to one gene target and replicates are factored into the MAGeCK gene score generation.

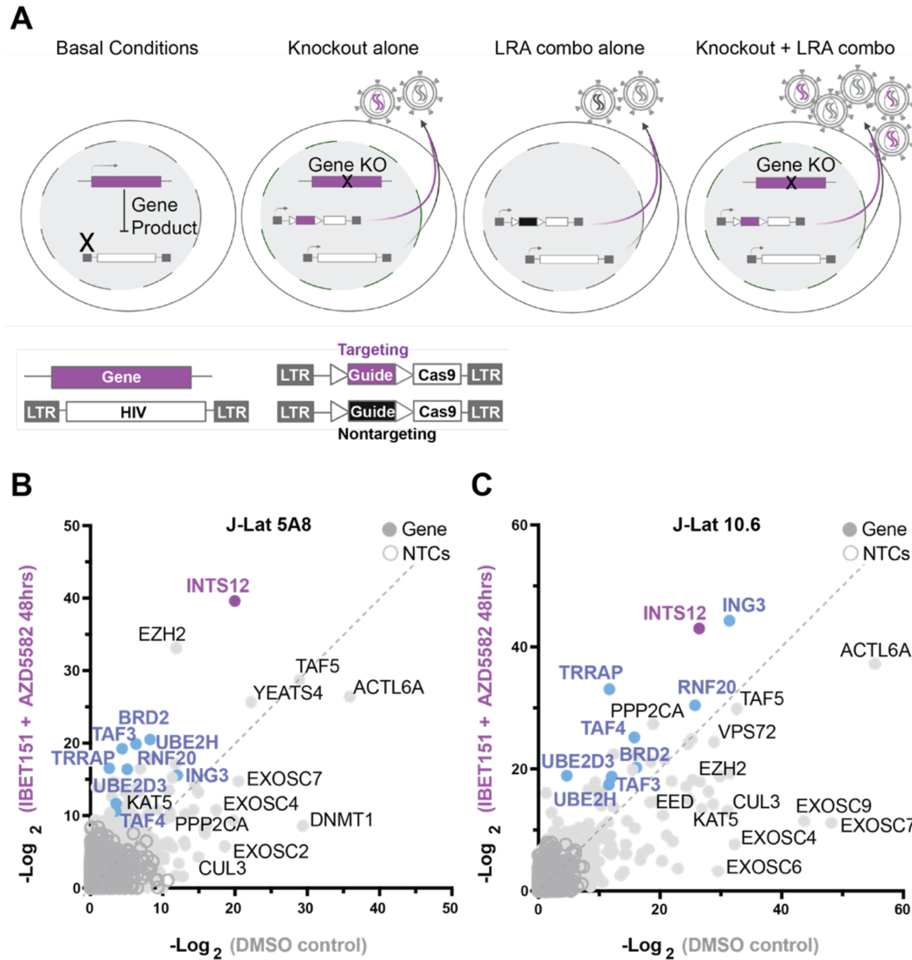


Figure 1: A screen to predict gene knockouts that will improve HIV reactivation from the combination of AZD5582 & I-BET151.
(A) Basal J-Lat conditions (left panel) followed by screen scenarios (middle to right panels). Screen overview: J-Lat cells containing an internal provirus are transduced with the HIV-CRISPR vector to generate a library of knockout cells for the human epigenetic library. Knockout cells are selected by puromycin and then either treated with LRAs or untreated. The HIV-CRISPR vector is packageable and will accumulate in the supernatant if the internal HIV provirus is reactivated. Sequencing the supernatant and cells allows for measurement of how enriched a guide is. (A, from left to right) Basal conditions show a gene product can block reactivation of HIV at the transcriptional level. Knockout alone shows that upon introducing the HIV-CRISPR some gene knockouts will result in latency reversal in the absence of LRA treatment. LRA combo alone shows the effect of LRA stimulation alone and how this will result in non-targeting guides (black) accumulating in the supernatant. Knockout + LRA combo shows that some gene knockouts will improve reactivation with LRA treatment and result in more virus accumulation. B-C are the results of the screens in J-Lat 5A8 **(B)** and J-Lat 10.6 **(C)**. Each are graphed as a comparison of the LRA combo AZD5582 & I-BET151 (Y-axis) treated screens to the untreated (DMSO) screens (X axis) as the -Log$_2$ MAGeCK gene scores for each gene target. Purple and blue genes are genes in common between J-Lat 10.6 and 5A8 that are predicted to improve AZD5582 & I-BET151 treatment specifically and purple is the top gene hit in common. NTC = nontargeting control guide.

We used a custom human epigenome guide library that contains 5,309 sgRNAs total (839 genes with 6 guides/gene) that comprises histone binders/modifiers and other general chromatin-associated proteins that has previously been validated as containing guides to multiple genes involved in HIV-1 latency (Hsieh et al., 2023). We conducted the screens in two cell lines, J-Lat 10.6 and J-Lat 5A8, that are clonal Jurkat T-lymphocyte cell lines that contain latent HIV. We chose these cells because they differ in integration site and HIV drug reactivation profiles (Chan et al., 2013; Jordan et al., 2003; Spina et al., 2013; Telwatte et al., 2019), and wanted to identify commonly enriched genes that were not integration site specific.

The screen results are shown as a comparison of a DMSO-treated screen compared to a screen carried out in the presence of 10nM AZD5582 & 100nM I-BET151 combined (Figure 1B-1C). Candidate knockouts that are predicted to improve the AZD5582 & I-BET151 combination specifically should fall above the dotted line in Figure 1B and 1C, as these genes are more highly enriched in the AZD5582 & I-BET151 screen compared to the DMSO screen. Genes that fall on the line are predicted to be drug-independent hits as they are equally enriched in both screens, and genes falling below the line are thought to be redundant to the function of the LRAs.

With this approach, we identified gene hits that have been validated previously using the same HuEpi library (Hsieh et al., 2023): KAT5, CUL3, ACTL6A, TAF5, DNMT1, DMAP1, VPS72, YEATS4, SRCAP, and ING3 (Figure 1B-1C, full screen list in supplemental file S1).The entire screen list is in supplemental file S1. Knockout of ING3 was previously shown to improve AZD5582 treatment for activation of latent HIV-1 (Hsieh et al., 2023), and also shows up as a candidate hit in the present screen done the presence of both AZD5582 & I-BET151 (Figure 1B and 1C). We also identified additional genes not found in our previous screen (Hsieh et al., 2023) that are predicted to improve the AZD5582 & I-BET151 combination specifically in both J-Lat lines tested including (shown in blue or purple, Fig. 1B and 1C): INTS12, TRRAP, RNF20,

BRD2, TAF3, TAF4, UBE2D3, and UBE2H. Some hits, EZH2 and PPP2CA, were only predicted to improve AZD5582 & I-BET151 treatment in one cell line. However, we focused the rest of this study on the most top ranking hit common to both J-Lat models that was enriched in the combination AZD5582 and I-BET151 treatment, Integrator Complex Subunit 12 (INTS12).

Integrator complex subunit 12 knockout specifically reactivates HIV independently and in combination with AZD5582 & I-BET151 treatment

The Integrator complex is known to play a role in the negative regulation of elongating transcripts (Elrod et al., 2019; Tatomer et al., 2019). Moreover, previous studies have shown that some Integrator members are recruited to the HIV LTR and regulate HIV transcript length (Stadelmayer et al., 2014), although INTS12 had not been previously implicated in HIV regulation. We validated INTS12 as a screen hit by either knocking out INTS12 with the HIV-CRISPR vector loaded with one guide and then selecting for knockout cells with puromycin (J-Lat 5A8 Figure 2A, J-Lat 10.6 supplemental figure S1) or by electroporation with a combination of three of guides to INTS12 (Figure 2B-2C). We used AAVS1 knockout as a CRISPR control where the guide targets within a safe harbor locus and knockout should have a minimal effect on transcription (Li et al., 2014). These knockouts were then treated with DMSO, a low dose of AZD5582 (10nM), a low dose of I-BET151 (100nM), or a combination of AZD5582 (10nM) & I-BET151 (100nM) (Figure 2A-2C) and the amount of HIV-1 released into the supernatant was measured by a reverse transcriptase activity assay (Vermeire et al., 2012). As expected, we find in AAVS1 knockout cells that there is minimal reactivation in the DMSO control, a small amount of HIV induction from AZD5582 or I-BET151 alone, and we observe synergy with AZD5582 & I-BET151 used in combination (Figure 2A-2C). Knocking out INTS12 alone in the absence of added LRAs also shows a modest increase in the amount of virus detected in the supernatant (Figure 2A-2B, DMSO), indicating that INTS12 itself is imposing a block to reactivation that is relieved upon knockout. In the presence of drug treatments, INTS12 knockout improves

reactivation of either AZD5582 or I-BET151 alone and we see the most HIV reactivation when INTS12 knockout is treated with AZD5582 & I-BET151 in combination (Figure 2A-2B), as the screen predicted (Figure 1B-1C). Flow cytometry data using the expression of GFP in the provirus gave similar results and showed a greater percentage of cells are activated from INTS12 KO alone and in combination with AZD5582 & I-BET151 supplemental figure S2).

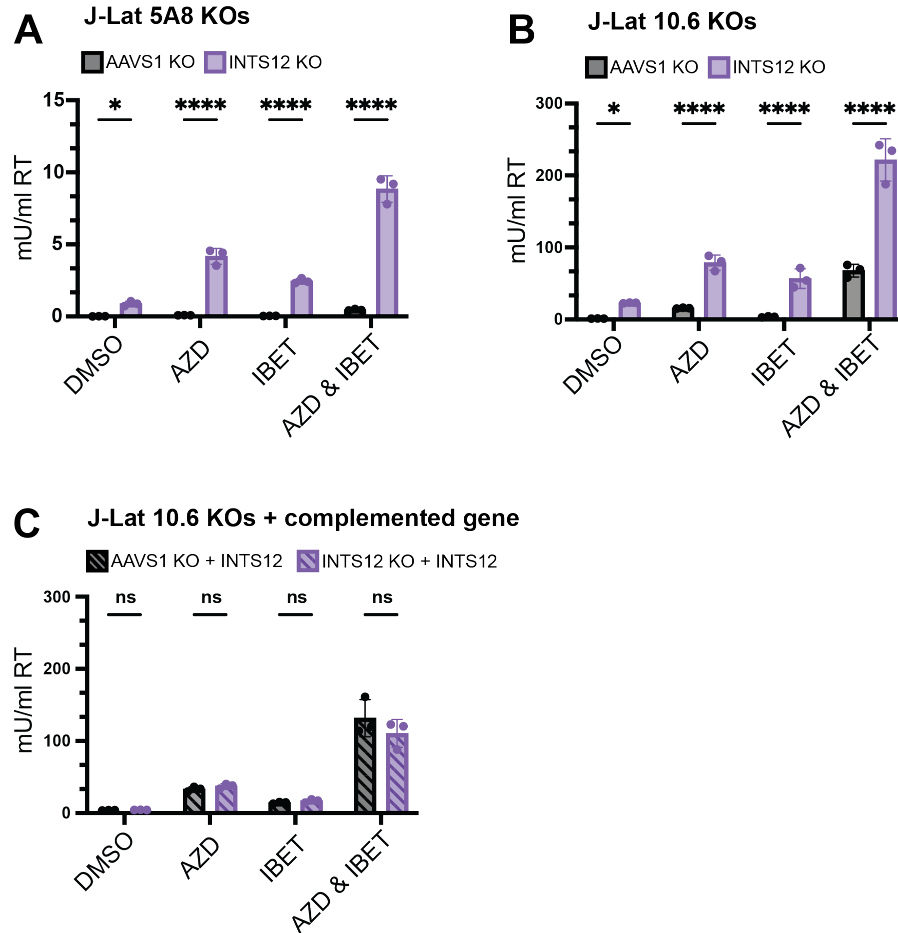
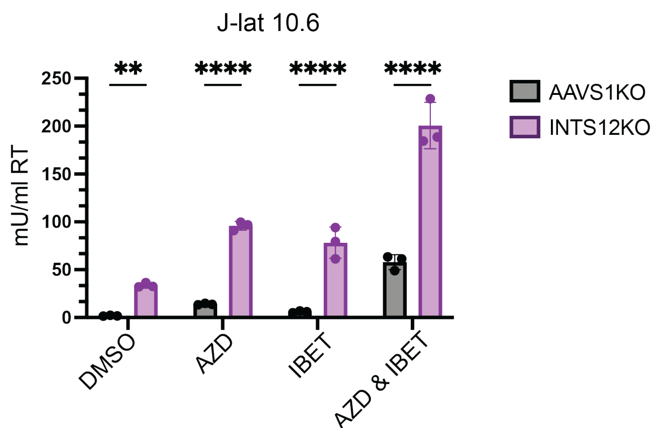


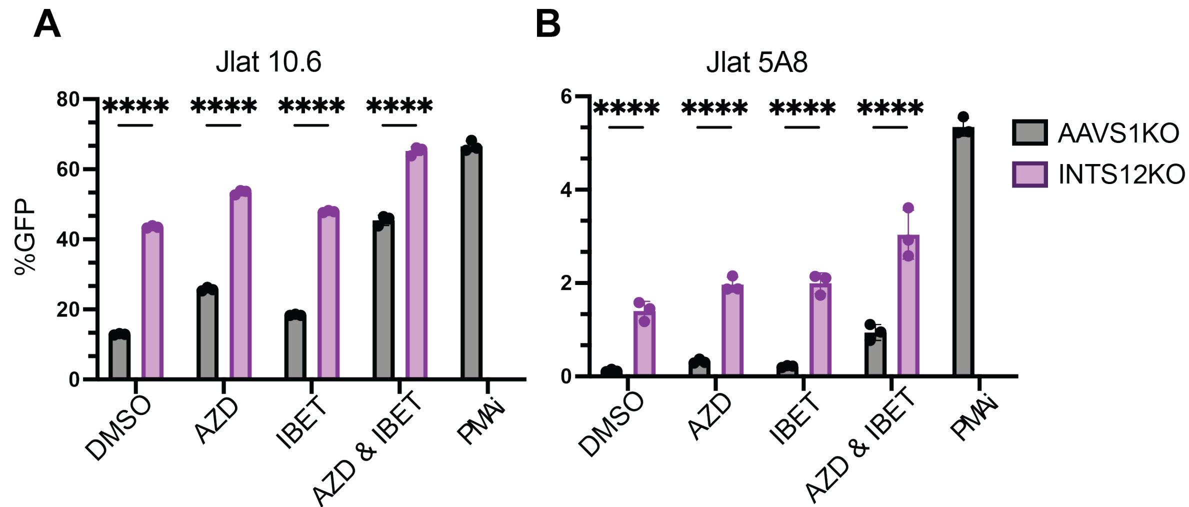
Figure 2: Validation of INTS12 knockout in HIV latency reversal both on its own and in the presence of AZD5582 & I-BET151.
J-Lat 5A8 **(A)** and J-Lat 10.6 **(B)** cells were knocked out for INTS12 or control locus, AAVS1. With a calculated INTS12 knockout score of 76% (for the one guide used) and 69% (for one of three guides used), respectively. Cells were then treated with 10 nM AZD5582 and or 0.1 µM I-BET151 for 24 hours (or an equivalent volume of DMSO), and HIV reverse transcriptase activity was measured from the supernatant (reported in mU/mL). **(C)** Complementation of cells knocked out for INTS12 or AAVS1 were transduced with a vector containing INTS12 before LRA treatments. These cells had an INTS12 knockout score of 55% for one of three guides used. Untreated = DMSO, AZD = AZD5582, IBET = I-BET151. For statistical analysis, all conditions are compared to the AAVS1 control. 2-way ANOVA, **(A-B)** uncorrected Fisher's LSD **(C),** Šídák's multiple comparisons test, p-value = <0.05 = *, = <0.01 = **, = <0.001 = ***, = <0.0001 = ****.

To ensure that this effect was due to knockout of INTS12 and not an off-target CRISPR effect, we performed INTS12 complementation experiments. We took pooled INTS12 KO cells and AAVS1 KO cells and transduced them with a lentiviral vector containing INTS12 and then measured reactivation by RT assay (Figure 2C) and found that reactivation of the INTS12 KO cells that were complemented with an INTS12 expression vector were now equivalent to the control AAVS1 KO cells with the same INTS12 expression vector (Figure 2C). These results show that INTS12 is responsible for a negative effect on HIV expression in the presence of latency reversal agents, AZD5582 and I-BET151.



Supplemental Figure S1: Additional validation of INTS12 knockout in HIV latency reversal both on its own and in the presence of AZD5582 & I-BET151.

J-Lat 10.6 cells were knocked out for INTS12 using the HIV-CRISPR vector with 1 guide targeting INTS12 (cells generated at the same time as Figure 2A). Cells were then treated with 10 nM AZD5582 and or 0.1 μM I-BET151 for 24 hours (or an equivalent volume of DMSO), and HIV reverse transcriptase activity was measured from the supernatant (reported in mU/mL). Untreated = DMSO, AZD = AZD5582, IBET = I-BET151. For statistical analysis, all conditions are compared to the AAVS1 control. 2-way ANOVA, uncorrected Fisher's LSD, p-value = <0.05 = *, = <0.01 = **, = <0.001 = ***, = <0.0001 = ****.



Supplemental Figure S2: Flow cytometry validation of INTS12 knockout in HIV latency reversal both on its own and in the presence of AZD5582 & I-BET151.

J-Lat 5A8 **(A)** and J-Lat 10.6 **(B)** cells were knocked out for INTS12 using the HIV-CRISPR vector with 1 guide targeting INTS12. Cells were then treated with 10 nM AZD5582 and or 0.1 μM I-BET151 for 48 hours (or an equivalent volume of DMSO), and flow cytometry was done to measure %GFP. Untreated = DMSO, AZD = AZD5582, IBET = I-BET151. For statistical analysis, all conditions are compared to the AAVS1 control. 2-way ANOVA, uncorrected Fisher's LSD, p-value = <0.05 = *, = <0.01 = **, = <0.001 = ***, = <0.0001 = ****.

INTS12 knockout increases HIV steady-state RNA levels with specificity for the LTR

To test the effects of INTS12 knockout on both HIV-1 and total host transcription, we performed RNA-seq on total RNA that has been ribosomal RNA depleted. RNA-seq was done on pooled INTS12 knockouts and pooled AAVS1 knockouts (as controls) in J-Lat 10.6 cells in biological triplicates, where knockout out of cells were generated three separate times and treated with either DMSO or AZD5582 & I-BET151. We first looked at how the sum of all transcripts mapping to HIV changed with the different conditions (Figure 3A). We observed that the RNA-seq trend matches what we saw with the virus release assay (Figure 2B) where the INTS12 KO alone reactivates HIV, and INTS12 KO further improves reactivation with AZD5582 & I-BET151. Reads were also mapped along the length of the HIV provirus (Figure 3B) using PMAi treated cells in this comparison as a positive control known to induce full length transcripts

(Falcinelli et al., 2022) (Figure 3B, bottom row). We observe the same differences quantified in (Figure 3A) as well as similar pileup trends along the length of the provirus. Because RNAs lacking a polyA tail are degraded rapidly (Biziaev et al., 2024) it is unclear whether those RNAs were successfully captured in our assay, and it is likely we are only looking at full length RNA detection. Nevertheless, the total RNA-seq results show that INTS12 KO increases HIV proviral activation at the transcriptional level.

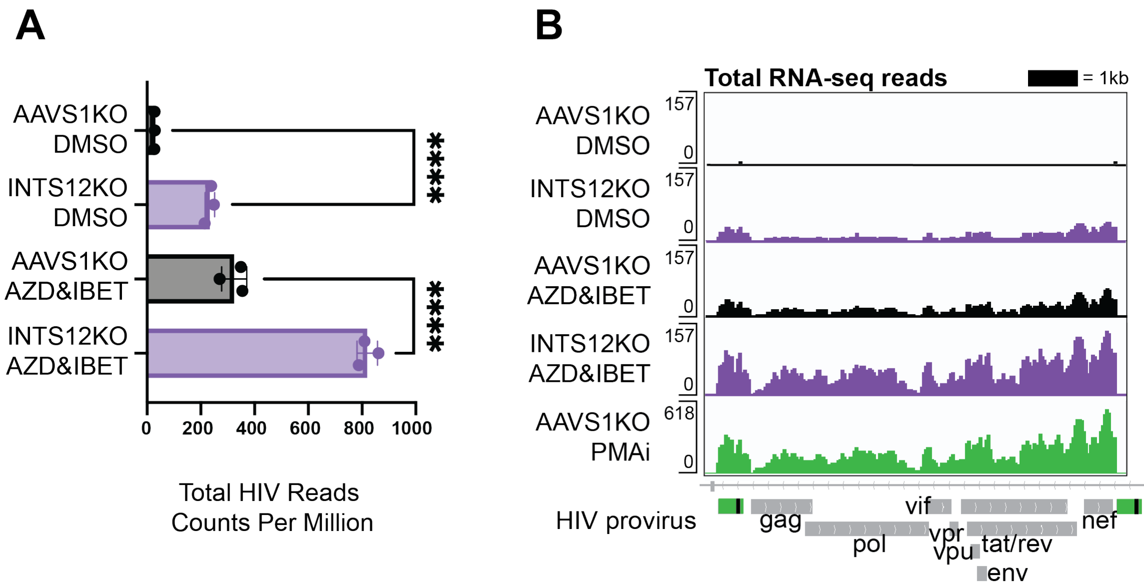


Figure 3: INTS12KO reactivates HIV alone and in combination with AZD5582 & I-BET151 at the level of steady-state viral transcripts.

(A) Total RNA-seq reads mapping to the HIV provirus were averaged for biological triplicates normalized to copies per million reads (CPM). One way ANOVA, Šídák's multiple comparisons tests, p-value = <0.05 = *, = <0.01 = **, = <0.001 = ***, = <0.0001 = ****. **(B)** Pileup graphs corresponding to each of the conditions represent averaged reads from biological triplicates that have been normalized to CPM, and the peaks correspond in location to the integrated provirus with viral genes labeled below. The scale of the pileups is in the top left of each row. Note that PMAi has a different scale than the other samples. The 3' LTR is masked so that reads will only map to the 5' LTR. AZD = AZD5582, IBET = I-BET151, PMAi = PMA & Ionomycin. All chromosome locations and quantified regions can be found in supplemental file S4. Cells used in this figure have an INTS12 knockout score of 75%.

We also investigated how INTS12 KO and AZD5582 & I-BET151 treatments alone and in combination affect transcription in the cell at a global level to assess the specificity to HIV compared to host genes. We first compared our AZD5582 & I-BET151 treatment condition to the DMSO treated AAVS1 KO cells and found that 80 genes were upregulated and 306 were downregulated, and that HIV was the most upregulated transcript with the highest fold-change (FC) and counts per million (CPM) (Figure 4A, left panel), similar to previous work (Falcinelli et al., 2022). Surprisingly, given the more wide-spread reported role of Integrator in transcription, we find that INTS12 KO alone is even more specific to HIV transcripts than AZD5582 & I-BET151 treatment, where we find the same level of enrichment of HIV as the previous comparison with a FC of 8, but there are only 70 upregulated genes and only 3 downregulated genes (Figure 4A, middle panel). This indicates that INTS12 is more important to HIV transcription than it is to any other transcriptional unit in J-Lat cells.

On the other hand, the transcriptional landscape of the combination of INTS12 KO cells treated with AZD5582 & I-BET151 compared to AAVS1 KO cells that were treated with DMSO (Figure 4A, right panel) showed an even greater enrichment of the HIV transcripts at a FC of ~28 (Log_2(Foldchange)=4.8). However, this gain of potency came with some loss of specificity as there was greater dysregulation of the host with 255 upregulated genes and 159 downregulated genes compared to the INTS12 knockout alone or the AZD5582 & I-BET151 treatment alone.

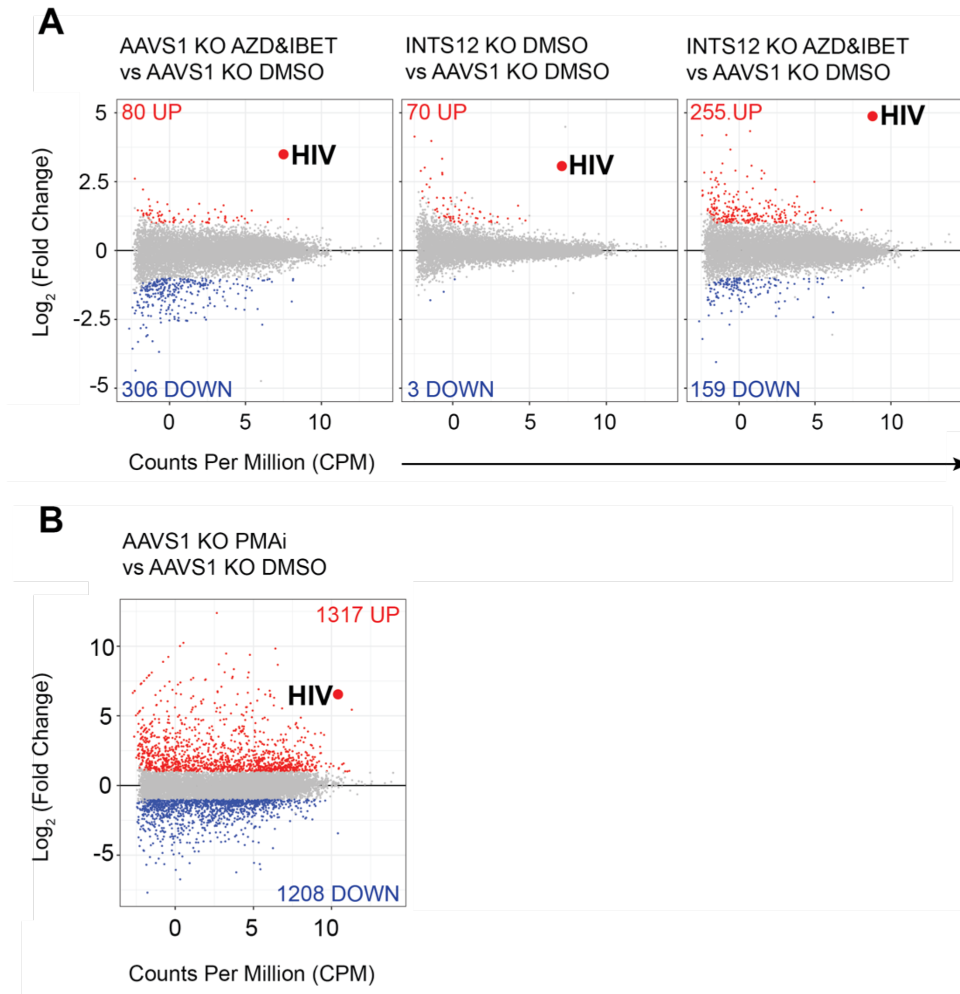


Figure 4: Specificity of INTS12KO for reactivation of HIV on its own and in combination with AZD5582 & I-BET151.

Differentially expressed genes are graphed by Log_2 (Fold Change) on the y axis, denoting how upregulated (red) or downregulated (blue), a gene is, and the average counts per million (CPM) on the x axis, denoting the expression of each gene. The big red dot denotes the average of all reads mapping to the HIV provirus. The red number at the top of each graph corresponds to the number of significantly upregulated genes in a comparison and the blue number in the bottom corresponds to number of significantly downregulated genes in a comparison. Grey genes are not statistically significant and not up or downregulated. **(A)**, left panel shows the effect of AZD5582 & I-BET151 treatment alone compared to the DMSO control. **(A)**, middle panel shows the effect of the INTS12 KO on gene expression compared to the AAVS1 KO control. **(A)**, right panel shows the effect of both AZD5582 & I-BET151 treatment of INTS12 KO cells compared to the control DMSO treated AAVS1 KO cells. **(B)** Is graphed using a different scale than A and shows the effect of PMAi treatment compared to the DMSO control. Cells used in this figure have an INTS12 knockout score of 75%.

Among the host genes most prominently affected by INTS12 knockout with AZD5582 & I-BET151 are MAFA, MAFB, and ID2 (full list of genes in supplemental file S3). Nonetheless, INTS12 knockout with AZD5582 & I-BET151 was much more specific to HIV-1 than PMAi, which can robustly activate HIV but at the cost of affecting thousands of host transcripts (Figure 4B). Thus, we find that INTS12 knockout acts at the transcriptional level in activating HIV from latency and is more specific for the HIV LTR than AZD5582 & I-BET151 treatment alone. We also find that INTS12 knockout indeed improves potency of reactivation of AZD5582 & I-BET151 but at the cost of some specificity.

Chromatin localization of INTS12 and RNAPII across the integrated HIV provirus

The effect of INTS12 on HIV-1 transcription could be direct (i.e. the Integrator complex directly affecting the steady-state levels of HIV-1 RNA transcription) or could be indirect by affecting the transcription of other gene products that subsequently act on the HIV-1 LTR. In order to partially distinguish these two hypotheses, we asked if INTS12 is present at the HIV-1 LTR using Cleavage Under Targets and Tagmentation (CUT&Tag) for profiling chromatin components (Janssens et al., 2021) using an antibody to INTS12 (Figure 5A). Using this approach, we observed that INTS12 is indeed at the LTR of HIV-1 as the signal over the LTR is greater in the AAVS1 KO cells than in cells with the partial INTS12 knockout (Figure 5A, left panel, compare top two rows with bottom two rows). We also saw this effect with a different INTS12 antibody (supplemental figure S3). We found that that INTS12 localization extends past the LTR, matching what has been seen previously where Integrator members localize to sites of pausing which are past the site of initiation (Elrod et al., 2019; Stadelmayer et al., 2014). In the right panel of Figure 5A we quantify all reads in the region specified by the dotted grey lines in the left panel (Figure 5A, right panel; exact coordinates in supplemental file S4). These results indicate

INTS12 binds to the HIV promoter suggesting that INTS12 represses HIV-1 activation from latency through a direct mechanism.

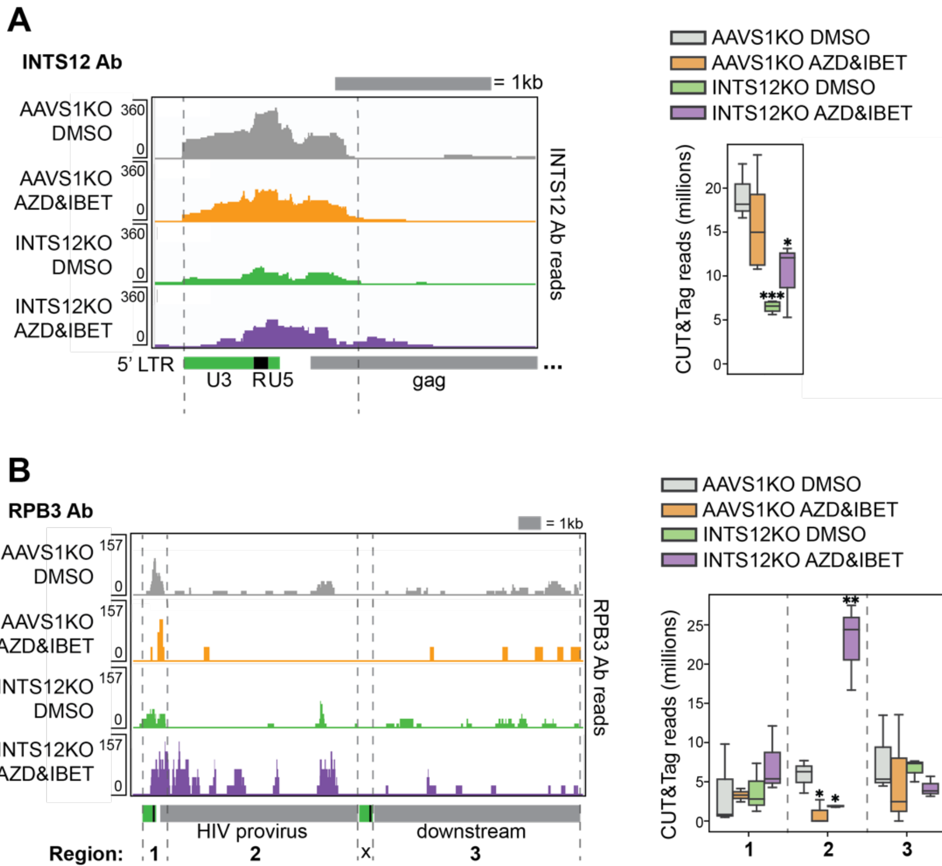
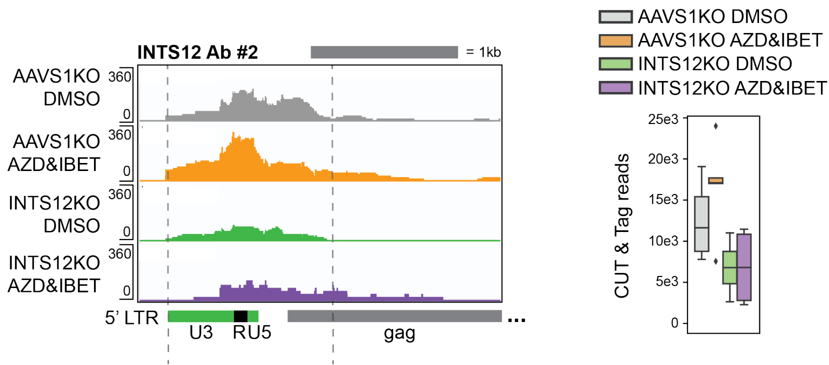


Figure 5: INTS12 binds the HIV promoter and KO with LRAs increase occupancy of RNAPII through the provirus.

CUT&Tag using antibodies to INTS12 **(A)** or total RNAPII (RPB3) **(B)** were used to generate pileup graphs that show where each are binding chromatin. The location of where reads are mapping is denoted below, and the scale are the numbers on the left of each row. Each row represents three to five technical replicates averaged together. (**A**, left panel) shows a zoom in of the HIV LTR and where INTS12 is binding. (**A**, right panel) quantifies total reads of INTS12 in the area denoted by the grey dashed lines in the left panel. (**B**, left panel) shows where RPB3 is binding across the HIV provirus and downstream. (**B**, right panel) quantifies total reads of RPB3 in the regions denoted by the grey dashed lines in the left panel, regions 1-3. All chromosome locations and quantified regions can be found in supplemental file S4. Two-tailed independent samples t-test p-value = <0.05 = *, = <0.01 = **, = <0.001 = *** , = <0.0001 = ****. Cells used in this figure have an INTS12 knockout score of 80%.



Supplemental Figure S3: INTS12 localization with a second antibody.

Left panel represents pile up graphs of sequencing reads corresponding to INTS12 binding corresponding to the genome location specified below. Right panel is the quantification of all reads between the grey dotted lines on the left. This region matches the same region quantified in Figure 5A for INTS12 Ab #1. All chromosome locations and quantified regions can be found in (supplemental file S4).

Given the Integrator complex's role in pausing and termination at promoters, we wanted to look at HIV-1 elongation by measuring RNAPII levels with CUT&Tag. We used an antibody against total RNAPII, RPB3, and generated pileup graphs of reads corresponding to where RPB3 is bound across the HIV-1 provirus and downstream (Figure 5B, left panel). Each row corresponds to at least 3 to 5 technical replicates. We quantified all reads of RPB3 in three regions (Figure 5B, right panel), exact coordinates in supplemental file S4. We see similar levels of RPB3 in region 1 for all four conditions. We defined region 2 as the remainder of the provirus excluding the LTR and the short 5' region of region 1. We found that there was a dramatic statistically significant increase in RPB3/RNAPII occupancy in the body of the provirus (region 2) only with both INTS12 KO and AZD5582 & I-BET151 together (Figure 5B). We did not see this increase in RNAPII when we examined the gene downstream of the integrated provirus (region 3). These data support the hypothesis that the combination of INTS12 with AZD5582 & I-BET151 specifically overcomes an elongation block that the INTS12 KO and AZD5582 & I-BET151 alone cannot overcome.

INTS12 knockout improves latency reversal ex vivo in T cells from virally suppressed people living with HIV (PLWH)

We next tested the role of INTS12 in HIV-1 latency reversal in an *ex vivo* system of CD4 T cells isolated from PBMCs of virally suppressed PLWH. CD4 T cells were electroporated with Cas9 complexed with either a non-targeting control guide (NTC), or a cocktail of 3 INTS12 guides and then treated with DMSO (vehicle) or AZD5582 & I-BET151 treatment for 24hrs (Figure 6A-6B). We used a low reactivating dose of AZD5582 (100nM) & I-BET151 (500nM) for primary cell experiments (Falcinelli et al., 2022). We tested six different donors with or without AZD5582 & I-BET151 treatment and measured cell-associated viral RNA expression by qPCR using primers in *gag* (Figure 6A-6B). In Figure 6A, when we treat donors with the vehicle control (DMSO), we find that INTS12 KO trends upwards for viral RNA expression in all six donors and this increase is statistically significant in four donors (Figure 6A). These same samples were treated with AZD5582 & I-BET151 (Figure 6B), and we found that although LRAs alone could activate viral RNA expression (compare samples in Figure 6A with 6B), the combination of AZD5582 & I-BET151 paired with the INTS12 KO shows an even further increase in HIV-1 reactivation that was statistically significant in four out of six donors (Figure 6B). For donor PH543 (upside down triangle), we found that there is an increase from the INTS12 KO itself (Figure 6A, right panel), and robust activation from AZD5582 and I-BET151 treatment itself (Figure 6B, right panel), however there was no further increase from INTS12 KO with AZD5582 & I-BET151 treatment. Degree of reactivation was correlated with reservoir size as donors PH504 (star symbol) and PH543 (upside down triangle) have the largest HIV reservoirs (supplemental file S5). This data shows that our screen was able to identify INTS12 as a barrier to HIV-1 reactivation in PLWH, as we find that there is reactivation from INTS12 knockout alone and improvement of AZD5582 & I-BET151 treatment with the addition of INTS12 knockout in a majority of virally suppressed PLWH tested *ex vivo.*

Because elongation of full-length HIV RNA has been a barrier to reactivation in PLWH previously, we not only wanted to look within the cells at *gag* RNA expression, but we sought to measure viral RNA in the supernatant by qPCR as a proxy for virus production. For this repeat assay, we chose three donors that had available materials (labelled in green in Figure 6). We used the same LRA concentrations we used in Figure 6A-6B, but extended the time of treatment to 72 hrs, and measured viral RNA in the supernatant (Figure 6C). Importantly, we observed for all three donors that INTS12 KO was able to improve reactivation either on its own or in the presence of AZD5582 & I-BET151. Excitingly, donor 543 (Figure 6C, right panel) showed a robust increase from the pairing of INTS12 KO with AZD5582 & I-BET151 treatment when measuring gag RNA in the supernatant. The differences between Figure 6C and Figure 6A-6B could be due to timing of the assay, or inherent variability with cells from PLWH, however we can conclude that the majority of these cells are responsive to INTS12 KO with or without AZD5582 & I-BET151 treatment and that this reactivation leads to full length HIV that can be detected in the supernatant.

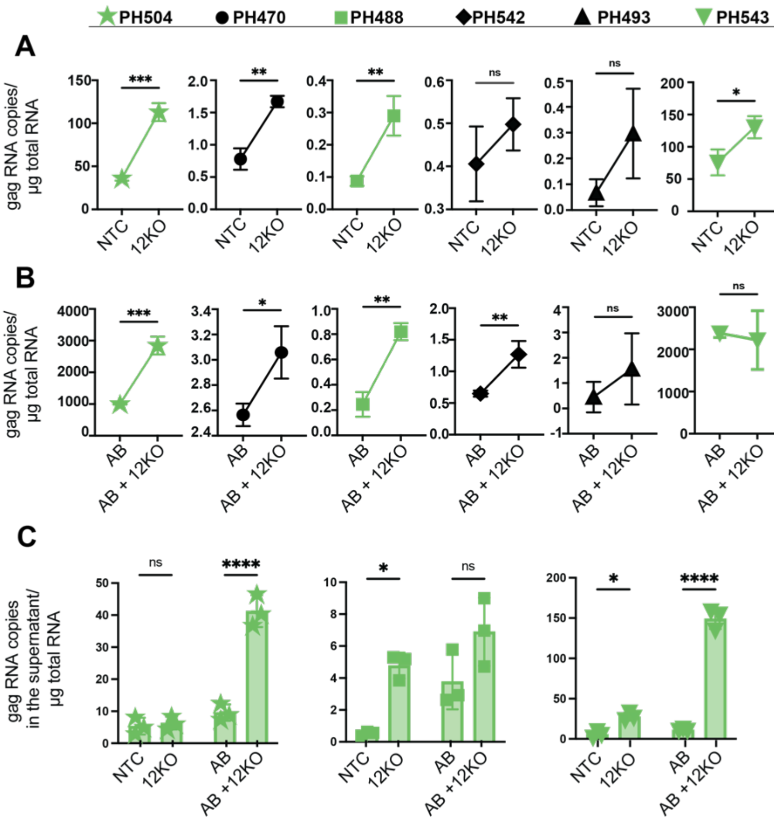


Figure 6: INTS12 knockout in an *ex vivo* primary cell system shows reactivation from knockout alone and in combination with AZD5582 & I-BET151.

Number of gag copies/ug of RNA were measured from the cell **(A-B)** or the supernatant **(C)** for different donors, marked by different symbols, above. **(A)** INTS12KO (12KO) was compared to a non-targeting control (NTC). **(B)** AZD5582 & I-BET151 treated INTS12 KO (AB + 12KO) was compared to AZD5582 & I-BET151 treatment (AB). Unpaired t-test, p-value = <0.05 = *, = <0.01 = **, = <0.001 = *** , = <0.0001 = ****. The green coloration denotes samples that are in common between A-C. **(C)** gag in the supernatant was measured for three different donors. Two-way ANOVA p-value = <0.05 = *, = <0.01 = **, = <0.001 = *** , = <0.0001 = ****.

Role of additional members of the Integrator complex in HIV latency reversal

Previous studies have shown that Integrator members are important for HIV latency (Stadelmayer et al., 2014), and some have done so in the context of combination LRA treatment (Li et al., 2020). In our initial screen of epigenetic factors (Figure 1), only INTS12 was included in the CRISPR guide library of the fifteen-member Integrator complex. In order to more

comprehensively interrogate the role of Integrator complex members in HIV latency, we remade a library of guides in the HIV-CRISPR vector where we included all Integrator members (with the exception of INTS15 that was not known at the time of library construction (Offley et al., 2023; Replogle et al., 2022)). The remainder of this library targets NF-kB related factors (supplemental file S6). We took this new library (herein, Screen #2) and treated J-Lat 10.6 cells with 1nM AZD5582 & 2.5µM I-BET151 for 24hrs (Figure 7) and found INTS12 is again predicted to improve the AZD5582 & I-BET151 treatment as it falls above the average NTCs. Importantly, however, all members of the cleavage module: INTS11, INTS9, INTS4; all members of the phosphatase module: INTS5, INTS6, INTS8; and all members of the core: INTS1, INTS2, INTS7 score positively in our screen. Thus, we predict that a large majority of the Integrator complex, consisting of the cleavage module, phosphatase module, core, and INTS12, but not the SOSS complex or the enhancer/arm module, likely play a role in HIV latency reversal in the presence of LRAs.

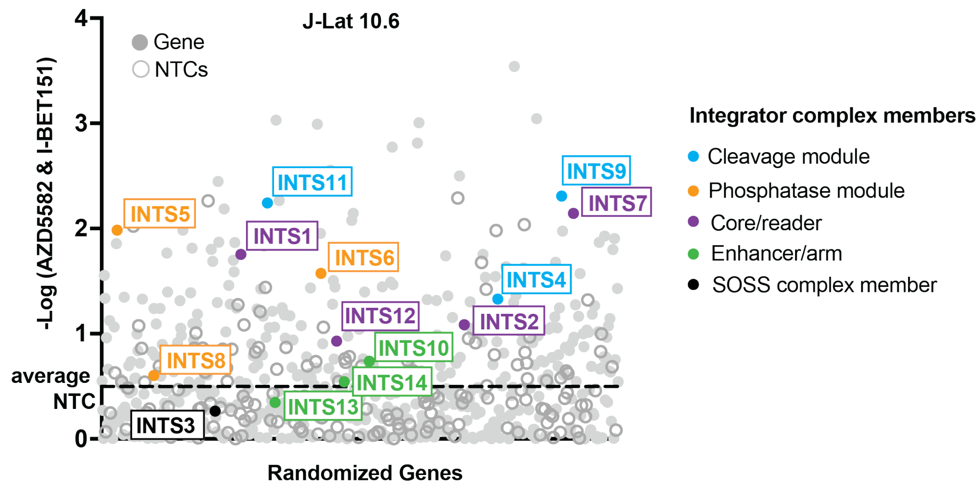


Figure 7: HIV-CRISPR screen with a library that includes Integrator complex members. Screen #2 was performed in J-Lat 10.6 cells of a library of NF-kB-related factors where all Integrator complex members were added. The y axis is the -log (MAGeCK gene score) which denotes how enriched a gene is. INTS12 has been shown to bind INTS1 (Fianu et al., 2021), so INTS12 was included in the core group coloration. The average NTCs are marked by a dotted line.

Discussion

We sought to improve HIV reactivation with a synergistic LRA combination AZD5582 & I-BET151 using an CRISPR screening approach. The ideal gene target would not only increase potency of this drug combination but maintain specificity to the virus and limit off-target effects. We used an epigenetic regulatory factor focused guide library in combination with AZD5582 & I-BET151 and identified INTS12. We validated that knockout of INTS12 results in increased latency reversal on its own but more in the presence of AZD5582 & I-BET151 treatment, as the screen predicted. We observed that INTS12 KO resulted in specific enrichment of HIV and minimal effects on host genes, and that INTS12 KO is more specific to HIV than AZD5582 & I-BET151 treatment alone. INTS12 KO paired with AZD5582 & I-BET151 treatment further increased potency of HIV reactivation, but some specificity was lost compared to AZD5582 & I-BET151 alone and INTS12 KO alone. However, INTS12 KO paired with AZD5582 & I-BET151 remains more specific to HIV than PMA & Ionomycin combination treatment. With CUT&Tag, we observed that INTS12 is at the HIV promoter which supports that it is acting directly on HIV-1 transcription, and we see an increase in RNAPII in the gene body of HIV only with the combination of INTS12 KO and AZD5582 & I-BET151, supporting that an elongation block is specifically overcome with this combination. Additionally, we validated INTS12 in an *ex vivo* primary cell model of latency where we see reactivation from INTS12 KO in a majority of donors tested with or without AZD5582 & I-BET151 treatment, and that reactivation upon INTS12 KO leads to detection of viral RNA in the supernatant indicating that full length HIV is likely generated. Lastly, we used the CRISPR-screen approach to predict which members of the Integrator complex could be targeted to improve the AZD5582 & I-BET151 combination and identified the cleavage module, phosphatase module, and core Integrator members, in addition to INTS12 as playing a role in HIV-1 latency. This study highlights the promise of using the

CRISPR-screening approach as a tool to identify blocks to HIV reactivation and improve LRA combinations, such as AZD5582 & I-BET151.

The role of Integrator complex in modulating HIV transcription

The Integrator complex is known to regulate and attenuate transcription at promoters that are bound by pausing factors NELF and DSIF (Skaar et al., 2015). HIV is known to be regulated by these pausing factors (Ping & Rana, 2001), and recently it has been shown that members of the Integrator are recruited to the HIV TAR loop and regulate processivity of HIV transcription (Stadelmayer et al., 2014). The Integrator contains 15 members that fall within different sub modules that perform various functions (Welsh & Gardini, 2023), and of these modules some members of the cleavage, phosphatase, and core have been shown to reverse HIV latency upon knockout (Li et al., 2020; Stadelmayer et al., 2014). Knockout of INTS3 of the SOSS complex was also not found to reverse latency in a previous study (Stadelmayer et al., 2014). Furthermore, members of the core and phosphatase have been shown to improve an LRA combination of a BET inhibitor with a PKC agonist (Li et al., 2020). We have recapitulated these previous findings (Figure 7), but we have also predicted that all members of these modules can be targeted to improve the AZD5582 & I-BET151 treatment including the cleavage module for which this has not been explored in the context of LRAs.

One study explored the different effects on gene transcription upon targeting the cleavage module vs targeting the phosphatase module of the Integrator complex (Hu et al., 2023). Selectively targeting the cleavage module of Integrator has been shown to result in a specific induction of lowly expressed genes, since these genes are regulated by pausing factors and Integrator (Hu et al., 2023). Given that HIV is regulated by these same factors and poorly expressed during HIV latency, it may not be surprising that targeting this module has shown an increase in HIV transcription. Since the Integrator complex has been shown to modulate genes

differently in the presence or absence of stress stimuli (Tatomer et al., 2019; Yue et al., 2017)) this may explain why targeting the Integrator complex in the presence of LRAs can improve reactivation. Moreover, Integrator is known to antagonize host PTEF-b function with its phosphatase module and enforce pausing at bound promoters. Therefore, targeting the phosphatase module can lead to more elongation at affected promoters. We find that INTS12 is located at the HIV-1 promoter (Figure 5A) and knockout fairly specifically reactivates HIV compared to host genes (Figure 4A, middle panel) which supports that this complex acts directly on HIV-1 transcription rather than indirectly though the modulation of other genes. However, it is also possible that INTS12 could function independently of the remainder of the Integrator complex.

Ex vivo Primary Cell system

To our knowledge, our study is the first to report an analysis of an Integrator complex member in an *ex vivo* experiment in cells from virally suppressed PLWH. We observed that INTS12 knockout reactivates HIV transcription in a majority of donors on its own and especially in the presence of AZD5582 & I-BET151 (Figure 6). This was in people with differing reservoir sizes, cells that have different HIV integration sites, and people on ART treatment for varying lengths of time. We were also able to show that HIV RNA could be detected in the supernatant, which is an aspect critical to consider with regard to the kill side of the shock-and-kill approach and has proven to be one of the more difficult elements of the approach so far with many studies showing reactivation but variable virus production. This result highlights the power in the CRISPR-screening approach and shows that even when cell line models are used, we can use LRA combinations and identify blocks to reactivation that can be used to improve reactivation in more clinically relevant cells from PLWH.

Synergy on multiple mechanisms

AZD5582 and I-BET151 are thought to synergize by acting to undo different blocks to latency reversal. AZD5582 working on the transcription initiation arm where non canonical NF-kB is upregulated and I-BET151 on the transcription elongation arm where it is thought to impact available cellular PTEF-b that can work in conjunction with HIV Tat for HIV transcription (Jang et al., 2005; Schroder et al., 2012; Turner, 2024; Yang et al., 2005). When Integrator is bound to the HIV promoter it can attenuate HIV transcription through its cleavage and phosphatase modules, as evidenced by knockout of members of these modules. As such, Integrator works on the elongation stage of transcription, and in our work, we have evidence that knocking out INTS12 may overcome a block to elongation. We not only see virus in the supernatant upon INTS12 KO in cells from PLWH (Figure 6), but we also see that specifically with the combination of INTS12 KO and AZD5582 & I-BET151 there is an increase in RNAPII downstream of the HIV promoter and throughout the HIV provirus (Figure 5). Therefore, our hypothesis is that targeting Integrator may allow for HIV transcription initiation events to not be aborted prematurely and allow for subsequent paused promoters to proceed to elongation from an unopposed PTEF-b. For this reason, removal of Integrator can lead to latency reversal on its own, but this also may explain why Integrator increases latency reversal with each LRA individually as well as improves the combination of INTS12 knockout with AZD5582 & I-BET151.

The Integrator complex itself is a fascinating target that needs to be considered for any cure approach involving transcription. Not only does it govern transcription regulation at the HIV promoter, but it appears to do so in ways that may be contrary to the ways other genes are affected given its relative specificity for HIV transcripts (Figure 4A). While it has been reported that Integrator can function differently in the presence of stress stimuli (Tatomer et al., 2019; Yue et al., 2017) we did not observe differential expression of any Integrator members upon AZD5582 & I-BET151 treatment in J-Lat cells (supplemental file S3) though this does not mean

that recruitment of the complex does not vary under stimulatory conditions. In Figure 5, we did not observe significantly different recruitment of INTS12 upon AZD5582 & I-BET151 treatment but did not look at recruitment of other Integrator members and how their recruitment may be impacted. It is possible the general stoichiometry of the various Integrator members is important for the function of the complex at various promoters, though this has not been explored. Understanding how the Integrator complex and its various modules are affected by different LRA stimuli may be crucial to optimizing the shock-and-kill strategy or even improving the reverse strategies of block-and-lock (Lyons et al., 2023) seeking to silence HIV more effectively at the HIV promoter. While, to our knowledge, small molecule drugs that target INTS12, or any member of the Integrator complex are not yet available, the development of such agents would be predicted to improve the efficacy of existing LRAs to achieve more effective viral reactivation as a step towards reservoir reduction.

Supplemental Files

https://pubmed.ncbi.nlm.nih.gov/39257755/

Competing interest statement

The authors declare they have no competing interests.

Author Contributions

Conceptualization: CNG,EH,ME

Methodology: CNG, MA, DHJ, JK, BA, EM, TLH

Investigation: CNG, MA, DHJ, JK, BA, EM, TLH, NMA, EPB, ME

Visualization: CNG, TLH, MA

Funding acquisition: NMA, EPB, ME

Project administration: NMA, EPB, ME

Supervision: NMA, EPB, ME

Writing – original draft: CNG, MA, EPB, ME

Writing – review & editing: CNG, MA, DHJ, TLH, EH, NMA, EPB, ME

Methods

Data Availability

All primary CUT&Tag sequencing data, RNA-seq data, and screen data can be found at GSE277306.

Ethics Statement

PBMC for this study were obtained from durably suppressed on ART people living with HIV under a University of North Carolina (UNC) institutional Review Board approved protocol (study number 08-1575). All Participants provided written consent.

Cell Culture and Maintenance

J-Lat 10.6 and J-Lat 5A8 cells were cultured with RPMI 1640 media (ThermoFisher, 11875093) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin/Streptomycin, and 10 mM HEPES (ThermoFisher, 15630080). HEK293T and TZM-bl cells were cultured in DMEM (ThermoFisher, 11965092) supplemented with Penicillin/Streptomycin and 10% FBS. All cells were maintained at 37ºC with 5% CO_2.

CRISPR screen: Human Epigenetic (HuEpi) Library

The HuEpi screen was performed as described in (Hsieh et al., 2023). Previously, J-Lat 10.6 and J-Lat 5A8 cells were prepared for the HIV-CRISPR latency screen by knocking out Zinc Antiviral Protein (ZAP), and the Human Epigenome Guide Library (HuEpi) was cloned into HIV-CRISPR vectors. Specific details for this paper below.

HuEpi screen: Lentivirus production

Lentivirus was made by adding 667 ng of previously prepped HuEpi HIV-CRISPR vectors (Hsieh et al., 2023), 500 ng psPAX2 (GagPol), and 333 ng MD2.g (VSVG) per well in 200 ul of serum-free DMEM and completed with 4.5 μl of TransIT-LT1 reagent (Mirus Bio LLC, MIR 2304). 20 x 6 well cell culture plates of 293Ts were used at a confluency of 40-60%. Media was replaced 24 hrs after transfection (and reduced to 1.5 ml/well) and the VSV-G pseudotyped lentivirus was harvested 48 hrs after that and filtered through a 0.22 μM filter (Thermo Scientific; 720–1320). Virus supernatant was combined (by cell type) and concentrated by ultracentrifugation. For ultracentrifugation, about 30 mL of supernatant was aliquoted into each polypropylene tube (Beckman Coulter; 326823) and sterile-filtered 20% sucrose (20% sucrose, 1 mM EDTA, 20 mM HEPES, 100 mM NaCl, distilled water) was added slowly underneath to form a sucrose cushion. Tubes were then placed in a pre-chilled swinging buckets SW 28 rotor at 23,000 rpm for 1 hour at 4°C in a Beckman Coulter Optima L-90K Ultracentrifuge. Supernatants were decanted, allowed to dry briefly, and pellets were resuspended overnight in 600 μL RPMI at 4°C (can do several hours). Concentrated lentivirus was then stored at -80°C in single use small aliquots (~150 μL) and two 50ul aliquots were stored at -80°C for titration.

HuEpi screen: HuEpi HIV-CRISPR cell generation

Virus was titered on TZM-bl cells (NIH AIDS Reagent Program; ARP-8129) in a colony-forming assay to calculate for a <1 multiplicity of infection (MOI) (0.54 was used in the HuEpi screens). Number of cells to transduce were calculated to be >500x (5029 HuEpi guides x 500 = 2.51e6 + error = 3e6 cells) so 3e6 J-Lat cells (ZAP KOs) were transduced per replicate. Cells were transduced with DEAE-Dextran (20 μg/mL final concentration, Sigma-Alrdich; D9885)) and spinoculated for 30 minutes at 1100 xg and incubated overnight. After 24 hrs, supernatant was replaced with fresh media containing 0.4 μg/mL of puromycin (Sigma) and cells were selected for 10-14 days (cells were grown for 21 days in this experiment to acquire enough for the desired screen coverage and all experimental conditions).

LRA resuspension

AZD5582 (MedChemExpress, HY-12600) and I-BET151 (SelleckChem, S2780) were resuspended in DMSO to make 1 mM stocks stored at -80 °C. Stocks were then diluted down to single-use working concentrations for experiments with RPMI.

HuEpi screen: LRA treatments

For the LRA treatments in the screen, we use a coverage of 5000x in case reactivation events are rare and we also account for viability. We calculated 25e6 cells were needed per replicate + error = 26e6 cells. We accounted for AZD5582's viability and calculated 35e6 cells/replicate for each condition. AZD5582 and I-BET151 were resuspended in DMSO to 1 mM stocks stored at -80°C. All other dilutions were performed in RPMI and used immediately. For the screen, 35e6 cells/replicate were grown at 5e5 cells/ml in a flask and treated with 10 nM AZD5582, 100 nM I-BET151, 10 nM AZD5582 & 100 nM I-BET151, or DMSO. Cells and supernatants were collected after 48 hrs of stimulation. Cells were spun down at 1500 x g for 3 min, supernatants were transferred to a new 50 ml conical, filtered through a 0.22 μm filter (Millipore Sigma, SE1M179M6), and stored at 4°C (can store overnight) for virus concentration. Cells were washed once with DPBS (Gibco; 14190144) and resuspended in 1 mL DPBS to store aliquots of 5e6 cells (approximately calculated, not counted) at -80°C. Finish concentrating virus by following steps detailed in lentivirus production (above) but resuspend the pellet in 150 μL at the end. Freeze viral supernatant at -80°C.

HuEpi screen: sample processing and prepping for sequencing

Genomic DNA from cell pellets were extracted with the QIAamp DNA Blood Midi Kit (Qiagen; 51183) and eluted in distilled water. vRNA from viral supernatant was extracted with the QIAamp Viral RNA Mini Kit (Qiagen, 52904). PCR 1: sgRNA sequences are in the gDNA and vRNA samples, they are amplified by PCR (Agilent; 600677) and RT-PCR (Invitrogen; 18064014), respectively, using HIV-CRISPR specific primers. PCR 2: adds barcodes and prepares the libraries for Illumina sequencing (examples of barcodes in (Hsieh et al., 2023)). Each amplicon

was cleaned up using double-sided bead clean-up (Beckman Coulter; A63880), quantified with a Qubit dsDNA HS Assay Kit (Invitrogen; Q32854), and pooled to 10 nM for each library. Library pools are sequenced on a single lane of an Illumina HiSeq 2500 in Rapid Run mode (Fred Hutch Genomics and Bioinformatics shared resource).

HuEpi screen: screen analysis

Analysis was performed as described in (Hsieh et al., 2023). Briefly, sequencing reads were demultiplexed and assigned to samples, trimmed, and aligned to the HuEpi library with Bowtie. Guide enrichment or depletion was determined with the MAGeCK statistical package.

Screen #2 containing all Integrator complex members

This screen was performed the same as the HuEpi screen was described above, with the following changes. The library contains 517 genes (6 guides/gene) + 163 NTCs = 3265 guides total. 1 nM AZD5582 & 2.5 μM I-BET151 was used for LRA treatment at 24 hrs. Coverage during transduction was 500x, the coverage during LRA treatments was 3600x. 50 bp sequencing was performed on the MiSeq V3 platform by the Fred Hutchinson Cancer Research Center Genomics Shared Resources.

CRISPR KOs/electroporations

J-Lat cells (in Figure 2A, Figure 3, and Figure 4) were knocked out for INTS12 or AAVS1 with single guides cloned in the HIV-CRISPR. Transduced cells were selected with 0.4 μg/mL puromycin selection for 10–14 days to generate CRISPR/Cas9-edited knockout pools. J-Lat cells (in Figure 2B-2C) were knocked out for INTS12 using a cocktail of three guides from IDT (Predesigned Alt-R™ CRISPR-Cas9 guide RNA) or AAVS1 using a cocktail of three guides from Synthego (ordered individually as gene knockout kit v2 and then pooled together) and Cas9 from IDT (Alt-R S.p. Cas9 Nuclease V3). Cells were electroporated using the SE Cell Line 4D-NucleofectorTM X Kit L from Lonza using the CL-120 pulse code and edited for at least 3 days before use. All guides used are located in supplemental file S2.

Vector for complementation

For the complementation experiment (Figure 2B-2C), after cells were electroporated and knocked out with the cocktails of 3 guides detailed above, half of the cells were transduced with a vector containing INTS12 generated on VectorBuilder (pLV[Exp]-Puro-EF1a>hINTS12[NM_020395.4]) and selected with 0.4 µg /ml puromycin for 10 days before LRA treatments. Cells were double checked for knock by ICE analysis and knock out scores only dropped by ~5% during this selection period.

ICE analysis

PCR products of a region containing the edited site in INTS12 knockout cells and not edited site in AAVS1 knockout cells were compared using the Synthego ICE analysis software to generate a knockout score. All primers can be found in supplemental file S2.

LRA treatments

AAVS1 KO and INTS12 KO cells were seeded at a density of 5e5 cells/ml in 96 well plates with 200 ul/well or in flasks. LRA treatments were performed in triplicate. Cells were seeded and then the calculated amount of LRA was added on top with mixing and cells were incubated. All J-Lat experiments used 10 nM AZD5582 & 100 nM I-BET151 for 48 hrs except Screen 2 (Figure 7) used 1nM AZD5582 & 2.5 uM I-BET151 for 24 hrs. Primary cell experiments used 100 nM AZD5582 and 500 nM I-BET151 for 24 hrs for qPCR and then treatment was extended to 72 hrs for gag RNA detection in the supernatant.

Virus measurement: RT assay

LRA treated cells were spun at 1500 x g for 3 minutes and 50 µl of supernatant was transferred to 96 well plates. Plates were either processed immediately or were frozen at -80oC for future processing. For the RT assay, 5 µl of supe for each condition was mixed with lysis buffer and then diluted in H_20 before use as template for qPCR. The detailed protocol can be found here (Vermeire et al., 2012).

RNA-seq run/analysis

Three biological triplicates were generated in J-Lat 10.6 cells for AAVS1 KO and INTS12 KO cells by knocking out three separate sets of cells and then cells were treated with LRAs. We included AAVS1 treated with DMSO, AAVS1 treated with AZD5582 & I-BET151, INTS12 KO treated with DMSO, and INTS12 KO treated with AZD5582 & I-BET151. Treated samples were spun down at 1.45e6 cells/tube (I used 2 tubes/sample), supernatant was aspirated, and cells were lysed with 700 µl of QIAzol (Qiagen, 1023537). Samples were spun through a QIAshredder (Qiagen, 79654) and then frozen at -80°C. Cells were processed with a miRNeasy kit (Qiagen, 217004), DNase treated with RNase-Free DNase Set (Qiagen, 79254), and then re-processed through the RNeasy Mini Kit (Qiagen, 74104) with the EtOH wash modified to 700 µL for small RNAs. Combine tubes for each sample. RNA quality was checked by TapeStation (all samples had a RIN of 10), and 10 µL of RNA at 50 ng/µL was sent for RNA-seq to the Fred Hutchinson Cancer Center Genomics Shared Resources where rRNA was removed to select for the rest of the total RNA with the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit (20040525) followed by indexing with IDT for Illumina RNA UD Indexes Set A Ligation (20040553). Samples were run using paired-end 2 x 50bp sequencing on the NovaSeq S1 by the Fred Hutchinson Cancer Center Genomics Shared Resources.

RNA-seq: Data Analysis

Fastq files were aligned to the custom genome assembly prepared by (Hsieh et al., 2023) in which chr9:136468439–136468594 of hg38 is replaced with the integrated HIV-1 sequence from MN989412.1 (Chung et al., 2020), and the second 634 bp copy of the LTR is masked by 'N's. GENCODE annotation v38 and HIV gene annotation were combined and genome coordinates were modified accordingly. STAR v2.7.7a (https://pubmed.ncbi.nlm.nih.gov/23104886/) with 2-pass mapping was used to align paired-end reads to the custom reference. FastQC 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), RNA-SeQC 2.3.4 (https://pubmed.ncbi.nlm.nih.gov/22539670/), RSeQC 4.0.0

(https://pubmed.ncbi.nlm.nih.gov/22743226/) were used to check various QC metrics including insert fragment size, read quality, read duplication rates, rRNA rates, gene body coverage and read distribution in different genomic regions. featureCounts (https://doi.org/10.1093/bioinformatics/btt656) in Subread 2.0.0 was used to quantify gene-level read counts in second-stranded fashion. Bioconductor package edgeR 3.36.0 (https://academic.oup.com/bioinformatics/article/26/1/139/182458) was used to detect differential gene expression between sample groups. Genes with low expression were excluded using edgeR function filterByExpr with min.count = 10 and min.total.count = 15. The filtered expression matrix was normalized by TMM method (https://doi.org/10.1186/gb-2010-11-3-r25) and subject to significance testing using quasi-likelihood pipeline implemented in edgeR. A gene was deemed differentially expressed if absolute log2 fold change was above 1 (i.e. fold change > 2 in either direction) and Benjamini-Hochberg adjusted p-values was less than 0.05.

CUT&Tag: sample preparation

Nuclei were prepared from J-Lat 10.6 cells. Samples included AAVS1 treated with DMSO, AAVS1 treated with AZD5582 & I-BET151, INTS12 KO treated with DMSO, and INTS12 KO treated with AZD5582 & I-BET151. Treated cells were pelleted in 1.5 mL microfuge tubes (10e6 cells/sample) spun at 300 x g for 5 min, and then resuspended in 1 mL of ice cold NE1 Buffer (20 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.5 mM Spermidine, 0.1% TritonX-100, 20% Glycerol, with Roche complete EDTA-free protease inhibitor tablet), and then incubated on ice for 10 min. Nuclei were centrifuged at 4°C at 1,300 x g for 4 min and then resuspended in 1 mL of Wash Buffer (20 mM HEPES pH7.5, 150 mM NaCl, 0.5 mM spermidine, supplemented with Roche complete EDTA-free protease inhibitor tablet). Nuclei were counted (10 µL was used) and diluted to a concentration of 1e6 nuclei/900 µL of wash buffer and aliquoted into cryovials with 100 µL of DMSO, which were quickly placed inside a Mr. Frosty Isopropanol chamber for slow freezing at -80°C. Nuclei were then stored at -80°C until use. For automated CUT&Tag processing, nuclei were thawed at room temperature, washed in wash buffer, and bound to

concanvalin-A (ConA) paramagnetic beads (Bangs Laboratories; BP531) for magnetic separation as described on the protocols.io website (https://doi.org/10.17504/protocols.io.bgztjx6n). Samples were then suspended in antibody binding buffer and split for overnight incubation with antibodies specific to INTS12 (Thermo Fisher, 16455-1-AP) (Figure 5) or INTS12 (Abbexa, abx234360) (supplemental figure S3) or RPB3 (Thermo Fisher, A303-771A), and IgG control (Abcam; 172730). Sample processing was performed in a 96 well plate using 25K Con-A bound nuclei per reaction on a Beckman Coulter Biomek liquid handling robot according to the AutoCUT&Tag protocol available from the protocols.io website (https://doi.org/10.17504/protocols.io.bgztjx6n) and described previously (Janssens et al., 2022) (Fred Hutch Genomics shared resource). Samples were run on the NextSeq P2 using paired-end 2 x 50 bp sequencing by the Fred Hutchinson Cancer Research Center Genomics Shared Resources.

CUT&Tag: Data Analysis

The same custom genome assembly as RNA-seq was used for CUT&Tag. Bowtie2 version 2.4.2 was used for alignments with the following parameters: —very-sensitive-local—soft-clipped-unmapped-tlen—dovetail—no-mixed—no-discordant -q—phred33 -I 10 -X 1000. For each target protein and each condition, 3-5 technical replicates were profiled. We removed duplicate reads from each replicate, and the combined reads for all replicates were pooled and used to generate the coverage-normalized bigwig files shown as genome-browser tracks. The number of unique reads for each replicate were quantified over the intervals indicated by the dotted grey lines in Figure 5, and we compared the read counts over the indicated intervals in the AAVS1KO AZD&IBET, INTS12KO DMSO and INTS12KO AZD&IBET conditions to the AAVS1KO DMSO control samples using a two-tailed independent samples t-test.

Primary cells: Isolation

CD4 T cells were isolated from PBMC obtained from ART suppressed PLWH and cultured in RPMI media supplemented with 10% FBS, Penicillin/Streptomycin, 10 mM HEPES, and 10 units

per mL of IL-2. All cells from PLWH were obtained from the UNC clinical HIV cohort (Study number: 08-1575)

Primary cells: knockout and LRA treatment

HIV infected CD4 cells from PLWH *in vivo* were electroporated using the P2 Primary Cell 96-well Nucleofector® Kit, (V4SP-2096, Lonza, Cologne, Germany), according to the manufacture's protocol with slight modification (Ashokkumar et al., 2024): briefly, 2-3× 10^6 cells were washed twice with phosphate-buffered saline by centrifuging at 90g for 5 min and resuspended in nucleofection buffer P2. The resuspended cells together with RNP complexes were immediately transferred into the cuvette of the P2 Primary Cell Nucleofector Kit (Lonza; V4SP-2096) and electroporated using the program code, EH-100 on the Lonza 4D-Nucleofector. Electroporated cells were resuspended with 200µL of prewarmed supplemented RPMI media with IL-2 (10U/mL) and IL-7 (4ng/mL) and expanded in a 37°C incubator. Three days post nucleofection, cells were treated with combination of AZD5582 (100nM) and I-BET151 (500nM) or DMSO and the expression of cell-associated (24hr) and cell-free (72hr) gag was quantified by RT-qPCR. The cells were maintained at 1x10^6 cells/mL with fresh media supplemented with IL-2 and IL-7 every 2-3 days. Pre-designed crRNA with higher off-target score targeting human gene, INTS12 (CTTTTGAGATGGACGGTAAC, CACAATACCACACCAGGCGA, and ACCAATGGATCTTTGACAGC), and/or scrambled guides (non-target control, NT) were obtained from IDT as controls. Annealing of crRNA and tracrRNA, preparation of CRISPR/Cas9 ribonucleoprotein complexes (RNPs). Three sgRNA targeting different regions of the target were multiplexed for more efficient target knockout.

Primary cell: RT-qPCR

Quantification of relative gene expression using quantitative-PCR

One-step RT-qPCR HIV Gag RNA gene expression quantifications were performed as previously described [2] Briefly, RNA from independent donors were extracted using

the RNeasy plus kit and QIAamp Viral RNA Mini Kit (Qiagen) for cell-associated and cell-free virus as per the manufacturer's instructions, respectively. 1ug of nanodrop quantified RNA from Non-target (NT) or INTS12 gRNA nucleofected cells were reverse transcribed and amplified using Fastvirus (Thermo, Waltham, MA) and primer sets for HIV Gag RNA (GAG-F: ATCAAGCAGCCATGCAAATGTT, GAG-R: CTGAAGGGTACTAGTAGTTCCTGCTATGTC, GAGProbe: FAM/ZEN-ACCATCAATGAGGAAGCTGCAGAATGGGA-IBFQ). Reactions were performed in 96-well plates using the Quant Studio 3 Real-Time PCR system (Applied Biosystems, Foster City, CA) real time thermocycler with a cycling parameters of 5 min reverse transcription step at 50°C, 95ºC for 20 sec for Taq activation, followed by 40 cycles of 95ºC (3 sec.), and 60ºC (30 sec.). All qPCR reactions were performed in triplicate. Normalized relative expression levels were calculated using the Prism software version 10.1.1 (GraphPad).

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Chapter 3: Perspectives and Future Directions

Summary

Strategies to improve specific and potent reactivation of HIV are essential to improving the shock-and-kill cure strategy. In Chapter 2, I described an expansion of a CRISPR-based screening approach developed in our lab, whereby two synergizing LRAs, AZD5582 & I-BET151, were interrogated with an epigenetic library to uncover additional blocks to reactivation that could be targeted to improve this LRA combination. I had the specific goal of identifying blocks that may prevent AZD5582 & I-BET151 from working effectively in reactivating HIV in *ex vivo* systems from cells from PLWH. I uncovered that INTS12 plays a role in latency maintenance *in vitro* and *ex vivo* since INTS12 knockout can indeed improve reactivation of latency reversal agents in these systems. Additionally, through RNA-seq experiments I have shown that HIV reactivation *in vitro* from INTS12 knockout alone is more specific than AZD5582 & I-BET151 treatment alone, and much more specific than other methods of latency reversal such as PMA & Ionomycin. These results validate INTS12 as a drug target that would be relatively specific to HIV-1 latency reversal. Furthermore, my thesis work has provided mechanistic insights into the role of INTS12 in HIV latency maintenance since, I have observed direct binding of INTS12 to the HIV promoter and have obtained results that are consistent with a role of INTS12 in decreasing the potency of latency reversal agents by establishing a transcriptional elongation block. In summary, these results demonstrate that I was able to use the CRISPR-screening approach in conjunction with two LRAs to predict additional blocks to latency reversal in relevant *ex vivo* systems.

Model for how Integrator improves LRA function

One hypothesis for why the knockout of INTS12 improves LRA function is based on the supposition that the absence of INTS12 affects the function of the Integrator complex as a whole. Therefore, knockout of INTS12 would affect the activities of both the cleavage (endonuclease) module or the phosphatase module of the complex. I hypothesize that targeting Integrator may improve AZD5582 treatment by preventing transcription termination from the cleavage module, which would allow for newly initiated HIV transcription events to be more likely to proceed, and targeting Integrator may improve I-BET151 treatment by removing antagonism of cellular PTEF-b from the phosphatase module, which would allow for the increase of available PTEF-b for HIV Tat to effectively aid in transcription elongation of HIV. These hypotheses may explain why I find an increase in latency reversal with INTS12 knockout paired with each LRA individually as well as the combination of LRAs. In this scenario, the more HIV transcriptional events that proceed will generate HIV Tat that will aid in efficient reactivation, whereas presence of the Integrator complex would impose a block to HIV reactivation at the elongation level and lead to transcription termination. A model detailing the interactions between AZD5582 & I-BET151 with the Integrator complex is shown in **Figure 3.1**. This model demonstrates why AZD5582 & I-BET151 synergize and proceed through elongation in vitro (**Figure 3.1, 3a**), and covers how Integrator may impose a block to reactivation in vitro, but more strongly in vivo, by engaging in the second scenario (**Figure 3.1, 3b**). In (**Figure 3.1, 3b**) knockout of Integrator would remove the block to elongation facilitate more of the (**Figure 3.1, 3a**) scenario. Additionally, even in the absence of LRAs, we have shown that INTS12 itself is imposing a block to latency reversal in Chapter 2. If the Integrator complex as a whole is affected by INTS12 knockout, some transcription events from stochastic activation are likely to proceed even if some other blocks are present.

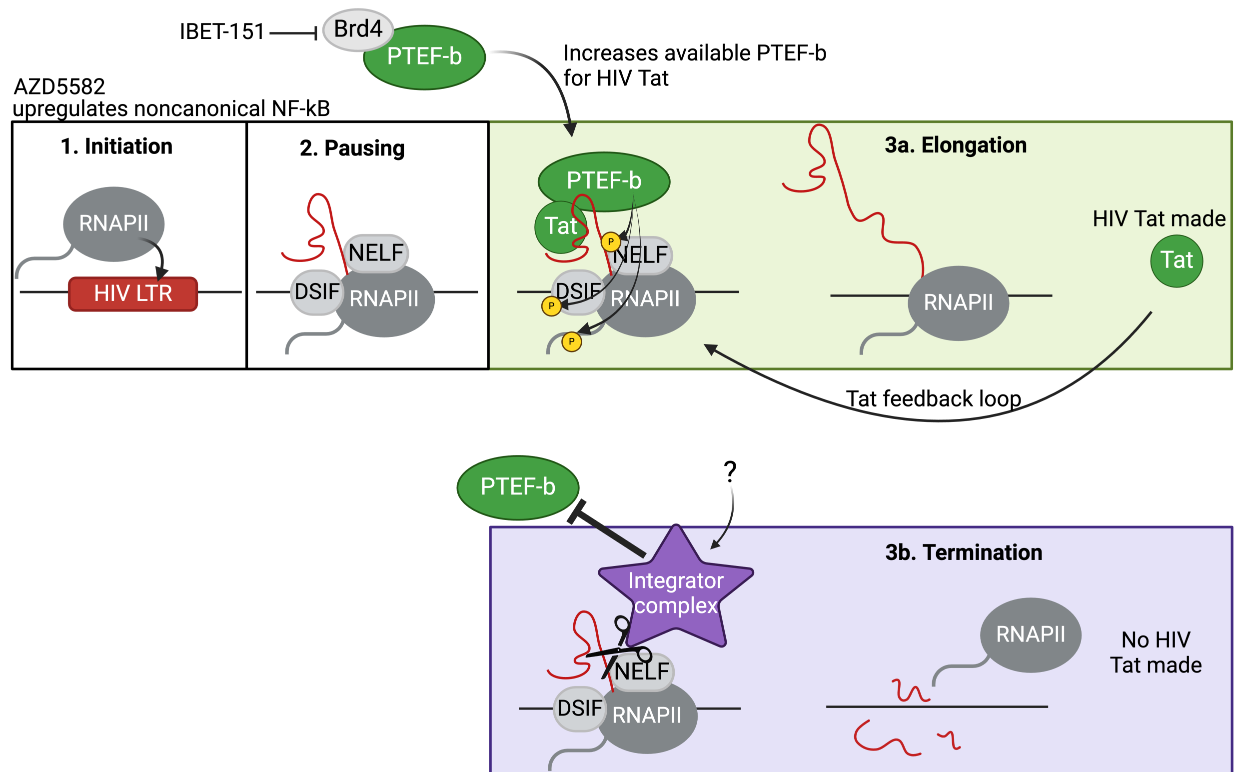

Figure 3.1. Model of the Integrator complex and AZD5582 & I-BET151 treatment. Shown are two scenarios (3a and 3b) that both begin with step 1 and 2. 1) Induced transcription initiation from treatment with AZD5582 that upregulates the ncNF-kB pathway 2) Pausing of the HIV promoter from binding of pausing factors NELF and DSIF, a step that allows for 5' RNA capping and recruitment of elongation factors to occur before elongation continues. 3a) A scenario predicted to occurs most of the time with AZD5582 & I-BET151 treatment *in vitro* and some of the time *in vivo*. Shows PTEF-b can phosphorylate pausing factors and RNAPII to facilitate elongation, which generates HIV Tat that can increase elongation efficiency. 3b) A scenario that is predicted to occur some of the time *in vitro* and more of the time *in vivo*. Shows the Integrator complex being recruited, through mechanisms unknown, to antagonize PTEF-b by dephosphorylating PTEF-b substrates and cleaving nascent RNA to terminate transcription.

However, there are also alternative hypotheses to explain my result wherein INTS12 alone may be performing a function separate from the enzymatic functions of the Integrator complex. For instance, there are characterized phenotypes of the knockout of each catalytic module of Integrator, and the phenotype I see from INTS12 knockout may be distinct. For example, in one study, when the phosphatase module was knocked out, the RNAPII became hyperphosphorylated with Ser2 in relation to total RNAPII levels (Hu et al., 2023). To explore

this model, I performed CUT&Tag on RNAPII Ser2 in **Figure 3.2** and total RNAPII in Chapter 2 Figure 5**,** on my INTS12 knockout cells compared to my control AAVS1 knockout cells. When I first looked at only how Ser2 phosphorylation (Ser2p) changed between different conditions, I saw that there were two conditions for which there was evidence of elongation competent RNAPII, i.e. higher Ser2 levels within HIV– AZD5582 & I-BET151 alone and paired with INTS12 KO (see Figure 3.2, A, region 2). Furthermore, there is evidence that there is perhaps more efficient elongation with the INTS12 KO paired with LRAs given there is a drop in Ser2 at the promoter (Figure 3.2, A, region 1) and higher levels detected within HIV (Figure 3.2, A, region 2), which would be expected if the phosphorylated and elongating RNAPII was efficiently leaving the promoter to transcribe HIV. We do not see this drop in Ser2 at the promoter for the AZD5582 & I-BET151 only condition, and we see less of an increase within HIV, which may point to more RNAPII being stalled in the promoter region and being unable to elongate (Compare **Figure 3.2, A, regions 1-2**). As a control for this experiment, I found no differences in RNAPII Ser2p levels in the gene that is downstream of HIV (**Figure 3.2, A, region 3**), nor did I see any differences for a more general marker on RNAPII, Ser5 phosphorylation, that I also tested via CUT&Tag (**Figure 3.2, B, regions 1-3**). When looking at the total RNAPII levels in Chapter 2 Figure 5, there is only one condition that shows a robust increase in total RNAPII– the INTS12 KO with AZD5582 & I-BET151, which could explain the increase in HIV transcription seen with this pairing, as there is not only elongation competent RNAPII but an increase in total RNAPII available. To tie these two findings into the hypothesis that INTS12 may be functioning separately from the phosphatase module of Integrator, when we compared the levels of Ser2p to total RNAPII, specifically the INTS12 KO with AZD5582 & I-BET151 condition, the levels are roughly the same, which means that we are not seeing the characteristic hyperphosphorylation phenotype seen from phosphatase module disturbance. Therefore, even though we know that the phosphatase module can be targeted to increase latency reversal, from (Li et al., 2020) as well as our own predictions in Chapter 2 Figure 7, it is possible that INTS12 knockout is not

affecting this module or at least not removing its function entirely. If the phosphatase module were still somewhat intact, this would mean that some of the kinase activity of PTEF-b would still be antagonized by Integrator, and to explain why we still see an improvement of reactivation with I-BET151 with INTS12 KO, perhaps I-BET151 can overcome some of the Integrator's phosphatase module antagonism simply by increasing the amount of available PTEF-b.

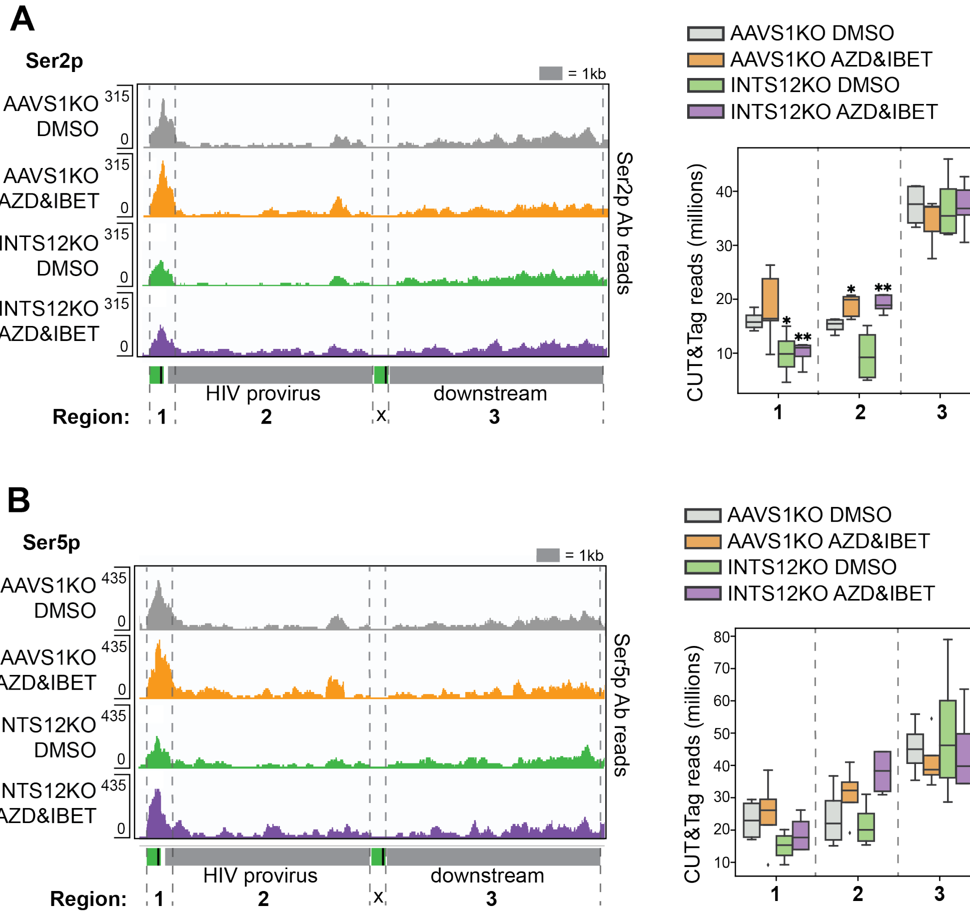

Figure 3.2. INTS12 knockout with AZD5582 & I-BET151 treatment increases RNAPII elongation.

CUT&Tag using antibodies to RNAPII Ser2 (A) or RNAPII Ser5 (B) were used to generate pileup graphs to show where each are binding chromatin under different conditions. The location of where reads are mapping is denoted below with the HIV LTR in green with the R region in black. The rest of HIV is denoted by grey in region 2, and the gene downstream is also grey but in region 3. The scale for each track is the number on the left of each row, and each row represents three to five technical replicates averaged together. (A and B, right panels) quantifies total reads in regions denoted by the grey dashed lines, regions 1-3. Two-tailed independent samples t-test p-value = <0.05 = *, = <0.01 = **, = <0.001 = ***, = <0.0001 = ****. Cells used in this figure have an INTS12 knockout score of 80%.

Additionally, I have reasons to believe that the cleavage module may also still be partially intact even in the absence of INTS12. The first phenotype reported upon cleavage module knockout is an increase in lowly expressed genes specifically (Hu et al., 2023). HIV is lowly expressed during latency and others have shown that knocking out the cleavage module leads to reactivation of HIV and affects processivity of transcription, where knockout leads to more full-length HIV transcripts (Stadelmayer et al., 2014). In Chapter 2 I detail how INTS12 knockout also leads to this reactivation phenotype, so it would appear that INTS12 may very well affect the cleavage module to result in HIV reactivation. However, a second phenotype reported with knockout of the cleavage module is read-through transcription and misprocessing of snRNAs. In my RNA-seq data from Chapter 2 Figure 3 and my CUT&Tag data from Chapter 2 Figure 5, INTS12 knockout does not lead to read-through transcription of HIV, as there are low transcripts and total RNAPII detected after the HIV provirus, suggesting the cleavage module may be somewhat intact in this function. Another group corroborates my INTS12 findings in a thesis by Blears et al., where they do not observe readthrough transcription of genes upon INTS12 knockout, nor do they observe snRNA misprocessing, which is rare amongst Integrator knockouts. Intriguingly, it is known that even the knockout of core member INTS1, INTS12's only predicted binding partner to the Integrator complex, shows strong misprocessing of snRNAs (Albrecht et al., 2018; Chen et al., 2012; Ezzeddine et al., 2011). Therefore, it is exciting that INTS12 knockout may result in reactivation of HIV and may be leaving some important functions of the Integrator intact, i.e. snRNA processing and transcription termination.

Because INTS12 knockout data does not fully recapitulate knockout of the cleavage or phosphatase modules, it is possible that these modules may be partially intact and perhaps INTS12 knockout only affects their stability or efficiency. Alternatively, this data supports that INTS12 may be functioning in an independent manner, while leaving other important Integrator functions intact. Both hypotheses could help explain the specificity seen for HIV reactivation

from INTS12 knockout in Chapter 2 Figure 4 and demonstrate why INTS12 may be a better target for small molecule drug development than the catalytic members of Integrator, or even the binding partner of INTS12, INTS1. While the Integrator complex as a whole is important for developmental processes and alterations of several members are associated with diseases such as cancer (Rienzo & Casamassimi, 2016), INTS12 polymorphisms in nature have more specifically been linked to lung functioning (Kheirallah et al., 2017). With these side-effects in mind, short-term modulation of the Integrator complex, even of the catalytic modules, has been shown to have no effect on snRNA production (Elrod et al., 2019). Therefore, targeting any member of Integrator for a short period of time may be effective at stimulating HIV, while limiting side-effects. However, since INTS12 itself is a more specific target, modulation of INTS12 short-term with the monitoring of lung function may be a strategy to employ inhibition of INTS12 in the shock-and-kill approach.

Integrator complex recruitment to the HIV LTR

Understanding how the Integrator complex is recruited to genes and HIV is imperative to finding ways to modulate its function. Several members of the Integrator complex itself have been implicated in recruitment, i.e. the arm/enhancer module and or the proposed reader INTS12, as well as a related elongation complex PAF1C. PAF1C is intriguing because it also functions as an elongation block to HIV reactivation and there is a known LRA targeting PAF1C that is actively being studied (Soliman et al., 2023). Some studies suggest that PAF1C may be responsible for recruiting Integrator to chromatin as it was demonstrated that Integrator members 1 through 12 were pulled down with PAF1C (Liu et al., 2022). Additionally, in this same study, when PAF1C is depleted, there is less pull down of selected Integrator members: core member INTS1 and endonuclease module member INTS11. It has yet to be elucidated if an

LRA targeting PAF1C phenocopies INTS12 knockout or other Integrator member knockout or if targeting both PAF1C and Integrator members would further increase HIV reactivation.

Recently, the arm module containing INTS10, INTS13, INTS14, and INTS15 has been implicated in Integrator recruitment, as it has been shown to bind transcription factors and modulate recruitment at some loci in a stimuli specific manner (Offley et al., 2023; Razew et al., 2024; Sabath et al., 2024). In our study, this modules importance to HIV reactivation is less clear than other modules, Chapter 2 Figure 7, though we did not include INTS15, which is thought to bridge the arm module with the rest of Integrator (**Figure 3.3**). This modules importance in recruiting Integrator needs to be explored further, because this may provide a more specific and subtle way of toggling Integrator without affecting other critical functions, especially if recruitment could be tied to a specific transcription factor.

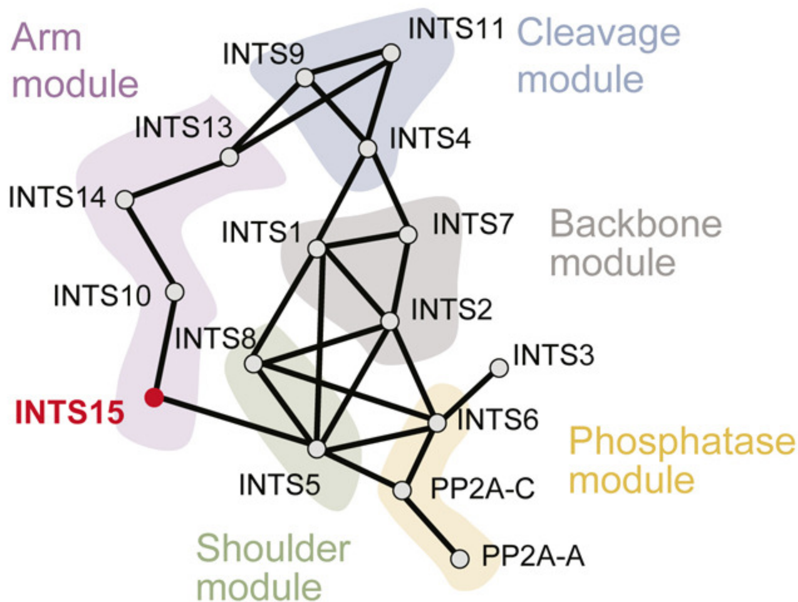

Figure 3.3. Overview of how the Integrator complex members interact.

The Integrator consists of the Cleavage module, Backbone (core) module, Phosphatase module, Shoulder module, and Arm module. INTS15 is in red as it is the newest member discovered that bridges the arm module to the rest of Integrator. INTS12 is not shown, however, it has been shown to crosslink with INTS1. **Figure 3.3** was reproduced from (Offley et al., 2023) with permission, Creative Commons Attribution License 4.0 International (CC BY 4.0).

Lastly, INTS12 itself is a candidate to consider for Integrator recruitment. It contains a plant homeodomain (PHD) finger, which is a domain that canonically binds to different methylations on histones and therefore, proteins containing this domain can serve as a histone reader for a complex (Welsh & Gardini, 2023). Interestingly, in work from collaborators not published, and recently reported in unpublished data from a thesis (Blears, 2023), it appears that the INTS12 PHD finger does not appear to bind to canonical histone methylations. Despite the lack of structural data on the presumably flexible INTS12, there have been studies that have uncovered heavy crosslinking between INTS12 and INTS1 as well as NELFA (Chen et al., 2013; Fianu et al., 2021; Zheng et al., 2020). It is possible that these interactions are sufficient for recruitment, rather than binding a histone mark. However, more work needs to be done to elucidate if INTS12 is binding marks other than methylations on histones, if binding INTS1 and NELFA are sufficient for recruitment, and or if the interactions between NELFA and INTS1 are crucial for the function of INTS12.

To further complicate investigating Integrator complex recruitment, it has been shown that stress stimuli can play a role. The main two types of stress explored are hyperosmotic stress and stimulation with EGF (Tatomer et al., 2019; Yue et al., 2017). In our study, we did not see differences in expression of Integrator members after stimulation with AZD5582 & I-BET151 treatment, however this does not mean that their recruitment was not altered, and this should be explored.

For all potential Integrator complex recruiters, it will be imperative to look at how the rest of the Integrator complex is affected by various Integrator or PAF1C depletions with or without stress stimuli, as it is possible that the stoichiometry of the complex may be altered and or different modules may be recruited in different ways to genes. Understanding the dynamics of the Integrator complex members with the paused RNAPII, PAF1C, and stress stimuli will inform small molecule inhibitor design to toggle these interactions.

Integrator and Latency Establishment

This thesis has largely explored the Integrator complex and its role as a block to latency reversal. However, given the Integrator complex governs HIV transcription, it could be possible that Integrator could also play a role in the dynamics of latency establishment. HIV infection is a highly stimulatory environment with a lot of cell death during acute infection, talked about in Chapter 1. These different types of stimuli should be explored, such as LPS that is largely generated from gut microbe translocation, to determine if these or other immune stimuli alter Integrator recruitment to the LTR. If Integrator recruitment is altered, this could prevent or promote HIV latency establishment and also affect cell death or survival accordingly. To test whether these stimuli alter Integrator recruitment, cells could be treated with different stimuli, and then CUT&Tag or other similar methods could be performed to look at select members from each module of Integrator and see if their recruitment is altered. It would be interesting if LPS was able to reduce Integrator recruitment, because this loss could potentially upregulate HIV expression and could help explain the cell death seen specifically in the gut during acute HIV infection. To test if the Integrator complex plays a role in latency establishment in general, primary T lymphocyte cells could be knocked out for INTS12 or a control gene AAVS1 and then infected with HIV that contains a reporter gene, such as GFP. After infection, the fluorescence could be measured by flow cytometry to ensure that the provirus integrated, and these cells can be selected for by sorting. Cytokines and culture conditions can be altered to allow the cells to enter a state of latency (Jefferys et al., 2021), and if Integrator normally helps the cell enter latency by turning off HIV transcription, I would expect that the INTS12 knockout cells will never enter latency or have delayed entry and that the fluorescence detected by flow will remain high whereas, the fluorescence in the control cells will wane overtime. These two experiments would help investigate if the Integrator complex is playing a role during HIV infection, where Integrator may upregulate HIV in the gut, due to the stimulatory environment, which may result in the

specific cell death seen in the gut, but also perhaps Integrator could lead to successful latency establishment and maintenance in a small population of cells by attenuating gene transcription resulting in the viral reservoir. It is possible that Integrator may be playing a role in both of these processes, and these experimental findings would have interesting implications for the biology of HIV infection.

CRISPR screen future directions

Our lab as well as others have demonstrated the power in the CRISPR-screening approach in improving LRAs and identifying novel targets for small molecule drug development. Ideally once a new inhibitor is developed, it tested in a NHP model where toxicity can be assessed, and viral reactivation and reservoir reduction can be measured in tissue reservoirs.

In work currently ongoing with collaborators, we are interested in improving ncNF-kB targeting. We have created a CRISPR library that targets proteins that bind to RelB, that is a part of the ncNF-kB pathway, and we are testing this library against different LRAs with the hopes of furthering our understanding of the ncNF-kB pathway itself, how LRAs targeting this pathway function, and how related LRAs function. This will allow for us to uncover the most specific way to target this pathway with respect to HIV, given that we already know its importance.

Integrator and the shock-and-kill approach as a whole

In an ideal scenario to improve the shock-and-kill approach a cocktail of LRAs would be devised that could specifically target all of the replication competent reservoir to functionally cure HIV. We know that transcription initiation is important and have identified fairly specific ways of targeting this pathway with LRAs such as AZD5582, and we also know that cellular PTEF-b is important to reactivation and have LRAs such as I-BET151 to target this factor.

However, we are only now uncovering the role of the Integrator complex in the shock-and-kill approach. Integrator is an important factor to consider because it can influence both initiation and elongation efforts and can respond differently under stimulatory conditions, therefore the Integrator complex likely plays a role in HIV reactivation for any LRA, and is a crucial factor to consider when trying to uncover the minimal or “base-set” of targets to achieve the most potent and specific treatment for the shock-and-kill approach.

INTS12 contains a highly conserved PHD finger that may be a good target for small molecule drug development (Kheirallah et al., 2017). Others are currently attempting to identify targetable residues, and have data to support that INTS12 is binding to NELFA with the PHD finger and that this interaction is important for Integrator function (Blears, 2023). Experiments that could be performed to help towards this effort are mass spec comparing INTS12 with an INTS12 PHD deleted mutant to predict additional binding partners of INTS12. Validation would consist of knocking out binding partners and observing if knockouts phenocopy INTS12 knockout and show an increase in HIV reactivation with and without AZD5582 & I-BET151. Additionally, to rule out binding of histone modifications other than methylations, a panel of histone modifications can be tested in INTS12 pull-down experiments to see if the domain is binding to a noncanonical mark for PHD fingers. Additionally, since the NELFA INTS12 interaction already has data to suggest importance, NELFA and INTS12 can be tagged to measure binding and this interaction can then be screened against a panel of small molecules to develop small molecule inhibitors that target this interaction and prevent binding. All of the above experiments would help find the most specific way to target INTS12 so that a small molecule drug could be developed and used in the shock-and-kill approach.

Altogether, the work in this thesis identifies INTS12 as being a target to specifically improve HIV-1 reactivation with AZD5582 & I-BET151 treatment *in vitro* and *ex vivo* systems. Future screens may explore the INTS12 knockout itself and what further factors can be targeted to improve HIV reactivation. The goal of these strategies is to develop small molecule drugs to

target novel hits such as INTS12, validate these new drugs *in vitro/ex vivo* to ensure that they function as LRAs, and then bring these new LRAs *in vivo* where dosing, combinations, and rounds of treatment can be tested to gain an understanding of safety and efficacy. Additionally, while this thesis covers improving the shock side of the shock-and-kill approach, the kill side is also being optimized towards this effort (Grasberger et al., 2024). In order for this strategy to work, we need to engage CD8 T cells and enhance clearing of infected cells through methods such as the introduction of antibodies that can target HIV antigens or stimulating the recruitment of effector cells to sites of reactivation. The goal of my thesis work is to provide a framework to improve multiple LRAs and expand upon what is known of latency maintenance so that we can move closer towards a functional cure for HIV.

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